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Antioxidant and anti-inflammatory activities of *Geranium robertianum* L. decoctions

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Abstract

Geranium robertianum L., commonly known as Herb Robert, is an herbaceous plant popularly known for its functional properties including antioxidant and anti-inflammatory. In this study, the phenolic profile of leaves and stems decoctions of *Geranium robertianum* L. was elucidated through UHPLC-DAD-ESI-MS^n analysis, and their antioxidant and anti-inflammatory potential were assessed *in vitro*. Importantly, and envisaging the use of these extracts in human diets, the potential toxicity of bioactive concentrations was also addressed in macrophages and hepatocytes.

Despite being both reach in ellagitanins, the extract from leaves was shown to be slightly more abundant than the one from stems. High radical scavenging activity against $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and $OH^{\bullet}$ were observed either for the leaves or the stems extract, as well as good activities towards ferric reducing antioxidant activity, lipid peroxidation and oxygen radical absorbance capacity methods. In addition, both extracts were very effective in scavenging $NO^{\bullet}$, as measured in a chemical model, while only the stems extract was able to decrease the production of this radical by stimulated macrophages. On the other hand, none of the extracts was able to modulate the activity of lipoxygenase or the expression of the inducible nitric oxide synthase. Overall, this data allowed to conclude that *G. robertianum* L. stems and leaves infusions are particularly rich in tannins. The strong scavenging effects displayed by the stems extract suggest that its anti-inflammatory activity may partially result from its anti-radical capacities towards $NO^{\bullet}$.

Keywords: *Geranium robertianum* L.; antioxidant; anti-inflammatory; tannins; phenolic compounds; herbal medicine.

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Introduction

Oxidative stress is defined as an imbalance between the production of free radicals and reactive metabolites, commonly known as reactive oxygen and nitrogen species (ROS and RNS), and their elimination by protective mechanisms, referred to as antioxidants.[1] This biological condition is closely associated to chronic inflammation and several pathological conditions including cancer, cardiovascular, hepatic and neurodegenerative diseases.[2]

In normal conditions, inflammation is of utmost importance to protect body tissues against harmful stimuli, manifesting rapidly and severely upon injury, but persisting only for a short period of time.[3] During this event, several signal transduction pathways are triggered promoting the activation of a great deal of pro-inflammatory mediators including cytokines, enzymes such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2) and lipoxygenase (LOX), and also more ROS and RNS species.[4] Notably, the up-regulation of iNOS deeply increases the production of $NO^{\bullet}$, which has very pleiotropic functions during inflammation. In turn, COX-2 and LOX are pivotal players in the arachidonic acid pathway, controlling the biosynthesis of pro-inflammatory prostaglandins and leukotrienes, respectively, considered as potent mediators locally released at the inflamed tissue.[5] These events result in an increase of leukocytes recruitment, consequently increasing the oxygen uptake leading to the oxidative burst, *i.e.*, the rapid release of ROS and RNS that will attack pathogenic organism and/or injured cells.[6] When not contained, this sustained inflammatory/oxidative environment leads to a vicious circle, which can damage healthy neighboring epithelial and stromal cells and over a long period of time may lead to chronic illnesses, namely cancer, diabetes, neurodegenerative and cardiovascular diseases.[7] Indeed, accumulation of ROS and NOS generated by inflammatory cells is thought to be one of the major factor by which chronic inflammation contributes to chronic diseases.[8] Since the overproduction of pro-inflammatory mediators raises and maintains inflammation, compounds targeting their expression are good candidates for attenuating inflammatory diseases.

On the other hand, edible plants are widely used for their health benefits. *Geranium robertianum* L. (Geraniaceae), also known as “Herb-Robert” or “Red Robin”, is one of such plants. Presently, it is possible to find in the market some dietary supplements based on extracts or compounds from this plant species. This is the case of NeuroCore®, or Glicobeter®; the former used as dietary supplements for fitness

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purposes focusing body building and/or fat loss, and the later indicated for controlling glycaemia and preventing inflammatory conditions in the pancreas. Besides, it is also possible to find in the market several tea sachets of aerial parts of this herb. Flowers infusions are consumed for the treatment of stomach disorders, headaches, and liver problems, while infusions and/or decoctions of leaves are considered to be good anti-diabetic, anti-inflammatory, anti-rheumatic, antioxidant and antidiarrhoeic.[9–11] Some studies have already shed a light on Herb-Robert claimed bioactivities namely antioxidant,[12–14] antimicrobial,[15–17] antidiabetic,[9] antiulcer,[18] neuroprotective[17] and cytotoxic or cytostatic against tumor cell lines.[19–21] Additionally, some authors have also shown the potential of *G. robertianum* L. aerial parts extracts against HOCl, a strong oxidant produced by neutrophils and a potent pro-inflammatory agent, and through inhibition of hyaluronidase and elastase activities, which are enzymes that degrade the extracellular matrix and play pivotal roles in the development of many diseases which possess inflammatory background[12,22]. Still, the majority of these studies have not performed a detailed analysis of bioactive components in the extracts or mostly focus on the flavonoids characterization,[12,20,21,23] despite Geraniaceae family is known to be particularly rich in tannins that are also largely known for a wide range of bioactivities. The exception to this was the recent work of Graça and co-workers,[19] whom reported several tannins in an acetone extract of *G. robertianum* L. that showed high antioxidant activity and cytotoxicity against several tumor cell lines.

In this context, the present work is intended to perform a detailed phenolic composition of aqueous extracts of *G. robertianum* L. obtained from the two most representative organs of the plant in terms of mass, i.e., leaves and stems (LAE and SAE, respectively), as well as to compare their respective potential in counteracting oxidant and inflammatory processes. The antioxidant ability of LAE and SAE was evaluated through a set of antioxidant assays including 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$), ferric reducing power, oxygen radical absorbance capacity (ORAC), lipid peroxidation and $OH^{\bullet}$ scavenging, in order to disclose distinct mechanisms of action, including radical scavenging, the ability to protect lipid oxidative damage and neutralization of the harmful reactivity of hydroxyl radical. Furthermore, the anti-inflammatory activity of the LAE and SAE was evaluated towards 5-LOX activity and LPS-triggered $NO^{\bullet}$ production, as well as on the total levels of iNOS expression, in RAW 264.7 macrophages. Moreover, the safety profile of *G. robertianum* L. was investigated in

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both hepatocytes and macrophages that constitute useful *in vitro* models to evaluate the potential cytotoxicity of the extracts, envisaging their possible usage in human diets.

1. Material and methods

1.1. Chemicals

Phosphate buffer saline (PBS) reagents (sodium salt, sodium chloride, potassium chloride, disodium hydrogen phosphate and potassium dihydrogen phosphate), iron(II) sulfate, potassium hexacyanoferrate(III), iron chloride(III), ABTS diammonium salt, trolox, butylated hydroxytoluene (BHT), trichloroacetic acid (TCA), DPPH$^{\bullet}$, dulbecco's modified eagle medium (DMEM), Tween® 20, penicillin G sodium salt, streptomycin sulfate salt, sodium bicarbonate, lipopolysaccharide (LPS) from *Escherichia coli* – serotype 026:B6, linoleic acid, soybean 5-lipoxygenase (LOX), gallic acid and ellagic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA), while fetal bovine serum (FBS) and trypsin were from Gibco (Paisley, UK). Fluorescein disodium salt, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), chlorogenic acid and thiobarbituric acid (TBA) were purchased from Acros Organics (Geel, Belgium). Ascorbic acid, formic acid, Folin–Ciocalteu reagent, sodium carbonate, sodium phosphate, potassium hydroxide and ethylenediamine tetraacetic acid (EDTA) were purchased from Panreac (Barcelona, Spain). Hydrogen peroxide and sodium hydroxide were purchased from Fisher Scientific (Hampton, USA). Boric acid was purchased from Chemlab (Zedelgem, Belgium), deoxyribose from Alfa Aesar (Massachusetts, USA) and mannitol from MERCK (New Jersey, USA). Solvents including ethanol, methanol and *n*-hexane of high performance liquid chromatography (HPLC) purity were purchased from Lab-Scan (Lisbon, Portugal).

1.2. Plant material

G. robertianum L. specimens were collected from the spontaneous plants growing in Cernache do Bonjardim and identified by Dr. Hélia Marchante from the Agriculture College of Coimbra. The botanical name and authority were further checked on 12th June 2014 in The Plant List database. After collection, leaves were separated from the stems and the two plant organs were separately dried for 5 days at 36 ºC in a ventilated incubator.

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1.3. Extraction of phenolic compounds

Dried stems and leaves of *G. robertianum* L. were separately grounded in a cross-beater mill SKl (Retsch, Haan, Germany), equipped with a sieve of 0.5 mm porosity. The plant material (10 g) was then submitted to a decoction with 400 mL of water during 15 min and the resulting solutions were filtered through G4 sintered plates filter. The residues were re-extracted two more times in the same conditions and the resulting filtrated solutions were combined and concentrated to approximately 100 mL in a rotary evaporator at 37 ºC, following defatting with equal volume of *n*-hexane. The aqueous defatted fraction was frozen, freeze-dried and kept under vacuum in a desiccator in the dark, for subsequent use.

1.4. Identification and quantification of phenolic compounds

Individual phenolic compounds were identified by UHPLC-DAD-ESI/MS^n, as described elsewhere.[24] The work was carried out in an Ultimate 3000 (Dionex Co., USA) apparatus with an ultimate 3000 Diode Array Detector (Dionex Co., USA) and coupled to a Thermo LTQ XL (Thermo Scientific, USA) ion trap mass spectrometer equipped with an ESI source. Analysis was run on a Hypersil Gold (Thermo Scientific, USA) C18 column (100 mm length; 2.1 mm i.d.; 1.9 μm particle diameter, end-capped) and its temperature was maintained at 30 ºC. The mobile phase for the separation of *G. robertianum* L extracts constituents was composed of (A) methanol and (B) 0.1% of formic acid (v/v). The solvent gradient started with 6–60% of solvent (A) over 60.8 min and from 60–100% over 5 min followed by the return to the initial conditions. The flow rate was 0.1 $mL.min^{-1}$ and UV–Vis spectral data for all peaks were accumulated in the range 200–500 nm while the chromatographic profiles were recorded at 280 and 320 nm.

Control and data acquisition of MS were carried out with the Thermo Xcalibur Qual Browser data system (Thermo Scientific, USA). Nitrogen above 99% purity was used and the gas pressure was 520 kPa (75 psi). The instrument was operated in negative-ion mode with ESI needle voltage set at 5.00 kV and an ESI capillary temperature of 275 ºC. The full scan covered the mass range from *m/z* 100 to 2000. CID–MS/MS and MS^n experiments were simultaneously acquired for precursor ions using helium as the collision gas with collision energy of 25–35 arbitrary units.

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For quantitative analysis, the limits of detection and quantification were calculated from the parameters of the calibration curves obtained by injection of known concentrations of different standard compounds, namely 5-caffeoylquinic acid (y=2×10^7x-53607; R^2=0.999), gallic acid (y=2×10^7x-149974; R^2=0.999) and ellagic acid (y=5×10^6x-93834; R^2=0.997).[25,26] Following a frequently adopted approach,[27,28] when phenolic reference compounds were not available, the calibration was based on structurally-related substances, and the results for each target phenolic compound were expressed in equivalents of the reference used.

1.5. Antioxidant properties

1.5.1. DPPH$^\bullet$ scavenging assay

The ability to scavenge DPPH$^\bullet$ was performed following the procedure previously described.[29]

1.5.2. ABTS$^{\bullet+}$ discoloration assay

This method was performed according to the procedure of Yang et al.,[30] with some modifications, as described elsewhere.[24] A stock solution of ABTS$^{\bullet+}$ was prepared by reacting the ABTS-NH_4 aqueous solution (7 mM) with 2.45 mM potassium persulfate (final concentration) and stored in the dark at room temperature for 12–16 h to allow the completion of radical generation. This solution was then diluted with ethanol so that its absorbance was adjusted to 0.70±0.02 at 734 nm. To determine the scavenging activity, solutions with concentrations ranging 0.13–1 mg/mL of *G. robertianum* L. extracts were prepared, and 100 µL of each were mixed with 1 mL of diluted ABTS$^{\bullet+}$ completing a final volume of 1.1 mL. Absorbance was then measured at 734 nm in a spectrophotometer (UVmini-1240 UV-VIS Spectrophotometer, SHIMADZU, Japan) against ethanol (used as blank) after 20 min of incubation in the dark at room temperature. The percentage of inhibition of ABTS$^{\bullet+}$, was calculated using Yen & Duh[31] as follows:

$$\%\ ABTS^{\bullet+}\ scavenging\ = \frac{(Ac - Ae)}{Ac} \times 100,$$

where Ac = Absorbance of the control (without extract addition); Ae = Absorbance of the extract. By plotting the percentage of ABTS$^{\bullet+}$ inhibition against extracts concentration it was possible to determine the IC_{50} (concentration of the extract able to inhibit the 50% of the ABTS$^{\bullet+}$) of each extract. Ascorbic acid was used as reference.

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1.5.3. $OH^{\bullet}$ scavenging assay

This assay was conducted following the procedure of Kumar et al.,[32] with slight modifications as previously described.[24] A solution (140 µL) composed of EDTA (300 µM), $FeCl_3$ (75 µM) and H_2O_2 (8.4 mM) prepared in 17.14 mM sodium phosphate buffer (pH 7.4), was mixed with 210 µL of *G. robertianum* L. extract solutions at different concentrations (56–336 µg/mL), 35 µL of ascorbate 1.2 mM and 35 µL of deoxyribose 33.6 mM. The reaction mixture was then incubated at 37 ºC over 60 min to allow the generation of hydroxyl radicals by ferric-ascorbate-EDTA-H_2O_2 interactions. Afterwards, 350 µL of 1% (w/v) TBA (prepared in 50 mM of NaOH) and equal volume of 5% (w/v) TCA were added and the solutions were placed in a boiling water bath for 15 min, to allow the formation of the pink chromogen. The reactions were then interrupted in an ice bath and the absorbance was measured in the spectrophotometer (UVmini-1240 UV-VIS Spectrophotometer, SHIMADZU, Japan) at 532 nm. The percentage of the inhibition of this reaction by the scavenging of $OH^{\bullet}$ was calculated again according to the equation of Yen & Duh,[31] and the IC_{50} determined through linear regression by plotting the percentage of inhibition against concentration of the extracts. Mannitol was used as reference compound.

1.5.4. Ferric reducing antioxidant power (FRAP) assay

For the reducing power assay, eight different concentrations of both extracts were prepared (0.01–0.09 mg/mL) and the method was carried out according to the procedure described before.[29]

1.5.5. *Lipid peroxidation inhibitory capacity in the presence of thiobarbituric acid reactive substances (TBARS)*

The lipid peroxidation method was performed as described by Catarino et al.[29] using five different concentrations (0.04–0.2 mg/mL) of each *G. robertianum* L. extract.

1.5.6. *Oxygen radical absorbance capacity (ORAC) assay*

The ORAC assay was performed according to the method of Rashidinejad et al.[33] with some modifications, as previously described.[24] In a 96-well, 150 µL of fluorescein (10 nM), prepared from a stock solution of 250 µM by diluting in 75 mM phosphate buffer (pH 7.4), were placed together with 25 µL of trolox standards (3.13–25 µM) and

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samples with final concentrations ranging between 1.25–12.5 μg/mL. For blanks, 25 μL of phosphate buffer were added instead of antioxidant solutions. After an incubation of 10 min at 37 ºC, 25 μL of 2,2'-azobisisobutyramidinium chloride (AAPH) (153 mM) solution was added to each well, to a final reaction volume of 200 μL. The plate was immediately placed in the plate reader (SLT, Austria) and fluorescence was monitored every minute over 60 min. The measurement was carried out at 37 ºC with automatic agitation for 5 s prior to each reading. Excitation was conducted at 485 nm with a 20 nm bandpass and emission was measured at 528 nm with a 20 nm bandpass.

Six concentration dependent kinetic curves were obtained for each sample, and for trolox as well. The area under the curve (AUC) of the fluorescence decay and Net AUC were calculated according to the following equations:[34]

$$AUC = 1 + \sum_{t_0=60\ min}^{t_i=60\ min} \frac{R_i}{R_0},$$

$$Net\ AUC = AUC_{Sample} - AUC_{Blank},$$

where R_0 is the fluorescence reading at the initiation of the reaction and R_i was the fluorescence read at the time i.

Linear regression analysis by plotting the Net AUC values against their correspondent concentration, allowed to obtain the slope (m) of the curve equations for each sample and standard. Antioxidant activities (ORAC value) were calculated by the following ratio:[34]

$$TE = \frac{m_{Sample}}{m_{Trolox}},$$

the final results were expressed in μM of Trolox equivalent/μg (TE μM/μg) of sample extract.

1.6. Anti-inflammatory properties

The anti-inflammatory properties of LAE and SAE were evaluated in two chemical assays, namely the inhibition capacity towards soybean 5-LOX and the $NO^{\bullet}$ scavenging activity, as well as the ability to inhibit $NO^{\bullet}$ production and expression of iNOS, in a cellular model of inflammation, namely Raw 264.7 macrophages stimulated with the *Toll-like* 4 receptor agonist lipopolysaccharide (LPS).

1.6.1. Soybean 5-Lipoxigenase (5-LOX) assay

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5-LOX assay was performed using linoleic acid as a substrate based on previous studies,[35] as previously described.[24] Linoleic acid (500 μM) was prepared by dilution of a stock solution (1M) in 0.2 M borate buffer (pH 9.0) containing 0.05% (v/v) Tween® 20. In a 96-well quartz plate, a mixture of 12.5 units of 5-LOX with 25 μL of each sample concentration (4.5–32 μg/mL) was prepared and incubated over 10 min at 37ºC. The reaction was initiated by the addition of 50 μL of linoleic acid and the plate was immediately placed in an UV/vis plate reader (UVmini-1240 UV-VIS Spectrophotometer, SHIMADZU, Japan) and the absorbance was recorded every 60 s over 10 min, at 234 nm. As the linoleic acid is converted to 1–3-hydroperoxy linoleic acid, the appearance of a conjugated diene promotes an increase in the absorbance that is proportional to the reaction time, thus generating a curve.[35] The value for inhibitory % of the enzyme activity was calculated as follows

$$\text{Inhibitory } \% = \frac{m_{Ac0} - m_{Aet}}{m_{Aet}} \times 100,$$

where m_{Ac0} is the slope of the straight line portion of the curve generated by the negative control and m_{Aet} the slope of the straight line portion of the curve generated by each sample. Three repetitions of this experiment were carried out for each sample and for ascorbic acid (4.5 – 17.5 μg/mL), which was used as standard compound.

1.6.2. Chemical NO• scavenging assay

This assay was performed according to the method of Bor et al.,[36] as previously described.[24] Briefly, incubation of 200 μL of sodium nitroprusside (3.33 mM) in PBS 100 mM (pH 7.4) with or without 200 μL of the different sample concentrations (3–23 μg/mL) was performed at room temperature under light irradiation for 10 min. After the addition of 200 μL of Griess reagent, the samples were incubated for another 10 min and subsequently measured spectrophotometrically at 562 nm (UVmini-1240 UV-VIS Spectrophotometer, SHIMADZU, Japan). The IC_{50} value for the NO• scavenging activity was determined by plotting the percentage of inhibition of nitrite generation in the presence of the plant extracts (also calculated through Yen & Duh[31] mentioned before) against the tested concentrations. Ascorbic acid was used as reference compound.

1.6.3. Cytotoxic effects

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The potential toxicity of the two extracts were evaluated in a human hepatic cell line (HepG-2—ATCC HB-8065) and in the macrophage cell line RAW 264.7. The assessment of metabolically active cells was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction colorimetric as previously described.[37]

1.6.4. Inhibition of inflammatory events in LPS-stimulated macrophages cell line RAW 264.7

For the assessment of the anti-inflammatory potential of LAE and SAE, $NO^{\bullet}$ production was measured by the accumulation of nitrites in the culture supernatants of LPS-stimulated macrophages (ATCC number: TIB-71), in the absence or in the presence of different concentrations (25–100 μg/mL) of both *G. robertianum* L. extracts, using the Griess reagent as previously described [37]. Since $NO^{\bullet}$ is synthesized from L-arginine by iNOS, the concentrations with higher bioactivity were further evaluated with regard to their effects on the levels of intracellular iNOS, through Western blot method as described by Bufalo et al.[37]

1.6.5. Statistical analysis

All data were expressed as mean ± standard deviation (SD) of three similar and independent experiments, with exception of cell viability, $NO^{\bullet}$ production and western blot assays for which data were expressed in mean ± standard error of the mean (SEM) of three similar and independent experiments. For the quantification of phenolic compounds, two-sided unpaired t-test was used. For the remaining assays one-way ANOVA followed by Tukey's post-hoc test was performed. The statistical tests were applied using GraphPad Prism, version 6 and the significance level was $p < 0.05$.

2. Results and discussion

2.1. Phenolic compounds of leaves and stems G. robertianum L. aqueous extracts

The aqueous extracts of *G. robertianum* L. accounted for 18 and 15% of dried stems and leaves of the plant, respectively. The UHPLC analysis revealed similar chromatographic profiles for LAE and SAE, though significant differences could be observed in between several peak intensities (Figure **1**). Indeed, the overall amount of

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phenolic compounds in the two extracts also varied, accounting for 649.2 and 536.4 mg/g of LAE and SAE, respectively (Table **1**), thus suggesting that this plant accumulates phenolic compounds preferentially in its leaves.

Amongst the detected phenolic compounds, the majority of them corresponded to ellagic acid (peak 20) or ellagitannins, overall accounting for approximately 63 and 50% of total quantified phenolic compound in LAE and SAE, respectively. This profile is coherent with the general accepted idea that Geraniaceae family is rich in tannins.[38] Indeed, these results are in agreement to those of Graça et al.[19] who found similar ellagitannin contents (approximately 70%) in acetone extracts of this species. However, other authors have reported that flavonoids constitute the major phenolic compounds of this species, possibly due to differences in the extraction procedures or variations resultant from seasonality, environmental conditions, geographical origin and other factors that have not been considered.[13,20,39]

It is important to note that ellagic acid has been reported to barely exist freely in leaves or other tissues[38] and to spontaneously be produced by HHDP esters upon acid hydrolysis in aqueous solutions. Therefore, the high amounts of ellagic acid herein quantified for *G. robertianum* L. aqueous extracts might be formed during their preparation, resulting in overestimated concentrations with respected to their real amounts in fresh plant, while those of ellagitannins might be underestimated.

Overall, 12 hydrolysable tannins were identified in LAE and SAE. These comprised isomers of geraniin (peaks 9, 10, 11 and 12) and corilagin (peak 14), that stood out as the most prevalent ones. These compounds had a UV_{max} at approximately 274 nm and showed MS spectra ($[M-H]^-$ at *m/z* 951→933→301 for geraniin isomers and $[M-H]^-$ at *m/z* 633→301 for corilagin) concordant with those previously described.[19,40,41] Together geraniin isomers accounted for 48.7 mg/g of LAE which is equivalent to 30% of the total quantified tannins, and 7.5% of the total quantified phenolics. The fact that these compounds were only detected in LAE suggests that *G. robertianum* L. accumulates them preferentially in its leaves. On the other hand, corilagin was detected in both extracts in significant amounts, though its abundance in SAE was higher than in LAE (57.1±0.5 and 40.7±0.3 mg/g extract, respectively). Both geraniin and corilagin have been previously reported in other *Geranium* species. In fact, geraniin was the first described tannin in *G. robertianum* L. and is the most abundant tannin in *Geranium*

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genus.[40,42,43] However, to our knowledge, this is the first time that corilagin is being described for this species.

Other hydrolysable tannins were also herein identified for the first time in *G. robertianum* L.. These correspond to two isomers of tris-galloyl-HHDP-hexose ($[M-H]^-$ at *m/z* 951), which eluted in peaks 7 (only in LAE) and 17, repandusidic acid A ($[M-H]^-$ at *m/z* 969) that eluted in peak 13, phyllanthusiin C ($[M-H]^-$ at *m/z* 925) that eluted in peak 15 and three isomers of phyllanthusiin B ($[M-H]^-$ at *m/z* 969) that eluted in peaks 11, 12 and 16. Although these compounds are not as prevalent as the ones aforementioned, together they accounted for 71.2 mg/g of LAE and 53.9 mg/g of SAE, which represents approximately 10% of total quantified phenolic compounds in each extract.

The compounds eluted in peaks 18 ($[M-H]^-$ at *m/z* 609) and 19 ($[M-H]^-$ at *m/z* 433) were also related to ellagic acid. These have been tentatively assigned on the basis of their MS fragmentation pattern that corresponded to that of ellagic acid-(*p*-coumaroyl)-hexose[44] and ellagic acid pentoside,[45] respectively. While the latter has been already described in *G. robertianum* L., to the best of our knowledge, ellagic acid-(*p*-coumaroyl)-hexose has never been described before for this species. The recovery of these two compounds was identical in both LAE (26.0±0.2 and 25.0±0.0 mg/g extract, respectively), and SAE (32.6±0.3 against 28.1±7.7 mg/g extract, respectively).

Gallic acid, a key unit of gallotannins (also classified as hydrolysable tannins), has been frequently described as one of the main phenolic acids of *G. robertianum* L.[18,21] However, this was not true for the extracts herein analyzed, since this compound (peak 3) only accounted for approximately 11 mg/g of extract either for LAE or SAE. Still, other three gallic acid derivatives were detected as trace elements of both *G. robertianum* L. extracts, namely galloylquinic acid (peak 2, $[M-H]^-$ at *m/z* 331), digalloyl-hexose (peak 4, $[M-H]^-$ at *m/z* 343) and digalloyl-hexose (peak 5, $[M-H]^-$ at *m/z* 483).

Besides ellagic acid and hydrolysable tannins, the leaves and stems aqueous extracts were also rich in brevifolin carboxylic acid (peak 8, UV_{max} at 277 and 355 nm, $[M-H]^-$ at *m/z* 291→247), i.e., an isocoumarin that accounted for approximately 23 and 33% of the total phenolic compounds quantified in the LAE and SAE, respectively. According to Okuda et al.,[43] brevifolin carboxylic acid is common to occur in *Geranium* genus, though to our knowledge it has never been described in *G. robertianum* L. up to now. Notably, important biological properties other than antioxidant have been shown for

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brevifolin carboxylic acid and its aglycone, including moderate antimicrobial effects, lipid peroxidation protective and remarkable hepatoprotective activity.[46,47] Besides, this compound has also been compared to ellagic acid due to their pharmacokinetics similarities, i.e., brevifolin is rapidly absorbed, distributed and eliminated from human organism as ellagic acid is.[48]

Chlorogenic acid ($[M–H]^-$ at *m/z* 353) and rutin ($[M–H]^-$ at *m/z* 609) were the only hydroxycinnamic acid and flavonoid, respectively, identified in the LAE and SAE herein analyzed. The former was eluted in peak 6 and was slightly more abundant in SAE (19.6±0.3 mg/mg of extract) than LAE (14.9±0.0 mg/mg of extract), while the second was only found in trace amounts. Interestingly, many authors have described the presence of several other hydroxycinnamic acids and flavonoids in *G. robertianum* L. extracts,[21,23,39] which was not verified in this study. Various variables including different solvents and preparative procedures of the plant extracts, timing of plant material collection, soil composition, geographical origin, variability between plants, and others not considered might be contributing for the differences of this phenolic profile comparing to others that have been previously described for this plant species.

2.2. Antioxidant properties of G. robertianum aqueous extracts

Since there is no universal method to proper determine antioxidant activity, the antioxidant potential of the *G. robertianum* L. aqueous extracts was estimated by different antioxidant assays, namely the $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and $OH^{\bullet}$ for assessing the extracts ability to trap the synthetic free radicals $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and $OH^{\bullet}$, respectively, along with FRAP, TBARS, and ORAC, in order to evaluate the extracts ability to reduce Fe^{3+} to Fe^{2+}, to inhibit the malondialdehyde formation resultant from the lipids oxidative degradation and to prevent the loss of fluorescence caused by the oxidative degradation of fluorescein, respectively.

Overall, a dose-dependent activity was observed for both samples in each assay performed (data not shown) and the corresponding IC_{50} values are resumed in table **2**. LAE was the extract that consistently demonstrated the most promising antioxidant potential obtaining lower IC_{50} values than SAE for almost all the antioxidant experiments, which is likely related to its higher content of total phenolics (649.2 mg/g of extract) compared to SAE (536.4 mg/g of extract). The only exception was observed in the ORAC assay in which no significant differences between LAE and SAE activities

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were observed (1.8±0.1 and 1.3±0.0 μM TE/mg sample, respectively). Interestingly, the IC_{50} values obtained for LAE were also close to those of ascorbic acid in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, and even lower than that of mannitol in $OH^{\bullet}$ scavenging, thus indicating a very strong scavenging capacity for this extract. Despite less effective than LAE, SAE has also revealed better activity on $OH^{\bullet}$ assay compared to mannitol, and relatively low IC_{50} values for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, also suggesting a good scavenging activity. Although the scavenging effects on $OH^{\bullet}$ was not reported in *G. robertianum* L. before, previous works have already reported $DPPH^{\bullet}$ scavenging activities of the aerial parts (mixtures of stems and leaves) of this plant. In their study, Graça et al.[19] performed this assay for several extracts of aerial parts of *G. robertianum* L. (aqueous, methanol, acetone, ethyl acetate, dichloromethane and *n*-hexane), revealing EC_{50} ranging from 54±1 to 1304±1 μg/mL, with the lowest value corresponding to the acetone extract. Other studies have reported lower values for aqueous extracts (EC_{50} of 6.53±0.58 μg/mL) and methanol extracts (IC_{50} of 14.93 μg/mL), which are in accordance to the results herein obtained.[17,49] Moreover, Neagu et al.[50] also reported good antioxidant activities for *G. Robertianum* L. on $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, although the extracts prepared in their study have undergone through a purification step first.

In contrast, regardless the good activity demonstrated on FRAP and TBARS, the IC_{50} values observed for the LAE and SAE are not as close to the standard compounds as in the previous assays. These same assays were performed for decoctions of *G. robertianum* L. aerial parts in Graça et al.[19] study revealing identical results for FRAP (61±3 μg/mL), but better values for TBARS (7.3±0.2 μg/mL). Moreover, Jemia et al.,[13] revealed an IC_{50} of 20±4.5 μg/mL for leaves methanol extracts corresponding to twice stronger activity than that of ascorbic acid used as reference in their work (40±1.31 μg/mL). This result suggest that methanol renders an extract with a stronger reducing power compared with the aqueous extract from leaves herein tested.

Despite LAE is higher in phenolic compounds, the results obtained regarding to the ORAC assay revealed that both extracts were equally effective inhibitors of the oxidative degradation of fluorescein, suggesting that these extracts might contain other compounds that are contributing for the antioxidant activity observed in this assay.

It is noteworthy that either ellagic acid or brevifolin carboxylic acid, i.e., the two major phenolic constituents of both LAE and SAE have been described several times for their promising antioxidant potential,[51,52] suggesting that they might be the main

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protagonists for the results observed. Not less important is the presence of other tannins, chlorogenic acid and gallic acids, which have also been proven to exert strong antiradical activities,[53–55] and therefore they are most likely to be contributing together for the overall antioxidant activity herein evidenced for *G. robertianum* L. extracts. Besides, several clinical studies have shown strong evidence that the presence of ellagitannins are intimately associated to the biological activities of ellagitannin-rich foods such as pomegranate juice,[56–58] therefore, the abundance of such compounds in *G. robertianum* is most likely one of the main reasons for the bioactivities herein described and other claimed health benefits reported for this species.

2.3. Evaluation of anti-inflammatory activities of G. robertianum aqueous extracts

2.3.1. Inhibition of soybean 5-Lipoxygenase activity and scavenging of chemical generated NO•

To evaluate the anti-inflammatory potential of the samples, *G. robertianum* L. aqueous extracts were firstly tested for their ability to inhibit the activity of 5-LOX and to scavenge the chemically-generated NO•. The former is an enzyme that catalyzes the oxidation of the arachidonic acid into leukotrienes, i.e., a group of chemical mediators closely related to inflammatory events, while the latter is an extremely important free radical released during inflammation as well, serving numerous signaling purposes.[59,60] Therefore, the ability to inhibit this enzyme and/or scavenge this radical is indicative of the anti-inflammatory potential of the samples in study.

As can be observed in figure **2A**, both *G. robertianum* L. extracts revealed no inhibitory effects towards 5-LOX activity up to 60 µg/mL. In fact, other species of *Geranium* have also been reported as weak inhibitors of this enzyme. This is the case of *Geranium wallichianum* D. Don ex Sweet from which methanol extracts of its rhizomes only reduced 5-LOX activity in 26.9%.[61]

The particularity of NO• is that in addition to its pro-inflammatory function, this is also a free radical. Consequently, as both *G. robertianum* L. extracts showed strong antiradical activities, promising effects against this radical could also be expected. Indeed, the IC_{50} values obtained for LAE and SAE (20.0±0.9 and 24.2±8.0 µg/mL) indicate that their activity against NO• is 10 times stronger than that of ascorbic acid which was used as standard compound (285.7±15.4 µg/mL). Furthermore, despite the difference between LAE and SAE was not found statistically significant, LAE displayed

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tendentiously better activity than SAE, which could be possibly related to its higher content in phenolic compounds.

Since both extracts demonstrated good potential at this level, further experiments were performed with biological systems in order confirm their anti-inflammatory properties.

2.3.2. Effects on the cell viability

The cytotoxicity evaluation of both extracts is an important step prior to their possible assessment for pharmacological/nutritional purposes. Therefore, in a first approach we evaluated their cytotoxicity towards macrophages and hepatocytes in order to select concentrations without cytotoxicity. The *in vitro* cytotoxic effect of *G. robertianum* L. aqueous extracts on macrophages RAW 264.7 are shown in figure **3**. Viability of the cells was not significantly altered with the treatment of the extracts in almost every concentration tested. An exception was noticed for the LAE at 100 µg/mL in LPS-stimulated cells. This suggests that the presence of LAE at 100 µg/mL does not induce toxicity for itself, but instead, it might potentiate the toxic effect that LPS naturally has on these cells.

As to HepG-2 cells, no cytotoxic effects were seen for any of the concentrations tested (figure **4**). Based on these data, further assays were performed using only non-toxic concentrations.

2.3.3. Scavenging of nitric oxide ($NO^{\bullet}$) and effects on iNOS and COX-2 expression

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$NO^{\bullet}$ is synthesized from L-arginine by inducible nitric oxide synthase (iNOS) expressed in numerous mammalian cells, such as macrophages, and large amounts of $NO^{\bullet}$ have been found in several inflammatory-related diseases, namely atherosclerosis, obesity, diabetes and neurodegenerative diseases.[62] For this reason, $NO^{\bullet}$ is a well-established marker of inflammation and inhibition of its production upon activation with an inflammatory stimulus, such as LPS, might be a useful strategy to disclose new anti-inflammatory compounds. Therefore, the effect of the extracts on $NO^{\bullet}$ production was analyzed by measuring the accumulation of nitrites in the culture medium of cells stimulated with the *Toll-like* receptor 4 agonist, LPS. In figure **5** it is observable that, under normal conditions, macrophages produce low nitrite levels (approximately 0.6±0.2 μM). Upon stimulation of these cells with LPS over 24 h, the production of nitrites increased about 20 fold the resting conditions, reaching to 24.2±0.5 μM.

Nitrite production by Raw 264.7 macrophages were evaluated using non-toxic concentrations of LAE (figure **5A**) and SAE (figure **5B**). Based on the results obtained for the scavenging ability of the chemically-generated $NO^{\bullet}$ scavenging, it was expected that the pre-treatment of cells with *G. robertianum* L. extracts would inhibit nitrite production from LPS-stimulated macrophages. Indeed, it was possible to notice that the nitrite production on the macrophages co-incubated with the target *G. robertianum* L. extracts was tendentiously decreased in a dose-dependent manner. Yet, only the SAE at 100 μg/mL has shown a statistically relevant inhibition of the LPS-induced nitrite release (18.7±1.1 μM). Few authors have reported the effects of *Geranium* on $NO^{\bullet}$ release. Choi et al.[63] have described that the hydroalcoholic extracts of *G. thunbergii* Siebold ex Lindl. & Paxton significantly inhibited the iNOS-dependent release of $NO^{\bullet}$ in LPS-stimulated Raw 264.7. However, Kim et al.[64] reported later that the hydroalcoholic extracts of the same species failed to inhibit $NO^{\bullet}$ release in the same cellular model.

Therefore, in an attempt to deeply explore the possible molecular mechanisms behind the decrement of nitrite levels released by Raw 264.7 macrophages cultured in the presence of SAE, further analysis through Western blot was carried out in order to disclose whether SAE (100 μg/mL) could block the LPS-induced iNOS protein expression. Note that the expression of iNOS is tightly associated with the $NO^{\bullet}$ released by macrophages upon a pro-inflammatory stimulus since this is the enzyme that catalyzes the conversion of arginine into citrulline which results in the production of this radical.[65]

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As observable on figure **6**, under normal conditions the murine macrophages do not express iNOS. In turn, when they are stimulated with LPS, the expression of iNOS is triggered, indicating that cells have entered in an inflammatory state. The addition of the SAE prior to the LPS stimulation was expected to inhibit the intracellular signaling pathways involved in iNOS expression. However, though strong inhibitory activity over iNOS has been previously described for ethanol extracts of other *Geranium* species, namely *G. sibiricum* L.,[66] this was not verified for *G. robertianum* L. stem aqueous extract, as the pre-treatment of murine macrophages with 100 μg/mL of SAE did not decrease the protein levels of iNOS triggered by LPS (figure **6A**) .

The fact that SAE decreased the $NO^{\bullet}$ levels but did not show any activity at an enzymatic level strongly suggests that this extract exerts its anti-inflammatory activity through its capacity to scavenge the target radicals.

3. Conclusion

The phenolic profile of the aqueous extracts of *G. robertianum* L. revealed a predominance of ellagic acid, brevifolin carboxylic acid and several other hydrolysable tannins. Both extracts revealed potent antioxidant activities, though LAE consistently showed the lowest IC_{50} values, which should be related to its higher content of total phenolic compounds compared to SAE. Nevertheless, since identical results were observed for ORAC, it is possible that these extracts contain other compounds that could be contributing for their antioxidant activity. Despite strong scavenging activity was observed for LAE and SAE extracts over the chemically generated $NO^{\bullet}$, only SAE at 100 μg/mL exhibited an effective inhibition of this radical produced by LPS-stimulated RAW 264.7 cells. The fact that, at this concentration, SAE was unable to modulate 5-LOX activity or iNOS expression suggests that its anti-inflammatory activity might be partially related to its scavenging effects against $NO^{\bullet}$. Our results also showed that only the highest concentration of LAE exhibited cytotoxic effects on Raw 264.7 cells, while no toxicity were detected for the HepG-2 for any of the concentrations tested.

Overall, this study allowed to disclose valuable data about the phenolic profile of *G. robertianum* L. aqueous extracts as well as partially corroborate the bioactivities claimed for this plant, in particular antioxidant and anti-inflammatory activities, opening new avenues for their further exploitation as active ingredients in nutraceutical and biomedical fields. Yet further studies are required to clarify the molecular

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mechanisms underlying the anti-inflammatory activity, as well as to identify the main contributors for their bioactivity. Moreover, despite the biotransformation and bioavailability of ellagitannins and their physiological effects are fairly well studied,[56] it is still necessary to perform further experiments, namely in more complex organisms *in vivo,* for a better understanding of the contribution of these compounds for *G. robertianum* bioactivities and to obtain a more reliable approach of the real physiological effects of these extracts.

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Conflits of interest

There are no conflicts of interest to declare

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Tables

Table 1 – Identification of LC-DAD-ESI/MS^n data of the most relevant fractions from the extracts of *G. robertianum*

Peak	RT (min)	$\lambda_{máx}$	MW	ESI-MS (*m/z*)	ESI-MS^n fragments[a]	mg/g extract		Compound	Ref
						Leaves	Stems		
1	3.14	213	192	191 [M-H]$^-$	MS^2[191]: **173**, 127, 111, 85, 93, 171, 109	<LOQ	<LOQ	Quinic acid[c]	67
2	4.79	216, 277	332	331 [M-H]$^-$	MS^2[331]: **169**, 193, 271, 211, 313, 125	<LOQ	<LOQ	Galloyl-hexoside[c]	68
3	6.03	214, 271	170	169 [M-H]$^-$	MS^2[169]: **125**	10.7±0.9[a]	10.9±0.7[a]	Gallic acid[b]	
4	6.71	215, 274	344	343 [M-H]$^-$	MS^2[343]: **191**, 169	<LOQ	<LOQ	Galloylquinic acid[c]	68
5	14.97	274	484	483 [M-H]$^-$	MS^2[483]: **313**, 331, 465, 439, 169	9.0±0.6	<LOQ	Digalloyl hexose[c]	69
6	16.72	236, 324	354	353 [M-H]$^-$	MS^2[353]: **191**, 179, 135	14.9±0.0[a]	19.6±0.3[b]	CQA[b]	
7	27.65	231, 280	952	951 [M-H]$^-$	MS^2[951]: **907**, 933, 799, 627, 301, 781; MS^3[907]: **301**, 431, 169, 393, 435	11.1±0.2	<LOD	Tris-galloyl-HHDP-hexose[c]	70
8	28.46	277, 355	292	291 [M-H]$^-$	MS^2[291]: **247**	153.4±0.3[a]	177.7±0.05[b]	Brevifolin carboxylic acid[d]	71
9	28.73	275	952	951 [M-H]$^-$	MS^2[951]: **933;** MS^3[933]: **301**, 431, 169, 393, 435	18.2±0.5	<LOD	Geraniin isomer[c]	40
			626	625 [M-H]$^-$	MS^2[625]: **463**	CO	<LOD	Ellagic acid-dihexoside	72
10	29.24	273	952	951 [M-H]$^-$	MS^2[951]: **933**, 301	11.5±0.1	<LOD	Geraniin isomer[c]	40
11	29.74	274	952	951 [M-H]$^-$	MS^2[951]: **933**	8.8±1.8	<LOD	Geraniin isomer[c]	40
			970	969 [M-H]$^-$	MS^2[969]: **925**, 633, 755, 881	CO	<LOD	Phyllanthusiin B	71
12	30.39	275	952	951 [M-H]$^-$	MS^2[951]: **933**, 301	10.2±0.6	<LOD	Geraniin isomer[c]	40

			970	969 [M-H]$^-$	MS^2[969]: **925**, 633, 755, 881; MS^3[925]: **301**, 463, 275, 437, 589	CO	<LOQ	Phyllanthusiin B	71
13	31.33	274	970	969 [M-H]$^-$	MS^2[969]: **633**, 247, 335; MS^3[633]: **301**, 463, 275, 419, 331	18.9±0.1 [a]	13.0±0.4 [b]	Repandusidic acid A[c]	40
14	31.66	269	634	633 [M-H]$^-$	MS^2[633]: **301**, 463, 275, 615, 419	40.7±0.3 [a]	57.1±0.5[b]	Corilagin[c]	70
15	35.34	229, 278	926	925 [M-H]$^-$	MS^2[925]: **301**, 605, 435, 907	14.3±0.0 [a]	12.2±1.1 [a]	Phyllanthusiin C[c]	71
16	36.04	226, 275	970	969 [M-H]$^-$	MS^2[969]: **925**, 755, 881, 633 MS^3[925]: **301**, 463, 275, 437, 589	19.5±0.3 [a]	21.8±0.9 [a]	Phyllanthusiin B[c]	40
17	40.91	239, 274	952	951 [M-H]$^-$	MS^2[951]: **907**, 933; MS^3[907]: **301**, 431, 169, 393, 435	7.4±0.0 [a]	6.9±0.3 [a]	Tris-galloyl-HHDP-hexose[c]	70
18	42.82	274, 357	610	609 [M-H]$^-$	MS^2[609]: **301**, 300, 445, 489, 463, 271, 255, 179	26.0±0.2 [a]	32.6±0.3[b]	Ellagic acid-(*p*-coumaroyl) hexose[d]	44
19	43.14	253, 360	434	433 [M-H]$^-$	MS^2[433]: **301**, 300	25.0±0.0 [a]	28.1±7.7 [a]	Ellagic acid pentoside[d]	45
20	44.92	253	302	301 [M-H]$^-$	MS^2[301]: **229**	249.6±0.5 [a]	156.5±0.9[b]	Ellagic acid[b]	
21	46.37	256, 295, 356	610	609 [M-H]$^-$	MS^2[609]: **301**	<LOQ	<LOQ	Rutin	73

CO – Co-eluted, <LOQ – not quantified, <LOD – not detected, [a] Fragments are arranged in descending order of relative abundance and the bold values correspond to the most abundant fragments. Underlined fragments correspond to double-charged fragments, [b] Identified using corresponding authentic standards, [c] Expressed in equivalents of gallic acid, [d] Expressed in equivalents of ellagic acid. Different letters in the same row indicate significant differences ($P<0.05$) according to the two-sided unpaired t-test.

Table 2 – Antioxidant potential of *G. robertianum* aqueous extracts, as evaluated in distinct assays.

Samples	DPPH$^{\bullet}$ (IC_{50} µg/mL)	ABTS$^{\bullet+}$ (IC_{50} µg/mL)	OH$^{\bullet}$ (IC_{50} µg/mL)	FRAP (IC_{50} µg/mL)	TBARS (IC_{50} µg/mL)	ORAC (µM TE/mg sample)
LAE	7.6±0.6	3.9±0.6	45.1±2.4**	63.3±5.4***	115.8±16.1	1.8±0.1
SAE	17.3±0.3***	5.8±0.5**	59.8±8.4**	93.5±5.5 ***	210.4±38.6***	1.3±0.0
Standard	4.8±0.3^{a}	1.3±0.2 ^{a}	196.2±16.4 ^{b}	20.0±0.2 ^{c}	41.1±5.2 ^{d}	-

Data represent mean ± SEM of three independent assays performed in triplicate (N=3). The standard compounds used were ^{a}ascorbic acid, ^{b}mannitol, ^{c}BHT and ^{d}trolox. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***P<0.001; **P<0.01, compared to the STD

Figures

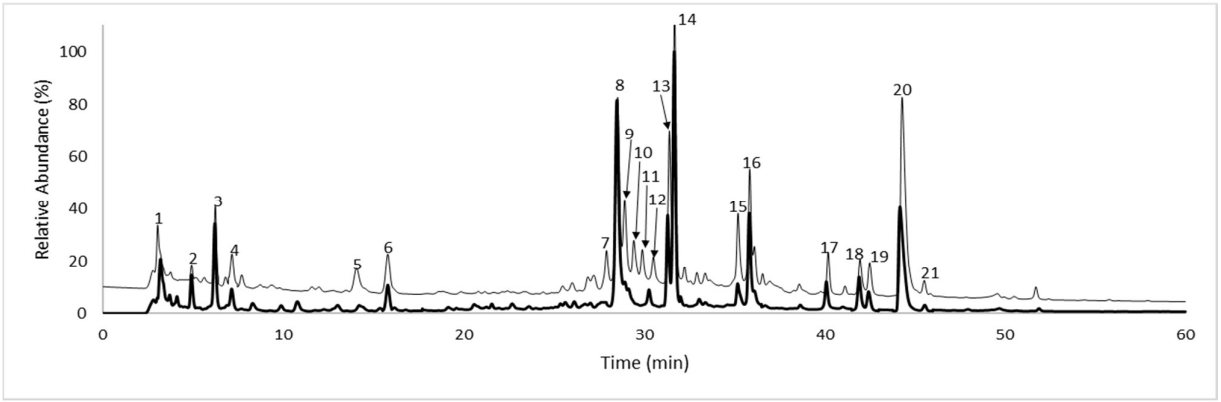

Figure 1 – Chromatographic profile of *G. robertianum* aqueous extracts at 280 nm. Chromatogram corresponding to stems extract is represented in bold lines, while the thin lines represent the chromatogram corresponding to the leaves extract. Numbers in figure correspond to the identified compounds, as represented in Table 2.

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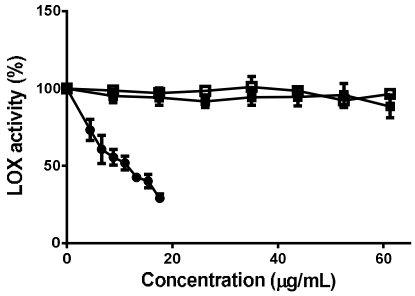

Figure 2 – Inhibition of 5-LOX in presence of different concentrations of LAE (■), SAE (□) and ascorbic acid (●).

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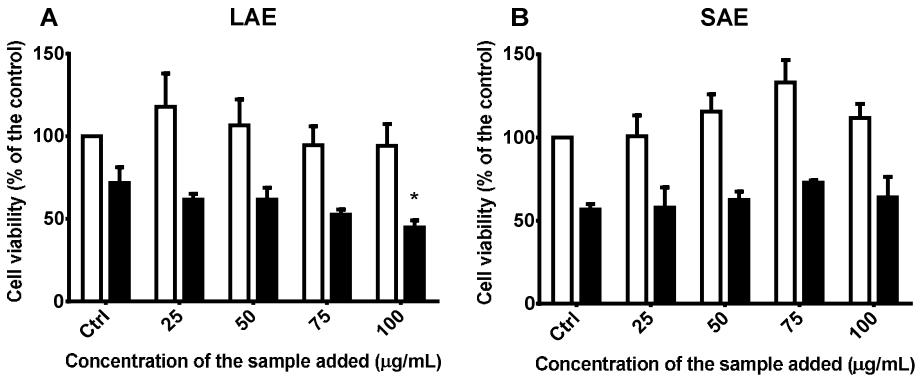

Figure 3 – Effects of the pre-treatment with LAE (A) and SAE (B) (25, 50, 75 and 100 µg/mL) on the cell viability (% of the control) of RAW 264.7 cells after 24h incubation with (■) or without (□) LPS. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. *P<0.05, compared to the control with LPS. Data represent mean ± SEM of 3 independent assays.

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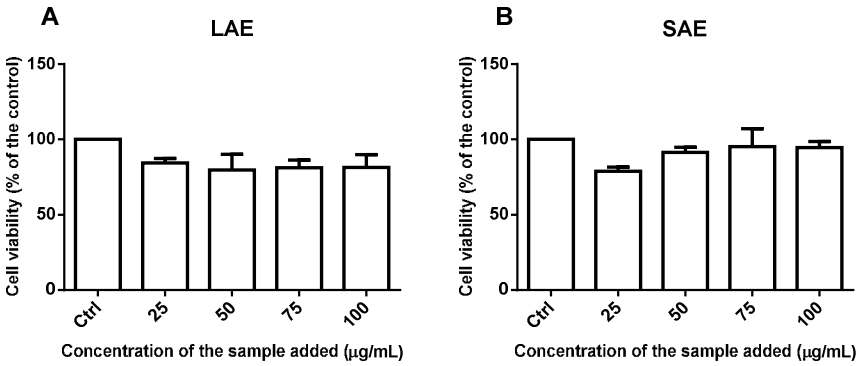

Figure 4 – Cell viability of HepG-2 cells treated with LAE (A), SAE (B) (25, 50, 75, and 100 µg/mL). Cell viability was assessed using the MTT assay and the results were expressed as percentage of control cells (Ctrl). Each value represents the mean ± SEM of at least 3 independent experiments.

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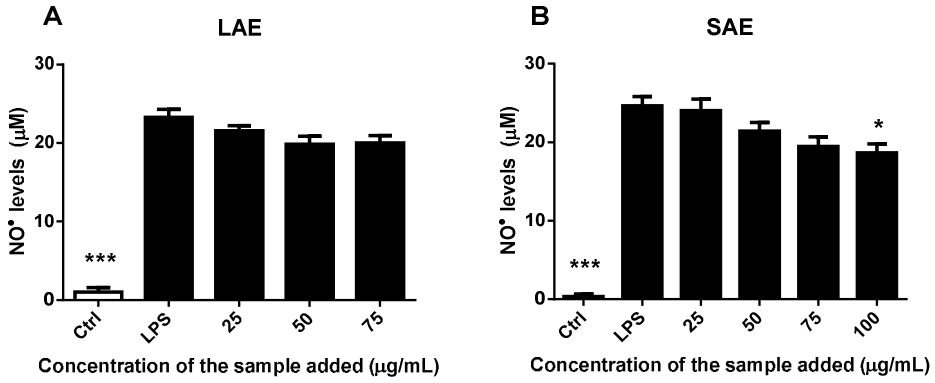

Figure 5 – Effects of the pre-treatment LAE (A) (25, 50 and 75 µg/mL) and SAE (B) (25, 50, 75 and 100 µg/mL) on the NO• levels (µM) on Raw 264.7 cells after 24h of incubation with (■) or without (□) LPS. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. *P<0.05; **P<0.01; ***P<0.001, compared to the control with LPS. Data represent mean ± SEM of 3 independent assays.

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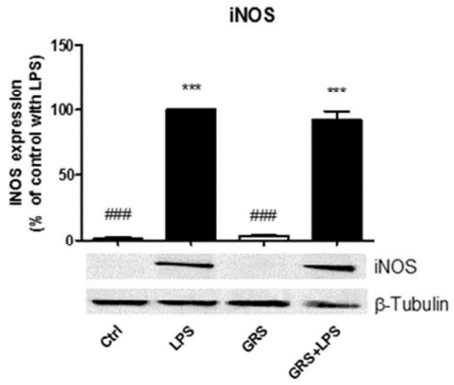

Figure 6 – Effect of the SAE extract (100µg/L) pre-treatment in the expression of iNOS enzyme (% of the LPS) in Raw 264.7 macrophages after 24h of incubation with (■) or without (□) LPS. An anti-β-tubulin antibody was used to confirm equal protein loading and normalize the data. Statistical analysis was performed by one-way ANOVA, followed by Tukey's post-hoc test. ***P<0.001, compared to the control without LPS; $^{###}$P<0.001, compared to the control with LPS. The blot is representative of 3 similar blots. iNOS=135 kDa