

Dehydration process influences the phenolic profile, antioxidant and antimicrobial properties of *Galium aparine* L.



Sylwia Senio^a, Carla Pereira^a, Josiana Vaz^a, Marina Sokovic^b, Lillian Barros^{a,*}, Isabel C.F.R. Ferreira^{a,*}

^a Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253, Bragança, Portugal

^b University of Belgrade, Department of Plant Physiology, Institute for Biological Research “Siniša Stanković”, Bulevar Despota Stefana 142, 11000, Belgrade, Serbia

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ABSTRACT

Galium aparine L. is a very disseminated plant in temperate zones, commonly known as clivers or bedstraw, belonging to the Rubiaceae family and it is traditionally used for its medicinal applications. In this study, *G. aparine* hydromethanolic extracts and infusions were prepared from air-dried and freeze-dried samples in order to assess their phenolic profile, antioxidant, antimicrobial, and cytotoxic properties. All the studied extracts revealed a similar phenolic profile, but the hydromethanolic extract obtained from the freeze-dried sample presented the highest concentration of phenolic compounds, followed by the respective infusion and the air-dried sample hydromethanolic extract. The major compound detected in the extracts was 5-*O*-caffeoylquinic acid (from 145 to 163 mg/g extract). Regarding the bioactivity, in general, the extracts presenting higher phenolic concentrations also revealed enhanced bioactive properties. The EC₅₀ values obtained in the antioxidant activity assays ranged from 13.5 to 884 µg/mL, with the freeze-dried sample hydromethanolic extract presenting the highest activity (13.5–555 µg/mL). Similar conclusions could be made in terms of antimicrobial properties, with this extract showing the lowest MIC (1.85–15 mg/mL), MBC (3.75–7.5 mg/mL), and MFC (3.75–20 mg/mL) values. None of the extracts revealed cytotoxicity. The results obtained in this study suggested that *G. aparine* extracts can be a good source of phenolic compounds with antioxidant and antimicrobial properties.

1. Introduction

There are 350,000 plant species in the world, among which about 80,000 edible and yet, it is estimated that only about 150 species are cultivated, directly for human consumption or as a feed for animals (Füleky, 2009). Numerous plant species that could provide excellent sources of foodstuff as part of a balanced diet, pharmaceutical products, insecticides, food additives such as colorants and flavourings, or even a raw ingredient for the preparation of beverages, remain underutilized (Haq, 1993).

It is well-known that polyphenol-rich foods and beverages may increase plasma antioxidant capacity, and plants are considered rich sources of these secondary metabolites, with a variety of more than 8000 such compounds identified from various plant species (Pandey and Rizvi, 2009). Moreover, epidemiological data indicates that the long term consumption of diets rich in plant polyphenols offers a protection against the development of cancers, diabetes, osteoporosis, cardiovascular and neurodegenerative diseases (Pandey and Rizvi, 2009; Young and Woodside, 2001). The ability of these natural

antioxidants to delay oxidation processes in foodstuffs and biological membranes has recently focused much attention (Lindsey et al., 2002; Vlase et al., 2014), which has led to an increased antioxidant assessment of many medicinal and food plant species (Abbasi et al., 2015). In this matter, the traditional knowledge plays an essential role on the identification of plants that may be useful, however, in many instances most of this knowledge survives in the people's memory and is now in danger of disappearing (Fabricant and Farnsworth, 2001; Fennell et al., 2004; Jyotsna and Katewa, 2016).

Galium aparine L. (Rubiaceae), commonly known as clivers or bedstraw is a common weed in temperate zones on all continents and in Europe, it occurs from Portugal in the west to Russia in the east, and from the UK in the north to Italy in the south (CABI, 2018). It is an unwanted and troublesome plant species in cereal, rapeseed and sugar beet fields where it is noted for its detrimental impact on the potential yields (Malik and Born, 1988). Traditionally, *G. aparine* has enjoyed a large number of medicinal applications for diverse health conditions. Briefly, the whole herb (stem, leaf, flower and seed) has been commonly used as cooling diuretic in fevers and for urinary tract infections,

* Corresponding authors.

E-mail addresses: lillian@ipb.pt (L. Barros), iferreira@ipb.pt (I.C.F.R. Ferreira).

in skin diseases such as eczema or psoriasis, ulcers, chronic sores, as a blood purifier i.e., to increase lymphatic flow, to reduce swellings, infection and inflammation, or to stop bleeding from wounds (Tobyn et al., 2016).

Beyond these applications, in Sweden, roasted seeds of *G. aparine* were used as a coffee substitute (Malik and Born, 1988) and in Turkey, young shoots of *G. aparine* are also eaten roasted (Taskin and Bitis, 2016) and used to coagulate milk, being known as “yogurt herb” (Aslantürk et al., 2017; Deliorman et al., 2001). Although the herb has long history of its use in phytotherapy, and the contemporary time’s herbalists continue to use it as a diuretic, the pharmacological evidence supporting this efficiency is scarce. According to some reports, this plant is a source of polyphenols (Moubasher et al., 2016; Vlase et al., 2014), phytosterols (Mocan et al., 2016), alkaloids, anthraquinones, saponins (Aslantürk et al., 2017), sesquiterpenoids, squalene, aromatic compounds, higher alkanes (and derivatives), fatty acids, chlorophylls, carotenoids and iridoids (Deliorman et al., 2001; Goryacha et al., 2014).

In order to establish scientific rationale for the use of *G. aparine*, the first aim of this study was to investigate the phenolic profile, antioxidant, antimicrobial and cytotoxic properties of hydromethanolic extracts and infusions prepared from this plant species. Furthermore, since medicinal plants are often dried and sold as semi- and processed products, in this study, a freshly harvested botanical material was prepared by air-drying and freeze-drying with the objective of examining the effect of the drying method on the phenolic composition and bioactive properties of *G. aparine*.

2. Material and methods

2.1. Samples and samples preparation

Galium aparine L. (Rubiaceae) was collected at various growth stages in order to prepare a homogeneous sample, in June 2017, from the campus of the Polytechnic Institute of Bragança, Portugal. The collected plant material was authenticated by Professor of Botany Carlos Aguiar and a specimen voucher was deposited in the herbarium of the School of Agriculture, Polytechnic Institute of Bragança (Portugal).

The vegetal material (leaves, stems, flowers and seeds) was subjected to two drying methods, i.e., air-drying (one week, at room temperature, in the dark) and freeze-drying (lyophilisation; FeeZone 4.5, Labconco, Kansas City, MO, USA). The dried samples were reduced to fine and homogeneous powder (~20 mesh) and stored at room temperature, protected from direct light, for further analysis.

2.2. Extracts preparation

For each sample, infusions and hydromethanolic extracts were prepared in order to compare the phenolic composition and bioactive properties of extracts obtained from different procedures, using thermal and nonthermal extractions, and solvents with different polarities, once the extracted compounds are dependent of these conditions. Regarding infusions, air-dried and freeze-dried samples (1 g) were infused with freshly boiled distilled water (100 mL), left aside for 5 min and subsequently filtered through filter paper. The resulting extracts were then freeze-dried and a yield of 21.9 and 27.4% was obtained for air-dried and freeze-dried samples, respectively.

For the preparation of the hydromethanolic extracts, the samples (1 g) were extracted by stirring (150 rpm) with 30 mL of methanol:water (80:20, v/v) at room temperature for 1 h, and subsequently filtered through filter paper. The residue was then extracted with an additional 30 mL portion of methanol:water (80:20, v/v) and filtered. Methanol was evaporated at 40 °C under reduced pressure (Rotavac Valve Tec, Germany) and the remaining supernatant was freeze-dried. The extraction yield was of 23.9 and 22.7% for air-dried and freeze-dried samples, respectively.

2.3. Phenolic compounds

The phenolic profile was determined by LC-DAD-ESI/MSn (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA). The lyophilized infusions and hydromethanolic extracts were re-dissolved in water and methanol/water mixture (80:20, v/v), respectively, at a concentration of 5 mg/mL. Double online detection was used using a DAD (280, 330, and 370 nm as preferred wavelengths) and in a mass spectrometer in negative mode, equipped with an ESI source (Linear Ion Trap LTQ XL mass spectrometer, Thermo Finnigan, San Jose, CA, USA), as previously described by Bessada et al. (2016).

The identification of the phenolic compounds was performed using standard compounds, when available, by comparing their retention times, UV-vis and mass spectra; and also comparing the obtained information with available data reported in the literature, giving a tentative identification, when no standards were available. For quantitative analysis, calibration curves (5–100 µg/mL) for each available phenolic standard (5-*O*-caffeoylquinic acid ≥ 99%, *p*-coumaric acid ≥ 90%, ferulic acid ≥ 90%, and quercetin-3-*O*-rutinoside ≥ 99% HPLC purity, Extrasynthèse, Genay, France) were constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of the most similar available standard (Table 1). The results were expressed as mg/g of extract.

2.4. Antioxidant activity

For the antioxidant activity assessment, the lyophilized infusions and hydromethanolic extracts were re-dissolved in water and methanol/water mixture (80:20, v/v), respectively, at a final concentration of 10 mg/mL. These stock solutions were further diluted to perform the below described assays.

2.4.1. DPPH radical-scavenging activity assay

DPPH radical-scavenging activity was assessed using BioTek ELX800 microplate Reader (Bio-Tek Instruments, Inc.; Winooski, USA). The reaction mixture in each of the 96 wells consisted of the infusions and hydromethanolic extracts at different concentrations (30 µL) and methanolic solution (270 µL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 60 min in the dark and at room temperature. The absorbance was measured at 515 nm to assess the reduction of DPPH radicals, which was calculated as a percentage of DPPH discoloration using the formula: $[(A_{\text{DPPH}} - A_{\text{S}})/A_{\text{DPPH}}] \times 100$, where A_{S} is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution (Rita et al., 2016).

2.4.2. Reducing power

In order to assess the capacity to convert Fe^{3+} into Fe^{2+} , samples solutions at different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The resulting mixtures were incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The supernatant (0.8 mL) was then poured in a 46-well microplate along with deionised water (0.8 mL) and ferric chloride (0.1% w/v). The reducing power was assessed through the absorbance at 690 nm, using the microplate reader mentioned above (Rita et al., 2016).

2.4.3. Inhibition of β -carotene bleaching assay

The β -carotene bleaching inhibition was assessed by measuring the capacity of the extracts and infusions to neutralise linoleate free radicals. A β -carotene solution was obtained by dissolving β -carotene (2 mg) in chloroform (10 mL), and evaporated (Rotavac Valve Tec, Germany) in a round bottom flask at 40 °C under reduced pressure. Linoleic acid (0.2 g), Tween 80 (2 g) and distilled water (0.5 L) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of the resulting emulsion were transferred into the test tubes containing

Table 1
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and tentative identification, and phenolic compounds quantification in *Galium aparine* L. infusions and hydromethanolic extracts (mean values \pm SD).

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion [M - H] ⁻ (m/z)	Main MS ² fragments (m/z)	Tentative identification	Freeze-dried		Air-dried		p-value (n = 36)	1-way ANOVA ^b
						Hydromethanolic extract (mg/g extract)	Infusion (mg/g extract)	Hydromethanolic extract (mg/g extract)	Infusion (mg/g extract)		
1	6.78	325	353	191(100),179(6),161(3),135(3)	5-O-Caffeoylquinic acid	163 \pm 2a	158 \pm 3b	157 \pm 2b	145 \pm 2c	0.792	< 0.001
2	8.11	320	353	191(100),179(5),161(3),135(3)	Caffeoylquinic acid	12.5 \pm 0.6a	10.5 \pm 0.4b	9.2 \pm 0.1c	5.2 \pm 0.2d	0.339	< 0.001
3	12.37	308	337	191(100),173(3),163(8),143(3)	5-O- Coumaroylquinic acid	2.10 \pm 0.09c	3.3 \pm 0.1a	2.22 \pm 0.07c	2.8 \pm 0.1b	0.795	< 0.001
4	13.03	323	367	193(8),191(100),173(3),135(2)	5-O-Feruloylquinic acid	3.53 \pm 0.05c	4.88 \pm 0.06b	5.37 \pm 0.04a	5.3 \pm 0.2a	0.220	< 0.001
5	16.32	276,310	519	357(44),339(100),327(45)	Phenylcoumaran hexoside	5.51 \pm 0.04a	4.6 \pm 0.1b	4.31 \pm 0.07c	4.04 \pm 0.08d	0.618	< 0.001
6	17.31	354	609	301(100)	Quercetin-3-O- rutinoside	4.6 \pm 0.1a	1.84 \pm 0.04c	3.07 \pm 0.06b	1.48 \pm 0.01d	0.280	< 0.001
TPC						191 \pm 3a	183 \pm 3b	181 \pm 2b	164 \pm 2c	0.840	< 0.001

nd – not detected.

For each different sample, different letters within a line means they differ significantly ($p < 0.05$).

Phenolic compound used for quantification: compounds 1 and 2-5-O-caffeoylquinic acid ($y = 161172 + 168823x$; $r^2 = 0.999$); compounds 3 and 5-*p*-coumaric acid ($y = 6966.7 + 301950x$; $r^2 = 0.999$); compound 4 – ferulic acid ($y = -185462 + 633126x$; $r^2 = 0.999$); and compound 6 – quercetin-3-O-rutinoside ($y = -76751 + 13343x$; $r^2 = 0.999$). TPC- total phenolic compounds.

^a Homoscedasticity among the different drying process and extraction method was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.

^b $p < 0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed).

different concentrations of infusions and hydromethanolic extracts, vortexed and the absorbance (T0) was measured at 470 nm. After 2 h of incubation at 50 °C in a water bath with agitation (100 rpm), the absorbance (T1) was measured again. β -carotene bleaching inhibition was calculated using the equation: Absorbance (T1) – Absorbance (T0) \times 100 (Rita et al., 2016).

2.4.4. Lipid peroxidation inhibition by thiobarbituric acid reactive substance (TBARS) assay

The porcine (*Sus scrofa*) brain tissue was obtained from local slaughter house, dissected and homogenized with cold Tris-HCl (20 mM, pH 7.4) buffer solution. The brain tissue homogenate was produced in ratio 1:2 w/v and centrifuged at 3000g for 10 min. An aliquot (100 μ L) of the supernatant was incubated with the sample solutions at different concentrations in the presence of FeSO₄ (10 mM, 100 μ L) and ascorbic acid (0.1 mM, 100 μ L) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 μ L) and a solution of thiobarbituric acid (TBA, 2% w/v, 380 μ L) was added. The samples were incubated at 80 °C for 20 min and centrifuged (3000g, 5 min) in order to remove precipitated proteins. The absorbance of the pink malondialdehyde (MDA)–TBA complex in the supernatant was measured at 532 nm, and the inhibition ratio (%) was determined using the equation: Inhibition ratio (%) = [(A–B) \times 100] where A is the absorbance of the control sample, and B is the absorbance of the sample solution (Svobodova et al., 2017).

2.5. Cytotoxicity

For the cytotoxicity assessment, the lyophilized infusions and hydromethanolic extracts were re-dissolved to a final concentration of 1000 μ g/mL. The hepatotoxicity was assessed using a non-tumour liver primary culture established in our laboratory, PLP2, prepared from a freshly harvested porcine liver obtained from a local slaughter house. The cell line was plated at 1.0×10^4 cells/well in 96-well plates and sulforhodamine B assay was performed according to a procedure previously described by the authors (Guimarães et al., 2013). The results were expressed in GI₅₀ values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

2.6. Antimicrobial activity

For the antimicrobial activity assessment, the lyophilized infusions and hydromethanolic extracts were re-dissolved in 5% DMSO, at a final concentration of 30 mg/mL. These stock solutions were further diluted to perform the bellow described assays.

2.6.1. Antibacterial activity

Antibacterial activity was assessed against Gram-negative bacteria: *Escherichia coli* (ATCC 35210), *Salmonella typhimurium* (ATCC 13311), *Salmonella enteritidis* (ATCC 13076), *Enterobacter cloacae* (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973), following a previously described procedure (Soković et al., 2010). The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined according to the referred reference. Streptomycin and ampicillin were used as positive controls.

2.6.2. Antifungal activity

Antifungal activity was evaluated against *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC 6275), *Candida krusei* (IBRS 1flac1), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112), and *Penicillium verrucosum* var. *cyclopium* (food isolate), following the procedure previously described by the authors (Soković and Van Griensven, 2006). The minimum inhibitory (MIC) and

minimum fungicidal (MFC) concentrations were obtained following the same reference. Ketokonazole and bionazole were used as positive controls.

2.7. Statistical analysis

For each drying treatment (air-dried and freeze-dried), three independent samples were analysed and all assays were carried out in triplicate. Data were expressed as mean \pm standard deviation. A 5% significance level, using IBM SPSS Statistics for Windows, version 23.0. (IBM Corp., Armonk, NY, USA), was applied to all statistical tests. The fulfilment of the one-way ANOVA requirements, specifically the normal distribution of the residuals (data not shown) and the homogeneity of variance, was tested by means of the Shapiro Wilk's and the Levene's tests, respectively. Depending on the homoscedasticity, the dependent variables were compared using Tukey's honestly significant difference (HSD; when homoscedastic) or Tamhane's T2 multiple comparison (when no homoscedastic) tests.

3. Results and discussion

3.1. Phenolic compounds

Phenolic compounds identification was carried out considering their retention times, whenever possible in comparison with commercially available standards, and both UV and MS spectra. Data obtained by HPLC-DAD-ESI/MS analysis (retention time, λ_{max} , pseudomolecular ion, main fragment ions in MS²), phenolic compounds identification, and respective quantification are present in Table 1. Six compounds were found in *G. aparine* hydromethanolic extracts and infusions, among which four phenolic acids, a flavonol and a lignin. All the extracts revealed similar profiles, only differing in the detected compounds content. The freeze-dried sample hydromethanolic extract was the one revealing the highest total phenolic compounds concentration (191 mg/g extract; chromatogram shown in Fig. 1), followed by the respective infusion and the air-dried sample hydromethanolic extract that presented similar phenolic amounts (183 and 181 mg/g extract, respectively). The most abundant compound in all the assessed extracts was 5-*O*-caffeoylquinic acid, in concentrations of about 85–88% of the total phenolic compounds content. All the compounds detected were present in higher amounts in the freeze-dried sample hydromethanolic extract, with the exception of 5-*O*-coumaroylquinic acid that was most abundant in this sample infusion (3.3 mg/g extract), and 5-*O*-feruloylquinic acid that was mostly detected in both air-dried sample extracts (5.37 mg/g of hydromethanolic extract and 5.3 mg/g of infusion).

Compounds 1 (5-*O*-caffeoylquinic acid) and 6 (quercetin-3-*O*-rutinoside) were positively identified according to their retention time, mass, and UV-vis characteristics by comparison with commercial standards. These compounds were also previously detected in samples of *G. aparine* ethanolic extracts (Vlase et al., 2014). Compounds 2 (caffeoylquinic acid), 3 (5-*O*-coumaroylquinic acid), and 4 (5-*O*-feruloylquinic acid) were identified taking into account the recommended IUPAC numbering system (IUPAC, 1976) as also the hierarchical keys previously developed by Clifford et al. (2005, 2003). Compound 5, was tentatively identified as a lignin, taking into account the fragmentation patterns reported by Morreel et al. (2014), that also observed MS² fragments at *m/z* 519, followed by *m/z* 357 (–162 u) resulting from hexose loss, and *m/z* 339 (–18 u) and 327 (–30 u) that could be explained by water and formaldehyde losses, respectively. According to the authors, these type I fragmentations are typically observed in the spectrum of phenylcoumarans, which has led to the tentative identification of this compound as phenylcoumaran hexoside.

In a study performed by Vlase et al. (2014), *G. aparine* ethanolic extracts presented six phenolic acids (caftaric, gentisic, caffeic, chlorogenic, *p*-coumaric, and ferulic acids) and five flavonoids (hyperoside, isoquercitrin, rutin, quercetin, and luteolin), with rutin,

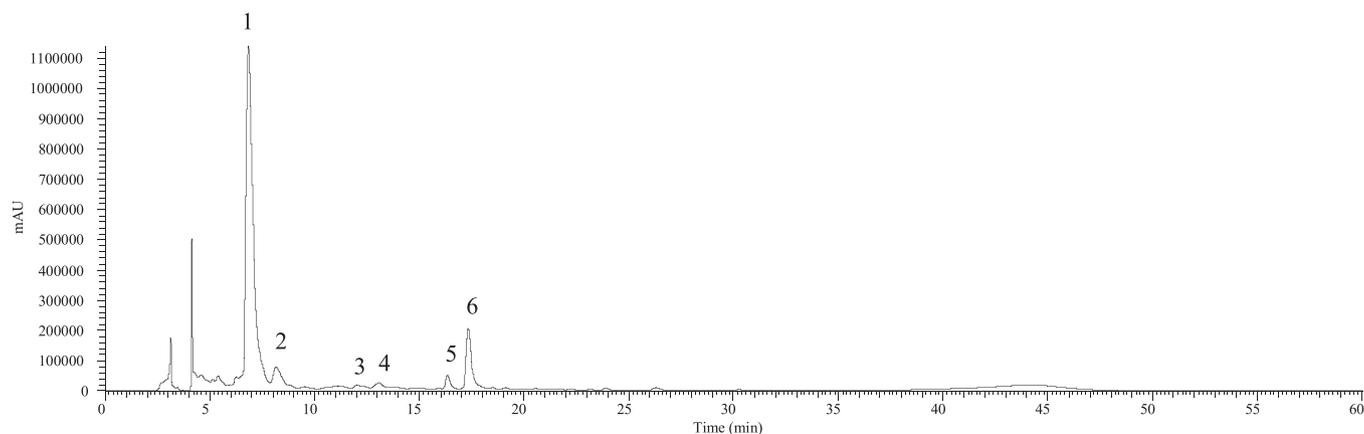


Fig. 1. Phenolic profile of *Galium aparine* L. freeze-dried sample hydromethanolic extract, recorded at 280 nm. Peak numbering is in accordance with Table 1.

quercetin, and ferulic acid (7.983, 5.679, and 3.793 mg/100 g dw, respectively) as the most abundant compounds. These authors also studied three other *Galium* species, namely *Galium verum*, *Galium mollugo*, and *Galium odoratum* and despite *G. aparine* presented the largest variety of phenolic compounds, *G. verum* was the species revealing the highest total phenolic concentration, followed by *G. mollugo*, *G. aparine*, and *G. odoratum* (894, 31, 21, and 17 mg/100 g dw, respectively). Other studies also evaluated *G. aparine* water extracts (Abbasi et al., 2015) and aqueous fractions (Bokhari et al., 2013) in terms of total phenolics and the water extracts contained 24 mg GAE/100 g fw, whereas the aqueous fractions revealed a slightly lower amount of 22.1 mg GAE/g of dried fraction.

3.2. Antioxidant activity and cytotoxicity

The antioxidant activity results of the *G. aparine* infusions and hydromethanolic extracts are presented in Table 2. In general, the freeze-dried sample hydromethanolic extract revealed lower EC₅₀ values (ranging from 13.5 to 555 µg/mL), indicating a stronger antioxidant activity, except for the DPPH scavenging activity assay, where the infusion obtained from the air-dried sample gave better results (EC₅₀ value of 467 µg/mL). This latest also revealed higher reducing power and β-carotene bleaching inhibition capacity (EC₅₀ values of 267.7 and 109 µg/mL, respectively) than the freeze-dried sample hydromethanolic extract and the air-dried sample infusion. Nevertheless, regarding the TBARS inhibition capacity, the air-dried sample hydromethanolic extract presented the best results (EC₅₀ value of 221 µg/mL), following the freeze-dried sample hydromethanolic extract. Comparing the results

obtained for the different extracts of *G. aparine* with those of trolox (positive control), the hydromethanolic extract of the freeze-dried sample was the only one revealing a lower EC₅₀ value (13.5 and 23 µg/mL for the extract and the positive control, respectively), in the TBARS inhibition assay. In a study performed with several *G. aparine* extract fractions, the butanol, methanol, and aqueous fractions revealed DPPH scavenging activity at 61.7, 75, and 58.3 µg/mL, respectively, which represent concentrations ten times lower than those obtained in the present study (Bokhari et al., 2013). On the other hand, in a study performed by Abbasi et al. (2015), water and acetone extracts of *G. aparine* showed approximately 69 and 55% DPPH scavenging activity, respectively, in concentrations of 100 mg/mL dw, but once again, it was not possible to compare these results to those achieved herein because of the methodology discrepancies along with the differences on the results analysis and presentation.

Regarding cytotoxicity evaluation (Table 2), none of the *G. aparine* extracts revealed toxicity in the assessed porcine liver cells primary culture, PLP2, at the studied concentration (1000 µg/mL), which support the use of this plant extracts without associated toxicity effects.

3.3. Antimicrobial activity

The results obtained in the screening of antimicrobial activity of *G. aparine* extracts against the studied bacteria and fungi are shown in Table 3. The antibacterial activity of the extracts was tested against eight bacterial strains: *B. cereus*, *M. flavus*, *S. aureus*, *L. monocytogenes*, *E. coli*, *E. cloacae*, *S. enteritidis*, and *S. typhimurium*. Generally, all the extracts revealed inhibitory and bactericidal capacity in concentrations

Table 2

Antioxidant activity and cytotoxicity of *Galium aparine* L. infusions and hydromethanolic extracts (mean values ± SD).

	Freeze-dried		Air-dried		p-value (n = 36)		Positive control
	Hydromethanolic extract	Infusion	Hydromethanolic extract	Infusion	Homoscedasticity ^a	1-way ANOVA ^b	
Antioxidant activity (EC ₅₀ values, µg/mL)							
DPPH scavenging activity	555 ± 4b	554 ± 11b	884 ± 12a	467 ± 3c	0.002	< 0.001	41 ± 1
Reducing power	175.2 ± 0.2d	327 ± 1b	365 ± 3a	267.7 ± 0.6c	0.001	< 0.001	41.7 ± 0.3
β-carotene bleaching inhibition	83 ± 1d	128 ± 4b	143 ± 8a	109 ± 2c	0.001	< 0.001	18 ± 1
TBARS inhibition	13.5 ± 0.8d	525 ± 2a	221 ± 3c	404 ± 6b	0.001	< 0.001	23 ± 1
Cytotoxicity (GI ₅₀ values, µg/mL)							
PLP2	> 1000	> 1000	> 1000	> 1000	–	–	3.2 ± 0.7

nd – not detected.

Trolox and ellipticine were used as positive control for the antioxidant activity and cytotoxicity assays, respectively.

^a Homoscedasticity among the different drying process and extraction method was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.

^b $p < 0.05$ indicates that the mean value of at least one component differs from the others (in this case, multiple comparison tests were performed). For each different sample, different letters within a line means they differ significantly ($p < 0.05$).

Table 3
Antimicrobial activity of *Galium aparine* L. infusions and hydromethanolic extracts.

	Freeze-dried		Air-dried		Positive control 1	Positive control 2
	Hydromethanolic extract	Infusion	Hydromethanolic extract	Infusion		
Antibacterial activity (MIC and MBC values, mg/mL)						
<i>Bacillus cereus</i>	3.75	3.75	1	3.75	0.1	0.25
	7.5	15	1.85	7.5	0.2	0.4
<i>Micrococcus flavus</i>	3.75	3.75	3.75	3.75	0.2	0.25
	7.5	15	7.5	7.5	0.3	0.4
<i>Staphylococcus aureus</i>	1.85	3.75	3.75	10	0.04	0.25
	3.75	15	7.5	20	0.1	0.45
<i>Listeria monocytogenes</i>	1.85	10	3.75	7.5	0.2	0.4
	7.5	20	15	20	0.3	0.5
<i>Escherichia coli</i>	3.75	> 30	10	> 30	0.2	0.4
	7.5	> 30	15	> 30	0.3	0.5
<i>Enterobacter cloacae</i>	1.85	15	3.75	7.5	0.2	0.25
	7.5	30	7.5	15	0.3	0.5
<i>Salmonella enteritidis</i>	1.85	15	7.5	3.75	0.15	0.3
	3.75	20	15	7.5	0.3	0.6
<i>Salmonella typhimurium</i>	1.85	15	3.75	7.5	0.25	0.4
	3.75	30	7.5	20	0.5	0.75
Antifungal activity (MIC and MFC values, mg/mL)						
<i>Aspergillus fumigatus</i>	15	> 30	7.5	30	0.25	0.15
	20	> 30	15	> 30	0.5	0.2
<i>Aspergillus versicolor</i>	1.85	7.5	3.75	15	0.2	0.1
	3.75	15	7.5	> 30	0.5	0.2
<i>Aspergillus ochraceus</i>	3.75	15	1.85	15	1.5	0.15
	7.5	30	3.75	> 30	2	0.2
<i>Aspergillus niger</i>	3.75	> 30	3.75	> 30	0.2	0.15
	7.5	> 30	7.5	> 30	0.5	0.2
<i>Candida krusei</i>	3.75	> 30	3.75	30	0.075	0.05
	7.5	> 30	7.5	> 30	0.15	0.1
<i>Penicillium funiculosum</i>	3.75	30	3.75	30	0.2	0.2
	7.5	> 30	7.5	> 30	0.5	0.25
<i>Penicillium ochrochloron</i>	3.75	> 30	10	30	2.5	0.2
	7.5	> 30	15	> 30	3.5	0.25
<i>Penicillium verrucosum</i> var. <i>cytopium</i>	7.5	> 30	10	30	0.2	0.1
	15	> 30	30	> 30	0.3	0.2

MIC: Minimum Inhibitory concentration; MBC: Minimum bactericidal concentration; MFC: Minimal fungicidal concentration.

As positive controls, streptomycin and ampicillin (positive control 1 and 2, respectively) were used for the antibacterial activity, while ketoconazole and bifonazole (positive control 1 and 2, respectively) were used for antifungal activity.

ranging from 1 to 15 and 1.85 to 30 mg/mL, respectively. Nevertheless, the infusions did not show the capacity to inhibit *E. coli*, at the studied concentrations (30 mg/mL). The extract revealing higher antibacterial properties, with lowest MIC and MBC values, was the freeze-dried sample hydromethanolic extract, which inhibited *S. aureus*, *L. monocytogenes*, *E. cloacae*, *S. enteritidis*, and *S. typhimurium* growth in a concentration of 1.85 mg/mL and *B. cereus*, *M. flavus*, and *E. coli* at 3.75 mg/mL. The MBC values presented by this extract varied from 3.75 to 7.5 mg/mL. *B. cereus* was the strain revealing the highest sensitivity to the extracts, with MIC and MBC values of 1 and 1.85 mg/mL when exposed to the air-dried sample hydromethanolic extract. This extract showed the second strongest antibacterial activity, considering all the studied bacteria, after the other hydromethanolic extract. As the most resistant bacterial strain, *S. typhimurium* needed MIC and MBC values ranging from 1.85 to 15 and 3.75 to 30 mg/mL, respectively. All the MIC and MBC values obtained for the positive controls were higher than those presented by the extracts. As far as we know, there are no reports on the antimicrobial activity of this plant infusions nor hydromethanolic extracts, which did not allow a comparison of the results obtained in the present study to other literature data. Nonetheless, an ethanol tincture of *G. aparine* desiccated stems produced an average zone of clearance of 2 mm (at a concentration of 27 mg/mL), while aqueous extracts did not reveal activity against *S. aureus*, in a study performed by Romero et al. (2005) using Kirby-Bauer disk diffusion susceptibility analysis to assess the antimicrobial activity. Otherwise, Zhao et al. (2006) investigated the antimicrobial activity of 2,5-dihydroxy-1,3-dimethoxyanthraquinone isolated from an *G. verum* ethanolic

extract, through the filter paper method, and reported a moderate activity against *E. coli* (12 mm), *Salmonella aureus* (14 mm), *Pseudomonas aeruginosa* (13 mm), and *S. typhimurium* (14 mm). Moreover, various *Galium mexicanum* aerial parts extracts (chloroform and methanol extracts) and fractions (hexane, chloroform, and methanol fractions) were found to possess antimicrobial activity against *Bacillus subtilis*, methicillin-resistant *S. aureus*, *S. aureus*, and *Streptococcus pyogenes*, in concentrations ranging from 166 to 666 µg/mL, except for the hexane fraction that inhibited *S. aureus* growth at 67 µg/mL (Bolivar et al., 2011).

In what concerns the antifungal activity of *G. aparine* extracts, eight fungi were assessed: *A. fumigatus*, *A. versicolor*, *A. ochraceus*, *A. niger*, *C. krusei*, *P. funiculosum*, *P. ochrochloron*, and *P. verrucosum*. Similar to the results obtained in the antibacterial activity assessment, the hydromethanolic extracts also revealed higher antifungal activity than the infusions, with the freeze-dried sample showing the lowest MIC and MFC values, which ranged from 1.85 to 15 and 3.75 to 20 mg/mL, respectively. The freeze-dried sample infusion showed the capacity to inhibit the growth of *A. versicolor*, *A. ochraceus*, and *P. funiculosum*, in concentrations of 7.5, 15, and 30 mg/mL, respectively, and also presented fungicidal properties in *A. versicolor* and *A. ochraceus* (MFC values of 15 and 30, respectively). On the other hand, the air-dried sample infusion showed MIC values of 15 mg/mL in *A. versicolor* and *A. ochraceus*, and 30 mg/mL in *A. fumigatus*, *C. krusei*, *P. funiculosum*, *P. ochrochloron*, and *P. verrucosum*, but did not present fungicidal activity at the tested concentration (30 mg/mL). All the studied fungi were sensitive to the hydromethanolic extracts and the major differences

between MIC and MFC values were observed for *P. verrucosum* in the presence of the air-dried sample hydromethanolic extract, where MFC (30 mg/mL) was the triple of the MIC value. The antifungal activity of the positive controls was higher than that of the extracts, but both hydromethanolic extracts presented MIC and MFC values close to those obtained for ketoconazole (positive control 1) in what concerns *P. ochrochloron* and *A. ochraceus*, respectively.

To the best of our knowledge, this is the first work on the antifungal activity of *G. aparine* extracts. Nevertheless, Bolivar et al. (2011) studied *G. mexicanum* extracts and fractions and demonstrated the ability of some hexane and methanol fractions to inhibit the growth of *Trichophyton rubrum* and *Cryptococcus neoformans*, when concentrated at 333–500 and 333–999 µg/mL, respectively, while a chloroform fraction exhibited activity in *Candida albicans* at 666 µg/mL; none of the assessed extracts (chloroform and methanol extracts) revealed antimicrobial properties. Similarly, *G. verum* methanolic extracts did not reveal antifungal activity in a study performed with several *Candida* species (Yiğit et al., 2009), contrarily to *G. aparine* hydromethanolic extracts assessed in the present study that showed the capacity to inhibit the growth of eight fungi, also revealing fungicidal properties.

4. Conclusion

Four extracts (two hydromethanolic extracts and two infusions) of *G. aparine* samples subjected to different drying procedures (freeze-drying and air-drying) were assessed for their phenolic profile and related bioactive properties. Generally, the samples presenting the highest levels of phenolic compounds also demonstrated higher bioactive properties. The freeze-dried sample hydromethanolic extract was the outstanding extract in terms of total phenolic concentration, antioxidant, antibacterial, and antifungal properties. None of the extracts revealed hepatotoxicity. The present study provides valuable information regarding *G. aparine*, contributing to the valorization of this *Galium* species, commonly known as an undesired and troublesome weed in several cultivars, and which previous reports are scarce.

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