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COMPARATIVE STUDY OF POLYPHENOLIC CONTENT, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF FOUR *GALIUM* SPECIES (*RUBIACEAE*)

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Four *Galium* species (*G. verum* L., *G. mollugo* L., *G. aparine* L. and *G. odoratum* L.) were analyzed in order to evaluate their polyphenolic content, antioxidant and antimicrobial activities. The identification and quantification of major phenolic compounds content was performed using a HPLC-MS/MS method. The total polyphenols, caffeic acid derivatives and flavonoids content was spectrophotometrically determined. The antioxidant activity was evaluated using the DPPH bleaching method and all data indicates that *G. verum* was the best source of antioxidants amongst the four studied species. Rutin was the compound found in largest amount in all analysed extracts. Luteolin was found only in the extracts of *G. mollugo* and *G. aparine* and kaempferol only in the extracts of *G. verum* and *G. odoratum*, but in different amounts. Chlorogenic acid, *p*-coumaric acid and ferulic acid were identified in all extracts in different amounts and caftaric acid only in *G. aparine* extract. The antimicrobial activity was determined using the diffusimetric method. *Galium* extracts showed inhibitory activity on various pathogenic bacteria and fungi tested. The quantitative and qualitative differences between the four species of *Galium* concerning the polyphenolic content, could serve as a taxonomic marker in order to distinguish species and to avoid adulterations.

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Keywords: *Galium* species; Polyphenols; Antioxidant activity; Antimicrobial activity; DPPH.

1. Introduction

Plant-origin polyphenols are antioxidant agents that have been suggested to exert beneficial pharmacological effects on neurodegenerative disorders. Plant polyphenols might also display anticarcinogenic, antimutagenic and cardioprotective effects assumed by their free radical scavenging properties. Polyphenols have been also reported as chemopreventive agents by lowering the cholesterol level and roughly limiting cell damage. Their ability to delay lipid oxidation in foodstuffs and biological membranes, in addition to their propensity to act as a prophylactic agent has motivated their research in food science and biomedicine [1,2].

The cosmopolitan genus *Galium*, belonging to *Rubiaceae*, consists of about 300 herbaceous plant species [3]. The species are used in food manufacturing and folk medicine all over the world. *Galium* species are herbaceous, annual or perennial, with thin rhizome and

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cylinder-form stem, that presents four prominent longitudinal lines. The leaves are in verticil but just two of these are genuine leaves, the others being stipules. The flowers are of four types in ended panicles and the fruits are twin nucules [4,5]. The European flora gathers more than 145 species [6]; in the Romanian flora, there are between 28 species quoted, most with white flowers and six with yellow flowers [7]. The aerial parts are gathered for medicinal purposes in the blossoming period. Because the identification of these plants is frequently vague or imprecise owing to their highly similar morphological characteristics, the results of chemotaxonomic investigations could be valuable for the systematic evaluation of this genus.

Galium verum L. – yellow "Lady's bedstraw", with golden yellow flowers, contains polyphenols, flavonoids, phenyl-propanoids compounds and iridoids. They are used as diuretic, depurative, light sedative, spasmolytic in kidney stones, and externally for injuries and skin damages as wound healing, psoriasis treatment and rheumatism [8]. In some countries, the drug is still used as a dye including the milk industries. In the same places with *G. verum*, vegetates *G. mollugo L.*, with white flowers, which is considered an adulteration for "Lady's bedstraw" [9,10,11,12].

Galium aparine L. is a typical scrambling climbing plant. Decoction of whole plant is locally used as a tea. Aerial parts of *G. aparine* contain anthraquinones, iridoids, alkanes, flavonoids, tannins, polyphenolic acids, and vitamin C. Some scientists have reported that the whole plant of *G. aparine* is used for the treatment of lymph swellings, tonsillitis, jaundice, wounds, cancer, fever, scurvy, hypertension and leukemia [13].

Galium odoratum L. – "sweet woodruff" (formerly known as *Asperula odorata L.*) is a mat-forming perennial plant that is most often seen as a ground cover in shady areas. Plants emit a strong odor of freshly mown hay when foliage is crushed or cut. Aromatic intensity of the foliage increases when dried, thus dried leaves are popularly used in sachets or potpourris. The plant has also been used commercially, in perfumes. Leaves are sometimes used to flavor teas and cold fruit drinks. "Sweet woodruff" was widely used in herbal medicine during the Middle Ages, gaining a reputation as an external application to wounds and cuts and also taken internally in the treatment of digestive and liver problems. In current day phytotherapy it is valued mainly for its tonic, diuretic and anti-inflammatory effect. The leaves are antispasmodic, cardiac, diaphoretic, diuretic, sedative and are used in the treatment of insomnia and nervous tension, varicose veins, biliary obstruction, hepatitis and jaundice. The plant contains coumarins, polyphenols, iridoids and flavonoids. It is commercially grown as a source of coumarin and it is not recommended to be used alongside conventional medicine for circulatory problems or in pregnancy state [14].

The aim of this study was to determine the polyphenolic content, and to evaluate antioxidant and antimicrobial activities of *G. verum L.*, *G. mollugo L.*, *G. aparine L.* and *G. odoratum L.* (*Rubiaceae*). None of these plants have subjected before to detailed studies in order to reveal their polyphenolic content or antioxidant and antimicrobial activities.

2. Experimental

2.1 Plant materials

Plant materials (aerial parts) from the four species were collected in 2013, during the blooming period (May-June) from the Province of Transylvania (NW of Romania) and identified by Professor Gianina Crișan from the Pharmaceutical Botany Department, one of the coauthors. Authenticated voucher specimens were deposited in the Herbarium of the Department of Pharmaceutical Botany, Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy. The vegetal material was air dried at room temperature in shade, separated and grinded to fine powder (300 μm).

2.2 Preparation of the extracts

2.0 g of plant material (aerial parts – *herba*) from each species, after being grinded to a proper degree of fineness (300 μm), were ultrasonicated with 20 ml of 70% ethanol (Merck,

Darmstadt, Germany), for 30 min at 60°C. The samples were then cooled down and centrifuged at 4500 rpm for 15 min, and the supernatant was recovered [2,15]. All the samples were filtered using Whatman filter paper and preserved at 4 ºC.

2.3 Chemicals and bacterial strains

Ferulic acid, sinapic acid, gentisic acid, gallic acid, patuletin, luteolin were products from Roth (Karlsruhe, Germany), cichoric acid, caftaric acid were products from Dalton (Toronto, ON, Canada), chlorogenic acid, *p*-coumaric acid, caffeic acid, rutin, apigenin, quercetin, isoquercitrin, quercitrin, hyperoside, kaempferol, myricetol, fisetin were products from Sigma Aldrich (St. Louis, MO, USA). HPLC grade methanol, analytical grade orthophosphoric acid, hydrochloric acid and Folin-Ciocâlteu reagent were purchased from Merck (Darmstadt, Germany), aluminum chloride, sodium acetate, sodium carbonate, ethanol (Merck, Darmstadt, Germany), DPPH (2,2-diphenyl-1-picrylhydrazyl) and BHT (butylated hydroxytoluene) were obtained from Alfa-Aesar (Germany). For the antibacterial potential assaying of the plant extracts, all microorganism strains were distributed by MicroBioLogics®: *Staphylococcus aureus* ATCC 49444 (Gram+), *Listeria monocytogenes* ATCC 13076 (Gram+), *Escherichia coli* ATCC 25922 (Gram-), and *Salmonella typhimurium* ATCC 14028 (Gram-) and one fungal strain, *Candida albicans* ATCC10231.

2.4 LC/MS analysis

2.4.1 Apparatus and Chromatographic Conditions

The experiment was carried out using an Agilent Technologies 1100 HPLC Series system (Agilent, Santa Clara, CA, USA) equipped with G1322A degasser, G13311A binary gradient pump, column thermostat, G1313A auto sampler and G1316A UV detector. The HPLC system was coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap SL). For the separation, a reverse-phase analytical column was employed (Zorbax SB-C18 100 × 3.0 mm i.d., 3.5 µm particle); the work temperature was 48°C. The detection of the compounds was performed on both UV and MS mode. The UV detector was set at 330 nm until 17.5 min, then at 370 nm. The MS system functioned using an electrospray ion source in negative mode. The chromatographic data were processed using ChemStation and DataAnalysis software from Agilent. The mobile phase was a binary gradient: methanol and acetic acid 0.1% (v/v). The elution started with a linear gradient, beginning with 5% methanol and ending at 42% methanol, for 35 minutes; then 42% methanol for the next 3 minutes [15,16]. The flow rate was 1 $mLmin^{-1}$ and the injection volume was 5 µL. The MS signal was used only for qualitative analysis based on specific mass spectra of each polyphenol. The MS spectra obtained from a standard solution of polyphenols were integrated in a mass spectra library. Later, the MS traces/spectra of the analyzed samples were compared to spectra from library, which allowed positive identification of compounds, based on spectral mach. The UV trace was used for quantification of identified compounds from MS detection. Four polyphenols cannot be quantified in current chromatographic conditions due overlapping (caftaric acid with gentisic acid and caffeic acid with chlorogenic acid). However, all four compounds can be selectively identified in MS detection (qualitative analysis) based on differences between their molecular mass and MS spectra. For all compounds, the limit of quantification was 0.5 µg/mL, and the limit of detection was 0.1 µg/mL. The detection limits were calculated as minimal concentration producing a reproductive peak with a signal-to-noise ratio greater than three. Quantitative determinations were performed using an external standard method. Calibration curves in the 0.5–50 µg/mL range with good linearity ($R^2 > 0.999$) for a five point plot were used to determine the concentration of polyphenols in plant samples [2,16,17].

2.5 Determination of total polyphenols, caffeic acid derivatives and flavonoids content

The total phenolic content (TPC) of the extracts was determined using the Folin-Ciocalteu method with some modifications [18,19]. 2 mL of each ethanolic extract wich was diluted 25 times

were mixed with 1.0 mL of Folin-Ciocalteu reagent, 10.0 mL of distilled water and diluted to 25.0 mL with a 290 g/L solution of sodium carbonate [1,2]. The samples were incubated in the dark for 30 min. The absorbance was measured at 760 nm. Gallic acid was used as standard for the calibration curve and was plotted at 0.02, 0.04, 0.06, 0.08, and 0.10 mg/mL, prepared in methanol:water (50:50, v/v). TPC values were determined using an equation obtained from the calibration curve of gallic acid graph ($R^2 = 0.999$).

The phenolic acids content in the vegetal materials was determined using the spectrophotometric method with Arnow's reagent [20]. The percentage of phenolic acids, expressed as caffeic acid equivalent on dry vegetal material (mg CAE/g vegetal material), was determined using an equation that was obtained from calibration curve of caffeic acid ($R^2 = 0.994$).

The spectrophotometric aluminum chloride method was used for flavonoids determination. 5 mL of each extract were mixed with 5.0 mL of sodium acetate 100 gL^{-1}, 3.0 mL of aluminum chloride 25 gL^{-1}, and filled up to 25 mL by methanol in a calibrated flask. The absorbance was measured at 430 nm [20]. Total flavonoids content values was determined using an equation obtained from calibration curve of the rutin graph ($R^2 = 0.999$). All analyses were done in triplicate.

2.6 DPPH• Radical Scavenging Assay

The free radical scavenging activity of the ethanolic extracts from the *herba* of four species of *Galium* was quantified in terms of hydrogen donating or radical scavenging ability using the stable DPPH radical method. The DPPH solution (0.1 gL^{-1}) in methanol was prepared and 4.0 ml of this solution were added to 4.0 ml of extract solution (or standard) in methanol at different concentrations (10-50 μg/ml). After 30 minutes of incubation at 40°C in a thermostatic bath, the decrease in the absorbance (n = 3) was measured at 517 nm. The percent of DPPH scavenging ability was calculated as: DPPH scavenging ability = $(A_{control} - A_{sample}/A_{control}) \times 100$, where $Abs_{control}$ is the absorbance of DPPH radical + methanol (containing all reagents except the sample) and Abs_{sample} is the absorbance of DPPH radical + sample extract. Afterwards, a curve of % DPPH scavenging capacity *versus* concentration was plotted and IC_{50} values were calculated. IC_{50} denotes the concentration of sample required to scavenge 50% of DPPH free radicals [17,19,21,22,23]. The positive controls were those using the standard solution quercetin and butylated hydroxytoluene (BHT). IC_{50} value is correlated with the antioxidant capacity. So, $IC_{50} \leq 50$ μg/mL value means a high antioxidant capacity; 50 μg/mL < $IC_{50} \leq 100$ μg/mL value means a moderate antioxidant capacity and $IC_{50} > 200$ μg/mL value means no relevant antioxidant capacity [24].

2.7 Antimicrobial activity test

The ethanolic extracts of *Gallium* species were tested for the antimicrobial activity against two Gram-positive bacterial strains: *Staphylococcus aureus* (ATCC 49444), *Listeria monocytogenes* (ATCC 13076), against two Gram-negative bacterial strains: *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028) and one fungal strain: *Candida albicans* (ATCC10231) by a previously described disc diffusion method, in Petri dishes. Each microorganism was suspended in Mueller Hinton (MH) broth and diluted approximately to 10E6 colony forming unit (cfu)/mL. They were "flood-inoculated" onto the surface of MH agar and MH Dextroxe Agar (MDA) and then dried. Six-millimeter diameter wells were cut from the agar using a sterile cork-borer, and 60 μL of each extract were added into the wells. The plates were incubated at 37°C and the diameters of the growth inhibition zones were measured after 24 h. Gentamicin (10 μg/well) and Fluconazole (25 μg/well) were used as standard drugs. The controls were performed only with sterile broth and overnight culture and 10 μL of 70% ethanol [25]. All tests were performed in triplicate, and clear halos greater than 10 mm were considered as positive results.

3. Results and discussions

3.1 LC – MS results

In this paper, 19 phenolic compounds have been investigated by HPLC-MS, in four 70% ethanolic extracts of *G. verum* L., *G. mollugo* L., *G. aparine* L. and *G. odoratum* L. *herba*. HPLC coupled with MS is a very powerful analytical technique, due to its high sensitivity and the structural information that can be obtained about the analytes. A high-performance liquid chromatographic (HPLC) method has been developed for the determination of 19 phenolic compounds (eight phenolic acids, four quercetin glycosides, and seven flavonol and flavone aglycones) from plant material. The applicability of the proposed analytical method and the qualitative and quantitative determination of the standard phenolic compounds have already been verified [2,15,16,]. This method allows a simultaneous analysis of different classes of polyphenols by a single pass column (the separation of all examined compounds was carried out in 35 min). The concentrations of identified polyphenolic compounds in the analyzed samples are presented in Table 1.

Table 1: The polyphenolic compounds in the extracts of Galium species (mg polyphenolic compounds/100 g dried vegetal material)

Polyphenolic compounds	**R_t±SD (min)**	*G. verum L.*	*G. mollugo L.*	*G. aparine L.*	*G. odoratum L.*
Caftaric acid	3.54 ± 0.05	NF	NF	<0,2	NF
Gentisic acid	6.52 ± 0.04	NF	NF	<0,2	<0,2
Caffeic acid	5.60 ± 0.04	NF	<0,2	<0,2	<0,2
Chlorogenic acid	5.62 ± 0.05	<0.2	<0,2	<0,2	<0,2
p-Coumaric acid	9.48 ± 0.08	0.983 ± 0.10	0.682 ± 0.06	1.404 ± 0.28	0.802 ± 0.08
Ferulic acid	12.8 ± 0.10	<0.2	<0,2	3.793 ± 0.31	0.658 ± 0.05
Sinapic acid	15.00 ± 0.10	NF	NF	NF	NF
Cichoric acid	15.96 ± 0.13	NF	NF	NF	NF
Hyperoside	18.60 ± 0.12	NF	NF	0.300 ± 0.03	NF
Isoquercitrin	19.60 ± 0.10	78.021 ± 0.95	<0,2	0.967 ± 0.13	NF
Rutin	20.20 ± 0.15	804.262 ± 1.89	24.312 ± 0.93	7.983 ± 0.30	11.248 ± 0.87
Myricetin	21.13 ± 0.12	NF	NF	NF	NF
Fisetin	22.91 ± 0.15	NF	NF	NF	NF
Quercitrin	23.64 ± 0.13	4.291 ± 0.21	4.478 ± 0.20	NF	2.048 ± 0.45
Quercetin	26.80 ± 0.15	2.651 ± 0.13	0.339 ± 0.04	5.679 ± 0.26	0.339 ± 0.37
Patuletin	29.41 ± 0.12	NF	NF	NF	NF
Luteolin	29.10 ± 0.19	NF	0.606 ± 0.08	0.467 ± 0.07	NF
Kaempferol	32.48 ± 0.17	3.069 ± 0.17	NF	NF	1.345 ± 0.09
Apigenin	33.10 ± 0.15	NF	NF	NF	NF

Notes: NF- not found, below limit detection. Values are the mean ± SD (n = 3).

The compounds were shown in Table 1 in the order of their retention time. The HPLC chromatogram of *G. verum* sample is presented in Figure 1, for *G. mollugo* sample in Figure 2, for *G. aparine* sample in Figure 3, and for *G. odoratum* in Figure 4, respectively. All quantitative determinations were performed using the external standard method. In the ethanolic extract of *G. verum*, three flavonoid glycosides were identified as main components. Rutin (quercetin 3-O-rutinoside) was the compound found in the largest amount (804.262 ± 1.89 mg/100 g) followed by isoquercitrin - quercetin 3-O-glucoside (78.021 ± 0.95 mg/100 g). Quercitrin (quercetin 3-O-rhamnoside), quercetin and kaempferol were detected at lower levels than major flavonoids (4.291 ± 0.21 mg/100 g, 2.651 ± 0.13 mg/100 g, 3.069 ± 0.17 mg/100 g,). *p*-coumaric acid was also detected but in a lower amount (0.983 ± 0.10 mg/100g). Ferulic acid and chlorogenic acid were also identified in this extract, but they were in too low concentration to be quantified (Table 1).

In the ethanolic extract of *G. mollugo* only two flavonoid glycosides were identified in large amount. Rutin was the compound found in the largest amount in the extract (24.312 ± 0.93 mg/100 g) followed by quercitrin (4.478 ± 0.20 mg/100 g). Luteolin and quercetin were detected at lower levels (0.606 ± 0.08 mg/100 g, 0.339 ± 0.04 mg/100g respectively) and isoquercitrin was also identified in the extract, but in too low concentration in order to be quantified (< 0.2 mg/100 g). Regarding the phenolic acids, *p*-coumaric acid was found in the largest amount (0.682 ± 0.06 mg/100g). Caffeic acid, chlorogenic acid, ferulic acid were identified, but they were in too low concentration (< 0.2 mg/100 g) to be quantified (Table 1). The presence of luteoline in the *G. mollugo extract* could serve as an important chemotaxonomic marker that could avoid the adulteration of *G. verum* with these taxa.

In the ethanolic extract of *G. aparine*, rutin was present in the largest amount (7.983 ± 0.30 mg/100 g) followed by quercetin (5.679 ± 0.26 mg/100 g). Isoquercitrin, luteoline and hyperoside were detected in lower amounts (0.967 ± 0.13 mg/100 g, 0.467 ± 0.07 mg/100 g and 0.300 ± 0.03 mg/100 g, respectively). In this extract were also identified three quercetin glycosides: rutin isoquercitrin and hyperoside and two free aglycones, quercetin (5.679 ± 0.26 mg/100 g) and luteolin (0.467 ± 0.07 mg/100 g). Regarding the phenolic acids, ferulic acid was found in the largest amount (3.793 ± 0.31 mg/100 g) followed by *p*-coumaric acid (1.404 ± 0.28 mg/100 g). Caftaric acid, gentisic acid, caffeic acid and chlorogenic acid were identified, but they were in too low concentration to be quantified (<0.2 mg/100 g) (Table 1). The presence of hyperoside and caftaric acid only in the *G. aparine* extract could constitute an important source of taxonomical differentiation.

In the ethanolic extract of *G. odoratum*, rutin was also the compound present in the largest amount (11.248 ± 0.87 mg/100 g) followed by quercitrin (2.048 ± 0.45 mg/100g). Two flavonoid glycosides were identified, e.g,. rutin and quercitrin and two free aglycones – kaempferol (1.345 ± 0.09 mg/100 g) and quercetin (0.339 ± 0.37 mg/100 g) in lower amounts than the flavonoid glycosides (Table 1). Regarding the phenolic acids composition, *p*-coumaric acid was detected in the largest amount (0.802 ± 0.08 mg/100 g) followed by ferulic acid (0.658 ± 0.05 mg/100 g). Other phenolic acids, like gentisic acid, caffeic acid, chlorogenic acid were only identified but not quantified due to their low concentration (<0.2 mg/100 g). Considering the 19 standard compounds used in this study (Table 1), some other peaks were not identified.

Thus, the comparative study showed large differences, especially quantitative, between the four *Galium* species. The major compound, found in all the analyzed extracts, was rutin. The richest species in rutin was *G. verum* (804.262 ± 1.89 mg/100 g) followed by *G. mollugo* (24.312 ± 0.93 mg/100 g). The aglycon, luteolin was detected only in *G.mollugo* and *G. aparine*. Kaempferol was detected in a larger amount in *G. verum* (3.069 ± 0.17 mg/100 g) than in *G. odoratum* (1.345 ± 0.09 mg/100 g). Quercetin was the aglycon present in all analysed extracts.

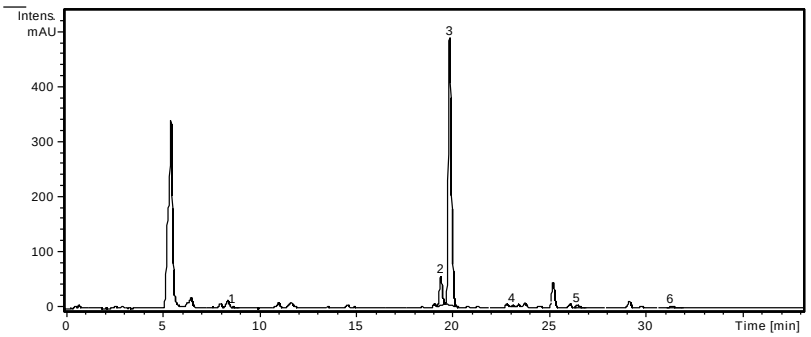

Fig. 1: HPLC chromatogram of G. verum sample
(1-p-coumaric acid, 2-isoquercitrin, 3-rutin, 4-quercitrin, 5-quercetin, 6-kaempferol)

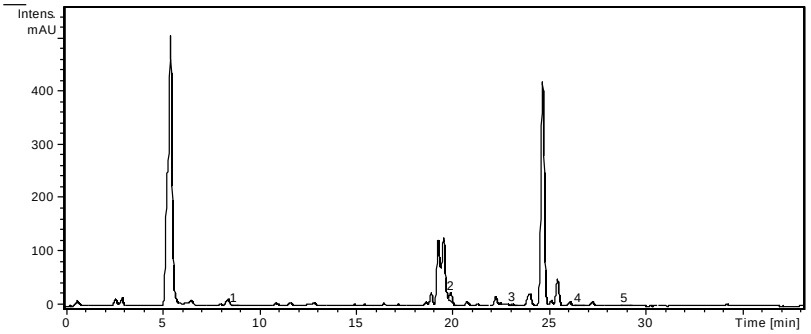

Fig. 2: HPLC chromatogram of G. mollugo sample
(1-p-coumaric acid, 2-rutin, 3-quercitrin, 4-quercetin, 5-luteolin)

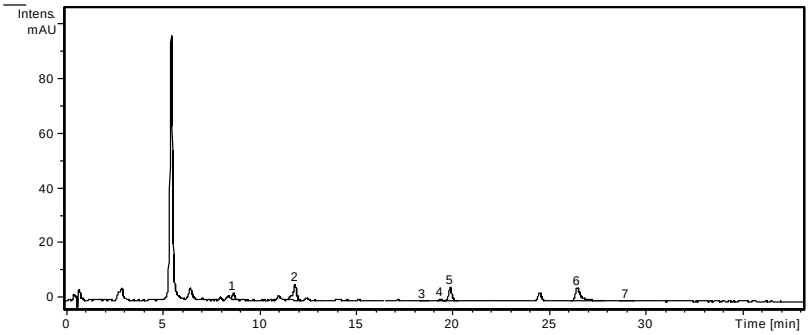

Fig. 3: HPLC chromatogram of G. aparine sample
(1-p-coumaric acid, 2-ferulic acid, 3-hyperoside, 4-isoquercitrin, 5-rutin, 6-quercetin, 7-luteolin)

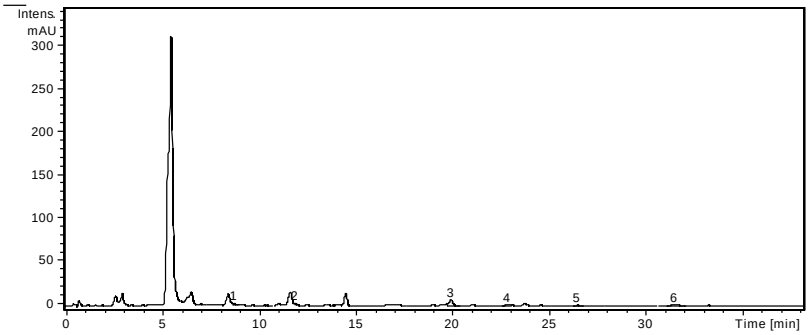

Fig. 4: HPLC chromatogram of G. odoratum sample
(1-p-coumaric acid, 2-ferulic acid, 3-rutin, 4-quercitrin, 5-quercetin, 6-kaempferol)

3.2 Determination of polyphenolic compounds content: total polyphenols, flavonoids and caffeic acid derivatives

Table 2: Quantitative determination of polyphenolic compounds content
(g compounds/100 g dried vegetal material)

Plant species	*G. verum L.*	*G. mollugo L.*	*G. aparine L.*	*G. odoratum L.*
Total polyphenolic compounds – TPC (g GAE/100 g dry mass)	2.6 ± 0.12	3.46 ± 0.13	2.40 ± 0.24	1.4 ± 0.35
Flavonoids (g RE/100 g dry mass)	5.21 ± 0.24	6.93 ± 0.44	1.60 ± 0.53	2.81 ± 0.54
Caffeic acid derivatives (g CAE/100 g dry mass)	1.329 ± 0.10	2.152 ± 0,19	0.348 ± 0.09	0.419 ± 0.17

Each value is the mean ± SD of three independent measurements. GAE: Gallic acid equivalents; RE: rutin equivalents; CAE: caffeic acid equivalents.

There are also quantitative differences in the total polyphenolic compounds, flavonoids and caffeic acid derivatives between these four *Galium* species. The highest amount of the total polyphenols was determined for *G. mollugo* (3.46 ± 0.13 g/100 g) followed by *G. verum* (2.6 ± 0.12 g/100 g), *G. aparine* (2.4 ± 0.24 g/100 g) and *G. odoratum* (1.4 ± 0.35 g/100 g) – Table 2. There is no literature data regarding the total polyphenolic compounds of these four species. Concerning the content of flavonoids, the highest amount was determined also for the extract of *G. mollugo* (6.93 ± 0.44g/100 g), followed by the extract of *G. verum* (5.21 ± 0.24 g/100 g), *G. odoratum* (2.81 ± 0.54g/100 g) and *G. aparine* (1.60 ± 0.53g/100 g). Tămaş et al. report that the total content of flavonoids was over three times higher for *G.verum* (2.24%) compared to *G. molugo* (0.72%). The difference in the extraction yield could be the result of using different extraction method in that work and ultrasonication in ours [4]. The phenolic acids values were expressed as caffeic acid equivalent (g CAE/100 g dried product). The highest amount of phenolic acids was determined for the ethanolic extract of *G. mollugo* (2.152 ± 0.19 g/100 g) followed by *G. verum* (1.329 ± 0.10 g/100 g). These results were in compliance with the determinations of the polyphenolic content and suggest that both *G. verum* and *G. mollugo* can be used as valuable sources of antioxidants.

3.3 In-vitro antioxidant activity

Determination of the free radical scavenging activity

Table 3: DPPH free radical scavenging activity

Samples	IC_{50} (μgmL^{-1})
BHT (standard control)	16 ± 0.54
Quercetin (standard control)	5.60 ± 0.35
G. verum	105.43 ± 0.15
G. mollugo	107.45 ± 0.53
G. aparine	116.43 ± 0.46
G. odoratum	264.42 ± 0.74

Each value is the mean ± SD of three independent measurements.

The antioxidant activity of these ethanol extracts was assessed by the DPPH radical bleaching method. BHT and quercetin (0.025 mg mL^{-1}) were used as the positive control (Table 3). The highest radical scavenging activity was showed by the extract of *G. verum* with IC_{50} = 105.43 ± 0.15 μgmL^{-1}, followed by the extract of *G. mollugo* with IC_{50} = 107.45 ± 0.53μgmL^{-1} and the extract of *G. aparine* with IC_{50} = 116.43 ± 0.46 μgmL^{-1}. The lowest radical scavenging activity was showed by the extract of *G. odoratum* with IC_{50} = 264.42±0.74 μgmL^{-1}. The $IC_{50(DPPH\bullet)}$ values of the extracts increased in the following order: *G. verum* < *G.mollugo* < *G. aparine* < *G. odoratum.* This was in good agreement with the total polyphenolic compounds values listed in Table 3. The lowest IC_{50} value means the most powerful antioxidant potential. According to this method, *G. verum* extract exhibited a moderate antioxidant capacity. Compared to the reference compounds, quercetin (IC_{50} = 5.60 ± 0.35 μgmL^{-1}) and BHT (IC_{50} = 16 ± 0.54 μgmL^{-1}), the ethanol extracts of *G.verum* showed lower antioxidant capacity. The results presented here constitute the first information on the antioxidant activities of Romanian *Galium* species ethanolic extracts. There is no literature data regarding the comparison of the antioxidant activities of these four *Galium* species.

3.4 Antibacterial activity

The disc-diffusion assay was used to determine the antimicrobial activity of the investigated ethanolic extracts of *Galium* against a panel of microorganisms including two gram-positive bacteria *Staphylococcus aureus* (*S. aureus*), and *Listeria monocytogenes* (*L. monocytogenes*), two gram-negative bacteria, *Salmonella typhimurium* (*S. typhimurium*) and *Escherichia coli* (*E. coli*), and the fungus *Candida albicans (C. albicans).* The results of the

antimicrobial activity of these extracts are shown in Table 4. The antibacterial activity is ranked from no activity (inhibition diameter < 10 mm), low (inhibition diameter between 10 and 15 mm), moderate (inhibition diameter between 15 and 20 mm) and high activity (diameter inhibition ≥ 20 mm) [26].

Table 4. Results of the antimicrobial activity of Galium extracts in agar diffusion method.

Samples	Inhibition zone in diameter (mm)				
	Staphylococcus aureus	*Listeria monocytogenes*	*Escherichia coli*	*Salmonella typhimurium*	*Candida albicans*
Galium verum	11 ± 0.05	16 ± 0.05	10 ± 0.05	12 ± 0.05	15 ± 0.05
Galium mollugo	-	10 ± 0.05	-	-	8 ± 0.05
Galium aparine	-	-	-	-	-
Galium odoratum	16 ± 0.05	16 ± 0.05	14 ± 0.05	10 ± 0.05	10 ± 0.05
Gentamicin	19 ± 0.05	18 ± 0.1	22 ± 0.00	18 ± 0.05	-
Fluconazole	-	-	-	-	25 ± 0.00

The values represent the average of three determinations ± standard deviations.
Gentamicin (10μg/disk) and Fluconazole (10μg/disk) were used as a positive control.

The results showed variation in the antimicrobial properties of these four extracts of *Galium*. As it can be seen from the Table 4, all investigated ethanolic extracts were active against all the bacteria tested. The antimicrobial activity of these extracts was exhibited mainly against the Gram-positive bacteria (*S. aureus*, *L. monocytogenes*). All four samples showed a low activity against Gram-negative bacteria (*S. typhimurium*, *E. coli*) with diameters of inhibition zones between 10 and 15 mm. The extract of *G. verum* showed a moderate antibacterial activity against *L. monocytogenes* (inhibition diameter - 16 mm), and limited activity against the other bacterial pathogens tested. The most pronounced activity with inhibition zones more than 14 mm was shown by the ethanolic extract of *G. odoratum*. Gentamicin was used as a reference antibiotic. The majority of these ethanolic extracts showed a weak antifungal activity against *C. albicans.* The extract of *G. mollugo* was inactive on this fungal strain with the diameter of inhibition zone from 8 mm. Fluconazole was used as reference antifungal agent.
The results of the present investigation suggest that various extracts of *Galium* exhibited an important activity against Gram-positive bacteria, especially.

3.4 Statistical analysis

All samples were analyzed in triplicate; average and relative SD were calculated using Excel software package.

4. Conclusions

We have determined the phenolic profile, the antioxidant and antimicrobial activities for four indigenous species of *Galium* genus (*Rubiaceae*) and we have completed the lack of literature data with new information concerning the polyphenolic compounds and their bioactivity. The simultaneous determination of a wide range of polyphenolic compounds was performed using a rapid, highly accurate and sensitive HPLC method assisted by mass spectrometry detection. The antioxidant activity evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) bleaching method indicate that *G. verum* was the best source of antioxidants amongst the four studied species, related with the total polyphenolic content. The antimicrobial tests underlined an important activity against Gram-positive bacteria for the extracts of *G. odoratum* and *G. verum*. The comparative

study showed significant differences, both qualitative and especially quantitative, among the four species of *Galium.* This study suggests that the aerial parts (*herba*) of *G. verum* and *G. mollugo* may be considered a source of important polyphenols with bioactive properties, a source that could be pharmaceutically exploited.

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