ORIGINAL ARTICLE

EVALUATION OF THE ANTIOXIDANT AND ANTIBACTERIAL POTENTIAL OF BIOACTIVE COMPOUNDS FROM *AJUGA REPTANS* EXTRACTS

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Abstract

The aim of this study was to evaluate the polyphenols and iridoids from *Ajuga reptans* (*Lamiaceae*) flower extracts, and their antioxidant and antibacterial activities. Total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC) and HPLC/UV/MS were employed for the identification and quantification of polyphenolic compounds. Total iridoid contents (TIC) were determined through a colorimetric method. The bioactive compounds were found in higher quantities in the ethanol extracts. Caffeic, *p*-coumaric and ferulic acids, quercitrin, luteolin and apigenin were identified by LC/MS in all samples. The antioxidant effects were evaluated by DPPH assay and TPC. *A. reptans* ethanol extract exhibited a higher antioxidant activity than the methanol extract, and a positive correlation between the antioxidant properties and the concentration of polyphenols was observed. The antimicrobial activity was evaluated by the use of dilution assays; the minimal inhibitory concentration and the minimal bactericidal concentration values were determined. These results indicate that *A. reptans* flowers contain active compounds with antioxidant and antibacterial potential.

Rezumat

Obiectivul studiului a constat în evaluarea conţinutului în polifenoli şi iridoide, precum şi a activităţii antioxidante şi antibacteriene pentru extractele obţinute din florile speciei *Ajuga reptans*, familia *Lamiaceae.* Conţinutul de polifenoli totali (TPC), de flavonoide totale (TFC), de antocianozide totale (TAC), precum şi o metodă HPLC/UV/MS au fost folosite pentru identificarea şi cuantificarea compuşilor polifenolici. Conţinutul de iridoide totale (TIC) a fost determinat printr-o metodă colorimetrică. Concentraţia compuşilor bioactivi a fost mai mare în extractele etanolice. În toate probele au fost identificaţi prin LC/MS: acidul cafeic, acidul *p*-cumaric, acidul ferulic, quercitrozida, luteolina şi apigenina. Evaluarea efectului antioxidant s-a realizat prin metoda DPPH şi determinarea TPC. Extractul etanolic de *A. reptans* a prezentat un efect antioxidant mai bun decât cel metanolic, în concordanţă cu conţinutul în polifenoli. Acţiunea antimicrobiană a fost evaluată prin tehnica microdiluţiilor, fiind determinate concentraţia minimă inhibitorie şi concentraţia bactericidă. Rezultatele cercetărilor arată că florile de *A. reptans* conţin compuşi bioactivi cu potenţial antioxidant şi antibacterian.

Keywords: *Ajuga reptans*, *polyphenols*, *antioxidant*, *antibacterial*

Introduction

Ajuga species (*Lamiaceae*) are perennial or annual herbaceous flowering plants, growing worldwide. Although several *Ajuga* sp. have economic importance and medicinal value, limited information is available about the phytochemistry and the therapeutic potential of Romanian plants. They are used in traditional medicine as a remedy for oedema, hypertension, fever, intestinal and biliary disorders, ulcer, as antipyretic, diuretic and astringent. *Ajuga reptans* is one of the most important species of the genus, known in ethno-medicine for its anti-inflammatory, wound healing, hepatoprotective properties [4, 9, 12]. Abroad studies on *Ajuga* plants have led to the identification of several bioactive compounds: phytoecdysteroids, triterpenes, sterols, diterpenes, anthocyanidins, iridoids, flavonoids, etc. [4, 9]. The quality of the natural products depends on the geographic origin, due to variations in pedoclimatic conditions, and the phyto-chemicals could vary in terms of quality and quantity [6].

Accordingly, the study aimed to evaluate the bioactive compounds (polyphenols and iridoids) and the pharmacological effects of *A. reptans* flowers harvested from Romanian spontaneous flora, for a proper use in phytotherapy.

Materials and Methods

Plant material: the plants were harvested at full flowering stage from wild populations, in June 2015 (Cluj County, Romania). The voucher specimen of the plants was stored in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, "Iuliu Haţieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania (accession number AR-33).

Extraction procedure: the air-dried powder of the vegetal product was extracted with different solvents; methanol extract (ME) was obtained from 0.5 g natural product and 50 mL 70% methanol for 30 min on a water bath at 60ºC [16]. The 10% tincture was prepared at room temperature from natural product and 70% ethanol (ethanol extract, EE) [17].

The total phenolic content (TPC) of the extracts was determined spectrophotometrically, by Folin-Ciocalteu method with adjustments [13]. The absorbance was evaluated using a JASCO UV-VIS spectrophotometer, at 760 nm. TPC was expressed as mg gallic acid/g dry material plant (mg GAE/g plant material).

The total flavonoid content (TFC) was determined by the method described in the Romanian Pharmacopoeia (X^{th} Ed.) at 430 nm, and was expressed as rutin equivalents (mg RE/g plant material) [17].

The total anthocyanin content (TAC) from the flowers of *Ajuga reptans* was measured by a colorimetric method at 535 nm, and was expressed as cyanidin-3-glucoside equivalents (mg CE/g plant material) [2].

The total iridoid content (TIC) was determined by a spectrophotometric method and the results were expressed as aucubin equivalents (mg AE/g dry weight) [10].

HPLC analysis of polyphenols

The determination of polyphenols was assessed using an Agilent 1100 HPLC Series system equipped with UV detector, and coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). The analysis was carried out in previously described conditions, and the detection and quantification were performed in UV assisted by MS detection [1, 5]. 18 polyphenolic compounds were used as standards (caftaric, gentisic, caffeic, chlorogenic, *p*-coumaric, ferulic, sinapic acids, hyperoside, isoquercitrin, rutin, myricetin, fisetin, quercitrin, quercetin, patuletin, luteolin, kaempferol, apigenin) [8, 16].

DPPH Radical Scavenging Activity

In order to evaluate the radical scavenging activity of the extracts, the DPPH (2,2-diphenyl-1-picryl-hydrazyl) assay was employed. 20 µL of diluted extracts were added to 980 µL DPPH solution (100 µM). The decrease in absorbance was measured after 30 min incubation period, at 517 nm (UV-VIS JASCO V-530 spectrophotometer). Quercetin and butylated hydroxytoluene (BHT) were used as standards. The inhibition percentage of the DPPH radical was determined after adding individual samples and using the following equation: $I = 100(A_c-A_s)/A_c$, (I - DPPH inhibition (%), A_c - absorbance of control sample, A_s - absorbance of tested sample). The anti-oxidant effects were also expressed as inhibitory concentration IC_{50}, and the values were calculated graphically. A lower value for IC_{50} reveals a higher antioxidant effect. All experiments were performed in triplicate [14, 15, 16].

Antibacterial activity

Microorganisms and culture conditions. Five aerobic bacterial strains were used, two Gram positive (*Staphylococcus aureus* - ATCC 49444 and *Listeria monocytogenes* - ATCC 19114) and three Gram negative (*Pseudomonas aeruginosa* - ATCC 27853, *Salmonella typhimurium* - ATCC 14028, and *Escherichia coli* - ATCC 25922). The microorganisms were purchased from Food Biotechnology Laboratory, Life Sciences Institute, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Romania. The bacteria were cultured on Müller-Hinton Agar and cultures were stored at 4°C [7, 15].

Microdilution method. A modified microdilution technique was employed. The bacterial cell suspensions were adjusted to a concentration of approximately 2.5 x 10^5 CFU/mL with sterile saline, and the inoculum was stored at +4°C. Minimum inhibitory concentrations (MICs) were determined by a serial dilution technique. A change from blue to pink indicates reduction of resazurin, thus bacterial growth. The MIC represents the lowest drug concentration that prevented the change of colour. The lowest concentration with no visible growth showing 99.5% killing of the original inoculum was defined as the minimum bactericidal concentration (MBC). The MBCs were determined by serial sub-cultivation of a 2 µL into microtitre plates containing 100 µL of broth per well and further incubation at 37°C, for 48 h. A 50% ethanol solution in water was used as negative control, whilst gentamycin (25 µL/well, 4 µg/mL) was used as positive control for bacterial growth [7, 15].

Statistical analysis

In all cases, the determinations were performed in triplicate. Data are presented as mean ± standard deviation (SD). Statistical analysis was carried out using Excel software package. One-way analysis of variance (ANOVA) and Duncan's multiple range tests were used to calculate significant differences ($p < 0.05$) between the means by SPSS (version 16.0). Correlation coefficients were determined by SPSS (version 16.0) Pearson correlation program.

Results and Discussion

The quantitative determination of the bioactive compounds

The use of different extraction methods and solvents may vary the quality and quantity of the active compounds [6]. Methanol/water and ethanol/ water mixtures were employed as solvents for the extraction of polyphenols and iridoids from the flowers of *A. reptans*.

The results showed that the amounts of total phenols, total flavonoids, total anthocyanins and total iridoids were higher in the ethanol extract. The amounts of active principles varied in accordance to the solvent and method used for extraction (maceration, reflux heating), with higher content in total polyphenols (20.86 - 24.11 mg GAE/g plant material), and iridoids (22.17 - 27.49 mg AE/g plant material) for ethanol extract of *A. reptans* flowers (Table I).

Table I

TPC, TFC, TAC and TIC in A. reptans flower extracts (± SD)

Extract	TPC (mg GAE/g)	TFC (mg RE/g)	TAC (mg CE/g)	TIC (mg AE/g)
Methanol extract (ME)	20.86 ± 0.53	12.38± 0.22	8.67± 0.19	22.17 ± 0.89
Ethanol extract (EE)	24.11 ± 0.57	13.75± 0.31	9.32± 0.26	27.49 ± 0.94

HPLC analysis of polyphenols

In order to determine the polyphenolic compounds from *A. reptans* extracts, an optimised LC/MS method for the identification and quantification of 18 polyphenols was employed. The extracts contain three cinnamic acid derivatives (caffeic acid, *p*-coumaric acid, ferulic acid), one flavonoid glycoside (quercitrin) and two free aglycones (luteolin, apigenin) [1, 7].

The HPLC chromatogram of *A. reptans* flower ethanol extract (Figure 1), and the amounts of polyphenols identified in the analysed extracts expressed as µg/g dried weight (dw) are presented (Table II).

Table II

Amounts of polyphenolic compounds in *A. reptans* extracts (µg/g dw)

No.	Polyphenolic Compounds	*m/z* Value	R_T ± SD (min)	*A. reptans* ME	*A. reptans* EE
1.	Caffeic acid	179	5.6 ± 0.04	34.39 ± 2.17	35.73 ± 2.72
2.	*p*-Coumaric acid	163	9.48 ± 0.08	26.02 ± 1.26	29.45 ± 1.99
3.	Ferulic acid	193	12.8 ± 0.10	20.15 ± 1.17	21.64 ± 1.73
4.	Quercitrin	447	23.64 ± 0.13	4.97 ± 0.81	5.52 ± 0.87
5.	Luteolin	285	29.64 ± 0.15	26.13 ± 1.93	27.81 ± 1.04
6	Apigenin	279	33.10 ± 0.17	23.56 ± 1.65	26.57 ± 1.49

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

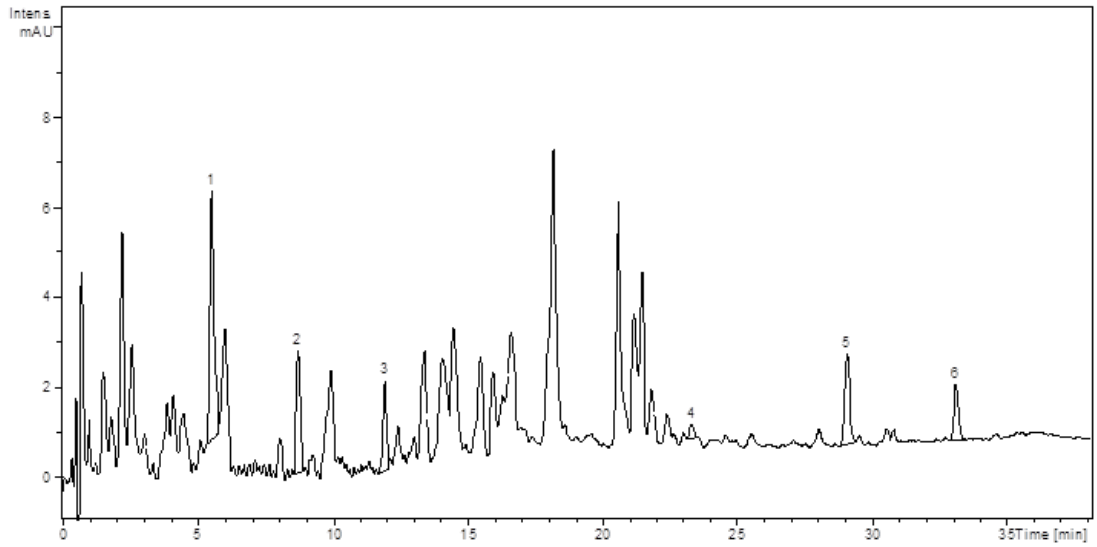

Figure 1.

HPLC chromatogram of *A. reptans* flower extract

Caffeic and p-coumaric acids were the main polyphenols found in the analysed extracts, with higher amounts in *A. reptans* ethanol extract (35.73 µg/g, and 29.45 µg/g respectively). Only quercitrin was identified and quantified as flavonoid glycoside in ME and EE, but in small amounts (4.97 - 5.52 µg/g). Two aglycones were determined in ME and EE from *A. reptans* flowers: luteolin

(26.13 μg/g, and 27.81 μg/g respectively), and apigenin (23.56 μg/g, and 26.57 μg/g respectively).
The obtained results revealed that *A. reptans* flowers contain high amounts of active compounds, particularly polyphenols and iridoids, which are generally used as important antioxidants and antimicrobial agents [1, 7].
To the best of our knowledge, this is the first report on polyphenols and iridoids from *A. reptans* flowers extracts; only the aerial parts of the plant were previously analysed by Ghita *et al*. [3], and smaller amounts of flavonoids (0.455 - 0.563% expressed in luteolin), and total iridoids (1.078 - 1.983% expressed in aucubin) were determined in the aerial parts collected from different Romanian regions.

Antioxidant activity assay

Concerning the evaluation of the antioxidant effects of the active compounds from *A. reptans* extracts *vs.* synthetic antioxidants, the DPPH method was employed. *A. reptans* flowers extracts reduced DPPH radical with different degrees of scavenging activity. A lower IC_{50} value indicates a higher bleaching effect, therefore better antioxidant properties. The results of the antioxidant activity evaluation by DPPH method are presented in Table III.
Quercetin was the strongest antioxidant (IC_{50} = 5.59 μg/mL). The extracts showed lower DPPH scavenging activity compared to the positive control compounds (quercetin, BHT). The ethanol extract of *A. reptans* flowers had the highest radical scavenging activity (49.35 ± 2.91 μg/mL), and the presence of significant high correlation ($p < 0.1$) for DPPH IC_{50} value *vs.* TPC and *vs.* TFC was determined. The antioxidant effects could be due to the high contents of polyphenols and flavonoids found in *A. reptans* extracts.

Table III

The antioxidant activity determined by DPPH method

Sample	IC_{50} (μg/mL)
A. reptans ME	83.16 ± 5.21
A. reptans EE	49.35 ± 2.91
Quercetin	5.59 ± 0.13
BHT	15.88 ± 1.06

Note: Values are the mean ± SD (n = 3). ME = methanol extract; EE = ethanol extract.

The antioxidant activities varied in the folowing order quercetin > BHT > *A. reptans* EE > *A. reptans* ME. The ethanol extract of *A. reptans* flowers demonstrated a high antioxidant activity ($IC_{50} \leq 50$ μg/mL) [1]. The biological activities of iridoids are mainly anti-inflammatory and anti-bacterial, thus their high content in vegetal product can complete the antioxidant effects of polyphenols. Therefore, *A. reptans flos* may have a wider potential than other flowers containing only polyphenolic compounds.

Antimicrobial activity

The results regarding the antibacterial assessment of *A. reptans* extracts and gentamicin (4 μg/mL) against Gram + and Gram – bacteria are presented in the Table IV. The antimicrobial effect was measured by microdilution assay, and we establish MIC (mg/mL) and MBC (mg/mL).

Table IV

The antimicrobial activity of *A. reptans* flower extracts

Bacterial Strains	MIC		MBC		Gentamycin
	A. reptans ME (mg/mL)	*A. reptans* EE (mg/mL)	*A. reptans* ME (mg/mL)	*A. reptans* EE (mg/mL)	(μg/mL)
S. aureus	1.56	0.78	3.12	1.56	0.038
P. aeruginosa	3.12	3.12	6.25	6.25	1.2
L. monocytogenes	6.25	6.25	12.5	12.5	0.076
E. coli	6.25	6.25	12.5	12.5	1.2
S. typhimurium	6.25	6.25	12.5	12.5	2.4

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

The MIC values obtained for the ethanol extract ranged from 0.78 to 6.25 mg/mL and from 1.56 to 6.25 mg/mL for the methanol extract of *A. reptans* flowers. Both extracts had comparable activities against some bacterial strains: *Pseudomonas aeruginosa, Listeria monocytogenes, Escherichia coli* and *Salmonella typhimurium*. *A. reptans* ethanol extract had the best antimicrobial activity against *S. aureus* (MIC value = 0.78 mg/mL and MBC value = 1.56 mg/mL), and against *Pseudomonas aeruginosa* (MIC value = 3.12 mg/mL and MBC value = 6.25 mg/mL). For both extracts we observed that the less susceptible strains were: *Listeria monocytogenes, Escherichia coli* and *Salmonella typhimurium*. According to Salvat *et al*., vegetal extracts with MIC value less than/or around 0.5 mg/mL indicate good antimicrobial effect [11]. Thus, our results showed moderate antibacterial activity for *A. reptans* flower extracts against the tested bacterial strains.

Conclusions

The comparative analysis of the extracts showed the presence of important biologically active compounds in *Ajuga reptans*. The research presents for the first time the content in natural compounds

(polyphenols and iridoids), and the biological activities of *A. reptans* flower extracts, with superior results revealed for the ethanol one. According to the obtained results, *Ajuga reptans* could be considered as a valuable source of natural products and it may have a good potential for possible applications in food and pharmaceutical industries.

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