

## LC/UV/MS PROFILE OF POLYPHENOLS, ANTIOXIDANT AND ANTIMICROBIAL EFFECTS OF *AJUGA GENEVENSIS* L. EXTRACTS

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### Abstract

The paper evaluated the polyphenolic content, antioxidant and antibacterial activities of *Ajuga genevensis* flower extracts. Total phenolic content, total flavonoid content and HPLC/UV/MS were used for the determination and quantification of polyphenols. Ethanol extracts were the richest in total phenols and flavonoids. Caffeic, *p*-coumaric and ferulic acids, hyperoside, quercitrin, luteolin and apigenin were identified by LC/MS in all samples. The DPPH assay was used to evaluate the antioxidant effects, and the results showed a better antioxidant activity for *A. genevensis* ethanol extract and a positive correlation between antioxidant effect and polyphenolic content. Antimicrobial activity was tested using dilution assays, minimal inhibitory concentration and minimal bactericidal concentration values were determined. The present study revealed that *A. genevensis* flowers contain bioactive compounds with good antioxidant and antibacterial properties for pharmaceutical and nutraceutical applications.

### Rezumat

Obiectivul studiului a constat în determinarea conținutului în polifenoli și evaluarea activității antioxidante și antibacteriene pentru extractele obținute din florile speciei *Ajuga genevensis*. Conținutul de polifenoli totali, flavonoide totale și o metoda HPLC/UV/MS au fost utilizate pentru analiza și identificarea compușilor polifenolici. Extractele etanolice au fost cele mai bogate în polifenoli totali și flavonoide. În toate probele au fost identificați: acidul cafeic, acidul *p*-cumaric, acidul ferulic, hiperozida, quercitrozida, luteolina și apigenina prin LC/MS. Pentru determinarea activității antioxidante s-a utilizat metoda cu DPPH, iar rezultatele au arătat efecte mai bune pentru extractul etanolic de *A. genevensis*, în concordanță cu conținutul în polifenoli. Acțiunea antimicrobiană a fost evaluată prin tehnica microdiluțiilor, cu determinarea concentrației minime inhibitorii și a concentrației bactericide. Rezultatele cercetărilor arată că florile de *A. genevensis* conțin compuși bioactivi cu efecte antioxidante și antibacteriene bune, cu aplicații potențiale în domeniul farmaceutic.

**Keywords:** *Ajuga genevensis* L., polyphenols, antioxidant, antimicrobial, HPLC/MS

### Introduction

The *Lamiaceae* family contains about 270 genera and 4000 species with a cosmopolitan distribution. *Ajuga* genus comprises evergreen, clump-forming rhizomatous perennial or annual herbaceous flowering plants. Six *Ajuga* species are mentioned in the spontaneous Romanian flora; they are used in our traditional medicine as anti-inflammatory, wound healing, hepatoprotective and anti-diarrhoeal. There are few data on the chemical composition of Romanian species, only *A. reptans* and *A. genevensis* have been briefly analysed [3, 5, 11, 14].

Various types of compounds within the genus have been reported by researchers worldwide, such as

phytoecdysteroids, *neo*-clerodane-diterpenes and diterpenoids, triterpenes, sterols, anthocyanidin-glucosides and iridoid glycosides, withanolides, flavonoids, triglycerides and essential oils [4, 6].

The active compounds of the same plant species might be different from one region to another in terms of chemistry, pharmacology and toxicology. Therefore, the aim of this research was to investigate the polyphenolic composition and to evaluate the therapeutic activity of *A. genevensis* flowers harvested from Romania, in order to improve the medicinal use.

## Materials and Methods

### Experimental

**Plant material:** the plants were harvested from wild populations from Cluj County on July 2014 at full flowering stage. The voucher specimen of the studied plants was stored in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, UMF "Iuliu Hațieganu" Cluj-Napoca, Romania, with the accession number AG-51.

**Extraction procedure:** the air-dried natural product was transformed to a powder and extracted with different solvents. Methanol extract was obtained using 0.5 g vegetal product and 50 mL 70% methanol for 30 min on a water bath at 60°C [10]; the tincture was prepared from 10 g natural product and 100 g 70% ethanol at room temperature [18].

The total phenolic content (TPC) of the extracts was measured using the Folin-Ciocalteu method, with some modifications [13]. The absorbance was measured at 760 nm, using a JASCO UV-VIS spectrophotometer. The standard curve was prepared by using different concentrations of gallic acid. TPC was expressed as mg gallic acid/g dry material plant (mg GAE/g plant material).

The total flavonoid content (TFC) was determined and expressed as rutin equivalents (mg RE/g plant material), using the method described in the Romanian Pharmacopoeia (X<sup>th</sup> Edition) [18]. The absorbance was measured at 430 nm.

### HPLC analysis of polyphenolic compounds

The identification and quantification of polyphenols was performed using an Agilent 1100 HPLC Series system equipped with UV detector, degasser, binary gradient pump, column thermostat, autosampler. The HPLC system was coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). The analysis was carried out in previously described conditions, using 18 polyphenolic compounds as standards: caftaric, gentisic, caffeic, chlorogenic, *p*-coumaric, ferulic, sinapic acids, hyperoside, rutin, isoquercitrin, myricetin, fisetin, quercitrin, quercetin, patuletin, luteolin, kaempferol, apigenin. The detection and quantification of polyphenols was performed in UV assisted by MS detection. Calibration curves in the 0.5 - 50 µg/mL range with good linearity ( $R^2 > 0.999$ ) for a five point plot were employed [1, 2, 16].

### DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to evaluate radical scavenging activity, by bleaching of the purple methanolic solution of the stable radical. The measure of antioxidant effect is the disappearance of the DPPH absorbance by the action of antioxidants. 20 µL of diluted extracts were added to 980 µL DPPH solution (100 µM). After 30 min of incubation, the decrease in absorbance was measured at 517 nm, using a UV-VIS JASCO V-530 spectrophotometer. Both hydrophilic and lipophilic

synthetic antioxidants, quercetin and butylated hydroxytoluene (BHT) were used as standards. The percentage inhibition of the DPPH radical after adding individual samples was calculated using the following equation:

$$I = 100(A_c - A_s)/A_c,$$

where I - DPPH inhibition (%),  $A_c$  - absorbance of control sample,  $A_s$  - absorbance of tested sample. The antioxidant activity was also expressed as inhibitory concentration  $IC_{50}$ , defined as the concentration of the sample required to cause a 50% decrease in initial DPPH radical absorbance.  $IC_{50}$  values in DPPH assay were calculated graphically. All experiments were performed in triplicate [1, 17].

### Antibacterial activity

#### Microorganisms and culture conditions

For the bioassay five aerobic bacterial strains were used, two Gram positive: *Staphylococcus aureus* (ATCC 49444), *Listeria monocytogenes* (ATCC 19114) and three gram negative: *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 14028) and *Escherichia coli* (ATCC 25922). All of the tested microorganisms were obtained from the Food Biotechnology Laboratory, Life Sciences Institute, University of Agricultural Sciences and Veterinary Medicine Cluj Napoca, Romania. The bacteria were cultured on Muller-Hinton Agar and cultures were stored at 4°C and subcultured once a month [9].

#### Microdilution method

In order to evaluate the antimicrobial activity, a modified microdilution technique was used. Bacterial species were cultured overnight in Tryptic Soy Broth (TSB) medium at 37°C. The bacterial cell suspensions were adjusted with sterile saline to a concentration of approximately  $2.5 \times 10^5$  CFU/mL (final volume of 100 µL per well). The inoculum was stored at +4°C. Determinations of minimum inhibitory concentrations (MICs) were performed by a serial dilution technique using 96-well microtiter plates. Into each well containing 100 µL of Tryptic Soy Broth (TSB), different extract dilutions and 10 µL of inoculum were added. The micro-plates were incubated for 24 - 48 h (37°C). The MIC of the samples was detected following the addition of 20 µL (0.2 mg/mL) of resazurin solution to each well. The plates were incubated again for 2 h (37°C). A change from blue to pink indicates the reduction of resazurin, thus the bacterial growth. The MIC was defined as the lowest drug concentration that prevented this colour change. The minimum bactericidal concentrations (MBCs) were determined by serial subcultivation of a 2 µL into microtiter plates containing 100 µL of broth per well and further incubation for 48 h at 37°C. The lowest concentration with no visible growth was

defined as MBC, indicating 99.5% killing of the original inoculum. Gentamycin was used as positive control for bacterial growth. A 50% ethanol solution in water was used as negative control [9].

#### Statistical analysis

In all cases, analyses were performed in triplicate. Data are presented as mean  $\pm$  standard deviation (SD). Statistical analysis was carried out using Excel software package.

## Results and Discussion

#### Polyphenolic compounds analysis

The amount of polyphenols and the composition of plant extracts are highly influenced by the type and polarity of solvent and extraction method [7].

In this study, ethanol/water and methanol/water mixtures were employed in order to evaluate the polyphenols from the *A. genevensis* flowers.

The total phenolic content and total flavonoid content of the extracts varied considering the extraction solvent and method used, with higher amounts in the ethanolic extract. The concentrations of total polyphenols (22.63 - 25.81 mg GAE/g plant material) and flavonoids (16.26 - 17.59 mg RE/g plant material) are presented in Table I.

An optimised HPLC/MS method for the separation and identification of 18 polyphenolic compounds (phenolic acids, flavonoid glycosides and aglycones) was used in order to analyse *A. genevensis* flower extracts. Seven polyphenols were identified and quantified in both extracts: three cinnamic acid derivatives (caffeic acid, *p*-coumaric acid, and ferulic acid), two flavonoid glycosides (hyperoside, quercitrin) and two flavones (luteolin, apigenin) by comparing retention times UV and MS data with those of the reference standards [1, 8].

**Table I**

TPC and TFC in *A. genevensis* flowers extracts ( $\pm$  SD)

Extract	TPC (mg GAE/g)	TFC (mg RE/g)
Methanolic extract (ME)	22.63 $\pm$ 0.61	16.26 $\pm$ 0.45
Ethanolic extract (EE)	25.81 $\pm$ 0.74	17.59 $\pm$ 0.52

The concentrations of the identified polyphenols in the analysed extracts are reported in Table II. The

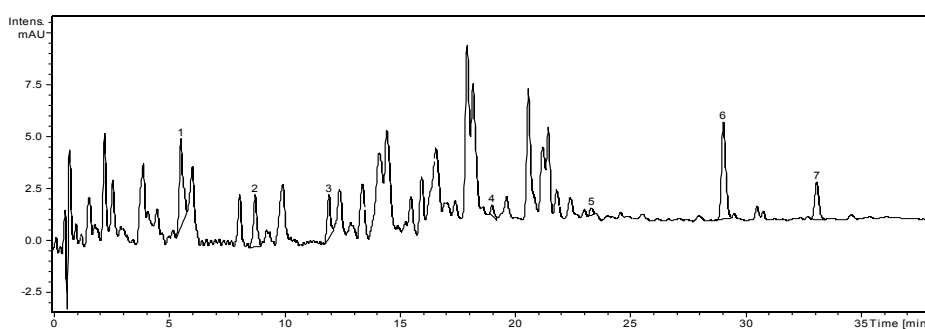
HPLC chromatogram of *A. genevensis* flower ethanol extract is presented in Figure 1.

**Table II**

Content of polyphenolic compounds in *A. genevensis* extracts ( $\mu$ g/100g)

Polyphenolic Compounds	<i>m/z</i> Value	<i>R<sub>T</sub></i> $\pm$ SD (min)	<i>A. genevensis</i> ME	<i>A. genevensis</i> EE
1. Caffeic acid	179	5.60 $\pm$ 0.04	1813 $\pm$ 5.46	2093 $\pm$ 7.61
2. <i>p</i> -Coumaric acid	163	9.48 $\pm$ 0.08	1143 $\pm$ 4.26	1645 $\pm$ 4.99
3. Ferulic acid	193	12.8 $\pm$ 0.10	1002 $\pm$ 1.17	1063 $\pm$ 3.72
4. Hyperoside	463	19.32 $\pm$ 0.12	309 $\pm$ 1.08	507 $\pm$ 2.63
5. Quercitrin	447	23.64 $\pm$ 0.13	179 $\pm$ 1.01	552 $\pm$ 2.87
6. Luteolin	285	29.64 $\pm$ 0.15	3217 $\pm$ 8.93	4132 $\pm$ 9.04
7. Apigenin	279	33.10 $\pm$ 0.17	2253 $\pm$ 7.65	2446 $\pm$ 8.49

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



**Figure 1.**

HPLC chromatogram of *A. genevensis* ethanol extract

Quantitative determinations revealed that luteolin and apigenin are the main compounds found in both extracts, with higher amounts in *A. genevensis* ethanolic extract (4132  $\mu$ g/100 g, and respectively 2446  $\mu$ g/100 g). Considering the flavonoid glycosides, only hyperoside and quercitrin were identified and quantified in methanolic and ethanolic extracts of

*A. genevensis* flowers in small quantities (309-507  $\mu$ g/100 g, and respectively 179 - 552  $\mu$ g/100 g).

Both ME and EE from *A. genevensis* flowers contain three phenolic acids; caffeic acid (1813 - 2093  $\mu$ g/100 g) was the compound found in the highest amount, followed by *p*-coumaric acid (1143 - 1645  $\mu$ g/100 g) and ferulic acid (1002 - 1063  $\mu$ g/100 g).

These results show that flowers of *A. genevensis* are rich in polyphenolic compounds, which are widely known as antioxidants and antimicrobial agents [1, 8]. No previous data were found regarding polyphenolic compounds analysis from *A. genevensis* flowers extracts, few researches studied only the aerial parts of the plant. A lower content in flavonoids was reported by Popescu *et al.* (0.8 - 1% expressed in rutin), and Ghita *et al.* (0.417 - 0.839% expressed in luteolin) in *A. genevensis herba* collected from different regions of Romania. Rosmarinic acid, oleanolic acid and maslinic acid were isolated and identified by Venditti *et al.* in aerial parts of *A. genevensis* growing in Italy [15].

#### Antioxidant activity assay

In order to evaluate the ability of *A. genevensis* extracts and synthetic antioxidants quercetin and BHT to donate the hydrogen atom, the stable free radical DPPH was used. Both extracts of *A. genevensis* flowers were able to reduce the DPPH radical with different degrees of scavenging activity. A higher bleaching effect reflected a better antioxidant effect, thus a lower IC<sub>50</sub> value.

The results obtained for the evaluation of the antioxidant activity using the DPPH bleaching assay are presented in Table III.

The positive control, quercetin, was the most potent antioxidant, with IC<sub>50</sub> value of 5.59 µg/mL. Examined extracts showed lower DPPH scavenging activity than the reference compounds, quercetin

and BHT. The highest radical scavenging activity was determined for *A. genevensis* ethanolic extract (46.45 ± 3.27 µg/mL), with positive correlation between scavenging activity on DPPH and total phenolic content and total flavonoid content. This indicates that polyphenolic compounds from *A. genevensis* flowers contribute to their antioxidant effects.

**Table III**

Antioxidant activity measured by DPPH method

Sample	IC <sub>50</sub> (µg/mL)
<i>A. genevensis</i> ME	72.08 ± 6.02
<i>A. genevensis</i> EE	46.45 ± 3.27
Quercetin	5.59 ± 0.13
BHT	15.88 ± 1.06

Note: Values are the mean ± SD (n = 3).

Considering the obtained results, the following order in antioxidant activities was established: *A. genevensis* ME < *A. genevensis* EE < BHT < quercetin. According to this method, the ethanolic extract of *A. genevensis* flowers showed a high antioxidant activity (IC<sub>50</sub> ≤ 50 µg/mL) [1].

#### Antimicrobial activity

The results of antibacterial evaluation of *A. genevensis* flower extracts and standard antibiotic gentamicin against both Gram-positive and Gram-negative bacteria are presented in Table IV. The *in vitro* antimicrobial potential was tested by the microdilution assay MIC (mg/mL) and MBC (mg/mL) were determined.

**Table IV**

Antimicrobial activity of *A. genevensis* extracts

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

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

The MIC values obtained ranged from 0.78 mg/mL to 6.25 mg/mL for ethanolic extract and from 1.56 to 6.25 mg/mL for methanolic extract of *A. genevensis* flowers. The results revealed that both extracts had similar effects against four bacterial strains: *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium*. The best antimicrobial activity was determined for *A. genevensis* ethanolic extract against *S. aureus*, with a MIC value of 0.78 mg/mL and a MBC value of 1.56 mg/mL, followed by *Pseudomonas aeruginosa* with a MIC value of 3.12 mg/mL and a MBC value of 6.25 mg/mL. Less sensitive strains were *Listeria monocytogenes*, *Escherichia coli* and *Salmonella typhimurium* for both *A. genevensis* extracts. Plant extracts with MIC values less than/or around 0.5 mg/mL indicate good antimicrobial effect, according to Salvat *et al.* [12].

The results of our study show moderate antibacterial effect for *A. genevensis* flower extracts against the tested bacterial strains.

#### Conclusions

The results of this study indicate an interesting polyphenolic composition of *Ajuga genevensis* flowers extracts. To our knowledge, our study presents for the first time the polyphenolic substances and the biological properties of *A. genevensis* flowers, with better results obtained for the ethanolic extract. The content in active polyphenols in correlation with good antioxidant and antimicrobial properties offer possibilities for further use of this natural product as an ingredient for functional food formulations.

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