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Ajuga reptans L. from Romanian flora as a promising candidate for novel food applications

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The increasing demand for natural ingredients with both functional and sensory properties has stimulated the exploration of underutilised plant species as potential sources for novel food applications. *Ajuga reptans* L. (Lamiaceae) remains relatively underexplored despite its phytochemical diversity and aromatic potential. This study evaluated *A. reptans* collected from four locations in Romania using an integrated analytical approach combining antioxidant screening with volatile and olfactometric profiling. Methanolic extracts were analysed for total phenolic content and antioxidant activity. Among the investigated samples, Sample 2 exhibited the highest phenolic content (8.67 ± 0.04 mg GAE/g d.w.) and consistently stronger antioxidant activity. Based on these results, this sample was selected for detailed characterisation of volatile and aroma-active compounds using HS-SPME-GC-MS and HS-SPME-GC-O. A total of 42 volatile compounds were identified, of which 16 were confirmed as odour-active contributors shaping the characteristic floral, herbal, and aromatic profile of the species. The integration of phytochemical and olfactometric data provides a multidimensional perspective on the relationship between chemical composition and aroma expression in *A. reptans*. Although exploratory in nature, the findings establish a baseline for further investigations into its suitability as a source of bioactive and aroma-active compounds relevant to novel food development.

Keywords *Ajuga reptans* L., Phytochemical profile, Volatile compounds, Aroma-active compounds, HS-SPME-GC-MS, HS-SPME-GC-O-MS, Antioxidant activity, Novel food potential

Aromatic and medicinal plants are widely recognised as important sources of bioactive compounds with potential applications in the food, pharmaceutical, and cosmetic industries[1]. In recent years, research has increasingly focused on plant-derived secondary metabolites, particularly phenolic compounds and terpenoids, have attracted increasing attention due to their functional properties and their relevance for the development of natural food ingredients and value-added products[2–5].

At the same time, the growing demand for natural and minimally processed foods has shifted research focus toward plant materials that combine functional properties with appealing sensory characteristics[6]. In this context, the volatile fraction of aromatic plants plays a central role in shaping flavour and aroma, which are key determinants of consumer perception and product acceptance. Recent trends in functional food development highlight the need to assess plant-based ingredients for both chemical and sensory properties to accurately gauge their usefulness[1].

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Chemical assays such as DPPH, ABTS, reducing power, and metal chelation are widely used in phytochemical research to provide an initial assessment of antioxidant-related properties. Although these methods are well established and allow comparison between plant materials, they primarily reflect chemical reactivity under controlled conditions and should therefore be interpreted as preliminary indicators of functional potential rather than direct evidence of biological effects[7–11].

In parallel, advances in analytical techniques have enabled more detailed characterisation of volatile compounds and their contribution to perceived aroma. Gas chromatography coupled with olfactometry (GC-O) represents a particularly valuable approach, as it allows the identification of odour-active compounds that significantly influence the sensory profile of plant-derived materials. The integration of chromatographic and olfactometric data has become increasingly important for linking chemical composition to aroma perception, particularly in the context of flavour science and novel food development[12–14].

The genus *Ajuga* (Lamiaceae) comprises several species characterised by a rich phytochemical composition, including phenolic compounds, iridoids, and terpenoids, and has been traditionally used for various medicinal purposes. Among these, *Ajuga reptans* L. is widely distributed in Europe and has attracted attention due to its reported phytochemical diversity and potential functional properties[2,4,5,8,11,15–17].

Despite these contributions, current knowledge on A. reptans remains incomplete, particularly regarding the relationship between its phytochemical composition and aroma-active volatile compounds. While several studies have focused on antioxidant-related properties and general phytochemical profiling, sensory-oriented investigations are still limited. To our knowledge, no thorough GC-O analysis of odour-active compounds in *A. reptans* originating from Romanian flora has been reported. This absence represents a significant gap in understanding the sensory potential of the species.

A.reptans has also gained increasing attention in the context of novel food research. Notably, extracts derived from *A. reptans* cell cultures have been included in the Union list of authorised novel foods under Regulation (EU) 2017/2470 and its amendments[18] reflecting their recognised safety and potential for use in food-related applications. However, wild-collected Romanian material has not yet been characterised from a sensory perspective, and its volatile and odour-active profile remains unexplored.

This aspect is of particular relevance for food-related applications, as the sensory profile of plant-derived ingredients is a critical factor influencing their acceptance and potential use in food formulations. In addition to compositional analysis, the identification of key aroma-active compounds provides valuable information for evaluating the suitability of plant materials as sources of flavour-relevant ingredients.

Therefore, the aim of the present study was to perform an integrated evaluation of *A.reptans* collected from different locations in Romania by combining antioxidant screening with volatile and olfactometric analysis. The study was designed as an exploratory investigation intended to provide a multidimensional baseline characterisation of the phytochemical profile, antioxidant-related properties, and aroma-active compounds of this species. By linking chemical composition with sensory-relevant attributes, this work contributes to the growing interest in underexplored plant species as potential candidates for the development of novel food ingredients[8,11,13,15–17].

Materials and methods

In this exploratory study, *Ajuga reptans* L. samples were collected during the flowering season from four locations in Neamț County, Romania. For each location, ten individual plants were harvested.

Three independent extract preparations were performed per location, and all measurements were conducted in duplicate to ensure internal consistency. Volatile and olfactometric analyses were carried out in nine replicates per sample.

The experimental design provides a controlled baseline characterisation of phytochemical and aroma profiles. The limited number of sampling sites and the single collection period means that the results should be interpreted as exploratory and not representative of broader ecological or seasonal variability.

Chemicals

Methanol (HPLC grade, CAS 67-56-1) was obtained from Scharlau Chemie S.A. (Barcelona, Spain). Deionised water was produced using a Millipore purification system. Folin-Ciocalteu reagent; sodium carbonate (≥ 99.99%, CAS 497-19-8); gallic acid (≥ 98.0%, CAS 149-91-7); ABTS (≥ 98%, CAS 30931-67-0); potassium persulfate (≥ 99.0%, CAS 7727-21-1); Trolox (97%, CAS 53188-07-1); DPPH (≥ 98%, CAS 1898-66-4); ferrous chloride (98%, CAS 7758-94-3); FerroZine™ reagent (97%, CAS 3451-29-6); EDTA (≥ 99.0%, CAS 60-00-4); phosphate buffer components (monobasic potassium phosphate, CAS 7558-80-7; dibasic potassium phosphate, CAS 7758-11-4); potassium ferricyanide (≥ 99.0%, CAS 13746-66-2); trichloroacetic acid (≥ 99.0%, CAS 76-03-9); and iron (III) chloride (> 99.99%, CAS 7705-08-0) were purchased from Sigma-Aldrich (Poznan, Poland)[9,19]. All reagents were of analytical grade and used as received.

Samples

Ajuga reptans L. was collected during the flowering stage (May 2018) from four natural populations located in the Eastern Carpathians, Western Moldavia, Romania: Potoci Village, Bicaz, Neamț County (Sample 1), Dodeni Village, Bicaz, Neamț County (Sample 2), Neamț Monastery, Vânători-Neamț Commune, Neamț County (Sample 3), and Ticoș-Floarea Village, Tașca Commune, Neamț County (Sample 4). For each location, ten individual plants were harvested. The aerial parts were air-dried at room temperature in shaded and well-ventilated conditions and subsequently ground to a fine powder[20].

The collected plant material was identified in the field by the corresponding author (Dr. Georgiana Gavril) using the authoritative botanical keys for the Romanian flora[21,22]. Identification was based on the diagnostic macro-morphological characteristics of *Ajuga reptans* L., particularly the presence of long rooting epigeal

stolons and bilateral stem pubescence, allowing reliable discrimination from closely related sympatric species. A voucher specimen was not deposited in a publicly accessible herbarium. The exact sampling localities, collection period, phenological stage, botanical identification procedure, and analytical methodology are reported in detail to facilitate traceability and reproducibility of the study.

The present study was conducted using dried aerial parts collected at the flowering stage. The use of a single phenological stage was intended to minimize biological variability and ensure reliable comparisons among the investigated populations.

Sample preparation

Dried plant material was prepared as described in Sect. 2.2. The aerial parts of *A. reptans* (5 g), previously ground, were extracted with absolute methanol (100 mL) in a water bath at 40 °C for 6 h. The extraction was performed in three successive cycles to ensure exhaustive recovery of soluble constituents. The combined extracts were filtered through a 0.45 µm membrane to remove particulate matter and stored at − 20 °C in sealed vials until further analysis. Methanol was selected as the extraction solvent due to its high efficiency in recovering phenolic compounds and its widespread use in comparative phytochemical screening studies. The limitations associated with solvent selectivity are acknowledged, as methanol does not extract all water-soluble or highly polar constituents[2,4,5].

Phenolics and antioxidant properties

Total phenolics content

Total phenolic content was determined using the Folin-Ciocalteu method. An aliquot of extract (0.05 mL) was mixed with distilled water (0.05 mL) and Folin-Ciocalteu reagent (0.2 mL, 1:5 dilution). After 3 min, sodium carbonate solution (1 mL, 10%) was added, and the mixture was incubated for 30 min. Absorbance was measured at 725 nm. Results were expressed as mg gallic acid equivalents (GAE) per g dry weight (d.w.). All measurements were performed in triplicate[2,4,5].

ABTS radical scavenging activity

The ABTS radical scavenging activity was determined using a modified ABTS decolorisation assay. The $ABTS^{\bullet+}$ radical cation was generated by reacting a 7 mmol/L ABTS solution with 2.45 mmol/L potassium persulfate and subsequently diluted with distilled water to an absorbance of 0.7 ± 0.05 at 734 nm.

Sample (5 µL) was mixed with $ABTS^{\bullet+}$ solution (0.25 mL), and absorbance was recorded after 30 min[19]. Radical scavenging activity was calculated as:

$$\text{Scavenging } (\%) = [1 - (A_s/A_C)] \text{ x } 100$$

Where, A*c* represents the absorbance of the control and A_s the absorbance in the presence of the sample. Results were expressed as mg Trolox equivalents per g dry weight (d.w.).

DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the literature described methods. DPPH solution (100 µM) was prepared in methanol (96%) and freshly prepared prior to analysis. The reaction mixture consisted of DPPH solution (0.25 mL) and extract (5 µL). Absorbance was measured at 517 nm after 10 min[9]. Radical scavenging activity was calculated as:

$$\text{Scavenging } (\%) = [1 - (A_s/A_C)] \text{ x } 100$$

Where, A_c represents the absorbance of the control and A_s the absorbance in the presence of the sample. Results were expressed as mg Trolox equivalents per g dry weight (d.w.).

Chelating power

Chelating power activity was determined according to previously described methods. Extract (5 mL) was mixed with $FeCl_2$ solution (0.1 mL, 2 mM) and FerroZine™ reagent (0.2 mL, 5 mM). The mixture was incubated at room temperature for 10 min. Absorbance was measured at 562 nm. Chelating activity was calculated as:

$$\text{Inhibition } (\%) = [1 - (A_s/A_C)] \text{ x } 100$$

Where, A*c* represents the absorbance of the control and A_s the absorbance in the presence of the sample. Results were expressed as mg EDTA equivalents per g dry weight (d.w.).

Reducing power

Reducing power was determined according to the literature described methods. Extract (0.04 mL) was mixed with phosphate buffer (0.04 mL, 200 mmol/L, pH 6.6) and potassium ferricyanide solution (0.04 mL, 1% w/v). The mixture was incubated at 50 °C for 20 min. Trichloroacetic acid (0.04 mL, 10% w/v) was then added, followed by centrifugation at 25 x g for 10 min. The supernatant (0.1 mL) was mixed with distilled water (0.1 mL) and $FeCl_3$ solution (0.04 mL, 0.1% w/v), and absorbance was measured at 700 nm. Reducing power was expressed as mg Trolox equivalents per g dry weight (d.w.)[10]. This assay reflects the electron donating capacity of the extract and provides a complementary indication of antioxidant behaviour under controlled chemical conditions.

HS-SPME-GC-MS conditions

Volatile compounds were analysed using headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS), a widely used technique for volatile profiling of plant matrices[23,24]. Extract (10 µL) was placed in a 20 mL glass vial and equilibrated at 80 °C for 30 min. A DVB/CAR/PDMS fibre (50/30 µm, Supelco, Bellefonte, PA, USA) was used for analyte extraction. Desorption was performed for 2 min in the GC injection port at 250 °C using a CTC Analytics autosampler (Agilent Technologies, Santa Clara, CA, USA).

The system consisted of an Agilent 6890 N gas chromatograph coupled to an Agilent 5975B mass-selective detector. Separation was carried out on an HP-5MS column (30 m x 0.25 mm internal diameter x 0.25 µm film thickness; Agilent Technologies). The oven temperature program was as follows: 40 °C (5 min), increased at 10 °C/min to 300 °C, and held for 2 min. Helium was used as carrier gas at a flow rate of 1.0 mL/min. The injector temperature was set to 250 °C. Mass spectra were acquired in SCAN mode over the range *m/z* 45–350. The MS source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. All samples were analysed in triplicate. These conditions were selected to ensure broad coverage of volatile constituents and reproducible chromatographic performance.

HS-SPME-GC-O-MS conditions

Aroma-active compounds were analysed using headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry and olfactometry (HS-SPME-GC-O-MS), which enables correlation of chromatographic peaks with sensory perception. Extract (10 µL) was placed in a 20 mL glass vial and equilibrated at 80 °C for 30 min. A DVB/CAR/PDMS fibre (50/30 µm; Supelco, Bellefonte, PA, USA) was used for analyte extraction. Thermal desorption was performed in the GC injection port at 250 °C for 2 min. The fibre was subsequently cleaned in a needle heater (Agilent Technologies, Santa Clara, CA, USA) at 270 °C for 2 min[23–25].

Analyses were carried out using an Agilent 7890 N gas chromatograph coupled to an Agilent 5977D mass detector (Agilent Technologies, Santa Clara, CA, USA). Separation was achieved on an HP-5MS column (30 m x 0.25 mm internal diameter x 0.25 µm film thickness). The oven temperature program was as follows: 40 °C (5 min), increased at 10 °C/min to 300 °C, and held for 2 min. Helium was used as carrier gas at a flow rate of 2.02 mL/min. Mass spectra were acquired in SCAN mode over the range *m/z* 45–350. The MS source and quadrupole temperatures were set at 230 °C and 150 °C, respectively.

Olfactometric analysis was performed using an OP275 olfactory detection port (GL Sciences, Germany). The sniffing port was connected via a four-port splitter using fused silica capillary transfer lines (0.25 mm internal diameter). The auxiliary gas flow was set to 5 mL/min, and the transfer line temperature was maintained at 110 °C. Humidified air was supplied to prevent drying of the nasal mucosa and to minimise analyte condensation.

Olfactometric evaluation was conducted by three trained panellists, who recorded odour descriptors for eluting compounds. Retention indices were calculated, and compound identification was supported by mass spectral libraries, retention index comparison, and the Flavornet database[26]. All analyses were performed in nine replicates per sample. Although the panel size was limited, the use of trained assessors is considered acceptable for exploratory GC-O studies focused on detecting odour-active compounds.

Optimisation of analytical method

The best HS-SPME extraction conditions were optimised through a two-step approach involving fibre selection and experimental design. Initially, three SPME fibres, polyacrylate (PA, 85 µm), polydimethylsiloxane (PDMS, 100 µm), and divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 µm) (Supelco, Bellefonte, PA, USA), were evaluated for analyte adsorption at 80 °C for 30 min. In addition, the DVB/CAR/PDMS fibre was tested at 40 °C for 30 min. The total chromatographic peak area was used as the response criterion. All fibres were conditioned prior to use according to the manufacturer's instructions.

Further optimisation was performed using a face-centred central composite design (FCCCD) combined with multiple linear regression (MLR) to evaluate the effects of extraction temperature and extraction time on the chromatographic response. Two independent variables were considered: extraction temperature (40, 60, and 80 °C) and extraction time (15, 20, and 30 min), each at three coded levels (− 1, 0, + 1).

The experimental design consisted of 15 runs, including two replicates at the central point (60 °C, 20 min) to estimate pure error and assess model reproducibility. Model fitting and statistical analysis were performed using MODDE 6.0 software (Umetrics, Umeå, Sweden). This optimisation ensured that extraction conditions maximised analyte recovery while maintaining method reproducibility.

Analysis of volatile compounds

Volatile and aroma-active compounds were analysed using HS-SPME-GC-MS and HS-SPME-GC-O-MS under comparable extraction conditions. Olfactometric evaluation was performed by three trained panellists using an olfactory detection port, and odour descriptors were recorded. Retention indices were calculated for all detected compounds using a homologous series of n-alkanes.

Compound identification was based on a multi-criteria approach, including mass spectral matching with the NIST library, comparison of experimental and literature retention indices, bibliographic data, and standard injection, where available. Confidence levels were assigned based on the agreement between multiple identification criteria, including mass spectral matching (NIST library), comparison of experimental and literature retention indices, bibliographic data, and standard injection, where available[23,24]. According to the criteria that a candidate fulfils, a percentage of confidence in the identification will be assigned according to Table 1.

Confirmed NIST match > 70%	Confirmed NIST match > 80%	Confirmed bibliography	Confirmed RI	Confirmed standard	Confidence level
✓					> 70%
✓		✓			> 75%
✓			✓		
✓		✓	✓		> 80%
	✓				
	✓	✓			> 85%
	✓		✓		
	✓	✓	✓		> 90%
				✓	100%

Table 1. Criteria for the confidence level of detected compounds.

Semi quantitative analysis

Semi-quantitative analysis of detected compounds was performed based on relative peak area normalisation. Results are expressed as the percentage contribution of individual peak areas to the total chromatographic area. This approach provides an estimate of the relative abundance of volatiles without requiring external calibration.

Statistics

Results are expressed as mean ± standard deviation (SD) of three independent replicates. Statistical differences among samples were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Differences were considered statistically significant at $p < 0.05$. The same statistical approach was applied to evaluate differences among extraction conditions during HS-SPME optimisation. Statistical analyses were performed using Microsoft Excel and GraphPad Prism software.

Results and discussion

The phytochemical and aroma profile of *A. reptans* was investigated using a combined analytical approach integrating antioxidant assays with volatile and olfactometric analyses. The exploratory design of the study and the limited sampling scope mean that the observed variability should be interpreted as indicative rather than representative of broader ecological or population-level patterns[8,11].

This integrated approach enabled a comprehensive characterisation of both bioactive compounds and aroma-active volatile constituents, providing complementary insights into the functional and sensory properties of the investigated samples[27]. The results highlight the presence of compounds with potential antioxidant activity alongside a diverse volatile profile contributing to the overall aroma signature of *A. reptans*[8,11].

The application of HS-SPME-GC-MS is widely recognised for its reliability in volatile profiling of plant matrices[23,24]. The integration of chromatographic and olfactometric data allowed the identification of key odour-active compounds, supporting the relevance of this species as a potential source of functional and sensory-active ingredients. Overall, the findings provide a baseline characterisation of the investigated samples and support further research on the phytochemical and aroma-related potential of *A. reptans*.

Phenolic content and antioxidant properties

The total phenolic content and antioxidant capacity of *A. reptans* extracts were evaluated using complementary spectrophotometric assays, including DPPH and ABTS radical scavenging activity, reducing power, and metal chelating activity. These assays primarily reflect chemical reactivity under controlled experimental conditions and should not be interpreted as direct indicators of biological efficacy or physiological relevance[2,4,5,8,10,11,19].

The obtained results indicate variability among samples, reflecting differences in phytochemical composition and potential functional properties of the investigated plant material. The combined use of multiple assays provides a broader characterisation of antioxidant behaviour, capturing different mechanisms such as radical scavenging, electron donation, and metal ion chelation[8,11]. Such variability is consistent with previously reported differences in plant-derived phytochemical profiles[4,5,28]. Table 2 presents the total phenolic content and antioxidant properties of the methanolic extracts of *A. reptans*[2,4,5,8,11].

After evaluating the four samples, Sample 2 showed the highest levels for most parameters, total phenolic content, ABTS and DPPH scavenging activity, and reducing power, except for metal chelating activity. The total phenolic content of Sample 2 reached 8.67 mg GAE/g d.w., which is higher than the value reported for ethanol extracts of *A. chamaecistus* subsp. *scoparia* (2.64 mg GAE/g d.w.), but lower than that reported for methanolic extracts of *A. chamaecistus* subsp. *tomentella* (18 mg/g d.w.) and previously reported values for *A. reptans* (17.6–22.6 mg/g d.w.)[2,4,5,10,19].

A similar trend was observed for antioxidant activity. The ABTS radical scavenging capacity was comparable to that reported for *A. chamaepitys*, whereas DPPH radical scavenging activity was lower. The reducing power values were also lower than those reported for Romanian *A. reptans* extracts (61 mg TE/g d.w.), which may be associated with differences in phenolic content[2,5,8,10,11,19]. Based on these results, Sample 2 was selected for further analysis of volatile and aroma-active compounds using HS-SPME-GC-MS and HS-SPME-GC-O-MS[23,24].

Activity	Sample 1	Sample 2	Sample 3	Sample 4
Total phenolics content [mg GE/g d.w.]	7.45 ± 0.06^{a}	8.67 ± 0.04^{b}	7.85 ± 0.07^{c}	8.59 ± 0.09^{b}
Ability to quench ABTS•$^{+}$ [mg TE/g d.w.]	7.09 ± 0.32^{a}	8.33 ± 0.14^{b}	7.58 ± 0.08^{c}	7.96 ± 0.17^{d}
Ability to scavenge DPPH• [mg TE/g d.w.]	6.85 ± 0.16^{a}	8.86 ± 0.28^{b}	7.92 ± 0.25^{c}	7.74 ± 0.09^{c}
Chelating power [mg EDTA/g d.w.]	3.64 ± 0.31^{a}	3.99 ± 0.14^{a}	2.45 ± 0.33^{b}	5.36 ± 1.02^{d}
Reducing power [mg TE/g d.w.]	18.65 ± 0.35^{a}	23.24 ± 0.46^{b}	20.66 ± 0.09^{c}	22.95 ± 0.47^{b}

Table 2. Phenolics and antioxidant properties of methanolic extracts of *Ajuga reptans* L. EDTA= methylenediaminetetraacetic acid; GE- gallic acid equivalents; TE= Trolox equivalents (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid equivalents), d.w. = dry weight; a, b, c, d = different letters within the same row indicate statistically significant differences among samples according to Tukey's multiple comparison test ($p < 0.05$).

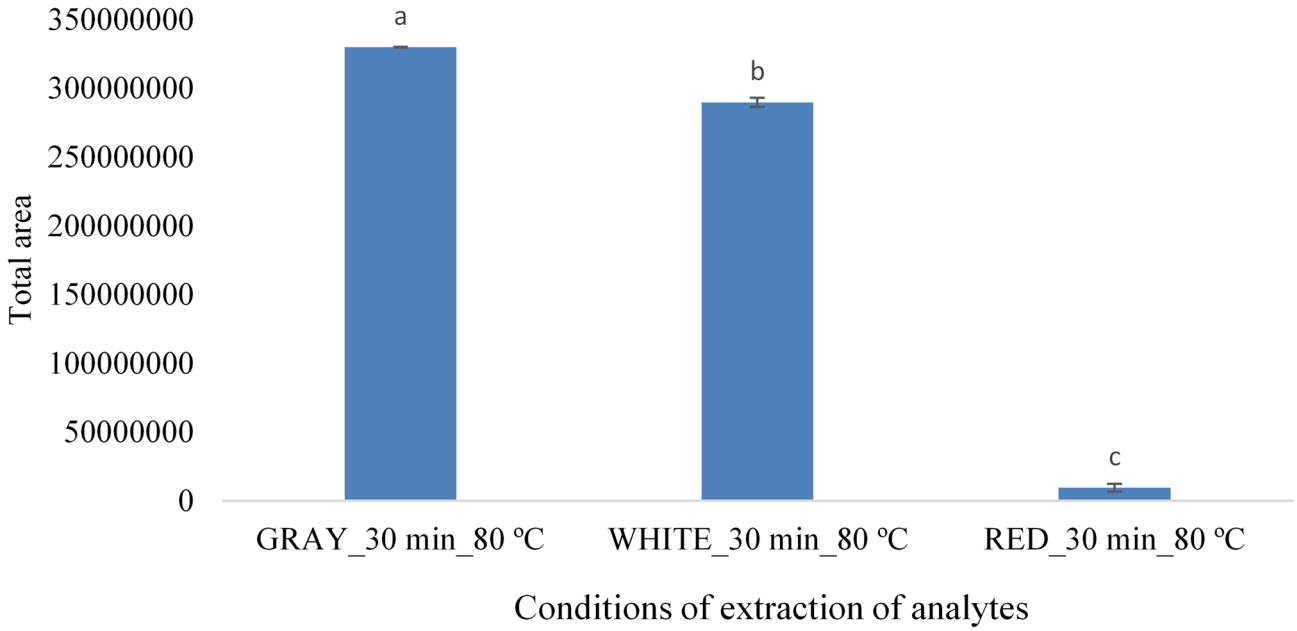

Fig. 1. **a**. Optimisation of HS-SPME extraction conditions based on fibre type and extraction temperature. Total chromatographic peak area was used as a response parameter. Different letters indicate statistically significant differences among extraction conditions according to Tukey's multiple comparison test ($p < 0.05$). **b** Results of contour plot for experimental optimisation of SPME-GC-MS method.

Optimisation of analytical method

Figure 1a illustrates the optimisation of HS-SPME-GC-MS and HS-SPME-GC-O-MS analytical conditions. Among the tested fibres, the DVB/CAR/PDMS fibre (50/30 µm) provided the highest chromatographic response, indicating superior extraction efficiency compared to polyacrylate (PA) and polydimethylsiloxane (PDMS) fibres. This behaviour can be attributed to the bipolar nature of the DVB/CAR/PDMS coating, which enables the adsorption of a broader range of volatile compounds, including both polar and non-polar analytes[23,24].

Regarding extraction conditions, higher temperatures (80 °C) resulted in an increased number of detected peaks and higher overall signal intensity. This trend is consistent with previous reports and can be explained by enhanced analyte volatility and improved mass transfer to the fibre coating. The optimisation results confirm that extraction temperature and adsorption time exert strong and synergistic effects on analyte recovery, supporting the selection of 80 °C and 30 min as optimal conditions for subsequent analyses.

The response surface contour plots (Fig. 1b) indicate that the relationship between extraction conditions (temperature and adsorption time) and the total chromatographic peak area is best described by a non-linear model. The highest peak areas were obtained at elevated temperatures and longer adsorption times, with optimal experimental conditions corresponding to 80 °C and 30 min. Variations in extraction parameters resulted in significant differences in peak area, confirming the strong influence of both factors on analyte adsorption efficiency.

Experimental data (Table 3) further demonstrated that increasing temperature and contact time enhanced the total chromatographic response, reflecting improved analyte volatility and fibre adsorption capacity. Statistical evaluation confirmed an excellent model fit ($R^2 = 0.93$, $Q^2 = 0.88$, model validity = 0.87, reproducibility = 0.91), with a non-significant lack of fit ($p > 0.05$), indicating strong explanatory and predictive performance. Response

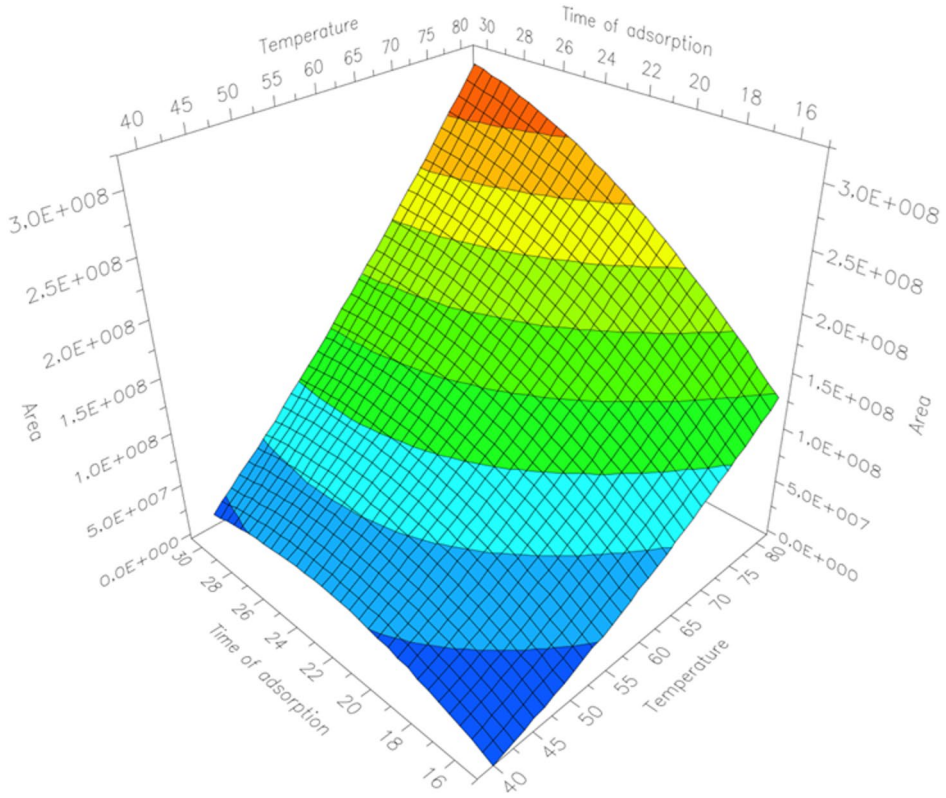

Fig. 1. (continued)

Run	Temperature (°C)	Temperature (coded)	Time (min)	Time (coded)	Peak Area
1	40	−1.0	15	−0.5	2.63×10^6
2	40	−1.0	15	−0.5	2.63×10^6
3	80	+1.0	15	−0.5	1.54×10^8
4	80	+1.0	15	−0.5	1.49×10^8
5	60	0.0	20	0.0	1.54×10^8
6	60	0.0	20	0.0	1.49×10^8
7	80	+1.0	20	0.0	1.65×10^8
8	40	−1.0	30	+1.0	2.63×10^7
9	40	−1.0	30	+1.0	2.63×10^7
10	60	0.0	30	+1.0	1.22×10^8
11	80	+1.0	30	+1.0	3.30×10^8
12	80	+1.0	30	+1.0	3.13×10^8
13	60	0.0	15	−0.5	6.05×10^6
14	40	−1.0	20	0.0	3.10×10^6
15	80	+1.0	30	+1.0	3.21×10^8
16	80	+1.0	30	+1.0	3.23×10^8
17	80	+1.0	30	+1.0	3.16×10^8

Table 3. Experimental design matrix (FCCCD) for method optimisation.

surface analysis revealed synergistic effects between temperature and time, particularly above 70 °C and 25 min, where peak area values exceeded 3.2×10^8. The model-predicted optimum (78 °C, 28 min) yielded a theoretical peak area of 3.28×10^8, closely matching the experimentally determined value ($3.23 \times 10^8 \pm 0.04 \times 10^8$), with a deviation below 2%.

These results confirm the validity of the optimised extraction conditions, which were subsequently applied in the analysis of volatile and aroma-active compounds.

Analysis of volatile compounds

A total of 42 volatile compounds were identified in Sample 2 of *A. reptans* (Table 4), reflecting a chemically diverse profile comprising aldehydes, acids, monoterpenes, sesquiterpenes, and fatty acid derivatives. Aromatic aldehydes such as benzaldehyde and its derivatives were among the detected compounds, contributing to characteristic sweet and almond-like notes, while carboxylic acids, including benzoic acid, were also identified, indicating potential roles in plant secondary metabolism. An example of the obtained chromatogram of Sample 2 analysed by HS-SPME-GC-MS is presented in Fig. 2.

Terpenoid compounds represented a major fraction of the volatile profile. Monoterpenes such as carvenone and thymol are typically associated with characteristic minty and herbal aromas, while sesquiterpenes including α-cubebene, β-elemene, and α-zingiberene contribute to woody and spicy notes commonly reported in essential oils. In addition, compounds such as α-cedrene and cedren-13-ol are known constituents of cedarwood-type aroma profiles. The application of HS-SPME-GC-MS is widely recognised for its reliability in volatile profiling of plant matrices[23,24].

No	KI	Compound	CAS	Match
1	985	Benzaldehyde	000100-52-7	91
2	1156	Benzaldehyde, 3-ethyl-	034246-54-3	70
3	1196	Benzoic acid	000065-85-0	90
4	1208	1-Dodecene	000112-41-4	89
5	1257	Carvenone	000499-74-1	90
6	1311	Thymol	000089-83-8	91
7	1357	alpha-Cubebene	017699-14-8	95
8	1396	beta-Longipinene	039703-25-8	70
9	1398	alpha-Cedrene	000469-61-4	98
10	1401	beta-Elemene	000515-13-9	95
11	1414	Benzenebutanal, gamma,4-dimethyl	004895-19-6	94
12	1416	alpha-Gurjunene	000489-40-7	96
13	1459	beta-Humulene	000116-04-1	70
14	1461	Cedren-13-ol, 8-	018319-35-2	70
15	1468	beta-*cis*-Caryophyllene	000087-44-5	90
16	1488	Valerena-4,7(11)-diene	351222-66-7	86
17	1493	gamma-Elemene	029873-99-2	91
18	1504	cis-Eudesma-6,11-diene	194607-93-7	70
19	1507	alpha-Curcumene	000644-30-4	95
20	1508	alpha-Zingiberene	000495-60-3	90
21	1516	gamma-Muurolene	030021-74-0	97
22	1521	beta-Bisabolene	000495-61-4	99
23	1535	cis-Calamenene	072937-55-4	91
24	1540	beta-Guaiene	000088-84-6	95
25	1573	Nerolidol 2	1000285-43-6	91
26	1621	Longipinocarveol, trans-	1000159-36-5	70
27	1626	alpha-Corocalene	020129-39-9	99
28	1648	tau-Cadinol	005937-11-1	83
29	1671	Alloaromadendrene oxide-(1)	1000156-12-8	70
30	1680	Longiverbenone	064180-68-3	70
31	1763	Xanthorrhizol	030199-26-9	92
32	1845	Neophytadiene	000504-96-1	91
33	1900	Nonadecane	000629-92-5	90
34	1919	Hexadecanoic acid, methyl ester	000112-39-0	98
35	1969	Gerany-p-cymene	055968-43-9	95
36	1995	Pentylcurcumene	1000413-12-5	96
37	2014	7-*epi*-*cis*-Sesquisabinene hydrate	58319-05-4	70
38	2056	Caparratriene	172549-29-0	74
39	2075	Thunbergol	025269-17-4	91
40	2119	Ledane	028580-43-0	90
41	2131	9-Octadecenoic acid, methyl ester	001937-62-8	97
42	2200	Docosane	000629-97-0	90

Table 4. Results of qualitative analysis of sample 2 of *A. reptans* extract obtained by HS-SPME-GC-MS.

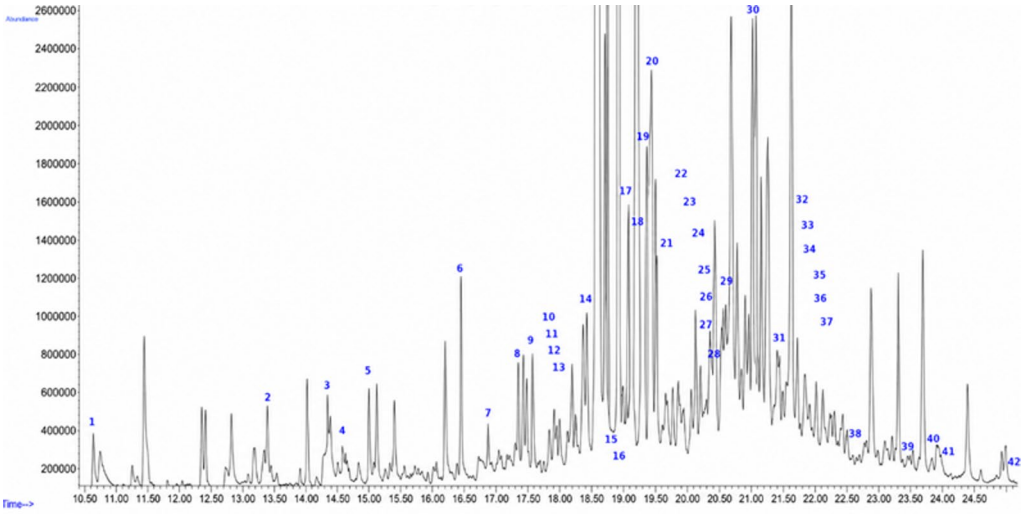

Fig. 2. Chromatogram of Sample 2 of *A. reptans* extract obtained by HS-SPME-GC-MS.

The presence of fatty acid derivatives, such as methyl 9-octadecenoate, further reflects the contribution of lipid-related metabolites to the overall volatile composition. Overall, the identified compounds contribute to a complex aroma profile and highlight the chemical diversity of *A. reptans*, supporting its potential as a source of bioactive and aroma-active constituents.

Analysis of odours

Odour analysis was conducted using HS-SPME-GC-O-MS, which identified eight classes of chemical compounds comprising forty-one individual components. Additionally, various aromas were detected through olfactometric evaluation (Table 5).

Semi-quantitative analysis

The pie charts in Fig. 3 illustrate the distribution of volatile compounds identified in *A. reptans* extracts according to odour descriptors and chemical classes, providing an integrated view of both sensory and compositional characteristics. The odour profile (Fig. 3a) is predominantly characterised by floral notes (56%), followed by herbal (37%), aromatic (5%), and mineral (2%) descriptors. This distribution suggests a complex and balanced sensory profile, in which floral and green-herbal attributes represent the dominant olfactory perception. Such profiles are typically associated with plant-derived materials rich in terpenoid compounds and may contribute to the overall sensory acceptability of the extract.

The compositional distribution (Fig. 3b), sesquiterpenes represent the major fraction (73%) of the identified volatile compounds, indicating a strong contribution of higher molecular weight terpenoids to the overall volatile profile. Monoterpenes account for 8%, while alcohols and esters each represent 4%, followed by aldehydes (3%), carboxylic acids (2%), and aromatic compounds (1%). The predominance of sesquiterpenes is consistent with their known role in shaping woody, spicy, and resinous aroma notes, while monoterpenes are generally associated with fresher, more volatile sensory attributes. This predominance aligns with previous findings on plant volatile composition[29].

The relationship between chemical composition and odour perception highlights the importance of terpenoid compounds in defining the aromatic signature of *A. reptans.* The high proportion of sesquiterpenes, together with the presence of selected monoterpenes and oxygenated derivatives, suggests a synergistic contribution to the observed floral and herbal sensory profile[30]. Such interactions between volatile compounds are well documented and can significantly influence the overall perception of aroma[31–33].

The present results are based on instrumental and olfactometric analyses performed under controlled experimental conditions. Although these findings provide valuable insight into the aroma profile, further studies are required to assess the contribution of these compounds under real food or biological conditions. Previous studies have reported that *A. reptans* contains phytoecdysteroids with insecticidal activity, particularly affecting larval development in certain insect species. At the same time, the absence of specific diterpenoid compounds has been noted in some reports, while related varieties such as *A. reptans* var. *atropurpurea* have been described as non-toxic and rich in ecdysteroids of potential biological relevance. These findings support the chemical complexity of the genus *Ajuga* and underline the importance of further investigations into both the bioactive and sensory-related properties of *A. reptans*[15–17].

The combined analysis of odour descriptors and chemical composition highlights the complexity of the volatile profile of *A. reptans* and supports its potential relevance as a source of aroma-active compounds, warranting further investigation in relation to its functional and sensory applications. The application of HS-SPME-GC-MS is widely recognised for its reliability in volatile profiling of plant matrices[23,24]. These findings justify further investigation into its functional and sensory applications.

Compound	Aroma	Aroma class	KI
Alcohols			
Cedren-13-ol, 8-	-	-	1461
Longipinocarveol, trans-	-	-	1621
7-epi-cis-Sesquisabinene hydrate	-	-	2014
Thunbergol	-	-	2075
Aldehydes			
Benzaldehyde	almond	floral	985
Benzaldehyde, 3-ethyl-	sweet	aromatic	1156
Benzenebutanal, gamma,4-dimethyl	-	-	1414
Alkanes			
Nonadecane	-	-	1900
Docosane	-	-	2200
Alkenes			
1-Dodecene	-	-	1208
Neophytadiene	-	-	1845
Aromatics			
Xanthorrhizol	-	-	1763
Ledane	-	-	2119
Pentylcurcumene	herb	herbs	1995
Carboxylic acid			
Benzoic acid	balsamic	aromatic	1196
Esteres			
Hexadecanoic acid, methyl ester	-	-	1919
9-Octadecenoic acid, methyl ester	-	-	2131
Monoterpenes			
Thymol	thyme	herbs	1311
Gerany-p-cymene	solvent, gasoline	mineral	1969
Sesquiterpenes			
alpha-Cubebene	herb	herbs	1357
beta-Elemene	-	-	1401
alpha-Cedrene	cedar, wood	aromatic	1398
beta-Longipinene	-	-	1396
gamma-Elemene	green, wood	aromatic	1493
alpha-Curcumene	herb	herbs	1507
alpha-Zingiberene	spice, fresh	herbs	1508
beta-Bisabolene	balsamic	aromatic	1521
gamma-Muurolene	herb, wood, spice	aromatic, herbs	1516
cis-Calamenene	-	-	1535
beta-Guaiene	wood, spice	aromatic, herbs	1540
Nerolidol 2	-	-	1573
beta-Humulene	wood	aromatic	1459
cis-Eudesma-6,11-diene	-	-	1504
Alloaromadendrene oxide-(1)	-	-	1671
Valerena-4,7(11)-diene	-	-	1488
alpha-Corocalene	-	-	1626
alpha-Gurjunene	wood, balsamic	aromatic	1416
tau-Cadinol	-	-	1648
Caparratriene	-	-	2056
Longiverbenone	-	-	1680
beta-*cis*-Caryophyllene	-	-	1468

Table 5. Results of olfactometric analysis of sample 2 of *Ajuga reptans L.* extract obtained by HS-SPME-GC-O-MS.

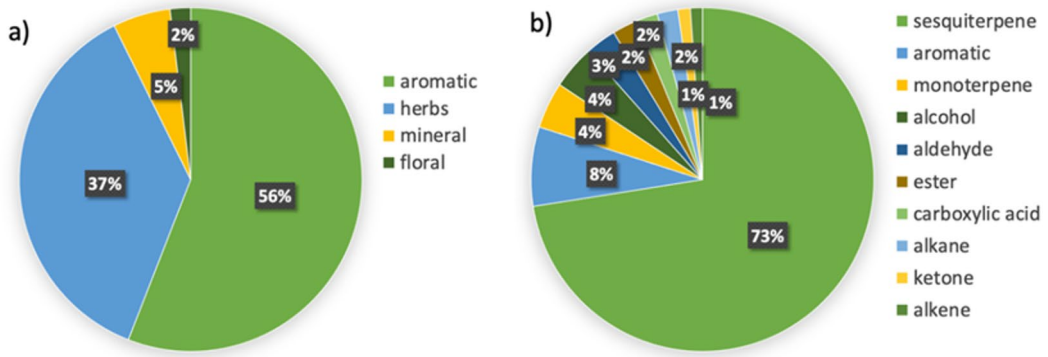

Fig. 3. A simple pie chart: Share of compounds classified according to (**a**) odour classes; (**b**) chemical classes.

Conclusions

This study provides, to the best of our knowledge, the first integrated characterisation of volatile and aroma-active compounds in *A. reptans* from Romanian flora, combining phytochemical screening with advanced chromatographic and olfactometric approaches. The applied analytical framework, integrating antioxidant assays, HS-SPME-GC-MS, and HS-SPME-GC-O, enabled a comprehensive assessment of both functional and sensory-related properties of the investigated samples. Comparative analysis showed that Sample 2 had the highest phytochemical content and antioxidant activity, except for metal chelating capacity. These differences highlight the influence of sample origin on the chemical profile, even within a limited sampling design.

The volatile profile of the selected sample was characterised by the identification of 42 compounds, encompassing a broad range of chemical classes, with a predominance of sesquiterpenes. The associated odour profile was mainly defined by floral and herbal notes, indicating a complex and balanced sensory signature. The integration of chromatographic and olfactometric data further enabled the identification of aroma-active contributors, emphasising the relevance of combining analytical and sensory approaches for a more accurate characterisation of plant-derived volatiles. The application of HS-SPME-GC-MS is widely recognised for its reliability in volatile profiling of plant matrices.

The predominance of terpenoid compounds, particularly sesquiterpenes, together with the presence of oxygenated derivatives, underlines the chemical complexity of *A. reptans* and its potential as a source of aroma-active constituents. In addition, the antioxidant screening provides an initial indication of the presence of compounds with functional relevance, although further targeted investigations are required to elucidate their specific roles.

While the findings are derived from an exploratory experimental design and a limited number of samples, they establish a robust baseline for the phytochemical and aroma-oriented characterisation of *A. reptans*. The combined methodological approach employed in this study demonstrates the value of integrating chemical and sensory analyses in the investigation of plant materials. These findings contribute to the growing body of research on underutilised plant species and support the potential of *A. reptans* as a candidate for the development of innovative food ingredients with combined functional and sensory relevance.

Future research should focus on expanding the sampling range, including seasonal and environmental variability, validating sensory perception in relevant application contexts, and further investigating the contribution of individual compounds to both aroma and functional properties. Such studies would contribute to a deeper understanding of the chemical-sensory relationships in *A. reptans* and support its potential applications.

Ethical considerations

In this study, the flowering aerial parts of *Ajuga reptans* L. were collected from naturally occurring populations in Romania exclusively for scientific research purposes. Harvesting was limited to the aerial flowering parts, while the stolons responsible for vegetative propagation were left undisturbed. *A. reptans* is abundant in the spontaneous flora of Romania, and the sampling locations were selected based on the natural abundance of the species. Consequently, harvesting did not threaten the reproduction or survival of the species in its native habitat. *A. reptans* is not listed on the IUCN Red List of Threatened Species. Collection of the plant material did not require specific permits or collection licenses under the applicable regulations in force at the time of collection.

Data availability

All data on the "*Ajuga reptans* L. from Romanian flora as a promising candidate for novel food applications" that support the findings of this study are included within this paper. Also, all data that support the findings of this study are available on request from the authors, in especially from corresponding authors: [georgiana.gavril@incdsb.ro](mailto: georgiana.gavril@incdsb.ro) (Georgiana-Luminita Gavril), [cnerin@unizar.es](mailto: cnerin@unizar.es) (Cristina Nerin), [nahar@ueb.cas.cz](mailto: nahar@ueb.cas.cz) (Lutfun Naharf).

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Author contributions

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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