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Pulmonaria mollis: Rediscovering a forgotten edible plant through phytochemical profiling and bioactivity assessment

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ABSTRACT

Pulmonaria species were historically valued as traditional remedies for respiratory ailments and as wild food, yet their culinary and medicinal applications have largely been forgotten. Among them, *Pulmonaria mollis* remains underinvestigated, with scarce phytochemical and pharmacological data. This study aimed to fill this gap by investigating the phenolic composition and biological activities of methanol extracts from the aerial parts (PMA) and roots (PMR) of *P. mollis*. UHPLC–MS^4 Orbitrap analysis identified 59 compounds, mostly phenolic acids. Quantitative profiling by UHPLC/DAD/(−)HESI-MS/MS showed PMA was rich in rosmarinic acid (4508.27 μg/g), *p*-hydroxybenzoic acid (605.70 μg/g), and caffeic acid (325.42 μg/g), whereas PMR contained higher levels of salvianolic acids A (2311.09 μg/g) and B (4697.98 μg/g). Along with significant antioxidant activity confirmed by standard spectrophotometric assays, where PMR showed slightly stronger radical scavenging capacity, both extracts effectively protected DNA from hydroxyl- and peroxyl-induced oxidative damage. In enzyme inhibition assays, both extracts demonstrated α-amylase inhibitory effects, while PMR showed particularly potent α-glucosidase inhibition (IC_{50} = 14.00 μg/mL), outperforming acarbose. Moderate antibacterial and antifungal activities were also observed, with PMR generally more effective, whereas cytotoxicity testing indicated selective activity against A431 (IC_{50} values of 178 ± 11 μg/mL and 64 ± 3 μg/mL for PMA and PMR, respectively) cells without affecting normal cells. Overall, the findings highlight *P. mollis* as a promising candidate for development into functional foods and nutraceuticals aimed at chronic disease prevention, providing valuable phenolic compounds and biological properties that support its potential use as an alternative to the commonly used *Pulmonaria officinalis*.

1. Introduction

Pulmonaria, commonly known as lungwort, is a genus of flowering plants belonging to the family Boraginaceae. It is native to Europe and Western Asia, with 10–18 recognized wild species depending on taxonomic treatment. One of the less commonly studied representatives of this genus is *Pulmonaria mollis* J.F. Wolff ex Hornem, also known as soft lungwort. This perennial herbaceous species is characterized by its typically unspotted leaves, in contrast to the more widely known *Pulmonaria officinalis*. *P. mollis* is distributed throughout large parts of Europe, where it inhabits deciduous forests and forest edges, ranging from lowland areas to mountainous regions at elevations up to approximately 1300 m. An interesting characteristic of this and many other species in the genus *Pulmonaria* is the change in flower color from pink to blue depending on the stage of flowering (Josifovic, 1974).

In recent years, global vegetable consumption has been steadily increasing, reflecting a growing awareness of the importance of healthy diets (Fidiyawati et al., 2024). Given that plants of the genus *Pulmonaria* are harvested in early spring, they may play a potentially important role in replenishing essential nutrients and bioactive compounds after the long winter months, when dietary diversity is often reduced. Historically, leaves of *P. officinalis* were consumed as a wild vegetable and

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brewed into herbal tea in various regions of Central and Eastern Europe. Young leaves of *P. obscura* were occasionally incorporated into spring soups, purées, and mixed salads, and were particularly used in early spring, when food shortages were common in colder regions (Łuczaj & Szymański, 2007). Moreover, recent studies have explored the incorporation of *P. mollis* extracts into food products, such as bread, highlighting its potential as a functional food ingredient (Borieva et al., 2019; Chauhan et al., 2022).

A review of publications in major databases from 2000 to 2024 indicates that studies on the chemical composition of the genus *Pulmonaria* are still scarce. Recent studies have primarily identified rosmarinic acid as the main compound in the aerial parts of *P. officinalis*, while literature on the chemical composition of the root remains scarce (Neagu et al., 2018). Krzyzanowska-Kowalczyk et al. (2018) identified 45 compounds in hydro-methanol extracts of *P. officinalis*, including caffeic acid derivatives and flavonols, with seasonal variation in phenolic content. Unlike the lesser-known *P. mollis*, *P. officinalis* has been traditionally used since the 16th century for various ailments. Following the doctrine of signatures, its spotted leaves were associated with lung diseases, supporting its use in herbal teas for respiratory conditions such as bronchitis, influenza, and cough (Krzyanowska-Kowalczyk et al., 2021). Other reports indicate its use in treating stomach, duodenal ulcers, and kidney diseases (Neagu et al., 2018). Those effects may be related to the high content of gamma-linolenic acid, which is transformed into precursors of prostaglandin PGE-1 and thromboxane TXA-1, known for their anti-inflammatory properties (Guil-Guerrero et al., 2003; Hernandez, 2016). Some sources highlight the traditional use infusions or decoctions of *P. officinalis* in wound healing on the Balkan Peninsula (Jarić et al., 2018). Supporting this traditional application, Pielesz and Paluch (2012) demonstrated that a hydrogel formulated from the aerial parts of *P. officinalis* is effective in managing wounds with profuse exudate. The research conducted by Neagu et al. (2018) demonstrated that both the aqueous and ethanolic extracts of the plant *P. officinalis* exhibit a significant inhibitory activity against acetylcholinesterase and tyrosinase, suggesting that this plant can be a potential natural source of bioactive compounds useful for human health, especially in neurodegenerative disorders.

To date, no study has employed such a broad range of methods to simultaneously investigate both the chemical composition and diverse biological activities of the *Pulmonaria* species, while almost no information is available on the phytochemistry of *P. mollis*, underscoring the novelty and significance of the present work. Taken together, the investigation of *P. mollis* addresses an important gap in the phytochemical and pharmacological characterization of this underexplored species. By combining detailed profiling of phenolic compounds with comprehensive biological evaluation, including antioxidant, DNA-protective, antidiabetic effects via α-amylase and α-glucosidase inhibition assays, antimicrobial, and cytotoxic assays, this study provides a multidimensional insight into its bioactivity. Such an integrative approach highlights the biomedical advantages of *P. mollis*, particularly its potential for chronic disease prevention through functional foods and nutraceuticals. Moreover, these findings support its consideration as a valuable alternative to the well-known *P. officinalis*, thereby expanding the therapeutic and dietary relevance of the genus *Pulmonaria*.

2. Materials and Methods

2.1. Chemicals

All standards and reagents used for determination the content of different classes of phenolic compounds and antioxidant activity of investigated extracts was provided by Sigma Aldrich (Deisenhofen, Germany) and Alfa Aesar (Karlsruhe, Germany). Roth (Karlsruhe, Germany) supplied all solvents necessary for high-performance liquid chromatography (HPLC) analyses, along with deoxyribonucleic acid extracted from salmon sperm used in antigenotoxic testing. The nutritional mediums used in microbiological studies were provided by the Torlak Institute of Virology, Vaccines, and Sera (Belgrade, Serbia).

2.2. Plant materials and extraction procedure

P. mollis was collected at the middle of March 2024 in the flowering phase in the village of Veliko Krčmare, near Kragujevac, Serbia. The plant material was identified by colleagues from the Institute of Biology and Ecology, Faculty of Science, University of Kragujevac, and has been deposited in the Herbarium of the Department of Biology and Ecology, under voucher specimen 140/24. The aerial part of the plant and the root were separated and dried at room temperature in the dark. The dried and crushed aerial part of the plant (60 g) and the root (90.78 g) were extracted with methanol by the maceration process, and the obtained extracts were filtered and evaporated with a vacuum evaporator (RV 10 basic, IKA, Staufen, Germany). After removing the solvent, 8.91 g of dried crude extract from the aerial parts and 13.90 g of *P. mollis* roots were obtained, corresponding to 14.85 % and 12.62 % w/w, respectively. The extracts were stored at 4 °C until further analysis.

2.3. Spectrophotometric determination of phenolic compounds in extracts

The total phenolic content was determined by the Folin–Ciocalteu method (Singleton et al., 1999) and expressed as mg gallic acid equivalents per gram of dry extract (mg GAE/g). Total flavonoids and flavonols were quantified after reaction with $AlCl_3$ (Quettier-Deleu et al., 2000; Yermakov et al., 1987) and expressed as mg quercetin equivalents (mg QUE/g). The total content of phenolic acids in the extracts was determined using Arnow's reagent, following the procedure described in the Polish Pharmacopoeia and detailed by Matkowski et al. (2008), and expressed as mg caffeic acid equivalents (mg CAE/g). Condensed tannins (mg GAE/g dry extracts) were determined based on the difference between total and unprecipitated phenolics after reaction with phloroglucinol and formaldehyde (Scalbert et al., 1989). Monomeric and total anthocyanins were quantified spectrophotometrically using the pH differential method (Giusti & Wrolstad, 2001), which is based on the color change of anthocyanins at various pH levels. The content of monomeric and total anthocyanins was calculated in cyanidin-3-glycoside equivalents (mg Cy 3-glc/g dry extract), as detailed by Srećković et al. (2020). All spectrophotometric assays were conducted in triplicate, and results are presented as mean values ± standard deviation. The complete experimental protocols for the spectrophotometric determination of phenolic compounds, including all assay conditions, are available in the Supplementary Material (S1).

2.4. LC/MS qualitative analysis of aerial parts and roots of P. mollis methanol extracts

Phenolic compounds were analyzed using a UHPLC system (Accela, ThermoFisher Scientific) with a Synchronis C18 column (100 × 2.1 mm, 1.7 μm) at 30 °C. The mobile phase consisted of 0.1 % acetic acid in water (A) and acetonitrile (B), with a flow rate of 300 μL/min and a gradient elution from 5 % to 95 % B over 15 min. Injection volume was 25 μL. Detection was performed using an LTQ Orbitrap mass spectrometer (ThermoFisher Scientific) equipped with a HESI-II ion source in negative mode. Source voltage was 4000 V, capillary temperature 300 °C, and CID energy 35 eV. Nitrogen was used as carrier and auxiliary gas (35 AU and 7 AU, arbitrary units). Compounds were identified based on accurate mass, retention time, and MSn fragmentation, with data processed using Xcalibur software (v2.1) and literature comparison. Additionally, a precise mass search was performed for their deprotonated molecules ($[M-H]^-$) and their MS^4 fragmentation patterns.

2.5. UHPLC-DAD/(−)HESI-MS/MS quantitative analysis of aerial parts and roots of P. mollis methanol extracts

Phenolic compounds of interest were quantified using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Bremen, Germany), which was equipped with a triple quadrupole mass spectrometer (TSQ Quantum Access Max, Thermo Fisher Scientific, Basel, Switzerland) that utilized electrospray ionization as described Mišić et al. (2015). Chromatographic separation was performed on a Hypersil Gold C18 column (50 × 2.1 mm, 1.9 μm) at 30 °C using a mobile phase of 0.1 % acetic acid in water (A) and acetonitrile (B), with a flow rate of 0.4 mL/min. MS parameters included an evaporator temperature of 450 °C, voltage of 4000 V, and collision energy of 30 eV (Argon). For quantification purposes mass traces of targeted compounds m/z were measured by using selected reaction monitoring (SRM) scanning mode with the compound-specific transitions of parent and product ions. Quantification was performed using the external standard method. The working standard solution was then created by mixing the stock solutions of the pure compounds in methanol to achieve a final concentration of 100 μg/mL. Methanol was utilized for the dilution of the working solution, effectively achieving a range of calibration levels from 20 to 0.002 μg/mL.

2.6. Antioxidant activity evaluation of aerial part and root methanol extracts of P. mollis

Antioxidant activity of methanolic extracts (aerial and root parts) of *P. mollis* was assessed using six complementary assays: phosphomolybdenum, DPPH, ABTS, ferrous ion chelation, lipid peroxidation inhibition, and reducing power. The phosphomolybdenum method was applied according to Prieto et al. (1999), with absorbance measured at 695 nm and results expressed as mg Trolox/g extract. DPPH and $ABTS^{\bullet+}$ radical scavenging assays were performed following Kumarasamy et al. (2007) and Re et al. (1999), respectively, with absorbances recorded at 517 and 734 nm, and antioxidant activity expressed as IC_{50} values. Metal-chelating activity was assessed using ferrozine/$FeSO_4$ method as described by Yau Yan (2006), and expressed as IC_{50}. Lipid peroxidation inhibition was evaluated in a linoleic acid emulsion via the thiocyanate method (Hsu et al., 2008), and results are expressed as IC_{50}. Reducing power was measured following Oyaizu (1986), and results were expressed as mg Trolox/g extract. Detailed protocols for these antioxidant assays are available in Srećković et al. (2020). Full protocols for the antioxidant activity assays, with complete details of experimental conditions, are available in the Supplementary Material (S2).

2.7. Inhibitory effects of extracts on α-amylase and α-glucosidase activities

Firstly, serial twofold dilutions of the extracts (250 μL), starting from a concentration of 4 mg/mL, were prepared in three sets. Then, 250 μL of α-amylase (dissolved in 0.02 M phosphate buffer, pH 6.9; 2U/mL) was added. The mixture was pre-incubated at 37 °C for 10 min. Subsequently, 250 μL of a 1 % starch solution prepared in 0.02 M sodium phosphate buffer (pH 6.9) was added, and the reaction mixture was further incubated at 37 °C for an additional 5 min. The enzymatic reaction was terminated by adding 500 μL of dinitrosalicylic acid (DNS) reagent. The mixtures were incubated in boiling water for 5 min and then cooled to room temperature. The reaction mixture was then diluted with 4 mL of distilled water, and the absorbance was measured at 540 nm using a spectrophotometer (Poovitha & Parani, 2016).

Extracts were evaluated for α-glucosidase inhibitory activity according to the method given by Poovitha and Parani (2016) with modifications. Serial twofold dilutions of the extracts were prepared in three sets for each sample, with a volume of 0.4 mL and an initial concentration of 2 mg/mL. To each test tube, 0.2 mL of α-glucosidase (0.067 U/mL) was added, and the tubes were incubated at 37 °C for 10 min. Following incubation, 0.4 mL of *p*-NPG (5 mM) was added, and the incubation was continued for an additional 5 min under the same conditions. The reaction was terminated by adding 2 mL of Na_2CO_3 (0.2 M), and the absorbance was measured at 405 nm.

The same procedures were applied to the standard drug, acarbose. The percentage of inhibition was calculated using the following equation. Additionally, the results are presented as IC_{50} values.

2.8. Antimicrobial activity

The antibacterial activity of methanolic extracts from aerial (PMA) and root (PMR) parts of *P. mollis* and chloramphenicol were evaluated against five Gram-negative and six Gram-positive bacterial strains. Antifungal effects of extracts and nystatin were tested against nine fungal species, including eight molds and *Candida albicans*. Bacterial strains were obtained from the Institute for Public Health, and fungal strains from the Laboratory for Microbiology, Faculty of Science, University of Kragujevac, Serbia. Nutrient agar and Sabouraud Dextrose Agar were used for bacterial and *C. albicans* cultivation, respectively. Potato Dextrose Agar was used for molds. The microdilution method in 96-well plates was applied to determine minimum inhibitory concentrations (MIC) following Sarker et al. (2007). Müller–Hinton broth and Sabouraud Dextrose Broth were used for bacteria and fungi, respectively. Microorganism suspensions were prepared at 1.0×10^6 CFU/mL (colony forming units/mL) for bacteria and *C. albicans*, and 5.0×10^4 CFU/mL for molds in accordance with NCCLS recommendations (CLSI, 2008; Nccls, 2002). Extracts, chloramphenicol, and nystatin solutions were added to wells at concentrations ranging from 0.156 to 20 mg/mL for extracts and 0.3125–40 mg/mL for reference drugs. Resazurin (0.6 mg/mL) was added as a bacterial growth indicator. MIC was defined as the lowest concentration with no visible growth or color change.

2.9. DNA protective activity of P. mollis extracts against damage induced by hydroxyl and peroxyl radicals

The protective effects of PMA and PMR on DNA against hydroxyl and peroxyl radicals were evaluated according to previously described methods (Srećković et al., 2020). Different concentrations of extracts and phenolic compounds were first prepared in methanol, followed by complete evaporation of the methanol. Subsequently, 45 μL of phosphate buffer (0.2 M, pH 7.4) and 10 μL of salmon sperm DNA (Carl Roth GmbH, Karlsruhe, Germany), at a concentration of 5 mg/mL and suspended in 0.9 % NaCl, were added to the dried extracts and standard compounds. Rosmarinic acid and catechin were used as reference standards, each at a concentration of 100 μM.

Hydroxyl radicals were generated by adding 0.9 μL of $FeSO_4$ (180 mM) and 3.6 μL of H_2O_2 (600 mM). After incubating the mixture for 15 min, the treated samples were subjected to electrophoresis. Peroxyl radicals were produced by adding 10 μL of 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 200 mM). The mixtures were vortexed and then incubated at 37 °C for 4 h. DNA samples were electrophoresed on a 1 % agarose gel containing ethidium bromide (10 mg/mL) in 1 × TAE (Tris-acetate-EDTA) buffer for DNA band visualization. Images of the gels were captured using a UV transilluminator (Vilber Lourmat, France) at 365 nm, and analyzed with ImageJ software (version 1.48, Softonic International, Barcelona, Spain).

2.10. Assessment of extract biocompatibility using the MTT assay

Cell viability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, as described by Srećković et al. (2020) with some modifications. The cytotoxicity of PMA and PMR extracts was performed on immortalized Balb C/3T3 mouse fibroblasts (Balb C/3T3) and their SVT2-transformed line (SVT2), human epidermoid carcinoma cells (A431), and immortalized human keratinocytes (HaCaT). Cells were cultured in Dulbecco's Modified Eagle's Medium

(DMEM) supplemented with 2 mM L-glutamine, 10 % fetal bovine serum (FBS), penicillin, and streptomycin at 37 °C in a 5 % CO_2 incubator. For MTT test, cells were plated in 96-well plates at 3×10^3 cells/well for immortalized lines and 2×10^3 cells/well for cancer cells. After 24 h, PMA and PMR were added to the cells (1–200 μg/mL). Following 48 h incubation, MTT solution (final concentration 0.5 mg/mL) was added, and plates were incubated for 4 h. Formed formazan crystals were dissolved in 2-propanol containing 0.01 N HCl, and absorbance was measured at 570 nm using a Microbeta Wallac 1420 plate reader. Cell survival was expressed as the percentage of viable cells in treated samples compared to controls. Controls included untreated cells and cells supplemented with identical volumes of buffer. Each sample was tested in three independent analyses, each carried out in triplicate.

2.11. Statistical analysis

The results are presented as the mean ± standard deviation (SD) of three independent experiments. For the determination of IC_{50} values, the percentage of inhibition at each tested concentration of the extracts was calculated, and the data were fitted using a sigmoidal dose-response inhibition curve in OriginPro 8 (OriginLab Corporation, Northampton, MA, USA). Statistical significance between group means was assessed by one-way analysis of variance (ANOVA), also performed using OriginPro 8.

3. Results and discussion

3.1. Phytochemical constituents of P. mollis methanol extracts

Determining the total phenolic content in *P. mollis* methanol extracts is crucial, as these compounds are known for their antioxidant properties and potential health benefits, including anti-inflammatory and anticancer effects. Understanding the phenolic profile of extracts can contribute to evaluating their nutritional value and therapeutic potential (Rahman et al., 2021). The amounts of total phenols, flavonoids, flavonols, phenolic acids, condensed tannins, total and monomeric anthocyanins in the methanolic extracts of the aerial part and root of *P. mollis* are presented in Table 1. The methanol extract of the root of *P. mollis* contained a slightly higher amount of total phenolic compounds (92.11 mg GAE/g) than the aerial part (79.85 mg GAE/g). However, the flavonoid content in the aerial part extract was 52.19 mg QE/g, while flavonoids were not quantified in the root extract using the applied method. Although both extracts were rich in phenolic acids and condensed tannins, the root extract demonstrated notably higher concentrations of these phenolic constituents. As expected, the content of monomeric and total anthocyanins was also higher in the aerial part (12.84 and 17.41 mg Cy-3-glc/g extract, respectively) compared with the root (5.77 and 8.55 mg Cy-3-glc/g extract, respectively). These results indicate a distinct distribution of phenolic compounds between the aerial part and the root of *P. mollis*. Both extracts exhibited a high phenolic content, with the root extract being particularly rich in total phenolics, condensed tannins, and phenolic acids. Based on the available literature, there is a limited number of studies investigating the chemical composition of *P. mollis*, in contrast to *P. officinalis*, which has been more extensively studied in this regard. A study conducted by Neagu et al. (2018) confirmed that ethanol extracts of *P. officinalis* are richer in total phenolics, flavonoids, and proanthocyanidins compared to aqueous extract. Furthermore, in the research conducted by Ivanova et al. (2005), *P. officinalis* demonstrated high antioxidant activity, with a TEAC value of 2.02 ± 0.14 mM and a quercetin equivalent of 673.39 ± 9.92 μM, indicating its significant content of bioactive compounds.

To identify the various compounds, present in the PMA and PMR extracts, the UHPLC–MS^4 Orbitrap metabolic fingerprinting technique was used. Based on the reviewed literature, this study represents the first comprehensive phenolic characterization of *P. mollis* aerial part and root extracts, providing valuable insights into its chemical composition and potential biological significance. The identification process was based on the mass of the deprotonated molecule $[M-H]^-$, as well as detailed fragmentation patterns obtained from MS^2, MS^3, and MS^4 analyses. A total of 59 compounds were identified, with their key MS data summarized in Table 2. Among these, phenolic acids were the most abundant group, comprising 40 compounds, primarily derivatives of caffeic acid. Both extracts contained a diverse array of phenolic acids characteristic of the *Salvia* genus, including danshensu, salvianolic acids A, C, C1, C2, C3, F1, and F2, as well as salviaflaside, sagecoumarin, methyl salvianolate H/I, and salpalaestinin (Piątczak et al., 2021; Srećković et al., 2022). The presence of these compounds in *P. mollis* may be attributed to shared biosynthetic pathways within the Lamiaceae family, convergent evolution, or ecological adaptations (Krzyzanowska-Kowalczyk et al., 2018). Additionally, 19 flavonoids were detected in the PMA extract, consisting of 9 flavonoid-*O*-glucosides and 10 flavonoid aglycones. The main *O*-glucoside flavonoids were quercetin and luteolin *O*-glucosides. In contrast, the PMR extract contained only luteolin 7-*O*-hexoside, which was present in significant quantities based on the calculated peak area in the chromatogram, whereas its presence in the PMA extract was at the detection limit.

Using a triple quadrupole mass spectrometer, a total of 19 compounds were quantified in the aerial part extract and 18 compounds in the root extract. The results of the phenolic compound quantification are presented in Table 3. Based on the obtained data, the PMA extract contained a higher total quantity of almost all identified compounds in comparison with PMR. The dominant compounds quantified in the PMA extract include the phenolic acids rosmarinic acid (4508.27 μg/g), *p*-hydroxybenzoic acid (605.70 μg/g), caffeic acid (325.42 μg/g), salvianolic acid B (480.37 μg/g), and salvianolic acid A (605.54 μg/g), as well as the flavonoids rutin (386.24 μg/g), quercetin 3-*O*-glucoside (784.70 μg/g), and apigenin (119.55 μg/g). Contrary to the results obtained in our study, the research conducted by (Krzyanowska-Kowalczyk et al., 2021) reported that rutin and caffeic acid was only present in trace amounts in the extracts derived from the aerial parts of *P. officinalis* and *P. obscura,* collected in Poland. Additionally, the aforementioned study found that the concentration of certain compounds, such as rosmarinic acid and lithospermic acid A, was twice as high in the *P. obscura* compared with *P. officinalis* extract. Before this study, the same group of scientists identified and confirmed high concentrations of rosmarinic acid, monardic acid A, lithospermic acid A, and globoidnan B in the methanolic extract of the aerial part of *P. officinalis*. Moreover, this study revealed that certain compounds, including rosmarinic acid, were present in higher concentrations in the extract prepared from plant samples collected during the autumn period (Krzyzanowska-Kowalczyk et al.,

Table 1
The content of different classes of phenolic compounds in the aerial part (PMA) and root (PMR) extracts of *P. mollis*.

Samples	Total phenolic content (mg GAE/g extract)	Total flavonoid content (mg QUE/g extract)	Total flavonol content (mg RUE/g extract)	Condensed tannins content (mg GAE/g extract)	Total phenolic acid content (mg CA/g extract)	Monomeric anthocyanins content (mg Cy-3-glc/g extract)	Total anthocyanins content (mg Cy-3-glc/g extract)
PMA	79.85± 2.12[a]	52.19 ± 5.28	11.01 ± 1.52	42.18 ± 2.53	50.57 ± 1.22	12.84 ± 2.01	17.41 ± 1.66
PMR	92.11 ± 3.17	n.d.	n.d.	61.03 ± 2.84	58.55 ± 2.15	5.77 ± 0.02	8.55 ± 0.04

[a] Results are mean values ± SD from three measurements; GAE – gallic acid equivalents; QUE – quercetin equivalents; RUE– rutin equivalents; CA – caffeic acid equivalents; Cy-3-glc – cyanidin-3-*O*-glucoside equivalents; n.d., not detected.

Table 2
UHPLC–MS^4 Orbitrap metabolic fingerprinting (negative ionization mode) of *Pulmonaria molis* aerial part (PMA) and root (PMR) extracts.

No	Compounds names	t_R, min	Molecular formula, $[M-H]^-$	Calculated mass, $[M-H]^-$	Exact mass, $[M-H]^-$	Δ mDa	MS^2 Fragments, (% Base Peak)	MS^3 Fragments, (% Base Peak)	MS^4 Fragments, (% Base Peak)	Heatmap*	
										PMA	PMR
	Phenolic acids										
1	Dihydroxybenzoyl hexoside 1	2.25	$C_{13}H_{15}O_9^-$	315.07216	315.06927	2.88	108(7), 109(12), 151(7), 152(43), **153**(100), 163(7), 165(11)	**109**(100)	**81**(100)	5854998	n.d.
2	Gallic acid	3.86	$C_7H_5O_5^-$	169.01425	169.01237	1.88	69(10), 84(4), 123(17), 124(11), **125**(100), 126(12), 150(4)	71(14), 79(30), **81**(100), 83(51), 97(78), 98(16), 107(15)	ND	13426150	n.d.
3	Galloyl hexoside	4.90	$C_{13}H_{13}O_{10}^-$	331.06707	331.06390	3.17	125(30), 150(10), 167(18), 168(98), 169(37), **313**(100), 314(17)	125(76), 137(22), 149(25), 150(54), **151**(100), 165(22), 193(58)	95(35), 107(47), **123**(100), 141(3)	10841688	n.d.
4	Danshensu hexoside	5.57	$C_{15}H_{19}O_{10}^-$	359.09837	359.09482	3.55	135(3), 179(14), **197**(100), 198(6)	73(17), 153(3), **179**(100)	107(3), **135**(100)	17819651	17422350
5	Vanillic acid	5.72	$C_8H_7O_4^-$	167.03498	167.03321	1.77	69(14), 121(3), **123**(100), 124(6), 137(3), 139(7), 149(6)	77(34), 79(42), 81(18), 93(8), **95**(100), 105(23), 108(9)	ND	13977825	18723316
6	Dihydroxybenzoyl hexoside 2	5.77	$C_{13}H_{15}O_9^-$	315.07216	315.06960	2.56	109(11), 152(11), **153**(100), 154(6), 285(20), 287(5), 298(4)	**109**(100)	65(46), **81**(100)	26386658	30214567
7	3,4-dihydroxyphenethyl alcohol 4-*O*-hexoside	5.88	$C_{14}H_{19}O_8^-$	315.10854	315.10434	4.20	123(5), **153**(100)	**123**(100)	77(22), 79(5), 81(14), 93(13), **95**(100), 105(37), 108(13)	1315370	n.d.
8	Protocatechuic acid	6.16	$C_7H_5O_4^-$	153.01933	153.01826	1.07	108(3), **109**(100), 123(27), 154(16)	80(13), **81**(100), 82(12)	ND	15697468	18420427
9	*p*-Coumaric acid	6.51	$C_9H_7O_3^-$	163.04007	163.03863	1.44	91(21), 99(62), 117(90), 118(27), **119**(100), 120(37), 131(22)	**91**(100)	ND	n.d.	1802298
10	Caffeoyl dihexoside	6.51	$C_{21}H_{25}O_{14}^-$	503.14063	503.13805	2.58	161(29), 179(57), 221(32), 251(59), **281**(100), 323(54), 341(56)	135(7), **179**(100), 221(31)	**135**(100)	220892	642485
11	Caffeoyl hexoside 1	6.63	$C_{15}H_{17}O_9^-$	341.08781	341.08478	3.02	135(7), **179**(100), 180(8), 181(21), 221(3), 251(9), 281(13)	**135**(100)	ND	9655150	611728
12	Caffeoyl threonic acid 1	7.05	$C_{13}H_{13}O_9^-$	297.06159	297.05923	2.36	**135**(100), 179(23)	59(4), 71(5), **75**(100), 89(77), 117(95)	ND	65605292	252019
13	*p*-Hydroxybenzoic acid	7.13	$C_7H_5O_3^-$	137.02442	137.02354	0.88	92(30), **93**(100), 108(27), 109(94), 137(42), 138(84), 139(24)	**66**(100), 182(78)	ND	12539815	15919150
14	Gentisic acid	7.27	$C_7H_5O_4^-$	153.01933	153.01821	1.12	107(8), 108(8), **109**(100), 110(4), 123(5), 125(5)	65(68), 66(6), 67(14), **81**(100), 83(35), 91(8), 123(10)	ND	15240223	33488353
15	Caffeoyl glycerol	7.35	$C_{12}H_{13}O_6^-$	253.07176	253.07058	1.18	**135**(100), 136(7), 161(55), 162(4), 179(38), 180(3)	79(7), **107**(100), 117(19), 135(56)	ND	6183540	n.d.
16	Caffeic acid	7.53	$C_9H_7O_4^-$	179.03498	179.03360	1.38	**135**(100)	75(8), 79(18), 91(21), 93(11), **107**(100), 117(15), 135(30)	ND	97841007	50739224
17	Caffeoyl threonic acid 2	7.60	$C_{13}H_{13}O_8^-$	297.06159	297.05982	1.77	**135**(100), 163(3), 179(21)	59(5), 73(3), 75(98), **89**(100), 117(88)	**59**(100)	7940883	n.d.
18	Salvianic acid C	7.60	$C_{18}H_{17}O_8^-$	377.08781	377.08439	3.41	197(3), 273(22), 289(5), 317(7), **359**(100), 360(7)	133(5), **161**(100), 179(21), 197(22), 223(8)	**133**(100)	1643842	n.d.
19	Ferulic acid	7.61	$C_{10}H_9O_4^-$	193.05063	193.04922	1.41	111(37), **134**(100), 135(9), 147(15), 149(34), 173(5), 178(4)	**106**(100), 107(11), 134(14)	ND	9642430	347441
20	Caffeoyl hexoside 2	7.76	$C_{15}H_{17}O_9^-$	341.08781	341.08509	2.71	**179**(100), 180(9), 193(5), 195(6), 223(7), 295(7), 323(3)	75(17), 81(9), 87(5), 99(31), 135(16), **143**(100), 161(45)	71(14), **81**(100), 99(29), 125(22)	8833785	n.d.
21	Yunnaneic acid F	8.09	$C_{29}H_{25}O_{14}^-$	597.12498	597.12358	1.40	197(18), 267(21), **311**(100), 312(15), 329(39), 355(10), 491(20)	249(4), 251(19), 252(3), 265(7), **267**(100), 268(7), 269(3)	223(38), 224(21), 239(52), 246(22), 249(78), 250(62), **252**(100)	n.d.	53035742
22	Salviaflaside	8.17	$C_{24}H_{25}O_{13}^-$	521.13007	521.12553	4.54	161(3), 323(3) **359**(100), 360(8)	133(5), 135(3), **161**(100), 179(21), 197(31), 223(7)	**133**(100)	5013748	11533588
23	Feruloyl threonic acid	8.37	$C_{14}H_{15}O_8^-$	311.07724	311.07473	2.51	135(3), **193**(100)	**134**(100), 149(18)	**106**(100), 134(7)	3995953	n.d.
24	Lithospermic acid	8.67	$C_{27}H_{21}O_{12}^-$	537.10385	537.10003	3.82	355(15), **359**(100), 360(15), 373(30), 491(14), 519(14), 519(29)	161(12), 179(17), **197**(100)	73(19), 153(4), **179**(100)	426106	5607517
25	Rosmarinic acid	9.02	$C_{18}H_{15}O_8^-$	359.07724	359.07323	4.01	133(3, **161**(100), 162(5), 179(13), 197(12), 223(5)	**133**(100)	**77**(100), 105(46)	45983167	659879623
26	Danshensu	9.04	$C_9H_9O_5^-$	197.04555	197.04394	1.61	73(19), 123(9), 151(48), 153(11), 167(7), **179**(100), 180(19)	91(3), 107(3), **135**(100)	**107**(100)	57061151	9984470
27	Salvianolic acid C 1	9.65	$C_{26}H_{19}O_{10}^-$	491.09837	491.09361	4.76	295(3), **311**(100), 312(11)	**267**(100), 268(7)	197(21), 211(23), 221(17), 238(18), **239**(100), 249(46), 267(35)	50532544	2388770
28	Sagecoumarin	9.70	$C_{27}H_{19}O_{12}^-$	535.08820	535.08391	4.29	161(15), 177(13), 179(14), 311(16), **329**(100), 330(13), 491(48)	283(4), **311**(100), 312(6)	133(17), 147(16), 221(7), 239(43), 249(36), 267(27), **283**(100)	179143557	916785
29	Salvianolic acid C 2	9.79	$C_{26}H_{19}O_{10}^-$	491.09837	491.09468	3.70	161(5), 179(3), 311(8), 313(3), 357(5), **359**(100)	133(5), **161**(100), 179(20), 197(23), 223(7)	**133**(100)	10035979	n.d.
30	Clinopodic acid A	9.82	$C_{18}H_{15}O_7^-$	343.08233	343.07916	3.17	135(15), 145(14), **161**(100), 162(10), 179(26), 197(14), 325(11)	**133**(100)	**77**(100), 105(98)	34913270	6922451
31	Methyl salvianolate H/I	9.94	$C_{28}H_{23}O_{12}^-$	551.11950	551.11601	3.49	321(97), 322(8), 339(12), 353(80), 507(25), **519**(100), 520(10)	279(5), 295(4), **321**(100), 339(60)	249(9), 265(3), 277(52), **279**(100), 293(20), 303(7)	557703	14914434
32	Salvianolic acid A	9.96	$C_{26}H_{21}O_{10}^-$	493.11402	493.10963	4.39	**359**(100), 360(4)	133(3), **161**(100), 179(12), 197(13), 223(5)	**133**(100)	98250646	529718953
33	Yunnaneic acid E	10.18	$C_{26}H_{21}O_{11}^-$	509.10894	509.10602	2.91	179(11), 311(31), 313(13), 329(34), **359**(100), 447(53), 491(9)	133(5), **161**(100), 179(19), 197(22), 223(7)	**133**(100)	990765	n.d.
34	Salvianolic acid F 1	10.24	$C_{17}H_{13}O_6^-$	313.07176	313.06905	2.71	**161**(100), 178(21), 267(74), 268(17), 283(26), 297(20), 300(23)	**133**(100)	**79**(100)	9799813	n.d.
35	Methyl lithospermate 1	10.39	$C_{28}H_{23}O_{12}^-$	551.11950	551.11596	3.54	161(11), 357(32), 358(11), 359(70), 360(13), **519**(100), 520(30)	161(3), **339**(100)	133(8), **161**(100), 177(15), 179(7), 295(3), 311(3)	1842294	72144880
36	Methyl rosmarinate	10.61	$C_{19}H_{17}O_8^-$	373.09289	373.08972	3.17	135(40), **179**(100), 180(3), 287(3), 313(30), 331(6), 331(28)	**135**(100)	106(57), **107**(100), 135(17)	21104377	16073081
37	Salvianolic acid F 2	11.09	$C_{17}H_{13}O_6^-$	313.07176	313.06870	3.06	**161**(100), 162(4)	**133**(100)	89(7), **105**(100), 107(20), 133(5), 140(8)	108520894	88625863
38	Methyl lithospermate 2	11.16	$C_{28}H_{23}O_{12}^-$	551.11950	551.11763	1.87	359(33), **389**(100), 390(13), 461(6), 505(11), 519(29), 520(6)	123(15), **151**(100), 179(10), 193(9), 195(4), 197(62), 357(32)	79(3), 81(4), 107(9), 108(3), 109(3), **123**(100), 133(4)	843982	11423201
39	Salvianolic acid C 3	11.23	$C_{26}H_{19}O_{10}^-$	491.09837	491.09555	2.82	179(14), 267(21), 293(11), **311**(100), 312(10), 329(19), 447(11)	174(4), 202(4), 223(4), 249(3), 267(42), **283**(100), 293(20)	173(40), **174**(100), 239(30), 255(14), 255(79), 265(13), 268(20)	14463315	n.d.
40	Salpalaestinin	11.47	$C_{28}H_{25}O_{12}^-$	553.13515	553.13407	1.08	177(43), 341(29), 343(53), 373(87), **477**(100), 489(31), 521(56)	267(25), 311(41), 312(16), 313(54), **343**(100), 433(16), 459(35)	133(12), 135(23), 177(24), 178(9), **179**(100), 267(22), 311(10)	306591	18981340
	Flavonoids										
41	Quercetin 3-*O*-(2''-rhamnosyl)hexoside	7.68	$C_{27}H_{29}O_{16}^-$	609.14611	609.14308	3.03	255(9), 271(18), **300**(100), 301(28), 445(13), 463(6), 489(9)	243(4), 254(8), 255(58), **271**(100), 272(12)	199(24), 215(26), 227(69), 229(13), 242(6), **243**(100), 271(19)	38838119	n.d.
42	Quercetin 3-*O*-(6''-rhamnosyl)hexoside	7.95	$C_{27}H_{29}O_{16}^-$	609.14611	609.14498	1.13	255(4), 271(8), 299(3), 300(42), **301**(100), 302(12), 343(6)	151(82), **179**(100), 255(8), 257(13), 271(9), 272(14), 273(17)	**151**(100)	44622980	n.d.
43	Quercetin 3-*O*-hexoside	8.21	$C_{21}H_{19}O_{12}^-$	463.08820	463.08444	3.76	299(3), 300(18), **301**(100), 302(5)	107(5), 151(79), **179**(100), 229(5), 257(11), 273(15), 283(5)	**151**(100)	85205179	n.d.
44	Kaempferol 7-*O*-(6''-rhamnosyl)hexoside	8.30	$C_{27}H_{29}O_{15}^-$	593.15119	593.14622	4.97	257(3), **285**(100), 286(9)	163(24), 213(30), 229(57), 241(38), 256(25), **257**(100), 267(50)	163(78), 185(15), 187(14), 189(11), 213(25), **229**(100), 239(39)	25088621	n.d.
45	Isorhamnetin 3-*O*-(6''-rhamnosyl)hexoside	8.37	$C_{28}H_{31}O_{16}^-$	623.16176	623.16128	0.48	255(4), 271(8), 272(3), 300(19), 301(3), **315**(100), 316(15)	272(6), 287(5), **300**(100), 301(3)	243(3), 254(10), 255(54), **271**(100), 272(38)	15330304	n.d.
46	Luteolin 7-*O*-hexoside	8.39	$C_{21}H_{19}O_{11}^-$	447.09329	447.08982	3.47	281(20), **285**(100), 286(11)	151(49), 175(96), **199**(100), 201(31), 217(73), 241(91), 243(61)	143(18), 153(5), 155(19), 157(5), **171**(100), 181(5), 182(10)	6536821	161251398

47	Quercetin 3-*O*-acetyl hexoside	8.42	$C_{23}H_{21}O_{13}^-$	505.09876	505.09465	4.11	179(3), 300(57), **301**(100), 302(8), 343(3), 445(3), 463(20)	151(86), **179**(100), 255(22), 271(34), 272(12), 273(17), 283(14)	**151**(100)	124883377	n.d.
48	Isorhamnetin 3-*O*-hexoside	8.72	$C_{22}H_{21}O_{12}^-$	477.10385	477.10176	2.09	271(7), 285(9), 286(4), 299(5), **314**(100), 315(37), 357(16)	243(27), 257(12), 271(79), **285**(100), 286(47), 299(17), 300(36)	**270**(100)	5102855	n.d.
49	Luteolin 7-*O*-acetyl hexoside	8.81	$C_{23}H_{21}O_{12}^-$	489.10385	489.10048	3.37	**285**(100), 286(7)	151(42), 175(98), 199(92), 217(74), **241**(100), 243(64), 257(27)	185(14), **197**(100), 198(80), 199(82), 212(8), 213(53), 226(14)	8712004	n.d.
50	Genkwanin	9.85	$C_{16}H_{11}O_5^-$	283.06120	283.05956	1.64	197(5), 211(5), 237(4), 239(32), **241**(100), 242(7), 255(12)	**197**(100), 198(8)	**141**(100), 151(48), 153(24), 169(20), 179(33), 180(30), 197(13)	3505416	n.d.
51	Luteolin	10.40	$C_{15}H_9O_6^-$	285.04046	285.03843	2.03	151(39), 175(91), 199(79), 217(65), **241**(100), 243(62), 285(56)	185(12), **197**(100), 198(82), 199(70), 213(44), 214(8), 226(14)	151(5), 165(10), **169**(100), 179(7), 182(7), 197(14), 329(3)	99252747	n.d.
52	Apigenin	11.30	$C_{15}H_9O_5^-$	269.04555	269.04395	1.60	149(48), 151(25), 201(27), **225**(100), 227(18), 269(35), 270(30)	157(9), 169(23), 180(27), **181**(100), 183(51), 196(24), 197(48)	122(18), **141**(100), 152(5), 153(20), 166(7), 180(8), 181(12)	66225521	n.d.
53	Chrysoeriol	11.55	$C_{16}H_{11}O_6^-$	299.05611	299.05356	2.55	**284**(100), 285(4)	**256**(100), 284(7)	188(29), 200(18), 211(26), 212(19), 214(13), **227**(100), 228(35)	27376312	n.d.
54	Quercetin dimethyl ether	11.93	$C_{17}H_{13}O_7^-$	329.06668	329.06239	4.28	197(21), 239(36), 291(39), 292(8), 309(18), **314**(100), 315(20)	285(10), **299**(100), 300(11)	**271**(100)	17390019	n.d.
55	Chrysin	12.31	$C_{15}H_9O_4^-$	253.05063	253.04870	1.93	157(9), **181**(100), 182(16), 209(22), 225(9), 253(27)	**153**(100), 155(11)	122(36), **127**(100), 134(37), 171(46), 238(14)	15074822	n.d.
56	Hispidulin	12.50	$C_{16}H_{11}O_6^-$	299.05611	299.05332	2.79	**284**(100), 285(9)	211(6), 227(7), 255(7), 256(75), 257(8), **284**(100), 285(23)	183(25), 188(27), 200(37), 212(49), **227**(100), 228(49), 239(31)	22062223	n.d.
57	Cirsimaritin	12.73	$C_{17}H_{13}O_6^-$	313.07176	313.06819	3.57	283(5), **298**(100), 299(10)	225(3), 269(30), 270(5), **283**(100)	163(8), 211(5), 227(12), 239(6), **255**(100)	21632857	n.d.
58	Quercetin trimethyl ether	13.11	$C_{18}H_{15}O_7^-$	343.08233	343.07865	3.68	313(6), **328**(100), 329(13)	282(15), 299(7), 309(15), 310(4), 312(3), **313**(100)	**285**(100)	78551789	n.d.
59	Acacetin	13.66	$C_{16}H_{11}O_5^-$	283.06120	283.05923	1.96	**268**(100), 269(11)	172(5), 200(5), 212(5), 239(19), 240(45), **268**(100), 269(24)	171(27), 172(46), 196(93), 210(21), **211**(100), 212(45), 240(26)	83355097	n.d.

*Heatmap showing distribution of phenolic compounds based on peak area. Red color boxes indicate the lower concentration while green color boxes showing higher concentration of compounds; n.d. – not detected.

Table 3
Quantification of metabolites in methanolic extracts of *P. mollis* aerial parts (PMA) and roots (PMR) using single reaction monitoring (SRM) scanning mode of the triple quadrupole mass spectrometer. Values are presented as μg per g of DE. Presented are pseudomolecular $[M-H]^-$ ions, diagnostic MS^2 fragments, and the chromatographic retention time (Rt).

No.	Rt (min)	Compounds	Pseudomolecular ion $[M-H]^-$ *m/z*	Diagnostic MS^2 fragments $[M-H]^-$ *m/z* (Intensity %)	PMA (μg/g)	PMR (μg/g)
1	0.48	Quinic acid[a]	191	171 (<5); 191 (40)	58.64	29.60
2	0.83	Protocatechuic acid[a]	153	108 (100); 109 (70)	24.15	18.97
3	0.94	*p*-Hydroxybenzoic acid[a]	137	65 (5); 93 (100)	605.70	480.84
4	1.99	5-*O*-Caffeoylquinic acid[a]	353	127 (<5); 191 (100)	23.81	81.32
5	2.04	Gentisic acid[a]	153	109 (100); 125 (10)	72.56	58.03
6	2.45	Caffeic acid[a]	179	134 (85); 135 (10)	325.42	75.05
7	4.01	Rutin[a]	609	300 (100); 301 (65)	386.24	3.82
8	4.22	Quercetin 3-*O*-glucoside[a]	463	243 (<5); 300 (100)	784.70	7.81
9	4.54	Ferulic acid[a]	193	134 (100); 178 (50)	n.d.	16.80
10	4.75	Kaempferol 3-*O*-glucoside[a]	447	255 (5); 284 (100)	193.02	7.39
11	5.01	Rosmarinic acid[a]	359	133 (10); 161 (100)	4508.27	2983.24
12	5.10	Salvianolic acid B[a]	717	321 (35); 519 (100)	480.37	4697.98
13	5.47	Salvianolic acid A[a]	493	185 (30); 295 (100)	605.54	2311.09
14	5.24	Eriodyctiol[a]	287	215 (5); 259 (100)	7.37	n.d.
15	5.70	Luteolin[a]	285	133 (100); 151 (40)	50.72	5.66
16	5.88	Apigenin[a]	269	117 (100); 149 (60)	119.55	6.43
17	5.83	Naringenin[a]	271	119 (100); 151 (55)	7.68	n.d.
18	6.15	Hispidulin[a]	299	284 (100); 285 (10)	43.68	4.31
19	6.70	Cirsimaritin[a]	313	183 (25); 298 (100)	39.16	4.79
20	7.72	Kaempferide[a]	299	284 (100); 285 (12)	9.44	3.51

[a] Authenticated by corresponding standards.

2018). Flavonoids, such as rutin and quercetin derivatives, were found in higher concentrations in the extract prepared from the sample collected in the spring. The seasonal variations in flavonoid content could be significant in understanding plant chemistry. Dresler et al. (2017) also reported that rosmarinic acid was the most abundant compound in the extract of the aerial part of *P. obscura*, while allantoin was present at the highest concentration in the root extract. Interestingly, the PMR extract exhibited a tenfold higher concentration of salvianolic acid B (4697.98 μg/g) and a fourfold higher concentration of salvianolic acid A (2311.09 μg/g) compared with the PMA extract. In contrast, all other compounds were present in much lower concentrations compared with the PMA extract. This technique did not quantify ferulic acid and naringin in the PMA extract. In addition to naringin, eriodyctiol and naringenin were also not quantified in the root extract. Corresponding TIC (Total Ion Current) chromatograms of PMA and PMR extracts are presented in Fig. 1S (Supplementary material).

3.2. Evaluation of antioxidant potential of methanol PMA and PMR extracts

Phenolic compounds found in plants are well-known for their potent antioxidant activity, primarily due to their ability to scavenge free radicals and neutralize oxidative stress. These compounds, including flavonoids, phenolic acids, and tannins, play a crucial role in protecting plant cells from damage and have been widely studied for their potential health benefits in humans (Oluwole et al., 2022; Srećković et al., 2020). While numerous studies have confirmed the strong antioxidant activity of *P. officinalis*, there is a lack of literature data supporting similar findings for *P. mollis*. This study is the first to provide a comprehensive analysis of the antioxidant potential of methanolic extracts from the aerial parts and roots of *P. mollis* using several methods.

Six *in vitro* methods were employed to assess the antioxidant potential of PMA and PMR extracts. Two of these methods, namely total antioxidant capacity and reductive activity, are based on the ability of the extracts to oxidize or reduce metal ions. The remaining four methods focus on evaluating the ability of the extracts to scavenge free radicals, such as $ABTS^{\cdot+}$ and $DPPH^{\cdot}$, inhibit lipid peroxidation, and chelate metal

ions. Results presented in Table 4 show that the PMR extract exhibited slightly higher antioxidant activity in nearly all the methods used compared with the PMA extract. The PMR extract demonstrated a higher neutralization potential against DPPH radicals and ABTS radical cations, with IC_{50} values of 84.25 μg/mL and 214.16 μg/mL, respectively. However, both extracts showed lower radical neutralization activity compared to the reference standards, ascorbic acid and BHT, which exhibited IC_{50} values ranging from 10.07 μg/mL to 29.06 μg/mL. Additionally, neither extract nor ascorbic acid exhibited activity in inhibiting lipid peroxidation, even at the highest applied concentrations, with respect to BHT, which showed an IC_{50} value of 11.98 μg/mL. Interestingly, the PMA extract exhibited a more pronounced chelating activity toward Fe^{2+} ions (IC_{50} 1902.22 μg/mL), likely due to its higher flavonoid content, which is known for its chelating properties. However, the chelating potential of the PMA extract was significantly lower compared to EDTA (IC_{50} of 24.65 μg/mL). The total antioxidant activity and reductive capacity of the extracts were evaluated, with the PMR extract demonstrating slightly better overall antioxidant activity. Specifically, 1 g of PMR extract exhibited an antioxidant activity equivalent to 434.13 mg of Trolox. On the other hand, both the PMA and PMR extracts showed similar reductive capacity.

The antioxidant activity of *P. officinalis*, the most well-known species in the genus, has been investigated using various methods. Ivanova et al. (2005) were the first to highlight the antioxidant potential of water extracts from *P. officinalis* leaves, which were collected in Bulgaria, using the ABTS assay. Their findings revealed significant antioxidant activity, with values exceeding 2 mM Trolox equivalent antioxidant capacity. Another study confirmed that the aqueous-ethanol extract of *P. mollis* aerial parts exhibits high DPPH radical scavenging activity (Oktyabrsky et al., 2009). In a recent study, the antioxidant potential of *P. officinalis* and *P. obscura* aerial part methanol extracts was assessed using the $ONOO^-$ scavenging assay. The resulting IC_{50} values for $ONOO^-$ scavenging activity were 32.66 μg/mL for *P. officinalis* and 36.71 μg/mL for *P. obscura*, indicating notable antioxidant capacities for both species. The same study investigated the effects of both extracts on the physiology of the cardiovascular system, particularly their antioxidant activity against peroxynitrite ($ONOO^-$). The results showed that the examined extracts partly prevented the peroxynitrite-induced decrease in the non-enzymatic antioxidant capacity of human blood plasma (Krzyanowska-Kowalczyk et al., 2021). Recently, a study reported by Sorescu et al. (2020) successfully synthesized gold and silver nanoparticles using an aqueous extract of the plant *P. officinalis* to enhance certain biological activities. Gold nanoparticles exhibited the highest antioxidant activity against DPPH radicals, followed by silver nanoparticles and the plant's aqueous extract. This suggests that bioactive compounds from *P. officinalis* were likely incorporated into the structure or surface of the synthesized nanoparticles, contributing to their enhanced antioxidant activity.

3.3. α-amylase and α-glucosidase inhibitory potential of extracts

Many medicinal plants exhibit inhibitory effects on α-amylase and α-glucosidase, key enzymes involved in carbohydrate digestion, thereby reducing postprandial glucose levels. This mechanism plays a crucial role in the prevention and management of type 2 diabetes by slowing glucose absorption and improving glycemic control. The exploration of plant-derived inhibitors offers a promising natural approach to diabetes therapy, with potential advantages over synthetic drugs due to their lower side effects and additional bioactive properties (Alam et al., 2019; Tundis et al., 2010). To the best of our knowledge, this is the first report on the analysis of the inhibitory potential of any plant species belonging to the genus *Pulmonaria* on the key carbohydrate-hydrolyzing enzymes *α*-amylase and *α*-glucosidase. The inhibitory effects of the tested extracts and the standard drug acarbose on α-amylase and α-glucosidase were evaluated and expressed as IC_{50} values (μg/mL) (Fig. 1A and B). The PMA extract exhibited moderate inhibition of *α*-amylase with an IC_{50} value of 1810.20 μg/mL, and a strong inhibitory effect on α-glucosidase, with an IC_{50} of 37.74 μg/mL. The PMR extract displayed lower α-amylase inhibitory activity (IC_{50} = 2510.74 μg/mL), while exerting pronounced inhibition of α-glucosidase (IC_{50} = 14.00 μg/mL). As expected, the standard drug acarbose exhibited high α-amylase inhibition potency (IC_{50} = 101.55 μg/mL) but was markedly less effective on α-glucosidase (IC_{50} = 215.26 μg/mL). Interestingly, both extracts showed significantly better α-glucosidase inhibitory activity compared with acarbose. Moreover, the PMR extract showed approximately 15 times higher inhibitory potential compared to acarbose. In contrast, both extracts showed much weaker *α*-amylase inhibitory potential compared with the standard, with the PMA extract being slightly more active. The findings presented herein provide novel insights into the antidiabetic potential of the *Pulmonaria* species and highlight their promise as natural sources of enzyme inhibitors. These results lay the groundwork for further phytochemical and pharmacological investigations aimed at isolating the bioactive constituents responsible for the observed effects, as well as for exploring the clinical relevance of these findings. However, these findings are based on *in vitro* evidence, and further *in vivo* and clinical studies are required to validate their efficacy and safety before it can be considered a therapeutic alternative.

3.4. Antibacterial and antifungal activity of PMA and PMR extracts

Although the genus *Pulmonaria* has been traditionally used in folk medicine for the treatment of respiratory ailments, emerging evidence suggests that certain species also possess antimicrobial properties. This study represents the first documented evaluation of the antibacterial and antifungal activity of *P. mollis* extracts, thus contributing novel insights to the existing body of knowledge. As shown in Table 5, both extracts exhibited measurable antibacterial activity against most tested bacterial strains. The PMA extract demonstrated relatively weaker activity, with minimum inhibitory concentration (MIC) values reaching 10 mg/mL. In contrast, the PMR extract showed slightly higher efficacy, with MIC values ranging from 1.25 to 10 mg/mL. Among all tested bacteria, *Bacillus cereus* was the most sensitive to both extracts, exhibiting the lowest MIC value of 0.625 mg/mL. The highest level of resistance to the PMR extract was recorded for *Escherichia coli* and *Klebsiella pneumoniae*, both

Table 4
Antioxidative activity of methanolic extracts of *P. mollis* aerial parts (PMA) and roots (PMR).

Extracts and standards	IC_{50} values (μg/mL)				Total antioxidant activity (mg Trolox/g extract)	Reducing capacity (mg Trolox/g extract)
	DPPH scavenging activity	$ABTS^+$ scavenging activity	Inhibition of lipid peroxidation	Metal chelating ability		
PMA	104.00 ± 4.19	265.20 ± 8.77	> 1000	1902.22	323.80 ± 15.38	366.89 ± 3.58
PMR	84.25 ± 1.77	214.16 ± 7.01	> 1000	>4000	434.13 ± 12.37	386.96 ± 3.91
Ascorbic acid	10.07 ± 0.46	14.36 ± 0.59	>100	–	–	–
BHT	23.51 ± 1.34	29.26 ± 2.02	11.98 ± 2.12	–	–	–
EDTA	–	–	–	24.65 ± 1.47	–	–

DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; -, not tested.

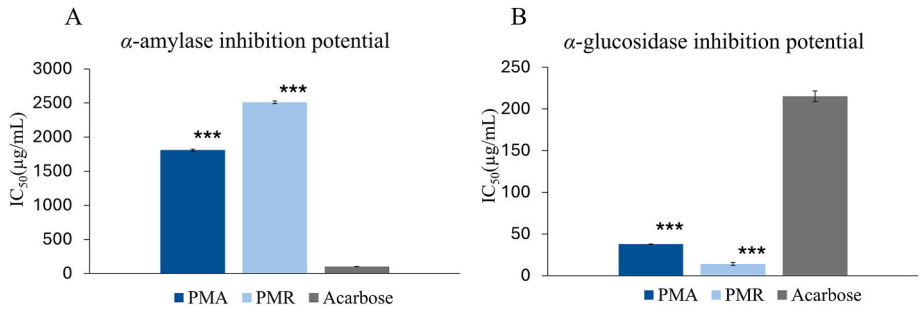

Fig. 1. Inhibitory activity (IC_{50}) of *P. mollis* aerial part (PMA) and root (PMR) extracts, and acarbose against α-amylase (A) and α-glucosidase (B). Data represent means ± SD. ***$p < 0.001$ in comparison to positive control acarbose.

Table 5
Antimicrobial activity of *P. mollis* aerial part (PMA) and root (PMR) methanol extracts.

Bacterial strains	MIC[a] (mg/mL)		MIC (μg/mL)
	PMA	PMR	Chloramphenicol
E. coli	20	>20	5
B. cereus	0.625	0.625	2.5
P. aeruginosa	20	20	20
E. faecalis	10	2.5	1.25
B. subtilis	20	2.5	5
M. lysodeikticus	20	10	2.5
S. typhimurium	20	2.5	5
S. enteritidis	20	10	0.625
S. epidermidis	20	1.25	2.5
S. aureus	20	2.5	5
K. pneumoniae	20	>20	2.5
Fungal strains			Nystatin
C. albicans	10	20	2.5
T. lougibrachiatum	10	10	1.25
T. harzianum	5	2.5	5
P. canescens	2.5	5	1.25
P. cyclopium	5	5	5
D. stemonitis	20	10	40
A. alternata	20	10	0.6125
F. oxysporum	5	0.625	1.25
A. brasiliensis	10	10	0.6125

[a] MIC – minimal inhibitory concentration.

of which displayed MIC values above 20 mg/mL. Compared with plant extracts, the reference antibiotic chloramphenicol displayed substantially greater antibacterial activity against all tested bacterial strains (MIC from 0.625 to 20 μg/mL). Due to the lack of comprehensive studies on *P. mollis*, insights into its antimicrobial potential are often inferred from investigations of the closely related *P. officinalis*. The study reported by Sadowska et al. (2019) investigated the antimicrobial potential of methanolic extracts from *P. officinalis* against *Staphylococcus aureus*, a key pathogen in lung infections. The minimum inhibitory concentration (MIC) was found to be 1–2 mg/mL, indicating moderate effectiveness. Additionally, the extract reduced several virulence factors of *S. aureus*, including anti-biofilm activity, protein A expression, α-hemolysin production, staphylococcal sortase A activity, and adherence to A549 lung cells.

The antifungal activity of the extracts was generally comparable (Table 5). The PMA extract exhibited the strongest effect against *Penicillium canescens*, with a MIC value of 2.5 mg/mL. In contrast, the most sensitive fungal strains to the PMR extract were *Fusarium oxysporum* (MIC 0.625 mg/mL) and *Trichoderma harzianum* (MIC 2.5 mg/mL). For most other fungi, the MIC values ranged between 5 and 10 mg/mL. On the other hand, nystatin exhibited exceptionally strong antifungal activity, except for *D. stemonitis* (MIC 40 μg/mL). Although specific studies on the antifungal activity of plants of the genus *Pulmonaria* are not widely available, numerous phenolic compounds present in them have shown significant antifungal activity. Flavonoids such as cirsimaritin, apigenin, and rutin are well known for their antimicrobial properties. Studies have shown that rutin and apigenin exhibit significant antifungal activity against various fungal strains, including *Candida albicans* and *Aspergillus niger* (Fang Tan et al., 2022; Han, 2009). It has also been documented those certain phenolic acids, which have been identified and quantified in current study in extracts of *P. mollis* such as rosmarinic and salvianolic acids, possess significant antifungal potential (Aydin et al., 2024; Sharma & Manhas, 2020). Sharma and Manhas (2020) reported a phylogenetical novel *Streptomyces* sp. M4 capable of producing salvianolic acid B, which exhibited strong antifungal activity against a range of phytopathogenic fungi.

3.5. The DNA-protective ability of the P. mollis extracts

The present study provides compelling evidence of the protective effects of *P. mollis* extracts, both from the aerial parts and roots, against oxidative DNA damage induced by hydroxyl and peroxyl radicals. Salmon sperm DNA was used as a model to evaluate the extent of strand breakage under oxidative stress, providing a reliable system to assess the radical scavenging capacity of the extracts. The PMA extract, tested at concentrations ranging from 25 to 400 μg/mL (Fig. 2A, lanes 4–8), exhibited a strong, dose-dependent inhibition of hydroxyl radical-induced DNA damage. Its protective effect even exceeded that of rosmarinic acid at 100 μM (Fig. 2A, line 3), a compound well known for its potent antioxidant capacity (Kola et al., 2024). In contrast, the root extract (Fig. 2B, lanes 4–8) showed slightly lower protective efficacy than the aerial part but was comparable to catechin (100 μM) (Fig. 2B, line 3), used as a reference compound, indicating that the root also contains bioactive constituents capable of mitigating oxidative damage. Considering that the PMA extract contains significantly higher flavonoid content, it is likely that its DNA protective activity results from a dual mechanism of hydroxyl radical neutralization. This involves both the donation of hydrogen atoms or electrons and the chelation of transition metals such as Fe^{2+}. Flavonoid compounds are particularly well recognized for this chelating function, thereby preventing radical formation through Fenton reactions (Kumar & Pandey, 2013). This combination of direct radical scavenging and metal chelation likely explains the slightly higher antioxidant activity observed in the aerial extracts.

Interestingly, both extracts showed their maximum protective effect at the lowest tested concentration (25 μg/mL) against peroxyl radical-induced damage (Fig. 2C and D, line 4). This indicates that relatively low concentrations are sufficient to neutralize the radical load, whereas higher concentrations do not provide additional benefits and may even slightly reduce efficacy. The protective effects of PMA and PMR were like those of the standard antioxidants rosmarinic acid and catechin, and in some cases even surpassed them, highlighting their strong radical-scavenging capacities.

To the best of our knowledge, our study provides the first direct evidence of the DNA-protective capacity of *P. mollis*, emphasizing the relevance of this species as a promising source of bioactive compounds

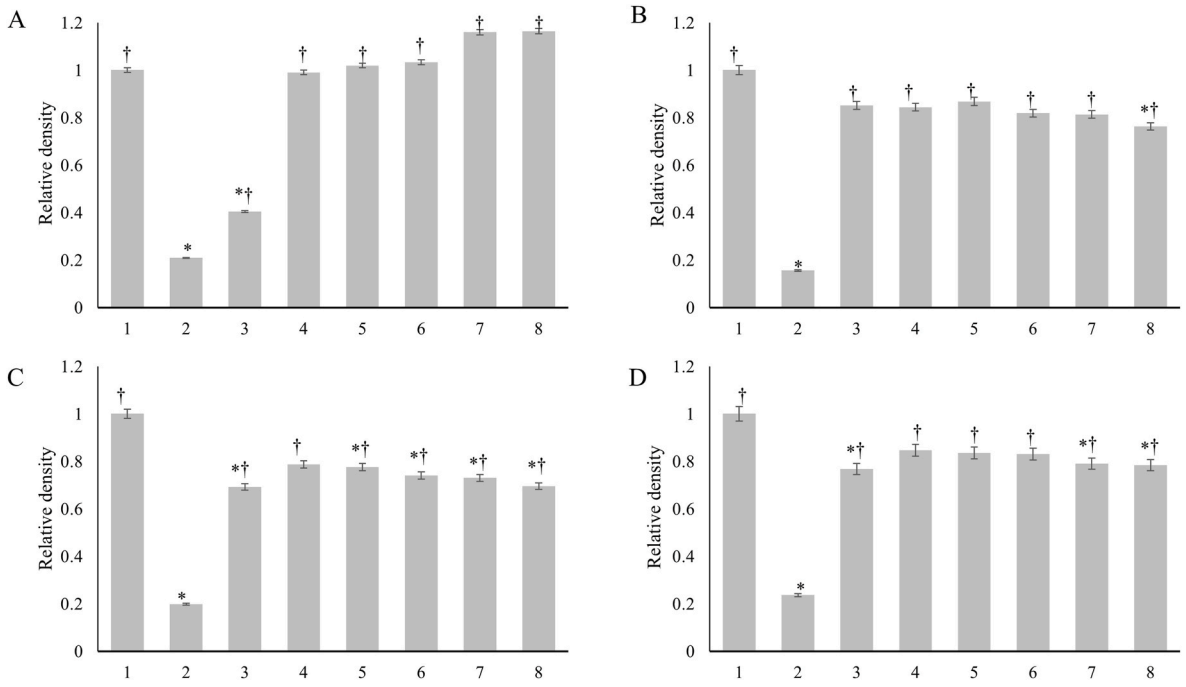

Fig. 2. Protective effect of the PMA (A and C) and PMR (B and D) methanol extracts against hydroxyl (A and B) and peroxyl (C and D) radicals-induced DNA damage. DNA from salmon sperm (1, negative control), DNA damage control (2, positive control), rosmarinic acid or catechin (3, standard), and extracts at concentrations of 25, 50, 100, 200, and 400 μg/mL (4, 5, 6, 7, and 8).*$p < 0.05$ when compared with the negative control group; †$p < 0.05$ when compared with the positive control group.

with antioxidant and genoprotective potential. Although *Pulmonaria* species have a long history of traditional medicinal use, scientific data on their effects against oxidative stress and DNA protection remain limited. The only previous investigation, conducted by Oktyabrsky et al. (2009), demonstrated that a water ethanol extract of *P. mollis* significantly reduced hydrogen peroxide (H_2O_2)-induced DNA damage in

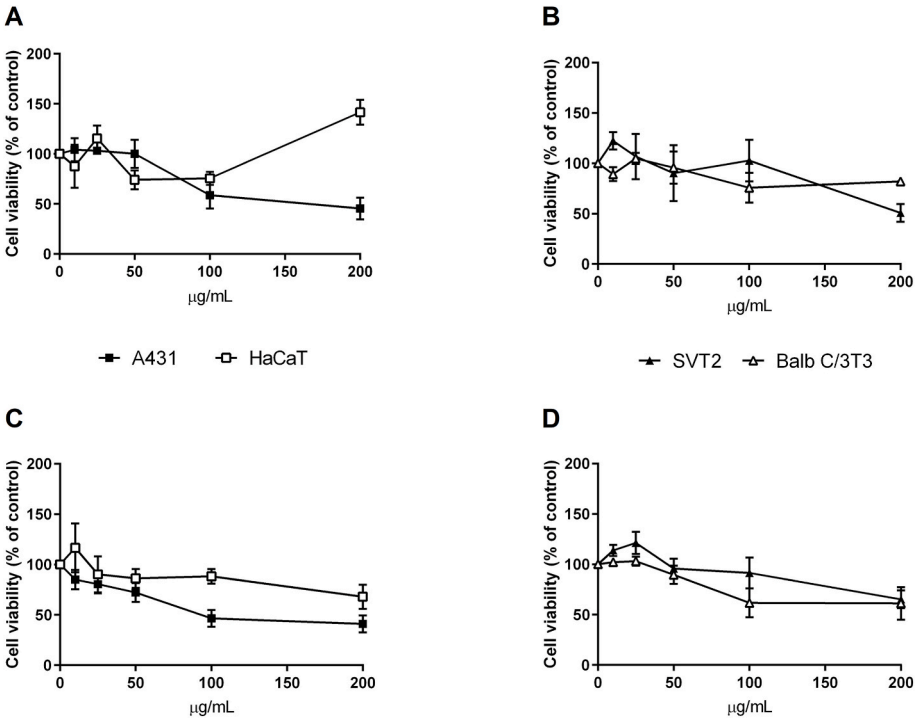

Fig. 3. Cytotoxic effect of PMA and PMR extracts on immortalized and cancer cell lines. A, PMA dose–response curves on HaCaT (white squares) and A431 cells (black squares); B, Balb C/3T3 (white triangles) and SVT2 cells (black triangles); C, PMR dose–response curves on HaCaT (white squares) and A431 cells (black squares); D, Balb C/3T3 (white triangles) and SVT2 cells (black triangles). Cell viability was evaluated following 48 h treatment with increasing concentrations (10–200 μg/mL) of PMA (A, B) or PMR (C, D) extracts using the MTT assay. Cell survival was expressed as described in the Materials and Methods section. Values are given as means ± SEM ($n \geq 3$).

Escherichia coli. In that study, the extract not only provided immediate chemical protection but also induced upregulation of the *kat*G and *sod*A genes, which encode key antioxidant defense enzymes.

3.6. Biocompatibility of PMA and PMR extracts

Cancer remains a major global health challenge, where prevention through diets rich in antioxidant compounds is increasingly recognized as essential (İpek et al., 2024). As such diets are becoming less common, exploring underutilized plants like *P. mollis* as novel sources of bioactive antioxidants may provide valuable support in cancer prevention. However, there is a notable lack of scientific studies investigating the anticancer potential of plant species belonging to this genus. The only available information refers to the traditional use of *P. officinalis* and *P. mollissima* in Russian folk medicine for the treatment of alimentary tract cancers, uterine malignancies, and leukemia (Spiridonov, 2008).

The cytotoxic activity of PMA and PMR extracts was investigated by MTT assay on two cancer cells (A431 and SVT2) and two immortalized cell lines (HaCaT and Balb C/3T3). The toxicity of the extracts was evaluated after 48 h incubation with increasing amounts of each extract. The results, shown in Fig. 3, indicate a dose-dependent decrease in cell viability, particularly evident in A431 cells, indicated by black squares in Fig. 3A–C. In particular, PMA extract exhibited an IC_{50} value (the concentration of extract able to induce 50 % cell death) of 178 ± 11 μg/mL, and PMR of 64 ± 3 μg/mL. As for SVT2 cells (Fig. 3B–D, black triangles), even if a dose-dependent effect was observed with both extracts, it was not possible to determine the IC_{50} value. Interestingly, neither PMA nor PMR influenced the viability of immortalized cells (Fig. 3, white symbols).

The findings that both extracts exert selective activity against epithelial carcinoma cells (e.g., A431), while remaining non-cytotoxic to fibroblasts, suggest their potential therapeutic topic use, reinforcing their safety margin and supporting further preclinical evaluation.

The observed cytotoxicity may be attributed to the presence of bioactive phytochemicals commonly found in medicinal plant extracts, including phenolic compounds, flavonoids, and terpenoids, which are known to induce apoptosis, inhibit proliferation, or modulate oxidative stress in tumor cells (Zhou et al., 2016). While the exact mechanisms underlying the differential sensitivity for epidermoid cancer cells remain to be elucidated, it is possible to speculate that cancer cells, due to their higher metabolic rate and altered redox balance, are more susceptible to the action of these compounds. However, further investigation into their phytochemical composition and molecular mechanisms of action are needed.

4. Conclusion

This study provides the first comprehensive insight into the phytochemical composition and bioactive properties of *P. mollis*. Both aerial and root extracts were found to be rich in phenolic compounds, particularly caffeic acid derivatives such as rosmarinic and salvianolic acids, which contributed to their strong antioxidant and enzyme-inhibitory activities. Notably, the extracts exhibited significant α-glucosidase inhibition, suggesting their potential for incorporation into glycemia-regulating functional food products. In addition, selective cytotoxic effects against cancer cells, moderate antimicrobial properties, and a favorable safety profile, including excellent DNA protective activity against oxidative damage, were observed. These findings support the reintroduction of *P. mollis* into the modern diet as a functional wild vegetable, in line with its traditional culinary uses in salads, soups, and purées. The demonstrated antidiabetic and anticancer potential highlight its value for the development of health-promoting foods and nutraceuticals.

CRediT authorship contribution statement

Nikola Srećković: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Investigation, Conceptualization. **Daria Maria Monti:** Writing – review & editing, Validation, Investigation, Formal analysis. **Davide Liberti:** Visualization, Investigation, Data curation. **Danijela Mišić:** Writing – review & editing, Validation, Investigation. **Uroš Gašić:** Investigation, Formal analysis, Data curation. **Sanja Lj Matić:** Visualization, Investigation, Formal analysis, Data curation. **Vladimir Mihailović:** Writing – review & editing, Visualization, Validation, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2025.107396.

Data availability

Data will be made available on request.

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