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ORIGINAL ARTICLE

Unveiling the phytochemical landscape of *Phlomis pungens* willd.: LC-MS/MS insights into antioxidant and multi-enzyme inhibitory potential

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Abstract: *Phlomis pungens* Willd. (syn. *Phlomis herba-venti* subsp. *pungens* (Willd.) Maire ex DeFilipps) is a medicinal and aromatic species that has been studied mainly for its essential oil composition, whereas its non-volatile phytochemical constituents have remained largely unexplored. In this study, the ethanolic extract of *P. pungens* was systematically characterized for the first time using a targeted LC–MS/MS method. In parallel, total phenolic and flavonoid contents, antioxidant potential, and multi-enzyme inhibitory activities were evaluated. LC–MS/MS profiling revealed a phenolic acid–rich composition, with quinic acid (14.146 mg/g extract) and chlorogenic acid (13.187 mg/g) identified as the predominant compounds. These were followed by cynaroside (1.541 mg/g), vanillic acid (1.453 mg/g), and cosmosiin (0.830 mg/g). The extract demonstrated considerable levels of total phenolics and flavonoids (37.18 ± 1.97 µg PEs/mg and 18.19 ± 0.90 µg QEs/mg extract, respectively). Antioxidant evaluation indicated moderate activity, particularly in the ABTS (IC_{50} = 32.05 ± 0.28 µg/mL) and CUPRAC ($A_{0.5}$ = 20.83 ± 0.37 µg/mL) assays. Enzyme inhibition studies revealed selective inhibitory effects against collagenase (24.73 ± 0.32%), tyrosinase (15.04 ± 0.11%), and butyrylcholinesterase (13.62 ± 0.12%) at 50 µg/mL, while no inhibition was detected toward acetylcholinesterase, urease, or elastase. These findings suggest that the biological activities of *P. pungens* may be associated with its non-volatile phenolic constituents. This work provides novel phytochemical data and highlights the potential of the species for future pharmaceutical and dermocosmetic applications.

Keywords: *Phlomis pungens*, LC–MS/MS, phenolic compounds, antioxidant activity, enzyme inhibition, medicinal plants

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1 Introduction

Medicinal and aromatic plants remain essential reservoirs of bioactive compounds with broad therapeutic potential (Hasimi et al., 2017; Inci et al., 2023; Karageçili et al., 2024; Özer et al., 2025; Labed et al., 2026). Their secondary metabolites, including phenolic acids, flavonoids, iridoids, terpenoids, and diverse glycosides, contribute to antioxidant, enzyme inhibitory, cardioprotective, antimicrobial, anti-inflammatory, and wound-healing activities, supporting their long-standing use in traditional medicine across cultures (Demirci Kayiran et al., 2019; Onder et al., 2022; Güven et al., 2023, 2024; Aslan et al., 2025). Oxidative stress is intricately associated with chronic illnesses, and plant-derived antioxidants mitigate reactive oxygen species (ROS)-driven cellular damage, making detailed phytochemical investigations an ongoing priority (Yilmaz et al., 2023; Yeniçeri et al., 2024; Hazman et al., 2024; Oyman et al., 2025; Yıldırım et al., 2025).

The genus *Phlomis* L. (Lamiaceae), comprising more than 100 species distributed largely across Europe, Asia, and North Africa, is widely used in traditional systems as herbal teas, stimulants, tonics, wound-healing agents, and treatments for gastrointestinal, inflammatory, respiratory, and metabolic conditions (Amri et al., 2022; El Atki et al., 2020; Amor et al., 2009). Phytochemical studies report a diverse spectrum of metabolites such as iridoid glycosides, phenylethanoid glycosides, flavonoids, diterpenes, lignans, and essential oils, reflecting rich medicinal potential (Amri et al., 2022; Li et al., 2010; Çalış & Başer, 2021). *Phlomis herba-venti* subsp. *pungens* (Willd.) Maire ex DeFilipps, commonly referred to as *Phlomis pungens* Willd., is a perennial medicinal species distributed across Türkiye, Iran, Azerbaijan, and neighboring regions.

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In Türkiye, the genus *Phlomis* is well represented in the flora and several species are traditionally used in Anatolian folk medicine (Uysal et al., 2021). Although specific ethnobotanical records for *Phlomis pungens* are limited, various *Phlomis* species are consumed as herbal teas prepared from their flowering aerial parts to alleviate gastrointestinal disorders and to promote liver, kidney, and cardiovascular health. In addition, they are traditionally employed in the management of diabetes, gastric ulcer, hemorrhoids, inflammation, and wounds, and are used as tonics and stimulants in Anatolia. These traditional indications are particularly relevant to the biological activities investigated in the present study, as inflammatory conditions and wound healing processes are closely associated with oxidative stress and enzyme-mediated pathways. Moreover, the traditional use of *Phlomis* species in diabetes management supports the relevance of evaluating enzyme inhibitory activities related to metabolic disorders. Collectively, these ethnobotanical data provide a rationale for exploring the antioxidant and multi-enzyme inhibitory potential of *P. pungens* (Amri et al., 2022; Kirimer et al., 2017).

Although numerous studies have examined its essential oil composition, identifying major volatile constituents such as germacrene D, α-pinene, bicyclogermacrene, and (E)-β-farnesene (Masoudi et al., 2006; Kirimer et al., 2017; Khalilzadeh et al., 2008), investigations of its non-volatile phytochemical composition remain limited. Still, some research has revealed the presence of iridoids such as lamiide and phenylethanoid glycosides including leucosceptoside B and forsythoside derivatives (Saracoglu et al., 2017). Several biological activities of *P. pungens* have been documented. Methanolic extracts exhibit anti-inflammatory effects and moderate antioxidant capacity (DPPH, CUPRAC, FRAP, ABTS) (Taşkın et al., 2018). Additional reports demonstrate antimicrobial, antioxidant, and wound-healing activities, with these effects attributed to both volatile and non-volatile constituents (El Atki et al., 2020; Okur et al., 2022). Essential oils also display inhibitory activity against enzymes such as acetylcholinesterase, butyrylcholinesterase, α-amylase, α-glucosidase, and tyrosinase, indicating relevance to neurodegenerative, metabolic, and dermatological disorders (Amri et al., 2022; Sarikurkcu et al., 2016). Despite these contributions, no study has comprehensively characterized the non-volatile phytochemical profile of *P. pungens* using a sophisticated sensitive technique such as LC-MS/MS. Existing research focuses primarily on volatile components, leaving its phenolic and flavonoid composition largely uncharacterized. Considering the key role of phenolics in antioxidant and enzyme-modulatory pathways, detailed metabolomic analysis is necessary to fully evaluate its therapeutic potential.

In this study, LC-MS/MS was employed to achieve the first comprehensive phytochemical characterization of *P. pungens*. In addition, the study assesses its total phenolic and flavonoid contents, antioxidant properties (CUPRAC, ABTS, DPPH), and enzyme-inhibitory activities against urease, tyrosinase, elastase, collagenase, AChE, and BChE. By integrating advanced analytical chemistry with comprehensive bioactivity evaluation, this research provides a detailed biochemical and functional characterization of this species, thereby supporting its potential applications in nutraceutical, pharmaceutical, and cosmeceutical fields.

2 Materials and Methods

2.1 Plant Material

Aerial parts of *P. pungens* were harvested in July 2023 from Akyayla village, located in the Bahçesaray district of Van Province, Türkiye. The collection was documented under collector number M. Fırat 40079. Botanical authentication of the species was performed by Dr. Mehmet Fırat based on established taxonomic keys and morphological characteristics. To ensure traceability and reproducibility, a voucher specimen was deposited and archived in the Virtual Herbarium of the Lake Van Basin (VHLV), where it remains accessible for future reference. Following field collection, the plant materials were carefully cleaned to remove foreign particles and then dried naturally under shaded conditions at room temperature. Direct sunlight was intentionally avoided to prevent degradation of heat- and light-sensitive phytochemicals. Once thoroughly dried, the plant material was pulverized into a coarse powder to increase extraction efficiency.

For preparation of the crude extract, 25 g of powdered whole-plant material was immersed in 250 mL of absolute ethanol. The extraction was performed using a maceration technique at ambient temperature for 8 h per cycle, and the procedure was repeated three consecutive times to maximize the recovery of soluble phytoconstituents. After each cycle, the mixture was filtered, and the filtrates were combined. The pooled solution was concentrated under reduced pressure at 40°C using a rotary evaporator until complete solvent removal was achieved. The resulting viscous crude extract was transferred into airtight containers and stored at 4°C in the absence of light to preserve chemical stability prior to LC–MS/MS profiling and biological activity assays. The use of ethanol as the extraction solvent was preferred due to its effectiveness in extracting diverse polar and semi-polar secondary metabolites, along with its safety, environmental acceptability, and compatibility with later analytical methods.

2.2 Chemicals and Reagents

Analytical-grade reagents and chemical compounds utilized in the study were sourced from Sigma-Aldrich (Darmstadt, Germany). Care was taken to ensure reagent quality and consistency in order to maintain reproducibility across experiments. For antioxidant activity determination, several chromogenic and radical-generating reagents were utilized, including ABTS (≥98%), DPPH (≥95%), Trolox (≥97%), ferrozine (≥97%), TPTZ (≥98%), neocuproine (≥98%), and ferrous sulfate hexahydrate (≥99%). These compounds were selected based on their established roles in electron-transfer and radical scavenging assays. Reference standards used for phenolic quantification included gallic acid (≥98%), rutin (≥95%), and caffeic acid (≥98%). These standards enabled calibration curve construction and

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quantitative comparison of phenolic constituents. Enzymatic bioassays required specific enzymes and substrates, including electric eel acetylcholinesterase (AChE: Type-VI-S, Sigma), butyrylcholinesterase (BChE: horse serum, Sigma), urease (from *Canavalia ensiformis*, Type III, Sigma), tyrosinase (mushroom-derived, Sigma), elastase (from Porcine pancreas, Type I, Sigma), collagenase (from *Clostridium histolyticum*, Type I, Sigma). Substrates and inhibitors such as acetylthiocholine iodide, butyrylthiocholine chloride, DTNB, galantamine hydrobromide, acarbose, and kojic acid were included to facilitate accurate enzyme activity measurements. Additional reagents used for spectrophotometric and colorimetric analyses included Folin–Ciocalteu reagent, ferric chloride, cupric chloride, sodium molybdate, ammonium molybdate, ammonium acetate, sodium nitrate, sodium carbonate, hydrochloric acid (37%), sodium hydroxide, EDTA, and ultrapure water. All routine laboratory chemicals were of analytical grade and prepared according to standardized laboratory protocols.

2.3 LC–MS/MS Analysis for Qualitative and Quantitative Phytochemical Profiling

An ultra-high-performance liquid chromatography system linked to a triple quadrupole mass spectrometer (Shimadzu LCMS-8040) was employed to perform detailed phytochemical profiling of the ethanolic extract. This advanced analytical platform enabled both qualitative identification and quantitative determination of targeted secondary metabolites within a single analytical run. The applied analytical approach relied on an earlier developed and extensively validated LC–MS/MS method for the concurrent detection of 53 phytochemical markers. These compounds encompassed various structural classes, including flavonoid aglycones, glycosylated flavonoids, phenolic acids, aldehydes, biflavonoids, stilbenoid derivatives, and benzopyrones. To enhance measurement precision and compensate for potential matrix suppression or analyte loss during extraction, preparation, and instrumental analysis, deuterium-labeled internal standards were incorporated into each sample. Ferulic acid-D_3 was used for non-flavonoid phenolics, rutin-D_3 for flavonoid glycosides, and quercetin-D_3 for flavonoid aglycones. These isotopically labeled compounds allowed normalization of signal fluctuations and improved quantification reliability. Previously published validation parameters (Yilmaz, 2020) included assessment of calibration linearity, detection and quantification limits, repeatability, reproducibility, relative standard deviation, and expanded uncertainty. Table 1 summarizes the retention times, ion transitions (precursor and product), and quantified levels of the detected analytes, while Supplementary Table S1 contains the detailed validation data.

2.4 Chromatographic and Mass Spectrometric Instrumentation

Separation of compounds was performed using a reversed-phase UHPLC configuration incorporating a degasser, dual high-pressure gradient pumps, an autosampler, and a thermostatically controlled column compartment. Chromatographic separation was performed using an Agilent Poroshell 120 EC-C18 column (150 mm × 2.1 mm, 2.7 µM particle size), with the column temperature maintained at 40°C to provide stable and reproducible retention characteristics. The mobile phase system consisted of two solvents: solvent A, composed of 5 mM ammonium formate in water with 0.1% formic acid, and solvent B, consisting of 5 mM ammonium formate in methanol supplemented with 0.1% formic acid. A gradient elution strategy was applied to improve chromatographic resolution. The analysis started with 20% solvent B, which was progressively increased to 100% over a period of 25 minutes. This composition was maintained until 35 minutes, after which the system was returned to the initial mobile phase conditions between 35 and 45 minutes to allow column re-equilibration. The flow rate was maintained at 0.5 mL/min, and all samples were introduced using an injection volume of 5 µL. Mass spectrometric detection was carried out using an electrospray ionization (ESI) source operating in both positive and negative ionization modes. Quantitative analysis relied on multiple reaction monitoring (MRM) to ensure high analytical sensitivity and selectivity. Instrument settings included a nitrogen nebulizing gas flow of 3 L/min, drying gas flow of 15 L/min, a heat block temperature of 400°C, and a desolvation line temperature of 250°C. Data acquisition and system operation were managed using Shimadzu LabSolutions software.

2.5 Performance and Validation Characteristics of the LC–MS/MS Method Used

The analytical methodology applied for phytochemical analysis was previously optimized and fully validated for fingerprint profiling of 53 selected compounds representing diverse phenolic subclasses. Validation procedures were performed using both external calibration standards and deuterated internal standards across fortified and non-fortified matrices. Calibration curves were constructed using eight concentration levels, each analyzed in triplicate. Linear regression was performed by plotting analyte-to-internal standard concentration ratios against corresponding peak area ratios. Determination coefficients (r^2) ranged between 0.957 and 0.989, indicating strong linear relationships. Limits of detection and quantification were calculated based on signal-to-noise criteria derived from repeated low-concentration measurements. Precision was evaluated by assessing intra-day and inter-day repeatability through standard addition experiments. Relative standard deviation values remained below 2.13% (intra-day) and 2.51% (inter-day), confirming high reproducibility. Accuracy was verified through recovery studies, which yielded values between 99.2% and 100.8%. Expanded uncertainty values were calculated according to EURACHEM guidelines (k = 2; 95% confidence interval). Overall, validation outcomes demonstrated that the method is robust, precise, and appropriate for routine quantitative phytochemical analysis.

2.6 Total Phenolic and Flavonoid Contents

The overall phenolic and flavonoid concentrations of the extract were determined using spectrophotometric assays adapted from previously described methodologies

Table 1. LC–MS/MS-based quantification results together with analytical method parameters for phytochemical constituents identified in the *P. pungens* ethanol extract

No	Analytes	RT[a]	MI (m/z)[b]	DI (m/z)[c]	Quantitative results (mg analyte/g extract)	No	Analytes	RT[a]	MI (m/z)[b]	DI (m/z)[c]	Quantitative results (mg analyte/g extract)
1	Quinic acid	3.0	190.8	93.0	14.15 ± 0.526	**29**	Salicylic acid	21.8	137.2	103.1	0.202 ± 0.003
2	Fumaric acid	3.9	115.2	40.9	0.726 ± 0.007	**30**	Cynaroside	23.7	447.0	193	1.54 ± 0.056
3	Aconitic acid	4.0	172.8	129.0	<LOD	**31**	Miquelianin	24.1	477.0	149	<LOD
4	Gallic acid	4.4	168.8	79.0	0.011 ± 0.001	**32**	Rutin-D3-IS[d]	25.5	612.2	152.1	NA
5	Epigallocatechin	6.7	304.8	219.0	<LOD	**33**	Rutin	25.6	608.9	93	0.053 ± 0.001
6	Protocatechuic acid	6.8	152.8	108.0	0.298 ± 0.010	**34**	Isoquercitrin	25.6	463.0	135.0/106.9	0.175 ± 0.004
7	Catechin	7.4	288.8	203.1	<LOD	**35**	Hesperidin	25.8	611.2	289	0.025 ± 0.001
8	Gentisic acid	8.3	152.8	109.0	<LOD	**36**	*o*-Coumaric acid	26.1	162.8	199	<LOD
9	Chlorogenic acid	8.4	353.0	85.0	13.19 ± 0.281	**37**	Genistin	26.3	431.0	151.1	<LOD
10	Protocatechuic aldehyde	8.5	137.2	92.0	0.166 ± 0.007	**38**	Rosmarinic acid	26.6	359.0	125	<LOD
11	Tannic acid	9.2	182.8	78.0	<LOD	**39**	Ellagic acid	27.6	301.0	166.9	<LOD
12	Epigallocatechin gallate	9.4	457.0	305.1	<LOD	**40**	Cosmosiin	28.2	431.0	134	0.830 ± 0.007
13	1,5-dicaffeoylquinic acid	9.8	515.0	191.0	<LOD	**41**	Quercitrin	29.8	447.0	108	<LOD
14	4-OH Benzoic acid	10.5	137.2	65.0	<LOD	**42**	Astragalin	30.4	447.0	203	<LOD
15	Epicatechin	11.6	289.0	203.0	<LOD	**43**	Nicotiflorin	30.6	592.9	65	<LOD
16	Vanillic acid	11.8	166.8	108.0	1.45 ± 0.021	**44**	Fisetin	30.6	285.0	191	<LOD
17	Caffeic acid	12.1	179.0	134.0	0.24 ± 0.004	**45**	Daidzein	34.0	253.0	305.1	<LOD
18	Syringic acid	12.6	196.8	166.9	<LOD	**46**	Quercetin-D3-IS	35.6	304.0	78	NA
19	Vanillin	13.9	153.1	125.0	<LOD	**47**	Quercetin	35.7	301.0	92	<LOD
20	Syringic aldehyde	14.6	181.0	151.1	<LOD	**48**	Naringenin	35.9	270.9	85	0.044 ± 0.050
21	Daidzin	15.2	417.1	199.0	<LOD	**49**	Hesperetin	36.7	301.0	109	<LOD
22	Epicatechin gallate	15.5	441.0	289.0	<LOD	**50**	Luteolin	36.7	284.8	203.1	0.096 ± 0.001
23	Piceid	17.2	391.0	135.0/106.9	<LOD	**51**	Genistein	36.9	269.0	108	<LOD
24	*p*-Coumaric acid	17.8	163.0	93.0	0.078 ± 0.002	**52**	Kaempferol	37.9	285.0	219	<LOD
25	Ferulic acid-D3-IS[d]	18.8	196.2	152.1	NA	**53**	Apigenin	38.2	268.8	79	0.068 ± 0.001
26	Ferulic acid	18.8	192.8	149.0	<LOD	**54**	Amentoflavone	39.7	537.0	129	<LOD
27	Sinapic acid	18.9	222.8	193.0	<LOD	**55**	Chrysin	40.5	252.8	40.9	<LOD
28	Coumarin	20.9	146.9	103.1	<LOD	**56**	Acacetin	40.7	283.0	93	0.017 ± 0.001

Note: [a]RT: Retention time, [b]MI (m/z): Molecular ions of the standard analytes (m/z ratio), [c]DI (m/z): Daughter ions, [d]IS: Internal standard, NA: Not applicable, <LOD: Lower than detection limit. *Values expressed are means ± S.D. of three parallel measurements.

(Onder et al., 2022). Total phenolics were expressed as pyrocatechol equivalents, whereas total flavonoids were calculated relative to quercetin standards. Calibration curves were generated using standard solutions, and regression equations were applied to convert absorbance values into concentration equivalents:

Absorbance = 0.0477 + 0.0422 pyrocatechol (μg) (r^2 = 0.9951)

Absorbance = 0.0374 + 0.0563 quercetin (μg) (r^2 = 0.9979)

To ensure consistent results and limit systematic bias, every measurement was carried out using identical experimental settings.

2.7 Assays for Antioxidant Activity

The antioxidant activity of the extract was assessed using three complementary in vitro spectrophotometric methods, each representing a different mechanism of antioxidant activity: DPPH free radical scavenging (Blois, 1958), ABTS radical cation decolorization (Re et al., 1999), and CUPRAC reducing power assays (Apak et al., 2004). α-Tocopherol and BHT were used as standard antioxidants for comparison. For the DPPH assay, a 0.1 mM ethanolic DPPH solution was combined with extract samples prepared at various concentrations. The reaction mixtures were incubated in the dark at room temperature for 30 minutes, after which the absorbance was measured at 517 nM. The scavenging effect on the DPPH radical was calculated as percentage inhibition relative to the control sample. For ABTS analysis, ABTS radical cations were generated by reacting ABTS with potassium persulfate and allowing the mixture to stand in darkness for 12 hours. The solution was diluted to an absorbance of approximately 0.700 at 734 nM before use. Following incubation with sample solutions, absorbance reduction was measured at 734 nM and inhibition calculated. In the CUPRAC method, the extract was combined with copper(II) chloride, ammonium acetate buffer (pH 7.0), and neocuproine. After incubation for 1 hour at room temperature, absorbance was measured at 450 nM, and antioxidant capacity expressed as percentage reduction.

2.8 Assays for Enzyme Inhibitory Activity

To determine its enzyme inhibitory potential, the extract was screened against collagenase, elastase, BChE, AChE, tyrosinase, and urease using validated spectrophotometric and colorimetric assays. All analyses were repeated three times, and the degree of inhibition was expressed as a percentage relative to control samples lacking the extract. Cholinesterase inhibition was determined using Ellman's method by measuring the formation of yellow 5-thio-2-nitrobenzoate at 412 nM (Ellman et al., 1961). Urease inhibition was assessed through quantification of ammonia production using a phenol-based detection system at 630 nM (Zahid et al., 2015). Tyrosinase activity was monitored by measuring dopachrome formation at 475 nM (Hearing & Jiménez, 1987). Elastase inhibition was evaluated using N-succinyl-$(Ala)_3$-nitroanilide substrate at 410 nM (Kraunsoe et al., 1996), while collagenase inhibition was determined by monitoring cleavage of a synthetic substrate at 340 nM (Thring et al., 2009). Appropriate positive controls were included in each assay to validate performance.

2.9 Statistical Analysis

The experimental data obtained from the study were presented as mean values accompanied by standard deviations, calculated from three independent experimental replicates. To evaluate differences between groups, one-way analysis of variance (ANOVA) was employed. In cases where the ANOVA test indicated statistically significant variation, Duncan's multiple range test was subsequently used to identify differences among group means. A significance threshold of $p < 0.05$ was adopted for all statistical evaluations. All statistical analyses were conducted using SPSS software (version 22.0; SPSS Inc., Chicago, IL, USA).

3 Results and Discussion

The efficiency of natural product separation and analysis is strongly influenced by the initial handling and processing of raw plant materials. In phytochemical investigations, extraction represents a critical preliminary step, as it enables the isolation and subsequent characterization of bioactive constituents present in medicinal plants. In recent years, substantial progress has been achieved in the biochemical exploration of therapeutically relevant plant species, leading to increased interest in natural products as potential sources of novel pharmacologically active compounds. Consequently, systematic screening and biological evaluation of extracts derived from underexplored or previously uninvestigated plant taxa have become a prominent strategy in drug discovery research. This growing interest is largely driven by the extensive structural and chemical diversity of naturally occurring secondary metabolites found in plant matrices. In the present study, a previously developed, rigorously validated, and robust LC–MS/MS analytical approach was employed to comprehensively assess the phytochemical composition of the ethanolic extract obtained from the whole plant material of *P. pungens*. Although several investigations have reported on the chemical composition and biological activities of related *P. pungens* species, to the best of our knowledge, a detailed characterization of the non-volatile phytochemical constituents of *P. pungens* using a highly sensitive and advanced technique such as LC–MS/MS has not yet been documented.

3.1 Phytochemical Identification of *P. pungens* EtOH Extract by LC-MS/MS

In the present study, a targeted LC–MS/MS approach enabled the comprehensive qualitative and quantitative characterization of 53 selected non-volatile phytochemicals in the ethanolic extract of *Phlomis pungens* (Figure 1). While previous studies on *P. pungens* have largely focused on essential oil composition and volatile terpenoids (Masoudi et al., 2006; Khalilzadeh et al., 2008; Kirimer et al., 2017; Sarikurkcu et al., 2016), compound-level quantitative profiling of its polar phenolic fraction has not been reported. In contrast, LC–MS-based investigations on other *Phlomis*

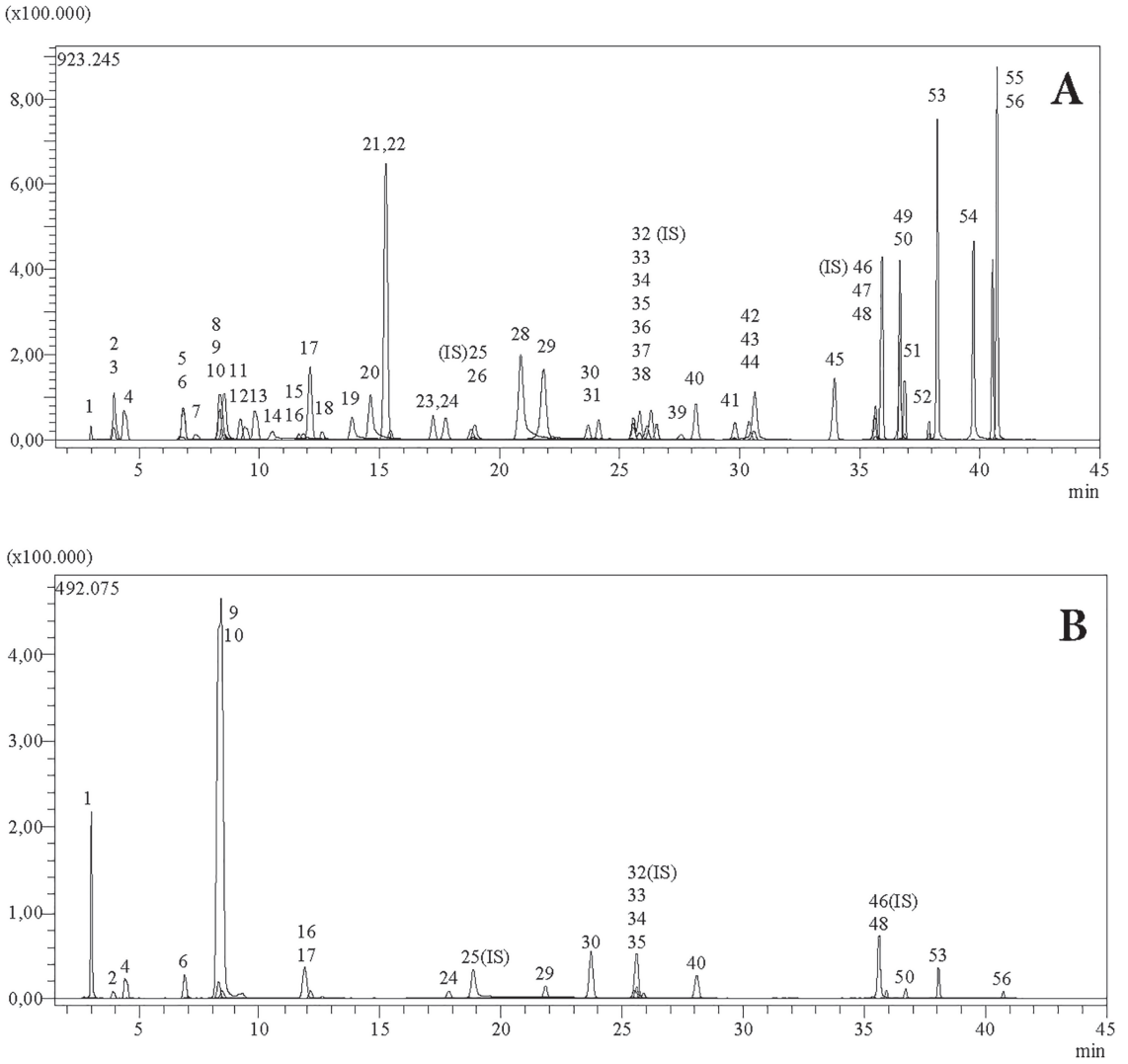

Figure 1. TIC (Total Ion Chromatogram) chromatogram of A: standard phenolic compounds and B: EtOH extract of *P. pungens* analysed by the LC-MS/MS method. (**1**: Quinic acid, **2**: Fumaric acid, **3**: Aconitic acid, **4**: Gallic acid, **5**: Epigallocatechin, **6**: Protocatechuic acid, **7**: Catechin, **8**: Gentisic acid, **9**: Chlorogenic acid, **10**: Protocatechuic aldehyde, **11**: Tannic acid, **12**: Epigallocatechin gallate, **13**: 1,5-dicaffeoylquinic acid, **14**: 4-OH Benzoic acid, **15**: Epicatechin, **16**: Vanillic acid, **17**: Caffeic acid, **18**: Syringic acid, **19**: Vanillin, **20**: Syringic aldehyde, **21**: Daidzin, **22**: Epicatechin gallate, **23**: Piceid, **24**: *p*-Coumaric acid, **25**: Ferulic acid D3, **26**: Ferulic acid, **27**: Sinapic acid, **28**: Coumarin, **29**: Salicylic acid, **30**: Cynaroside, **31**: Miquelianin, **32**: Rutin, **33**: Rutin D3, **34**: Isoquercitrin, **35**: Hesperidin, **36**: o-Coumaric acid, **37**: Genistin, **38**: Rosmarinic acid, **39**: Ellagic acid, **40**: Cosmosiin, **41**: Quercitrin, **42**: Astragalin, **43**: Nicotiflorin, **44**: Fisetin, **45**: Daidzein, **46**: Quercetin D3, **47**: Quercetin, **48**: Naringenin, **49**: Hesperetin, **50**: Luteolin, **51**: Genistein, **52**: Kaempferol, **53**: Apigenin, **54**: Amentoflavone, **55**: Chrysin, **56**: Acacetin)

species have emphasized iridoids, phenylethanoid glycosides, and flavonoids as dominant metabolite classes (Li et al., 2010; Çalış & Başer, 2021), highlighting the relevance of advanced LC–MS/MS profiling for chemotaxonomic and functional interpretation within the genus.

The LC–MS/MS results demonstrated that the phytochemical architecture of *P. pungens* is strongly dominated by quinic acid (14.146 mg/g extract) and chlorogenic acid (13.187 mg/g). Together, these two metabolites account for a substantial proportion of the quantified phenolic pool, indicating that caffeoylquinic acid-related metabolism represents a major biosynthetic axis in this species (Figure 2). Previous phytochemical investigations in the genus have reported quinic acid derivatives and caffeoylquinic acid esters as recurrent constituents. For example, quinic acid esters and chlorogenic acid derivatives were described as characteristic metabolites in several *Phlomis* taxa (Li et al., 2010; Çalış & Başer, 2021). However, most of these studies relied on isolation-based phytochemistry rather than quantitative LC–MS-based metabolomics, and therefore did not provide concentration data. In this context, the present finding that chlorogenic acid reaches 13.187 mg/g in *P. pungens* provides, for the first time, a quantitative benchmark for caffeoylquinic acid abundance within this taxon. Chlorogenic acid is widely recognized for its antioxidant, anti-inflammatory, and enzyme-modulatory properties, and its high concentration in the extract likely underpins the ABTS and CUPRAC antioxidant performance observed in the present study.

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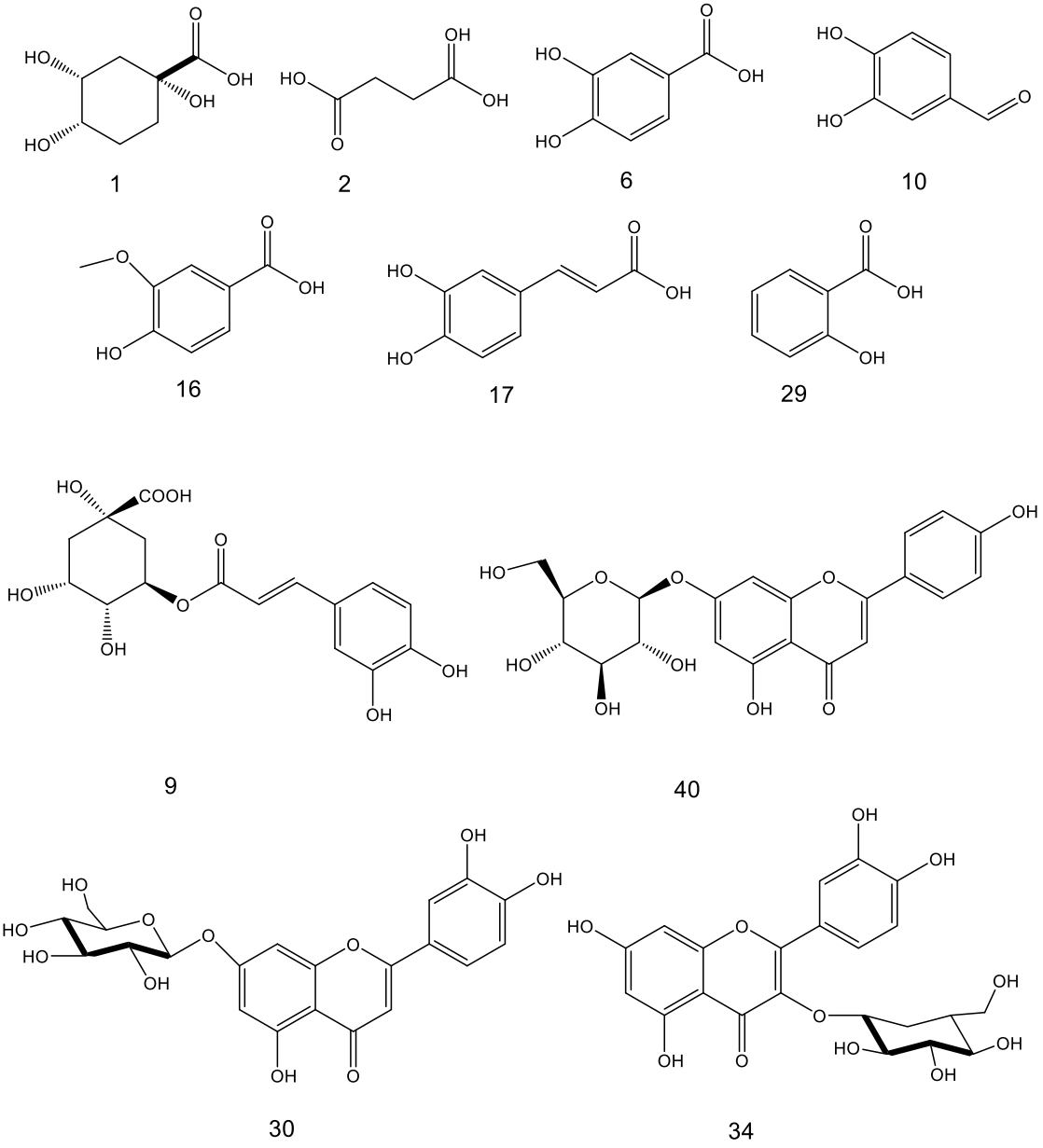

Figure 2. Identified phytochemicals in the studied extracts of *P. pungens* by LC-MS/MS

Although Taşkın et al. (2018) reported antioxidant activity in methanolic extracts of *P. pungens*, no LC–MS-based quantification of chlorogenic acid or related phenolics was provided, limiting chemical–biological correlation in earlier work.

Among flavonoids, cynaroside (luteolin-7-O-glucoside) was quantified at 1.541 mg/g, making it the most abundant flavonoid in the extract. Cosmosiin (apigenin-7-O-glucoside) was present at 0.830 mg/g, while isoquercitrin was detected at 0.175 mg/g. These results align with previous LC–MS-based reports that identify flavonoid glycosides as characteristic constituents of *Phlomis* species (Li et al., 2010; Çalış & Başer, 2021). In a comparative LC–MS/MS investigation of other Lamiaceae species, flavonoid glycosides such as rutin, isoquercitrin, and luteolin derivatives were frequently reported as dominant compounds (Onder et al., 2022; Yilmaz et al., 2023). However, in those studies, the relative abundance of flavonoids often exceeded that of simple phenolic acids. In contrast, *P. pungens* exhibits a phenolic-acid-dominant profile, with flavonoids forming a secondary but still biologically meaningful fraction. This compositional balance distinguishes *P. pungens* from several other Lamiaceae taxa and suggests species-specific metabolic prioritization toward hydroxycinnamic acid pathways. The detection of luteolin (0.096 mg/g), apigenin (0.068 mg/g), and naringenin (0.044 mg/g) further strengthens the flavonoid scaffold profile of the extract. Flavone aglycones and their glycosides are frequently associated with tyrosinase and metalloproteinase inhibition (Çalış & Başer, 2021), which

is consistent with the selective collagenase (24.73%) and tyrosinase (15.04%) inhibition observed in this study.

Comprehensive reviews of *Phlomis* phytochemistry emphasize iridoid glycosides (e.g., lamiide), phenylethanoid glycosides (e.g., forsythoside derivatives), and flavonoids as hallmark constituents of the genus (Li et al., 2010; Çalış & Başer, 2021). Indeed, iridoids and phenylethanoid glycosides have been isolated from *P. pungens* collected in Azerbaijan (Saracoglu et al., 2017). However, these metabolites were not included in the targeted LC–MS/MS panel used in the present study, which focused primarily on phenolic acids and flavonoids. Therefore, while previous isolation studies highlighted iridoid and phenylethanoid scaffolds (Saracoglu et al., 2017), the current work complements this knowledge by establishing the quantitative predominance of quinic and caffeoylquinic acid derivatives in the ethanol extract. Importantly, earlier studies on *P. pungens* essential oils reported germacrene D as the major volatile component, reaching 31.1–39.2% in leaf and flower oils of related subspecies (Khalilzadeh et al., 2008), and similar terpenoid dominance was observed in samples from Iran and Azerbaijan (Masoudi et al., 2006; Kirimer et al., 2017). These volatile-focused studies positioned sesquiterpenes as primary chemical markers of the species. In contrast, the present LC–MS/MS results demonstrate that the non-volatile phytochemical fraction is chemically distinct and dominated by polar phenolics rather than terpenoids. This highlights the importance of solvent selection and analytical strategy when defining the chemotype of *P. pungens*.

From a chemotaxonomic perspective, the predominance of quinic acid derivatives and flavonoid glycosides observed in *P. pungens* is consistent with broader phytochemical trends in the genus *Phlomis*, where hydroxycinnamic acid derivatives and flavonoids are frequently reported (Li et al., 2010; Çalış & Başer, 2021). However, the unusually high quantitative contribution of free quinic acid (14.146 mg/g) relative to flavonoid glycosides may represent a distinguishing chemical feature of this species, at least under the ecological and geographical conditions of collection. Functionally, the phenolic-acid-dominant profile observed here provides a more direct mechanistic basis for the antioxidant capacity and selective enzyme inhibition than previously available in the literature. Earlier biological studies on *P. pungens* reported antioxidant and enzyme inhibitory activities without compound-level quantification (Sarikurkcu et al., 2016; Taşkın et al., 2018; Okur et al., 2022). By contrast, the present LC–MS/MS data enable correlation of specific metabolites—particularly chlorogenic acid, caffeic acid, and luteolin derivatives—with the observed bioactivities.

Compared to previous LC–MS-based phytochemical studies on Lamiaceae species (Onder et al., 2022; Yilmaz et al., 2023), which typically report diverse but moderately distributed phenolic profiles, *P. pungens* demonstrates a more concentrated and structurally coherent phenolic signature dominated by caffeoylquinic acid metabolism. Moreover, unlike prior LC–MS-based work on *Phlomis* that primarily focused on qualitative identification of iridoids and phenylethanoid glycosides (Li et al., 2010; Saracoglu et al., 2017), the present study provides quantitative data expressed in mg/g extract, enabling direct biochemical interpretation and inter-study comparability. Taken together, the LC–MS/MS findings position *P. pungens* as a phenolic-acid-dominant member of the genus, chemically distinct from essential-oil-defined chemotypes and quantitatively characterized at the compound level for the first time. This structured comparison with previous LC–MS and isolation-based studies clarifies that while iridoids and phenylethanoid glycosides are important genus-level markers, caffeoylquinic acid derivatives and flavonoid glycosides represent the major non-volatile quantitative contributors in the ethanol extract of *P. pungens*. This integrated perspective strengthens the phytochemical rationale of the study and more clearly situates the present findings within the broader literature on *Phlomis* species.

3.2 Total Phenolic and Flavonoid Analysis

To complement the compound-level LC–MS/MS analysis, the total phenolic content (TPC) and total flavonoid content (TFC) of the ethanolic extract were determined spectrophotometrically. As presented in Table 2, the extract exhibited a considerable phenolic content (37.18 ± 1.97 µg PEs/mg extract) along with a moderate flavonoid content (18.19 ± 0.90 µg QEs/mg extract). These findings indicate that phenolic compounds represent a major fraction of the extract, while flavonoids contribute to a lesser but still significant extent. The TPC and TFC values are in strong agreement with the LC–MS/MS results (Table 1), which demonstrated the predominance of phenolic acids, particularly quinic and chlorogenic acids, alongside flavonoid glycosides such as cynaroside and cosmosiin. The relatively high TPC can therefore be largely attributed to the abundance of low-molecular-weight phenolic acids, whereas the moderate TFC reflects the prevalence of glycosylated flavonoids rather than aglycones. Because spectrophotometric assays provide a global estimation and may be influenced by matrix effects, the parallel use of LC–MS/MS strengthens the reliability and interpretability of these results. Previous studies on *P. pungens* and other *Phlomis* species have reported variable phenolic and flavonoid contents depending on extraction conditions and geographical origin (Taşkın et al., 2018; Okur et al., 2022). However, most of these studies did not support bulk phenolic data with compound-specific quantification. The present study therefore offers a more robust assessment by linking TPC and TFC values directly to identified metabolites.

3.3 Antioxidant Capacities

The antioxidant potential of the ethanolic extract of *P. pungens* was evaluated using complementary in vitro assays that reflect different antioxidant mechanisms, including free radical scavenging and reducing power. The extract demonstrated measurable activity in all applied assays, with DPPH radical scavenging activity expressed as an IC_{50} value of 87.71 ± 1.23 µg/mL and ABTS radical scavenging activity with an IC_{50} value of 32.05 ± 0.28 µg/mL (Table 2). Additionally, the studied extract exhibited a notable reducing capacity in

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Table 2. Total phenolic and flavonoid contents and antioxidant activities of *Phlomis pungens* species

Samples	Total phenolic (μg PEs/mg)[3]	Total flavonoids (μg QEs/mg)[4]	Antioxidant activity (μg/mL)		
			DPPH (IC_{50})	ABTS (IC_{50})	CUPRAC (IC_{50})
P. pungens	37.18 ± 1.97[a]	18.19 ± 0.90[a]	87.71 ± 1.23[a]	32.05 ± 0.28[a]	20.83 ± 0.37[a]
BHT[5]	–	–	50.72 ± 1.21[b]	16.01 ± 0.77[b]	8.99 ± 0.32[b]
α-TOC[5]	–	–	14.98 ± 0.76[c]	9.05 ± 0.29[c]	14.98 ± 0.39[c]
Oleanolic acid[5]	–	–	–	–	–
Epicatechin gallate[5]	–	–	–	–	–

Note: [1]Data are presented as mean values with standard deviations derived from three independent replicates, normalized to the negative control.
[2]Not active.
[3]PEs, pyrocatechol equivalents (y = 0.0477 + 0.0422 (r^2: *0.9951*)).
[4]QEs, quercetin equivalents (y = 0.0374 + 0.0563 (r^2: *0.9979*)).
[5]Standard compound.
[a,b,c]Different superscript letters within the same column indicate statistically significant differences between samples ($p < 0.05$, one-way ANOVA followed by Tukey's post hoc test).

the CUPRAC assay ($A_{0.5}$ = 20.83 ± 0.37 μg/mL), indicating an efficient electron-donating ability. While these values reflect moderate to good antioxidant activity, they do not indicate exceptionally strong radical scavenging when compared with standard antioxidants, and therefore should be interpreted within the context of a phenolic-rich crude extract rather than as evidence of extraordinary potency.

The stronger performance observed in the ABTS and CUPRAC assays compared to the DPPH assay suggests that the antioxidant behavior of *P. pungens* is primarily driven by hydrophilic phenolic compounds capable of rapid electron transfer (Apak et al., 2022). This pattern is fully consistent with the LC–MS/MS profile, which revealed high concentrations of quinic acid and chlorogenic acid (Table 1). Chlorogenic acid, in particular, is widely recognized as a potent antioxidant and free radical scavenger and has been reported to contribute significantly to the antioxidant capacity of many plant-derived extracts (Alcázar Magaña et al., 2021; Huang et al., 2023). Mechanistically, chlorogenic acid contains a catechol moiety within its caffeoyl structure, enabling efficient hydrogen atom donation and stabilization of phenoxyl radicals through resonance delocalization. Its ability to undergo electron transfer reactions aligns well with the relatively stronger ABTS and CUPRAC responses observed in the present study. Quinic acid, although often considered a weaker antioxidant on its own, can enhance overall antioxidant performance when present at high concentrations and in combination with hydroxycinnamic acids (Alcázar Magaña et al., 2021). In this matrix, quinic acid may act synergistically by influencing solubility, redox cycling, or regeneration dynamics of more active phenolics, thereby indirectly contributing to the observed antioxidant response.

Medium-abundance phenolic acids such as caffeic acid (0.244 mg/g), protocatechuic acid (0.298 mg/g), protocatechuic aldehyde (0.166 mg/g), salicylic acid (0.202 mg/g), and fumaric acid (0.726 mg/g) further strengthen the antioxidant matrix through additive and potentially synergistic effects (Skroza et al., 2022). Caffeic and protocatechuic derivatives are particularly known for their strong radical scavenging properties due to their catechol structures, which facilitate hydrogen atom transfer and metal-chelating capacity, while salicylic acid and vanillic acid may contribute to redox modulation and stabilization of reactive species (Magiera et al., 2025; Skroza et al., 2022). Flavonoid glycosides such as cynaroside (1.541 mg/g), cosmosiin (0.830 mg/g), and isoquercitrin (0.175 mg/g), together with flavonoid aglycones detected at moderate levels (luteolin, apigenin, and naringenin), likely further enhance antioxidant performance, especially in assays based on electron transfer mechanisms (Apak et al., 2022). Structurally, the presence of hydroxyl substitutions on the B-ring of flavones facilitates electron donation and radical stabilization, and glycosylation may influence solubility and bioavailability within the assay system. However, given their moderate concentrations, these flavonoids are more likely to contribute cumulatively rather than individually dominating the antioxidant response.

Earlier investigations of *P. pungens* have reported antioxidant activity mainly in the context of essential oils or crude extracts without detailed chemical correlation (Sarikurkcu et al., 2016; Masoudi et al., 2006; Kirimer et al., 2017; Taşkın et al., 2018; Okur et al., 2022). Essential oil-based studies attributed antioxidant effects largely to terpenoid constituents, whereas the present results demonstrate that the antioxidant capacity of *P. pungens* is more plausibly associated with its non-volatile phenolic fraction. By directly linking antioxidant outcomes with LC–MS/MS-quantified metabolites, this study provides a more chemically substantiated—yet conservatively interpreted—explanation for the antioxidant potential of the species.

3.4 Enzyme Inhibitory Activities

The enzyme inhibitory potential of the ethanolic extract of *Phlomis pungens* was evaluated against a broad panel

Table 3. Collagenase, elastase, tyrosinase, cholinesterase, and urease enzyme inhibitory activities of *Phlomis pungens* species

Samples	Enzyme activity[1] (Inhibition%, at 50 µg/mL concentration)					
	AChE	BChE	Urease	Tyrosinase	Elastase	Collagenase
P. pungens	*nd*[a]	13.62 ± 0.12[a]	*nd*[a]	15.04 ± 0.11[a]	*nd*[a]	24.73 ± 0.32[a]
Galanthamine[2]	86.26 ± 1.33[b]	80.99 ± 2.03[b]	–	–	–	–
Thiourea[2]	–	–	98.50 ± 1.21[b]	–	–	–
Kojic acid[2]	–	–	–	84.64 ± 1.44[b]	–	–
Oleanolic acid[2]	–	–	–	–	50.89 ± 1.01[b]	–
Epicatechin gallate[2]	–	–	–	–	–	90.07 ± 1.03[b]

Note: [1]Data are presented as mean values with standard deviations derived from three independent replicates, normalized to the negative control.
[2]Standard compound.
nd: not active.
[a,b] Different superscript letters within the same enzyme column indicate statistically significant differences between samples ($p < 0.05$, one-way ANOVA followed by Tukey's post hoc test).

of enzymes with pharmacological and cosmeceutical relevance, including collagenase, butyrylcholinesterase (BChE), acetylcholinesterase (AChE), elastase, tyrosinase, and urease. At a concentration of 50 µg/mL, the extract exhibited selective inhibitory activity, showing measurable inhibition of BChE (13.62 ± 0.12%), tyrosinase (15.04 ± 0.11%), and collagenase (24.73 ± 0.32%), while no significant inhibition was observed against AChE, urease, or elastase (Table 3). Although the inhibition levels are moderate, the selectivity profile provides important mechanistic clues regarding the phytochemical drivers of activity. It should be emphasized that these inhibition percentages represent preliminary, screening-level activities obtained at a single concentration and do not reflect detailed potency parameters such as IC_{50} values. Therefore, the present results should be interpreted as an initial indication of bioactivity that warrants further dose–response evaluation and bioassay-guided fractionation rather than as evidence of strong enzyme inhibition.

Among the tested enzymes, collagenase inhibition was the most pronounced, indicating potential relevance of *P. pungens* for applications related to skin aging and extracellular matrix degradation. To date, collagenase inhibitory activity has not been previously reported for *P. pungens*. Earlier studies on this species primarily focused on essential oil composition and antioxidant or anti-inflammatory properties, without evaluating matrix metalloproteinase-related enzymes (Kirimer et al., 2017; Sarikurkcu et al., 2016). In contrast, some *Phlomis* species have been reported to exhibit collagenase or elastase inhibitory effects, often attributed to phenolic acids and flavonoids (Islam et al., 2024; Çalış & Başer, 2021). In the present study, the observed collagenase inhibition can be mechanistically associated with chlorogenic acid, caffeic acid, protocatechuic acid, and flavonoid glycosides such as cynaroside and cosmosiin. Phenolic acids containing catechol or hydroxycinnamic scaffolds are capable of chelating the catalytic zinc ion present in matrix metalloproteinases and forming hydrogen-bond interactions within the enzyme active site, thereby interfering with substrate binding (Islam et al., 2024). Flavone derivatives such as luteolin-based glycosides may further contribute through π–π stacking and hydrogen bonding interactions within the enzyme pocket. Although the concentrations of individual flavonoids are moderate, their combined presence alongside high levels of chlorogenic acid supports a cumulative inhibitory effect. The detection of collagenase inhibition therefore represents a novel biological feature of *P. pungens* and expands its potential dermocosmetic relevance beyond previously reported activities.

The extract also demonstrated moderate tyrosinase inhibitory activity. Tyrosinase inhibition has been investigated previously for *P. pungens*, but mainly in the context of essential oil or crude extract screening, often reporting weak to moderate effects (Sarikurkcu et al., 2016; Okur et al., 2022). The present findings are consistent with these earlier observations in terms of magnitude but provide a more mechanistically informative explanation. The LC–MS/MS profile (Table 1) revealed the presence of flavonoids such as luteolin, naringenin, apigenin, and their glycosides, which are widely recognized as natural tyrosinase inhibitors due to their ability to interact with the copper-containing active site of the enzyme (Hassan et al., 2023). Structurally, the hydroxylated flavone backbone facilitates coordination with the binuclear copper center of tyrosinase, while additional hydrogen-bonding interactions stabilize binding within the active site. Therefore, even at moderate concentrations, these flavonoids may exert measurable inhibition, explaining the observed activity profile.

With respect to cholinesterase inhibition, the extract exhibited mild but selective inhibition of BChE, while showing no activity against AChE. Previous studies on *P. pungens* and related *Phlomis* species have reported cholinesterase inhibition mainly for essential oils or selected extracts (Sarikurkcu et al., 2016; Okur et al., 2022). The present study confirms and extends these findings by demonstrating that *P. pungens* preferentially inhibits BChE rather than AChE. This selectivity is pharmacologically relevant, as BChE activity increases in the later stages of Alzheimer's

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disease, and selective BChE inhibitors are increasingly considered promising therapeutic leads (Fernández-Bolaños et al., 2022; Sang et al., 2025). Phenolic acids and flavonoid scaffolds are capable of interacting with the peripheral anionic site and catalytic active site gorge of cholinesterases through π–π interactions and hydrogen bonding. Although their inhibitory potency is generally lower than that of alkaloid-type inhibitors, cumulative weak interactions from multiple phenolics may account for the modest yet selective BChE inhibition observed. The absence of alkaloid-type compounds in the LC–MS/MS profile may partly explain the lack of AChE activity (Çalış & Başer, 2021).

Urease inhibition was not observed for *P. pungens* in the present study, suggesting that the phenolic composition identified may not effectively interfere with the nickel-containing catalytic site of urease under the tested conditions. Similarly, elastase inhibition was not detected, which is consistent with the lack of previous reports describing elastase inhibition for *P. pungens* (Kirimer et al., 2017). While some *Phlomis* species have been reported to exhibit elastase inhibitory effects (Çalış & Başer, 2021), such activity is often linked to specific flavonoid aglycones present at higher concentrations than those observed here.

Overall, the enzyme inhibitory profile of *P. pungens* should be interpreted as selective and moderate rather than broadly potent. Nevertheless, the mechanistic consistency between the identified phenolic compounds—particularly chlorogenic acid and flavone derivatives—and the observed inhibition of collagenase, tyrosinase, and BChE strengthens the biochemical plausibility of the results. By integrating compound-level LC–MS/MS data with enzyme inhibition outcomes, the present study provides a more mechanistically grounded interpretation of bioactivity than previous reports relying solely on crude extract screening. Future studies involving purified fractions, isolated compounds, and full kinetic characterization will be necessary to confirm the strength and specificity of these inhibitory effects.

4 Conclusions

This study establishes *Phlomis pungens* as a notably phenolic-rich medicinal plant when evaluated through a modern non-volatile profiling lens. Unlike earlier research emphasizing essential oils, the present work provides the first LC–MS/MS-based quantitative assessment of *P. pungens* and demonstrates that its chemistry is dominated by quinic acid (14.146 mg/g) and chlorogenic acid (13.187 mg/g), supported by flavonoid glycosides such as cynaroside (1.541 mg/g) and cosmosiin (0.830 mg/g). This metabolite architecture aligns well with the extract's measurable TPC/TFC and consistent antioxidant performance across ABTS, DPPH, and CUPRAC assays. Bioactivity screening further indicated selective enzyme inhibition, with the strongest activity against collagenase (24.73%) and moderate inhibition of tyrosinase (15.04%) and BChE (13.62%), suggesting potential relevance to dermocosmetic and health-related applications where oxidative stress and extracellular-matrix or melanogenesis-associated enzymes are implicated. Collectively, these findings expand the scientific understanding of *P. pungens* beyond its volatile fraction and provide a chemically grounded explanation for its antioxidant and enzyme inhibitory tendencies. Future studies should prioritize (i) broader metabolomics to capture phenylethanoid glycosides/iridoids not covered in the targeted panel, (ii) bioassay-guided fractionation to identify the most active contributors to collagenase/tyrosinase inhibition, and (iii) mechanistic, in vivo, and in silico validation to translate this phytochemical potential into safe and effective phytopharmaceutical or cosmeceutical candidates.

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Author Contributions

Enes Arica: Supervision, Writing original draft, Investigation, Data curation, Funding acquisition. Mehmet Firat: Data curation.

Availability of Data and Materials

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Conflicts of Interest

The authors declare that they have no competing financial interests.

Supporting Information

Supplementary information for this article is available online at http://www.acgpubs.org/journal/records-of-natural-products.

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