

RESEARCH ARTICLE

Comprehensive Organ-Specific Phenolic Characterization of Endemic *Hedysarum candidissimum* by Liquid Chromatography–Tandem Mass Spectrometry: Antioxidant, Enzyme-Inhibitory, and Antiproliferative Activities Supported by Chemometric Analysis

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ABSTRACT

Hedysarum candidissimum (HC), a species endemic to Türkiye, represents a potential source of bioactive phytochemicals with pharmacological applications. This study provides the first comprehensive investigation of the species, integrating organ-specific phytochemical profiling with biological activity evaluations. Methanolic extracts from leaf, stem, and root were analyzed for their phytochemical composition and biological activities. Liquid Chromatography–Tandem Mass Spectrometry analysis quantified 53 phenolic compounds and revealed organ-dependent differences in metabolite distribution. Quinic acid was the major phenolic acid, with the highest content in the stem (91.81 mg/g extract), while flavonoid glycosides such as isoquercitrin (90.72 mg/g extract) and astragalín (43.61 mg/g extract) were higher in leaves. Antioxidant activity was determined by DPPH, FRAP, TPC, and TFC assays. The leaf extract showed the strongest activity, with the lowest DPPH IC₅₀ (5.43 ± 0.18 µg/mL), highest reducing power (192.47 ± 0.91 mg TE/g extract), and highest phenolic and flavonoid contents (220.84 ± 1.07 mg GAE/g extract and 107.26 ± 0.77 mg QE/g extract). Enzyme inhibition assays demonstrated strong activity against acetylcholinesterase, butyrylcholinesterase, and α-glucosidase (2.44 ± 0.11, 3.35 ± 0.37, and 3.40 ± 0.20 µg/mL). Antiproliferative activity showed moderate growth inhibition in lung (A549, Calu1, H1650) and breast (MCF-7, MDA-MB-231) cancer cell lines (GI₅₀: 2.40–19.15 µg/mL), with higher sensitivity in breast cells. The stem extract exhibited the highest tumor selectivity index (1.83), while LDH assays indicated low toxicity. Multivariate analyses confirmed organ-specific metabolite patterns and relationships between phenolic composition and biological activities. These findings indicate that the species, particularly the leaf extract, represents a source of phenolic metabolites for further phytochemical and biological investigation.

1 | Introduction

Medicinal plants continue to be recognized as sources of bioactive natural products with potential pharmaceutical and nutraceutical applications. Plant-derived secondary metabolites are important resources in drug discovery because of their structural diversity and diverse biological activities (Harvey et al. 2015; Newman and Cragg 2020; Atanasov et al. 2021). Among these metabolites, phenolic compounds constitute one of the most widespread and biologically relevant classes in plants and include phenolic acids, flavonoids, tannins, lignans, and related derivatives (Dai and Mumper 2010). These compounds are of particular interest because they are associated with antioxidant effects and with the modulation of biological targets involved in chronic disorders (Shahidi and Ambigaipalan 2015; Panche et al. 2016).

Phenolic compounds have been widely investigated because oxidative stress is closely associated with the development of disorders such as cancer, diabetes mellitus, cardiovascular diseases, and neurodegenerative conditions (Liguori et al. 2018). In addition to their radical-scavenging properties, many phenolics have shown inhibitory activity against enzymes involved in metabolic and neurodegenerative diseases. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are among the therapeutic targets in Alzheimer's disease, while α -glucosidase inhibition is relevant to the control of postprandial hyperglycemia in type 2 diabetes mellitus (Lane et al. 2006; Tundis et al. 2010). Phenolic constituents have also been associated with antiproliferative activity by mechanisms involving apoptosis induction, modulation of oxidative stress, and cell-cycle regulation, and phenolic plant extracts therefore represent promising materials for multifunctional bioactivity studies (Dai and Mumper 2010; Cragg and Newman 2013).

The genus *Hedysarum* L. (Fabaceae) comprises about 300 species distributed mainly in temperate regions of Asia, Europe, and North Africa, and several members of the genus have long been used in traditional medicine (Dong et al. 2013). Phytochemical studies on *Hedysarum* species have revealed a wide variety of metabolites, including flavonoids, isoflavonoids, phenolic acids, triterpenoids, coumarins, lignans, and polysaccharides, together with diverse biological activities such as antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, antidiabetic, and anticancer effects (Dong et al. 2013; Gao et al. 2023; Baltürk et al. 2025). *H. candidissimum* (HC), an endemic species of Türkiye, has also been reported as a potentially valuable source of bioactive phytochemicals. In addition, HC has a geographically restricted distribution in the Upper Kızılırmak and Upper Fırat regions of Türkiye and has been reported from calcareous steppe habitats and fallow fields at elevations of about 800–1500 m (Hedge 1970). Previous studies on HC have reported certain phenolic constituents and biological activities in crude extracts (Altay et al. 2022); however, the phytochemical composition and biological properties of its individual plant organs have not yet been investigated in detail. The biosynthesis and distribution of secondary metabolites may vary among leaves, stems, and roots; therefore, organ-based evaluation can provide information on the localization of bioactive constituents and on plant parts with higher concentrations of pharmacologically relevant metabolites. In this context, LC–MS/MS-based phytochemical profiling combined with antioxidant, enzyme inhibition, and antiproliferative

assays provides a useful approach for clarifying how organ-specific differences in phenolic composition are reflected in biological activity.

To the best of our knowledge, this study represents the first comprehensive in vitro investigation of organ-specific variations in phenolic composition and their association with antioxidant, enzyme inhibitory, and antiproliferative activities in HC. Accordingly, the present study aimed to characterize the phytochemical profile and evaluate the biological activities of methanolic extracts obtained from different plant organs (leaf, stem, and root). Phenolic constituents were identified and quantified using LC–MS/MS, while biological activities were evaluated using a series of in vitro assays, including antioxidant capacity, inhibition of AChE, BChE, and α -glucosidase, and antiproliferative effects against selected human cancer cell lines. By integrating detailed phytochemical profiling with multiple bioactivity assessments, this study provides new information on the organ-specific phenolic composition of HC and its in vitro biological activities, thereby contributing to the phytochemical and bioactivity-based characterization of this species.

2 | Materials and Methods

2.1 | Plant Material

The plant material of HC was collected from the Soğuksular locality of Kemah (Erzincan, Türkiye) in July 2024 during the flowering period. The collected specimens were separated into root, stem, and leaf parts and dried under laboratory conditions in the shade, protected from direct sunlight. The plant was taxonomically identified by Prof. Dr. Ali Kandemir (Department of Biology, Faculty of Arts and Sciences, Erzincan Binali Yıldırım University). A voucher specimen has been deposited in the EBYU Herbarium (Herbarium No: 0000046).

2.2 | Extraction Procedure

The dried plant materials (root, stem, and leaf) were ground into a fine powder. Ten grams of each sample were extracted with 100 mL of methanol by maceration overnight at room temperature. Methanol was selected as the extraction solvent because the present study was designed to compare the phenolic composition and biological activities of different plant organs under standardized extraction conditions. The metabolites included in the analysis were phenolic acids, flavonoids, and related medium-polarity constituents to be quantified by LC–MS/MS; therefore, a polar solvent capable of extracting these compounds was required. Methanol is widely used in phytochemical studies because it provides extraction of phenolic acids, flavonoids, and related phenolic constituents from plant materials (Alara et al. 2021; Kiliç et al. 2025). In addition, the use of a single solvent for leaf, stem, and root samples minimized extraction-related variability and allowed the differences observed among organs to be interpreted more consistently in relation to organ-specific phytochemical composition instead of solvent-dependent selectivity. The extraction was repeated three times, and the resulting extracts were combined. The mixtures were filtered to remove plant residues,

and the solvent was evaporated under reduced pressure to obtain crude extracts. The extracts were stored in dark vials at 4 °C until further analysis. The extraction yields, calculated relative to the initial dry plant material, were 14.9% for leaf, 10.1% for stem, and 7.4% for root. The differences in extraction yield among leaf, stem, and root samples may be attributed to organ-specific variation in the concentration of methanol-soluble constituents and to differences in tissue composition.

2.3 | LC-MS/MS Conditions

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used for the quantitative analysis of 53 phytochemicals in the methanolic extracts of HC leaves, stems, and roots using a previously developed and validated analytical method; detailed validation parameters, including limit of detection (LOD), limit of quantification (LOQ), recovery, and precision, are provided in the corresponding method studies (Akman, Şimşek, Akşit, Akşit et al. 2024; Yılmaz et al. 2025). Analyses were performed on a Shimadzu Nexera ultrahigh-performance liquid chromatograph (UHPLC) coupled with a tandem mass spectrometer. A reversed-phase UHPLC system was used, equipped with an auto-sampler (SIL-30AC), a column oven (CTO-10ASvp), binary pumps (LC-30AD), and a degasser (DGU-20A3R). Chromatographic separation was performed on a reversed-phase Agilent Poroshell 120 EC-C18 analytical column (150 mm × 2.1 mm, 2.7 µm) at a column temperature of 40 °C. The elution was performed using a binary solvent system, where eluent A consisted of water with 5 mM ammonium formate and 0.1% formic acid, and eluent B consisted of methanol with 5 mM ammonium formate and 0.1% formic acid. The gradient elution program was applied as follows: 20%–100% B from 0 to 25 min, 100% B from 25 to 35 min, and 20% B from 35 to 45 min. The flow rate was set to 0.5 mL/min, and the injection volume was 5 µL.

Mass spectrometric analysis was performed using a Shimadzu LCMS-8040 tandem mass spectrometer equipped with an electrospray ionization (ESI) source operating in both positive and negative ion modes (Akman, Şimşek, Akşit, Aydın et al. 2024). Data acquisition and evaluation were performed using LabSolutions software (Shimadzu). The multiple reaction monitoring mode was used for the selective identification and quantification of phytochemicals, based on optimized precursor-to-product ion transitions. Collision energies (CE) were set to provide proper fragmentation and to improve the transmission of target product ions. The MS operating conditions were as follows: drying gas (N₂) flow rate of 15 L/min, nebulizing gas (N₂) flow rate of 3 L/min, desolvation line temperature of 250 °C, heat block temperature of 400 °C, and interface temperature of 350 °C.

2.4 | Antiproliferative Activity

2.4.1 | Cell Lines and Cell Culture

Human lung cancer cell lines A549 (ATCC CCL-185), Calu1 (ATCC HTB-54), and H1650 (ATCC CRL-5883), and breast cancer cell lines MCF7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26), together with the normal lung cell line Beas-2B (RRID: CVCL-0168) and the normal breast cell line MCF10A (ATCC

CRL-10317), were used in this study. All cell culture procedures were conducted in a laminar flow cabinet. Cells were cultured in DMEM or RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 2% penicillin–streptomycin and incubated at 37 °C in a 5% CO₂ atmosphere. Cells were plated in 96-well plates at a density of 1 × 10⁴ cells per well. After 16 h of incubation to allow cell attachment, samples were added, and cells were incubated for an additional 24 h before analysis (Köksal et al. 2021; Akman, Simsek, Akşit et al. 2025).

2.4.2 | Measurement of Cell Proliferation

The MTT assay was used to evaluate the effects of the plant extracts on cell proliferation after 24 h of incubation with cancer cell lines (Akşit et al. 2025; Gözcü et al. 2025). Results were expressed as percentage inhibition, with the optical density of solvent-treated (DMSO) cells defined as 100%. Percentage inhibition was calculated using the formula: % inhibition = $[1 - (A_{\text{test}}/A_{\text{control}})] \times 100$. IC₅₀ values were calculated from absorbance data obtained after treatment with increasing concentrations of the extracts (1.96–125.0 µg/mL). Dose-response curves were generated using logarithmic regression analysis in Microsoft Excel. GI₅₀ values were calculated based on cell growth according to the following equations: $[(T_i - T_z)/(C - T_z)] \times 100$ for $T_i \geq T_z$ (cytostatic effect) or $[(T_i - T_z)/T_z] \times 100$ for $T_i < T_z$ (cytotoxic effect), where T_z represents the absorbance at time zero, C denotes control growth, and T_i indicates growth in the presence of the test samples. The antiproliferative effects were expressed as the concentration required for 50% growth inhibition (GI₅₀). The tumor selectivity index (TSI) was calculated using the IC₅₀ value obtained for normal cells relative to that of cancer cells.

2.4.3 | Cytotoxicity Test

Cytotoxic and cytostatic effects of the extracts were determined using the lactate dehydrogenase (LDH) release assay (Şimşek et al. 2023; Gözcü et al. 2024b). LDH is a stable cytoplasmic enzyme that is released into the culture supernatant after damage to the plasma membrane, and increased LDH levels therefore reflect increased cell death. The LDH cytotoxicity assay was performed in accordance with the manufacturer's instructions. Formazan formation resulting from LDH enzymatic activity was quantified spectrophotometrically, and percentage cytotoxicity was calculated using the following equation

$$\% \text{Cytotoxicity} = \frac{(A_{\text{sample}} - A_{\text{low control}}) / (A_{\text{high control}} - A_{\text{low control}})}{\times 100}.$$

2.5 | Enzyme Assays

2.5.1 | Anti-Cholinesterase Activity

The inhibitory effects of the plant extracts on AChE and BChE were determined spectrophotometrically using a previously described procedure with minor modifications (Tüfekçi et al. 2023; Akman, Simsek, Erken et al. 2025; Köksal et al. 2025). Plant extracts and tacrine (positive control) were diluted with deionized water to obtain different concentrations. The experimental reaction mixture consisted of 100 µL of Tris-HCl buffer

(1 M, pH 8.0), 50 μ L of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and 20 μ L of enzyme solution. The reaction was initiated with acetylthiocholine iodide for AChE and butyrylthiocholine iodide for BChE as substrates. Following enzymatic hydrolysis of the substrates, thiocholine reduced DTNB and generated the yellow 5-thio-2-nitrobenzoate anion. The absorbance at 412 nm was measured spectrophotometrically. Results are expressed as IC₅₀ values, defined as the concentration of the extract required to inhibit enzyme activity by 50%. All experiments were performed in triplicate, and results are reported as mean $\pm$ standard deviation (SD).

2.5.2 | α -Glucosidase Inhibition Activity

A previously described procedure with minor modifications was used to determine α -glucosidase inhibitory activity (Akman et al. 2023). Acarbose and the plant extracts used as positive controls were diluted with deionized water to the concentrations required for the assay. Phosphate buffer (1 M, pH 7.4), sample, and enzyme solution were added to the test tube and incubated at 35 °C for 10 min. Following incubation, *p*-nitrophenyl- α -D-glucopyranoside (*p*-NPG) was added, and the mixture was incubated again at 35 °C. The absorbance at 405 nm was measured spectrophotometrically. IC₅₀ values were determined from concentration-response curves. All experiments were performed in triplicate, and results are reported as mean $\pm$ standard deviation (SD).

2.6 | Antioxidant Activity

2.6.1 | DPPH Free Radical Scavenging Activity

The DPPH[•] radical scavenging activity of the extracts was determined according to a previously described method (Albayrak et al. 2024; Akşit and Şimşek 2025; Şimşek 2026). Briefly, 3 mL of the test sample prepared at different concentrations was mixed with 1 mL of a 0.26 mM DPPH[•] solution. The mixtures were vortex-mixed and incubated in the dark at room temperature for 30 min. The absorbance values were determined at 517 nm. The radical scavenging activity was determined according to the formula: RSA (%) = [(A₀ - A₁)/A₀] $\times$ 100, where A₀ is the control absorbance, and A₁ is the absorbance obtained with extracts or standards. The recorded absorbance values were converted to percentage activity, and the IC₅₀ (μ g/mL) values for the extracts were then calculated. The results were compared with standard antioxidants such as Trolox, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and ascorbic acid. All analyses were performed in triplicate, and the results are expressed as mean $\pm$ standard deviation.

2.6.2 | Ferric Reducing Antioxidant Power (FRAP)

The reducing power activity of HC root, stem, and leaf extracts was evaluated following a previously described procedure (Alkan et al. 2025; Şimşek 2025b; Şimşek et al. 2026). Following the preparation of extract stock solutions (1 mg/mL), 250 μ L was mixed with phosphate buffer (0.2 M, pH 6.6) to obtain a final volume of 1.25 mL. Incubation at 50 °C for 20 min was performed after adding 1.25 mL of 1% K₃Fe(CN)₆. After incubation, the reaction mixture was mixed with 1.25 mL of 10% TCA and 0.25 mL of 0.1% FeCl₃. Absorbance of the final mixture was determined at 700 nm, and

the results were expressed as mg TE/g extract according to a calibration curve obtained with Trolox (10–100 μ g/mL). The results were compared with the standard antioxidants Trolox, BHT, BHA, and ascorbic acid. All assays were conducted in triplicate, and the results are expressed as mean $\pm$ standard deviation.

2.6.3 | Total Phenolic Content

The total phenolic content of the extracts was determined by the Folin–Ciocalteu method according to a previously reported procedure (Akşit et al. 2022; Gözcü et al. 2024a; Şimşek 2025a). Briefly, distilled water (4.5 mL) was mixed with 100 μ L of extract and standard solutions (1 mg/mL). Following the addition of 100 μ L of Folin–Ciocalteu reagent to the mixtures, 300 μ L of 2% Na₂CO₃ was added. After vortexing, the mixtures were incubated at room temperature for 120 min, and the absorbance was determined spectrophotometrically at 760 nm. Various concentrations of gallic acid (1–1000 μ g/mL) were used to generate the calibration curve ($y = 0.117x - 0.011$). Phenolic content was expressed as mg gallic acid equivalents (GAE) per g extract. All measurements were performed in triplicate.

2.6.4 | Total Flavonoid Content

Total flavonoid content was determined according to a previously reported procedure (Varol et al. 2023; Adigüzelli et al. 2024; Şimşek et al. 2025). First, 4.7 mL of methanol was mixed with 100 μ L of extract and standard solutions (1 mg/mL). The mixtures were vortexed and incubated at room temperature for 45 min following the addition of 100 μ L NH₄CH₃COO (1 M) solution and 100 μ L AlCl₃ (10%). Then, the absorbance of the mixture was determined at 415 nm. Using a calibration curve constructed with quercetin (1–1000 μ g/mL), the results were expressed as mg quercetin equivalents (QE) per g extract. All tests were performed in triplicate.

2.7 | Statistical Analyses

Statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). All experiments were conducted in triplicate, and the results are presented as mean $\pm$ standard deviation (SD). Differences among the extracts obtained from different plant organs were evaluated by one-way analysis of variance, followed by Duncan's multiple range test for post-hoc comparisons among group means. Differences were considered statistically significant at $p < 0.05$. In addition, multivariate analyses, including principal component analysis (PCA) and hierarchical heatmap analysis, were performed using the quantitative phytochemical and bioactivity data to determine organ-dependent distribution patterns and their associations with biological activities.

3 | Results and Discussion

3.1 | Phytochemical Investigation of the Extracts by LC–MS/MS

Phytochemical profiling of the HC extracts was performed using a previously developed and validated LC–MS/MS method. The

extracts were analyzed for their bioactive phytochemical composition, and 53 phenolic compounds were quantified. The quantitative data are presented in Table 1, while Figure 1 shows the LC-MS/MS TIC chromatograms of the HC extracts.

LC-MS/MS profiling of HC showed significant differences in the distribution of secondary metabolites among root, stem, and leaf organs, providing a more detailed chemical perspective than previous whole-plant extract analyses (Altay et al. 2022). While previous research quantified phenolics in methanol, ethyl acetate, and water extracts obtained from the whole aerial parts, the present study evaluates the phytochemical composition according to plant part, allowing differences in compound distribution to be observed. This plant part-based approach provides additional information on metabolite distribution in HC and supports the interpretation of its biological activity.

Quinic acid was the major component of the phytochemical profile and showed its highest concentration in stems (91.81 mg/g

extract), followed by leaves (35.457 mg/g extract) and roots (29.135 mg/g extract). The high concentrations observed in the aerial parts indicate increased activity of the shikimate pathway in this organ. Within the genus *Hedysarum*, quinic acid has frequently been reported as a major metabolite with recognized antioxidant and hepatoprotective implications (Dong et al. 2013). The concentrations observed in the stems suggest that this fraction could be considered for antioxidant-related nutraceutical or functional food applications.

Evaluation of phenolic acids reveals a contrasting distribution pattern. Protocatechuic acid shows the highest concentration in roots (1.237 mg/g extract), followed by stems (0.858 mg/g extract), and the lowest concentration in leaves (0.214 mg/g extract), indicating a higher concentration in root organs. Salicylic acid showed its highest concentration in stems (1.57 mg/g extract), followed by roots (0.955 mg/g extract) and leaves (0.771 mg/g extract). Gallic acid exhibits relatively balanced concentrations across organs (root: 0.219 mg/g extract;

TABLE 1 | LC-MS/MS quantitative results of extract of HC (mg analyte/g extract).

No	Compound name	RT	Leaf	Stem	Root	No	Compound name	RT	Leaf	Stem	Root
1	Quinic acid	3.0	35.457	91.81	29.135	29	Salicylic acid	21.8	0.771	1.57	0.955
2	Fumaric acid	3.9	N.D.	0.821	0.993	30	Cyanoside	23.7	N.D.	N.D.	N.D.
3	Aconitic acid	4.0	N.D.	N.D.	N.D.	31	Miquelianin	24.1	N.D.	N.D.	N.D.
4	Gallic acid	4.4	0.304	0.297	0.219	32	Rutin-D3-IS	25.5	N.A.	N.A.	N.A.
5	Epigallocatechin	6.7	N.D.	N.D.	N.D.	33	Rutin	25.6	1.38	0.229	0.1
6	Protocatechuic acid	6.8	0.214	0.858	1.237	34	Îsoquercitrin	25.6	90.719	21.945	4.569
7	Catechin	7.4	11.007	14.803	N.D.	35	Hesperidin	25.8	1.538	0.146	0.072
8	Gentisic acid	8.3	0.203	0.16	0.123	36	o-Coumaric acid	26.1	N.D.	N.D.	N.D.
9	Chlorogenic acid	8.4	N.D.	0.031	0.035	37	Genistin	26.3	N.D.	N.D.	N.D.
10	Protocatechuic aldehyde	8.5	N.D.	N.D.	0.534	38	Rosmarinic acid	26.6	N.D.	N.D.	N.D.
11	Tannic acid	9.2	0.971	0.347	0.14	39	Ellagic acid	27.6	N.D.	N.D.	N.D.
12	Epigallocatechin gallate	9.4	N.D.	N.D.	N.D.	40	Cosmosiin	28.2	N.D.	N.D.	N.D.
13	Cynarin	9.8	N.D.	N.D.	N.D.	41	Quercitrin	29.8	0.056	N.D.	N.D.
14	4-OH Benzoic acid	10.5	N.D.	N.D.	N.D.	42	Astragalin	30.4	43.613	5.818	0.797
15	Epicatechin	11.6	N.D.	N.D.	N.D.	43	Nicotiflorin	30.6	0.254	N.D.	N.D.
16	Vanillic acid	11.8	N.D.	N.D.	N.D.	44	Fisetin	30.6	N.D.	N.D.	N.D.
17	Caffeic acid	12.1	0.026	0.074	0.144	45	Daidzein	34.0	N.D.	N.D.	0.018
18	Syringic acid	12.6	N.D.	N.D.	N.D.	46	Quercetin-D3-IS	35.6	N.A.	N.A.	N.A.
19	Vanillin	13.9	N.D.	N.D.	N.D.	47	Quercetin	35.7	0.471	0.261	N.D.
20	Syringic aldehyde	14.6	N.D.	N.D.	N.D.	48	Naringenin	35.9	0.017	0.073	0.173
21	Daidzin	15.2	N.D.	N.D.	N.D.	49	Hesperetin	36.7	0.048	0.063	0.036
22	Epicatechin gallate	15.5	N.D.	N.D.	N.D.	50	Luteolin	36.7	N.D.	0.003	0.007
23	Piceid	17.2	N.D.	N.D.	N.D.	51	Genistein	36.9	0.003	0.013	0.067
24	p-Coumaric acid	17.8	0.004	0.03	0.012	52	Kaempferol	37.9	0.083	N.D.	N.D.
25	Ferulic acid-D3-ISh	18.8	N.A.	N.A.	N.A.	53	Apigenin	38.2	N.D.	N.D.	0.006
26	Ferulic acid	18.8	N.D.	N.D.	N.D.	54	Amentoflavone	39.7	N.D.	0.007	N.D.
27	Sinapic acid	18.9	N.D.	N.D.	N.D.	55	Chrysin	40.5	N.D.	N.D.	N.D.
28	Coumarin	20.9	N.D.	N.D.	N.D.	56	Acacetin	40.7	0.039	0.176	0.983

Abbreviations: IS, internal standard; NA, not applicable; ND, not detected.

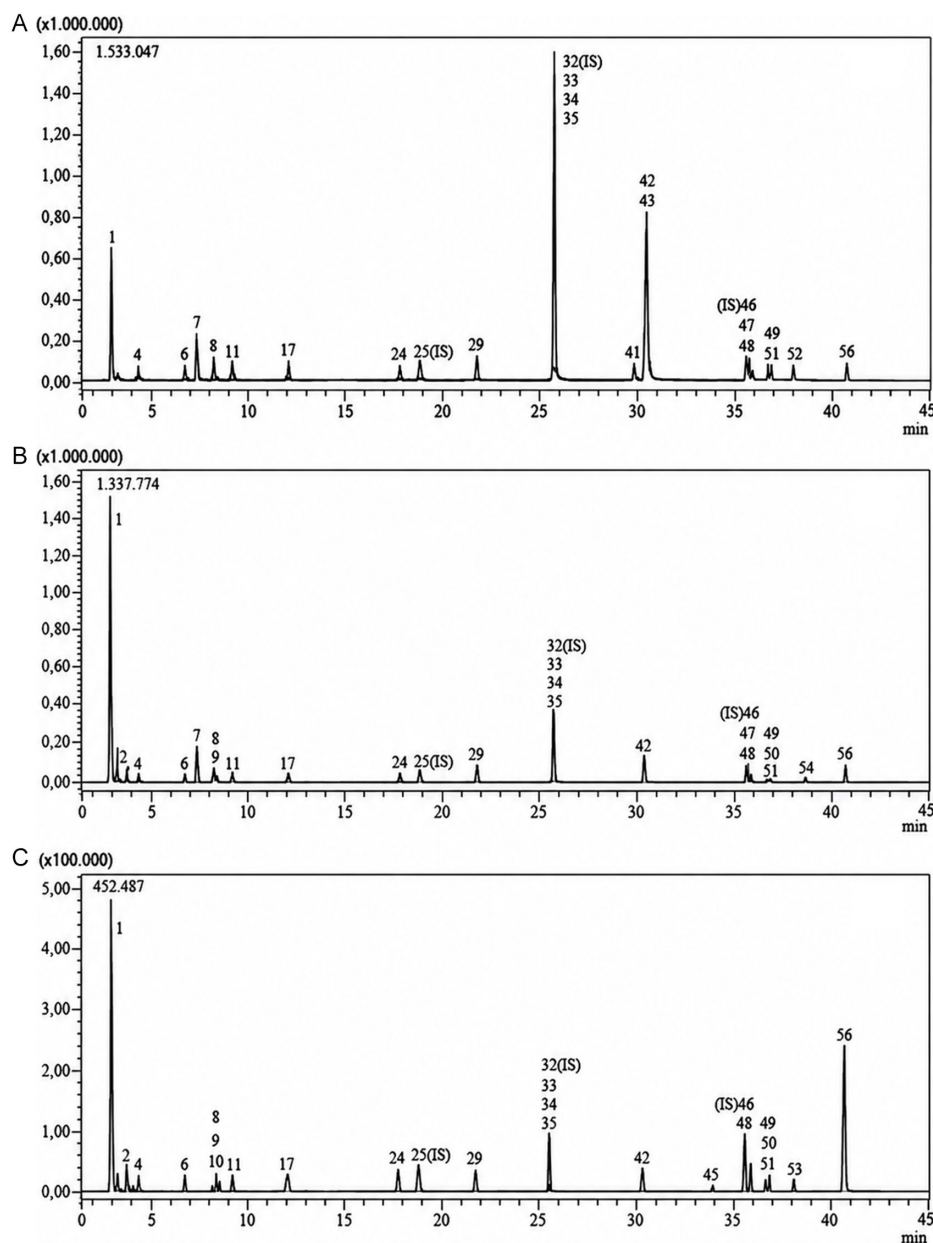


FIGURE 1 | Total ion chromatograms of the (A) leaf, (B) stalk, and (C) root extracts of HC.

stem: 0.297 mg/g extract; leaf: 0.304 mg/g extract), while caffeic acid is higher in roots and lower in leaves (0.144 and 0.026 mg/g extract, respectively). These hydroxybenzoic and hydroxycinnamic derivatives are widely documented for antimicrobial, anti-inflammatory, and radical-scavenging activities in *Hedysarum* species and may synergize with flavonoids in contributing to the bioactivities previously reported for HC extracts (Dong et al. 2013; Altay et al. 2022).

A major quantitative feature of the present data is the high concentration of flavonoid glycosides in leaves. Isoquercitrin reaches 90.719 mg/g extract in leaves, compared with 21.945 mg/g extract in stems and 4.569 mg/g extract in roots. Astragalin follows a similar gradient, with 43.613 mg/g extract in leaves, 5.818 mg/g extract in stems, and 0.797 mg/g extract in roots. Rutin is also present at higher concentrations in leaves (1.38 mg/g extract) compared to stems (0.229 mg/g extract) and roots (0.1 mg/g

extract). Hesperidin was present at higher concentrations in leaves (1.538 mg/g extract) compared with stems (0.146 mg/g extract) and roots (0.072 mg/g extract). The higher concentration of glycosylated flavonols in leaves suggests that photosynthetically active organs are an important site of antioxidant flavonoid content. Such compounds are recognized as bioactive markers in the genus and are associated with α -glucosidase and cholinesterase inhibition reported previously for this species (Altay et al. 2022). From a food science perspective, isoquercitrin and rutin are valued for vascular protective effects and their role in improving oxidative stability, supporting their potential incorporation into functional food systems (Dong et al. 2013).

Flavan-3-ols show a different distribution pattern. Catechin was not observed in roots but was present at high concentrations in stems (14.803 mg/g extract) and leaves (11.007 mg/g extract). This suggests increased tannin biosynthesis in aerial organs,

consistent with reports that aerial parts of *Hedysarum* species often contain higher concentrations of tannins (Burlando et al. 2017). Given the strong antioxidant and collagen-protective properties of catechin derivatives, this distribution may be associated with enzyme inhibition and skin-protective effects described in related species (Burlando et al. 2017).

In contrast, aglycone flavonoids and isoflavones were present at higher relative concentrations in roots. Genistein is highest in roots (0.067 mg/g extract), followed by stems (0.013 mg/g extract) and leaves (0.003 mg/g extract). Daidzein was present only in roots (0.018 mg/g extract). Naringenin shows the highest concentration in roots (0.173 mg/g extract) compared with stems (0.073 mg/g extract) and leaves (0.017 mg/g extract). Hesperetin presents moderate distribution (root: 0.036 mg/g extract; stem: 0.063 mg/g extract; leaf: 0.048 mg/g extract). The higher concentration of isoflavonoids in roots is consistent with patterns reported for other *Hedysarum* species and reflects their documented estrogenic, anti-cancer, and antidiabetic relevance (Keleke et al. 2025).

When these findings are directly compared with the previous whole-plant LC–MS/MS analysis of HC, several important distinctions are observed (Altay et al. 2022). In a previous study, isoquercitrin was quantified in the methanol extract at 7.581 mg/g extract, rutin at 1.915 mg/g extract, and hesperidin at 2.308 mg/g extract. In contrast, the present leaf-focused analysis shows isoquercitrin at 90.719 mg/g extract and astragalin at 43.613 mg/g extract, values higher than those reported for the whole-plant extract. Similarly, quinic acid was previously reported in the methanol extract at 28.377 mg/g extract, while the value observed in stems (91.81 mg/g extract) indicates that certain plant parts contain about threefold higher concentrations. These differences show how whole-plant extraction can dilute highly concentrated metabolites from specific plant organs. In addition, previous research reported high total phenolic content and strong enzyme inhibition in methanol extracts; the present plant part-based data show that this activity is associated mainly with isoquercitrin and astragalin in leaves, while stem fractions contain higher concentrations of quinic acid and catechin (Altay et al. 2022).

Comparison with additional *Hedysarum* species further supports these findings. The genus is widely characterized by the presence of flavonoids and medicinal relevance in traditional applications (Dong et al. 2013). Reports on *H. pallidum* describe glycosylated kaempferol derivatives (Dob et al. 2024), and *H. vicioides* similarly contains quercetin glycosides (Murai and Nakamura 2022). The current results are consistent with these trends and show high concentrations of isoquercitrin and astragalin in the leaves of HC.

To investigate metabolite distribution across plant organs, heatmap analysis (Figure 2) and PCA (Figure 3) were conducted using quantitative LC–MS/MS results. The heatmap shows relative metabolite profiles across leaves, stems, and roots, indicating organ-specific distribution patterns. Flavonoid glycosides such as isoquercitrin, astragalin, hesperidin, and rutin were present at higher concentrations in leaves, consistent with their quantitative values. Catechin was also present at high concentrations in both leaves and stems, while quinic acid was highest in stems, followed by roots and leaves. Several phenolic acids, including

protocatechuic acid, fumaric acid, and caffeic acid, exhibited relatively higher concentrations in roots, together with aglycone-type compounds such as acacetin and protocatechuic aldehyde, which were present mainly or only in the root. The heatmap thus indicates that flavonoid glycosides are mainly present in aerial organs, particularly in leaves, while phenolic acids and aglycone flavonoids show a root-associated or widely distributed pattern. The PCA (Figure 3) showed that PC1 and PC2 accounted for 70.8% and 29.2% of the total variation, respectively, and clearly separated the three plant organs according to their phytochemical composition. The position of the leaf extract on PC1 was associated mainly with high concentrations of flavonoid glycosides, particularly isoquercitrin, astragalin, hesperidin, rutin, and nicotiflorin, together with quercetin and tannic acid, indicating that these variables contributed most to leaf differentiation. In contrast, the stem extract was positioned toward PC2 and was associated with quinic acid, catechin, salicylic acid, *p*-coumaric acid, and amentoflavone, suggesting that these metabolites contributed more strongly to the stem-specific phytochemical profile. The root extract was located in the negative region of PC1 and was characterized by protocatechuic acid, caffeic acid, naringenin, fumaric acid, luteolin, and acacetin, indicating that aglycone-type flavonoids and selected phenolic acids were more closely associated with root separation. The heatmap analysis together with these results indicated that organ discrimination was associated mainly with the contrast between leaf flavonoid glycosides and the more acid-/aglycone-oriented metabolite patterns of stem and root extracts.

Overall, this LC–MS/MS analysis shows a differential distribution in HC, where leaves concentrate flavonoid glycosides, stems show higher concentrations of quinic acid and catechin, and roots contain relatively higher proportions of isoflavonoid aglycones and selected phenolic acids. By separating and quantifying individual plant parts, the present study provides more detailed phytochemical data than the previous whole-plant investigation and highlights the importance of tissue-resolved analysis for pharmacological, nutraceutical, and functional food development strategies.

3.2 | Enzyme Inhibition Activity of Extracts

This study provides the first comprehensive evaluation of the inhibitory activities of leaf, stem, and root extracts of HC against AChE, BChE, and α -glucosidase enzymes. The results were compared with the standard inhibitors, tacrine and acarbose. The findings are presented in Figures 4–6, and the corresponding IC₅₀ values are presented in Table 2. The data demonstrate that enzyme inhibition is strongly dependent on plant part, with the leaf extract exhibiting the highest activity across all tested enzymes.

In vitro inhibition of AChE was highest for the leaf extract ($2.44 \pm 0.11 \mu\text{g/mL}$; $R^2 = 0.9254$), followed by the stem ($5.37 \pm 0.17 \mu\text{g/mL}$; $R^2 = 0.9918$) and root extracts ($6.42 \pm 0.12 \mu\text{g/mL}$; $R^2 = 0.9894$). Tacrine, used as the reference inhibitor, showed an AChE IC₅₀ value of $3.43 \pm 0.40 \mu\text{g/mL}$ ($R^2 = 0.9981$, $p < 0.05$). The leaf extract therefore showed stronger AChE inhibition than tacrine, while the stem and root extracts showed lower activity. Compared with tacrine, the leaf extract shows an IC₅₀ value ~29% lower

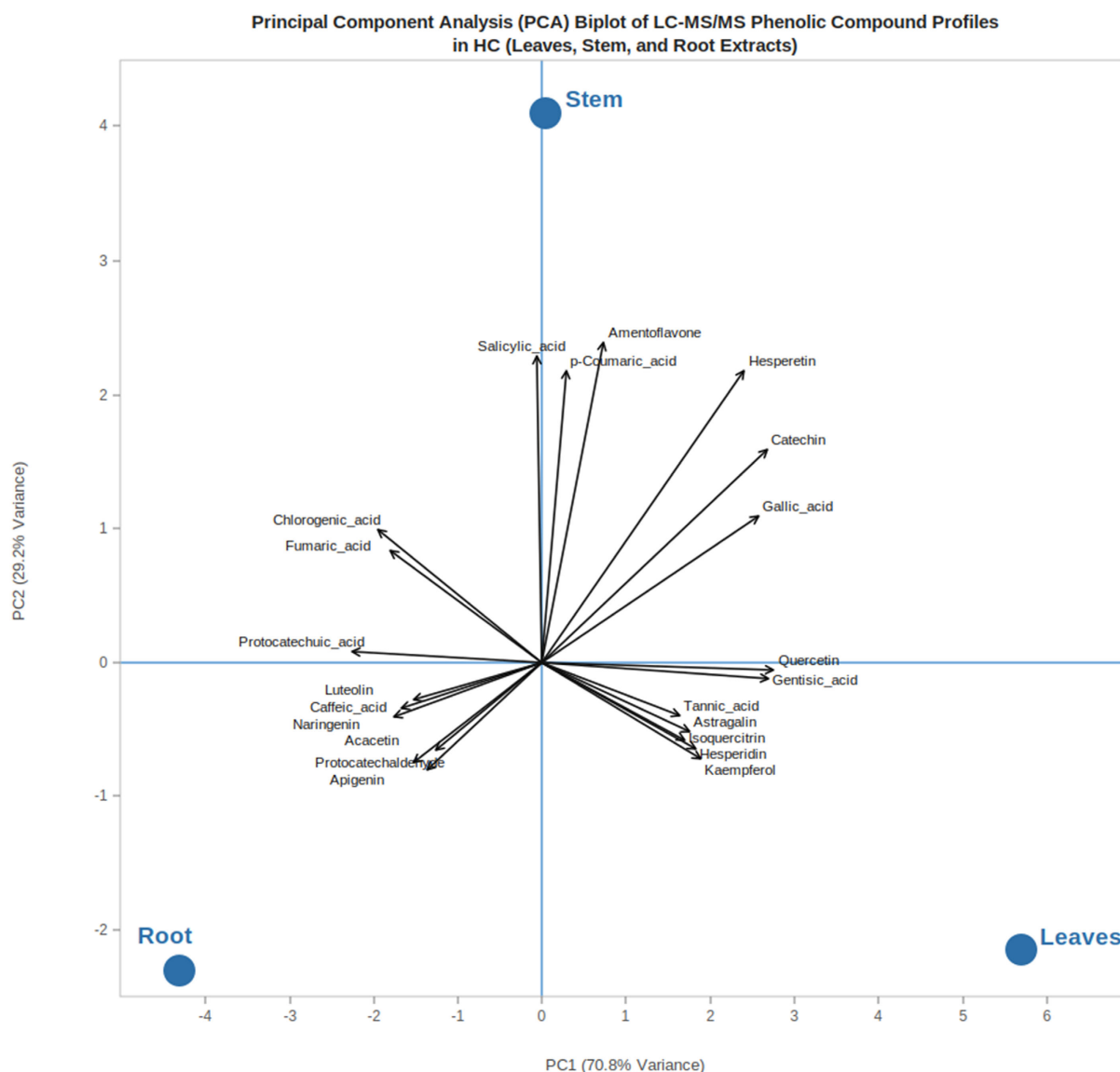


FIGURE 2 | Heatmap of LC-MS/MS-derived phenolic compounds in different organs (leaf, stem, and root) of HC.

($3.43 \rightarrow 2.44 \mu\text{g/mL}$), while the stem and root show values $\sim 57\%$ and $\sim 87\%$ higher, respectively. These quantitative differences indicate that HC leaves contain an effective mixture of anticholinesterase constituents, while the stem and root organs contain lower concentrations and/or different phenolic profiles associated with lower inhibitory activity.

A similar pattern was observed for BChE. The leaf extract again showed the lowest IC_{50} ($3.35 \pm 0.37 \mu\text{g/mL}$; $R^2 = 0.9630$). The root ($5.17 \pm 0.04 \mu\text{g/mL}$; $R^2 = 0.9539$) and stem ($5.54 \pm 0.39 \mu\text{g/mL}$; $R^2 = 0.9544$) extracts showed moderate inhibition and had similar values. Tacrine inhibited BChE at $3.18 \pm 0.20 \mu\text{g/mL}$ ($R^2 = 0.9768$), indicating that the leaf extract is within a similar potency range to the reference compound, while stem and root extracts showed lower inhibitory activity. These results indicate that the leaf extract is anticholinesterase-active and shows a stronger preference for AChE than the other plant organs.

For α -glucosidase, the leaf extract showed the strongest inhibition ($3.40 \pm 0.20 \mu\text{g/mL}$; $R^2 = 0.9927$), followed by the stem

($4.17 \pm 0.21 \mu\text{g/mL}$; $R^2 = 0.9507$) and root extracts ($4.88 \pm 0.07 \mu\text{g/mL}$; $R^2 = 0.9551$). Acarbose, used as a positive control, showed an IC_{50} of $3.55 \pm 0.042 \mu\text{g/mL}$ ($R^2 = 0.9992$), indicating that the leaf extract exhibited potency comparable to acarbose. The α -glucosidase regression models showed high correlation ($R^2 \approx 0.95\text{--}0.99$), supporting the reliability of the calculated IC_{50} values and indicating that the observed order reflects biological differences and not model-related effects.

When compared with previous reports on *Hedysarum*, the IC_{50} values obtained here indicate that the HC leaf extract is among the most potent reported for the genus and more active than the only directly comparable study on HC. In a previous study, ethyl acetate, methanol, and water extracts of HC were evaluated, and the methanol extract showed AChE inhibition with an IC_{50} of $93.26 \mu\text{g/mL}$ (Altay et al. 2022). The same study also reported α -glucosidase inhibition of $28.57 \pm 0.82 \mu\text{g/mL}$. In comparison, the leaf extract in the present study (AChE = $2.44 \mu\text{g/mL}$; α -glucosidase = $3.40 \mu\text{g/mL}$) is ~ 38 -fold and ~ 8 -fold more potent, respectively. These differences suggest that plant organ

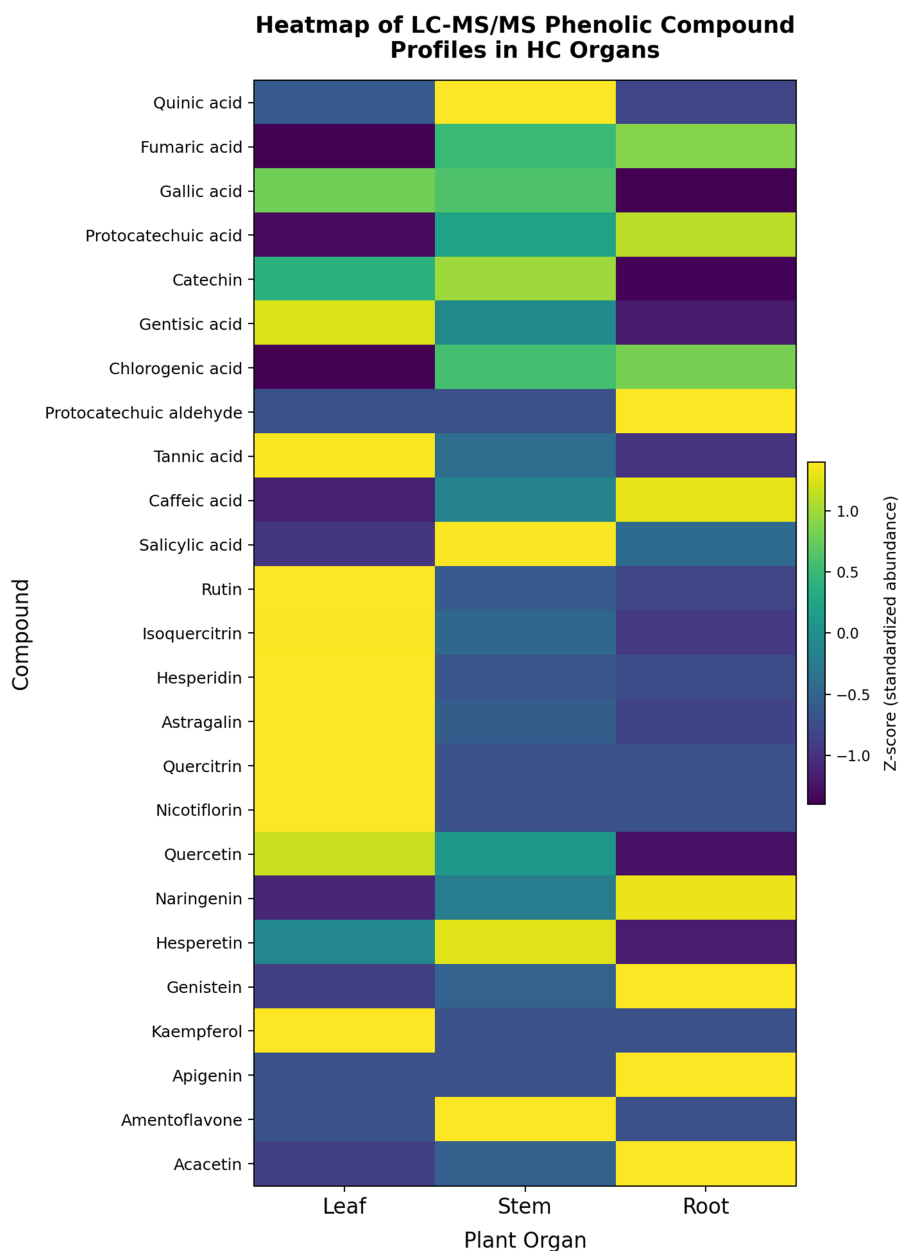


FIGURE 3 | PCA biplot of LC-MS/MS-derived phytochemicals in different organs (leaf, stem, and root) of HC.

selection, extraction parameters, and ecological or seasonal chemotype variation may strongly influence the concentrations of enzyme-active secondary metabolites in HC. LC-MS/MS analysis in that report indicated phenolic acids and flavonoid glycosides as the main metabolites (Altay et al. 2022).

The stronger enzyme-inhibitory activity of the leaf extract was closely associated with its organ-specific flavonoid profile. LC-MS/MS analysis showed that the leaf extract contained higher concentrations of isoquercitrin, astragalin, rutin, and hesperidin, together with catechin, compared with the stem and root extracts. Among these compounds, isoquercitrin and astragalin were present at the highest concentrations in the leaf extract, indicating that they may contribute to the observed AChE, BChE, and α -glucosidase inhibition. This interpretation is also supported by the PCA analysis, which showed that the leaf extract clustered with enzyme inhibition activity and with these

flavonoid glycosides, while the lower IC_{50} values were observed in the extract containing higher concentrations of isoquercitrin, astragalin, rutin, and hesperidin. Flavonoids of this type have been reported to inhibit cholinesterases and carbohydrate-hydrolyzing enzymes by interacting with catalytic and peripheral binding regions via hydrogen bonding, hydrophobic contacts, and π - π interactions (Katalinić et al. 2010; Taslimi et al. 2017; Caglayan et al. 2019; Goudjil et al. 2024; Al-Hilo 2025). Therefore, although the biological effects observed in the present study should be considered the result of a multicomponent extract, the enzyme-inhibitory activity of HC leaves can be associated with isoquercitrin, astragalin, rutin, hesperidin, and catechin, which were present at higher concentrations in this organ than in the stem and root extracts.

To further investigate the relationship between enzyme-inhibitory activity and phytochemical composition, PCA was

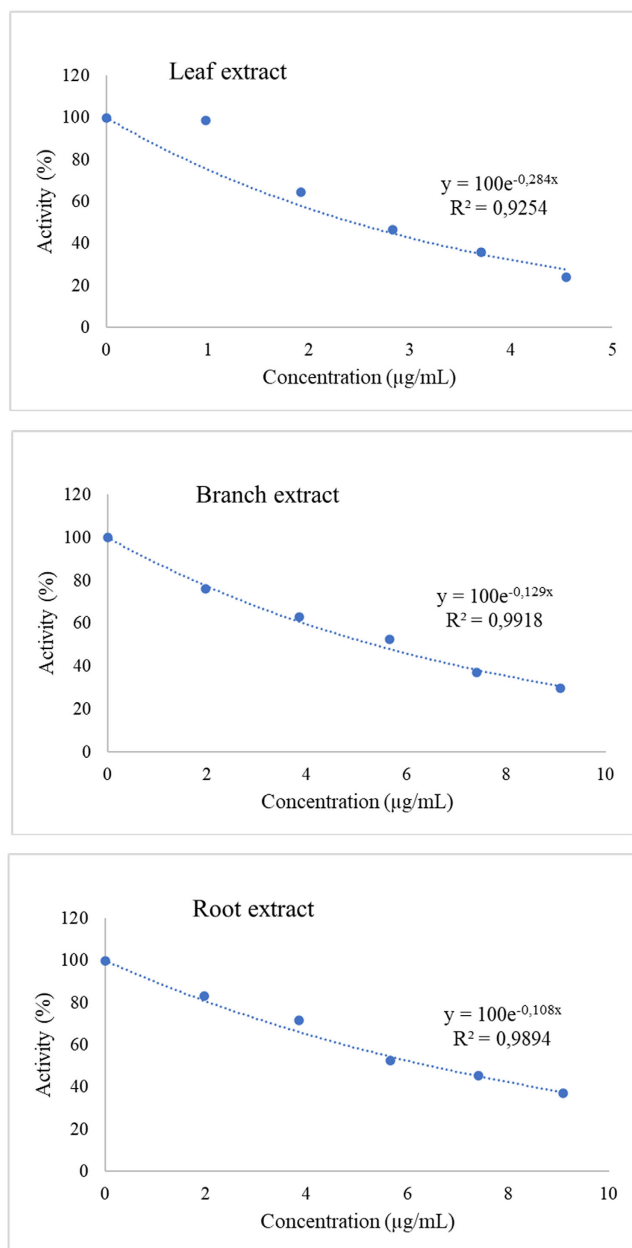


FIGURE 4 | Activity (%) concentration graph of methanolic extracts of leaves, stems, and roots of HC for the enzyme acetylcholinesterase (AChE).

performed by integrating AChE, BChE, and α -glucosidase IC_{50} values with the LC-MS/MS metabolite profiles of the leaf, stem, and root extracts (Figure 7). The PCA showed a clear separation of the three plant organs according to their combined chemical and biological characteristics. The leaf extract was positioned in the region opposite to the IC_{50} variables and was closely associated with flavonoid glycosides such as isoquercitrin, astragalgin, rutin, hesperidin, miquelianin, and nicotiflorin, together with quercetin and kaempferol. Because lower IC_{50} values correspond to stronger enzyme inhibition, this distribution indicates that these variables contributed strongly to the leaf-associated inhibitory profile observed against AChE, BChE, and α -glucosidase. In contrast, the stem extract showed a distinct position and was associated more closely with quinic acid, salicylic acid, catechin, and *p*-coumaric acid, suggesting that these metabolites contributed

more to the stem-specific phytochemical pattern than to the stronger inhibitory activity observed in the leaf extract. The root extract showed a separate distribution from the aerial organs and was associated with aglycone-type flavonoids and isoflavonoids, including naringenin, genistein, acacetin, and luteolin, together with comparatively higher IC_{50} values. This positioning suggests that the lower enzyme-inhibitory activity of the root extract was associated with a phytochemical profile characterized by lower concentrations of the flavonoid glycosides more closely associated with the leaf extract. The PCA confirmed organ-dependent separation of the HC extracts and indicated that the strongest enzyme-inhibitory profile was associated mainly with the flavonoid glycosides concentrated in the leaf extract, while stem and root extracts were characterized by

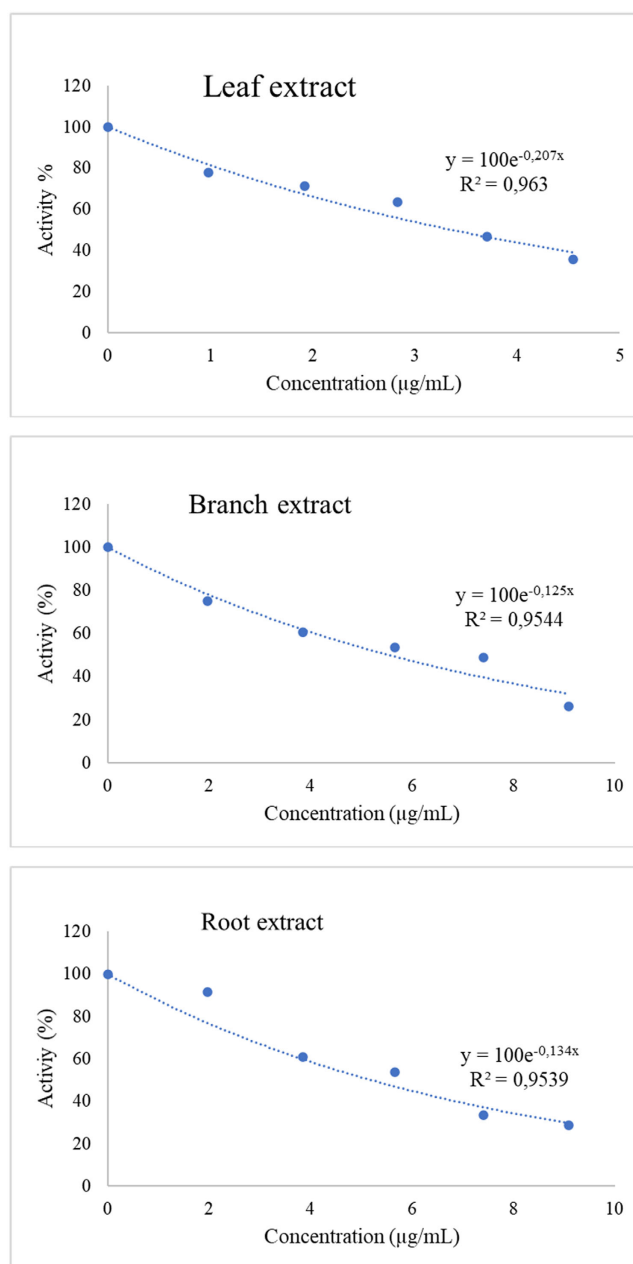


FIGURE 5 | The activity % concentration graph shows the inhibitory activity of methanol extracts obtained from leaves, stems, and roots of HC on butyrylcholinesterase (BChE).

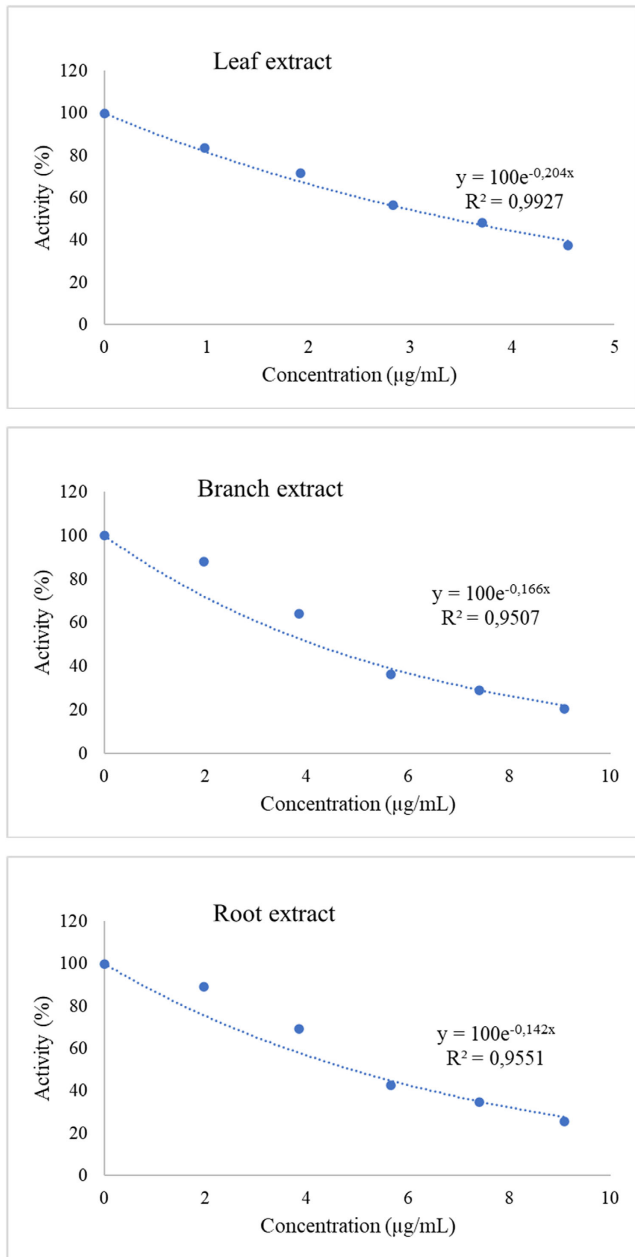


FIGURE 6 | Inhibitory activity of methanol extracts from the leaves, stems, and roots of HC against the enzyme α -glucosidase.

TABLE 2 | IC_{50} (μ g/mL) values of the inhibitory effects of HC on AChE, BChE, and α -glucosidase enzymes.

Inhibitor	IC_{50} (μ g/mL)				α -Glucosidase	R^2
	AChE	R^2	BChE	R^2		
Leaf	2.44 ± 0.11^a	0.9254	3.35 ± 0.37^a	0.9630	3.40 ± 0.20^a	0.9927
Stem	5.37 ± 0.17^c	0.9918	5.54 ± 0.39^c	0.9544	4.17 ± 0.21^b	0.9507
Root	6.42 ± 0.12^d	0.9894	5.17 ± 0.04^b	0.9539	4.88 ± 0.07^c	0.9551
Tacrine	3.43 ± 0.40^b	0.9981	3.18 ± 0.20^a	0.9768	—	—
Acarbose	—	—	—	—	3.55 ± 0.042^a	0.9992

Note: Values are expressed as mean $\pm$ SEM ($n = 3$). Statistical significance was evaluated by one-way ANOVA. Different superscript (a–d) letters within the same column denote statistically significant differences among groups at $p < 0.05$. R^2 values represent the coefficients of determination obtained from dose–response curve analysis.

metabolite groups showing a different relationship with the tested enzyme activities.

A broader genus-level comparison further indicates the unusually high potency observed in this study. Zengin et al. (2015) investigated *H. varium* extracts and reported IC_{50} values for the most active ethyl acetate extract (AChE: 1.44 ± 0.001 mg/mL; BChE: 3.29 ± 0.018 mg/mL) (Zengin et al. 2015). Similarly, Baltürk et al. (2025) reported that the water extract of *H. nitidum* showed AChE and BChE inhibition (47.62 ± 1.45 μ g/mL; 106.1 ± 1.48 μ g/mL, respectively) (Baltürk et al. 2025). In addition, *H. coronarium* leaf extracts have been shown to inhibit AChE and BChE and were proposed as potential candidates for Alzheimer’s disease management (D’Errico et al. 2025).

Research on the antidiabetic potential of *Hedysarum* species is comparatively limited; however, available data indicate that methanol extracts often show the highest α -glucosidase inhibitory activity. Reported values include *H. nitidum* (39.53 ± 0.27 μ g/mL) and *H. varium* (2.88 ± 0.051 mg/mL), while acarbose showed an IC_{50} of 6.67 ± 0.20 mg/mL in the same assay systems (Zengin et al. 2015; Altay et al. 2022; Baltürk et al. 2025). In comparison, the leaf extract evaluated here (3.40 μ g/mL) showed stronger inhibition and potency comparable to acarbose.

Overall, the findings of the present study are consistent with previous reports on *Hedysarum* species but show higher activity values. Variations in inhibitory activity within and between species may be influenced by harvesting time, geographic origin, climatic conditions, soil composition, and both qualitative and quantitative differences in secondary metabolite composition. In conclusion, HC, particularly the leaf extract, exhibited strong in vitro inhibitory activity against cholinesterase and α -glucosidase. These findings suggest that the species contains constituents for further investigation in terms of enzyme-targeted bioactivity; however, additional in vivo and mechanistic studies are required before conclusions regarding therapeutic relevance in neurodegenerative or metabolic disorders can be considered.

3.3 | Antiproliferative Activity of Extracts

Cancer is a major contributor to mortality globally, and although numerous chemotherapeutic agents are available, many

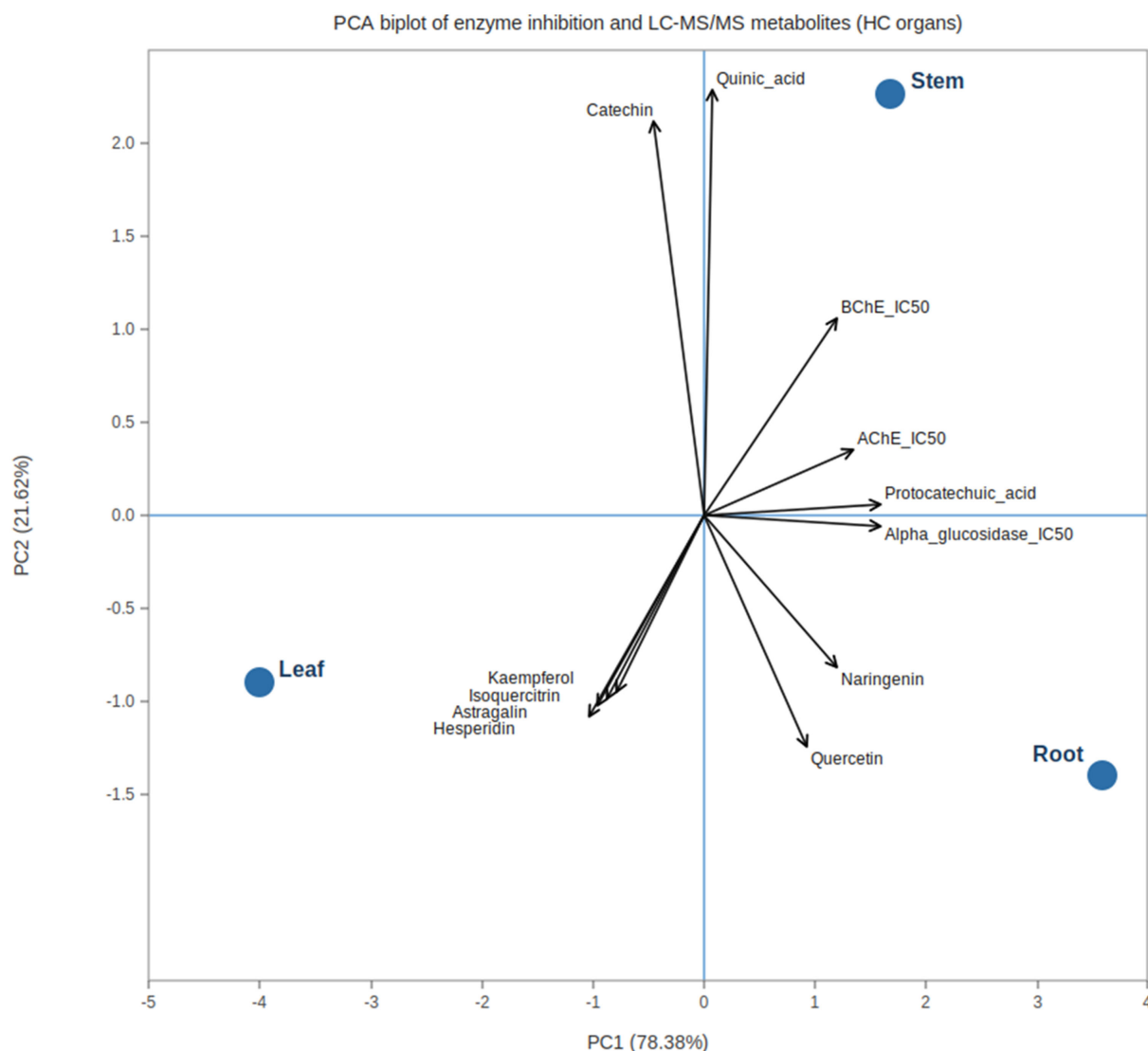


FIGURE 7 | PCA biplot integrating enzyme inhibitory activities and LC-MS/MS-derived metabolites in different organs (leaf, branch, and root) of HC.

treatments show limited therapeutic benefit because of tumor heterogeneity, genetic alterations, and drug resistance mechanisms. In addition, severe toxicity associated with many chemotherapeutic agents limits their long-term use. Therefore, the discovery of new natural compounds with antiproliferative potential and relatively low toxicity represents an important research area. In this context, the antiproliferative effects of plant extracts obtained from different plant organs (leaf, stem, and root) were evaluated against lung cancer (A549, Calu1, and

H1650) and breast cancer (MCF7 and MDA-MB-231) cell lines using the MTT assay. In addition, normal cell lines (Beas2B and MCF10A) were used to evaluate the safety profile of the extracts. The calculated GI_{50} and IC_{50} values are presented in Tables 3 and 4.

The GI_{50} values indicated moderate growth inhibitory activity against A549, Calu1, and H1650 cells, with values between 13.71 and 19.15 $\mu\text{g/mL}$ depending on the extract and the cell line.

TABLE 3 | GI_{50} and IC_{50} values of HC extracts in lung cancer and normal cell lines.

Extracts ($\mu\text{g/mL}$)	A549		Calu1		H1650		Beas2B		TSI
	GI_{50}	IC_{50}	GI_{50}	IC_{50}	GI_{50}	IC_{50}	GI_{50}	IC_{50}	
Leaf	15.17	270.59	19.15	229.53	18.10	211.06	15.29	214.49	0.90
Stem	14.85	285.22	10.06	274.26	15.14	133.08	2.75	222.58	0.96
Root	18.65	296.58	15.09	247.27	13.71	112.00	11.15	227.81	1.04
5FU	2.10	95.77	2.23	96.43	1.98	94.55	2.01	94.38	0.99

TABLE 4 | GI₅₀ and IC₅₀ values of HC extracts in breast cancer and normal cell lines.

Extracts ($\mu\text{g/mL}$)	MCF7		MDA-MB-231		MCF10A		TSI
	GI ₅₀	IC ₅₀	GI ₅₀	IC ₅₀	GI ₅₀	IC ₅₀	
Leaf	17.76	151.37	9.95	148.21	14.25	171.74	1.15
Stem	16.49	127.21	2.40	56.85	4.52	168.03	1.83
Root	6.82	167.72	10.70	151.04	12.10	159.00	1.00
5FU	1.83	91.35	2.14	95.82	1.90	92.40	0.99

Among the tested extracts, the stem extract demonstrated the strongest growth inhibition against Calu1 cells (GI₅₀ = 10.06 $\mu\text{g/mL}$), while the leaf extract showed the highest GI₅₀ value in the same cell line (19.15 $\mu\text{g/mL}$), indicating a lower inhibitory effect. The root extract showed relatively stronger inhibitory activity against H1650 cells (GI₅₀ = 13.71 $\mu\text{g/mL}$) compared with the other extracts. When compared with the reference chemotherapeutic drug 5-FU, which exhibited GI₅₀ values of about 2 $\mu\text{g/mL}$, the extracts showed lower potency in terms of direct antiproliferative activity. However, the IC₅₀ values of the extracts were between 112.00 and 296.58 $\mu\text{g/mL}$, which are higher than those observed for 5-FU (95 $\mu\text{g/mL}$). The lowest IC₅₀ value was observed in H1650 cells treated with the root extract, while the highest value was observed in A549 cells treated with the same extract. These findings suggest that the extracts exhibit a cytostatic mode of action, characterized by inhibition of cellular proliferation without significant cytotoxicity, which may represent a beneficial characteristic for natural anti-cancer candidates.

Compared with lung cancer cells, breast cancer cells showed higher sensitivity to the extracts. The GI₅₀ values were between 6.82 and 17.76 $\mu\text{g/mL}$ for MCF7 cells and 2.40 and 10.70 $\mu\text{g/mL}$ for MDA-MB-231 cells. The strongest inhibitory activity was observed in MDA-MB-231 cells treated with the stem extract (GI₅₀ = 2.40 $\mu\text{g/mL}$). Although this value is slightly higher than that of 5-FU (GI₅₀ $\approx$ 2 $\mu\text{g/mL}$), it indicates considerable antiproliferative activity, particularly against triple-negative breast cancer cells. In MCF7 cells, which represent estrogen receptor-positive breast cancer, the root extract demonstrated the strongest growth inhibition (GI₅₀ = 6.82 $\mu\text{g/mL}$) compared with the other extracts. The IC₅₀ values were between 56.85 and 167.72 $\mu\text{g/mL}$, while the IC₅₀ value of 5-FU was 90 $\mu\text{g/mL}$, indicating that although the extracts show lower potency than the reference drug, they still exhibit moderate antiproliferative activity.

The TSI was determined to evaluate the selectivity of the extracts toward cancer cells relative to normal cells. TSI values were between 0.90 and 1.04 in lung cancer models and between 1.00 and 1.83 in breast cancer models (Tables 3 and 4). Among the tested extracts, the stem extract showed the highest selectivity in breast cancer cells (1.83), suggesting that this extract may inhibit tumor cell proliferation with comparatively lower toxicity toward normal mammary cells. When compared with the reference chemotherapeutic drug 5-FU, the extracts demonstrated moderate selectivity toward cancer cells. Although the selectivity values were moderate, these findings indicate that

certain plant extracts may contain bioactive compounds capable of targeting cancer cells more strongly than normal cells, which may be advantageous for potential anticancer agents because it could help reduce adverse effects on healthy tissues. Similar selective antiproliferative properties have been reported for several natural products containing flavonoids and phenolic compounds, which are known to target cancer cells through mechanisms involving oxidative stress modulation and apoptosis induction.

The antiproliferative activity observed in the present study is consistent with previous reports describing the biological activities of species within the *Hedysarum* genus. Phytochemical investigations have demonstrated that *Hedysarum* species contain a wide variety of bioactive compounds, including flavonoids, phenolic acids, triterpenoids, coumarins, and lignans, many of which are associated with antioxidant and antitumor properties (Dong et al. 2013). These secondary metabolites have been reported to influence multiple cellular pathways involved in cancer progression, including apoptosis induction, oxidative stress regulation, and inhibition of tumor cell proliferation. Previous reports on HC have described significant cytotoxic activity against several cancer cell lines, including colon and breast cancer models, supporting the results obtained in the present study (Altay et al. 2022). Similarly, investigations on *H. gmelinii* have shown that prenylated chalcones isolated from plant roots exhibit moderate antiproliferative activity against several cancer cell lines, including HepG2, A549, and HCT116 (Liu et al. 2018). These findings are consistent with the relatively strong inhibitory activity observed for the root extract in the present study.

In addition, pharmacological studies on *H. multijugum* have revealed that flavonoids such as quercetin, kaempferol, and formononetin may regulate important molecular targets involved in tumor growth, including signaling pathways related to apoptosis and cell proliferation (Wang et al. 2025). These compounds have been shown to influence genes such as TP53, AKT1, CASP3, and VEGFA, which are associated with tumor development (Wang et al. 2025). Flavonoids identified in *Hedysarum* species have also been reported to inhibit cancer cell proliferation, including mechanisms such as induction of apoptosis, modulation of oxidative stress, and regulation of the cell cycle. Therefore, the antiproliferative effects observed in the present study may be associated with similar classes of phytochemicals present in the investigated extracts.

To investigate the relationship between antiproliferative activity and phytochemical composition of HC extracts, PCA was

performed by integrating the growth-inhibitory data obtained from lung (A549, Calu1, and H1650) and breast (MCF7 and MDA-MB-231) cancer cell lines with the LC-MS/MS metabolite profiles (Figure 8). The PCA showed distinct grouping of leaf, stem, and root extracts according to their combined phytochemical and antiproliferative profiles, indicating that the biological responses were associated with organ-dependent differences in metabolite composition. The leaf extract was positioned in association with flavonoid glycosides, particularly isoquercitrin, astragaloside, rutin, hesperidin, miquelianin, and nicotiflorin, which were present at higher concentrations in this organ. The association of these variables with the activity pattern observed in several cancer cell models suggests that flavonoid glycosides contributed significantly to the antiproliferative profile of the leaf extract. In contrast, the stem extract showed an intermediate position in the PCA space and was associated more closely with quinic acid, catechin, salicylic acid, and *p*-coumaric acid, indicating that the growth-inhibitory activity of this organ may reflect the contribution of a different phenolic subgroup than that

observed in the leaf extract. The root extract showed a separate distribution and was associated with aglycone flavonoids and iso-flavonoids, including naringenin, genistein, daidzein, acacetin, and luteolin. This distribution suggests that the antiproliferative profile of the root extract may be associated with these metabolites and not with the flavonoid glycosides that characterized the leaf extract. Accordingly, the PCA supports the interpretation that the differences observed in antiproliferative activity among HC organs were associated with total phenolic concentration as well as with variation in the phenolic classes associated with each plant part. In this respect, leaf-associated flavonoid glycosides appear to contribute more strongly to the antiproliferative pattern of the aerial organs, while the root profile reflects the contribution of aglycone-type flavonoids and isoflavonoids.

Combined evaluation of the LC-MS/MS and PCA data suggests that different groups of phenolics may be associated with the antiproliferative responses observed in different HC organs. The leaf extract, which showed strong activity in several cancer

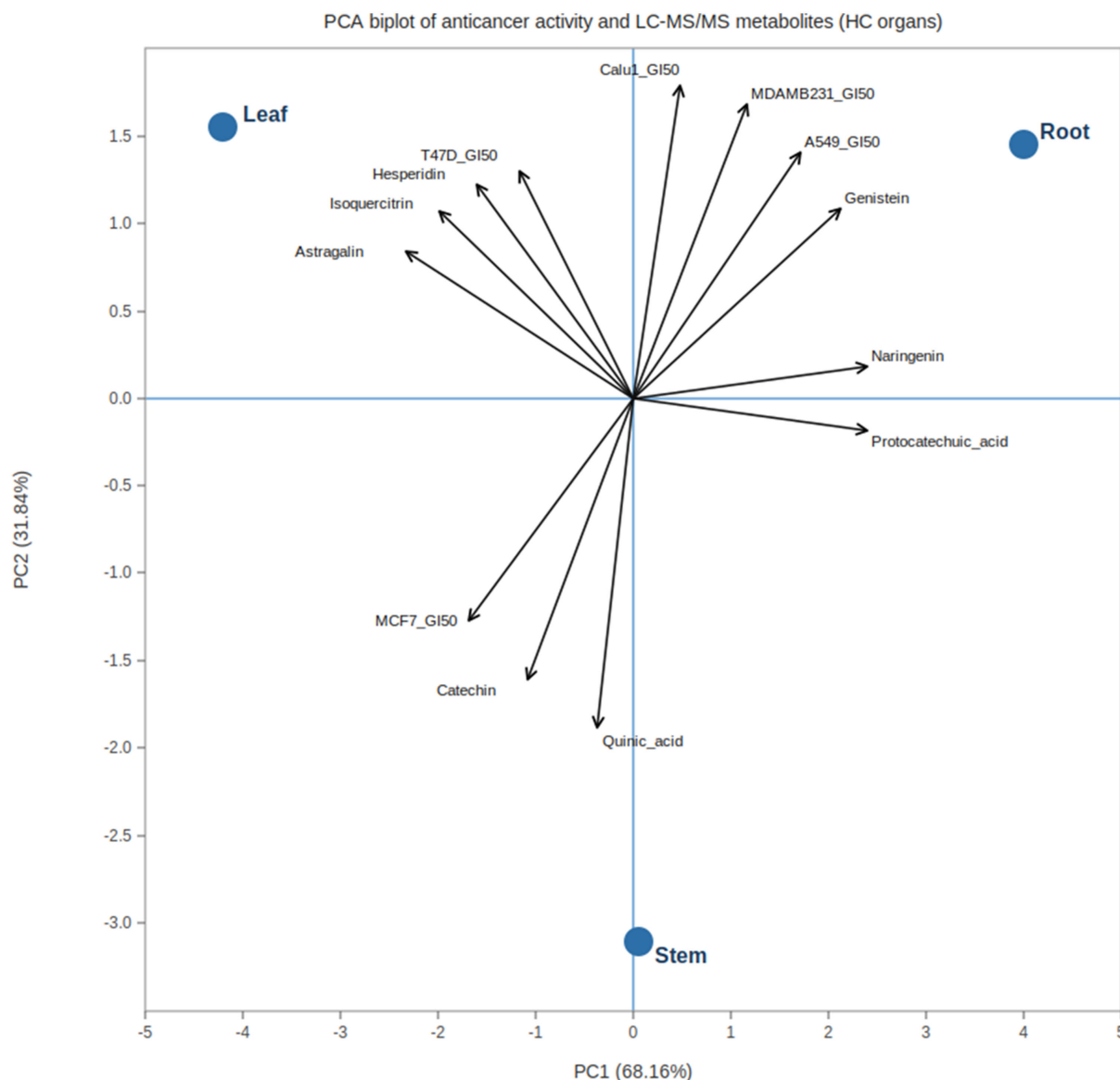


FIGURE 8 | PCA biplot integrating antiproliferative activity (GI_{50} values) and LC-MS/MS-derived metabolites in different organs (leaf, stem, and root) of HC.

cell models, was characterized by high concentrations of isoquercitrin, astragalin, rutin, and hesperidin; these flavonoid glycosides have previously been associated with growth inhibition, apoptosis induction, and oxidative stress modulation in cancer cells. In contrast, the root extract contained relatively higher concentrations of genistein, daidzein, naringenin, and acacetin, flavonoids and isoflavonoids that have been reported to exert antiproliferative effects, with genistein and daidzein also being associated with hormone-responsive anticancer activity (Bhat et al. 2021; Motallebi et al. 2022). The comparatively stronger response of MCF7 cells to the root extract may therefore be related, at least in part, to its isoflavonoid content, while the broader activity of the leaf extract may reflect the contribution of flavonoid glycosides present at much higher concentrations. Thus, the antiproliferative activity observed in the present study was associated mainly with leaf flavonoid glycosides (isoquercitrin, astragalin, rutin, hesperidin) and root isoflavonoid/aglycone-type constituents (genistein, daidzein, naringenin, and acacetin); however, these relationships should be interpreted as correlation-based compound–activity associations and not as direct evidence of causality, because the biological responses may reflect the combined effects of multiple metabolites present in the extracts.

Overall, the present findings are consistent with previous reports indicating that *Hedysarum* species contain bioactive secondary metabolites with in vitro antiproliferative activity. In the current study, the moderate growth-inhibitory effects observed for HC extracts, together with their relatively low cytotoxicity toward normal cells, indicate that this species may be considered for further investigation from a natural product perspective. However, these findings should be interpreted as preliminary in vitro evidence, and additional studies including phytochemical isolation, apoptosis- and cell cycle-based assays, and in vivo validation are required before conclusions regarding anticancer potential can be considered.

3.4 | Cytotoxicity Analysis

The cytotoxic effects of the extracts were further evaluated using the LDH release assay, which measures the activity of lactate dehydrogenase released from damaged cell membranes into the extracellular environment. LDH activity is widely used as an indicator of membrane damage and cellular toxicity. The cytotoxicity results are presented in Table 5. According to the results, the extracts exhibited relatively low cytotoxicity across all tested cell lines, with values between 2.96% and 12.54% depending on the extract and the cell line. In lung cancer cell lines, cytotoxicity values were between 2.96% and 5.65%, while the cytotoxicity value of 5-FU was considerably higher. Similarly, normal lung

cells (Beas-2B) showed relatively low cytotoxic responses (4.10%–5.40%), indicating minimal toxicity toward non-cancerous cells compared with the standard chemotherapeutic drug. Breast cancer cell lines exhibited slightly higher cytotoxic responses, with the highest cytotoxicity value observed in MDA-MB-231 cells treated with the stem extract (12.54%). In contrast, cytotoxicity values in normal mammary cells (MCF10A) were relatively low (3.32%–6.46%). Overall, when the antiproliferative activity obtained from the MTT assay and the cytotoxicity findings obtained from the LDH assay are evaluated together, the extracts demonstrate moderate antiproliferative activity accompanied by relatively low toxicity toward normal cells and acceptable tumor selectivity index values. Although their activity was lower than that of the standard chemotherapeutic drug 5-FU, the ability of the extracts to inhibit cancer cell proliferation with limited toxicity toward healthy cells supports their relevance for further in vitro and mechanistic investigation. These findings should not be interpreted as direct evidence of therapeutic anticancer activity.

3.5 | Antioxidant Activity of Extracts

The antioxidant activities of HC extracts from different plant organs (leaf, stem, and root) were evaluated using multiple in vitro assays, including DPPH radical scavenging activity, FRAP, TPC, and TFC. The results are presented in Table 6 and indicate differences among plant organs, suggesting that antioxidant metabolites are distributed among the organs of the species. The aerial plant parts exhibited higher antioxidant potential than the root extract, suggesting organ-dependent distribution of bioactive compounds.

The DPPH radical scavenging activity of the extracts was determined and expressed as IC₅₀ values. Among the tested samples, the leaf extract exhibited the strongest radical scavenging activity (5.43 ± 0.18 µg/mL), followed by the stem extract (11.40 ± 0.62 µg/mL), while the root extract showed lower activity (156.30 ± 1.78 µg/mL). The IC₅₀ value of the leaf extract was about 29-fold lower than that of the root extract and less than half that of the stem extract, indicating higher DPPH radical scavenging activity. When compared with reference antioxidants, including BHA (8.04 ± 0.69 µg/mL), Trolox (8.50 ± 0.77 µg/mL), ascorbic acid (9.91 ± 0.87 µg/mL), and BHT (10.70 ± 0.73 µg/mL), the leaf extract demonstrated comparable and even superior activity. Statistical analysis revealed significant differences among the samples ($p < 0.05$), with the leaf extract forming a separate group and the root extract showing the lowest activity. The high DPPH radical scavenging activity observed in the leaves of HC indicates a comparatively strong antioxidant capacity within the genus. In other *Hedysarum* species, including

TABLE 5 | % Cytotoxicity values of HC extracts in lung and breast cancer and normal cell lines.

% Cytotoxicity	A549	Calu1	H1650	Beas2B	MCF7	MDA-MB-231	MCF10A
Leaf	3.02	4.43	5.65	5.40	3.56	5.07	4.94
Stem	3.48	2.96	5.06	5.02	3.73	12.54	3.32
Root	3.27	4.29	4.35	4.10	6.58	3.27	6.46
5FU	7.25	12.53	7.82	8.86	7.58	11.80	9.90

TABLE 6 | Total phenolic, flavonoid contents, and antioxidant activity of HC.

Sample	DPPH [*] scavenging IC ₅₀ (μg/mL)	Total phenolic (mg GAE/g extract)	Total flavonoids (mg QE/g extract)	Reducing power (mg TE/g extract)
Leaf	5.43 ± 0.18 ^{a*}	220.84 ± 1.07 ^a	107.26 ± 0.77 ^a	192.47 ± 0.91 ^d
Stem	11.40 ± 0.62 ^c	107.74 ± 2.05 ^b	71.67 ± 0.43 ^b	170.44 ± 0.42 ^d
Root	156.30 ± 1.78 ^d	37.09 ± 0.97 ^c	13.69 ± 1.10 ^c	90.46 ± 0.24 ^e
Trolox	8.50 ± 0.77 ^b	—	—	—
BHA	8.04 ± 0.69 ^b	—	—	338.57 ± 0.31 ^b
BHT	10.70 ± 0.73 ^c	—	—	257.80 ± 1.24 ^c
Ascorbic acid	9.91 ± 0.87 ^{bc}	—	—	394.17 ± 0.98 ^a

^{a–e}Different letters in the same column indicate statistical difference ($p < 0.05$).

H. polybotrys, *H. scoparium*, and *H. carnosum*, DPPH IC₅₀ values reported for aerial parts are generally higher, depending on solvent polarity and extraction conditions (Dong et al. 2013; Zhao et al. 2014; Salah et al. 2016), compared with the value observed for HC leaves in the present study. Similarly, antioxidant activity reported for *H. formosum* and *H. atropatanum* also indicates variability among species within the genus (Babayeva et al. 2025). Although detailed antioxidant investigations on HC are limited in the literature, available phytochemical screenings report the presence of phenolic acids and flavonoid derivatives in its aerial parts, supporting the strong radical scavenging activity observed in this study (Altay et al. 2022). The lower activity observed in the root extract is consistent with patterns reported for other *Hedysarum* species, where roots generally contain lower phenolic concentrations and show reduced antioxidant activity compared with aerial parts (Dong et al. 2013; Keleke et al. 2025). These comparisons suggest that HC, particularly its leaves, may represent one of the more active representatives of the genus in terms of DPPH radical scavenging potential, which may reflect quantitative and qualitative differences in phenolic composition among species.

The TPC of HC extracts from different plant organs was quantified and expressed as mg gallic acid equivalent phenolic component (GAE) per gram of extract (Table 6). Among the plant organs, the leaf extract exhibited the highest phenolic content (220.84 ± 1.07 mg GAE/g extract), followed by the stem extract (107.74 ± 2.05 mg GAE/g extract), while the root extract contained lower concentrations (37.09 ± 0.97 mg GAE/g extract). Statistical analysis confirmed significant differences among all samples ($p < 0.05$), with leaves, stems, and roots classified into different groups. Phenolic compounds are well-known contributors to antioxidant activity due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and quench singlet oxygen (Kumar and Goel 2019; Bouymajane et al. 2024). The significantly higher TPC observed in the leaves of HC may contribute to their superior performance in radical scavenging assays such as DPPH and correlate with the stronger antioxidant activity observed in this organ. Previous studies on *Hedysarum* species have reported considerable variation in total phenolic content depending on species, plant organ, and extraction solvent. Reported values in different *Hedysarum* species are generally between about 55 and 122 mg GAE/g extract, and aerial parts often show higher phenolic concentrations than roots

(Dong et al. 2013; Uyar et al. 2017; Malfa et al. 2024; Babayeva et al. 2025). These values are generally lower than those reported for the leaves of HC, indicating that this species has a higher phenolic content compared with other *Hedysarum* species. In contrast, some *Hedysarum* species have been reported to contain moderate or lower phenolic concentrations. The water extract of *H. cappadocicum* contained 50.96 ± 1.01 mg GAE/g extract (Bektaş et al. 2018), while the total phenolic contents of different extracts of *H. varium* were reported as 20.96, 45.11, and 37.97 mg GAE/g extract for ethyl acetate, methanol, and water extracts, respectively (Zengin et al. 2015). Such interspecific differences highlight the considerable variability in phenolic composition within the genus, which may be influenced by genetic, environmental, and ecological factors. The higher TPC values in the leaves of HC, compared with its stems and roots and with reports for other *Hedysarum* species, indicate higher phenolic concentrations in this organ and are consistent with its strong antioxidant activity.

A similar distribution pattern was observed for TFC in HC, which showed significant differences among plant organs ($p < 0.05$), with leaves containing the highest content (107.26 ± 0.77 mg QE/g extract), followed by stems (71.67 ± 0.43 mg QE/g extract) and roots (13.69 ± 1.10 mg QE/g extract). The flavonoid concentration in leaves was 1.5-fold higher than in stems and eightfold higher than in roots, indicating higher concentrations in aerial parts. Flavonoids are well-recognized redox-active secondary metabolites that function as free radical scavengers and metal chelators, contributing significantly to overall antioxidant capacity (Pietta 2000; Zahra et al. 2024). The higher flavonoid content in the leaves is consistent with their superior DPPH radical scavenging activity and reflects the preferential distribution of UV-protective and defense-related phenolics in photosynthetically active organs. Phytochemical investigations of HC report the presence of phenolic and flavonoid compounds in methanolic extracts, including the identification of flavonoid glycosides by LC–MS/MS analysis (Altay et al. 2022), supporting the flavonoid concentrations observed in the present study. Across the genus *Hedysarum*, considerable interspecific variation in flavonoid composition has been reported, and these compounds show differential distribution among plant organs, with aerial parts generally containing higher phenolic and flavonoid content than roots (Vysochina et al. 2011; Dong et al. 2013; Dall'Acqua et al. 2022; Keleke et al. 2025). In this context, the comparatively

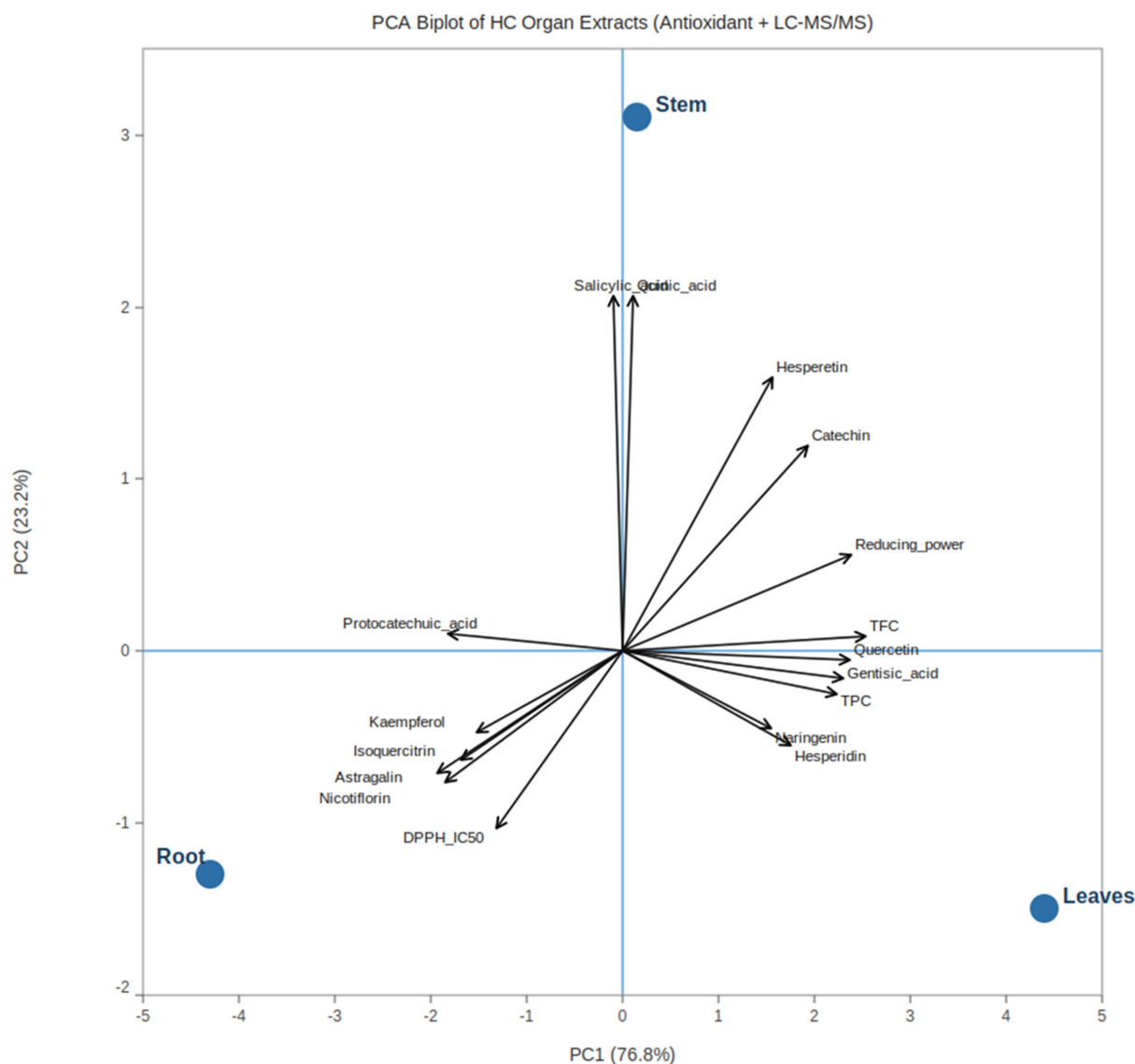


FIGURE 9 | PCA biplot integrating antioxidant parameters and LC–MS/MS-derived metabolites in different organs (leaf, stem, and root) of HC.

high TFC in the leaves of HC suggests that this species contains relatively high flavonoid concentrations among its congeners. Together with the stronger antioxidant activity of the leaf extract, these findings indicate that flavonoid concentrations contribute to the organ-level differences in antioxidant activity observed in HC, reflecting both genetic and environmental influences on biosynthetic processes.

The FRAP assay further confirmed the antioxidant capacity of the investigated extracts. The results showed significant differences among plant parts ($p < 0.05$). The leaf extract exhibited the highest reducing power (192.47 ± 0.91 mg TE/g extract), followed by the stem extract (170.44 ± 0.42 mg TE/g extract), while the root extract showed lower activity (90.46 ± 0.24 mg TE/g extract). These findings are consistent with the DPPH radical scavenging results and indicate that aerial plant organs show stronger antioxidant potential than roots. When compared with standard antioxidants, the reducing power of the leaf extract was lower than that of ascorbic acid (394.17 mg TE/g extract) and BHA (338.57 mg TE/g extract), but it approached the activity of

BHT (257.80 mg TE/g extract), indicating a considerable electron-donating capacity. The higher reducing capacity observed in leaves may be associated with their higher phenolic and flavonoid contents, indicating a contribution of phenolic metabolites to the antioxidant activity of the species. When these results are compared with previously reported species within the genus *Hedysarum*, the reducing power of HC is relatively high. Antioxidant investigations on several *Hedysarum* species have reported comparable FRAP values mainly in extracts with high polyphenolic content. Consistent with this trend, aerial extracts of *H. coronarium*, *H. semenowii*, and *H. nitidum* have been reported to exhibit considerable ferric reducing capacity associated with their phenolic composition (Burlando et al. 2017; Panigrahy et al. 2017; Baltürk et al. 2025; Keleke et al. 2025). In addition, recent phytochemical investigations on *H. yilmazunalii* indicated that extracts containing higher concentrations of phenolic constituents exhibited stronger FRAP activity, highlighting the close relationship between phenolic composition and reducing capacity (Şimşek and Varol 2025). In comparison with previous reports, the FRAP value obtained

for the leaves of HC is comparable or slightly higher than those reported for several other species within the genus, suggesting that this species may represent a relatively strong source of redox-active phytochemicals. This observation is consistent with previous reports indicating that plant extracts rich in phenolic compounds generally exhibit stronger reducing power, largely attributed to hydroxyl groups capable of donating electrons and stabilizing oxidized intermediates (Dai and Mumper 2010; Shahidi and Ambigaipalan 2015; Ahmad et al. 2025). Therefore, the relatively high reducing capacity observed in the leaf extract of HC further supports the contribution of polyphenolic metabolites to the antioxidant potential of this species and suggests that the aerial parts may serve as an important source of natural antioxidants.

The antioxidant profile of the leaf extract is also consistent with its phytochemical composition. The leaf extract was characterized by high concentrations of isoquercitrin, astragalin, hesperidin, rutin, and catechin, together with higher total phenolic and flavonoid contents than the stem and root extracts. These compounds have been reported to possess hydrogen-donating, radical-scavenging, and electron-transfer capacities, and their presence in the leaf extract may contribute to its stronger DPPH scavenging activity and ferric reducing power. Accordingly, the antioxidant activity of HC leaves can be associated mainly with the combined contribution of isoquercitrin, astragalin, hesperidin, rutin, and catechin, rather than with a single compound.

The relationship between antioxidant activity and phenolic composition among different plant organs of HC was analyzed by PCA by integrating antioxidant variables with the LC–MS/MS profiles of the identified phenolic compounds (Figure 9). The PCA showed a clear separation of leaf, stem, and root extracts according to their combined phytochemical composition and antioxidant characteristics. The first two principal components, PC1 and PC2, accounted for 76.8% and 23.2% of the total variation, respectively. The leaf extract was located in the positive PC1 region and was associated with higher total phenolic content, total flavonoid content, and ferric reducing antioxidant power, together with several flavonoid compounds present at higher concentrations in this organ. In particular, isoquercitrin, astragalin, catechin, hesperidin, and quercetin were positioned close to the leaf extract and antioxidant variables, indicating that these metabolites contributed strongly to the antioxidant profile of the leaf extract. The close association of these flavonoids with antioxidant variables in the PCA supports the interpretation that the superior antioxidant activity of the leaf extract was associated mainly with its flavonoid glycoside phenolic composition; however, these relationships should be interpreted as correlation-based compound–activity associations and not as direct mechanistic evidence, because the antioxidant response may reflect the combined contribution of multiple phenolic constituents present in the extract. In contrast, the stem extract was distributed mainly along PC2 and was associated more closely with quinic acid, protocatechuic acid, and intermediate phenolic and flavonoid content, indicating that these variables contributed to the distinct antioxidant profile of the stem extract and may account for its intermediate activity observed in the DPPH and FRAP assays. The root extract was positioned in the negative PC1 region and was associated with higher DPPH IC₅₀ values together with comparatively lower concentrations of phenolic and flavonoid

compounds, indicating that its weaker antioxidant profile was related to the lower representation of antioxidant-associated metabolites compared with the aerial organs. The PCA supports the interpretation that the antioxidant differences observed among HC organs were associated with variation in total phenolic and flavonoid content, as well as with the distribution of specific flavonoid compounds that contributed more strongly to the antioxidant profile of the leaf extract.

4 | Conclusion

This study investigated the phytochemical composition and in vitro biological activities of methanolic extracts obtained from different organs (leaf, stem, and root) of HC. LC–MS/MS analysis revealed clear organ-dependent differences in phenolic composition. The leaf extract was characterized by flavonoid glycosides such as isoquercitrin, astragalin, rutin, hesperidin, and related flavonoids, while the stem extract showed higher concentrations of quinic acid, catechin, and selected phenolic acids, and the root extract contained relatively higher proportions of aglycone flavonoids and isoflavonoids. These compositional differences were reflected in the biological activity profiles of the investigated plant parts. Among the tested extracts, the leaf extract exhibited the strongest antioxidant activity, as demonstrated by DPPH radical scavenging activity, FRAP, and higher TPC and TFC values, and also showed the strongest inhibitory activity against AChE, BChE, and α -glucosidase. The extracts also demonstrated moderate antiproliferative activity against lung and breast cancer cell lines, together with relatively low cytotoxicity toward normal cells. In addition, the stem extract showed the highest TSI in breast cancer models, while the low LDH-based cytotoxicity supported a growth-inhibitory effect with limited membrane damage. The combined LC–MS/MS and chemometric analyses further indicated that the antioxidant and enzyme-inhibitory profiles of the leaf extract were associated mainly with its flavonoid glycoside composition, while the antiproliferative responses reflected the contribution of different phenolic groups distributed among the plant organs, including aglycone flavonoids and isoflavonoids in the root extract. These findings contribute to the current phytochemical and bioactivity-related information available for HC and indicate that this species, particularly the leaf extract, may be considered for further phytochemical and biological investigation. However, the present results should be interpreted as initial in vitro findings, and additional studies involving the isolation of active constituents, mechanistic assays, and in vivo validation are required before conclusions regarding pharmacological or therapeutic relevance can be considered.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data supporting the findings of this study are included in the published article.

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