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First Report on the Phytochemical and Pharmacological Properties of *Hedysarum yilmazunalii*

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ABSTRACT

This study presents the phytochemical and biological evaluation of the species *Hedysarum yilmazunalii* Kandemir, F. Yıldız & H.İ. Turkoğlu. Methanolic extracts from leaves, stems, and roots were characterized by LC-MS/MS, and their antioxidant, enzyme inhibitory, and antiproliferative activities were investigated. The analyses showed organ-specific differences in phenolic composition, with quinic acid as the major compound in leaves together with higher levels of flavonoid glycosides, including astragalin and isoquercitrin. Aerial extracts showed strong enzyme inhibitory activity. Leaf and stem extracts exhibited cholinesterase inhibition, with IC₅₀ values of 5.82 and 6.42 µg/mL for AChE and 5.41 and 5.50 µg/mL for BChE, respectively. The leaf extract showed α-glucosidase inhibitory activity (4.10 µg/mL), comparable to acarbose, and antioxidant activity (DPPH: 6.46 µg/mL), together with the highest phenolic (145.43 mg GAE/g extract) and flavonoid contents (87.20 mg QE/g extract). Antiproliferative assays demonstrated growth inhibition of lung and breast cancer cell lines, with the lowest GI₅₀ values of 1.00 µg/mL for H1650 and 2.34 µg/mL for MDA-MB-231 cells. LDH cytotoxicity remained low (2.25%–12.59%), indicating limited membrane damage in both cancer and normal cells. These findings identify *H. yilmazunalii* as a source of compounds with antioxidant, neuroprotective, antidiabetic, and anticancer potential.

1 | Introduction

Natural products are among the most important sources of bioactive compounds in drug discovery, particularly for the development of multitarget therapeutic agents with potentially improved safety profiles. Plant-derived secondary metabolites, including polyphenols, flavonoids, terpenoids, alkaloids, and polysaccharides, are recognized for their diverse pharmacological

properties, including antioxidant, anti-inflammatory, enzyme inhibitory, neuroprotective, and antiproliferative effects [1–5]. This interest is associated with the growing global burden of chronic noncommunicable diseases, including type 2 diabetes mellitus, neurodegenerative disorders, cardiovascular diseases, and cancer, as well as limitations associated with many synthetic drugs, including side effects, reduced therapeutic effectiveness during long-term use, and drug resistance [1, 2]. In this

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context, medicinal plants continue to represent valuable sources of structurally diverse metabolites with potential pharmaceutical relevance.

Oxidative stress is an important factor in the pathogenesis of many chronic diseases and results from an imbalance between reactive oxygen species (ROS) production and antioxidant defense systems. Elevated ROS levels can damage lipids, proteins, and nucleic acids and contribute to the progression of disorders such as Alzheimer's disease, diabetes, atherosclerosis, and cancer [6, 7]. Phenolic compounds and flavonoids are among the major plant metabolites associated with antioxidant activity via radical scavenging, metal-chelating, and redox-modulating mechanisms [4]. In addition to their antioxidant properties, these compounds may inhibit therapeutically relevant enzymes, including acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and α -glucosidase. Inhibition of AChE and BChE is a widely used strategy in the symptomatic management of Alzheimer's disease, while α -glucosidase inhibition is relevant to the control of postprandial hyperglycemia in type 2 diabetes [8, 9]. Polyphenolic compounds have also been reported to show antiproliferative effects against different cancer cell lines via mechanisms including apoptosis induction, cell cycle regulation, and modulation of signaling pathways [10, 11].

Fabaceae is one of the largest families of flowering plants and includes numerous medicinally important species characterized by diverse phytochemical composition [12–14]. Within this family, the genus *Hedysarum* L. comprises 154–200 species distributed mainly in temperate and semiarid regions of Eurasia and North Africa [15, 16]. In Türkiye, the genus is represented by 21 native species, several of which are endemic [17, 18]. Phytochemical studies on *Hedysarum* species have identified flavonoids, isoflavones, triterpenoids, coumarins, lignans, alkaloids, sterols, fatty acids, and polysaccharides, many of which are associated with antioxidant, anti-inflammatory, antidiabetic, anticancer, and immunomodulatory activities [19–21]. Recent studies on different *Hedysarum* species, including *Hedysarum candidissimum*, *Hedysarum nitidum*, *Hedysarum varium*, and *Hedysarum pallidum*, have also reported antioxidant, enzyme inhibitory, and antiproliferative activities, largely associated with their phenolic and flavonoid constituents [17, 18, 22]. These studies also indicate interspecific and organ-dependent variation in phytochemical composition and biological activity, highlighting the importance of species-specific and organ-focused investigations.

The HY is a recently described endemic species from Erzincan Province in northeastern Türkiye and was identified in 2023 based on morphological and molecular analyses. To the best of our knowledge, the phytochemical composition and biological activities of HY have not previously been investigated; accordingly, this study provides the first comprehensive evaluation of its phytochemical profile and in vitro biological activities. Methanolic extracts obtained from leaves, stems, and roots were analyzed by LC-MS/MS for quantitative phenolic profiling, and their antioxidant properties, AChE, BChE, and α -glucosidase inhibitory activities, as well as antiproliferative and cytotoxic effects against selected lung and breast cancer cell lines, were investigated.

2 | Results and Discussion

2.1 | Phytochemical Investigation of the Extracts by LC-MS/MS

Phytochemical profiling of the HY extracts was performed using a previously developed and validated LC-MS/MS method, and a total of 53 phenolic compounds were quantitatively determined (Table 1 and Figure S1). The present study demonstrated significant phytochemical variation among different anatomical parts of HY. The molecular level characterization of the species phenolic profile, together with its correlation to functional bioassays, indicates that this metabolic heterogeneity is closely associated with antioxidant capacity, enzyme inhibition kinetics, and antiproliferative activity.

LC-MS/MS-based phytochemical analysis demonstrated organ-specific variation in the phenolic composition of HY and provided the first comprehensive chemical characterization of this species, for which no phytochemical or biological activity studies have been reported in the literature. Flavonoids, isoflavones, phenolic acids, and other polyphenolic compounds constitute the major bioactive metabolites reported in the genus *Hedysarum*; however, the quantitative distribution observed in HY indicates a species-specific metabolic profile [23]. One of the most significant findings from the data obtained is the high content of quinic acid in all plant organs. This compound was present at a high concentration in the stem extract (191.254 mg/g extract), followed by the leaf (45.037 mg/g extract) and root (19.576 mg/g extract) extracts. The high quinic acid concentration observed in the stem suggests that the shikimate metabolic pathway is highly active in this organ. Cinnamic acid has been associated with antioxidant, anti-inflammatory, antiviral, and enzyme inhibitory effects, and its high content, particularly in the aerial parts, suggests a potential contribution to the biological activities of these plant organs [17].

In addition to cynarin, the phytochemical profile of the plant is largely characterized by flavonoid glycosides, particularly in the leaves. Among the analyzed flavonoids, astragalin was identified as the compound with the highest concentration; it was determined at 113.433 mg/g extract in the leaves, 27.813 mg/g extract in the stems, and 1.183 mg/g extract in the roots. Isoquercitrin showed a similar distribution pattern and was determined at 58.775 mg/g extract in leaves, 18.267 mg/g extract in stems, and 2.173 mg/g extract in roots. The high concentrations of kaempferol- and quercetin-derived flavonoid glycosides in photosynthetically active organs suggest that these compounds may contribute significantly to photoprotection and the reduction of oxidative stress. Astragalin and isoquercitrin have been reported to exhibit antioxidant, anti-inflammatory, antihypertensive, and antidiabetic effects. This suggests that the leaf extracts of HY may be the fraction with the highest biological activity potential of the species [17].

The stem extract exhibited a concentrated polyphenolic composition. In addition to quinic acid, catechin (18.271 mg/g extract) and isoquercitrin (18.267 mg/g extract) were determined at high concentrations in the stems. This indicates that both compounds belonging to the flavan-3-ol group and flavonol glycosides are

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

No	Compound name	RT	Leaf	Stem	Root	No	Compound name	RT	Leaf	Stem	Root
1	Quinic acid	3.0	45.037	191.254	19.576	29	Salicylic acid	21.8	0.56	1.189	0.178
2	Fumaric acid	3.9	0.327	1.002	0.396	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	0.231	0.411	0.178	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	0.031	0.016	0.078
6	Protocatechuic acid	6.8	0.834	1.645	0.496	34	Îsoquercitrin	25.6	58.775	18.267	2.173
7	Catechin	7.4	5.972	18.271	N.D.	35	Hesperidin	25.8	1.389	0.233	0.079
8	Gentisic acid	8.3	0.13	N.D.	N.D.	36	o-Coumaric acid	26.1	N.D.	N.D.	N.D.
9	Chlorogenic acid	8.4	0.028	0.052	N.D.	37	Genistin	26.3	N.D.	N.D.	N.D.
10	Protocatechuic aldehyde	8.5	N.D.	N.D.	0.134	38	Rosmarinic acid	26.6	N.D.	N.D.	N.D.
11	Tannic acid	9.2	0.413	0.262	0.059	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	N.D.	N.D.	N.D.
14	4-OH Benzoic acid	10.5	N.D.	N.D.	N.D.	42	Astragalin	30.4	113.433	27.813	1.183
15	Epicatechin	11.6	N.D.	N.D.	N.D.	43	Nicotiflorin	30.6	1.021	0.141	N.D.
16	Vanilic acid	11.8	N.D.	N.D.	N.D.	44	Fisetin	30.6	N.D.	N.D.	N.D.
17	Caffeic acid	12.1	0.036	0.084	0.049	45	Daidzein	34.0	N.D.	N.D.	0.026
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	0.399	0.272	0.046
20	Syringic aldehyde	14.6	N.D.	N.D.	N.D.	48	Naringenin	35.9	0.026	0.099	0.419
21	Daidzin	15.2	N.D.	N.D.	N.D.	49	Hesperetin	36.7	0.031	0.053	0.052
22	Epicatechin gallate	15.5	N.D.	N.D.	N.D.	50	Luteolin	36.7	N.D.	N.D.	0.007
23	Piceid	17.2	N.D.	N.D.	N.D.	51	Genistein	36.9	N.D.	0.012	0.162
24	p-Coumaric acid	17.8	0.011	0.01	0.016	52	Kaempferol	37.9	0.3	0.116	0.017
25	Ferulic acid-D3-ISh	18.8	N.A.	N.A.	N.A.	53	Apigenin	38.2	N.D.	N.D.	0.01
26	Ferulic acid	18.8	N.D.	N.D.	N.D.	54	Amentoflavone	39.7	N.D.	N.D.	0.005
27	Sinapic acid	18.9	N.D.	N.D.	0.209	55	Chrysin	40.5	N.D.	N.D.	N.D.
28	Coumarin	20.9	N.D.	N.D.	N.D.	56	Acacetin	40.7	0.038	0.075	1.671

*N.D.: Not detected; N.A.: Not applicable; IS: Internal standard

present at high concentrations in the same organ. Catechins have been reported to exhibit potent free radical scavenging, cardio-protective, and antimicrobial activities, suggesting that the stem may represent a biologically important reservoir of polyphenols. In addition, protocatechuic acid (1.645 mg/g extract) and salicylic acid (1.189 mg/g extract) also showed their highest levels in the stems. This finding suggests that phenylpropanoid metabolism is active in the stem and that this organ may contribute significantly to the plant's defense mechanisms.

Although leaves have lower quinic acid values compared to stems, they were determined to have the highest total flavonoid content. In addition to astragalins and isoquercitrins, hesperidin (1.389 mg/g extract), nicotiflorin (1.021 mg/g extract), and quercetin (0.399 mg/g extract) were determined at high concentrations in the leaves. Among glycosylated flavonoid derivatives, the total flavonoid content was higher in the leaves than in the root and stem extracts. A similar distribution pattern has been reported in *Hedysarum semenowii*, where the aerial organs were found to exhibit higher antioxidant activity [19]. The metabolic profile observed in HY is consistent with this trend across the genus, but shows a quantitative difference, particularly in terms of the high astragalins concentration determined in the leaves.

The root extract, while more limited in total phenolic content, is characterized by the presence of certain flavonoid aglycones and some phenolic acids at higher concentrations. According to the analysis results, acacetin was determined at the highest concentration in the roots (1.671 mg/g extract), with lower concentrations in the stems (0.075 mg/g extract) and leaves (0.038 mg/g extract). In addition, genistein was determined in the roots at 0.162 mg/g extract, while daidzein (0.026 mg/g extract) and sinapic acid (0.209 mg/g extract) were also determined in this organ. Isoflavones and methoxylated flavones have been associated with estrogenic, anticancer, and enzyme inhibitory properties and have been widely reported in *Hedysarum* species [19]. The concentration of these compounds in the roots suggests that these metabolites may contribute to root-associated interactions, including plant–soil relationships and allelopathic activity.

The combined distribution of phenolic acids and flavonoids indicates metabolic differentiation among plant organs. Within the phenolic acid fraction, quinic acid was present at the highest concentration, particularly in stems, and leaves contained higher concentrations of flavonoid glycosides, including astragalins and isoquercitrins, than other phenolic compounds. In contrast, roots exhibit a metabolic profile with a more balanced distribution of phenolic acids and flavonoid aglycones. These findings indicate functional differences among plant organs in relation to metabolic and ecological roles. Leaves are characterized by flavonoid-mediated antioxidant and UV-protective mechanisms, while stems are characterized by high concentrations of defensive and structural phenolic compounds. Roots were characterized by a more limited composition of bioactive aglycones and signaling-related compounds.

Comparisons with previously studied species also indicate that HY has a distinct phytochemical profile. Although quinic acid and certain flavonoids have been reported as the main components in *H. candidissimum*, *H. semenowii* is characterized by

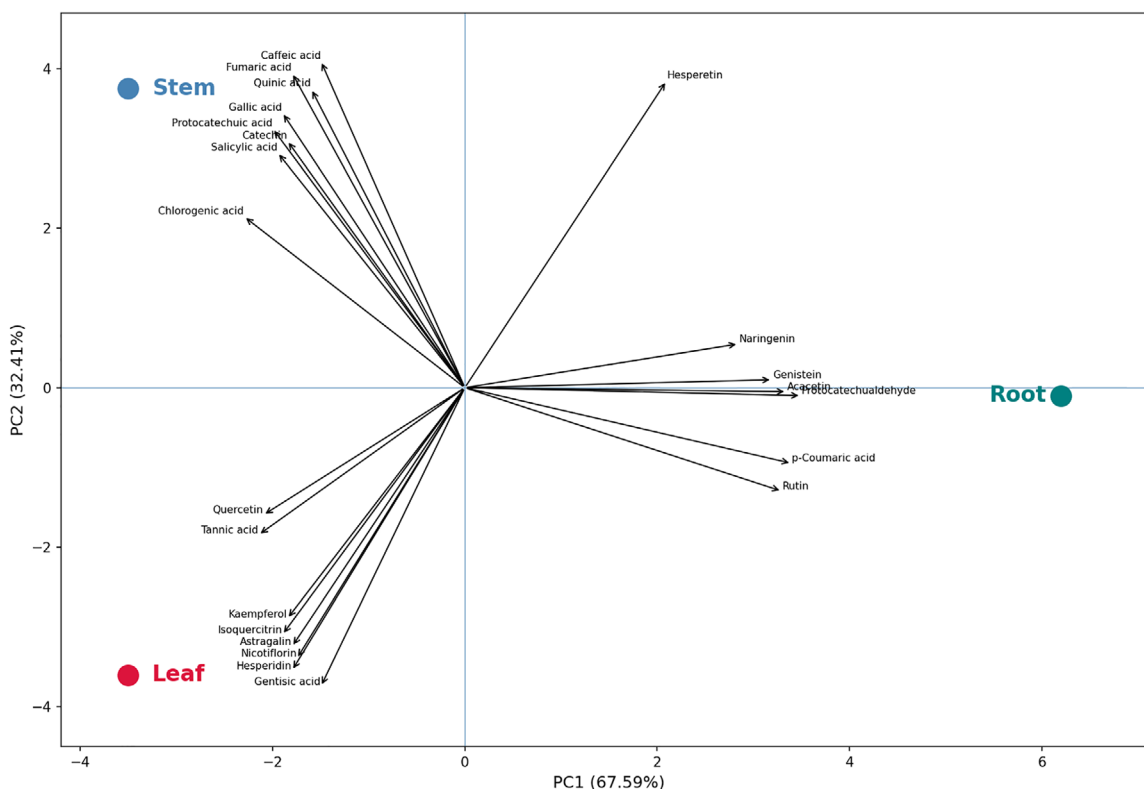
flavones in the aerial parts and isoflavones in the roots. HY generally follows the trend of flavonoid distribution in aerial organs observed within the genus, but shows a quantitative difference from previously studied species, particularly in terms of the high astragalins concentration determined in leaves and the high catechin concentration determined in stems [17, 19].

Based on the integrated evaluation of the PCA biplot (Figure 1) and heatmap (Figure 2), a clear organ-specific distribution pattern of phenolic compounds in HY samples was observed. The heatmap (Figure 2) indicated that several phenolic acids, including quinic acid, gallic acid, protocatechuic acid, catechin, caffeic acid, and salicylic acid, were present at higher concentrations in the stem, suggesting that this organ functions as a major reservoir for intermediate phenolic metabolites. In contrast, the root was characterized by relatively higher concentrations of compounds such as protocatechuic aldehyde, *p*-coumaric acid, rutin, naringenin, and sinapic acid, indicating a metabolic profile associated with flavonoid-related and phenylpropanoid derivatives. Leaf samples exhibited higher concentrations of compounds including gentisic acid, isoquercitrin, hesperidin, astragalins, nicotiflorin, quercetin, and kaempferol, indicating their association with flavonol biosynthesis and photoprotective functions. PCA analysis (Figure 1) strongly supported these findings, with the first two components (PC1: 65.08%, PC2: 34.92%) accounting for most of the total variance and separating the three plant organs. Root samples were positioned at positive PC1 values and were associated with vectors such as naringenin, rutin, protocatechuic aldehyde, and enzyme inhibition parameters (AChE-IC₅₀ and BChE-IC₅₀), indicating a relationship with bioactivity related metabolites. Stem samples were located in the positive PC2 and negative PC1 regions and were correlated with phenolic acids, including quinic acid, gallic acid, protocatechuic acid, catechin, chlorogenic acid, and salicylic acid. Leaf samples were distributed at negative PC1 and PC2 values and were associated with flavonoid derivatives such as quercetin, kaempferol, isoquercitrin, astragalins, nicotiflorin, and hesperidin. The separation of the organs and the directional distribution of metabolite vectors indicate metabolic differentiation among plant parts. Overall, both multivariate analyses indicate that phenolic biosynthesis and distribution in HY are organ-dependent, with stems showing higher concentrations of phenolic acids, roots associated with bioactivity related phenolics and flavonoids, and leaves characterized by flavonol glycosides, indicating different physiological and biochemical roles.

2.2 | Enzyme Inhibition Activity of Extracts

In this study, the inhibitory effects of methanol extracts obtained from different parts of HY, such as roots, stems, and leaves, on cholinesterase and alpha-glucosidase enzymes were investigated (Figures S2, S3, and S4). According to the IC₅₀ values presented in Table 2, HY extracts showed statistically significant differences in all enzymes ($p < 0.05$). The strongest inhibitory effect against AChE was observed for the reference inhibitor tacrine ($3.43 \pm 0.40 \mu\text{g/mL}$), followed by the stem extract ($5.82 \pm 0.34 \mu\text{g/mL}$), leaf extract ($6.42 \pm 0.36 \mu\text{g/mL}$), and root extract ($9.00 \pm 0.13 \mu\text{g/mL}$) (Table 2, Figure S2). The difference between stem and leaf extracts was limited, with both extracts showing significantly higher AChE inhibitory activity compared to the root extract.

PCA biplot of LC-MS/MS phytochemicals (HY organs)

**FIGURE 1** | PCA biplot of LC-MS/MS phytochemicals in the leaf, stem, and root of HY.**TABLE 2** | Inhibitory effects of HY extracts on cholinesterase (AChE, BChE) and α -glucosidase enzymes.

Inhibitor	IC ₅₀ (μ g/mL)					
	AChE	R ²	BChE	R ²	α -glucosidase	R ²
Leaf	6.42 $\pm$ 0.36 ^c	0.9217	5.50 $\pm$ 0.15 ^c	0.9988	4.10 $\pm$ 0.25 ^b	0.8641
Stem	5.82 $\pm$ 0.34 ^b	0.8926	5.41 $\pm$ 0.51 ^b	0.9946	4.50 $\pm$ 0.45 ^c	0.9834
Root	9.00 $\pm$ 0.13 ^d	0.9094	10.19 $\pm$ 0.95 ^d	0.9859	11.74 $\pm$ 0.84 ^d	0.9632
Tacrine	3.43 $\pm$ 0.40 ^a	0.9981	3.18 $\pm$ 0.20 ^a	0.9768	—	—
Acarbose	—	—	—	—	3.55 $\pm$ 0.04 ^a	0.9992

* Values are expressed as mean $\pm$ SD ($n = 3$). Statistical significance was evaluated by one-way ANOVA. Different superscript (a–d) letters within the same column denote statistically significant differences among groups at $p < 0.05$. R² values represent the coefficients of determination obtained from dose–response curve analysis.

A similar trend was observed on the BChE enzyme. Tacrine exhibited the strongest inhibitory effect with the lowest IC₅₀ value (3.18 $\pm$ 0.20 μ g/mL), while the highest activity among the extracts was determined in the stem extract (5.41 $\pm$ 0.51 μ g/mL). The leaf extract (5.50 $\pm$ 0.15 μ g/mL) showed activity very close to that of the stem extract. The root extract exhibited a lower inhibitory effect on BChE (10.19 $\pm$ 0.95 μ g/mL) (Table 2, Figure S3).

In the α -glucosidase inhibition assay, the leaf extract (4.10 $\pm$ 0.25 μ g/mL) showed inhibitory activity comparable to that of the reference inhibitor acarbose (3.55 $\pm$ 0.04 μ g/mL), with only a limited difference between them. The stem extract showed similar

activity (4.50 $\pm$ 0.45 μ g/mL), but was significantly lower than that of the leaf extract. The root extract showed the lowest inhibitory activity with the highest IC₅₀ value on the α -glucosidase enzyme (11.74 $\pm$ 0.84 μ g/mL) (Table 2, Figure S4).

Cholinesterase enzymes are associated with the pathogenesis of Alzheimer's disease. Therefore, AChE and BChE inhibitors are among the target enzymes used in symptomatic treatment [24]. The side effects associated with synthetic drugs have increased interest in plant-derived natural compounds with AChE inhibitory (AChEI) potential, particularly in recent years. These plants contain a wide variety of phytochemicals with

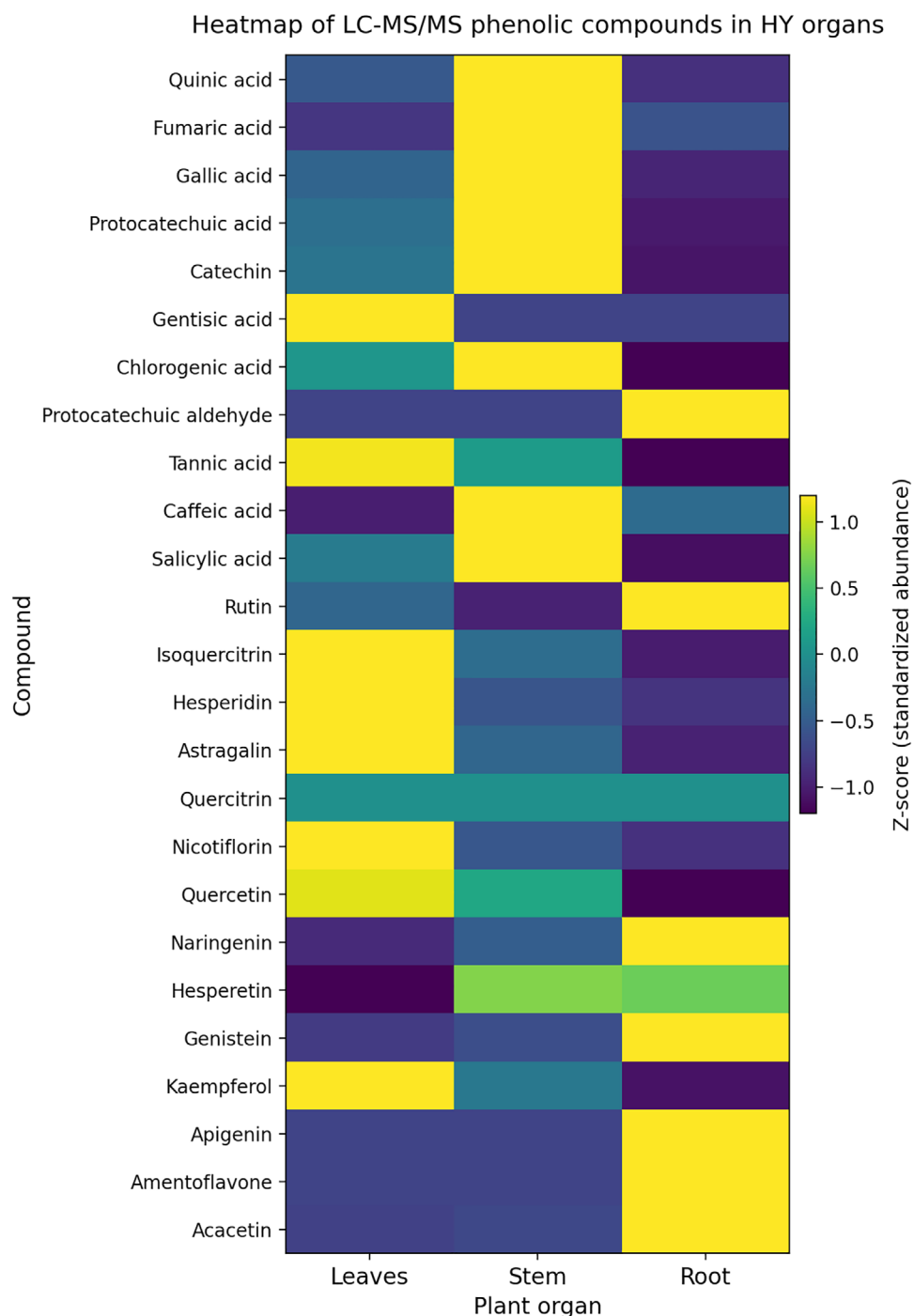


FIGURE 2 | Heatmap of LC-MS/MS phenolic compounds in HY organs.

antioxidant, anti-inflammatory, and other pharmacological properties, in addition to proven AChEI activity, such as phenolics, flavonoids, terpenoids, and alkaloids [25]. This study was conducted to contribute to the identification of new plant sources of AChE inhibitors in this context.

The results obtained in our study show that the leaf and stem extracts of HY exhibit strong inhibitory effects on AChE, BChE, and α -glucosidase. These biological activities are consistently supported by the phytochemical profile determined by LC-MS/MS analysis. LC-MS/MS analyses showed that quinic acid was the phenolic compound present at the highest concentration

in all organs of HY. Quinic acid was present at the highest concentration in the stem extract, followed by the leaf and root extracts. Quinic acid has been associated with antioxidant, anti-inflammatory, antiviral, and enzyme inhibitory activities in *Hedysarum* species, and its high concentration, especially in aerial organs, in this study indicates a significant contribution to the biological potential of these organs [17, 26].

The strong inhibitory activity of leaf extracts, characterized by high concentrations of flavonoid glycosides, on AChE ($6.42 \pm 0.36 \mu\text{g/mL}$), BChE ($5.50 \pm 0.15 \mu\text{g/mL}$), and α -glucosidase ($4.10 \pm 0.25 \mu\text{g/mL}$) enzymes may be attributed to the high

concentrations of flavonoid glycosides such as astragalín, isoquercitrín, hesperidín, and nicotiflorín. The antioxidant, anti-inflammatory, antihypertensive, and antidiabetic effects of these compounds have been reported in detail in the literature. These findings suggest that HY leaf extracts are one of the most bioactive fractions of the species [17, 27–29].

The significant inhibitory effect of stem extracts on the enzymes AChE ($5.82 \pm 0.34 \mu\text{g/mL}$) and BChE ($5.41 \pm 0.51 \mu\text{g/mL}$) can be attributed to the high quinic acid content determined by LC-MS/MS analysis (191.254 mg/g extract). The presence of cinchonic acid alongside biologically active flavonoid compounds such as catechin and isoquercitrín provides an important phytochemical basis for the potent inhibitory effect of stem extracts on cholinesterase enzymes. The synergistic presence of these compounds may contribute to the comparable inhibitory activity of stem extracts on cholinesterase enzymes compared to leaf extracts [30–32]. In contrast, the relatively lower inhibitory effect of stem extracts on the α -glucosidase enzyme ($4.50 \pm 0.45 \mu\text{g/mL}$) may be related to the more limited flavonoid glycoside diversity and total flavonoid load compared to leaf extracts. The similarities and differences observed between leaf and stem extracts reveal that organ-specific distribution of phenolic acids and flavonoids contributes to enzyme inhibitory activity. These findings strongly support the potential of the aerial organs of HY, particularly with respect to neuroprotective and antidiabetic biological activities.

Similar trends have been documented in investigations of cholinesterase and α -glucosidase inhibitory activities across different *Hedysarum* species. In particular, the methanolic leaf extract of *H. candidissimum* has demonstrated significant AChE inhibitory activity [17]. In a study on *H. varium*, the highest inhibitory activity was reported in the ethyl acetate extract [22]. In contrast, a study on *H. nitidum* showed that the water extract exhibited the highest cholinesterase inhibitory activity [18]. These findings indicate that the highest inhibitory activity observed in *Hedysarum* species may vary depending on the extraction solvent used, the geographical and ecological conditions in which the plants grow, and the resulting variations in phytochemical composition. However, when the current literature is considered as a whole, *Hedysarum* species show inhibitory activity against cholinesterase enzymes. These findings indicate that the genus may represent a potential biological resource, particularly with respect to neurodegenerative diseases and diabetes-related metabolic pathways. In this context, the strong AChE and BChE inhibition exhibited by the leaf and stem extracts of HY suggests that this species could be a promising resource for the development of neuroprotective agents. In this study, the leaf extract of HY exhibited the highest inhibitory effect on the α -glucosidase enzyme, suggesting a potential antidiabetic activity comparable to acarbose. In the literature, the IC_{50} value for α -glucosidase in the methanolic leaf extract of *H. candidissimum* has been reported as $28.57 \pm 0.82 \mu\text{g/mL}$, for *H. nitidum* as $39.53 \pm 0.27 \mu\text{g/mL}$, and for *H. varium* as $2.88 \pm 0.05 \text{ mg/mL}$ [17, 18, 22]. In particular, the methanolic extract of *H. varium* has been reported to exhibit a stronger inhibitory effect compared to the standard inhibitor acarbose ($6.67 \pm 0.20 \text{ mg/mL}$). The lower IC_{50} values obtained in this study indicate that HY may have comparable or even higher inhibitory activity than other *Hedysarum* species. The high biological activity observed in leaf extracts is likely associated with their high phenolic and flavonoid content.

To further evaluate the relationship between enzyme inhibitory activity and phytochemical composition in HY, PCA was performed using the IC_{50} values obtained for AChE, BChE, and α -glucosidase inhibition together with the quantitative phytochemical profile of the leaf, stem, and root extracts (Figure 3). The first two components represented 100.0% of the total variance, with PC1 and PC2 accounting for 65.08% and 34.92% of the variation, respectively, indicating a clear separation of the three organs according to both metabolite composition and enzyme inhibitory response. The root extract was positioned in the positive region of PC1, close to the α -glucosidase IC_{50} and BChE IC_{50} variables, and was associated with genistein, naringenin, protocatechualdehyde, and vanillin. This distribution indicates that the root extract was more closely related to these IC_{50} variables and therefore exhibited lower inhibitory potency against α -glucosidase and BChE than the leaf extract, as lower IC_{50} values correspond to stronger enzyme inhibition. The stem extract was located in the negative region of PC1 and the positive region of PC2 and was mainly associated with quinic acid, gallic acid, protocatechuic acid, catechin, salicylic acid, chlorogenic acid, caffeic acid, and fumaric acid, indicating a profile characterized by higher levels of phenolic acids compared with the other organs. In contrast, the leaf extract was positioned in the negative region of both PC1 and PC2 and was associated with AChE IC_{50} together with several flavonoids, including rutin, quercetin, kaempferol, isoquercitrín, astragalín, nicotiflorín, hesperidín, tannic acid, and gentisic acid. The position of the leaf extract relative to the AChE IC_{50} variable, together with its separation from the root-associated IC_{50} variables, indicates that the leaf had a phytochemical composition associated with stronger cholinesterase inhibitory activity. The PCA findings support the organ-dependent nature of enzyme inhibition in HY and indicate that the leaf and stem extracts showed phytochemical–bioactivity relationships different from those of the root extract, with the leaf associated mainly with flavonoids and the stem with phenolic acids.

2.3 | Antioxidant Activity of Extracts

The DPPH[•] free radical scavenging activity, total phenolic content (TPC), total flavonoid content (TFC), and reducing power of HY extracts obtained from leaf, stem, and root were determined and compared with synthetic antioxidant standards, and the results are presented in Table 3. Among the three plant parts, the leaf extract demonstrated the highest DPPH radical scavenging capacity, with an IC_{50} value of $6.46 \pm 0.83 \mu\text{g/mL}$, followed by the stem extract ($9.16 \pm 0.30 \mu\text{g/mL}$) and the root extract ($112.96 \pm 2.19 \mu\text{g/mL}$). The leaf extract exhibited stronger radical scavenging activity than all reference standards tested, including Trolox ($8.50 \pm 0.77 \mu\text{g/mL}$), BHA ($8.04 \pm 0.69 \mu\text{g/mL}$), BHT ($10.70 \pm 0.73 \mu\text{g/mL}$), and ascorbic acid ($9.91 \pm 0.87 \mu\text{g/mL}$), while the stem extract showed comparable activity to Trolox and BHA, and the root extract showed significantly lower DPPH[•] scavenging activity relative to all standards. Statistical analysis indicated significant differences among the samples ($p < 0.05$), with the leaf extract in one group and the root extract exhibiting the lowest activity. The superior radical scavenging capacity of the aerial parts is consistent with their higher phenolic and flavonoid content, as phenolic hydroxyl groups are widely recognized as important hydrogen donors in free radical neutralization

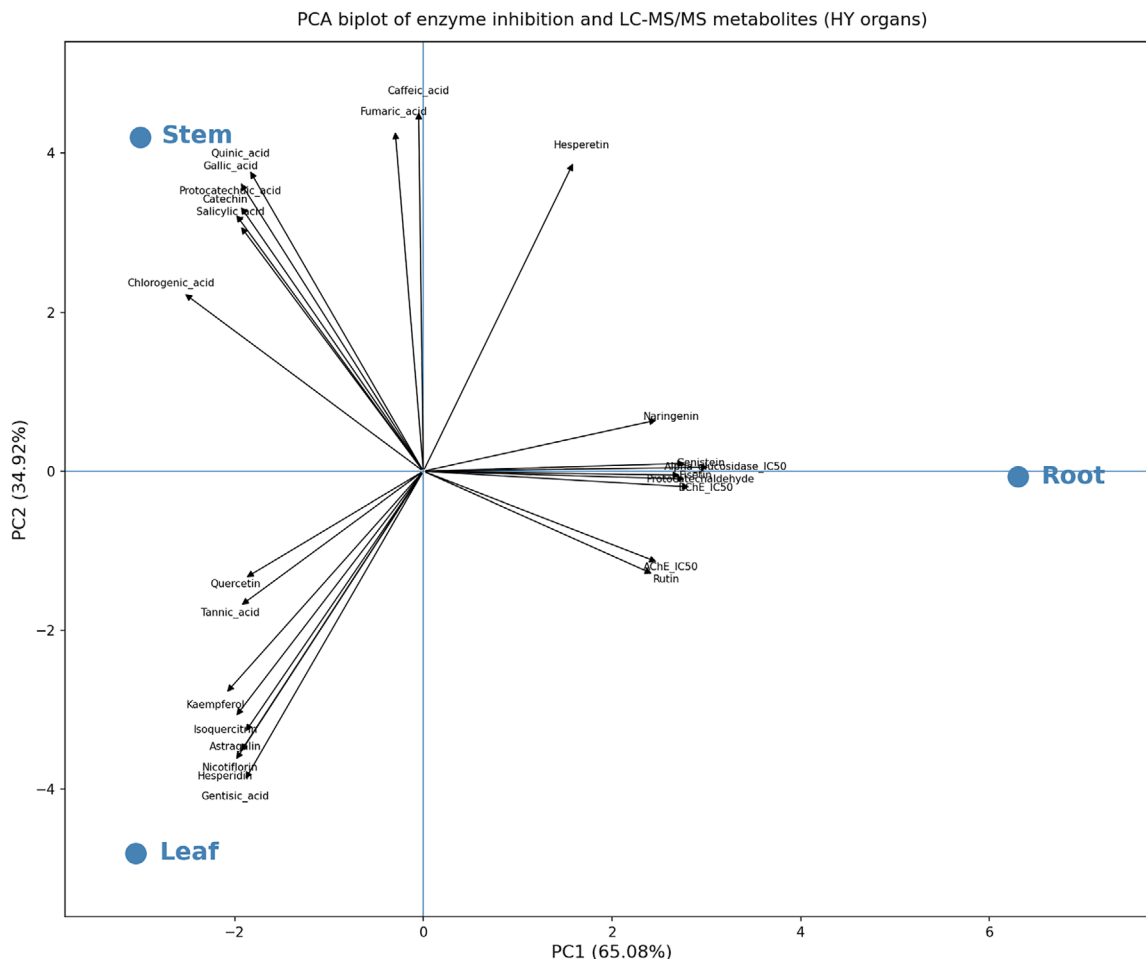


FIGURE 3 | PCA biplot of enzyme inhibition and LC-MS/MS metabolites (HY organs).

TABLE 3 | Total phenolic, flavonoid contents, and antioxidant activity of HY.

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	6.46 ± 0.83 ^a	145.43 ± 1.00 ^a	87.20 ± 0.89 ^a	188.18 ± 0.22 ^d
Stem	9.16 ± 0.30 ^c	130.15 ± 1.77 ^b	67.20 ± 1.34 ^b	176.80 ± 0.53 ^{de}
Root	112.96 ± 2.19 ^e	46.07 ± 1.84 ^c	14.82 ± 0.67 ^c	106.78 ± 0.18 ^f
Trolox	8.50 ± 0.77 ^{bc}	—	—	—
BHA	8.04 ± 0.69 ^b	—	—	338.57 ± 0.31 ^b
BHT	10.70 ± 0.73 ^d	—	—	257.80 ± 1.24 ^c
Ascorbic acid	9.91 ± 0.87 ^{cd}	—	—	394.17 ± 0.98 ^a

* a-f Different letters in the same column indicate statistical difference ($p < 0.05$).

reactions [33, 34]; in this context, HY shows higher activity compared to other *Hedysarum* species. Recent studies on *H. nitidum* have shown that aerial-part extracts have high phenolic and flavonoid content along with strong antioxidant activity in DPPH, FRAP, and CUPRAC assays [18]. In contrast, the DPPH IC₅₀ values reported for *Hedysarum carnosum* in Tunisia were reported between 16–37 μg/mL, indicating that the species has moderate to strong antioxidant capacity; however, the IC₅₀ value in HY leaves is lower than all fractions reported for *H. carnosum*,

and even the stem extract value (9.16 μg/mL) indicates stronger activity than most *H. carnosum* fractions [35].

The total phenolic content of HY extracts followed a similar trend, with the leaf showing the highest value (145.43 ± 1.00 mg GAE/g extract), followed by the stem (130.15 ± 1.77 mg GAE/g extract) and root (46.07 ± 1.84 mg GAE/g extract). Statistical analysis confirmed significant differences among all samples ($p < 0.05$), with leaves, stems, and roots classified into different

groups. These values are considerably higher than those reported for other *Hedysarum* species from Turkey; reported TPC values between 20.90–83.25 mg GAE/g extract for *H. varium* extracts [22], while the butanol extract of the endemic species *Hedysarum aucheri* was reported to have 122.4 mg GAE/g dry weight [36]. The reported TPC value (46.85 mg GAE/g) for *H. candidissimum* is also much lower than that determined in the present study, indicating higher phenolic content, and the TPC values of leaf and stem extracts are higher than this value, highlighting the high phenolic content of this newly described endemic species. The lower phenolic content in the root may be related to organ-specific biosynthesis and translocation patterns of secondary metabolites characteristic of the genus *Hedysarum*. In *H. coronarium* (Italy) samples analyzed in a honey matrix, the total phenolic content was reported between 47.9 and 248.3 mg/kg; although honey samples cannot be directly compared with plant extracts, these data provide additional context for phenolic variability within the genus [37].

In parallel, the total flavonoid content was highest in the leaf (87.20 ± 0.89 mg QE/g extract), followed by the stem (67.20 ± 1.34 mg QE/g extract) and root (14.82 ± 0.67 mg QE/g extract). These values were also higher than those reported for some *Hedysarum* species, including *H. candidissimum* (35.42 mg QE/g extract) and *H. varium* (2.41–39.71 mg RE/g extract) [17, 22]. Flavonoids constitute a major class of polyphenols in the genus *Hedysarum*; 79 flavonoid compounds, including flavones, isoflavones, flavanones, chalcones, flavanols, and pterocarpanes, have been identified from various species of this genus [19, 23]. The TFC of the HY leaf extract (87.20 mg QE/g) is higher than that reported for the n-butanol extract of *H. aucheri* (66.3 mg QE/g DW), highlighting the notable phytochemical potential of HY [36]. Organ specific differences in phytochemical composition have also been reported in *H. semenowii*, with variations in the distribution of flavonoid subclasses between aerial parts and roots, where isoflavonoid type compounds are particularly associated with the roots [19].

The FRAP assay indicated the antioxidant capacity of the investigated extracts, with significant differences observed among plant parts ($p < 0.05$). The reducing power of HY extracts, expressed as mg Trolox equivalents per gram extract, was highest in the leaf extract (188.18 ± 0.22 mg TE/g), followed by the stem extract (176.80 ± 0.53 mg TE/g) and the root extract (106.78 ± 0.18 mg TE/g). Only FRAP activity has been reported for *Hedysarum cappadocicum* (429.21 μ M FeSO₄/g). This value is higher than the reducing capacity reported for HY leaves (188.18 mg TE/g); however, direct comparison is not possible because the units used in the two studies (mg TE/g and μ M FeSO₄/g) are not directly comparable. The available data indicate that *H. cappadocicum* also has high reducing capacity [38]. The reported FRAP (62.21 mg TE/g) value for *H. candidissimum* is also considerably lower than that of HY leaves (188.18 mg TE/g), confirming the stronger reducing power of HY [17]. All plant extracts showed lower reducing capacity than the synthetic standards ascorbic acid (394.17 ± 0.98 mg TE/g), BHA (338.57 ± 0.31 mg TE/g), and BHT (257.80 ± 1.24 mg TE/g); however, the leaf and stem extracts still demonstrated a considerable electron donating ability. The reducing power assay measures the capacity of antioxidants to donate electrons and reduce Fe³⁺ to Fe²⁺, which is associated with the number of phenolic hydroxyl groups capable of electron

donation [39, 40]. In addition, the FRAP reported for *H. carnosum* is lower than the reducing capacity observed in HY extracts, further supporting the strong antioxidant profile of this species [35]. Similarly, “high antioxidant activity” has been reported in *H. coronarium* honey from Algeria; however, quantitative data are not available, which limits comparison [41], although these findings are consistent with the general antioxidant potential observed within the genus.

To further evaluate the relationship between antioxidant activity and phenolic composition of different HY organs, a PCA was performed by combining antioxidant assay results with LC-MS/MS-derived phenolic profiles (Figure 4). PC1 (66.53%) and PC2 (33.47%) together represented 100% of the total variance, indicating discrimination among the samples. The leaf extract was positioned on the positive side of PC1 and the negative side of PC2 and was strongly associated with antioxidant-related variables such as TFC and reducing power, as well as flavonoid compounds including quercetin, tannic acid, kaempferol, isoquercitrin, astragalin, nicotiflorin, hesperidin, and gentisic acid. This pattern indicates that flavonoid content contributes to the antioxidant capacity of the leaf. The stem extract was located in the positive regions of both PC1 and PC2 and showed strong correlations with phenolic acids such as quinic acid, gallic acid, protocatechuic acid, catechin, salicylic acid, chlorogenic acid, as well as caffeic acid and fumaric acid, indicating a profile characterized by phenolic acids and different from other organs. In contrast, the root extract was positioned on the negative side of PC1 and slightly negative along PC2 and was associated with higher DPPH IC₅₀ values, along with compounds such as protocatechuic aldehyde, genistein, naringenin, hesperetin, and rutin. The association with DPPH IC₅₀ suggests a comparatively lower antioxidant potential of the root extract. The PCA demonstrates that the antioxidant capacity of HY is organ-dependent and strongly influenced by differences in phenolic composition, with leaves characterized by flavonoid-related antioxidant activity, stems by phenolic acid content, and roots by compounds associated with lower radical scavenging activity.

The results of the present study demonstrate a consistent relationship between the phenolic and flavonoid content of HY organ extracts and their antioxidant performance across all assays, and numerical comparisons across species indicate that HY shows higher phenolic and flavonoid content and stronger antioxidant activity, particularly in leaves, than many other species within the genus. The leaf extract, with the highest TPC and TFC, exhibited the strongest DPPH' scavenging activity and reducing power, while the root extract, with the lowest secondary metabolite content, showed the lowest antioxidant capacity, suggesting that phenolic compounds, and flavonoids in particular, are the principal contributors to the antioxidant activity of HY. These findings highlight HY as a promising natural source of antioxidants, particularly in its aerial parts, and support the need for further detailed investigations of its phytochemical profile and potential applications in food, pharmaceutical, and nutraceutical fields.

2.4 | Antiproliferative Activity of Extracts

The antiproliferative activity of HY leaf, stem, and root extracts was investigated in human cancer cell lines, including three lung

TABLE 5 | GI₅₀ and IC₅₀ values (μg/mL) of the extracts in breast cancer and normal cell lines.

Extracts (μg/mL)	MCF7		MDA-MB-231		MCF10A		TSI
	GI ₅₀	IC ₅₀	GI ₅₀	IC ₅₀	GI ₅₀	IC ₅₀	
Leaf	3.04	64.37	3.82	130.28	1.02	141.58	1.45
Stem	3.91	67.45	1.98	30.99	1.02	102.46	2.08
Root	1.69	138.98	1.13	99.64	1.02	133.92	1.12
5FU	1.96	92.35	2.04	85.82	1.93	90.49	1.02

In the breast cancer panel, all extracts demonstrated antiproliferative activity against both MCF-7 and MDA-MB-231 cell lines, with GI₅₀ values lower than those observed in the normal MCF-10A line. For MCF-7, GI₅₀ values were 3.04 μg/mL (leaf), 3.91 μg/mL (stem), and 1.69 μg/mL (root), with the root extract showing higher activity than 5-FU (1.96 μg/mL). Against the MDA-MB-231 triple-negative breast cancer cell line, characterized by the absence of ER, PR, and HER2 expression, the root extract again showed the lowest GI₅₀ value (1.13 μg/mL), lower than that of 5-FU (2.04 μg/mL) [45, 46]. Although the stem extract showed the highest calculated TSI (2.08), this value should be interpreted cautiously because it was calculated using an extrapolated IC₅₀ value for the normal MCF10A cell line. Therefore, this result should be regarded as a preliminary indication of selectivity that requires confirmation using higher concentration ranges. The IC₅₀ values were between 31 and 141 μg/mL, and the stem extract showed an IC₅₀ value of 30.99 μg/mL against MDA-MB-231. This activity is consistent with previously reported cytotoxic effects of plant extracts against breast cancer cell lines and supports the relevance of HY as a potential source of bioactive compounds [44, 47].

The antiproliferative effects in HY are supported by previous studies on other *Hedysarum* species. The genus contains a wide range of bioactive compounds, including flavonoids, triterpenoids, and polysaccharides, many of which have demonstrated cytotoxic and proapoptotic effects in different cancer models [23]. In particular, polysaccharides isolated from *Hedysarum polybotrys* have been shown to inhibit the proliferation of hepatocellular carcinoma (HepG2) and gastric cancer (MGC-803) cells, and this effect increased with concentration [48, 49], while total flavonoids from the same species suppress leukemia K562 cell proliferation by modulating cell-cycle progression [50]. More recent evidence indicates that *Hedysarum polysaccharides* can also induce apoptosis and inhibit tumor growth in lung adenocarcinoma A549 cells by modulating the Bax/Bcl-2 signaling pathway [21, 51]. Further support is provided by studies on Turkish endemic *Hedysarum* species. The dichloromethane extract of *H. aucheri* has been reported to exhibit strong cytotoxic activity against multiple cancer cell lines, including A549 and MCF-7, with IC₅₀ values between 0.64 and 24 μg/mL, while showing low toxicity toward normal cells [36]. Similarly, *H. candidissimum* extracts showed cytotoxic effects against colon and breast cancer cell lines, although at higher IC50 values [17]. The GI₅₀ values for HY in both lung (1.00–16.80 μg/mL) and breast (1.13–3.91 μg/mL) cancer models are within or lower than these reported ranges, indicating that this species shows comparable or higher antiproliferative activity.

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

performed by integrating cytotoxicity data obtained from lung (A549, Calu-1, and H1650) and breast (MCF-7 and MDA-MB-231) cancer cell lines with LC-MS/MS-derived metabolite profiles (Figure 5). PC1 (62.95%) and PC2 (37.05%), which together represent 100% of the total variance, discriminated leaf, stem, and root extracts based on their phytochemical composition and biological activity. The leaf extract was positioned in the negative regions of both PC1 and PC2 and was characterized by flavonoid compounds, particularly quercetin, kaempferol, isoquercitrin, astragalin, nicotiflorin, hesperidin, and tannic acid. These compounds are widely reported to exhibit antiproliferative and proapoptotic activities, and their association with MDA-MB-231 and Calu-1 in the PCA suggests that they may contribute to the observed cytotoxic effects, particularly against aggressive cancer cell lines. In contrast, the stem extract showed an intermediate distribution in the negative region of PC1 and the positive region of PC2 and was mainly associated with phenolic acids such as gallic acid, protocatechuic acid, catechin, salicylic acid, chlorogenic acid, quinic acid, and caffeic acid. Although these compounds have been associated with antioxidant and moderate antiproliferative properties, their association with TSI_{Breast} and partial association with MCF-7 suggests a more selective and comparatively moderate contribution to cytotoxic activity. The root extract was positioned on the positive side of PC1 and was associated with compounds such as naringenin, genistein, acacetin, protocatechualdehyde, sinapic acid, daidzein, *p*-coumaric acid, and rutin. These metabolites, particularly flavonoid aglycones and isoflavonoids, have been reported to show anticancer activity involving apoptosis induction and cell cycle modulation, and their association with H1650 and Beas-2B suggests a stronger relevance to lung-related cellular responses. Additionally, A549 and TSI_{Lung} were located in a different region from most phytochemicals, suggesting that the antiproliferative response in A549 cells may be influenced by different compounds or synergistic interactions not strongly represented by individual variables in the PCA analysis. These findings demonstrate that differences in antiproliferative activity among HY extracts are closely associated with the organ-specific distribution of phenolic and flavonoid compounds identified by LC-MS/MS analysis.

Overall, HY extracts exhibit strong antiproliferative activity across multiple cancer cell lines, with potency comparable to or higher than that of 5-FU in certain models. The strongest effects were identified in H1650 and MDA-MB-231 cell lines, where the root extract showed the lowest GI₅₀ values, indicating the highest potency. The stem extract showed the highest calculated TSI; however, because this parameter partly relied on extrapolated IC₅₀ values, it should be considered an exploratory observation rather than definitive evidence of tumor selectivity. The observed

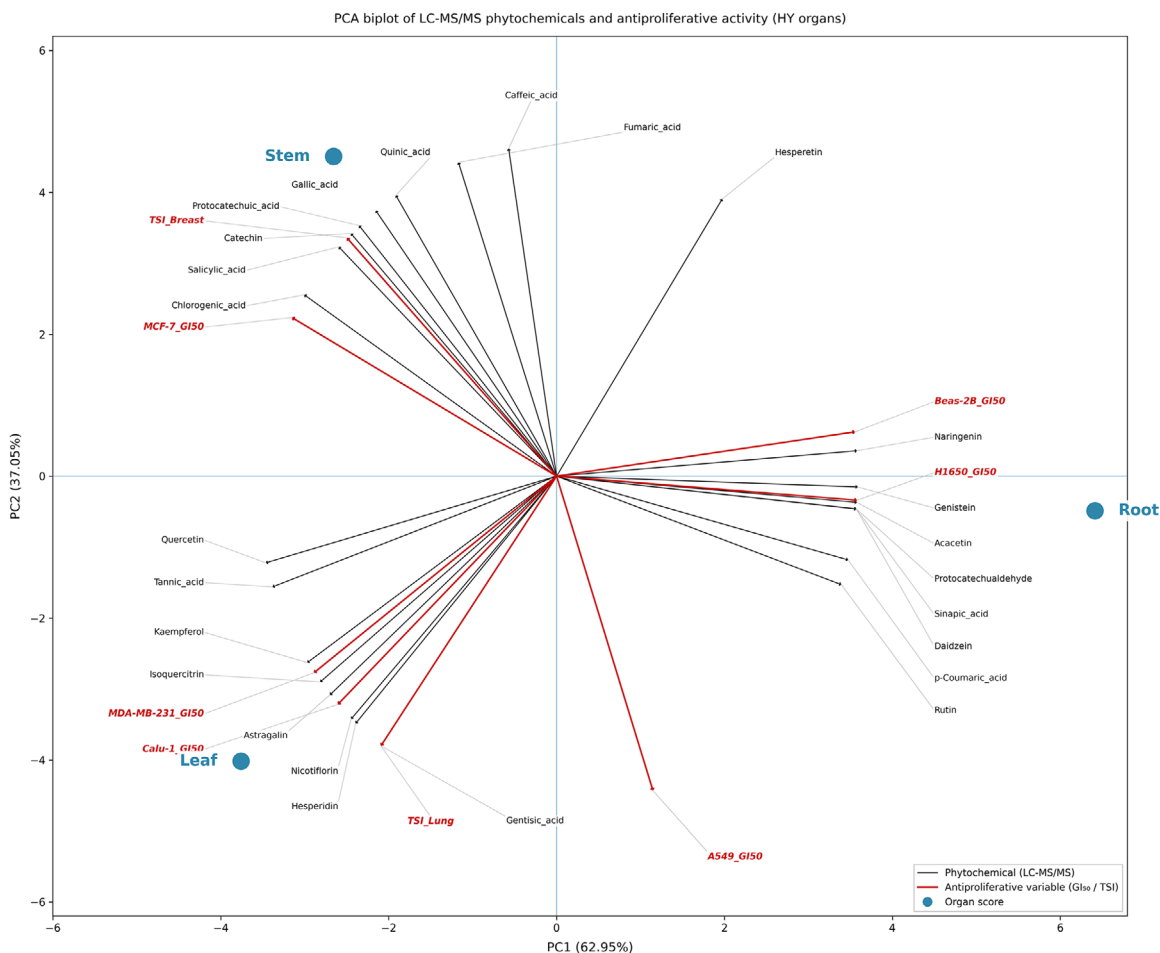


FIGURE 5 | PCA biplot of LC-MS/MS phytochemicals and antiproliferative activity.

activity, together with moderate selectivity indices, suggests the presence of biologically active secondary metabolites. The activity profiles are consistent with those reported for other *Hedysarum* species; however, the HY root extract showed higher potency against breast cancer cell lines than other Turkish *Hedysarum* species examined in previous studies. These findings identify HY as a pharmacologically relevant endemic species and provide a strong scientific basis for further studies focused on bioactivity-based fractionation, identification of active compounds, and elucidation of mechanisms associated with anticancer activity.

2.5 | Cytotoxicity Analysis

The cytotoxic effects of the extracts were further investigated using the LDH release assay, which determines lactate dehydrogenase activity present in the extracellular medium as a result of cell membrane damage. As LDH is a cytosolic enzyme present in the extracellular medium following membrane disruption, its extracellular levels indicate membrane damage and necrotic or late-stage apoptotic cell death. Therefore, this assay provides complementary information to proliferation-based assays by directly reflecting cellular damage rather than metabolic activity. The cytotoxicity data are presented in Table 6.

In lung cancer cell models (A549, Calu-1, and H1650), cytotoxicity values were between 2.25% and 6.85%, indicating a low cytotoxic profile. The lowest LDH release was determined in H1650 cells treated with the leaf extract (2.35%), indicating minimal membrane damage, while the highest value was determined in the same cell line treated with the root extract (6.85%). These findings indicate that although certain extracts show antiproliferative effects, they are not associated with high levels of membrane damage, which may be related to cytostatic or apoptosis-mediated mechanisms and not necrotic cell death. In contrast, the reference drug 5-FU demonstrated higher cytotoxicity, particularly in A549 cells (12.88%), reflecting its well-documented mechanism of action involving DNA synthesis inhibition and induction of cell death. Importantly, in normal lung epithelial cells (Beas-2B), all extracts exhibited low cytotoxicity (3.34%–4.54%), lower than that of 5-FU (8.68%), indicating lower off-target toxicity and a comparatively limited effect on nontumorigenic cells.

In breast cancer cell lines (MCF-7 and MDA-MB-231), the extracts showed comparatively higher cytotoxic responses, with values as high as 12.59%. The stem extract showed the highest cytotoxicity in MCF-7 cells (12.59%) and a similarly high effect in MDA-MB-231 cells (12.25%), suggesting that bioactive constituents in the stem may be associated with stronger membrane damage or cell death related effects in breast cancer models. This variation in sensitivity may be associated with the molecular characteristics of

TABLE 6 | % Cytotoxicity values of extracts in lung and breast cancer and normal cell lines.

% cytotoxicity	A549	Calu1	H1650	Beas2B	MCF7	MDA-MB-231	MCF10A
Leaf	5.32	5.99	2.35	3.34	8	4.18	5.89
Stem	3.59	2.25	6.58	4.54	12.59	12.25	3.16
Root	3	3.44	6.85	4.43	6.21	8.04	3.43
5FU	12.88	6.23	6.2	8.68	9.35	8.54	7.39

these cell lines, such as hormone receptor status and invasiveness, particularly in triple-negative MDA-MB-231 cells. Leaf and root extracts exhibited lower cytotoxicity, indicating a more moderate effect on membrane integrity. Cytotoxicity in normal mammary epithelial cells (MCF10A) was low (3.16%–5.89%), suggesting that the extracts act more strongly on cancer cells while showing limited toxicity toward healthy cells.

When the LDH-based cytotoxicity findings are interpreted together with the antiproliferative results from the MTT assay, a consistent activity pattern is observed. The extracts demonstrate moderate inhibition of cancer cell proliferation while inducing only limited membrane damage, suggesting the involvement of cytostatic effects, cell-cycle modulation, or apoptosis. This is particularly important in the context of anticancer drug discovery, where selective cytotoxicity toward cancer cells with minimal toxicity to normal tissues is an important consideration. Although the cytotoxic potency of the extracts is lower than that of 5-FU, their effects on cancer cell growth, together with low cytotoxicity in noncancerous cells, suggest a degree of selectivity. These findings indicate that HY extracts may be considered for further investigation as natural anticancer agents, particularly with respect to their safety profile and potential selective effects on tumor cells.

3 | Conclusions

This study provides the first comprehensive phytochemical and biological characterization of HY, a recently described endemic species from Türkiye, and demonstrates that this species represents a promising source of bioactive secondary metabolites. LC-MS/MS profiling demonstrated an organ-specific distribution of phenolic compounds, with flavonoid glycosides mainly present in the leaves, phenolic acids at higher levels in the stems, and certain aglycones and isoflavonoids in the roots, suggesting a metabolic differentiation associated with the bioactivity patterns. The leaf extract exhibited the highest antioxidant capacity, in line with its total phenolic and flavonoid content, while both leaf and stem extracts showed inhibitory activity against AChE, BChE, and α -glucosidase, suggesting potential neuroprotective and antidiabetic effects; moreover, the α -glucosidase inhibition of the leaf extract was comparable to that of acarbose. In antiproliferative assays, all extracts showed growth inhibition in lung and breast cancer cell lines, with stronger effects in H1650 and MDA-MB-231 cells, while low LDH cytotoxicity in normal cells. Low LDH cytotoxicity in normal cell lines together with favorable GI_{50} values suggests that HY extracts warrant further investigation for their antiproliferative potential. Overall, the findings indicate that HY is a biologically active species within

the genus *Hedysarum*, showing multitarget effects associated with its phenolic composition; however, further studies including bioactivity-guided isolation of active compounds, mechanistic investigations, and in vivo studies are needed to determine its biological effects and pharmacological relevance.

4 | Experimental Section

4.1 | Plant Material

The plant material of HY was collected from its natural habitat in Eriç village, Kemah (Erzincan, Türkiye) in 2024. The collected specimens were separated into root, stem, and leaf organs, and each organ was analyzed individually for phytochemical and biological properties. The species 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: EBYU-0000049).

4.2 | Extraction Procedure

The plant materials (root, stem, and leaf) were dried and ground into a fine powder. Each sample (10 g) was extracted with 100 mL of methanol by maceration at room temperature overnight. Methanol was selected as the extraction solvent because of its polarity and its ability to extract phenolic and flavonoid compounds [52]. The extraction was performed three times, after which the extracts were combined.

4.3 | LC-MS/MS Conditions

Quantitative profiling of 53 phytochemicals in the methanolic extracts obtained from HY leaves, stems, and roots was performed using LC-MS/MS, based on a previously validated analytical procedure [53, 54], with a Shimadzu Nexera ultrahigh performance liquid chromatography (UHPLC) system coupled to a tandem mass spectrometer. The analyses were performed on a UHPLC system equipped with an autosampler (SIL-30AC), column oven (CTO-10ASvp), binary pump system (LC-30AD), and degassing unit (DGU-20A3R). An Agilent Poroshell 120 EC-C18 column (150 mm $\times$ 2.1 mm, 2.7 μ m) was used for chromatographic separation at 40°C. A binary mobile phase system was used, consisting of solvent A (water with 5 mM ammonium formate and 0.1% formic acid) and solvent B (methanol with 5 mM ammonium formate and 0.1% formic acid). The mobile phase gradient was programmed from 20% to 100% B over 25 min, followed by 100%

B for 10 min and 20% B over the next 10 min. The flow rate was 0.5 mL/min, and the injection volume was 5 μ L.

Using a Shimadzu LCMS-8040 tandem mass spectrometer equipped with an electrospray ionization (ESI) source, mass spectrometric analysis was performed in both positive and negative ion modes [55]. With LabSolutions software (Shimadzu), data acquisition and evaluation were performed. For the selective identification and quantification of phytochemicals, the multiple reaction monitoring (MRM) mode was used based on optimized ion transitions. Collision energies (CE) were optimized for fragmentation and transmission of target product ions. As follows, the MS operating conditions were: drying gas (N_2) flow rate of 15 L/min, nebulizing gas (N_2) flow rate of 3 L/min, desolvation line (DL) temperature of 250°C, heat block temperature of 400°C, and interface temperature of 350°C.

4.4 | Anticholinesterase Activity

The inhibitory activity of HY extracts against AChE and BChE was determined spectrophotometrically using a previously described method with minor modifications [56–58]. With deionized water, plant extracts, and tacrine (positive control) were diluted to obtain different concentrations. The experimental reaction mixture included 100 μ L 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. By adding the substrates acetylthiocholine iodide for AChE and butyrylthiocholine iodide for BChE, the reaction was initiated. Enzymatic hydrolysis of the substrates generated thiocholine, which reacted with DTNB to produce the yellow 5-thio-2-nitrobenzoate anion. The absorbance was measured spectrophotometrically at 412 nm. Results were expressed as IC₅₀ values, defined as the concentration required to inhibit 50% of enzyme activity. In triplicate, all experiments were performed, and as mean $\pm$ standard deviation (SD), results are reported.

4.5 | α -Glucosidase Inhibition Activity

The α -glucosidase inhibitory activity was determined using a previously described procedure with minor modifications [59]. Acarbose was used as the positive control. The plant extracts and acarbose were diluted with deionized water to appropriate concentrations. Phosphate buffer (1 M, pH 7.4), enzyme solution, and sample were mixed and incubated at 35°C for 10 min. After incubation, *p*-nitrophenyl- α -D-glucopyranoside (*p*-NPG) was added, and the mixture was again incubated at 35°C. The absorbance was measured spectrophotometrically at 405 nm. IC₅₀ values were calculated from percent activity and concentration data. In triplicate, all experiments were performed, and as mean $\pm$ standard deviation (SD), results are reported.

4.6 | DPPH Free Radical Scavenging Activity

According to a previously described procedure, the DPPH[•] radical scavenging capacity of the extracts was determined [60–62]. Stock solutions of the extracts (1 mg/mL) and a 0.26 mM DPPH[•] solution were prepared, and the extracts were diluted in methanol

to concentrations of 20–1000 μ g/mL. Each diluted extract (3 mL) was mixed with 1 mL of DPPH[•] solution, vortexed, and incubated in the dark at room temperature for 30 min. Absorbance was measured at 517 nm, and the percentage of scavenging activity was calculated. With standard antioxidants such as Trolox, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and ascorbic acid, the results were compared. In triplicate, all analyses were performed, and the results are expressed as mean $\pm$ standard deviation.

4.7 | Ferric Reducing Antioxidant Power (FRAP)

The reducing power activity of HY root, stem, and leaf extracts was determined according to a previously described procedure [63–65]. Stock solutions of the extracts and standards were prepared at 1 mg/mL. For the assay, 100 μ L of each extract was diluted to 1.25 mL with 0.2 M phosphate buffer (pH 6.6), followed by the addition of 1.25 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50°C for 20 min. After incubation, 1.25 mL of 10% trichloroacetic acid (TCA) and 0.25 mL of 0.1% $FeCl_3$ were added, and the absorbance was measured at 700 nm. The results were expressed as mg Trolox equivalents per gram of extract (mg TE/g extract) using a Trolox calibration curve. The antioxidant activity of the samples was compared with that of ascorbic acid, BHA, and BHT. In triplicate, all analyses were performed, and the results are expressed as mean $\pm$ standard deviation.

4.8 | Determination of Total Phenolic Content

The total phenolic content of the extracts was determined using the Folin–Ciocalteu method [66–68]. 100 μ L of each extract solution was diluted with 4.5 mL of distilled water, followed by the addition of 100 μ L of Folin–Ciocalteu reagent and 300 μ L of 2% Na_2CO_3 solution. The mixture was incubated at room temperature for 2 h, and absorbance was measured at 760 nm. The results were expressed as mg of gallic acid equivalent per gram of extract (mg GAE/g extract). All analyses were performed in triplicate.

4.9 | Determination of Total Flavonoid Content

Total flavonoid content was determined according to a previously reported procedure [69–71]. 100 μ L of each extract and standard was diluted to 4.8 mL with methanol. Then, 100 μ L of 1 M ammonium acetate (NH_4CH_3COO) and 100 μ L of 10% aluminum chloride ($AlCl_3$) were added. The mixture was vortexed and incubated at room temperature for 45 min. Absorbance was measured at 415 nm, and the total flavonoid content was expressed as mg quercetin equivalents per gram of extract (mg QE/g extract). Analyses were systematically performed in triplicate.

4.10 | Cell Lines and Cell Culture

In this study, human lung cancer cell lines A549 (ATCC CCL-185), Calu1 (ATCC HTB-54), and H1650 (ATCC CRL-5883), as well as 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. All cell culture procedures were performed in a laminar flow cabinet. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ in DMEM or RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 2% penicillin–streptomycin. Cells (1 × 10⁴ per well) were added to 96-well plates. After a 16 h incubation to allow cell attachment, samples were added, followed by an additional 24 h incubation before analysis [72].

4.11 | Measurement of Cell Proliferation

After 24 h incubation with cancer cell lines, the effects of the plant extracts on cell proliferation were determined by the MTT assay [73, 74]. Results were expressed as percentage inhibition, using the optical density of solvent-treated (DMSO) cells as the 100% reference. Using the formula % inhibition = $[1 - (A_{\text{test}} / A_{\text{control}})] \times 100$, percentage inhibition was calculated. From absorbance data obtained following treatment with increasing concentrations of the extracts (1.96–125.0 µg/mL), IC₅₀ values were calculated. Using logarithmic regression analysis in Microsoft Excel, dose response curves were generated. Based on cell growth, GI₅₀ values were calculated 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. GI₅₀ corresponds to the concentration resulting in 50% growth inhibition. As the ratio of the IC₅₀ concentration in normal cells to the mean IC₅₀ concentration in cancer cells, TSI values were calculated. When 50% inhibition was not experimentally reached within the tested concentration range (1.96–125 µg/mL), IC₅₀ values were estimated by nonlinear regression extrapolation. Accordingly, TSI values calculated from these IC₅₀ estimates were considered exploratory and interpreted with caution.

4.12 | Cytotoxicity Test

The cytotoxic and cytostatic effects of the extracts were determined using the lactate dehydrogenase (LDH) release assay [75, 76]. LDH is a stable cytoplasmic enzyme present in the culture supernatant following plasma membrane damage; therefore, increased LDH levels indicate increased cell death. In accordance with the manufacturer's instructions, the LDH cytotoxicity assay was performed. Spectrophotometrically, formazan formation resulting from LDH enzymatic activity was quantified, and using the following equation, percentage cytotoxicity was calculated:

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

4.13 | Statistical Analyses

Statistical analyses were performed using SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). All experiments were performed in triplicate, and the data are presented as mean

± standard deviation (SD). Differences among extracts from different plant organs were compared using one-way ANOVA, followed by Duncan's multiple range test for post hoc comparisons, and $p < 0.05$ was considered statistically significant. Relationships between phytochemical parameters and biological activities were determined using Pearson correlation analysis. Multivariate analyses, including principal component analysis (PCA) and heatmap analysis, were used to investigate the distribution of phytochemicals and their associations with biological activities.

Author Contributions

Etem Osma and **Müjgan Elveren**: conceived the project, performed the experiments, and methodology. **Samet Şimşek**: performed the experiments, analyzed the data (formal analysis and statistics), and wrote the manuscript. **Sevgi Altın**, **Mustafa Abdullah Yılmaz**, and **Ali Aydın**: performed the experiments and revised the manuscript. **Ali Kandemir**: performed the experiments and provided technical assistance. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data will be made available on request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting File 1: cbdv71659-sup-0001-SuppMat.docx