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A Comprehensive Investigation of *Equisetum ramosissimum*: Phytochemical Composition, Antioxidant Potential, and Enzyme Inhibition Activity

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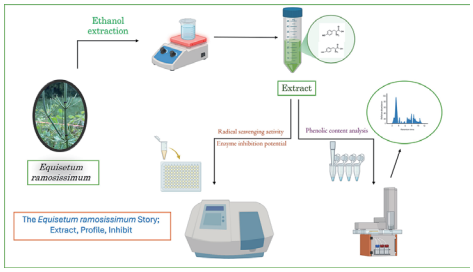

Abstract: This study provides a comprehensive investigation of the phytochemical composition, antioxidant capacity, and enzyme inhibition properties of *Equisetum ramosissimum*, a medicinal plant traditionally used in Turkey. The main aim was to characterize its bioactive compounds and evaluate their biological relevance using modern analytical techniques. Ethanolic extracts of the plant were analyzed by LC-MS/MS, enabling the identification and quantification of 13 phenolic compounds, including kaempferol (1458.11 µg/L), vanillic acid (1002.26 µg/L), and resveratrol (424.12 µg/L). Antioxidant activity was assessed through DPPH, ABTS, FRAP, and CUPRAC assays, where the extract demonstrated strong radical scavenging and metal reducing capacity. Enzyme inhibition studies revealed significant inhibitory effects on AChE, BChE, and α-glucosidase, with IC_{50} values comparable to standard inhibitors. These findings indicate that *E. ramosissimum* is a rich source of phenolic compounds with strong antioxidants and enzyme inhibitory properties. The originality of this work lies in its systematic evaluation of Turkish *E. ramosissimum* populations, highlighting their potential as natural therapeutic agents for managing oxidative stress-related diseases, neurodegenerative disorders, and diabetes.

Key words: antioxidant, bioactivity, enzyme inhibition, LC-MS/MS, phytochemical profiling, *Equisetum ramosissimum*

1 Introduction

The importance of plant-derived natural compounds in the pharmaceutical and food industries has been clearly demonstrated by numerous studies in recent years[1-3]. These compounds play a role in the prevention of many chronic diseases by combating oxidative stress caused by free radicals[4-6]. In particular, secondary metabolites such as phenolic compounds, flavonoids, tannins, and saponins possess antioxidant, anti-inflammatory, anticarcinogenic, and antimicrobial effects[7, 8]. In summary, these bioactive components have been used both in drug synthesis and as inspiring model molecules directly as therapeutic agents[9]. Therefore, the identification of new species and consequently the discovery of new bioactive compounds keep hope alive in drug development processes. In addition, elucidating the mechanisms of action of plant-derived substances is considered an excellent source for potential drug candidates[10]. The richness and diversity of the Turkish flora offer significant potential for the discovery and investigation of plant-based natural products. These plants, which have held a place in traditional medicine for many years, are also at the center of modern scientific research[11]. The genus Equisetum (horsetail) belongs to the family Equisetaceae and includes perennial plants that have existed since prehistoric times[12, 13]. *Equisetum ramosissimum*, which is included in this genus, is an important species commonly grown in Turkey and known as "kırkkilit otu" (horsetail) among the public[14, 15]. It is known that horsetail has been used in traditional medicine for many years due to its diuretic, wound healing, diuretic, and hemostatic effects[13, 16]. In addition, due to its high silica content, it is also recommended for supporting bone

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health[17]. Modern phytochemical analyses have shown that *E. ramosissimum* offers a rich phenolic profile, mainly composed of components such as quercetin, kaempferol, isoquercitrin, chlorogenic acid, and caffeic acid[3, 4, 18]. Each of these components plays an active role in the neutralization of free radicals and thus contributes to antioxidant activity[19, 20]. Among the in vitro analytical methods commonly used to evaluate antioxidant potential are 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), cupric reducing antioxidant capacity (CUPRAC), oxygen radical absorbance capacity (ORAC), and total phenolic content (TPC) determination[19, 21]. Through these analyses, the scavenging effect of plant extracts on oxygen radicals can be quantitatively measured[22]. In the study conducted by Saeed-Abadi et al.[12], it was reported that high antioxidant capacity and phenolic content were obtained as a result of optimizing Equisetum arvense extracts using the ultrasound-assisted extraction (UAE) method. Similarly, Mohamed & Ibrahim (2024) emphasized that extraction conditions (type of solvent, temperature, duration) have a determining effect on the phytochemical composition. Some studies have revealed that phenolic compounds can be more efficiently isolated in extractions performed with polar solvents (ethanol, methanol, and water)[4, 17]. The use of modern techniques increases extract yield and bioactivity compared to traditional solvent-based methods[8, 23]. The high flavonoid content of *E. ramosissimum* brings forward not only its traditional uses but also its potential use in modern pharmaceutical formulations[24, 25]. The existing literature contains significant information regarding the chemical and biological properties of plants belonging to the Equisetum genus; however, these studies have mostly remained superficial or included limited biological analyses[14].

For example, Al-Bayati et al.[26] determined the total phenolic and flavonoid content of *E. ramosissimum*, but a comprehensive analysis regarding its antioxidant and enzyme inhibition activities was not conducted. Sureshkumar et al.[27], on the other hand, only examined DPPH and FRAP activities and did not present a detailed evaluation of the relationship between chemical profile and biological activities. This study, however, focuses on the relationship between phenolic content and biological activities and presents a more comprehensive analysis in this context. In addition, research conducted on this species indicates that its areas of use may expand both as a dietary supplement and as an alternative therapeutic tool[18, 21]. Although *Equisetum ramosissimum* (horsetail) is known in traditional medicine for its diuretic, anti-inflammatory, and antioxidant properties, comprehensive experimental studies on its populations in Turkey remain limited, and especially its phenolic, antioxidant, and enzyme inhibition properties need to be investigated more thoroughly.

This study aims to fill this gap in the literature by systematically examining samples of *E. ramosissimum* from Turkey.

Considering the role of phenolic compounds and antioxidant activities in biological systems, a detailed investigation into the chemical and biological profiles of this species will not only contribute to the literature but also allow the evaluation of its potential therapeutic use[28]. Additionally, increasing the knowledge regarding the biological diversity of this species among local plants is also among the objectives of this study. A detailed investigation of the phytochemical and biological properties of local species such as *E. ramosissimum* may open new research areas for biotechnological and pharmacological applications. The main research question of the study is whether there is a significant relationship between the phenolic and flavonoid profile of *Equisetum ramosissimum* and its antioxidant activities, and to what extent these contents affect enzyme inhibition activities.

For this purpose, a comprehensive chemical analysis was conducted, and the obtained data were supported by biochemical tests and modern analytical methods such as LC-MS/MS. Such an approach allows for an understanding of the chemical mechanisms underlying the biological effects of plant extracts. In the study, antioxidant activities (via DPPH, ABTS, FRAP, and CUPRAC methods) and enzyme inhibition activities (particularly on AChE, BChE, and α-glucosidase enzymes) were investigated.

The combined use of these methods enables a holistic evaluation of the biological activity of *Equisetum ramosissimum*.

2 Materials and Methods

2.1 Materials

This study utilized various analytical-grade chemicals, including 1,1-diphenyl-2-picryl-hydrazyl (DPPH), α-tocopherol, methanol (gradient grade for liquid chromatography), ethanol (isocratic grade), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), butylated hydroxyanisole (BHA), butylated hydroxy-toluene (BHT), DTNB (Ellman's Reagent; 5,5-dithio-bis-(2-nitrobenzoic acid)), 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris-HCl), cholinesterase, and *p*-nitrophenyl-D-glucopyranoside (*p*-NPG). All chemicals, including the LC-MS/MS reference compounds listed in Table 4, were supplied by Sigma-Aldrich (Darmstadt, Germany).

2.2 Plants materials

The plant material used in this study was collected in May 2024 from the Kirazdere area of Kocaeli province, located in the Marmara region of Turkey (Latitude: 40.6761, Longitude: 29.9702 Altitude: 109 m). The species identifi-

cation of the collected plant was carried out by Prof. Dr. Mustafa Korkmaz from the Department of Biology at Erzincan University, and the plant was identified as *Equisetum ramosissimum*, a subspecies belonging to the Equisetum family.

The collected plant materials were subjected to drying at 40℃, and the dried and powdered samples were kept in a moisture-free environment to preserve their stability prior to analysis.

2.3 Preparation of sample extract

An amount of 20 grams from the dried and pulverized plant material was measured and subjected to extraction using 400 mL of a 70% ethanol–water mixture. The process was carried out in a sealed vessel under continuous stirring with a magnetic stirrer for 24 hours. Upon completion, the mixture was passed through Whatman No. 1 filter paper (Sigma-Aldrich, Germany) to separate the solid plant residues. The filtrate was then concentrated using a rotary evaporator (Heidolph 94200, Bioblock Scientific, Germany) to remove the solvent. The obtained extract was exposed to cold air drying and maintained at −20℃ until analytical procedures were performed[29, 30]. The extraction process yielded a product recovery rate of approximately 20–25% (w/w).

2.4 DPPH· scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was determined according to a modified version of the Blois method, as adapted by Ndhlala et al.[31, 32]. Initially, a 0.1 mM stock solution of DPPH—a stable radical characterized by its intense purple coloration—was prepared. Reference antioxidants and the ethanol extract of *Equisetum ramosissimum* were each dissolved in ethanol at a concentration of 1 mg/mL to form separate stock solutions. Various volumes from these solutions were transferred into test tubes, and the final volume in each was adjusted to 2 mL using 96% ethanol. Subsequently, the DPPH solution (0.5 mL) was added to each mixture, vortexed and allowed to react at ambient temperature for 30 minutes. After incubation, the absorbance values were recorded at 517 nm, and the radical scavenging activity was quantified based on the reduction in absorbance.

2.5 ABTS radical scavenging assay

Compounds with oxidative potential can transform ABTS into its chromophoric radical form, $ABTS^{\cdot+}$. Antioxidant-containing samples have the capability to reduce the intensity of this green-blue radical by donating electrons or hydrogen atoms[33]. In the present study, the $ABTS^{\cdot+}$ radical scavenging capacity was evaluated according to the procedure described by Gulcin et al.[34]. For radical generation, a 7 mM ABTS stock solution was combined in equal parts with a 2.45 mM potassium persulfate solution and kept in the dark at room temperature for 12–16 hours to allow full oxidation. Following this incubation period, the $ABTS^{\cdot+}$ solution was diluted with ethanol until the absorbance at 734 nm reached 0.750 ± 0.025, forming the working solution. Ethanol extracts of the sample (1 mg/mL) and reference compounds (1 mg/mL) were initially prepared, and pipetted into separate tubes in increasing concentrations. Each sample was then brought to a final volume of 2 mL using 96% ethanol. Subsequently, 0.5 mL of the prepared $ABTS^{\cdot+}$ working solution was added to each tube, vortexed and left to react in darkness for 30 minutes. Absorbance readings were taken at 734 nm, and the antioxidant activity was calculated by comparing the decrease in absorbance against that of the standards, with results expressed as percent radical scavenging activity.

2.6 Phenolic compound analysis by LC-MS/MS

The phenolic composition of the *Equisetum ramosissimum* ethanol extract was assessed using Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS; Shimadzu, Kyoto, Japan). Initially, method optimization was performed using 25 high-purity standard phenolic compounds to establish accurate detection and quantification parameters. Upon completion of the optimization process, quantitative analysis of the phenolic constituents in the plant extract was carried out. The analytical protocol, including compound-specific optimization and detec-tion, was based on a modified version of the method described by Altay et al.[35].

For the LC-MS/MS analysis, a gradient elution program was employed using a binary mobile phase consisting of solvent A (deionized water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid), as detailed in Table 1. The gradient flow profile used for compound separation is provided in Table 2. The total duration of the chromatographic run was 15 minutes, which included a 3-minute re-equilibration phase at the end of the sequence.

The LC-MS/MS analyses were conducted in MRM (Multiple Reaction Monitoring) mode using Lab Solutions software (Shimadzu, Kyoto, Japan) (Division Shimadzu Corporation, 2024), and all quantifications were performed accordingly. In this acquisition mode, the mass spectrometer selectively monitors specific precursor and production transitions for each target compound, thereby enhancing specificity by excluding signals from non-target molecules. This approach significantly minimizes background interference and improves analytical accuracy and sensitivity[36]. Detailed specifications of the modules that constitute the liquid chromatography system used in this study are summarized in Table 3.

2.7 Fe^{3+} reducing antioxidant power assay (FRAP)

FRAP of the *Equisetum ramosissimum* ethanol extract was assessed by adapting the procedure originally pro-

Table 1 The working method of LC/MSMS.

Mobile Phase	A: 0.1% formic acid and deionize water,
	B: 0.1% formic acid and methanol
Flow	Gradient
Flow Rate	0.5 mL/min
Injection Volume	5 μL
Column Property	C18 Inertsil ODS-4 (2 μM, 3.0 mm × 100 mm)
Column Temperature	40℃

Table 2 Gradient method mobile phase flow data used in LC/MS MS analysis.

Mobile Phase	% A (0.1% formic acid and di water)	% B (0.1% formic acid and methanol)
0 min.	80	20
8 min.	50	50
12 min.	5	95
15 min.	80	20

Table 3 Modules of the liquid chromatography device where the analyses are performed.

Column Furnace	CTO-10ASVP
Degasser	DGU-20A3R
Dual pumps	LC-30AD
Autosampler	SIL-30AC
Column Property	C18 Inertsil ODS-4 column (3.0 mm × 100 mm, 2 μM)

posed by Oyaizu[37], with modifications based on the method outlined by Dikici and Köksal[28]. For the analysis, 1 mg/mL stock solutions of both the standard antioxidants and the plant extract were prepared. Measured volumes of these solutions were transferred into glass test tubes using a micropipette. Then, 1.25 mL of 0.2 M phosphate buffer (pH 6.6) was added, followed by 1.25 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution. The resulting mixtures were incubated at 50℃ for 20 minutes. After incubation, 1.25 mL of 10% trichloroacetic acid (TCA) and 0.1% ferric chloride ($FeCl_3$) solution were sequentially added. The absorbance of each final mixture was recorded at 700 nm. The antioxidant capacity was expressed in terms of mg Trolox equivalent (TE) per gram of extract.

2.8 Cu^{2+} reducing assay (CUPRAC)

CUPRAC of the *Equisetum ramosissimum* ethanol extract was evaluated using a modified form of the method developed by Apak et al.[38]. In this procedure, 1 mL of 0.01 M copper (II) chloride solution, 1 mL of 7.5 mM neocuproine (prepared in ethanol), and 1 mL of 1.0 M ammonium acetate buffer (pH 7.0) were added sequentially into test tubes. Afterward, varying concentrations of the plant extract (ranging from 10 to 40 μg/mL) were introduced into the tubes, and the total volume of each mixture was brought to 4 mL with ultrapure water.

The prepared reaction mixtures were vortexed thoroughly and incubated for 30 minutes at room temperature (25℃) in the absence of light. Following incubation, the absorbance was measured at 450 nm using a UV-Vis spectrophotometer. Trolox was employed as the reference standard antioxidant, and results were reported as Trolox Equivalent Antioxidant Capacity (TEAC). An increase in absorbance was interpreted as a higher capacity of the extract to reduce cupric ions (Cu^{2+}), indicating greater antioxidant potential.

2.9 Cholinesterase inhibition assay

The inhibitory activity of the *Equisetum ramosissimum* ethanol extract on cholinesterase enzymes, specifically AChE and BChE, was determined via a spectrophotometric approach based on the Ellman method, with slight procedural modifications[39]. The reaction mixture consisted of 50 μL of DTNB, Tris-HCl buffer (1 M, 100 μL, pH 8.0), and cholinesterase enzyme solution (5.32×10^{-3} U, 50 μL), which was incubated at 30℃ under mild agitation for 15 minutes. Subsequently, 50 μL of butyrylthiocholine iodide (for BChE) or acetylthiocholine iodide (for AChE) was

added to initiate the enzymatic reaction. Hydrolysis of the respective substrates was monitored spectrophotometrically by measuring absorbance at 412 nm. The ethanol extract was tested at various concentrations to evaluate its inhibitory effect, and IC_{50} values were calculated by plotting the percentage of enzyme inhibition against the logarithmic concentration of the extract[11, 40].

2.10 α-GLY inhibition assay

In line with previously established experimental procedures, *p*-nitrophenyl-D-glycopyranoside (*p*-NPG) was employed as the substrate to determine the inhibitory effect of the *Equisetum ramosissimum* extract on α-glucosidase (α-GLY) activity[41]. The enzymatic reaction was monitored by measuring the absorbance of the resulting product at 405 nm using a spectrophotometer. The extract was tested at different concentrations to evaluate its inhibition potential, and the IC_{50} values were derived by constructing dose-response curves, which plotted the percentage of enzyme inhibition as a function of inhibitor concentration[11].

3 Result and Discussion

3.1 Detailed characterization of phenolic constituents in the ethanol extract of *Equisetum ramosissimum* via LC-MS/MS

Horsetail, which has long been used in traditional medicine, is a medicinal plant with high pharmacological potential. This species is rich in secondary metabolites and is particularly notable for its phenolic compounds[42, 43]. In this study, the phenolic content of the ethanolic extract of *Equisetum ramosissimum* was comprehensively evaluated using LC-MS/MS analysis. A total of 20 different standard phenolic compounds were analyzed, and among them, 7 were confirmed to be present in the extract and their quantities were determined (Table 4).

Phenolic compounds are chemical structures that play a protective role against oxidative stress in plants and are associated with antioxidant, anti-inflammatory, anticancer, antimicrobial, and neuroprotective activities in terms of human health[44]. In this study, the phenolic compounds present in the ethanol extract of *E. ramosissimum* were quantitatively analyzed using the LC-MS/MS method in Multiple Reaction Monitoring (MRM) mode, and high accuracy and sensitivity were achieved by focusing only on the

Table 4 The Phenolic content of *Equisetum ramosissimum* ethanol extract.

Compounds	[a]LOD/LOQ (μg/L)	Recovery (%)	[b]RT	Conc. (μg/L)
Quercetin	22.5/25.7	1.001	6,091	39,89
Acetohydroxamic Acid	2.8/8.2	1.000	1,986	144,22
Catechin hydrate	8.2/11.4	0.994	4,958	339,50
Vanillic Acid	125.5/142.2	1.001	6,026	1002,26
Resveratrol	9.0/13.6	0.998	5,713	424,12
Fumaric Acid	25.2/31.3	0.997	3,674	542,09
Gallic acid	0.90/1.6	1.000	4,134	N.D.
Caffeic Acid	6.3/10.7	1.009	5,283	513,71
Phloridzin dihydrate	61.0/207.0	1.000	5,646	445,18
Oleuropein	0.05/1.0	0.997	5,643	N.D.
Ellagic Acid	0.101/0.333	1.002	5,895	N.D.
Myricetin	55.4/59.6	0.999	5,858	16,79
Protocatechuic acid	30.3/35.4	1.011	5,875	N.D.
Butein	22.7/28.6	0.096	6,084	107,03
Naringenin	5.4/6.4	0.998	6,104	N.D.
Luteolin	0.5/2.5	1.007	6,19	177,69
Kaempferol	206.6/214.3	0.999	6,288	1458,11
Alizarin	65.2/77.5	0.966	6,8	N.D.
4-Hydroxybenzoic Acid	30.5/40.25	0.996	6,13	N.D.
Salicylic acid	0.83/1.7	1.004	6,104	1,08

[a] LOD/LOQ (μg/L): Limit of detection/limit of quantitation.
[b] RT: Retention time. N.D.: Not detected

ions of the target molecules during the analyses[27, 45]. Previous studies have reported the antioxidant potential of *E. ramosissimum*[27, 45]; however, the scope and methodology of these works differ from the present study. For instance, Sureshkumar et al.[27] characterized the plant's phytochemical profile mainly by GC-MS and evaluated its antioxidant potential through several in vitro assays but did not provide targeted quantitative analysis by LC-MS/MS nor a direct correlation with enzyme inhibition. On the other hand, Yilmaz[45] reported a validated LC-MS/MS methodology, yet its application to *E. ramosissimum* was limited and the study did not integrate detailed biological activity assessments. In contrast, our work combines validated MRM-based LC-MS/MS quantification of 13 phenolic compounds with four complementary antioxidant assays (DPPH, ABTS, FRAP, CUPRAC) and enzyme inhibition analyses (AChE, BChE, and α-glucosidase). This integrative approach not only quantifies dominant compounds such as kaempferol (1458.11 μg/L) and vanillic acid (1002.26 μg/L) but also reveals novel findings such as the strong inhibitory effect against α-glucosidase (IC_{50} = 2.95 μg/mL), a result not previously reported. These distinctions highlight the originality of the present study and extend current knowledge on the pharmacological potential of *E. ramosissimum*.

This methodology is based on a protocol modified by Altay et al.[35] and optimized for phenolic analysis in plant extracts.

Among the compounds detected at the highest levels were kaempferol (1458.11 μg/L), vanillic acid (1002.26 μg/L), fumaric acid (542.09 μg/L), caffeic acid (513.71 μg/L), resveratrol (424.12 μg/L), and phloridzin dihydrate (445.18 μg/L).

Kaempferol is a flavonoid that stands out particularly for its anticancer and anti-inflammatory effects; it has been shown to be effective against various types of cancer such as colon, breast, and prostate cancer[46]. Vanillic acid is known for its hepatoprotective and antimicrobial effects and plays a protective role against liver damage caused by oxidative stress[47, 48]. Resveratrol is a stilbene derivative known for its protective effects against cardiovascular diseases and its anti-aging properties. Neuroprotective effects against Alzheimer's disease have also been reported[49–51]. Caffeic acid, with both antioxidant and anti-inflammatory properties, supports the immune system and may be effective against metabolic disorders such as diabetes and obesity[52, 53]. Phloridzin dihydrate and fumaric acid are among metabolites with significant physiological effects and, in this respect, make important contributions to the therapeutic potential of the extract[54, 55]. Phloridzin dihydrate is a dihydrochalcone glycoside belonging to the flavonoid class, and recent studies have indicated that this compound exhibits promising effects particularly on type 2 diabetes. It acts as an inhibitor of Sodium/glucose cotransporter 1 (SGLT1) and Sodium/glucose cotransporter 2 (SGLT2), thereby inhibiting glucose absorption from the intestine and reabsorption from the kidney, thus reducing postprandial glycemia[56].

In addition, the strong antioxidant activity of phloridzin, through its free radical scavenging capacity at the cellular level, has been reported to provide protective effects against cardiovascular diseases and certain types of cancer[57].

Fumaric acid is a natural dicarboxylic acid known as an intermediate metabolite of the citric acid cycle and is found in many plants[58]. The health effects of fumaric acid have been mainly investigated in terms of anti-inflammatory, neuroprotective, and anticarcinogenic aspects[59]. In particular, dimethyl fumarate, which is used in the treatment of multiple sclerosis (MS), is a derivative of this compound and suppresses the inflammatory process by enhancing antioxidant defense.

Furthermore, in animal experiments, fumaric acid has been reported to show antiproliferative effects on colon, lung, and breast cancer cell lines[60, 61]. The high levels of these compounds in *Equisetum ramosissimum* extract reveal that this plant has significant potential for the prevention of diabetic complications, suppression of inflammatory diseases, and reduction of oxidative stress-induced cellular damage. Moreover, it is recommended that the synergistic effects of these compounds be examined in more detail in future in vivo and clinical studies.

3.2 Antioxidant activity of *Equisetum ramosissimum* ethanol extract

Natural antioxidants obtained from plants, especially phenolic compounds, play an important role in the prevention of oxidative stress-related diseases by neutralizing reactive oxygen species (ROS)[62].

The antioxidant properties of *Equisetum ramosissimum* ethanol extract were evaluated using DPPH, ABTS, FRAP, and CUPRAC assays. The results of these analyses are presented in Table 5.

In the DPPH· assay, the free radical scavenging activity of the extract was evaluated by measuring the decrease in absorbance resulting from the reduction of the deep, purple-colored DPPH· radical. The extract exhibited 79.74 ± 6.43% inhibition in the DPPH free radical scavenging test, indicating a high free radical scavenging capacity, particularly due to its phenolic compound content. This value was found to be comparable to that of the reference antioxidant Trolox (81.23 ± 7.08%) and significantly higher than BHT (48.37 ± 3.87%). This indicates that the extract has a strong effect on the DPPH radical. The high DPPH scavenging activity of the extract (79.74 ± 6.43%; Table 5) correlates well with the presence of hydrogen-donating phenolic compounds quantified by LC-MS/MS (Table 4), particularly kaempferol, phloridzin and caffeic acid, which are known to act as potent radical scavengers in single-

Table 5 Radical removal and metal reduction activity of *Equisetum ramosissimum*.

Antioxidants	DPPH[a] (0.2 mg mL^{-1})	ABTS[a] (0.2 mg mL^{-1})	FRAP Assay[b] (0.2 mg mL^{-1})	CUPRAC Assay[b] (0.2 mg mL^{-1})
Equisetum ramosissimum	79.74 ± 6.43	42.99 ± 3.86	0.422 ± 0.03	0.662 ± 0.04
BHT	48.37 ± 3.87	46.97 ± 3.74	0.72 ± 0.06	0.68 ± 0.05
Trolox	81.23 ± 7.08	84.52 ± 6.47	0.32 ± 0.01	0.56 ± 0.03

Standard antioxidants (BHT; butylated hydroxytoluene, trolox).

[a] Values are expressed as percent radical scavenging activity.

[b] Values are expressed as absorbance. A higher absorbance value reflects a stronger capacity for metal ion reduc-tion

electron or hydrogen-transfer based assays. This observation is consistent with previous reports that flavonoids and hydroxycinnamic acids contribute substantially to DPPH activity[63, 64]. In the ABTS assay, the extract showed 42.99 ± 3.86% inhibition. This value is similar to that of BHT (46.97 ± 3.74%) but significantly lower than that of Trolox (84.52 ± 6.47%).

Since the ABTS test is particularly sensitive to hydrophilic antioxidants, the relatively lower activity of the extract compared to Trolox can be explained by the compositional balance between its hydrophilic and lipophilic constituents. While hydrophilic compounds are more reactive in aqueous radical environments such as $ABTS^{\cdot+}$, lipophilic antioxidants typically exhibit higher efficiency in lipid or hydrophobic phases. Therefore, the presence of both polar and non-polar phenolics in the extract may have reduced its overall effectiveness in the ABTS assay, despite the strong antioxidant activity demonstrated in DPPH, CUPRAC, and FRAP tests[33]. Nevertheless, this rate suggests that *E. ramosissimum* exhibits a moderate scavenging effect against various types of radicals[65]. In the FRAP test, which measures iron ion reducing capacity, the extract exhibited an absorbance value of 0.422 ± 0.03, indicating that the antioxidant properties may function to inhibit free radical formation through metal ions. This property associated with the electron-donating ability of phenolic hydroxyl groups and influenced by both the substitution pattern of flavonoids and the overall phenolic load. Compared to the values of Trolox (0.32 ± 0.01) and BHT (0.72 ± 0.06), the ferric reducing power of *E. ramosissimum* extract was observed to be higher than that of Trolox but lower than that of BHT. This result indicates that the extract contains phenolic compounds with strong reducing potential[37, 42]. The absorbance value of 0.662 ± 0.04 obtained in the CUPRAC assay revealed that the extract's capacity to reduce Cu^{2+} ions is quite close to that of BHT (0.68 ± 0.05) and higher than that of Trolox (0.56 ± 0.03). The CUPRAC method is sensitive to both hydrophilic and lipophilic antioxidants; in this context, the result obtained indicates that the extract is effective in both types of radical environments[38, 66]. Taken together, the complementary patterns across DPPH, CUPRAC and FRAP suggest that the extract contains a mixture of phenolics that effectively donate hydrogen atoms and electrons under the conditions of the different assays, producing high radical scavenging in DPPH and substantial reducing capacity in FRAP/CUPRAC[42, 66, 69].

When these findings are evaluated overall, the ethanol extract of *E. ramosissimum* showed high activity, especially in the DPPH and CUPRAC analyses. This situation can be explained by the high content of bioactive compounds such as flavonoids, phenolic acids, and tannins in the extract[67, 68]. Many studies have indicated that antioxidant activities are directly related to the concentration of phenolic compounds[8, 69]. The results obtained support the consideration of *E. ramosissimum* as a natural source of antioxidants.

Although the phenolic composition and antioxidant activities of *E. ramosissimum* have been previously studied, the present results provide stronger support and context by directly linking quantitative LC-MS/MS data with multiple antioxidant assays. While earlier works reported the general antioxidant potential of the plant, our findings demonstrate comparatively higher levels of key phenolics such as kaempferol and vanillic acid, which correlate with the strong radical scavenging and reducing power observed in DPPH, CUPRAC, and FRAP assays (Tables 4 and 5). Moreover, the integration of enzyme inhibition data with phenolic profiling allows a clearer interpretation of the extract's pharmacological potential, particularly its strong α-glucosidase inhibitory effect. This integrative approach underscores the superiority of our study, as it not only confirms previous reports but also advances current knowledge by providing a composition–activity relationship that had not been explicitly addressed before.

3.3 Cholinesterase and α-GLY inhibition assay

The inhibition of enzymes that play an important role in the pathophysiology of neurodegenerative and metabolic diseases has attracted great interest in recent years in terms of pharmaceutical research.

In particular, the biochemical roles of enzymes such as AChE, BChE, and α-GLY are crucial in the pathogenesis of metabolic diseases such as type 2 diabetes. In this context, the investigation of natural compounds capable of inhibiting these enzymes is important for the discovery of poten-

Table 6 Inhibition effect of *Equisetum ramosissimum* ethanol extract on AChE, BChE, and α-glucosidase.

Inhibitors	AChE IC_{50} (μg/mL)	R^2	BChE IC_{50} (μg/mL)	R^2	α-glucosidase IC_{50} (μg/mL)	R^2
E. ramosissimum	5.92	0.9713	5.82	0.9664	2.95	0.9939
Tacrine	3.19	0.9943	2.51	0.9915		
Acarbose					3.24	0.9944

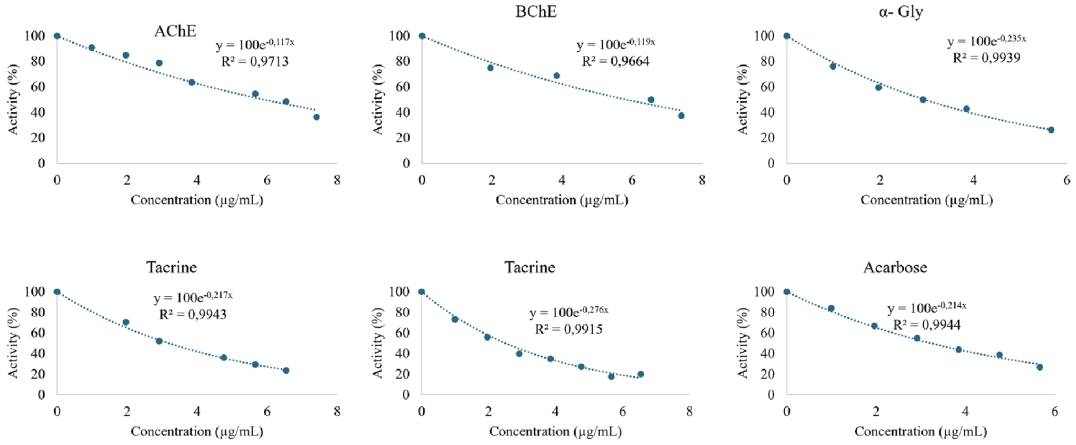

Fig. 1 %Activity-concentration graph for α-GLY, AChE and BChE enzymes of *E. ramosissimum* extract and standards.

tial pharmacological agents[4, 70, 71]. The inhibition potentials of *E. ramosissimum* ethanol extract against metabolic enzymes such as AChE, BChE, and α-GLY were evaluated using spectrophotometric analyses, and the findings obtained are presented in Table 6 and Fig. 1. The inhibition potential of the ethanolic extract of *Equisetum ramosissimum* on these enzymes was evaluated by spectrophotometric analysis based on the Ellman method[39].

As a result of the spectrophotometric analyses, the extract was found to exhibit significant inhibition against AChE, BChE, and α-glucosidase enzymes. The IC_{50} values were calculated as 5.92 μg/mL (R^2: 0.9713) for AChE, 5.82 μg/mL (R^2: 0.9664) for BChE, and 2.95 μg/mL (R^2: 0.9939) for α-GLY. These values indicate that the extract possesses a significant inhibitory potential on both cholinesterases and α-glucosidase. The positive control compound tacrine exhibited IC_{50} values of 3.19 μg/mL for AChE and 2.54 μg/mL for BChE, while the reference inhibitor acarbose used for comparison against α-GLY showed an IC_{50} value of 3.24 μg/mL.

These results reveal that the *E. ramosissimum* extract exhibits a stronger inhibitory effect on the α-GLY enzyme compared to acarbose.

In similar studies in literature, plant-derived phenolic compounds and flavonoids have been reported to exhibit significant inhibitory effects on cholinesterase and α-glucosidase enzymes[64, 72]. In particular, the enzyme inhibitory effects of phenolic compounds such as quercetin, kaempferol, caffeic acid, and phloridzin have been emphasized in numerous studies[73]. Considering the LC-MS/MS analysis results of the *E. ramosissimum* species given in Table 4, it is thought that the compounds detected in high amounts, such as phloridzin, fumaric acid, and kaempferol, contribute to enzyme inhibition[74]. These compounds, which structurally contain phenolic hydroxyl groups, can bind to the active sites of enzymes through hydrogen bonds or π–π interactions, thereby inhibiting enzyme activity[75, 76]. Obtaining cholinesterase inhibitors from natural sources that can help maintain acetylcholine levels in Alzheimer's disease is important in terms of having lower side effect profiles[72].

As a result, *E. ramosissimum* extract, with its strong inhibitory activity against both AChE and BChE as well as α-glucosidase enzymes, can be considered a potential neuroprotective and antidiabetic herbal agent.

These data were obtained with the contribution of the relevant phenolic components and should be supported by future in vivo and clinical studies.

4 Conclusion

This study comprehensively evaluated the antioxidant and enzyme inhibition activities as well as the phenolic compound content of the ethanol extract of *Equisetum ramosissimum*. Among the 20 phenolic standards analyzed by LC-MS/MS, 13 were positively identified, and the detection of compounds such as phloridzin dihydrate and

fumaric acid at high concentrations indicated the plant's therapeutic potential. The extract exhibited strong free radical scavenging properties in DPPH and ABTS assays, and high metal reducing capacity in CUPRAC and FRAP assays. These results revealed that the extract possesses remarkable antioxidant activity.

In addition, the inhibitory effects of the extract on AChE, BChE, and α-GLY enzymes were tested at various concentrations, and IC_{50} values were calculated; these values were found to be similar to or even stronger than some standard inhibitors. These findings suggest that the extract may have complementary therapeutic potential against metabolic disorders such as neurodegenerative diseases and diabetes.

When all the data obtained are evaluated together, it is understood that *E. ramosissimum* is a natural source containing multifunctional bioactive compounds. In this respect, it shows promise for the development of new pharmacological agents that can be used in the prevention or supportive treatment of oxidative stress-related diseases, nervous system disorders, and metabolic syndromes.

To confirm these in vitro findings, advanced in vivo and clinical studies are recommended.

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Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Emrah DİKİCİ: Methodology, conceptualization, investigation, project administration, writing - original draft, writing - review & editing.

Data Availability Statement

Data will be made available on request.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess25175

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