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https://doi.org/10.31925/farmacia.2026.3.16 **ORIGINAL ARTICLE**

EVALUATION OF ANTIOXIDANT, α-GLUCOSIDASE, AND ACETYLCHOLINESTERASE INHIBITORY ACTIVITIES OF EXTRACTS OF FIVE ETHNOBOTANICALLY VALUABLE PLANTS OBTAINED BY GREEN CHEMISTRY PRINCIPLES

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Abstract

Green chemistry, which has become popular in recent years and emphasizes its benefits to human health, is manifesting in every aspect of life. Products developed using green chemistry principles, especially those used in the health sector, are in greater demand. This study reports the research results of the antioxidant, α-glucosidase, and acetylcholinesterase inhibitor activities of ethanol extracts obtained according to green chemistry principles from the species *Chaerophyllum byzantinum, Echium vulgare, Euphrasia pectinata, Trifolium pratense*, and *Telekia speciosa*, collected from the plateaus of Giresun province in the Black Sea Region. The ethanol extracts of the collected species, obtained *via* Soxhlet extraction in accordance with green chemistry principles, were evaluated for antioxidant activity using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Subsequently, they were evaluated for α-glucosidase and acetylcholinesterase inhibitory activities. The DPPH radical scavenging activity of the *E. pectinata* species has been demonstrated for the first time in this study. The α-glucosidase and acetylcholinesterase activities of *E. pectinata, Chaerophyllum byzantinum*, and *Echium vulgare* species have been demonstrated for the first time in the literature with this study.

Rezumat

Acest studiu a investigat activitățile antioxidante, de inhibare a α-glucozidazei și a acetilcolinesterazei ale extractelor etanolice obținute conform principiilor chimiei verzi din speciile *Chaerophyllum byzantinum*, *Echium vulgare*, *Euphrasia pectinata*, *Trifolium pratense* și *Telekia speciosa*, colectate de pe platourile provinciei Giresun, situată în regiunea Mării Negre. Extractele etanolice ale speciilor colectate, obținute prin extracție Soxhlet în conformitate cu principiile chimiei verzi, au fost evaluate din punctul de vedere al activității antioxidante cu ajutorul testului cu DPPH. Ulterior, a fost evaluat și potențialul de inhibare a α-glucozidazei și a acetilcolinesterazei. Activitatea de captare a radicalului DPPH a speciei *E. pectinata* a fost demonstrată pentru prima dată în cadrul acestui studiu. Totodată, activitățile de inhibare a α-glucozidazei și a acetilcolinesterazei pentru speciile *E. pectinata*, *Chaerophyllum byzantinum* și *Echium vulgare* sunt raportate pentru prima dată în literatura de specialitate în acest studiu.

Keywords: green chemistry, medicinal plants, antioxidant, α-glucosidase, acetylcholinesterase

Introduction

Green Chemistry, established in the early 1990s, has led to advancements in eco-friendly processes, particularly in the development of environmentally friendly solvents. Research focuses on aqueous catalysis and supercritical fluids, essential for chemical reactions. The environmental impacts of ionic liquids and fluorous media depend on their health and ecological features, while the sustainability of bio-based solvents needs validation. Ongoing development of novel catalytic processes and innovative methods, such as microwave and ultrasonic synthesis, along with *in situ* spectroscopic techniques, is being effectively applied in research, reflecting the extensive exploration in green chemistry globally (1). The principles of green chemistry are essential for promoting sustainable practices in both laboratory and industrial settings. Key strategies include using safer, less toxic solvents, minimizing reagent consumption, and reducing waste generation. Additionally, energy efficiency is improved by conducting reactions under milder conditions and eliminating unnecessary derivatization. A critical component is the use of renewable raw materials to reduce reliance on non-renewable resources. Together, these

approaches foster the creation of environmentally friendly chemical processes that support sustainable development goals (2).

Plants have played a crucial role in human health, providing nourishment and medicinal benefits throughout history. Recent research into the phytochemistry of these plants has highlighted the effectiveness of traditional treatments, underscoring their long-standing use in addressing various ailments and infections (3, 4).

The genus *Chaerophyllum* (*Apiaceae*) includes 70 species, of which 4 are native to Türkiye. *Chaerophyllum byzantinum* is present in northern Türkiye and the southeastern Balkans, where it is cultivated for vegetable consumption. Known as Mendek in Giresun, it serves as a digestive aid in dishes. Other species are also used in culinary applications despite the presence of chaerophyllin, a toxin that can cause diarrhea and incoordination. Some *Chaerophyllum* species are noted for antibacterial, antioxidant, stimulant, and expectorant properties. Few reports detail their essential oils, with sabinene, p-cymen-8-ol, and terpinolene being the main components identified in *C. byzantinum*'s essential oil (5-9).

The genus *Echium* comprises about 68 species, mainly annual and biennial herbs native to the Mediterranean region. *Echium vulgare* L., known for its traditional medicinal uses, exhibits sedative, anti-inflammatory, antioxidant, and anxiolytic properties, used to treat conditions such as skin fissures and snakebites. Due to extensive colonization, *Echium* species are now found globally. The plant contains pyrrolizidine alkaloids and naphthoquinones, such as shikonins, which have demonstrated antibacterial, antifungal, and anticonvulsive effects. In Tuscany, *E. vulgare* leaves are used to treat animal dermatosis, and the plant is consumed in various forms, such as tea and tincture, to treat urinary tract issues, with caution advised due to possible hepatotoxicity from prolonged use (10-14).

The genus *Euphrasia* belongs to the *Orobanchaceae* family and includes around 219 species of hemiparasitic plants worldwide, with 10 species in Türkiye. *Euphrasia pectinata* Ten. is notably distributed and is used in Anatolian traditional medicine for wound healing. Key compounds identified in *E. pectinata* include iridoid glucosides like boschnaloside and 7-hydroxyboschnaloside, along with six additional iridoid glucosides and two phenylethanoid glycosides from previous studies (15-17).

Telekia speciosa (Heartleaf oxeye) is a perennial plant from Southeastern Europe and Asia Minor, belonging to the *Asteraceae* family. It is traditionally used in Balkan countries to treat bronchial asthma, notably by inhaling smoke from its underground parts. Phytochemical studies have revealed that it is a significant source of isoalantolactone, and extracts from its aerial parts contain several fatty acids and sterols, while its underground parts contain essential oil, bitter substances, and inulin (18-22).

The genus *Trifolium*, prevalent in temperate and subtropical regions, is particularly well established in the Mediterranean, particularly Türkiye, which is home to 104 species. *Trifolium pratense*, or red clover, is noted for its medicinal properties, containing flavones, flavonols, and estrogenic isoflavones like formononetin, biochanin A, daidzein, and genistein. It is widely recommended for alleviating menopausal symptoms and is used traditionally in Turkish medicine for various ailments, including as an expectorant and analgesic. Additionally, several *Trifolium* species are vital forage plants for livestock in the Mediterranean. Historically, red clover has been valued across various cultures, including by Native Americans, for the treatment of dermatological and respiratory conditions (23-26).

In this study, we aimed to evaluate the antioxidant, α-glucosidase, and acetylcholinesterase inhibitory activities of ethanol extracts from 5 species under green chemistry conditions.

Materials and Methods

Plant materials

In our study, we used *Chaerophyllum byzantinum* Boiss., *Echium vulgare* L., *Euphrasia pectinata* Ten., *Telekia speciosa* (Schreb.) Baumg., and *Trifolium pratense* L. collected from the highlands of Giresun province, located in the Black Sea region of Türkiye, and recorded in the Herbarium of the Faculty of Pharmacy at Istanbul University with the codes ISTE118747, ISTE118749, ISTE118737, ISTE118746, and ISTE118748, respectively, identified by Dr. Hüseyin Onur Tuncay.

Extraction

The aerial parts of the collected plants were dried in a cool, shaded place. The dried material was crushed and packaged in filter paper for Soxhlet extraction. We used a Soxhlet apparatus and ethanol as the solvent to do the extraction. We used 500 mL of ethanol to extract dried plant materials from *Chaerophyllum byzantinum* Boiss. (100 g), *Echium vulgare* L. (177.4 g), *Euphrasia pectinata* Ten. (167.5 g), *Telekia speciosa* (Schreb.) Baumg. (185.3 g), and *Trifolium pratense* L. (98.5 g) for 3 hours in separate batches. The ethanol extracts weighed 4.27 g, 6.77 g, 46.77 g, 16.64 g, and 11.63 g, corresponding to extraction yields of 4.27%, 3.82%, 27.9%, 8.98%, and 11.81%, respectively. The obtained extracts were stored at + 4°C.

Determination of total phenolic content (TPC)

We applied the Cao *et al*. (2020) 96-well microplate method with minor modifications (27). We diluted the Folin-Ciocâlteu reagent (FCR) with Milli-Q® water at a 1:3 ratio. We dissolved the plant extracts in methanol at 5 concentrations (0.5 - 5 mg/mL).

We established a calibration curve using six concentrations of gallic acid (0.04 - 0.2 mg/mL). In short, 5 µL of samples, 225 µL of Milli-Q® water, 5 µL of diluted FCR, and 15 µL of 2% sodium carbonate solution were added to each well. The microplate was then left at room temperature for 2 hours. After the incubation period, we measured absorbance at 760 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, CA, USA). We express the total phenolic content of the plant extracts as mg gallic acid equivalent (GAE)/g dry extract and gallic acid equivalent/g dry plant material. Wells containing 5 µL of methanol instead of a sample were used as blanks.

DPPH scavenging assay

We slightly modified the Fu *et al*. (2010) method (28). A 375 µM stock solution of DPPH free radical was prepared in methanol. Plant extracts were dissolved in DMSO (240 µg/mL), and DMSO served as a negative control. 10 µL of samples and 200 µL of DPPH solution were mixed in a 96-well microplate and incubated at 37°C for 30 min. We measured absorbance at 515 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, CA, USA) after incubation. We calculated the DPPH scavenging activity (%) using the following formula:

DPPH scavenging activity (%) = [1 - (Absorbance of sample at 450 nm/Absorbance of negative control at 450 nm)] * 100.

In vitro acetylcholinesterase inhibitory activity

Plant extracts (100 µg/mL) and galanthamine hydrobromide (positive control; 6 final concentrations between 500 and 7500 nM) were dissolved in DMSO. We performed the colorimetric assay using Ellman's method, with minor modifications (29). To sum up, 40 µL of assay buffer (Tris-HCl pH = 8.0), 20 µL of samples, and 20 µL of acetylcholinesterase (AChE) from electric eel (1 U/mL, dissolved in assay buffer) were mixed in a 96-well microplate. We incubated the microplate at 25°C for 10 minutes. After the incubation period, 20 µL of acetylthiocholine iodide (ATChI) solution (1.25 mM, dissolved in assay buffer) and 100 µL of 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) (0.33 mM, dissolved in a mixture of assay buffer, 1 M NaCl, and 0.2 M $MgCl_2*6H_2O$ solutions) were added to each well, and the absorbance was measured at 412 nm *via* a microplate reader (Multiskan GO, ThermoFisher, CA, USA) immediately. For the reagent blank and the sample blank, DMSO and extract were used, respectively. We used 20 µL of assay buffer instead of enzyme solution in the blank wells. We monitored absorbance changes for 40 minutes. We calculated the AChE inhibition (%) of plant extracts using the following equation:

AChE inhibition (%) = [1 - (Enzyme rate of sample/Enzyme rate of negative control)] * 100.

In vitro α-glucosidase inhibitory assay

Plant extracts (625 µg/mL) and acarbose (positive control, 6 final concentrations between 90 - 560 µM) were prepared in DMSO and Milli-Q® water, respectively. We performed the negative control using both DMSO and Milli-Q® water. We slightly modified the Bothon *et al*. (2013) method (30). In brief, 75 µL of assay buffer (0.1 M sodium phosphate buffer, pH = 6.8), 25 µL of samples, and 50 µL of α-glucosidase from Saccharomyces cerevisiae (500 U/L in assay buffer) were put into a 96-well microplate. DMSO and extracts were utilized for the reagent blank and the sample blank, respectively, and 50 µL of assay buffer was used instead of the enzyme solution. The plate was then heated to 37°C and left there for 10 minutes. After adding 50 µL of p-nitrophenyl-α-glucopyranoside (pNPG) to each well, the Multiskan GO (Thermo Fisher, CA, USA) was used to monitor absorbance changes at 405 nm for 45 minutes. We calculated the α-glucosidase inhibition (%) of plant extracts using the following equation:

α-glucosidase inhibition (%) = [1 - (Enzyme rate of sample/Enzyme rate of negative control)] * 100

Statistical analysis

One-way ANOVA followed by Tukey's multiple comparison test and Pearson's correlation test was performed using GraphPad Prism v10.2.3 software (GraphPad Software, Boston, Massachusetts, USA). Each experiment was performed in triplicate, with each replicate representing an independent biological replicate. Results were expressed as the mean ± SEM.

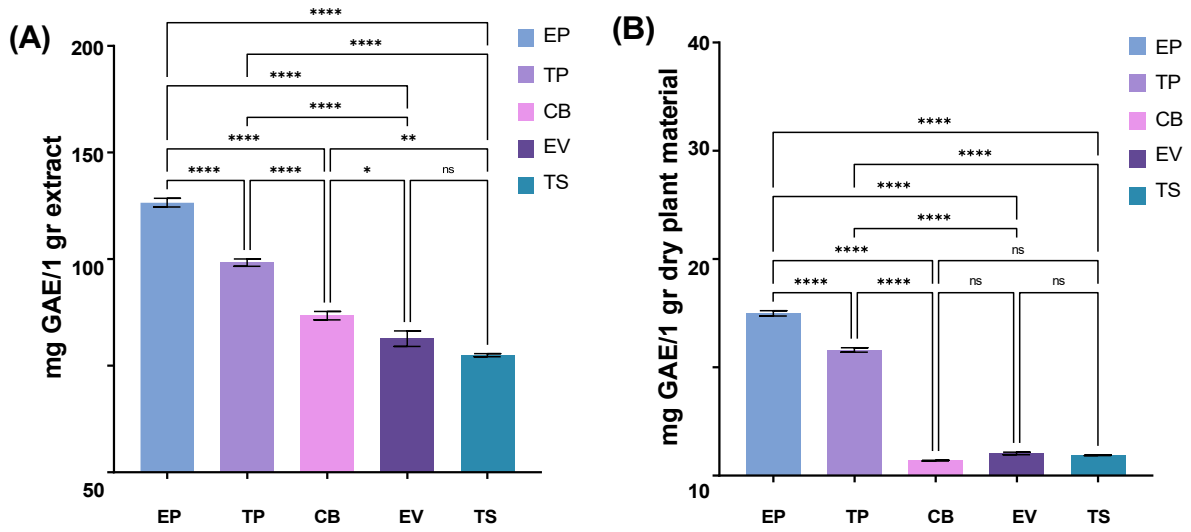
Results and Discussion

Total phenolic content

Post-hoc comparison results of total phenolic (mg GAE/g dry extract and mg GAE/g dry plant material) contents of ethanolic extracts are presented in Figure 1. The differences between the total phenolic content of plants and their respective extracts were significant ($p < 0.0001$) except for *C. byzantinum*, *E. vulgare*, and *T. speciosa*, according to Tukey's post-hoc pairwise comparisons (Figure 1).

Euphrasia pectinata Ten. ethanolic extract showed the highest phenolic content (126.45 ± 2.041 mg GAE/g dry extract and 14.97 ± 0.242 mg GAE/g dry plant material) among the tested extracts. The distribution of phenolic acids can vary among *Euphrasia* L. species. According to the Polish Pharmacopeia (2002), Gawenda-Kempczyńska *et al.* (2022) found out how much of 17 phenolic acids were present in three types of *Euphrasia*: *E. nemorosa*, *E. rostkoviana*, and *E. stricta*. They also measured the total phenolic content of these plants. The researchers reported that the total polyphenol content of the tested *Euphrasia* species ranged from 37.7 to 50.1 mg pyrogallol equivalent/g. It was also said that

phenolic acid, along with salicylic acid, protocatechuic acid, and ferulic acid, can be used to tell different *Euphrasia* species apart (16). Another study investigated eight phenolics in *E. pectinata* (leaves, flowers, and stems) during the vegetative, flowering, and fruiting periods. Mirovich *et al*. (2017) found that acteoside, a phenylethanoid glucoside, was most abundant during the vegetative and fruiting phase, while cynaroside was most abundant during the flowering phase (31). Benedec *et al*. (2024) examined two *Euphrasia* species from Romania: *E. stricta* and *E. officinalis* subsp. *pratensis*. They used a 70% ethanolic extract to study them and measured the total phenolic content, total flavonoid content, and caffeic acid derivatives (CAD) using spectrophotometry and LC-MS/MS. The study reported that *E. stricta* had 74.91 mg GAE/g, 10.81 mg RE/g, and 55.02 mg CAE/g of total polyphenols, total flavonoids, and CAD, while *E. officinalis* subsp. *pratense* had 92.10 mg GAE/g, 24.72 mg RE/g, and 45.08 mg CAE/g. The study also revealed that the two species' most abundant phenolics were chlorogenic acid and rutin (32).



EP: *Euphrasia pectinata*; TP: *Trifloium pratense*; CB: *Chaerophyllum byzantinum*; EV: *Echium vulgare*; TS: *Telekia speciosa*

EP: *Euphrasia pectinata*; TP: *Trifloium pratense*; CB: *Chaerophyllum byzantinum*; EV: *Echium vulgare*; TS: *Telekia speciosa*

Figure 1.

Total phenolic content of EtOH extracts, (A) mg gallic acid equivalent/1 g dry extract; (B) mg gallic acid equivalent/1 g dry plant material. Asterisks indicate statistically significant differences according to Tukey's post-hoc test (p value: > 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****))

Trifolium pratense L., which is known for its isoflavones, is also reported as a source of phenolics. We found the total phenolic content in our study to be 98.25 ± 1.738 mg GAE/g dry extract (or 11.60 ± 0.205 mg GAE/g dry plant material). Akbaribazm *et al*. (2020) conducted a study using an ethanolic extract of *T. pratense* leaves and stems. The study reported a total polyphenol content of 58.12 mg GAE/g in the dried plant. Akbaribazm *et al*. (2020) reported the discovery of 37 polyphenols in *T. pratense*, which included phenolic acids, flavones, flavonols, and isoflavones (33). Another study focused on microwave-assisted methanolic extract of *T. pratense* at three different vegetative stages (30 cm growth, 50 cm growth, and bud). The study showed that the total phenolic content was 41.96 mg GAE/g dry extract. It was found that all the extracts contained isoflavonoids (genistein and daidzein) and other phenols (p-hydroxybenzoic and caffeic acids, kaempferol 3-O-glucoside, quercetin 3-O-glucoside, and hyperoside). However, the earliest vegetative phase had the most phenolic compounds (34). Albulescu *et al*. (2024) looked at two types of extracts from the aerial parts of *T. pratense*: a heat-reflux water extract and a hydroethanolic (50% ethanol) extract, helped by ultrasound. The water and hydroethanolic extracts had a total phenolic content of 6.49 and 9.66 mg GAE/g, respectively. An HPLC-UV test showed that both extracts contained substantial levels of isoflavones, especially biochanin A and formononetin (35). A different study extracted six varieties of *T. pratense* (Tenia, Milena, Atlantis, Lucrum, Magellan, and Lemmon) using 50% ethanol in an ultrasonic bath. The Lemmon variety stood out among the tested extracts, with total phenolic content values of plant parts (leaves and flowers) ranging from 12.758 to 56.178 mg GAE/g dry plant material (36).

Along with papers on its essential oil components (*i.e*., monoterpene hydrocarbons, sesquiterpenoids), phenylpropanoids, polyacetylenes, phenolic acids,

and flavonoids have been reported for *Chaerophyllum* sp. (37, 38). While our study found the number of polyphenols in *C. byzantinum* Boiss. to be 73.34 ± 1.963 mg GAE/g dry extract, it yielded the lowest amount of phenolics *per* gram of dried material (1.39 ± 0.037 mg GAE/g dried plant material). In literature, the total phenolic content of *Chaerophyllum* sp. varies depending on extraction parameters and the plant part used. Koca *et al*. (2018) stated that the phenolic content of *C. byzantinum* leaves macerated in 80% methanol at + 4°C was 2890.2 mg/kg (39). In another study, it was reported that the hydroalcoholic (80% ethanol) extract of *C. byzantinum* leaves contained 23.2 mg GAE/g dry plant material (40). On the other hand, Çoruh *et al*. (2007) worked with aerial parts of *C. macropodum* and extracted with methanol at 50°C. It was reported that the obtained extract contained 34.0 mg GAE/g dry extract total phenolics (41). Petrović *et al.* (2017) studied the leaves and roots of *C. aromaticum*, which were extracted with methanol in an ultrasonic bath. Total polyphenols were reported as 57.69 mg GAE/mg fresh weight and 45.03 mg GAE/mg fresh weight for roots and leaves, respectively (38). Another study focused on Apiaceae plants, including *C. macrospermum*. According to LC-ESI-MS, *C. macrospermum* contains 33 phenolic compounds (phenolic acids, coumarins, and flavonoids), yielding a total phenolic content of 35.75 mg GAE/g (37).

The present work found the total phenolic content of *Echium vulgare* L. to be 62.53 ± 3.616 mg GAE/g dry extract (or 2.06 ± 0.119 mg GAE/g dry plant material). Similar values were reported by Nićiforović *et al*. (2010). It was stated that the methanol Soxhlet extract of *E. vulgare* (aerial parts) yielded 64 mg GAE/g dry extract (42). Another study focused on the aerial parts of *E. vulgare*, first defatting them with petroleum ether before extracting them with various solvents, including chloroform, ethyl acetate, acetone, and ethanol. It was reported that the total phenolic content of extracts ranged from 85.20 to 100.21 mg GAE/g dry extract, and ethanol extract had the highest total phenolic content (43). In addition to *E. vulgare*, *E. amoenum* was also assessed in terms of total phenolics. Abd Haghighi *et al*. (2024) reported that the 70% ethanol macerate of *E. amoenum* yielded 8.3754 mg GAE/g dry extract (44). Asghari *et al*. (2019) conducted a study on the flowers of *E. amoenum*, resulting in the production of various alcoholic and aqueous extracts, including Soxhlet and maceration extracts in methanol, as well as 70% methanol extracts by decoction and infusion. Asghari *et al*. (2019) noted that the decoction yielded the highest amount of phenolics (296.2 mg GAE/g dry extract), while the methanol maceration yielded the lowest amount at 90.2 mg GAE/g dry extract (45).

The *Telekia speciosa* (Schreb.) Baumg extract, a single-type *Telekia* plant (46), had the lowest amount of total phenolics (54.77 ± 0.718 mg GAE/g dry extract or 1.88 ± 0.025 mg GAE/g dry plant material) in our study. Literature shows that *T. speciosa* aerial parts and roots contain hydroxybenzoic and hydroxycinnamic acids, acylated hydroxycinnamic acid glucosides, caffeoylhexaric acids, and flavonoids (19, 47,48). The study extracted the flower heads (anthodium), aerial parts, and roots of *T. speciosa* using 80% methanol in an ultrasonic bath, followed by a total phenolic assay. Gevrenova *et al*. (2020) reported total phenolic contents of 31.40, 37.24, and 58.60 mg GAE/g dry extract for anthodium, aerial parts, and roots, respectively (47). Another study extracted the aerial parts and underground parts of *T. speciosa* using methanol at 50°C under reflux. Total phenolic content was reported as 63.02 mg GAE/g dry extract and 162.84 mg GAE/g dry extract for aerial parts and underground parts, respectively (49).

DPPH scavenging assay

Post-hoc comparison results of DPPH free radical scavenging activity of extracts (at 240 µg/mL) were presented in Figure 2. The difference between the DPPH free radical scavenging activity of the extracts was statistically significant ($p < 0.0001$) according to Tukey's post-hoc pairwise comparisons. However, the difference between the DPPH radical scavenging activities of *E. vulgare* and *T. speciosa* extracts was not significant (Figure 2).

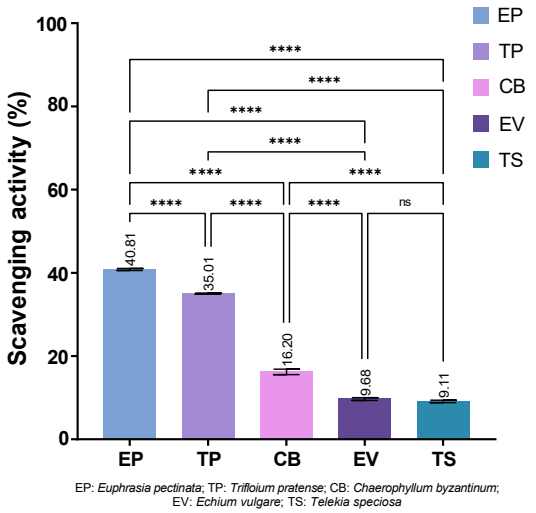


Figure 2.

DPPH scavenging activities (%) of EtOH extracts. Asterisks indicate statistically significant differences according to Tukey's post-hoc test (p value: > 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****))

As well as having a high total phenolic content, *E. pectinata* showed the highest DPPH scavenging activity (40.81 ± 0.249 %) among the tested samples. Literature shows that some of *E. pectinata*

constituents (*i.e.*, ixoroside, acteoside, and leucosceptoside A) have DPPH scavenging activities (17, 50, 51). For instance, the DPPH scavenging activity of ixoroside (an iridoid glycoside, which was reported for aerial parts of *E. pectinata* previously) was stated (Ixoroside IC50 = 21.65 ± 0.26 µg/mL, ascorbic acid IC50 = 3.11 ± 0.07 µg/mL) (51). Another study reported that acteoside (IC_{50} = 49 µM) and leucosceptoside A (IC_{50} = 76 µM) exhibited greater DPPH inhibitory activity than ascorbic acid (IC_{50} = 112 µM) (50). Researchers also reported DPPH scavenging activities in other *Euphrasia* species. In a study, conducted with the aerial parts of two *Euphrasia* species (*E. officinalis* subsp. pratensis and *E. stricta*), IC_{50} values of the 70% ethanolic extract (60°C, 30 min) against DPPH were reported as 50.93 ± 2.19 and 71.57 ± 3.42 µg/mL for *E. officinalis* subsp. *pratensis* and *E. stricta*, respectively (32). A different study assessed the 50% ethanol extract and infusion of *E. rostkoviana*. Teixeira and Silva (2013) reported that the IC_{50} values for the 50% ethanol extract and infusion were 0.087 and 0.048 mg/mL, respectively (52). Also, a full study on *E. officinalis* extracts was released, and the DPPH-scavenging abilities of these extracts were found (defatted plant material was extracted with dichloromethane, methanol in Soxhlet, and water at 75°C; the methanol extract was split up with diethyl ether, ethyl acetate, and butanol, and the rest of the watery phase was stored). It was reported that ethyl acetate showed the strongest activity (EC_{50} = 0.23 ± 0.01 mg extract/mg DPPH radical) and the highest phenolic content among the extracts (53).

In this study, the ethanolic extract of *Trifolium pratense* had slightly lower DPPH scavenging activity (35.01 ± 0.152%) than the extract of *Echium pectinata*. *Trifolium* sp. may exhibit different antioxidant activities depending on species or even variety. For example, *T. pratense* shows higher antioxidant activity than its total phenolic content (34-36, 54). Tundis *et al.* (2015) studied the ethanolic extracts of *T. pratense* and *T. repens* flowers. It was reported that the DPPH (IC_{50}) activity of the extracts was 34.0 ± 1.6 and 10.3 ± 1.2 µg/mL for *T. pratense* and *T. repens*, respectively (54). An ethanolic extract at 50% from six types of *T. pratense* plants (Tenia, Milena, Atlantis, Lucrum, Magellan, and Lemmon) was used in a separate study to test their ability to scavenge DPPH. It was reported that flowers of the Lemmon variety gave the strongest DPPH inhibitory activity (IC_{50} = 1.393 mg/mL) and the highest phenolic content, while leaves of the Magellan variety possessed the weakest DPPH activity (IC_{50} = 7.924 mg/mL) and the lowest phenolic content (36). In addition to botanical classification, plant growth stage can also affect antioxidant activity. Microwave-assisted methanolic extracts of *T. pratense*, which were collected at different growth stages (30 cm growth, 50 cm growth, and bud), were subjected to DPPH inhibitory activity. A 30 cm growth sample was found to have the most phenolics and the best antioxidant activity (IC_{50} = 22.98 ± 0.14 µg/mL against DPPH) (34). Another study focuses on aqueous and hydroalcoholic (50%) extracts of *T. pratense* aerial parts. Hydroalcoholic extract showed higher total polyphenolic content than water extract, as well as higher activity against DPPH (2.02 ± 0.05 and 4.61 ± 0.12 mg BHT/g extract for aqueous and hydroethanolic extracts, respectively) (35). It was reported that leaves and stems of *T. pratense* gave 149 ± 11.61 µmol eq. Trolox/10 g dried plant against DPPH free radical (33).

In the present work, *C. byzantinum* ethanolic extract exhibited relatively low radical scavenging activity (16.20 ± 0.667%) compared to *E. pectinata*. Limited studies on the DPPH activity of *C. byzantinum* have shown that extraction temperature affects antioxidant activity and phenolic content. In a study, an 80% ethanolic Soxhlet extract of *C. byzantinum* leaves gave 23.2 ± 1.1 mg pyrocatechol/g dried materials and showed 40 - 60% inhibition at 85 µg/mL (40). Another study found that *C. byzantinum* leaves extracted with 80% methanol at 4°C had a total phenolic content of 2890.2 ± 884.3 mg/kg and DPPH scavenging activity with an EC_{50} value of 1.00 ± 0.57 mg/mL (39). Molo *et al*. (2021) performed isolation studies on different extracts (hexane, chloroform, acetone, and methanol) of *C. bulbosum* aerial parts and tested the DPPH-scavenging activity of nine isolated compounds. It was reported that quercetin-3-O-β-D-glucopyranoside gave the strongest activity with a 6.94 ± 0.29 µg/mL IC_{50} value (55). Studies also evaluated methanol extracts of other *Chaerophyllum* sp. It was found that the edible parts of *C. macropodum* had an IC_{50} value of 0.623 mg/mL, and the aerial parts had an IC_{50} value of 54.22 ± 0.64 mg Trolox equivalent/g extract (37, 41). Petrović *et al.* (2017) studied the roots and aerial parts of *C. aromaticum* and obtained essential oils and methanol macerates. It was stated that the DPPH activity of essential oils remained relatively low in methanol extracts, and the methanolic extract of *C. aromaticum* roots had the highest DPPH activity and total phenolic yield (2.31 ± 0.08 µg TE/mg fresh weight and 57.69 ± 0.64 µg GAE/mg fresh weight, respectively) (38).

Ethanolic extract of *Echium vulgare* displayed low DPPH scavenging activity (9.68 ± 0.333%) in this study, which is also relatively low compared to the literature data (42, 43). Methanol Soxhlet extracts of *E. vulgare* and *E. rubrum* aerial parts were investigated in terms of DPPH scavenging activity. It was noted that IC_{50} values were 290 ± 18 µg/mL for *E. rubrum* and 243 ± 14 µg/mL for *E. vulgare* (42). Root extracts of *E. vulgare* (petroleum ether, ethyl acetate, chloroform, acetone, and ethanol Soxhlet

extracts) were also examined against the DPPH radical. It was reported that extracts showed DPPH scavenging activity with a 70.01 - 78.76 µg/mL IC_{50} value (43). In their 2020 study, Al-Rimawi *et al*. used leaves of *E. angustifolium* and extracted chemicals with hexane, acetone, methanol, and water in an incubator shaking at 400 rpm for 72 hours. It was reported that methanol extract showed comparable activity with Trolox (methanol extract IC_{50} = 1.95 ± 01.26 µg/mL, Trolox IC_{50} = 1.88 ± 1.57 µg/mL) (56). Another study assessed the DPPH scavenging activity of *E. amoenum* flowers extracted using various solvents, including crude methanol extracts and 70% methanol extracts obtained *via* the Soxhlet and maceration methods, as well as water decoctions and infusions. The decoction was found to have the most total phenolics and the strongest DPPH scavenging activity (IC_{50} = 22.8 ± 3.7 µg/mL) (45). In addition to flowers, the roots of *E. amoenum* have been assessed for radical scavenging activity. It was reported that the 70% ethanolic macerate yielded an IC_{50} value of 279.867 µg/mL against the DPPH free radical (44).

Like *Echium vulgare*, *Telekia speciosa* also showed minimal activity (9.11 ± 0.282) against the DPPH radical. As far as we know, there is only one report on the radical-scavenging activity of this taxon (47). The anthodium, aerial parts, and roots of *T. speciosa* were extracted with 80% methanol in an ultrasonic bath. The extracts demonstrated DPPH scavenging activity with 77.50, 98.76, and 104.14 mg Trolox equivalent/g for anthodium, aerial parts, and roots, respectively (47).

In vitro acetylcholinesterase inhibitory activity

Figure 3 presents the post-hoc comparison results of acetylcholinesterase inhibitory activities of EtOH extracts. According to results, extracts of *E. pectinata* and *T. speciosa* showed the highest inhibitory activity among tested extracts (22.98 ± 0.236% and 21.01 ± 0.257%, respectively), but Tukey's multiple comparison test revealed that the difference between them was statistically significant (p = 0.0002). On the other hand, *T. pratense* exhibited slightly lower activity (19.96 ± 0.159%) than *T. speciosa*, but it was also found statistically different (p = 0.0332). All of the other comparisons were significantly different (p < 0.0001) (Figure 3).

Ixoroside is an iridoid glycoside that has been shown to block AChE (IC_{50} values are 19.06 ± 0.51 µg/mL for ixoroside and 38.47 ± 0.001 µg/mL for galanthamine). It was also discovered in *E. pectinata* (17, 51). There are a limited number of studies on the AChE-inhibitory activities of *Euphrasia* sp. extracts. It was reported that the infusion and the 50% ethanolic extract of *E. rostkoviana* leaves exhibited inhibitory activity against AChE, with IC_{25} values of 0.759 and 0.978 mg/mL, respectively (52). Another report focuses on different extracts of *E. officinalis* aerial parts. According to Ververis *et al*. (2024), the most active extract was butanol, which was separated from the methanol Soxhlet extract through partition. It had an IC50 value of 646.93 ± 33.03 µg/mL (53).

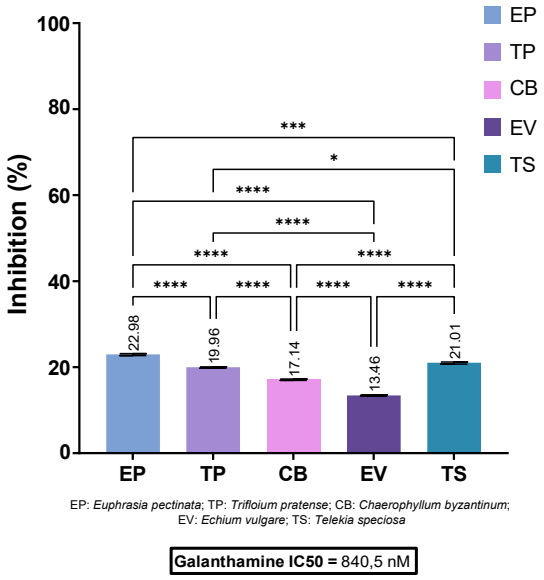


Figure 3.

AChE inhibitory activities of EtOH extracts (at 100 µg/mL). Asterisks indicate statistically significant differences according to Tukey's post-hoc test (p value: > 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****))

A study subjected ultrasound-assisted 80% methanol extracts of different parts of *Telekia speciosa* to an AChE inhibitory assay. The study reported that the AChE inhibitory activity of the extracts was 4.47, 4.82, and 4.71 mg galanthamine equivalent/g for the anthodium, aerial parts, and roots, respectively. Researchers also noted that *T. speciosa* extracts were rich in eudesmanolides and esters of caffeic/ferulic acid hexosides (47). Seo *et al*. (2017) and Gevrenova *et al*. (2020) reported that *T. speciosa* also exhibited dose-dependent inhibition of AChE in the brain tissues of cognitively impaired mice (47, 57).

In our study, *T. pratense* ethanolic extract inhibited AChE enzyme by 19.96 ± 0.159%. The literature demonstrates the activity of *T. pratense*'s isoflavones (genistein, daidzein, biochanin A, formononetin, and pratensein) on *in vivo* models (58-60). The TLC bioautography assay reported the AChE inhibitory activity of caffeoylmalic acid, in addition to isoflavones (genistein, daidzein, formononetin, and their glycosides; biochanin A glycoside) in ultrasound-assisted 50% ethanol samples of *T. pratense* and *T. medium* (61). Extracts of *Trifolium* sp. were also evaluated in terms of AChE inhibitory activity. A study evaluated the AChE inhibitory activity of different extracts (hexane, chloroform, ethanol, methanol, and water) of *T. repens*. It was reported that chloroform

extract gave the highest inhibitory activity against AChE (IC_{50} = 21 µg/mL) (62). Another study tested crude extracts (sequentially macerating with petroleum ether, acetone, methanol, and water) of *T. angustifolium* var. *angustifolium* as AChE inhibitors. It was reported that the water extract exhibited the highest AChE inhibitory activity at 200 µg/mL (89.06% inhibition) (63).

Chaerophyllum byzantinum inhibited 17.14 ± 0.159% AChE in our study. Other *Chaerophyllum* species have been reported to exhibit AChE inhibitory activity in the literature. It was found that a methanol extract of the aerial parts of *C. macrospermum* could stop AChE activity (4.53 ± 0.15 mg galanthamine equivalent *per* g extract) (37). Another study tested extracts of *C. bulbosum* (hexane, chloroform, acetone, methanol, and hot water) and isolated nine compounds against AChE. Molo *et al*. (2021) said that hexane extract and its compound n-octacosane could stop AChE from working (hexane extract IC_{50} = 10.71 ± 0.10 µg/mL, n-octacosane IC_{50} = 73.73 ± 0.65 µg/mL) (55). Petrović *et al.* (2017) worked with essential oils and methanol extracts from the roots and aerial parts of *C. aromaticum*. It was stated that both essential oils exhibited similar inhibitory effects on AChE (47.65 - 50.88% inhibition); on the contrary, the aerial parts methanol extract inhibited AChE, while the roots methanol extract activated AChE (36% inhibition and 8.40% activation, respectively) (38).

Echium vulgare ethanolic extract exhibited the lowest AChE inhibitory (13.46 ± 0.165%) activity among the tested extracts. Asghari *et al*. (2019) evaluated the water and alcoholic extracts of *E. amoenum* flowers (crude extracts with methanol and 70% methanol obtained *via* the Soxhlet and maceration methods; decoctions and infusions with water) and found that they could inhibit AChE. It was reported that decoction and infusion extracts gave the highest AChE inhibition compared to alcoholic and hydroalcoholic extracts (0.76 and 0.90 mg galantamine equivalent/g extract, respectively) (45). Researchers conducted another study on the whole plant of *E. confusum*, macerating it with ethanol for 72 hours at room temperature and then fractionating it to extract pyrrolizidine alkaloids. Then, crude extracts, partitioned fractions, and isolated pyrrolizidine alkaloids were tested for AChE inhibitory activity. It was reported that crude extract (IC_{50} = 268.33 ± 2.15 µg/mL), fraction C, and fraction D (IC_{50} = 176.86 ± 6.38 µg/mL and 206.13 ± 2.86 µg/mL, respectively) were found to be active against AChE. 7-O-angeloyllycopsamine N-oxide was found to be the most active pyrrolizidine alkaloid from the fractions (IC_{50} = 0.276 ± 0.018 mM) (64).

In vitro α-*glucosidase inhibitory activity*

Post-hoc comparison results of *in vitro* α-glucosidase inhibitory activity of ethanolic extracts at 625 µg/mL were presented in Figure 4. All of the extracts other than *E. pectinata* and *T. pratense* showed statistically significant differences ($p < 0.0001$) in their activity.

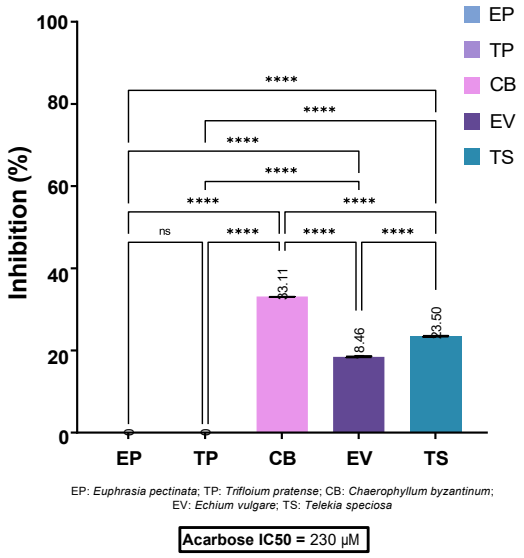


Figure 4.

α -glucosidase inhibitory activity of ethanolic extracts at 625 µg/mL. Asterisks indicate statistically significant differences according to Tukey's post-hoc test (p value: > 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****))

Euphrasia pectinata and *Trifolium pratense* didn't show inhibitory activity on the α-glucosidase enzyme. Some natural compounds from *E. pectinata*, like ixoroside and leucosceptoside A, have been shown to be effective against α-glucosidase. It was reported that the IC_{50} value of ixoroside was 4.11 ± 0.36 µg/mL (Acarbose IC_{50} = 13.50 ± 0.04 µg/mL), and for leucosceptoside A, two IC_{50} values were stated as 0.7 mM (Acarbose IC_{50} = 14.4 mM) and 273.0 µM (Acarbose IC_{50} = 204.2 µM) (17, 51, 65). Researchers found no activity in the extracts, despite promising reports of high activity of isolated compounds. Teixeira and Silva (2013) studied with 50% ethanolic extract and infusions of *E. rostkoviana* leaves, and they stated that the tested extracts were found to be inactive against α-glucosidase (52).

T. pratense isoflavonoids, pterocarpans, and polysaccharides were mentioned with their α-glucosidase inhibitory activities. In 2016, Wang *et al*. used a 60% ethanolic extract of *T. pratense* (defatted with petroleum ether) to screen for possible α-glucosidase inhibitors using ultrafiltration liquid chromatography (66). Another study involved extracting *T. pratense* with deionized water at 90°C for 120 min, followed by an isolation study to obtain polysaccharides. It was mentioned that *T. pratense* polysaccharides (10 mg/mL) showed α-glucosidase inhibitory activity with 86.72% of the activity of acarbose (67). It was reported that three isoflavonoids (genistein, formononetin, and

irilone) and one pterocarpan (maackiain) exhibited inhibitory activity against α-glucosidase (66). Gościniak *et al.* (2023) studied six varieties of *T. pratense* (Tenia, Milena, Atlantis, Lucrum, Magellan, and Lemmon) and tested the ultrasound-assisted 50% ethanolic extracts of leaves and flowers against α-glucosidase. It was reported that IC_{50} values were 4.481 - 24.935 mg/mL for leaves and 2.044 - 17.006 mg/mL for flowers. Despite their higher isoflavone content, leaves showed lower activity than flowers (36). In another study, ethanolic extracts of *T. repens* and *T. pratense* were tested in terms of α- glucosidase inhibition; IC_{50} values were given as 69.5 ± 3.1 and 70.8 ± 3.9 µg/mL, respectively (Acarbose IC_{50} = 35.5 ± 1.2 µg/mL) (54).

Chaerophyllum byzantinum ethanol extract showed the highest α-glucosidase inhibitory activity (33.11 ± 0.059%) in this study. A limited number of studies reported on α-glucosidase activity of *Chaerophyllum* sp. extracts and isolated compounds. A study used α-glucosidase to test different extracts (hexane, chloroform, acetone, methanol, and hot water) of *C. bulbosum* aerial parts, as well as 9 isolated compounds. It was reported that only hexane extract (IC_{50} = 58.63 ± 0.79 µg/mL) and luteolin-7-O-β-D-glucopyranoside (IC_{50} = 352.63 ± 0.23 µg/mL) showed notable inhibitory activity against α-glucosidase (acarbose IC_{50} = 378.66 ± 0.14 µg/mL) (55). Another study was conducted with the aerial parts of *C. macrospermum*. It was stated that the methanol extract of *C. macrospermum* exhibited 1.93 ± 0.26 mmol acarbose equivalent/g extract (37).

Echium vulgare ethanol extract also exhibited α-glucosidase inhibitory activity (18.46 ± 0.140%) in the present work. In literature, the α-glucosidase inhibitory activity of *Echium* sp. extracts varies. Asghari *et al.* (2019) obtained various alcoholic and aqueous extracts (Soxhlet and maceration extracts of methanol and 70% methanol; decoction and infusion) from *E. amoenum* flowers and tested them against α-glucosidase. It was reported that decoction (IC_{50} = 16.9 ± 0.7 µg/mL) and 70% methanol Soxhlet extract (IC_{50} = 18.1 ± 2.9 µg/mL) showed comparable activity with acarbose (IC_{50} = 19.7 ± 3.8 µg/mL) (45). In another study, hexane, acetone, methanol, and water extracts of *E. angustifolium* leaves were tested for α-glucosidase inhibitory activity. It was stated that all extracts displayed lower activity than acarbose (IC_{50} = 37.15 ± 0.33 µg/mL), and the acetone extract (IC_{50} = 247.52 ± 2.58 µg/mL) was the most active one (56).

The ethanol extract of *Telekia speciosa* showed moderate inhibition (23.50 ± 0.152%) against α-glucosidase. There is one paper on the α-glucosidase inhibitory activity of *T. speciosa*, which reported that ultrasound-assisted 80% methanol extracts of the anthodium, aerial parts, and roots of *T. speciosa* displayed inhibitory activity against α-glucosidase of 0.42 ± 0.12, 0.41 ± 0.01, and 1.27 ± 0.01 mmol acarbose equivalent/g, respectively. Researchers have reported acylquinic acids (chlorogenic, caffeoylferuloylquinic acids, etc.), sesquiterpenes (helenin, a synonym for alantolactone), and flavonoids (luteolin, hispidulin, etc.) as valuable inhibitors of key enzymes in carbohydrate digestion (47, 68).

Conclusions

In the present work, AChE inhibitory and α-glucosidase inhibitory activities of *Euphrasia pectinata*, *Chaerophyllum byzantinum*, and *Echium vulgare*, in addition to DPPH scavenging activity of *E. pectinata* have been reported for the first time. According to Pearson's test, there is a significant correlation between TPC and DPPH activity (r = 0.9740). However, no correlation was found between TPC and enzyme-inhibitory activities (AChE and α-glucosidase). Our findings showed that DPPH activity was strongly related to total phenolic content, which is also consistent with the literature (17, 34, 36, 38, 53). Even though some phenolic compounds contribute to enzyme inhibitory activities (55, 58-61, 65, 66, 68), non-polar components of the tested plants (*i.e.*, volatile essential oil terpenes, iridoids, pyrrolizidine alkaloids, and sesquiterpene lactones) also may play a role in AChE inhibitory and α-glucosidase inhibitory activity, according to our results, which are supported by literature (38, 48, 51, 55, 57, 64).

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Conflict of interest

The authors declare no conflict of interest.

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