



OPEN Comprehensive phytochemical, anatomical and biological evaluation of *Ziziphora clinopodioides* Lam. (Lamiaceae), used as a traditional tea from Türkiye

Ömer Çeçen

This study provides a comprehensive investigation of *Ziziphora clinopodioides* Lam. (Lamiaceae) from Türkiye, focusing on its phytochemical composition, biological activities, and anatomical characteristics relevant to its potential as a functional food ingredient. Methanol and aqueous extractions yielded 28.09% and 30.91%, respectively, calculated based on the dry weight of the plant material. Essential oil analysis identified 98.3% of the total composition, with oxygenated monoterpenes, particularly 1,8-cineole (28.2%), terpinen-4-ol (12.0%), and pulegone (7.9%), as dominant constituents. Enzyme inhibition assays revealed α -amylase inhibition of 29.3% by the essential oil, calculated based on the tested sample concentration, and moderate cholinesterase inhibition by the methanolic extract; however, all activities were lower than those of the reference drugs. Morphological analysis confirmed the plant as a mat-forming perennial with dense hirsuteness and capitate inflorescences. Leaf anatomy showed amphistomatic, bifacial structure with diverse trichome types and a well-organized vascular system. The stem anatomy exhibited a quadrangular structure with varied glandular and non-glandular trichomes, developed collenchyma, and a distinct endodermis. The findings of this research could shed light on the medicinal value of *Z. clinopodioides*, and open up new possibilities for its use in various food products or therapeutic applications.

Keywords Antioxidant activity, Enzyme inhibition, Essential oil composition, Leaf and stem anatomy, *Ziziphora clinopodioides*

The Lamiaceae family is a large group of flowering plants that includes many plant genera, one of which is *Ziziphora* L. This genus is found in regions of Afghanistan, Anatolia, Armenia, Caucasia, Iraq, Middle Asia, Pakistan, Syria, Türkiye, Turkmenistan, and West Siberia. It has 17 species in World, 6 species in Türkiye^{1–3}. *Z. clinopodioides* Lam. is an edible medicinal plant widely distributed in Anatolia, with its leaves, flowers, and stem frequently used as a wild vegetable or food additive for flavor and aroma in Türkiye. The plant, locally known as "Kirnanesi," is commonly used in the preparation of aromatic and traditional teas for its properties related to gastrointestinal disorders, appetite stimulation, gas relief, antiseptic effects, and wound healing. The plant, known locally as "Kirnanesi," is commonly used in the preparation of aromatic tea for gastrointestinal disorders and as an aperitif, carminative, antiseptic, and wound-healing agent. Additionally, in the eastern part of Türkiye, it is added to a special cheese known as "herby cheese"⁴. Infusion of aerial parts of the plant used by the people for the treatment of carminative, colds and flu, orexigenic, stomachache in Türkiye⁵. Four species of *Ziziphora* are to Iran, including *Z. capitata*, *Z. clinopodioides*, *Z. persica*, and *Z. tenuior*. *Ziziphora* extracts have also been found to be potential agents for wound healing and edema treatment. *Z. clinopodioides* and *Z. tenuior* frequently prescribed in different countries to treat cold, bronchitis, cough, headache, dysentery, diarrhea, nausea, typhus, and cardiovascular diseases in folkloric medicine. These species have also been recognized as effective remedies against inflammation, arrhythmia, insomnia, and recommended as sedatives, anti-spasmodics, antiseptics,

Kamil Özdağ Faculty of Science, Department of Biology, Karamanoğlu Mehmetbey University, Karaman 70100, Türkiye. email: omercecen@kmu.edu.tr

analgesics, carminatives, aphrodisiacs, and flavoring agents⁶. It has traditionally been used to relieve digestive disorders such as diarrhea, bloating, and nausea, and is also known for its appetitive, antiseptic, sedative, and wound-healing properties. In addition to its medicinal uses, it is widely employed to enhance the flavor, aroma, and appearance of meat and traditional meat products. Recent studies highlight its potential as a natural antioxidant and antimicrobial agent, effectively extending the shelf life of raw foods during refrigerated storage without affecting sensory quality⁷. *Z. clinopodioides* has been found to contain menthol, (+)-pulegone, 1,8-cineole, and limonene as its major essential oil components. Additionally, the plant can produce phenolic compounds like caffeic acid and flavonoid derivatives such as luteolin, 7-methylsudachitin, and thymonin. The essential oils of *Z. clinopodioides* have exhibited antimicrobial, antifungal, antioxidant, and sedative activities in previous pharmacological investigations^{8–10}.

This study seeks to explore the potential therapeutic benefits of *Z. clinopodioides* by evaluating the antidiabetic and anticholinesterase properties of its methanolic and aqueous extracts, as well as essential oils extracted from its flowering aerial parts. In addition to this, the study analyzes the chemical composition of the extracted essential oils, and investigates the morphological and anatomical characteristics of the plant. The findings of this research could shed light on the medicinal value of *Z. clinopodioides* and open up new possibilities for its use in various therapeutic applications.

Results and discussion

Extraction

The extraction yields were determined to be 28.09% for the methanol extract and 30.91% for the aqueous extract, indicating a slightly higher recovery of soluble compounds in the aqueous medium compared to methanol.

Methanol and water are widely used as solvents in chemical analysis and traditional decoction, respectively. They are the most common choices for extracting bioactive compounds from herbal medicines, with methanol frequently used in LC analysis and water preferred for traditional preparations. A previous study reported that the water extract exhibited stronger antioxidant effects compared to ethanol extracts¹¹.

The slightly higher extraction yield in the aqueous extract (30.91%) compared to the methanol extract (28.09%) suggests that water may be more effective in extracting polar and water-soluble compounds. This difference could be attributed to the better solubility of certain bioactive components in water or the ability of water to penetrate the sample matrix more effectively. However, methanol's efficiency in extracting a broad range of polar and non-polar compounds should not be overlooked. Further analysis of the extracts' chemical profiles could provide deeper insights into the specific compounds responsible for the yield variation.

Essential oils

The analysis of the essential oil composition revealed a total of 98.3% identified compounds. The predominant class was oxygenated monoterpenes, accounting for 67.9% of the total composition. The most abundant compound in this group was 1,8-cineole (28.2%), followed by terpinen-4-ol (12.0%), pulegone (7.9%), and isomenthone (5.8%). Monoterpene hydrocarbons represented 21.5% of the total oil content, with β -pinene (5.1%), limonene (4.9%), and γ -terpinene (3.6%) as the major constituents. Sesquiterpene hydrocarbons made up 5.5%, with germacrene D (2.8%) and bicylogermacrene (0.9%) as the key components. Meanwhile, oxygenated sesquiterpenes constituted 2.8%, including caryophyllene oxide (1.6%) and spathulenol (1.2%). Other minor components, classified under "Others," accounted for 0.6% of the total composition. Overall, the oil exhibited a high proportion of oxygenated monoterpenes, particularly 1,8-cineole, terpinen-4-ol, and pulegone, which are known for their biological and pharmacological properties (Fig. 1) (Table 1).

Ziziphora species are traditionally used in Iran for their medicinal properties. This study analyzed the chemical composition and antibacterial effects of *Z. clinopodioides* essential oil. GC–MS results identified carvacrol (64.2%), thymol (19.2%), p-cymene (4.8%), and γ -terpinene (4.6%) as the dominant compounds¹².

The essential oil of *Z. clinopodioides* from Iran was obtained via hydrodistillation and analyzed by GC–MS, identifying 27 compounds. The major constituents were pulegone (44.5%), terpineol (14.5%), methyl acetate (10.9%), iso-neomenthol (7.1%), and 1,8-cineole (4.1%)¹³.

The essential oil composition of *Z. clinopodioides* was analyzed using capillary GC and GC/MS. A total of 44 compounds were detected, with 35 identified, accounting for 96.62% of the oil. Among these, 24 compounds were reported for the first time. The dominant components were 1,8-cineole (14.05%) and pulegone (21.92%)¹⁴.

The essential oil composition of *Ziziphora clinopodioides* from current study showed a high proportion of oxygenated monoterpenes (67.9%), with 1,8-cineole, terpinen-4-ol, and pulegone as the predominant compounds. In comparison, previous studies identified different key components: Shahbazi¹² reported carvacrol (64.2%) and thymol (19.2%) as the major compounds, while Behravan et al.¹³ found pulegone (44.5%) and terpineol (14.5%) as the main constituents in the Iranian sample. Baser et al.¹⁴ highlighted 1,8-cineole (14.05%) and pulegone (21.92%) as dominant compounds in their analysis. These findings underline the variability in essential oil profiles of *Ziziphora clinopodioides* depending on geographic origin, with consistent presence of 1,8-cineole and pulegone.

Enzyme inhibition assays

The antidiabetic and anticholinesterase potential of essential oil and various extracts of *Ziziphora clinopodioides* were evaluated in vitro (Table 2). The aqueous extract demonstrated moderate α -glucosidase inhibition activity at $8.77 \pm 0.11\%$, whereas this activity was not determined for the essential oil and methanol extract. In terms of α -amylase inhibition, the essential oil exhibited the highest activity among the plant samples, with 29.30%, followed by the methanol (20.89%) and aqueous (11.92%) extracts. Compared to the standard antidiabetic agent acarbose, which inhibited α -glucosidase and α -amylase by 74.72% and 67.87%, respectively, the plant samples showed lower inhibitory potential.

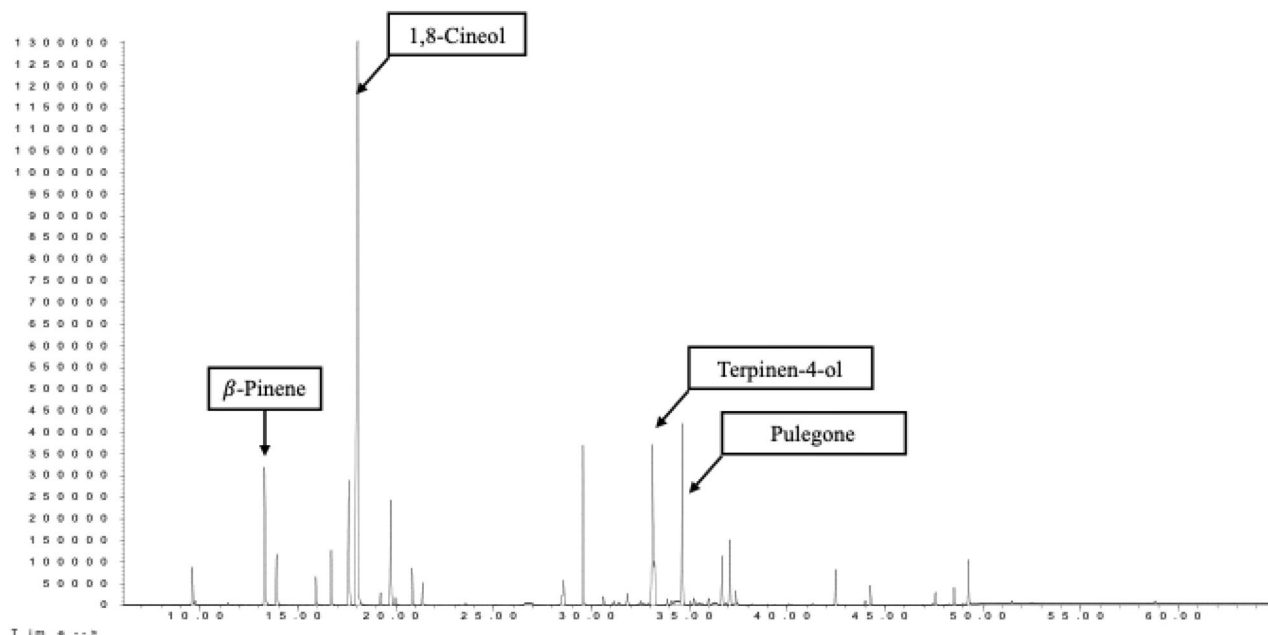


Fig. 1. GC-MS chromatograms of *Ziziphora clinopodioides*.

Regarding cholinesterase inhibition, the methanolic extract exhibited the highest acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition at 12.35% and 21.82%, respectively. The essential oil and aqueous extract showed lower AChE inhibition (5.94% and 9.18%, respectively) and comparable BChE inhibition (10.66% and 10.03%, respectively). In contrast, the reference drug donepezil exhibited nearly complete inhibition of AChE and BChE at 100% and 98.61%, respectively.

A study investigated the antidiabetic potential of phytochemicals isolated from *Ziziphora clinopodioides* subsp. *bungeana*, focusing on α -glucosidase inhibition and GLUT4 translocation. A total of 20 compounds were isolated, including a unique monoterpene diperoxy dimer and several triterpenoids. Among the tested compounds, ursolic acid (IC_{50} = 80.9 μ M), oleanolic acid (IC_{50} = 135.3 μ M), betulinic acid (IC_{50} = 45.3 μ M), and 11-oxoursolic acid (IC_{50} = 162.7 μ M) exhibited stronger α -glucosidase inhibitory activity than the standard inhibitor acarbose (IC_{50} = 264.7 μ M). Notably, betulinic acid was the most active compound, demonstrating nearly six-fold greater potency than acarbose. In addition to enzyme inhibition, the study assessed GLUT4 translocation in C2C12 myotubes. Pomolic acid was the only compound that significantly enhanced GLUT4 translocation ($p < 0.05$), suggesting insulin-mimetic behavior¹⁵.

Another study evaluated the α -amylase inhibitory potential of ethyl acetate, methanolic, and aqueous extracts of *Ziziphora taurica* subsp. *cleonoides*. Among the tested samples, the ethyl acetate extract exhibited the strongest α -amylase inhibitory activity with an IC_{50} value of 1.95 mg/mL, which was closest to the positive control acarbose (IC_{50} = 1.21 mg/mL). The methanol extract showed moderate activity (IC_{50} = 3.97 mg/mL), while the aqueous extract was the least active (IC_{50} = 36.99 mg/mL), indicating relatively weak inhibition. A strong correlation was observed between α -amylase inhibition and total phenolic content ($r = 0.931$), suggesting phenolic compounds may be primarily responsible for the observed enzyme inhibitory effects¹⁶.

The α -amylase inhibitory activity of *Ziziphora taurica* subsp. *taurica* was evaluated using ethyl acetate, methanol, and water extracts. The ethyl acetate extract (ZTT-EtOAc) exhibited the strongest inhibitory effect with an IC_{50} value of 1.82 mg/mL, followed by the methanol extract (ZTT-MeOH) at 2.69 mg/mL. The water extract (ZTT-W), however, showed significantly weaker inhibition with an IC_{50} of 62.56 mg/mL. Compared to the reference inhibitor acarbose (IC_{50} = 1.21 mg/mL), the ethyl acetate extract demonstrated a relatively comparable level of inhibition¹⁷.

Although the antidiabetic activity of *Z. clinopodioides* has not been comprehensively studied before, our results revealed modest α -glucosidase and α -amylase inhibitory effects, particularly in the aqueous extract (8.77%) and essential oil (29.30%), respectively. In comparison, *Z. clinopodioides* subsp. *bungeana* showed significantly stronger α -glucosidase inhibition by isolated triterpenes such as betulinic acid and ursolic acid, outperforming acarbose¹⁵. Additionally, extracts of *Z. taurica* subsp. *cleonoides* and *taurica* demonstrated much higher α -amylase inhibition with IC_{50} values around 1.8–2.0 mg/mL^{16,17}. The higher α -amylase inhibition observed in *Z. taurica* extracts may be due to differences in solvent polarity, extraction efficiency, or species-specific phytochemical composition. According to the literature, this is the first detailed report evaluating whole plant extracts of *Z. clinopodioides* from Türkiye in terms of both α -glucosidase and α -amylase inhibition.

In a study, the ethanol extracts of both aerial and root parts of *Z. clinopodioides* were evaluated for their inhibitory effects against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes using Ellman's method. However, no inhibition activity was detected against either enzyme at the tested concentration (200 μ g/mL)¹⁸. A study evaluated the acetylcholinesterase (AChE) inhibitory activities of aqueous extracts from

RRI	Component	%	IM
1032	α -Pinene	1.1	t_R , MS
1035	α -Thujene	0.1	MS
1076	Camphene	0.1	t_R , MS
1118	β -Pinene	5.1	t_R , MS
1132	Sabinene	1.6	t_R , MS
1174	Myrcene	1.0	t_R , MS
1188	α -Terpinene	1.6	t_R , MS
1203	Limonene	4.9	t_R , MS
1213	1,8-Cineole	28.2	t_R , MS
1246	(Z)- β -Ocimene	0.4	MS
1255	γ -Terpinene	3.6	t_R , MS
1266	(E)- β -Ocimene	0.2	MS
1280	<i>p</i> -Cymene	1.1	t_R , MS
1290	Terpinolene	0.7	t_R , MS
1393	3-Octanol	0.5	MS
1474	<i>trans</i> -Sabinene hydrate	0.8	MS
1475	Menthone	0.4	MS
1503	Isomenthone	5.8	MS
1535	β -Bourbonene	0.5	MS
1553	Linalool	0.2	t_R , MS
1556	<i>cis</i> -Sabinene hydrate	0.3	MS
1571	<i>trans-p</i> -Menth-2-en-1-ol	0.6	MS
1590	Bornyl acetate	tr	t_R , MS
1611	Terpinen-4-ol	12.0	t_R , MS
1612	β -Caryophyllene	1.1	t_R , MS
1638	<i>cis-p</i> -Menth-2-en-1-ol	0.3	MS
1638	Menthol	0.3	t_R , MS
1662	Pulegone	7.9	t_R , MS
1668	(Z)- β -Farnesene	0.2	MS
1682	δ -Terpineol	0.5	MS
1706	α -Terpineol	0.5	t_R , MS
1719	Borneol	0.2	t_R , MS
1726	Germacrene D	2.8	MS
1755	Bicyclogermacrene	0.9	MS
1804	Piperitone	3.0	t_R , MS
1857	Geraniol	tr	t_R , MS
1949	Piperitenone	3.6	MS
2006	Dehydrothymol	tr	t_R , MS
2008	Caryophyllene oxide	1.6	t_R , MS
2144	Spathulenol	1.2	MS
2198	Thymol	0.9	t_R , MS
2239	Carvacrol	2.4	t_R , MS
2384	Farnesyl acetone	0.1	MS
	Monoterpene Hydrocarbons	21.5	
Continued			

RRI	Component	%	IM
	Oxygenated Monoterpenes	67.9	
	Sesquiterpene Hydrocarbons	5.5	
	Oxygenated Sesquiterpenes	2.8	
	Others	0.6	
	Total	98.3	

Table 1. The Composition of the Essential Oil of *Ziziphora clinopodioides*. RRI: Relative retention indices calculated against *n*-alkanes; %: calculated from FID data; tr: Trace (< 0.1%); IM: Identification method tR, identification based on the retention times of genuine compounds on the HP Innowax column; MS, identified on the basis of computer matching of the mass spectra with those of the Wiley and MassFinder libraries and comparison with literature data.

Samples	Antidiabetic Activities		Anticholinesterase Activities	
	α -Glucosidase Inhibition (%) (5000 μ g/mL) (mean $\pm$ std)	α -Amylase Inhibition (%) (5000 μ g/mL) (mean $\pm$ std)	Acetylcholinesterase Inhibition (%) (5 μ g/mL) (mean $\pm$ std)	Butyrylcholinesterase Inhibition (%) (500 μ g/mL) (mean $\pm$ std)
Essential oil	ND	29.30 $\pm$ 5.92	5.94 $\pm$ 2.73	10.66 $\pm$ 1.51
MeOH	ND	20.89 $\pm$ 2.49	12.35 $\pm$ 1.32	21.82 $\pm$ 5.27
Aqueous	8.77 $\pm$ 0.11	11.92 $\pm$ 0.59	9.18 $\pm$ 1.76	10.03 $\pm$ 4.21
Acarbose ^a	74.72 $\pm$ 1.41	67.87 $\pm$ 2.74	-	-
Donepezil ^b	-	-	100 $\pm$ 1.14	98.61 $\pm$ 0.56

Table 2. In vitro antidiabetic and anticholinesterase activities of essential oil and extracts of *Ziziphora clinopodioides*. ^a Positive control for antidiabetic activities. ^b Positive control for anticholinesterase activities. N.D.: Not Determined.

five medicinal plants, including *Z. clinopodioides*, in vitro using human erythrocyte and serum enzymes. The results revealed that *Z. clinopodioides* and *Chrysophthalmum montanum* exhibited competitive inhibition on erythrocyte AChE, while *Melissa officinalis* and *Plantago lanceolata* showed non-competitive inhibition. In contrast, *Valeriana officinalis* demonstrated non-competitive inhibition on serum AChE¹⁹. In another study, ethanol extracts prepared from different parts (flower, leaf, branch, root, and mixed parts) of *Z. capitata* were evaluated for their inhibitory effects on cholinesterase enzymes using Ellman's method. The results showed that none of the extracts exhibited significant activity against acetylcholinesterase (AChE), with most considered inactive (AChE inhibition < 10%). In contrast, moderate butyrylcholinesterase (BChE) inhibitory activity was observed in the leaf (40.25%), root (40.56%), and mixed (40.25%) extracts at 200 μ g/mL. The flower and branch extracts showed weaker BChE inhibition at 24.87% and 20.09%, respectively. These values were significantly lower than the reference standard galantamine, which showed 89.12% (AChE) and 76.10% (BChE) inhibition¹⁹.

In the present study, the methanol extract of *Z. clinopodioides* exhibited the highest anticholinesterase activity, with 12.35% AChE and 21.82% BChE inhibition, whereas the aqueous extract and essential oil showed weaker inhibition, particularly against AChE. Compared to the standard donepezil, which showed nearly complete inhibition, the effects of the extracts were modest. Previous studies reported varying results for this species and its relatives. Özkan et al.⁹ found no cholinesterase inhibition at 200 μ g/mL in ethanol extracts of *Z. clinopodioides*, while Özdemir et al.¹⁸ reported that aqueous extracts of the same species exhibited competitive inhibition on erythrocyte AChE. Similarly, Yiğitkan et al.¹⁹ demonstrated that *Z. capitata* showed moderate BChE inhibition in root and leaf extracts, but very limited AChE inhibition. The differences in activity among studies may be attributed to factors such as taking samples from plants grown in different habitats, extraction solvents, plant parts used, and phytochemical composition. Overall, this study is among the first to report moderate dual inhibition of AChE and BChE by *Z. clinopodioides* methanol extract from Türkiye, suggesting potential for further investigation through compound isolation and structure–activity studies.

Morphology

The plant is a suffruticose, mat-forming perennial characterized by stems that range from prostrate to erect, emerging from a branched base and reaching up to 30 cm in height. The stems exhibit a hirsute texture. Leaves measure between 1.5 and 2.5 cm in length and vary from lanceolate to ovate in shape; their surfaces range from scabrous to pilose, with notable pubescence concentrated at the base of the lamina. The bracts are typically broader than the leaves and are sessile. The inflorescence is capitate, consisting of densely flowered verticillasters. The calyx is straight, narrowly tubular, and measures 6–7 mm, with dense hirsuteness externally and a ring of hairs on the inner surface at the base of the calyx teeth. The corolla is bilabiate with a notched upper lip, purple in color, measuring 10–12 mm, and is hirsute on its outer surface. The corolla tube, measuring 5–6 mm, is enclosed within the calyx. Both stamens and style are exserted; there are two fertile stamens with adherent anthers. The stigma is either bifid or united. Nutlets are ovoid in shape and possess a smooth surface²⁰ (Fig. 2).

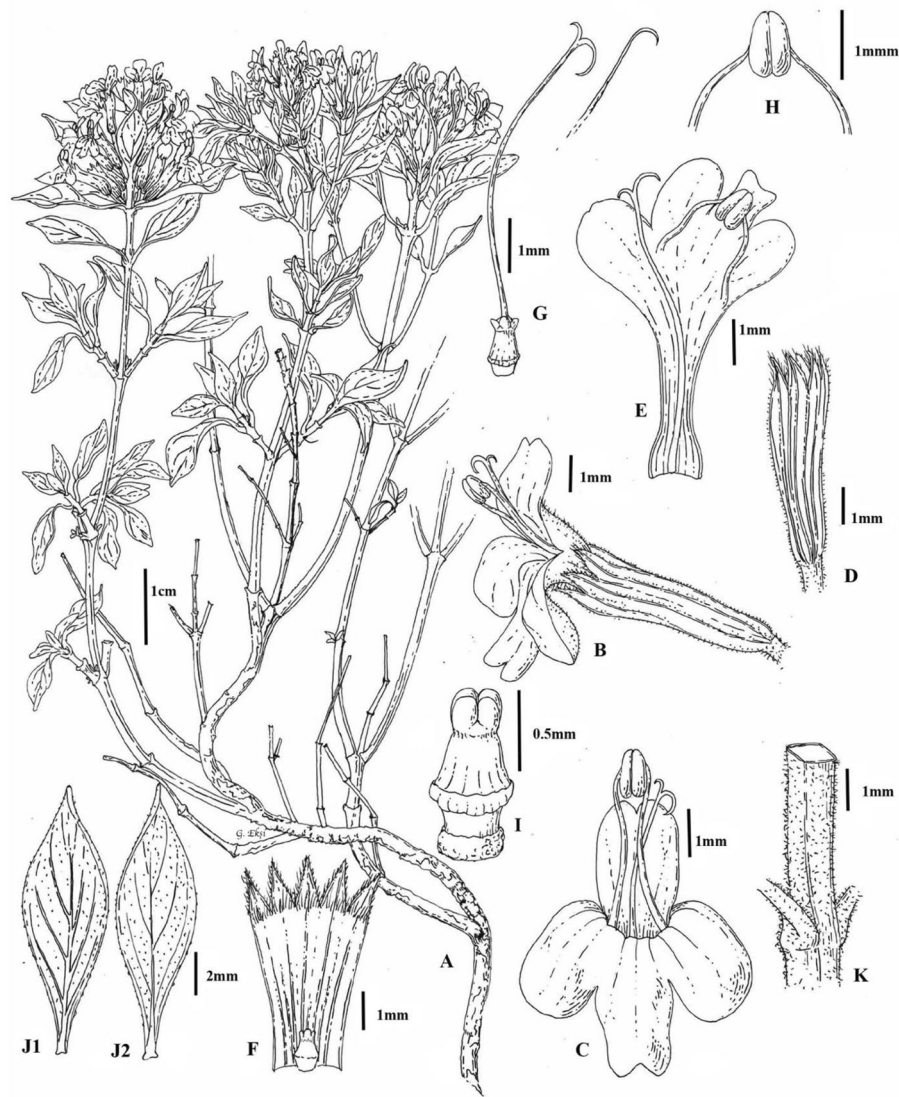


Fig. 2. *Ziziphora clinopodioides*. Plant (A), flower (B), front view of corolla (C), calyx (D), corolla longitudinal section (E), calyx longitudinal section and ovary (F), style (G), stamens (H), pistil (I), leaves upper and lower surface (J), stem cross-section (K). Drawn by Gülnur Ekşi Bona.

Anatomy

Leaf anatomy

In transverse section, the leaf displays a thin cuticular layer covering the epidermis on both adaxial and abaxial surfaces. The epidermis is composed of a single layer of cells ranging from elliptical to spherical in shape (Fig. 3 D, E, G, H, I, O). Both surfaces of the leaf bear trichomes, which include unicellular to multicellular non-glandular types and multicellular glandular types (Fig. 3 A–E, G, H, L–O). Glandular trichomes are characterized by a stalk composed of one to three cells, terminating in a unicellular, spherical head (Fig. 3 B, D, E, G, H, L, M). Non-glandular trichomes are morphologically variable, consisting of one to five cells and may be acicular or curved in form (Fig. 3 A, B, D, E, G, H, M–O). The midrib generally contains a single large vascular bundle, occasionally accompanied by a smaller secondary bundle (Fig. 3 A, B, I, J). Diacytic stomata are present on both the upper and lower epidermal layers (Fig. 3 F, O), indicating that the leaf is amphistomatic and bifacial in structure (Fig. 3 A–O). The palisade parenchyma consists of three to four layers of rectangular to elliptically elongated cells, while the spongy parenchyma comprises two to three layers of loosely arranged cells with intercellular spaces (Fig. 3 D–O). The vascular bundles are of the collateral type (Fig. 3 A–D, H–K, N).

Stem anatomy

In transverse section, the stem exhibits a quadrangular shape (Fig. 4 A–B). The epidermis is composed of a single layer of elliptical to rectangular cells (Fig. 4 D, F, H, J), and the stem surface is densely covered with both glandular and non-glandular trichomes (Fig. 4 A–D, F–H, J). Four distinct types of glandular trichomes are identified: (1) peltate trichomes, comprising a unicellular stalk with a broad, multicellular head (Fig. 4 J); (2)

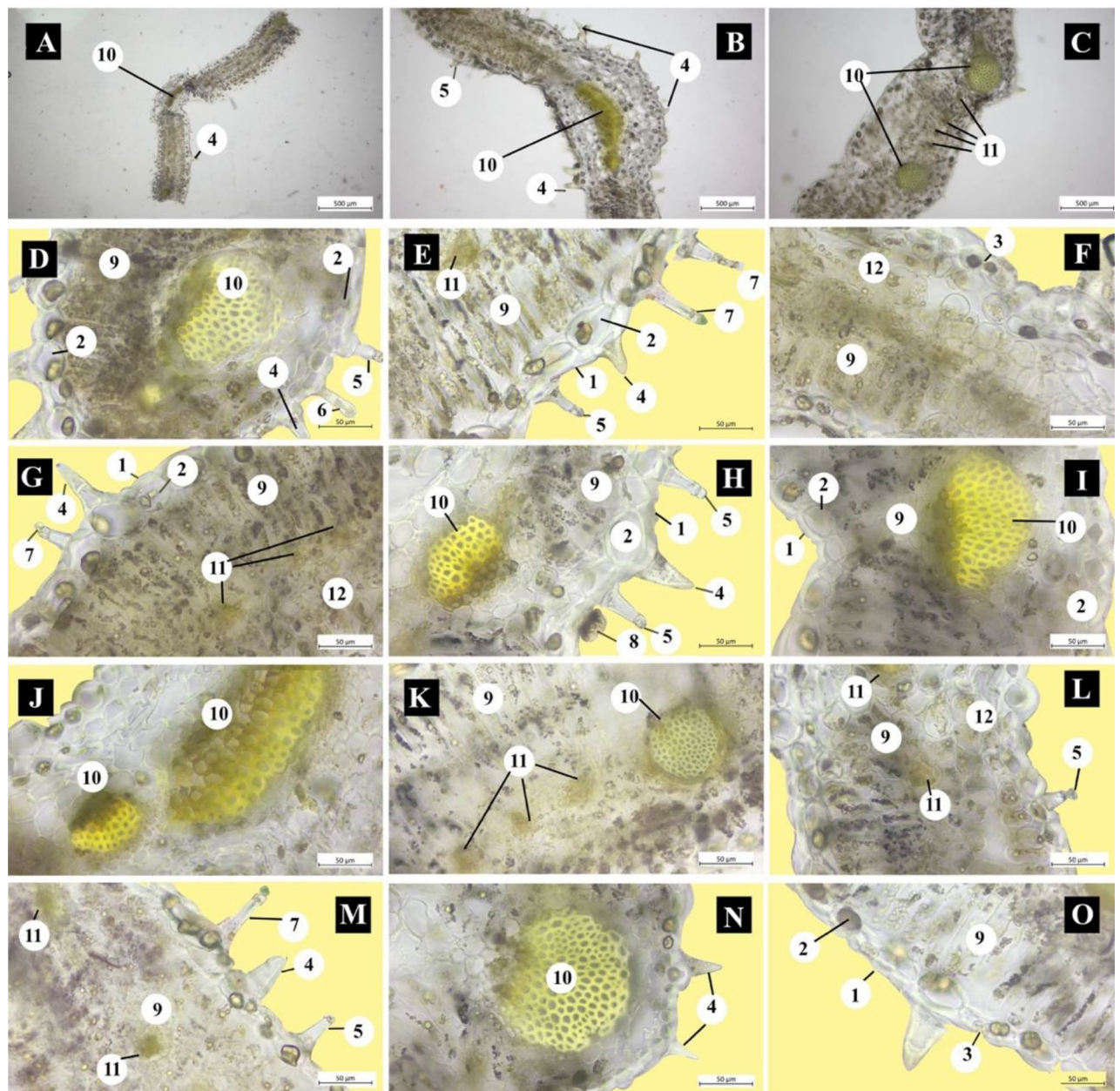


Fig. 3. Leaf transverse-section of *Ziziphora clinopodioides* (A–O). Cuticle (1), epidermis (2), stoma (3), one to five celled non-glandular trichome (4), head and stalk unicellular glandular trichome (5), head unicellular longer than stalk cell, stalk one celled glandular trichome (6), head unicellular, stalk two or three celled glandular trichome (7), Lamiaceae type glandular trichome (8), palisade parenchyma (9), central vascular bundle (10), lateral vascular bundle (11), spongy parenchyma (12).

pyriform glandular trichomes, made up of two cells (Fig. 4 G); (3) capitate trichomes with a unicellular stalk and a bicellular spherical head (Fig. 4 H); and (4) capitate trichomes with a two-celled stalk and an elliptic to spherical bicellular head (Fig. 4 J). Non-glandular trichomes are acicular or curved, and consist of one to five cells (Fig. 4 A–D, F–H, J). The collenchyma forms one to two layers beneath the epidermis, consisting of 9–11 layers of thick-walled cells located at the angles (Fig. 4 A–D, F–H, J). The parenchyma is arranged in two to six layers (Fig. 4 A, B, D, E, G, H, J). The endodermis and cambium are clearly differentiated (Fig. 4 A, B, D, E, H, J, K). The central pith is composed of both lignified and unlignified parenchymatous cells, typically large, polygonal or orbicular in shape (Fig. 4 A, B, I, K).

Morphological analysis of 8 *Ziziphora clinopodioides* populations revealed key traits for distinguishing subspecies in Iran. Micro-morphological features of the leaf epidermis and pollen supported these findings. PCA highlighted the most variable characters. Pollen and epidermal traits proved valuable for infraspecific taxonomy, and combining macro- and micro-morphological data is recommended for accurate classification²¹. In a previous study, the morphological and chemical diversity of *Z. clinopodioides* subsp. *bungeana* was investigated

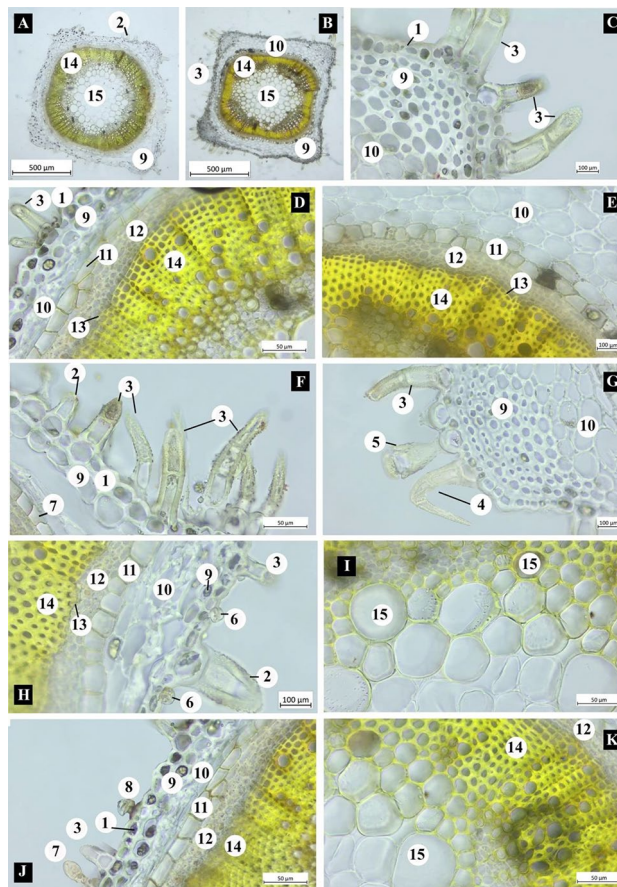


Fig. 4. Stem transverse-section of *Ziziphora clinopodioides* (A–K). Epidermis (1), simple trichome (2), one to five celled acicular non-glandular trichome (3), one to four celled curved non-glandular trichome (4), two cellular pyriform glandular trichome (5), glandular trichome with two celled head and unicellular stalk (6), glandular trichome with two celled head and two celled stalk (7), glandular trichome with multicellular head and unicellular stalk (8), collenchyma (9), parenchyma (10), endodermis (11), phloem (12), cambium (13), xylem (14), pith (15).

across four populations from varying bioclimatic zones in Iran. The authors reported significant morphological differentiation linked to ecological conditions, particularly between semi-arid and sub-humid regions. Pulegone was identified as the main essential oil component in all populations, while iso-menthone and thymol contents varied with climate. The study emphasized that limited morpho-chemical diversity may threaten the subspecies' long-term viability without appropriate habitat protection²². A previous pharmacognostic study focused on the aerial parts of *Z. Clinopodioides*. The researchers identified key macroscopic diagnostic features, including leaf structure, stem morphology, and the color and form of the calyx and corolla. Microscopic examination revealed diacytic stomata, multicellular trichomes, and large essential oil glands distributed across various plant organs. The mesophyll was clearly differentiated into palisade and spongy tissues. Histochemical staining further confirmed the localization of specific metabolites, contributing to the anatomical and chemical characterization of the species²³.

Compared to previous studies on *Z. clinopodioides*, current findings confirmed several shared diagnostic features, such as diacytic stomata, multicellular trichomes, and large essential oil glands in leaves and floral organs. However, this study provided more detailed characterization, particularly in trichome diversity and stem anatomy, including the presence of distinct glandular types and a well-differentiated vascular system. Additionally, the bifacial leaf structure with clearly layered palisade and spongy tissues was consistent, further supporting its taxonomic identification and potential pharmacognostic value.

The differences observed in extraction yields and enzyme inhibitory activities among the extracts and essential oil of *Ziziphora clinopodioides* can be primarily attributed to solvent polarity and phytochemical diversity. Polar solvents such as water and methanol are known to extract phenolics and other hydrophilic compounds that are frequently associated with enzyme inhibitory activities, whereas essential oils are mainly composed of volatile terpenoids that generally exhibit moderate biological effects. The dominance of oxygenated monoterpenes in the essential oil is consistent with previous reports on *Ziziphora* species, although quantitative differences among studies reflect the influence of geographical and environmental factors. Moreover, the presence of abundant glandular trichomes observed in the anatomical analysis supports the chemical findings, as these structures are recognized as the primary sites of essential oil biosynthesis in Lamiaceae species^{14,24–26}.

The variation observed in extraction yields and enzyme inhibitory activities among the methanolic, aqueous, and essential oil fractions of *Ziziphora clinopodioides* is consistent with previous reports emphasizing the strong influence of solvent polarity and phytochemical diversity on biological outcomes. Polar solvents such as water and methanol are known to preferentially extract phenolics, flavonoids, and triterpenoids, which have frequently been associated with α -glucosidase, α -amylase, and cholinesterase inhibition in *Ziziphora* species and other members of the Lamiaceae family. In contrast, essential oils are dominated by volatile monoterpenes, particularly oxygenated monoterpenes such as 1,8-cineole, terpinen-4-ol, and pulegone, which are more often linked to moderate enzyme modulation rather than strong inhibition.

The modest antidiabetic and anticholinesterase activities observed in the present study are therefore in agreement with earlier findings reporting stronger effects for isolated compounds or semi-purified fractions compared to whole extracts or essential oils. Moreover, the pronounced variability in essential oil composition reported in the literature for *Z. clinopodioides*—driven by geographical origin, ecological conditions, and phenological stage—further supports the need to interpret biological activities within a chemotypic and ecological framework. From a pharmacognostic perspective, the detailed anatomical features identified in this study, particularly the diversity and abundance of glandular trichomes, provide a structural basis for the observed chemical profiles and support the species' potential as a functional food or medicinal resource, while also highlighting the necessity of further phytochemical fractionation and compound-level investigations.

While detailed phytochemical characterization was conducted only for the essential oil, the methanolic and aqueous extracts were included as comparative references to provide a broader biological context rather than detailed compound–activity relationships. Essential oils and plant extracts are both known to contain diverse classes of bioactive compounds, but their chemical profiles and relative constituent abundances can differ substantially depending on the extraction method and solvent polarity, making direct comparisons of specific activities difficult without comprehensive phytochemical data for each fraction²⁷.

In this study, due to resource and scope considerations, the major focus was placed on the volatile constituents of the essential oil, which were fully characterized and discussed in relation to biological activity, whereas the crude extracts were used to illustrate general activity trends and to contextualize the essential oil results.

Materials and methods

Plant materials

The plant material was collected from the Kayaönü Village vicinity (1710 m, 36° 38' 08" N 32° 56' 25" E) 10 km north of Ermenek district, Karaman province, Türkiye, by the author on 22 July 2024. Voucher specimens have been deposited at the Herbarium of Biodiversity Application and Research Center, Karamanoglu Mehmetbey University (KMUB 7712).

Extraction

To obtain the methanolic and aqueous extracts from *Z. clinopodioides*, the dried flowering aerial parts were finely powdered and mixed with methanol using a mechanical mixer. The mixture was left to macerate at room temperature for 8 h and 3 days, respectively. Once maceration was complete, the extract was dried and weighed. For the aqueous extract, 50 g of dried plant material were boiled in water for 2 h, and the resulting mixture was filtered and frozen at -80 degrees Celsius. The frozen extract was then subjected to lyophilization and the resulting weight was recorded. This process allowed for the extraction of bioactive compounds from the plant material, which could potentially possess therapeutic properties.

Essential oil extraction and analysis

To extract essential oils from the flowering aerial parts of *Z. clinopodioides*, the Clevenger apparatus was utilized, with the addition of water during the 3–4 h extraction process. The resulting oils were then subjected to gas chromatography-mass spectrometry (GC–MS) analysis using an Innovax FSC column (60 m × 0.25 mm, 0.25 μ m film thickness), with helium gas used as the carrier at a flow rate of 0.8 mL/min. The GC oven was initially set at 60 °C for 10 min, followed by a gradual increase at a rate of 4 °C per minute until reaching 220 °C, where it was held for 10 min before being further increased to 240 °C at a rate of 1 °C per minute. The injector temperature was maintained at 250 °C, while the mass spectra were recorded across a range of m/z 35 to 450 at 70 eV. The GC analysis was conducted using an Agilent 6890N GC system, with the flame ionization detector (FID) temperature set at 300 °C. This analytical approach allowed for the identification and quantification of the chemical components present in the essential oils, which could be linked to the potential therapeutic benefits of *Z. clinopodioides*. Computer matching against commercial (Wiley GC/MS Library, MassFinder Software 4.0)^{28,29} and in-house “Başer Library of Essential Oil Constituents” built up by genuine compounds and components of known oils.

α -Amylase inhibition assay

The inhibitory activity against α -amylase was determined using a modified method based on Nampoothiri et al.³⁰ as refined by Yuca et al.³¹. Test samples and acarbose were dissolved in dimethyl sulfoxide. In a reaction mixture, 100 μ L of the sample solution was combined with 100 μ L of a 1% starch solution prepared in 20 mM sodium phosphate buffer (pH 6.9, containing 6 mM sodium chloride) and incubated at 25 °C for 10 min. Subsequently, 100 μ L of α -amylase (from porcine pancreas) solution (0.5 mg/mL) was added, followed by an additional 10-min incubation. The reaction was terminated using a dinitro salicylic acid (DNS) reagent, and the absorbance was recorded at 540 nm. Acarbose (at a final concentration of 5 mg/mL) served as the positive control, while the buffer alone used as the negative control. All experiments were performed in triplicate to ensure accuracy and reproducibility. The percentage inhibition was computed as $(1 - \Delta A_{540\text{sample}} / \Delta A_{540\text{control}}) \times 100$.

α -Glucosidase inhibition assay

The α -glucosidase (from *Saccharomyces cerevisiae*) inhibition assay was performed following a modified protocol adapted from Bachhawat et al.³² and further refined by Yuca et al.³³. Test samples and acarbose were dissolved in a 50 mM potassium phosphate buffer (pH 6.9). The reaction mixture, prepared in a 96-well plate, consisted of 20 μ L of the sample solution, 10 μ L of α -glucosidase enzyme (1 U/mL), and 50 μ L of potassium phosphate buffer. After a 5-min incubation at 37 °C, the reaction was initiated by adding 20 μ L of *p*-nitrophenyl- α -D-glucopyranoside (3 mM) and allowed to proceed for 30 min at the same temperature. The reaction was terminated by adding 50 μ L of 0.1 M sodium carbonate. Acarbose (at a final concentration of 5 mg/mL) was used as the positive control, while the buffer served as the negative control. The formation of *p*-nitrophenol was quantified by measuring absorbance at 405 nm using a microplate reader. All experiments were conducted in triplicate. The percentage inhibition was calculated as $(1 - \Delta A_{405\text{sample}} / \Delta A_{405\text{control}}) \times 100$.

Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) inhibition assay

The inhibitory activities against acetylcholinesterase (AChE) (from electric eel) and butyrylcholinesterase (BChE) (from equine serum) were evaluated using a modified version of the method described by Ingkaninan et al.³⁴ as adapted by Karakaya et al.³⁵. Test samples and donepezil (at a final concentration of 5 mg/mL) were dissolved in dimethyl sulfoxide (DMSO). In a 96-well microplate, a reaction mixture containing 125 μ L of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent), 25 μ L of the respective substrate (acetylthiocholine for AChE or butyrylthiocholine for BChE), 50 μ L of Tris-HCl buffer, and 25 μ L of the sample solution was prepared and incubated. The reaction was initiated by adding the corresponding enzyme (AChE or BChE), and the enzymatic activity was assessed by measuring absorbance at 405 nm. Donepezil was used as the positive control, while the buffer alone served as the negative control. All experiments were conducted in triplicate to ensure reliability and reproducibility. The percentage inhibition was calculated as $(1 - \Delta A_{405\text{sample}} / \Delta A_{405\text{control}}) \times 100$.

Morphological and anatomical studies

Morphological characterization of *Z. clinopodioides* was conducted using a Leica S8APO stereo microscope. Ten specimens were examined to document the structural features of stems, leaves, flowers, and roots. Comprehensive descriptions were recorded, accompanied by detailed scientific illustrations.

For anatomical investigations, plant material was initially fixed in 70% ethanol to preserve tissue integrity. Transverse sections of both stems and leaves were then prepared and analyzed using Sartur's reagent and chloral hydrate to enhance tissue contrast and visibility under the microscope.

Statistical analysis

All experiments were carried out in triplicate, and the results are expressed as mean values accompanied by standard deviations (SD). The statistical significance of differences among samples was assessed using the Kruskal–Wallis's test, with a significance level set at $p < 0.05$.

The Kruskal–Wallis's test was selected because the data did not consistently meet the assumptions required for parametric tests, particularly normal distribution and homogeneity of variances, which is common in biological assays conducted with small sample sizes ($n = 3$). As a non-parametric test, Kruskal–Wallis compares median values rather than means, making it more robust against deviations from normality and the influence of outliers. Therefore, this test was considered more appropriate for reliable comparison of the experimental groups under the present conditions.

Conclusion

This study presents a comprehensive evaluation of *Ziziphora clinopodioides* Lam. collected from Türkiye, highlighting its potential as a source of bioactive compounds with relevance to food and health applications. The high content of oxygenated monoterpenes, especially 1,8-cineole, terpinen-4-ol, and pulegone, suggests strong antioxidant and antimicrobial potential. Although the enzyme inhibition activities were moderate compared to standard drugs, the essential oil and methanolic extract exhibited measurable inhibitory effects against α -amylase, acetylcholinesterase, and butyrylcholinesterase, indicating possible functional roles in managing metabolic and neurodegenerative conditions. The detailed morphological and anatomical findings, including specialized trichomes and well-organized vascular structures, provide essential taxonomic and pharmacognostic data. Overall, the results support the traditional uses of *Z. clinopodioides* and suggest that it may serve as a valuable natural additive or preservative in functional food formulations. Further studies including in vivo assessments and formulation-based research are warranted to validate its efficacy and safety in food systems.

Overall, the present findings provide preliminary in vitro support that may inform future formulation strategies and in vivo studies, rather than constituting direct evidence for functional food or therapeutic applications.

Data availability

All data are included in the manuscript.

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Author contributions

All study was carried out by Ömer Çeçen.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to Ö.Ç.

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