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# EFFECTS OF SOME TURKISH PLANT EXTRACTS ON CARBONIC ANHYDRASE AND CHOLINESTERASE ENZYMES

**İbrahim Seyda URAS[a,*], Murat KÜRŞAT[b], Belma KONUKLUGIL[c], Murat ŞENTÜRK[d]**

**ABSTRACT.** Cholinesterase inhibitors are valuable compounds that can be used in many different therapeutic applications, including Alzheimer's disease. Carbonic anhydrase (CA) inhibitors constitute a pharmacological intervention employed for the management and alleviation of various medical conditions, including glaucoma, idiopathic intracranial hypertension. Turkey has a large and diverse flora, home to thousands of plant species. Hundreds of compounds of medicinal importance have been identified from the many plants in this flora. In this study, the inhibitory properties of seven different plant extracts on CA I and II, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes were investigated. The tested extracts showed AChE inhibitory activity with values ranging from 1.26 to 4.20 µg/mL, BChE inhibitory activity with values ranging from 1.32 to 4.24 µg/mL, CA I inhibitory activity with values ranging from 0.74 to 1.82 µg/mL and CA II inhibitory activity with values ranging from 0.033 to 0.067 µg/mL. The extract of *Zosima absinthifolia* showed a very active inhibition profile against both AChE and BChE ($IC_{50}$ 1.26 ± 0.01 µg/mL for AChE and 1.32 ± 0.02 µg/mL for BChE). The results indicate that these extracts are potent cholinesterases inhibitors and specifically *Zosima absinthifolia* extract could be evaluated for further studies.

***Keywords:*** *enzyme inhibition, Turkey flora, Zosima absinthifolia.*

[a] *Agri Ibrahim Cecen University, Faculty of Pharmacy, Department of Pharmacognosy, Ağrı, Türkiye*
[b] *Bitlis Eren University, Faculty of Science and Arts, Department of Biology, Bitlis, Türkiye*
[c] *Lokman Hekim University, Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Türkiye*
[d] *Agri Ibrahim Cecen University, Faculty of Pharmacy, Department of Biochemistry, Ağrı, Türkiye*
[*] *Corresponding author: isuras@agri.edu.tr*

## INTRODUCTION

In this study, the enzyme inhibitory properties of seven plants growing in Turkey, one of which is endemic, were investigated. It was aimed to investigate the various enzyme activities of plants grown in Bitlis province and used among the public to relieve various symptoms. The selection of plants carried out based on their traditional use. *Diplotaenia cachrydifolia* is used traditionally for treating diabetes and rheumatism [1]. In Turkish folk medicine, the mixture made from the *Zosima absinthifolia* leaves is used in the treatment of diabetes [2]. In European folk medicine, *Salvia* species has been used to treat gastrointestinal disorders (dyspepsia, flatulence, abdominal spasms, diarrhea, inflammation of intestinal mucosa), inflammation of the mouth and throat [3]. Significant medicinal benefits from *Fumaria* species have been reported in traditional medicine, especially as a remedy for hepatobiliary disorders, cough, eczema treatment, and skin diseases [4]. *Anarrhinum* species [5], *Ferulago species* [6], and *Rhabdosciadium* species [7] are used in the treatment of various diseases in folk medicine.

The *Zosima* genus, which belongs to the Apiaceae family and spreads over a wide area from the Middle East to Turkey, Iran and Afghanistan, has been used as a medicinal plant for centuries. Essential oils and extracts of this plant have many biological activities such as anti-inflammatory, antimicrobial and cytotoxic activity. The essential oil of “peynir otu” (*Zosima absinthifolia)* fruits has a high antibacterial effect and it was found effective against some bacteria such as *Bacillus pumilus* and *B. subtilis* [8]. Another study concluded that flower and fruit extracts of *Z. absinthifolia* have high antioxidant and anticholinesterase properties.

Bahadir, Çitoğlu [9] stated that n-hexane extract obtained from the aerial part of *Z. absinthifolia* has anti-inflammatory properties in rats. In another study, it was found that the methanol extract of *Z. absinthifolia* fruits exhibited significantly higher free radical scavenging, antibacterial, anti-inflammatory, and cytotoxic activities [9]. Additionally, studies on the essential oil compositions of *Z. absinthifolia* fruits have shown that the main components of the essential oil are octyl acetate, octyl octanoate, octyl hexanoate and 1-octanol [10, 11].

*Salvia* species are represented by about a hundred species, half of which are endemic, in Turkey. In a study on “fırat şalbası” (*Salvia pseudeuphratica)*, the main components of the plant’s essential oil were found to be camphor, 1,8 cineole and linalool by GC-MS analysis. It was thought that the essential oil of the plant could be used as an insect repellent due to its high camphor content [12]. In another study, researchers focused on the anticholinesterase

activity of *S. pseudeuphratica* essential oil. The study found that the essential oil of the plant showed high anticholinesterase activity with an $IC_{50}$ value of 26 µg/mL [13].

*Fumaria* L. (Papaveraceae) has about 60 species and is found on the European continent, especially in the Mediterranean region and Eastern and Western Europe. Extracts obtained from *Fumaria spp*. has been used traditionally to treat rheumatism, abdominal pain, abdominal cramps, fever, diarrhoea, some skin diseases (rash or conjunctivitis), syphilis and leprosy.

Studies have presented extremely rich sources in terms of the alkaloid content of *Fumaria* species. In addition to the alkaloid content, the presence of different types of flavonoids, steroid structures and organic acids is also constant. Studies conducted with *Fumaria* extracts have also found that they have strong antihypertensive, hepatoprotective, diuretic, laxative effects, and antifungal, antibacterial, anti-inflammatory activities. These biological activities have mostly been associated with the presence of isoquinoline alkaloids [14].

The result of the GC-MS analysis of “akşahtere” *(Fumaria asepala)* essential oil conducted by Yılmaz Sancar in 2023, Phytol (20.74 %) was the major substance, followed by Thymol (20.42%), Benzyl Benzoate (15.89 %) and Hexahydrofarnesyl acetone (12.92 %). As a result of the antimicrobial analysis, *F. asepala* essential oil showed the best antimicrobial effect against *S. aureus*-ATCC 25923 (24 mm) and *K. pneumoniae* (24 mm). To determine antioxidant effects, total antioxidant level (TAS) and total oxidant level (TOS) were examined and it was found that the total oxidant level was high [15].

“Köse otu” *(Diplotaenia cachrydifolia)* is a member of the Apiaceae family and a perennial plant that grows wild in the eastern parts of Turkey. In previous studies, compounds such as jatamansin, xanthotoxin, bergapten and isopimpinellin were isolated from different parts of this plant [16]. In another study, alpha phellandrene and isomyristin substances were found in high amounts in the oil obtained from the roots of the plant, while terpinolene isodillapiol substances were found in high amounts in the oil obtained from the leaves and fruits. The researchers compared their results with those of other studies and evaluated that the difference in the major substances could be due to climatic factors, plant collection location, plant nutrition status and genetic differences [17]. In a more recent study, antimicrobial, antioxidant and antigenotoxic activity analyses were performed on the extracts prepared with different solvents (ethanol, acetone, hexane) from the plant. The highest antioxidant activity (DPPH $IC_{50}$: 2.5234 µg/mL) and phenol content (55.36 ± 0.035 µg/mL) were observed in the ethanol extract, which also showed protective effect against genotoxicity induced by mitomycin C [1].

The *Rhabdosciadium* genus, belonging to the Umbelliferae family, is distributed with two species in Turkey. “Som handok” (*Rhabdosciadium microcalycinum)* is a plant native only to Turkey. In Turkey, it can only be seen in the upper Euphrates region, in the provinces of Bingöl and Elazığ. In a GC-MS analysis on the composition of the essential oil obtained from *Rhabdosciadium microcalycinum* plant collected from the Elazığ region, the major component of the oil was found to be germacrene D [18]. In a recent study, different activities of the ethanolic extract of *R. microcalycinum* plant collected from the Bingöl region were investigated. The plant extract showed remarkable inhibitory effects on AChE and alpha glucosidase enzymes, and the $IC_{50}$ values were found to be 35.86 mg/mL and 10.14 mg/mL, respectively [19].

Plants belonging to the genus *Ferulago* are members of the Apiaceae family. When the essential oil of “yıldız kişnişi” (*Ferulago stellata)* plant collected from Iran was investigated, GC-MS analysis revealed that 2,4,5-trimethyl benzaldehyde and alpha and beta pinene compounds were the major components of the oil [20]. In another study, the inhibitory effects of the ethanolic extract of the plant collected from the Çatak region of Van province on AChE, alpha glucosidase and alpha amylase enzymes were remarkable, and the IC50 values were 1.772 µg/mL, 33.56 µg/mL, and 0.639 µg/mL, respectively [6].

The treatment of various diseases is greatly influenced by enzyme inhibitors. AChE (EC 3.1.1.7) plays a crucial role in terminating cholinergic signaling by hydrolyzing acetylcholine (ACh), a vital neurotransmitter for memory and motor function [21].

Located postsynaptically, AChE terminates neuronal signaling by rapidly hydrolyzing ACh. Unlike AChE, butyrylcholinesterase (BChE) (EC 3.1.1.8) is primarily synthesized in the liver and distributed throughout the body, including blood plasma and the nervous system [22]. Clinical evidence demonstrates that AChE inhibitors enhance cholinergic activity by elevating ACh levels within cholinergic synapses [23]. While AChE primarily mediates ACh hydrolysis, BChE contributes to regulating ACh levels and plays a crucial role in drug metabolism and detoxification [24]. Selective inhibitors for both AChE and BChE are valuable therapeutic tools for managing motor neuron diseases like dementia, myasthenia gravis, and Alzheimer’s disease [21].

CAs (EC 4.2.1.1) are metalloenzymes catalyzing the conversion of $CO_2$ to $HCO_3^-$ and $H^+$. These ubiquitous enzymes are encoded by six distinct gene families across diverse species [25]. Humans possess fifteen CA isoforms, with cytosolic hCA I/II being most prevalent across tissues. Understanding CA modulation holds therapeutic potential for various clinically significant disorders [26, 27]. Notably, specific CA inhibitors have enabled the development of novel drugs for treating epilepsy, edema, and glaucoma. Therefore, exploring and identifying novel CA isoenzyme inhibitors represents a promising avenue for therapeutic discovery [26, 28, 29].

## RESULTS AND DISCUSSION

An evaluation of seven plant extracts against the AChE enzyme identified extract of *Z. absinthifolia* as possessing the most potent inhibitory activity, exhibiting an $IC_{50}$ value of 1.26 µg/mL (Figure 1).

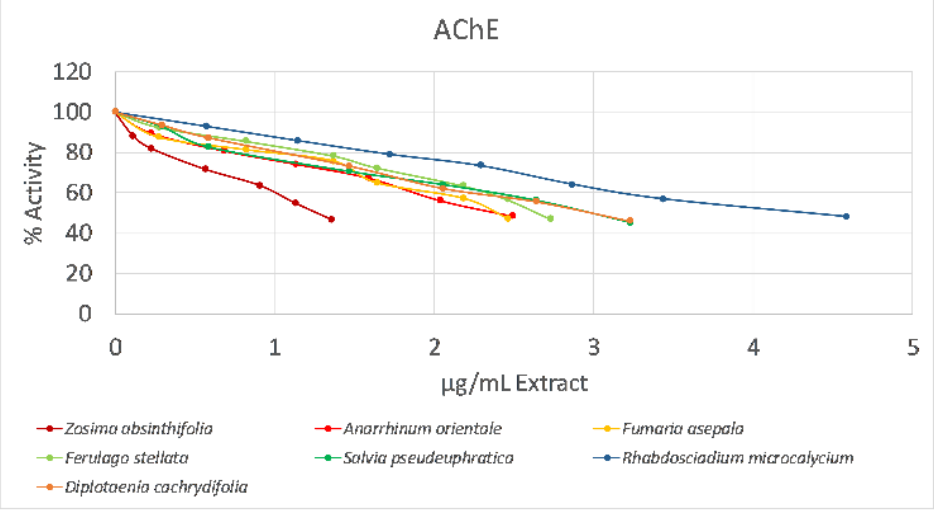


**Figure 1.** Decreasing in the AChE enzyme activity against tested extracts.

Conversely, extract of *R. microcalycinum* demonstrated the weakest inhibitory effect, as reflected by its $IC_{50}$ value of 4.2 µg/mL. Notably, all tested extracts displayed close inhibitory activity compared to the reference molecule, galantamine, which possessed an $IC_{50}$ value of 0.4 µg/mL (Table 1).

**Table 1.** Inhibition values of AChE and BChE enzymes with the tested extracts.

| Plant Extracts | AChE $IC_{50}$ value (µg/mL) | Galantamine Equivalents for AChE (µg/mL) | BChE $IC_{50}$ value (µg/mL) | Galantamine Equivalents for BChE (µg/mL |
|---|---|---|---|---|
| *Zosima absinthifolia* | 1.26 ± 0.01 | 3.15 | 1.32 ± 0.02 | 0.6 |
| *Anarrhinum orientale* | 2.47 ± 0.03 | 6.175 | 2.92 ± 0.03 | 1.327 |
| *Fumaria asepala* | 2.42 ± 0.03 | 6.05 | 2.43 ± 0.03 | 1.104 |
| *Ferulago stellata* | 2.81 ± 0.03 | 7.025 | 2.96 ± 0.03 | 1.345 |
| *Salvia pseudeuphratica* | 2.87 ± 0.03 | 7.175 | 2.95 ± 0.03 | 1.34 |
| *Rhabdosciadium microcalycinum* | 4.20 ± 0.04 | 10.5 | 4.24 ± 0.04 | 1.927 |
| *Diplotaenia cachrydifolia* | 2.90 ± 0.03 | 7.25 | 2.90 ± 0.03 | 1.318 |
| Galantamine[a] | 0.40 ± 0.10 | | 2.20 ± 0.30 | |

[a] [30].

An assessment of the inhibitory potential of various plant extracts against the BChE enzyme revealed that extract of *Z. absinthifolia* exhibited the most pronounced inhibitory activity, with an $IC_{50}$ value of 1.32 µg/mL (Figure 2).

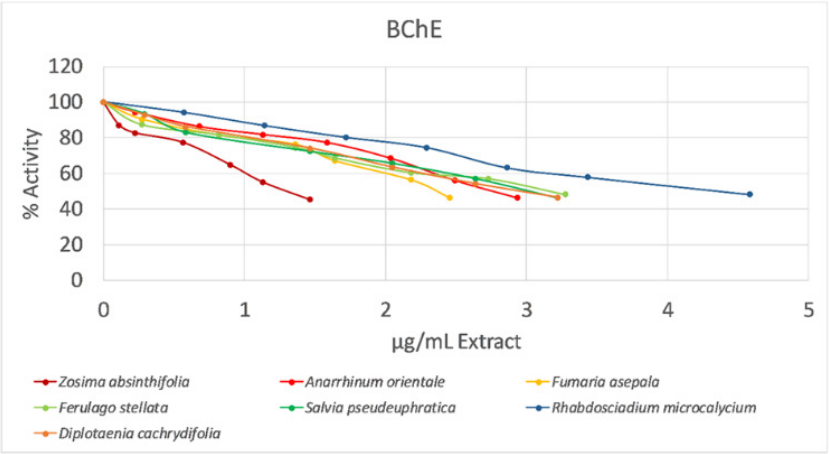


**Figure 2.** Decreasing in the BChE enzyme activity against tested extracts.

Among the seven extracts tested against the AChE enzyme, R. microcalycinum ($IC_{50}$ = 1.82 µg/mL) showed the weakest inhibitory effect among the seven extracts against the hCA I enzyme. However, extract *Z. absinthifolia* (0.74 µg/mL) showed the best inhibitory profile among all plant extracts (Figure 3).

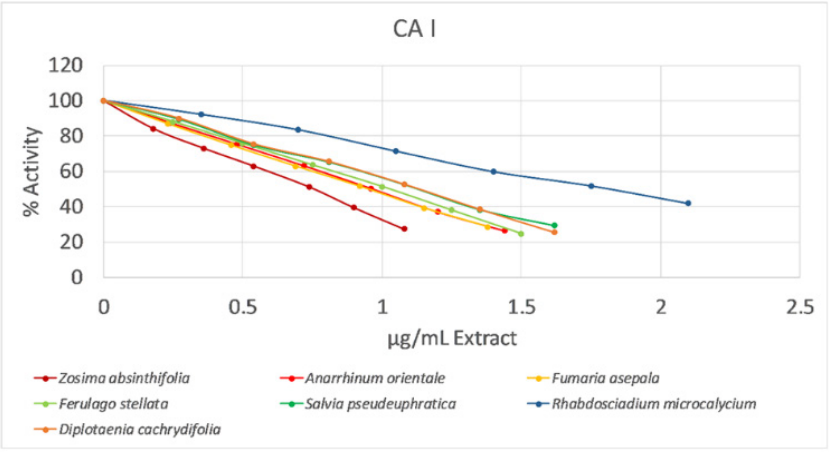


**Figure 3.** Decreasing in the CA I enzyme activity against tested extracts.

Additionally, in this study, the values obtained for the hCA I enzyme (0.74-1.82 µg/mL) were compared with the reference molecule acetazolamide (1.652 µg/mL). All of these plant extracts showed better or similar values than acetazolamide (Table 2).

**Table 2.** Inhibition values and acetazolamide equivalents (AE) of CA I and CA II enzymes with the tested extracts.

| Plant Extracts | CA I $IC_{50}$ value (µg/mL) | Acetazolamide Equivalents for CA I (µg/mL) | CA II $IC_{50}$ value (µg/mL) | Acetazolamide Equivalents for CA II(µg/mL) |
|---|---|---|---|---|
| *Zosima absinthifolia* | 0.74 ± 0.02 | 0.456 | 0.033 ± 0.001 | 2.062 |
| *Anarrhinum orientale* | 0.97 ± 0.03 | 0.587 | 0.041 ± 0.001 | 2.562 |
| *Fumaria asepala* | 0.95 ± 0.03 | 0.575 | 0.038 ± 0.001 | 2,375 |
| *Ferulago stellata* | 1.01 ± 0.03 | 0.611 | 0.047 ± 0.001 | 2.937 |
| *Salvia pseudeuphratica* | 1.13 ± 0.03 | 0.684 | 0.051 ± 0.001 | 3.187 |
| *Rhabdosciadium microcalycinum* | 1.82 ± 0.08 | 1.123 | 0.067 ± 0.001 | 4.187 |
| *Diplotaenia cachrydifolia* | 1.11 ± 0.04 | 0.671 | 0.053 ± 0.001 | 3.312 |
| Acetazolamide | 1.652 ± 0.03 | | 0.016 ± 0.001 | |

*R. microcalycinum* extract ($IC_{50}$ = 0.067 µg/mL) showed the weakest inhibitory effect among the seven extracts against the hCA II enzyme. However, extract *Z. absinthifolia* (0.033 µg/mL) showed the best inhibitory profile among all plant extracts (Figure 4).

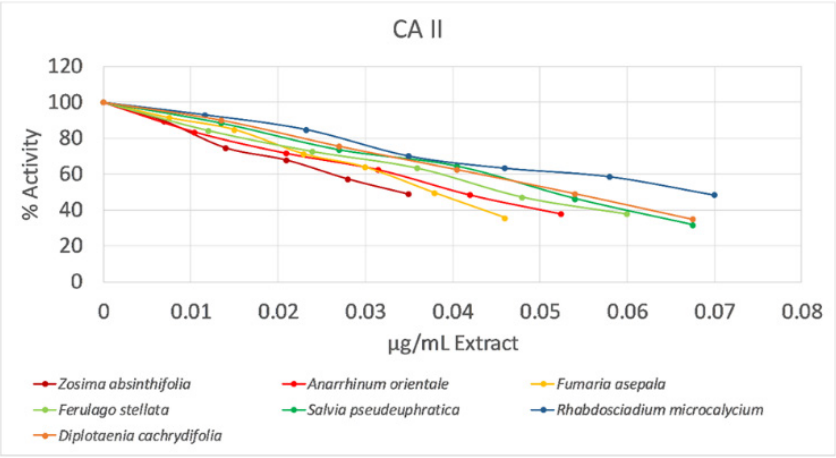


**Figure 4.** Decreasing in the CA II enzyme activity against tested extracts.

Additionally, in this study, the values obtained for the hCA II enzyme (0.033-0.067 µg/mL) were compared with the reference molecule acetazolamide (0.016 µg/mL). All of these plant extracts showed values close to acetazolamide (Table 2).

When comparing our results with those from other studies on the same plants, we observe significant variations in the outcomes. For AChE inhibition, the experiment using *Fumaria asepala* extract at a concentration of 1 µg/mL resulted in 21 % inhibition in our study. However, the same plant's extract prepared using a chloroform: methanol (1:1) solvent system exhibited 9.76 % inhibition at the same concentration. Since both plants were extracted using solvents of similar polarity, the observed differences in results may be attributed to solvent selection for extraction or the geographical origin of the plants in different studies (Sivrihisar, Ankara – Baskil, Elazığ) [31].

Another group working with *Zosima absinthifolia* conducted a comprehensive study using different parts of the plant collected from Erzurum. In their research, extracts prepared from different parts of the plant using various solvents such as methanol, hexane, dichloromethane, and ethyl acetate and some of the secondary metabolites of plant isolated. Results showed significant variations in terms of cholinesterase inhibition. The highest inhibitory activity among extracts was observed in dichloromethane fruit extracts at 20 µg/mL, against both AChE and BChE (31.46% and 82.27% respectively). Among the isolated compounds pimpinellin showed highest activity against BChE (66.55% inhibition at 20 µg/mL) while umbelliferon presented best results against AChE (61.09% inhibition at 20 µg/mL) [32]. This results aligned with our findings and it points that the enzyme inhibitory activity of *Z. absinthifolia* could be related to its coumarin compounds.

In a study conducted on the ethanolic extract of *Ferulago stellata* collected from the Çatak region in Van province, it was determined that it exhibited activity with a lower $IC_{50}$ value compared to our results ($IC_{50}$: 2.42 and 1.77 µg/mL) [6].

In a conducted study, the acetylcholinesterase inhibitory activity of the ethanolic extract obtained from *R. microcalycinum* collected in Bingöl was found to be lower compared to our own results. This endemic species, which grows exclusively in Bingöl and Elazığ, yielded significantly different results when samples were collected from two distinct regions ($IC_{50}$: 35.86 and 4.20 µg/mL). The observed variation in activity is likely due to compositional differences in the bioactive compounds, influenced by solvent selection or the specific geographical location of plant growth [19].

In another study, the $IC_{50}$ value obtained from the AChE inhibitor activity test on the essential oil of *S. pseudeuphratica* was significantly higher than the $IC_{50}$ value demonstrated by plant's methanol extract in our study ($IC_{50}$: 26 and

1.13 µg/mL). Since both studies utilized plants collected from the same region, the difference in results is attributed to the fact that the activity test was performed on volatile oil in the initial study and on methanol extract in our study [13].

## CONCLUSIONS

The evaluation of the inhibitory effects of Turkish plant extracts against various key enzymes, including AChE, BChE, CA I and II, has yielded significant insights into their potential therapeutic applications.

The anticholinesterase and carbonic anhydrase inhibitor activity analysis on *Diplotaenia cachrydifolia* and *Anarrhinum orientale* plant extracts has been conducted for the first time in this study. High activity was observed at the reference substance level in both butyrylcholinesterase and carbonic anhydrase inhibition. These findings highlight the potential pharmacological significance of these plant species.

## EXPERIMENTAL SECTION

### *Chemicals and laboratory*

All enzymes and chemicals used for experiments were bought from Sigma Aldrich (Germany). Extract preparation and enzyme inhibiton studies were carried out in Central Research Laboratory of Agri İbrahim Cecen University.

### *Sample collection*

All plants collected and identified by Murat Kürşat in 2020 from different regions of Turkey. *Diplotaenia cachrydifolia* Boiss. was collected in September from Karz Mountain, Bitlis at 2250 m. *Ferulago stellata* Boiss. and *Rhabdosciadium microcalycinum* Hand.-Mazz. were collected in September from Kambos Mountain, Bitlis at 1750 m. *Anarrhinum orientale* Benth. was collected in August from Kambos Mountain, Bitlis at 1600 m. *Fumaria asepala* and *Zosima absinthifolia* (Vent.) Link was collected in May from the Baskil region of Elazığ. *Salvia pseudeuphratica* Rech.f. was collected in July from Keban, Elazığ. Voucher specimen for each plant is available at Bitlis Eren University.

### *Preparation of plant extracts*

For each plant, 50 g of dried plant material was taken. The coarsely ground parts were extracted with methanol at room temperature. The extraction process was repeated 4 times, and plants were extracted with a magnetic

stirrer for 12 hours by adding 1 L of fresh methanol each time. The methanolic phases obtained at the end of the extraction were combined and the methanol was evaporated with a rotavapor and the dry crude extracts were obtained for each plant. Extracts of *Z. absinthifolia*, *A. orientale*, *F. asepala*, *F. stellata*, *S. pseudeuphratica*, *R. microcalycinum* and *D. cachrydifolia* were numbered 1 to 7, respectively.

### *Carbonic anhydrase I/II inhibition*

The activities of carbonic anhydrase I and II (CA I/II) were assessed spectrophotometrically at 348 nm by monitoring the conversion of 4-nitrophenyl acetate (NPA) to 4-nitrophenolate (NP) per minute over 3 minutes incubation at 25 °C. Each reaction mixture comprised:

- 1.4 mL of 50 mM Tris-SO4 buffer (pH 7.4)
- 1 mL of 3 mM NPA
- 0.5 mL of deionized water
- 0.1 mL of enzyme solution

for a total volume of 3.0 mL [33].

### *Cholinesterase enzymes inhibition*

The inhibitory activity against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) was evaluated using a standardized assay system. Each reaction mixture contained:

- 5-60 µL of inhibitor sample solution
- 200 µL of buffer (1 M Tris-HCl for AChE, PB for BChE; pH 8.0)
- 50 µL of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (0.5 mM)
- 50 µL of acetylthiocholine iodide/S-butrylthiocholine chloride (10 mM)
- 10 µL of enzyme solutions at concentration of 0.28 U/mL for AChE and 0.32 U/mL for BChE [34].

The reaction was initiated by adding the enzyme, and the absorbance at 412 nm was monitored at 25º every two minutes for a total of 6 minutes incubation. A control lacking the inhibitor was included for comparison [35].

### *General enzyme inhibition studies*

The inhibitory activities of plant extracts against carbonic anhydrases I and II (CA I/II), acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) were determined using spectrophotometric methods (Agilent BioTek Epoch Microplate Spectrophotometer, California, USA). Acetazolamide and galantamine served as reference molecules for CA I/II and AChE/BChE, respectively. Meticulously prepared stock solutions of the investigated extracts

and reference molecules were dissolved in dimethyl sulfoxide at a concentration of 1 mg/mL. Subsequent dilutions using distilled water were performed to achieve a 1000-fold dilution. The inhibitory activity of these extracts on the aforementioned enzymes was assessed at seven distinct concentration points. The detailed methodology employed in this study adheres to procedures established in previously published works. Inhibitory properties for all extracts and standard drugs were calculated as IC50 values determined graphically from inhibition curves against the log inhibitor concentration and the percentage of inhibition. $IC_{50}$ values represent the inhibitor concentration required for 50% inhibition of the enzyme. [29, 36, 37].

### *Statistical analysis of data*

The SPSS program was used for the analysis of the experimental data. The standard deviation for $IC_{50}$ values were calculated and results were reported as mean ± standard deviation.

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