S. Altın and E. Köksal / Hacettepe J. Biol. & Chem., 2024, 52 (1), 1-9

Research Article

Hacettepe Journal of Biology and Chemistry

journal homepage: www.hjbc.hacettepe.edu.tr

Glaucium cappadocicum: *in vitro* Antioxidant, Antimicrobial, Anticholinesterase Inhibition Properties

Glaucium cappadocicum: *in vitro* Antioksidan, Antimikrobiyal, Antikolinesteraz İnhibisyon Özellikleri

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ABSTRACT

This study evaluated the total phenolic and flavonoid material content as well as the antioxidant, antibacterial, and anticholinesterase activities of extracts prepared from the endemic *Glaucium cappadocicum* plant. The extracts' antioxidant properties were assessed using the DPPH and FRAP methods, their antibacterial effects were assessed using the disk diffusion method on Gram positive and Gram negative bacteria, and their ability to inhibit cholinesterase activity was assessed using the Ellman method. In all biological activity assays, the alkaloid extract had the maximum activity. Especially in enzyme inhibition studies, alkaloid extract showed stronger inhibition than the standard inhibitor tacrine. These findings are the first report showing that high concentrations of compounds with antioxidant, anti-acetylcholinesterase, and antibacterial activity can be found in extracts from Glaucium cappadocicum.

Key Words

Glaucium cappadocicum, enzyme inhibition, antimicrobial, antioxidant.

ÖZ

Bu çalışmada Türkiye'ye endemik *Glaucium cappadocicum*'dan hazırlanan ekstrelerinin toplam fenolik ve flavonoid madde içeriği belirlenerek, antioksidan, antimikrobiyal ve antikolinesteraz aktiviteleri değerlendirildi. Ekstrelerin antioksidan aktiviteleri DPPH ve FRAP yöntemleriyle, antimikrobiyal testler Gram pozitif ve Gram negatif bakteriler üzerinde disk difüzyon yöntemiyle, antikolinestreaz inhibisyon çalışmaları ise Ellman yöntemiyle belirlenmiştir. Tüm biyolojik aktivite testlerinde en yüksek aktiviteyi alkaloid ekstresi sergilemiştir. Özellikle enzim inhibisyon çalışmalarında alkaloid ekstresi standart inhibitör olan takrinden daha güçlü inhibisyon sergilemiştir. Bu bulgular, Glaucium cappadocicum'dan yapılan ekstraktlarda antioksidan, anti-asetilkolinesteraz ve antibakteriyel aktiviteye sahip bileşiklerin yüksek konsantrasyonlarda bulunabileceğini gösteren ilk rapordur.

Anahtar Kelimeler

Glaucium cappadocicum, enzim inhibisyonu, antimikrobiyal, antioksidan.

Article History: Received: Jul 25, 2023; Revised: Oct 12 16, 2023; Accepted: Oct 19, 2023; Available Online: Dec 1, 2023.

DOI: https://doi.org/10.15671/hjbc.1332418

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INTRODUCTION

The search for herbal drugs, herbal extracts, or herbal medicine raw materials is advancing quickly due to the adverse effects of synthetic drugs on humans that have emerged in recent years and the fact that, in some situations, such drugs are not sufficient against pain or inflammation treatments. As a result, drug delivery systems are constantly exploring for all-natural, more efficient medical treatments and medications. Turkey's rich botanical endowment, which includes a wide array of medicinal and fragrant plants, has contributed to its ascent to prominence. Currently, the most effective elements of traditional treatment approaches are medicinal herbs. The therapeutic value of a plant rises depending on the active ingredients of the medicinal and aromatic plants employed. The *Papaveraceae* family is one of these priceless plants. Because of the significant alkaloids and other bioactive substances, they contain, members of the *Papaveraceae* family have long piqued the interest of scientists. One of these important members is the ***Glaucium*** genus, which produces flowers in the *Papaveraceae* family that are fragrant and colorful. It comprises 28 species worldwide and is widespread in Central Asia, North Africa, and Europe [1-3]. Turkey is home to almost one-third of all species on earth [4]. The ***Glaucium*** genus is abundant in alkaloids, just like other members of the *Papaveraceae* family. The genus is dominated by aporphine group alkaloids, which are pharmacologically active [5–6].

Due to its rich alkaloid content, it is used in both traditional medicine and pharmacology. The seeds and aerial parts of *Glaucium* have historically been employed in traditional medicine as a laxative, anti-diabetic, and treatment for dermatology [6-9]. At the same time, diabetic, antimicrobial [10], antiviral, antifungal, anti-inflammation [11], antiulcerogenic, antioxidant, antitussive, hypoglycemi [5], antiproliferative, cytotoxic, anticancer [12], anticholinesterase [13-14], antinociceptive [15], with its pharmacological activities have also been reported. *G. cappadocicum* is an endemic species found only in Erzincan, and the only investigations on it have been morphological. Even though the ***Glaucium*** genus has a large number of secondary metabolites, it is a significant shortcoming that this species has not been studied pharmacologically. In this study, we aimed to fill the gap in the literature by determining the pharmacological or phytochemical properties of the species. Additionally, as this will be the first report for the *G. cappadocicum* species, all the findings from our study that will be presented are crucial.

MATERIALS and METHODS

Herbal source

Glaucium cappadocicum was collected from the Erzincan-Kemah-Yahşiler Road region in June 2021. Coupon samples were kept in Erzincan Binali Yıldırım University Biology Department Herbarium with Altın S.7 code. The drying process of the plant was carried out in a sun-free room and at room temperature. After the dried plant was pulverized with liquid nitrogen, it was used in biological activity tests.

Preparation of the extract

Two different extraction processes were performed on the strain: sequential extraction and alkaloid extraction. 100 g from the powdered material was weighed for sequential extraction, and 2x500 mL each of hexane, ethyl acetate, methanol, and water were added for 24 hours of extraction in order of increasing polarity. Four different extracts were prepared by evaporation of the solvents. Alkaloid extract; 10 g of the plant was extracted in methanol (3 times x100 mL). The methanol was removed from the extract after filtering, and the remaining material was dissolved in 50 mL of 3N hydrochloric acid. The filter was then used to remove it. The filtrate was extracted three times with dichloromethane (25 mL) after the pH was brought down to 8 with NaOH (3N). After drying the obtained extract with $MgSO_4$, the solvent was removed to create crude alkaloid extract. Until they were employed, all extracts were kept at +4 °C [16].

Antioxidant Activities

Activity of DPPH in eliminating free radicals

As detailed in our earlier study [17-18], the free radical (DPPH•) scavenging activities of extracts were assessed by adapting the method reported from Blois [19] to ELISA spectroscopy. Antioxidant Activities of ethyl acetate (GCE), methanol (GCM), water (GCW), and alkaloid (GCA) extracts prepared from the aerial parts of ***G. cappadocicum*** (GC) plants were tested DPPH method. Samples at different concentrations (20, 40, 80, 120, 160, 240, 320 and 400 µg/mL) were prepared from 1 mg/mL (extract and standard) stock solutions and to make the volume 3 ml, methanol was used. Then, a 0.26 mM DPPH solution (1 mL) was added, vortexed, and incubated for half an hour in the dark. IC_{50} (µg/mL) values were calculated by determining the % activity values of

the extracts. Trolox was used as the standard substance.

FRAP, or ferric reducting antioxidant capability

By applying ELISA spectroscopy to the technique described by Oyaizu M. [20], the reducing power activity test was carried out [17,18]. The reducing power activity of ethyl acetate (GCE), methanol (GCM), water (GCW) and alkaloid (GCA) extracts prepared from the aerial parts of the *G. cappadocicum* (GC) plant was tested by the FRAP method. Stocks (1 mg/mL) were prepared from the extracts and the standard substance. The test tubes received 100 µL of stock solution, 1.15 mL of a buffer with phosphate (0.2 M, pH 6.6), and 1.25 mL of potassium ferric cyanide [$K_3Fe(CN)_6$] (1%) in that order. It was left to incubate at 50 °C for 20 minutes. After incubation, the reaction medium was added 1.25 mL of 10% trichloroacetic acid and 0.25 mL of 0.1% $FeCl_3$ solutions. At 700 nm, absorbance was measured. The outcomes are presented as µg trolox equivalent/g extract.

Determination of total phenolic contents

Folin-Ciocalteu reagent was used to assess total phenolic content as described in our prior studies [17-18]. 4.5 mL of distilled water, 100 mL of the Folin-Ciocalteu reagent, and 100 µL of the sample (made at a concentration of 1 mg/mL) were put in sequentially. The mixture was then let to sit at ambient temperature for 10 minutes. The 300 mL of 2% Na_2CO_3 solution was then added, vortexed, and incubated for two hours at room temperature. At 760 nm, absorbance was measured following incubation. Total phenolic contents were determined as mg of gallic acid equivalent / g extract [21].

Determination of total flavonoid contents

The aluminum chloride colorimetric method was used to quantify the total flavonoid concentration [22]. 100 µL of the stock solutions prepared in 1 mg/mL methanol were taken, and over 4.7 mL of methanol was added. Then, NH_4CH_3COO (100 µL, 1 M) and $AlCl_3$ (100 µL, 10%) solutions were added, respectively. Absorbance was measured at 415 nm following a 45-minute incubation period at room temperature. Total flavonoid content was given as mg quercetin equivalent/g extract [18].

Antimicrobial Activity

Bacterial isolates

Antimicrobial activities of ethyl acetate (GCE), methanol (GCM), water (GCW), and alkaloid (GCA) extracts prepared from the aerial parts of *G. cappadocicum* (GC) plants were tested on standard bacterial isolates. For studies, Gram (-) of these bacteria were *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 700603, colistin-resistant *Escherichia coli* ATCC 19846, Gram (+) *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumonia* ATCC 45616, *Staphylococcus aureus* ATCC 25922, and additionally *Candida albicans* were used.

Determination of antimicrobial activity: Inoculum preparation and disk diffusion method

Inoculum preparations [17-18] were prepared as detailed in our article. The Kirby-Bauer disk diffusion method

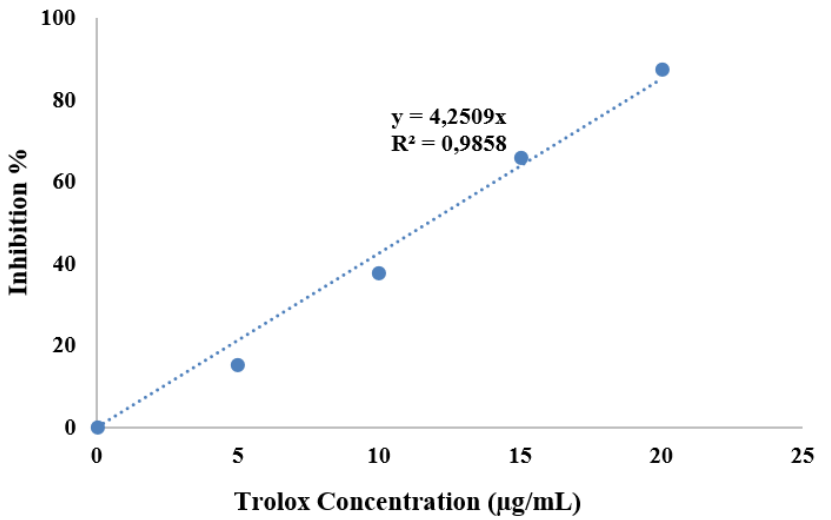

Figure 1. Trolox standard curve.

was used to determine the antibacterial activity studies of [23]. With the aid of a cotton swab, the produced bacterial solutions were applied to Mueller Hinton agar medium (Biomerieux, France) and allowed to dry for 15 minutes. For the isolation of Streptococcus pneumoniae, Mueller Hinton Fastidious (5% defibrinated horse blood and 20 mg/l -NAD) agar was utilized. 200 mg/mL stocks were prepared from all extracts. 20 µL of these prepared stocks were added to 6 mm diameter standard blank discs (Bioanalyse Turkey) and left to dry (15 minutes). After the treated discs were dried, they were placed on the medium in a sterile manner, and the petri dishes were incubated (at 37 °C for 18 hours). Following incubation, the non-bacterial zone's diameter surrounding the discs was calculated. The same procedures were repeated three times for each bacterium. Vancomycin 5 µg/mL and Erythromycin 15 µg/ml for Gram (+) bacteria and Amikacin 30 µg/mL disc for Gram (-) bacteria were used as standard materials.

AChE/BChE activity determination and inhibition studies

AChE and BuChE inhibitory activities were determined spectrophotometrically according to Ellman's colorimetric method [24]. The substrates used were acetylthiocholine iodide (AChI) and butyrylthiocholine iodide (BChI). Ellman's reagent DTNB (5,50-dithio-bis (2-nitrobenzoic acid), was used for the measurement of AChE and BChE activities. 100 µL of buffer for activity measurement (1 M, pH 8.0: Tris-HCl buffer for AChE and phosphate buffer for BChE), 10 µL of sample solution prepared at different concentrations in deionized water, 50 µL of DTNB (10 mM), 10 µL of enzyme (0.385 U/mL for the AChE test and 0.880 U/mL for the BChE test). The reaction was started by adding and finally 50 µL of AChI /BChI solution. The absorbance of the yellow color 5-thio-2-nitrobenzoic acid formed as a result of the reaction is measured at 412 nm. Results are given as % activity and IC_{50} [25-27].

RESULTS and DISCUSSION

Antioxidant activity

Natural antioxidants derived from plants serve as the primary source of defense against oxidative stress and illnesses brought on by free radicals. Free radicals are produced more often, which leads to oxidative stress. Biomolecules are oxidized as a result of oxidative stress, which results in cellular harm and death. As a result, oxidative stress is to blame for the aging process as well as a number of illnesses, including cancer, diabetes, cardiovascular, neurodegenerative, and inflammatory disorders. The extracts from *G. cappadocicum*, on which the antioxidant data in this study are based, may be able to prevent oxidative stress because they can scavenge certain free radicals.

DPPH is frequently used to determine the antioxidant activity of natural products thanks to its faster analysis advantage compared to other methods [28]. The free radical scavenging activity of *G. cappadocicum* extracts (Table 1) was determined by the DPPH method. The free radical scavenging power of the samples increased in a dose-dependent manner. While all extracts showed good activity, alkaloid and especially water extracts showed quite strong antioxidant activity. The DPPH radical scavenging activity was determined as IC_{50}:13.73±0.17 µg/mL for the water extract and IC_{50}=37.14±0.4 µg/mL for the alkaloid extract. *Glaucium flavum* ethanol extract's a prior investigation was established to be IC_{50} :140 µg /mL [29]. In another study on *Glaucium grandiflorum* var. *Grandiflorum*, the DPPH IC_{50} results for ethyl acetate and methanol extracts were found to be 250 µg/mL and 55 µg/mL, respectively [30].

Flavonoids are special groups of phenolic compounds isolated from various plants. It has many beneficial properties, such as antioxidant, antimicrobial, and anticancer properties. Therefore, they constitute the most important source for antioxidant studies [28]. Total phenolic and flavonoid results for *G. cappadocicum* are presented (Table 1). Total phenolic content was determined as GCM (285,037), GCE (243.19), GCA (149.67), and GCS (140.96) mg GAE/g, respectively. Total flavonoid content was found to be GCE (195.52), GCM (106.47), GCW (44.57), and GCA (29.57) mg QE/g. The total phenolic content of *Glaucium flavum* was determined to be 158.3 mg GAE/g for the ethanol extract, and the total flavonoid content was determined to be 128.43 mg CE/g [29]. Another study uses *G. grandiflorum* methanol extract, total phenolic compounds were found to be 3.54 mg/g gallic acid equivalents, and total flavonoids were found to be 3.15 mg/g catechin equivalents [17].

Antimicrobial activity

S.aureus is a bacterium that frequently infects people. They are primarily discovered in the nose and throat, in acne, and in human and animal feces. They are a common source of staphylococcal food poisoning and are also present in hospitals, hospital employees, and hand

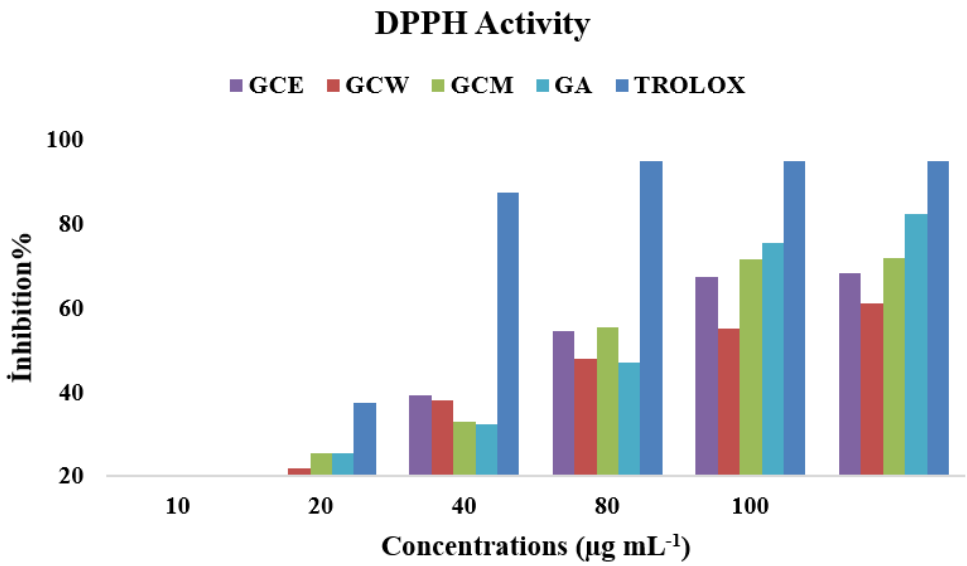

Figure 2. DPPH radical scavenging activity of *G. cappadocicum* exracts.

Table 1. Antioxidant activities of aerial parts of *G. cappadocicum.*

Extract/Positive Control	DPPH• scavenging IC_{50} (µg mL^{-1})	Total phenolics mg GAE g^{-1} Extract	Total flavonoids mgQEg^{-1} Extract	Reducing power mgTE g^{-1} Extract
GCH	-	-	-	-
GCE	44.87±0.4	243.19 ±1.89	195.52±0.7	159,1667±1.40
GCM	48.79±0.12	285,037±0.71	106.47±1.03	141.70±0.63
GCW	13.73±0.17	140.96±0.14	44,57±0.43	48.85±0.83
GCA	37.14±0.4	149.67±0.12	29.57±14	115.39±0.2
Trolox	11.95±0.15			

Table 2. Antimicrobial activity results of *G. cappadocicum* extracts for gram (+) and gram (-) bacterial strains

Extracts and standards	Concentration (mg/ disk)	Zone Diameter of Standard Strains (mm ± SD)			
		S. aureus	*E. faecalis*	*S. pneumoniae*	*Cd.alb.*
GCA	4	13.6±0.23	-	12.3±0.57	13±0.57
GCE	4	10.5±0.57	-	-	
GCM	4	10.5±0.20	-	-	10±0.21
GCW	4	6.0±0.57	-	-	6.0±0.57
Control	mcg /disk				
Amikacin	30	-	-	-	
Erythromycin	15	21.3±0.57	-	-	-
Vancomycin	5	-	14.3±0.57	25.3±0.57	

food preparation areas [31].

A deadly gram positive bacteria called *Streptococcus pneumoniae* causes meningitis, bronchitis, pneumonia, and otitis media. The need for new antibiotics grows as *S. pneumoniae* develops acquired antimicrobial resistance [32]. Multiple antibiotic resistance of microorganisms complicates treatment. This increases the search for new antibiotic sources.

In our study, we used the disk diffusion method, which is a common method for antimicrobial activity tests. For antimicrobial studies of various extracts of the plant, studies were carried out using seven bacteria and one fungus. *Staphylococcus aureus* was significantly inhibited by all extracts. Akaloid extract significantly inhibited the growth of *C.albicans* fungus and bacteria *S.aureus* and *S.pneumoniae*. The antimicrobial activity results of *G. cappadocicum* revealed that the alkaloid extract showed a strong inhibitory effect (Table 2). This was followed by methanol and ethyl acetate extracts, while the water extract exhibited moderate activity.

In a study on ***Glaucium*** species, studies were conducted in the concentration range of 125-1000 mg/mL for *G. vitellinum* and *G. aucheri* alkaloid extracts. Three gram-negative bacteria, including *E. coli, S. typhi, P. aeruginosa,* and *S. aureus,* were determined by the disk diffusion method on *C. albicans* as gram positive bacteria and yeast. No inhibitory effect was detected with this method [33]. In another study, 9, 10, 12, 17, and 18 mm inhibition was determined against *S. enteritidis, E. coli, S. aureus, B. anthracis,* and *Proteus*, respectively, *at G. elegans* 750 µg/disc concentration [34].

Enzymes results

Alzheimer's disease is a disease that causes a decline in neurotransmitters in the brain. Acetylcholine release is reduced as a result of the disease. Low acetylcholine release causes issues with information transfer and nerve conduction. Suppressing the AChE enzyme, which degrades acetylcholine, is one approach to solving these issues. Cholinesterase enzyme inhibitors are typically employed for this purpose. The capacity of cholinesterase enzyme inhibitors to boost cholinergic function by lowering acetylcholine breakdown serves as the basis for their mode of action. For the symptomatic management of individuals with mild to moderate Alzheimer's disease, numerous plant-derived alkaloids that act as AChE inhibitors (AChE-I) are available. These compounds can be used as models for the development of anti-Alzheimer medications. Previous research has demonstrated that a number of plant extracts and the bioactive substances they contain represent intriguing alternatives to currently used therapies for neurodegenerative illnesses. Many significant age-related illnesses, including AD, share the characteristics of elevated oxidative stress and decreased cellular energy metabolism. Proteins, lipids, and DNA with oxidative modifications are found in excessively high concentrations in the cells of AD patients' brains. Compounds that are not only AChE inhibitors but also have anti-inflammatory or antioxidant properties are considered attractive due to the possibility of creating multitarget drugs that act by different mechanisms [35].

Treatment for Alzheimer's disease must include the inhibition of cholinesterase enzymes. All of the extracts we produced were utilized to treat Alzheimer's disease and produced outcomes that were extremely similar to those of the standard drug we used in our study, tacrine. Additionally, the plant's alkaloid extract demonstrated a significantly stronger inhibition than tacrine (Figure 3 and Table 3). The enzyme inhibition values of the alkaloid extract were determined as AChE IC_{50}: 0.55 and BChE IC_{50}: 0.65 µg/mL, while the IC_{50} values for tacrine were determined as 3.19 µg/mL and 2.54 µg/mL, respectively.

CONCLUSION

Alkaloids are highly significant secondary metabolites that have extensive pharmacological effects and strong biological activity. Alkaloids with biological significance make up 50% of all natural compounds with plant origin.

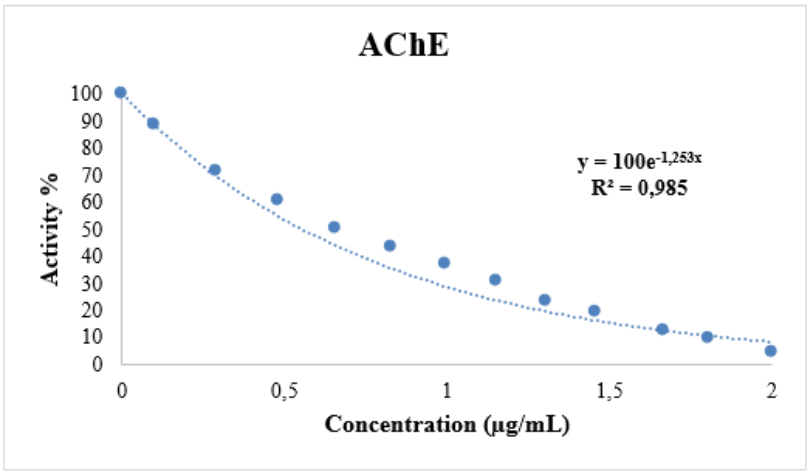

Figure 3. *G. cappadocicum*'s graphs of % activity against cholinesterase enzymes.

Table 3. IC_{50} values for acetylcholinesterase and butyrylcholinesterase.

	IC_{50} (µg/mL)			
Inhibitor	AChE	R^2	BChE	R^2
GCH	3.27	0.8741	4.15	0.9665
GCE	3.48	0.966	3.81	0.9294
GCM	3.74	0.9499	2.92	0.9409
GCW	4.05	0.9574	3.65	0.9576
GCA	0.55	0.985	0.65	0.9353
Tacrine	3.19	0.9896	2.54	0.9878

Alkaloids are employed as medicines to treat ailments as a result of these characteristics. With its analgesic effects, morphine is remarkable, as are vincristine and vinblastine's effectiveness in the treatment of cancer. Alkaloids are crucial to the search for novel medications for all of these reasons. Due to the bioactive alkaloids they contain, plants in the *Glaucium* family are extremely significant. Biological activity studies were carried out on extracts of *G. cappadocicum* prepared by different processes. Sequential extraction and alkaloid extracts were prepared, following antioxidant, antimicrobial, and anticholinesterase activities were evaluated for these extracts. It has been demonstrated with data that *G. cappadocicum* is a powerful antioxidant source. Alkaloid, ethyl acetate, and methanol extracts show strong antimicrobial effects for *S. aureus, S. pneumoniae,* and *C. albicans*, while water extract is moderately inhibitory. All extracts of *G. cappadocicum* showed potent anticholinesterase activity. In particular, the alkaloid extract exhibited higher inhibition than tacrine, used as a drug in the treatment of AD. This draws attention to the strong inhibitory activity of the alkaloid extract of *G. cappadocicum*, especially for the AChE/BChE enzyme. All these results indicate that *G. cappadocicum* extracts are not only a potent cholinesterase inhibitor, but also highlight the possibility of creating multi-target drugs with antimicrobial and antioxidant properties.

ACKNOWLEDGEMENTS

For the identification of plant materials, the authors are especially grateful to Prof. Dr. Ali Kandemir (Erzincan Binali Yıldırım University, Biology Department).

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