Chemical Profiling of *Stachys cretica* subsp. *anatolica* Rech. f. (Endemic) Essential Oils and their Methanol Extracts with Evaluation as Enzyme Inhibitors, Antioxidant, and Antimicrobial Agents

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Enzyme inhibition activities, phenolic compounds, antioxidant activities, bioactive compounds, antimicrobial activities, and chemical components of essential oil and methanol extracts obtained from the aerial parts of *S. cretica* subsp. *anatolica* were investigated. The main phenolic compounds of aerial parts were catechin, oleuropein, and epicatechin. The determined enzyme inhibitor activities highlight the potential of *S. cretica* subsp. *anatolica* as a source of bioactive compounds, particularly for carbonic anhydrase and cholinesterase inhibition. The essential oil and methanol extract exhibited remarkable activities against CA-II, AChE, and BChE, although they were less potent than standard inhibitors. The essential oils generally showed stronger antimicrobial activity compared to the 30% methanol extracts across most bacterial and fungal strains, as evidenced by minimum lethal concentration (MLC) and lower minimum inhibitory concentration (MIC) values and larger inhibition zones. Chloramphenicol used alone exhibited the highest antimicrobial efficacy, with the lowest MIC and MLC values and the largest inhibition zones. The essential oils of *S. cretica* subsp. *anatolica* were determined as esters, oxygenated sesquiterpenes, and aldehydes in aerial parts. The main components were found to be hexahydrofarnesyl acetone in the aerial parts.

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INTRODUCTION

The Lamiaceae family has a wide distribution in Anatolia. One of the largest genera of this family is *Stachys* L., which contains about 300 taxa. This genus is generally found in the temperate Irano-Turanian and the Mediterranean regions. Türkiye is one of the richest countries in terms of *Stachys* taxa, with 83 recorded species and a 48% endemism rate (Bhattacharjee 1980; Mabberley 1987; Goren *et al.* 2011).

Members of the *Stachys* genus are used as traditional medicine and consumed as wild tea in Anatolia as well as in Iran. It is known as mountain tea. It is used for the same

purpose as sage, to treat skin infections, asthma, rheumatic and respiratory disorders, digestive problems, inflammatory disorders, as a wound healing agent, antiphlogistic, antianxiety, cholagogic, sedative, throat pains, tumors, coughs, and kidney diseases (Kartsev *et al.* 1994; Yesilada *et al.* 1999; Maleki *et al.* 2001; Rabbani *et al.* 2003; Amirghofran *et al.* 2006; Maleki-Dizaji *et al.* 2008; Khanavi *et al.* 2009; Ozturk *et al.* 2009; Goren *et al.* 2011).

Stachys taxa include at least nine natural product chemicals, which include alkaloids, carbohydrates, essential oils, flavonoids, iridoids, lipids, phenylpropanoid glycosides, steroids, and terpenoids (Radulović *et al.* 2006; Ahmad *et al.* 2007; Kotsos *et al.* 2007; Radulović *et al.* 2007; Soliman *et al.* 2007; Toshihiro *et al.* 2008; Giuliani *et al.* 2009).

The main components of most *Stachys* species were found to be caryophyllene oxide, *β*-caryophyllene, linalyl acetate, linalool, *β*-pinene, and germacrane D (Harmandar *et al.* 1997; Kaya *et al.* 2001; Skaltsa *et al.* 2001; Radulovic´ *et al.* 2007; Goren *et al.* 2011). In the meantime, the presence of diterpenoids, for instance kaurane, pimarane, labdane, and abietanes, were reported to be minor compounds of some *Stachys* essential oils (Piozzi and Bruno 2009; Goren *et al.* 2011). *S. cretica* subsp. *symrnaea* is an endemic and widespread species in northwestern, western and southern Anatolia. In the study of the chemical composition and antimicrobial activity of *S. cretica* subsp. *symrnaea* essential oil, it was reported that the main component of the oil was determined to be *β*-caryophyllene (51.0%) (Ozturk *et al.* 2009; Goren *et al.* 2011).

This plant is well known for its antibacterial and antioxidant effects in medicine and pharmacology (Grujic-Jovanovic *et al.* 2004; Erdemoglu *et al.* 2006). It can also help in continuous bioactive extraction of natural products. Moreover, this plant species could be beneficially used for extraction of useful medicinally important metabolites (Ozdemir *et al.* 2017). In the meantime, the emergence and spread of pathogenic bacteria has led to an increase in bacterial infections, raising concerns about the development of new antimicrobial meditations. In addition, because these bacteria form microbial biofilms and exhibit resistance to various drugs, infection control and healthcare face a significant global challenge (Selim *et al.* 2024).

The aim of this study was to determine and compare the enzyme inhibition, phenolic compound, antioxidant and antimicrobial activities of essential oil and methanol extracts obtained from the aerial parts of *S. cretica* subsp. *anatolica* plant. The article differs from other studies in that the region where the plant sample was taken, the analysis and comparison of methanol extract and essential oil samples are considered for the first time.

EXPERIMENTAL

Plant Material

In this study, samples of *S. cretica* subsp. *anatolica* (endemic) were collected from the Bayburt province (Türkiye), Gümüşhane road, near the Bayburt (coordinates: 40°20'44"N, 40°01'09"E, altitude: 1632 m, date: 22 June 2024, Habitat: hill sides). Figure 1 illustrates habitus, flowers, and leaves of the plant. A total of 1000 g of plant (dry weight) material was collected for analysis. The taxonomic identification of the plant was verified by Associate Professor Abdurrahman Sefalı from the Department of Primary Education, Faculty of Education, Bayburt University, Bayburt, Türkiye. The collected sample was

deposited in the Herbarium of Bingöl University, where it was assigned the reference number BIN Sefalı 1202.

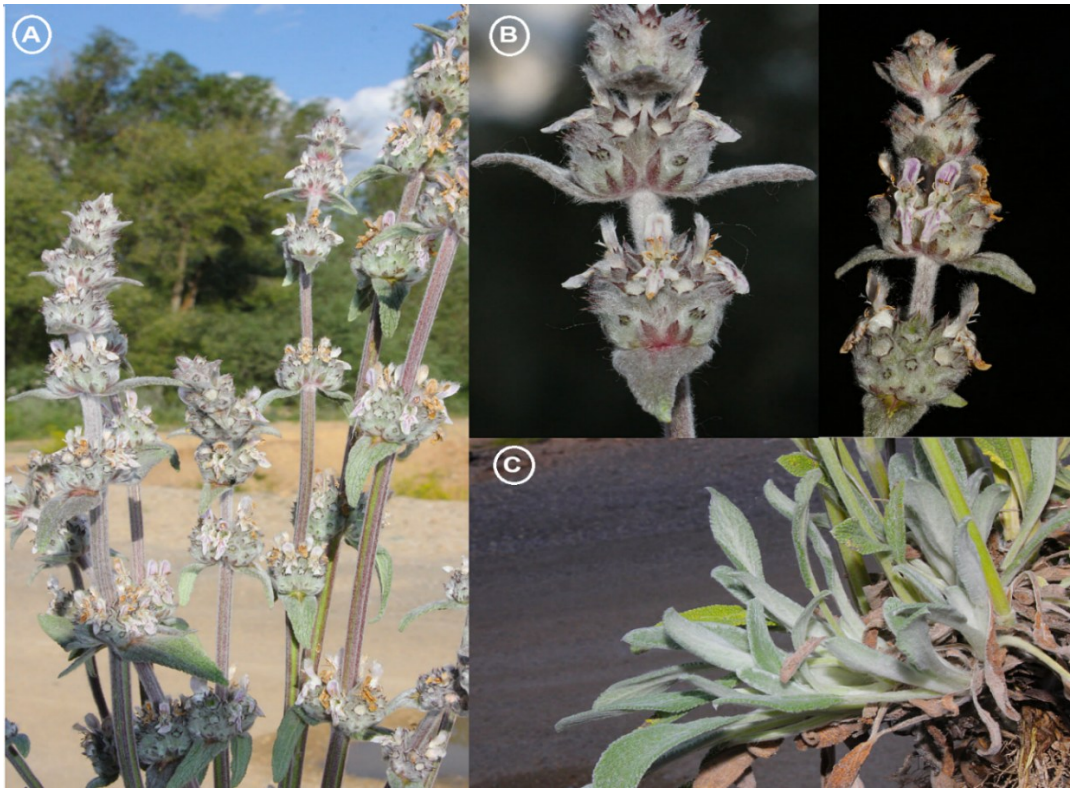

Fig. 1. S. *cretica* subsp. *anatolica*: A. Inflorescence, B. Flowers, and C. Basal leaves

Extraction Procedure

Extraction essential oil

A 500 g sample of the plant material was dried under shaded conditions, finely ground, and passed through a 250-micron sieve. From this processed material, 100 g was selected for essential oil extraction. The extraction was carried out using hydrodistillation at 100 °C and 4 h, employing a modified Clevenger apparatus equipped with both internal and external cooling systems. The resulting essential oil was dissolved in hexane, filtered through a 0.45-micron membrane, and securely stored in amber-colored vials to preserve its integrity (Öz *et al.* 2023).

Methanolic extraction

The extraction process was performed using a 3-L, 320 W ultrasonic bath (Bandelin Ultrasonic Bath). Initially, 10 g of the ground plant material was weighed, and 50 mL of a 30% aqueous methanol (MeOH) solution was added. The mixture was then subjected to ultrasound-assisted extraction for 60 min at a controlled temperature of 40 °C. After extraction, the solution was filtered twice using Whatman 1 filter paper and subsequently centrifuged at 4000 rpm for 10 min to separate the plant extracts. The resulting supernatant was carefully transferred to a beaker, and the methanol-water mixture was fully evaporated at 40 °C to obtain the final extract (Öz *et al.* 2023).

Enzyme Inhibitory Activities of the Extracts

Activity assay for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes

The activities of AChE and BChE enzymes were spectrophotometrically assessed using the Ellman method. In this assay, acetylthiocholine iodide-a thioester-is used as a substrate instead of acetylcholine. Upon enzymatic hydrolysis by acetylcholinesterase, thiocholine is released, which subsequently reacts with DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid)). This reaction produces a yellow-colored compound, 5-thio-2-nitrobenzoic acid (TNB), whose formation is monitored by measuring absorbance at 412 nm (Ellman *et al.* 1961). For butyrylcholinesterase, the procedure is identical except that butyrylthiocholine iodide is used as the substrate. Enzyme activities were recorded at various inhibitor concentrations, and percentage activity was calculated. IC_{50} values were determined from the inhibition curves using Lineweaver–Burk plots (Lineweaver and Burk 1934).

Carbonic anhydrase enzyme activity assay

This method is based on the esterase activity of carbonic anhydrase, which catalyzes the hydrolysis of *p*-nitrophenyl acetate-used as the substrate-into *p*-nitrophenol or *p*-nitrophenolate, both of which absorb at 348 nm. Because these two forms exhibit identical absorbance at this wavelength, the dissociation of a proton from the phenolic OH group does not influence the measurement (Landolfi *et al.* 1997).

α-Glucosidase enzyme activity assay

α-Glucosidase activity was determined following the method described by Tao *et al.* (2013), using *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) as the substrate. The enzymatic reaction was monitored by measuring absorbance at 405 nm. Inhibitory effects were evaluated by testing various concentrations, and the percentage of residual activity was plotted against inhibitor concentration. IC_{50} values were derived from the resulting inhibition curves based on the Lineweaver–Burk model (Lineweaver and Burk 1934).

α-Amylase enzyme activity assay

α-Amylase inhibitory activity was assessed using a modified version of the Caraway-Somogyi iodide/potassium iodide (IKI) method, adapted from Yang *et al.* (2012). Briefly, 25 µL of each sample was added to a 96-well microplate, followed by 50 µL of α-amylase solution prepared in phosphate buffer (pH 6.9, containing 6 mM NaCl). After a 10-min incubation at 37 °C, 50 µL of 0.05% starch solution was added. A blank without enzyme was also prepared in parallel. The plate was then incubated for another 10 min at 37 °C. To stop the reaction, 25 µL of 1 M HCl was added, followed by 100 µL of IKI reagent to develop color. Absorbance was measured at 630 nm using a microplate reader. Acarbose served as the reference inhibitor, and IC_{50} values were calculated to express the inhibitory potential of the samples.

Determination of Phenolic Profiles Using LC-MS/MS Analysis

The LC-MS/MS analyses were performed using a Thermo Scientific Dionex Ultimate 3000 UHPLC system coupled with a TSQ Quantum Access Max tandem mass spectrometer (TSQ Quantum Access Max, Thermo Fisher Scientific, San Jose, CA, USA). The liquid chromatography system was equipped with an autosampler, degasser, dual pump, and column compartment. Chromatographic separation was performed on a C18

reversed-phase Inertsil ODS HYPERSIL analytical column (250 mm × 4.6 mm, 5 µm), maintained at a constant temperature of 30 °C.

The mobile phase consisted of two components: phase A was ultrapure water containing 0.1% formic acid, and phase B was methanol. The gradient elution program was applied as follows: 0 to 1 min, 0% B; 1 to 22 min, 95% B; 22 to 25 min, 95% B; and 25 to 30 min, 100% B. The total run time, including re-equilibration, was set to 34 min. The injection volume was 20 µL, and the flow rate was adjusted to 0.7 mL/min.

Following extensive optimization trials to ensure effective ionization and separation of the target phenolic compounds, this mobile phase composition and gradient program were selected. The phenolic compounds listed in Tables 2 and 3 were analyzed using this liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method, and a chromatogram of the standard phenolic compounds is also provided. The analytical method applied in this study was adapted from the protocol developed by Kayir *et al.* (2023).

Determination of Antioxidant Capacity

The methanol extract and essential oil of *S. cretica* subsp. *anatolica* was evaluated for its antioxidant potential using multiple methods. The ferric ion (Fe^{3+}) reducing capacity was measured through the FRAP assay, while its ability to neutralize free radicals was assessed using ABTS and DPPH assays. Furthermore, the extract's bioactive composition was quantified by determining the total antioxidant capacity (TAC), total flavonoid content (TFC), and total phenolic content (TPC). These analyses provided a comprehensive understanding of the extract's antioxidant properties and its underlying bioactive constituents.

The methanol extracts and essential oil were analyzed for their ferric ion reducing antioxidant power (FRAP) according to the procedure outlined by Fidan *et al.* (2023). A FRAP reagent was prepared, and distilled water (500 µL) served as the blank. Standard solutions (250 µL) were processed under identical conditions. The FRAP values of the samples were determined using a calibration curve ($y = 0.012x + 0.0516$, $R^2 = 0.998$) generated from $FeSO_4$ solutions. The results were expressed as milligrams of $FeSO_4$ equivalents per 100 g of sample, reflecting the total iron-reducing capacity.

The ABTS radical scavenging activity was evaluated following the procedure described by Kobya *et al.* (2024). An ABTS solution was prepared, and methanol (150 µL) was used as the blank. Both standard Trolox solutions (150 µL) and the sample extract were processed identically. The absorbance of the resulting mixtures was measured at 734 nm using a spectrophotometer (UV 1800, Shimadzu, Kyoto, Japan). The scavenging activity of the ABTS cation radicals was quantified using a calibration curve ($y=-0.0144x+0.615$, $R^2 = 0.997$) constructed from Trolox standards. The results were expressed in terms of mg Trolox equivalents (TRE) per 100 g.

The DPPH radical scavenging activity of the methanol extract and essential oil derived from the plant was evaluated following the procedure outlined by Yilmaz *et al.* (2023). In this approach, the extract was combined with 2,2-diphenyl-1-picrylhydrazyl (DPPH) solutions at predetermined concentrations. The mixtures were thoroughly vortexed and then kept in the dark at room temperature for 30 min. After the incubation period, the absorbance of the samples was measured at 517 nm. The percentage inhibition of DPPH radicals was determined using Eq. 1. The results were reported as milligrams of ascorbic acid (AA) equivalents per 100 g (based on the calibration curve $y = -0.0093x + 0.945$, $R^2 = 0.998$) and as the percentage of free radical scavenging activity.

$$\text{Inhibition (\%)} = 100 \times \frac{\text{Control Absorbance} - \text{Example Absorbance}}{\text{Control Absorbance}} \quad (1)$$

The total antioxidant capacity (TAC) of the plant's methanol extract and essential oil were measured using a molybdate reagent, as per the methodology outlined by Yilmaz *et al.* (2023). In this procedure, pure water (250 µL) served as the blank in place of the sample. The absorbance of the reaction mixtures was recorded at 695 nm using a spectrophotometer. For the standard solutions, 500 µL aliquots were prepared and processed under identical conditions. The TAC content in the methanol extract was quantified as milligrams of Ascorbic acid equivalents (AAE) per 100 grams, based on a calibration curve (y = 0.0022x - 0.057, R^2 = 0.998) generated from ascorbic acid standards (Yilmaz *et al.* 2023).

The total flavonoid content (TFC) in the methanol extracts and essential oil of the plant were assessed using the protocol established by Yilmaz *et al.* (2023). The absorbance of the final reaction mixture was measured at 506 nm with a spectrophotometer. Pure water (500 µL) was employed as the blank, while 500 µL of standard solutions were prepared and processed similarly. The TFC in the samples was expressed as milligrams of quercetin equivalents (QEE) per 100 g, utilizing a calibration curve (y = 0.0038x + 0.0164, R^2 = 0.997) constructed from quercetin solutions dissolved in ethanol (Yilmaz *et al.* 2023).

The total phenolic content (TPC), a key bioactive component in the methanol extracts and essential oil, was measured following the procedure detailed by Yilmaz *et al.* (2023) with the Folin-Ciocalteu reagent. After preparing the reaction mixture, it was thoroughly vortexed and then incubated in the dark at room temperature for 120 min. The absorbance of the mixture was subsequently recorded at 760 nm. A blank was prepared by combining 3.7 mL of water, 500 µL of methanol, 100 µL of Folin-Ciocalteu reagent, and 600 µL of a 10% sodium carbonate (Na_2CO_3) solution. The phenolic content in the samples was quantified as milligrams of gallic acid equivalents (GAE) per 100 g, based on a calibration curve (y = 0.0052x + 0.0074, R^2 = 0.997) generated from gallic acid standards.

Determination of Antimicrobial Activity

Agar diffusion method

The antimicrobial activity of essential oils of *S. cretica* subsp. *anatolica* was evaluated using a modified agar diffusion method based on CLSI (2017) guidelines. Test organisms included Gram-positive bacteria (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Bacillus cereus* ATCC 9634), Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, and *Escherichia coli* ATCC 25922), and the yeast *Candida albicans* ATCC 18804. Fresh microbial cultures were grown on Müller-Hinton Agar (MHA) and standardized to 0.5 McFarland turbidity (approximately 1.5 × 10^8 CFU/mL) using 0.9% sterile saline. The inoculum was uniformly spread over MHA plates with sterile swabs.

Sterile 5.5-mm disks were placed onto the inoculated agar surfaces, and 15 µL of each sample (25 mg/mL in DMSO) was applied. Plates were kept at 4 °C for 2 h to allow compound diffusion. Chloramphenicol, nalidixic acid and nystatin (512 µg/mL) served as positive controls, while DMSO was used as the negative control. Following diffusion, bacterial plates were incubated at 37 °C for 24 h and yeast plates at 28 °C for 48 h. Inhibition zones were measured with a digital caliper. All experiments were conducted in triplicate, and the results were analyzed statistically.

Determination of MIC and MLC *values*

The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values of *S. cretica* subsp. *anatolica* essential oils against selected pathogens were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017). MIC testing was performed using the broth microdilution method in sterile 96-well microplates with Mueller-Hinton Broth (MHB) as the culture medium. The first wells were filled with double-strength MHB, while subsequent wells contained standard MHB. Essential oil samples prepared at 25 mg/mL were added to the first wells to achieve a starting concentration of 12.5 mg/mL, followed by serial two-fold dilutions. Nalidixic acid, chloramphenicol, and nystatin (each at 256 µg/mL, dissolved in DMSO) were used as positive controls.

Each well received 10 µL of microbial inoculum standardized to 0.5 McFarland turbidity. Wells containing only media or media with extracts served as negative controls. The plates were incubated at 37 °C for 24 h for bacteria and at 28 °C for 48 h for fungi. The MIC was defined as the lowest concentration of the essential oil that showed no visible microbial growth.

To determine MLC, 50 µL aliquots from the MIC, 2×MIC, and 4×MIC wells were plated onto Mueller-Hinton Agar (MHA) using a Drigalski spatula. Following incubation under the same conditions as above, colony-forming units (CFUs) were counted. The MLC was recorded as the lowest concentration that resulted in a ≥99.9% reduction in viable cell count compared to the initial inoculum. The methodology for antifungal assessment was adapted in part from Melkoumov *et al.* (2013), who reported enhanced *in vitro* and *in vivo* antifungal efficacy using nanosized nystatin formulations.

The Analysis GC-MS/FID of Essential Oil

For analysis with a gas chromatography-mass spectrometry/flame ionization detector (GC-MS)/FID, 1 µL of the essential oil solution was injected with a 1:5 split ratio, utilizing helium (> 99.999%) as the carrier gas at a constant flow rate of 1 mL/min. The analysis was conducted using an HP-5 apolar capillary column (30 m x 0.32 mm, 0.25 µm film thickness) on an Agilent 7890A gas chromatograph coupled with an Agilent 5975C mass spectrometer (GC-MS/FID; Agilent Technologies Inc, Santa Clara, CA, USA). The injector, ion source, and quadrupole temperatures were maintained at 250, 230, and 150 °C, respectively. The GC oven temperature program began at 60 °C, was held for 2 min, and then ramped up to 240 °C at a rate of 3 °C per minute. The FID detector was operated at 250 °C, with hydrogen and air flows set to 35 mL/min and 350 mL/min, respectively. Mass spectra were recorded in the range of m/z 45 to 450, following a 4.000-minute solvent delay (Öz *et al.* 2023). The identification of essential oil components was achieved by comparing their mass spectra with reference data from the Willey and NIST libraries. Additionally, the Kovats indices were used to confirm the identities of the compounds, as described in previous studies (Adams 2007).

RESULTS AND DISCUSSION

The Results of Enzyme Inhibitory Activities

The enzyme inhibition activities of the methanol extract and essential oil of *S. cretica* subsp. *anatolica*, as well as their comparison with standard inhibitors, such as acetazolamide, tacrine, and acarbose, provide valuable insights into the plant's

pharmacological potential. The methanol extract of *S. cretica* subsp. *anatolica* demonstrated significant (SPSS, IBM, version 29.0, Armonk, NY, USA) activity in inhibiting CA-II (IC_{50} = 0.048 ± 0.002 µg/mL), although it was less potent than the standard inhibitor acetazolamide (IC_{50} = 0.0023 ± 0.0002 µg/mL). Carbonic anhydrases are a family of enzymes that play critical roles in physiological processes such as pH regulation, CO_2 transport, and electrolyte secretion (Supuran 2016). Inhibitors of CA-II are particularly important in the treatment of glaucoma, epilepsy, and altitude sickness (Supuran 2016). The moderate activity of the methanol extract suggests that it contains compounds with potential CA-II inhibitory properties, which could be further explored for therapeutic applications. The weaker activity of the essential oil (IC_{50} = 0.279 ± 0.003 µg/mL) may be attributed to differences in the chemical composition of the two extracts. Previous studies have shown that phenolic compounds and flavonoids, often abundant in methanol extracts, exhibit significant CA-II inhibitory activity (Ekinci *et al.* 2013).

The essential oil of *S. cretica* subsp. *anatolica* showed stronger AChE inhibition (IC_{50} = 0.029 ± 0.008 µg/mL) compared to the methanol extract (IC_{50} = 0.23 ± 0.06 mg/mL). AChE inhibitors are widely used in the treatment of neurodegenerative diseases, such as Alzheimer's disease, as they prevent the breakdown of acetylcholine, a neurotransmitter essential for memory and cognitive function (Greig *et al.* 2001). The higher efficacy of the essential oil may be due to the presence of terpenoids and other volatile compounds, which have been reported to exhibit cholinesterase inhibitory activity (Mukherjee *et al.* 2007). However, both extracts were less potent than the standard inhibitor tacrine (IC_{50} = 0.012 ± 0.001 µg/mL), a well-known AChE inhibitor. This suggests that while *S. cretica* has potential, further optimization or isolation of active compounds may be required to enhance its efficacy.

The essential oil of *S. cretica* subsp. *anatolica* also exhibited stronger BChE inhibition (IC_{50} = 0.107 ± 0.0014 µg/mL) compared to the methanol extract (0.64 ± 0.008 mg/mL). BChE, like AChE, is involved in the hydrolysis of acetylcholine and has been implicated in neurodegenerative diseases (Darvesh *et al.* 2003). The essential oil's higher activity may be attributed to its complex mixture of terpenes and phenolic compounds, which have been shown to possess cholinesterase inhibitory properties (Mukherjee *et al.* 2007). However, the standard inhibitor tacrine (IC_{50} = 0.0017 ± 0.0003 µg/mL) remains significantly more potent. These findings align with previous studies on other plant species, where essential oils have demonstrated moderate cholinesterase inhibition but often fall short of synthetic inhibitors (Loizzo *et al.* 2008).

The methanol extract of *S. cretica* subsp. *anatolica* showed moderate *α*-glucosidase inhibition (1.41 ± 0.01 mg/mL), while the essential oil exhibited no activity. *α*-Glucosidase inhibitors are used in the management of type 2 diabetes, as they delay carbohydrate digestion and glucose absorption, thereby reducing postprandial blood glucose levels (Van de Laar *et al.* 2005). The activity of the methanol extract, though lower than that of the standard inhibitor acarbose (0.061 ± 0.002 µg/mL), suggests the presence of bioactive compounds, such as flavonoids or phenolic acids, which are known to inhibit *α*-glucosidase (Tadera *et al.* 2006). The lack of activity in the essential oil may be due to the absence of these compounds in its volatile fraction.

Both the essential oil (7.47 ± 0.17 mg/mL) and the methanol extract (12.77 ± 0.20 mg/mL) of *S. cretica* subsp. *anatolica* showed limited activity against *α*-amylase. *α*-Amylase inhibitors are also used in diabetes management, as they reduce starch digestion and glucose absorption (Van de Laar *et al.* 2005). The weak activity of both extracts compared to acarbose (IC_{50} = 33.27 ± 0.12 µg/mL) suggests that *S. cretica* may not be a

strong candidate for α-amylase inhibition. This is consistent with findings from other plant species, where α-amylase inhibition is often less pronounced than α-glucosidase inhibition (Tadera *et al.* 2006).

The findings from this study highlight the potential of *S. cretica* subsp. *anatolica* as a source of bioactive compounds, particularly for cholinesterase and carbonic anhydrase inhibition. The methanol extract and essential oil exhibited notable activities against CA-II, AChE, and BChE, although they were less potent than standard inhibitors. These results are consistent with previous studies on other *Stachys* species, which have demonstrated significant biological activities due to their rich content of phenolic compounds, flavonoids, and terpenoids (Stegăruș *et al.* 2021).

The essential oil's stronger activity against AChE and BChE suggests its potential as a natural candidate for the treatment of neurodegenerative diseases. However, further studies are needed to isolate and identify the active compounds responsible for these effects. Additionally, the moderate α-glucosidase inhibition by the methanol extract indicates its potential role in diabetes management, although its activity is not as strong as that of synthetic inhibitors.

In conclusion, *S. cretica* subsp. *anatolica* shows promise as a source of natural enzyme inhibitors, particularly for cholinesterase and carbonic anhydrase. Future research should focus on the isolation and characterization of its bioactive compounds, as well as *in vivo* studies to evaluate its therapeutic potential.

Table 1. Enzyme Activity Results of *S. cretica* subsp. *anatolica* Methanol Extract and Essential Oil

	CA-II IC_{50}	AChE IC_{50}	BChE IC_{50}	α-Glucosidase IC_{50}	α-Amylase IC_{50}
Methanol extract	0.048 ± 0.002^{b} µg/mL	0.23 ± 0.06^{c} mg/mL	0.64 ± 0.008^{c} mg/mL	1.41 ± 0.01^{b} mg/mL	12.77 ± 0.20^{c} mg/mL
Essential Oil	0.279 ± 0.003^{b} µg/mL	0.029 ± 0.008^{b} µg/mL	0.107 ± 0.0014^{b} µg/mL	No inhibition observed	7.47 ± 0.17^{b} mg/mL
Asetazolamid	0.0023 ± 0.0002^{a} µg/mL	-	-	-	-
Tacrine	-	0.012 ± 0.001^{a} µg/ml	0.0017 ± 0.0003^{a} µg/mL	-	-
Acarbose	-	-	-	0.061 ± 0.002^{a} µg/mL	33.27 ± 0.12^{a} µg/mL

Different letters within the same column indicate significant differences (Duncan, $p<0.05$). Lower IC_{50} indicates stronger inhibition

Results for Phenolic Compounds

Figures 2 and 3 present the chromatograms of the 31 standards used in the analysis and the chromatogram of the methanol extract obtained from the aerial parts of *S. cretica* subsp. *anatolica*. This comprehensive analysis, conducted using LC-MS/MS, highlights

the extract's rich phenolic profile, providing detailed insights into its chemical composition.

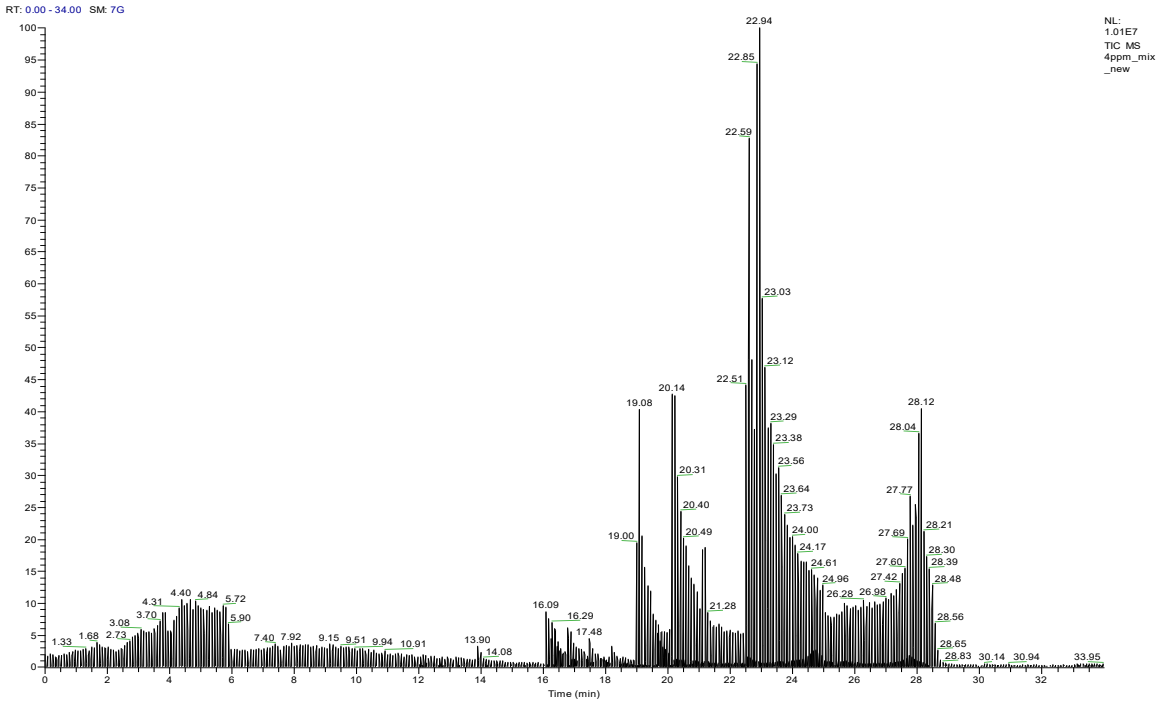

Fig. 2. Chromatogram of standard phenolic compounds analyzed by LC/MS/MS

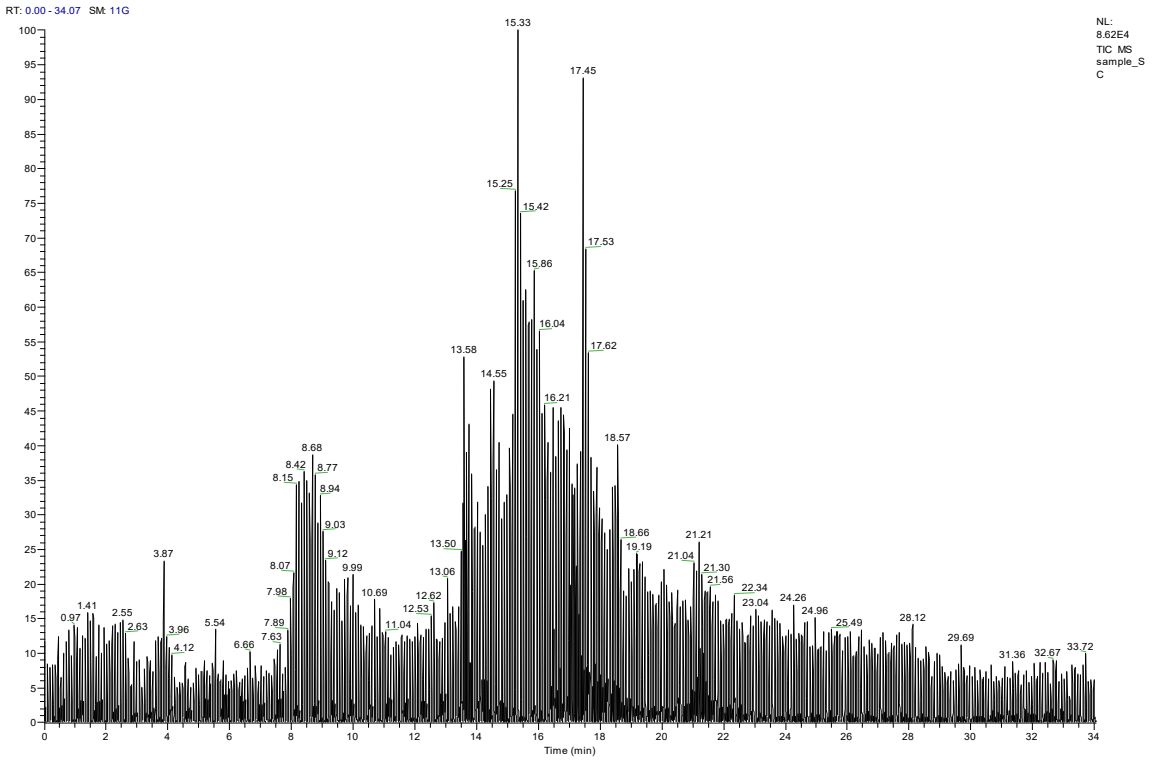

Fig. 3. Chromatogram of extracts of aerial parts of *S. cretica* subsp. *anatolica* by LC/MS/MS

The chromatographic profiles presented in Fig. 2 and 3 are total ion chromatograms (TIC) obtained using a TSQ Quantum Access Max tandem mass spectrometer coupled to a Thermo Scientific Dionex Ultimate 3000 UHPLC system. Unlike chromatograms that display specific peaks corresponding to individual compounds, TIC outputs represent the sum of all ion intensities detected at each point in time. As such, these chromatograms provide an overall profile of the sample's ion content across the retention time range rather than isolating and labeling each analyte peak. This is a standard output format for tandem mass spectrometry systems operating in multiple reaction monitoring (MRM) mode.

Table 2. Amounts of Phenolic Compounds in the Extracts of Aerial Parts of *S. cretica* subsp. *anatolica*

No.	Phenolic Compound	*S. cretica subsp. anatolica* Extracts (µg/g)	
1	Gallic Acid	86.95	± 2.52
2	Protocatechuic Acid	n.d.	
3	Protocatechuic Aldehyde	n.d.	
4	Sesamol	22.29	± 1.21
5	Gentisic Acid	n.d.	
6	Catechin	24483.71	± 24.11
7	Chlorogenic Acid	n.d.	
8	Epicatechin	153.12	± 5.71
9	Caffeic Acid	n.d.	
10	Vanillin	n.d.	
11	Syringic Acid	n.d.	
12	*p*-Coumaric Acid	n.d.	
13	Taxifolin	115.47	± 3.34
14	Ferulic Acid	3.77	± 0.11
15	Salicylic Acid	n.d.	
16	4-Hydroxybenzoic Acid	n.d.	
17	Hesperidin	56.91	± 1.62
18	Rosmarinic Acid	n.d.	
19	Oleuropein	1426.32	± 10.32
20	Luteolin-7-Glucoside	12.49	± 0.12
21	Rutin	n.d.	
22	Resveratrol	93.97	± 6.72
23	Ellagic Acid	n.d.	
24	Naringenin	5.25	± 0.61
25	Quercetin	6.26	± 0.66
26	Luteolin	64.32	± 1.93
27	Apigenin	n.d.	
28	Pinocembrin	n.d.	
29	Chrysin	n.d.	
30	Galangin	n.d.	
31	Flavone	86.96	± 2.26

n.d.: Not detected

According to the data presented in Table 2, the most abundant compound identified in the extract was catechin (24484 µg/g dw), a flavonoid known for its potent antioxidant, anti-inflammatory, and cardioprotective properties. The high concentration of catechin suggests that *S. cretica* subsp. *anatolica* may have significant protective effects against oxidative stress-related diseases. This finding aligns with studies on other *Stachys* species, such as *S. germanica*, *S. officinalis*, *S. byzantina*, *S. inflata*, *S. sylvatica*, *S. palustris*, and *S. recta*, where catechin and similar flavonoids have also been detected (Bahadori *et al.* 2020; Benedec *et al.* 2023).

Another notable compound in *S. cretica* subsp. *anatolica* was oleuropein (1426.3 µg/g dw), a phenolic compound commonly found in olive leaves. Oleuropein is recognized for its antidiabetic, antioxidant, and antimicrobial properties, which enhance the therapeutic potential of *S. cretica* subsp. *anatolica* in addressing metabolic disorders and infectious diseases. While oleuropein is not frequently reported in other *Stachys* species, its presence in *S. cretica* subsp. *anatolica* highlights the unique phytochemical profile of this plant.

Other flavonoids detected in significant amounts include epicatechin (153.1 µg/g dw) and taxifolin (115.5 µg/g dw). Epicatechin is particularly known for its cardiovascular health benefits, while taxifolin exhibits strong antioxidant and anti-inflammatory properties. These compounds further underscore the potential of *S. cretica* subsp. *anatolica* in managing chronic diseases. Similar flavonoids have been identified in other *Stachys* species, such as *S. lavandulifolia*, where epicatechin and taxifolin were also found in notable quantities (Bingol and Bursal 2018).

The extract also contained gallic acid (87.0 µg/g dw), a phenolic acid renowned for its strong antioxidant and antimicrobial properties, and hesperidin (56.9 µg/g dw), a flavonoid glycoside noted for its cardiovascular health benefits. Additionally, luteolin (64.3 µg/g dw), a flavonoid with antioxidant, anti-inflammatory, and neuroprotective effects, and its glycosylated form, luteolin-7-glucoside (12.49 µg/g dw), were identified. Luteolin-7-glucoside, a flavonoid derivative, is particularly noteworthy for its enhanced bioavailability and potent antioxidant activity, which further supports the therapeutic potential of *Stachys* species.

Another flavonoid detected in the extract was flavone (87.0 µg/g dw), a simple flavonoid with antioxidant and anti-inflammatory properties. Flavone is known to modulate cellular signaling pathways and has been studied for its potential in cancer prevention and treatment. Additionally, sesamol (22.3 µg/g dw), a phenolic derivative with strong antioxidant and anti-inflammatory properties, was identified. Sesamol is particularly known for its ability to scavenge free radicals and protect against oxidative stress-related damage. These compounds further enhance the therapeutic profile of *S. cretica* subsp. *anatolica*.

Compounds detected in lower concentrations, such as ferulic acid (3.77 µg/g dw), a phenolic acid with antioxidant and cardioprotective effects, and naringenin (5.25 µg/g dw) and quercetin (6.26 µg/g dw), both flavonoids, also exhibit significant biological activities. Quercetin, in particular, is known for its antioxidant, anti-inflammatory, and antidiabetic properties, and it may play a role in reducing insulin resistance. These compounds, although present in smaller amounts, contribute to the overall bioactivity of *S. cretica* subsp. *anatolica*.

However, certain phenolic compounds, such as chlorogenic acid, caffeic acid, rutin, ellagic acid, and rosmarinic acid, were not detected in the *S. cretica* subsp. *anatolica* extract. This suggests that the phenolic profile of the plant may vary depending on factors

such as species, growing conditions, and extraction methods. For instance, previous studies have reported the presence of compounds such as rutin and chlorogenic acid in other *Stachys* species, such as *S. lavandulifolia* (Bingol and Bursal 2018). These variations indicate that the chemical composition of *Stachys* species can be influenced by ecological and genetic factors.

Studies on the phenolic content and biological activities of other *Stachys* species further support the therapeutic potential of this genus. For example, the methanol extract of *S. lavandulifolia* was found to contain high levels of naringenin and luteolin (Bingol and Bursal 2018). Similarly, *S. germanica*, *S. officinalis*, *S. byzantina*, *S. inflata*, *S. sylvatica*, *S. palustris*, and *S. recta* have been reported to contain gallic acid, catechin, ferulic acid, hesperidin, luteolin, and luteolin-7-glucoside (Bahadori *et al.* 2020; Benedec *et al.* 2023). These studies demonstrate that while there are common phenolic compounds across *Stachys* species, the specific composition and concentrations can vary significantly depending on the species and environmental conditions.

The methanol extract of the aerial parts of *S. cretica* subsp. *anatolica* exhibits significant antioxidant, anti-inflammatory, and antidiabetic potential, primarily due to the high concentrations of phenolic compounds, such as catechin and oleuropein, as highlighted in Table 2. Additionally, compounds including gallic acid, hesperidin, luteolin, luteolin-7-glucoside, resveratrol, flavone, and sesamol, further enhance the plant's therapeutic value. However, the absence of certain phenolic compounds, such as chlorogenic acid and rutin, suggests that the chemical profile of *S. cretica* subsp. *anatolica* may vary depending on various factors. These findings support the traditional uses of *S. cretica* subsp. *anatolica* but more comprehensive pharmacological and clinical studies are needed to fully understand its therapeutic potential and how it compares to other *Stachys* species.

The Results of Antioxidant Capacity and Bioactive Component

This study evaluated the antioxidant activity and bioactive components of the methanol extract and essential oil of *S. cretica* subsp. *anatolica*, a plant known for its potential health benefits. Antioxidant activity was assessed using DPPH, ABTS, and FRAP assays, while bioactive components were quantified through TPC, TFC, and TAC. The results, as presented in Table 3, highlight significant differences in antioxidant potential and bioactive composition between the methanol extract and essential oil.

The DPPH assay, a widely used method for evaluating free radical scavenging activity, revealed that the methanol extract of *S. cretica* subsp. *anatolica* exhibited significantly stronger antioxidant activity (279.8 mg AAE/100 g) compared to its essential oil (65.6 mg AAE/100 g). This was further supported by the higher inhibition percentage of the methanol extract (63.8%) relative to the essential oil (9.94%). The IC_{50} values, which indicate the concentration required to scavenge 50% of free radicals, further underscored this finding. The methanol extract demonstrated a much lower IC_{50} value (44.8 mg/mL) compared to the essential oil (957.6 mg/mL), highlighting its superior efficiency in neutralizing free radicals. Ascorbic acid, used as a reference standard, exhibited an even lower IC_{50} value (92.6 µg/mL), confirming its exceptional antioxidant capacity, consistent with previous studies (Brand-Williams *et al.* 1995; Kedare and Singh 2011).

Similarly, the ABTS assay, which measures the ability to scavenge ABTS radicals, also demonstrated the methanol extract's stronger antioxidant potential (73.9 mg TRE/100 g) compared to the essential oil (19.9 mg TRE/100 g). This aligns with findings by Re *et al*. (1999), who emphasized that ABTS radical scavenging activity is a reliable indicator

of antioxidant capacity, particularly for extracts rich in phenolic compounds. The FRAP assay, which evaluates ferric ion reduction capacity, further corroborated these results, showing that the methanol extract (974 mg $FeSO_4$/100 g) had significantly higher reducing power than the essential oil (652 mg $FeSO_4$/100 g). This suggests that the methanol extract has a greater ability to donate electrons and neutralize free radicals, a characteristic often associated with high antioxidant activity (Benzie and Strain 1996).

Table 3. Antioxidant Activity and Bioactive Component Profile of *S. cretica* subsp. *anatolica* Methanol Extract and Essential Oil

Antioxidant Activity Levels						
	Essential Oil and Methanol Extract Yield	DPPH			ABTS	FRAP
	g/100 g	mg AAE/100 g	Inhibition (%)	IC_{50} (mg/mL)	mg TRE/100 g	mg $FeSO_4$ /100 g
Methanol Extract	15.31 ± 0.11[a]	279.82 ± 2.07[a]	63.81 ± 0.47[b]	44.77 ± 0.33[b]	73.86 ± 0.21[a]	974.12 ± 5.76[a]
Essential Oil	0.19 ± 0.04[b]	65.56 ± 3.11[b]	9.94 ± 0.47[c]	957.57 ± 45.60[c]	19.89 ± 0.02[b]	651.53 ± 5.50[b]
Ascorbic Acid			98.32 ± 0.01[a]	92.65 ± 0.11[a] µg/mL		
Bioactive Components						
	TPC mg GAE/100 g		TFC mg QEE/100 g		TAC mg AAE/100 g	
Methanol Extract	1005.22 ± 52.92[a]		132.85 ± 2.58[a]		841.25 ± 18.71[a]	
Essential Oil	63.24 ± 008[b]		17.76 ± 0.90[b]		588.17 ± 5.51[b]	

Different letters within the same column in the table indicate significant statistical differences in pairwise comparisons ($p < 0.05$). n:3. Duncan's test was applied; AAE: Ascorbic Acid Equivalent, TRE: Trolox Equivalent, GAE: Gallic Acid Equivalent, QEE: Quercetin Equivalent

The superior antioxidant activity of the methanol extract can be attributed to its significantly higher levels of bioactive components. The methanol extract contained substantially more total phenolic content (1005 mg GAE/100 g) compared to the essential oil (63.2 mg GAE/100 g). Phenolic compounds are well-documented for their strong antioxidant properties, as they can donate hydrogen atoms or electrons to stabilize free radicals (Huang *et al.* 2006). Similarly, the methanol extract had a much higher total flavonoid content (133 mg QEE/100 g) than the essential oil (17.8 mg QEE/100 g). Flavonoids are another class of bioactive compounds known for their potent antioxidant effects, including free radical scavenging and metal chelation (Middleton *et al.* 2000). Additionally, the methanol extract exhibited a higher total antioxidant capacity content (841 mg AAE/100 g) compared to the essential oil (588 mg AAE/100 g), further confirming its enhanced antioxidant potential.

The methanol extract's superior performance can be explained by its higher extraction efficiency for polar compounds, such as phenolics and flavonoids, which are more soluble in methanol. This is consistent with studies by Dai and Mumper (2010), who demonstrated that methanol is an effective solvent for extracting a wide range of

antioxidant compounds from plant materials. The lower IC_{50} value of the methanol extract in the DPPH assay further supports its greater antioxidant efficiency, as a lower IC_{50} value indicates higher antioxidant activity (Prior *et al.* 2005).

The Results of Antimicrobial Activities

The antimicrobial activity of the 30% methanol extract and essential oil was evaluated against a range of bacterial and fungal strains, with their performance compared to the standard antibiotics chloramphenicol and nalidixic acid. Antimicrobial efficacy is inversely related to the MIC and MLC values, where lower values indicate higher potency. Additionally, the diameter of the inhibition zone (DDT in mm) provides a visual measure of antimicrobial effectiveness, with larger zones reflecting stronger activity. The detailed antimicrobial results, including DDT, MIC, and MLC values for all tested strains, are presented in Table 4.

The antimicrobial activity of the 30% methanol extract and essential oil was evaluated against a range of bacterial and fungal strains, with their performance compared to the standard antibiotics chloramphenicol and nalidixic acid. Antimicrobial efficacy is inversely related to the MIC and MLC values, where lower values indicate higher potency. Additionally, the diameter of the inhibition zone (DDT in mm) provides a visual measure of antimicrobial effectiveness, with larger zones reflecting stronger activity (Balouiri *et al.* 2016).

B. cereus, the 30% methanol extract demonstrated a DDT of 7.83 mm, with MIC and MLC values of 0.41 mg/mL and 0.48 mg/mL, respectively. The essential oil exhibited stronger activity, with a DDT of 11.6 mm and significantly lower MIC (0.24 mg/mL) and MLC (0.81 mg/mL) values. Chloramphenicol, however, showed the highest antibacterial activity, with a DDT of 21.1 mm and the lowest MIC (0.04 mg/mL) and MLC (0.07 mg/mL) values. These results are consistent with previous studies indicating that essential oils often exhibit moderate antimicrobial activity, while conventional antibiotics such as chloramphenicol remain highly effective (Bakkali *et al.* 2008).

E. coli, the methanol extract recorded a DDT of 7.80 mm, with MIC and MLC values of 0.81 mg/mL and 0.24 mg/mL, respectively. The essential oil performed better, with a DDT of 10.9 mm and lower MIC (0.12 mg/mL) and MLC (1.63 mg/mL) values. Chloramphenicol displayed the strongest activity, with a DDT of 19.9 mm and the lowest MIC (0.02 mg/mL) and MLC (0.07 mg/mL) values. This aligns with findings by Nazzaro *et al.* (2013), who noted that essential oils can disrupt bacterial cell membranes, but their efficacy is often lower than synthetic antibiotics.

K. pneumoniae, the methanol extract showed a DDT of 7.30 mm, with MIC and MLC values of 1.63 mg/mL and 0.95 mg/mL, respectively. The essential oil exhibited greater efficacy, with a DDT of 9.43 mm and lower MIC (0.48 mg/mL) and MLC (3.25 mg/mL) values. Chloramphenicol achieved the highest inhibition, with a DDT of 29.9 mm and the lowest MIC (0.02 mg/mL) and MLC (0.07 mg/mL) values. These findings align with studies highlighting the resistance of *K. pneumonia* to natural extracts and its susceptibility to broad-spectrum antibiotics (Piddock 2012).

In the case of *P. aeruginosa*, the methanol extract demonstrated a DDT of 7.67 mm, with MIC and MLC values of 0.81 mg/mL and 0.96 mg/mL, respectively. The essential oil performed slightly better, with a DDT of 10.73 mm and lower MIC (0.46 mg/mL) and MLC (1.63 mg/mL) values.

Table 4. Antimicrobial Activities of *S. cretica* subsp. *anatolica* Methanol Extract and Essential Oil

	MeOH 30%			Essential Oil			Chloramphenicol			Nalidixic Acid			Nystatin		
	DDT (mm)	MIC (mg/mL)	MLC (mg/mL)	DDT (mm)	MIC (mg/mL)	MLC (mg/mL)	DDT (mm)	MIC (mg/mL)	MLC (mg/mL)	DDT (mm)	MIC (mg/mL)	MLC (mg/mL)	DDT (mm)	MIC (mg/mL)	MLC (mg/mL)
Bacillus cereus ATCC 9634	7.83	0.41	0.48	11.59	0.24	0.81	21.12	0.04	0.07	16.78	0.06	0.13	NT	NT	NT
	± 0.12	± 0.04	± 0.11	± 015	± 0.01	± 0.11	± 0.16	± 0.01	± 0.01	± 0.06	± 0.01	± 0.01	NT	NT	NT
Escherichia coli ATCC 25922	7.80	0.81	0.24	10.90	0.12	1.63	19.88	0.02	0.07	22.65	0.06	0.15	NT	NT	NT
	± 0.10	± 0.05	± 0.08	± 0.13	± 0.01	± 0.08	± 0.14	± 0.01	± 0.01	± 0.14	± 0.01	± 0.01	NT	NT	NT
Klebsiella pneumoniae ATCC 13883	7.30	1.63	0.95	9.43	0.48	3.25	29.88	0.02	0.07	20.77	0.02	0.07	NT	NT	NT
	± 0.10	± 0.08	± 0.12	± 0.02	± 0.03	± 0.12	± 0.17	± 0.01	± 0.01	± 0.16	± 0.01	± 0.01	NT	NT	NT
Pseudomonas aeruginosa ATCC 27853	7.67	0.81	0.96	10.73	0.46	1.63	10.51	0.02	0.07	26.66	0.01	0.05	NT	NT	NT
	± 0.12	± 0.11	± 0.01	± 0.20	± 0.02	± 0.07	± 0.14	± 0.01	± 0.01	± 0.28	± 0.01	± 0.01	NT	NT	NT
Staphylococcus aureus ATCC 25923	7.53	0.42	0.08	10.94	0.04	0.81	18.99	0.001	0.005	20.54	0.02	0.05	NT	NT	NT
	± 0.09	± 0.06	± 0.01	± 0.07	0.01	± 0.05	± 0.11	± 0.001	± 0.001	± 0.13	± 0.01	± 0.01	NT	NT	NT
Enterococcus faecalis ATCC 29212	8.27	0.10	0.24	0.12	0.46	0.20	11.12	0.018	0.035	21.65	0.01	0.03	NT	NT	NT
	± 0.06	± 0.01	± 0.02	± 0.02	± 0.02	± 0.02	± 0.11	± 0.001	± 0.001	± 0.16	± 0.01	± 0.01	NT	NT	NT
Candida albicans ATCC 18804	7.93	1.63	1.90	11.93	0.95	3.20	NT	NT	NT	NT	NT	NT	26.34	0.004	0.008
	± 0.08	± 0.03	± 0.04	± 0.11	± 0.11	± 0.09	NT	NT	NT	NT	NT	NT	0.12	±0.001	±0.001

The discs have a diameter of 5.5 mm; Samples: 25 mg/mL, Antibiotic: ± 0.51 mg/mL; 15 µL were pipetted onto each disc, n = 3; DDT: Disk Diffusion Test, NT: Not tested

Chloramphenicol showed the highest effectiveness, with a DDT of 10.51 mm and the lowest MIC (0.02 mg/mL) and MLC (0.07 mg/mL) values. This is in line with research by Stratev *et al.* (2016), who reported that *P. aeruginosa* is often resistant to natural extracts but remains sensitive to conventional antibiotics.

For *S. aureus*, the methanol extract recorded a DDT of 7.53 mm, with MIC and MLC values of 0.42 mg/mL and 0.08 mg/mL, respectively. The essential oil exhibited better performance, with a DDT of 10.9 mm and significantly lower MIC (0.04 mg/mL) and MLC (0.81 mg/mL) values. Chloramphenicol demonstrated exceptional activity, with a DDT of 19.0 mm and the lowest MIC (0.001 mg/mL) and MLC (0.005 mg/mL) values. These findings are supported by studies indicating that *S. aureus* is highly susceptible to chloramphenicol but shows variable resistance to natural extracts (Tong *et al.* 2015).

In the case of a fungal strain, *E. faecalis*, the methanol extract showed a DDT of 8.27 mm, with MIC and MLC values of 0.10 mg/mL and 0.24 mg/mL, respectively. The essential oil performed better, with a DDT of 0.12 mm and lower MIC (0.46 mg/mL) and MLC (0.20 mg/mL) values. Chloramphenicol exhibited moderate activity, with a DDT of 11.12 mm, MIC of 0.018 mg/mL, and MLC of 0.035 mg/mL. These results align with research by Fisher and Phillips (2009), who noted that essential oils can exhibit antifungal activity, though often at higher concentrations compared to antibiotics.

Evaluation of *C. albicans* showed that the methanol extract recorded a DDT of 7.93 mm, with MIC and MLC values of 1.63 mg/mL and 1.90 mg/mL, respectively. The essential oil demonstrated superior efficacy, with a DDT of 11.9 mm and lower MIC (0.95 mg/mL) and MLC (3.20 mg/mL) values. Nystatin showed significant antifungal activity, with a DDT of 26.3 mm, MIC of 0.004 mg/mL, and MLC of 0.008 mg/mL.

The results indicate that essential oils generally exhibit stronger antimicrobial activity compared to the 30% methanol extracts across most bacterial and fungal strains, as evidenced by lower MIC and MLC values and larger inhibition zones. However, chloramphenicol consistently demonstrated the highest antimicrobial efficacy, with the lowest MIC and MLC values and the largest inhibition zones.

This is consistent with the findings of Aminov (2010), who emphasized the superior performance of synthetic antibiotics in combating microbial infections. Essential oils, particularly against *B. cereus*, *E. coli*, and *S. aureus*, showed notable antimicrobial potential, suggesting their viability as natural therapeutic agents. Nevertheless, their activity was generally inferior to chloramphenicol, underscoring the need for further research to optimize their efficacy. This could include refining extraction methods or combining essential oils with other compounds to enhance their antimicrobial properties, as suggested by studies such as that by Bassolé and Juliani (2012).

GC-MS/FID Conditions for Essential Oil Analysis

The GC-MS/FID analysis of the essential oil obtained from the aerial parts of the endemic *S. cretica* subsp. *anatolica* plant identified 155 compounds. The structures of 151 of these compounds were identified, but the structures of 4 compounds could not be determined. When Table 5 is examined, it can be seen that the identified components constituted 98.60% of the essential oil. It was understood that the most prominent compounds in the essential oil extracted from the aerial parts of the plant were hexahydrofarnesyl acetone (7.83%), hexadecanoic acid (7.76%), benzaldehyde (5.76%), diisobutyl phthalate (5.70%), and *cis*-chrysanthenol acetate (4.98%). The main compound of the essential oil of *S. cretica* subsp. *anatolica* was hexahydrofarnesyl acetone (Table 5).

Also when considering Table 5, it can be seen that the 151 compounds identified in the essential oil were grouped into 13 different chemical classes. The number of compounds and their percentage distributions within these classes are as follows: esters (25.1%; 28 compounds), with diisobutyl phthalate as the main component; oxygenated sesquiterpenes (18.5%; 15 compounds), with hexahydrofarnesyl acetone; aldehydes (13.1%; 22 compounds), with benzaldehyde; oxygenated monoterpenes (8.50%; 23 compounds), with eucalyptol; oil acids (7.70%; 2 compounds), with hexadecanoic acid; sesquiterpene hydrocarbons (7.34%; 18 compounds), with *β*-bourbonene; hydrocarbons (5.76%; 13 compounds), with cyclotetradecane; monoterpene hydrocarbons (4.50%; 9 compounds), with *α*-pinene; alcohols (2.59%; 5 compounds), with 1-octen-3-ol; others (2.07%; 5 compounds), with 1,2,3,4-tetrahydro-2-methylquinoline; ketones (1.98%; 9 compounds), with benzophenone; oxygenated diterpenes (1.15%; 1 compound), with phytol; diterpene hydrocarbons (0.06%; 1 compound), with dehydroabietane; and unidentified compounds (1.60%; 4 compounds). Among these, esters were found to be the most dominant chemical class in terms of both their relative percentage and number of compounds.

In a study conducted on *Stachys cretica* subsp. *anatolica* samples collected in the Koçtepe region of Isparta in 2017 to 2018, the above ground parts of the plant during the flowering period were analyzed by solid phase microextraction (SPME). As a result of the analysis carried out using the GC-MS device, 58 volatile compounds were detected. Among the main components, germacrene D (34.56%), *β*-caryophyllene (21.04%), and (*E*)-2-hexenal (12.58%) stand out (Sarıkaya 2018).

In another study published in 2024, the changes in volatile components in the flowers and leaves of *Stachys cretica* subsp. *anatolica* at different altitudes were examined. A total of 79 components were identified in the analyses performed by the Headspace-Solid Phase Micro-Extraction (HS-SPME) method. Benzaldehyde and *α*-pinene were determined as the main components in both lower and upper altitudes in flowers. In leaves, benzaldehyde and *α*-pinene were determined as the main components in the lower altitudes, and benzaldehyde and germacrene D were determined as the main components in the upper altitudes (Tekeş 2024).

Table 5. The Essential Oil Components of Aerial Parts in *S. cretica* subsp. *anatolica*

No	RT (min)	Compounds	% Area	Kovats Index	Literatür Kovats Index
1	4.186	3-Methyl-hexane	0.06	671	671
2	4.537	2-Ethyl-furan	0.58	700	700
3	4.983	Methyl-cyclohexane	0.33	719	719
4	6.832	Hexanal	0.76	799	799
5	8.587	(*E*)-2-Hexenal	0.81	849	849
6	9.59	1,3-*trans*,5-*cis*-Octatriene	0.06	878	879
7	10.42	Heptanal	0.12	901	901
8	11.726	*α*-Pinene	2.12	933	933
9	12.327	Camphene	0.20	947	947
10	12.827	**Benzaldehyde**	**5.76**	960	960

11	13.502	*β*-Pinene	1.36	976	976
12	13.640	1-Octen-3-ol	1.25	979	979
13	13.829	2,3-Octanedione	0.15	984	984
14	13.941	3-Octanone	0.15	987	987
15	14.136	2-Pentyl-furan	0.34	991	991
16	14.322	3-Octanol	0.07	996	996
17	14.612	Octanal	0.15	1003	1003
18	14.946	(*E,E*)-2,4-Heptadienal	0.04	1011	1011
19	15.193	*α*-Terpinene	0.06	1017	1017
20	15.527	*p*-Cymene	0.33	1024	1024
21	15.811	Eucalyptol	2.11	1031	1031
22	16.162	3-Octen-2-one	0.11	1039	1040
23	16.334	Benzeneacetaldehyde	0.17	1043	1043
24	16.978	γ-Terpinene	0.27	1059	1059
25	17.292	Acetophenone	0.30	1066	1066
26	17.508	(*E,E*)-3,5-Octadien-2-one	0.20	1071	1072
27	18.028	*p*-Mentha-2,4(8)-diene	0.03	1084	1084
28	18.241	*α*-Terpinolen	0.13	1089	1089
29	18.447	3,5-Octadien-2-one	0.05	1093	1093
30	18.734	Linalool	0.71	1100	1100
31	18.902	Nonanal	0.60	1104	1104
32	19.115	1-Methyl-4-(1-methylpropyl)-benzene	0.07	1109	1100
33	19.233	1-Octen-3-yl-acetate	0.02	1112	1112
34	19.311	Fenchol	0.01	1114	1115
35	19.638	*p*-Menth-2-en-1-ol	0.06	1122	1122
36	19.733	3-Octanyl acetate	0.03	1124	1124
37	19.831	*α*-Campholenal	0.36	1127	1127
38	20.371	*trans*-Pinocarveol	0.86	1140	1140
39	20.610	Borneol (Camphor)	0.69	1146	1146
40	20.776	*p*-Mentha-1,5-dien-8-ol	0.07	1150	1150
41	20.924	(*E,Z*)-2,6-Nonadienal	0.06	1153	1153
42	21.083	Rose furan epoxide	0.03	1157	1160
43	21.201	(*E*)-2-Nonenal	0.15	1160	1160
44	21.349	Pinocarvone	0.61	1164	1164
45	21.511	Isoborneol	0.37	1168	1164
46	21.677	1-Nonanol	0.08	1172	1172
47	21.954	Terpinen-4-ol	0.41	1178	1178
48	22.227	*p*-Methylacetophenone	0.29	1185	1186
49	22.507	*α*-Terpineol	0.34	1192	1192
50	22.646	Methyl salicylate	0.33	1195	1195
51	22.740	Myrtenol	0.86	1198	1198
52	22.868	Safranal	0.06	1201	1201
53	23.057	Decanal	0.24	1206	1206

54	23.297	2,4-Nonadienal	0.74	1212	1212
55	23.624	trans-Carveol	0.26	1220	1220
56	24.117	*cis*-3-Hexenyl 2-methylbutanoate	0.14	1232	1231
57	24.296	Hexyl 2-methylbutyrate	0.20	1237	1237
58	24.475	Cuminal	0.04	1242	1242
59	24.610	Carvone	0.10	1245	1245
60	24.954	Carveol	0.03	1254	1252
61	25.336	***cis*-Chrysanthenol acetate**	**4.98**	1263	1263
62	25.660	Cinnamal	0.19	1272	1273
63	25.818	Phellandral	0.06	1276	1276
64	25.977	Isopseudocumenol	0.08	1280	1279
65	26.261	Isobornyl acetate	0.20	1287	1287
66	26.402	Lavandulyl acetate	0.17	1290	1290
67	26.510	Sabinyl acetate	0.28	1293	1293
68	26.699	Perillal	0.03	1298	1295
69	26.794	*trans*-Pinocarvyl acetate	0.23	1300	1300
70	27.023	Undecanal	0.05	1307	1307
71	27.185	3-methylbutyl 2-furoate	3.35	1311	1318
72	27.357	(*E,E*)-2,4-Decadienal	0.09	1316	1316
73	27.445	Mesitaldehyde	0.05	1318	1323
74	27.756	Myrtenyl acetate	0.04	1326	1326
75	28.080	Nerol acetate	0.07	1335	1335
76	28.427	Octyl isobutyrate	0.04	1344	1343
77	28.778	2-methylpropanoic acid-2,2-dimethyl-1-(2-hydroxy-1-methylethyl)-propyl ester	1.27	1354	1365
78	28.941	Durylaldehyde	1.25	1358	1357
79	29.133	(*E*)-2-Undecenal	0.18	1363	1364
80	29.342	Decanoic acid	0.07	1369	1369
81	29.572	2-Ethyl-3-hydroxyhexyl 2-methylpropanoate	1.36	1375	1375
82	29.680	*α*-Copaene	0.47	1378	1378
83	30.034	*β*-Bourbonene	1.99	1387	1387
84	30.260	*β*-Elemene	0.15	1393	1393
85	30.318	Benzylcarbinol isobutyrate	0.17	1395	1395
86	30.446	Dihydro- γ-ionone	0.35	1398	1396
87	30.608	1,2,3,4-Tetahydro-2-methylquinoline	1.04	1403	1406
88	30.756	Dodecanal	0.19	1407	1407
89	31.060	*α*-Cedrene	0.22	1416	1416
90	31.273	Caryophyllene	0.57	1422	1422
91	31.654	Octyl 2-methyl butyrate	0.70	1433	1434
92	31.860	2-Dodecenal	0.06	1439	1439
93	32.049	*α*-Farnesene (isomer 2)	0.57	1444	1444

94	32.154	Germacrene D	0.14	1447	1448
95	32.238	4-(2,3,6-Trimethylphenyl)-2-butanone	0.03	1449	1445
96	32.336	*trans*-Geranylacetone	0.22	1452	1452
97	32.481	*trans*-*β*-Farnesene	0.29	1456	1456
98	32.630	2,6,10-Trimethyltridecane	1.04	1460	1461
99	32.927	Alloaromadendrene	0.07	1469	1469
100	33.069	1-Dodecanol	0.79	1473	1473
101	33.325	*γ*-Muurolene	0.39	1480	1480
102	33.443	*α*-Curcumene	0.95	1484	1484
103	33.609	Phenethyl 2-methylbutyrate	2.18	1488	1488
104	33.791	*β*-Bisabolene	0.04	1494	1494
105	33.922	Pentadecane	0.24	1497	1500
106	34.064	*α*-Muurolene	0.10	1501	1501
107	34.311	*α*-Farnesene	0.19	1509	1509
108	34.425	*β*-Curcumene	0.35	1512	1512
109	34.571	*γ*-Cadinene	0.05	1517	1517
110	34.874	Calamenene	0.68	1526	1526
111	35.121	Dihydroactinidiolide	0.06	1533	1532
112	35.256	Dibutyl maleate	0.23	1537	1556
113	35.519	Sesquisabinene hydrate	0.69	1545	1544
114	35.904	Germacrene B	0.25	1557	1557
115	36.363	1,5-Epoxysalvial-4(14)-ene	0.36	1570	1571
116	36.484	*γ*-Undecalactone	0.58	1574	1574
117	36.737	(-)-Spathulenol	2.40	1582	1582
118	36.923	Caryophyllene oxide	0.63	1587	1587
119	37.051	Ledene alcohol	0.28	1591	1577
120	37.261	Hexadecane	1.24	1597	1600
121	37.396	Globulol	0.05	1602	1607
122	37.625	*α*-Acorenol	0.10	1609	1612
123	37.821	Alloaromadendrene oxide	0.47	1615	1613
124	38.067	Junenol	0.15	1623	1622
125	38.239	Benzophenone	0.73	1628	1628
126	38.891	Amylcinnamic aldehyde	0.44	1649	1645
127	39.093	Methyl dihydrojasmonate	0.88	1655	1650
128	39.195	*α*-Cadinol	0.81	1659	1659
129	39.711	Cyclotetradecane	1.36	1675	1673
130	39.833	(+)-Valeranone	3.17	1679	1684
131	40.049	Levomenol	0.74	1686	1685
132	40.187	Vulgarol B	0.52	1690	1688
133	40.390	Heptadecane	0.39	1697	1700
134	41.078	Methyl quinaldate	0.08	1719	1711

135	41.962	*α*-Hexylcinnamaldehyde	1.20	1749	1750
136	42.320	(+)-*β*-Costol	0.64	1760	1756
137	43.394	Octadecane	0.05	1796	1800
138	44.180	Isopropyl myristate	0.03	1822	1823
139	44.835	**Hexahydrofarnesyl acetone**	**7.83**	1846	1846
140	45.533	**Diisobutyl phthalate**	**5.70**	1870	1870
141	45.716	Cyclohexadecane	0.47	1877	1880
142	46.266	Nonadecane	0.17	1896	1900
143	47.008	Methyl hexadecanoate	0.20	1922	1922
144	47.987	**Hexadecanoic acid**	**7.76**	1955	1955
145	48.109	Dibutyl phthalate	1.97	1959	1959
146	49.152	Eicosane	0.38	1995	2000
147	51.322	Dehydroabietane	0.06	2054	2054
148	52.173	1-Octadecanol	0.44	2077	2077
149	52.834	Heneicosane	0.08	2094	2100
150	53.017	*γ*-Palmitolactone	0.10	2099	2104
151	53.472	Phytol	1.17	2111	2111

Compound Classification		**Compound Number**	**Main Compound**
Esters (No: 33, 36, 50, 56, 57, 61, 65-67, 69, 71, 74-77, 81, 85, 91, 103, 112, 116, 127, 134, 138, 140, 143, 145, 150)	25.13	28	Diisobutyl phthalate
Oxygenated sesquiterpenes (No: 113, 115, 117-119, 121-124, 128, 130-132, 136, 139)	18.54	15	Hexahydrofarnesyl acetone
Aldehydes (No: 4, 5, 7, 10, 17, 18, 23, 31, 41, 43, 53, 54, 62, 70, 72, 73, 78, 79, 88, 92, 126, 135)	13.09	22	Benzaldehyde
Oxygenated monoterpenes (No: 21, 30, 34, 35, 37-40, 42, 44, 45, 47, 49, 51, 52, 55, 58-60, 63, 68, 86, 96)	8.50	23	Eucalyptol
Oil acids (No: 80, 144)	7.70	2	Hexadecanoic acid
Sesquiterpene hydrocarbons (No: 82-84, 89, 90, 93, 94, 97, 99, 101, 102, 104, 106, 107-110, 114)	7.34	18	*β*-Bourbonene
Hydrocarbons (No: 1, 3, 6, 98, 105, 129, 133, 137, 141, 142, 146, 149)	5.76	13	Cyclotetradecane
Monoterpene hydrocarbons (No: 8, 9, 11, 19, 20, 24, 27, 28, 32)	4.50	9	*α*-Pinene
Alcohols (No: 6, 25)	2.59	5	1-Octen-3-ol
Others (No: 12, 16, 46, 100, 148)	2.07	5	1,2,3,4-Tetahydro-2-methylquinoline
Ketones (No: 13, 14, 22, 25, 26, 29, 48, 95, 125)	1.98	9	Benzophenone
Oxygenated diterpenes (No: 151)	1.15	1	Phytol
Diterpene hydrocarbons (No: 147)	0.06	1	Dehydroabietane
Total	98.40	151	
Unidentified compounds	1.60	4	

RT: Retention time
Kovats index: Retention indices calculated against
Literatür kovats index: Literature retention indices based on Adams 2007, NIST, and WILLEY

In the present study, only the aerial parts of *S. cretica* subsp. *anatolica* were utilized to obtain both essential oil and phenolic-rich extracts. Essential oil was extracted through classical hydrodistillation, while the methanolic extract was obtained using ultrasound-assisted extraction with 30% methanol. This modern extraction technique offers significant advantages in terms of efficiently isolating phenolic compounds. Unlike many previous studies that employed conventional solvent extraction methods, the use of ultrasound-assisted extraction enhances extraction yield and selectivity. Moreover, the simultaneous evaluation of both essential oil and methanol extract in terms of comprehensive biological activity sets our study apart from other reports in the literature.

In related studies, Bahadori *et al.* (2020) prepared aqueous infusions from the leaves of *S. byzantina*, *S. inflata*, and *S. lavandulifolia*, and evaluated their phenolic content and antioxidant activities. Benedec *et al.* (2023) used methanolic extracts of the leaves of various *Stachys* species (notably *S. palustris*) for LC-MS/MS-based profiling and biological activity analysis. Similarly, Bingol and Bursal (2018) investigated the phenolic composition and antioxidant properties of *S. lavandulifolia var. brachydon* using LC-MS/MS on methanolic extracts. Stegăruș *et al.* (2021) analyzed the aerial parts of three Romanian *Stachys* species, obtaining methanolic extracts for phytochemical and biological activity evaluation.

In terms of antioxidant assays, the interpretation here was guided by standard approaches in the literature. The ABTS assay, as reported by Re *et al.* (1999), is a well-established method for determining antioxidant capacity, particularly in phenolic-rich extracts. The DPPH radical scavenging method, introduced by Brand-Williams *et al.* (1995) and further elaborated by Kedare and Singh (2011), is also widely applied to assess antioxidant potential. The alignment of our methodology with these validated approaches reinforces the comparability and reliability of our results.

CONCLUSIONS

1. The aim of the study was to compare the essential oil and methanol extractions obtained from the aerial parts of *S. cretica* subsp. *anatolica* plant with different analysis methods.
2. The enzyme inhibition activities of the methanol extract and essential oil of *S. cretica* subsp. *anatolica*, the methanol extract of *S. cretica* subsp. *anatolica* demonstrated significant activity in inhibiting CA-II, although it was less potent than the standard inhibitor acetazolamide. The weaker activity of the essential oil may be attributed to differences in the chemical composition of the two extracts. The essential oil of *S. cretica* subsp. *anatolica* showed stronger AChE inhibition compared to the methanol extract. However, both extracts were less potent than the standard inhibitor tacrine, a well-known AChE inhibitor. The essential oil of *S. cretica* subsp. *anatolica* also exhibited stronger BCHE inhibition compared to the methanol extract. However, the standard inhibitor tacrine remains significantly more potent. The methanol extract of *S. cretica* subsp. *anatolica* showed moderate α-glucosidase inhibition, while the essential oil exhibited no activity. Both the essential oil and the methanol extract of *S. cretica* subsp. *anatolica* showed limited activity against α-amylase. The weak activity of both extracts compared to acarbose suggests that *S. cretica* subsp. *anatolica* may not be a strong candidate for α-amylase inhibition.

3. The most abundant phenolic compounds in the extracts of aerial parts of *S. cretica* subsp. *anatolica* were catechin, oleuropein, epicatechin, taxifolin, flavone, and gallic acid.
4. In this study, the antioxidant capacities and bioactive component of aerial part samples were found. As the DPPH of the antioxidant capacities, the methanol extract of *S. cretica* subsp. *anatolica* exhibited substantially stronger antioxidant activity compared to its essential oil. The ABTS assay also demonstrated the methanol extract's stronger antioxidant potential compared to the essential oil. For the FRAP assay, the methanol extract had significantly higher reducing power than the essential oil. In bioactive components, the methanol extract contained substantially more TPC compared to the essential oil. The methanol extract had a much higher TFC than the essential oil. The methanol extract exhibited a higher TAC compared to the essential oil. The methanol extract of *S. cretica* subsp. *anatolica* demonstrated significantly higher antioxidant activity and bioactive component levels compared to the essential oil. These findings highlight the potential of methanol extracts as a rich source of natural antioxidants, which could be further explored for applications in food preservation, pharmaceuticals, or nutraceuticals. However, the essential oil, while less effective in antioxidant assays, may still hold value in other applications, such as antimicrobial or anti-inflammatory uses. Further research could focus on optimizing extraction methods or combining these extracts to enhance their overall bioactivity and potential therapeutic applications.
5. The detailed antimicrobial results were studied including MLC, MIC, and DDT values for all tested strains. The results demonstrate that essential oils usually exhibit stronger antimicrobial activity compared to the 30% methanol extracts across most fungal and bacterial strains, as evidenced by lower MLC and MIC values and larger inhibition zones. Nonetheless, chloramphenicol consistently showed the highest antimicrobial efficacy, with the lowest MLC and MIC values and the largest inhibition zones.
6. In the GC-MS/FID analysis of the attained essential oils, 151 compounds were found in aerial parts. The chemical classes with the most constituents in the essential oils of *S. cretica* subsp. *anatolica* were determined as esters (25.13%), oxygenated sesquiterpenes (18.54%), and aldehydes (13.09%) in aerial parts. The chemical classes with the highest percentage of components in essential oils of plant constituents were detected as esters. The main component found in essential oils of plant parts was determined as hexahydrofarnesyl acetone (7.83%).

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