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A Comparative Study on the Phenolic Compositions and Biological Activities of *Stachys annua*, *Stachys cretica*, and Endemic *Stachys tmolea* from Türkiye

Raziye Ozturk Urek, [*,a] ***Leman Tarhan,*** [a] ***Aylin Oner,*** [b] ***Yildizay Ayyildiz*** [c] ***and Mahmure Nakiboglu*** [d]

[a]*Department of Chemistry-Biochemistry, Faculty of Science, Dokuz Eylül University, 35390 Buca-Izmir, Türkiye*

[b]*Graduate School of Natural and Applied Sciences, Dokuz Eylül University, 35390 Buca-Izmir, Türkiye*

[c]*Torbali Vocational School of Higher Education, Dokuz Eylül University, 35860 Izmir, Türkiye*

[d]*Department of Biology, Faculty of Science, Dokuz Eylül University, 35390 Buca-Izmir, Türkiye*

In this study, the phenolic compound profiles and bioactivity properties of aerial part extracts obtained from *Stachys annua*, *Stachys cretica*, and the endemic species *Stachys tmolea*, all of which are recognized as medicinal plants in Türkiye, were investigated. In this context, phenolic components in the flavonoid-subgroup extracts like flavanones, flavonols, flavan-3-ols, flavan-3-ols acid hydrolysis, flavones, and in the phenolic acids and their liquid-solid phase acid-base hydrolysis extracts were determined by reversed-phase high-performance liquid chromatography coupled with a diode array detector (RP-HPLC-DAD). Also, the total-phenolic, total-flavonoid, and total-tannin contents of the *Stachys* species were estimated. Among the evaluated antioxidant capacities of the flavonoid and phenolic acid extracts, *S. annua* exhibited the strongest scavenging capacities against 2,2-diphenyl-1-picrylhydrazyl and hydroxyl-radicals with half-maximal inhibitory concentration (IC_{50}) values of 5.53 ± 0.32 and 1.09 ± 0.02 μg mL^{-1}, respectively. *S. tmolea* indicated the highest nitric-oxide radical scavenging and metal-chelating capacities. The highest enzyme inhibitory activities were determined in *S. annua* for acetylcholinesterase and in *S. cretica* for tyrosinase. The phenolic acid extracts displayed moderate cytotoxic activity against ACC-201, OE-33, HeLa, and HepG2 cells with IC_{50} values ranging from 20.1 ± 1.25 to 45.3 ± 1.35 μg mL^{-1}. The results show that the phenolic composition and bioactivity properties of investigated *Stachys* species are potentially promising for applicability in the food and pharmaceutical industries.

Keywords: antioxidant, cytotoxicity, enzyme inhibition, phenolics, *Stachys* spp.

Introduction

Phenolics with bioactive properties produced by plants are secondary metabolites, and their applications in fields such as food and medicine are of great importance due to their antioxidant, anti-inflammatory, antibacterial, antitumor, antimutagenic, and anticancer properties.[1-3] They act as antioxidants by scavenging a variety of free radicals, inhibiting primary-chain radical initiation reactions, binding metal ion catalysts, and decomposing primary oxidation products into non-radical species.[2,4,5] In conditions where the balance between intracellular free radical levels and antioxidant systems is disrupted, various oxidations in biomolecules such as proteins, lipids, and DNA, neurodegenerative and cardiovascular diseases, increased apoptosis, aging, cancer, and other disorders play a triggering role.[1,3,5] Therefore, the consumption of natural plant sources containing phenolic compounds is of great importance for a healthy life.[1-4]

In this context, *Stachys* L. is one of the largest genera of the family Lamiaceae, comprising approximately 300 species.[6-8] It is widely distributed throughout the temperate regions of the Mediterranean, and Southwest Asia, North and South America, and South Africa. In Türkiye, *Stachys* L. is represented by 81 taxa, 51 of which are endemic.[7] Previous studies[8-11] have shown that *Stachys* species contain bioactive components such as

*e-mail: raziye.urek@deu.edu.tr
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phenolics, volatile oils, iridoids, and diterpenoids, which are responsible for many of their biological activities. Plants of this genus are widely used in folk medicine, as food additives, and herbal teas. Historically, they have been used to treat various diseases, including influenza, ulcers, infections, asthma, diabetes, rheumatism, and neuropathy.[9,12] Previous studies[8,10,13] reported chlorogenic acid, verbascoside, and apigenin 7-glucoside as the major phenolic compounds in *Stachys tmolea* and *Stachys cretica* subsp. *anatolica*, whereas hesperidin, kaempferol, and apigenin were identified in *S. cretica* subsp. *smyrnaea*. In addition, the essential oil profiles of aerial part extracts of *Stachys officinalis* collected in Serbia have been investigated.[14] Another study indicated significant antioxidant, antimicrobial, and cytotoxic activities in extracts of *Stachys acerosa* Boiss., *Stachys nenthamiana* Boiss., *Stachys byzantina* K. Koch, *Stachys lavandulifolia* Vahl., *Stachys obtusicrena* Boiss., *Stachys persica* S. G. Gmel. ex C. A. Mey., *Stachys pilifera* Benth., *Stachys pubescens* Ten., and *Stachys spectabilis* Choisy ex DC. collected from Iran.[15]

In the present study, the phenolic compound profiles and biological activities of aerial part extracts of *Stachys annua*, *S. cretica*, and the endemic species *S. tmolea*, which are known as medicinal plants in Türkiye were investigated. Phenolic profiling included flavanones, flavonols, flavan-3-ols, acid hydrolysed flavan-3-ols (AH), flavones, phenolic acids, and their liquid- and solid- phase acid-base hydrolysis extracts. In addition, the total-phenolic content (TPC), total-flavonoid content (TFC), and total-tannin content (TTC) of the methanolic extracts were determined. The antioxidant properties of the extracts were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), hydroxyl (HO$^{\bullet}$), and nitric oxide (NO$^{\bullet}$) radical scavenging assays, as well as metal chelating activity, total reducing power (TRP), and ferric reducing antioxidant power (FRAP) assays. The inhibitory effects of the extracts on acetylcholinesterase (AChE) and tyrosinase were also investigated. Furthermore, the cytotoxic activities of the *Stachys* extracts against human cervical carcinoma (HeLa), lung carcinoma (ACC-201), oesophageal adenocarcinoma (OE-33), hepatocellular carcinoma (HepG2), and breast adenocarcinoma (MCF-7) cell lines were determined.

Experimental

Materials and reagents

In this study, all chemicals were of analytical or high-performance liquid chromatography (HPLC) grade and purchased from Sigma-Aldrich. Ultrapure water obtained from a Milli-Q purification system was used for the preparation of all solutions. The ACC-201 (23132/87) and OE-33 (ACC-706) cancer cell lines were obtained from the German collection of microorganisms and cell cultures (DSMZ), whereas HeLa (CCL-2), MCF-7 (HTB-22), and HepG2 (HB-8065) cell lines were obtained from the American type culture collection (ATCC). Roswell Park Memorial Institute (RPMI) 1640 and Dulbecco Modified Eagle Medium (DMEM) culture media, as well as fetal bovine serum, were purchased from GIBCO-BRL.

Plant materials

S. annua (L.), *S. cretica* subsp. *smyrnaea* Rech. Fil., and *S. tmolea* Boiss. were collected from Acıpayam village (37°22'31"N, 29°13'14"E) (FAMER 1418) in Denizli Province, and Bozdağlar village (38°14'31"N, 28°10'27"E) (FAMER 1466) and Bozdağlar-Ödemiş (38°18'20"N, 28°07'44"E) (FAMER 1459) in Izmir Province, Türkiye, respectively. Taxonomic identification of the collected specimens was carried out by Dr. M. Nakiboglu. Voucher specimens were deposited in the Herbarium of the Fauna and Flora Research and Application Centre (FAMER), Dokuz Eylül University, Türkiye. All *Stachys* samples were collected during the flowering period. The flowers and leaves of each species were separated, air-dried at room temperature in the dark, and subsequently lyophilized. Equal amounts (15 g each) of the dried flowers and leaves were mixed, ground using a mechanical grinder, and sieved to obtain a particle size of 0.30-1.0 mm. A 20 g portion of the resulting powder mixture from each *Stachys* species was subjected to Soxhlet extraction with *n*-hexane for 5 h to remove lipophilic constituents. After extraction, the *n*-hexane phase was discarded, and the defatted plant material was dried using a rotary evaporator to remove residual solvent. The resulting samples were lyophilized and stored at –20 °C until preparation of phenolic extracts. Flavonoid subgroup and phenolic acid extractions were subsequently performed with 3 and 7 g portions of the defatted plant material, respectively.[16-21]

Preparation of flavonoid subgroup extractions

For the flavanone extraction, 3 g of defatted dry plant material was extracted with 120 mL of ethanol/water (4:1, v/v) medium in a water bath at 90 °C for 2 h. After cooling, the extract was centrifuged at 5,000 rpm for 10 min, and the supernatant was concentrated under reduced pressure using a rotary evaporator to obtain the dried extract.[16]

For flavonol extraction, 3 g of defatted dry plant material was extracted under reflux in a Soxhlet apparatus using 150 mL of 85% aqueous methanol and 45 mL of 25% HCl for 1 h.[17] After cooling the extract was concentrated using a rotary evaporator and dried under reduced pressure.

For flavan-3-ols extraction, 3 g defatted dry plant material was extracted with 60 mL of methanol in an ultrasonic bath at 65 °C for 2 h. This extraction procedure was repeated twice, after which the combined extracts were centrifuged at 5,000 rpm for 10 min. The solvent was removed under reduced pressure using a rotary evaporator.[18]

Flavan-3-ol-acid hydrolysis (AH) extracts were prepared by sequential liquid-liquid extraction of flavan-3-ol extracts with 45 mL of diethyl ether followed by an equal volume of ethyl acetate. The combined organic extracts were then subjected to acid hydrolysis in 100 mL of 2.5 M HCl/methanol (4:1, v/v) at 100 °C for 2 h in a water bath.[19]

For flavone extraction, 3 g defatted dry plant material was stirred in 150 mL of diethyl ether at 25 °C for 20 min.[20] The mixture was filtered, and the solid residue was extracted twice in 75 mL portions of diethyl ether for 10 min each. The filtrates were combined and concentrated under reduced pressure using a rotary evaporator.

For each *Stachys* species, five flavonoid subgroup extracts were obtained. The extracts were subsequently dried by rotary evaporation followed by lyophilization to obtain powdered samples for subsequent analyses.

Preparation of phenolic acid extractions

Initially, 7 g of powdered defatted *Stachys* material was extracted by stirring with 120 mL of methanol/water (4:1, v/v) at 25 °C for 1 h. After solvent evaporation and lyophilization, half of the resulting extract was dissolved in 14 mL of water adjusted to pH 2.0. Extraction with 14 mL of diethyl ether liquid-liquid was then performed three consecutive times. The ether phase, containing the free phenolic acids (FPAs) was collected and concentrated under reduced pressure.[21] The remaining aqueous phase was adjusted to pH 7.0 with 2 M NaOH and concentrated under reduced pressure using a rotary evaporator. The resulting residue was redissolved in 14 mL of 2 M NaOH and stirred at 25 °C for 4 h. The solution was then adjusted to pH 2.0 with 6 M HCl and extracted three times with diethyl ether as mentioned above. The ether phase obtained at this stage contained the base-hydrolysed phenolic acids (BHPAs). Subsequently the remaining aqueous phase was treated with 14 mL of 6 M HCl at 95 °C for 20 min and extracted with ethyl ether. The resulting ether fraction was designated as the acid-hydrolysed phenolic acids (AHPA) extract. The FPA, BHPA, and AHPA extracts were concentrated under reduced pressure and subsequently lyophilized to obtain a dry powder for further analyses.

The remaining half of the original extract was used for solid phase hydrolysis. The solid residue remaining after the FPA extraction was hydrolysed with 65 mL of 2 M NaOH at 25 °C for 4 h. After separation of the liquid phase, the remaining solid residue was further hydrolysed with 6 M HCl at 95 °C for 1 h. Both liquid phases were separately extracted with diethyl ether. The resulting extracts were designated as phenolic acid-solid, base hydrolysis 1 ($PASB_1$) and as phenolic acid-solid, acid hydrolysis 2 ($PASA_2$), respectively. Finally, the remaining solid residue was subjected first to acid hydrolysis and subsequently to base hydrolysis under the same experimental conditions. The corresponding extracts were designated as phenolic acid: solid-acid hydrolysis 1 ($PASA_1$) and phenolic acid: solid-base hydrolysis 2 ($PASB_2$), respectively.

In total, 12 extracts were prepared from for each *Stachys* species, comprising five flavonoid subgroup extracts and seven phenolic acid extracts.

RP-HPLC-DAD analysis of phenolic compounds

Analyses of phenolic compounds in the *Stachys* extracts were performed using a high-performance liquid chromatography system (HPLC, Agilent 1200 Series, USA) equipped with a ultraviolet diode array detector (UV-DAD) and a fluorescence detector. HPLC was carried out on a C18 reverse-phase column (250 mm × 4.6 mm, 5 µm particles). Dried extracts and phenolic standards were dissolved in methanol (99.7%), filtered through 0.45 µm membrane filters, and analysed in triplicate using the reversed-phase high-performance liquid chromatography coupled with a diode array detector (RP-HPLC-DAD) system. Phenolic standards solutions were prepared in methanol at a concentration of 1 mg mL^{-1}. Determination of phenolic compounds was achieved considering the peak areas of calibration curves generated according to the retention times (t_R) of the standard chromatograms. The standards used, their corresponding t_R values, and the calibration curve equations (Supplementary Information (SI) section, Table S1) are provided as supplementary data, together with the chromatograms of the extracts (Figures S1-S26, SI section) and the flavonoid and phenolic acid profiles (Tables S2-S27, SI section). The injection volume was 20 µL, and the column temperature was 30 °C. The limit of detection (LOD) and limit of quantification (LOQ) were determined using the standard deviation of the response and the slope of the calibration curve or estimated based on signal-to-noise ratios of 3 and 10 for LOD and

LOQ, respectively. Method sensitivity was evaluated by calculating the relative standard deviation (RSD %) from three replicate measurements at the LOD and LOQ levels using the following equation:

$$RSD = (\text{standard deviation / mean}) \times 100 \quad (1)$$

Analysis of flavonol extracts was carried out according to the method described by Olszewska.[17] The mobile phase consisted (A) 0.5% orthophosphoric acid in water and (B) methanol, using the following gradient program at room temperature: 0-10 min, 40-60% B; 10-21 min, 60% B; 21-23 min, 60-40% B; 23-26 min, 40% B; 26-30 min, 40% B. The flow rate was 1 mL min^{-1}, and detection was performed at 370 and 254 nm. Analysis of flavan-3-ols and flavan-3-ol-(AH) compounds were performed according to the method describing by de Villiers *et al.*[18] A mobile phase consisted (A) 2% acetic acid in water and (B) acetonitrile/water (70:30, v/v), using the following gradient program: 0-3 min, 5% B; 3-8 min, 5-15% B; 8-10 min, 15-20% B; 10-12 min, 20-25% B; 12-20 min, 25-40% B; 20-30 min, 40-80% B. UV detection was carried out at 280 nm with a flow rate of 1.2 mL min^{-1}. For the analysis of flavanones, a mobile phase consisted (A) 0.6% acetic acid in water and (B) methanol, using the following gradient program: 0-5 min, 20-40% B; 5-8 min, 40% B; 8-12 min, 40-60% B; 12-25 min, 60% B; 25-30 min, 60-20% B.[16] The flow rate was 0.4 mL min^{-1}, and UV detection was performed at 285 nm. For flavone analysis, the mobile phase consisted (A) water/formic acid (19:1, v/v) and (B) methanol, using the following gradient program: 0-5 min, 50% B; 5-30 min, 80% B; 30 min, 80% B. The flow rate was 1 mL min^{-1}, and detection was performed at 350 nm.[20] For the analysis of phenolic acids, the mobile phase consisted (A) acetonitrile and (B) 2% acetic acid in water, using the following gradient program: 0-30 min, 85-100% B; 30-55 min, 50-85% B; 55-60 min, 0-50% B; 60-65 min, 0-100% B.[21] The flow rate was 1 mL min^{-1}. Hydroxybenzoic acid and hydroxycinnamic acid derivatives were estimated at 280 and 320 nm, respectively.

Total phenolic, flavonoid, and tannin contents

TPC of the *Stachys* extracts was determined using the Folin-Ciocalteu method. The reaction mixture consisted Folin-Ciocalteu reagent (2 M), sodium carbonate (7%, m/v), and *Stachys* extracts at concentrations ranging from 10 to 100 μg mL^{-1}. After incubation at room temperature for 2 h, the absorbance was measured at 765 nm using gallic acid as the calibration standard. TPC was expressed as mg gallic acid equivalents *per* g dry weight extract ($mg_{gae}\ g_{dwe}^{-1}$).[22] TFC was determined using the aluminium chloride method.[23] Extract samples were mixed with $AlCl_3.6H_2O$ (10%, m/v) and potassium acetate (1 M), followed by vortex mixing. The reaction mixture was incubated at room temperature in the dark for 30 min, after which the absorbance was measured at 415 nm. TFC was expressed as mg quercetin equivalents *per* g dry weight extract ($mg_{qe}\ g_{dwe}^{-1}$). TTC was determined using the vanillin-HCl assay.[24] The reaction mixture consisted of the extract, vanillin solution (4%, m/v), and concentrated HCl was used. After vortex mixing, the mixture was incubated at room temperature in the dark for 20 min, and the absorbance was measured at 500 nm. TTC was expressed as mg tannic acid equivalents *per* g dry weight extract ($mg_{tae}\ g_{dwe}^{-1}$).

Antioxidant properties

The antioxidant capacities of the *Stachys* extracts were evaluated by determining their scavenging activities against reactive oxygen species, reactive nitrogen species, and synthetic free radicals using spectrophotometric methods. The radical scavenging activities of the phenolic extracts were expressed as half-maximal inhibitory concentration (IC_{50}) values, representing the concentration required to inhibit 50% of the radicals. The $DPPH^{\bullet}$ assay based on the reduction of the stable nitrogen centred free radical $DPPH^{\bullet}$ by electron- or hydrogen-donating antioxidant compounds. For the determination of $DPPH^{\bullet}$ radical scavenging activity, extract solutions prepared in methanol of concentrations ranging 1 to 300 μg mL^{-1} were mixed with $DPPH^{\bullet}$ solution (1 mM) and incubated in the dark at room temperature for 30 min. The absorbance was measured at 517 nm.[25] Vitamin-C (0-35 μg mL^{-1}) and butylated hydroxytoluene (BHT) (0-10 μg mL^{-1}) were used as positive controls.

$HO^{\bullet}$ scavenging activity was determined using the 2-deoxyribose assay, which evaluate the ability of antioxidant compounds to compete with 2-deoxyribose for hydroxyl radicals generated by the Fenton reaction. This competition inhibits the degradation of 2-deoxyribose to malondialdehyde, which is subsequently quantified spectrophotometrically.[26] The reaction mixture was (final volume, 1.0 mL) contained $FeCl_3$ (100 μM), ethylenediaminetetraacetic acid (EDTA, 104 μM), H_2O_2 (100 μM), vitamin-C (100 μM), deoxyribose (2.8 mM), phosphate buffer (20 mM, pH 7.4), and *Stachys* extracts at concentrations ranging from 1 to 100 μg mL^{-1}.[26] The mixtures were incubated at 37 °C for 60 min, followed by the addition of 1 mL thiobarbituric acid (1%, m/v) and 1 mL trichloroacetic acid (TCA, 2% m/v). The reaction mixtures were then incubated in a boiling water bath for

15 min, and the absorbance was measured at 535 nm. BHT (0-10 μg mL^{-1}) was used as the positive control.

$NO^{\bullet}$ scavenging activity was determined using the Griess assay, which evaluates the ability of antioxidant compounds, to inhibit the formation of nitrite ions generated from sodium nitroprusside. The resulting nitrite ions were quantified colorimetrically.[27] The reaction mixture consisted of sodium nitroprusside (10 mM), phosphate-buffered saline (20 mM, pH 7.4), and the *Stachys* extracts (10-300 μg mL^{-1}). After incubation at 25 °C for 150 min, 0.5 mL of the reaction mixture was mixed with 0.5 mL of Griess reagent, prepared as a 1:1 (v/v) mixture of 2% (m/v) sulphanilamide and 0.2% (m/v) naphthyl ethylenediamine dihydrochloride. The resulting mixtures were incubated at 25 °C for 30 min.[27] The control A_o was prepared by replacing the extract with phosphate buffer. Absorbance was measured at 546 nm. Vitamin-C (0-65 μg mL^{-1}) was used as the positive control.

Metal chelating activity was determined using the ferrozine assay, which is based on the formation of a stable, water-soluble coloured complex between ferrozine and Fe^{II} ions.[28] The chelating capacity of the extracts was evaluated by measuring the inhibition of Fe^{II}-ferrozine complex formation. The reaction mixture consisted of $FeCl_2$ (1 mM), extracts (10-300 μg mL^{-1}), and acetate buffer (100 mM, pH 4.0). After incubation at room temperature for 30 min, ferrozine (5 mM) was added, and the absorbance was measured at 562 nm.[28] EDTA (0-10 μg mL^{-1}) was used as the positive control.

The TRP and FRAP of the *Stachys* extracts were evaluated to determine their electron-donating capacities. Both assays are based on the reduction of Fe^{III} to a Fe^{II} by antioxidant compounds, resulting in a measurable colour change.[29,30] TRP was determined according to the method of Oyaizu.[29] The reaction mixture consisted of $K_3Fe(CN)_6$ (1%, m/v), phosphate buffer (0.2 M, pH 6.6), and extracts at varying concentrations (10-100 μg mL^{-1}). After incubation at 50 °C for 30 min, TCA (10%, m/v) was added, and the mixture was centrifuged. An aliquot of the supernatant was then mixed with $FeCl_3$ (0.1%, m/v), and the absorbance was measured at 700 nm. Increased absorbance indicated greater TRP. The TRP values were expressed as mg vitamin-C equivalents *per* g dry weight extract ($mg_{vce}\ g_{dwe}^{-1}$). For the FRAP assay, 75 μL of *Stachys* extract (10-100 μg mL^{-1}) was mixed with 1425 μL of FRAP reagent consisting of 25 mL (300 mM, pH 3.6) acetate buffer, 2.5 mL of 10 mM 2,4,6-tris(2-pyridyl)-s-triazine, and 2.5 mL of 20 mM $FeCl_3$.[30] The reaction mixtures were incubated at 37 °C in the dark for 30 min, and the absorbance was measured at 593 nm. FRAP values were expressed as mg vitamin-C equivalents *per* g dry weight extract ($mg_{vce}\ g_{dwe}^{-1}$).[31]

Inhibition of AChE and tyrosinase activities

The inhibitory activities of the *Stachys* extracts against AChE and tyrosinase activities were determined according to the methods described by Uysal *et al.*[32] Galantamine (0-400 μg mL^{-1}) and kojic acid (0-500 μg mL^{-1}) were used as positive controls for AChE and tyrosinase assays, respectively. For the AChE inhibition assay, the reaction mixture consisted of 50 μL of extract, 125 μL of 3 mM 5,5-dithio-bis(2-nitrobenzoic acid) and 25 μL of 15 mM acetylthiocholine iodide. Subsequently, 25 μL of AChE solution (0.28 U mL^{-1}) was added, and the mixture was incubated for 15 min. Absorbance was then measured at 405 nm. For the tyrosinase inhibition assay, the reaction mixture consisted of 25 μL of extract, 40 μL of tyrosinase solution (200 U mL^{-1}), 100 μL of phosphate buffer (40 mM, pH 6.8), and 40 μL of L-DOPA (10 mM). After incubation at 25 °C for 5 min, the absorbance was measured at 492 nm. Enzyme inhibitory activities were expressed as IC_{50} value (μg mL^{-1}).

MTT cell proliferation assay

The cytotoxic effects of *Stachys* extracts against HeLa (ATCC® CCL-2), ACC-201 (DSMZ 23132/87), OE-33 (DSMZ ACC706), HepG2 (ATCC HB-8065), and MCF-7 (ATCC HTB-22) cancer cell lines were evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide (MTT) assay according to the method described by Mosmann.[33] Cells were seeded to 96-well plates at 1×10^5 cells *per* well and cultured at 37 °C in a humidified atmosphere of 5% CO_2. The cells were treated with *Stachys* extracts at concentrations ranging from 10 to 150 μg mL^{-1} prepared in 0.4% dimethyl sulfoxide (DMSO) for 24 and 48 h. Cells treated with culture medium containing 0.4% DMSO alone served as the negative control, while mitomycin-C was used as the positive control. Following incubation, 20 μL MTT solution (5 mg mL^{-1}) was added to each well, and the plates were incubated for 4 h at 37 °C. The resulting formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as the percentage of absorbance relative to the untreated control. IC_{50} values, defined as the extract concentrations causing 50% inhibition of cell viability, were calculated from dose-response curves generated for each cancer all line. The negative control (0.4% DMSO) showed no significant effect on cell viability, whereas cancer cells, the positive control mitomycin-C (50 μg mL^{-1}) inhibited the proliferation of HeLa, ACC-201, OE-33, and MCF-7 cells by 90.84, 87.64, 82.35, and 76.86%, respectively, after 48 h treatment.

Statistical analyses

All experiments were performed in triplicate and the results are represented as the mean ± standard deviations (SD). Statistical significance was analysed using the Student's *t*-test, with $p < 0.05$ considered statistically significant.

Results and Discussion

Phenolic compounds of *Stachys* extracts

Flavonoids and phenolic acids, which represent major classes of natural phenolic antioxidants, play essential roles in healthy nutrition by maintaining intracellular redox homeostasis and contributing to the prevention of various disorders, including aging-related diseases and cancer.[1,3] The major flavonoid and phenolic acid compounds were estimated in total 12 subgroup extracts obtained from the aerial parts of *S. annua*, *S. cretica*, and *S. tmolea* (Tables 1 and 2). Estimation was performed based on the peak areas from calibration curves generated according to the t_R of the corresponding standard chromatograms.

As shown in Table 1, epicatechin and epigallocatechin were the predominant among the 5 flavonoid subgroup extracts, and detected in the flavan-3-ol-(AH) fractions of all three *Stachys* species. The third most abundant flavonoid differed among the species, being hesperidin in the flavanone fraction of *S. annua*, hesperetin in the flavanone fraction of *S. cretica*, and isoquercitrin in the flavonol fraction of *S. tmolea*. Comparison of flavonoid contents revealed the following order: *S. tmolea* > *S. annua* > *S. cretica* as 468.72 ± 0.56; 262.38 ± 0.33; 126.70 ± 0.25 mg g_{dwe}^{-1}, respectively.

Table 1. Flavonoid compounds in the subgroup extracts of the *S. annua*, *S. cretica*, and *S. tmolea* samples determined by RP-HPLC-DAD analysis. Results were expressed as mean ± standard deviation of three independent replicates

Subgroup extracts of flavonoids / nm	*S. annua* / (mg g_{dwe}^{-1})	*S. cretica* / (mg g_{dwe}^{-1})	*S. tmolea* / (mg g_{dwe}^{-1})
Flavanone / 285 nm			
Hesperidin	41.44 ± 1.26	1.78 ± 0.07	10.99 ± 0.85
Naringenin	1.11 ± 0.05	3.33 ± 0.14	0.98 ± 0.04
Hesperetin	0.62 ± 0.01	9.53 ± 0.26	5.31 ± 0.12
Flavonol / 254, 370 nm			
Rutin	1.92 ± 0.07	nd	nd
Myricetin	1.15 ± 0.03	nd	0.34 ± 0.03
Quercetin	1.34 ± 0.05	0.25 ± 0.02	nd
Kaempferol	0.75 ± 0.07	2.31 ± 0.07	9.88 ± 0.23
Isorhamnetin	3.05 ± 0.10	nd	1.99 ± 0.06
Isoquercitrin	9.75 ± 0.22	nd	40.89 ± 1.03
Quercitrin	1.72 ± 0.08	nd	0.57 ± 0.02
Flavan-3-ol / 280 nm			
Catechin	nd	nd	nd
Epicatechin	3.02 ± 0.15	1.26 ± 0.05	1.33 ± 0.01
Epigallocatechin	nd	8.94 ± 0.14	6.04 ± 0.09
Flavan-3-ol-(AH) / 280 nm			
Catechin	11.37 ± 0.77	nd	nd
Epicatechin	116.63 ± 1.52	10.42 ± 1.01	312.02 ± 4.65
Epigallocatechin	52.72 ± 1.10	88.19 ± 1.33	66.66 ± 1.17
Flavone / 350 nm			
Apigenin	3.27 ± 0.10	0.12 ± 0.01	0.45 ± 0.03
Luteolin	0.83 ± 0.07	0.34 ± 0.05	0.55 ± 0.04
Eupatorin	3.99 ± 0.11	0.23 ± 0.02	0.24 ± 0.01
Diosmin	7.70 ± 0.15	nd	nd
Chrysoeriol	nd	nd	nd
Total amount	262.38 ± 0.33	126.70 ± 0.25	468.72 ± 0.56

mg g_{dwe}^{-1}: mg g dry weight extract^{-1}; nd: not detected.

In the study by Bursal *et al.*[12] epigallocatechin and catechin were not detected in the leaf extracts of *S. annua* collected from Türkiye whereas epicatechin was estimated at 4.5 ± 0.5 and 62.7 ± 6.4 μg g_{ext}^{-1} in the methanolic and aqueous extracts, respectively. Similarly, catechin and epicatechin were not detected in the methanolic and aqueous extracts of the aerial parts of *S. cretica* subsp. *anatolica* collected from Türkiye.[8] Bahadori *et al.*[9] reported that kaempferol, hesperidin, quercetin, and apigenin were the major flavonoids in the methanolic extract of aerial parts of *S. cretica* subsp. *mersinaea* from Türkiye with concentrations of 1910, 25770, 1900, and 2360 μg g_{ext}^{-1}, respectively. In the present study, hesperetin was identified for the three investigated *Stachys* species. In addition, epigallocatechin was detected in *S. cretica* and *S. tmolea*, while diosmin was detected in *S. annua*. Likewise, myricetin, quercitrin, and isoquercitrin were identified in *S. annua* and *S. tmolea*.[8-10,12,13,34] Furthermore, the flavan-3-ol-(AH) fractions, which were investigated in the present study exhibited the highest catechin levels following acid hydrolysis.

The hydroxybenzoic and hydroxycinnamic acid derivatives identified in the aerial part extracts of *S. annua*, *S. cretica*, and *S. tmolea* are presented in Tables 2 and 3. Benzoic acid, protocatechuic acid, and vanillic acid were the three predominant phenolic acids in both *S. cretica* and *S. tmolea*. No significant difference was observed between the protocatechuic acid content of *S. tmolea*-$PASA_1$ and *trans*-cinnamic acid content of *S. tmolea*-$PASB_2$ ($p > 0.05$). In contrast, the major phenolic acids identified in *S. annua* were rosmarinic acid, *trans*-cinnamic acid, and protocatechuic acid.

Kocak *et al.*[34] previously quantified phenolic acids in the methanolic extracts of aerial parts of *S. annua* subsp. *annua* var. annua collected from Türkiye and reported chlorogenic acid (6.28 ± 0.153 mg g_{ext}^{-1}), benzoic acid (6.12 ± 0.128 mg g_{ext}^{-1}), and rosmarinic acid (1.58 ± 0.077 mg g_{ext}^{-1}) as the predominant compounds. Chlorogenic acid was not detected in any of the *Stachys* species analyzed in the present study; however, rosmarinic acid and benzoic acid were present at relatively high concentrations in the FPA and BHPA extracts, respectively. Previous studies[9] have also shown that extracts of *S. cretica* subsp. *mersinaea* contain high levels of benzoic acid (3080 μg g^{-1}) and gallic acid (520 μg g^{-1}) in the ethyl acetate extract, as well as chlorogenic acid (1800 μg g^{-1}) in the methanolic extract. Likewise, aerial part extracts of *S. tmolea* collected from Afyonkarahisar, Türkiye, were reported to contain chlorogenic acid (1120.14 ± 41.24 μg g^{-1} dry plant in the methanolic extract), together with 4-hydroxybenzoic acid and protocatechuic acid (29.66 ± 0.12 and 11.82 ± 0.05 μg g^{-1} dry plant, respectively) in the aqueous extract.[10] Overall, the concentrations of phenolic compounds determined in the present study (Tables 1-3) were generally higher than those reported in previous studies.[9,10,34] These discrepancies are likely attributable to differences in plant material, extraction procedures, solvent systems, geographical origin, and climatic conditions affecting the investigated *Stachys* species.[8-10,12,13,35] Consistent with these findings, Khanavi *et al.*[36] reported that although *Stachys* species are generally rich in phenolic compounds, their composition and abundance are strongly influenced by the extraction method and solvent employed.

Total-phenolic, total-flavonoid, and total-tannin contents of *Stachys* extracts

Phenolic compounds are among the most abundant secondary metabolites in plants and comprise a wide range of structurally diverse compounds, from simple to complex, such as tannins. The TPC, TFC, and TTC of the methanolic extracts of *S. annua*, *S. cretica*, and *S. tmolea* are presented in Table 4.

Among the investigated species, *S. tmolea* exhibited the highest TPC (397.04 ± 4.72 $mg_{gae}\ g_{dwe}^{-1}$), TFC (80.07 ± 1.26 $mg_{qe}\ g_{dwe}^{-1}$), and TTC (208.90 ± 3.84 $mg_{tae}\ g_{dwe}^{-1}$). TPCs followed the order *S. tmolea* > *S. annua* > *S. cretica*. Although no significant difference was observed between TFCs of *S. annua* and *S. cretica* ($p > 0.05$), a slight but statically significant difference was found in their TTCs. TPCs determined for the three *Stachys* species examined in the present study were higher than these previously reported for *S. byzantina*, *S. lavandulifolia* Vahl., *S. acerosa* Boiss., *S. obtusicrena* Boiss., *S. persica* and *S. cretica* L. subsp. *vacillans* extracts.[15] Stegăruș *et al.*[37] reported total polyphenol contents of 197 ± 0.27, 222 ± 0.34, and 232 ± 43 $mg_{gae}\ g^{-1}$ in for the ethanolic extracts of *S. sylvatica*, *S. byzantine*, *S. officinalis*, respectively, values that are generally comparable with those obtained in the present study. Similarly, the TTC of *S. sieboldii* Miq. from Korea was reported to be 40.41 ± 2.54 $mg_{tae}\ g_{dwe}^{-1}$.[38] Although studies investigating the TPC, TFC, and TTC of *Stachys* species remain limited, the values reported in the available literature are generally lower than those obtained in the present study.[27,36,37,38]

Antioxidant properties of *Stachys* extracts

In recent decades, increasing attention has been given to the studies on the antioxidant properties of phenolic

Table 2. Hydroxybenzoic acid components in the extracts of the *S. annua*, *S. cretica*, and *S. tmolea* samples determined by RP-HPLC-DAD analysis. Results were expressed as mean ± standard deviation of three independent replicates

	Hydroxybenzoic acid derivatives					
Phenolic acid extracts / 280, 320 nm	Gallic / (mg g_{dwe}^{-1})	Benzoic / (mg g_{dwe}^{-1})	Vanillic / (mg g_{dwe}^{-1})	4-Hydroxy-benzoic / (mg g_{dwe}^{-1})	Syringic / (mg g_{dwe}^{-1})	Proto-catechuic / (mg g_{dwe}^{-1})
Free phenolic acids (FPA)						
S. annua	0.24 ± 0.01	3.71 ± 0.19	2.16 ± 0.05	0.74 ± 0.03	1.35 ± 0.06	0.41 ± 0.01
S. cretica	2.99 ± 0.16	3.28 ± 0.07	5.31 ± 0.18	4.02 ± 0.28	1.26 ± 0.04	1.63 ± 0.06
S. tmolea	6.96 ± 0.39	nd	7.06 ± 0.29	3.84 ± 0.16	7.06 ± 0.09	2.26 ± 0.17
Base-hydrolysed phenolic acids (BHPA)						
S. annua	nd	55.15 ± 1.12	1.42 ± 0.04	28.97 ± 0.90	14.15 ± 0.31	0.62 ± 0.01
S. cretica	1.29 ± 0.04	172.80 ± 2.65	57.76 ± 1.52	31.35 ± 1.02	nd	27.88 ± 0.87
S. tmolea	0.11 ± 0.01	53.60 ± 1.13	7.39 ± 0.68	10.58 ± 0.14	27.40 ± 0.85	15.01 ± 0.31
Acid-hydrolysed phenolic acids (AHPA)						
S. annua	1.42 ± 0.04	7.35 ± 0.32	58.49 ± 0.72	26.05 ± 0.85	11.89 ± 0.14	2.83 ± 0.05
S. cretica	1.79 ± 0.04	nd	32.52 ± 0.35	15.89 ± 0.33	15.89 ± 0.31	nd
S. tmolea	10.91 ± 0.12	nd	58.34 ± 0.86	47.08 ± 1.11	18.67 ± 0.38	4.41 ± 0.07
Phenolic acids of solid hydrolysis solid phase hydrolysis: first acid (A_1) then base (B_2)						
$PASA_1$						
S. annua	nd	nd	2.08 ± 0.05	nd	5.84 ± 0.08	148.32 ± 2.37
S. cretica	5.13 ± 0.18	nd	3.18 ± 0.07	1.03 ± 0.04	3.04 ± 0.07	93.53 ± 1.51
S. tmolea	13.92 ± 0.54	nd	10.35 ± 0.62	3.95 ± 0.07	6.11 ± 0.09	73.01 ± 1.21
$PASB_2$						
S. annua	nd	nd	10.84 ± 0.74	7.37 ± 0.13	9.01 ± 0.31	15.28 ± 0.87
S. cretica	4.43 ± 0.12	5.44 ± 0.18	20.64 ± 0.81	4.72 ± 0.07	11.86 ± 0.45	32.69 ± 0.93
S. tmolea	3.56 ± 0.07	nd	0.86 ± 0.02	8.48 ± 0.24	7.87 ± 0.10	2.07 ± 0.04
$PASB_1$						
S. annua	nd	24.44 ± 0.84	1.53 ± 0.06	1.26 ± 0.07	3.86 ± 0.05	0.73 ± 0.03
S. cretica	nd	nd	nd	nd	nd	nd
S. tmolea	nd	95.50 ± 1.45	5.37 ± 0.18	1.40 ± 0.04	nd	9.99 ± 0.11
$PASA_2$						
S. annua	nd	nd	5.04 ± 0.17	nd	9.22 ± 0.12	2.44 ± 0.10
S. cretica	nd	nd	nd	nd	nd	nd
S. tmolea	nd	nd	2.19 ± 0.04	3.57 ± 0.07	11.24 ± 0.34	2.25 ± 0.05

mg g_{dwe}^{-1}: mg g dry weight extract $^{-1}$; nd: not detected.

compounds, which may synergistically, additively, or antagonistically in scavenging reactive radical species in biological systems.[3,4] In the present study, the DPPH•, HO•, and NO• radical scavenging activities, as well as metal chelating capacity, TRP, and FRAP of the *Stachys* extracts, were evaluated and presented in Table 5. Among the tested samples the highest DPPH• scavenging activity (IC_{50} = 5.53 ± 0.32 μg mL^{-1}) was observed for the *S. annua*-FPA extract. In comparison, the IC_{50} values for the *S. cretica*-AHPA and *S. tmolea*-flavan-3-ol-(AH) extracts were 29.62 ± 0.95, and 30.31 ± 0.92 μg mL^{-1}, respectively, with no statically significant difference ($p > 0.05$). The most active *S. annua* extract exhibited 3.39 and 4.87-fold higher activity than vitamin-C and BHT, respectively ($p < 0.05$), indicating a stronger radical scavenging capacity. In a previous study, the IC_{50} values for the DPPH• scavenging activity of aqueous and methanolic extracts of *S. annua* were reported as 8.9 ± 0.2 and 7.8 ± 0.2 μg mL^{-1}, respectively.[12] Similarly, methanolic extracts of several *Stachys* species from different geographical regions have shown higher IC_{50} values, including *S. anisochila* (17.90 μg mL^{-1}), *S. plumosa* (101.61 μg mL^{-1}), *S. alpina* subsp. *dinarica* (26.14 μg mL^{-1}), and *S. beckeana* (20.90 μg mL^{-1}).[39] In another study IC_{50} values for DPPH• scavenging activity of methanolic extracts of nine *Stachys* species ranged from 454.4 ± 26.8 μg mL^{-1} (*S. spectabilis*) to 135.1 ± 4.5 μg mL^{-1}

Table 3. Hydroxycinnamic acid components in the extracts of the *S. annua, S. cretica*, and *S. tmolea* samples determined by RP-HPLC-DAD analysis. Results were expressed as mean ± standard deviation of three independent replicates

	Hydroxycinnamic acid derivatives						
Phenolic acid extracts / 280, 320 nm	Rosmarinic / (mg g_{dwe}^{-1})	*o*-Coumaric / (mg g_{dwe}^{-1})	*p*-Coumaric / (mg g_{dwe}^{-1})	Caffeic / (mg g_{dwe}^{-1})	Ferulic / (mg g_{dwe}^{-1})	Sinapic / (mg g_{dwe}^{-1})	*t*-Cinnamic / (mg g_{dwe}^{-1})
Free phenolic acids (FPA)							
S. annua	244.62 ± 7.42	0.99 ± 0.07	0.47 ± 0.02	2.46 ± 0.12	0.75 ± 0.06	201.23 ± 3.95	nd
S. cretica	5.42 ± 0.18	0.22 ± 0.01	0.81 ± 0.05	0.32 ± 0.01	0.45 ± 0.02	16.41 ± 0.65	nd
S. tmolea	9.05 ± 0.21	0.10 ± 0.01	1.31 ± 0.07	0.82 ± 0.05	0.67 ± 0.04	8.23 ± 0.17	nd
Base-hydrolysed phenolic acids (BHPA)							
S. annua	7.58 ± 0.19	nd	7.05 ± 0.20	2.05 ± 0.05	0.78 ± 0.03	15.13 ± 0.92	1.35 ± 0.03
S. cretica	4.69 ± 0.13	1.29 ± 0.04	0.71 ± 0.03	nd	2.11 ± 0.06	10.14 ± 0.27	35.62 ± 1.07
S. tmolea	0.83 ± 0.02	0.58 ± 0.01	6.46 ± 0.23	5.02 ± 0.21	35.35 ± 1.03	3.36 ± 0.06	2.66 ± 0.05
Acid-hydrolysed phenolic acids (AHPA)							
S. annua	2.61 ± 0.05	0.21 ± 0.01	5.07 ± 0.31	5.53 ± 0.28	3.64 ± 0.19	5.19 ± 0.28	0.49 ± 0.02
S. cretica	5.13 ± 0.28	3.68 ± 0.09	5.59 ± 0.27	16.13 ± 0.95	3.86 ± 0.07	44.37 ± 1.34	0.41 ± 0.01
S. tmolea	5.18 ± 0.19	0.83 ± 0.06	2.90 ± 0.05	5.27 ± 0.24	1.73 ± 0.03	9.11 ± 0.13	0.68 ± 0.04
Phenolic acids of solid hydrolysis solid phase hydrolysis: first acid (A_1) then base (B_2)							
$PASA_1$							
S. annua	2.08 ± 0.07	0.59 ± 0.04	nd	1.40 ± 0.07	0.31 ± 0.01	12.63 ± 0.23	nd
S. cretica	5.67 ± 0.22	1.13 ± 0.09	1.34 ± 0.08	2.09 ± 0.11	1.54 ± 0.07	23.46 ± 0.85	nd
S. tmolea	3.95 ± 0.13	2.24 ± 0.08	0.91 ± 0.03	4.79 ± 0.17	0.49 ± 0.03	23.27 ± 1.15	nd
$PASB_2$							
S. annua	1.25 ± 0.03	nd	5.31 ± 0.29	3.46 ± 0.16	1.08 ± 0.03	22.46 ± 0.90	nd
S. cretica	2.30 ± 0.11	0.79 ± 0.05	0.52 ± 0.03	0.68 ± 0.03	2.14 ± 0.12	21.01 ± 1.12	1.19 ± 0.04
S. tmolea	2.11 ± 0.08	0.61 ± 0.02	0.26 ± 0.01	nd	3.01 ± 0.06	70.30 ± 1.47	nd
Phenolic acids of solid phase hydrolysis: first base (B_1), then acid (A_2)							
$PASB_1$							
S. annua	0.43 ± 0.02	nd	1.35 ± 0.04	1.20 ± 0.05	2.87 ± 0.12	6.06 ± 0.22	nd
S. cretica	nd	nd	nd	nd	nd	nd	nd
S. tmolea	1.21 ± 0.07	0.33 ± 0.01	1.11 ± 0.06	nd	nd	7.03 ± 0.16	19.63 ± 0.88
$PASA_2$							
S. annua	0.58 ± 0.03	0.76 ± 0.04	2.34 ± 0.15	nd	nd	34.34 ± 1.03	nd
S. cretica	nd	nd	nd	0.13 ± 0.01	tr	nd	nd
S. tmolea	0.77 ± 0.01	0.37 ± 0.01	1.36 ± 0.07	nd	nd	6.16 ± 0.23	nd

mg g_{dwe}^{-1}: mg g dry weight extract; nd: not detected; tr: trace (< 0.001 mg g_{dwe}^{-1}).

Table 4. Total-phenolic, -flavonoid and -tannin contents of *Stachys* species. Results were expressed as mean ± standard deviation of three independent replicates

Stachys species	Total phenolics / (mg_{gae} g_{dwe}^{-1})	Total flavonoids / (mg_{qe} g_{dwe}^{-1})	Total tannins / (mg_{tae} g_{dwe}^{-1})
S. annua	228.41 ± 3.92	65.38 ± 1.52	173.00 ± 4.17
S. cretica	147.75 ± 6.35	68.88 ± 3.45	157.00 ± 2.25
S. tmolea	397.04 ± 4.72	80.07 ± 1.26	208.90 ± 3.84

mg_{gae} g_{dwe}^{-1}: mg gallic acid equivalent g dry weight extract^{-1}; mg_{qe} g_{dwe}^{-1}: mg quercetin equivalent g dry weight extract^{-1}; mg_{tae} g_{dwe}^{-1}: mg tannic acid equivalent g dry weight extract^{-1}.

(*S. byzantine*).[15] Overall, the IC_{50} value obtained for *S. annua*-FPA in the present study was lower than most values reported in the literature, indicating a comparatively stronger DPPH$^{\bullet}$ scavenging capacity.[12,15,39]

HO$^{\bullet}$ radicals are among the most reactive oxygen species and can cause severe damage to biomolecules;

therefore, their neutralisation is of particular biological importance. In this study, the HO• scavenging activities of the *Stachys* extracts were evaluated using the 2-deoxyribose assay, and the results are presented in Table 5. The highest HO• scavenging activity was observed for the flavonol and $PASB_1$ extracts of *S. annua*, with identical IC_{50} values of 1.09 ± 0.02 μg mL^{-1}, showing no statically significant difference ($p > 0.05$). Similarly, IC_{50} values of 1.17 ± 0.01 and 1.18 ± 0.02 μg mL^{-1} were obtained for *S. tmolea*-$PASB_1$ and *S. cretica*-$PASB_2$ extracts, respectively, with no significant difference between them ($p > 0.05$). These values were considerably lower than that of BHT (5.10 ± 0.20 μg mL^{-1}), indicating a stronger HO• scavenging activity of the investigated extracts ($p < 0.05$) compared with the literature, the HO• scavenging activity of *Stachys* species has been relatively less studied.[39] In a previous report, the methanolic extract of *S. anisochila* showed the highest activity among tested species, with an IC_{50} value of 6.25 μg mL^{-1}.[39] Overall, the *Stachys* extracts examined in the present study exhibited notably strong HO• scavenging activities, suggesting their potential as effective natural antioxidants against highly reactive radical species. are promising.

NO• is an important signalling molecule involved in various physiological and pathological processes; however, excessive production of NO• may lead to the formation of cytotoxic reactive nitrogen species and contribute to the development of several diseases. In this study, the NO• scavenging activities of the *Stachys* extracts were evaluated using the Griess assay, and the results are presented in Table 5. The highest NO• scavenging activity was observed for the *S. tmolea*-$PASA_1$ extract, with an IC_{50}

Table 5. Antioxidant activity results of obtained *S. annua*, *S. cretica* and *S. tmolea* extracts. Results were expressed as mean ± standard deviation of three independent replicates

Phenolic extracts	IC_{50} / (μg mL^{-1})				$mg_{vce}\ g_{dwe}^{-1}$	
	DPPH•	HO•	NO•	Metal chelating	TRP	FRAP
Flavanone						
S. annua	29.16 ± 0.88	15.00 ± 0.79	58.72 ± 2.16	71.70 ± 1.18	0.98 ± 0.02	0.30 ± 0.01
S. cretica	36.76 ± 1.03	15.40 ± 0.77	57.10 ± 1.14	59.16 ± 1.11	0.71 ± 0.05	0.10 ± 0.002
S. tmolea	46.15 ± 1.09	1.25 ± 0.02	54.46 ± 1.10	86.84 ± 1.34	0.82 ± 0.03	0.17 ± 0.005
Flavonol						
S. annua	37.32 ± 1.03	1.09 ± 0.02	44.08 ± 1.26	108.58 ± 1.55	1.02 ± 0.03	0.24 ± 0.01
S. cretica	72.67 ± 2.14	11.89 ± 0.07	78.89 ± 1.18	108.90 ± 2.72	0.64 ± 0.01	0.09 ± 0.003
S. tmolea	36.75 ± 1.03	13.90 ± 0.85	42.62 ± 1.10	106.80 ±1.61	0.85 ± 0.04	0.28 ± 0.01
Flavan-3-ol						
S. annua	46.33 ± 1.18	13.15 ± 0.87	52.27 ± 1.13	108.70 ± 1.62	0.90 ± 0.08	0.23 ± 0.01
S. cretica	100.66 ± 1.52	13.03 ± 0.06	44.15 ± 1.10	89.40 ± 1.45	0.69 ± 0.02	0.06 ± 0.001
S. tmolea	53.10 ± 1.10	11.64 ± 0.28	58.62 ± 1.12	74.40 ± 1.16	0.90 ± 0.05	0.16 ± 0.007
Flavan-3-ol-(AH)						
S. annua	6.55 ± 0.35	16.70 ± 0.80	57.45 ± 1.12	177.81 ± 2.75	1.85 ± 0.04	1.11 ± 0.02
S. cretica	57.30 ± 1.11	16.40 ± 1.04	102.02 ± 1.59	126.01 ± 1.75	0.79 ± 0.02	0.47 ± 0.01
S. tmolea	30.31 ± 0.92	17.24 ± 0.65	57.01 ± 2.26	184.16 ± 2.02	1.92 ± 0.11	1.13 ± 0.08
Flavone						
S. annua	16.65 ± 2.55	28.86 ± 1.90	131.44 ± 1.72	50.94 ± 1.15	0.57 ± 0.01	0.05 ± 0.001
S. cretica	172.18 ± 2.62	15.64 ± 0.85	275.50 ±3.97	30.23 ± 0.90	0.52 ± 0.03	0.02 ± 0.001
S. tmolea	252.65 ± 3.92	30.50 ± 2.21	183.55 ± 2.65	27.73 ± 0.85	0.50 ± 0.02	0.004 ± 0.0001
Free phenolic acids (FPA)						
S. annua	5.53 ± 0.32	13.27 ± 0.66	88.56 ±1.33	112.08 ± 1.53	2.08 ± 0.13	1.46 ± 0.07
S. cretica	120.74 ± 1.61	16.00 ± 0.74	50.30 ± 1.10	88.96 ± 1.35	0.61 ± 0.02	0.06 ± 0.002
S. tmolea	101.28 ± 1.52	12.28 ± 0.61	128.21 ± 1.70	95.71 ±1.44	0.77 ± 0.03	0.09 ± 0.003
Base-hydrolysed phenolic acids (BHPA)						
S. annua	97.14 ± 1.45	13.40 ± 0.68	61.97 ± 1.13	60.83 ± 1.11	0.73 ± 0.03	0.09 ± 0.001
S. cretica	36.12 ± 1.01	14.40 ± 0.71	36.34 ± 1.01	86.50 ± 1.32	1.13 ± 0.08	0.36 ± 0.02
S. tmolea	94.39 ± 1.44	12.02 ± 0.65	46.18 ± 1.17	107.53 ± 1.60	0.72 ± 0.04	0.12 ± 0.05

Table 5. Antioxidant activity results of obtained *S. annua*, *S. cretica* and *S. tmolea* extracts. Results were expressed as mean ± standard deviation of three independent replicates (cont.)

Phenolic extracts	IC_{50} / (μg mL^{-1})				$mg_{vce}\ g_{dwe}^{-1}$	
	DPPH•	HO•	NO•	Metal chelating	TRP	FRAP
Acid-hydrolysed phenolic acids (AHPA)						
S. annua	80.21 ± 1.25	13.56 ± 0.70	60.66 ± 1.18	121.15 ± 2.67	0.96 ± 0.04	0.22 ± 0.01
S. cretica	29.62 ± 0.95	17.24 ± 0.46	58.64 ± 2.11	50.00 ± 1.10	1.03 ± 0.03	0.41 ± 0.03
S. tmolea	31.98 ± 1.22	12.48 ± 0.63	86.68 ± 1.33	108.44 ± 3.61	0.82 ± 0.05	0.29 ± 0.02
Phenolic acids of solid hydrolysis solid phase hydrolysis: first acid (A_1) then base (B_2)						
$PASA_1$						
S. annua	58.17 ± 2.11	13.82 ± 0.71	86.10 ± 1.35	138.70 ± 2.29	1.49 ± 0.04	0.29 ± 0.02
S. cretica	93.93 ± 1.45	16.00 ± 0.75	77.08 ± 1.21	100.89 ± 1.58	1.28 ± 0.03	0.23 ± 0.01
S. tmolea	46.36 ± 1.19	12.08 ± 0.58	18.82 ± 0.75	158.78 ± 3.26	1.03 ± 0.01	0.39 ± 0.02
$PASB_2$						
S. annua	86.64 ± 2.34	12.40 ± 1.57	57.59 ± 2.25	91.00 ± 2.43	0.80 ± 0.05	0.14 ± 0.005
S. cretica	58.04 ± 1.10	1.18 ± 0.02	42.09 ± 1.12	71.68 ± 0.15	0.84 ± 0.04	0.20 ± 0.016
S. tmolea	67.93 ± 1.62	12.58 ± 0.55	44.24 ± 0.69	81.94 ± 1.30	0.94± 0.07	0.18 ± 0.010
Phenolic acids of solid phase hydrolysis: first base (B_1), then acid (A_2)						
$PASB_1$						
S. annua	88.03 ± 1.35	1.09 ± 0.05	41.54 ± 1.10	75.77 ± 1.11	0.43 ± 0.03	0.08 ± 0.003
S. cretica	155.18 ± 2.45	10.76 ± 0.47	41.82 ± 2.32	62.70 ± 2.41	0.41 ± 0.01	0.06 ± 0.004
S. tmolea	70.11 ± 1.18	1.17 ± 0.01	22.29 ± 0.87	50.00 ±1.38	0.57 ± 0.04	0.13 ± 0.001
$PASA_2$						
S. annua	90.40 ± 1.42	12.76 ± 0.62	128.00 ± 1.71	74.25 ± 1.67	0.83 ± 0.06	0.17 ± 0.008
S. cretica	90.94 ± 2.48	12.68 ± 0.28	188.11 ± 2.67	107.00 ± 3.51	0.61 ± 0.02	0.01 ± 0.001
S. tmolea	78.86 ± 2.24	13.99 ± 0.64	58.23 ± 2.11	65.70 ± 1.13	0.88 ± 0.07	0.16 ± 0.010
Positive control						
BHT	26.80 ± 1.40	5.10 ± 0.20	–	–	–	–
Vitamin-C	18.72 ± 0.17	–	36.76 ± 1.03	–	–	–
EDTA	–	–	–	5.50 ± 0.30	–	–

$mg_{vce}\ g_{dwe}^{-1}$: mg vitamin-C equivalents *per* g dry weight $extract^{-1}$; BHT: butylated hydroxytoluene; EDTA: ethylenediaminetetraacetic acid; TRP: total reducing power; FRAP: ferric reducing antioxidant power.

value of 18.82 ± 0.75 μg mL^{-1}. In comparison, the IC_{50} values for *S. cretica*- BHPA and *S. annua*-$PASB_1$ extracts were 36.34 ± 1.01 and 41.54 ± 1.10 μg mL^{-1}, respectively. When compared with the positive control (vitamin C, IC_{50} = 36.76 ± 1.03 μg mL^{-1}), the *S. tmolea*-$PASA_1$ extract exhibited approximately two-fold higher activity, indicating a stronger NO• scavenging effect ($p < 0.05$). Notably, no previous studies have reported the NO• scavenging activity of *Stachys* species; therefore, direct comparison with the literature is not possible. Excessive levels of NO• can react with biological macromolecules, leading to cellular damage and contributing to the progression of disorders such as neurodegenerative diseases, including Parkinson's disease.[40]

In the present study, isoquercitrin, isorhamnetin, rutin (flavonols), and rosmarinic, trans-cinnamic, and caffeic acids (phenolic acids) were among the predominant compounds detected in the extracts, all of which are known to possess strong antioxidant properties.[4,6] A previous study[41] reported that the order of phenolic acids with strong DPPH• scavenging activity was rosmarinic acid > caffeic acid > chlorogenic acid > α-tocopherol > ferulic acid. Overall, the radical scavenging activities observed in the present study were generally higher than those of the positive controls, highlighting the potential of these extracts for further applications, particularly in the food industry. Synthetic antioxidants such as BHT and butylated hydroxyanisole (BHA), which are widely used as food preservatives, have been associated with potential health risks.[42] Consequently, interest in natural antioxidant alternatives has increased in recent years.[43] As shown in Table 5, the highest metal chelating activity (lowest IC_{50} values) was observed for the flavone fractions of *S. tmolea*

and *S. cretica* (27.73 ± 0.85 and 30.23 ± 0.90 μg mL^{-1}, respectively), with no statistically significant difference between them ($p > 0.05$). In contrast, *S. annua*-flavone extract showed a higher IC_{50} value (50.94 ± 1.15 μg mL^{-1}), indicating lower activity. All extracts were less effective than the positive control EDTA (IC_{50} = 5.50 ± 0.20 μg mL^{-1}) ($p < 0.05$). Ferhat *et al.*[44] reported 48.00 ± 1.71% metal chelating activity for the *n*-butanol extract of *S. guyoniana* aerial parts at 100 μg mL^{-1}. In another study, Kirkan[35] reported ferrous ion chelating capacities of methanolic and aqueous extracts of *S. cretica* as 17.53 ± 1.31 and 68.72 ± 0.80 $mg_{edtae}\ g_{extract}^{-1}$, respectively. Overall, the metal chelating activities of *S. annua*, *S. cretica*, and endemic *S. tmolea* observed in the present study are comparable to or higher than those reported in the literature.[36,44]

TRP and FRAP assays, which are based on the reduction of Fe^{III} to Fe^{II}, are widely used to evaluate the electron-donating capacity of antioxidant compounds. Iron also plays an essential role in various physiological processes, including neuronal function and cellular electron-transfer reactions.[45] Thus, these assays provide important insight into the reducing potential of plant extracts. As presented in Table 5, the highest TRP and FRAP values were obtained for *S. annua*-FPA extract (2.08 ± 0.13 and 1.46 ± 0.07 $mg_{vce}\ g_{dwe}^{-1}$, respectively). The best TRP values for the other species were 1.28 ± 0.03 $mg_{vce}\ g_{dwe}^{-1}$ for *S. cretica*-$PASA_1$ and 1.92 ± 0.11 $mg_{vce}\ g_{dwe}^{-1}$ for *S. tmolea*- flavan-3-ol-(AH), with no significant difference between the highest values of *S. annua* and *S. tmolea* ($p > 0.05$). For FRAP activity, the highest values for *S. cretica* and *S. tmolea* were observed in the flavan-3-ol-(AH) fractions (0.47 ± 0.01 and 1.13 ± 0.08 $mg_{vce}\ g_{dwe}^{-1}$, respectively). Bursal *et al.*[12] reported Fe^{III} reducing capacities of *S. annua* methanol and water extracts as 1.956 ± 0.004 and 1.731 ± 0.010, respectively, while the standard BHA showed a higher value (2.404 ± 0.013). In another study, methanolic extracts of *S. persica* and *S. fruticulosa* exhibited strong FRAP values (61.42 and 62.02 mmol Fe^{II} 100 g^{-1}, respectively).[36] Additionally, FRAP values of *S. cretica* methanol and water extracts were reported as 254.40 ± 8.58 and 160.01 ± 4.14 $mg_{TroloxE}\ g_{extract}^{-1}$, respectively.[36] The FRAP value of *S. sieboldii* extract (0.78 ± 0.04 $mg_{vce}\ g_{dwe}^{-1}$) was previously reported to be 1.87-fold lower than that of the best-performing *S. annua*-FPA extract in the present study.[46] These differences may be attributed to variations in phenolic content, particularly the number and position of hydroxyl groups, which directly influence electron-donating capacity and antioxidant activity. In general, comparison of TRP and FRAP values across studies remains limited due to differences in expression units and reference standards. Nevertheless, the results of the present study indicate reducing capacities of the investigated *Stachys* extracts.

Overall, *S. annua*-FPA showed superior performance in DPPH$^{\bullet}$ scavenging, TRP, and FRAP assays, while *S. tmolea* extracts exhibited stronger NO$^{\bullet}$ scavenging and metal chelating activities. No significant differences were observed between the three species for HO$^{\bullet}$ scavenging activity, nor between *S. annua* and *S. tmolea* for TRP ($p > 0.05$). Consequently, *S. annua* and *S. tmolea* emerged as the most active species in terms of four antioxidant parameters. These findings are consistent with the higher TPC, TFC, and TTC observed in *S. annua* and endemic *S. tmolea* compared with *S. cretica*. Overall, the antioxidant activities of *Stachys* extracts appear to associate well with their phenolic composition, supporting the role of phenolic compounds as key contributors to radical scavenging and reducing properties. Recent studies have highlighted the complex synergistic, additive, and antagonistic interactions of phenolic compounds in biological antioxidant systems.[2,47]

Inhibition of AChE and tyrosinase activities

Enzyme inhibitors have attracted increasing attention due to their potential applications in pharmaceutical, food, and cosmetic industries. AChE inhibitors are particularly important for improving cognitive function and food-related bioactivities, while tyrosinase inhibitors are widely used to prevent hyperpigmentation and to manage melanin-related disorders, including certain neurological conditions.[13] The inhibitory effects of the *Stachys* extracts against AChE and tyrosinase are presented in Table 6. No inhibitory activity was observed for extracts not included in this table. The highest AChE inhibitory activities (IC_{50} values) were obtained for *S. annua*, *S. tmolea*, and *S. cretica*-FPA extracts, with values of 181 ± 9.21, 200 ± 8.73, and 210 ± 10.34 μg mL^{-1}, respectively.

In a previous study, the IC_{50} values for methanolic and aqueous extracts of *S. annua* were reported as 119.8 ± 2.4 and 150.1 ± 3.0 μg mL^{-1}, respectively, while tacrine, used as a positive control, showed an inhibitory effect (0.12 ± 0.02 μM).[12] Kocak *et al.*[34] reported AChE inhibitory activities of *S. annua* extracts (ethyl acetate, methanol, and water) as 1.89 ± 0.01, 1.58 ± 0.01, and 0.46 ± 0.02 $mg_{galae}\ g_{extract}^{-1}$, respectively. Similarly, Bahadori *et al.*[13] reported AChE inhibition values for *S. cretica* extracts ranging from 21.54 ± 1.74 to 343.78 ± 10.79 $\mu g_{galae}\ g_{dryplant}^{-1}$. Overall, although the inhibitory activity observed in the present study is lower than that of galantamine, it is comparable with previously reported values for *Stachys* species.

Regarding tyrosinase inhibition, the highest IC_{50} values were recorded for *S. annua*-flavanone (107 ± 8.41 μg mL^{-1}), *S. cretica*-FPA (170 ± 9.01 μg mL^{-1}), and *S. tmolea*-FPA (233 ± 5.88 μg mL^{-1}). These activities correspond to approximately 88, 56, and 41% of the activity of the positive control kojic acid, respectively (Table 6), which is noteworthy. Interestingly, the most active fractions were flavanone and FPA extracts, which are rich in compounds containing α-keto functional groups. Badria reported that flavonoids bearing α-keto groups exhibit strong tyrosinase inhibitory activity, supporting the findings of the present study.[48]

In a previous study, methanolic and aqueous extracts of *S. cretica* from Antalya, Türkiye, showed IC_{50} values of 1.14 and 2.4 mg mL^{-1} for tyrosinase inhibition, respectively.[36] Kocak *et al*.[34] reported tyrosinase inhibitory activities of *S. annua* extracts ranging from 8.13 ± 0.87 to 23.95 ± 1.34 mg_{kae} $g_{extract}^{-1}$, while Bahadori *et al*.[13] reported values of 0.42 ± 0.07 to 2.45 ± 0.05 mg_{kae} $g_{dryplant}^{-1}$ for *S. cretica* extracts. Overall, the results obtained in the present study indicate that specific fractions of *Stachys* species exhibit measurable inhibitory activity against both AChE and tyrosinase enzymes.

Cytotoxic effects of *Stachys* extracts against HeLa, ACC-201, OE-33, HepG2, and MCF-7 cancer cell lines

The cytotoxic and antiproliferative effects of *Stachys* extracts were evaluated against HeLa, ACC-201, OE-33, HepG2, and MCF-7 cancer cell lines following 24 h and 48 h of incubation. The effectiveness of the extracts was expressed as IC_{50} values, defined as the concentration required to reduce cell viability by 50%. According to the plant screening program of the United States National Cancer Institute (NCI), crude plant extracts are considered promising when IC_{50} values are below 30 μg mL^{-1} in *in vitro* cytotoxicity assays.[49] Therefore, in the present study, extract concentrations ranging from 10 to 150 μg mL^{-1} were used.

As shown in Figure 1, the most potent cytotoxic activity against the HeLa cell line was observed for *S. tmolea*-$PASA_1$, with an IC_{50} value of 24.5 ± 1.62 μg mL^{-1} after 48 h of incubation. For the ACC-201 cell line, the highest cytotoxic effects were recorded for *S. annua*-FPA (IC_{50} = 20.1 ± 1.25 μg mL^{-1} at 24 h) and *S. cretica*-AHPA (IC_{50} = 20.5 ± 1.35 μg mL^{-1} at 48 h). Against the OE-33 cell line, *S. annua*-FPA and *S. tmolea*-$PASA_1$ exhibited IC_{50} values of 22.0 ± 3.72 and 24.1 ± 2.02 μg mL^{-1}, respectively (24 h), with no statistically significant difference between them ($p > 0.05$). Overall, *S. annua*-FPA showed strong activity against both ACC-201 and OE-33 cell lines without significant variation in IC_{50} values ($p > 0.05$), while *S. tmolea*-$PASA_1$ was active against both OE-33 and HeLa cell lines under different incubation periods. For the HepG2 cell line, the highest cytotoxic activity was observed for *S. cretica*-$PASA_1$ (IC_{50} = 45.3 ± 1.35 μg mL^{-1}, 48 h), whereas for the MCF-7 cell line, the effect was recorded for *S. tmolea*-AHPA (IC_{50} = 120.0 ± 2.50 μg mL^{-1}, 24 h). These values exceed the NCI threshold of 30 μg mL^{-1}, indicating relatively weak activity against these two cell lines.

Phenolic acid-rich extracts showing IC_{50} values below 30 μg mL^{-1} against HeLa, ACC-201, and OE-33 cell lines contained compounds such as *trans*-cinnamic, caffeic, vanillic, syringic, rosmarinic, 4-hydroxybenzoic, protocatechuic, gallic, and *o*-/*p*-coumaric acids. Notably, the first four of these compounds were consistently present in all active extracts. Previous studies have indicated that these phenolic acids may exert cytotoxic effects individually or in combination through synergistic mechanisms.[2,50]

Literature data[51] indicate that cytotoxic effects of *Stachys* extracts against HeLa, HepG2, and MCF-7 cell lines are generally moderate or weak. For instance, IC_{50} values exceeding 500 μg mL^{-1} were reported for ethanol and methanol extracts of *S. lavandulifolia* against HeLa cells, while a methanol extract of *S. cretica* subsp. *vacillans* showed an IC_{50} value of 759 ± 1.53 μg mL^{-1}.[51,52] Saravanakumar *et al*.[11] reported that ethyl acetate extract

Table 6. Enzyme inhibitory effects of the phenolic extracts of the *Stachys* species. Results were expressed as mean ± standard deviation of three independent replicates

Enzyme	*Stachys* species	IC_{50} / (μg mL^{-1})					
		Flavanone	Flavan-3-ol	Flavan-3-ol-(AH)	Flavone	FPA	Positive control
AChE	*S. annua*	750 ± 15.72	ni	1073 ± 16.09	ni	181 ± 9.21	galantamine 8.12 ± 0.02
	S. cretica	983 ± 17.43	403 ± 11.64	611 ± 12.68	322 ± 9.46	210 ± 10.34	
	S. tmolea	1104 ± 19.85	ni	ni	310 ± 8.27	200 ± 8.73	
Tyrosinase	*S. annua*	107 ± 8.41	684 ± 10.72	531 ± 17.03	273 ± 6.85	1084 ± 17.96	kojic acid 94.70 ± 1.38
	*S. cretic*a	201 ± 8.55	213 ± 7.83	402 ± 8.15	190 ± 7.09	170 ± 9.01	
	S. tmolea	373 ± 7.13	ni	1224 ± 18.27	272 ± 5.16	233 ± 5.88	

AChE: acetylcholinesterase; FPA: free phenolic acid; ni: not inhibited.

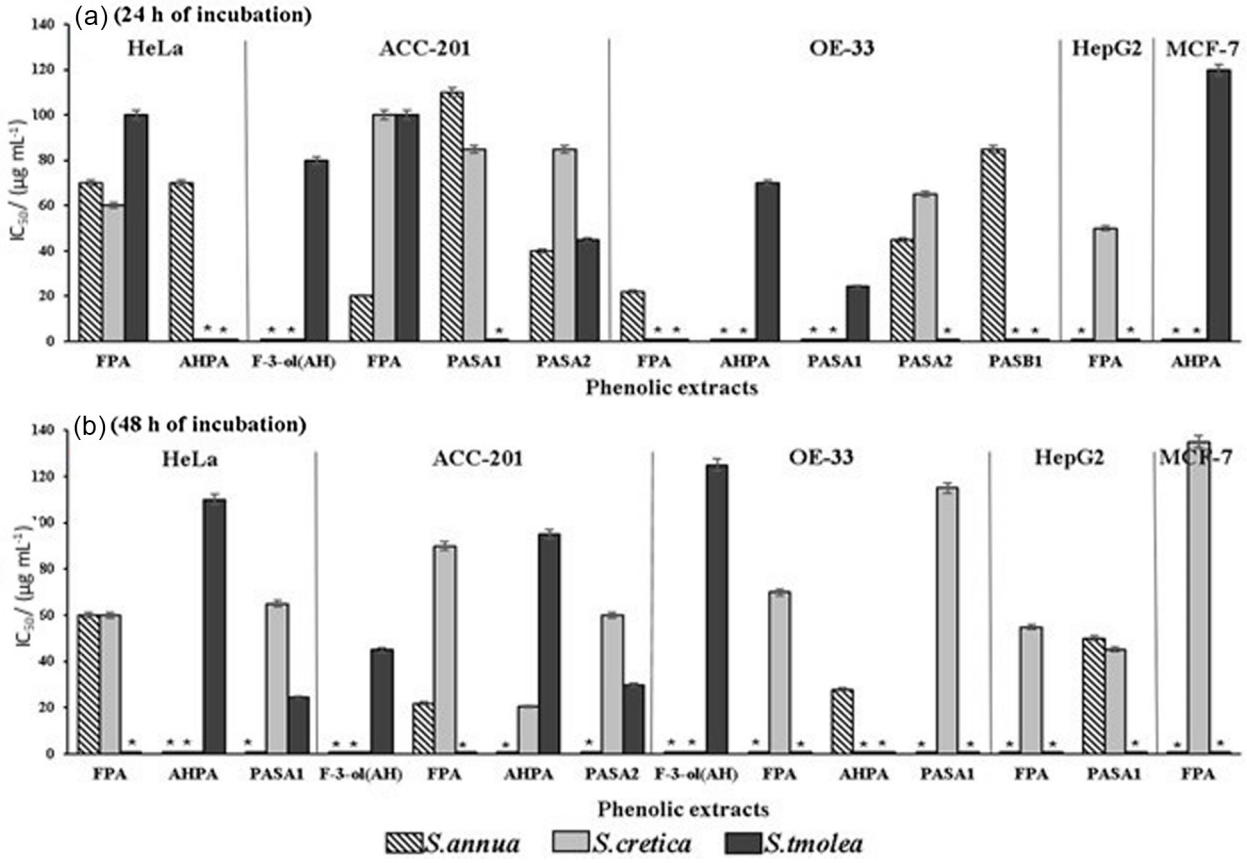


Figure 1. The IC_{50} values of the *S. annua*, *S. cretica*, and *S. tmolea* extracts against the HeLa, ACC-201, OE-33, HepG2, and MCF-7 cancer cell lines after 24 h (a) and 48 h (b) incubations. FPA: free phenolic acids; AHPA: acid-hydrolysed phenolic acids; flavan-3-ol-(AH); $PASA_1$, phenolic acid: solid phase-acid hydrolysis 1; $PASA_2$, phenolic acid: solid phase-acid hydrolysis 2; $PASB_1$, phenolic acid: solid phase-base hydrolysis 1. IC_{50} values of 140 µg mL^{-1} and above are not considered and symbolised (*). Results were expressed as mean ± standard deviation of three independent replicates.

of *S. riederi* var. *japonica*, despite being rich in rosmarinic and caffeic acids, did not exhibit significant cytotoxicity against HepG2 or NIH3T3 cells. In MCF-7 cell line studies, hydroalcoholic extract of *S. setifera* showed an IC_{50} value of 827.52 µg mL^{-1}, while other *Stachys* species such as *S. recta*, *S. palustris*, *S. germanica*, and *S. byzantina* showed inhibition rates between 28-55% at 10 mg mL^{-1}.[53,54] Leporini *et al.*[55] reported inhibition rates of 23-37% at 200 µg mL^{-1} for *S. glutinosa* ethanol extract against MCF-7 and HepG2 cell lines. Importantly, reports on the cytotoxic effects of *Stachys* species against OE-33 and ACC-201 cell lines are extremely limited. Therefore, the cytotoxic activities observed in the present study for *S. annua*, *S. cretica*, and *S. tmolea* against these cell lines are particularly noteworthy when compared with existing literature.[50] Overall, phenolic compounds derived from natural plant sources have attracted increasing attention as potential candidates for anticancer drug development, combination therapies, and chemotherapy strategies.[56,57] However, further mechanistic studies are still required to fully elucidate their therapeutic potential.

Conclusions

In the present study, flavonoid subgroups, phenolic acids, and their acid- and base-hydrolysed fractions (obtained from both liquid and solid phases) were extracted from three *Stachys* species using specific extraction procedures. RP-HPLC-DAD analysis revealed several phenolic compounds that are reported in the genus *Stachys*, including hesperetin (*S. annua*, *S. cretica*, and *S. tmolea*), myricetin, isoquercitrin, and quercitrin (*S. annua* and *S. tmolea*), epigallocatechin (*S. cretica* and *S. tmolea*), and diosmin (*S. annua*). Among the phenolic acids, benzoic, protocatechuic, and vanillic acids predominated in *S. cretica* and *S. tmolea*, whereas rosmarinic, *trans*-cinnamic, and protocatechuic acids were the major constituents of *S. annua*. The investigated extracts exhibited remarkable antioxidant activities. In particular, *S. annua* showed $DPPH^{\bullet}$ scavenging activity that was 4.87-fold stronger than that of BHT, while all three species displayed similarly high $HO^{\bullet}$ scavenging capacities. Furthermore, *S. tmolea* indicated $NO^{\bullet}$ scavenging activity that was approximately two-fold greater than that of vitamin C.

These findings highlight the potential of *Stachys* extracts as natural antioxidant sources and suggest their possible application as alternatives to synthetic antioxidants in food-related products. The extracts also exhibited moderate inhibitory activities against AChE and tyrosinase, indicating their potential for further investigation in neuroprotective and cosmetic applications. In addition, several phenolic acid-rich fractions indicated promising cytotoxic activity, particularly against ACC-201 and OE-33 cancer cell lines, with IC_{50} values below the threshold proposed by the NCI for promising crude plant extracts. Overall, the results indicate that *S. annua*, *S. cretica*, and especially the endemic species *S. tmolea* represent valuable natural sources of phenolic compounds with antioxidant, enzyme inhibitory, and cytotoxic properties. These findings provide a scientific basis for the further exploitation of *Stachys* species in food, cosmetic, and pharmaceutical applications.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

Data Availability Statement

All data are available in the text.

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Author Contributions

R. O. U.: data curation, formal analysis, investigation, methodology, validation, visualization, writing (original draft, review & editing); L. T.: conceptualization, collection of *Stachys* species, investigation, methodology, supervision, writing (original draft, review & editing); A. O.: formal analysis, investigation, validation, visualization; Y. A.: formal analysis, investigation, visualization; M. N.: investigation, collection and identification of the *Stachys* species.

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