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MICROBIOLOGY

Comparative analysis of phenolic compositions and biological activities of three endemic *Teucrium* L. (Lamiaceae) species from Turkey

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Abstract: This study investigates the chemical compositions and biological activities of the methanol extracts of three endemic *Teucrium* species (*T. ekimii*, *T. pestalozzae* and *T. semrae*) collected from Turkey. Total phenolic and flavonoid contents were assessed spectrophotometrically. The total phenolic and flavonoid content in the *T. ekimii* methanolic extract were importantly higher than other both extracts. The polyphenolic components of the extracts were identified by liquid chromatography. Seven phenolic compounds were identified namely catechin, rutin, luteolin, apigenin chlorogenic acid, sinapic acid and rosmarinic acid. Antioxidant activities were determined by five *in vitro* assays namely phosphomolybdenum assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, β-carotene bleaching assay, ferric ions reducing antioxidant power (FRAP) and cupric ions reducing antioxidant capacity (CUPRAC). The total antioxidant activity method exhibited that *T. ekimii* methanol extract exerted better antioxidant activity. The methanol extract of *T. ekimii* showed better antiradical scavenging activity as measured by DPPH assay. The antimicrobial capacities were determined by agar diffusion assay. Three endemic *Teucrium* species tested showed slight antibacterial activity only against *Aeromonas hydrophila, Klebsiella pneumoniae* and *Streptococcus pneumoniae.* The findings showed that three endemic *Teucrium* species may be utilized as natural sources of antioxidant and antimicrobial compounds in food and farmacy products.

Key words: *Teucrium*, antimicrobial, antioxidant, phenolic compounds, bioactivity.

INTRODUCTION

Reactive oxygen species (ROS) are endogenously produced in living organisms during normal cellular processes (Gulcin 2020, Huyut et al. 2017). ROS are mainly composed of non radical species and free radicals (Anbudhasan et al. 2014, Güneş et al. 2019, Huyut et al. 2017, Perron & Brumaghim 2009). Atoms, molecules or ions containing one or more unpaired electrons are called free radicals that are very reactive species (Anbudhasan et al. 2014). They are quickly atatck the molecules in neighbouring cells, and possibly can be harmful to lipids, carbohydrates, DNA, and proteins (Gulcin 2020, Perron & Brumaghim 2009). Excessive ROS cause some harmful effects. For example, imbalance between ROS production with antioxidant defences causes oxidative stress (Gulcin 2020, Güneş et al. 2019). Oxidative stress is related to causing a large number of diseases including cardiovascular, Alzheimer, Parkinson, ulcerative colitis, aging, cancer and atherosclerosis (Alam et al. 2013, Güneş et al. 2019, Huyut et al. 2017, Tsao 2010). Thus, prevention of oxidative stress has important for the prevention and treatment of these diseases (Perron & Brumaghim 2009).

Antioxidants are organic compounds that inhibit and/or reduce the oxidation processes of free radical in both food systems and the human body (Gulcin 2020, Ozgen et al. 2016). Antioxidant compounds can retard lipid peroxidation and thereby prevent deterioration of pharmaceutical products and food during processing and storage (Gulcin 2020). Synthetic antioxidants including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ), propyl gallate (PG) and octyl gallate (OG) are added to fats, oils, and lipid-containing foods because of inhibit or delay the lipid oxidation (Gulcin 2020). However, their acceptability for consumers is decreased because of the consumer doubt regarding the safety of using synthetic antioxidants in food products. Synthetic antioxidants have been limited by legislative rules because of their carcinogenic and toxic effects (Gulcin 2020). Hence, there has been increasing interest in search for alternative, safe and natural antioxidant resources, especially of plant origin such as fruits, vegetables, spices, grains, and herbs (Anbudhasan et al. 2014, Gulcin 2020).

Depending on the World Health Organization (WHO), around three-quarters of the world's population use herbs to cure diseases, and there are several examples of new pharmaceuticals generated from wild plant species (Soliman et al. 2021). Polyphenols are secondary metabolites and present extensively in plants, have attracted the attention in the food field in the recent years (Cory et al. 2018, Gulcin 2020, Tsao 2010). Polyphenolic compounds are known to have many biological activities include anticancer, antioxidant, anti-inflammatory, anti-microbial and antiviral effects (Abdel-Shafy & Mansour 2017, Güneş et al. 2019, Perron & Brumaghim 2009). In respect of preventing ands/or treating chronic diseases, polyphenols that show antioxidant activity are extremely important in terms of human health. Polyphenols protect cells and tissues against oxidative damage by acting as antioxidants (Güneş et al. 2019). As antioxidants, polyphenols are the most abundant in Man diet (Abdel-Shafy & Mansour 2017) and preserve food from oxidative rancidity (Gulcin 2020). Current literature suggests that high intake of diets rich in polyphenols may help decrease the incidence of many chronic diseases. This effect may be due to their antioxidant capacity (Cory et al. 2018, Gulcin 2020, Rasouli et al. 2017, Tsao 2010).

Teucrium L. (Lamiaceae) is composed of approximately 300 species. They distributed mainly in Europe, America, Asia, Australia and the Mediterranean area. It is represented by 48 taxa, which are 18 species endemic in Turkey (Aksoy-Sagirli et al. 2015). This genus has been utilized as medicinal herbs for over 2000 years (Küçük et al. 2006). Plants of this genus have long been used medicinally in folk medicine as expectorant, antiseptic, diuretic, hypoglycemic, antispasmodic, antipyretic and antihelmintic, diaphoretic, antirheumatic, carminative agents and antiulcer activities, to asthma, coughs, chronic bronchitis, stomach pain, amenorrhea, diabetes and gout (Aksoy-Sagirli et al. 2015, Grujičić et al. 2020, Kaska et al. 2019, Menichini et al. 2009, Tarhan et al. 2016, Ulubelen et al. 2020). Many biological activities such as analgesic, hypolipidemic, antiulcer, antioxidant, antimicrobial, antiviral, antifungal, anticonvulsant, anticancer, antitumor, antidiabetic, hepatoprotective, antipyretic, hypoglycaemic, insect antifeedant, cytotoxic, proapoptotic, antihypertensive, anti-inflammatory, anorexic, antiallergic and antinociceptiv effects have been described for different *Teucrium* species (Abdollahi et al. 2003, Aksoy-Sagirli et al. 2015, Chabane et al. 2021, El-Shazly & Hussein 2004, El Atki et al. 2019, Grujičić et al. 2020, Kaska et al. 2019, Khaled-Khodja et al. 2014, Maccioni et al. 2007, Menichini et al. 2009,

Rizvi et al. 2019). These activities are atributed to presence of rich poyphenolic compounds in the *Teucrium* species (Grujičić et al. 2020, Ulubelen et al. 2020).

Phytochemical investigations showed that some species belonging to the genus *Teucrium* contain *neo*-clerodane or abietane diterpenes, triterpenes, sesquiterpenes, and steroids. Flavonoids and aromatic compounds, although not as abundant as furan-containing neo-clerodane diterpenoids, are also determined (Ulubelen et al. 2020). This genus is one of the richest sources of monoterpenes, diterpenes, sesquiterpenes, sterols, iridoids, saponins, tannins, polyphenols, flavonoids and alkaloids (De Marino et al. 2012, El-Shazly & Hussein 2004, Khaled-Khodja et al. 2014, Rizvi et al. 2019).

Some of *Teucrium* species are presently used in the production of flavoured wines, bitters, liqueurs and herbal teas as nutritional plants. Infusions of leaves and flowers are utilized for flavouring beers in some regions. Their several biological properties such as antimicrobial, antioxidant and antifungal activities make them important in food industries as as natural preservative agents (Menichini et al. 2009).

In Turkish folk medicine, *T. chamaedrys, T. flavum* and *T. montanum* have been consumed to treatment of ulcer and diabetes and to fight obesity (Küçük et al. 2006). In the literature, there are many papers for antioxidant activities of *T. polium* (Ardestani & Yazdanparast 2007, Bakari et al. 2015, De Marino et al. 2012, El Atki et al. 2019, Khaled-Khodja et al. 2014, Panovska et al. 2005, Sharififar et al. 2009, Tepe et al. 2011), *T. sandrasicum* (Aksoy-Sagirli et al. 2015, Kaska et al. 2019, Tarhan et al. 2016), *T. chamaedrys, T. montanum* (Panovska et al. 2005), *T. montbretii* subsp. *pamphylicum* (Özkan et al. 2007), *T. trifidum* (Mazhangara et al. 2020) and *T. arduini* (Šamec et al. 2010). Several previous studies were caried out on the phytochemical composition of extracts from *T. polium* (De Marino et al. 2012, Panovska et al. 2005, Proestos et al. 2006, Sharififar et al. 2009, Tepe et al. 2011), *T. ramosissimum* (Ben Sghaier et al. 2011), *T. montanum* (Djilas et al. 2006), *T. chamaedrys, T. montanum* (Panovska et al. 2005), *T. arduini* (Grujičić et al. 2020, Šamec et al. 2010) and *T. flavum* (Grujičić et al. 2020). However, to the best of our knowledge, there are'nt no study on the phytochemical constitutions, antioxidant and antimicrobial activities of extracts from *T. ekimi* H. Duman (Duman 1998), *T. pestalozzae* Boiss. and *T. semrae* Aksoy, Dirmenci & Özcan (Aksoy et al. 2020) which are wild growing in Turkey. Therefore, the main goal of the present work was to determine, for the first time, phytochemical compositions, total phenolics, total flavonoids and antioxidant and antimicrobial activities of Turkish endemic *T. ekimi*, *T. pestalozzae* and *T. semrae* extracts as new potential sources of natural antioxidant and antimicrobial agents.

MATERIALS AND METHODS

Plant material and extraction

The plant materials were collected in the area of Antalya (Turkey), in summer 2019. Taxonomic identification was made by Prof. Dr. Ahmet AKSOY (Akdeniz University, Department of Biology, Antalya, Turkey). The voucher specimens were stored at the herbarium of Erciyes University (AA2993, AA3015 and AA3013).

1- *Teucrium ekimii* H. Duman- Antalya, Beldibi, limestone rocks, 100m, 07.06.2019, Aksoy 3013 (Turkish name; Erkurtaran)- Endemic

2- *Teucrium pestalozzae* Boiss.- Antalya, Döşemealtı, Çubukbeli Passage, limestone rocks, 990m, 07.06.2019, Aksoy 3015 (Turkish name: Oğlanotu)- Endemic

3- Teucrium semrae Aksoy, Dirmenci & Özcan- Muğla, Seydikemer, Minare Village, Pınara Ancient City, limestone rocks, 400-750

m, 23.05.2019, Aksoy 3004 (Turkish name: Kaya güzeli) - Endemic

The plants were dried at at room temperature (about 25 °C) and grinded by a mill. Powdered plant sample was macerated with methanol (1:10) for three days. The collected extracts were filtered (Whatman No. 1) and then concentrated to dryness under vacuum using Rotary evaporator (Rotavator, Buchi, Switzerland; *T*< 40 °C). Extract yield was determined and saved at 4 °C until analyzed (El-Mougy & Abdel-Kader 2007).

Liquid chromatography (LC) analysis

The extract was dissolved in methanol at the concentration of 10 mg/mL. A liquid chromatograph (Shimadzu) was equipped with HPLC pumps (LC-2030-C) and a DAD detector (284 nm). Eclipse XDB-C18 (5µm) column (250 x 4.60 mm) (Agilent) was used. The flow rate was 0.8 mL/min and the injection volume 20 µL. The analyses of the phenolic compounds were carried out at 30 °C using two linear gradients of methanol. Identification and quantitative analysis were made by comparison with standards. 19 compounds including gallic acid, protocatechuic acid, catechin, epicatechin, chlorogenic acid, caffeic acid, vanilic acid, syringic acid, *p*-coumaric acid, *o*-coumaric acid, ferulic acid, sinapic acid, rutin, ellagic acid, rosmarinic acid, luteolin, quercetin, kaempferol and apigenin were used as standard (Albayrak et al. 2010).

Total phenolic content

Folin-Ciocalteu method was used for determination of total phenolic contents in the extracts (Singleton & Rossi 1965). 0.04 mL of the methanol solution of the extract (1 mg/mL) and 600 µL of 20% sodium carbonate solution were mixed with 200 µL of Folin-Ciocalteau reagent. The absorbance was read at 765 nm (Shimadzu UV-Vis 1240, Japan) after 2h incubation in the dark at room temperature. Gallic acid as standard was used. Results were expressed as mg of gallic acid equivalents (GAE) per gram dried extract.

Total flavonoids

Total flavonoid contents of extracts were determined using the aluminum chloride colorimetric assay (Pourmorad et al. 2006). The methanol solution of the extract (1 mg/mL, 0.5 mL) was added into volumetric flask containing of aluminum chloride (10%, 0.1 mL), potassium acetate (1 M, 0.1 mL) and distilled water (2.8 mL) to react for 30 min. The absorbance of the mixture was read at 415 nm. Total flavonoids content was expressed as mg quercetin equivalents (QE) per gram dried extract.

Total antioxidant activity

The total antioxidant activity of the extracts was detected using the phosphomolybdenum method (Prieto et al. 1999). The assay measure green phosphate Mo (V) complex formed result of the reduction of Mo (VI) to Mo (V) in acid pH s(Prieto et al. 1999). 0.4 mL of the extract (2 mg/ml) was added to 4 ml of reagent solution (4 mmol/L ammonium molybdate, 0.6 mol/L sulphuric acid and, 28 mmol/L sodium phosphate). The solution was incubated at 95 °C for 90 min and then cooled to room temperature. The absorbance was read at 695 nm. Ascorbic acid was used to prepare the calibration curve and findings were expressed as mg ascorbic acid equivalents (AAE) per gram diried extract.

β-Carotene bleaching assay

The capability of the extract to prevent the bleaching of the β-carotene-linoleic acid emulsion was detected (Cao et al. 2009). A solution of β-carotene and linoleic acid was prepared with 2 mg of β-carotene in 10 mL chloroform, 20 mg linoleic acid, and 200 mg

Tween 40. The chloroform was removed in vacuo and 50 mL of aerated distilled water was added to the residue. 0.2 mL of each extract (1 mg/mL) was added to 5 mL of the above mixture. The tubes were incubated at 50 °C. After 2 h, the absorbance values were determined at 470 nm. Inhibition percentage of bleaching (%) was calculated. BHT (Butylated Hydroxytoluene) was used as the positive control.

DPPH radical scavenging ability

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activities of the extracts were measured (Lee et al. 1998). 50 µL of the extracts at 0.1- 2 mg/mL concentrations was added to 1 mL of the methanolic solution of 0.1 mmol of DPPH. The solution was incubated for 30 min at room temperature in dark. Then, the absorbance of the solutions was read at 517 nm. The percent inhibition of DPPH radical was calculated by the following equation:

$$\frac{\text{Percentage inhibition (I \%) = Absorbance of control -Absorbance of sample x 100}}{\text{Absorbance of control}}$$

BHT was used as a synthetic control. The IC50 value was calculated as the concentration of causing a 50% inhibition of DPPH radical.

Ferric cyanide (Fe^{3+}) reducing antioxidant power method

The capacity of the extracts to reduce ferric ions was determined using the Ferric Reducing Antioxidant Power (FRAP) assay (Tuberoso et al. 2010). The FRAP assay is based on the reduction of ferric 2,4,6-tris(2-pyridyl)-1,3,5-triazine [Fe(III)-TPTZ] to the ferrous complex at low pH. FRAP reagent was prepared by mixing of 300 mM acetate buffer (pH 3.6), 20 mM FeCl3. 6^{H2O} with 10 mM TPTZ in 40 mM HCl. An aliquot of the extract was mixed with diluted FRAP reagent and incubated at 37 °C for 30 min. The absorbance was measured at 595 nm. The quantitative analysis was made using the external standard assay (Fe^{+2} sulphate, 0.1-2 mmol), correlating the absorbance at 595 nm with the concentration. The results were expressed as mmol/L of Fe^{2+}.

Cupric-ion-reducing antioxidant capacity (CUPRAC) method

One mL of each solution of 7.5 mM neocuproine, 10 mM CuCl2and 1 M NH^{4Ac} buffer (pH 7.0) was mixed. Then, 0.5 mL of extracts at 0.2-1 mg/mL concentrations were added to this mixture. Then, 0.6 mL of deionised water was added and incubated for 30 min incubation at room temperature. The mixture absorbance was read at 450 nm. Trolox as the positive control was used (Apak et al. 2006).

Antimicrobial activity

In this study, the antimicrobial activity of three *Teucrium* species was tested against *Aeromonas hydrophila* ATCC 7965, *Yersinia enterocolitica* ATCC 1501, *Salmonella typhimurium* NRRLE 4463, *Listeria monocytogenes* 1/2B, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Bacillus cereus* ATCC 11778, Methicillin resistant *Staphylococcus aureus* ATCC 43300 (MRSA), *Streptococcus pneumoniae* ATCC 10015 and *Candida albicans* 10231. Agar-well diffusion method was used to determination of antimicrobial capacity (Albayrak et al. 2010). The inoculum suspension was adjusted to 106 -107 colony-forming units (cfu)/mL and suspended in sterile growth medium. Mueller Hinton agar for bacteria and Malt extract for yeast inoculated with 0.1% microbial suspension was poured over the Petri dishes (9 cm). The wells (5 mm) were cut from the agar. 50 µL of extract (30 mg/mL) was added to the wells. The methanol was used as a control. Microbial growth inhibition was determined as the diameter of the inhibition

zones around the wells. Tetracycline (10 mg/mL) and natamycin (30 mg/mL) were used as standard antibiotics.

Statistical analysis

Data from the analyses were subjected to analysis of variance (ANOVA) using SPSS (2022) for Windows. Means were separated at the 5% significance level by the least significant difference (LSD) test. Bivariate correlations were analysed by Pearson's test using SPSS 22.0 on Windows. The data were presented as mean ± standard deviation.

RESULTS AND DISCUSSION

The percent yields of the methanol extracts were found to be 16.78%, 21.6% and 17.13% (w/w) for *T. ekimii*, *T. pestalozzae* and *T. semrae*, respectively (Table I). Similarly, the yields of methanol, chloroform and aqueous extracts obtained by Soxhlet extraction and boiling water from *T. ramosissimum* were reported previously as 16.78%, 6.53% and 21.6%, respectively (Ben Sghaier et al. 2011). The yields of petroleum ether, chloroform, methanol and aqueous extracts from *T. polium* were reported as 5.9%, 3.7%, 14.9% and 8%, respectively (Sharififar et al. 2009). The extraction efficiency may vary according to the extraction method (Khaled-Khodja et al. 2014).

The phenolic components of three *Teucrium* species tested were identified using the LC apparatus. The results are given as ppm in Table II. Phenolic components couldn't be identified in the extracts not given in the Table II. Seven phenolic compounds were identified namely catechin, rutin, luteolin, apigenin, chlorogenic acid, sinapic acid and rosmarinic acid. The major compound existing in the extracts of *T. ekimii* and *T. pestalozzae* was identified as chlorogenic acid (13.957 and 53.367 ppm, respectively) while the least abundant compound was rosmarinic acid (173 and 400 ppm, respectively). Rutin was the major phenolic compound (14.311 ppm), while the minor compound was catechin (986 ppm) in the extract of *T. semrae*.

Similar findings have been published by other authors analyzing other *Teucrium* species. Sesquiterpenoids, iridoids, di and triterpenoids, and phenolic compounds were identified in *Teucrium* genus. The *neo*-clerodane diterpenoids as potential chemotaxonomic markers were the main compounds of this genus (Sadeghi et al. 2022). Grujicic (2020) identified *p*-coumaric acid, chlorogenic acid, vanilic acid, caffeic acid, syringic acid, ferulic acid, catechin, epicatechin, rutin and quercetin in the extracts of *T. adunini* and *T. flavum*. Caffeic acid (0.65 mg/100 g), ferulic acid (0.95 mg/100 g) and luteolin (0.48 mg/100 g) were determined in *T. polium* methanol extract (Proestos et al. 2006). The methanol, chloroform and aqueous extracts from *T. ramosissimum* were reported the existence of various quantities of tannins, coumarins, sterols and

Table I. Extraction yields, total phenolic, flavonoid contents of three *Teucrium* species.

Plants	Extraction Yields %	Total phenolics (mg GAE/g extract)	Total flavonoids (mg QE/g extract)
T. ekimii	16.78 ± 0.0	20.86 ± 1.5 [a]	8.37 ± 0.0 [a]
T. pestalozzae	21.60 ± 0.1	8.18 ± 0.3 [c]	6.76 ± 0.1 [c]
T. semrae	17.13 ± 0.0	17.65 ± 0.0 [b]	7.57 ± 0.0 [b]

Note: In each column, means of three independent experiments (± SD) with different superscript letters are significantly different ($p < 0.05$). Total phenolic content expressed as Gallic acid equivalent (GAE), total flavonoid content expressed as Quercetin equivalent (QE).

particularly, flavonoids (Ben Sghaier et al. 2011). Diterpenoids, flavonoids and phenolic acids were previously isolated classes of constituents from *T. montanum* (Djilas et al. 2006). Protocatechuic, 4-hydroxybenzoic, salicylic, gentisic, ferulic, vanillic, caffeic, syringic, sinapic, 4-coumaric were determined in flower and leaf infusion of *T. arduini* (Šamec et al. 2010). The chemical composition analysis of different extracts (diethyl ether, ethyl acetate and *n*-butanol) obtained from *T. chamaedrys, T. monta*num, *T. polium* were demonstrated the existence of flavonoids luteolin, apigenin and/or diosmetin (Panovska et al. 2005). Rutin and apigenin from *T. polium* extracts were identified (Sharififar et al. 2009). Caffeic acid, phenylethanoid glycoside, luteolin 7-*O*-glycoside, luteolin 7-*O*-rutinoside, teucreoside, verbascoside, diosmetin 7-*O*-glycoside, apigenin 7-*O*-glucuronide, tetrahydroxyflavone 7-*O*-glycoside, dihydroxymethoxyflavone glycoside, luteolin, diosmentin were identified from *T. polium* water extract by UV and MS spectral data (Tepe et al. 2011). The genus *Teucrium* is a rich source of *neo*-clerodane diterpenoids (Bozov & Penchev 2019). New natural *neo*-clerodane diterpenoid, namely 20-*O*-acetyl-teucrasiatin was isolated from *T. polium* collected in Nothern Iran (Venditti et al. 2017a). In other study, cirsilineol, apigenin 7-*O*-rutinoside (isorhoifolin), cirsimaritin, diosmetin, apigenin, cirsiliol and lastly poliumoside were identified in ethanolic extract of *T. polium* collected in Southern Iran (Venditti et al. 2017b). Similarly, 14 flavonoids and phenylethanoid glycosides were determined in the methanolic extract of *T. polium* from Algeria (Chabane et al. 2021). Also, the presence of iridoids and phenyl-ethanoid glycosides including verbascoside, forsythoside, samioside, alyssonoside, harpagide, 8-*O*-acetyl-harpagide, cirsiliol and β-arbutin in the *T. chamaedrys* were reported (Frezza et al. 2018). Same author and co-aouthors showed that pheophytin *a*, poliumoside, apigenin, luteolin, cirsimaritin, cirsiliol, 8-*O*-acetyl-harpagide and teucardoside were identified from *T. capitatum* (Frezza et al. 2022). These differences could be related to differences of species, extraction technique, the distinct habitat in which the plant has been collected and also standard compounds used.

Total phenolic and flavonoid contents of the methanol extracts obtained from different three *Teucrium* species were determined. Folin-Ciocalteu colorimetric assay was used to determination of total polyphenols and given as mg gallic acid equivalent per g dried extract. Aluminum chloride method was used to estimate of total flavonoids in the extracts.

Table II. The quantity (ppm) of phenolic compounds determined in three *Teucrium* by LC-MS.

Compounds	*T. ekimii*	*T. pestalozzae*	*T. semrae*
Catechin	262,0	5.899,0	986,0
Chlorogenic acid	13.957,0	53.367,0	4.295,0
Sinapic acid	5.196,0	11.799,0	5.528,0
Rutin	596,0	9.545,0	14.311,0
Rosmarinic acid	173,0	400,0	1.448,0
Luteolin	-	409,3	1.582,3
Apigenin	2.775,0	5.715,0	6.130,0

-: Not detected.

The total flavonoid contents of the methanol extracts were given as mg quercetin equivalent per g dried extract. Table I shows the findings of total phenolic and flavonoids in the methanolic extracts of three *Teucrium* species investigated in the existing study. The phenolic and flavonoid contents of the methanol extracts of three *Teucrium* species tested were significantly different ($p < 0.05$). The findings exhibited that the methanolic extracts contained phenolic contents in the following order: *T. ekimii* (20.86 ± 1.5 mg GAE/g)> *T. semrae* (17.65 ± 0.0 mg GAE/g) > *T. pestalozzae* (8.18 ± 0.3 mg GAE/g). The findings, as given in Table I, exhibit that the total flavonoids in the methanol extracts have the following order: *T. ekimii* (8.37 ± 0.0 mg QE/g)> *T. semrae* (7.57 ± 0.0 mg QE/g) > *T. pestalozzae* (6.76 ± 0.1 mg QE/g). The methanolic extract of *T. ekimii* showed to have a higher concentration of both total phenolic and total flavonoid content compared to other two investigated *Teucrium* species. According to these results, it can be concluded that the methanol extracts studied here possesses high content of phenolics and flavonoids.

The total phenolic and flavonoids in the three *Teucrium* species tested were lower if compared to methanol and other extracts of *T. polium*. Total phenolic and flavonoids of the different extracts of *T. polium* were in the range of 77.1-268.2 mg GAE/g and 21.4-197.4 mg catechin equivalents/g extract, respectively (Ardestani & Yazdanparast 2007). Total phenolic and flavonoids contents in *T. polium* water extract were found as 54.95 µg GAE/mg extract and 11.08 µg QE/mg extract, respectively (Tepe et al. 2011). Total phenolic and flavonoids of *T. polium* methanolic extract 45.65 mg GAE/g and 10.98 mg QE/g (Khaled-Khodja et al. 2014). Total phenolic and flavonoids of *T. polium* extracts ranged from 48.88 to 400.00 mg of GAE/g and 2.75 to 38.85 mg of QE/g, respectively (Bakari et al. 2015). Total phenolic contents of methanolic, ethanolic, water and ethyl acetate extracts of *T. polium* were determined as 95.53, 70.28, 40.6 and 29.25 mg GAE/g. Also, in the same study total flavonoid contents of these extracts were found to be 101.9, 65.83, 82.66 and 43.22 mg RE/g, respectively (El Atki et al. 2019). Total phenolic and flavonoids of *T. polium* methanolic extract were reported as 86.63 mg GAE/g and 24.43 mg QE/g (Chabane et al. 2021). *T. polium* ethanolic extract showed DPPH scavenging activity and FRAP due to its high phenol (155.2 mg GAE/g) and flavonoids contents (67.2 mg catechin equivalent/g) (Qabaha et al. 2021).

When the extracts of three *Teucrium* species tested were compared with *T. sandrasicum* (Aksoy-Sagirli et al. 2015, Kaska et al. 2019, Tarhan et al. 2016), *T. arduini* and *T. flavum* (Grujičić et al. 2020), *T. montbretii* subsp. *pamphylicum* (Özkan et al. 2007) and *T. ramosissimum* (Ben Sghaier et al. 2011) extracts , it was clear that the phenol and flavonoid contents were lower than these species.

The *T. sandrasicum* methanol extract was reported to be a rich source of phenolic and flavonoids (113.73 mg GAE/ g, 104.39 mg catechin equivalents/g) (Aksoy-Sagirli et al. 2015). Total phenolic and flavonoids of different extracts from *T. sandrasicum* leaves and flowers ranged from 33.37 to 81.15 mg of GAE/g and from 30.23 to 95.12 mg of catechin equivalents/g, respectively (Tarhan et al. 2016). Total phenolic and flavonoids of the hydromethanolic and the hydroethanolic extract of *T. sandrasicum* had been found as 145.71, 150.18 mg GAE/g and 48.04, 47.26 mg QE/g extract, respectively (Kaska et al. 2019). The contents of total phenolics and flavonoids in *T. arduini* and *T. flavum* extracts were 200.35 and 171.08 mg GA/g, and 96.32 and 78.14 mg RU/g, respectively (Grujičić et al. 2020). Total phenolic content of *T. montbretii* subsp. *pamphylicum* methanolic extract were reported

as 99.4 mg gallic acid equivalents (GAE)/g (Özkan et al. 2007). Total phenolic content of the methanol, chloroform and aqueous extracts from *T. ramosissimum* were reported as 120, 60 and 121.66 µg GAE/ mg, respectively. Theirs flavonoid contents were also determined as 565, 85 and 320 µg QE/ mg extract, respectively (Ben Sghaier et al. 2011).

On the other hand, the total phenol contents found in this study were similar with *T. arduini* and *T. trifidum* whose total phenol contents were in the range of 6.24-30.49 mg GAE/g (Šamec et al. 2010) and 14.1087 to 21.7977 mg GAE/g (Mazhangara et al. 2020), respectively. The total phenolic and flavonoid contents of the various *Teucrium* species may be different according to plant part, method and solvents used for extraction (Grujičić et al. 2020).

Many methods have been established for the evaluation of antioxidant capacity of the extracts including total antioxidant activity by the phosphomolybdenum, inhibition of β-carotene bleaching, FRAP assay and scavenging of DPPH radical. Table III shows the findings of antioxidant activities of the extracts expressed as mg AAE/g, DPPH inhibition (IC^{50}), percent inhibition of β-Carotene bleaching and FRAP (mM/L) values.

The phosphomolybdenum assay measures the reduction of Mo (VI) to Mo (V) in the presence of antioxidants and the production of green Mo (V) complex (Prieto et al. 1999). The total antioxidant activities of the extracts determined by the phosphomolybdenum assay were in the range of 26.30-43.43 mg ascorbic acid equivalents (AAE)/g extract. The findings showed that *T. ekimii* extract had the highest antioxidant activity with a value of 43.43 ± 1.2 mg AAE/g dried extract. *T. pestalozzae* and *T. semrae* extracts showed lower activity with values of 33.97 ± 0.2 and 26.30 ± 0.4 mg AAE/g dried extract, respectively ($p < 0.05$) (Table III). Similar to our findings, researchers reported that the extracts of *T. polium* (Ardestani & Yazdanparast 2007, El Atki et al. 2019), *T. montbretii* subsp. *pamphylicum* (Özkan et al. 2007) and *T. sandrasicum* (Kaska et al. 2019) had a substantial total antioxidant activity in phosphomolybdenum assay. The antioxidant activities of various extracts of *T. polium* were found in the range of 78.3-318.4 mg AAE/g (Ardestani & Yazdanparast 2007). The different extracts of *T. polium* showed antioxidant activity especially, the highest level of total antioxidant capacity was found in water extract (129.5 ± 3.19 mg AAE/g), while the ethyl acetate extract showed significantly the lowest activity (21.70 ± 2.20 mg AAE/g) (El Atki et al. 2019). Antioxidant activity of methanolic extract of *T. montbretii* subsp. *pamphylicum* tested by the phosphomolybdenum assay was 191.5 AAE mg/g (Özkan et al. 2007). In a previous study, the hydromethanolic and the hydroethanolic extract of *T. sandrasicum* showed strong antioxidant activity with 143.97 ± 4.96 and 102.25 ± 8.60 µg AAE/mg, respectively (Kaska et al. 2019). Moreover, three *Teucrium* species tested in this

Table III. Antioxidant activities of three endemic *Teucrium* species.

Plants	Total antioxidant activity (mg AAE/g extract)	DPPH assay IC_{50} (µg/mL)	β-Carotene assay % Inhibition	FRAP mM/L
T. ekimii	43.43 ± 1.2 [a]	26.40 [c]	58.94 [a]	3.34 ± 0.0 [a]
T. pestalozzae	33.97 ± 0.2 [b]	41.03 [a]	58.57 [a]	2.68 ± 0.0 [c]
T. semrae	26.30 ± 0.4 [c]	37.90 [b]	34.94 [b]	3.27 ± 0.0 [b]

Note: In each column, means of three independent experiments (± SD) with different superscript letters are significantly different ($p < 0.05$). Total antioxidant activity expressed as Ascorbic acid equivalent (AAE).

study showed lower total antioxidant capacity than these species.

In the present work, the β-Carotene/linoleic acid assay was used to determine the inhibition of linoleic acid oxidation by the extracts from three *Teucrium* species. The β-carotene bleaching assay measures spectrophotometrically on loss of the yellow colour of β-carotene in result of a reaction with radicals that are formed by linoleic acid oxidation in the absence of an antioxidant (Bakari et al. 2015). The extracts inhibited moderate and slightly lipid peroxidation. *T. ekimii* extract showed the highest degree of inhibition (58.94%), followed by *T. pestalozzae* extract (58.57%) and *T. semrae* extract (34.94%), at 2 mg/ml concentration ($p < 0.005$) (Table III). *T. ekimii* and *T. pestalozzae* extracts exhibited the statistically same inhibition potential against oxidation of linoleic acid. These inhibitory effects of all extracts tested were significantly lower than the value for the BHT (105.47%), at same concentration. A similar inhibition was determined for an extracts of *T. polium*. Aqueous, hydroalcoholic, ethanol, acetone, and dichloromethane extracts of *T. polium* exerted different degree of inhibitor effect in the range of 40-60% against oxidation of linoleic acid (Bakari et al. 2015). Similarly, it has been reported that water extracts of *T. polium* exerted good antioxidant capacities at the β-carotene/linoleic acid method (Tepe et al. 2011). The different extracts (diethyl ether, ethyl acetate and n-butanol) obtained from *T. chamaedrys, T. montanum, T. polium* were exerted relatively high inhibitory effect (36-43%) in the β-carotene/linoleic acid model system (Panovska et al. 2005). However, the hydromethanolic and hydroethanolic extract of *T. sandrasicum* showed the higher β-carotene bleaching activity than methanolic extracts of three *Teucrium* species tested in this study with values to 90.60% and 86.58%, respectively (Kaska et al. 2019).

The DPPH radical scavenging abilities of the extracts and standard BHT are presented in Figure 1. The extracts displayed a concentration dependent radical scavenging capacity ($p < 0.05$). BHT demonstrated the highest DPPH scavenging activity (92.15%), while *T. ekimii* (68.07 %), *T. pestalozzae* (66.81 %), and *T. semrae* (66.71 %) followed in decreasing order at the highest concentration (2 mg/mL) ($p < 0.05$). The three *Teucrium* species studied in the present study exerted a moderate hydrogen donating ability in the presence of DPPH radical. The concentrations (µg/mL) required to scavenge 50 % of the radical (IC^{50}) were in the following order: *T. pestalozzae* (41.03) > *T. semrae* (37.90)> *T. ekimii* (26.40)> (BHT (3.35) (Table III). The least IC^{50} indicate the strongest DPPH radical scavenging activity.

Our results were consistent with the earlier studies that various *Teucrium* species including *T. polium* (Ardestani & Yazdanparast 2007, Bakari et al. 2015, De Marino et al. 2012, El Atki et al. 2019, Khaled-Khodja et al. 2014, Panovska et al. 2005, Sharififar et al. 2009, Tepe et al. 2011), *T. sandrasicum* (Aksoy-Sagirli et al. 2015, Kaska et al. 2019, Tarhan et al. 2016), *T. montbretii* subsp. *pamphylicum* (Özkan et al. 2007), *T. chamaedrys, T. montanum* (Panovska et al. 2005) and *T. trifidum* (Mazhangara et al. 2020) have inhibitory effect on stable DPPH radicals. The methanolic extracts from three *Teucrium* species in the present study exerted the higher DPPH scavenging activity than that reported for methanolic extract of *T. polium* (IC^{50} =0.095 mg/mL) by (Khaled-Khodja et al. 2014), while it was the lower than that reported for methanol extract of *T. polium* (IC50= 20.1 µg/mL) by (Sharififar et al. 2009). Similar to our fingings, various extracts of *T. polium* showed different degree antiradical activity with in the range of 13-32 µg/mL IC^{50} values in DPPH assay (Bakari et

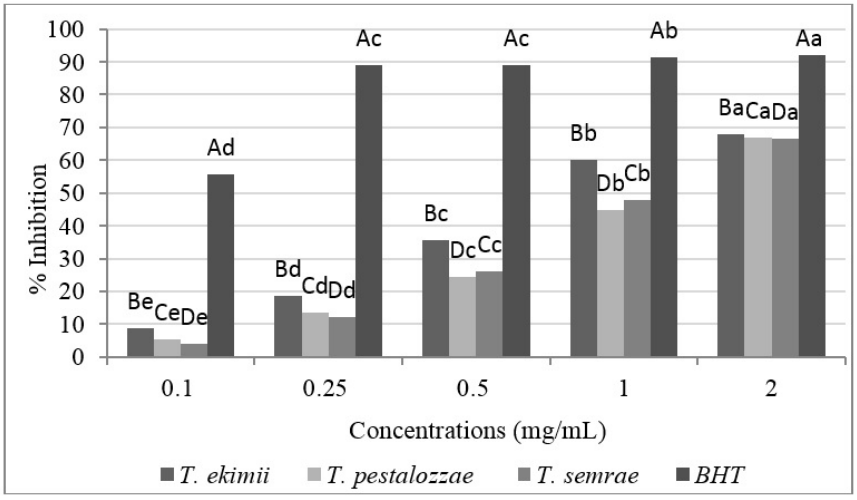

Figure 1. %Inhibition values of three endemic *Teucrium* species and BHT as positive control by DPPH assay.

al. 2015). The DPPH scavenging capacities of the various extracts of *T. polium* were determined previously with IC^{50} values in the range of 0.41-1.62 mg/mL (El Atki et al. 2019). IC^{50} values of different extract (*n*-hexane, *n*-butanol and aqueous) of *T. polium* were ranged from 7.8 to >300 µg/mL in DPPH assay (De Marino et al. 2012). IC50 values of different extract of *T. polium* were found to be 9.8->100 µg/mL (Ardestani & Yazdanparast 2007). The high DPPH scavenging activity was recorded for *T. polium* water extract with 83.95% inhibition at 1.0 mg/mL (Tepe et al. 2011).

T. sandrasicum methanol extract have inhibitory activity against stable DPPH radicals (IC50= 1.05 mg/mL) (Aksoy-Sagirli et al. 2015). IC^{50} values of different extracts from *T. sandrasicum* leaves and flowers were reported ranged from 5.85 to 62 µg/mL in DPPH assay (Tarhan et al. 2016). IC^{50} values of the hydroethanolic and the hydromethanolic extracts of *T. sandrasicum* were determined previously as 76.94 and 67.93 µg/mL in DPPH assay (Kaska et al. 2019).

DPPH radical scavenging effect of of *T. montbretii* subsp. *pamphylicum* methanolic extract was 58.6% at 100 ppm (Özkan et al. 2007). DPPH scavenging activities of different extracts from *T. chamaedrys, T. montanum* and *T. polium* were determined with IC^{50} values in the range of 10-70 mg/mL (Panovska et al. 2005). At the 0.08 mg/mL concentration, *T. trifidum* demonstrated the high DPPH scavenging activity with 92.67 % for acetone extract, 92.15 % for ethanol extract, 91.65 % for methanol extract and 39.64 % for the aqueous extract. In the same study, IC^{50} values of these extracts were found as 0.012-0.095 mg/mL (Mazhangara et al. 2020). A strong correlation between the IC^{50} value of DPPH and total phenolic (R^2= -0852) and flavonoid (R^2= -0.965) contents of *T. ekimii*, *T. pestalozzae* and *T. semrae* extracts was found.

Based on the standard (Fe^{2+}), the FRAP values of the extracts, at 2 mg/mL concentration, were 3.34 ± 0.0, 2.68 ± 0.0 and 3.27 ± 0.0 mM/L for *T. ekimii*, *T. pestalozzae* and *T. semrae*, respectively which was comparable to that of L-ascorbic acid at 4.15 ± 0.0 mg/mL ($p< 0.005$) (Table III). This results indicate potential of the methanol extracts of three *Teucrium* sprecies tested as a potential antioxidant. Our results were similar to the other researchers who reported that *Teucrium* species have high reducing power. The methanol extract of *T. sandrasicum* acted as a reductant with 2.66 ± 0.21 mM/L Fe^{2+}FRAP value (Aksoy-Sagirli et al. 2015). FRAP values of leaf and flower infusions of *T. arduini* were recorded in the range of 37.58-171.08 µmoL Fe^{+2}/g (Šamec et al. 2010). *T. polium* extracts revealed antioxidant activity with IC^{50} values of 0.21-4.25 mg/mL in

FRAP method (El Atki et al. 2019). A significant positive correlation between ferric reducing activity and phenolic (R^2= 0.980) and flavonoid (R^2= 0.905) contents of *T. ekimii*, *T. pestalozzae* and *T. semrae* extracts was found.

The reduction of the copper (II)-neopurin complex to the copper (I)-neucuproin complex in the presence of the extract was analysed using CUPRAC assay (Apak et al. 2006). The formed solution gives maximum absorbance at 450 nm. The copper (II) reducing abilities of three *Teucrium* species are given as absorbance values at 450 nm and compared with the values of standard Trolox (Figure 2). Reducing activity increased with increasing concentration. High absorbance values reflect high reducing activity. Copper (II) reducing activity of the methanol extarcts have the following order: *T. ekimii* (2.12) > *T. semrae* (2.03) > *T. pestalozzae* (1.51) at 1 mg/mL ($p< 0.005$). The absorbance values of the extracts from *T. ekimii* and *T. semrae* were similar to value of the trolox (2.85) at the same concentration. To the best of our knowledge, there are no reports in the literature for copper reducing power of *Teucrium* species. This is the first time that the *in vitro* antioxidant activity of three endemic *Teucrium* species (*T. ekimii*, *T. pestalozzae* and *T. semrae*) is reported in the literature.

The antimicrobial activity of the methanolic extracts of *T. ekimii*, *T. pestalozzae* and *T. semrae* was tested by agar diffusion assay using nine bacteria and one yeast strain. In this study, the antimicrobial activities of the extracts were compared with standard antibiotics. Three *Teucrium* species tested showed slight antibacterial activity (7-8.5 mm) only against *Aeromonas hydrophila, Klebsiella pneumoniae* and *Streptococcus pneumoniae* at 30 mg/ml. No effect was observed aginst *Y. enterocolitica, S. thyphimurium, L. monocytogenes, E. coli, S. aureus* (MRSA) *and B. cereus*. The methanol extracts exhibited very lower inhibitory zones against all tested bacteria than tetracycline. None of the investigated extracts exerted inhibitory effect on the yeasts namely *C. albicans*. Natamycin had 23 mm inhibitory zone on *C. albicans* at 10 mg/mL concentration (Table IV). *n*-hexane-ether extract of *T. leucocladum* exerted potent inhibitory activity against *E. coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Candida albicans* (15-19 mm) while the

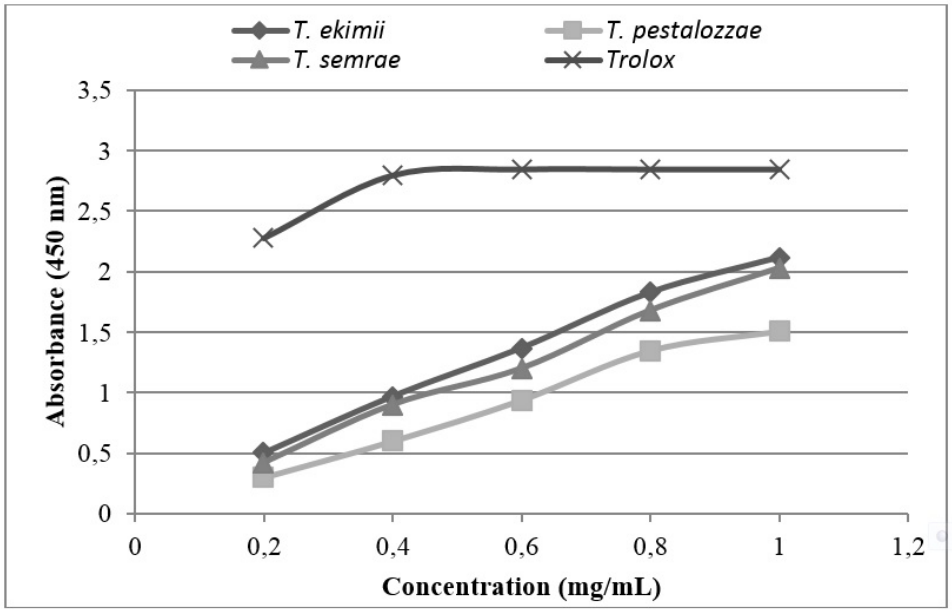

Figure 2. CUPRAC results of three endemic *Teucrium* species and Trolox.

extract was inactive against *S. aureus* at 20 mg/mL (El-Shazly & Hussein 2004). *T. montanum* extracts exhibited antibacterial activity against *Pseudomonas aeruginosa*, *S. aureus*, *Sarcina lutea* and *Bacillus sp*. (Djilas et al. 2006). Antibacterial activity of *T. montbretii* subsp. *pamphylicum* methanolic extract was tested using the agar diffusion assay. The extract had no effect against any of the bacteria at 1% and 2.5%. *Salmonella typhi* was the most resistant bacterium, but *L. monocytogenes* was the most sensitive (Özkan et al. 2007). Antibacterial activity of ethanol and methanol extracts of *T. polium* was previously reported (Darabpour et al. 2010). *T. arduini* infusions had no inhibitory activity on *E. coli, P. aeruginosa, C. albicans* and *Aspergillus niger* at 66.66 mg/mL concentration (Šamec et al. 2010). *T. trifidum* extracts had an appreciable broad-spectrum antibacterial activity against tested pathogenic bacteria including *S. aureus, S. thyphimurium, Vibrio cholerae, K. pneumoniae, Streptococcus pyogenes, B. cereus, B. subtilis* and *Pseudomonas aeruginosa* (Mazhangara et al. 2020). According to literature survey, this is the first time that *in vitro* antimicrobial activity of three endemic *Teucrium* species (*T. ekimii*, *T. pestalozzae* and *T. semrae*) is reported in the literature.

In conclusion, the results reported in the present study exerted that three endemic *Teucrium* species collected from Turkey contain a considerable amount of phenolic compound and have a significant antioxidant activity. It is noteworthy that this activity was shown herein for the first time to three endemic *Teucrium* species (*T. ekimii*, *T. pestalozzae* and *T. semrae*). As the results were compared in terms of the antioxidant activity, the highest total antioxidant activity, DPPH scavenging and inhibitory activity on the bleaching of the β-carotene-linoleic acid,

Table IV. The antimicrobial activities of three *Teucrium* species and standard antibiotics (zone size, mm).

	Extracts (30 mg/mL)			**Tetracycline (10 mg/mL)**
Bacteria	***T. ekimii***	***T. pestalozzae***	***T. semrae***	**mm**
A. hydrophila	7.75*	7.75	8.5	27.0
Y. enterocolitica	-	-	-	23.0
S. thyphimurium	-	-	-	15.0
L. monocytogenes	-	-	-	29.0
E. coli	-	-	-	24.0
K. pneumoniae	7.0	8.0	8.0	48.0
S. aureus (MRSA)	-	-	-	25.0
B. cereus	-	-	-	27.0
S. pneumoniae	8.0	7.5	9.0	24.0
Yeast				Natamycin (10 mg/mL)
C. albicans	-	-	-	23.0

*: **Inhibition zone include diameter of hole (6 mm). Sample amount is 50 µL. -: Not detected.**

ferric and copper reducing activity were observed in *T. ekimii* extract. A significant correlation was also recorded between DPPH scavenging activity and FRAP values with the presence of high phenolics and flavonoids, which were apparently the main responsible for antioxidant activity. This is the first time that catechin, rutin, luteolin, apigenin chlorogenic acid, sinapic acid and rosmarinic acid were determined in the extracts of *T. ekimii*, *T. pestalozzae* and *T. semrae*. Morever, the methanolic extracts from *T. ekimii*, *T. pestalozzae* and *T. semrae* exerted slight antibacterial activity only against *Aeromonas hydrophila*, *Klebsiella pneumoniae* and *Streptococcus pneumoniae*. We recorded for the first time, the antimicrobial effects of Tukish endemic *Teucrium* extracts on nine bacteria and one yeast strain. The results reported in our study offer that *T. ekimii*, *T. pestalozzae* and *T. semrae* are promising candidates for natural antioxidant and antimicrobial agents in food, medical, therapeutic, and pharmacological industries.

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Collection and taxonomic identification were made by Ahmet Aksoy. Sevil Albayrak and Ahmet Aksoy contributed to the study design and performed experiments and acquired and analyzed the data. Both authors contributed in part to writing and editing the manuscript and approved the final version.