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When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

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Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

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One author: (Ergenç 2000)

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Book Section: Benn MH, Jacyno JM (1983) The toxicology and pharmacology of diterpenoid alkaloids. In: Pelletier SW (ed./eds.) *Alkaloids: Chemical and Biological Perspectives*, Vol. 1, John Wiley & Sons, New York, pp. 153-210.

Books with a Single Author: Bremer K (1994) *Asteraceae: Cladistics and Classification*. 1st ed, Timber Press, USA.

Editor(s) as Author: Huizing EH, de Groot JAM, editors (2003) *Functional reconstructive nasal surgery*. Stuttgart-New York: Thieme.

Conference Proceedings: Bengissson S, Sothemin BG, (1992) Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors. *MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics*; 1992 Sept 6-10; Geneva, Switzerland. Amsterdam: North-Holland; pp.1561-5.

Scientific or Technical Report: Cusick M, Chew EY, Hoogwerf B, Agrón E, Wu L, Lindley A, Ferris FL 3rd; Early Treatment Diabetic Retinopathy Study Research Group. Early Treatment Diabetic Retinopathy Study Research Group. Risk factors for renal replacement therapy in the Early Treatment Diabetic Retinopathy Study (ETDRS), Early Treatment Diabetic Retinopathy Study. *Kidney Int*: 2004. Report No: 26.

Dissertation (Thesis): Gürdal B (2010) *Ethnobotanical Study in Marmaris District (Muğla)*. Unpublished MSc Thesis, İstanbul University, Institute of Health Science, İstanbul.

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Treglia G, Ceriani L, Sadeghi R, Giovacchini G, Giovannella L. (2014) Relationship between prostate-specific antigen kinetics and detection rate of radiolabelled choline PET/CT in restaging prostate cancer patients: A meta-analysis. *Clin Chem Lab Med*. <http://www.reference-global.com/toc/cclm/current> Accessed 16.09.2014.



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The anatomical properties of *Scabiosa atropurpurea* L. (Caprifoliaceae)

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ABSTRACT

Scabiosa atropurpurea L. (Caprifoliaceae), a medicinal plant traditionally known as “Mor uyuzotu or Şeytanotu” in Turkey, has been used for treating acne, bronchitis, cold, and cough. Although phytochemical studies are available, there are no taxonomic studies related to this plant. The aim of this study was to investigate the anatomy of leaf, stem, and root of *S. atropurpurea*. Detailed photographs of plant parts were taken, and measurements of the leaf, stem, and root were carried out. The cross section of the stem revealed a single-layered epidermis containing one-celled simple eglandular trichomes and rarely one-celled hook-shaped eglandular trichomes. The cortex showed multilayered parenchymatous cells and a single-layered endodermis. The phloem was followed by the cambium, and the xylem was lignified. The pith was made up of parenchymatous cells. The leaves are bifacial and amphistomatic and stomata are mesomorphic in the cross section and also stomata are anomocytic in the surface section.

The cross section of the root showed that the epidermis was replaced with the periderm. Under the phloem, which had few layers, the xylem was composed of tracheary elements surrounded with sclerenchymatous cells.

Keywords: *Scabiosa atropurpurea*, Caprifoliaceae, Şeytanotu, plant anatomy, Anatolia.

INTRODUCTION

The genus *Scabiosa* L. is a member of the Caprifoliaceae Juss. family and it is represented by ca. 100 species all over the world. Most of *Scabiosa* species grow in the Mediterranean region (Carlson et al. 2012). This genus is represented by 32 species in Turkey (Göktürk 2012). One of them, *S. atropurpurea* L., is a biennial or perennial plant which is 20-60 cm in height. This species is separated from other species with its bluish-lilac flower color and the characteristic shape of its fruit. It is native to Turkey and located around roadsides, dry fields, and dunes. The flowering time of this plant is from May to October and it shows a wide distribution in Turkey (Mathew 1972; Ekici 2010; Akalın Uruşak et al. 2013; Köse and Özen 2017).

S. atropurpurea known as “Mor uyuzotu, Şeytan otu” in Turkey (Asal and Yaşarkan 2017; Tuzlacı 2006) is known as “Ambarina” in Northern Peru and “Escabiosa” in Iberian Peninsula (Bussman and Glenn 2010; Bussman et al. 2010). It has been used traditionally in several diseases like acne, bronchitis, cold, cough by means of its analgesic, antipyretic anti-inflammatory and antibacterial activities (Marhuenda-Requena et al. 1987; Saenz-Rodrigues et al. 1987; Bonet et al. 1999; Bussman and Glenn 2010). In Iberian Peninsula, a kind of herbal tea is prepared from its aerial parts and it is used as a veterinary diuretic. In Northern Peru, it is used orally or by inhalation for menstrual regulation (Bonet and Vallès 2007; Bussman and Glenn 2010). In Egypt, it is known as an ornamental plant (Elhawary et al. 2011). Many studies revealed that the main chemical constituents of this species are iridoid

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glucosides, flavonoids and phenolic compounds (Polat et al. 2010; Elhawary et al. 2011).

Anatomical studies were not found in the literature review of the *S. atropurpurea*. In this study, the stem, leaf and root parts of the taxon were examined and the anatomical structure was revealed for the first time. Moreover, a distribution map of the species was located (Figure 1).

MATERIALS AND METHODS

The study material, *S. atropurpurea* was collected from İstanbul, Validebağ Korusu grove on October 2017. The collected specimens were identified by Zeynep Büşra Erarslan and dried specimen of the plant was kept in the Herbarium of İstanbul University Faculty of Pharmacy (ISTE) by herbarium number 115040. Leaves, stems and roots stored in 70% ethanol for anatomical examination, and then all cross sections and surficial sections were cut by hand with blade. Samples were examined in Sartur reagent. Photographs were taken by Canon Power shot A640 and measurements of stems, leaves and roots were made by program of KAMERAM©.

RESULTS

Leaf

From the cross-section of leaf was found to be bifacial, amphistomatic and mesomorphic. The upper and lower epidermises are composed of single-layered, rectangular-rounded cells. Upper epidermal cells have larger sizes than lower epidermal cells. The epidermal cells on both surfaces are surrounded by a thin layer of cutin. On the upper and lower epidermis, there are one-celled eglandular trichomes and multi-celled glandular trichomes. The measurements of the glandular trichomes are $20.65 - 28.41 \times 30.47 - 51.49 \mu\text{m}$ and average is $- 40.55$

μm . The mesophyll is composed of 3-4 layers of palisade parenchyma cells under the upper epidermis and spongy parenchyma cells with wide intercellular spaces. Leaf thickness is between $314.39 - 356.69 \mu\text{m}$ and average is $335.55 \mu\text{m}$ (Figure 2 a, b, c).

In the cross section of the main vein, 1-2 layers of collenchyma are seen under the lower epidermis. There are thin-walled parenchyma cells of different sizes between the collenchyma layer and the vascular bundles. A few druse crystals were seen in this area. Veins are collateral, with the xylem located upper side and phloem located lower side. The leaf main vein thickness is $523.77-686.19 \mu\text{m}$ and average is $601.27 \mu\text{m}$ (Figure 2 d, e, f).

On the surface section, lower epidermal cells seem to be wavier than the upper epidermal cells. On both sides of the lamina there are oval shaped and various sized stomata (Table 1). Stomata anomocytic, 2-3 cells surrounding each stoma which are not recognizably distinct from the remaining cells in the mature epidermis. The stomatal index for the upper surface of the lamina 24.52; the stomatal index for the lower surface is 26.23. The stomatal index ratio was calculated as 0.934 (Figure 3 a, b).

Stem

The outermost part of the herbaceous stem's cross section is composed of epidermal cells with a single layer and the cells are covered by a thin cuticular layer. Width of the epidermal cells is ranging from 16.94 to $18.61 \mu\text{m}$, length is ranging from $14.68 \mu\text{m}$ to $19.10 \mu\text{m}$. One-celled simple eglandular trichomes and rarely one-celled hook-shaped eglandular trichomes are seen on the epidermis. Just below this layer, there are varying sizes of multilayered parenchymatous cells which are forming the cortex part of the stem. A single layered en-

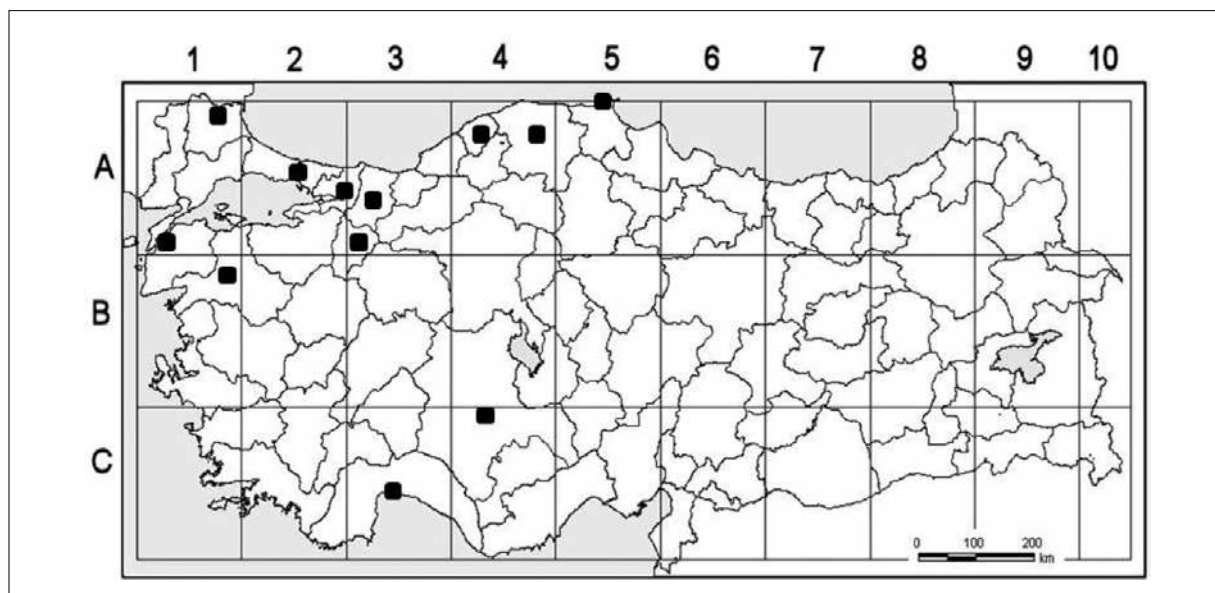


Figure 1. Distribution map of *S. atropurpurea* L. in Turkey.

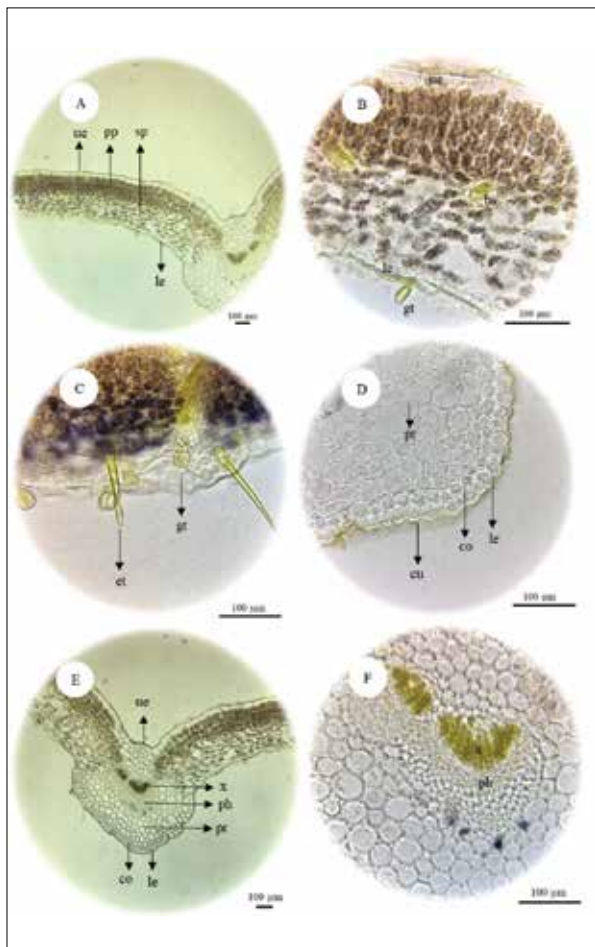


Figure 2. a-f. (a) The cross sections of leaves of *S. atropurpurea*, (b) intervascular part of leaf (mesophyll), (c) trichomes, (d) the main vein, (e) vascular bundle; bs bundle sheath, co collenchyma, cu cuticle, et eglandular trichome, gt glandular trichome, le lower epidermis, ph phloem, pp palisade parenchyma, pr parenchyma, st stomata, sp spongy parenchyma, ue upper epidermis x xylem.

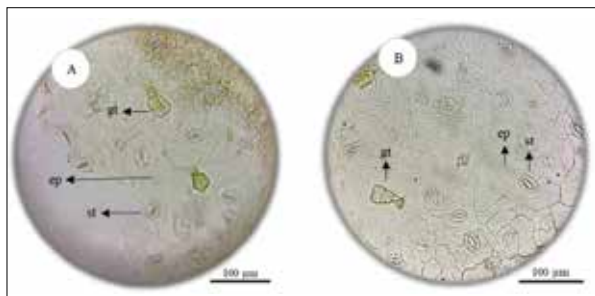


Figure 3. a,b. The surface sections of leaves of *S. atropurpurea*; (a) upper surface of leaf, (b) lower surface of leaf; ep epidermis, gt glandular trichome, st stomata.

dodermis is located under parenchyma cells. The phloem is followed by cambium. The xylem lignified and forms a wider layer than phloem. The pith comprises of large parenchymatous cells which have got starch grains and thicken on the walls as they approach the xylem layer. Parenchyma cells vary in diameter (Table 1, Figure 4 a-d).

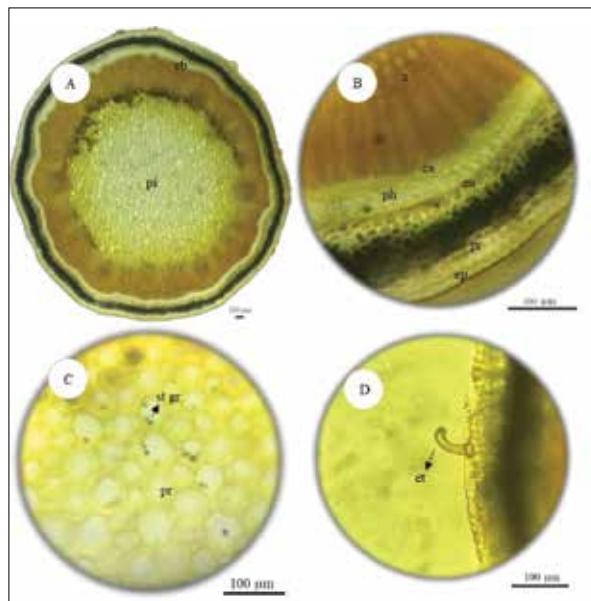


Figure 4. a-d. The cross sections of stem of *S. atropurpurea*; (a) general view, (b) cortex and vascular bundles, (c) pith, (d) trichome; ep epidermis, en endodermis, pr parenchyma, et eglandular trichome, ph phloem, pi pith, st gr starch grain, x xylem, vb vascular bundle.

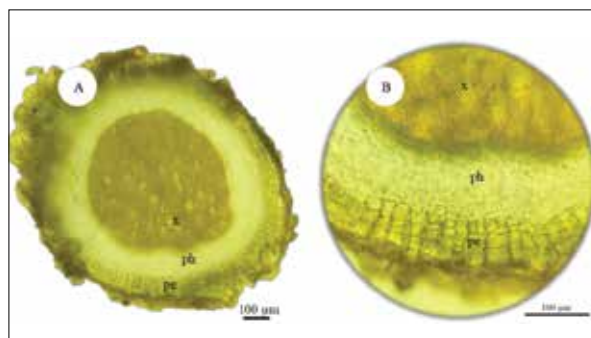


Figure 5. a,b. The cross section of root of *S. atropurpurea*; (a) general view, (b) cortex and vascular bundles; pe periderm, ph phloem, x xylem.

Root

On the cross section, the cells of epidermis are replaced by 2-3 layers of periderm. Endodermis is not seen. Under a few layered phloem, xylem is composed of tracheary elements with surrounded by sclerenchymatous cells in root. Diameters of trachea cells are 13.84 – 34.75 μm (Table 1, Figure 5 a, b).

DISCUSSION

The stem, leaf and root anatomy of *S. atropurpurea* were examined and the results were compared with literatures in this study.

The anatomical study's with *Scabiosa rotata* M.Bieb, it is stated that the eglandular trichomes are present in the stem and leaf, and also a small amount of glandular trichomes are present in the leaf. In addition, it was observed that *S. atropurpurea*

Table 1. Anatomical measurements of *S. atropurpurea*.

	Width (µm)				Length (µm)			
	Min. - Max.		Avr. ± Sd		Min. - Max.		Avr. ± Sd	
Stem								
Epidermis cell	16.94	18.61	17.70	0.74	14.68	19.10	17.21	1.84
Pith cell (diameter)	30.34	76.03	51.28	17.83				
Trachea (diameter)	8.85	18.33	14.67	3.75				
Endodermis cell	6.81	13.04	10.67	2.78	18.74	39.37	25.49	8.31
Leaf								
Upper epidermis cell	8.78	26.01	18.01	7.37	24.16	42.42	34.10	8.44
Upper stomata	18.38	20.31	19.05	0.79	21.79	34.53	28.35	4.52
Trachea (diameter)	4.66	11.24	7.03	2.08				
Lower stomata	12.07	17.10	14.17	2.15	18.48	26.52	22.74	3.10
Lower epidermis cell	7.73	14.22	11.62	2.97	26.23	16.94	33.17	9.33
Root								
Periderm cell	9.54	15.26	13.12	2.41	16.17	22.80	20.46	2.14
Trachea (diameter)	13.84	34.75	22.01	7.71				

Min: minimum, Max: maximum, Avr: average, Sd: standard deviation.

leaves and stem have eglandular trichomes (Panayır and Baykal 1997). Additionally, in an anatomical study with *S. hispidula* Boiss., it is stated that glandular trichomes are present in the stem and especially in the leaf. Therefore, *S. atropurpurea* is similar to *S. hispidula* with the appearance of glandular trichome in the leaf (Akyol et al. 2016).

It is also stated that all of these features in the cross sections of *S. rotata* and *S. hispidula*, are found in the members of the subfamily (Panayır and Baykal 1997; Akyol et al. 2016). It is reported that *S. hispidula* has hydathode. On the other hand, hydathode was not observed in *S. atropurpurea* anatomy.

In the other species of plant, clustered crystals (Akyol et al. 2016) or druse and salt crystal sands (Panayır and Baykal 1997) are found in the leaf and root anatomy of the species. Only druse crystals were observed in the leaf anatomy of *S. atropurpurea* species.




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Leaf indumentum in some Turkish species of *Teucrium* (Lamiaceae)

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ABSTRACT

Trichome micromorphology can be used as a discriminating character in the separation of species and subspecies. The micromorphological characteristics of foliar trichomes from five *Teucrium* taxa (*T. scordium* subsp. *scordium*, *T. sirnakense*, *T. chasmophyticum*, *T. andrusi* and *T. spinosum*) were investigated by scanning electron microscope. Seven types of trichomes were identified, including glandular and non-glandular. The glandular trichomes were recorded in three types; short clavate, long clavate, and sessile. The non-glandular trichomes were identified with four types; unicellular thin-walled, 2(5)-celled thin-walled, 3–7(11)-celled flexuose, elongated thin-walled and 2-celled thick-walled trichomes. Generally, the trichome types were similar on both sides of the leaves, except in *T. spinosum*. Sessile glandular trichomes were the most common type and were occurred in all the investigated species. Also clavate glandular trichomes were found in all the species, except *T. scordium* subsp. *scordium*. All trichomes were distinctively thick-walled, whereas thin-walled trichomes were observed in only *T. chasmophyticum* species. The leaf micromorphology of *T. sirnakense* and *T. chasmophyticum* has also been reported in detail for the first time in this study.

Keywords: *Teucrium*, Lamiaceae, trichome, micromorphology, SEM, leaf indumentum

INTRODUCTION

Teucrium L. belong to the Lamiaceae family is a well-known, wide-distributed and also one of the largest genus with more than 260 species distributed all around the world. (Kastner 1989; Abu-Assab and Cantino 1993). Mediterranean region, the center of diversity of the genus, has about 96% of all taxa (Cantino et al. 1992; Navarro and El Oualidi 2000).

Teucrium species have been divided into ten sections all over the world regarding their calyx shapes and the inflorescence structures (McClintock and Epling 1946; Tutin and Wood 1972). These are the sections: sect. *Teucriopsis* Benth., sect. *Teucrium*, sect. *Chamaedrys* Miller Schreber, sect. *Polium* Miller Schreber, sect. *Isotriodon* Boissier, sect. *Pycnobotrys* Bentham, sect. *Scorodonia* (Hill) Schreber, sect. *Stachyobotrys* Bentham, sect. *Scordium* Reichenbach, and sect. *Spinularia* Boissier. The species of the *Teucriopsis* and *Pycnobotrys* sections are not distributed in Turkey. (Ekim 1982). The eight sections of the genus, consist of 49 taxa (36 species) and 18 of them endemic are naturally grown in Turkey (Govaerts 1999; Duman 2000; Dönmez 2006; Parolly and Eren 2007; Dönmez et al. 2010; Dinç et al. 2011, Dirmenci 2012; Özcan et al. 2015; Vural et al. 2015; Dinç and Doğu 2016).

Teucrium sirnakense Özcan and Dirmenci (endemic) and *T. scordium* L. subsp. *scordium* (Sect. *Scordium*), *T. Andrusi* Post (endemic) and *T. chasmophyticum* Rech. f. (Sect. *Isotriodon* and *T. spinosum* L. (Sect. *Spinularia*) were examined in this study. Sect.

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Scordium has toothed leaves, rectangular stems, subgibbous calyces and subequal calyx teeth, and Sect. *Isotriodon* has dentate or entire leaves, terete stems, gibbous and bilabiate calyces. Sect. *Spinularia* is quite different than these two sections. *T. spinosum* is the only annual species in the Turkish *Teucrium*, and has resupinate corolla according to Flora of Turkey (Ekim 1982).

Teucrium species has traditionally been used in Turkey for abdominal pain, antidiabetic, antipyretic, stomachache, common cold, high fever and rheumatic pain (Aksoy-Sagirli et al. 2015).

Plant trichomes are important to descriptive and experimental botanists and data about them and their indumenta are routinely included in many studies. Many authors, such as Behnke (1984); Navarro and El Oualidi (2000); Beyrouthy et al. (2009); Moon et al. (2009); Kaya et al. 2012; Khalik and Hassan (2012); Osman (2012); Ecevit-Genç et al. (2017); Genç et al. (2017); Zareh et al. (2017) emphasize the importance of trichomes in taxonomy.

In many genera of Lamiaceae, the trichome morphology is very useful for the classification of all taxonomic levels (Marin et al. 1994; Navarro and El Oualidi 2000; Moon et al. 2009; Salmaki et al. 2009; Ecevit-Genç et al. 2015, 2017).

Trichomes are widely distributed over the different parts of the Lamiaceae genus and they are generally distinguished as glandular and non-glandular trichomes. Micromorphological features, especially trichomes, are available taxonomic characters in *Teucrium*. Trichomes have an important role in the infrageneric classification of the genus. There have been many studies on the trichomes of *Teucrium* species in recent years (Navarro and El Oualidi 2000; Grubescic et al. 2007; Dinç et al. 2011; Eshratifar et al. 2011; Doğu et al. 2013, Ecevit et al. 2015, 2017).

The main purposes of this paper are to provide a detailed description of the leaf micromorphology of five *Teucrium* species belonging to three different sections.

MATERIALS AND METHODS

The material was collected from different localities in Turkey by the authors. Voucher specimens have been deposited in the ISTE. A list of taxa included in the study was given in Table 1.

For the micromorphological study, the materials were obtained from collected specimens and micromorphological investigations were conducted using scanning electron microscope (SEM). For SEM analysis, leaves parts were mounted on the stubs and coated with gold layer. They were studied

Table 1. Collection data of *Teucrium* taxa studied.

Taxon	Collection data
<i>T. scordium</i> subsp. <i>scordium</i>	Edirne, İpsala border gate, 01.viii.2014, T.Dirmenci, ISTE 101 691
<i>T. sirmakense</i>	Şırnak, Taşdelen village, rocky slopes, 10.vi.2013, T.Dirmenci, E.Akçiçek, Ö.Güner, ISTE 101 694
<i>T. chasmophyticum</i>	Siirt, Between Eruh-Gölgelikonak village, 11.vi.2013, T.Dirmenci, E.Akçiçek, Ö.Güner, ISTE 101 711
<i>T. andrusi</i>	Mardin, Bakırkırı hill, cliffs, 23.vi.2013, T.Özcan, M.Açar, ISTE 101 712
<i>T. spinosum</i>	Diyarbakır, Diyarbakır-Ergani roadsides, 09.vi.2014, T.Dirmenci, E.Akçiçek, Ö.Güner, ISTE 101 719

ISTE: Herbarium of the Faculty of Pharmacy of İstanbul University

Table 2. Trichome types (Navarro and El Oualidi 2000)

Glandular trichomes	
A	Clavate glandular trichomes
A1	Short clavate glandular trichomes. Generally with two, large and thin stalk cells.
A2	Long clavate glandular trichomes. Generally with long 3-5 stalk cells.
B	Subsessile glandular trichomes, peltate trichomes
Non-glandular trichomes	
C	Thin-walled trichomes
C1	Triangular, large and very thin -walled unicellular hairs.
C2	Large, thin-walled, 2[5]-celled trichomes which are acute apical cell. Internodes have ridges or marked.
C3	Flexuose and elongated, 3-7(11)-celled trichomes, with internodes distinct, the apical cell acute with micro-papillae.
D	Short and slightly conical, generally 2-celled thick-walled trichomes which are pointed short or elongated apical cell, erect or sometimes slightly curved.

with a scanning electron microscope (FEI Quanta 450 FEG-EDS). All leaves were scanned from adaxial and abaxial surfaces. Terminology of the indumenta on leaves were based on Navarro and El Oualidi (2000), (Table 2).

RESULTS

The micromorphological characteristics and distribution of the trichomes on leaves of five *Teucrium* taxa were examined in this study. Different type of indumentum shows considerable among species (Table 3). SEM micrographs of all indumentum types are presented in Figure 1.

In the sect. *Scordium*, *T. scordium* subsp. *scordium* leaves have sparsely B, C1 and C2 trichome types on both surface, although *T. sirnakense* leaves have A1, A2, B type glandular trichomes and C2 and C3 non-glandular trichomes on the adaxial and abaxial surface. Dense indumentum appears on the abaxial surface than the adaxial surface of this species leaves (Figure 1).

In the Sect. *Isotriodon*, *T. chasmophyticum* has A1, B, D trichome types on both surface of the leaves. The lower surface of the leaf has a much denser indumentum than the upper surface (Figure 1). *T. andrusi* has A1, A2, B, C2, C3 trichome types adaxial and abaxial surface of the leaves. Dense indumentum appears on both surface of the leaves (Figure 1). *Teucrium spinosum* belongs the sect. *Spinularia*, A2, B, C2 trichome types are observed at the abaxial surface of leaves; A2, B, C3 trichome types were found on the adaxial surface of leaves (Figure 1).

DISCUSSION

Trichome morphology is the useful taxonomic markers in some genera of Lamiaceae. Their absence or presence can be used as taxonomic characters in the infrageneric classification of some genera (Metcalf and Chalk 1950; Navarro and El Oualidi 2000; Moon et al. 2009). For the classification of trichome in *Teucrium*, distinction into thin and thick-walled provides taxonomic support to the delimitation of the species, could be regarded as a valid taxonomic character (Navarro

and El Oualidi; Eshratifar et al. 2011; Ecevit-Genç et al. 2015, Ecevit-Genç et al. 2017).

As a result of our work, seven trichome types were observed in five species belong to three different section of *Teucrium* (Table 2). The trichome types located on the adaxial and abaxial sides of leaves are same except *T. spinosum*. Among them subsessile glandular trichomes are most widespread in all taxa examined. Clavate glandular trichomes are generally found all of the species except *T. scordium* subsp. *scordium*. 2-celled thick-wall non-glandular trichomes are common trichome types but only *T. chasmophyticum* have thin-wall non-glandular trichome. In this work, the leaves trichome micro-morphology of *T. sirnakense*, *T. chasmophytum* are reported in detail by SEM for the first time.

Many studies have been presented about leaves indumentum of *Teucrium* species. For example, 56 *Teucrium* species related to the nine sections were investigated by Navarro and El Oualidi (2000). The authors analyzed five species of the sect. *Spinularia* including *T. spinosum* and they found different trichome types between species. According to their results, *T. spinosum* has flexuose and elongated thin-walled non-glandular trichomes and subsessile glandular trichomes. In addition to this species, long clavate glandular and large thin-walled non-glandular trichome types were identified in this study. The author have investigated four species of sect. *Isotriodon*. Thick-walled non-glandular and long clavate glandular trichomes are most common trichomes and flexuose and elongated thin-walled trichome types are less frequent in this section according to Navarro and El Oualidi (2000). But elongated 5-7(8)-celled thick-wall non-glandular trichomes was not observed on any taxa of studied in this study. Also long clavate glandular trichomes were not observed on *T. chasmophytum*.

We have observed subsessile glandular trichomes and large thin-walled non-glandular trichomes on *T. scordium* subsp. *scordium* leaves. The other subspecies of *T. scordium* subsp. *scordioides* leaves indumentum was investigated many previous studies and long clavate glandular trichomes, subsessile glandular trichomes and flexuose and elongated thin-walled

Table 3. Trichome types and distribution on the adaxial-abaxial leaf surfaces of studied *Teucrium* taxa

Section	Taxon	Leaves trichomes (According to Navarro & El Oualidi 2000)	
		Adaxial surface	Abaxial surface
Scordium	<i>T. scordium</i> subsp. <i>scordium</i>	B, C1, C2	B, C1, C2
	<i>T. sirnakense</i>	A1, A2, B, C2, C3	A1, A2, B, C2, C3
Isotriodon	<i>T. chasmophyticum</i>	A1, B, D	A1, B, D
	<i>T. andrusi</i>	A1, A2, B, C2, C3	A1, A2, B, C2, C3
Spinularia	<i>T. spinosum</i>	A2, B, C2	A2, B, C2

non-glandular trichomes were found by Navarro and El Oualidi (2000) and Ecevit-Genç et al. (2017). Peltate, capitate acicular and flagelliform trichome types were found by Jurišić Grubešić et al. (2007). According to all these findings trichome types are show some differences between two sub-species.

T. scordium investigated morpho-anatomically by Lakusic et al. (2010) from Balkan peninsula and they found glandular (peltate, unicellular capitate, multicellular capitate), and non-

glandular (unicellular unbranched, multicellular unbranched) trichomes on the leaves of this species. But the unicellular trichomes were not observed on the leaves of *T. scordium* subsp. *scordium* in this study.

Clavate glandular trichomes are ordinarily observed on the leaves of taxa of sect. *Isotriodon* by our team's previous paper (Ecevit-Genç et al. 2017). The results of this study about the species of the sect. *Isotriodon* are overlapped with the previous study of our team. *T. andrusi* leaves were examined by

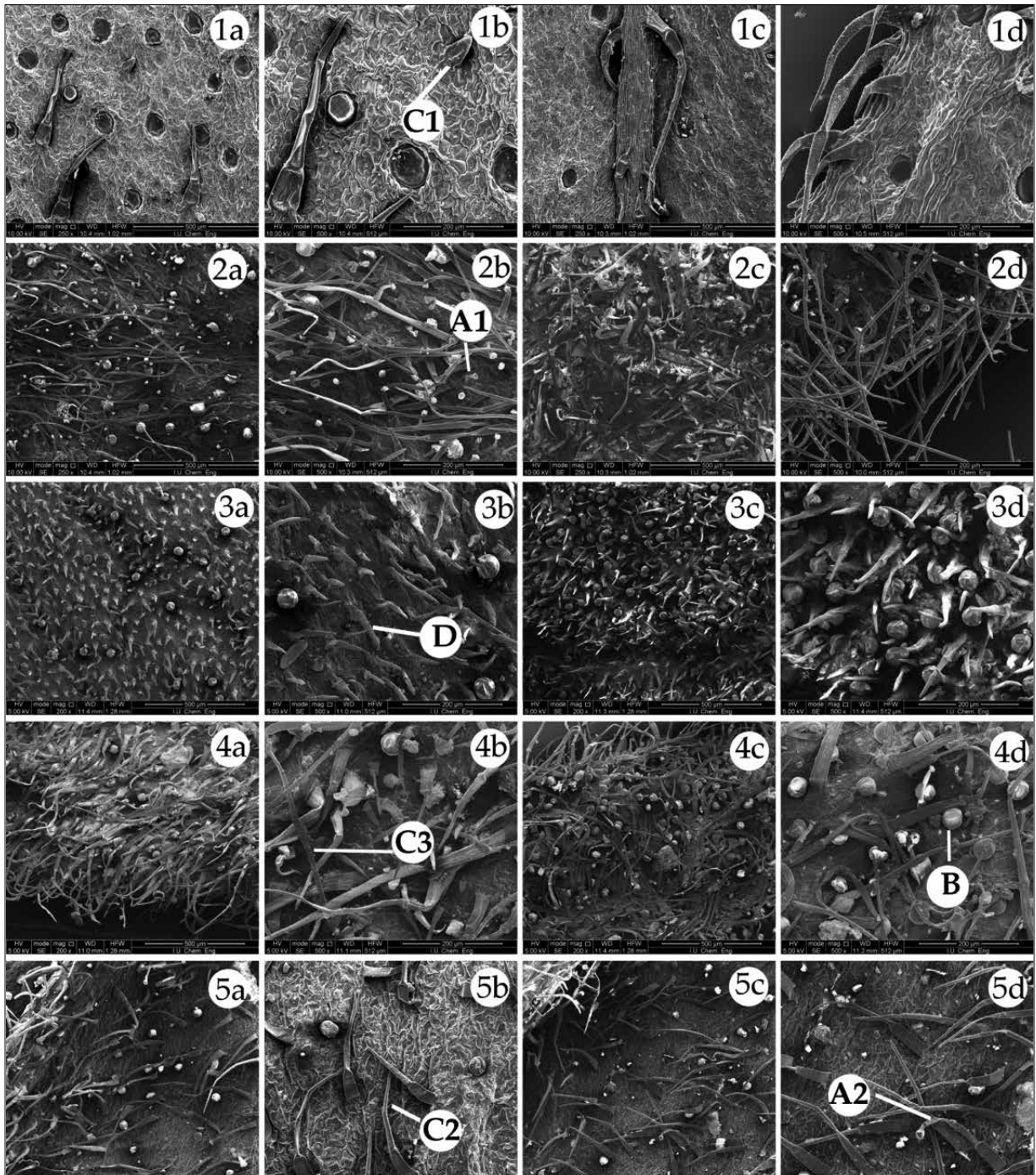


Figure 1. a-d. SEM micrographs of leaves of *Teucrium*. Adaxial surface (a, b), abaxial surface (c, d). *T. scordium* subsp. *scordium* (1), *T. sirnakense* (2), *T. chasmophytum* (3), *T. andrusi* (4), *T. spinosum* (5). (scale bars: a, c =500 micrometer (µm); b, d =200 µm).

Dinç et al. (2011) and their results are corresponding to our results.

This paper gives detailed information on the micromorphological features of the *T. spinosum*, *T. scordium* subsp. *scordium*, *T. sirnakense*, *T. chasmophytum* and *T. andrusi* species. We concluded that trichome types are useful for specific delimitation of *Teucrium* species. However, micromorphological features must be supported by other morphological, molecular, biogeographical characters.

Acknowledgements

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Purification and partial characterization of thioredoxin reductase from the hepatopancreas of the mollusc *Mytilus galloprovincialis* Lam.

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ABSTRACT

Thioredoxin reductase (TrxR, EC 1.6.4.5) is a ubiquitous flavoenzyme that is present from Archaea to humans, and it is the only enzyme capable of catalyzing the reduction of thioredoxin (Trx) by nicotinamide adenine dinucleotide phosphate (NADPH). Although TrxR has been purified and characterized from different bacteria, plants, and mammalian organisms, a survey of the literature revealed no studies on the purification and characterization of TrxR from the mussel *Mytilus galloprovincialis* Lam. In this study, TrxR was purified to homogeneity from the hepatopancreatic tissue of *M. galloprovincialis* Lam. by extraction, ammonium sulfate precipitation, and DEAE-Sepharose CL-6B anion and 2',5'-ADP-agarose chromatographies, and some of its kinetic properties were examined. Molar mass determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed only a single protein band corresponding to a molecular weight of 35 kDa. Optimum pH and temperature were found to be 7.0 and 60°C, respectively. K_m and V_{max} values for NADPH were found to be 85 μmol and 4.82 $\mu\text{mol}/\text{min}/\text{mg}$, respectively. For 5,5'-dithiobis (2-nitrobenzoic) acid (DTNB), the K_m and V_{max} values were 193 μmol and 1.32 $\mu\text{mol}/\text{min}/\text{mg}$, respectively. Increasing the knowledge on the kinetic properties of TrxR will significantly increase the prospects of enzyme application as an oxidative stress biomarker in mussels and fishes for monitoring contamination in coastal environments.

Keywords: Enzyme purification, kinetic properties, *Mytilus galloprovincialis* Lam., thioredoxin reductase

INTRODUCTION

Thioredoxin (Trx), nicotinamide adenine dinucleotide phosphate (NADPH) and thioredoxin reductase (TrxR) comprise a thioredoxin system which exists in nearly all living cells (Arnér and Holmgren 2006). Trx, the physiological substrate for TrxR, occurs in either an oxidized or a reduced form. Reduced Trx prevents oxidation of various proteins by donating hydrogen atoms from two of the cysteine residues at its active site. Oxidized Trx is reduced by TrxR using NADPH as an electron donor (Seo and Lee 2010).

TrxR belongs to the pyridine nucleotide-disulfide oxidoreductase family and is a dimeric flavoenzyme (Lu et al. 2009). The preliminary purification and characterization studies were performed with TrxR from archaea, bacteria and anaerobic amino-acid-utilizing bacteria (Moore et al. 1964; Williams 1995; Harms et al. 1998; Horecká et al. 1998; Seo and Lee 2010; Yang and Ma 2010), fungi, some eucaryotes including plant (Reicheld et al. 2005) and intracellular parasites (Brown et al. 1996; Coombs et al. 2004; Maggioli et al. 2004; Arias et al. 2010; Kapoor and Banyal 2011). Extensive studies have been made also upon mammalian Trx system. Mammalian TrxR was purified to homogeneity from rat liver (Larsson 1973; Luthman and Holmgren 1982; Lu et al. 2009), bovine adrenal cortex (Watabe et al. 1999) and human placenta (Gromer et al. 1998).

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Bivalve molluscs like mussels, clams and oysters are highly nutritive commercially valuable seafood species on the worldwide basis. Populations of bivalves living in coastal areas are subject to many investigations for their possible use as enzyme resources. Many references can be found in the literature to the enzymes present in the digestive system of bivalves (Yalvaç and Kuşçu, 1993; Arisan-Ataç et al., 1994; Özsoy and Berkkan, 1997; Somar et al., 2000; Can et al. 2000; Dönmez et al. 2014). As TrxR is known to be involved in maintenance of redox homeostasis and antioxidant defense by reducing disulphide sites in oxidized proteins, it was used as a biomarker in the digestive gland of wild mussels (*M. galloprovincialis*) for biomonitoring the marine pollution (Sureda et al. 2011). However, no report has been found in the literature on the isolation and purification of TrxR from the mussel *M. galloprovincialis*. This work describes for the first time the purification and characterization of TrxR from the mussel *M. galloprovincialis*.

MATERIAL AND METHODS

The mussels belonging to the species *Mytilus galloprovincialis* Lam. were obtained from the Yenikapi seaboard on the day of experiment.

Purification of thioredoxin reductase

The purification of TrxR from hepatopancreas tissue of *M. galloprovincialis* involved four steps:

1. Isolation: 73.5 g hepatopancreas of freshly collected 60 mussels were homogenized in 200 mL of 10 mM Tris-HCl containing 1 mM EDTA, pH 7.5 buffer (TE buffer) by means of a homogenizer (Art-MICCRA D-1, Heitesheim, Deutschland). The homogenate obtained was centrifuged at 13,000 rpm for 20 min in a refrigerated centrifuge (Heraeus-Megafuge 1.OR, Thermo Fisher Scientific, Waltham, MA, USA) and the supernatant (the crude extract) was collected.
2. Ammonium sulphate precipitation: The crude extract was precipitated by ammonium sulphate at 80% saturation and left overnight in the refrigerator. The precipitate, separated by means of centrifugation at 13,000 rpm for 20 min was dissolved in TE buffer and dialyzed against the same buffer to remove the salt. The dialyzed solution was heated at 56°C in a water bath for 10 min, cooled, and after centrifugation at 13,000 rpm for 20 min the precipitate was discarded and the supernatant was used for further purification procedure.
3. DEAE-Sepharose Chromatography: The dialysate was applied to a column of DEAE-Sepharose CL-6B (1.5x25 cm) previously equilibrated with 10 mM TE buffer. The column was washed with approximately 150 mL of equilibration buffer until no protein could be detected in the effluent. The enzyme was eluted with a linear gradient of 0-0.5 M NaCl in 10 mM TE buffer. The eluate

was collected in 0.5 mL fractions and assayed for absorbance at 280 nm and for enzyme activity. The fractions showing TrxR activity were pooled and concentrated by ultrafiltration with a stirred cell (Millipore Corporation, Bedford, MA 01730 USA) equipped with a PM 10 membrane (Amicon, Inc., Beverly, Mass.) under nitrogen pressure of 20 lb/in². The concentrated enzyme fractions were subsequently dialyzed against TE buffer. All operations were performed at 4°C.

4. Affinity chromatography: The main activity peak was applied to a 2',5'-ADP-agarose column (1x10 cm) equilibrated with 10 mM TE buffer. The column was eluted with a gradient of 0 to 0.5 M NaCl in TE buffer. The purified enzyme solution was concentrated by ultrafiltration and dialyzed against 10 mM TE buffer. The purified enzyme was stored at -80°C until used.

Determination of protein concentration

Protein concentration was determined either by the Bradford method (1976) using bovine serum albumin (BSA) as a standard or measurement of absorbance at 280 nm.

Determination of thioredoxin reductase activity

TrxR activity was measured by the reduction of DTNB with NADPH to 5-thio-2-nitrobenzoic acid (TNB), which produces a strong yellow color that is measured at 412 nm (Holmgren and Bjornstedt 1995) using Thioredoxine reductase Assay Kit (Abcam, Cambridge, MA, USA) according to the manufacturer's instructions. TrxR was determined also by the insulin-dependent reduction assay described by Arnér and Holmgren (2000). Enzyme activity was expressed as $\mu\text{mol}/\text{min}/\text{mL}$ of formation of TNB by using extinction coefficient of 6.35 mM^{-1} . One unit is defined as NADPH-dependent production of 2 μmol of TNB per minute.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12.5% polyacrylamide gel slabs at pH 8.3 by using 1 M Tris-glycine buffer containing 0.1% (w/v) SDS according to Laemmli's method (1970). Subunit molar mass was analyzed under reduced conditions. The purified sample was prepared by heating a protein solution in a sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol, 5% 2-mercaptoethanol) at 100°C for 5 min. A low-molecular-weight calibration mixture (Amersham Biosciences, Buckinghamshire, UK) was used as standard.

Kinetic properties of thioredoxin reductase

The relationship between the pH variation and TrxR activity was investigated at a pH range of 6-10 by using 500 mM potassium phosphate buffer containing 1 mM EDTA. The effect of temperature on the activity of the enzyme was studied between 30°C and 85°C under assay conditions. The effect

Table 1. Purification of thioredoxin reductase of *Mytilus galloprovincialis* from 73.5 g of wet hepatopancreas tissue

Purification Step	Volume (mL)	Total Protein (mg)	Total Activity (U)*	Specific Activity (U/mg)**	Yield (%)	Purification Fold
1-Crude extract	200	3 642.4	112 298.0	30.8	100	-
2-80% ammonium sulphate fraction	12.5	371.3	1 336.3	3.6	10.2	0.1
3-DEAE-Sepharose CL-6B (10 mM)	10	10.6	775.4	73.2	0.3	2.4
4-2',5'-ADP-agarose (400 mM)	1	0.04	1 189.8	29 745.8	0.001	965.7

* $\mu\text{mol TNB}/\text{min}$
** $\mu\text{mol TNB}/\text{min}/\text{mg protein}$

of substrate concentration on the velocity of the enzyme reaction was investigated by using varying concentrations of NADPH (0.015-0.24 mM) and DTNB (0.19-3 mM) as substrates. Km and Vmax were calculated by means of the equation of Lineweaver-Burk plot.

RESULTS AND DISCUSSION

The thioredoxin and glutathione systems are the two major thiol-dependent reductases that maintain a reducing intraenvironment in the presence of oxygen (Lu et al. 2009). In this study, approximately 40 μg of the purified enzyme was obtained from 73.5 g hepatopancreas of the mollusc *M. galloprovincialis*. The enhanced purification was primarily due to the use of 2,5-ADP as an affinity ligand for purification of NADPH-binding proteins, a procedure previously described by Brodelius et al. (1974). The use of the affinity step resulted in a 0.001% yield of enzyme with a specific activity of 29 745.8U/mg protein (Table 1). Earlier purification schemes for Trx and TrxR involved anion exchange and affinity column chromatography steps. Affinity column chromatography was used as the initial purification step by Pigiet and Conley (1977), who purified both TrxR and glutathione reductase (GR) 300-fold in one step. However, it was reported that the use of affinity chromatography after several initial purification steps resulted in greater column yield. Williams et al. (1967) reported that, GR and TrxR, each purified by a two-step chromatographic procedure including anion-exchange chromatography and affinity chromatography, bind to the affinity gel at the extent of 600 and 570 units/mL of gel respectively; while using a crude extract, only 116 and 26 units/mL gel of each enzyme bind to the affinity gel. This may be due to the presence of several NADPH-binding proteins competing with the desired proteins in the crude extract (Pigiet and Conley 1977). By a combination of anion exchange and affinity chromatography, TrxR was purified to homogeneity from *Streptomyces aureofaciens* 3239 (Horecká et al. 1998), a protozoan parasite *Giardia duodenalis* (Brown et al. 1996), and the worm *Fasciola hepatica* (Maggioli et al. 2004). TrxR from rat liver with specific activity of 625 U/mg was obtained by chromatography on Sephadex and on DEAE-cellulose (Larsson 1973). The method described here for the purification of TrxR from the hepatopancreas

tissue of *M. galloprovincialis* has resulted in a preparation with higher specific activity.

The procedure used for the purification of the TrxR from *M. galloprovincialis*, was similar to that used for the purification of this enzyme from anaerobic amino-acid utilizing bacteria (*Eubacterium acidaminophilum*, *Clostridium litorale*, *C. sticklandii*, *C. sporogenes*, *C. cylindrosporum* and *Tissierella creatinophilla*) as described by Harms et al. (1998). The proteins isolated in this study did not bind to DEAE-Sepharose and were eluted with the washing buffer (Figure 1). However, they were bound tightly to the affinity gel material and eluted with 0.4 M NaCl (Figure 2) as reported by Harms et al. (1998). The affinity step permitted the rapid and high yield purification of large quantities of enzyme for subsequent use in structural studies.

All described purification schemes for the TrxR involve a heat denaturation step; 65°C, 5 min (Moore et al. 1964); 70°C, 8 min (Williams et al. 1967); 60°C (Maggioli et al. 2004); 56°C, 10 min (Lu et al. 2009). In this study, heat treatment was also an important step for the enrichment of the enzyme, in which otherwise difficult-to-remove contaminating proteins were eliminated. The heat treatment was effective in simplifying the purification procedure and increasing the yield.

Optimum pH for TrxR have been reported to exist generally at pH 7.5 (Watabe et al. 1999), 7.4 (Kapoor and Banyal 2011), 7.7 (Williams 1995) and 6.5 (Yang and Ma 2010). Optimum pH values for TrxR determined in the present study was 7.0, which is within the mentioned range (Figure 3).

The optimum temperature of TrxR was found to be 60°C (Figure 4). However, the enzyme activity was reduced at 70°C. Similarly, the activity of TrxR from *D. radiophilus* was drastically reduced at 80°C and completely inactivated at 90°C (Seo and Lee 2010). TrxR from the hyperthermophilic bacterium *Thermotoga maritima* was reported to display a relatively high thermostability (up to 95°C) (Yang and Ma 2010). Thermostable enzymes have considerable potential in biotechnical applications because of their resistance to heat denaturation and consequently lower replacement costs when enzymes are integrated into high

temperature processes. TrxR enzymes with optimum temperature as low as -4°C and 0°C has been reported by Kapoor and Banyal (2011) and Özgençli and Çiftçi (2016), respectively. It might be the result of the necessity for these organisms to adapt itself to environmental conditions.

Km and Vmax values for NADPH were $85\ \mu\text{mol}$ and $4.8\ \mu\text{mol}/\text{min}/\text{mg}$, respectively (Figure 5). Also, Km and Vmax

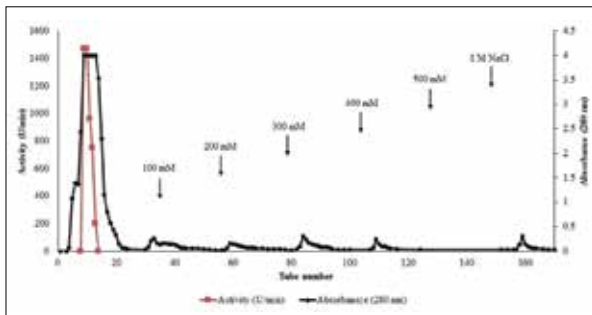


Figure 1. DEAE-Sephacel ion exchange chromatography. Elution profile of the 80% ammonium sulphate fraction of *Mytilus galloprovincialis* hepatopancreas tissue crude extract.

Column: $25 \times 1.5\ \text{cm}$, sample volume: $12.5\ \text{mL}$ ($371.3\ \text{mg}$ protein), flow rate: $48\ \text{mL}/\text{hour}$, the enzyme was eluted with a linear gradient of $0-1\ \text{M}$ NaCl in $10\ \text{mM}$ TE (pH 7.5) buffer.

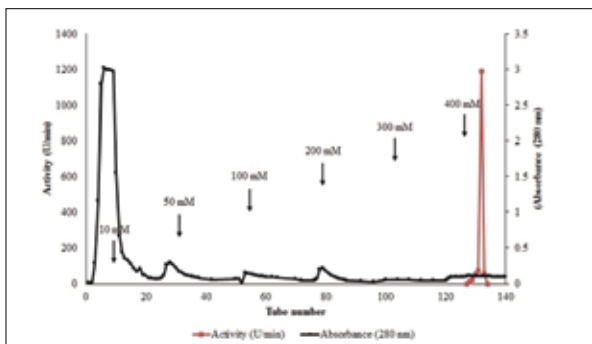


Figure 2. 2',5'-ADP-agarose chromatography of thioredoxin reductase after DEAE-Sephacel purification.

Column: $1 \times 10\ \text{cm}$, sample volume: $1\ \text{mL}$ ($10.6\ \text{mg}$ protein), flow rate: $1\ \text{mL}/\text{min}$, the enzyme was eluted with a linear gradient of $0-0.5\ \text{M}$ NaCl in $10\ \text{mM}$ TE (pH 7.5) buffer.

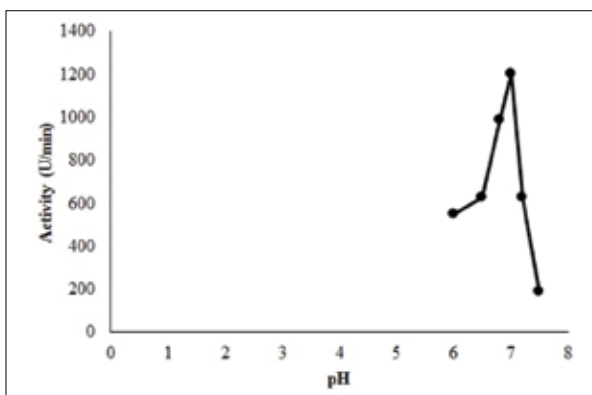


Figure 3. Effect of pH on *Mytilus galloprovincialis* hepatopancreas tissue thioredoxin reductase activity.

values for DTNB were found to be $193\ \mu\text{mol}$ and $1.32\ \mu\text{mol}/\text{min}/\text{mg}$, respectively (Figure 6). Km and Vmax values for DTNB were lower or comparable to that reported for *Plasmodium berghei* ($K_m=125\ \mu\text{mol}$; $V_{\text{max}}=100\ \mu\text{mol}/\text{min}$) (Kapoor and Banyal 2011). *Deinococcus radiophilus* ($K_m=463\ \mu\text{mol}$; $V_{\text{max}}=756\ \mu\text{mol}/\text{min}$) (Seo and Lee 2010) and rat liver ($K_m=660\ \mu\text{mol}$) (Luthman and Holmgren 1982) and higher than that reported for rainbow trout ($K_m=0.828$

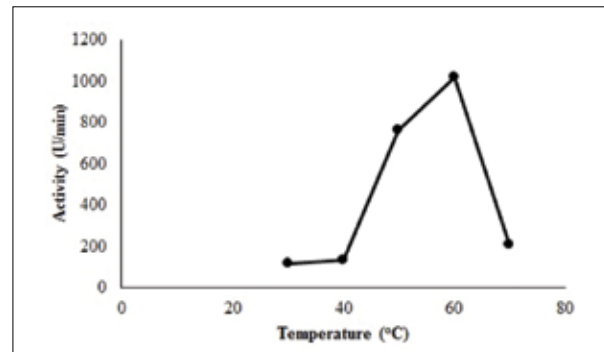


Figure 4. Effect of temperature on *Mytilus galloprovincialis* hepatopancreas tissue thioredoxin reductase activity.

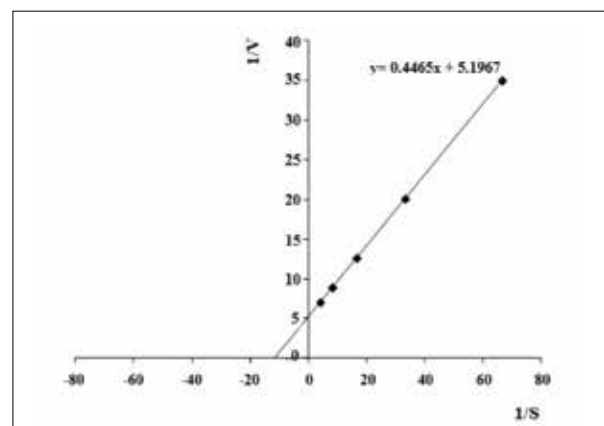


Figure 5. Effect of different concentrations of NADPH on the activity of *Mytilus galloprovincialis* hepatopancreas tissue thioredoxin reductase activity ($-1/K_m = -11.76$).

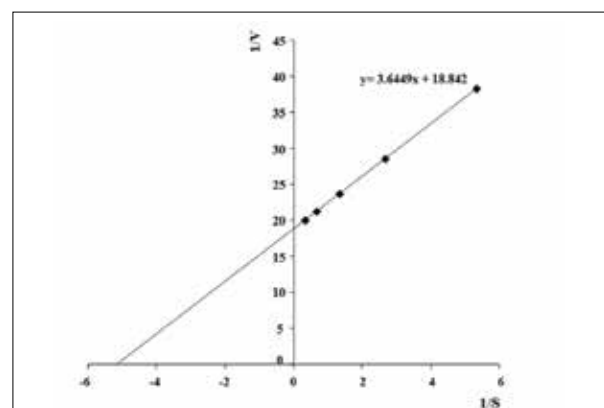


Figure 6. Effect of different concentrations of DTNB on the activity of *Mytilus galloprovincialis* hepatopancreas tissue thioredoxin reductase activity ($-1/K_m = -5.18$).

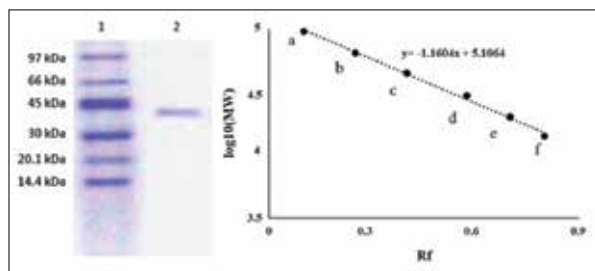


Figure 7. SDS-PAGE electrophoresis of the purified thioredoxin reductase after affinity chromatography.

Line 1: Low molecular weight proteins [a: phosphorylase 97 kDa, b: albumin 66 kDa, c: ovalbumin 45 kDa, d: carbonic anhydrase 30 kDa, e: trypsin inhibitor 20 kDa and f: α -lactalbumin 14,4 kDa; Line 2: Purified enzyme after affinity chromatography.

μmol) (Özgençli and Çiftçi (2016). Km and Vmax values for NADPH were higher than that reported for *Deinococcus radiophilus* (Km=12.5 μmol ; Vmax= 25 $\mu\text{mol}/\text{min}$) (Seo and Lee 2010), rat liver (Km=6 μmol) (Luthman and Holmgren 1982) and rainbow trout (Km=12.65 μmol) (Özgençli and Çiftçi (2016).

The purity of the enzyme was confirmed by SDS-PAGE showing a single band with a molecular mass of about 35 kDa (Figure 7). This value is the same with that reported for TrxR in prokaryotes, archaea and lower eukaryotes, but different from a protein in higher eukaryotes that was found have a MW of 55 kDa (Williams et al. 2000).

CONCLUSION

The TrxR enzyme from mussel *M. galloprovincialis* was purified to homogeneity, and its properties were investigated. The results may contribute to a great number of studies applying oxidative biomarkers in mussels and fishes for monitoring environmental pollution.

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In vitro multiple pharmacological targets of *Colutea cilicica* Boiss. & Balansa against key enzymes linked to neurodegenerative diseases, diabetes, and hyperpigmentation

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ABSTRACT

Prevention and treatment of noncommunicable diseases such as neurodegenerative diseases, diabetes, and hyperpigmentation using medicinal plants has attracted increasing attention during the past few decades. In this study, *Colutea cilicica* Boiss. & Balansa extracts (ethyl acetate, methanol, and water) were evaluated against key enzymes involved in neurodegenerative diseases, diabetes, and hyperpigmentation. The antioxidant (free radical scavenging, reducing power, β -carotene/linoleic acid, and phosphomolybdenum) and metal chelation properties were also investigated. The methanol extracts of *C. cilicica* vigorously inhibited the activities of acetylcholinesterase and butyrylcholinesterase (1.33 and 0.68 mg galantamine equivalents [GALAE]/g extract, respectively). It was observed that *C. cilicica* extracts possessed a higher inhibitory potential for α -glucosidase (2.71–1.23 mmol acarbose equivalents [ACAE]/g extract) than that for α -amylase (0.57–0.12 mmol ACAE/g extract). The water extract of *C. cilicica* showed potent radical scavenging capacity against DPPH (2, 2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] [42.46 and 57.70 mg trolox equivalents (TE)/g extract, respectively). Phytochemical determination showed that *C. cilicica* water extract (17.26 mg rutin equivalents [RE]/g extract) was rich in flavonoids compared with ethyl acetate and methanol extracts (2.78 and 2.83 mg RE/g extract, for the respective extracts). These findings reveal the interesting potential of *C. cilicica* as a valuable source of phytochemicals that can be used against common noncommunicable diseases, particularly against enzymes involved in neurodegenerative diseases.

Keywords: *Colutea cilica*; Alzheimer's disease; diabetes mellitus; natural agents; phytopharmaceuticals.

INTRODUCTION

Non communicable diseases such as diabetes, and neurodegenerative diseases such as Alzheimer disease (AD) have become a major global health burden (Moreno Cervantes et al 2017). Though the exact cause of AD remains uncertain, hypotheses of the possible pathological pathways have been suggested to affecting mainly the elderly segment of the global population. Factors implicated in the pathogenesis of AD include reduced level of the neurotransmitter acetylcholine, oxidative stress, aggregation of amyloid β peptide, and tau protein, and transition metal action (Amadoruge and Barnham 2011; Butterfield et al 2007; Zhao and Zhao 2013). Based on the above mentioned evidences, scientists are aiming at finding therapeutic strategies to manage this debilitating condition. On the other hand, the prevalence of diabetes is on a sharp rise, irrespective of age, affecting younger

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adults and even children. Panoply of risk factors have been identified such as drastic rise in level of obesity, sedentary lifestyle including low levels of physical activity and familial cases of diabetes (Mutie et al 2017).

Plants have a long and well-known history in the treatment of various human ailments. The diversity of phytoconstituents which exhibit wide spectrum of pharmacological activities, make them ideal candidate for the discovery of novel lead compounds (Jambocus et al 2017). The *Colutea* genus comprises of about 28 species, forming part of the Fabaceae family (Peşin Süntar et al 2011). These deciduous flowering plants are native to southwest Asia, North Africa, and Southern Europe. *Colutea cilicica* is chiefly cultivated as an ornamental plant for its attractive yellow flowers and papery like inflated pods containing the seeds (Davis 1997). In Turkey *C. cilicica* fruits are used to treat abscesses, wounds, and inflammation (Sezik et al 2001). *C. cilicica* is used against helminthiasis and hypertension in Iraq (Molan et al 2012). D-pinitol isolated from *C. cilicica* was found to significantly reduce inflammation (Eser et al 2017).

Yet, there is a lack of scientific information regarding the potential of *C. cilicica* in the management of AD. Thus in the present study, we aimed at assessing the possible inhibitory function of *C. cilicica* extracts on enzymes targeted in the management of AD, namely cholinesterases. Additionally, we studied the possible inhibitory action of *C. cilicica* extracts on enzymes related to diabetes, a condition which has been associated to AD. Finally, using a set of *in vitro* assays we determined the antioxidant potential of *C. cilicica* extracts. This study was carried out in an endeavor to provide baseline data on the biological properties of a traditionally used medicinal plant for further studies in an attempt of discovering new lead molecules to manage global health problems.

MATERIAL AND METHODS

Plant Material and extraction procedure

Aerial parts of *Colutea cilicica* L. (Soguksu national park, Kızılcahamam, Ankara) was collected during summer 2014 and allowed to air dry at the room temperature. Taxonomic identification was carried out by Dr. Murad Aydın Sanda, senior taxonomist of the Department of Biology, Selcuk University, Turkey.

To produce solvent extracts, the air-dried samples (5 g) were macerated with 100 mL of ethyl acetate and methanol at room temperature for 24 h. Further, the extracts were concentrated under vacuum at 40 °C by using a rotary evaporator. To obtain water extracts, the powdered samples were boiled with 100 mL distilled water for 20 min. The water extract was lyophilized (-80°C, 48 h). All samples were stored at +4°C in dark until use.

Total phenolics and flavonoids content

The total phenolics content was determined by Folin-Ciocalteu method (Slinkard and Singleton 1977). Sample solution (0.25

mL) was mixed with diluted Folin-Ciocalteu reagent (1 mL, 1:9, v/v) and shaken vigorously. After 3 min, Na₂CO₃ solution (0.75 mL, 1%) was added and the sample absorbance was read at 760 nm after a 2 h incubation at room temperature. The results were expressed as gallic acid equivalents (mg GAE/g extract).

For total flavonoid content: sample solution (1 mL) was mixed with one milliliter of aluminum trichloride (2%) in methanol. Blanks for each extracts were prepared by adding sample solution (1 mL) to methanol (1 mL) without AlCl₃. The sample and blank absorbances were read at 415 nm after a 10 min incubation at room temperature. The results were evaluated as milligrams of rutin equivalents (mg RE/g extract) (Zengin et al 2016).

Biological activities evaluation

Antioxidant capacity and enzyme inhibitory effects of *C. cilicica* extracts were detected for biological abilities. The assays were performed as described by our previous study (Grochowski et al. 2017), and they are summarized in the below.

Antioxidant assays

DPPH scavenging activity

After combining 1.0 mL of extract solution with 4 ml of DPPH (0.267 mM), samples were incubated for 30 minutes in room temperature in darkness. Afterwards, absorbance of samples were measured at 517 nm. Results were calculated as milligrams of trolox equivalents per gram of dry extract (TEs/g).

ABTS radical cation scavenging activity

Formation of ABTS⁺ radical cation is an effect of incubation in darkness in room temperature mixture of 7 mM ABTS with 2.45 mM potassium persulfate. Prepared solution was diluted with methanol until its absorbance reached 0.700 ± 0.02 at 734 nm. 1 mL of extract solutions were combined with previously prepared 2 mL of ABTS⁺ solution and after 30 min of incubation, absorbance at 734 nm was measured. Results were expressed as milligrams of trolox equivalents per gram of dry extract.

Phosphomolybdenum method

0.3 mL of tested extract solutions were added to reagent mixture, containing 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate and after 90 min incubation in 95 °C absorbances were read in 695 nm against blank sample (0.3 mL methanol with 3 mL reagent mixture). Millimoles of trolox per gram of dry extract were the measurement unit.

B-carotene/linoleic acid method

A stock solution of β-carotene–linoleic acid mixture was prepared as following: 0.5 mg β-carotene was dissolved in chloroform (1 mL). 25 µL linoleic acid and 200 mg Tween 40 was added. Chloroform was completely evaporated using a vacuum evaporator. Then 100 mL of oxygenated distilled water was added with vigorous shaking; 1.5 mL of this reaction mixture

was dispersed to test tubes and sample solution (0.50 mL, 2 mg/mL) were added and the emulsion system was incubated for up to 2 h at 50°C. The same procedure was repeated with the standard (Butylated hydroxytoluene (BHT)) and a blank. After this incubation period, the sample absorbance was read at 490 nm. Measurement of absorbance was continued until the color of β -carotene disappeared. The bleaching rate (R) of β -carotene was calculated according to Eq. (1).

$$R = \left[\frac{\ln(a/b)}{t} \right] \quad (1)$$

Where, ln=natural log, a=absorbance at time 0, b=absorbance at time t (30, 60, 90, 120 min). The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control using Eq. (2).

$$AA = \left[\frac{(R_{\text{Control}} - R_{\text{Sample}})}{R_{\text{Control}}} \right] \times 100, \quad (2)$$

Cupric ion reducing (CUPRAC) method

Extract solutions (0.5 mL) were added to reaction mixture [CuCl₂ (1 mL, 10 mM), neocuproine (1 mL, 7.5 mM), NH₄Ac buffer (1 mL, 1 M, pH 7.0)] and the absorbance was recorded at 450 nm after 30 min of incubation at room temperature. Similarly, a blank sample (prepared in the same manner but without the extract) was prepared and analysed according to this procedure. Milligrams of trolox equivalents per gram of dry extract (TEs/g extract) were the measurement unit.

Ferric reducing antioxidant power (FRAP) method

Extract solution (0.1 mL) was added to reagent (2 mL) in acetate buffer (0.3 M, pH 3.6), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (10 mM) in 40 mM HCl and ferric chloride (20 mM) in a final ratio of 10:1:1 (v/v/v). Then, the absorbance at 593 nm was read after 30 min of incubation at room temperature. Similarly, a blank sample (prepared in the same manner but without the extract) was prepared. Milligrams of trolox equivalents per gram of dry extract (TEs/g extract) were the measurement unit.

Metal chelating activity on ferrous ions

Extract solution (2.0 mL) was added to FeCl₂ (0.05 mL, 2 mM), and the reaction was started using 0.2 mL of 5 mM ferrozine. Similarly, a blank sample for each sample (prepared in the same manner but without ferrozine) was prepared, and all the absorbances were recorded after 10 min of incubation (room temperature) at 562 nm. Milligrams of EDTA equivalents per gram of dry extract (EDTAEs/g extract) were the measurement unit.

Enzyme inhibitory assays

Cholinesterase inhibition

After 15 min of incubation at 25 °C the reaction mixture composed by the extract solution (50 mL), DTNB (3 mM 125 mL) and enzyme solution (0.265 u/mL AChE or 0.026 u/mL BChE) solution (25 mL) in Tris-HCl buffer (pH 8.0) was added to the substrates [acetylthiocholineiodide (15 mM ATCI) or butyrylthiocholine chloride (1.5 mM BTCl, 25 mL)]. Likewise,

a blank sample (prepared in the same manner but without the extract) was prepared and all the absorbances were recorded at 405 nm after 15 min. Milligrams of galantamine equivalents per gram of dry extract (GALAEs/g extract) were the measurement unit.

α -Amylase inhibition

After 10 min of incubation at 37 °C the reaction mixture comprising the extract solution (25 mL), α -amylase solution (10 u/mL, 50 mL) in phosphate buffer (pH 6.9 with 6 mM sodium chloride) was added to the starch solution (50 mL, 0.05%). The reaction was stopped with the addition of HCl (25 mL, 1 M), and then the iodine-potassium iodide solution was added (100 mL). Likewise, a blank sample (prepared in the same manner but without the extract) was prepared, and all the absorbances were recorded at 630 nm after 10 min of incubation at 37 °C. Millimoles of acarbose equivalents per gram of dry extract (ACAEs/g extract) were the measurement unit.

α -Glucosidase inhibition

After 15 min of incubation at 37 °C the reaction between the extract solution (50 mL) glutathione (0.5 mg/mL, 50 mL), α -glucosidase solution (0.2 u/mL 50 mL) in phosphate buffer (pH 6.8) and PNPg (10 mM, 50 mL) was stopped with sodium carbonate (50 mL, 0.2 M). Likewise, a blank sample (prepared in the same manner but without the extract) was prepared, and all the absorbances were recorded at 400 nm after 15 min of incubation at 37 °C. Millimoles of acarbose equivalents per gram of dry extract (ACAEs/g extract) were the measurement unit.

Tyrosinase inhibition

Extract solution (25 mL) was added to a tyrosinase solution (200 u/mL, 40 mL) and phosphate buffer (40 mM, 100 mL, pH 6.8) in a 96-well microplate and then incubated for 15 min at 25 °C. The reaction was started using L-DOPA (10 mM, 40 mL), and after 10 min of incubation at room temperature all the absorbances were recorded at 492 nm. Similarly, a blank sample (prepared in the same manner but without the extract) was prepared and analysed according to this procedure. Milligrams of kojic acid equivalents per gram of dry extract (KAE/g extract) were the measurement unit.

Statistical Analysis

All the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The differences between the different extracts were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference post hoc test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 14.0 program.

RESULTS and DISCUSSION

Enzyme inhibitory effects

Maintaining the level of acetylcholine in the brain by inhibiting cholinesterase enzymes, is an important strategy for treating AD (Shariffar et al 2012). Cholinesterases namely, acetyl and

butyryl cholinesterase, terminate neuronal transmission by hydrolyzing acetylcholine, the main neurotransmitter involved in the cholinergic system (Samaradivakara et al 2016). Currently used medication to alleviate AD symptoms carry numerous side effects such as nausea, vomiting, diarrhea, muscle cramps, fatigue, weight loss, confusion, constipation, and dizziness (NI 2017). The need for novel molecules deprived of side effects is of utmost importance. Galantamine used in the treatment of mild to moderate AD, is an alkaloid which inhibits acetyl cholinesterase enzyme (Olin and Schneider 2002). With regards to the therapeutic potential of plants, we have studied the cholinesterase inhibition capacity of the ethyl acetate, methanol, and water extracts of *C. cilicica* using *in vitro* methods. In the present study, the methanol extract of *C. cilicica* actively inhibited AChE and BChE (1.33 and 0.68 mg GALAE/g extract, respectively), compared to a lower activity for ethyl acetate extract (0.99 and 0.09 GALAE/g extract, for respective enzymes) while no activity was recorded for the water extract (Table 1). The observed inhibitory action of *C. cilicica* methanol extract on cholinesterase enzymes might be ascribed to other phytochemicals such as alkaloids, terpenes, and sterols as previously described by Ahmed et al (2013). D-pinitol identified in the leaves of *C. cilicica* (Eser et al., 2017) was reported to interfere with the accumulation of beta amyloid, involved in Alzheimer's disease (National Library of Medicine, 2017).

Although, tyrosinase was chiefly associated to melanin synthesis and skin hyperpigmentation therapy, evidences highlight that this copper-containing enzyme is linked to Parkinson's disease (PD), the second most common neurodegenerative disorder after AD (Neagu et al 2015). PD is a chronic, progressive movement disorder which affects 6.3 million people worldwide (Coomber et al 2017). PD involves the malfunctioning and apoptosis to neurons in the substantia nigra, which controls movement and coordination (Ellis and Fell 2017). Histological data highlight the presence of neuromelanin in the substantia nigra. Indeed, the dual protective and toxic function of neuromelanin have been reported (Zucca et al 2017). Evidences of the possible role of tyrosinase in the biosynthesis of neuromelanin make its modulation a popular target for the treatment of PD (Cespedes et al 2017). From Table 1 it was observed that *C. cilicica* extracts possessed variable degree of inhibition against tyrosinase; ethyl acetate extract (54.70 mgKAE/g extract) being the most

active and water extract being the least active (42.58 mgKAE/g extract). The observed difference might be associated to the different phytochemical composition of the different extracts (Chigayo et al 2016; Thouri et al 2017).

Magnetic resonance imaging of the brain of elderly suffering from diabetes showed a decrease in the hippocampus size, resulting from reduced neurogenesis and enhanced neuronal death (Pugazhenthil et al 2017). In fact, epidemiological data have revealed that elevated glucose level, the hallmark of diabetes, increases the risk of developing dementia and prompts the exacerbation of mild cognitive impairment of AD. Hyperglycemia was reported to increase amyloid- β levels in the brain by altering neuronal activity via K_{ATP} channels (Macauley et al 2015). Epidemiological data also suggest that insulin resistance impaired glucose absorption by neurons, impairing neuronal transmission and cognition (Kandimalla et al 2017). Thus, controlling glucose level in elderly diabetic patients might prevent the onset and/or worsening of cognitive impairments. Alpha-Amylase and α -glucosidase are two enzymes which have been extensively studied for the management of diabetes. α -Amylase is responsible for the hydrolysis of polysaccharides at the early stage of digestion, while α -glucosidase cleaves disaccharides, producing glucose which is absorbed by the enterocytes of the intestinal villi (Zhang et al 2017). From Table 1, it was observed that *C. cilicica* extracts possessed higher α -glucosidase (2.71-1.23 mmol ACAE/g extract) inhibitory potential compared to α -amylase (0.57-0.12 mmol ACAE/g extract). The order of inhibition against α -amylase was as follows ethyl acetate>methanol>water. The same trend was observed against α -glucosidase. From Table 2, it was noted that the ethyl acetate extract (24.77 mgGAE/g extract) of *C. cilicica* contained the highest amount of phenolic. Indeed, Ademiluyi and Oboh (2013) and Jiang et al (2017) also reported the potent inhibitory effect of phenolic rich extracts on α -amylase and α -glucosidase.

Antioxidant properties

Oxidative stress is known to play a key role in AD. Oxidative stress refers to the increased production of reactive oxygen species accompanied by the reduced efficiency of the innate antioxidant defense mechanism (Tramutola et al 2017). Reactive oxygen species participate in cellular signaling pathways *in vivo*, but overproduction induces oxidation of membrane lipids, inactivate enzymes and normal cellular

Table 1. Enzyme inhibitory effects of *C. cilicica* extracts.

Extracts	AChE inhibition (mgGALAE/g extract)	BChE inhibition (mgGALAE/g extract)	Tyrosinase (mgKAE/g extract)	α -amylase (mmolACAE/g extract)	α -glucosidase (mmolACAE/g extract)
Ethyl acetate	0.99±0.01*	0.09±0.01	54.7±2.28	0.57±0.076	2.71±0.05
Methanol	1.33±0.03	0.68±0.02	52.28±0.38	0.38±0.03	2.60±0.09
Water	na	na	42.58±0.72	0.12±0.01	1.23±0.10

* Values expressed are means ± S.D. of three parallel measurements. AChE: acetylcholinesterase; BChE: butyrylcholinesterase; GALAE: galantamine equivalents; ACAE: acarbose equivalents; KAE: kojic acid equivalents; na: not active.

Table 2. Total phenolic and flavonoid contents and total antioxidant, and metal chelating abilities of *C. cilicica* extracts

Extracts	TPC (mgGAE/g extract)	TFC (mgRE/g extract)	DPPH scavenging (mgTE/g extract)	ABTS scavenging (mgTE/g extract)	FRAP (mgTE/g extract)	CUPRAC (mgTE/g extract)	β -carotene/ linoleic acid assay (inhibition [%] at 2 mg/ml concentration)	Phospho- molybdenum (mmolTE/g extract)	Metal chelating activity (mgEDTAE/g extract)
Ethyl acetate	24.77 \pm 1.88*	2.78 \pm 0.21	34.26 \pm 1.09*	22.67 \pm 0.34	32.00 \pm 1.92	56.21 \pm 4.25	91.96 \pm 0.20	1.88 \pm 0.01	6.78 \pm 0.03
Methanol	17.82 \pm 1.87	2.83 \pm 0.10	12.89 \pm 2.75	16.22 \pm 1.25	21.36 \pm 1.26	49.97 \pm 1.99	91.44 \pm 0.50	1.61 \pm 0.01	0.65 \pm 0.01
Water	20.25 \pm 0.73	17.26 \pm 0.16	42.46 \pm 0.08	57.70 \pm 1.22	58.48 \pm 0.34	74.68 \pm 0.66	86.12 \pm 0.40	0.91 \pm 0.04	2.72 \pm 0.02
BHT	nt	nt	nt	nt	nt	nt	91.70 \pm 0.39	nt	nt

* Values expressed are means \pm S.D. of three parallel measurements. TPC: Total phenolic content; TFC: Total flavonoid content; GAE: gallic acid equivalents; RE: rutin equivalents; TE: trolox equivalents; EDTAE: EDTA equivalents; nt: no tested.

functioning, damage proteins and DNA, thus contributing to AD (García-Blanco et al 2017). In the present study, we assessed the antioxidant potential of *C. cilicica* extracts by using a set of antioxidant assays. Free radicals are unsw molecules containing one or more unpaired electrons, making them highly reactive (Poprac et al 2017). ABTS and DPPH radicals have been extensively used *in vitro* to provide an insight of the radical scavenging capacity of antioxidant molecules. The water extract of *C. cilicica* showed potent radical scavenging capacity against DPPH and ABTS (42.46 and 57.70 mgTE/g extract). Phytochemical determination showed that *C. cilicica* water extract (17.26 mg RE/g extract) was rich in flavonoids as compared to ethyl acetate and methanol extracts (2.78 and 2.8326 mg RE/g extract, for respective extracts). This finding is in accordance with the statements of Pizzino et al (2017) and Kumar and Pandey (2013). The FRAP and CUPRAC assays were employed to evaluate the reducing potential of *C. cilicica* extracts. The redox potential of phytochemicals makes them potent reducing agents (Al-Rimawi et al 2016). As noted from Table 2, water extract of *C. cilicica* was a potential reducing agent (58.48 and 74.68 mg TE/g extract, for FRAP and CUPRAC assays respectively). This extract contained the highest amount of flavonoids, which was previously reported to act as a potent reducing agent (Ghasemzadeh and Ghasemzadeh 2011). β -carotene/linoleic acid and phosphomolybdenum assays are also currently used to provide an insight of the oxidant scavenging potential of plant phytochemicals. From Table 2, it was observed that the extracts of *C. cilicica* (value ranging from 91.96 to 89.12% inhibition as compared to 91.70% inhibition for BHT) prevented β -carotene discoloration induced by linoleic acid oxidation in the following order ethyl acetate>methanol>water. The same trend was observed for the phosphomolybdenum assay.

Metal ions such as copper, zinc, and iron, are involved in neuron signaling, apoptosis, cell proliferation, inflammation, and oxidative stress control (Kepp 2017). Disruption of the homeostasis of metal ions was associated to amyloid- β deposits and tau phosphorylation, resulting in the formation of neurofibrillary tangles and senile plaque formation, which

fuel AD (Wang and Wang 2017). Finding molecules possessing metal chelating abilities might serve in the management of AD. From the present study ethyl acetate extract (6.78 mg EDTAE/g extract) of *C. cilicica* showed the most potent metal chelating activity followed by water extract (2.72 mg EDTAE/g extract) and methanol extract (0.65 mg EDTAE/g extract). Total phenolic content determination followed the same trend (Table 2), suggesting that phenolic composition of the extracts obtained from solvents of different polarities affected the observed chelating potential.

CONCLUSION

AD is a complex, multifactorial disorder affecting the elderly segment of the world's population. While existing treatments are palliative and offer no improvement of the disease conditions, the major concern is the discovery of new effective agents. This study provides an insight on the potential of *C. cilicica* in the management of AD. *C. cilicica* acted as cholinesterase inhibitor. *C. cilicica* was also found to modulate the action of other enzymes namely α -amylase and α -glucosidase, which are the main targets of glycaemic control. Indeed, chronic dyshomeostasis of blood glucose level was linked to AD. Data collected from the present study showed that *C. cilicica* water extract was rich in flavonoids and possessed potent reducing potential. *C. cilicica* previously reported to be used in traditional medicine, might also be used as a natural source of antioxidants. Therefore, *C. cilicica* merits further investigation as it is a source of valuable multi-target compounds for the management of AD.

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