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Research Article

Biological activity studies on *Klasea serratuloides* (DC) Greuter & Wagenitz subsp. *karamanica* B. Dogan & A. Duran extracts obtained with different extraction methods

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Abstract: The aim of this study was to investigate the antioxidant and enzyme inhibition activities of methanol extracts prepared from *Klasea serratuloides* (DC) Greuter & Wagenitz subsp. *karamanica* B. Dogan & A. Duran with Soxhlet, ultrasonic extraction, and maceration methods. 1,1-Diphenyl-2-picryl hydroxyl (DPPH) quenching assay and 2,2'-azinobis-3- ethylbenzothiozoline-6-sulfonic acid (ABTS) cation decolorization test were used to evaluate *in vitro* radical scavenging activity. The total phenolic content was determined with the Folin-Ciocalteu method while the total flavonoid content was evaluated by the aluminum chloride colorimetric method. According to the results, DPPH, ABTS radical scavenging activity and iron chelating activity of methanol of *Klasea serratuloides* were shown concentration-dependent manner. The extract obtained from maceration was found to be higher than the other extracts. It suggests that the maceration technique was more effective than the other extraction methods for the determination of the phenolic content. The methanol extract of KS using soxhlet (61.17 ± 3.62) and ultrasonic extraction (58.76 ± 1.46) showed higher inhibition than the extract prepared with maceration methods (34.54 ± 0.73) against BChE. All extracts displayed moderate inhibition activity against AChE. As for enzyme inhibition activity, the extract from soxhlet method was found to be more potent tyrosinase, acetylcholinesterase and butyrylcholinesterase inhibitors than the other extracts prepared by ultrasound assisted extraction and maceration methods. The present study suggests that *K. serratuloides* should be given special attention to conduct further investigation for its phytochemical constituents that attribute to their antioxidant potentials, and enzyme inhibition activities.

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1. INTRODUCTION

Klasea Cass. (Asteraceae) is represented by 46 species, and 10 sections. Although it is formerly defined as a section of the *Serratula* genus, it is sometimes accepted as an independent genus (Annales *et al*., 2012; Dogan *et al*., 2014). It is located in Himalayas, Central Asia, Mediterranean basin, SE Europe, China, southern Russia, Iran, and Turkiye. The genus *Klasea,* is distributed in Mediterranean and Irano – Turanian phytogeographic regions of Turkiye, with 15 species. Five of these species are endemic to Turkiye (Dogan *et al*., 2014). *K. serratuloides*

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(DC.) Greuter & Wagenitz is located in Van, Tunceli (Babacan *et al*., 2017; Tel *et al*., 2015). *Klasea serratuloides* subsp. *karamanica* (KS) is endemic to the Ayrancı, Ermenek, Ereğli and Ulukışla, Anatolia. It is morphologically similar to *K. serratuloides* subsp. *serratuloides* (Dogan *et al*., 2014). There is a limited number of biological activity studies on both species and genus. Flowering part of *Klasea pusilla* (Labill.) Greuter &Wagenitz is traditionally used treatment of insect bites in Mount Hermon-Lebanon (Baydoun *et al*., 2015).

Klasea serratuloides (KS), perennial, 8-42 cm tall, arachnoid, 5-8 mm in diameter below. Stout, unbranched stem that emerges from thick fleshy roots. Leaves are obovate in shape, coriaceous, petiolate, conspicuously veined, typically simple, rarely lyrate-pinnatifid, 10–30 (–38) 5-12 (16) cm in length, with sinuate-lobulate margins. Capitula solitary on peduncles ranging in length from 7 to 12 cm, with a diameter of 3.0 to 6.0 cm and a height of 3.5 to 5.5 cm. 3.0–5.0 3.5–4.5 cm subglobose involucre Phyllaries are 8-seriate, greenish with a few dark striations, ovate acuminate with a strongly reflexed, pale-colored apical section; Outer phyllaries are 8-10 5-7 mm, sparsely arachnoid; inner phyllaries are 20-25 6- 8 mm, glabrous. Petals are pale and straight, about 15-20 mm in length. 5-lobed mauve corolla with glandular hairs on the lobes; tubes 30-35 mm long. Achenes are 10-13 mm in length. Pappus plumose, dirty-white, persistent, 15–18 mm. Flowers in May-July, and fruits in July –August (Doğan *et al*., 2014).

Oxidative stress caused by reactive oxygen species plays a role in the pathogenesis of many chronic and degenerative diseases such as multiple sclerosis, immune disorders, cardiovascular disease, Alzheimer, Parkinson, cancer, diabetes mellitus, skin disorder, and dementia (Mao *et al*., 2013; Szymanska *et al*., 2018; Eruygur *et al*., 2020). The antioxidants obtained from medicinal plants, and dietary supplements can prevent the formation of possible diseases by inhibiting free radicals. In recent years, the natural antioxidants have been preferred as a priority, therefore the investigation on the discovering of new phytochemicals with antioxidant activity has still been maintaining by researchers. According to the literature, phenolic compounds, which are common in plants, are the most important components with their high antioxidant activity and free radical scavenging effect among the secondary metabolites (Rice-Evans *et al*., 1997; Gan *et al*., 2010). Therefore, determining of total phenolic contents of the medicinal plants is mainly remarkable for the antioxidant capacities of the plants.

Alzheimer's disease is characterized by social behavioral disorder, loss of memory, and cognitive performances. It is known that more than one mechanism may be effective in the pathogenesis of this disease. Cholinergic hypothesis, which is one of these, has been suggested that in Alzheimer's patients the deficits in cholinergic function may contribute to the cognitive decline associated with delirium. When acetylcholinesterase that hydrolyzes acetylcholine is inhibited by the chemical agents, they can be most promising to take place in the treatment of the disease (Orhan *et al*., 2009; Senol *et al*., 2013; Eruygur *et al*., 2019). The discovery of novel molecules or extracts originated from plants that inhibit acetylcholinesterase and butyrylcholinesterase could be model for the development of new drug candidates for the treatment.

Polyphenol oxidase, or tyrosinase, is a copper-containing enzyme. This enzyme is found in bacteria, fungi, plants, and mammals. It catalyzes the hydroxylation of tyrosine (monophenol) to 3,4-dihydroxyphenylalanine or DOPA (o-diphenol) and the oxidation of DOPA to dopaquinone (o-quinone) in the human body. Melanin is a color pigment that protects our skin from UV light damage. But excessive production of melanin causes pigmentation disorders. The purpose on usage of tyrosinase inhibitors is to prevent melanin production by eliminating the enzyme function (Likhitwitayawuid, 2008; Liyanaarachchi *et al*., 2018). Tyrosinase inhibitors are used in the treatment of hyperpigmentation, as well as included in the cosmetic preparations as skin whitening agents.

In this study, we have evaluated the enzyme inhibitory, and antioxidant activities of the methanol (MeOH) extracts which were prepared using different methods from aerial parts of *Klasea serratuloides* (DC) Greuter & Wagenitz subsp. *karamanica* B. Dogan & A. Duran *(*KS). We have also reported that total phenol and flavonoid contents, as well as the biological activities of the MeOH extracts differed from each other depending on the using extraction techniques, such as maceration, Soxhlet extraction, and ultrasound assisted extraction.

2. MATERIAL and METHODS

2.1. Plant Material

KS aerial parts were taken from the natural flora of Karaman, Southern Turkiye (C4: Karaman, Ermenek, on slopes of Tepebaşı village, 1100 m, 21.06.2017, S. Doğu 3456 Y. Bağcı). The plant material was identified by botanist Yavuz Bağcı and Süleyman Doğu. The voucher specimen was held at Selcuk University's Herbarium in Konya (KNYA) Turkiye. In the year 2020, the antioxidant and enzyme inhibition activities were carried out in the laboratories of Selcuk University's Pharmacy Faculty in Konya.

2.2. Chemicals and Equipment

2-2′-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), EDTA (Ethylenediaminetetraacetic acid), sodium carbonate, quercetin, gallic acid, ferrozin, AChE, BChE, tyrosinase, L-tyrosine, galantamine, and kojic acid were purchased from Sigma–Aldrich (St. Louis, MO). Folin–Ciocalteu's phenol reagent, hydrochloric acid, and methanol were obtained from Merck (Darmstadt, Germany). All chemicals used in the experiments were of analytical grade. To evaporate the combined solvents using Rotary evaporator (Buchi R-300, Switzerland). Spectrophotometric measurements were performed by a microplate reader (Multiskan Go; Thermo Scientific Inc.).

2.3. Preparation of The Extracts

The aerial parts of KS were dried in the shade until they reached a constant weight, then pulverized in a blender. In separate bottles, 20 g of dried plant material was extracted for 6 h with 180 mL of methanol using the Soxhlet, ultrasonic extraction, and maceration techniques. The extract was then filtered via Whatman No. 1 filter paper. A rotating evaporator at 40 ° C was used to concentrate the filtrate under reduced pressure. Table 1 shows the yield of KS extracts obtained by Soxhlet, Ultrasonic extraction, and maceration techniques.

Table 1. % Yield of the extracts from *Klasea serratuloides.*

Sample	Extraction Methods	%Yield of the extacts (w/w)
		MeOH
Klasea serratuloides	Soxhlet	37.28
	Ultrasonic extraction	22.57
	Maceration	20.05

2.4. Determination of Total Phenolic Content (TPC)

For the measurement of the TPC in the extracts of KS, the spectrophotometric Folin-Ciocalteu (F-C) method was used according to the method of Clarke *et al*., (2013) with slight modification. 10 µL of extract, suitably diluted with DMSO, were combined with 100 µL of freshly 10-fold diluted F–C reagent in distilled water. After 5 min, 100 µL of 7.5% Na_2CO_3 was added, and the absorbance was measured at 650 nm with a microplate reader after 60 min of incubation (Multiscan Sky). In parallel, appropriate blanks (DMSO) were run. All analyses were done in triplicate, and the results were represented by means of standard deviation (SD).

The TPC was estimated as mg GAE (gallic acid equivalent)/ g extract using a calibration curve of concentration vs absorbance.

2.5. Determination of Total Flavonoid Content (TFC)

The total flavonoid concentration of KS extracts was determined using the aluminum chloride colorimetric technique (Yang *et al*., 2011). On a 96-well plate, the test solution (150µL, 0.3 mg/mL) produced with ethanol was combined with 2% $AlCl_3$. In a microplate reader, the absorbance was measured at 435 nm after 15 m of room temperature incubation. The total flavonoid content was calculated as mg QE (quercetin equivalent)/ g extract dry weight basis.

2.6. *In vitro* Antioxidant Activity

2.6.1. *DPPH radical scavenging activity*

The radical scavenging activity of extracts was determined using the DPPH radical scavenging assay. After mixing 20 µL of test solution with 180 µL of DPPH solution in a 96-well plate. The plate was measured at 540 nm using an Elisa reader (Multiscan Sky, USA) after 15 m of incubation in the dark (Eruygur *et al*., 2019). Ascorbic acid was used as positive control. All analyses were done in triplicate, and the results were represented by means of standard deviation (SD). The following equation (1) was used to calculate the percent DPPH scavenging effect:

$$\% \text{ DPPH Scavenging Effect} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}} x100 \quad (1)$$

2.6.2. *ABTS radical scavenging activity*

According to Re *et al*., the extracts' ABTS cation radical decolorization activity was performed with minor modifications (Re *et al*., 1999). Allowing 15 mL of 7 mM ABTS and 264 µL of 140 mM potassium persulfate solution to stand in the dark at room temperature for 16 h before the experiment yielded the ABTS•+ radical stock solution. The ABTS•+ working solution was made fresh by diluting the stock solution with 80%MeOH and measuring the absorbance at 734 nm to get 0.70 ± 0.02. 50 µL of sample solution were combined with 100µL of ABTS•+ working solution in a 96-well plate. The mixture was then allowed to stand at room temperature for 10 minutes, after which the absorbance was also measured at 734 nm. All of the activities were repeated three times, with the results reported as a means of standard deviation. For comparison of the ABTS+ scavenging activity, ascorbic acid was utilized as an antioxidant standard. The following equation (2) was used to calculate the percent ABTS scavenging effect:

$$\% \text{ ABTS Scavenging Effect} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}} x100 \quad (2)$$

2.6.3. *Iron Chelating Activity*

The iron chelating activity of the extracts was determined based on the interaction of ferrozin-Fe^{2+}-complex (Chai *et al*., 2014). In summary, a mixture of 0.4 mL of 0.2 mM ferrozine, 0.2 mL of 0.1 mM $FeSO_4$ and 0.2 mL of extract was incubated at room temperature. The absorbance was measured at 562 nm after a 10-minute incubation period. As a positive control, EDTA was utilized.

2.7. Enzyme Inhibition Activity

2.7.1. *Acetylcholinesterase / butyrylcholinesterase inhibition assay*

This experiment was carried out according to the Ellman al., method with some modifications (Ellman *et al*.,1961). A mixture of 20 µL of test sample/reference standard at various concentrations, 140µL of 200 mM phosphate buffer (pH 7.7), 10 µL of 5,5-dithio-bis-(2-nitrobenzoic acid (DTNB) and 20µL of enzyme (0.22 U/mL for acetylcholinesterase/0.1 U/mL

for butyrylcholinesterase prepared in PBS buffer) was incubated for 15 min at 25 °C. Following the addition of 10 µL of 0.5 mM DTNB, 10 µL of substrate (0.71 mM acetylthiocholine iodide / 0.2 mM butyrylthiocholine iodide) was mixed and incubated for another 5 min. When substrate was added, the absorbance of the plate was measured at 0 and 5 min, yielding a yellow color at 412 nm. As a positive control, galantamine was used. The following equation (3) was used to express the results:

$$\% \text{ Inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} x100 \quad (3)$$

2.7.2. *Tyrosinase enzyme inhibition activity*

Tyrosinase inhibitory activity of the extracts was evaluated according to the method previously reported (Yang *et al*., 2012). 20µL of sample solution diluted with buffer, 100µL of phosphate buffer, and 20µL of tyrosinase (250 U/mL) were combined in each well of a 96 well plate and incubated for about 10 min at 25 °C. The mixture was incubated for 30 min at 25 °C after adding 20µL of 3 mM L-tyrosine as a substrate. After incubation period, the absorbance was read at 492 nm. In place of the sample, kojic acid and phosphate buffer (100 mM PBS, pH 6.8) were employed as positive and negative controls, respectively. The equation (3) was used to calculate the extracts' inhibitory effects on tyrosinase.

3. RESULTS / FINDINGS

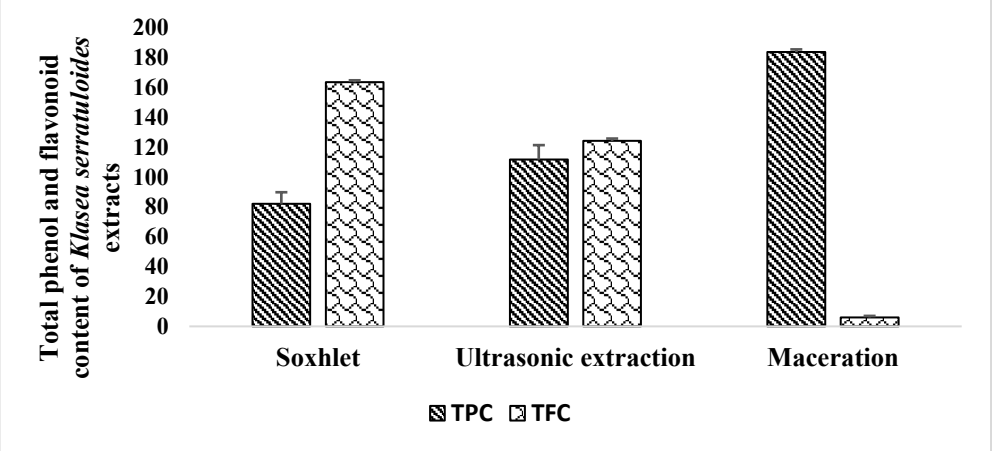
3.1. Total Phenolic and Flavonoid Content

Many chemical components in medicinal plants have various biological activities. Flavonoid and phenolic compounds are among the most important classes. Flavonoids such as kaempferol, quercetin, and rutin are polyphenolic compounds and they are known for their potent free radical scavenging effects (Rice-Evans *et al*., 1997; Atanassova *et al*., 2011). Total phenol content of extracts was calculated as mg gallic acid equivalent /g extract with the calibration equation of y=0.0027x+0.0084 (r^2= 0.9966). Total flavonoid content of extracts calculated as mg Quercetin equivalent (QE)/g extract equivalent as y=0.0346x+ 0.2221 (r^2= 0.9773).

When the total phenol and total flavonoid contents were evaluated in this study, the total phenol content of extracts prepared from KS with Soxhlet, ultrasonic extraction and maceration methods were determined as 82.19±7.71, 111.80±9.69, and 183.87±1.76 mg, respectively.

According to the results, total phenol content was found to be the highest in the extract prepared with maceration, it was followed by ultrasonic extraction and the least in Soxhlet extract. Total flavonoid content was expressed as quercetin equivalent (QE). The total flavonoid content of methanol extracts prepared from *KS* with Soxhlet, ultrasonic extraction and maceration methods was determined as 163.65±1.29, 124.33±1.55, and 6.07±0.94 mg QE/g, respectively (Figure 1). It can be thought to be that the maceration technique was more effective for extraction of phenolic compounds, the Soxhlet technique was more effective for extraction of flavonoids compounds in this plant.

Figure 1. Total phenol and total flavonoid content of KS extracts prepared with Soxhlet, ultrasonic extract and maceration methods.



3.2. Antioxidant Activity

The *in vitro* antioxidant activities have been determined by DPPH, ABTS and iron chelating activity methods. Radical scavenging activities of KS were compared with synthetic antioxidant compounds. All of extracts exerted high radical scavenging activity with both DPPH and ABTS assay and showing scavenging activity over 50%. The scavenging ability of these samples showed a concentration dependent activity profile. The best antioxidant activity was observed in the extract of KS with maceration methods in DPPH (IC_{50}: 24.32 ± 1.02 µg/mL) and ABTS (IC_{50}: 13.44 ± 0.29 µg/mL). It was observed to have higher antioxidant activity compared to positive control. The extract of KS obtained with soxhlet (39.15 ± 2.44%) was more active than other extract with ultrasonic extraction (36.15±0.61%) and maceration (30.56±2.28%) in terms of iron-chelating activity. As a result, KS has antioxidant activity in all three methods as given in the Table 2. In a previous study, *K. serratuloides* (DC.) Greuter &Wagenitz were found to have antioxidant activity in DPPH, CUPRAC, and ferrous ion-chelating methods (Tel *et al*., 2015).

Table 2. ABTS and DPPH radical scavenging effects, and iron chelating activities of the extracts of *K. serratuloides*.

Extraction Methods	ABTS (percentage± S.D.[a]) 83.33 µg/mL[b]	DPPH (percentage± S.D.[a]) 50 µg/mL[b]	Iron-chelating activities (percentage± S.D.[a]) 250 µg/mL[b]
Soxhlet	80.93 ± 3,71 (IC_{50}: 45.79 ± 0.67 µg/mL)	60.87 ± 4.9 (IC_{50}: 19.81 ± 0.69 µg/mL)	39.15 ± 2.44
Ultrasonic extraction	54.63 ± 3.07 (IC_{50}: 47.27 ± 2.21 µg/mL)	72.21 ± 2.85 (IC_{50}: 40.78 ± 2.17 µg/mL)	36.15±0.61
Maceration	82.05 ± 0.90 (IC_{50}: 13.44 ± 0.29 µg/mL)	87.51 ± 0.33 (IC_{50}: 24.32 ± 1.02 µg/mL)	30.56 ± 2.28
Reference	87.51 ± 0.17[c]	93.91 ± 0.14[c]	87.06 ± 0.34[d]

a: Standard deviation, b: Final concentration, c: Ascorbic acid (2 mg/mL) d: EDTA (2 mg/mL)

3.3. Enzyme Inhibition Activity

The methanol extracts obtained from the aerial parts of KS were tested for their acetylcholinesterase (AChE), and butyrylcholinesterase (BChE) tyrosinase (TYR) inhibitory activity at the concentration of 50, 100, and 200 µg/mL. Inhibitory activity of extracts given at different concentration in Table 3. All extracts displayed moderate inhibition activity against AChE, while all extracts exhibited high inhibition activity against BChE. The methanol extract of KS using soxhlet (61.17 ± 3.62) and ultrasonic extraction (58.76 ± 1.46) showed higher inhibition than the extract prepared with maceration methods (34.54 ± 0.73) against BChE at 50µg/mL of concentration. As can be seen from the Table 3, the extract prepared with soxhlet showed 45.57% inhibition against AChE and 61.17% inhibition against BChE at 50 µg/mL. All of them have moderate inhibition activity against TYR at the concentration of 125µg/mL and 250 µg/mL. The methanol extracts of KS prepared with soxhlet, ultrasonic extraction and maceration technique was showed 29.49%, 25.12%, and 52.80% inhibition against TYR at concentration of 125 µg/mL. According to Tel *et al*. (2015), methanol extract of *K. serratuloides* showed completely good anti-tyrosinase activity at 50 µg/mL (31.1%) and hexane extract showed high anti-acetylcholinesterase activity (IC_{50}: 134.7 ± 2.1 µg/mL) (Tel *et al*., 2015).

Table 3. Enzyme inhibition effects of the extracts of *K. serratuloides*.

Inhibitory activity	Concentration	Soxhlet extraction	Ultrasonic extraction	Maceration	Reference [d.e]
AChE (percentage ± S.D.[a])	50 µg/mL[b]	45.57±0.96	27.44±0.13	41.39±0.73	99.10±1.18[d]
	100 µg/mL[b]	75.19±1.63	50.84±0.37	46.40±4.00	
	200 µg/mL[b]	93.86±3.26	86.63±2.67	90.07±2.83	
BChE (percentage ±S.D.[a])	50 µg/mL[b]	61.17±3.62	58.76±1.46	34.54±0.73	84.34±4.85[d]
	100 µg/mL[b]	69.59±2.19	-[c]	-[c]	
	200 µg/mL[b]	-[c]	-[c]	-[c]	
TYR (percentage ± S.D.[a])	62.5 µg/mL[b]	20.53±3.34	7.27±4.68	15.52±1.18	80.96±0.51[e]
	125 µg/mL[b]	29.49±2.09	25.12±2.65	52.80±2.35	
	250 µg/mL[b]	38.04±1.50	30.52±2.68	-[c]	

KS: *Klasea serratuloides* [a]Standard deviation, [b]Final concentration [c]Not detected [d]Galanthamine hydrobromide [e]Kojic acid

When the results of this study are evaluated, in DPPH and ABTS methods, the antioxidant activity of the KS extracts prepared by maceration technique was higher than the others. The maceration technique is more suitable for the extraction of phenolic compounds that play a role in antioxidant activity.

The highest iron chelation activity was observed extract of KS with Soxhlet. For enzyme inhibition, the extract from Soxhlet was found to be more effective against both cholinesterase and tyrosinase. The yield of KS extract obtained by Soxhlet was higher than the yield of other extracts. Therefore, it can be said that the natural compounds with strong cholinesterase and TYR inhibitory activity can be extract with soxhlet technique from KS.

4. CONCLUSION

As a conclusion, antioxidant activity was found to be high in all extracts of KS prepared by different methods. Especially the extract prepared with soxhlet demonstrated the highest inhibitory activity against all enzymes. Although all the extracts show good inhibition against tested enzymes, the chemical composition analysis of the extracts should be conducted to determine which compound is responsible for the activity. It has been seen as promising for the related diseases in the future.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship contribution statement

Authors are expected to present author contributions statement to their manuscript such as. **Nuraniye Eruygur:** Investigation, Supervision, Arrangement and Analysis, reviewing and correcting the original draft. **Fatma Ayaz:** Investigation, Arrangement and Analysis. **Yavuz Bağcı:** Plant collection and Diagnosis. **Süleyman Doğu:** Plant collection and Diagnosis. **Tuğsen Doğru:** Writing, Analysis, creating of Table and Graphics. **Merve Koçak:** Writing, Arrangement, and Analysis.

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