Cumhuriyet Sci. J., 45(4) (2024) 756-762
DOI: https://doi.org/10.17776/csj.1568111

Cumhuriyet Science Journal

| csj.cumhuriyet.edu.tr | Founded: 2002 ISSN: 2587-2680 e-ISSN: 2587-246X Publisher: Sivas Cumhuriyet University

In Vitro Tyrosinase and Collagenase Inhibitory and Antioxidant Potential of *Smyrnium rotundifolium* Mill. and *Euphorbia virgata* Waldst.&Kit. from Türkiye

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Research Article

History
Received: 19/10/2024
Accepted: 19/12/2024

ABSTRACT

Antioxidants help prevent signs of aging and skin tone inequalities by protecting our skin from free radicals. High tyrosinase inhibition has a whitening effect on the skin, while collagenase inhibition has an anti-sagging effect on the skin. Antioxidant activity and tyrosinase/collagenase enzyme inhibition capacities have mutually supporting effects. The aim of this study was to determine the antioxidant activities, tyrosinase and collagenase inhibitory potentials of ethanol extracts of two medicinal plants from Turkey (*Smyrnium rotundifolium* and *Euphorbia virgata*). In the study, 6 different reference substances and their chemical contents were investigated. Myricetin, quercetin and kaempferol were observed in *S. rotundifolium* extract, and quercetin was observed in *E. virgata* extract. According to the antioxidant capacity results measured by both analyses; *S. rotundifolium* (IC_{50} $DPPH^{\bullet}$: 4.9±0.15 µg/mL, IC_{50} $ABTS^{\bullet+}$: 4.3±0.2 µg/mL) and *E. virgata* (IC_{50} $DPPH^{\bullet}$: 4.6±0.11 µg/mL, IC_{50} $ABTS^{\bullet+}$: 4.1±0.13 µg/mL) extracts were observed to have antioxidant capacities similar to each other. It was determined that *S. rotundifolium* had higher anti-collagenase (27.9±0.13% inhibition) and anti-tyrosinase (11.1±0.14% inhibition) activities compared to *E. virgata* extract. These results showed us that *S. rotundifolium* can be considered as a strong candidate for the management of epidermal hyperpigmentation and skin elasticity and deserves further study.

Keywords: Antioxidant, Collagenase, *Euphorbia virgata, Smyrnium rotundifolium,* Tyrosinase.

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Introduction

Enzymes can show their effects outside their natural environment when suitable conditions are provided, such as laboratory or artificial environments. In this way, enzymes and enzyme inhibitors are used in many areas, especially in medicine, food and cosmetics industry, pesticide production and agriculture industry [1]. Tyrosinase enzyme is a copper-containing enzyme that catalyzes the oxidation of monophenols and is also involved in melanin biosynthesis. It is possible to find melanin pigment in many living things (animals, plants, fungi, bacteria) in nature, especially animals [2]. In humans, melanin plays a very important role in both absorbing free radicals and protecting the organism from harmful UV rays [3]. It is known that hereditary and environmental factors are among the main causes of skin color problems. These dermatological events include melasma, solar melanosis, ephelides, senile lentigos and acne scars [4]. Tyrosinase inhibitors are frequently used in the skin care sector, especially as a lightener for blemished skin. Skin products containing synthetic tyrosinase inhibitors have adverse effects such as redness, itching and, in the future, skin cancer [5]. Tyrosinase inhibitors have strong antioxidant activity. Because plants that receive high amounts of sunlight activate oxygen radicals to protect themselves from the sun's harmful rays. Therefore, plants need to have strong antioxidant effects to protect themselves from damage [6]. Due to health concerns regarding synthetic molecules, there has been a resurgence of interest in natural products in the development of new compounds for the treatment of hyperpigmentation. Discovery of new phytochemical compounds with similar biological effects, enzyme inactivation and antioxidant activity is desirable [3].

Collagenases are proteolytic enzymes involved in extracellular matrix remodeling and degradation, which are responsible for the physiological processes of organ development and tissue regeneration, and play a direct role in skin aging. Collagenases are also involved in the degradation of many matrix and non-matrix proteins, such as growth factors, which can control cell growth and survival [7,8]. Skin aging is an inevitable biological process in the human body and is not only due to increasing age, but also due to both chronological and photo-aging. Examples of photo-aging include exposure to ultraviolet rays, pollution or nicotine. During this aging process, the epidermis becomes thinner and blood vessels become fewer. It is known that this damage occurs as a result of the degradation of elastic fibers, collagen fibers and hyaluronic acid, and significant degenerative changes in the upper dermal connective tissue. It is thought that this

is due to the increased expression of elastase, collagenase and hyaluronidase produced in fibroblasts and various inflammatory cells [9,10].

Oxidative stress occurs as a result of an imbalance between free radicals and antioxidants in the human body. It is known that this imbalance has important consequences on human health. The accumulation of free radicals in the body and their high levels due to uncontrolled production damage cellular components such as lipids, proteins and DNA and can eventually lead to neuronal dysfunction and death. According to the results of experimental studies, it has been observed that oxidative stress accelerates the progression of cancer, cardiovascular system diseases, neurological diseases and many diseases related to aging [7,11–13].

The *Smyrnium* genus, which belongs to the Apiaceae family and has 38 species in the world distribution, is represented by 6 taxa in Turkey, including *S. rotundifolium*. It is a biennial herbaceous plant that grows in stony places, bushes and forest edges [14]. The roots of *Smyrnium* taxa are often used in the treatment of different illnesses as diuretic, depurative and laxative. The fruit has carminative and stomachic effects [15].

The *Euphorbia* genus is the genus with the most species in the Euphorbiaceae family. *Euphorbia* species contain latex. This genus, which has nearly 2000 species, is usually seen in Africa or Madagascar. There are 91 *Euphorbia* species growing in Turkey. It has been known for many years that it has been used externally to heal wounds and skin diseases such as warts and eczema, and internally to cure migraines and intestinal parasites [16,17]. Studies have shown that *Euphorbia* species have cytotoxic, antitumor, antibacterial, anti-inflammatory and anti-HIV activities [18]. Despite the high vitamin C content of *Smyrium* species [14], the lack of evidence for their use in skin diseases among the public and the use of the aboveground parts of *Euphorbia* species in eczema and warts among the public [16,19] led us to examine these plants in terms of dermatological activity.

The purpose of our study was to determine the antioxidant activities and tyrosinase and collagenase enzyme inhibition potentials of ethanol extracts of two medicinal plants from Turkey (*Smyrnium rotundifolium* and *Euphorbia virgata*).

Materials and Methods

Plant Materials

S. rotundifolium and *E. virgata* plants used in the study were collected from the area at coordinates 39°41'40"N, 37°02'25"E, at an altitude of 1400 m from height, within the borders of Sivas İmaret Village between June and July 2023. The taxonomic description of the collected samples was made by Anadolu University Faculty of Pharmacy Faculty Member Professor Yavuz Bülent KÖSE. The collected plant samples were preserved in the Anadolu University Faculty of Pharmacy Herbarium as *S.rotundifolium* (ESSE: 16196), *E.virgata* (ESSE: 16197).

Preparation of Plant Extract

In our study, plant extracts prepared from the aerial parts of *S. rotundifolium* and *E. virgata* were used. The samples were first washed with tap water and then with pure water to prevent possible contamination, then dried on blotting papers, ground in a grinder and 100 grams were taken and 1000 ml of ethanol was added. It was kept at room temperature in a shaker at 150 rpm for 24 hours. At the end of the extraction process, the extract was filtered through filter paper, and then the solvent was removed in a rotary evaporator at 40°C. The obtained extracts was placed in a dark glass bottle and stored at -20 °C to be used in experimental procedures [20].

High Pressure Liquid Chromatography (HPLC)

HPLC analysis was performed using an Agilent 1100 HPLC system equipped with a UV-DAD detector. Extracts of *S. rotundifolium* and *E. virgata* were prepared in ethanol at a concentration of 10 mg/mL and filtered through a 0.22 µm membrane filter. Phenolic reference compounds, including quercetin, myricetin, gallic acid, kaempferol, rosmarinic acid, and apigenin, were dissolved in methanol prior to HPLC analysis. A C18 column (250 x 4.6 mm, 5 µm) was utilized. The mobile phases consisted of Phase A (acetonitrile: distilled water: formic acid (10:89:1, v/v)) and Phase B (acetonitrile: distilled water: formic acid (89:10:1, v/v)), which were applied using a gradient elution. Mobile Phase B was varied from 15% to 100% over a 40-minute analysis period. The flow rate was maintained at 1.0 mL/min, and all samples were injected in triplicate. Detection was carried out at 330 nm, with an injection volume of 20 µL and a column temperature set at 40°C [21].

Antioxidant Capacity

Scavenging of DPPH free radical (2,2-Diphenyl-1-picrylhydrazyl) test

The total antioxidant capacity of the ethanol extracts of *S. rotundifolium* and *E. virgata* were determined using the DPPH free radical (DPPH$^{\bullet}$) scavenging method, as described by Blois and colleagues [22]. In this method, the reaction mixture consisted of 100 µM DPPH$^{\bullet}$ prepared in methanol, along with various concentrations of the test formulations. After an incubation period of 30 minutes at a controlled temperature of 25 ± 2 °C, the absorbance of the mixture was measured at a wavelength of 517 nm using a UV spectrophotometer (UV-1800, Shimadzu, Japan) to assess the scavenging activity of the extract [23]. Ascorbic acid, a well-known antioxidant, was used as a positive control to compare the efficacy of the extract in neutralizing free radicals. The results of the assay were expressed as IC_{50} values (µg/mL), which represent the concentration of the extract required to inhibit 50% of the DPPH$^{\bullet}$ in the reaction mixture, indicating the antioxidant potency of the sample.

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) Antioxidant Activity

The antioxidant capacity of the samples was evaluated using the ABTS radical ($ABTS^{\bullet+}$) cation decolorization assay, following the protocol described by [24]. In this method, a solution of 7 mM $ABTS^{\bullet+}$ was prepared in water

and then mixed with 2.45 mM potassium persulfate to generate the ABTS radical cation. This mixture was allowed to stand in a dark room at 25°C for 16 hours to ensure complete formation of the radical cation before it was used in the assay. Subsequently, ethanol was added to the reaction mixture, and the absorbance was measured at 734 nm at 25°C to determine the extent of decolorization, which indicates the scavenging activity of the antioxidants in the sample. Each measurement was performed in triplicate to ensure accuracy and reproducibility of the results. In this assay, ethanol was used as the negative control, while Trolox served as the positive control to provide a reference for antioxidant activity [23]. The results were expressed as IC_{50} values (µg/mL).

Tyrosinase Inhibition

The *in vitro* anti-tyrosinase activity of the ethanol extracts of *S. rotundifolium* and *E. virgata* (20 µg/mL) was assessed following the method outlined by Hearing and Jimenez [25]. This method first involves evaluating the ability of the compounds to inhibit the diphenolase activity of tyrosinase, with L-DOPA serving as the substrate. Tyrosinase from mushroom origin (E.C. 1.14.18.1) (30 U, 28 nM) was dissolved in a sodium phosphate buffer (pH 6.8, 50 mM), and the test compounds were added to the solution, followed by a pre-incubation period of 10 minutes at room temperature. The enzymatic reaction was then initiated by adding 0.5 mM L-DOPA to the mixture, and the change in absorbance at 475 nm was monitored at 37°C. Kojic acid was used as a positive control to benchmark the inhibitory effect of the extract [26].

Collagenase Inhibition

For the evaluation of anti-collagenase activity, the ethanol extracts of *S. rotundifolium* and *E. virgata* (20 µg/mL) was initially dissolved in ethanol. The enzyme inhibition assay was conducted according to the manufacturer's instructions provided in the "Collagenase Activity Assay Kit (Colorimetric, Abcam 196999)." The inhibition of collagenase by the test substances was measured kinetically using a multi-mode microplate reader (SpectraMax i3) at 345 nm and 37°C. The degree of enzyme inhibition by the extract was calculated by comparing it against the standards included in the kit. The results were expressed as the percentage of inhibition, and the mean inhibition values were calculated for the samples. All assays were conducted in duplicate to ensure reliability and reproducibility of the data [26].

Results and Discussion

High Pressure Liquid Chromatography (HPLC)

Six different reference compounds which includes quercetin, myricetin, gallic acid, kaempferol, rosmarinic acid, and apigenin, were used for phytochemical analysis of *S. rotundifolium* and *E. virgata.* As a result, Table 1 represents the retentions times of the standard compounds based on the related HPLC chromatogram.

Table 1. Retention times of the standard compounds

Reference compounds	t_R
Gallic acid	3.748
Rosmarinic acid	9.576
Myricetin	10.425
Quercetin	14.324
Apigenin	16.998
Kaempferol	18.342

According to the HPLC chromatogram of *S. rotundifolium* (Figure 1), myricetin, quercetin and kaempferol standards were present in the extract.

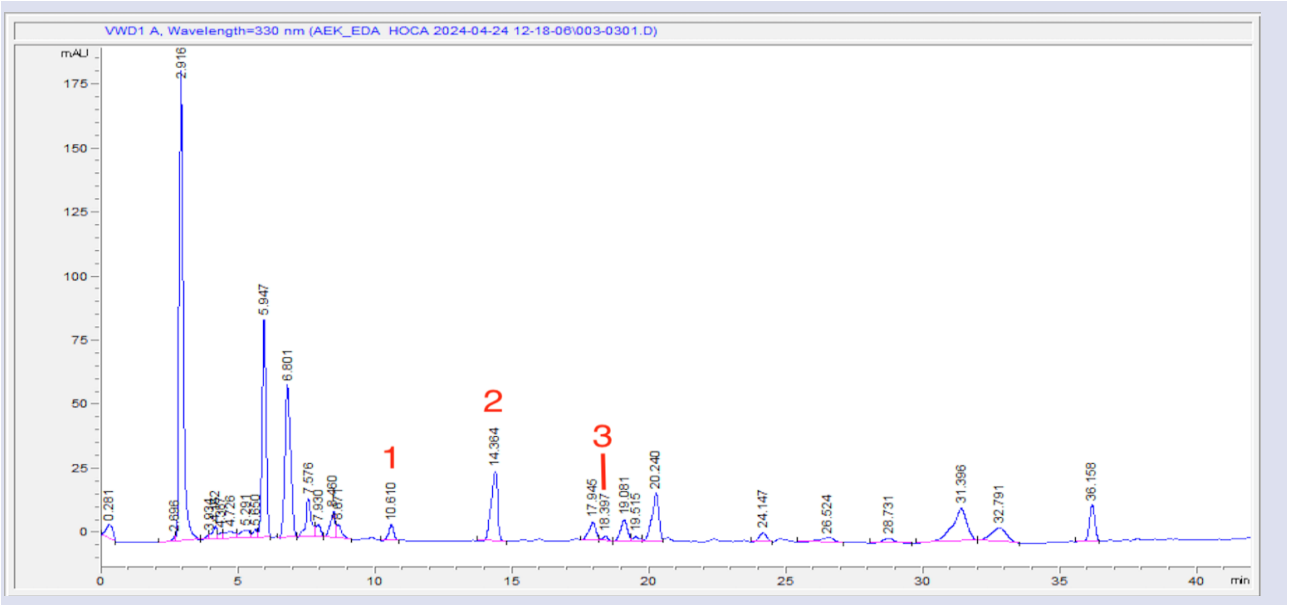

Figure 1. HPLC chromatogram of *S. rotundifolium* ethanol extract (1. myricetin tR: 10.610, 2. quercetin tR: 14.364, 3. kaempferol tR: 18.397)

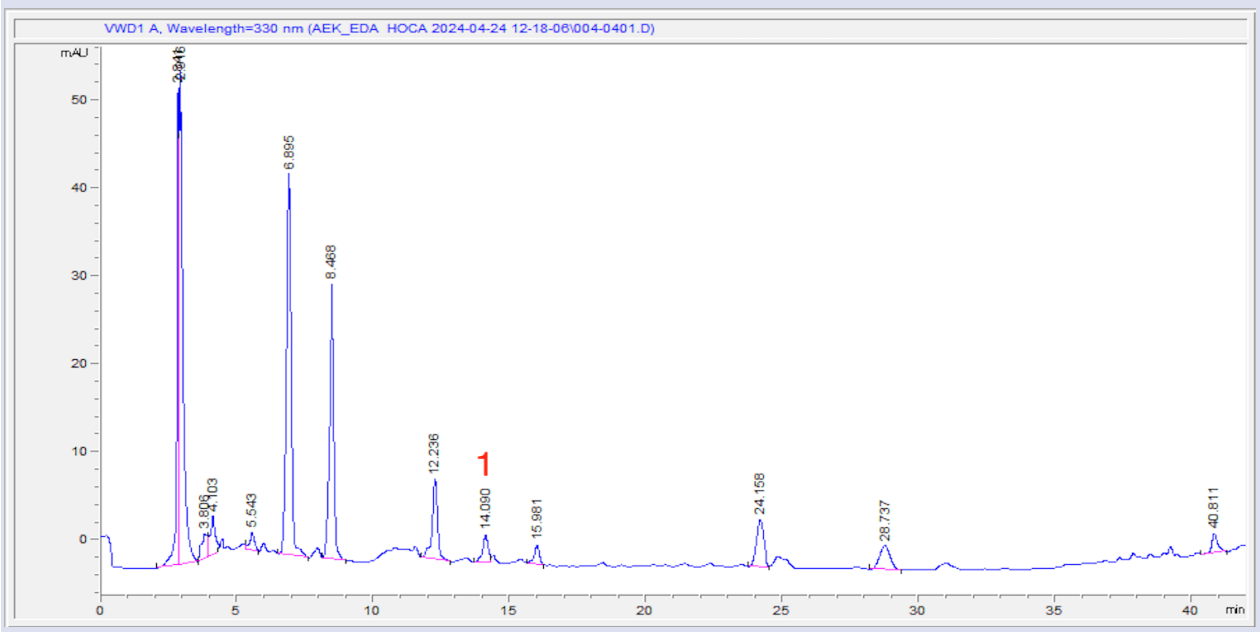

Figure 2. HPLC chromatogram of *E. virgata* ethanol extract (1. quercetin tR: 14.090)

According to the HPLC chromatogram of *E. virgata* (Figure 2), the ethanolic extract was found to contain only quercetin among the standards analyzed.

In 1984 and 1985, Goren et al. examined the phytochemistry of *S. rotundifolium* and isolated sesquiterpene lactones such as α-selinene, germacrone, and furodiene from the fruits. They also isolated oxepine derivatives such as smyrnicordiolide, and isosmyrnicordiolide from the roots of the *S. rotundifolium* [27,28].

Previous studies on *Euphorbia* genus showed that they contain phenolic compounds and flavonoids such as myricetin, rutin, kaempferol, and quercetin [29]. Our HPLC results supported the literature.

Antioxidant Capacity

ABTS$^{\bullet+}$ antioxidant activity tests

The antioxidant capacity of the ethanolic extracts of *S. rotundifolium* and *E. virgata* was investigated using DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ antioxidant assays. Based on the results of these analyses, *S. rotundifolium* and *E. virgata* extracts demonstrated similar antioxidant capacities as measured by both assays. The *S. rotundifolium* extract exhibited antioxidant capacity with the IC_{50} values of 4.9±0.15 µg/mL and 4.3±0.2 µg/mL for the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays, respectively. Similarly, the *E. virgata* extract showed antioxidant capacity with the IC_{50} values of 4.6±0.11 µg/mL and 4.1±0.13 µg/mL for the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays. Detailed results were given in Table 2.

Table 2. DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assay results of the *S. rotundifolium* and *E. virgata* ethanolic extracts

	S. rotundifolium	*E. virgata*	Positive control
	IC_{50} (µg/mL)		
DPPH$^{\bullet}$	4.9±0.15	4.6±0.11	0.002±0.0001 (Ascorbic acid)
ABTS$^{\bullet+}$	4.3±0.2	4.1±0.13	0.01±0.0001 (Trolox)

The antioxidant capacity of both extracts may be related to the phenolic compounds they had according to the HPLC analysis results.

A previous study reported the antioxidant activities of 47 different Mediterranean plant species extracts, which one of them was the leaves of *S. rotundifolium*. According to the results, *S. rotundifolium* extract exhibited a low antioxidant capacity compared to other extracts with the value of 152.4 µg ascorbic acid equivalent per 1 g of extract (total water-soluble antioxidant activity) [30].

Tyrosinase and Collagenase Inhibition

Tyrosinase and collagenase enzyme inhibition capacities of the *S. rotundifolium* and *E. virgata* ethanolic extracts were investigated. According to the results, *S. rotundifolium* extract showed 11.1±0.14% inhibition on tyrosinase enzyme, and 27.9±0.13% inhibition on collagenase enzyme. On the other hand, *E. virgata* extract exhibited 8.9±0.18% inhibition on tyrosinase enzyme, and 8.1±0.1% inhibition on collagenase enzyme. Based on the results, *S. rotundifolium* was found to have higher anti-collagenase and anti-tyrosinase activity compared to *E. virgata* extract. Kojic acid and quercetin were used as positive standards for anti-tyrosinase activity and anti-collagenase activity, respectively (Table 3).

Table 3. Anti-tyrosinase and anti-collagenase results of the *S. rotundifolium* and *E. virgata* ethanolic extracts

	S. rotundifolium	*E. virgata*	Positive control
	% inhibition		
ATA*	11.1±0.14	8.9±0.18	98.9±0.002 (Kojic acid)
ACA**	27.9±0.13	8.1±0.1	91.4±0.007 (Quercetin)

*Anti-tyrosinase activity
** Anti-collagenase activity

Although the medicinal potential of plants in Turkey is important, the knowledge in this area and the studies on these plants are limited. Since antioxidant and enzyme inhibition studies on *S. rotundifolium* and *E. virgata* species are quite limited, studies on different species are also included.

Flavonoids are secondary metabolites that exhibit pharmacological activities in many areas including antioxidant, antimicrobial, spasmolytic, diuretic, capillary protection, cytostatic, etc. The source of most of these activities is quercetin, the most important flavonol known. In addition to quercetin, kaempferol and myricetin are also common flavonoids in plants responsible for the activities. If we look at our HPLC results, we can see that our plants contain myricetin, rutin, kaempferol and quercetin, which are responsible for antioxidant activity [31].

In a study, antioxidant and antimicrobial activities of 6 different *Smyrnium* were investigated. The antioxidant properties of methanol extracts were examined by DPPH$^{\bullet}$ scavenging method. *S. olusatrum* indicated the strongest radical scavenging activity at a concentration of 1.08 mg/mL (96.75 ± 0.47%). The DPPH$^{\bullet}$ scavenging activity of the extract belonging to the *S. rotundifolium* species was observed as 96.15 ± 0.00% at a concentration of 0.99 mg/mL [32]. In a study conducted on several *Smyrnium* species, it was determined that *S. olansatrum* had low inhibitory potential against acetylcholinesterase, butyrylcholinesterase and tyrosinase enzymes [33]. In a different study, it was determined that the methanol extract of *S. cordiifolium* had good tyrosinase inhibition potential (137.54 mg kojic acid equivalent/g extract) [3].

In a recent study conducted with the species *S. connatum*, acetylcholinesterase, butylcholinesterase, tyrosinase inhibition and antioxidant activities of the prepared plant extracts were investigated. It was observed that the aerial parts of *S. connatum* were generally more active in terms of antioxidant activity determination. The aerial parts and roots of *S. connatum* showed high inhibitory activity against both cholinesterase enzymes. All extracts showed moderate inhibitory activity against tyrosinase (54.87% and 44.31%). As a result of the findings, it was determined that the biological activity of the aerial parts of *S. connatum* was generally more active than the roots of *S. connatum* [15]. In a study conducted with leaf extracts of the species *S. rotundifolium*, the total phenol content was found to be 157.3 mg GAE/g extract and exhibited low antioxidant activity [30]. In a different study investigating its chemical content, kaempferol, kaempferol 3-β-D-galactoside, kaempferol 3-methyl ether 7-β-D-glucoside and kaempferol 3-diglucoside were found in its content [34].

Since no research on antioxidant and enzyme activity was found in our literature search for the *E. virgata* species, our study is the first in this sense. In a previous study; quercetin-3-O-glucoside, kaempferol, kaempferol-3-O-glucoside, kaempferol-3-rutinoside and rutin flavonoids were isolated from *E. virgata* [35]. In another study conducted to investigate the wound healing effect of aerial parts of some *Euphorbia* species, hexane, ethyl acetate and methanol solvents were used. The results showed that aerial parts of *E. characias* have wound healing and anti-inflammatory activities in different models [16]. Antimicrobial effects of methanol extracts and latex of some *Euphorbia* species used for medical purposes in Turkey were investigated. The results showed that the extracts of *Euphorbia* species inhibited the growth of the tested microorganisms at different rates. In addition, the MIC (Minimum Inhibitor Concentration) values of the extracts were determined as 31.2-1000 µg [36].

It has been reported that quercetin, kaempferol and ellagic acid isolated from the distillation water formed during the extraction of essential oil from *Rosa damascena* flowers inhibited tyrosinase enzyme 10 times more strongly than the control group kojic acid (56.1 µM) [37]. In the study investigating the tyrosinase enzyme inhibition of quercetin, a flavonoid compound with potential as a skin-lightening agent; it was concluded that quercetin compounds obtained by maceration from *Moringa oleifera* L. leaves could be potential skin-lightening agents [38]. Among the plants in our study, the presence of myricetin, quercetin and kaempferol in *S. rotundifolium* and quercetin in *E. virgata* support our tyrosinase inhibition results.

Conclusion

The cosmetics sector is showing significant growth, especially in skin aging. For this reason, it is known that studies on natural products that prevent aging and pigmentation are popular research areas. Our work; it was targeted to define whether it can be used in dermatological diseases caused by the differentiation of melanin pigment in the skin, in neurological diseases and in the food/cosmetics sector with the anti-tyrosinase activity test; and in the content of topical formulations prepared for anti-wrinkle and skin aging with the anti-collagenase activity test. Since it is known that oxidative stress plays a role in degenerative processes associated with aging, the antioxidant activity of the extracts was also examined in our study.

According to the results, *S. rotundifolium* was found to have higher anti-collagenase and anti-tyrosinase activity compared to *E. virgata* extract. *S. rotundifolium* can be considered as a strong candidate for the development of plant-derived products for the management of hyperpigmentation and skin aging, which deserves further study. To the best of our knowledge, this study can be considered as the first report from Turkey investigating the antioxidant, anti-tyrosinase and anti-collagenase inhibitory effects of *S. rotundifolium* and *E. virgata*.

Conflicts of interest

No conflicts of interest have been declared by the authors.

Acknowledgment

We would like to thank Prof. Dr. Yavuz Bülent KÖSE for identifying the plant.

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