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The relationship between the chemical components and skin-related enzyme inhibition, antioxidant activities and toxicity profile of *Helichrysum armenium* subsp. *armenium*

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ABSTRACT

The aerial parts of *Helichrysum armenium* subsp. *armenium* were subjected to methanol, ethyl acetate and *n*-hexane extraction. The extracts were evaluated for their *in vitro* enzyme inhibitory activity [lipoxygenase (LOX), tyrosinase and elastase], antioxidant activity and toxicity (on fibroblast cells). All the extracts displayed a weak effect on LOX and elastase. The ethyl acetate extract showed the highest inhibition on tyrosinase enzyme with IC_{50} = 460 µg/mL, while kojic acid had IC_{50} = 30 µg/mL. The methanol and ethyl acetate extracts compared to the *n*-hexane extract exhibited stronger DPPH$^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity, as well as iron(II)-chelating potential, and were shown to have rich phenolic and flavonoid contents. The methanol extract was toxic to healthy fibroblast cells at a higher concentration with IC_{50} > 500 µg/mL compared to the other extracts. Phytochemical analysis of the extracts was made quantitatively by LC–MS/MS. The major components identified in the methanol and the ethyl acetate extracts were astragalin, quinic acid, apigenin-7-glycoside, isoquercitrin, chlorogenic acid, naringenin, apigenin and luteolin-7-glucoside. The compounds detected in the *n*-hexane extract were much fewer. Based on these findings, *H. armenium* subsp. *armenium* can be considered to be a natural raw material with the potential to be used in the cosmetic industry.

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Introduction

Helichrysum (Asteraceae) species are represented in the flora of Turkey by 27 taxa, 14 of which are endemic (Davis 1975; Davis et al. 1988; Erik 2000). These species are commonly called "altın otu" or "ölümsüz çiçek" and are utilized as tea (Baytop 1994). Ethnobotanical studies have shown that the species including *Helichrysum arenarium* (L.) Moench, *Helichrysum armenium* DC., *Helichrysum orientale* (L.) Vaill. and *Helichrysum plicatum* DC. are the most commonly utilized species. *Helichrysum* species have been used for centuries as a spice in various foods, as a folk remedy (wound curative), and for cosmetic intention (Viegas et al. 2014). The flowers of *Helichrysum* species have long been used in traditional European medicine as a cholagogic, choleretic, hepatic and detoxifying herbal remedy (Pljevljakušić et al. 2018). Decoctions made from flowers or flower heads are used to treat conditions such as kidney stones, abdominal pain, shortness of breath and asthma. Capitulums are also used to relieve earache through inhalation, while olive oil macerate is used as a cough suppressant and to treat the common cold (Tuzlacı and Erol 1999; Demirci and Özhatay 2012; Gürdal and Kültür 2013; Saraç et al. 2013; Tetik et al. 2013; Akbulut and Karaköse 2016).

A number of *Helichrysum* species have been reported to have antibacterial (Taglialatela-Scafati et al. 2013; Kutluk et al. 2018), antiviral and antifungal (Aslan et al. 2006), antioxidant (Tepe et al. 2005; Aslan et al. 2007a, 2007b), anti-inflammatory (Minaiyan et al. 2014; Hussain et al. 2020; Taşkın et al. 2020), anti-allergic (Carini et al. 2001), anti-diabetic (Aslan et al. 2007a, 2007b), genotoxic and mutagenic (Eroğlu et al. 2009, 2010), anti-urease and anti-tyrosinase (Aydın 2019), anti-cholinesterase, anti-butyrylcholinesterase and anti-neurodegenerative activities (Aydın 2020; Taşkın et al. 2020; Zengin et al. 2020). Carbonic anhydrase and α-glucosidase enzyme inhibition and skin protective effects were among the reported activities (Aydin 2020; Granger et al. 2020; Jovanović et al. 2020).

Regarding the chemical profile of the members of the genus *Helichrysum*, the main compounds were pyrons, phloroglucinols, benzofurans, phthalides, terpenes, phenolic acids, flavonoids, chalcones and essential oils (Eroğlu et al. 2009; Öztürk et al. 2014; Akaberi et al. 2019; Bahşi 2020). It has been claimed that phenolics, found in abundance in

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Helichrysum species, are powerful anti-inflammatory agents and could be used to treat skin conditions (Viegas et al. 2014).

Although its use in ethnobotanical research is comparable to other species, *H. armenium* subsp. *armenium* is used as an infusion for blood thinning and anti-inflammatory purposes (Demirci and Özhatay 2012; Bak and Çifçi 2020). The species has also been found to be effective against pathogenic microorganisms such as bacteria, viruses and fungi (Aslan et al. 2006; Kutluk et al. 2018).

The use of raw materials of natural origin in cosmetic formulations has recently become more and more common in the cosmetics market. Botanical ingredients are widely used due to their numerous anti-aging, anti-inflammatory and UV protective effects (Cavinato et al. 2017). In addition, plants high in polyphenolic compounds are known to have antioxidant and anti-aging properties (Pan et al. 2012). Polyphenols are particularly common in topically applied formulations because, in addition to their cosmetic properties, they also have powerful antioxidant effects and act in a variety of ways (Malinowska 2013). In light of all of these factors, it is of the utmost importance to investigate the possible pharmacological potential of new herbal compounds and the mechanisms by which they exert their effects.

With this study, we therefore aim to determine the anti-aging and skin lightening effects of the extracts of *H. armenium* subsp. *armenium*. For this reason, lipoxygenase (LOX), tyrosinase and elastase inhibitory activities of the aerial extracts of the plant were tested *in vitro*. Toxicity assessments of the extracts were performed on the healthy mouse fibroblast cell line L929. 1,1-Diphenyl-2- picrylhydrazil (DPPH) and 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging potential and iron(II)-chelating tests were used to determine the antioxidant capacity of the extracts. The compositions of the extracts were determined by LC–MS/MS analysis and total phenol and flavonoid content tests.

Material and methods

Plant material

Helichrysum armenium DC. subsp. *armenium* was gathered in June 2019 from the rocky areas at approximately a 950 m altitude around Gümenek village on the Tokat-Niksar road in the Tokat province of Turkey. Identification of the plant material was carried out by Prof. Dr Yavuz Bülent Köse. The plant specimens are kept in the Van Yuzuncu Yil University Herbarium (Herbarium No: 16264).

Extraction procedure

The extracts were obtained from the maceration of the dried aerial parts of the plant with three different polarity solvents, namely methanol (MeOH), ethyl acetate (EtOAc) and *n*-hexane; each extraction was performed twice with an interval of 48 h using a shaker mixer. The obtained extracts were concentrated using an evaporator. The extracts were lyophilized and then stored at −18 °C until use.

Enzyme inhibition experiments

LOX inhibitory activity

The LOX inhibitory test was executed spectrophotometrically, modifying the procedure described by Baylac and Racine (2003). The samples were mixed with a potassium phosphate buffer (100 mM, pH 9.0) and 20 µL of LOX solution and incubated at 25 °C for 10 minutes and then, the reaction started with 50 µL of linoleic acid. The change in absorbance was taken at 234 nm for 10 minutes. A quartz cuvette was used for kinetic measurements and the assay was repeated in triplicate.

Tyrosinase inhibitory activity

Tyrosinase inhibition of the samples was performed according to the procedure of Chang et al. (2007) with certain modifications. The positive standard in the test was kojic acid. The assay was performed on a 96-well microplate. Test solutions were added with 2 mM L-DOPA prepared in phosphate buffer (50 mM, pH 6.8) and kept at 25 °C for two minutes at room temperature in darkness. Next, the reaction started with the addition of tyrosinase enzyme (50 U/mL in phosphate buffer). Finally, incubation of the test mix was carried out at 25 °C for 10 minutes. The kinetic absorbance change in the enzyme was recorded at 475 nm for 10 minutes at intervals of every 30 seconds by means of a microplate reader during the dopachrome formation.

Elastase inhibitory activity

Elastase inhibitory assay was applied by modifying the Melzig et al. (2001) method. Analysis samples, 17 mU/mL human neutrophil elastase (HNE) and Tris-HCl buffer (0.1 M) were pipetted into the same well and then were then incubated at 25 °C for five minutes. MAAPVN (N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide) was added to the mixture then incubated at 37 °C for one hour. Spectrophotometric measurement was taken at 405 nm after the addition of oleanolic acid to halt the enzymatic reaction.

Enzyme inhibition studies were performed with 100 and 500 µg/mL concentrations of the samples. In addition, the IC_{50} value of the samples exhibiting more than 50% inhibition effect was calculated. The obtained data were compared with the standards.

Enzyme inhibition data were obtained from the following equation:

$$\%\text{Inhibition} = \left[\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right) / \text{Abs}_{\text{control}}\right] \times 100$$

Where $\text{Abs}_{\text{control}}$ is the absorbance of control and $\text{Abs}_{\text{sample}}$ is the absorbance of sample.

Cell culture experiment

Twenty-four hours before starting the experimental work, cells in the flask were counted and inoculated into a 96-well microplate with 10×10^3 cells in 100 µL per well. After 24 hours, the medium on the cells that had adhered to the plate was removed. Analysis materials were prepared at different concentrations, i.e. 7.81, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL. One hundred microliters of the samples were added to the

wells by diluting them in the medium. In this state, it was stored for 24 hours at 37 °C in a carbon dioxide incubator. The medium in the wells were emptied just after 24 hours. The 3-(4,5-dim ethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was dissolved in phosphate-buffered saline at 5 mg/mL and transferred to a bottle by sterile filtration. After this, 100 µL of MTT solution diluted 1/10 from the MTT stock was distributed to the wells and kept in a carbon dioxide environment for three hours. At the end of three hours, 100 µL of dimethyl sulfoxide (DMSO) was added to the wells so that the crystals formed with the MTT were dissolved. After waiting for 10 minutes, each well was read with a microplate reader at 540 nm.

The toxicity levels were calculated using the following formula:

$$\%\,\text{Viability} = \left[\left(\text{Abs}_{\text{sample}} \times 100\right) / \text{Abs}_{\text{control}}\right]$$

Where $\text{Abs}_{\text{control}}$ is the absorbance of control and $\text{Abs}_{\text{sample}}$ is the absorbance of sample.

Antioxidant activities

DPPH radical scavenging activity

The samples were tested for DPPH$^{\cdot}$ scavenging activity according to the procedure by Gyamfi et al. (1999). Rosmarinic acid was used as a positive reference. Tris-HCI buffer (50 nM, pH 7.4), DPPH$^{\cdot}$ solution (0.1 mM) and sample materials were blended. After incubating the mixture for 30 minutes at room temperature and without light, spectrophotometric measurements were taken at 517 nm.

ABTS radical scavenging activity

$ABTS^{\cdot+}$ scavenging power of the samples was conducted in accordance with the Re et al. (1999) procedure. The samples were blended with an $ABTS^{\cdot+}$ solution (7 mM) and $K_2S_2O_8$ (2.45 mM) and incubated in the dark for 12–16 hours.

Kinetic absorbance measurement was taken at 734 nm for 30 minutes at room temperature. The percentage inhibition was calculated against the concentration as Trolox equivalents (TEAC). Rosmarinic acid was utilized as a reference substance.

Iron(II)-chelating activity

Iron(II)-chelating activity was assessed as the Na_2EDTA equivalent iron-chelating activity ($mg_{Na2EDTA}/g_{sample}$) by using the method of Carter (1971). Samples dissolved in a suitable solvent were added with $FeCl_2$–$4H_2O$ (2.0 mmol/L) and methanol and then the mixture was incubated for five minutes. The analysis reaction was begun by the addition of ferrozine (5.0 mM). After a 10-minute incubation period, absorbance values were taken at 562 nm.

All antioxidant activity experiments were performed in triplicate. The antioxidant activities of the samples were calculated using the following formula:

$$\%\text{Inhibition} = \left[\left(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}\right) / \text{Abs}_{\text{control}}\right] \times 100$$

Where $\text{Abs}_{\text{control}}$ is the absorbance of control and $\text{Abs}_{\text{sample}}$ is the absorbance of sample.

Chemical composition analysis

Total phenol and flavonoid contents

The total phenol content of the samples was tested using the Folin-Ciocalteau method (Singleton et al. 1999) and expressed as gallic acid equivalent (GAE). To this purpose, distilled water (3.95 mL), Folin-Ciocalteu reagent (250 µL) and 20% Na_2CO_3 (750 µL) were added to the samples. After this, the cuvette containing the mixture was incubated at 25 °C for two hours. At the end of the period, the absorbances of all samples and gallic acid were read in a spectrophotometer at 760 nm.

Calculation of the total flavonoid content of the extracts, expressed as catechin equivalent (CAE), was determined by the aluminum chloride colorimetric test (Zhishen et al. 1999). An aliquot of the samples, 4 mL of distilled water and 0.3 mL of 5% $NaNO_2$ were mixed. After five-minute incubation period, 0.3 mL of 10% $AlCl_3 6H_2O$ and 2 mL of 1 M NaOH were added to the mixture. Finally, the total volume was made up to 10 mL with distilled water. Spectrophotometric measurements of the samples were taken at 510 nm. All the tests were repeated three times.

Chemical analysis of extracts by LC–MS/MS

A previously developed and validated LC–MS/MS method was utilized for the qualitative and quantitative evaluation of phytochemicals in the methanol, ethyl acetate and *n*-hexane of the studied species (Yilmaz 2020). Using the recently developed LC–MS/MS technique, 53 phytochemical molecules, including 14 flavonoid aglycones, 13 flavonoid glycosides, 20 phenolic acids, 3 phenolic aldehydes, 1 benzopyrone, 1 stilbenoid glucoside and 1 biflavonoid, were detected and quantified in the investigated species. For the flavonoid glycosides, flavonoids and non-flavonoid substances, rutin D3, quercetin D3 and ferulic acid D3 were utilized as the deuterated internal standards, respectively. In-depth procedures of the method validation have been previously described in the literature in terms of inter-day and intra-day precision (repeatability), linearity, accuracy (recovery), determination and quantification limits (LOD/LOQ) and relative standard uncertainty (U% at 95% confidence level (k = 2)) (Yilmaz 2020). The utilized instrumentation comprised an ultrahigh performance liquid chromatograph (UHPLC) of the Shimadzu Nexera type in conjunction with a triple quadrupole mass spectrometer. The autosampler (SIL-30AC model), column oven (CTO-10ASvp type), binary pumps (LC-30CE model) and degasser were all included in the reversed-phase UHPLC (DGU- 20A3R model). The reversed-phase Agilent Poroshell 120 EC-C18 model (150 mm × 2.1 mm × 2.7 m) analytical column was used for the chromatographic separation. The temperature in the column was fixed at 40 °C. Eluent A (water + 5 mM ammonium formate + 0.1% formic acid) and Eluent B (methanol + 5 mM ammonium formate + 0.1% formic acid) made up the elution gradient. Furthermore, 20–100% B (0, 25 minutes), 100% B (25, 35 minutes) and 20% B (35, 45 minutes) were the

employed gradient elution profile. Additionally, 0.5 mL/min and 5 µL, respectively, were chosen as the solvent flow rate and injection volume.

A Shimadzu brand LCMS-8040 tandem mass spectrometer supplied with an electrospray ionization (ESI) source operable in both negative and positive ionization modes was utilized for the mass spectrometric determination. LabSolutions software (Shimadzu) was used to harvest and process the LC–ESI-MS/MS data. The phytochemicals were quantified using the multiple reaction monitoring (MRM) technique. Based on the screening of specific precursor phytochemical-to-fragment ion transitions, the MRM method was improved to define and quantify phytochemical substances alone. The MS operating settings were as follows: 15 L/min of drying gas (nitrogen) flow, 3 L/min of nebulizing gas (nitrogen) flow, 250 °C for the DL, 400 °C for the heat block and 350 °C for the interface.

Statistical tests

ANOVA was used for the statistical analysis. The Levene test was used to investigate the homogeneity of the variance. Multiple comparisons at the $p < 0.05$ level were analyzed using the Dunnett test and the Tukey test.

Results and discussion

Enzyme inhibition activities

All samples showed inhibition activity on the tyrosinase enzyme. When the samples were compared, the EtOAc extract exhibited remarkable inhibitory activity with an IC_{50} = 460 µg/mL. The more polar MeOH extract gave inhibition with an IC_{50} = 482 µg/mL. The IC_{50} value of kojic acid, which is a standard inhibitor, was found to be 30 µg/mL. The *n*-hexane extract (500 µg/mL) showed a weaker effect compared to the others, with a 26% inhibition of tyrosinase. In the elastase enzyme assay, the concentration of the studied extracts was 100 µg/mL. All the extracts had weaker inhibition on the elastase enzyme and displayed inhibition in the range of 13–24%. All the samples were determined to have no inhibitory action on LOX. The test results are summarized in Table 1.

Cell line and culture analysis

The toxic effects of the extracts in healthy L929 mouse fibroblast cell lines were compared using the IC_{50} (µg/mL) values. The findings of the study are given in Table 2 and Figure 1. The effect of the MeOH extract on the cell viability at 125 and 500 µg/mL concentration was 94.47% and 67.87%, respectively. It was observed that the MeOH extract became toxic at a higher concentration (>500 µg/mL) compared with the other samples. The cytotoxic effect concentration was calculated as IC_{50} = 295 µg/mL for the EtOAc extract and IC_{50} = 117 µg/mL for the *n*-hexane extract. Accordingly, the MeOH extract was found to be less toxic than the other extracts.

The use of the essential oil of *Helichrysum italicum* (Roth) G.Don is common in the cosmetics industry (Ascrizzi et al. 2017; Combes et al. 2017; Fraternale et al. 2019). However, there are studies on the extracts of other *Helichrysum* species in cosmetic drug raw material research. Sagbo and Otang-Mbeng (2021) reported that the anti-aging effect of the ethanolic extract of the leaves of *Helichrysum petiolare* Hilliard & B.L.Burtt showed weak inhibition on collagenase, elastase and tyrosinase enzymes and strong inhibition on glycation protein, but did not show cytotoxic effects against human dermal fibroblast (MRHF) cells. In another study, 10 flavonoids were detected from the methanol extract of the aerial parts of *Helichrysum teretifolium* (L.) D.Don, which were found to be effective in the inhibition of lipid peroxide, hydroxy and peroxyl radical absorbance capacity, as well as *in vitro* anti-tyrosinase and anti-elastase activities (Olugbenga et al. 2015). Süntar et al. (2013) investigated the wound healing effect of *Helichrysum graveolens* (M.Bieb.) Sweet *in vivo; in vitro* elastase, hyaluronidase and collagenase inhibitory effects were also reported. The authors reported that apigenin detected in the plant exhibited notable anti-inflammatory, antioxidant, anti-hyaluronidase and anti-collagenase properties. It was discovered that the molecules found in *H. italicum* and *Helichrysum kraussii* Sch.Bip. inhibited LOX, cyclooxygenase-1 (COX-1) and COX-2 enzymes and had anti-inflammatory properties (Sala, Recio, Schinella, Máñez, Giner, Cerdá-Nicolás, et al. 2003; Sala, Recio, Schinella, Máñez, Giner, Ríos 2003; Koeberle et al. 2008; Bauer et al. 2011; Legoale et al. 2013).

Table 2. Toxicity effects of the extracts.

Sample	IC_{50} (µg/mL)
MeOH	>500
EtOAc	295.83 ± 7.18
n-Hexane	117.80 ± 5.21

Values are given as mean ± standard deviation with a ±95% confidence interval (n = 3).

Table 1. LOX, tyrosinase and elastase inhibitory effects of the extracts.

	LOX	Tyrosinase				Elastase
Sample	% Inhibition (100 µg/mL)	% Inhibition (500 µg/mL)	% Inhibition (100 µg/mL)	% Inhibition (500 µg/mL)	IC_{50} (µg/mL)	% Inhibition (100 µg/mL)
MeOH	–	–	24.79 ± 0.64^{b}	51.19 ± 0.40^{c}	482.06 ± 0.80	24.00 ± 0.31^{a}
EtOAc	–	–	22.18 ± 0.76^{b}	64.05 ± 0.53^{b}	460.00 ± 1.63	13.55 ± 0.10^{c}
n-Hexane	–	–	14.96 ± 0.49^{c}	26.15 ± 0.12^{d}		16.25 ± 0.30^{b}
NDGA (20 µg/mL)	99.50 ± 0.19					
Kojic acid			55 ± 0.57^{a}	95.98 ± 0.20^{a}	30.00 ± 0.05	
Oleanolic acid (1 mM)						54.40 ± 1.07

Values = mean ± standard error (n = 3); –: no inhibition; Mean with different letters (a–d) within the same group is significantly different at $p < 0.05$.

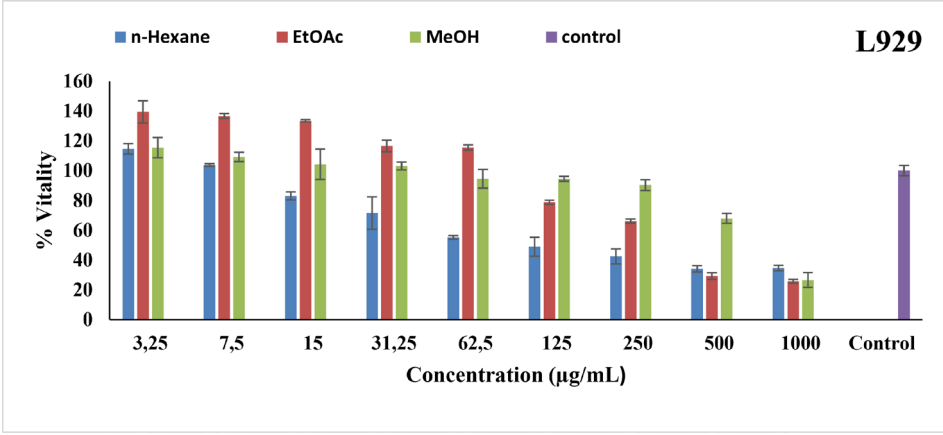


Figure 1. Evaluation of the toxicity profile of the extracts in the L929 mouse fibroblast cell line. Values are given as a mean ± standard deviation with a ±95% confidence interval (n = 3).

Antioxidant activity

DPPH$^{\cdot}$ scavenging activity

EtOAc and MeOH extracts had strong DPPH$^{\cdot}$ scavenging power, with IC_{50} (µg/mL) values of the extracts at 21.72 and 30.57 µg/mL, respectively. This IC_{50} data is approximately three to five times the IC_{50} (6.21 µg/mL) of rosmarinic acid used as a positive reference compound (Table 3).

ABTS$^{\cdot+}$ scavenging activity

All the extracts were studied at a concentration of 4–0.5 mg/mL and were active in scavenging the ABTS$^{\cdot+}$ radical. The ABTS$^{\cdot+}$ scavenging potential of the extracts was MeOH > EtOAc > n-hexane. The MeOH extract (TEAC = 2.46 ± 0.00 mmol/L Trolox) showed almost the same potent effect as rosmarinic acid (TEAC = 2.51 ± 0.01 mmol/L Trolox) at 1 mg/mL (Table 4).

Iron(II)-chelating activity

When Table 5 results were examined, the MeOH and EtOAc extracts had more iron-chelating activity, but not as much as Na_2EDTA.

Chemical content analysis

Phenolic and flavonoid contents of the extracts

As shown in Table 6, the MeOH and EtOAc extracts had the highest total phenolic content (294.80 ± 6.56 and 278.45 ± 5.42 $mg_{GAE}/g_{extract,}$ respectively). It was also determined that the MeOH extract (118.12 ± 2.70 $mg_{CAE}/g_{extract}$) had a higher flavonoid content than the other extracts.

LC–MS/MS analysis of the extracts

The chemical composition of the extracts was evaluated qualitatively and quantitatively for 53 compounds using LC–MS/MS. The results are shown in Table 7. Among the compounds detected in the MeOH extract, those with the highest concentration and their mg/g extract values were as follows; astragalin (33.65 ± 0.38), quinic acid (27.99 ± 1.04), apigenin-7-glycoside (20.61 ± 0.17), isoquercitrin (15.15 ± 0.33), chlorogenic acid (15.09 ± 0.32) naringenin (12.20 ± 0.48), apigenin (4.45 ± 0.08) and luteolin-7-glucoside (4.53 ± 0.17). The major compounds of the EtOAc extract were as follows: apigenin-7-glycoside (13.79 ± 0.11), astragalin (12.95 ± 0.15), naringenin (7.13 ± 0.28), apigenin (5.23 ± 0.09) and acacetin (4.80 ± 0.17). Fewer compounds were detected in the n-hexane

Table 3. DPPH$^{\cdot}$ scavenging activity of the extracts.

Extracts	DPPH IC_{50} (µg/mL)
MeOH	30.57 ± 1.23^{b}
EtOAc	21.72 ± 3.14^{b}
n-Hexane	350.00 ± 7.18^{c}
Rosmarinic acid	6.21 ± 0.01^{a}

Data are calculated as means ± standard deviation. Different letters show significantly different at $p < 0.05$ in the same column (n = 3).

Table 4. ABTS$^{\cdot+}$ scavenging activity of the extracts.

Extracts	4 mg/mL	2 mg/mL	1 mg/mL	0.5 mg/mL
	TEAC mmol/L Trolox			
MeOH	2.48 ± 0.01^{a}	2.46 ± 0.013^{a}	2.46 ± 0.05^{a}	1.72 ± 0.07^{c}
EtOAc	$2.30 \pm 0.04^{a,b}$	2.04 ± 0.10^{b}	1.87 ± 0.19^{c}	1.38 ± 0.17^{d}
n-Hexane	$2.38 \pm 0.23^{a,b}$	$1.91 \pm 0.32^{b,c}$	1.45 ± 0.26^{d}	1.14 ± 0.16^{e}
Rosmarinic acid			2.51 ± 0.01^{a}	2.49 ± 0.03^{a}

Data are given as mean ± standard deviation (n=3). The bars with the same lower case letter (a–e) are not significantly ($p > 0.05$) different, n = 3.

Table 5. The iron(II)-chelating activity of the extracts.

	Chelating activity (%)			
Extracts	4 mg/mL	2 mg/mL	1 mg/mL	0.5 mg/mL
MeOH	43.52 ± 1.17^{b}	43.05 ± 1.31^{b}	41.56 ± 1.26^{b}	$37.80 \pm 1.80^{b,c}$
EtOAc	44.14 ± 1.34^{b}	43.06 ± 1.39^{b}	41.98 ± 0.95^{b}	41.03 ± 0.88^{b}
n-Hexane	21.18 ± 1.42^{d}	20.53 ± 2.25^{d}	17.48 ± 1.69^{e}	14.56 ± 2.63^{e}
Na_2EDTA			97.48 ± 0.24^{a}	95.72 ± 1.14^{a}

Data are presented as means ± standard deviation. Mean with different letters (a–e) within the same sample is significantly different at $p < 0.05$ (n = 3).

Table 6. The total phenol and total flavonoid contents of the extracts.

Extracts	Total phenol ($mg_{GAE}/g_{extract}$)	Total flavonoid ($mg_{CAE}/g_{extract}$)
MeOH	294.80 ± 6.56^{a}	118.12 ± 2.70^{a}
EtOAc	278.45 ± 5.42^{b}	68.87 ± 0.66^{b}
n-Hexane	157.50 ± 3.71^{c}	35.86 ± 2.94^{c}

Values = mean ± standard deviation. Mean with different letters (a–c) within the same sample is significantly different at $p < 0.05$ (n = 3).

Table 7. Phytochemical content of the extracts by LC–MS/MS.

No.	Analytes	RT[a]	MI (*m/z*)b	FI (*m/z*)c	*U*d	Quantification (mg analyte/g extract) MeOH	EtOAc	*n*-Hexane	No.	Analytes	RT[a]	MI (*m/z*)b	FI (*m/z*)c	*U*d	Quantification (mg analyte/g extract) MeOH	EtOAc	*n*-Hexane
1	Quinic acid	3.0	190.8	93.0	0.0372	27.99 ± 1.04	0.18 ± 0.00	*	29	Salicylic acid	21.8	137.2	65.0	0.0158	0.08 ± 0.00	0.02 ± 0.00	*
2	Fumaric acid	3.9	115.2	40.9	0.0091	*	*	*	30	Luteolin 7-glucoside	23.7	447.0	284.0	0.0366	4.53 ± 0.17	1.06 ± 0.04	*
3	Aconitic acid	4.0	172.8	129.0	0.0247	*	*	*	31	Miquelianin	24.1	477.0	150.9	0.0220	*	*	*
4	Gallic acid	4.4	168.8	79.0	0.0112	0.03 ± 0.00	*	*	32	Rutin-D3-ISe	25.5	612.2	304.1	NA	–	–	–
5	Epigallocatechin	6.7	304.8	219.0	0.0184	*	*	*	33	Rutin	25.6	608.9	301.0	0.0247	0.04 ± 0.00	*	*
6	Protocatechuic acid	6.8	152.8	108.0	0.0350	0.513 ± 0.02	0.21 ± 0.00	*	34	isoquercitrin	25.6	463.0	271.0	0.0220	15.15 ± 0.33	2.51 ± 0.06	*
7	Catechin	7.4	288.8	203.1	0.0221	*	*	*	35	Hesperidin	25.8	611.2	449.0	0.0335	0.01 ± 0.00	*	*
8	Gentisic acid	8.3	152.8	109.0	0.0167	0.132 ± 0.00	*	*	36	*o*-Coumaric acid	26.1	162.8	93.0	0.0147	*	*	*
9	Chlorogenic acid	8.4	353.0	85.0	0.0213	15.09 ± 0.32	0.84 ± 0.02	0.02 ± 0.00	37	Genistin	26.3	431.0	239.0	0.0083	*	*	*
10	Protocatechuic aldehyde	8.5	137.2	92.0	0.0396	0.05 ± 0.00	0.08 ± 0.00	*	38	Rosmarinic acid	26.6	359.0	197.0	0.0130	0.08 ± 0.00	*	*
11	Tannic acid	9.2	182.8	78.0	0.0190	0.01 ± 0.00	*	*	39	Ellagic acid	27.6	301.0	284.0	0.0364	*	*	*
12	Epigallocatechin gallate	9.4	457.0	305.1	0.0147	*	*	*	40	Apigenin 7-glucoside	28.2	431.0	269.0	0.0083	20.61 ± 0.17	13.79 ± 0.11	*
13	1,5-dicaffeoylquinic acid	9.8	515.0	191.0	0.0306	0.25 ± 0.00	*	*	41	Quercitrin	29.8	447.0	301.0	0.0268	0.02 ± 0.00	*	*
14	4-OH Benzoic acid	10.5	137.2	65.0	0.0237	0.35 ± 0.00	0.23 ± 0.00	*	42	Astragalin	30.4	447.0	255.0	0.0114	33.65 ± 0.38	12.95 ± 0.15	*
15	Epicatechin	11.6	289.0	203.0	0.0221	*	*	*	43	Nicotiflorin	30.6	592.9	255.0/284.0	0.0108	*	*	*
16	Vanillic acid	11.8	166.8	108.0	0.0145	*	*	*	44	Fisetin	30.6	285.0	163.0	0.0231	*	*	*
17	Caffeic acid	12.1	179.0	134.0	0.0152	0.57 ± 0.00	0.26 ± 0.00	*	45	Daidzein	34.0	253.0	223.0	0.0370	*	*	*
18	Syringic acid	12.6	196.8	166.9	0.0129	*	*	*	46	Quercetin-D3-ISe	35.6	304.0	275.9	–	–	–	–
19	Vanillin	13.9	153.1	125.0	0.0122	*	0.03 ± 0.00	*	47	Quercetin	35.7	301.0	272.9	0.0175	1.13 ± 0.02	1.08 ± 0.02	*
20	Syringic aldehyde	14.6	181.0	151.1	0.0215	*	*	*	48	Naringenin	35.9	270.9	119.0	0.0392	12.20 ± 0.48	7.13 ± 0.28	0.01 ± 0.00
21	Daidzin	15.2	417.1	199.0	0.0202	*	*	*	49	Hesperetin	36.7	301.0	136.0/286.0	0.0321	0.53 ± 0.02	0.29 ± 0.00	*
22	Epicatechin gallate	15.5	441.0	289.0	0.0229	*	*	*	50	Luteolin	36.7	284.8	151.0/175.0	0.0313	2.85 ± 0.09	2.17 ± 0.07	0.01 ± 0.00
23	Piceid	17.2	391.0	135/106.9	0.0199	*	*	*	51	Genistein	36.9	269.0	135.0	0.0337	0.03 ± 0.00	0.04 ± 0.00	*
24	*p*-Coumaric acid	17.8	163.0	93.0	0.0194	0.63 ± 0.01	0.25 ± 0.00	*	52	Kaempferol	37.9	285.0	239.0	0.0212	0.50 ± 0.01	0.48 ± 0.01	0.05 ± 0.00
25	Ferulic acid-D3-IS[e]	18.8	196.2	152.1	0.0170	–	–	–	53	Apigenin	38.2	268.8	151.0/149.0	0.0178	4.45 ± 0.08	5.23 ± 0.09	0.01 ± 0.00
26	Ferulic acid	18.8	192.8	149.0	0.0181	*	*	*	54	Amentoflavone	39.7	537.0	417.0	0.0340	*	*	*
27	Sinapic acid	18.9	222.8	193.0	0.0317	*	*	*	55	Chrysin	40.5	252.8	145.0/119.0	0.0323	0.01 ± 0.00	0.01 ± 0.00	tr
28	Coumarin	20.9	146.9	103.1	0.0383	*	*	*	56	Acacetin	40.7	283.0	239.0	0.0363	0.54 ± 0.02	4.80 ± 0.17	0.28 ± 0.01

*: not detected, –: not applicable. tr: <0.01.
[a]RT: retention time.
[b]MI (*m/z*): molecular ions of the standard analytes (*m/z* ratio).
[c]FI (*m/z*): fragment ions.
[d]*U* (%): percent relative uncertainty at 95% confidence level ($k = 2$).
[e]IS: Internal standard, these numbers indicate which IS stands for which phenolic compound.

extract of *H. armenium* subsp. *armenium* and the amount of these compounds was <1 mg/g of extract. The total ion chromatograms (TICs) of 53 compounds mixture and extracts of *H. armenium* subsp. *armenium* are shown in Figures 2 and 3.

Helichrysum armenium has two subspecies, *H. armenium* subsp. *armenium* and *H. armenium* subsp. *araxinum* (Kirp.) Takht. Cubukcu and Yuksel (1982) collected these two subspecies from Kars and Bingöl-Tunceli regions and determined the phytochemical contents of the capitulum and leafy stems qualitatively and quantitatively. Of all the detected compounds, galangin 3-methyl ether was found only in the capitulum and leafy stems of *H. armenium* subsp. *araxinum*. Except for this compound, the presence of apigenin, luteolin, naringenin, apigenin 4'-glucoside, apigenin 7-glucoside, astragalin, quercetin 3-glucoside, helichrysin A, helichrysin B and isosalipurposide in the capitulum; kaempferol, quercetin, astragalin and quercetin 3-glucoside in the leafy stems were determined in both subspecies. In the same study, *H. armenium* subsp. *armenium* capitulums were determined to have naringenin derivatives dominantly; helichrysin A ((–)-naringenin-5-glucoside) and helichrysin B ((±)-naringenin-5-glucoside) (Cubukcu and Yuksel 1982). The LC–MS/MS findings agreed with those reported by Cubukcu and Yuksel (1982) in that apigenin, luteolin, naringenin, astragalin, kaempferol, apigenin 7-glucoside and quercetin were determined from the above-ground parts of *H. armenium* subsp. *armenium*. These components were previously reported to possess antioxidant, anti-inflammatory and skin protection effects (Wang et al. 2020; Tian et al. 2021). In other comprehensive research conducted by Albayrak et al. (2010), phenolic substances of methanol extracts of sixteen *Helichrysum* species (*H. arenarium* (L.) Moench subsp. *aucheri* (Boiss.) P.H.Davis & Kupicha, *H. armenium* subsp. *armenium*, *H. artvinense* Davis & Kupicha, *H. chionophilum* Boiss. & Bal., *H. compactum* Boiss., *H. goulandriorum* E.Georgiadou, *H. graveolens*, *H. heywoodianum* P.H.Davis, *H. kitianum* Yıldız, *H. noeanum* Boiss., *H. orientale*, *H. pallasii* (Spreng.) Ledeb., *H. peshmenianum* S. Erik, *H. plicatum* DC. subsp. *plicatum*, *H. plicatum* DC. subsp. *polyphyllum* (Ledeb.) Davis & Kupicha, *H. stoechas* subsp. *barrelieri* (Ten.) Nyman) were determined. According to the results obtained, chlorogenic acid, caffeic acid and apigenin-7-glucoside were identified in all species. However, the remaining phenolic compounds such as ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, syringic acid, apigenin, epicatechin, eriodictyol, hesperidin, luteolin, naringenin, quercetin and resveratrol showed qualitative and quantitative differences in these species. Of these compounds, chlorogenic acid, apigenin and apigenin-7-glucoside were found to be the main component in all the species extracts. Chlorogenic acid, caffeic acid, apigenin, apigenin-7-glycoside, epicatechin, hesperidin, luteolin and naringenin were detected in *H. armenium* subsp. *armenium* (Albayrak et al. 2010). This research was the first record of 19 components, including quinic acid, isoquercitrin, quercitrin, *p*-coumaric acid, hesperetin, genistein, chrysin, acacetin, gallic acid, protocatechuic acid, gentisic acid, protocatechuic aldehyde, tannic acid, 1,5-dicaffeoylquinic acid, 4-OH benzoic acid, salicylic acid, luteolin-7-glucoside, rutin and rosmarinic acid in the aerial parts of *H. armenium* subsp. *armenium* that brings originality to the literature. In the same study, it was reported that the total phenolic content of the MeOH extract of *H. armenium* subsp. *armenium* was found to be $89.02 \pm 0.8\,mg_{GAE}/g_{extract}$ and its DPPH radical scavenging activity was found to be IC_{50} = 16.61 µg/mL (Albayrak et al. 2010). Based on the literature reports, this study is the first to present data on antioxidant potential using $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ scavenging activities and iron(II)-chelating activity and total phenolic and flavonoid contents of different extracts of *H. armenium* subsp. *armenium*.

Relationship between biological activity and chemical content

When evaluated in terms of the contents and biological activities of the extracts, the EtOAc and MeOH extracts showed more effective inhibition on the tyrosinase enzyme and were found to be more potent in radical scavenging and metal chelating than the *n*-hexane extract. In addition to these positive effects, the toxicity of MeOH extract on healthy fibroblast cells is in a wider confidence interval. In support of these results, the high phenolic and flavonoid contents of these extracts (EtOAc and MeOH) were also confirmed by LC–MS/MS. Recently, research on the biological effects of phenolic and flavonoid compounds has been the focus in

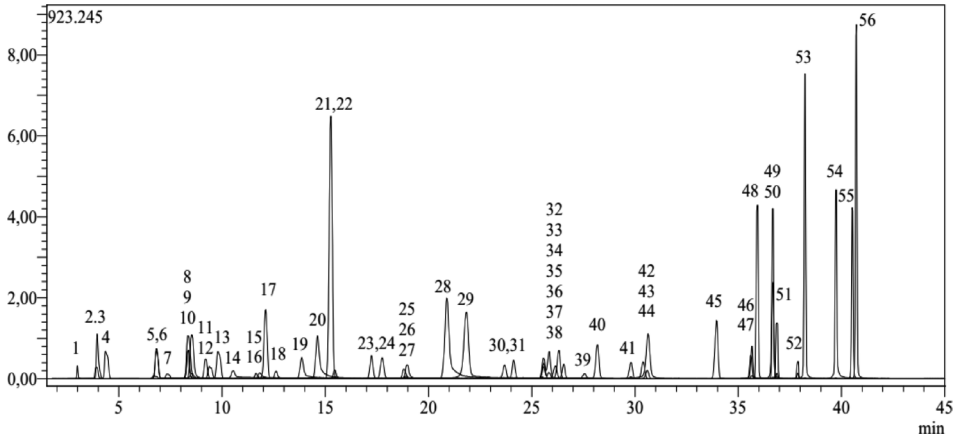


Figure 2. The TIC of standard phenolic and non-phenolic compounds. Numbers 1–56 represent the number of standard phytochemicals given in Table 7.

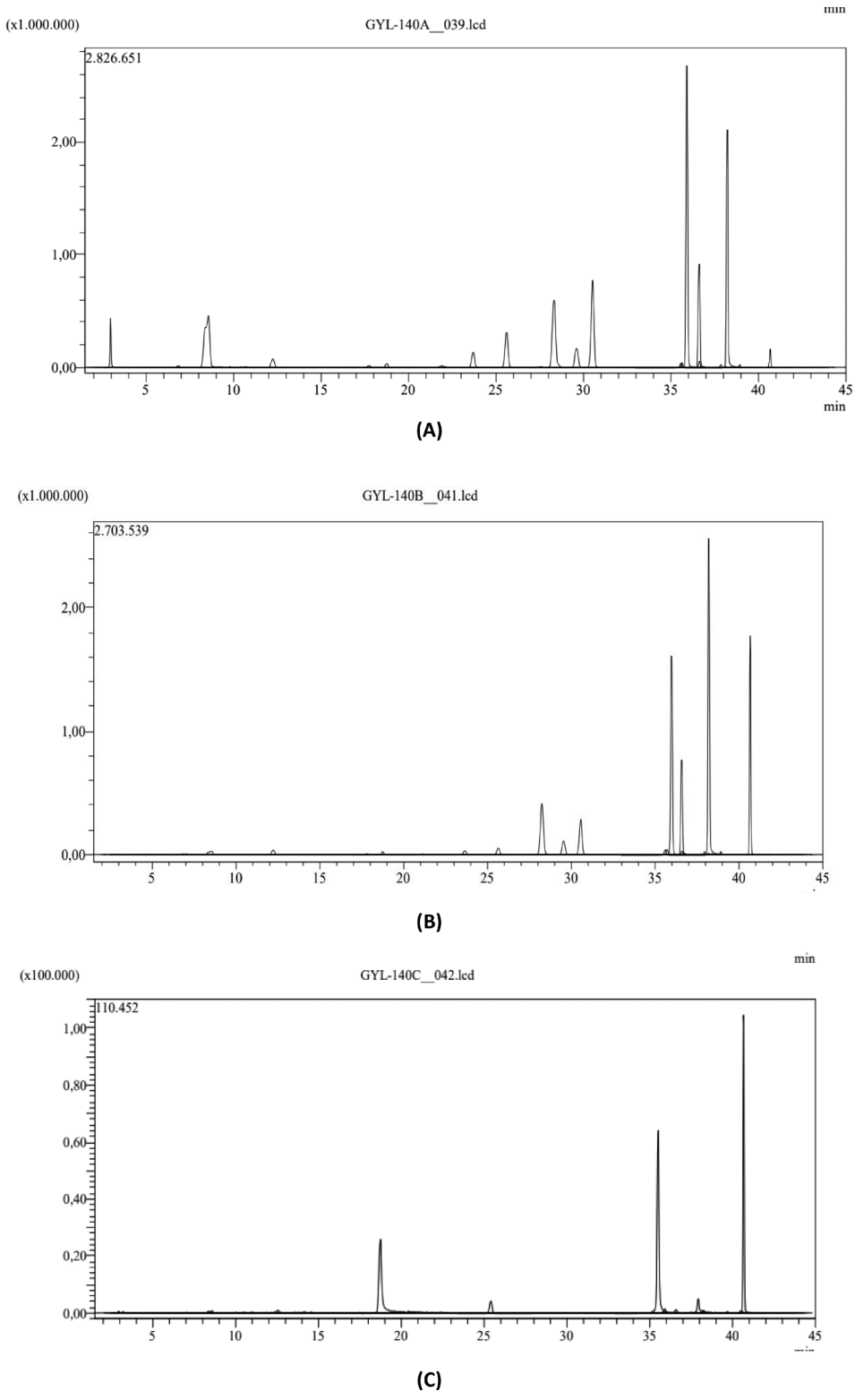


Figure 3. The TIC of *H. armenium* subsp. *armenium* extracts. (A) MeOH extract; (B) EtOAc extract; and (C) *n*-Hexane extract.

phytochemistry. In our study, it was observed that extracts rich in these compounds had a more active effect. Interestingly, although the amount of predominant compounds in the MeOH extract was higher than in the EtOAc extract, the anti-tyrosinase activity of the MeOH extract was slightly weaker than that of the EtOAc extract. Furthermore, although these main substances were less abundant in the EtOAc than the MeOH extract, they caused more significant toxicity (IC_{50} = 295.83 µg/mL) in fibroblast cells. Astragalin (kaempferol-3-glucoside) flavonoid, one of these main compounds, has been reported to have multiple pharmacological activities such as antioxidant, anticancer, anti-inflammatory, neuroprotective and cardioprotective. Considering the cosmetic use of astragalin, it has been determined that it inhibits melanin synthesis

by inhibiting collagenase. Additionally, it reduces skin darkening and has an anti-wrinkle effect. Moreover, its antioxidant effect indirectly contributes to this feature. The combination of astragalin with quercetin is known to have a protective effect against UV radiation. It has also been reported that astragalin may be a major flavonoid that can be used as a photoprotective agent for the skin to reduce UV damage (Riaz et al. 2018). There are studies in which quinic acid derivatives are effective in anti-melanogenesis (Choi et al. 2021). *Morus nigra* leaf extract, standardized on isoquercitrin, chlorogenic and rutin, has been reported to have a high tyrosinase inhibiting effect and to show no cytotoxic effects on murine melanoma, human keratinocytes and mouse fibroblasts (de Freitas et al. 2016). The positive effects on the skin and the safe effects of these compounds on the mentioned cells support the study. Contrary to other compounds, naringenin and apigenin-7-glycoside were found to increase tyrosinase activity and can be used for the tanning effect in cosmetic products. In the same study, it was reported that naringenin demonstrated a meaningful proliferative effect against mouse melanoma B16F10 cells (Bouzaiene et al. 2016). It can be said that naringenin and apigenin-7-glycoside negatively affected the desired result in the study and suppressed the effects of other compounds that may affect tyrosinase and elastase enzymes. When the effects of other dominant compounds in the effective extracts (MeOH and EtOAc) on the skin were examined, it was reported that apigenin significantly inhibited tyrosinase (Ha et al. 2002) and that acacetin strongly suppressed melanin synthesis (Kim et al. 2017).

To date, the effects of polyphenols containing large groups, such as phenolic acids, flavonoids, lignans, stilbenes, anthocyanins, phenolic alcohols and terpenes on the skin have been associated with their antioxidant, anti-elastase, anti-collagenase, anti-hyaluronidase, oxidative DNA damage inhibition, UV photoprotection, anti-inflammatory effect on MMP-1, -2 and -3 enzymes, anticancer and antimicrobial properties (Zillich et al. 2015; Bharadvaja et al. 2023). In particular, these multifunction properties contribute to the preference of polyphenols in many cosmetic formulas. Previous publications support the findings in explaining the effect of *H. armenium* subsp. *armenium*, which is rich in polyphenolic substances, combating various skin-related conditions. In the light of the results of this study, it is clear that the investigation of *H. armenium* subsp. *armenium* may create a new herbal source for bioactive substances effective for skin-related pathologies.

Conclusion

Based on the results of the study, *H. armenium* subsp. *armenium* can be considered as a potential replacement for other *Helichrysum* species in the cosmetic industry due to its high polyphenolic substance content. Compounds derived from *H. armenium* subsp. *armenium* extracts not only have a healing effect on the skin, but also have a less toxic and strong antioxidant effect, which can extend the compound's shelf life and eliminate the need for additional antioxidants in cosmetic formulations. This suggests that *H. armenium* subsp. *armenium* may be used as a natural raw material in the creation of safe and effective anti-aging and skin-whitening cosmetic formulations. These findings show that *H. armenium* subsp. *armenium* is worthy of investigation and will lead to further studies related to the medical and cosmetic industry.

Author contributions

Conception/design of study—G.Y. and G.Ş.K.; data acquisition—G.Y., G.Ş.K., S.İ., M.A.Y. and Y.B.K.; data analysis/interpretation—G.Y. and G.Ş.K.; drafting manuscript—G.Y. and G.Ş.K.; review and editing—G.Y., G.Ş.K. M.A.Y. and G.Z.; final approval and accountability—G.Y., G.Ş.K., S.İ., M.A.Y. G.Z. and Y.B.K.

Disclosure statement

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