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In Vitro Enzyme Inhibitory Activity of Ten *Ferulago* W. Koch Species Growing in Turkey

Esra Küpeli Akkol[1*]
https://orcid.org/0000-0002-5829-7869

Hayri Duman[2]
https://orcid.org/0000-0003-2795-9791

Tuğba Günbatan[1]
https://orcid.org/0000-0002-1138-3145

Ceyda Sibel Kılıç[3]
https://orcid.org/0000-0003-2905-7628

İlhan Gürbüz[1]
https://orcid.org/0000-0002-3670-0899

Mert İlhan[4]
https://orcid.org/0000-0001-5042-3915

[1]Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Turkey; [2]Gazi University, Faculty of Science, Department of Biology, Ankara, Turkey; [3]Ankara University, Faculty of Pharmacy, Department of Pharmaceutical Botany, Ankara, Turkey; [4]Van Yüzüncü Yıl University, Faculty of Pharmacy, Department of Pharmacognosy, Van, Turkey.

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[*]Correspondence: esrak@gazi.edu.tr; Tel.: +90 312 2023185 (E.K.A.)

HIGHLIGHTS

- Ethanol extract of *F. mascrosciadia* significantly inhibits collagenase and elastase.
- Ethanol extract of *F. syriaca* significantly inhibits collagenase and elastase.
- Other studied *Ferulago* species do not show activity on collagenase and elastase enzymes.

Abstract: *Ferulago* species are traditionally used for the treatment of skin diseases, bronchitis, and depression, to increase body strength, as an immunostimulant, aphrodisiac and sedative in Anatolia. In this study, hyaluronidase, collagenase and elastase inhibitory potential relevant to wound healing activities of ethanol extracts from the roots of ten *Ferulago* species [*F. cassia* Boiss., *F. humilis* Boiss., *F. isaurica* Peşmen, *F. longistylis* Boiss., *F. mascrosciadia* Boiss. & Balansa, *F. sandrasica* Pesmen & Quézel, *F. setifolia* K. Koch., *F. silaifolia* (Boiss.) Boiss., *F. trojana* Akalın & Pimenov formerly known as *F. sylvatica* (Besser) Rchb. and *F. syriaca* Boiss.] were investigated. Ethanol extracts of *F. mascrosciadia* and *F. syriaca* were the most active ones against collagenase and elastase, respectively. *In vitro* wound healing activity of ten *Ferulago* species is reported for the first time in the current study.

Keywords: *Ferulago*; Collagenase; Elastase; Hyaluronidase; Wound.

INTRODUCTION

In terms of plant diversity and endemism, 9753 species and 11466 taxa grow naturally in Turkey and the endemism rate is quite high (31,12%). Considering the entire number of vascular plant taxa in whole European continent, biodiversity that Turkish flora possesses could be understood better. Turkey's geographical location, being under the influence of different climate types, having different bedrock and soil

types, being at the intersection of three phytogeographical regions (Irano-Turanian, Euro-Siberian and Mediterranean phytogeographical regions), having vast altitude differences are some of the factors contributing to this biodiversity [1-4].

Ferulago W. Koch genus (Apiaceae) that we have selected as the research topic is represented by approximately 50 species around the world and 35 of them grow naturally in Turkey [5-10]. While species that are gathered in 3 sections are seen in Turkey, Crete, Iran, Iraq; members of the other two sections grow naturally in Bulgaria, South Romania, East Greece, Central and Northern Turkey, Syria, Iran, Iraq and Lebanon [5,11]. In addition to this distribution pattern and having more than %50 endemism ratio, Turkey is considered to be the gene center for this genus.

Since ancient times, *Ferulago* species have been utilized for the treatment of stomach-intestine disorders, intestinal worms, haemorrhoids and as sedative, tonic, stimulant, digestive, carminative, analgesic and food in the regions that they grow. In addition, they are reported to be used against snake bites, splenic disorders, and headaches. Gums obtained from the roots of some *Ferulago* species are used as spice and have carminative effect, as well. However, these species are mainly known with their aphrodisiac effects and are used as animal fodder to improve animal productivity [12-14]. They have typical smell due to having secretions at their fruits and roots, which possess essential oil-resina-gum mixture. Because of this typical smell, they are used as spice, food and especially goats love to eat them [15]. Furthermore, they are utilized as strong protective agents in perfumery and cream preparations [16].

Ferulago species are known with the names “asaotu, çakşırotu, çağşır, geyikotu, kılkuyruk, kişniş, kimyonotu, kurtkulağı, kuyrukotu, kuzubaşı, kuzukemirdi, kuzukişnişi, kuzukulağı, mayasılotu, tilkikuyruğu” in different parts of Turkey [12, 15]. To the best of our knowledge, traditional usage of *Ferulago* species in Turkey is not less and they are being used for skin diseases, bronchitis, depression, surd mutism, to increase body strength, as immunostimulant, aphrodisiac and sedative [17-20]. Some parts of *Ferulago* species are available in the market to increase sex drive as “çakşır otu” in Turkey. These products are being sold in the market as food supplements, and are used for disorders like fatigue, lack of sex drive, and also to increase the quality and quantity of sperm [11].

Like most of Apiaceae family members, *Ferulago* species are rich in essential oil and coumarins. In previous studies, many different coumarin derivatives like 4'-*O*-3-methyl-2-butenoyl-rutaretin, 2,4,5-trimethylbenzaldehyde, aegelinol benzoate, agasyllin, angelicin, auraptene, bergapten, crenulatin, felamedin, ferulol, grandivitinol, hydroxy grandivittin, imperatorin, isoimperatorin, isopimpinellin, marmesin, meranzine hydrate, ostenol, osthol, peucedanin, peucedanol-2’-benzoate, prantschimgin, psoralen, suberosin, umbelliferone and xanthotoxin have been isolated from *Ferulago* species [21-32]. Their essential oils mainly consist of bornyl acetate, camphene, carene-*δ*-3, *β*-caryophyllene, caryopyllene oxide, cis-chrysanthenyl acetate, ar-curcumene, *β*-germacrene, 4,6-guaiadiene, *α*-humulene, isobutyl acetate, limonene, myrcene, ocimene, *α*-pinene, *β*-pinen, sabinene, terpinolene, 2,3,6-trimethyl benzaldehyde, 2,4,5-trimethyl benzaldehyde, and E-verbenol. Flavonoids, anthocyanins, steroids and tannins are other secondary metabolites that are confirmed to be present in this genus. Literature search yielded studies focusing on cytotoxic, antilipidemic, antidiabetic, antibacterial, antiulcerogenic, antifungal, antioxidant, anticholinesterase, anti-inflammatory, aphrodisiac, *α*-amylase and *α*-glucosidase inhibitory effects of *Ferulago* species, however wound healing activity of *Ferulago* species has not been studied before [11-12, 25-26, 32-45].

Hyaluronidase, elastase and collagenase are responsible for the degradation of the main components of the extracellular matrix, namely the hyaluronic acid, elastin and collagen [46]. In addition to having a role in wound healing, these enzymes have a function in skin aging, as well. For example, it is well known that hyaluronidase, collagenase and elastase enzymes are the major enzymes responsible for dehydration of the skin and collagen and elastin are the major components of the connective tissue and hyaluronic acid keeps the moist and inhibition of these enzymes could therefore improve skin aging [47]. Elastin accounts for only about 1-2% of the dry weight of skin but it is important for the maintenance of skin’s elasticity and resilience. Hyaluronic acid is mucopolysaccharide that holds the water and keeps the body moist, lubricated and smooth. These connective tissue proteins are constantly attacked by several enzymes like collagenases, elastases and matrix metalloproteinases, which leads to decrease in thickness of skin and it becomes dry and wrinkled [48]. There are many studies on the involvement of these enzymes in the aging process of the skin [49-55] and also some secondary metabolites and plant species have been tested for their anti-collagenase and anti-elastase activities [47-48, 52-56]. To name a few, polyphenols such as catechin and epigallocatechin gallate have been found to be inhibitors of collagenase and elastase. And some other Apiaceae species like *Angelica* and *Anise* were demonstrated to have anti-collagenase activity (~32%) [57]. Therefore, screening these 10

Ferulago species for their wound healing effects may also enable us to make predictions on their anti-aging effects, as well.

MATERIALS AND METHODS

Plant materials

Roots of 10 *Ferulago* species were collected from different parts of Turkey and were dried under shade at room temperature. Voucher specimens were identified by Hayri Duman (Gazi University, Faculty of Science, Division of Biology) and herbarium specimens were deposited in the Herbarium of Ankara University Faculty of Pharmacy (AEF). Collection dates, localities and herbarium numbers of voucher specimens are presented in Table 1.

Table 1. The collected plant, collection sites and extraction yields.

Plant name	Collection site	Herbarium number	Extract yield (w/w, %)
***Ferulago cassia* Boiss.**	Konya, Beyşehir, at 1st km of Tınaztepe-Bozkır old road, serpentine areas, *Pinus nigra* forest opening, 1555 m, 20/6/2016, (37° 13.785'N / 31° 5.537'W)	AEF 28775	19.45
***Ferulago humilis* Boiss.**	Muğla, Milas, North of İkizköy, under *Pinus brutia* openings, 250 m, 9/6/2016 (35S 577097 / 4114855)	AEF 28773	17.66
***Ferulago isaurica* Peşmen**	Antalya-Alanya, between Durbannaz-Banlıca, under *Pinus brutia* forest, calcerous rocks, 837 m, 21/6/2016, (36° 39.095'N / 32° 7.497'W)	AEF 28778	20.45
***Ferulago longistylis* Boiss.**	Erzincan-Refahiye road, Sakaltutan locality, high mountain steppe, 2030 m, 13/7/2016, (39° 52.885'N / 39° 9.292'W)	AEF 28777	24.02
***Ferulago mascrosciadia* Boiss. & Balansa**	Balıkesir, Bigadiç, North of Düğüncüler village, under *Quercus* trees and in *Cistus laurifolius* openings, 8/6/2016 (39° 16.333'N / 28° 32.141'E)	AEF28776	22.35
***Ferulago sandrasica* Pesmen & Quézel**	Denizli, Beyağaç, Beyağaç-Köyceğiz road, at Kartal Lake intersection, 1390m, 9/6/2016, (37° 7.674'N / 28° 50.267'E)	AEF 28772	19.48
***Ferulago setifolia* K. Koch**	Erzincan, Üzümlü district, Karakaya Town, Karakaya Town, between Karakaya-Tekçam Highland, high mountain steppe, 2047 m, 13/7/2016, (39° 42.127'N / 39° 45.874'W)	AEF 28770	16.37
***Ferulago silaifolia* (Boiss.) Boiss.**	Bursa, Uludağ road, 10 km to the National Park, under *Castanea sativa* and *Pinus nigra* forest, 837 m 7/6/2016 (40° 8.69'K / 29° 1.019'D)	AEF 28771	16.02
***Ferulago syriaca* Boiss.**	Hatay, Harbiye-Şenköy road, maquis openings, 452 m 22/6/2016452 m (36° 7.194'N / 36° 9.988'W)	AEF 28779	16.37
***Ferulago trojana* Akalın & Pimenov**	Çanakkale, Çan, Terzialan-Bayramiç road, 5. km, under the oak forest, schistose soil, 365 m, 8/6/2016, (39° 55.928'K / 27° 0.544'D)	AEF 28774	18.84

Preparation of plant extracts

30 g of roots were grounded and placed in separate vessels. 200 mL of 80% ethanol (Tekkim) were added to each vessel and macerated in a shaker for 48 hours at 140 rpm. At the end of this period, extracts were filtered and the residues of all extracts were subjected to the same process for 6 times more. Filtered extracts were combined and the solvent was evaporated with the help of a rotary evaporator at a temperature not exceeding 45°C. The extract yields (w/w) are given in Table 1.

Hyaluronidase inhibitory activity assessment

Inhibition of hyaluronidase enzyme was evaluated by measuring the amount of *N*-acetylglucosamine released from sodium hyaluronate. 50 µL of bovine hyaluronidase (7900 units/mL) was dissolved in 0.1 M acetate buffer (pH 3.6) and was mixed with 50 µL of different concentrations of the extracts dissolved in 5% DMSO. For control group, 50 µL of 5% DMSO was added instead of the extracts. After 20 minutes of incubation at 37ºC, 50 µL of calcium chloride (12.5 mM) was added to the mixture and again incubated for another 20 minutes at 37ºC. 250 µL sodium hyaluronate (1.2 mg/mL) was added and incubated for 40 minutes at 37ºC. After incubation, the mixture was treated with 50 µl of 0.4 M NaOH and 100 µL of 0.2 M sodium borate and then incubated for 3 minutes inside boiling water bath. 1.5 mL of *p*-dimethylaminobenzaldehyde solution was added to the reaction mixture after cooling to room temperature and was further incubated at 37ºC for 20 minutes for the development of color. The absorbance of this colored solution was measured at 585 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA) [53, 58].

Collagenase inhibitory activity assessment

Samples were dissolved in DMSO. *Clostridium histolyticum* (ChC) was dissolved in 50 mM Tricine buffer (with 0.4M NaCl and 0.01M $CaCl_2$, pH 7.5). Then, 2 mM N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) solution was prepared in the same buffer. 25 µL buffer, 25 µL test sample and 25 µL enzyme were added to each well and incubated for 15 minutes. 50 µL substrate was added to the mixture to immediately measure the decrease of the optical density (OD) at 340 nm using a spectrometer.

The ChC inhibitory activity of each sample was calculated according to the following formula:

$$\text{ChC inhibition activity (\%)} = OD_{Control} - OD_{Sample} \times 100 / OD_{Control}$$

Where $OD_{control}$ and OD_{sample} represent the optical densities in the absence and presence of sample, respectively [59].

Elastase inhibitory activity assessment

Sample solution and human neutrophil elastase enzyme (HNE) (17 mU/mL) were mixed in 0.1 M Tris-HCl buffer (pH 7.5), then incubated at 25ºC for 5 minutes. N-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (MAAPVN) was added to the mixture and incubated at 37ºC for 1 hour. The reaction was stopped by the addition of soybean trypsin inhibitor (1 mg/mL) and the optical density due to the formation of *p*-nitroaniline was immediately measured at 405 nm. The HNE inhibitory activities were calculated as described in the ChC inhibitory activity [60-61].

Statistical analysis of the data

The data on percentage wound healing was statistically analyzed using one-way analysis of variance (ANOVA). The values of $p \leq 0.05$ were considered statistically significant.

RESULTS

In this study, *in vitro* inhibitory activities of the ethanolic extracts from some *Ferulago* species growing in Turkey on hyaluronidase, collagenase and elastase enzymes, all of which are involved in the wound healing process, were studied. *In vitro* hyaluronidase inhibitory activity assay results showed that none of the extracts obtained from the roots *Ferulago* species exerted a significant inhibitory activity on hyaluronidase enzyme at 100 µg/mL concentration (Figure 1).

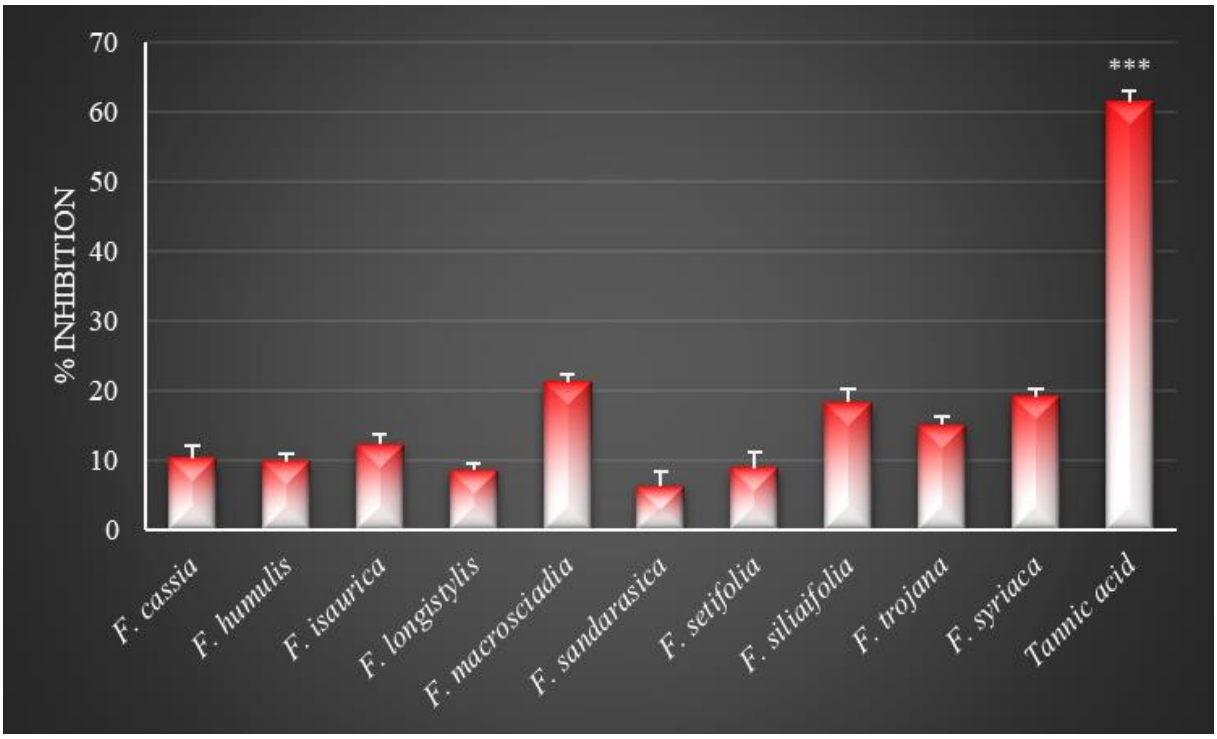

Figure 1. Hyaluronidase inhibitory activity of the ethanolic extracts of *Ferulago* species.

On the other hand, the ethanolic extract prepared from *F. mascrosciadia* and *F. syriaca* displayed inhibitory effect on collagenase enzyme with the inhibition values of 34.83% and 28.84%, respectively. Similar results were obtained from elastase inhibitory activity. *F. mascrosciadia*, *F. syriaca* and *F. trojana* were found to have significant elastase inhibitory activity with the values of 41.20%, 34.15%, and 30.02% respectively. In addition, rest of the extracts was also neither effective nor had high activity on both collagenase and elastase enzymes (Figure 2 and 3).

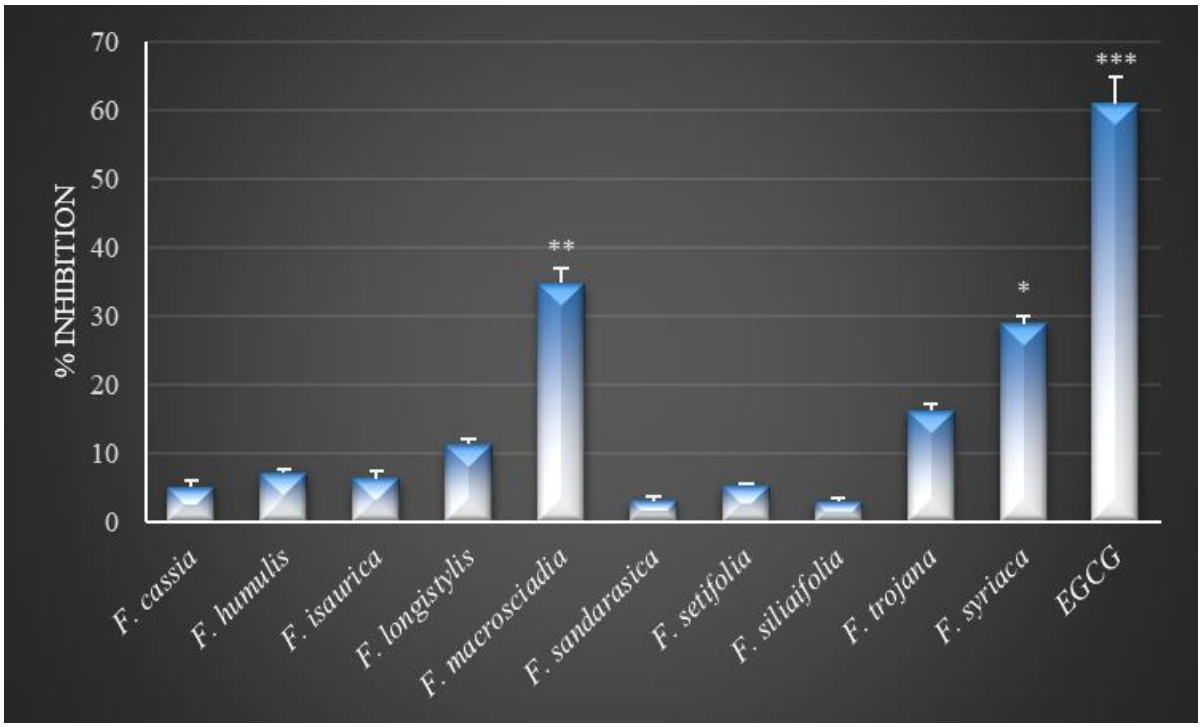

Figure 2. Collagenase inhibitory activity of the ethanolic extracts of *Ferulago* species.

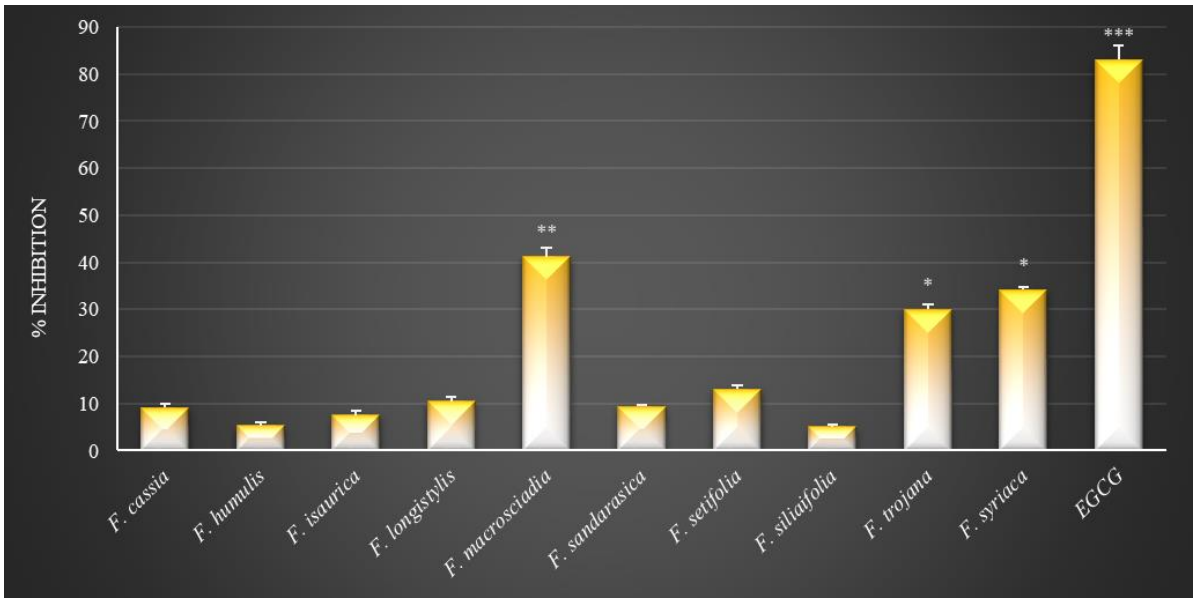

Figure 3. Elastase inhibitory activity of the ethanolic extracts of *Ferulago* species.

DISCUSSION

Wounds are physical injuries that result in the opening or rupture of skin, which can cause anatomical and functional disorders. Wound healing is a dynamic, difficult process that leads to the reestablishment of tissue integrity and homeostasis and includes inflammation, reepithelization, granulated tissue formation, neovascularization, wound contraction and remodeling of the extracellular matrix [62].

Wound healing is comprised of convoluted processes with successive reactions. Healing processes are classically divided into four phases: (1) Hemostasis phase; (2) Inflammatory phase; (3) Proliferative phase; and (4) Maturation and remodeling phase, although they also overlap each other [63-64].

Chronic, non-healing wounds and burn wounds have been shown to have high levels of elastase which degrade cytokine growth factors, fibronectin and endogenous levels of protease inhibitors. Furthermore, it has also been shown that minimal levels of elastase and matrix metalloproteinases, which are originated in acute wounds, might be essential for an appropriate healing response [65]. Collagen is known to play a significant role in all phases of wound healing process whereas elastin, another protein found in the extracellular matrix (ECM) gives skin and other tissues elasticity.

Inflammation is formed by cytokines and chemokines, which arise largely from resident cells (epithelium, endothelium, fibroblasts, etc.). Metalloproteinases (MMPs) activate the mediators by cleaving them from the cell surface or processing them to increase their activity, or degrade them, thus inhibiting inflammatory signals. Moreover, MMPs are able to cleave components of cell-cell junctions and cell-matrix contacts within the epithelium to support re-epithelialization. Also, MMPs are involved in remodeling the scar ECM either openly by proteolytic degradation of proteins, such as collagens, or indirectly via their ability to affect cell behavior. Variation of the ECM is integral to the resolution of wound healing but also has implications in regulation of inflammation [66-73].

MMPs similarly play a pivotal role in normal wound healing via degradation of several ECM components, which facilitates the migration of cells and remodeling of the wound [68,72]. It is when the balance between ECM degradation and deposition is dislocated, though, in part, owing to a disruption in the equilibrium of the production or activation of proteases and their individual inhibitors that wounds become chronic. Chronic wounds are continued in a state of determined inflammation, characterized by abundant levels of proteases and neutrophils, which themselves secrete proteases (including collagenase and elastase), which additional augment connective tissue breakdown [70].

During cutaneous wound healing, interstitial collagenase (MMP-1) is conveyed by macrophages, endothelial cells, and fibroblasts in the dermis [74]. The dermal expression of collagenase is thought to direct remodeling of the extracellular matrix concluded the controlled degradation of fibrillar collagens [75]. The regulation of collagenase in the epidermis seems to be independent of dermal collagenase [76], suggesting a dual role for proteinase during healing [77-81].

Hyaluronic acid (HA) being a universal megadalton linear polysaccharide, it principally connects and begins contact between cells that are physically separated in a tissue. It can bind to hundreds of ECM proteins

and as well as cell surface HA receptors and might provide autocrine and paracrine stimulation. Furthermore, as HA can hold larger volume of water and as well as metal ions, it has the ability to bear compressive loads in tissues and joints *in vivo*, and provides lubrication to articulating surfaces. Due to the versatility and varied functionality of HA, it can be manipulated in an extensive variability of medical applications, with, for example ophthalmology, soft tissue regeneration, wound healing, visco-supplementation of joints, bone and tendon regeneration, drug delivery, embryo and organ protection, surface coating and conditioning agents [82-85]. So, presence of combination inhibitors of hyaluronidases in HA and HA based materials/formulations might reduce the risk of tissue inflammation at the management site and might also provide antiangiogenic or antiproliferative or anticoagulant effects.

The balanced regulation of HA metabolizing enzymes, including HAases and HASs is essential for the normal tissue organization and ECM facilitated purposes. The biochemical assets of the HAases, the pH optima, substrate specificity, and their site of expression in the cell vary owing to the type of tissue, age, disease and development. The HA degradation by HAases is very serious in a number of vital regulatory processes ranging from fertilization to aging and the augmented level of HAases activity was perceived in several pathophysiological conditions. In addition, the HA degradation products may be associated with several significant biological and pharmacological functions, such as the stimulation of angiogenesis, immunostimulation, inflammation, and reduction of viscosity in joints. Therefore, the use of HAase inhibitors could be imperative to control some of the serious pathologies that promote uncontrolled degradation of HA. There are a good number of studies focused on the HAases inhibitors of biological implication. Identification of plant based natural inhibitors and generation of synthetic structural analogs may serve as respected anti-aging, antitumor, and antimicrobial contraceptive agents, and similar alternatives to complement anti-venom as first aid agents in venomous bite/sting.

To the best of our knowledge, wound healing activity of *Ferulago* species has not been studied previously. According to Akalın (1999), quercetin and kaempferol are present in leaves of *F. mascrosciadia;* while quercetin and luteolin are present in leaves of *F. trojana* [15]. Wound healing effect of kaempferol was investigated on diabetic and non-diabetic rats by incisional and excisional wound models. Best wound healing effect was detected in the diabetic excisional and nondiabetic incisional wound (92.12% and 94.17%, respectively) with 1% (w/w) kaempferol ointment. Statistically higher hydroxyproline levels were showed on nondiabetic excisional and incisional wounds treated with same ointment (2.84 and 2.07 mg/mg, respectively, $p < 0.01$). Additionally, significantly higher reepithelialization scores were observed in diabetic and nondiabetic excisional and incisional wounds models [86]. Luteolin ointment was investigated in the same way by the same research group. In this study, the best wound healing activity was recorded in incision and excision wounds (97.6 %, 96.1 %, respectively) with 0.5 % (w/w) luteolin ointment. Increase in tensile strength and epithelization induction were observed in both non-diabetic and diabetic groups with luteolin treatment [87]. In a different research, quercetin was showed to accelerate wound closure in cell scratch assay (60% wound closure rate at 36 hours post- scratch). In ischemia-reperfusion injury model, faster wound healing, reduced immune cell infiltration (30%-50% reduction at myeloperoxidase + neutrophil accumulation) and proinflammatory cytokines production by suppressing MAPK pathway were observed with quercetin application [88]. Other than these *in vivo* studies, there are investigations showing that these flavonoids and their derivatives have inhibitory activity against hyaluronidase, collagenase and elastase enzymes [61, 89-93]. Although the activity of pure compounds is not examined in this present work, obtained significant collagenase and elastase inhibition with *F. mascrosciadia* and *F. trojana* could be possibly due to these flavonoids.

It is well known that *Ferulago* species are rich in essential oil. According to Demetzos and coauthors (2000), caryophyllene oxide is one of the major components of *F. trojana* essential oil [34]. In another research examining the essential oil composition of *F. mascrosciadia* and *F. trojana* fruits, p-cymene was recorded as one of major compounds [94]. Its *in vitro* inhibitory activity on human neutrophil elastase was investigated and determined to inhibit human neutrophil elastase with 25 µM IC_{50} value [95]. *Anethum graveolens* L. essential oil that determined to have *p*-cymene as one of major compounds was shown to inhibit growth of methicillin resistant *Staphylococcus aureus* and accelerate wound healing in comparison to the control group. It produced decrease in tissue edema and inflammatory cell infiltration ($P < 0.05$). On the contrary, it produced increase in fibroblasts count and collagen density [96]. Considering that these essential oil components could be present at least in a small amount in ethanol extract, they could possibly have effect on enzyme inhibitory of activity *F. trojana* and *F. mascrosciadia*.

CONCLUSION

In this study, ethanol extracts from the roots of ten *Ferulago* species have been screened for their wound healing activity. According to the data that we have obtained herein, the most effective species in respect to collagenase and elastase inhibition tests was found to be *F. mascrosciadia* and *F. syriaca*. To the best of our knowledge, we herein report wound healing activity of ten *Ferulago* species (*F. cassia*, *F. humilis*, *F. isaurica*, *F. longistylis*, *F. mascrosciadia*, *F. sandrasica*, *F. setifolia*, *F. silaifolia*, *F. syriaca* and *F. trojana*) for the first time. Among these plants, ethanol extract of *F. mascrosciadia* and *F. syriaca* were determined to be the most active ones in the inhibition of collagenase and elastase enzymes. We can conclude that these species may be used in wound healing and also can be utilized in anti-aging products which also proceed with a similar pathway via the action of collagenase, elastase and hyaluronidase enzymes. Further studies are needed to elucidate the active substances responsible for these activities.

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Conflicts of Interest: The authors declare no conflict of interest.

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