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ORIGINAL ARTICLE

The Importance of *Asphodeline* Species on Enzyme Inhibition: Anti-Elastase, Anti-Hyaluronidase and Anti-Collagenase Potential

Asphodeline Türlerinin Enzim İnhibisyonundaki Önemi: Anti-Elastaz, Anti-Hyalüronidaz ve Anti-Kollajenaz Potansiyeli

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ABSTRACT

Asphodeline species are widespread in the inner Anatolia region in Turkey and used for the treatment of skin disorders, earaches and haemorrhoids. The aim of the present study is to investigate *in vitro* inhibitory effects of the extracts prepared from the stems, seeds, leaves and roots of *Asphodeline brevicaulis* subsp. *brevicaulis* var. *brevicaulis*, *A. baytopae* and *A. cilicica* on hyaluronidase, collagenase and elastase enzymes. Hyaluronidase, collagenase and elastase inhibitory effects of the extracts were performed by using *in vitro* enzyme inhibitory assays based on spectrophotometric evaluation. The methanol extract of the roots of *A. cilicica* displayed the highest hyaluronidase, collagenase and elastase inhibitory activities. On the other hand, the acetone extract of the roots of *A. cilicica*, the acetone extract of the leaves of *A. brevicaulis* subsp. *brevicaulis* var. *brevicaulis*, the acetone and the methanol extracts of seeds of *A. baytopae*, acetone and the methanol extract of the roots of *A. baytopae*, the aqueous extract of the stems of *A. cilicica*, the acetone and the methanol extracts of the leaves of *A. cilicica*, the aqueous extract of the roots of *A. cilicica* possessed significant collagenase, hyaluronidase and elastase inhibitory activities. In the present study, extracts of *Asphodeline* species significantly inhibited collagenase, elastase and hyaluronidase enzymes, suggesting their utilization for the treatment of wounds, cancer, cardiovascular diseases, inflammation, bone destruction and fibrosis, as well as skin aging.

Key words: *Asphodeline*, Xanthorrhoeaceae, Collagenase, Elastase, Hyaluronidase

ÖZ

Asphodeline türleri Türkiye'de İç Anadolu Bölgesi'nde geniş bir yayılış göstermektedir ve halk arasında deri hastalıkları, kulak ağrıları ve hemoroit gibi rahatsızlıklara karşı kullanılmaktadır. Bu çalışmanın amacı, *Asphodeline brevicaulis* subsp. *brevicaulis* var. *brevicaulis*, *A. baytopae* ve *A. cilicica* bitkilerinin gövde, tohum, yaprak ve köklerinden hazırlanan ekstrelerin hyalüronidaz, kollajenaz ve elastaz enzimleri üzerindeki *in vitro* inhibitor etkinliklerini araştırmaktır. Bu ekstrelerin inhibitor etkileri spektrofotometrik yöntemlerle değerlendirilmiştir. *A. cilicica* köklerinden hazırlanan metanol ekstresinin en yüksek hyalüronidaz, kollajenaz ve elastaz inhibitor aktivitelere sahip olduğu belirlenmiştir. Diğer yandan, *A. cilicica* köklerinden hazırlanan aseton ekstresinin, *A. brevicaulis* subsp. *brevicaulis* var. *brevicaulis* yapraklarından hazırlanan aseton ekstresinin, *A. baytopae* köklerinden ve tohumlarından hazırlanan aseton ve metanol ekstrelerinin, *A. cilicica* gövdesinden hazırlanan sulu ekstrenin, *A. cilicica* yapraklarından hazırlanan aseton ve metanol ekstrelerinin, *A. cilicica* köklerinden hazırlanan sulu ekstrenin önemli ölçüde kollajenaz, elastaz ve hyalüronidaz etki gösterdiği belirlenmiştir. Sonuç olarak bu çalışmada *Asphodeline* türlerinden hazırlanan ekstrelerin kollajenaz, elastaz ve hyalüronidaz enzimlerini önemli ölçüde inhibe ettiği ortaya koyulmuştur. Bu nedenle bu sonuçlar bitkinin yara, kanser, kardiyovasküler rahatsızlıklar, enflamasyon, kemik yıkımı ve fibrozis gibi hastalıkların tedavisinde kullanılabileceğini destekler niteliktedir.

Anahtar kelimeler: *Asphodeline*, Xanthorrhoeaceae, Kollajenaz, Elastaz, Hyalüronidaz

INTRODUCTION

Asphodeline Rchb. genus (Xanthorrhoeaceae), represented by 14 species in the world, is growing wild in south-west Asia and Middle-Eastern countries as well as in Mediterranean region. In Turkey, *Asphodeline* genus comprises of 20 taxa, 12 of which are endemic to Turkey showing that Turkey is one of the gene centres of this genus (1-3). In rural areas in Turkey, several *Asphodeline* species are known in the name of "çiriş" and are widespread in inner Anatolia region. Especially *Asphodeline cilicica* E. Tuzlacı, *A. damascena* (Boiss.) Baker, *A. globifera* J. Gay Ex Baker, *A. lutea* (L.) Rchb. and *A. taurica* (Pallas) Kunth are consumed as food in salads (4). This genus

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was recorded to have economical and nutritional importance with high levels of essential amino acids and polyphenols (3-7). Besides their nutritional properties, *Asphodeline* species also possess medicinal features and are employed in medical practices in traditional medicine. For instance, *A. globifera* has been used for alleviating the symptoms of haemorrhoids; *A. damascena* and *A. cilicica* were recorded to be utilized for the treatment of earaches (8); and *A. lutea* has been used for the treatment of skin diseases (9). Due to their mentioned use above, many phytochemical researches have been conducted on *Asphodeline* species revealing the presence of secondary metabolites such as antraquinones, sesquiterpenes, flavonoids and naphthalene type compounds (6,10-13). Enzymes have very important roles in the pathogenesis of several diseases including cancer, inflammation, Alzheimer's and Parkinson's diseases, familial hypercholesterolemia, myasthenia gravis etc. (14-17). Due to the important roles of these enzymes on several diseases, novel drugs that display inductive or inhibitory effects should be developed.

In the present research, *in vitro* inhibitory effects of the extracts obtained from the stems, seeds, leaves and roots of *A. brevicaulis* (Bertol.) J. Gay ex Baker subsp. *brevicaulis* (Bertol.) J. Gay ex Baker var. *brevicaulis* (Bertol.) J. Gay ex Baker, *A. baytopae* E. Tuzlacı and *A. cilicica* on hyaluronidase, collagenase and elastase enzymes, which are the major enzymes responsible for dehydration of the skin, were investigated.

EXPERIMENTAL

Plant materials

Asphodeline species were collected at the end of flowering stage (May-July) and information regarding the collection sites of the plants and herbarium numbers were presented in Table 1. Taxonomic identification of the plant materials were confirmed by the senior taxonomist Dr. Murad Aydın Sanda, from the Department of Biology, Selçuk University. The voucher specimens were deposited at the KNYA Herbarium of Department of Biology, Selçuk University, Konya, Turkey.

Preparation of the plant extracts

The plant materials (stem, root, seed and leaf) were dried at the room temperature. The dried parts were ground to a fine powder using a laboratory mill. For each of the powdered parts (10 g) were separately extracted with 250 mL acetone and methanol in a Soxhlet apparatus for 6-8 h. The residue was extracted with 250 mL hot distilled water for 30 min and the extracts were filtered concentrated under vacuum at 40°C by using a rotary evaporator. The aqueous extracts were lyophilized (-80°C, 48 h). Yields of the extracts were given in Table 1.

In vitro enzyme inhibitory assays

Hyaluronidase inhibiton assay

Hyaluronidase inhibiton assay was performed according to the methods described by Lee & Choi (1999) and Sahasrabudhe & Deodhar (2010) with some modifications (18-20).

An amount of 50 µL an aliquot of bovine hyaluronidase (7900 units/mL) was dissolved in 0.1 M acetate buffer (pH 3.6). This mixture then was mixed with 50 µL of different concentrations of the extracts prepared in 5% DMSO. An aliquot of 50 µL of 5% DMSO was added instead of the extracts in the control group. After incubation at 37°C for 20 min, 50 µL of calcium chloride (12.5 mM) was added to the mixture and reincubated for another 20 minutes at 37°C. 250 µL sodium hyaluronate (1.2 mg/mL) was added and incubated for 40 minutes at 37°C.

Table 1. The collection cites and herbarium numbers of Asphodeline species; parts used for the extraction, extract types and percentage yields of the extracts

Plant name	Collection site	Herbarium no	Extract type	Parts used	Yield (w/w, %)
A. brevicaulis subsp. *brevicaulis* var. *brevicaulis*	Mersin, Arslanköy, between Arslanköy and Yeniköy, 37° 00' 20.9"N, 34° 29' 24.6 E, alt. 1077 m	KNYA-GZ1004	Acetone	Sm/ R/ Lf / Sd	1.73/ 1.28/ 2.71/ 3.91
			Methanol		12.64/ 16.04/ 11.21/ 10.27
			Aqueous		18.05/ 14.1/ 20.35/ 20.41
A. baytopae	Mersin, Gulnar, between Gulnar and Aydincik, 36° 16' 07" N, 33° 22' 11" E, alt. 751 m	KNYA-GZ1003	Acetone	Sm/ R/ Lf / Sd	0.88/ 4.68/ 2.66/ 2.71
			Methanol		4,16/ 39.06/ 22.38/ 6.42
			Aqueous		4.32/ 27.56/ 25.91/ 10.00
A. cilicica	Adana, between Catalan and Aladag, 37° 27'37'' N, 35° 20' 12'' E, alt. 1080 m	KNYA-GZ1005	Acetone	Sm/ R/ Lf / Sd	2.88/ 2.42/ 4.07/ 3.52
			Methanol		8.85/ 7.39/ 13.32/ 14.47
			Aqueous		10.78/ 9.35/ 13.04/ 24.49

Abbreviations: alt.: altitude; Sm: Stem, R: Root, L: Leaf, Sd: Seed

Table 2. Collagenase and elastase inhibitory activity of the extracts of *Asphodeline* species

Material	Parts used	Extract type	Concentration (µg/mL)	Collagnease inhibition (%) ± S.E.M.	Elastase inhibition (%) ± S.E.M.
A. brevicaulis subsp. *brevicaulis* var. *brevicaulis*	Stem	Acetone	100	15.79±1.83	19.43±1.95
		Methanol	100	14.66±1.48	12.63±1.59
		Aqueous	100	15.99±1.64	19.47±1.93
	Seed	Acetone	100	13.44±2.14	8.12±1.66
		Methanol	100	17.06±1.64	16.42±1.82
		Aqueous	100	14.73±2.14	10.17±2.34
	Leaf	Acetone	100	**31.38±1.14****	**39.39±1.61****
		Methanol	100	10.93±2.26	8.37±2.11
		Aqueous	100	8.66±1.22	6.30±1.82
	Root	Acetone	100	20.34±2.42	22.49±2.22
		Methanol	100	23.64±2.31	28.78±1.98
		Aqueous	100	14.38±1.91	15.85±1.80
A. baytopae	Stem	Acetone	100	19.31±2.64	25.46±1.64
		Methanol	100	13.43±2.54	20.10±1.76
		Aqueous	100	7.56±1.48	9.29±1.84
	Seed	Acetone	100	**30.55±1.28****	**40.22±1.46****
		Methanol	100	**37.22±1.40*****	**42.32±1.76****
		Aqueous	100	9.53±1.82	11.83±1.80
	Leaf	Acetone	100	16.33±1.77	18.29±1.85
		Methanol	100	18.89±1.82	21.16±2.34
		Aqueous	100	8.46±2.54	9.68±1.49
	Root	Acetone	100	**31.70±1.56****	**41.51±1.40****
		Methanol	100	**29.85±2.49***	**31.88±1.92***
		Aqueous	100	10.36±1.99	14.87±1.55
A. cilicica	Stem	Acetone	100	12.26±2.83	14.64±1.72
		Methanol	100	9.41±1.34	7.84±1.90
		Aqueous	100	**35.38±1.43*****	**45.97±1.44****
	Seed	Acetone	100	16.66±2.04	20.66±1.89
		Methanol	100	15.36±1.92	18.54±2.34
		Aqueous	100	8.52±1.78	9.44±1.62
	Leaf	Acetone	100	**36.71±1.21****	**43.32±1.58****
		Methanol	100	**34.22±1.49****	**47.42±1.41****
		Aqueous	100	8.33±1.63	13.83±1.63
	Root	Acetone	100	**37.61±1.73*****	**48.44±1.53****
		Methanol	100	**39.90±1.15*****	**53.78±1.33****
		Aqueous	100	**28.77±1.70***	**30.15±1.70***
Epigallocathecin gallate			100	**48.62±1.14*****	**84.31±1.24*****

*: p<0.05; **: p<0.01; ***: p<0.001; S.E.M.: Standard error of the mean

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 min in the boiling water. *p*-Dimethylaminobenzaldehyde solution (1.5 mL) was added to the reaction mixture after cooling to room temperature and was further incubated at 37°C for 20 minutes to develop a color. The absorbance of this colored solution was measured at 585 nm by using Beckmann Dual Spectrometer (Beckman, Fullerton, CA, USA).

Collagenase inhibiton assay

The method for the collagenase inhibition was performed according to the method of Barrantes & Guinea (2003) with some modifications (20,21).

The 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 into the mixture. The decrease of the optical density (OD) was immediately measured at 340 nm using a spectrophotometer.

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

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.

Elastase inhibiton assay

According to the method described by Melzig et al. (2001), the sample solution and human neutrophil elastase enzyme (HNE) (17 mU/mL) were mixed in 0.1 M Tris-HCl buffer (pH 7.5). The mixture was incubated at 25°C for 5 min. N-Methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (MAAPVN) was added into the mixture and incubated at 37°C for 1 h. By the addition of 1 mg/mL soybean trypsin inhibitor, the reaction was stopped and the optical density was immediately measured at 405 nm. The HNE inhibitory activities were calculated according to the equation given in the ChC inhibitory assay (20,22).

Statistical analysis of the data

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

RESULTS

Turkey is considered as one of the gene centers of *Asphodeline* genus with 20 taxa, as it is represented in Europe by only three species. Plants of the genus *Asphodeline* have traditionally been used as either food or therapeutical agent in various parts of the world as well as in Turkey (1,2). In the previous study, *Asphodeline* species were found to possess acetylcholinesterase, butyrylcholinesterase, amylase, glucosidase and tyrosinase inhibitory activities (3). Indeed, enzymes are known to be involved in the pathogenesis of several diseases, for instance hyaluronidase, collagenase and elastase enzymes involves in the pathogenesis of wound, cancer, cardiovascular diseases, inflammation, bone destruction and fibrosis (20,23-26). Collagen and elastin are the major components of the connective tissue and hyaluronic acid keeps the moist.

Table 3. Hyaluronidase inhibitory activity of the extracts of *Asphodeline* species

Material	Parts used	Extract type	Concentration (µg/mL)	Hyaluronidase inhibition (%) ± S.E.M.
A. brevicaulis subsp. *brevicaulis* var. *brevicaulis*	Stem	Acetone	100	20.16±1.42
		Methanol	100	15.72±1.93
		Aqueous	100	14.78±2.16
	Seed	Acetone	100	20.16±1.42
		Methanol	100	15.72±1.93
		Aqueous	100	14.78±1.46
	Leaf	Acetone	100	21.49±1.32
		Methanol	100	15.72±1.93
		Aqueous	100	14.78±2.16
	Root	Acetone	100	27.55±1.82
		Methanol	100	28.12±1.74
		Aqueous	100	14.39±1.44
A. baytopae	Stem	Acetone	100	27.48±2.69
		Methanol	100	12.64±1.81
		Aqueous	100	7.74±1.21
	Seed	Acetone	100	29.95±1.86
		Methanol	100	28.67±1.72
		Aqueous	100	10.46±2.18
	Leaf	Acetone	100	22.83±1.39
		Methanol	100	28.37±2.66
		Aqueous	100	18.53±2.28
	Root	Acetone	100	**35.14±1.44***
		Methanol	100	18.22±1.93
		Aqueous	100	13.82±2.94
A. cilicica	Stem	Acetone	100	17.26±1.95
		Methanol	100	9.50±1.38
		Aqueous	100	**45.51±1.25****
	Seed	Acetone	100	32.29±2.12
		Methanol	100	16.23±2.19
		Aqueous	100	12.21±1.63
	Leaf	Acetone	100	**39.26±1.11****
		Methanol	100	*42.30±1.14***
		Aqueous	100	16.48±1.86
	Root	Acetone	100	**47.20±1.01****
		Methanol	100	**49.49±1.17****
		Aqueous	100	25.14±1.81
Tannic acid			100	**87.33±0.94*****

*: p<0.05; **: p<0.01; ***: p<0.001; S.E.M.: Standard error of the mean

Inhibition of hyaluronidase, collagenase and elastase enzymes could therefore improve skin aging (20,27-29). In the present study we aimed to investigate *in vitro* inhibitory effects of the extracts prepared from the different parts of *A. baytopae, A. brevicaulis* subsp. *brevicaulis* var. *brevicaulis*, *A. cilicica* on hyaluronidase, collagenase and elastase enzymes.

The results revealed that the methanol extract prepared from the roots of *A. cilicica* (ACRM) displayed the highest hyaluronidase, collagenase and elastase inhibitory activity with the inhibition value of 49.49%, 39.90% and 53.78%, respectively. It was also found that acetone extract of the roots of *A. cilicica* (ACRAc) demonstrated 37.61% and 48.44% inhibitiory effect on collagenase and elastase enzymes, respectively. In addition, acetone extract of the leaves of *A. brevicaulis* subsp. *brevicaulis* var. *brevicaulis* (ABrLAc) demonstrated inhibitiory effect with the values of 31.38% and 39.39% on collagenase and elastase enzymes, respectively. Acetone and methanol extracts of the seeds of *A. baytopae* (ABaSdAc and ABaSdM), acetone and methanol extracts of the roots of *A. baytopae* (ABaRAc and ABaRM), aqueous extract of the stems of *A. cilicica* (ACSmAq), acetone and methanol extracts of the leaves of *A. cilicica* (ACLAc and ACLM), aqueous extract of the roots of *A. cilicica* (ACRAq) possessed significant collagenase and elastase inhibitory activities. On the other hand, ABaRAc, ACSmAq, ACLAc, ACLM and ACRAc were detected to possess significant hyaluronidase inhibitory effect (Table 2 and 3).

DISCUSSION

According to the ethnobotanical studies, *Asphodeline* species such as *A. cilicica*, *A. damascena*, *A. globifera*, *A. lutea*, and *A. taurica* are consumed as food in salads (4). Due to the information regarding its consumption as food, the nutritional features of these species were investigated in our previous research revealing their high amount amino acid composition (4). *Asphodeline* species are used not only as food, but also as therapeutic agents for earaches, skin disorders and haemorrhoids in folk medicine (8,9,13). Due to their several medicinal utilization by people living in rural areas, *Asphodeline* species recently have attracted the researchers' attention to either verify the therapeutical usage in scientific platform or to investigate the phytochemical ingredients.

There have been several studies indicating the enzyme inhibitory activities of phenolic compounds and anthraquinones. Sawabe et al. (1998) investigated the inhibitory effects of water extracts obtained from sixty-six natural medicines on hyaluronidase, elastase and tyrosinase enzymes. The study pointed out that the enzyme inhibitory effect is positively correlated with high amount of phenolic content (30). Moreover, Lee et al. (2001) isolated a new phenolic compound, encoded CC-517, from *Areca catechu* L. and revealed its significant anti-hyaluronidase and anti-elastase activities. The compound inhibited human neutrophil elastase with the IC_{50} value of 60.8 µg/mL; hyaluronidase with the IC_{50} value of 210 µg/mL. It also exhibited more potent elastase inhibitory effect than oleanolic acid and ursolic acid (31).

Tanaka et al. (1990) conducted a study on collagenase inhibitory effect of 44 anthraquinone type compounds. Results of the

study demonstrated the inhibitory activity of anthraquinones, amongst emodin being the most potent active inhibitor with the IC_{50} value of $4x10^{-5}$ M (32). Furthermore, Zembower et al. (1992) synthesized several anthraquinone analogues and evaluated their elastase inhibitory activity on human leukocyte. Consequently, it was reported that 1,8 dihydroxyanthraquinone analogues possess elastase inhibitory effect (33).

Previous researches reported that *Asphodeline* species have high antioxidant capacity and phenolic content (7,13,34). Similarly, Zengin et al. (2015) recently investigated the antioxidant and enzyme inhibitory effects as well as anthraquinone profile of the methanol extracts obtained from the roots of eight *Asphodeline* species. According to the results, *A. cilicica* was found to possess the highest total phenolic content, while *A. brevicaulis* subsp. *brevicaulis* var. *brevicaulis* and *A. baytopae* had the highest total anthraquinone content. Therefore, the inhibitory effects of *A. cilicica, A. brevicaulis* subsp. *brevicaulis* var. *brevicaulis* and *A. baytopae* could be attributed to its phenolic and anthraquinone contents (3).

CONCLUSION

In the present study, ACRM, ACRAc, ABrLAc, ABaSdAc, ABaSdM, ABaRAc, ABaRM, ACSmAq, ACRAq significantly inhibited collagenase, elastase and hyaluronidase enzymes, suggesting that these extracts could be used for the treatment of several diseases including wound, cancer, cardiovascular diseases, inflammation, bone destruction and fibrosis as well as potential ingredients for the cosmetic formulations to avoid skin aging.

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