In Vitro Anti-Aging Activitiy of Arum Dioscoridis with In Silico Prediction of Its Active Compounds

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(Received on 27th June 2023, accepted in revised form 1st February 2024)

Summary: *Arum dioscoridis* is a plant that has been used by people for centuries for shortness of breath, asthma, bronchitis, expectorant, intestinal laziness, digestive system, malaria and especially hemorrhoid diseases. The aims of the study are to determine the efficient extraction method by extracting the plant with different methods, to investigate the antioxidant and anti-aging effects of the extracts, and to perform in silico prediction analyzes of the active molecules of the plant. The plant was extracted using ethanol by maceration, ultrasonic wave extraction, and soxhlet extraction. The antioxidant and anti-aging potential of the obtained extracts were investigated. To determine antioxidant activity, DPPH free radical scavenging, cupric ion reduction and iron ion reduction methods were applied. Anti-aging activity was determined by inhibiting the enzyme elastase. Additionally, the active components of the ethanol extract of the plant were determined using PASSonline, LAZAR, ADMETlab 2.0 and CLC-pred online prediction programs. The yields obtained from the maceration, ultrasonic wave extraction and soxhlet extraction methods are 8.99 g/100g, 19.94 g/100g and 13.2 g/100g, respectively. The IC_{50} values from the DPPH method obtained from the maceration ethanol extract, ultrasonic wave ethanol extract, and soxhlet ethanol extract were 3.943 µg/mL, 2.797 µg/mL, and 2.273 µg/mL, respectively. The IC_{50} values of the extracts obtained from the elastase inhibition method were 3.112 µg/mL, 3.357 µg/mL and 2.964 µg/mL, in the same order. Additionally, the extracts showed inhibition of cupric ions at 1.8 nm, 0.81 nm and 1.79 nm, in the same order. *In silico* biological activity was determined with the PASSonline program, toxicity with LAZAR and ADMETlab programs, and the effects of active molecules against some cell lines were determined with the CLC-Pred program. As a result, it was determined that the plant has biological activities to protect the skin against aging *in silico* and *in vitro*.

Keywords: *Arum dioscoridis;* Anti-aging; Antioxidant; PASSonline; LAZAR; ADMETlab.

Introduction

Arum dioscoridis, belonging to the Araceaa family, Arum class, is an angiosperm, monocotyledonous, perennial herb. It grows in grasslands, meadows, pastures, rocky, stony, gravelly, arid slopes, fields. *A. dioscoridis* species is widely grown in Antalya region, but it can also be grown in Burdur, Antep, Mersin, Adana, Hatay regions. Although there are 28 species of Arum genus in total, there are 20 Arum species grown in Turkey. *A. dioscoridis* is a plant that has been used among the people for centuries. Although the healing properties of the plant have not been proven, it is used in Anatolia for shortness of breath, asthma, bronchitis, expectoration, intestinal laziness, digestive system, mumps, snakebite, urinary tract diseases, malaria and especially hemorrhoid diseases [1, 2].

In the phytochemical content screening of plants belonging to the Arum family, it has been shown that these plants contain various secondary metabolites [3]. Among these, the *A. dioscoridis* species that was the subject of the study was found to contain six flavonoids, one coumarin glycoside and two phenolic acids. The identified constituents ($mg.mL^{-1}$) are isoorientin (88.31), vitexin (36.27), luteolin (4.97), apigenin (0.35), quercetin-3-O-β glucoside (0.63), quercetin (0.15), esculin (0.30), ferulic acid (4.09) and caffeic acid (2.72) [14]. These active ingredients have anti-aging, antioxidant, antibacterial, anti-inflammatory and cytotoxic effects [38-42]. Therefore, it is important to choose the most efficient method for the extraction of these vital components. Current techniques have been developed for the extraction of these antioxidant compounds from plant sources. Conventional methods provide the extraction process efficiently. However, due to the long duration of the processes and the temperature applied according to the method, the deterioration of the structure of the molecules has led to the development of new methods. Maceration is carried out at room temperature. In this method, the extraction process can take a long time. The Soxhlet extraction method allows to obtain more molecules. However, if the applied temperature cannot be controlled, it can

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damage the structure of the molecules. Ultrasonic assisted extraction (UAE) technique is among the non-conventional methods. This method has the characteristics of high reproducibility in a short time, minimal solvent consumption, and lower energy requirement and temperature [4, 5]. Ultrasound assisted extraction occurs with the help of the disintegrating effects of ultrasonic waves. Ultrasound has some advantages in this extraction method. They are to intensify mass transfer, reduce cell disruption, increase penetration and capillary effects [6]. This method is applied in the extraction of many chemical components due to its high efficiency, low energy requirement, less solvent and less time-consuming method.

Skin aging is not due to a single condition. Aging is a biologically complex phenomenon caused by various external factors. The largest organ in the body is the skin. Due to its size and footprint, the skin is exposed to high levels of sunlight and UV rays. Under the influence of UV rays, reactive oxygen species are formed in the skin. Reactive oxygen species activate enzymes that break down extracellular matrix proteins in the dermis and alter the integrity of the skin [7]. Proteins such as collagen and elastin play a role in maintaining the elastic structure of the skin. One of the most important causes of skin aging is the deterioration of the extracellular matrix structure, which contains many proteins, especially collagen and elastin [8]. As the activity of collagenase and elastase enzymes increase, the structure of the extracellular matrix is disrupted. One of the currently accepted approaches to prevent skin aging is to inhibit these enzymatic activities by secondary metabolites extracted from plants [9]. The elastase enzyme is involved in the physiological degradation of elastin, which is responsible for skin elasticity. For example, there is an increase in elastase activity that is closely related to wrinkle formation in various diseases such as psoriasis, dermatitis, inflammatory processes and premature skin aging [10].

In the study, *A. dioscoridis* plant was extracted by maceration, ultrasonic wave and soxhlet methods. Antioxidant and anti-aging activities of extracts obtained from three methods were examined. Then, the structure-activity relationships of the active components in the ethanol extract of the plant were interpreted using PASSonline, LAZAR, ADMETlab and CLC-pred online prediction programs. Different methods were chosen to determine which extraction method yielded the highest yield from the plant. Experiments were carried out at room temperature with maceration, sonication interaction with the ultrasonic wave method, and at high temperatures with the soxhlet method. In addition, it was determined by different analysis methods which of the extracts obtained from these methods showed higher *in vitro* biological effects. To determine the *in silico* pharmacological potential of the plant, the active ingredients contained in the ethanol extract were determined from the literature and their web-based analysis was performed. In this regard, it was determined which method the plant produced extract with higher yield and which extract was biologically effective.

Experimental

Equipments

UV Spectrophotometer (Hitachi U-2900, Japan), Eliza Reader (Biotek Synergy H1 Hybrid Reader, USA), Rotary Evaporator (Heidolph Hei-VAP Value, Germany), pH-meter (Mettler Toledo, Switzerland), Magnetic stirrer (Heidolph MR) Hei-Standard, Germany), Centrifuge (Nüve NF800, Turkey), micro pipettes (2-20 µL, 10-100 µL, 20-200 µL, 100-1000 µL, 1000-5000 µL) (Eppendorf, Germany), precision balance (Shimadzu AUW2200, Japan), ultrasonic bath (VNR Ultrasonic Cleaner, Germany), ultrapure water device (Younglin Instrument, aquaMAX-Ultra, Korea), Water Bath (Wisd WiseBath, Korea), Vortex (Bio Vortex V1 – BIOSAN, Latvia).

Chemicals

Gallic acid, quercetin, 1,1-diphenyl-2-picrylhydrazil (DPPH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol, nitroblutetrazolium (NBT), potassium peroxy disulphate ($K_2S_2O_8$), copper (II)) chloride, tween-40, chloroform, dichloromethane, methanol, ethanol, aluminum nitrate, potassium acetate, neocuproin (2,9-dimethyl-1,10-phenanthroline), t-butanol, ammonium sulfate, Folin-Ciocalteu reagent, sodium carbonate, ammonium acetate, sodium hydrogen phosphate and sodium dihydrogen phosphate were obtained from Sigma-Aldrich. The chemicals used and all solvents are of analytical purity.

Plant Material

The *A. dioscoridis* plant was collected from the Kaş district of Antalya province at the coordinates 36° 12′ 6″ North and 29° 38′ 15″ East. It was dried under sunlight at 30^{0}C in May. Dried plant was stored at room temperature until studied. The plant samples were collected from the field by paying attention to the

presence of vegetative and generative organs such as leaves, flowers, fruits, underground parts (tubers, rhizomes, onions, etc.) required for identification. In addition, information such as location, habitat characteristics, elevation, and collection date for the collected plant samples were recorded in the field. These collected specimens were identified using the work titled "Flora of Turkey and the East Eagean Islands" [11-13]. The chemical composition of this plant investigated using HPLC in previous studies [14, 15]. Six flavonoids, one coumarin glycoside and two plant acids were detected in the ethanol extract of *A. dioscoridis* by Afifi *et al.* [14]. Identified components, isoorientin (88.31 mg/mL), vitexin (36.27 mg/mL), luteolin (4.97 mg/mL), apigenin (0.35 mg/mL), quercetin-3-O-β-glucoside (0.63 mg/mL), quercetin (0.15 mg/mL), esculin (0.30 mg/mL), ferulic (4.09 mg/mL) and caffeic acids (2.72 mg/mL).

Extraction Methods

Maceration

100 g of the aerial parts of the powdered *A. dioscoridis* plant was extracted with 250 mL ethanol in a shaker at 160 rpm for 12 hours at room temperature. After filtering twice through Whatman No.1 filter paper, the solvent was removed by means of a rotary evaporator. The extract obtained was weighed, its yield was calculated and stored at -18°C [4, 15].

Ultrasound-assisted extraction

A. dioscoridis powder was weighed 100 g and sonicated with 250 mL of ethanol for 5, 15, 30 and 60 min. in an ultrasonic bath (Diamond 30 H model, operating at 20 kHz frequency) at 45°. The extracts were filtered and the solvent was removed using a rotary evaporator. The yield as in the previous section was calculated and stored at -18°C [16].

Soxhlet extraction

Soxhlet extraction method was used for the extraction of *A. disocoridis* plant and the extraction process was carried out using distilled ethanol. The ethanol used was of analytical grade. 100 g of powdered herb was weighed and placed in a soxhlet cartridge and placed in the device. The extraction was carried out at a constant temperature of 60°C for 6 hours. The post-processing extract was evaporated using a rotary evaporator (Heidolph-insturments, Rotavapor, Germany). After evaporation of the solvent, the weight of the extract was measured and stored in an airtight container for analysis [17].

Antioxidant activity determination methods

DPPH free radical scavenging activity assay

The free radical scavenging activities of extracts from *A. dioscoridis* plant were determined using 1,1-Diphenyl-2-picrylhydrazil free radical according to the method developed and slightly modified by Blois [18] and is published elsewhere [19]. The free radical scavenging activity is calculated as % inhibition from the equation below.

$$\text{Inhibition } \% = [(A_{control} - A_{extract})/A_{control}]x100$$

A: Absorbance

Each sample was investigated in three parallels. BHA and BHT were used as standard.

Cupric ion reducing antioxidant capacity (CUPRAC) assay

The cupric ion (Cu^{2+}) reducing capacities of the extracts obtained from the *A. dioscoridis* plant were applied by slightly modified Apak *et al.* [20] and is published elsewhere [19]. The cupric ion reducing capacity was determined by examining the absorbance values obtained. BHA and BHT were used as standard.

Anti-aging Activity Determination Method

Elastase inhibitory activity assay

The antiaging potential of the A. dioscoridis plant was determined following the protocol in Enzo Life Science [21] and published elsewhere [19]. The percentage of elastase inhibition was calculated using the following equation.

$$\text{Inhibition } (\%) = [(A_{control} - A_{sample}) / A_{control}] \text{ x } 100$$

A: Absorbance

Half maximum inhibitory concentration (IC_{50}) values were determined using a graph of inhibitor concentration versus percent inhibition. N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (100 μM) was used as a positive control and all tests were performed three times.

In silico Prediction Programs

PASSonline, LAZAR, ADMETlab 2.0 and CLC-pred web-based programs were used for in silico prediction analysis of active ingredients of *A.*

dioscoridis plant. The chemical structures of these active ingredients were drawn using ChemDraw Professional 16.0 (CambridgeSoft) and saved in MOL file format. Qualitative evaluation of in silico predictions of potential metabolite structures was made by comparing the values of each program with those characterized in available biomonitoring studies in the literature.

In silico toxic risks prediction by PASSonline

A computer-based program PASS (Prediction of Activity Spectra for Substances) was used to screen the antioxidant, antibacterial, antifungal, anti-inflammatory and antimutagenic potential of phenolic compounds in the ethanolic extract of *A. dioscoridis*. The software aims to predict the biological activities of other molecules based on the structure-activity relationship with a chemical whose biological activity is known [22]. There are more than 205,000 molecules in the library that the software uses for prediction. Estimation is made using more than 3750 biological activities provided by the same library. Activity estimates are made in Pa (probable activity) and Pi (probable inactivity). Pharmacological activity is seen in molecules where Pa is higher than Pi [23].

Access link for PASSonline: https://www.way2drug.com/passonline/index.php

In silico toxicity prediction by LAZAR

LAZAR (Lazy Structure-Activity Relationships) is an online open code software developed by the European Union OpenTox Project and recommended for amateur users. It is a local QSAR model that predicts mutagenicity, carcinogenicity, and acute toxicity [24]. By using the Lazar virtual web program, estimations of toxic properties such as acute toxicity (Daphnia magna), carcinogenicity (Rat), lowest observed side effect level (Rat), mutagenicity (*Salmonella typhimurium*) of the active ingredients of *A. dioscoridis* plant were obtained [25].

Access link for LAZAR: https://lazar.in-silico.ch/predict

In silico toxicity prediction by ADMETlab 2.0

ADMETlab is a state-of-the-art computer program designed to predict the specific absorption, distribution, metabolism, elimination and toxicity properties of a chemical from 2D and 3D molecular structures. The software uses predictive descriptive values for the above ADMET features based on independent mathematical models. There are numerous studies in the US EPA's Distributed Searchable Toxicity database used to examine the receptor binding patterns of the ADMET prediction program [26]. Acute toxicity (Rat), carcinogenicity, skin sensitization and CYP1A2, CYP2C19, CYP2C9, CYP2D6 and CYP3A4 inhibition potentials of active ingredients of *A. dioscoridis* plant were investigated using ADMETlab 2.0 program.

Access link for ADMETlab 2.0: https://admetmesh.scbdd.com/service/evaluation/index

In silico prediction of cell line cytotoxicity with CLC-Pred

The CLC-Pred online program is used to predict the cytotoxic activities of cancerous cells. The basic principle of the software is based on the construct-cell line cytotoxicity examination designed by PASS. The anticancer activity of the active molecules of *A. dioscoridis* can be performed and optimized using the CLC-Pred database. Pa values are obtained from the web-based tool to determine the predicted cytotoxicity. These values give results against a variety of human cell lines. If the Pa value is >0.5, the probability of the molecule having an inhibitory effect against the cell line is quite high. The Pi value indicates that the molecule has no effect [27]. Using the Pa and Pi values on the online CLC-pred website, the effectiveness of the compounds against which cell type, which tissue, and which tumor type was determined.

Access link for CLC-pred: https://www.way2drug.com/cell-line/index.php

Statistical Analysis

Statistical differences between IC_{50} value groups obtained from DPPH and ELAS methods were determined by One-Way ANOVA and Tukey Post-Hoc test. These analyses were performed using GraphPad Prism 9.0 for Windows (GraphPad Software, San Diego, CA, USA). Significant differences of absorbance value groups obtained from DPPH, ELAS and CUPRAC methods were determined by One Way ANOVA and Tukey Post-Hoc test in IBM SPSS Statistics 22. Data were studied for three independent determinations and expressed as n=3. In ANOVA tests, $p < 0.05$ values were accepted as the limit of significance.

Table-1: Yields of *A. dioscoridis* plant using ethanol according to different extraction methods*.

Maceration yield (g/100 g)	Soxhlet extraction yield (g/100 g)	Ultrasonic wawe extraction yields (g/100 g)			
		5 min	15 min	30 min	60 min
8,99±0,02	13,20±0,13	1,6±0,06	19,94±0,28	2,89±0,08	3,44±0,04

* The amount of extract obtained from 100 g of sample was given in g.

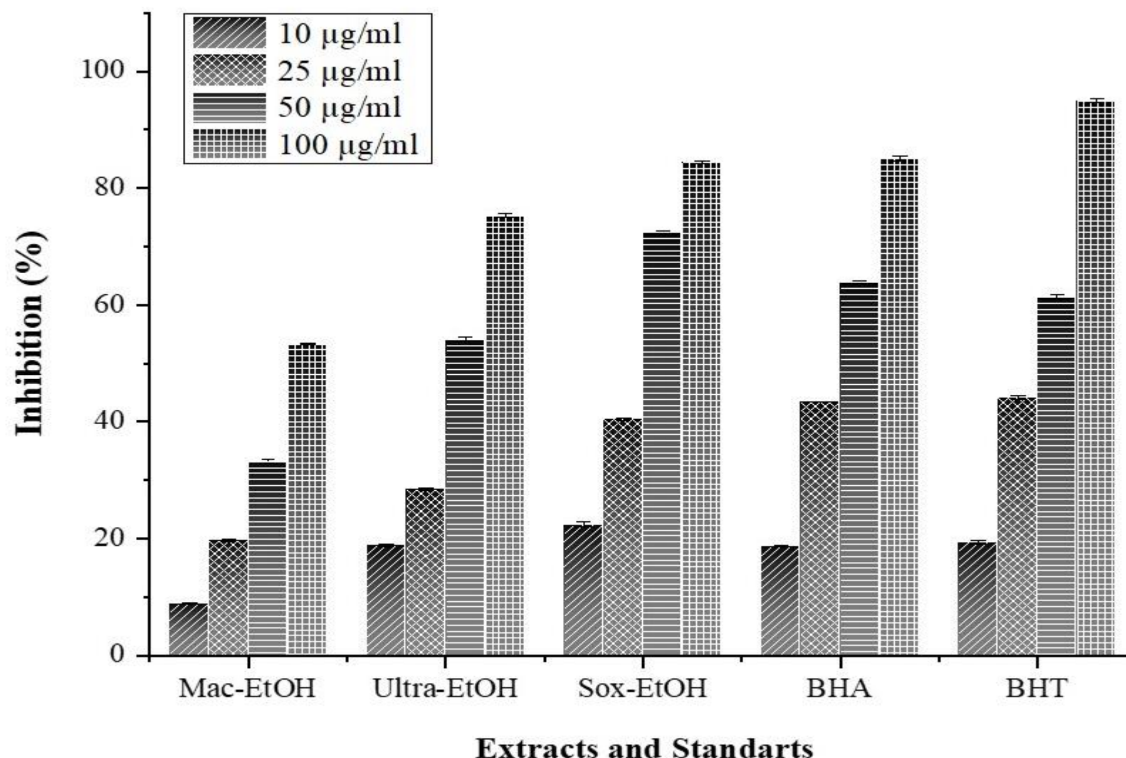

Fig. 1: DPPH radical scavenging activity graph. Mac-EtOH: Extract obtained using ethanol by maceration method, Ultra-EtOH: Extract obtained using ethanol by ultrasonic wave method, Sox-EtOH: Extract obtained using ethanol by the soxhlet method, BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene.

Result and Discussion

Extraction Yields

A. dioscoridis plant was extracted by three different extraction methods: maceration, ultrasonic wave extraction and soxhlet extraction. Ethanol was used as the solvent for all three methods. Yields were calculated as the amount of g extract obtained from 100 g of sample. A yield of 8.99 g/100 g was obtained with the maceration method and 13.20 g/100 g with the soxhlet method. A yield of 1.6 g/100 g in 5 min, 19.94 g/100 g in 15 min, 2.89 g/100 g in 30 min and 3.44 g/100 g in 60 min was obtained from ultrasonic wave extraction. The yields obtained from the extractions are collectively given in Table-1. The highest extraction efficiency was obtained from the ultrasonic wave extraction [28] method applied for 15 minutes. In the research, a more effective yield was obtained by using the ultrasonic wave extraction method compared to the soxhlet and maceration methods. It was observed that ultrasonic waves dissolved molecules in ethanol at a high rate.

Antioxidant Activity Results

DPPH free radical scavenging activity results

In the study, extracts were obtained by maceration, ultrasonic wave extraction and soxhlet extraction methods using ethanol. The inhibition effects of these extracts against DPPH free radical were investigated. The results of the extracts and standards are given in Fig 1.

The extract obtained by using the soxhlet method (Sox-EtOH) showed the highest inhibition effect with an inhibition power of 84.5% at a

concentration of 100 µg/mL. At the same concentration value, 75% inhibition potential was found with extract obtained by using ultrasonic wave method (Ultra-EtOH) and 53% inhibition potential was found with extraction method at room temperature (Mac-EtOH). Standard antioxidant BHA with 85% inhibition power and standard BHT with 95% inhibition power scavenged DPPH free radical. Compared with the standards, Sox-EtOH extract and Ultra-EtOH extract were found to be more effective than the maceration extract. When the IC_{50} values were examined, it was found that it was 3.94 for Mac-EtOH extract, 2.79 for Ultra-EtOH extract, and 2.27 for Sox-EtOH extract. The IC_{50} values of BHA and BHT standard antioxidants were determined as 2.37 and 2.29, respectively. These values showed that the extracts inhibited DPPH free radical as high as the standards.

CUPRAC results

The cupric ion reducing power helps to partially interpret the metal reduction potential of the phenolic compounds in the extract. BHA and BHT molecules were used as standards in the study. The cupric ion reducing power results of the extracts and standards are given in Fig 2. As absorbance values, BHA and BHT showed reduction potential with values of 3.3 and 2.8, respectively. With this method, Sox-EtOH and Mac-EtOH extracts showed very close inhibition potential with absorbance values of 1.8 and 1.79, respectively. Ultra-EtOH extract, on the other hand, exhibited a lower metal inhibition potential compared to other extracts with an absorbance value of 0.8. These evaluations were made at a concentration of 100 µg/mL.

Anti-aging Activity Results

To interpret the anti-aging potential of Mac-EtOH, Ultra-EtOH and Sox-EtOH extracts, elastase enzyme inhibition was applied. The elastase enzyme inhibitory results of the extracts and the positive standard are shown in Fig 3.

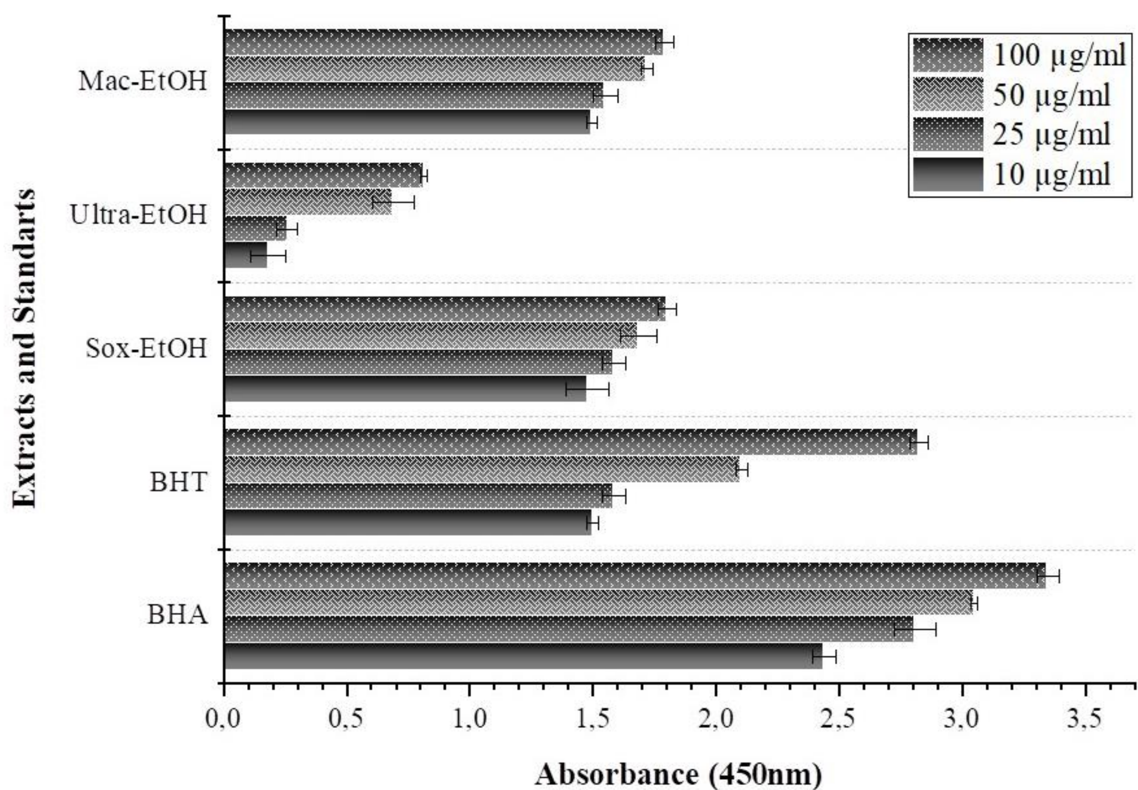

Fig. 2: Cupric ion reducing capacity graph. Mac-EtOH: Extract obtained using ethanol by maceration method, Ultra-EtOH: Extract obtained using ethanol by ultrasonic wave method, Sox-EtOH: Extract obtained using ethanol by the soxhlet method, BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene.

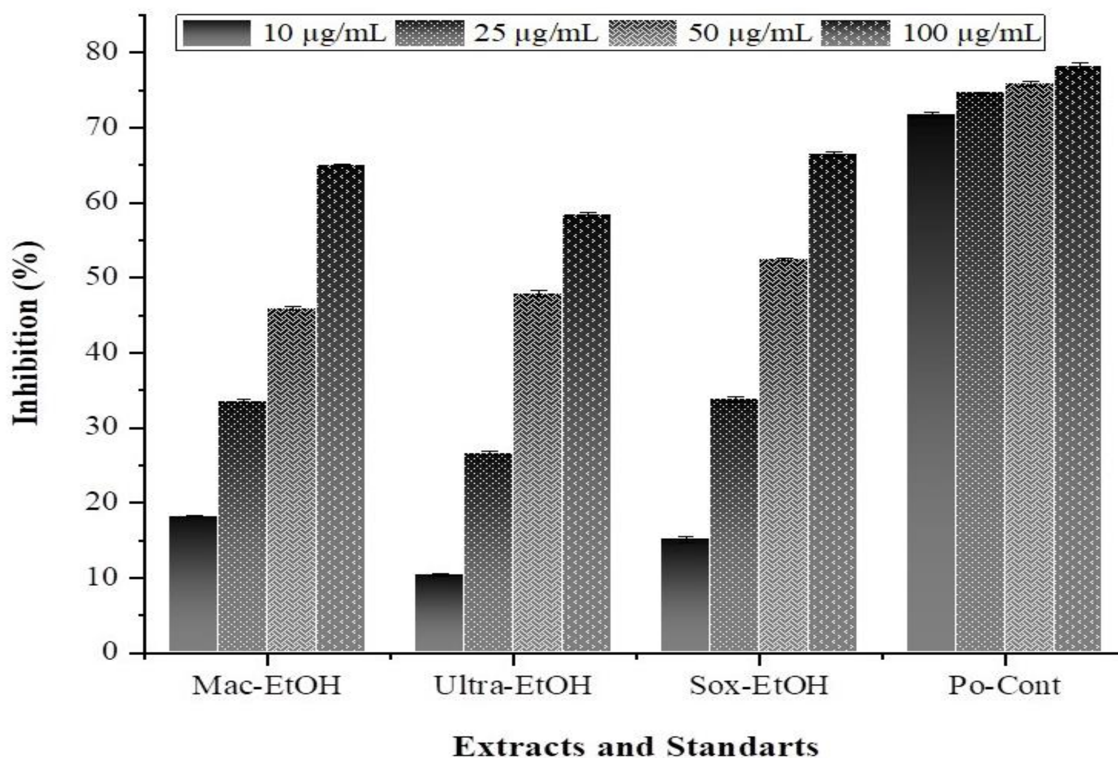

Fig. 3: Elastase enzyme inhibitory graph. (Mac-EtOH: Extract obtained using ethanol by maceration method, Ultra-EtOH: Extract obtained using ethanol by ultrasonic wave method, Sox-EtOH: Extract obtained using ethanol by the soxhlet method, Po-Cont: Positive control).

N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone was used as a positive control. Compared with the standard, Sox-EtOH and Mac-EtOH were found to inhibit the elastase enzyme at approximately the same rate. Ultra-EtOH extract was found to be less effective than the other two extracts. The IC_{50} value for Mac-EtOH was found to be 3.11, the IC_{50} value for Ultra-EtOH was 3.36, and the IC_{50} value for Sox-EtOH was found to be 2.96. When the IC_{50} values of all three extracts were examined, they were found to be quite successful in elastase enzyme inhibition.

In silico Prediction Analysis Results

The active components of the *A. dioscoridis* plant were identified as isoorientin, vitexin, caffeic acid, luteolin, apigenin, ferulic acid, esculin, quercetin and quercetin-3-O-β-glucoside [14]. Chemical structures of molecules were drawn using ChemDraw Professional 16.0 for in silico predictive analysis (Fig 4).

The hydroxyl and ketone groups in the structures of chemical molecules enable them to interact with other molecules. The active ingredients of *A. dioscoridis* were determined from the literature and their structural formulas are given in Fig 4.

The PASSonline software program is one of the most widely used web-based programs to predict the biological activities of chemical molecules [29, 30]. The results were obtained by applying PASSonline according to the structure-based comparison of the *in silico* antioxidant, antibacterial, anti-radical, anti-inflammatory, anti-mutagenic effects of the active molecules of *A. dioskoridis*. PASSonline results of active molecules are given in Table-2. Isoorientin showed the highest Pa values for antioxidant, antiradicalic and antimutagenic activity, while vitexin showed the highest Pa value for antiradicalic potential; caffeic acid, luteolin, apigenin and ferulic acid showed high antimutagenic activity. Quercetin showed high antioxidant, antiradical and antimutagenic activity, while Quercetin-3-O-β-glucoside provided high inhibition power against oxidation and free radicals. Quercetin and isoorientin were found to be the molecules with the most comprehensive effect.

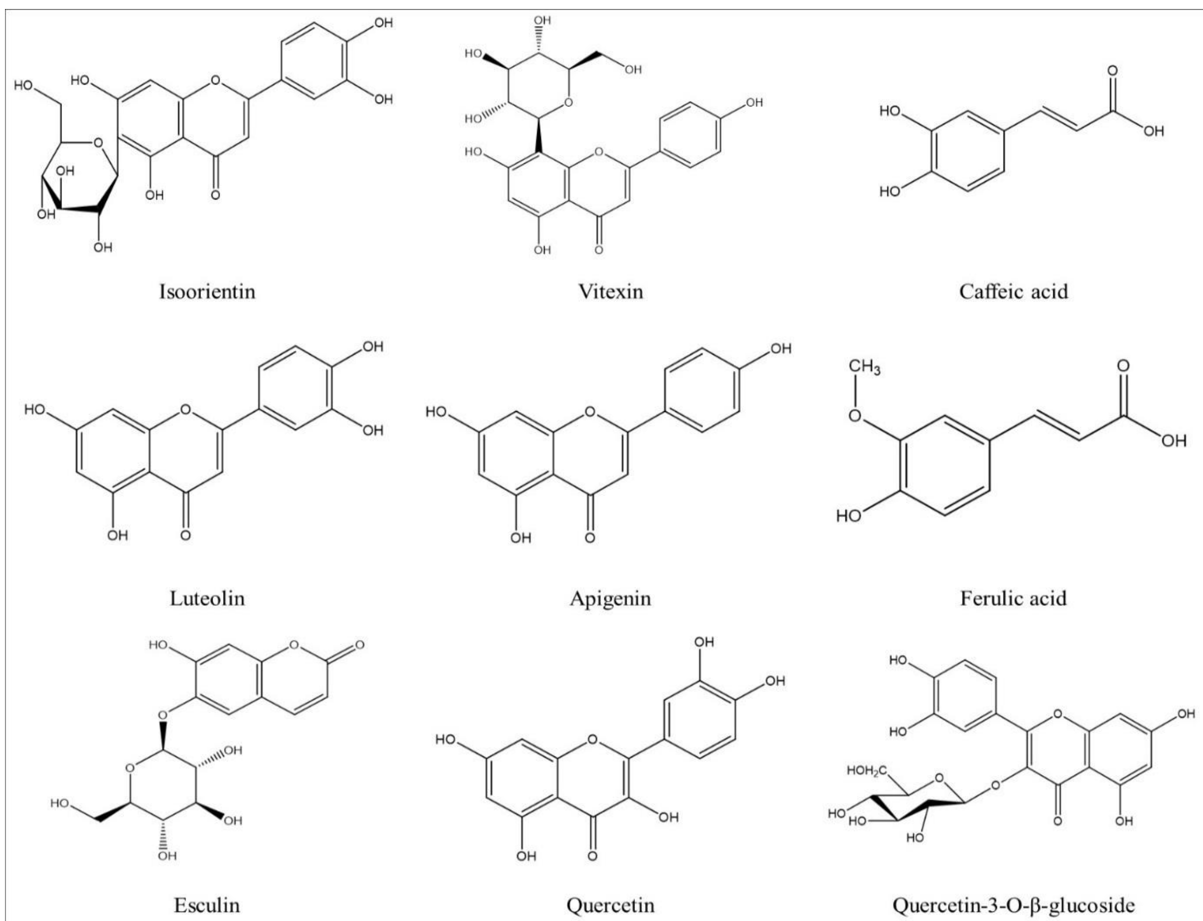

Fig. 4: Molecular structures of the major components of *A. dioscoridis.*

Table-2**:** Pharmacological activities predicted for major phenolic compounds of *A. dioscoridis* ethanolic extracts by PASSonline

Molecules	Antioxidant		Free radical scavenger		Antibacterial		Antifungal		Anti-inflammatory		Antimutagenic	
	Pa*	Pi**	Pa*	Pi**	Pa*	Pi**	Pa*	Pi**	Pa*	Pi**	Pa*	Pi**
Isoorientin	0,818	0,003	0,871	0,002	0,511	0,015	0,663	0,012	0,496	0,058	0,813	0,004
Vitexin	0,780	0,004	0,901	0,002	0,541	0,013	0,722	0,009	0,606	0,030	0,820	0,004
Caffeic acid	0,603	0,005	0,647	0,005	0,358	0,041	0,450	0,039	0,651	0,023	0,845	0,003
Luteolin	0,775	0,004	0,755	0,003	0,388	0,033	0,520	0,027	0,661	0,021	0,940	0,001
Apigenin	0,732	0,004	0,719	0,004	0,391	0,032	0,524	0,027	0,644	0,024	0,921	0,002
Ferulic acid	0,540	0,005	0,917	0,002	0,333	0,048	0,430	0,044	0,604	0,031	0,900	0,002
Esculin	0,788	0,003	0,941	0,001	0,620	0,008	0,705	0,009	0,733	0,012	0,547	0,012
Quercetin	0,872	0,003	0,811	0,003	0,387	0,033	0,490	0,032	0,689	0,017	0,940	0,001
Quercetin-3-O-β-glucoside	0,913	0,003	0,978	0,001	0,599	0,009	0,714	0,009	0,739	0,011	0,763	0,004

*Pa: Probable activity; **Pi: Probable inactivity. Pa > 700: probable activity greater than 70%. The PASS prediction results were interpreted and used as follows: (i) only activities with Pa > Pi are considered as possible for a particular compound; (ii) if Pa > 0.7, the chance to find the activity experimentally is high.

Using LAZAR (Table-3), it was observed that these substances, with the exception of quercetin and caffeic acid, are unlikely to have carcinogenic potential, and that quercetin may also be mutagenic. According to Table-4, research-related data via ADMETlab, it was determined that not all substances examined had carcinogenic potential and there was a possibility of skin sensitization to substances other than esculin and Quercetin-3-O-β-glucoside. It was concluded that quercetin is an inhibitor of CYP1A2 and CYP2C9, luteolin cannot inhibit CYP2C19, but it can be an inhibitor of CYP1A2, CYP2C9, CYP2D6, CYP3A4 together with apigenin. It was determined that substances other than quercetin, luteolin and apigenin could not inhibit these enzymes.

Table-3: Prediction of toxicity for major phenolic compounds of *A. dioscoridis* ethanolic extracts using LAZAR.

Molecules	Acute toxicity (Daphnia magna) (mmol/L) (mg/L)	Carcinogenicity (Rat)	Lowest observed adverse effect level (Rat) (mmol/kg_bw/day) (mg/kg_bw/day)	Mutagenicity (*Salmonella typhimurium*)
Isoorientin	CF	Non-carcinogenic	CF	Non-mutagenic
Vitexin	CF	Non-carcinogenic	CF	Non-mutagenic
Caffeic acid	0.103 18.6	Carcinogenic	CF	Non-mutagenic
Luteolin	0.0269 7.7	Non-carcinogenic	4.52 1290.0	Non-mutagenic
Apigenin	0.0404 10.9	Non-carcinogenic	4.34 1170.0	Non-mutagenic
Ferulic acid	0.0279 5.42	Non-carcinogenic	0.428 83.2	Non-mutagenic
Esculin	CF	Non-carcinogenic	2.9 986.0	Non-mutagenic
Quercetin	0.0151 4.56	Carcinogenic	6.73 2030.0	Mutagenic
Quercetin-3-O-β-glucoside	CF	Non-carcinogenic	4.45 2060.0	Non-mutagenic

CF: Could not find similar substances for threshold 0.2 with experimental data in the training dataset.

Table-4: Prediction of toxicity and pharmacokinetic properties for major phenolic compounds of *A. dioscoridis* ethanolic extracts using ADMETLab 2.0.

Molecules	Acute toxicity (Rat)	Carcinogenicity	Skin Sensitization	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
Isoorientin	---	---	++	---	---	---	---	---
Vitexin	---	---	+	--	---	---	---	---
Caffeic acid	++	--	+++	---	---	--	---	---
Luteolin	---	---	+++	+++	--	+	+	+
Apigenin	---	--	+++	+++	+	+	++	++
Ferulic acid	++	-	+++	---	---	--	---	---
Esculin	---	-	-	--	---	---	---	---
Quercetin	---	---	+++	+++	---	+	-	-
Quercetin-3-O-β-glucoside	---	---	-	--	---	---	---	---

Note: For the classification endpoints, the prediction probability values are transformed into six symbols: 0-0.1(---), 0.1-0.3(--), 0.3-0.5(-), 0.5-0.7(+), 0.7-0.9(++), and 0.9-1.0(+++).

CLC-Pred is a web-based tool developed for estimating cell line toxicity of plant active compounds. The tool is used in chemiinformatics and medicinal chemistry to predict cell line type and tissue based on the tumor type of interest. In the study, estimations were made for the compounds indicated as active in *A. dioscoridis* (Table-5). Predictive results with a Pa value of >0.5 indicate a more active state for the predicted cancer cell line. Nine compounds were examined for cytotoxic activity prediction in different cell lines using the CLC-Pred tool. Except for Quercetin and Quercetin-3-O-β-glucoside, seven compounds showed successful results (Table-5).

In silico predictions of different cell lines were evaluated by examining the respective cancer type, probability and cell type. Isoorientin showed a significant cytotoxic effect against promyeloblast leukemia cancer (0.576/HL-60) and non-small cell lung cancer (0.513/NCI-H838). Likewise, vitexin showed strong activity against promyeloblast leukemia cancer (0.696/HL-60). Caffeic acid exhibited potent cytotoxic activity against erythroleukemia cancer (0.538/K562) and ovarian adenocarcinoma (0.607/IGROV-1). Luteolin and apigenin both showed cytotoxic activity against oligodendroglioma cancer (0.523/Hs 683 and 0.587/Hs 683). Ferulic acid, like caffeic acid, showed a cytotoxic effect against both ovarian adenocarcinoma cancer (0.529/IGROV-1) and erythroleukemia cancer (0.542/K562). Esculin exhibited cytotoxic activity against promyeloblast leukemia cancer (0.529/HL-60) and non-small cell lung cancer (0.529/NCI-H838) as well as embryonic lung fibroblast cancer (0.556/WI-38 VA13).

Statistical analysis results

Table-6 shows the IC_{50} values of the extracts obtained using DPPH and ELAS methods. The IC_{50} values of the extracts were found to be close to the standards. It was concluded that all three methods were effective in extracting phenolic compounds. However, in terms of inhibitory power, Sox-EtOH extract showed the highest effect against both DPPH radicals and elastase enzyme. Since there was no significant difference between the IC_{50} values obtained, the groups were not compared with each other.

Table-5**:** Cancer cell line prediction for major phenolic compounds of *A. dioscoridis* ethanolic extracts using CLC-Pred.

Molecules	Pa	Pi	Cell line	Cell line full name	Tissue	Tumor type
Isoorientin	0.576	0.019	HL-60	Promyeloblast leukemia	Haematopoietic and lymphoid tissue	Leukemia
	0.513	0.043	NCI-H838	Non-small cell lung cancer. 3 stage	Lung	Carcinoma
Vitexin	0.696	0.009	HL-60	Promyeloblast leukemia	Haematopoietic and lymphoid tissue	Leukemia
Caffeic acid	0.607	0.010	IGROV-1	Ovarian adenocarcinoma	Ovarium	Adenocarcinoma
	0.538	0.021	K562	Erythroleukemia	Haematopoietic and lymphoid tissue	Leukemia
Luteolin	0.523	0.049	Hs 683	Oligodendroglioma	Brain	Glioma
Apigenin	0.587	0.029	Hs 683	Oligodendroglioma	Brain	Glioma
Ferulic acid	0.542	0.020	K562	Erythroleukemia	Haematopoietic and lymphoid tissue	Leukemia
	0.529	0.016	IGROV-1	Ovarian adenocarcinoma	Ovarium	Adenocarcinoma
Esculin	0.529	0.024	HL-60	Promyeloblast leukemia	Haematopoietic and lymphoid tissue	Leukemia
	0.529	0.036	NCI-H838	Non-small cell lung cancer. 3 stage	Lung	Carcinoma
Esculin*	0.556	0.018	WI-38 VA13	Embryonic lung fibroblast	Lung	ND
Quercetin	ND	ND	ND	ND	ND	ND
Quercetin-3-O-β-glucoside	ND	ND	ND	ND	ND	ND

*Non-tumor cell line prediction. ND: Not detected.

Table-6: Antioxidant and Anti-aging activity results of *A. dioscoridis* by the DPPH and Elastase inhibitory assays[a].

Samples	DPPH[b] IC_{50} (μg/mL)	ELAS[b] IC_{50} (μg/mL)
Mac-EtOH[c]	3,943 ± 0,68	3,112 ± 0,907
Ultra-EtOH[c]	2,797 ± 1,46	3,357 ± 1,425
Sox-EtOH[c]	2,273 ± 0,24	2,964 ± 0,198
BHA[d]	2,374 ± 0,52	NT
BHT[d]	2,296 ± 1,08	NT
Po-Cont[e]	NT	ND

[a]Statistical analysis for IC_{50} values were performed using One Way Anova test and Tukey test in GraphPad Prism 9.0 program. According to the results, no significant difference was found between the IC_{50} values of the DPPH group and the IC_{50} values of the ELAS group ($p > 0.05$, F=20137). [b]Methods: DPPH (DPPH free radical scavenging activity method) and ELAS (Elastase enzyme inhibitory activity method). [c]Extracts: Mac-ETOH (Extract obtained using ethanol by maceration method), Ultra-EtOH (Extract obtained using ethanol by ultrasonic wave method), Sox-EtOH (Extract obtained using ethanol by the soxhlet method). [d]Reference compounds: BHA (Butylated hydroxyanisole) and BHT (Butylated hydroxytoluene). [e]Positive control for elastase enzyme inhibition: N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone. ND: Not detected. NT: Not tested.

According to the Table-7, there were significant differences between Mac-EtOH, Ultra-EtOH, Sox-EtOH, BHA, BHT and Po-Cont groups when DPPH, ELAS and CUPRAC methods were applied to ethanol extracts obtained from *A. dioscoridis* ($p < 0,05$). Within the applied methods, significant differences between the groups showed which group was more active than the other group or groups. The p value allowed to determine whether there was a significant difference in the study.

In the DPPH method, there was a significant difference between the control group and Mac-EtOH, Sox-EtOH, BHA and BHT groups (Mean: 0.8000, $p < 0.05$), there was no significant difference with the Ultra-EtOH group ($p > 0.05$). There was a significant difference between the Mac-EtOH group and the BHT group (Mean: 0.3700, $p < 0.05$), there was no significant difference with the other groups ($p > 0.05$). There was a significant difference between Ultra-EtOH group and Mac-EtOH, Sox-EtOH, BHA and BHT groups (Mean: 0.7100, $p < 0.05$).

Significant differences were found between the control group and Mac-EtOH, Ultra-EtOH, Sox-EtOH and Po-Cont groups in the ELAS method (Mean: 3244.6667, $p < 0.05$). While a significant difference was found between the Ultra-EtOH group and Mac-EtOH and Po-Cont groups (Mean: 1702, 6667, $p < 0.05$), there was no significant difference with the other groups ($p > 0.05$). A significant difference was found between the Sox-EtOH group and the Mac-EtOH and Po-Cont groups (Mean: 1739, 3333, $p < 0.05$) but there was no significant difference with the other groups ($p > 0.05$).

Table-7: One Way ANOVA and Tukey test analysis using the absorbance values of the extracts and standarts read in DPPH free radical scavenging, elastase enzyme inhibition and CUPRAC activitiy methods.

Method - Group		N	Mean	Std. error	Source of Variance	Sum of Squares	df	Mean Square	F	p	Sig. Dif.
DPPH	1,00	3	0,8000	0,00000	Betw. Groups	1,570	6	0,262	43,718	0,000	1-2,4,5,6;
	2,00	3	0,3700	0,11533	Within Groups	0,084	14	0,006			2-6;
	3,00	3	0,7100	0,02082	Total	1,654	20				3-2,4,5,6.
	4,00	3	0,2933	0,00333							
	5,00	3	0,2233	0,00882							
	6,00	3	0,1267	0,01202							
	7,00	3	0,0000	0,00000							
	Total	21	0,3605	0,06275							
ELAS	1,00	3	3244,6667	128,54355	Betw. Groups	22542175,143	6	3757029,190	330,536	0,000	1-2,3,4,7;
	2,00	3	1403,6667	36,40665	Within Groups	159130,667	14	11366,476			3-2,7;
	3,00	3	1702,6667	29,16810	Total	22701305,810	20				4-2,7.
	4,00	3	1739,3333	87,46491							
	5,00	3	0,0000	0,00000							
	6,00	3	0,0000	0,00000							
	7,00	3	1372,0000	13,11488							
	Total	21	1351,7619	232,48812							
CUPRAC	1,00	3	0,0600	0,00000	Betw. Groups	10,890	6	1,815	660,582	0,000	2-1;
	2,00	3	0,4667	0,00882	Within Groups	0,038	14	0,003			3-1,2;
	3,00	3	0,8133	0,04910	Total	10,929	20				4-1,2,3;
	4,00	3	1,4533	0,02603							5-1,2,3,4;
	5,00	3	1,7900	0,05033							6-1,2,3,4.
	6,00	3	1,8067	0,02667							
	7,00	3	0,0000	0,00000							
	Total	21	0,9129	0,16131							

*N: Number of experiments, df: degree of freedom, F: Fisher ratio, p: probability.
**Statistical analyzes were made using absorbance values of the samples at a concentration of 100 mg/mL.
***DPPH: DPPH free radical scavenging activity method, ELAS: Elastase enzyme inhibitory activity method, CUPRAC: Cupric ion reducing antioxidant capacity method.
****Group 1: Control, Group 2: Mac-ETOH (Extract obtained using ethanol by maceration method), Group 3: Ultra-EtOH (Extract obtained using ethanol by ultrasonic wave method), Group 4: Sox-EtOH (Extract obtained using ethanol by the soxhlet method), Group 5: BHA (Butylated hydroxyanisole), Group 6: BHT (Butylated hydroxytoluene), Group 7: Po-Cont (Positive control as N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone).

When the CUPRAC method was examined, there was a significant difference between the Mac-EtOH group and the control group (Mean: 0.4667, $p < 0.05$), but there was no significant difference with the other groups ($p > 0.05$). There were significant differences between the Ultra-EtOH group and the Mac-EtOH and control groups (Mean: 0.8133, $p < 0.05$), but there was no significant difference with the other groups ($p > 0.05$). There were significant differences between the Sox-EtOH group and the Ultra-EtOH, Mac-EtOH and control groups (Mean: 1, 4533, $p < 0.05$), but there was no significant difference with the other groups ($p > 0.05$). There were significant differences between the BHA and BHT groups and the Sox-EtOH, Ultra-EtOH, Mac-EtOH and control groups (BHA and BHT Means respectively: 1.7900 and 1.8067, $p < 0.05$).

The DPPH molecule is a free radical. The molecule accepts an electron or a hydrogen radical to achieve stable structure. The DPPH free radical scavenging method is one of the widely used methods for the determination of antioxidant activity, and it is an easy, fast and sensitive method to investigate the antioxidant activities of phenolic compounds in plant extracts [31]. Most plant extracts have strong antioxidant potential due to their phyto-components such as phenolic acids and flavonoids [32, 33].

In this study, it was observed that *A. dioscoridis* extracts significantly reduced DPPH radicals at increasing concentrations (Fig. 1). The free radical scavenging activity of Sox-EtOH extract with an IC_{50} value of 2.273±0.24 µg/mL measured by the DPPH method (Table-2) was higher than that of Ultra-EtOH (IC_{50}=2.797 ± 1.46 µg/mL) and Mac-EtOH (IC_{50}=3.943 ± 0.68 µg/mL) extracts. found to be high ($p < 0.05$). It was determined that the IC_{50} values of BHT and BHA standards were lower than the Sox-EtOH extract and higher than the other two extracts. In addition, it was observed that *A. dioscoridis* extracts significantly inhibited the elastase enzyme at increasing concentrations (Fig 3). The elastase enzyme inhibition activity of the Sox-EtOH extract with an IC_{50} value of 2.964±0.24 µg/mL given in Table 6 was found to be higher than that of the Ultra-EtOH (IC_{50}=3.357 ± 1.425 µg/mL) and Mac-EtOH (3.112 ± 0.907 µg/mL) extracts ($p < 0.05$).

Karahan *et al.* [34] extracted the leaves of *A. dioscoridis* plant with acetone, ethanol, methanol and water. The antioxidant activity of the extracts they obtained were determined using the DPPH scavenging method and the iron reducing power method. They found that methanol and ethanol extracts showed the highest antioxidant effect. They determined that the ethanol extract showed 15% inhibitory power at a concentration

of 1 mg/mL and 5% inhibitory power at a concentration of 125 mg/mL. In the study, they showed 53% to 84% inhibition power using ethanol with three extraction methods. Therefore, it was concluded that the results of the research showed higher antioxidants. It was found that the reason for this was the aerial parts of the plant in this study, and only the leaves in their studies. Uguzlar *et al.* [15] extracted seeds of *A. dioscoridis* plant with methanol, acetone and hexane in their study. They investigated the antioxidant activity with the DPPH method and the β-carotene linoleic acid system. They found that methanol extract showed higher antioxidant effect than acetone and hexane extracts. They determined IC_{50} values of 3.03±0.5 mg/mL, 2.85±0.21 mg/mL and 0.50±0.19 mg/mL for hexane, acetone and methanol extracts, respectively [15]. When the IC_{50} results of the Uguzlar *et al.* [15] were compared with this study, it was observed that higher values were obtained from the hexane and acetone extracts, and lower values from the methanol extract. They used the DPPH and β-carotene linoleic acid system to determine antioxidant activity, while DPPH and CUPRAC methods were used in this study. While Uguzlar et al. applied the DPPH method for methanol, acetone and hexane extracts, this researcher applied it for ethanol extract in this study. In addition, while the seeds of the plant were examined in the study of the Uguzlar *et al.* [15], the aerial part of the plant was studied in this study. Both studies provided valuable information for *A. dioscoridis* to the literature.

Cu^{+2} reducing capacity of the extracts were applied to the CUPRAC method developed by Apak *et al.* [20]. When the organism's natural defenses are suppressed by the overproduction of reactive oxygen and nitrogen species, a state of oxidative stress occurs in which cellular and extracellular macromolecules can undergo oxidative damage and cause tissue damage [35]. In the study, at a concentration of 100 μg/mL, Mac-EtOH extract and Sox-EtOH extract reduced copper ions more than Ultra-EtOH extract. When the literature was examined, it was understood that the CUPRAC activity of the ethanolic extract of *A. dioscoridis* plant was performed for the first time in this study.

When the PASSonline results of nine main compounds identified in the ethanolic extract of *A. dioscoridis* plant were examined, it was determined that quercetin and isoorientin molecules had the highest Pa values as antioxidant, antiradicalic, antibacterial, antifungal, anti-inflammatory and antimutagenic. Quercetin and caffeic acid were detected as carcinogenic by LAZAR tool. In addition, Quercetin was found to be mutagenic. Other molecules in the study were detected as non-carcinogenic and non-mutagenic. Quercetin was also stated as carcinogenic and mutagenic. This showed that dose studies are needed for the Quercetin molecule in subsequent studies. According to the ADMETlab tool, it was determined that *A. dioscoidis* ethanol extract did not have carcinogenic molecules, but had molecules that affect skin sensitivity. It was also observed that some of the main components contained in it were able to inhibit CYP1A2, CYP2C19, CYP2C9, CYP2C6 and CYP3A4 enzymes. Molecules such as luteolin, apigenin and quercetin have been reported to be skin sensitive and to be inhibitors of CYP450 enzymes. Quercetin specifically inhibits CYP3A4 and CYP1A2 enzymes [36]. In this study, it was determined that quercetin molecule inhibited CYP1A2 and CYP2C9 enzymes. This showed that the study correlated with the literatures.

Chinnasamy and Arumugam selected 23 molecules from 20 plant species and interpreted their bioactivity in the PASS database [37]. They interpreted Pa values against many cell lines such as Stomach adenocarcinoma cells, gastric epithelial carcinoma cells, non-small cell lung carcinoma cells, Oligodendroglioma cells. In the study, the effect of phenolic and flavonoid compounds against cell lines similar to theirs was examined by interpreting Pa values. According to the results, it was determined that the molecules showed high activity against cancer cells in both studies.

There is no statistically significant difference between the IC_{50} values obtained from the DPPH free radical scavenging activity and elastase inhibitory activity methods ($p > 0.05$, F=20137). In the statistical analysis made with absorbance values, significant differences were found between Mac-EtOH, Ultra-EtOH, Sox-EtOH, BHA, BHT and Po-Cont groups when DPPH, ELAS and CUPRAC methods (F_{DPPH}: 43.718, F_{ELAS}: 330.536, F_{CUPRAC}: 660.582, $p < 0.05$).

Conclusions

A. dioscoridis plant is used in medical applications for various diseases. In the study, the extraction of the plant with three different methods and the antioxidant and anti-aging activities of these extracts were investigated. Higher efficiency was obtained with ultrasonic wave extraction method. The extracts were found to inhibit free radicals and the elastase enzyme. It was also found to have the ability to reduce copper ions.

PASSonline, LAZAR, ADMETlab and CLC-pred tools were used to examine the in silico biological activities and toxic profiles of the active molecules of *A. dioscoridis*. In addition to drug and cosmetic preparation development, pharmacological evaluation is also needed in the food field. Also, these tools are preferred to save time, money, protocol improvement and many animals used in the study. In addition, PASS supports the ethics of modification, reduction and improvement for an

animal used in research. Prediction supporting in vitro antioxidant activities were obtained in the PASSonline program. It was determined that some of the active molecules from the LAZAR and ADMETlab programs could show toxic, carcinogenic and mutagenic effects. It was found that the molecules could be further tested in leukemia and carcinoma cell lines.

As a result, it was concluded that the plant has antioxidant and anti-aging effects, but it is necessary to do dose studies in cell lines in vitro in order to determine the minimum toxic concentration. With the data to be obtained, it can be used in cosmetic products.

Acknowledgements

The study was carried out with the project number 1.2017/32 supported by the Scientific Research Unit of Bezmialem Vakif University. The author extends his sincere thanks to Bezmialem Vakif University for financial support.

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