

Evaluation of biological activities of onion from Turkey and determination of phytochemical contents

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ABSTRACT: *Allium cepa* L. belonging to Amaryllidaceae, is considered to be one of the most cultivated and consumed vegetable in the world. Besides nutritional value, as medicinal usage has also been reported. Onion has been cultivated widely and used as a vegetable mainly, in Turkey. Treatment of wounds, abscess, hemorrhoids, arteriosclerosis, gynecological problems, urinary inflammation, headache, cough by usage of onion and its diuretic and hypoglycemic activities are recorded in Turkish folk medicine. In this study, antimicrobial, antioxidant, anti-inflammatory activities and total phenolic-flavonoid contents of red, white and yellow onion samples from Ankara-Turkey, were investigated. The peels and tissues of onion samples were extracted by sonication with 80% ethanol at room temperature. Phytochemical contents were investigated by High Performance Liquid Chromatography. The highest amount of quercetin was detected in red onion peel (0.3169 mg/100 mg); and protocatechuic acid in yellow onion peel (0.3492 mg/100 mg). Determining the antimicrobial activities against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* by using microbroth dilution method, peel extracts exhibited higher activity than tissues. Yellow onion peel displayed the best 2,2'-azino bis (3-ethylbenzothiazoline-6-sulphonic acid) and 2,2-diphenyl-1-picrylhydrazyl scavenging activity (IC₅₀=0.0125 and 0.0433 mg/ml, respectively) and also human red blood cell membrane stabilization activity which was used as a marker of anti-inflammatory activity (IC₅₀=0.5363 mg/ml). Supporting these biological activities, the highest flavonoid content was determined in yellow onion peel (186.9250 mg QE/g crude extract). Additionally, red onion peel displayed the highest superoxide anion radical scavenging activity (IC₅₀=0.0987 mg/ml). All results have revealed that peel extracts possessed higher activity than tissues.

KEYWORDS: *Allium cepa*; anti-inflammatory activity; antimicrobial activity; antioxidant activity.

1. INTRODUCTION

Allium cepa L. (onion) belongs to the *Allium* genus from Amaryllidaceae family, is recorded around 918 species over the World. Only one bulb is produced by each onion plant, therefore the "Onion" word is originated from the "unio" word which refers to "one", "single" in Latin [1]. *A. cepa* has been cultivated for many years for both agricultural and medical purposes. From ancient times usage as medicinal plant for treatment of various illnesses such as digestive, skin and metabolic ailments, insect bites has also been reported [2, 3]. In ancient Greek it was consumed as blood cleanser by runners. Gladiators used onion juice to strengthen their muscles. Phoenician and Greek sailors used it to prevent scurvy. Additionally, Hippocrates prescribed onion for wound healing, diuretic, and management of pneumonia. In India, onion was defined as one of the significant spice, vegetable, and therapeutic plant [2]. Onion, is also used to treat infectious diseases, in Asian countries especially in India and Pakistan. In Turkish folk medicine, bulbs are used for abscesses, running sores, against hemorrhoids, arteriosclerosis, gynecological disorders, urine inflammation, headache and cough; seeds are added in some pastes to improve sexual capability; skins are used to dye wool yarns [4,5].

Many phytochemical studies revealed *A. cepa* is enriched in seconder metabolites including phenolic acids, flavonoids, organosulfide compounds, and these compounds are responsible for medicinal properties

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and specific flavour [6]. Many flavonoids including quercetin and quercetin glycosides, delphinidin 3,5-diglycosides, isorhamnetin 3,4'-diglucoside were determined in various onion types [7]. S-alk(en)yl-L-cysteine sulfoxides, a class of biologically active organo-sulfur compounds, dipropyl disulphide and dipropyl trisulfide were described as major components in onion oil by Vazquez-Armenta et al [8]. After crushing the bulb; diallyl sulphide and diallyl disulphide, allicin, iso-alliin, methiin, propiin are released. These compounds are also responsible for onion's characteristic flavour and smell. When onion is chopped, enzyme alliinase activated and irritating lachrymatory factor is released [9]. Thiopropal S-oxide, another sulfur volatile, is a lachrymator factor that converts to methylpentanols which is an another tear up factor and exists in only onion [10]. Additionally, tartaric, ascorbic, oxalic, malic, succinic and citric acid were detected in onion extracts [7]. Following the alkenyl-cysteine sulfoxide enzymatic degradation, pyruvic acid occurs and pungency of onion is measured by pyruvic acid content [11]. According to the previous phytochemical studies significant differences were observed phenolic as well as organosulfur contents of onion types [6].

A. cepa is known as a potent antimicrobial agent for infectious diseases. The principle compounds in onion, responsible for antimicrobial activity were found as sulfur compounds [12]. On the other hand, allicepin (an antifungal compound) was isolated from onion [13]. *A. cepa* contains other antimicrobial phenolic substances such as protocatechuic acid, ferulic acid, p-coumaric acid and catechol. Kaempferol and quercetin were proved to contribute to this activity [14]. *cis*-2,3-Dimethyl-5,6-dithiabicyclohexane 5-oxide (Zwiebelane A) is another compound isolated from *A. cepa* bulbs and was observed to increase the potential activity of fungicidal antibiotic Polymyxin B [15]. Antidiabetic activity has also been reported by several preclinical studies. Sulfur containing compounds were suggested as responsible compounds for antidiabetic activity. S-methylcysteine sulfoxide (SMCS) and S-allylcysteinesulfoxide were found to be active on pancreas and induce increasing on blood insulin levels by Akash et al [16]. Moreover, *A. cepa* displayed antihyperlipidemic activity by decreasing total lipoprotein lipase activity and serum cholesterol level in adipose tissue, the free fatty acid level in tissue and serum [7,17].

A. cepa which is one of the oldest cultivated and the most used vegetable in the world has importance as vegetable due to its nutritional properties which is strongly correlated with phytochemical content (water, sugar, protein, fats, fiber, vitamin and mineral) and as medicinal plant because of the biological activities. Onion has several varieties with red, yellow and white colours and phytochemical content of onion differs due to variety. Phytochemical content which is directly correlated with biological activity of the onion can also vary due to different factors including genotype (cultivar and variety), geographical region, climatic conditions (sun exposure, rainfall, etc.), soil microenvironment, agricultural conditions, pre-harvest and post-harvest factors, degree of ripeness [18,19]. Therefore, present study aimed to investigate biological activities and phytochemical contents of red, yellow and white onion samples supplied from Turkey-Ankara region, where is one of the important culture area of the onion in Turkey.

2. RESULTS

Crude extract quantities and amounts of plant material used for extraction were given in Table 1. Our results have revealed that *A. cepa* samples supplied from Ankara-Turkey displayed significant antimicrobial, antioxidant and anti-inflammatory activities. 2,2'-azino bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide (SO) radical scavenging activities were utilized to determine antioxidant activity of the extracts. IC₅₀ values were calculated for per extract and compared with standard compound butylated hydroxytoluene (BHT) as shown in Table 2.

Table 1. Crude extract quantities with amounts of plant material.

		Plant material (g)	Crude extract (mg)
Yellow onion	Peel	5.26	672.32
	Tissue	5.17	2703.44
Red onion	Peel	2.93	526.56
	Tissue	5.13	3108.03
White onion	Peel	3.06	359.00
	Tissue	23.46	3291.66

Table 2. ABTS, DPPH and SO radical scavenging activities of *A. cepa* extracts (IC₅₀).

Extracts		ABTS-IC ₅₀ (mg/ml)	DPPH-IC ₅₀ (mg/ml)	SO-IC ₅₀ (mg/ml)
Yellow onion	Peel	0.0125±0.0008*	0.0433±0.0026*	0.2290±0.0285*
	Tissue	0.1454±0.0061*	1.6351±0.0457*	0.8846±0.0578*
Red onion	Peel	0.0126±0.0015*	0.0559±0.0047*	0.0987±0.0155*
	Tissue	0.1288±0.0075*	1.4433±0.1221*	0.2724±0.0169*
White onion	Peel	0.0225±0.0005*	0.1071±0.0149*	0.5625±0.0399*
	Tissue	0.0482±0.0003*	0.6003±0.0028*	2.3032±0.1771*
BHT		0.0017±0.0001*	0.0092±0.0005*	0.0600±0.0051*

(*) Statistically significant as compared to control, p<0.05. Each test repeated at least three times. Data represent mean±SD.

Anti-inflammatory activity of the samples was recorded by measuring protective capacity against human red blood cells (HRBC) membrane hemolysis. IC₅₀ values of the extracts were calculated and compared with each other and standard compound acetylsalicylic acid (ASA). All results were given in Table 3.

Table 3. Human red blood cell membrane stabilizing effects of *A. cepa* extracts (IC₅₀).

Extracts		IC ₅₀ (mg/ml)
Yellow onion	Peel	0.5363±0.0475*
	Tissue	14.0121±0.2479*
Red onion	Peel	0.8909±0.0307*
	Tissue	18.6185±0.2469*
White onion	Peel	2.1289±0.0217*
	Tissue	7.4782±0.0967*
ASA		0.3289±0.0215*

(*) Statistically significant as compared to control, p<0.05. Each test repeated at least three times. Data represent mean±SD.

Among the extracts, peel extract of red onion showed the best antibacterial activity against tested bacteria. Considering to results, it can be said that peel extracts usually exhibited higher antibacterial activity than tissue extracts. However, the effect can be considered as weak compared to standard antibiotics (Table 4).

Table 4. Minimum inhibitory concentration (MIC) values (mg/ml) of the *A. cepa* extracts against tested bacteria.

Extracts		Test bacteria			
		<i>S. aureus</i> ATCC 29213	<i>E. faecalis</i> ATCC 29212	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853
Yellow onion	Peel	75	75	75	75
	Tissue	150	150	-	150
Red onion	Peel	9.4	18.8	18.8	18.8
	Tissue	150	150	150	150
White onion	Peel	75	75	75	75
	Tissue	37.5	75	150	150
Ampicillin		0.0003	0.0005		
Ofloxacin				0.001	0.008

"-"= represents no activity

Standard compounds, quercetin, kaempferol, rutin, hyperoside, isoquercitroside, quercitroside, orientin, isoorientin, protocatechuic acid, ferulic acid, gallic acid, *p*-coumaric acid, were analyzed by High Performance Liquid Chromatography (HPLC) qualitatively and quantitatively. Among these compounds only quercetin and protocatechuic acid were detected in peel extracts of yellow and red onions while white onion tissue extract was found to contain only quercetin. Limit of detection (LOD) and limit of quantification (LOQ) values were determined for two compounds and given in Table 5. The highest amount of protocatechuic acid was determined in yellow onion peel extract while quercetin was found highest in red onion peel extract (Table 5).

Table 5. Quantity of quercetin and protocatechuic acid in *A. cepa* extracts, LOD and LOQ values of quercetin and protocatechuic acid (mg/100 mg).

Extracts		Quercetin	Protocatechuic acid
Yellow onion	Peel	0.2946±0.0002	0.3492±0.0002
Red onion	Peel	0.3169±0.0003	0.2227±0.0001
White onion	Tissue	0.0132±0.0000	-
LOD		0.00009	0.00008
LOQ		0.00030	0.00020

“-”= not detected

Total phenolic contents of the extracts were calculated as mg gallic acid equivalent (GAE) for per g crude extract and total flavonoid contents were presented as mg quercetin equivalent (QE) for per g crude extract, in this study (Table 6). It has been observed that all peel extracts have higher phenolic and flavonoid contents than tissue extracts.

Table 6. Total phenolic and total flavonoid content of *A. cepa* extracts (mg GAE/ g crude extract and mg QE/g crude extract).

Extracts		Total phenolic content mg GAE/ g crude extract	Total flavonoid content mg QE/ g crude extract
Yellow onion	Peel	14.1825±0.2443	186.9250±0.2830
	Tissue	0.9325±0.0427	13.5000±0.0258
Red onion	Peel	11.5575±0.0350	118.3750±0.3376
	Tissue	0.7575±0.2165	15.3750±1.1236
White onion	Peel	27.1491±0.7234	29.8500±0.9416
	Tissue	5.9325±0.1984	21.1750±0.5560

Each test repeated at least three times. Data represent mean±SD.

3. DISCUSSION

A. cepa has been proposed to obtain numerous biological properties such as antimicrobial, anti-inflammatory, antioxidant, antiplatelet, anti-diabetic, analgesic, hypolipidemic and antihypertensive activity as well as the protective effect against the cardiovascular diseases and cancer risk including breast, colon, skin and cervix cancer [12].

According to ABTS and DPPH tests, onion peel extracts exhibited higher activities than tissue extracts. Extracts with the highest and lowest activity for both methods showed correlation. The antioxidant capacities of the extracts examined by scavenging effects on ABTS and DPPH free radicals. The results were found as yellow onion peel > red onion peel > white onion peel > white onion tissue > red onion tissue > yellow onion tissue. The maximum ABTS and DPPH scavenging activity was measured in yellow onion peel extract with the IC₅₀ values of 0.0125 and 0.0433 mg per ml, respectively. Peel extract of red onion followed yellow onion peel with the IC₅₀ value of 0.0559 mg per ml for DPPH and 0.0126 mg per ml for ABTS radical scavenging activity. The lowest activity was found in yellow onion tissue both DPPH (IC₅₀ = 1.6350 mg/ml), and ABTS (IC₅₀ = 0.1454 mg/ml) methods. Additionally, in SO method red onion peel extract displayed the highest potential with the IC₅₀ value of 0.0987 mg/ml which is similar to standard compound, BHT (IC₅₀ = 0.0600 mg/ml). Secondary inhibitory activity was observed in yellow onion peel (IC₅₀ = 0.2290 mg/ml). At 0.5 mg/ml concentration levels, red onion peel showed 94.93%, white onion peel 44.42%, red onion tissue 86.85% and yellow onion tissue 49.48% inhibitor activity. White onion tissue did not exhibit any inhibitory activity at the same concentration. Moreover, the lowest inhibitory activity was shown in white onion tissue (IC₅₀ = 2.3032 mg/ml).

According to recent studies, *A. cepa* displays antioxidant activity by different mechanisms including radical scavenging activity, inhibiting xanthine oxidase activity, increasing plasma superoxide dismutase and glutathione peroxidase activities [20, 21]. Qadir et al. tested methanolic, ethanolic, acetone and aqueous extracts of onion bulbs in DPPH assay and found IC₅₀ values in 58.4-67.6 µg/ml range with the highest activity in aqueous extract and the lowest with ethanolic extract [22]. 70% ethanolic and aqueous extracts of red and yellow onion peels were compared in terms of their DPPH and ABTS radical scavenging capacity. IC₅₀ values belonging to DPPH method was found as 10.60 and 9.86 µg/ml for red onion peel's ethanolic and water extract

while for yellow onion extracts were 4.50 and 6.77 $\mu\text{g}/\text{ml}$. ABTS radical scavenging capacity of ethanolic and aqueous extracts of yellow and red onion peels were measured as 6.64, 12.03, 7.00, 29.04 $\mu\text{g}/\text{ml}$ (IC_{50}), respectively [23].

Nuutila et al. compared antioxidant activity of yellow and red onions peel and tissue by using lipid peroxidation and DPPH radical scavenging inhibition methods. While extract of yellow onion peel (80 mg/ml) inhibited lipid peroxidation 20-80% in rat hepatocytes; extract of yellow onion peel (1000 mg/ml) inhibited 40%. According to DPPH radical scavenging assay, extracts of red onion showed better activity than yellow onion extracts and peel extracts exhibited higher activity than tissue extracts [24].

Anti-inflammatory activity results indicated membrane stabilizing capacity of onion peels was higher than tissues. Among all extracts, the best activity was observed in yellow onion peel with 0.5363 mg/ml IC_{50} value followed by red onion peel ($\text{IC}_{50} = 0.8909$ mg/ml). The lowest anti-inflammatory activity was observed in red onion tissue extract with IC_{50} value of 18.6185 mg/ml.

Anti-inflammatory and analgesic activities have also been tested by carrageenan-induced paw edema method and formalin test. Formation of thromboxanes and leukotrienes by inhibition of lipoxygenase (LOX) and cyclooxygenase (COX) pathways are suggested as mechanisms of anti-inflammatory activity [25].

Numerous studies have been indicated antimicrobial activity of different onion types and extracts. Ethanol extract, aqueous extract and essential oil of red onion inhibited *Salmonella typhimurium* [26]. Benkeblia tested antimicrobial activity of onion essential oil on *Aspergillus niger*, *Fusarium oxysporum*, *Penicillium cyclopium*, *Salmonella enteritidis*, *Staphylococcus aureus*. Antibacterial activity of red onion oil showed higher activity than yellow and white onion oils [27]. Methanolic extracts of white and red onion inner and outer layers showed inhibitory effects on *E. coli*, *P. aeruginosa* and *S. aureus* [28].

HPLC analyses showed while yellow onion peel and red onion peel extracts contain protocatechuic acid, white onion tissue extract was found to contain quercetin.

Previous studies demonstrated that different types of onions contain phenolic acids such as citric, ascorbic, malic, tartaric, succinic, oxalic, ferulic, p-coumaric, synapic, p-hydroxybenzoic, chlorogenic acid and flavonoids including quercetin, quercetin-3-glucoside, quercetin-4'-glucoside, quercetin-3,4'-diglucoside, quercetin-7-glucoside, quercetin-7,4'-diglucoside, quercetin-3,7-diglucoside, quercetin-3,7,4'-triglucoside, quercetin-3-rutinoside, quercetin-3-rhamnoside, kaempferol, kaempferol-4'-glucoside, kaempferol-3-glucoside, kaempferol-7,4'-diglucoside, isorhamnetin-3,4'-diglucoside [6, 29].

The highest value for phenolic content was shown in white onion peel extract (27.1491 mg GAE/g crude extract) followed by yellow onion peel extract (14.1825 mg GAE/g crude extract) and red onion peel extract (11.5575 mg GAE/g crude extract), respectively. The highest total flavonoid content was found in yellow onion peel extract (186.9250 mg QE/g crude extract) followed by red onion peel extract (118.3750 mg QE/g crude extract) and white onion peel extract (29.8500 mg QE/g crude extract). Total phenolic, flavonoid content of all onion peel extracts were found higher than tissues. Benmalek et al. measured total flavonol content of inner and outer layers of white and red onions. While the minimum flavonol content was observed in inner layers of white onion (in significant), outer layers of red onion were detected as 2.37 $\mu\text{g}/\text{g}$; outer layers of white onion 17.3 $\mu\text{g}/\text{g}$ and inner layers of red onion 17.3 $\mu\text{g}/\text{g}$ [28].

The usage of onion for its antimicrobial and antioxidant properties is particularly important in folk medicine which have been corroborated by many literature data. On the other hand, the potential effects of diabetes, inflammation and Alzheimer's treatment are also very noticeable. Researches have revealed that there are some differences in the biological activity results of the extracts prepared from the *A. cepa*. Antimicrobial activity is one of the most studied activity, which even the activity results on the same microorganism races produce quite different results. As with all plant species, it is thought that phytochemical content varies depending on factors such as the region where the *A. cepa* grows, climate conditions, soil type, process during and after harvest. As mentioned earlier the onion has also different varieties as: red, yellow and white with different chemical contents. For these reasons, in this study it is aimed to investigate *in vitro* biological activities and examine phytochemical ingredients of white, yellow and red onion varieties supplied from Ankara-Turkey.

Although some activity studies were found, there has not been any studies in which both activity and phytochemical content were evaluated for onion samples from Turkey. The findings exhibited that all three onion extracts have remarkable biological activities. Peel extracts have been detected as the higher than the tissue extracts. The red and yellow onion peel extracts were detected as the highest for their DPPH, SO and ABTS radical scavenging activities. It has been observed that the peel extract of yellow onion displayed higher anti-inflammatory activity than other extracts. Additionally, the red onion peel extract has the highest

antimicrobial activity against tested microorganisms. Phytochemical studies have revealed that, white onion peel extract and yellow onion peel extract has phenolics and flavonoids in high amount respectively when compared with the other extracts. Quercetin and protocatechuic acid amounts of the extracts were determined by validated HPLC analysis and quantified as the highest in red and yellow onion peel extracts, respectively.

4. CONCLUSION

In conclusion, our results are supported to the findings of literature. The biological activities of onions are suggested to closely related with their phytochemical contents including flavonoids. Onion can improve human health owing to high biological activities derived from the crucial phytochemical compounds it contains.

5. MATERIALS AND METHODS

5.1. Plant material

Plant materials (red, white, yellow onion) were bought from local market from Ankara, Turkey. Peel and tissue were separated, dried at room temperature and powdered.

5.2. Chemicals

Quercetin, kaempferol, rutin, hyperoside, isoquercitrin, quercitrin, orientin, isoorientin, ferulic acid, gallic acid, p-coumaric acid, protocatechuic acid; as standard compounds for HPLC analyses were bought from Sigma-Aldrich and Fluka companies. Acetylsalicylic acid (ASA), aluminium chloride, butylated hydroxytoluene (BHT), potassium persulfate, Folin&Ciocalteu's phenol reagent, sodium chloride, sodium nitrite, sodium carbonate, trolox, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), dimethyl sulfoxide (DMSO) were bought from Sigma-Aldrich. Ethanol, methanol, acetonitrile and phosphoric acid were bought from Merck and Fluka.

5.3. Extraction

Dried plant materials were extracted with ethanol:water (80:20) mixture at room temperature during 24 hours by maceration, then stirred in an ultrasonic bath for 30 minutes and filtered. This procedure was repeated 3 times. Filtrates were combined and vaporized under vacuum at 40 °C then lyophilized to obtain crude extracts. The yield of the extracts prepared from plant materials were given in Table 1.

5.4. Phytochemical analyses

5.4.1. High performance liquid chromatography (HPLC)

HPLC analyses were observed by using Agilent LC 1260 (Agilent Technologies, Darmstadt, Germany). The diode array detector was used with 210 and 270 nm wavelengths; peak areas were integrated using Agilent Software. ACE 5 C18 column (250 mm; 4.6 mm; 5 µm) was accepted for separation and heated to 40°C. The solvent flow was set to 1ml/min and injection volume 10 µl. Gradient elution of three solvents were used; Solvent A (0.2% phosphoric acid in water), Solvent B (Acetonitrile) and Solvent C (Methanol). Eluting was started with A:B (90:10); followed by A:B (50:50) with linear change during 36 minutes and from min 36.01 to min 40 isocratic flow was used with Solvent C.

Extracts were dissolved in mixture of water:methanol mixture (1:1), concentrations were set to 25 mg/ml. Standards were dissolved in methanol and injected to HPLC with 7 different concentrations (0.01; 0.025; 0.05; 0.1; 0.25; 0.5; 1 mg/ml) then calibration curves were made up.

5.4.2. Determination of total phenolic contents of the extracts

Total flavonoid content

Total flavonoid content of the extracts was examined by aluminum chloride colorimetric method on 510 nm using a microplate reader [30]. Total amount of flavonoids was calculated as quercetin equivalent (QE) per g crude extract on the basis of a standard curve of quercetin.

Total phenolic content

Folin-Ciocalteu method was utilized to determine total phenolic content of the extracts. Wavelengths were measured at 765 nm using a microplate reader [31]. Total amount of phenolics was expressed as gallic acid equivalent (GAE) per g of crude extract on the basis of a standard curve of gallic acid.

5.5. Biological activity tests

5.5.1. Antioxidant activity

Strong free radical scavenging activity is directly related to higher antioxidant potential. Due to the fact that the scavenging ability of DPPH, ABTS and SO free radicals of the plant extracts are widely used to analyze the antioxidant potentials [14, 22, 32].

DPPH free radical scavenging activity

The activity of antioxidant substances on DPPH free radical is determined because of their donating ability of hydrogen. Due to the fact that, scavenging ability of the plant extracts on DPPH free radical was assessed [33]. In briefly; various concentrations of extracts were mixed thoroughly with DPPH in methanol (100 μ M). The mixture was stand for 30 minutes and incubated in the dark and at the room temperature. Then the absorbance of the samples was determined at 517 nm using a microplate reader. BHT was assessed as a standard compound. Each experiment was repeated in three times. The percentage inhibition values of DPPH by the samples and then the half maximal inhibitory concentrations (IC_{50}) were calculated (Eq. 1). The results were described as mean $IC_{50} \pm$ Standard Deviation (SD).

$$\text{percentage of inhibition} = [(A_{\text{control}} - A_{\text{standard/sample}}) / A_{\text{control}}] \times 100 \quad (\text{Eq. 1})$$

ABTS free radical scavenging activity

The antioxidant activity of the extracts on ABTS free radical was investigated as described previously [34]. ABTS radical solution was formed by reacting aqueous solution of ABTS (7 mM) and potassium persulfate (2.45 mM). The reaction mixture was left to stand overnight at room temperature (in the dark). ABTS solution was diluted until the absorbance was 0.700 ± 0.02 at 734 nm with ethanol. Various concentrations of extracts and ABTS working solution was stirred, and then stand for 6 min. The absorbance was measured at 734 nm using a microplate reader. BHT was chosen as a standard compound. Each experiment was repeated in triplicate. The percentage inhibition of ABTS by the samples was calculated. Then IC_{50} values were calculated (Eq. 2) and the results were described as mean $IC_{50} \pm$ SD.

$$\text{percentage of inhibition} = [(A_{\text{control}} - A_{\text{standard/sample}}) / A_{\text{control}}] \times 100 \quad (\text{Eq. 2})$$

Superoxide anion radical scavenging activity

Cytochrome c is reduced by reaction with superoxide. Superoxide anion radical scavenging activity of extracts was performed according to the method of McCord and Fridovich with small modifications [35]. The reaction mixture was comprised of phosphate buffer (pH=8.9), cytochrome C (50 mM), xanthine (50 μ M), xanthine oxidase (0.32 units/ml) and various concentrations of samples. Xanthine oxidase was put into this reaction mixture and then the reaction started. The mixture was incubated at 30°C and the absorbance was measured at 550 nm using a microplate reader for cytochrome C reduction. BHT was utilized as a reference compound. Each experiment was repeated in triplicate. The percentage inhibition of superoxide radical by the samples was calculated according to Eq. 3. The results were described as mean $IC_{50} \pm$ SD.

$$\text{percentage of inhibition} = [(\Delta A_{\text{control}} - \Delta A_{\text{standard/sample}}) / (\Delta A_{\text{control}} - \Delta A_{\text{blank}})] \times 100 \quad (\text{Eq. 3})$$

5.5.2. Anti-inflammatory activity

In vitro human red blood cell membrane stabilization method was used to determine anti-inflammatory activity of samples [36]. The protocol was reviewed and approved by Ankara University Faculty of Medicine Human Research Ethics Committee (14.05.2020/I5-273-20). Human blood was collected from healthy volunteers, care to fresh blood. It was noted that the volunteers did not take any anti-inflammatory, analgesic or steroid medications for 4 weeks before the experiment. The collected blood was centrifuged. Then the packed cells were washed with sterile isosaline (0.85%, pH=7.2). Various concentrations of samples and 10% red blood cell suspension was mixed and then incubated at 56°C for 30 minutes. Afterwards, the temperature

of the tubes was reduced to room temperature and the tubes were centrifuged at 2500 rpm for 5 min. The absorbance was determined at 560 nm using a microplate reader. ASA was used as a reference compound. Each experiment was repeated in triplicate. The percentage protection of cell membrane by the samples was calculated (Eq. 4). The results were described as mean $IC_{50} \pm SD$.

$$\text{percentage of protection} = 100 - [(A_{\text{standard/sample}} / A_{\text{control}}) \times 100] \quad (\text{Eq. 4})$$

5.5.3. Antibacterial activity

The following bacterial strains were utilized for determining antibacterial activity: Gram (+) bacteria: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213. Gram (-) bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853.

Minimum inhibitory concentrations (MIC) of the extracts was determined by micro-broth dilution method [37,38]. Bacterial cultures were obtained in Mueller Hinton Broth (Difco, Difco Laboratories, Detroit, MI, USA) and serial two-fold dilutions (ranging from 150 mg/ml to 1.2 mg/ml) were prepared in the medium. A set of wells containing only inoculated broth, ampicillin and ofloxacin were utilized as control. The mixtures were incubated for 18-24 hour at $35 \pm 1^\circ\text{C}$. The last tube with no microbial growth was documented to represent MIC value.

5.6. Statistical analysis

Experimental data were analyzed statistically using SPSS v25.0 software. Each experiment was conducted at least in three times. The results were presented as mean \pm SD. Oneway analysis of variance (ANOVA) with Tukey post-test was performed to compare the biological activities of the samples. Statistically significant was stated as p values < 0.05 .

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