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In vitro enzyme inhibitory properties, antioxidant activities, and phytochemical studies on *Juniperus drupacea*

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ABSTRACT: *Juniperus drupacea* Labill. is used for medicinal purposes and to obtain a traditional food product "pekmez" for centuries. We aimed to evaluate antidiabetic and antioxidant activities of the extracts prepared from *J. drupacea* fruits, leaves and branches. Therefore, *a*-amylase and *a*-glucosidase inhibitory effects and antioxidant activities of the water, ethyl acetate, and methanol extracts were evaluated. Additionally, total phenolic and total flavonoid contents of the extracts were investigated. Standardization of the methanol extracts on amentoflavone and umbelliferone was performed using a RP-HPLC-DAD method. All extracts showed excellent and dose dependent inhibitory effect on *a*-glucosidase enzyme. Moreover, fruit methanol extract (99.92 ± 0.30%) and leaf methanol extract (99.44 ± 1.78%) were more effective than reference drug Acarbose (98.88 ± 0.07%) at 1 mg/ml concentration. On the other hand, the extracts rich in flavonoids and phenolics showed remarkable antioxidant activity. Results of HPLC analysis revealed that amentoflavone was detected in serious amount especially in the leaves as 0.148±0.001 g/100 g dry weight. Umbelliferone was determined in minor amount in both leaves and branches.

KEYWORDS: *Juniperus*; Cupressaceae; antidiabetic; antioxidant; amentoflavone.

1. INTRODUCTION

Juniperus drupacea Labill. (Cupressaceae) is native to the Eastern Mediterranean Region and distributed in Greece, Turkey, Syria, Lebanon and Israel [1]. It is called "andız" in Turkey and used in folk medicine as well as source of pix and a molasses called "pekmez" [2]. Fruits of the plant are used as traditional medicine to treat helminthes infections, stomachache, against hemorrhoids and decoction of fresh shoots is used for urinary inflammations, gout and to treat abdominal pain [3-6]. The pix of *J. drupacea* is prepared by burning the stems of the plant and is used topically for alopecia, eczema and wounds; internally for cold, cough, urinary infections and to treat diarrhea [2, 5]. Andız pekmezi is a kind of fruit juice concentrate prepared from the ripe fruits of *J. drupacea* which is a well-known food product widely consumed in Anatolia. It has a high nutritional value thus it is a good energy source and used to improve psychologic and immune functions. Additionally it is believed to be aphrodisiac and beneficial for asthma and hemorrhoid treatment [2, 7].

Many of the traditional therapeutic applications of *J. drupacea* have been confirmed by the positive bioactivities of the extracts in experimental studies [8-12].

Our comprehensive studies on the antidiabetic activity of traditional medicines of Turkey is going on and due to strong antidiabetic effect of other *Juniperus* species (*J. oxycedrus, J. communis, J. foetidissima* and *J. sabina*) in our *in-vitro* and *in-vivo* studies [13-16], *J. drupacea* is selected as the subject of this study. Thus, *in-vitro* antidiabetic effect of water, ethyl acetate and methanol extracts of leaf, fruit and branches were investigated by using *a*-amylase and *a*-glucosidase enzyme inhibitory activities. On the other hand, antioxidant activities of the extracts were determined by metal chelating capacity, ferric-reducing antioxidant power, phosphomolybdenum, superoxide anion scavenging, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical cation and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity assays. Total phenolic and total flavonoid contents of the extracts were determined by using common

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spectrophotometric techniques. High performance liquid chromatography (HPLC) analysis was performed to determine the amounts of umbelliferone and amentoflavone in the methanol extracts for standardization.

2. RESULTS

α-Glucosidase inhibitory activities of *J. drupacea* extracts were evaluated at 3 different logarithmic concentrations (3,1 and 0.3 mg/ml) and the calculated inhibitory percentages are given in Table 1. All of the extracts showed excellent and dose dependent inhibitory effect on *a*-glucosidase enzyme and inhibitory activity of methanol extracts of branch, fruit and leaves were higher than ethyl acetate and water extracts. Moreover, fruit methanol extract (99.92± 0.30 %) and leaf methanol extract (99.44 ± 1.78 %) were more effective than the reference antidiabetic drug Acarbose (98.88 ± 0.07 %) at 1 mg/ml concentration. Fruit ethyl acetate extract exerted the lowest enzyme inhibitory activity with inhibition percentage 76.42 at 3 mg/ml concentration. *a*-Amylase inhibitory activities of the plant extracts were evaluated at the same logarithmic concentrations (3,1 and 0.3 mg/ml) and results are given in Table 1. Surprisingly, only leaf water extract, showed a poor inhibitory effect on *a*-amylase enzyme at 3 mg/ml concentration. The inhibition percentage of the reference drug Acarbose was found between 44.72-26.26 at tested concentrations (1-0.1 mg/ml).

Table 1. Enzyme inhibitory effects of the *J. drupacea*.

Plant Part	Extract	α-Glucosidase Inh. Inh. % ± S.D.			α-Amylase Inh. Inh. % ± S.D.		
		3 mg/ml	1 mg/ml	0.3 mg/ml	3 mg/ml	1 mg/ml	
Branch	Water	99.01 ± 0.30	60.15 ± 4.49	19.75 ± 3.66	-	-	
	MeOH	99.94 ± 0.16	96.96 ± 0.31	20.13 ± 2.06	-	-	
	EA	89.19 ± 1.71	93.30 ± 2.32	61.09 ± 2.96	-	-	
Fruit	Water	98.21 ± 0.35	63.34 ± 1.92	2.42 ± 1.97	-	-	
	MeOH	> 100.00	99.92 ± 0.30	34.97 ± 2.59	-	-	
	EA	76.42 ± 2.06	67.49 ± 0.57	48.41 ± 1.11	-	-	
Leaf	Water	97.55 ± 0.34	67.76 ± 2.39	14.01 ± 4.15	9.14 ± 0.99	-	
	MeOH	> 100.00	99.44 ± 1.78	60.30 ± 2.23	-	-	
	EA	87.69 ± 3.36	80.70 ± 0.44	71.10 ± 1.26	-	-	
Reference		**1 mg/ml**	**0.3 mg/ml**	**0.1 mg/ml**	**1 mg/ml**	**0.3 mg/ml**	**0.1 mg/ml**
Acarbose		98.88 ± 0.07	97.98 ± 0.03	96.37 ± 0.56	44.72 ± 2.76	31.51 ± 3.18	26.26 ± 5.74

-: No activity, S.D.: Standard Deviation

Antioxidant activities of *J. drupacea* extracts were evaluated by using six different techniques and the results are given in Table 2 and 3. Metal chelating activity of fruit and leaf water extracts were calculated to be higher than 100% at 3 mg/ml. Branch water, fruit methanol and fruit ethyl acetate extracts were found to be active in varying proportions (54.94, 18.88 and 25.59 % respectively) at 3 mg/ml. The chelating agent EDTA was used as positive control and showed strong activity between 98.87-95.75 % at 1-0.1 mg/ml concentrations. Ferric reducing power of all extracts were promising (except ethyl acetate extracts) at 3 mg/ml. Leaf methanol extract exerted the highest ferric reducing power with absorbance 3.4400 ± 0.1107 while the absorbance of reference (ascorbic acid) was 3.5870 ± 0.0874 at the same concentration (3 mg/ml). Phosphomolybdenum method is a spectrophotometric method that has been developed for the quantitative determination of total antioxidant capacity. According to our results, total antioxidant capacity of ethyl acetate extracts of fruit, leaf and branches were found to be very high (1056.87, 931.08 and 852.46 mg ascorbic acid equivalent/g extract, respectively). Superoxide anion scavenging activities of all extracts at 3 mg/ml concentration were excellent (between >100 and 83.66%). However their ABTS radical scavenging activities were poor ranging between 38.86 and 13.16 %. DPPH radical scavenging activities of fruit methanol (43.18 %) and branch ethyl acetate extracts (42.68 %) were similar to the reference compound BHT (50.67 %) at 1 mg/ml.

Total phenolic contents (TPC) of the branch, fruit and leaf extracts were ranged from 46.11 to 340.67 mg gallic acid equivalent/g extract (Table 4). For all tested plant parts, TPC of the extracts were in the following order; ethyl acetate extracts > methanol extracts > water extracts. The highest TPC was determined in the ethyl acetate extract of the fruits and the lowest were in the water extract of the fruits of *J. drupacea*. The highest total flavonoid content was determined in the ethyl acetate extract of the leaves with 117.25 mg quercetin equivalent/g extract and the second was the ethyl acetate extract of the fruits (63.55 mg quercetin equivalent/g

extract). The flavonoid contents of the other extracts were similar to each other ranging from 18.31-42.43 mg quercetin equivalent/g extract.

Table 2. Metal chelating activity, ferric reducing power and total antioxidant capacity *J. drupacea* extracts.

Plant Part	Extract	Metal Chelating Activity % ± S.D.			Ferric Reducing Power Absorbance ± S.D.			Total Antioxidant Capacity
		3 mg/ml	1 mg/ml	0.3 mg/ml	3 mg/ml	1 mg/ml	0.3 mg/ml	
Branch	Water	54.94 ± 2.05	30.32 ± 5.41	-	1.9770 ± 0.0508	1.7543 ± 0.0751	0.5647 ± 0.0461	-
	MeOH	-	-	-	1.8237 ± 0.0491	1.7587 ± 0.0692	1.0010 ± 0.0425	-
	EA	-	-	-	1.8860 ± 0.0327	0.5553 ± 0.0560	0.1670 ± 0.0139	852.46 ± 24.02
Fruit	Water	> 100.00	22.64 ± 8.08	-	1.9817 ± 0.0978	1.9260 ± 0.0700	0.6470 ± 0.0216	-
	MeOH	18.88 ± 3.30	-	-	2.1903 ± 0.1187	1.9407 ± 0.0881	-	-
	EA	25.59 ± 3.44	-	-	2.0404 ± 0.0786	0.3273 ± 0.0100	0.0923 ± 0.0060	1056.87 ± 18.67
Leaf	Water	> 100.00	62.26 ± 2.66	-	2.0453 ± 0.1086	1.9754 ± 0.1731	0.6370 ± 0.0381	-
	MeOH	-	-	-	3.4400 ± 0.1107	2.0190 ± 0.0454	0.8247 ± 0.0634	53.20 ± 9.08
	EA	-	-	-	0.5487 ± 0.0171	0.2470 ± 0.0573	0.0720 ± 0.0170	931.08 ± 9.08
Reference		**1 mg/ml**	**0.3 mg/ml**	**0.1 mg/ml**	**3 mg/ml**	**1 mg/ml**	**0.3 mg/ml**	
EDTA		98.87 ± 0.14	96.60 ± 3.39	95.75 ± 0.08	NT	NT	NT	NT
Ascorbic acid		NT	NT	NT	3.5870 ± 0.0874	3.4547 ± 0.0852	3.0787 ± 0.0587	

NT: Not tested, -: No activity, S.D.: Standard Deviation
Total antioxidant capacity is expressed as mg ascorbic acid equivalent/g extract ± S.D.

Table 3. Superoxide anion, DPPH and ABTS radical scavenging activities of *J. drupacea* extracts.

Plant Part	Extract	Superoxide anion scavenging activity Inhibition % ± S.D.			DPPH radical scavenging activity Inhibition % ± S.D.			ABTS radical scavenging activity Inhibition % ± S.D.		
		3 mg/ml	1 mg/ml	0.3 mg/ml	3 mg/ml	1 mg/ml	0.3 mg/ml	3 mg/ml	1 mg/ml	0.3 mg/ml
Branch	Water	97.78 ± 3.73	53.89 ± 0.98	2.41 ± 1.00	-	-	31.57 ± 3.89	38.86 ± 2.33	8.27 ± 1.02	6.90 ± 2.06
	MeOH	96.40 ± 0.77	73.55 ± 3.02	31.99 ± 3.61	43.18 ± 2.00	38.38 ± 4.87	35.61 ± 3.03	38.81 ± 2.08	13.72 ± 1.14	1.79 ± 1.89
	EA	> 100.00	46.99 ± 3.56	-	33.33 ± 3.14	42.68 ± 1.16	48.99 ± 1.58	28.30 ± 6.84	9.18 ± 3.66	3.79 ± 0.74
Fruit	Water	83.66 ± 0.39	51.73 ± 4.78	15.28 ± 1.69	-	-	31.57 ± 3.89	18.24 ± 2.26	10.01 ± 2.35	4.12 ± 0.39
	MeOH	91.51 ± 2.51	76.88 ± 1.69	19.69 ± 2.72	33.08 ± 1.16	43.18 ± 0.76	43.94 ± 0.76	32.11 ± 2.37	8.77 ± 1.03	9.18 ± 1.69
	EA	84.94 ± 4.10	40.67 ± 8.83	19.30 ± 1.80	48.70 ± 1.58	36.62 ± 3.06	23.99 ± 8.34	23.29 ± 1.19	10.38 ± 2.80	5.71 ± 1.67
Leaf	Water	97.65 ± 2.78	53.63 ± 1.36	-	-	-	-	13.16 ± 2.75	6.34 ± 1.59	6.12 ± 0.97
	MeOH	89.19 ± 4.24	64.69 ± 0.21	25.91 ± 3.83	-	17.68 ± 2.44	9.60 ± 1.86	36.21 ± 1.87	14.22 ± 0.29	7.06 ± 4.02
	EA	92.92 ± 7.19	78.84 ± 6.10	4.63 ± 0.05	-	25.51 ± 2.31	13.38 ± 4.57	17.38 ± 2.46	11.05 ± 2.52	8.02 ± 1.43
Reference		**1 mg/ml**	**0.3 mg/ml**	**0.1 mg/ml**	**1 mg/ml**	**0.3 mg/ml**	**0.1 mg/ml**	**3 mg/ml**	**1 mg/ml**	**0.3 mg/ml**
Quercetin		86.52 ± 3.26	75.19 ± 3.22	68.45 ± 0.27	NT	NT	NT	NT	NT	NT
BHT		>100	62.74 ± 2.08	18.07 ± 2.40	50.67 ± 3.40	47.17 ± 4.31	40.83 ± 7.69	NT	NT	NT
Gallic acid		NT	NT	NT	NT	NT	NT	88.91 ± 0.15	88.37 ± 0.26	83.24 ± 1.47

NT: Not tested, -: No activity, S.D.: Standard Deviation

Results of HPLC analysis revealed that amentoflavone was detected in serious amount especially in the leaves as 0.148±0.001 g/100 g dry weight, and umbelliferone was determined in minor amount in both leaves and branches (Table 5). Any of the other investigated phenolics such as chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, myricetin, quercetin, luteolin and apigenin were determined in *J. drupacea*. A sample chromatogram is given in Figure 1. In our previous study on *J. foetidissima* and *J. sabina*, we obtained similar results in terms of the absence of these phenolic acids and flavonoids. Moreover, only amentoflavone and umbelliferone were also detected and quantified in *J. foetidissima* and *J. sabina*. The method validation parameter results were displayed in our previous study [16].

Table 4. Yield percentages (% w/w), total phenolic and flavonoid contents of *J. drupacea* extracts.

Plant Part	Extract	Yield % (w/w)	Total Phenolic Content (Mean ± S.D.)	Total Flavonoid Content (Mean ± S.D.)
Branch	Water	9.41	76.32 ± 5.78	18.31 ± 0.30
	MeOH	7.48	109.18 ± 4.73	17.78 ± 0.30
	EA	6.07	330.73 ± 3.85	37.85 ± 0.61
Fruit	Water	22.18	46.11 ± 1.60	22.89 ± 0.61
	MeOH	26.89	122.56 ± 6.05	27.99 ± 2.94
	EA	3.76	340.67 ± 5.29	63.55 ± 1.10
Leaf	Water	14.05	85.93 ± 2.95	25.88 ± 2.30
	MeOH	20.64	112.88 ± 3.56	42.43 ± 1.85
	EA	3.04	280.70 ± 2.62	117.25 ± 3.46

Total flavonoid content was expressed as mg quercetin equivalent/g extract and total phenolic content was expressed as mg gallic acid equivalent/g extract

Table 5. Amentoflavone and umbelliferone contents of branches, fruits and leaves of *J. drupacea*.

Plant Part	Amentoflavone (g/100 g dw ± SD)	Umbelliferone (g/100 g dw ± SD)
Branch	0.0058 ± 0.0001	0.0008± 0.0001
Fruit	0.0360 ± 0.0010	ND
Leaf	0.1480 ± 0.0010	0.0056 ± 0.0001

Results are expressed as Mean ± Standard Deviation (n=3); ND: Not detected

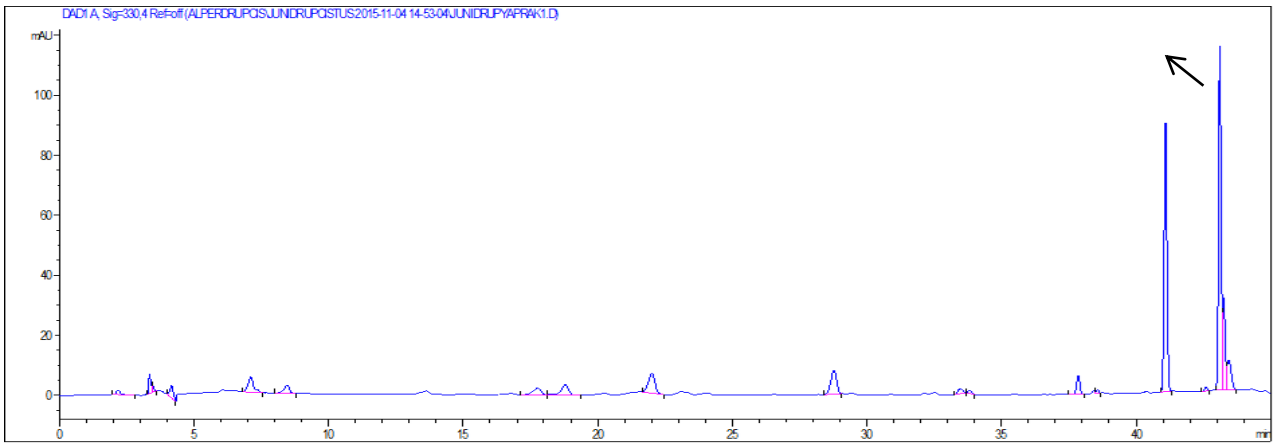

Figure 1. HPLC chromatogram of *J. drupacea* leaf methanol extract. The arrow marked peak belongs to amentoflavone (Retention time=43.1 min) in the chromatogram.

3. DISCUSSION

Juniperus species are used for their medicinal, cosmetic and nutritional properties in many countries. They are mainly used to lower high blood glucose levels in Turkey as folk medicine. On the other hand, many researches carried out a number of *in vitro* and *in vivo* studies and have proven that berries, leaves and essential oils of different *Juniperus* species have promising antidiabetic activity compatible with their folkloric usage. Beside reducing blood glucose levels directly, junipers have defined to have impact on blood glucose homeostasis by different mechanisms such as increasing *in vitro* insulin secretion [17], elevating zinc levels [18], decreasing hemoglobin and insulin glycation [19, 20], inhibiting pancreatic lipase, *a*-amylase and *a*-glucosidase [15, 16, 21]. Additionally, their antioxidant, lipid peroxidation inhibitory, cholesterol and triglyceride lowering and antiobesity activities contribute to their positive effects on diabetic complications [14-16, 18, 22].

Chemical studies revealed that *J. drupacea* contain secondary metabolites from different classes such as coumarins (umbelliferone), flavonoids (amentoflavone, cupressuflavone, catechin, (-)-epicatechin, hikoniflavone, procyanidin dimers B1, B2, B3, B4, B5, B6, quercetin-3-*a*-*O*-L-rhamnofuranoside, rutin), phenolic acids (gallic acid, chlorogenic acid, protocatechic acid), terpenes (4-epi-abietic acid, *δ*-cadinene, *β*-caryophyllene, *trans*-communic acid, *a*-copaene, ferruginol, *a*-humulene, limonene, *β*-phellandrene, *a*-pinene, *β*-pinene, sandaracopimaric acid, (+)-totarol) [11, 23, 24]. Beside, El-Ghorab et al. studied on the chemical composition of the volatile chemicals in dichloromethane extract from a steam distilate of *J. drupacea* berries. Hydrocarbons, alkyl-alcohols, -aldehydes, -ketones, and –acids, alkyl esters, aromatic compounds, terpenes and terpenoids were determined by gas chromatography/mass spectrometry. The major chemicals identified in the extract were *a*-pinene (23.73%), thymol methyl ether (17.32%), camphor (10.12%), (*Z*)-nerolidyl acetate (5.64%), *β*-farnesol (5.64%) [8].

In our previous studies, antidiabetic activity of different *Juniperus* species (*J. oxycedrus, communis, foetidissima* and *sabina*) used as traditional medicine in Turkey was evaluated by using *in-vitro* and *in-vivo* methods. Fatty acids, such as palmitic, linoleic and linolenic acid were found as the major compounds in antidiabetic non-polar subfractions of *J. oxycedrus* leaves through bioactivity guided isolation technique (BGIT) [13]. Through *in vivo* BGIT, shikimic acid, 4-*O*-*β*-D-glucopyranosyl ferulic acid and oleuropeic acid-8-*O*-*β*-D-glucopyranoside have been isolated from the *n*-butanol subextract of *J. oxycedrus* berries by silica gel and reverse phase column chromatography as the main active ingredients of the active subfraction [14]. Furthermore, *in vitro* and *in vivo* antidiabetic activities of *J. foetidissima* (Jf) and *J. sabina* (Js) berry and leaf extracts have been determined. Extracts have had high inhibitory activity on *a*-glucosidase enzyme and moderate inhibitory activity on *a*-amylase. Additionally, extracts have lowered blood glucose concentrations of STZ-diabetic animals significantly after oral administration. Amentoflavone has been determined as the major constituent of the leaf and berry extracts and umbelliferone has been identified in the leaf extracts. The antidiabetic activity of the extracts has been attributed to amentoflavone which has significant inhibitory activity on carbohydrate digestive enzymes as well as its positive effects on insulin resistance [16]. Similar results were obtained in this study and *J. drupacea* extracts inhibited α-glucosidase enzyme strongly beside their high antioxidant activities. Additionally, HPLC analyses have shown that both amentoflavone and umbelliferone were found in the *J. drupacea* extracts. Amentoflavone was identified as the major compound in the leaf methanol extract and umbelliferone was determined in minor amounts in leaf and branch methanol extracts.

Amentoflavone is a biflavonoid that has strong antidiabetic activity and have been isolated as the active principle of many plants up to the present [25-27]. Amentoflavone has strong inhibitory activity against *a*-glucosidase and *a*-amylase enzymes, it may enhance insulin-induced intracellular signaling possibly through inhibition of PTP1B activity and improve insulin resistance [28-30].

As known, standardization is required for assurance of quality, efficacy and safety of plant extracts used in phytotherapy. If the compounds responsible for the activity of the extract are unknown, marker substances that are chemically defined constituents of herbal extracts can be used for standardization. According to the results of current study, similar to our previous study [16], we can clearly indicate that amentoflavone as the major constituent of the *J. drupacea*, should be selected as marker compound for the standardization of the *J. drupacea* extracts and preparations.

4. CONCLUSION

Juniperus drupacea having strong enzyme inhibitory activity had also showed strong antioxidant activity. It is well known that antioxidants combat with oxidants and free radicals and play an important role in the inhibition of development of tissue and organ damage in diabetic patients. Amentoflavone is the main constituent of the *J. drupacea* extracts and should be mainly responsible for their antioxidant and antidiabetic activities. So that, standardization of the *Juniperus* extracts and preparations could be performed by using this biflavonoid compound, and new natural products to struggle with diabetes should be introduced to the medicinal field and phytotherapy.

5. MATERIALS AND METHODS

5.1. Plant material

Juniperus drupacea Labill. (Cupressaceae) was collected near Akseki-Antalya, Turkey in August, 2015 by Nilufer and Caglar Orhan. Plant was identified by Assoc. Prof. Dr. N. Orhan and voucher specimen has been deposited in the Herbarium of Gazi University Faculty of Pharmacy under the herbarium code of GUEF 3388.

5.2. Preparation of the plant extracts

Ethyl acetate (EA) and methanol (MeOH) extracts: Five grams of dried and powdered plant parts (leaves, fruits, and branches) were extracted with 200 ml methanol and ethyl acetate (2.5% a/h) on a shaker for 18 hours at room temperature. Extracts were then filtered from filter paper. This procedure was repeated for two more times, extracts were pooled and condensed by a rotary evaporator.

Water extract: Dried and powdered plant parts (leaves, fruits, and branches) (5 g) were extracted with 50 ml hot water (4% a/h) on a heating-magnetic stirrer for 6 hours. Extracts were then filtered from filter paper and the residues were extracted with 50 ml water with the same procedure again. Filtered water extracts were combined and freeze-dried. Yields of the extracts were calculated and given in Table 4.

5.3. Assay for *a*-glucosidase inhibitory activity

a-Glucosidase inhibitory activities of the extracts were determined using the already published method [15]. Acarbose (Bayer Group, Turkey), a potent alpha-glucosidase inhibitor, was used as positive control. *Bacillus stearothermophilus* originated α-Glucosidase type IV enzyme (Sigma Co., St. Louis, USA) was dissolved in phosphate buffer (0.5 M, pH 6.5). Extracts were dissolved in ethanol at different logarithmic concentrations (3000, 1000, and 300 µg/ml). The enzyme solution and test extracts were preincubated in a 96-well microtiter plate for 15 min at 37 °C. Then, 20 mM p-nitrophenyl-α-d-glucopyranoside (NPG), (Sigma) was added to the wells. The microtiter plate was incubated at 37 °C for 35 min. The increase in the absorption at 405 nm due to the hydrolysis of NPG by α-glucosidase was measured by an ELISA (VersaMax, Molecular Devices, USA) microtiter plate reader. The inhibition percentage (%) was calculated by the equation: Inhibition %=[($A_{Control}$-A_{Sample})/$A_{Control}$]×100

5.4. Assay for *a*-amylase inhibitory activity

a-Amylase inhibitory effects of the extracts were determined using our previously published method [15]. Experiments were carried out with three replicates and Acarbose (Bayer, Turkey) was used as the positive control. Porcine pancreatic α-amylase type VI (EC 3.2.1.1, Sigma) was dissolved in distilled water. Potato starch (0.5 %, w/v) in phosphate buffer (pH 6.9) was used as substrate solution. Plant extract was dissolved in DMSO at logarithmic concentrations (3, 1, 0.3 mg/ml). After the addition of the enzyme solution, mixtures were incubated at 37 °C for 3 min. Then, substrate solution was added and the mixtures were incubated at 37 °C for 5 min. DNS colour reagent solution (96 mM 3,5-dinitrosalicylic acid, 5.31 M sodium potassium tartrate in 2 M NaOH) was added to the mixtures and the tubes were put into a 85 °C heater. After 15 min, distilled water was added to the tubes and the tubes were cooled on ice. Absorbances of the mixtures were read at 540 nm. Acarbose was used as the positive control. Standard maltose calibration graph was prepared. The amount of maltose generated was calculated by using the standard maltose calibration graph (0-0.1% w/v) and the obtained net absorbance. Percent of inhibition was calculated as: Inhibition %=[(Maltose $_{Control}$ -Maltose $_{Sample}$)/Maltose $_{Control}$)]×100

5.5. Metal chelating capacity

The chelating activity of *J. drupacea* extracts on Fe^{+2} was determined by the method of Dinish et al. [31]. Extracts were incubated with $FeCl_2$ (2 mM). The reaction was initiated by the addition of 0.2 ml of ferrozine (5 mM) and the total volume was adjusted to 4 ml with ethanol. After 10 min, the absorbance was measured at 562 nm. EDTA (ethylenediaminetetraacetic acid) was used as a reference compound. The control contained $FeCl_2$ and ferrozine. The percentage of inhibition of the ferrozine-Fe^{+2} complex formation was calculated using this formula: Metal chelating activity (%)=[($A_{Control}$– A_{Sample})/$A_{Control}$]×100. Analyses were carried out in triplicate and the results were averaged.

5.6. Ferric-reducing antioxidant power

Different logarithmic concentrations of the extracts (3, 1, and 0.3 mg/ml) and ascorbic acid as reference were mixed with phosphate buffer (0.2 mol/l, pH 6.6) and $K_3Fe(CN)_6$. Tubes were incubated at 50°C for 20 min, then trichloro acetic acid was added and the mixture was vortexed. Following centrifugation, the supernatant was mixed with same amount of distilled water and $FeCl_3$ and the absorbance at 700 nm was measured [16]. Analyses were run in three replicates and the results were averaged.

5.7. Total antioxidant activity by phosphomolybdenum assay

Distilled water and moybdate reagent solution were added to the plant extracts and tubes were vortexed. After the incubation at 90◦C for 90 min tubes were cooled. The absorbances of the samples were measured at 695 nm and the results were expressed as mg ascorbic acid equivalent/g extract [16].

5.8. Superoxide anion scavenging activity

The influence of the plant extracts on the generation of superoxide radicals was determined by spectrophotometric measurement of the product formed on reduction of NBT (Nitro blue tetrazolium chloride). In brief, superoxide radicals were generated in a non-enzymatic system and the reaction mixture containing 25-2000 µg/ml of the test fraction in ethanol, 1 ml of PMS (phenazine methosulphate), 1 ml of β-NADH (nicotinamide adenine dinucleotide) and 1 ml of NBT in phosphate buffer (pH 7.4) was incubated at ambient temperature for 5 min. The resulting colour was read at 560 nm against blank samples. Analyses were run in three replicates and the results were averaged. Quercetin was used as reference in this assay [32, 33].

5.9. Assay for scavenging activity of ABTS radical cation

ABTS radical cation ($ABTS^{\cdot+}$) scavenging assay was generated by using a spectrophotometric method that was described in Orhan et al., 2014 [15]. Samples were vortexed and their absorbances were read at 734 nm. Gallic acid was used as the positive control.

5.10. Estimation of DPPH radical scavenging activity

The DPPH radical scavenging activity of the extracts was determined in a 96 well-plate [34]. 160 µl of extract was mixed with 40 µl of DPPH solution and incubated in darkness for 30 min. Then the absorbance was measured at 520 nm utilizing a 96-well ELISA microplate reader (VersaMax, Molecular Devices, USA). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software. BHT (Butylated hydroxytoluene) was used as a positive control at 0.57, 1 and 3 mg/ml concentrations.

5.11. Determination of total phenolic content

The extracts were mixed with Folin-Ciocalteu reagent (10%) and samples were incubated for 5 minutes at room temperature. Then, sodium carbonate solution was added and samples were vortexed immediately. The absorbance of mixture was measured at 735 nm after 30 minutes at room temperature in a dark place. The mean of three readings was used and the total phenolic content was expressed in mg of gallic acid equivalents (GAE)/g extract [35]. Calibration curve equation was; y(Abs.)=5.306x(Conc.)+0.0587 and the coefficient of determination was r^2= 0.9986.

5.12. Determination of total flavonoid content

The method of Kosalec et al. was used to determine total flavonoid contents of the extracts [36]. Dried extracts were dissolved in ethanol. Ethanol, sodium acetate and aluminium chloride solution were added to the samples and the mixture was diluted to 5 ml by distillated water. After 30 minutes incubation at room temperature, the absorbance of yellow mixtures was measured at 415 nm. Results were expressed in mg of quercetin equivalents (QE)/g extracts. Calibration curve equation was; y(Abs)=1.8934x(Conc)-0.025 and the coefficient of determination was r^2 =0.9996.

5.13. Standardization of the extracts by using RP-HPLC-DAD analysis

The qualitative and quantitative analyses of the phenolic compounds in the branch, fruit, and leaf methanol extracts were performed using our previously validated HPLC method according to the following procedure [16]: Chlorogenic acid (C3878), caffeic acid (C0625), ferulic acid (128708), *p*-coumaric acid (C9008), myricetin (70050), quercetin (Q0125), luteolin (L9283), apigenin (10798), amentoflavone (40584) and umbelliferone (H24003) were purchased from Sigma-Aldrich, Germany. Protocatechuic acid was purchased from HWI Analytik GmbH, Germany. All other chemicals were analytical grade and obtained from either Sigma or Merck. HPLC system consisting of a HP Agilent 1260 series quaternary pump, degasser and photo-diode array detector was used for analysis. The samples were injected using HP Agilent 1260 Autosampler, and ACE column (5 µm, 250 mm x 4.6 mm) was used for the separation process at 30°C. Data analysis was performed with Agilent ChemStation software. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of the peak areas. Gradient elution was applied with a flow rate of 0.8 ml/min.

The mobile phase was a mixture of trifluoroacetic acid 0.1% in water (solution A), trifluoroacetic acid 0.1% in methanol (solution B), and trifluoroacetic acid 0.1% in acetonitrile (solution C). The gradient composition was (A:B:C), 80:12:8 at 0 min, 75:15:10 at 8 min, 70:18:12 at 16 min, 65:20:15 at 24 min, 50:35:15 at 32 min, 25:60:15 at 40 min and 80:12:8 at 45 min.

All solvents were filtered through a 0.45 µm filter before use and degassed in an ultrasonic bath. From each solution and sample 10 µl was injected into the column and the chromatograms were recorded from 200 to 400 nm. Standard solutions were analyzed and three-dimensional chromatograms (wavelength; time; absorbance) were obtained to select the optimum wavelength for detection of the phenolics with maximum sensitivity. Quantification was performed by measuring at 330 nm for amentoflavone and umbelliferone using a photo-diode array detector. Retention times (min) were 18.7 for umbelliferone and 43.1 for amentoflavone. Standart curves were calculated as y=72936x-19.485 (R^2=0.993) for umbelliferone and y=26854x-12.591 (R^2=0.999) for amentoflavone. Amentoflavone and umbelliferone content of *J. drupacea* is expressed as g/100 g dry weight of fruits, leaves and branches.

5.14. Statistical analysis

All analyses were carried out in triplicates and the results were averaged. All values are expressed as the mean ± standard deviation (S.D.), linear regression analyses and calculations were done by using Microsoft Excel and GraphPad Instat softwares.

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