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Evaluation of fruit extracts of six Turkish *Juniperus* species for their antioxidant, anticholinesterase and antimicrobial activities

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Abstract

BACKGROUND: *Juniperus* L. (Cupressaceae) species are mostly spread out in the Northern Hemisphere of the world, and some of them are used as folkloric medicines. The fruits of some species are eaten. Since oxidative stress is one of the reasons for neurodegeneration and is associated with the Alzheimer's disease (AD), the extracts prepared from the fruits of six *Juniperus* species were screened for their antioxidant activity. Therefore, the extracts were also evaluated against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), which are chief enzymes in the pathogenesis of AD. In addition, antimicrobial activity was also evaluated.

RESULTS: In the β-carotene–linoleic acid assay, acetone extracts of *J. oxycedrus* subsp. *oxycedrus, J. sabina* and *J. excelsa*, and methanol extracts of *J. phoenicea* and *J. sabina*, effectively inhibited oxidation of linoleic acid. The hexane extracts of *J. oxycedrus* subsp. *oxycedrus, J. foetidissima* and *J. phoenicea* showed remarkable inhibitory effect against AChE and BChE.

CONCLUSION: Because of their high antioxidant activity, *J. excelsa, J. oxycedrus* subsp. *oxycedrus, J. sabina* and *J. phoenicia* might be used in the food industry as preservative agents or extension of the shelf-life of raw and processed foods. Since the hexane extracts of *J. oxycedrus* subsp. *oxycedrus* and *J. foetidissima* demonstrated significant anticholinesterase activity they should be considered as a potential source for anticholinesterase agents.

Keywords: *Juniperus foetidissima*; *Juniperus phoenicea*; *Juniperus excelsa*; *Juniperus oxycedrus* subsp. *oxycedrus*; *Juniperus communis* subsp. *nana*; *Juniperus sabina*; antioxidant activity; anticholinesterase activity; antimicrobial activity

INTRODUCTION

In the food industry, the synthetic antioxidants butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate and *tert*-butylhydroquinone have been used because of their high antioxidant capacity. However, these antioxidants have been reported to be responsible for liver damage and carcinogenesis in F344 rats.[1] For these reasons, use of synthetic antioxidants is limited in some countries.[2] Thus interest in naturally occurring antioxidants, consumed safely by people and animal for years,[3] has been increasing because of their protective effects on oxidative stress damage in humans. On the other hand, excess amounts of free radical species which causes oxidative stress are also associated with pathology of many diseases including Alzheimer's disease (AD), which is a progressive neurological disorder characterized by cognitive deficit and behavioural abnormalities in the patient.[4] It is reported that reactive oxygen species contribute to cellular ageing and neuronal damage.[5] Therefore the use of antioxidants may reduce the progression of AD and minimize neuronal degeneration.[6] It is an advantage for an antioxidant food to exhibit inhibitory activity against acetylcholinesterase and butyrylcholinesterase, which are chief enzymes in the pathogenesis of Alzheimer's disease. Thus the development and utilization of more effective antioxidants of natural origin as well as anticholinesterase compounds are desired.

Juniperus L. (Cupressaceae) number almost 70 species throughout the world and are mostly distributed in the Northern Hemisphere.[7] The widespread areas extend from Japan and East Asia to Europe, as well as from North and East Africa to North America. *Juniperus* wood, essential oil and berries (fruits) have

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been used from ancient civilizations, and it was known as a traditional remedy for many illnesses in the Middle Ages, including use as a diuretic and digestive. In particular, *Juniperus* berries have been used as a fragrance and were thought to purify the air where it was used. Commercial interest is in the distilled sprits from fermented juniper berries, often mixed or redistilled with grain or fruit sprits.

In Turkey, the *Juniperus* genus is represented by 10 taxa under seven species, namely, *J. communis* L. subsp. *hemisphaerica* (Presl.) Nyman, *J. communis* L. subsp. *communis* Sp.Pl., *J. communis* L. subsp. *nana* Syme, *J. oxycedrus* L. subsp. *macrocarpa* (Sibth. & Sm.) Ball, *J. oxycedrus* L. subsp. *oxycedrus* L., *J. foetidissima* Willd., *J. excelsa* Bieb, *J. oblonga* Bieb., *J. sabina* L., *J. phoenicea* L.[8] *Juniperus* fruits and leaves, besides their wood, have also been used by Anatolian people since ancient times. The coniferous parts and leaves of *Juniperus* are utilized in medicine and the cosmetic industry as an antihelmintic, diuretic, stimulant and antiseptic. Furthermore, the berries of *J. communis* L. are used in gin production.[9] *J. oxycedrus* is commonly used for the preparation of traditional medicinal brandy in Dalmatia. *J. excelsa* is used as a medicine against tuberculosis and jaundice, besides its uses as a wood preservative material.[7] The local names, parts used, usages/ailments and preparation of these *Juniperus* species are given in Table 1.[9–11]

In previous studies, the major phenolic constituents have been identified in extracts of *Juniperus* species as lignans, coumarins, sesquiterpenes, abietane, labdane and pimarane diterpenes, flavonoids, biflavonols, flavone glycosides and tannins. Although some *Juniperus* species growing in Anatolia have been previously studied for their volatile[12] and non-volatile secondary metabolites,[7] only a few studies have investigated their more polar compounds, such as lignans, flavonoids and other phenolics.[12] The literature survey indicates that *Juniperus* species are rich in volatile constituents,[13] as well as in tannins, flavonoids,[14] lignans,[15] phenylpropanoids,[16] abietane[17] pimarane and labdane diterpenes.[7]

Because the berries of *J. oxycedrus, J. phoenicea* and *J. communis* are eaten in Turkey,[18,19] and noting the above-mentioned uses of *Juniperus* species, particularly the use of their berries in the medicinal, food and cosmetic industries, we focused our studies on *Juniperus* species and initiated a study of berries of six *Juniperus* species growing in Turkey for their potential antioxidant and anticholinesterase activities, presented here. The antimicrobial activity of the extracts was also evaluated in this work.

In the present study, 18 extracts prepared from the berries of *J. communis* subsp. *nana, J. excelsa, J. foetidissima, J. oxycedrus* subsp. *oxycedrus, J. phoenicea* and *J. sabina* were investigated for their antioxidant, anticholinesterase and antimicrobial potential. For the determination of antioxidant activity, two main complementary tests; namely, 1,1-diphenyl-2-picrylhydrazyl and β-carotene–linoleic acid assays, were carried out. In addition, total phenolic and flavonoid contents were also determined as pyrocatechol and quercetin equivalents, respectively. Anticholinesterase activity was evaluated according to Ellman's method, and antimicrobial activity was screened against a series standard and non-standard bacteria with different strains, as well as against two fungi.

MATERIALS AND METHODS

Chemicals

Methanol, acetone, hexane, pyrocatechol, aluminium nitrate and quercetin were obtained from E. Merck (Darmstadt, Germany). β-Carotene, linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween-40), Folin–Ciocalteu's reagent (FCR), α-tocopherol, butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), electric eel acetylcholinesterase (AChE, type VI-S, EC 3.1.1.7, 425.84 U mg^{-1}), horse serum butyrylcholinesterase (BChE, EC 3.1.1.8, 11.4 U mg^{-1}), 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide, butyrylthiocholine chloride and galanthamine were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were of analytical grade.

Plant material and preparation of *Juniperus* extracts

Information concerning the collection dates, collection localities and their herbarium numbers is as follows:

- *J. communis* L. subsp. *nana* Syme and *J. oxycedrus* L. subsp. *oxycedrus* L. were collected from Karabük province, Gölveren to Keltepe, at 1180 m altitude in September 2004, and herbarium numbers were given as BOF 300 and BOF 301, respectively.
- *J. excelsa* Bieb. and *J. foetidissima* Willd. were collected from İçel province, Kargagediği to Erdemli, at 200 m altitude in October 2004, and herbarium numbers were given as BOF 304, and BOF303, respectively.
- *J. phoenicea* L. was collected from Muğla province, Yokuşbaşı to Bodrum, at 150 m altitude in September 2004, and herbarium number was given as BOF 305.
- *J. sabina* L. was collected from Karabük province, Sorkun to Keltepe, at 1750 m altitude in October 2004, and herbarium number was given as BOF 302.

The *Juniperus* species were identified by Dr Barbaros Yaman, Faculty of Forestry at Bartın University, Bartın, Turkey. Voucher specimens of these species were deposited at the Herbarium of Faculty of Foresty, Bartın, Turkey.

Each sample (100 g) was extracted with 200 mL hexane (four times at room temperature). After filtration of the hexane extract the residue was extracted with 200 mL acetone (four times at room temperature) and then 200 mL methanol (four times at room temperature), successively. Each filtrated solvent was evaporated to dryness *in vacuo*. Yields (w/w) of the crude extracts are given in Table 2.

Bioassays

Determination of total phenolic content

Phenolic content in all extracts was determined with FCR and expressed as micrograms of pyrocatechol equivalents (PEs).[20] One millilitre of the solution (containing 500 µg) extracts in methanol was added to 46 mL distilled water and 1 mL FCR and mixed thoroughly. After 3 min, 3 mL of 2% sodium carbonate was added to the mixture and shaken intermittently for 2 h at room temperature. Absorbance was measured at 760 nm. Concentrations of phenolic content were calculated according to the following equation, obtained from a standard pyrocatechol graph:

$$\text{Absorbance} = 0.0517 \text{ pyrocatechol } (\mu g) - 0.0283 \ (r^2\ 0.9993)$$

Determination of total flavonoid compounds

Measurement of flavonoid content of the extracts was based on the aluminium nitrate method[21] with slight modifications, and results were expressed as micrograms of quercetin equivalents (QEs). An aliquot of 1 mL of the solution (containing 500 µg)

Table 1. Local names, parts used, uses/ailments treated and preparation of *Juniperus* plants used in Turkey

Botanical name	Local names	Part used	Use/ailment treated	Preparation
J. communis L. subsp. *nana* Syme.	Bodur Ardıç	Berries and leaves	• Cold, gout, urethral and indigestion diseases, odorant and appetizing	• As infusion (1–2%) or decoction
			• For the treatment of rheumatism and haemorrhoids	• 10 g essential oil is mixed with 100 g olive oil, externally (also berries decoction internally)
				• As dietary tea (infusion)
J. excelsa Bieb.	Boylu Ardıç	Berries and leaves	• Bronchitis, cold	• As decoction
			• Cough	• As infusion
J. foetidissima Willd.	Kokulu Ardıç	Berries and leaves	• Cough and cold	• As infusion
J. oxycedrus L. subsp. *oxycedrus* L.	Katran Ardıcı	Berries and herba	• Hair and other skin diseases (itch, psoriasis, eczema, wound healing)	• Pix juniperi: vaselin (1 : 9) mixture is used against itch and some skin diseases (external and internal)
			• Cold, cough and bronchitis and expectorant	• As berries infusion (also as decoction and inhalant)
			• Prostate cancer and haemorrhoids, rheumatism, urethral diseases including kidney stones	• As decoction (in general) (also pix used against haemorrhoids)
				• Essential oil
				• As dietary tea
				• Berries are also eaten
			• Emmenagogue, cold, cough, rheumatism, ulcer	• As decoction
			• Diarrhea	• Berries are eaten
J. sabina L.	Sabin Ardıcı	Herba	• Diuretic, emmenagogue, abortive and in the treatment of diabetes mellitus	• Herba sabinae is powdered and 0.3–0.6 g taken per day

extracts in methanol was added to test tubes containing 0.1 mL of 10% aluminium nitrate, 0.1 mL of 1 mol L^{-1} potassium acetate and 3.8 mL methanol. After 40 min at room temperature, the absorbance was determined spectrophotometrically at 415 nm. The concentrations of flavonoid content were calculated according to the following equation, obtained from a standard quercetin graph:

$$\text{Absorbance} = 0.0788\ \text{quercetin}\ (\mu g) - 0.0088\ (r^2\ 0.9995)$$

Antioxidant activity

Determination of antioxidant activity with β-carotene bleaching method

Antioxidant activity of the extracts was evaluated using a β-carotene–linoleic acid test system[22] with slight modifications. β-Carotene (0.5 mg) in 1 mL chloroform was added to 25 µL linoleic acid and 200 mg Tween 40 emulsifier mixture. After evaporation of chloroform under vacuum, 100 mL distilled water saturated with oxygen was added with vigorous shaking. 4 mL of this mixture was transferred to a series of test tubes containing different concentrations of the sample. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. The emulsion system was incubated for 2 h at 50 °C. A blank, devoid of β-carotene, was prepared for background subtraction, and (+)-catechin, quercetin, BHA and α-tocopherol were used as standards. The bleaching rate (R) of β-carotene was calculated according to the following equation:

$$R = \frac{\ln \frac{a}{b}}{t}$$

where ln = natural log, a = absorbance at time zero and b = absorbance at time t (120 min). Antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control, using following equation:

$$\text{AA} = \frac{R_{\text{control}} - R_{\text{sample}}}{R_{\text{control}}} \times 100$$

DPPH free radical scavenging activity

The free radical scavenging activity of extracts was determined by the DPPH assay described by Blois[23] with slight modification. In its radical form DPPH absorbs at 517 nm, but on reduction by an antioxidant or a radical species its absorption decreases. Briefly, a 0.1 mmol L^{-1} solution of DPPH in methanol was prepared and 4 mL of this solution was added to 1 mL of sample solution in methanol at different concentrations. Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical of an antioxidant was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Anticholinesterase activity

AChE and BChE inhibitory activity was measured by slightly modifying the spectrophotometric method developed by Ellman *et al.*[24] AChE from electric eel and BChE from horse serum were used, while acetylthiocholine iodide and butyrylthiocholine chloride were employed as substrates of the reaction. DTNB was used for measurement of cholinesterase activity. Ethanol was used as a solvent to dissolve test samples and the controls. In order to dissolve hexane extract in ethanol, an ultrasonic bath (Elmasonic, Singen, Germany) was used, keeping the sample in the bath for 2 min at 35 °C. Briefly, 150 µL of

Table 2. Yield percentages and total phenolic and total flavonoid contents of extracts of *Juniperus* species[a]

Juniperus species	Extract	Yield (%)	Phenolic content (μg PEs mg^{-1} extract)[b]	Flavonoid content (μg QEs mg^{-1} extract)[c]
J. communis subsp. *nana*	Hexane	2.96	n.t.[d]	n.t.
	Acetone	2.96	15.76 ± 1.75	2.62 ± 0.14
	Methanol	2.46	14.87 ± 0.48	2.30 ± 0.01
J. excelsa	Hexane	1.56	n.t.	n.t.
	Acetone	2.12	30.04 ± 1.27	4.59 ± 0.29
	Methanol	2.24	14.97 ± 0.47	0.91 ± 0.10
J. foetidissima	Hexane	2.22	n.t.	n.t.
	Acetone	2.60	20.45 ± 0.37	3.67 ± 0.25
	Methanol	2.80	33.46 ± 0.43	4.41 ± 0.19
J. oxycedrus subsp. *oxycedrus*	Hexane	2.68	n.t.	n.t.
	Acetone	2.13	27.69 ± 0.86	6.17 ± 0.21
	Methanol	1.92	19.98 ± 0.85	1.63 ± 0.28
J. phoenicea	Hexane	1.96	n.t.	n.t.
	Acetone	2.22	19.87 ± 1.25	2.64 ± 0.38
	Methanol	2.01	32.64 ± 0.64	13.53 ± 0.54
J. sabina	Hexane	1.35	n.t.	n.t.
	Acetone	2.15	30.77 ± 0.45	11.81 ± 0.22
	Methanol	1.01	31.58 ± 0.45	8.83 ± 0.12

[a] Values expressed are means ± SD of three parallel measurements ($P < 0.05$).
[b] PEs, pyrocatechol equivalents.
[c] QEs, quercetin equivalents.
[d] n.t., not tested.

100 mmol L^{-1} sodium phosphate buffer (pH 8.0), 10 μL sample solution dissolved in ethanol at different concentrations, and 20 μL AChE (5.32×10^{-3} U) or BChE (6.85×10^{-3} U) solution were mixed and incubated for 15 min at 25 °C, and 10 μL of 0.5 mmol L^{-1} DTNB was added. The reaction was then initiated by the addition of 10 μL acetylthiocholine iodide (0.71 mmol L^{-1}) or butyrylthiocholine chloride (0.2 mmol L^{-1}). The hydrolysis of these substrates was monitored spectrophotometrically by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride, respectively, at a wavelength of 412 nm utilizing a 96-well microplate reader (SpectraMax PC340, Molecular Devices, Sunnyvale, CA, USA). Measurements and calculations were evaluated using Softmax PRO v5.2 software. Percentage of inhibition of AChE or BChE was determined by comparison of reaction rates of samples relative to a blank sample (ethanol in phosphate buffer pH 8) using the formula $(E-S)/E \times 100$, where E is the activity of enzyme without the test sample and S is the activity of enzyme with the test sample. The experiments were carried out in triplicate. Galanthamine was used as reference compound.

Antimicrobial activity

In this study, *Enterobacter aerogenes* RSKK 720, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Micrococcus luteus* NRRL B-4375, *Proteus vulgaris* RSKK 96026, *Bacillus subtilis* ATCC 6633, *Streptococcus mutans* CNCTC 8/77, *Staphylococcus aureus* ATCC 25923, *Candida albicans* ATCC 10239 and *C. tropicalis* RSKK 665 were used. Also, multiple-antibiotic-resistant bacteria *Stenotrophomonas maltophila* MU 23, *S. maltophila* MU 25, *S. maltophila* MU 52, *S. maltophila* MU 53, *S. maltophila* MU 63, *S. maltophila* MU 64, *S. maltophila* MU 69, *S. maltophila* MU 94, *S. maltophila* MU 99, *S. maltophila* MU 136, *S. maltophila* MU 137, *Staphylococcus xylosus* MU 34, *S. xylosus* MU 35, *S. xylosus* MU 37, *S. xylosus* MU 42, *S. aureus* MU 46, *S. capitis* MU 27, *S. lentus* MU 43 and *Staphylococcus* sp. MU 28 and methicillin-resistant *S. aureus* (MRSA) MU 38 and oxacillin-resistant *S. epidermidis* MU 30 were used.

RSKK coded strains were obtained from the Institute of Refik Saydam Hıfzıssıhha Culture Collection, ATCC coded strains from the American Type Culture Collection, CNCTC coded strain from the Czechoslovak Collection of Type Cultures and NRRL coded strain from the Northern Regional Research Laboratory. MU coded strains were obtained from the Muğla University Culture Collection.

S. xylosus, S. aureus, S. capitis, S. epidermidis, S. lentus, Staphylococcus sp., *E. coli, M. luteus, B. subtilis, E. aerogenes* and *P. vulgaris* were cultured in Nutrient Broth (NB) (Difco Laboratories, Detroit, MI, USA) at 37 ± 0.1 °C; *S. mutans* was cultured in Brain Heart Infusion Broth (BHIB) (Difco) at 37 ± 0.1 °C; *P. aeruginosa* and *S. maltophila* were cultured in Nutrient Broth (NB) (Difco) at 30 ± 0.1 °C; *C. albicans* and *C. tropicalis* were cultured in Sabouraund Dextrose Broth (SDB) (Difco) at 30 ± 0.1 °C.

Inocula, prepared by adjusting the turbidity of the medium to match the 0.5 McFarland Standard Dilutions of this suspension in 0.1% peptone (w/v) solution in sterile water, were inoculated on NB, BHIB, and SDB to check the viability of the preparation. The cultures of bacteria and yeast were maintained in their appropriate agar slants at 4 °C throughout the study and used as stock cultures.

The dried plant extracts were dissolved in the same solvent (acetone or methanol) to a final concentration of 20 mg mL^{-1}. The antimicrobial activities of the both acetone and methanol extracts of *Juniperus* species were assayed by the disc diffusion

Table 3. Antioxidant activity of the extracts of *Juniperus* species[a]

Species	Extract	β-Carotene–linoleic acid assay (% inhibition) 25 μg	50 μg	100 μg	IC_{50} (μg mL^{-1})	DPPH assay (% Inhibition) 25 μg	50 μg	100 μg	IC_{50} (μg mL^{-1})
J. communis subsp. *nana*	Hexane	n.t.[b]	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	Acetone	14.11 ± 0.11	32.75 ± 1.03	55.95 ± 0.57	87.22	4.23 ± 0.26	7.25 ± 0.29	13.30 ± 0.51	>100
	Methanol	33.90 ± 5.28	38.84 ± 0.50	46.09 ± 1.65	>100	4.13 ± 0.36	7.28 ± 0.46	13.58 ± 0.66	>100
J. excelsa	Hexane	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	Acetone	29.75 ± 0.27	44.21 ± 0.92	67.51 ± 0.68	64.04	19.05 ± 0.37	32.53 ± 0.36	58.49 ± 1.03	83.77
	Methanol	25.90 ± 0.77	36.06 ± 0.85	50.15 ± 1.09	98.12	2.58 ± 0.12	5.72 ± 0.18	12.00 ± 0.51	>100
J. foetidissima	Hexane	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	Acetone	n.a.[c]	10.39 ± 0.34	12.55 ± 0.46	>100	6.18 ± 0.45	10.98 ± 0.39	20.58 ± 0.65	>100
	Methanol	25.81 ± 1.54	38.10 ± 1.22	54.58 ± 2.84	86.26	8.85 ± 0.63	15.29 ± 0.22	32.15 ± 0.93	>100
J. oxycedrus subsp. *oxycedrus*	Hexane	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	Acetone	44.57 ± 1.17	57.01 ± 0.71	73.06 ± 0.68	36.23	10.27 ± 0.61	18.47 ± 0.19	34.86 ± 0.66	>100
	Methanol	18.35 ± 0.84	31.23 ± 5.01	48.84 ± 0.85	>100	1.56 ± 0.11	3.25 ± 0.25	6.76 ± 0.25	>100
J. phoenicea	Hexane	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	Acetone	35.47 ± 1.89	45.06 ± 0.57	58.79 ± 0.64	69.99	15.01 ± 0.32	23.84 ± 0.34	39.49 ± 0.53	>100
	Methanol	45.94 ± 0.53	60.86 ± 1.12	81.14 ± 0.65	30.85	6.43 ± 0.26	11.33 ± 0.21	21.13 ± 0.93	>100
J. sabina	Hexane	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	Acetone	47.17 ± 2.44	52.98 ± 0.94	67.61 ± 0.25	36.84	10.74 ± 0.14	17.97 ± 0.17	32.42 ± 0.65	>100
	Methanol	53.04 ± 1.91	60.82 ± 0.44	74.13 ± 1.29	12.95	24.66 ± 0.81	34.94 ± 0.57	55.49 ± 0.95	86.78
α-Tocopherol[d]		60.90 ± 0.07	62.92 ± 0.79	64.14 ± 0.21	3.10	61.19 ± 0.56	92.69 ± 0.07	95.23 ± 0.07	7.06
BHT[d]		63.53 ± 0.93	63.66 ± 0.58	65.21 ± 0.91	7.34	40.43 ± 0.05	53.18 ± 0.51	73.91 ± 0.11	45.11

[a] Values expressed represent the means ± standard deviation of three parallel measurements ($P < 0.05$).
[b] n.t., not tested; [c] n.a., not active.
[d] Antioxidant standard.

method.[25] Mueller Hinton Agar (MHA) (Difco) and Sabouraund Dextrose Agar (SDA) (Difco) were sterilized in a flask and cooled to 45–50 C and distributed to sterilized Petri dishes with a diameter of 9 cm (15 mL) after injecting cultures (0.5 mL) of bacteria and yeasts and distributing medium in Petri dishes homogeneously. The plates were held for 15–20 min at room temperature. The discs (6 mm in diameter) were impregnated with 20 μL of the 20 mg mL^{-1} extracts (400 μg per disc) placed on the inoculated agar. For the control groups, pure acetone and methanol without the extract were used. Plaques impregnated with yeasts were incubated at 30 °C for 48 h, and plaques impregnated with *S. xylosus, S. aureus, S. capitis, S. epidermidis, S. lentus, Staphylococcus* sp., *E. coli, M. luteus, B. subtilis, E. aerogenes, P. vulgaris* and *S. mutans* were incubated at 37 °C for 24 h, and plaques impregnated with *P. aeruginosa* and *S. maltophila* were incubated at 30 °C for 24 h. At the end of the period, inhibition zones formed on the MHA and SDA were evaluated in millimetres. Discs of tetracycline (30 μg), penicillin (10 U), ampicillin (10 μg), amoxicillin + clavulanic acid (20 μg/10 μg), imipenem (10 μg), cefaperazon (75 μg), methicillin (5 μg), oxacillin (1 μg), gentamicin (10 μg) and nystatin (30 μg) were used as positive controls. Studies were performed in triplicate and the developing inhibition zones were compared with those of reference discs.

Statistical analysis

All data on both antioxidant activity and anticholinesterase activity tests are the average of triplicate analyses. The data were recorded as mean ± standard deviation. Analysis of variance (ANOVA) was performed. Significant differences between means were determined by Student's *t*-test; *P*-values <0.05 were regarded as significant.

RESULTS AND DISCUSSION

Antioxidant activity results

In this study, *in vitro* antioxidant activity of the various extracts obtained from the berries of six *Juniperus* species, compared to those of BHT and α-tocopherol, were assessed by two complementary tests; namely, β-carotene–linoleic acid assay for total antioxidant activity and DPPH assay for free radical scavenging activity. In addition, total phenolic and total flavonoid contents in the extracts were determined as pyrocatechol and quercetin equivalents, respectively.

Since the phenolic compounds are very important constituents of plants and known as powerful chain-breaking antioxidants,[26] total phenolic content of the extracts was investigated and expressed as micrograms of pyrocatechol per milligram of extract, as shown in Table 2. Methanol extracts of *J. foetidissima, J. phoenicea, J. sabina* and acetone extracts of *J. sabina* and *J. excelsa* had higher phenolic contents than the other extracts. All acetone extracts of the studied *Juniperus* species, except for *J. foetidissima*, were found to be more effective than the methanol extracts. Flavonoids are natural phenolic compounds and well-known antioxidants, and their concentration in the extracts were expressed as micrograms of quercetin equivalents per milligram of extract, as shown in Table 2. The flavonoid contents of the acetone

Table 4. Anticholinesterase activity of the extracts of *Juniperus* species[a]

Species	Extract	AChE assay (% inhibition)				BChE assay (% inhibition)			
		25 µg	50 µg	100 µg	200 µg	25 µg	50 µg	100 µg	200 µg
J. communis subsp. *nana*	Hexane	n.a.[b]	n.a.	n.a.	21.65 ± 0.50	64.30 ± 2.62	70.56 ± 0.06	73.87 ± 0.50	79.92 ± 0.47
	Acetone	n.a.	n.a.	n.a.	11.33 ± 1.08	n.a.	n.a.	4.24 ± 1.88	11.92 ± 1.39
	Methanol	34.17 ± 2.66	36.17 ± 0.24	42.03 ± 0.48	51.61 ± 0.73	n.a.	n.a.	1.73 ± 0.80	6.65 ± 0.34
J. excelsa	Hexane	1.76 ± 1.33	7.18 ± 1.66	18.81 ± 0.29	26.85 ± 0.51	32.41 ± 4.11	39.87 ± 0.77	47.36 ± 1.24	58.35 ± 0.78
	Acetone	n.a.	n.a.	0.78 ± 0.15	8.76 ± 3.45	n.a.	1.31 ± 1.35	7.58 ± 2.06	15.91 ± 1.17
	Methanol	n.a.	n.a.	n.a.	2.01 ± 1.10	2.20 ± 0.52	3.11 ± 0.06	5.76 ± 0.81	10.41 ± 0.64
J. foetidissima	Hexane	62.10 ± 0.09	63.80 ± 0.15	74.81 ± 1.60	75.25 ± 0.77	49.29 ± 0.52	61.72 ± 0.49	71.36 ± 0.73	77.12 ± 1.43
	Acetone	41.88 ± 1.53	49.49 ± 1.67	53.57 ± 0.90	60.03 ± 1.00	23.57 ± 1.92	28.56 ± 0.52	40.92 ± 0.75	54.94 ± 0.54
	Methanol	33.59 ± 2.63	47.64 ± 2.37	39.18 ± 2.99	52.32 ± 1.64	6.96 ± 1.14	9.70 ± 2.34	18.19 ± 1.37	28.77 ± 0.22
J. oxycedrus subsp. *oxycedrus*	Hexane	65.17 ± 3.52	74.93 ± 0.40	79.64 ± 0.36	81.40 ± 0.26	84.92 ± 0.30	87.50 ± 2.87	89.57 ± 1.64	95.75 ± 2.79
	Acetone	4.47 ± 1.04	9.29 ± 1.58	20.07 ± 0.43	33.48 ± 0.18	27.09 ± 0.10	35.72 ± 0.01	38.80 ± 0.89	49.06 ± 0.17
	Methanol	n.a.	n.a.	n.a.	n.a.	4.70 ± 1.38	10.50 ± 1.23	20.62 ± 2.03	25.79 ± 0.74
J. phoenicea	Hexane	36.46 ± 1.69	44.41 ± 1.08	62.39 ± 0.24	75.25 ± 2.70	55.43 ± 1.32	64.20 ± 1.32	78.88 ± 0.45	81.40 ± 1.81
	Acetone	36.94 ± 5.46	38.90 ± 1.63	46.52 ± 1.88	52.98 ± 1.98	39.54 ± 1.46	48.95 ± 0.63	51.02 ± 1.00	60.18 ± 0.98
	Methanol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.32 ± 0.89	6.60 ± 0.19
J. sabina	Hexane	22.91 ± 0.76	33.93 ± 0.36	45.13 ± 1.05	59.38 ± 1.11	28.36 ± 0.05	37.11 ± 1.49	44.50 ± 0.22	53.65 ± 0.86
	Acetone	39.04 ± 0.46	41.90 ± 1.03	47.51 ± 1.11	54.08 ± 0.98	40.15 ± 1.46	45.90 ± 1.53	49.01 ± 1.56	60.08 ± 1.58
	Methanol	12.44 ± 0.06	16.61 ± 0.92	24.26 ± 0.15	33.96 ± 0.27	2.40 ± 0.41	8.05 ± 1.78	20.87 ± 0.50	37.82 ± 1.28
Galantamine[c]		68.36 ± 1.10	74.38 ± 0.65	78.59 ± 0.47	81.41 ± 0.03	40.59 ± 0.88	48.73 ± 0.90	65.02 ± 0.44	75.54 ± 1.05

[a] Values expressed represent the means ± standard deviation of three parallel measurements ($P < 0.05$).
[b] n.a., not active.
[c] Standard drug, in μmol L^{-1} concentration.

extracts were found to be higher than the methanol extracts of *Juniperus* species, except for *J. foetidissima* and *J. phoenicea*. Most flavonoid-rich extracts were found to be methanol extracts of *J. phoenicea* (13.53 ± 0.54 µg QEs mg^{-1} extract) and acetone extracts of *J. sabina* (11.81 ± 0.22 QEs mg^{-1} extract), while the methanol extract of *J. excelsa* (0.91 ± 0.10 QEs mg^{-1} extract) was the poorest one.

Table 3 shows the antioxidant activity of acetone and methanol extracts of six *Juniperus* species, compared with α-tocopherol, and BHT used as positive controls. In the β-carotene–linoleic acid assay, oxidation of linoleic acid was effectively inhibited by methanol extracts of *J. sabina* (IC_{50} = 12.95 µg mL^{-1}) and *J. phoenicia* (IC_{50} = 30.85 µg mL^{-1}), followed by acetone extracts of *J. oxycedrus* subsp. *oxycedrus* (IC_{50} = 36.23 µg mL^{-1}), *J. sabina* (IC_{50} = 36.84 µg mL^{-1}) and *J. excelsa* (IC_{50} = 64.04 µg mL^{-1}). Under the same conditions α-tocopherol and BHT exhibited IC_{50} values of 3.10 and 7.34 µg mL^{-1}, respectively. In the DPPH assay, acetone extract of *J. excelsa* demonstrated the highest DPPH radical scavenging activity, which was close to that of methanol extract of *J. sabina* (Table 3).

Anticholinesterase activity results

In some investigations, radical scavenging activity was found to be related to anticholinesterase activity.[10] According to Atta-ur-Rahman and Choudhary, the use of antioxidants may minimize neuronal degradation.[6] Therefore, 18 extracts prepared with hexane, acetone and methanol from six *Juniperus* species of Turkish origin were screened *in vitro* against AChE and BChE enzymes at 25, 50, 100 and 200 µg mL^{-1}. As listed in Table 4, the most active extract against AChE was found to be the hexane extract of *J. oxycedrus* subsp. *oxycedrus*, having 81.40% of inhibition at 200 µg mL^{-1}, followed by hexane extracts of *J. foetidissima* (75.25%) and *J. phoenicea* (75.25%) at the same concentration. Only the methanol extracts of *J. communis* subsp. *nana* and *J. foetidissima* possessed moderate inhibition of AChE as polar extracts. Against BChE, hexane extract of *J. oxycedrus* subsp. *oxycedrus* was also the most active extract, with 95.75% inhibition. Interestingly, hexane extracts of *J. communis* subsp. *nana* and *J. excelsa* demonstrated activity against BChE.

Antimicrobial activity results

Antimicrobial activity of both acetone and methanol extracts of berries of six *Juniperus* species growing in Anatolia has been evaluated *in vitro* against 29 bacterial species, including multiple-antibiotic-resistant bacteria, and two yeasts which are known to cause infections in humans. As summarized in Table 5, the extracts exhibited more or less antimicrobial activity against some of the tested microorganisms. The inhibition zones of the reference antibiotics are given in Table 6 against a series microorganisms. All extract concentrations (400 µg per disc) used in this study were low compared to the amount of reference antibiotics (10 µg, 30 µg, 75 µg etc.).

Acetone and methanol extracts of *J. foetidissima* caused 7–10.0 mm and 6.5–11.0 mm inhibition zones on some of tested bacteria. The acetone extract produced 7.5, 8.0 and 9.0 mm inhibition zones on multiple-antibiotic-resistant *S. maltophilia* MU 64, MU 69 and MU 99, respectively. Methanol extract of *J. foetidissima* inhibited all of the antibiotic-resistant staphylococci,

Table 5. Antimicrobial activity of *Juniperus* species (400 µg/disk) against the bacterial strains tested based on disc-diffusion method

	Inhibition zone diameter (mm)[a]																	
	J. communis subsp. *nana*			*J. excelsa*			*J. foetidissima*			*J. oxycedrus* subsp. *oxycedrus*			*J. phoenicea*			*J. sabina*		
Microorganism	Hexane	Acetone	Methanol	Hexane	Acetone	Methanol	Hexane	Acetone	Methanol	Hexane	Acetone	Methanol	Hexane	Acetone	Methanol	Hexane	Acetone	Methanol
E. aerogenes RSKK 720	–	–	8	–	–	–	–	–	–	–	–	–	–	–	–	–	8	8
P. aeruginosa ATCC 27853	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	8
E. coli ATCC 25922	–	–	10	–	–	–	–	–	–	–	–	–	–	–	9	–	7.5	–
M. luteus NRRL B-4375	–	–	–	6.5	–	–	–	–	9	–	–	–	–	–	–	7.5	–	–
P. vulgaris RSKK 96026	8	–	–	–	8.5	–	–	–	–	–	–	–	7.5	6.5	7.5	–	8	–
B. subtilis ATCC 6633	–	–	9	–	–	–	7	8.5	11	–	–	–	8.5	–	9.5	–	9	–
S. mutans CNCTC 8/77	–	–	–	–	–	–	–	–	–	–	–	–	7	–	–	7	7	–
S. aureus ATCC 25923	–	–	–	7.5	–	–	–	–	8	–	–	–	–	–	–	–	–	8.5
C. albicans ATCC 10239	6.5	–	8.5	–	6.5	–	–	–	7.5	–	–	–	8	–	9	–	–	6.5
C. tropicalis RSKK 665	–	–	9	–	–	–	6.5	–	–	–	–	–	6.5	–	–	6.5	7	–
S. maltophila MU 23	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7	9.5	–
S. maltophila MU 25	–	–	7	–	–	–	–	–	–	–	–	–	–	–	7.5	–	–	7.5
S. maltophila MU 52	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6.5	8.5	–
S. maltophila MU 53	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7.5	9	–
S. maltophila MU 63	–	–	–	8	–	–	–	–	–	–	–	–	7	–	7	–	–	–
S. maltophila MU 64	–	–	–	8	–	–	7.5	7.5	–	–	–	–	–	–	–	–	8	–

Table 5. (*Continued*)

	Inhibition zone diameter (mm)[a]																	
	J. communis subsp. *nana*			*J. excelsa*			*J. foetidissima*			*J. oxycedrus* subsp. *oxycedrus*			*J. phoenicea*			*J. sabina*		
Microorganism	Hexane	Acetone	Methanol	Hexane	Acetone	Methanol	Hexane	Acetone	Methanol	Hexane	Acetone	Methanol	Hexane	Acetone	Methanol	Hexane	Acetone	Methanol
S. maltophila MU 69	–	–	–	8	–	–	7	8	–	–	–	–	9	–	–	–	–	–
S. maltophila MU 94	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	8
S. maltophila MU 99	–	–	–	10	–	–	7	9	–	–	–	–	9	–	–	–	–	–
S. maltophila MU 136	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S. maltophila MU 137	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S. capitis MU 27	–	–	8	9	–	–	8	10	8.5	7	–	–	9	–	8.5	8	8.5	–
Staph. sp. MU 28	–	–	8	8	–	–	8.5	9	8	8	–	–	8.5	–	8	8	8	8.5
S. epidermidis MU 30	7.5	–	10	–	7.5	–	–	–	7	7.5	–	–	–	–	8	–	–	7
S. xylosus MU 34	–	–	–	–	–	–	–	–	7	–	–	–	–	–	–	7	8.5	7.5
S. xylosus MU 35	–	–	–	7	–	–	–	7	7	–	–	–	–	8	–	–	7	7.5
S. xylosus MU 37	–	–	–	8	–	–	7	7	7	–	–	–	7	–	–	–	–	–
S. aureus MU 38	–	–	–	–	–	–	–	–	6.5	–	–	–	7	9.5	–	9	8	8.5
S. xylosus MU 42	–	7.5	–	7.5	–	–	7	7.5	8.5	7	–	6.5	–	–	–	7	9	8
S. lentus MU 43	–	8.5	–	–	–	8	8	7	7	–	–	8.5	6.5	–	–	–	–	7
S. aureus MU 46	–	–	7.5	–	–	–	–	7	7	–	–	–	–	–	–	–	–	–

Table 6. Inhibition zone diameter of the reference antibiotics to Gram-positive and Gram-negative test bacteria and yeasts

Microorganism	Antibiotic								
	P	AM	AMC	IPM	CFP	ME	OX	CN	NYS
	Inhibition zone diameter (mm)								
E. aerogenes RSKK 720	8	–	n.t.	n.t.	n.t.	n.t.	–	n.t.	n.t.
P. aeruginosa ATCC 27853	–	–	–	23	21	n.t.	n.t.	n.t.	n.t.
E.coli ATCC 25922	–	–	16	21	21	n.t.	n.t.	n.t.	n.t.
M. luteus NRRL B-4375	32	29	32	36	26	n.t.	n.t.	n.t.	n.t.
P. vulgaris NRRL B-4375	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	14	n.t.
B. subtilis ATCC 6633	11	–	23	48	19	n.t.	n.t.	n.t.	n.t.
S. mutans CNCTC 8/77	15	12	20	20	14	n.t.	n.t.	n.t.	n.t.
S.aureus ATCC 25923	21	17	20	n.t.	n.t.	15	12	n.t.	n.t.
C. albicans ATCC 10239	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	19
C. tropicalis RSKK 665	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	15

P, penicillin (10 U); AM, ampicillin (10 μg); AMC, amoxicillin + clavulanic acid (20 μg/10 μg); IPM, imipenem (10 μg), CFP, cephaperazone (75 μg); ME, methicillin (5 μg); OX, oxacillin (1 μg); CN, gentamicin (10 μg); NYS, nystatin (30 μg); n.t., not tested; (–), no zone.

and the acetone extract also inhibited these bacteria, except for *S. epidermidis* MU 30, *S. xylosus* MU 34 and *S. aureus* MU 38.

The acetone extract of *J. phoenicea* showed antibacterial activity only on *P. vulgaris, S. xylosus* MU 35 and *S. aureus* MU 38. The methanol extract had more effect on bacteria than the acetone extract. The antibacterial activities of essential oils from the leaves and berries of *J. oxycedrus* subsp. *oxycedrus* and *J. phoenicea* have been previously studied, and none of the extracts showed antimicrobial activity. In another study, the essential oil of *J. phoenicea* ssp. *turbinata* showed activity against *C. albicans* and *S. aureus*.[27] When the results were taken into general consideration, it was seen that the extracts of *J. excelsa* and *J. oxycedrus* subsp. *oxycedrus* had the lowest activity. The acetone extract of *J. oxycedrus* subsp. *oxycedrus* had no effect on any of the microorganisms tested. According to some other studies, the essential oil of *J. oxycedrus* subsp. *oxycedrus*[27] and *J. excelsa*[28] have shown antibacterial activities. Some other antimicrobial activity studies were carried out on *J. oxycedrus*, collected from various region of Turkey.[29,30]

The acetone extract of *J. communis* subsp. *nana* showed only an inhibitory effect on *S. xylosus* MU 42 and *S. lentus* MU 43 among the tested bacteria, while methanol extract of the same plant was not found to be effective against these two bacteria (Table 5).

The inhibition effects of acetone and methanol extracts of *J. sabina* on bacteria varied among species, and the inhibition zones were found to be 7.0–9.5 mm and 6.5–8.5 mm, respectively. Both extracts showed inhibition effects on multiple-antibiotic-resistant staphylococci. The extracts also inhibited growth of some strains of multiple-antibiotic-resistant *Stenotrophomonas maltophilia* (Table 6), in general.

Several methanol extracts including *J. foetidissima, J. phoenicea, J. communis* subsp. *nana, J. sabina* and the hexane extracts of *J. communis* subsp. *nana* and *J. phoenicea* and only one acetone extract belongs to *J. excelsa* demonstrated antifungal activity against *C. albicans*. Hexane extracts of the three species *J. foetidissima, J. phoenicia* and *J. sabina* exhibited week to moderate activity, while only one acetone and one methanol extract of two species showed moderate activity against the yeast *C. tropicalis* (Table 5).

CONCLUSIONS

Among the 18 extracts studied, prepared from six species, five – namely methanol extracts of *J. sabina* and *J. phoenicea*, and acetone extracts of *J. oxycedrus* subsp. *oxycedrus, J. sabina* and *J. excelsa* – showed high lipid peroxidation inhibitory activity compared to the other extracts. Although the acetone extract of *J. oxycedrus* subsp. *oxycedrus* showed high lipid peroxidation inhibitory activity, it has shown any antimicrobial activity; therefore no correlation was observed between antioxidant and antibacterial activity. However, the acetone extract of *J. oxycedrus* subsp. *oxycedrus* exhibited moderate AChE and BChE inhibitory activity. On the other hand, the hexane extracts of *J. oxycedrus* subsp. *oxycedrus, J. foetidissima* and *J. phoenicea* demonstrated significant AChE and BChE inhibitory activities; however, since none of the hexane extracts were tested for antioxidant activity, there was no opportunity to make any correlation for their antioxidant–anticholinesterase activity.

The results presented in this study should be considered as the first information on the anticholinesterase activity of *Juniperus* plants. Similarly, except for an antioxidant activity report on *J. communis*,[31] the results should further be considered as the first information on the antioxidant activity of *Juniperus* plants growing in Turkey.

Based on our results, *Juniperus* berry extracts, obtained particularly from *J. sabina* and three other species – *J. excelsa, J. oxycedrus* subsp. *oxycedrus* and *J. phoenicea* – appeared as high-potential natural sources of antioxidant activity which may be used in the food industry as preservative agents or to extend the shelf-life of raw and processed foods. All hexane extracts, containing non-polar components of the studied *Juniperus* species, exhibited high anticholinesterase activity, particularly against BChE. Conversely, hexane extracts of *J. communis* subsp. *nana and J. excelsa* exhibited fairly weak activity against AChE, while the former exhibited high and the latter moderate BChE inhibitory activity.

In conclusion, the hexane extract of *J. oxycedrus* subsp. *oxycedrus* was found to be an important source for potential anti-Alzheimer drugs, with high anticholinesterase activity against both AChE and BChE enzymes. Thus further studies on *Juniperus* species, urgently on *J. oxycedrus* subsp. *oxycedrus, J. sabina* and *J. phoenicea* targeting to determine the active compounds responsible for antioxidant and anticholinesterase activity from both polar and non-polar extracts, are needed.

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