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Bioactivity Screening of the Selected Turkish Marine Sponges and Three Compounds from *Agelas oroides*

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Abstract: The extracts of various marine sponges (*Agelas oroides* and *Axinella damicornis*, *Axinella cannabina*, *Ircinia spinulosa*, *I. fasciculata*, and *I. variabilis*, *Dysidea avara*, and *Sarcotragus spinulosus*) collected from different spots of the Turkish cost of the Mediterranean Sea have been evaluated for their antibacterial, antifungal, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, and acetylcholinesterase (AChE) inhibitory activities. Three compounds (oroidin, 4,5-dibromopyrrol-2-carboxylic acid, and 25-hydroxy-24-methylcholesterol) were isolated from *A. oroides* and tested in the same manner. The sponge extracts showed notable antimicrobial and anti-AChE activity and low DPPH scavenging activity. Oroidin was found to have moderate anti-AChE and strong radical scavenging activities. The results demonstrated that the sponge extracts exerted a variable degree of antibacterial, anti-radical, and anti-AChE activity, whereas they seemed to have similar antifungal effect. Our findings point out to the fact that the collection site has an important influence on bioactivity of the sponges.

Keywords: Marine sponge; antimicrobial; anticholinesterase; radical scavenger; collection site.

1. Introduction

The last 30 years have seen an explosive augmentation of natural products from marine organisms that have been characterized by the isolation and structure elucidation of unusual and beautiful structures with no precedent in terrestrial natural products. Among the marine organisms; marine sponges (Phylum *Porifera*) are the oldest and simplest multicellular animals on earth, having originated over a billion years ago. As they are sessile organisms, sponges have been known to produce biologically active compounds, which are assumed to be used as a kind of defense mechanism against their predators.

Acetylcholinesterase (AChE) inhibition is one of the most accepted treatment approaches for Alzheimer's disease (AD) and unfortunately, there is no cure, yet, to stop the disease, but only

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symptomatic treatment is available for mild to moderate types of AD generally by AChE inhibitors, all of which are nitrogenous compounds such as rivastigmine, donepezil, and galanthamine, an alkaloid isolated from the snowdrop plant, *Galanthus nivalis* (Amaryllidaceae) [1]. Therefore, there is still great need to find new molecules with AChE inhibitory activity. For this purpose, we also screened some of the sponge extracts as well as three pure compounds (**1-3**) isolated from *Agelas oroides*.

Turkey is a country of peninsula surrounded by the Black Sea at the north, the Aegean Sea at the west, and the Mediterranean Sea at the south. Despite of its long coastal line, which is 8300 km in total, there have been limited works on its marine prosperity from the view point of isolation of biologically active compounds. For this purpose, we have started a joint project on isolation and various bioactivity evaluations of the marine organisms from the Turkish waters and collected a good variety of the marine sponges. Then, antibacterial, antifungal, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, and acetylcholinesterase (AChE) inhibitory activities of the methanol extracts obtained from marine sponge species collected from different coastal sites of Turkey; namely *Agelas oroides* and *Axinella damicornis* from four locations, *Axinella cannabina*, *Ircinia spinulosa*, *I. fasciculata*, and *I. variabilis* from two locations, *Dysidea avara*, and *Sarcotragus spinulosus* have been determined. Antibacterial activity of the sponge extracts was assayed against following bacteria and their isolated strains; *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Bacillus subtilis*, while they were screened by microdilution method against two fungi; *Candida albicans* and *C. parapsilosis*. Antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test. Their AChE inhibition was determined by the spectrophotometric method of Ellman using an ELISA microplate reader at 50, 100, and 200 μg mL^{-1}. The methanol extract of *A. oroides* was subjected to further separation, which led to isolation of three known compounds identified as oroidin **(1)**, 4,5-dibromopyrrol-2-carboxylic acid **(2)**, and 25-hydroxy-24-methylcholesterol **(3)**. The compounds **(1-3)** were also tested for their DPPH radical scavenging and AChE inhibitory effects in the same manner.

2. Materials and Methods

2.1. Sponge Materials

The sponges were collected by SCUBA diving from the four main locations including Antalya, Kemer, and Hatay provinces on the Mediterranean cost and Ayvalik town on the Aegean coast during the year of 2007 by biologist Bülent Gözcelioglu and transferred immediately to the laboratory in Ankara while kept in ethanol (70%) during transfer and later on put in deepfreeze until the experimental process. The sponges collected were identified by Prof.Dr. Rob van Soest (Zoology Museum, University of Amsterdam, Holland) as *Agelas oroides* (Kemer, two samples from Hatay, Ayvalik), *Axinella damicornis* (Kemer, Hatay, Antalya, Ayvalik), *Axinella cannabina* (Hatay and Ayvalik), *Ircinia spinulosa* (Kemer and Hatay), *I. fasciculata* (Kemer and Hatay), and *I. variabilis* (two samples from Antalya), *Dysidea avara* (Ayvalik), and *Sarcotragus spinulosus* (Antalya).

2.2. Extraction Procedure

The sponge samples were chopped by a knife into small pieces, weighed accurately (approximately 100 g for each) and later extracted individually with methanol (1000 mL) for several times. The methanol phases were filtered and evaporated *in vacuo* until dryness. The obtained methanol extracts were employed in the activity and isolation experiments.

2.3. Determination of Antibacterial and Antifungal Activity

2.3.1. Preparation of the Test Materials

The sponge extracts were prepared in dimethylsulphoxide (DMSO) at a final concentration of 256 µg mL^{-1} and sterilized by filtration using 0.22 µm Millipore (MA 01730, USA) and used as the stock solutions. Reference antibacterial agents of ampicillin (AMP; Fako Pharmaceutical Company) and ofloxacin (OFX; Hoechst Marion Roussel Pharmaceutical Company) were obtained from their respective manufacturers and dissolved in phosphate buffer solution (AMP; pH: 8.0, 0.1 mol L^{-1}), and in distilled water (OFX). The stock solutions of these agents were prepared in medium according to as CLSI (formerly National Committee for Clinical Laboratory Standards recommendations-NCCLS) [2,3].

2.3.2. Microorganisms and Inoculum Preparation

Antibacterial activity tests were carried out against standard (ATCC; American type culture collection, RSKK; Culture collection of Refik Saydam Central Hygiene Institute) and isolated (clinical isolate and obtained from Department of Microbiology, Faculty of Medicine, Gazi University, Ankara, Turkey) strains of Gram-negative type; *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 10145, *Proteus mirabilis* ATCC 7002, *Klebsiella pneumoniae* RSKK 574, *Acinetobacter baumannii* RSKK 02026, and the strains of Gram-positive type; *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, and *C. parapsilosis* ATCC 22019 were employed for determination of antifungal activity. Mueller Hinton Broth (MHB, Difco) and Mueller Hinton Agar (MHA, Oxoid) were applied for growing and diluting of the bacterium suspensions as described beforehand [4]. The synthetic medium RPMI-1640 with *L*-glutamine was buffered to pH:7 with 3-[*N*-morpholino]-propansulfonic acid and culture suspensions were prepared. The microorganism suspensions used for inoculation were prepared at 10^5 cfu (colony forming unit) mL^{-1} by diluting fresh cultures at McFarland 0.5 density (10^8 cfu mL^{-1}). Suspensions of bacteria and fungi were added in each well of the diluted samples, density of 10^5 cfu mL^{-1} for fungi and bacteria. The bacterial suspensions used for inoculation were prepared at 10^5 cfu mL^{-1} by diluting fresh cultures at McFarland 0.5 density (10^8 cfu mL^{-1}). The fungus suspensions were prepared by the spectrophotometric method of inoculum preparation at a final culture suspension of $2.5x10^3$ cfu mL^{-1} [2,3].

2.3.3. Antibacterial and Antifungal Tests

The microdilution method was employed for antibacterial and antifungal activity tests as described in our previous study [4]. Media were placed into each well of 96-well microplates. Sample solutions at 512 µg mL^{-1} were added into first rows of microplates and two fold dilutions of the compounds (256-0.125 µg mL^{-1}) were made by dispensing the solutions to the remaining wells. Culture suspensions (10 µL) were inoculated in all of the wells. The sealed microplates were incubated at 35ºC for 24 h and 48 h in a humid chamber. The lowest concentration of the samples that completely inhibited macroscopic growth was determined and minimum inhibitory concentrations (MICs) were calculated.

2.4. DPPH Radical Scavenging Activity Test

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined by the method of Blois [5]. The samples and references dissolved in ethanol (75%) were mixed with DPPH solution (1.5×10^{-4} M). Remaining DPPH amount was measured at 520 nm using a Unico 4802 UV-visible double beam spectrophotometer (USA). Gallic acid and butylated hydroxyanisol (BHA) were employed as the references. Inhibition of DPPH in percent (I%) was calculated as given below:

$I\% = [(A_{blank}-A_{sample}) / A_{blank}] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of the extracts/references.

2.5. AChE Inhibitory Activity Test

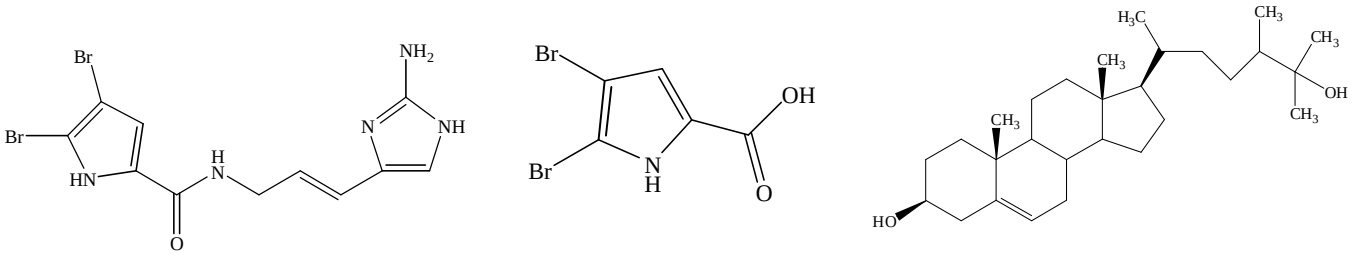
AChE inhibition was assayed by the spectrophotometric method of Ellman et al [6]. Electric eel AChE (Type-VI-S; EC 3.1.1.7, Sigma) was employed as the enzyme source, while acetylthiocholine iodide (Sigma, St. Louis, MO, USA) as substrate and 5,5´-dithio-*bis*(2-nitrobenzoic)acid (DTNB; Sigma, St. Louis, MO, USA) were also used in the anti-AChE activity determination. All reagents and conditions were same as described in our previous publication [7]. The experiments were performed in triplicate. Galanthamine purchased from Sigma (St. Louis, MO, USA) was the reference in this study.

2.6. Isolation Studies on Agelas oroides

The methanol extract of *A. oroides* (10.54 g) was dissolved with 500 mL of distilled water and taken into a separatory funnel. After shaking, ethyl acetate (500 mL) was added in the aqueous phase (lower phase) each time and the ethyl acetate phase (upper phase) was collected. The ethyl acetate and aqueous phases were separately collected, evaporated under vacuum until dryness, then, they were put in vials and left under nitrogen stream and weighed accurately (ethyl acetate phase: 2.44 g; aqueous phase: 3.39 g). The ethyl acetate phase was subjected to vacuum liquid chromatography (VLC) using *n*-hexane, dichloromethane, ethyl acetate, and methanol, which led to collection of 9 main fractions. Following thin layer chromatography (TLC) monitoring of the VFC fractions, fr.7 (0.753 g) was further loaded on Sephadex column chromatography (CC) eluting with methanol, which afforded 256 subfractions. After TLC monitoring, the similar subfractions were combined and reduced to 26 subfractions. Among them, two major spots were isolated from subfr.5 by liquid chromatography-mass spectrometry [LC-MS; HP-Agilent (HPLC), Thermoquest (MS)] using acetonitrile:water (0.1% formic acid) as eluent on reversed-phase column (RP-18; Europher 100, C-18A, Knauer) with a flow rate of 0.4 mL min^{-1}, leading isolation of compounds **1** and **3**.

The methanol extract of *A. oroides* (4.06 g) at the start was then again dissolved in methanol:distilled water mixture (100 mL) and taken into a separatory funnel. After shaking, *n*-hexane (90 mL) was added in the methanol phase (lower phase) each time and the *n*-hexane phases (upper phase) were collected for 5 times. After that, the *n*-hexane phases were collected, evaporated under vacuum until dryness, kept in vials and weighed precisely. The *n*-hexane phase was subjected to silica gel (Si 60) CC eluting with dichloromethane and methanol mixtures in increasing polarity, which led to 14 fractions. TLC analysis reduced number of the fractions to 6 after combining the similar fractions. Formation of a white precipitate was observed in fr.3, washed several times with methanol, left in deepfreeze for 15 minutes, and crystal form of the precipitated compound (**3**) was obtained.

Chemical structures of compounds **1-3** were identified by means of spectral methods including nuclear magnetic resonance techniques (NMR) such as ^{1}H, ^{13}C NMR, H-H COSY, and MS (Sektorfeld-Massenspektrometer, Finnigan MAT 8200) as well as their TLC comparison with the authentic compounds. According to the spectral data and TLC evaluation; compounds **1, 2**, and **3** were elucidated as oroidin, 4,5-dibromopyrrol-2-carboxylic acid, and 25-hydroxy-24-methylcholesterol, respectively (Figure 1).



1 **2** **3**

Figure 1. Chemical structures of the compounds (**1-3**) isolated from *Agelas oroides*

3. Results and Discussion

3.1. Antimicrobial Activity

Table 1. Antibacterial activity of the sponge extracts and references against Gram (-) bacteria expressed as minimum inhibitory concentrations (MICs; µg mL^{-1})

	Microorganisms									
	E. coli		***P. aeruginosa***		***P. mirabilis***		***K. pneumonaei***		***A. baumannii***	
Extracts tested	**ATCC[a] 35218**	IS[b] ESβL+	**ATCC 10145**	IS	**ATCC 7002**	IS ESβL+	**RSKK 574**	IS ESβL+	**RSKK 02026**	IS
Agelas oroides (Kemer)	4	16	2	16	8	32	8	16	16	32
Agelas oroides (Hatay-I)	16	32	8	32	8	32	16	32	32	64
Agelas oroides (Hatay-II)	8	32	8	64	16	64	8	8	16	64
Agelas oroides (Ayvalik)	8	16	8	32	8	32	8	16	16	32
Axinella damicornis (Hatay)	16	32	8	32	8	32	16	32	16	32
Axinella damicornis (Kemer)	4	16	2	16	16	32	16	32	16	64
Axinella damicornis (Ayvalik)	8	16	8	32	4	16	8	16	32	64
Axinella cannabina (Ayvalik)	16	32	2	16	8	32	8	16	32	64
Axinella cannabina (Hatay)	4	16	4	16	8	32	4	8	8	64
Ircinia spinulosa (Kemer)	16	32	8	64	16	64	16	32	16	64
Ircinia spinulosa (Hatay)	4	16	2	16	4	16	16	32	32	64
Ircinia fasciculata (Hatay)	2	16	2	16	4	16	16	32	16	64
Ircinia fasciculata (Kemer)	8	32	4	64	4	16	8	16	16	64
Dysidea avara (Ayvalik)	16	32	8	32	8	32	8	16	32	64
References										
AMP[d]	2	>128	-[c]	-	2	>128	2	>128	2	>128
GM[e]	-	-	0.5	2	-	-	-	-	-	-
OFX[f]	0.12	0.5	1	64	<0.12	1	<0.12	0.5	0.12	64

[a]American Type of Culture Collection [b]Isolated Strain, [c]Not tested, [d]Ampicillin, [e]Gentamicin, [f]Ofloxacin

The methanol extracts of the Turkish marine sponges; namely *Agelas oroides* (Kemer, two samples from Hatay, Ayvalik), *Axinella damicornis* (Kemer, Hatay, Antalya, Ayvalik), *Axinella cannabina* (Hatay and Ayvalik), *Ircinia spinulosa* (Kemer and Hatay), *I. fasciculata* (Kemer and Hatay), and *I. variabilis* (two samples from Antalya), *Dysidea avara* (Ayvalik), and *Sarcotragus spinulosus* (Antalya) were screened for their antibacterial and antifungal activities against the standard and isolated

strains of Gram-negative type; *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 10145, *Proteus mirabilis* ATCC 7002, *Klebsiella pneumoniae* RSKK 574, *Acinetobacter baumannii* RSKK 02026, and the strains of Gram-positive type; *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231 and *C. parapsilosis* ATCC 22019 (Tables 1 and 2).

Table 2. Antibacterial activity against Gram (+) bacteria and antifungal activity of the sponge extracts and references expressed as minimum inhibitory concentrations (MICs; µg mL^{-1})

Extracts	**Microorganisms**							
	S. aureus		*E. faecalis*		*B. subtilis*		*C. albicans* ATCC 10231	*C. parapsilosis* ATCC 22019
	ATCC[a] 25923	IS[b] MRSA	ATCC 29212	IS	ATCC 6633	IS		
Agelas oroides (Kemer)	0.5	64	8	>128	16	32	16	16
Agelas oroides (Hatay-I)	1	128	16	>128	32	64	16	16
Agelas oroides (Hatay-II)	1	128	8	128	16	32	16	16
Agelas oroides (Ayvalik)	0.5	64	4	64	16	32	16	16
Axinella damicornis (Hatay)	1	128	16	>128	16	32	16	16
Axinella damicornis (Kemer)	2	128	16	128	16	32	16	16
Axinella damicornis (Ayvalik)	0.5	64	16	128	32	64	16	16
Axinella cannabina (Ayvalik)	2	128	16	128	32	64	16	16
Axinella cannabina (Hatay)	0.5	128	8	128	8	16	16	16
Ircinia spinulosa (Kemer)	1	128	8	64	16	32	16	16
Ircinia spinulosa (Hatay)	0.5	64	4	64	32	64	16	16
Ircinia fasciculata (Hatay)	2	128	16	>128	16	32	16	16
Ircinia fasciculata (Kemer)	1	128	8	>128	16	32	16	16
Dysidea avara (Ayvalik)	2	128	16	128	32	64	16	16
References								
AMP[d]	<0.12	>128	0.5	>128	0.12	0.5	-[c]	-
OFX[e]	0.25	64	1	32	-	-	-	-
FLU[f]	-	-	-	-	-	-	2	4

[a]American Type of Culture Collection, [b]Isolated Strain, [c]Not tested, [d]Ampicillin, [e]Gentamicin, [f]Ofloxacin

The extracts were the most active against ATCC strain of *S. aureus* having the MIC values between 0.5 – 1 μg mL^{-1}, where the references; ofloxacin and ampicillin had MIC at 0.25 and <0.12 μg mL^{-1}, respectively. *I. spinulosa* and *I. fasciculata* samples of Hatay collections exhibited similar potent antibacterial properties towards the tested microorganisms. Generally, the extracts were much more moderate against the clinically isolated strains of bacteria. Interestingly, occurrence of a locational variety in antibacterial activity was observed among the extracts of the same sponge species collected from different coastal sites. The extracts were found to be effective towards *C. albicans* and *C. parapsilosis* with MIC value at 16 μg mL^{-1}.

3.2. DPPH Radical Scavenging Activity

Considering DPPH radical scavenging activity of the extracts as listed in Table 3, only *I. variabilis* of Antalya-I collection exerted the best scavenging effect (65.92%) at 2000 μg mL^{-1} as compare to the references; butylatedhydroxyl anisol (BHA) and gallic acid. Interestingly, another sample of *I. variabilis* collected from different part of Antalya (Antalya-2) did not show a noticeable effect in the same test. Most of the extracts seemed not to have scavenging activity.

Table 3. DPPH radical scavenging activity (inhibition %±S.E.M.) of the sponge extracts

	Percentage of inhibition±S.E.M.[a] against DPPH radical		
Extracts	500 μg mL^{-1}	1000 μg mL^{-1}	2000 μg mL^{-1}
Agelas oroides (Kemer)	-[b]	19.31±0.78	29.76±4.66
Agelas oroides (Hatay-I)	-	-	8.79±0.57
Agelas oroides (Hatay-II)	-		11.78±0.63
Agelas oroides (Ayvalik)	17.03±0.99	23.02±2.59	47.69±4.08
Axinella damicornis (Hatay)	-		12.89±2.25
Axinella damicornis (Kemer)	17.56±0.77	19.66±0.66	38.29±1.01
Axinella damicornis (Ayvalik)	-	-	16.58±0.75
Axinella damicornis (Antalya)	-	-	-
Axinella cannabina (Ayvalik)	-	-	14.25±0.81
Axinella cannabina (Hatay)	8.51±1.79	7.95±0.47	17.10±0.68
Ircinia spinulosa (Kemer)	-	-	11.68±0.65
Ircinia spinulosa (Hatay)	-	-	-
Ircinia fasciculata (Hatay)	-	-	7.30±0.28
Ircinia fasciculata (Antalya)	13.09±1.04	22.47±1.34	39.27±1.93
Ircinia variabilis (Antalya-I)	19.14±1.71	38.63±3.92	65.92±1.86
Ircinia variabilis (Antalya-II)	-	-	7.18±0.88
Dysidea avara (Ayvalik)	-	-	15.34±0.18
Sarcotragus spinulosus (Antalya)	-	8.03±0.00	10.62±0.65
References			
Butylated hydroxy anisol (BHA)	77.99±0.48	81.66±1.67	82.94±0.68
Gallic acid	91.61±0.06	92.57±0.10	93.19±0.00

[a]Standard error mean (n=3), [b]= No activity observed

3.3. AChE Inhibitory Activity

Only the methanol extracts of *A. oroides* (Kemer), *A. damicornis* (Kemer and Ayvalik), *A. cannabina* (Ayvalik), *I. spinulosa* (Kemer), *I. fasciculata* (Kemer and Hatay), and *D. avara* (Ayvalik) were tested *in vitro* for their AChE inhibitory effect at 50, 100, and 200 μg mL^{-1} using galanthamine as the reference in this assay (Table 4). The extracts exhibited a moderate inhibition in concentration-dependent manner. The most active extracts at 200 μg mL^{-1} belonged to *I. fasciculata* of Hatay collection (68.64%), *I. spinulosa* of Kemer collection (68.19%), and *D. avara* (66.90%).

The three compounds (oroidin (**1**), 4,5-dibromopyrrol-2-carboxylic acid (**2**), and 25-hydroxy-24-methylcholesterol (**3**) isolated from *A. oroides* were also screened for their DPPH radical scavenging and AChE inhibitory activity (Table 5). In both tests, only oroidin showed a weak inhibitory effect against AChE having 26.24% at 100 μg mL^{-1}, while it showed a high radical scavenging activity at 50, 100, and 200 μg mL^{-1} (89.96, 93.07, and 93.91%, respectively) in concentration-dependent manner.

Table 4. Acetylcholinesterase (AChE) inhibitory activity of the sponge extracts

Extracts	**Percentage of inhibition±S.E.M[a] against AChE**		
	50 μg mL^{-1}	100 μg mL^{-1}	200 μg mL^{-1}
Agelas oroides (Kemer)	42.89±0.47	47.02±0.88	49.78±1.01
Axinella damicornis (Kemer)	41.09±0.32	41.35±0.77	51.89±0.93
Axinella cannabina (Ayvalik)	36.69±0.72	39.46±0.82	39.28±1.32
Ircinia spinulosa (Kemer)	52.80±0.99	56.08±1.14	68.19±0.89
Ircinia fasciculata (Kemer)	55.65±0.76	54.81±1.28	57.60±1.02
Ircinia fasciculata (Hatay)	56.85±0.65	60.06±0.68	68.64±0.99
Dysidea avara (Ayvalik)	39.04±1.05	57.87±0.39	66.90±0.92
Reference			
Galanthamine	98.88±0.93 at 100 μg mL^{-1}		

[a]Standard error mean (n=3)

Up to date, there have been many reports on chemistry of the marine sponges of *Agelas*, *Axinella*, *Ircinia*, *Dysidea*, and *Sarcotragus* genera, which are the materials of this study. *Agelas* species have been reported to have bromopyrrol-alkaloids in major amounts [8-10], while *Axinella* species have been known to contain various terpene derivatives, alkaloids, cyclopeptides, etc. [11-13]. *Ircinia* species have been one of the most chemically investigated species since 1970's, which were found to be quite rich in linear furanoterpenes in particular [14,15]. *Dysidea avara* has been reported to contain avarol and avarone, the major sesquiterpenes with several biological activities [16-18].

A number of studies on antimicrobial activity of these sponges as well as their secondary metabolites have been reported up to date. For instance; bromopyrrole-type of several compounds isolated from *A. conifera* were stated to be active against *B. subtilis*, *E. coli*, *Herpes simplex* and *vesicular stomatitis* viruses,

Table 5. Acetylcholinesterase (AChE) inhibitory activity and DPPH radical scavenging activity (inhibition %±S.E.M.) of the compounds (**1-3**) isolated from *Agelas oroides*

Compounds	**Percentage of inhibition±S.E.M[a] against AChE**					
	Concentration (μg mL^{-1})					
	6.25	12.5	25	50	100	200
Oroidin (**1**)	-[b]	-	-	18.95±1.84	26.24±0.19	55.21±2.94
4,5-dibromopyrrol-2-carboxylic acid (**2**)	-	-	-	-	-	-
25-hydroxy-24-methylcholesterol (**3**)	-	-	-	-	-	7.85±2.42
Reference Galanthamine	--[c]	--	--	--	98.88±0.93	--
Percentage of inhibition±S.E.M.[a] against DPPH radical						
Oroidin (**1**)	16.11±0.54	18.33±0.58	55.27±0.35	89.96±0.60	93.07±0.83	93.91±0.46
4,5-dibromopyrrol-2-carboxylic acid (**2**)	2.43±0.72	3.51±0.01	5.27±1.06	5.88±0.90	11.23±0.28	16.58±0.89
25-hydroxy-24-methylcholesterol (**3**)	2.09±0.11	2.43±0.36	3.09±0.12	3.60±0.47	4.04±1.26	6.44±0.97
References						
BHA	--	--	--	77.99±0.48	81.66±1.67	82.94±0.68
Gallic acid	--	--	--	91.61±0.06	92.57±0.10	93.19±0.00

[a]Standard error mean (n=3), [b] No activity observed, [c] Not tested

Penicillium atrovenetum, and *Saccharomyces cerevisiae* [19]. In similar studies on various *Agelas* species, bromopyrrole alkaloids such as oroidin, sceptrine, agelorine A and B, chlatramide A and B were found to display antimicrobial activity [20-23]. According to aforementioned antimicrobial reports; we may consider that bromopyrrole alkaloids might be contributing to the antimicrobial activity of *A. oroides* extracts tested herein (Tables 1 and 2). Several antimicrobial studies were also carried out on some species of the marine sponge *Axinella*. Although the crude extract of *A. corrugata* exerted a potent antibacterial effect against the opportunistic pathogen *Vibrio parahaemolyticus*, its major metabolite stevensine (an alkaloid) caused a weak activity, which led to the suggestion that this major compound is not solely responsible for the antibacterial effect of the extract of *A. corrugata* [23]. The ethyl acetate extracts of seven marine sponges collected from Tunisia were screened for their antibacterial activity against eight human pathogenic bacteria and six human pathogenic fungi using the agar disk diffusion method [24] and among them, the most active ones were concluded to be the extracts belonging to *A. oroides* and *A. damicornis*. The genus *Ircinia* is known to be rich in linear furanoterpenes and an antimicrobial study on twenty-three linear hydroquinones either isolated from *I. spinulosa* or synthesized suggested a structure-antibacterial relationship that optimum length of the side chain of these hydroquinones should be in the range of five to fifteen carbon atoms [25]. In another study to investigate antibacterial activity of *I. ramosa* collected during two periods in January and May [25], it was found out that while the non-polar fractions of the sponge extract of January collection had antibacterial effect, the non-polar fractions obtained from the sponge sample collected in May were more active. Therefore, it was concluded that the chemical composition of the sponge or its associated bacteria appears to be influenced by the environmental and seasonal factors. The extracts from *I. spinulosa* and *I. fasciculata* obtained from two different Mediterranean spots were observed to exert a variable antibacterial effect among them. As mentioned on *Ircinia*; this might be due to difference in their chemical nature which may be resulted from locational diversity. Avarol, a major sesquiterpenoid hydroquinone isolated from *D. avara*, was reported to exert a

strong antibacterial activity [26]. In a wide-ranged screening study on forty marine sponges collected from San Diego (California, USA) were screened for their antimicrobial activity [27] and occurrence of antimicrobial effect was observed in twenty-six of those sponges including *D. amblia* and *Axinella* sp. In our study, the methanol extract of *D. avara* was moderately active against the tested bacteria as compared to the other extracts as listed in Tables 1 and 2.

Considering antioxidant activity of the marine sponge species screened herein; our literature survey indicated that there has been only one former study describing antioxidant potential of the compounds; 2-octaprenyl-1,4-hydroquinone and 2-(24-hydroxy)-octaprenyl-1,4-hydroquinone isolated from *I. spinosula* and its eight synthetic derivatives [28]. Their antioxidant activity was tested *in vitro* by scavenging of free radical DPPH and inhibition of the lipid peroxidation induced by the Fe^{++}/ascorbate system. The two natural hydroquinone derivatives were found to display high antioxidant effect in both tests, which was in accordance with our results. In our DPPH radical scavenging assay, the highest scavenging effect (65.92%) was observed in *I. variabilis* of Antalya-I collection (Table 3) and linear terpenes including hydroquinones could be suggested to cause antioxidant activity. However, the same sponge species collected at another part of Antalya (II) did not show scavenging effect as well as *I. spinulosa* and *I. fasciculata* (Table 3). As we have mentioned above, it might be explained by locational differences which may cause variability in activity depending on the chemical nature. Interestingly, oroidin, an alkaloid isolated from *A. oroides* in this study, exerted a great anti-radical potential against DPPH at higher concentrations (Table 5) although the extract had insignificant antiradical effect (Table 3).

Among the tested sponge extracts, particularly the MeOH extracts prepared from the *Ircinia* species displayed promising results in AChE inhibition test over 50% and these extracts are worth to investigate further (Table 4). Among the isolated compounds, only oroidin was found to have a remarkable anti-AChE activity (Table 5).

In conclusion, marine organisms constitute an immense biodiversity in search for novel drug candidates as we have shown in this study. Our project is still in progress to screen more sponges from the Turkish waters for their biological activities and further isolation and identification of their active molecule(s).

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