https://doi.org/10.31925/farmacia.2021.1.9 **ORIGINAL ARTICLE**

PHENOLIC CONTENT, ANTIOXIDANT AND *IN VITRO* ANTIDIABETIC EFFECTS OF THIRTEEN MARINE ORGANISMS FROM MEDITERRANEAN SEA

DIDEM DELIORMAN ORHAN [1]*, NILUFER ORHAN [1], OZGE DEMIR [2], BELMA KONUKLUGIL [2]

[1]*Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, 06330, Etiler Ankara, Turkey*
[2]*Ankara University, Faculty of Pharmacy, Department of Pharmacognosy, 06100, Tandoğan Ankara, Turkey*

**corresponding author: didemdeliorman@gmail.com*

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Abstract

The aim of the study was to evaluate the total phenolic content, *in vitro* antidiabetic and antioxidant potential of marine organisms collected from Mediterranean coast. Methanol extracts of one soft coral (*Eunicella singularis*) and twelve sponge species (*Agelas oroides*, *Aplysina aerophoba*, *Axinella cannabina*, *A. polypoides*, *Cliona viridis*, *Dictyonella incisa*, *Dysidea avara*, *Ircinia incisa*, *I. oros*, *I. variabilis*, *Petrosia ficiformis*, *Sarcotragus spinulosa*) were investigated for their enzyme inhibitory activities, total phenolic content, total antioxidant capacity, ferric reducing antioxidant power, metal chelating and radical scavenging activities. *Dysidea avara* was found to be the most active extract on *α*-glucosidase enzyme (94.66 - 4.87% for 3000 - 100 µg/mL). Therefore, *α*-glucosidase inhibitory activity of its major compounds avarol and avarone was tested and found to be 86.18 ± 1.76% and 78.94 ± 1.38% respectively, at 10 µM. The present study indicated that the sponge *Dysidea avara* can be evaluated as a new natural source in the treatment of diabetes mellitus.

Rezumat

Scopul studiului a fost evaluarea conținutului fenolic total, potențialul antidiabetic *in-vitro* și cel antioxidant al organismelor marine colectate de pe coasta mediteraneană. Extractele metanolice dintr-o specie de coral (*Eunicella singularis*) și douăsprezece specii de burete (*Agelas oroides*, *Aplysina aerophoba*, *Axinella cannabina*, *A. polypoides*, *Cliona viridis*, *Dictyonella incisa*, *Dysidea avara*, *Ircinia incisa*, *I. oros*, *I. variabilis*, *Petrosia ficiformis*, *Sarcotragus spinulosa*) au fost investigate privind activitățile lor inhibitoare enzimatice, conținutul fenolic total, capacitatea antioxidantă totală, potența reducătoare, chelarea metalelor și activitatea de *scavenger*. *Dysidea avara* s-a dovedit a fi cel mai activ extract, cu acțiune asupra enzimei α-glucozidază (94,66 - 4,87% pentru 3000 - 100 µg/mL). Activitatea inhibitorie asupra α-glucozidazei a compușilor avarol și avaronă s-a dovedit a fi de 86,18 ± 1,76% și, respectiv 78,94 ± 1,38% la concentrația de 10 µM. Studiul a arătat că *Dysidea avara* ar putea fi evaluat ca o sursă naturală în tratamentul diabetului zaharat.

Keywords: antidiabetic, antioxidant, avarol, avarone, coral, sponge

Introduction

Marine sponges have attracted a great interest in the scientific communities and they have been the subject of hundreds of phytochemical and biological activity studies in the last 60 years. Sponge secondary metabolites possess a large range of bioactivities and until now, more than 5000 different compounds have been isolated from about 500 sponge species [6, 22]. Sponges are mainly a source of unusual nucleosides, bioactive terpenes, sterols, cyclic peptides, alkaloids, fatty acids, peroxides and amino acid derivatives. Secondary metabolites of sponges have been found to interfere with pathogenesis of a wide range of diseases. Their biological activities can be classified as antiinflammatory, antitumor, immunosuppressive or neurosuppressive, antiviral, antimalarial, antibiotic and antifouling [35]. Turkey is surrounded by Black sea, the Aegean Sea and the Mediterranean Sea and has a long coastline which is 4200 km long. The total number of the sponge species in the Turkish marine fauna was reported to be 131 [40]. Although there are many studies on the biodiversity of marine organisms of Turkey, there are only a few studies on their biological activities and phytochemical profile [1-3, 13, 16, 18, 27]. Therefore, biological activity and phytochemical studies were conducted on the thirteen marine organisms from coasts of Turkey in this study. *α*-Amylase and *α*-glucosidase inhibitory activities, total antioxidant capacity, ferric reducing antioxidant power, metal chelating and [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (ABTS) radical scavenging activities of coral and sponge extracts were investigated and their total phenolic content was determined.

Materials and Methods

Collection of marine organisms. Sponge and coral samples were collected by scuba divers from reef in habitats at depths of 9 - 21 m from different locations

of Turkish coasts. Foreign materials and/or organisms were removed from samples with a knife. Samples were transferred as soon as possible to the laboratory in Ankara while kept in ethanol (70%) during transfer and later on put in deepfreeze until the experimental process. Samples were deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Ankara University, Turkey. The species investigated in this study are listed in Table I.

Table I

Collection sites, date and extraction yield of marine organisms

Sample Name	Family	Location, City, Country	Collection Date	Yield %
Sponge Species				
Agelas oroides	*Agelasidae*	Kemer, Antalya, Turkey	October, 2014	1.84
Aplysina aerophoba	*Aplysinidae*	Gölcük, Kocaeli, Turkey	April, 2013	2.23
Axinella cannabina	*Axinellidae*	Turgut Reis, Muğla, Turkey	April, 2013	2.55
Axinella polypoides	*Axinellidae*	Gökova, Muğla, Turkey	April, 2013	2.97
Cliona viridis	*Clionaidae*	Gölcük, Kocaeli, Tukey	April, 2013	3.34
Dictyonella incisa	*Dictyonellidae*	Seferhisar, İzmir, Turkey	October, 2013	3.91
Dysidea avara	*Dysideidae*	Ayvalık, Balıkesir, Turkey	October, 2013	3.50
Ircinia incisa	*Irciniidae*	Fethiye, Muğla, Turkey	July, 2013	3.03
Ircinia oros	*Irciniidae*	Fethiye, Muğla, Turkey	October, 2013	1.75
Ircinia variabilis	*Irciniidae*	Turgut Reis, Muğla, Turkey	April, 2012	1.29
Petrosia ficiformis	*Petrosiidae*	Gökova, Muğla, Turkey	April, 2013	2.71
Sarcotragus spinulosa	*Irciniidae*	Kemer, Antalya, Turkey	April, 2013	1.65
Coral Species				
Eunicella singularis	*Gorgoniidae*	Ayvalık, Balıkesir, Turkey	October, 2013	1.23

Extraction. Dried and chopped coral/sponge samples were extracted individually with pure methanol (3 x 50 mL). Each extraction was developed with mechanical shaking, in glass flasks at room temperature. Extracts were filtered and concentrated under vacuum on a rotary evaporator and lyophilized by a freeze dryer. The obtained methanol extracts were employed in the activity experiments and isolation procedures.

Isolation of avarone and avarol. Avarol and avarone were isolated from the methanol extract of *Dysidea avara* as previously described. The structures of the compounds were established on the basis of spectroscopic data (^{1}H NMR) and comparison with the standards on TLC [3].

Determination of total phenolic content

Total phenolic content was measured according to a previously published spectrophotometric protocol [41] by using Folin-Ciocâlteu reagent. The total phenolic content was expressed in mg of gallic acid equivalents/g extracts. Calibration curve equation was y(Abs.) = 5.306 x (Conc.) + 0.0587 with r^2 = 0.9986.

Bioactivity methods

Assay for scavenging activity of ABTS radical cation. ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation ($ABTS^{\cdot+}$) scavenging assay was achieved by using the spectrophotometric methods of Re *et al.* and Meot-Duros *et al*. with slight modifications [20, 32]. Gallic acid was used as the positive control.

Ferric-reducing antioxidant power. The reducing power of the coral/sponge extracts was determined by the reducing power assay of Oyaizu [26]. Ascorbic acid was used as the positive control.

Metal chelating activity. Extracts were incubated with $FeCl_2$ (2 mM). The reaction was initiated by the addition 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 was used as a reference compound. The control contained $FeCl_2$ and ferrozine [12].

Total antioxidant activity by phosphomolybdenum assay. Extracts were added to test tubes containing distilled water and molybdate reagent solution. Vortexed tubes were incubated at 90°C for 90 min. Then, tubes were cooled to room temperature and the absorbances of the samples were measured at 695 nm. Results were expressed as ascorbic acid equivalent [30].

Assay for α-amylase and α-glucosidase inhibitory activity. The *α*-amylase inhibitory activity of the extracts was determined by the chromogenic method of Ali *et al*. [4]. Acarbose (Bayer Group, Turkey) was used as the positive control. α-Glucosidase inhibitory activity of the extracts was determined by the method of Lam *et al*. [19]. Acarbose was used as positive control.

Statistical analysis

All experiments were carried out with three replicates. Values were presented as means ± standard deviation (S.D.) or standard error of the mean (S.E.M.). Statistical differences between the treatments and the controls were tested by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test using the "Instat" statistic computer program. A difference in the mean values of $p < 0.05$ was considered to be statistically significant. Linear regression analyses were done using MS-DOS software (GraphPad InStat statistical program). Pearson's correlation coefficient was calculated using Microsoft excel 2016.

Results and Discussion

Total phenolic content of the sponge extracts ranged from 83.51 to 117.24 mg GAE/g extract. The highest total phenolic content was determined in *Axinella polypoides* extract (Table II).

Table II

Total phenolic content, ferric reducing power, total antioxidant capacity and ABTS radical scavenging activity of coral/sponge extracts

Sample Name	Total Phenolic Content	Ferric-Reducing Power (Absorbance at 700 nm ± S.D.)		TAC * (Mean ± S.D.)	ABTS Radical Scavenging Activity (% ± S.D.)	
	mg GAE/g ± S.D.	3 mg/mL	1 mg/mL		3 mg/mL	1 mg/mL
A. oroides	101.25 ± 12.41	0.3940 ± 0.0075	0.1557 ± 0.0347	619.35 ± 24.83	51.34 ± 0.61	20.94 ± 3.97
A. aerophoba	98.63 ± 3.68	0.8327 ± 0.0190	0.2444 ± 0.0104	105.62 ± 16.55	22.39 ± 2.78	6.02 ± 0.95
A. cannabina	95.13 ± 13.47	0.0374 ± 0.0031	0.0060 ± 0.0012	76.94 ± 14.72	1.15 ± 0.31	4.11 ± 1.98
A. polypoides	117.24 ± 0.06	1.8050 ± 0.0617	0.6174 ± 0.0196	210.75 ± 35.84	38.18 ± 1.89	17.67 ± 1.60
C. viridis	86.57 ± 2.47	0.0860 ± 0.0059	0.0134 ± 0.0042	227.48 ± 25.46	6.85 ±1.81	5.61 ± 1.97
D. incisa	96.83 ± 11.68	0.4517 ± 0.0298	0.1190 ± 0.0087	139.07. ± 57.35	17.85 ± 0.40	6.40 ± 1.00
D. avara	111.95 ± 6.11	0.7600 ± 0.0242	0.2710 ± 0.0200	743.61 ± 20.28	27.01 ± 1.19	23.76 ± 2.20
E. singularis	101.75 ± 4.98	0.5034 ± 0.0368	0.1294 ± 0.0097	136.68 ± 10.61	24.40 ± 3.01	12.03 ± 0.80
I. incisa	96.28 ± 3.60	0.3150 ± 0.0074	0.0860 ± 0.0055	12.43 ± 1.55	16.08 ± 1.07	12.11 ± 1.00
I. oros	97.08 ± 8.85	0.3920 ± 0.0296	0.0970 ± 0.0072	461.65 ± 12.42	16.54 ± 1.35	5.86 ± 2.06
P. ficiformis	89.79 ± 5.66	0.0817 ± 0.0167	0.0310 ± 0.0302	155.79 ± 8.28	17.62 ± 0.00	4.95 ± 1.90
S. spinulosa	83.51 ± 8.19	0.6970 ± 0.0057	0.2390 ± 0.0068	686.26 ± 27.14	26.78 ± 0.87	11.12 ± 2.55
Reference		**3 mg/mL**	**1 mg/mL**		**3 mg/mL**	**1 mg/mL**
Gallic Acid	NT	NT	NT	NT	98.93 ± 0.23	98.47 ± 0.40
Ascorbic A.		0.1928 ± 0.0042	0.1830 ± 0.0064	382.5 ± 17.0	NT	NT

NT: Not tested; S.D.: Standard Deviation; TAC: Total antioxidant capacity; * Total antioxidant capacity is expressed as mg ascorbic acid equivalent/g extract

Except *Axinella cannabina*, *Petrosia ficiformis* and *Cliona viridis*, all extracts (0.3150 - 1.8050) exhibited stronger antioxidant activity than ascorbic acid (0.1928) in ferric reducing power assay. In phosphomolybdenum assay, total antioxidant capacity of *Ircinia variabilis* (619.35 ± 24.83), *Dysidea avara* (743.61 ± 20.28), *Sarcotragus spinulosa* (686.26 ± 27.14) and *Ircinia oros* (461.65 ± 12.42) extracts was found higher than or close to those of Trolox (382.5 ± 17.0) (Table II).

With regard to ABTS radical scavenging activity, among all tested extracts, *Agelas oroides* extract (51.34 ± 0.61%) exerted the highest ABTS radical scavenging activity at 3 mg/mL concentration. The other extracts did not show promising radical scavenging activity compared to the reference compound gallic acid. As seen in Table III, *Cliona viridis* extract had the highest chelating activity (84.64 ± 1.73%). But, *Axinella cannabina*, *Dysidea avara* and *Sarcotragus spinulosa* extracts (5.19 ± 1.65 to 69.09 ± 0.86) at 3 mg/mL concentration exhibited a low metal chelating activity in comparison to EDTA. As a result, antioxidant activity of all tested extracts was observed to vary depending on the method used. Because of solubility problems, we could not achieve antioxidant activity tests and evaluate total phenolic content of *Ircinia variabilis* extract.

Table III

Metal chelating capacities of coral/sponge extracts

Sample Name	Metal Chelating Capacity (% ± S.D.)		
	3 mg/mL	1 mg/mL	0.3 mg/mL
A. oroides	-	-	-
A. aerophoba	-	-	-
A. cannabina	5.19 ± 1.65	-	-
A. polypoides	-	-	-
C. viridis	84.64 ± 1.73	84.26 ± 5.35	84.02 ± 1.90
D. incisa	-	-	-
D. avara	61.17 ± 5.03	50.90 ± 8.42	49.38 ± 2.95
E. singularis	-	-	-
I. incisa	-	-	-
I. oros	-	-	-
P. ficiformis	-	-	-
S. spinulosa	69.09 ± 0.86	63.90 ± 1.41	56.02 ± 1.30
Reference	**2 mg/mL**	**1 mg/mL**	**0.5 mg/mL**
EDTA	98.87 ± 0.49	98.02 ± 1.73	97.46 ± 0.00
Gallic Acid	NT	NT	NT

Results of the *in vitro* α-glucosidase and α-amylase inhibitory studies were presented in Table IV Among all tested marine samples, *Dysidea avara* showed a strong α-glucosidase enzyme inhibitory activity with percentage inhibitions ranging from 94.66 - 4.87% for 3000 - 100 µg/mL. We observed that the rest of the extracts did not inhibit α-glucosidase enzyme remarkably at 3000 µg/mL. Therefore, the lower concentrations of these extracts were not studied for their enzyme inhibitory activities. In this study, the reference substance, acarbose had 98.05 ± 0.03% α-glucosidase inhibition at 30 µg/mL. According to our results, *Dysidea avara* was found to be the most active sponge, hence α-glucosidase inhibitory activity of avarone and avarol which were previously isolated from *Dysidea avara* was investigated [5]. Both avarone (78.94%) and avarol (86.18%) exhibited strong inhibitory activities against α-glucosidase enzyme in a dose-dependent manner. It is thought that these compounds having sesquiterpene hydroquinone skeleton are responsible for the strong enzyme inhibitory activity of *Dysidea avara* extract.

On the other hand, α-amylase enzyme inhibitory activity of the coral/sponge extracts ranged from 1.32% to 14.93% at 3000 µg/mL. The inhibition percentage of the reference substance acarbose (3000 µg/mL) on α-amylase enzyme was found to be 80.74 ± 2.53%.

Table IV

α-Glucosidase and α-amylase inhibitory activity of coral/sponge extracts

Sample Name	**α-Glucosidase Inhibitory Activity Inhibition% ± S.D.**				**α-Amylase Inhibitory Activity Inhibition % ± S.D.**	
	3000 µg/mL	**1000 µg/mL**	**300 µg/ml**	**100 µg/mL**	**3000 µg/mL**	
A. oroides	7.55 ± 2.36	-	-	-	10.50 ± 0.26	
A. aerophoba	7.75 ± 4.05	-	-	-	2.89 ± 1.39	
A. cannabina	6.63 ± 5.52	-	-	-	7.24 ± 2.23	
A. polypoides	5.24 ± 2.01	-	-	-	1.32 ± 1.79	
C. viridis	7.45 ± 0.00	-	-	-	5.87 ± 3.81	
D. incisa	16.80 ± 4.38	-	-	-	6.98 ± 1.30	
D. avara	94.66 ± 0.62	68.03 ± 2.56	30.81 ± 8.11	4.87 ± 1.44	3.58 ± 3.39	
E. singularis	15.18 ± 4.89	-	-	-	4.52 ± 0.28	
I. incisa	6.34 ± 1.89	-	-	-	4.34 ± 0.56	
I. oros	7.81 ± 2.02	-	-	-	10.70 ± 0.85	
I. variabilis	4.29 ± 6.49	-	-	-	5.63 ± 1.17	
P. ficiformis	7.35 ± 5.14	-	-	-	3.29 ± 2.39	
S. spinulosa	2.57 ± 0.00	-	-	-	14.93 ± 1.97	
Compounds		**10 µM**	**3 µM**	**1 µM**		
Avarone		78.94 ± 1.38	72.32 ± 3.73	42.02 ± 2.51	NT	
Avarol		86.18 ± 1.76	85.51 ± 2.68	69.15 ± 1.56	NT	
Reference	**30 µg/mL**	**10 µg/mL**	**3 µg/mL**	**1 µg/mL**	**3000 µg/mL**	**1000 µg/mL**
Acarbose	98.05 ± 0.03	96.13 ± 0.62	92.40 ± 1.05	88.45 ± 3.35	80.74 ± 2.53	64.50 ± 1.72

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

The relationship between total phenol content, anti-oxidant and enzyme inhibitory activities of the samples was investigated by Pearson analysis (Table V). Except for total antioxidant activity, a significant, but not very strong relationship was determined between total phenol contents of the samples and ABTS, metal chelating capacities and ferric reducing power. There was generally a negative correlation among the α-amylase inhibitory activities of the samples, their antioxidant activities and total phenol content. On the other hand, the α-glucosidase enzyme inhibitory activities of the samples were significantly, moderately and positively correlated with their total phenol contents. A positive, significant and moderate relationship was observed between α-glucosidase enzyme inhibitory activity and total antioxidant activities of the samples.

Diabetes mellitus is a common metabolic disorder in which there are high blood sugar levels over a prolonged period. There are different types and classes of drugs that work in different ways used in the treatment of diabetes such as sulfonylureas, biguanides, meglitinides, thiazolidinediones, α-glucosidase inhibitors, bile acid sequestrants. Inhibition of α-amylase and α-glucosidase, enzymes that play a role in digestion of starch and glycogen, is considered a strategy for the treatment of disorders in carbohydrate uptake, such as diabetes and obesity. Plants are an important source of chemical constituents with potential for inhibition of carbohydrate digestive enzymes and can be used as therapeutic or functional food sources [33]. Many studies were conducted on the enzyme inhibitory effects of marine organisms. Extracts prepared from *Echinodictyum pykei*, *Cymbastela* sp., *Haliclona* sp. and *Raspaila* sp., and secondary metabolites of various sponge species like callyspongynic acid (from *Callyspongia truncate*), schulzeines A-C (from *Penares schulzei*) and penasulfate A (from *Penares* sp.) are found to be potent inhibitors of α-glucosidase [24, 25, 31, 34, 37].

Table V

Pearson's correlation coefficients between total phenol contents, antioxidant and enzyme inhibitory acitivities of the samples

	ABTS	Metal chelating capacity	Ferric reducing power	Total antioxidant capacity	Total phenol content
Total phenol content	0.5035**	-0.3290*	0.7064**	0.0869	
Total phenol content activity	0.1217	0.3915*	0.1386	0.5220**	0.4608*
α-amylase inhibitory activity	-0.0348	-0.2363	-0.6045**	0.1746	-0.2585

* Correlation is significant at the 0.05 level; ** Correlation is significant at the 0.01 level.

Hitherto, there have been numerous reports on chemical constituents of the tested marine sponges in this study. The genus *Petrosia* has been reported to have sterols, alkaloids and polyacetylenic molecules [15]. The preliminary chemical screening of *Eunicella singularis* showed the presence of alkaloids, glycosides, terpenoids, steroids and saponins [10, 11]. *Agelas* species have been reported to have bromopyrrol-alkaloids in major amounts. Several terpene derivatives, alkaloids and cyclopeptides in *Axinella* species have been identified by spectral analysis. On the other hand, *Ircinia* species were found to be quite rich in linear furanoterpenes [13]. Bary *et al*. reported the presence of tannins, alkaloids, sterols, saponins, flavonoides, free quinones and polyphenols in *Cliona viridis* [5]. Some brominated isoxazoline alkaloids including aplysinamisin-1, aerophobin-2, isofistularin-3 and aerothion were isolated from the Mediterranean sponges *Aplysina aerophoba* [38]. *Dictyonella* species are rich in sterols, fatty acids, saponins and triterpenoids [7]. Sesquiterpenoids such as avarol and avarone having several biological activities were isolated from *Dysidea avara* by Ferrandiz *et al*. [14].

On the other hand, oxidative stress, is one of the major problems observed in diabetic patients that leads to severe complications. Therefore, discovery of antidiabetic compounds/extracts having antioxidant effects is one of the targets for finding new generation antidiabetic drugs. Erdogan Orhan *et al*. [13] and Aktaş *et al*. [2] have studied on the antioxidant activities of marine sponges collected from Mediterranean coast of Turkey. 2,2-diphenyl-1-picrylhydrazil (DPPH), superoxide (SO) and nitric oxide (NO) radical scavenging activities of the sponge extracts have been determined and dose dependent radical scavenging activity has been observed. In our study, total antioxidant capacity, ferric reducing power and metal chelating capacity of *Dysidea avara* extract was found to be promising compared to the standard antioxidant compounds EDTA, Trolox and ascorbic acid.

This is the first report on the *in vitro*antidiabetic potentials of Turkish marine organisms. In this study, *Dysidea avara* showed a strong *α*-glucosidase enzyme inhibitory activity with percentage inhibitions ranging from 94.66 - 4.87% for 3000 - 100 µg/mL. During the course of our studies on Turkish marine sponges, avarol and avarone isolated from the methanol extract of *D. avara* [3] exhibited a potent *α*-glucosidase inhibitory activity. Previous studies also showed that avarol has highly effective *α*-glucosidase enzyme inhibitory activity [17].

Avarol is a marine natural product known for more than 40 years [8, 21] that is present in large amounts only in the sponge *Dysidea avara* [23]. This molecule possesses a rigid sesquiterpene skeleton and a reactive hydroquinone moiety, which can interfere with reactive oxygen species production and the redox status of cells. Avarol and avarone exhibits a wide array of biological activities including antibacterial, antifungal, antioxidant, antiplatelet, antipsoriatic, antiviral and antitumour effects [3, 9, 36, 39]. Additionally, some alkyl(aryl)thio and alkyl(aryl)amino derivatives of avarol and avarone (oxidised form of avarol) have exhibited moderate acetylcholinesterase inhibitory activity which has been shown to be essential to delay the onset of Alzheimer's disease [28, 29].

Conclusions

In this study, total phenolic content, antioxidant activities (total antioxidant capacity, ferric reducing power and metal chelating capacity) and *in vitro* antidiabetic effects of various marine organisms from the Mediterranean were tested, and *Dysidea avara* was found to be the most promising organism in terms of these activities. On the other hand, its avarol and avarone content have been found to be responsible for strong α-glucosidase enzyme inhibitory activity of *D. avara*. In conclusion, the sponge *Dysidea avara* found in Mediterranean Sea coasts can be evaluated as a new natural source in the treatment of diabetes mellitus.

Conflict of interest

The authors declare no conflict of interest.

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