https://doi.org/10.31925/farmacia.2020.2.22 **ORIGINAL ARTICLE**

BIOACTIVITY SCREENING AND ISOLATION OF THREE FATTY ACID ETHYL ESTERS FROM *ANEMONIA VIRIDIS*

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Abstract

Different habitats of seas and oceans make these ecosystems enormous sources of bioactive natural compounds. Marine species like terrestrial species produce terpenes, steroids, fatty acids, etc. In this study, we describe the isolation and structure identification of three fatty acids ethyl esters, 9-pentadecenoic acid ethyl ester, 9-heptadecenoic acid ethyl ester and 5,8,11,14,17-docosapentaenoic acid ethyl ester from *Anemonia viridis* coast of Bodrum (Turkish coast). The structure identification was made by NMR and Mass spectroscopic methods. Furthermore, the antioxidant and cytotoxic activity of extract were determined using the superoxide radical scavenging method and the MTT assay, respectively. Acetylcholinesterase inhibition and tyrosinase inhibition activities of methanolic extract of *A. viridis* were also screened. As a result, *A. viridis* methanolic extract possesses dose-dependent cytotoxic activity, moderate superoxide radical scavenging and tyrosinase inhibition activity. However, it was inactive against acetylcholinesterase enzyme. This is the first study on isolation of secondary metabolites and bioactivity screening of *A. viridis* from Turkey.

Rezumat

Diferitele habitate ale mărilor și oceanelor fac din aceste ecosisteme surse enorme de compuși naturali bioactivi. Speciile marine produc terpeni, steroizi, acizi grași. În acest studiu, descriem izolarea și identificarea structurii a trei esteri etilici ai acizilor grași din *Anemonia viridis*, originară de pe coasta marină din Turcia. Identificarea structurii a fost făcută prin metode RMN și spectroscopie de masă. S-a evaluat activitatea antioxidantă și citotoxică, precum și efectul asupra activității acetilcolinesterazei și tirozinazei.

Keywords: *Anemonia viridis*, acetylcholinesterase inhibition, cytotoxic activity, tyrosinase inhibition

Introduction

Currently, more than 100 natural product-derived compounds are used in the clinical and preclinical stages. These compounds have a wide range of therapeutic properties such as anti-cancer, anti-infective, and anti-diabetic activities [1]. Approximately half of the new drugs have natural origin or have been designed on the basis of natural product structures. In this frame, marine natural compounds from the point of chemical novelty are predominant to terrestrial natural products [2]. Some of the marine invertebrates are used as food and medicine from immemorial time. Marine invertebrates were used for the treatment of some disorders like digestive, genitourinary and skin disorders [3]. Over the past several decades, there has been a wide interest in drug discovery from natural products, especially from marine sources. Secondary metabolites isolated from marine species showed their therapeutic activity as microtubule-interfering agent, DNA-interactive agents and target the ion channels and enzymes [4]. Some of the short and long chain fatty acids that are isolated from natural sources, especially polyunsaturated fatty acids have valuable pharmaceutical and biomedical potential. For example, some fatty acids have potential role in decreasing brain-related disorders such as dementia and Alzheimer's disease, therapeutic management of colorectal cancer and anti-inflammatory activity [5]. In this study, we investigated the isolation and structure elucidation of three fatty acids ethyl esters from *Anemonia viridis* and screened the bioactivity of the methanolic extract of *A. viridis* like antioxidant, cytotoxicity, acetylcholine esterase and tyrosinase inhibition activities. *A. viridis* belong to the *Actiniidae* family and *Anemonia* genus [6]. According to the old records reported by Hippocrates, Athenaeus, Xenocrates and Athenaeus, *A. viridis* was used as a laxative agent in broth form and was used as a diuretic, abdominal bloating and pain reliever in the cooked flesh form [3].

Materials and Methods

General

NMR spectra were recorded in $CDCl_3$ on a Bruker DRX 600 spectrometer equipped with an inverse TCI CryoProbe. Chemical shifts values are reported in ppm (δ) and referenced to internal signals of residual protons ($CDCl_3$ ^1H δ 7.35, ^{13}C 77.0 ppm). High resolution mass spectra were acquired on a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Milan, Italy). 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), quercetin, ascorbic acid, galantamine, kojic acid was obtained from Sigma-Aldrich Chem Co (St. Louis, MO). HEP-2 (human larynx epidermoid carcinoma) were provided by Refik Saydam Hygiene Center, Virology Laboratory, Ankara, Turkey.

Extraction and isolation of fatty acids

A. viridis was collected from Bodrum, Turkey in March 2016, by a scuba diver and was identified by Dr. Gözcelioğlu. A voucher specimen was deposited at the Pharmacognosy Department of Faculty of Pharmacy, Ankara University. The sample was cut to small pieces and then extracted by methanol. The extract was dried under vacuum; methanolic extract (5.4933 g) was partitioned successively between water, diethyl ether and n-butanol. The diethyl ether fraction (0.4649 g) was loaded to Silica gel column chromatography and eluted by petroleum ether and diethyl ether to obtained 15 fractions. Afterward for more purification first fraction was loaded to preparative TLC plates and eluted by petroleum ether: diethyl ether (9.5:0.5). Compounds **I**, **II** and **III** were isolated and identified by ^1H and ^{13}C NMR.

Superoxide radical scavenging activity by alkaline DMSO method

Sodium hydroxide was added to the air saturated dimethyl sulfoxide (DMSO) for generating the super-oxide radical. The stable, nitroblue tetrazolium (NBT) in solution is reduced to formazan dye at room temperature. 10 µL NBT (1 mg/mL) was added to 100 µL alkaline DMSO and 30 µL of extract at different concentrations, to give the final volume of 1.4 mL. The absorbance was measured at 560 nm, the experiment was performed in triplicate. Quercetin and ascorbic acid were used as positive controls [7].

In-vitro cytotoxic activity assay (MTT test)

Hep-2 human cells (Human epithelial type 2 cell line) were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% foetal bovine serum, glutamine (2 mM) and 1% streptomycin in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Cells were plated in a 96-well-plate with 1×10^5 cells/well of concentration. After 48 hours of incubation methanolic extract (25 - 200 mg/mL) of *A. viridis* was added to the cell in different concentrations. Subsequently, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide (MTT) reagent (0.5 mg/mL in sterile phosphate buffer) was added directly to the wells and incubated for 4 hrs. The absorbance was measured at 570 nm. The percentage growth inhibition was calculated using the following formula, 200 µL of cells (Hep-2) was added without extract as the control group [8].

$$\%\text{Cell Inhibition} = (100 - Abs_{sample}/Abs_{control}) \times 100.$$

In vitro acetylcholinesterase (AChE) inhibition assay

The methanolic extract of *A. viridis* was tested for its AChE inhibitory activity. For this purpose, 25 µL of acetylthiocholine iodide (15 mM), 125 µL of DTNB (3 mM), and 25 µL (1000 µg/mL) of extract solution at the different concentration (0.1 - 0.01 mg/mL) was added to 96 well plate and incubated for 15 min at 30ºC. The absorbance was measured 10 times (each 13 s) at 415 nm. Then 25 µL of AChE (0.3 U/mL) was added to the mixture and the absorbance was determined. For control well added all components except the extract. As positive control galantamine was used. All treatments were performed in triplicate [9].

The percentage of AChE inhibitory activity (%IA) was calculated using the following equation:

$$\%IA = ((Cc - Ce)/Cc) \times 100,$$

where: Cc is the control kinetic (containing all reactants, except the AChE enzyme) and Ce is the experimental kinetic for extract.

Inhibition of tyrosinase activity

Four wells were designed as A, B, C, and D that each well has 180 µL reaction mixture. Well A contain 20 µL of tyrosinase (480 units/mL) solution in 20 mM phosphate buffer, 140 µL 20 mM phosphate buffer (pH 6.8), and 20 µL methanol. Well B contain 160 µL 20 mM phosphate buffer (pH 6.8) and 20 µL methanol; well C contain; 20 µL tyrosinase solution (480 units/mL), 140 µL 20 mM phosphate buffer (pH 6.8), and 20 µL sample solution in different concentration (0.1 - 0.01 mg/mL); well D contain; 160 µL 20 mM phosphate buffer (pH 6.8) and 20 µL sample solution. These wells were incubated at room temperature for 120 min. Then, 20 µL L-DOPA (0.85 mM) were added and incubated at room temperature for 20 min. The absorbance of wells was measured at 490 nm. Kojic acid was used as a positive control [10].

$$\%\text{Inhibition} = (((A - B) - (C - D))/(A-B)) \times 100$$

A is the absorbance of well A, B is the absorbance of well B, C is the absorbance of well C, D is the absorbance of well D.

Results and Discussion

In this study we isolated secondary metabolites from *A. viridis*. According to the NMR and Mass spectrometry data, 9-pentadecenoic acid ethyl ester, 9-heptadecenoic acid ethyl ester and 5,8,11,14,17-docosapentaenoic acid ethyl ester were isolated. Figure 1shows the structures and compounds.

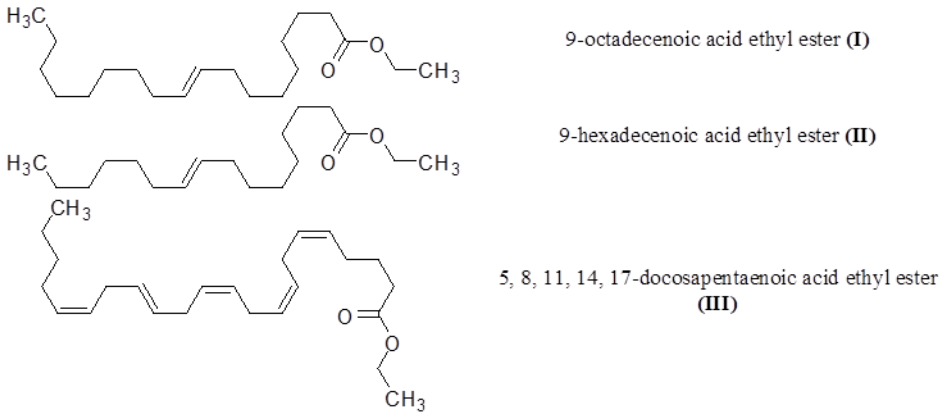

Figure 1.
Structure of fatty acid ethyl esters isolated from *A. viridis*

9-octadecenoic acid ethyl ester (I): White amorphous powder. HRESIMS *m/z* 333.5049 $[M+Na]^+$ (calculated for $C_{20}H_{38}O_2Na$, 333.5043).
9-hexadecenoic acid ethyl ester (II): White amorphous powder. HRESIMS *m/z* 305.4506 $[M+Na]^+$ (calculated for $C_{18}H_{34}O_2Na$, 305.4511).
5, 8, 11, 14, 17-docosapentaenoic acid ethyl ester (III): White amorphous powder. HRESIMS *m/z* 381.5475 $[M+Na]^+$ (calculated for $C_{24}H_{38}O_2Na$, 381.5471). The NMR data are presented in Table I.

Table I
^1H-^{13}C NMR of isolated compounds (400 MHz, $CDCl_3$)

	I		II		III	
Position H	**δ_H (*J* in Hz)**	**δ_c**	**δ_H (*J* in Hz)**	**δ_c**	**δ_H (*J* in Hz)**	**δ_c**
5, 6, 8, 9, 11, 12, 14, 15, 17, 18					5.41 - 5.32 (m, 10H)	128.1 - 128.9
9, 10	5.35-5.39 (m, 2H)	129.4 - 129.6	5.38-5.35(m, 2H)	129.3 - 129.7		
2’	4.13 (q, *J* = 6.1 Hz, 2H)	61.1	4.12 (q, *J* = 6.1 Hz, 2H)	61.4	4.15 (q, 6.2, 2H)	61.3
1’	1.25 (t, *J* = 6.1 Hz, 3H)	18.4	1.24 (t, *J* = 6.1 Hz, 3H)	18.7	1.31 (t, *J* = 6.2 Hz, methyl, 3H)	19.2
7, 10, 13, 16					2.89-2.82 (bisallylic, 8H)	25.7 - 25.9
2	2.31 (t, *J* = 7.1 Hz, α-methylenes of acyl, 2H)	34.5	2.31 (t, *J* = 7.1 Hz, α-methylenes of acyl, 2H)	34.8	2.32 (t, 7.2, αmethylenes of acyl, 2H)	34.0
4, 19					2.15-2.09 (m, 4H)	27.2 - 27.5
8, 11	2.03 (m, 4H)	26.9 - 27.5	2.02 (m, 4H)	27.0 - 27.4		
3	1.62 (β-methylenes of acyl, 2H)	24.9	1.62 (β-methylenes of acyl, 2H)	25.1	1.62 (β-methylenes of acyl, 2H)	25.0
20,21					1.37 - 1.25 (m, aliphatic protons)	28.0 - 31.2
22					1.01 (t, *J* = 6.8 Hz, methyl, 3H)	14.2
4, 5, 6, 7, 12, 13, 14, 15, 16, 17	1.37-1.25 (m, aliphatic protons)	29.9 - 30.4				
4, 5, 6, 7, 12, 13, 14, 15			1.38 - 1.24 (m, aliphatic protons)	29.2 - 29.8		
16			0.88(t, 7.0, methyl, 3H)	14.6		
18	0.89 (t, 7.0, methyl, 3H)	14.3				
1		171.3		171.5		172.1

In this research, we also determined the antioxidant activity of *A. viridis* through superoxide radical scavenging method. According to the results, *A. viridis* extract showed 20% inhibition of superoxide radical scavenging at 800 µg/mL concentration, while ascorbic acid and quercetin were used as standards.

The results are shown in Figure 2. Cytotoxicity activity of the methanolic extract of *A. viridis* against Hep-2 cell lines was measured performing the MTT assay and has showed dose dependent cytotoxic activity (IC_{50}: 120.1 µg/mL) where adriamycin was used as a standard with IC_{50}: 0.362 ± 0.76 µg/mL. *A. viridis* methanolic extract was inactive against acetylcholinesterase where galantamine was used as a standard by IC_{50}: 85.84 ± 2.50 µg/mL inhibition activity. Tyrosinase inhibition activity of *A. viridis* has showed IC_{50}: 81.28 ± 4.41 µg/mL, where kojic acid was used as standard by IC_{50}: 63.09 ± 0.95 µg/mL, the results are presented in Table II.

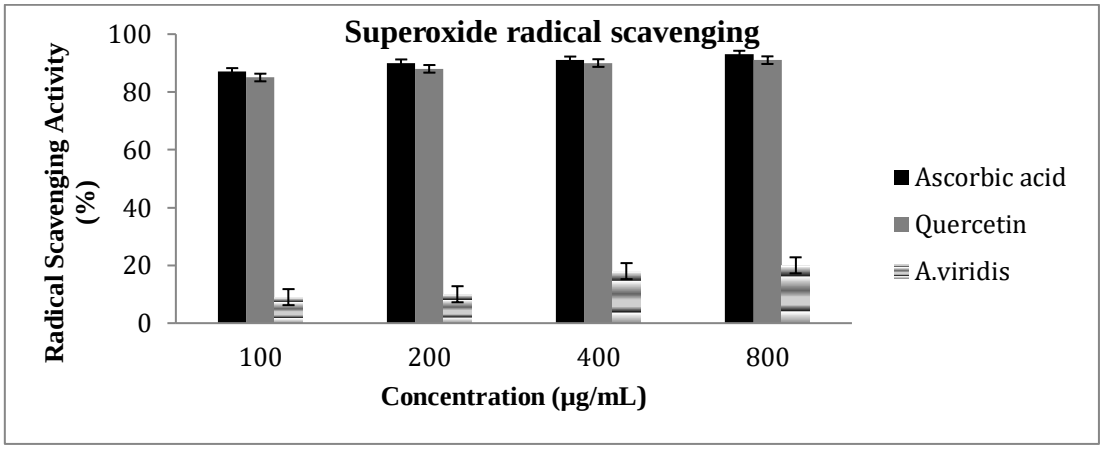

Figure 2.
Superoxide radical scavenging activity of *A. viridis*

Table II
Cytotoxic, acetylcholinesterase and tyrosinase inhibition activity of *A. viridis*

	IC_{50} (µg/mL)		
	Cytotoxicity activity (Hep-2 cell line)	Acetylcholinesterase inhibition activity	Tyrosinase inhibition activity
A. viridis	120.1 ± 0.98	-	81.28 ± 4.41
Adriamycin	0.362 ± 0.76	*	*
Galantamine	*	85.84 ± 2.50	*
Kojic acid	*	*	63.09 ± 0.95

- not active, * not determined

Seas and oceans contain a broad diversity of the species with biologically active metabolites representing a valuable source with great potential not only in the pharmaceutical industry but also in the cosmetic and nutraceutical industries as well. There are many studies about the isolation and identification of proteins and peptides from sea anemones [11-13]. However, few studies are available on the isolation of secondary metabolite from sea anemones. A great number of marine natural products from marine organisms have been extensively investigated for their bioactive properties and demonstrated interesting anti-inflammatory, cytotoxic, immunomodulating, antimicrobial, antiviral, neurosuppresive, antioxidant and analgesic activities [14, 15]. *Anemonia viridis* formerly known as *A. sulcate* is a widespread and extensively studied Mediterranean species of sea anemone from which a large number of toxins have been isolated. Cytotoxicity and anti-proliferative activities of these isolated proteins have also been investigated [16, 17].

According to the literature survey, there have only been a small number of studies regarding the bioactivity of sea anemones. Crude extracts of *Aiptasia mutabilis* (anemone) were shown to possess significant cytotoxic activity against Vero and HEp-2 cells. In the study conducted by Ramezanpour *et al.*, they found that *Heteractis magnifica* extract showed very high cytotoxic activity on HT47D and MCF7 human breast cancer cells [18, 19]. In another study, the researchers isolated 5 toxins from *Anthopleura elegantissima*, a species with previously studied cardiostimulatory, cytotoxic and cytolytic activities. These compounds showed cardiotoxic and neurotoxic activities [20]. Well-known voltage-gated Na^+ channel toxins isolated from sea anemone venoms act on neurotoxin receptor and inhibit the deactivation of these channels [21]. The crude extract of *Bunodosoma caissarum* was found to inhibit glutamate binding to cerebral cortical membranes and enhanced glutamate release from cortical synaptosomes [22]. In another study, antioxidant, antimicrobial effects of *Heteractis aurora*, *Heteractis crispa* and *Stichodactyla haddoni* were examined and acetic acid-17-acetoxy-4,4,10,13-tetramethyl7-oxo-2,3,4, 7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-

cyclopenta (a) phenanthren-3-yl (ester) and 4 [-4-di-ethylamino1-methylbutylamino] - 1,2 dimethoxy-6-bromonaphthalene was isolated from *H. aurora* [23]. In a study conducted in India, saturated and unsaturated fatty acids were detected in 4 sea anemones (*Heteractis magnifica*, *H. aurora*, *Stichodactyla haddoni* and *S. gigantea*) [24]. Yatkın and *et al.* examined the seasonal changes of fatty acid content in Turkish sea anemone *Actinia equina* [25].

Conclusions

Turkish coastline is almost 8400 km long in total and there is not enough research about marine species found on this coastline. In this study we investigated *A. viridis* collected from Bodrum coasts, leading to isolation and characterization of three fatty acids ethyl esters by NMR and mass analysis. During the course of our studies, while the crude methanolic extract of the organism has shown significant tyrosinase inhibition activity, it has showed moderate super-oxide radical scavenging and cytotoxic activity and it was inactive against acetylcholinesterase enzyme. Further study could be carried out using more bio-activity screening, such as *in vivo* tyrosinase inhibition activity and cytotoxicity effect of *A. viridis* on more cancer cell lines, and isolation and identification of more related secondary metabolites from this sea anemone. To the best of our knowledge, this study is the first study on secondary metabolites and bio-activities of *A. viridis* from Turkey.

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Conflict of interest

The authors declare no conflict of interest.

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