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.

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

Rezumat

Diferitele habitat 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 acizi lor 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 aceticolinesterazei 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 CDCl₃ 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 (CDCl₃ 1H δ 7.35, 13C 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. Gözceliöglu. A voucher specimen was deposited at the Pharmacognosy Department of Faculty of Pharmacy, Ankara University. The sample was cut at the Pharmacognosy Department of Faculty of Pharmacy, Ankara, Turkey.

**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% CO₂, 95% air at 37°C. Cells were plated in a 96-well-plate with 1 × 10⁵ 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].

\[
\text{%Cell Inhibition} = \left( 100 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100.
\]

**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 1 shows the structures and compounds.
9-octadecenoic acid ethyl ester (I): White amorphous powder. HRESIMS m/z 333.5049 [M+Na]+ (calculated for C20H38O2Na, 333.5043).

9-hexadecenoic acid ethyl ester (II): White amorphous powder. HRESIMS m/z 305.4506 [M+Na]+ (calculated for C18H34O2Na, 305.4511).

5, 8, 11, 14, 17-docosapentaenoic acid ethyl ester (III): White amorphous powder. HRESIMS m/z 381.5475 [M+Na]+ (calculated for C24H38O2Na, 381.5471).

The NMR data are presented in Table I.

Table I

| Position | δH (J in Hz) | δC | δH (J in Hz) | δC | δH (J in Hz) | δC |
|----------|--------------|----|--------------|----|--------------|----|
| 5, 6, 8, 9, 11, 12, 14, 15, 17, 18 | 5.35-5.39 (m, 2H) | 129.4 - 129.6 | 5.38-5.35 (m, 2H) | 129.3 - 129.7 | 4.13 (q, J = 6.1 Hz, 2H) | 61.1 |
| 2 | 4.12 (q, J = 6.1 Hz, 2H) | 61.4 |
| 1 | 1.25 (t, J = 6.1 Hz, 3H) | 129.3 - 129.7 | 4.15 (q, 6.2, 2H) | 61.3 |
| 2 | 2.31 (t, J = 7.1 Hz, α-methylene of acyl, 2H) | 34.5 | 2.31 (t, J = 7.1 Hz, α-methylene of acyl, 2H) | 34.8 | 2.32 (t, 7.2, α-methylene of acyl, 2H) | 34.0 |
| 4, 19 | 2 | 2.15-2.09 (m, 4H) | 27.2 - 27.5 |
| 8, 11 | 2 | 2.03 (m, 4H) | 26.9 - 27.5 | 2.02 (m, 4H) | 27.0 - 27.4 |
| 3 | 1.62 (β-methylene of acyl, 2H) | 24.9 | 1.62 (β-methylene of acyl, 2H) | 25.1 | 1.62 (β-methylene of acyl, 2H) | 25.0 |
| 20, 21 | 1 | 1.37 - 1.25 (m, aliphatic protons) | 28.0 - 31.2 | 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 |
| 18 | 0.89 (t, 7.0, methyl, 3H) | 14.3 |
| 1 | 171.3 | 171.5 |

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 \( A. \text{viridis} \) 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. \( A. \text{viridis} \) 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 \( A. \text{viridis} \) 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.

Seas and oceans contain a broad diversity of the species with biologically active metabolites representing a valuable source 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, neurosuppressive, antioxidant and analgesic activities [14, 15]. \( Anemone \text{a viridis} \) formerly known as \( A. \text{saluca} \) 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 \( Aiptasia \text{mutabilis} \) (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 \( Heteractis \text{magnifica} \) 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 \( Anthopleura \text{elegantissima} \), 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 \( Bunodosoma \text{caissarum} \) 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 \( Heteractis \text{aurora}, Heteractis \text{ crispa} \) and \( Stichodactyla \text{haddoni} \) were examined and acetic acid-17-acetoxy-4,4,10,13-tetramethy17-oxo-2,3,4, 7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-

### Table II

|          | Cytotoxicity activity (Hep-2 cell line) IC\(_{50}\) (µg/mL) | Acetylcholinesterase inhibition activity IC\(_{50}\) (µg/mL) | Tyrosinase inhibition activity IC\(_{50}\) (µg/mL) |
|----------|----------------------------------------------------------|----------------------------------------------------------|--------------------------------------------------|
| \( A. \text{viridis} \) | 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

![Figure 2.](image-url)

**Figure 2.**

Superoxide radical scavenging activity of \( A. \text{viridis} \)

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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 hadaloni and S. gigantea) [24]. Yatkan 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 superoxide radical scavenging and cytotoxic activity and it was inactive against acetylcholinesterase enzyme. Further study could be carried out using more bioactivity 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 bioactivities of A. viridis from Turkey.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Harvey AL, Natural products in drug discovery. Drug Discov Today, 2008; 13(19-20): 894-901.
2. Montaser R, Luesch H, Marine natural products: a new wave of drugs? Future Med Chem., 2011; 3(12): 1475-1489.
3. Voultsiadou E, Therapeutic properties and uses of marine invertebrates in the ancient Greek world and early Byzantium. J Ethnopharmacol., 2010; 130(2): 237-247.
4. Haefner B, Drugs from the deep: marine natural products as drug candidates. Drug Discov Ther., 2003; 8(12): 536-544.
5. Aluko RE, Functional Foods and Nutraceuticals, Food Science Text Series. Springer New York Dordrecht Heidelberg London, 2012.
6. WoRMS - World Register of Marine Species, www.marinespecies.org.
22. Gondran M, Eckeli AL, Migues PV, Gabilan NH, Rodrigues AL. The crude extract from the sea anemone, *Bunodosoma caissarum* elicits convulsions in mice: possible involvement of the glutamatergic system. *Toxicon*, 2002; 40(12): 1667-1674.

23. Thangaraj S, Bragadeeswaran S, Gokula V. Sea anemones as potential source for bioactive metabolites. *Int J Pep Res Ther.*, 2019; 25: 591-604.

24. Thangaraj S, Bragadeeswaran S, Gokula V. Fatty acid composition of select sea anemones from Mandapam Coast, Tamil Nadu. *Indian J Geo Marine Sci.*, 2019; 48(8): 1232-1237.

25. Yatkın K, Ayas D, Ali Rüza Köşker AR, Durmuş M, Yılmaz Uçar Y. Seasonal changes in the chemical composition of the headlet anemones (*Actinia equina*) from Mersin Bay, Northeastern Mediterranean coast of Turkey. *NESciences*, 2017; 2(2): 11-20.