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RESEARCH PAPER

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Isolation and Bioactivities Screening of Turkish *Microcosmus vulgaris*

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Abstract

During the course of our investigation for isolation of biological active compounds from marine organisms of the Turkish seas, secondary metabolites and bioactivities of, *Microcosmus vulgaris* collected from (Turkish coasts) İzmir coast was studied. In the study 5α- 6α- epoxyergosta 7-en- 3β- ol was isolated from *Microcosmus vulgaris.* Furthermore, antioxidant and antiproliferative activity of crude extract and 5α- 6α- epoxyergosta 7-en- 3β- ol were determined by DPPH, superoxide radical scavenging and nitric oxide radical scavenging and MTT assay respectively. Antimicrobial activity of crude extract was also determined against some Gram positive, Gram negative and yeast strains. Stearic acid, palmitic acid and myristic acid were also detected by GC-MASS analysis. This is the first study about isolation and bioactivity determination of Turkish *M. vulgaris.*

Introduction

Oceans are a unique source of natural products with wide range of bioactivity. For this purpose, numerous secondary metabolites have been isolated and some of them are used as pharmaceuticals, cosmetics, nutritional supplements, molecular probes and fine chemicals (Kijjoa and Sawangwong, 2004). Isolation of natural products from marine organisms is a new trend in the world and especially in Turkey. In recent years, many new pharmacological active metabolites have been explored from the marine organisms (Martins et al., 2014). Tunicates are marine invertebrate animals

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divided into three main groups as sessile ascidians, pelagic appendicularians and thaliaceans. They are rich sources of secondary metabolites with biofuels and pharmaceutical potential. Didemnin B, patellamide D, patellazole C which isolated from tunicate were shown unique mechanisms of action against diverse targets and against cancer-relevant cell lines (~100 pM) (Schmidt et al., 2012; Mehbub et al., 2014, Raslan et al., 2017).

Trabectedin is an alkaloid and it is antitumor substance of Yondelis® which is isolated from a tunicate *Ecteinascidia turbinata*. Aplidin® and Lurbinectedin 173 are compounds isolated from *Aplidium albicans* and *Ecteinascidia turbinata* that are still in Phase III and Phase II studies for cancer treatment (Kim, 2012; Mehbub et al., 2014).

Steroids, terpenoids, carotenoids and a significant number of acetogenins, macrocyclic lactones, polyethers, cyclic peroxides, and simple alkyl sulfates are found mainly in tunicate. Different modified peptides and depsipeptide, sometimes different types of amino acids are nitrogenous metabolites that are found in tunicate. Heterocyclic alkaloids include pyridoacridines, tryptophan-derived alkaloids, alkaloids derived from phenylalanine and tyrosine, and lysine-derived alkaloids. Non-nitrogenous secondary metabolites are less in number (Menna et al., 2012).

Ascididemnin from *Didemnum sp.* (Kobayashi et al., 1988), Aplidin from *Aplidium albicans* (Cooper et al., 2012) and Didemnin B from *Tridemnum solidum* (Chun et al., 1986; Cooper et al., 2012) are cytotoxic active secondary metabolites with marine origin. Trabectedin (Yondelis) which is currently used as an antitumor drug for soft tissue sarcomas is isolated from *Ecteinascidia turbinate* (Patel, 2011) Vitilevuamit is also a natural product which has antitumor activity and is isolated from *Didemnum cuculiferum* and *Polysyncranton lithostrotum* (Edler, et al., 2002; Fojo, 2008).

Turkey, is surrounded by Black Sea, Aegean Sea and Mediterranean Sea. Despite of its long coastal line there have been limited works on marine bioactive compounds.

About 61 tunicate species are living in Turkey's coasts, but there are not any studies about secondary metabolites and bioactivity of these species (Çinar, 2014; Konuklugil, 2016). In this study, secondary metabolites and antioxidant, antiproliferative activities of *M. vulgaris* collected from Aegean Sea were investigated.

Materials and Methods

General

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), quercetin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazoliumbromide (MTT), sulfanilamide and napthylethylenediamine dihydrochloride were purchased from Sigma Aldrich. McCoy's 5A medium, fetal bovine serum (FBS), streptomycin and glutamine were from PAA (PAsching, Austaria), HCT 116 colon cancer cells were kindly provided by Bert Vogelstein.Molecular devices Spectra MAX 190 Microplate Reader helps to get absorbance. GC-MS, GC-Focus PolarisQ, Thermo Fisher Scientific was used.

Preparation of Extracts

M. vulgaris belongs to Microcosmus genus, Pyuridae family. *M. vulgaris* was collected by scuba-diving from İzmir, Agean Sea 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 choloform-methanol (1:1) three times. The extract was dried under vacuum (40 g) and kept at 4°C until its use.

Isolation and Identification of 5α- 6α- Epoxyergosta 7-En- 3β- Ol

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The crude extract of *M.vulgaris* was partitioned between methanol:water (90:10) and n-hexan, ethyl acetate, n- butanol respectively.

n- Butanol fraction was applied to vacuum liquid chromatography (VLC). Totally four fractions were obtained. First fraction was selected for isolation of secondary metabolites. Silica and Sephadex column chromatography was used for further purification. Obtained compound (white amorphous) was washed with methanol several times then was dissolved in chloroform and sent to NMR (Nuclear Magnetic Resonance). The structures of the compounds were identified by ^{1}H and ^{13}C NMR.

GC_MASS Analysis

Fatty acid analysis was performed in the other three VLC fractions, using a GC-MS on an ion trap mass spectrometer equipped with Electron ionization source (70 eV) (Polaris Q; ThermoScientific) coupled with a ThermoScientific GC system. The GC conditions were as follows: 5% phenyl column (Trace TR-5, 30 m × 0.25 mm × 0.25 µm; ThermoScientific) oven temperature program: the column held initially at 60 °C for 3 min after injection, then increased to 60 °C for 3 min, followed by a 4 °C min^{-1} gradient up to 165 °C, then 1 °C min^{-1} up to 180 °C, and, finally, 35 °C min^{-1} up to 310 °C, holding for 5 min. carrier gas: helium. Samples were directly injected (2 µL) in split (1:10) mode, with a blink window of 3 min, inlet temperature of 210 °C, transfer line set at 250 °C, and ion source temperature of 230 °C.

DPPH Antioxidant Activity Determination

Different concentrations of crude extract and 5α- 6α- epoxyergosta 7-en- 3β- ol were prepared and added to equal volume of DPPH solution which was prepared freshly in methanol (0.1 mM). After 30 min at room temperature, the absorbance was recorded at 517 nm. quercetin was used as as standard (Shirwaikar et al., 2006). Radical scavenging activity was calculated by the following formula:

Inhibition%= [(Absorbance $_{control}$- Absorbance $_{sample}$)/ Absorbance control]×100

Superoxide Radical Scavenging Activity by Alkaline DMSO Method (SO)

Superoxide radical scavenging activity of the crude extract and 5α- 6α- epoxyergosta 7-en- 3β- ol were determined by alkaline DMSO method. To 1 mL of alkaline DMSO (5 mM NaOH in 0.1 mL water) 10 µL of NBT (1 mg/mL) and 30 µL of different concentration of extracts or standard compounds were added. DMSO was added to the reaction mixture to give a final volume of 140 µL. The absorbance was measured at 560 nm using microplate reader (Harput et al., 2011). Radical scavenging activity was calculated by the following formula:

Inhibition%= [(Absorbance $_{control}$- Absorbance $_{sample}$)/ Absorbance $_{control}$]×100

The IC_{50} were obtained through extrapolation from regression analysis. The antioxidant activity was evaluated based on this IC_{50} value.

Nitric Oxide Radical Scavenging Activity (NO)

NO radical scavenging activity of the crude extract and 5α- 6α- epoxyergosta 7-en- 3β- ol were determined. Brifley, 60 µL of sodium nitroprusside (10 mM) dissolved in phosphate buffered saline were added to 60 µL of serial diluted extracts and was incubated under light at room temperature for 150 min. Finally, an equal volume of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dihydrochloride, 2.5% H_3PO_4) was added into each well in order to measure the nitrite content. After10 minutes, absorbance was measured at 577 nm in a microplate reader (Senthil

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Kumar et al., 2012)

Radical scavenging activity was calculated by the following formula:

Inhibition%= [(Absorbance $_{control}$- Absorbance $_{sample}$)/ Absorbance $_{control}$]×100

Antiproliferative Activity

Effect of *M. vulgaris* crud extract and 5α- 6α- epoxyergosta 7-en- 3β- ol on cell viability of HCT 116 colon cancer cell line was determined by MTT assay.

HCT 116 cells were incubated with extracts for 48h, then cell viability was analyzed using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Assay. Insoluble formazan cyrstals were dissolved by adding DMSO to the wells. The absorbance (550nm) was measured with spectrophotometer. Untreated cells were used as negative control. Cell viability was calculated as relative to control. Docetaxel (1 µM) was used as a positive control. Untreated cells were used as a negative control (C). Data are shown as mean ± SD of three independent experiments.

Antimicrobial Activity

Antibacterial activity tests were carried out against *Acinetobacter haemolyticus* (ATCC 19002), *Acinetobacter septicum* (NRB 239), *Klebsiella pneumoniae* (CDC 529), *Staphylococcus aureus* (JCSC 4744), *Staphylococcus epidermidis* (ATCC 35984), *Candida glabrata* (ATCC 90030), *Cryptococcus neoformans* (NIH 68) and *Cryptococcus gattii* (NIH 112) strains. Antimicrobial activity was determined by a modified microdilution method as described in CLSI M07-A9 standard for bacteria and CLSI M27-A3 standard for yeasts. 8,9 The tested two fold serial dilutions of the extracts were between 256 and 0.5 µg mL $^{-1}$. and were incubated at 35ºC for 24 for bacteria and 48 hours for yeasts. Minimum concentration of the extract that completely inhibited macroscopic growth of the microorganism was accepted as minimum inhibitory concentration.

Results

In this study *M. vulgaris* was selected to isolate secondary metabolites. According to NMR results 5α- 6α- epoxyergosta 7-en- 3β- ol was isolated from methanol and chloroform extract. The structure 5α- 6α- epoxyergosta 7-en- 3β- ol and its ^{1}H and ^{13}C NMR data were presented in Figure1, Table 1 and 2 respectively. Antioxidant activity of *M. vulgaris* and 5α- 6α- epoxyergosta 7-en- 3β- ol were determined by DPPH, SO and NO assays. According to the results 5α- 6α- epoxyergosta 7-en- 3β- ol has shown higher antioxidant activity than methanolic extract of *M. vugaris*. Quercetin was used as standard in these assays. Results are presented in Table 3.

According to the antiproliferative effects of *M. vulgaris* and 5α- 6α- epoxyergosta 7-en- 3β- ol on HCT 116 cell line decreased cell viability dose dependently and significantly ($p<0.001$). In addition to this, *M. vulgaris* extracts (1-200µM) inhibited colon cancer cell viability significantly ($p<0,05$). Docetaxel was used as standard in this assay. Results are presented in Table 4.

According to the GC-MASS results, palmitic acid was the major fatty acid in three Butanol fractions. The results are presented in Table 5.

Antimicrobial activity of crude extract was tested against Gram positive, Gram negative and yeast strains. According to the results presented in Table 6, the crude extract has shown significant activity against *Staphylococcus aureus, Acinetobacter haemolyticus, Cryptococcus neoformans* and *Cryptococcus gattii strains* (MIC: 32 µg/ml) .

Discussion

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The marine environment hosts a huge number of organisms that produce unique secondary metabolites with wide range of bioactivity. Some of these metabolites are used in pharmaceutical and cosmeceutical industries. Eight marine-origin compounds in different phases of the clinical pipeline are approved by Food and Drug Admnistration (FDA) or European Medicines Agency (EMEA). From the eight compounds currently on the market (Adcetris®,Cytosar-U®, Halaven®, Yondelis®, Carragelose®, Vira A®, Lovaza®, Prialt®) only Prialt® , Yondelis® and Carragelose®, have used the original natural molecule without any modification. Other compounds have lead optimization, in different steps of their development. Furthermore, the search for new source and new bioactive compounds are still ongoing (Martins et al., 2014). There are fifty tunicate species belonging to twelve families in Turkish seas. We collected *M.vulgaris* from İzmir, Agean Sea during our continuing research projects focusing on searching for new bioactive substances from marine organisms,.

According to the literature search there were only four studies of *M. vulgaris.* Sulcatin is the first pyridine alkaloid that was isolated from tunicates. A new antiproliferative alkaloid, n-methylprydinium was isolated from *M. vulgaris* (Aiello et al., 2000). In 2002 and 2003, sulcaceramit, a glicosphingolipid, and its perasetate form and three new glycosphingolipids were isolated from *M.vulgaris* (Aiello et al., 2002; 2003). In this research we isolated 5α- 6α- epoxyergosta 7-en- 3β- ol methanolic extract of *M. vulgaris*. If we compare our results with previous studies, although we could not detect alkaloid in this species, we isolated and detected steroid and fatty acids respectively. This is first study about isolation and identification of Turkish tunicates. As a result of the antioxidant activity, crude extract has shown better radical scavenging activity and antiproliferative effect than the 5α- 6α- epoxyergosta 7-en- 3β- ol. which may be the synergistic interactions of the compounds in the crude extract (Ulrich- Merzenich et al., 2010).

Antimicrobial activity of crude extract has shown significant activity against Gram positive, Gram negative and yeast strains. Furthermore, this is the first record about cytotoxic activity of *M.vulgaris* against colon cancer cells. As a result of GC-MASS analysis, stearic acid, palmitic acid and myristic acid were detected in Butanol fraction of *M.vulgaris*. Palmitic acid has high ratio in Butanol fractions (50%). Several reports have shown that tunicates are rich of steroids and fatty acids (Zlatanos et al., 2009; Maoufoud et al., 2009). The results of this study have been proven by the previous studies about tunicate contents.

In this study, 5α- 6α- epoxyergosta 7-en- 3β- ol was isolated and three fatty acids were detected from *M.vulgaris.* Antioxidant and antiproliferative activities of crude extract and isolated compound were investigated, furthermore antimicrobial activity of crude extract was determined. There are so many tunicate species with secondary metabolites contents and bioactivities that await to be analyzed. Despite the large number of species, it is likely that new substances will be obtained in future studies. To the best of our knowledge, totally 50 tunicate species were detected in Turkey, among them *M. vulgaris* is the first and only species that was analyzed for secondary metabolites and bioactivities.

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Table 1.^{1}H NMR data for 5α- 6α- epoxyergosta 7-en- 3β- ol

NO	δc
H- 1,2,3,4,9,11,12,14,15,16,17 M (21-H)	1.30-2.29
5	-
6 M (1-H)	3.5
7 M (1-H)	5.14- 5.36
8	-
10	-
13	-
18 M (3-H)	0.69
19, 24 M (4-H)	1.00-1.13
20	-
21,22,23,26,27,28	0.81-0.95
25	1.58

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Table 2. ^{13}C NMR data for 5α- 6α- epoxyergosta 7-en- 3β- ol

NO	δc
1	35.47 t
2	30.9 t
3	56.79 d
4	39.52 t
5	71.81 s
6	73.39 d
7	121.69 d
8	140.77 s
9	42.17 d
10	37.27 s
11	23.83 t
12	39.3 t
13	50.32 s
14	54.40 d
15	22.1 t
16	140.77 s
17	28.0 t
18	56.19 d
19	11.85 q
20	18.9 q
21	36.52 q
22	18.72 q
23	35.78 t
24	30.7 t
25	39.1 d
26	31.56 d
27	20.2 q
28	21.27 q

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Table 3. Antioxidant activity of *M.vulgaris* and 5α- 6α- epoxyergosta 7-en- 3β- ol

	IC_{50} µg/ml ± SD		
Assay	5α- 6α- epoxyergosta 7-en- 3β- ol	*M.vulgaris*	Quercetin
SO	502.15± 0.22	332.2 ±0.11	11.2 ± 0.21
DPPH	498.1 ± 0.54	314.4 ± 0.81	8.23 ± 0.17
NO	519.9 ± 0.41	341.3 ± 0.52	13.8 ± 0.12

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Table4. Antiproliferative effects of crude extract and 5α- 6α- epoxyergosta 7-en- 3β- ol against HTC-116 cell line

IC_{50} mg/ml ± SD	
5α- 6α- epoxyergosta 7-en- 3β- ol	6.76 ± 4.02
Crude extract	0.69 ± 4.4
Docetaxel	0.008±2.9

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Table5. GC-MASS analysis results

	Stearic acid%	Palmitic acid%	Myristic acid%
Butanol fraction 1	9	50	11
Butanol fraction 2	12	45	19
Butanol fraction 3	8	40	18

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Table 6. Antimicrobial activity of *M. vulgaris*

Strains	MIC (µg/ ml)
Acinetobacter haemolyticus ATCC 19002	32
Acinetobacter septicum NRB 239	128
Klebsiella pneumoniae CDC 529	128
Staphylococcus aureus JCSC 4744	32
Staphylococcus epidermidis ATCC 35984	128
Candida glabrata ATCC 90030	128
Cryptococcus neoformans NIH 68	32
Cryptococcus gattii NIH 112	32

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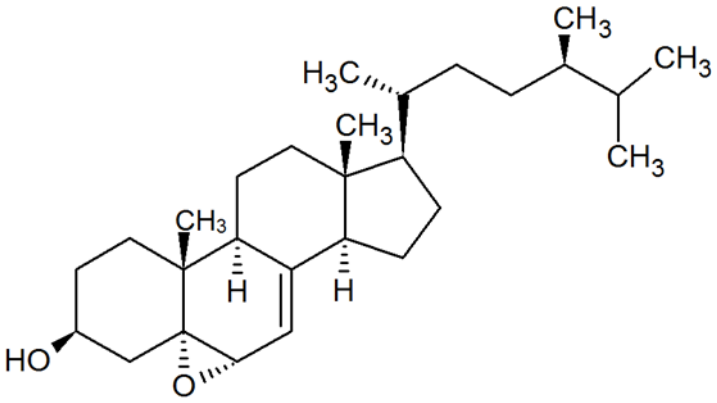

Figure 1. Structure of 5α- 6α- epoxyergosta 7-en- 3β- ol