ORIGINAL ARTICLE

BIOACTIVITY SCREENING OF SOME MARINE SPECIES FROM TURKEY'S COASTS

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Abstract

Different habitats of seas and oceans made them an enormous source of biodiversity. Seven different marine species (Coral, Anemon, Tunicate) were tested in a screening platform for the identification of their potential bioactivity related to antimicrobial, cytotoxicity, tyrosinase inhibitory activity. Antimicrobial activity was carried out by broth dilution methods, used to determine the minimum inhibitory concentration (MIC). The cytotoxic activity of the samples was carried out by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The tyrosinase inhibitory potentials of selected species extracts were determined using ELISA microtiter assays. The tyrosinase inhibitory activity of these species is the most important result obtained within the experiment.

Rezumat

Diferitele habitate ale mărilor şi oceanelor reprezintă o sursă enormă de biodiversitate. Au fost analizate şapte specii marine (Coral, Anemon, Tunicate) într-o platformă de screening, pentru identificarea activităţii microbiene, citotoxicităţii activităţii inhibitoare a tirozinazei. Activitatea antimicrobiană a fost testată prin metoda diluţiilor, în vederea obţinerii concentraţiei minime inhibitorii. Activitatea citotoxică a probelor a fost testată prin testul MTT (bromură de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazoliu), Potenţialul inhibitor al tirozinazei pentru speciile selectate a fost determinat folosind metoda ELISA, evidenţiindu-se cele mai importante rezultate experimentale.

Keywords: antimicrobial activity, cytotoxic activity, marine species, tyrosinase inhibitory

Introduction

Considering the fact that marine species have a wide biodiversity, in the last decades, bioactivity screening of these species was of great interest [11]. The seas and oceans are different and complex habitats, therefore marine species produce a wide spectrum of potent of active and unique compounds [10]. Furthermore, some of these compounds have biological properties as antioxidant [12], antimicrobial [22], anticancer [28], tyrosinase inhibitory [5] activities. Since ancient times, natural products had relevant roles in cancer treatment; nowadays, many compounds from natural sources are used as anticancer agents. Citarabine is the first drug reported of marine origin [21]. Dieckol that was isolated from marine brown alga had shown tyrosinase inhibitory activity three times higher than kojic acid [15]. For this reason, screening new sources for potential bioactive compounds from marine is a substantial research area. Turkey has more than 8500 km of coastline, and a great diversity of marine species, but there are few studies about their bioactivity screening. In this study there were investigated the antimicrobial, cytotoxic, tyrosinase inhibitory activities of methanolic extracts of *Parazoanthus axinellae*, *Halocynthia papillosa*, *Styela clava*, *Cladocora caespitosa*, *Cerianthus membranaceus*, *Eunicella cavolinii*, *Ascidiella aspersa*. The aim of this study was screening the bioactivity of marine species which are found in Turkey's coasts. It is a first stage in screening for bioactive secondary metabolites from marine species.

Materials and Methods

General

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazoliumbromide (MTT) (98%), 3,4-dihydroxy-L-phenylalanine (L-DOPA) (≥ 98%), tyrosinase from mushroom, kojik acid (≥ 98.5%) were purchased from Sigma Aldrich Chem Co (St. Louis, MO). Mueller Hinton broth, Sabouraud dextrose broth and ethyl acetate were purchased from Merck, Germany. Hep-2 cell line was provided by Refik Saydam Hygiene Center, Ankara Turkey. The determinations were performed on molecular devices Spectra MAX 190 Microplate Reader.

Preparation of Extracts

Samples were collected from the South of Turkey in June 2014, at 10 m depth and transferred immediately

to the laboratory of Ankara while kept in 70% ethanol during transfer and later on put in deep freeze until the experimental process. The samples were identified by Dr. Gözcelioğlu, based on conventional macroscopic and microscopic marine sponge identification procedures. Voucher specimens were deposited at the Pharmacognosy Department of the Faculty of Pharmacy, Ankara University, Turkey. Samples and their genus information are summarized in Table I.

Table I

Selected marine species and their genus information

Species	Family	Genus	Location
Parazoanthus axinellae (Coral)	*Parazoanthidae*	*Parazoanthus*	Kesan
Halocynthia papillosa (Tunicate)	*Pyuridae*	*Halocynthia*	Kesan
Cladocora caespitose (Coral)	*Scleractinia İncertae Sedis*	*Cladocora*	Ayvalik
Cerianthus membranaceus (Anemone)	*Cerianthidae*	*Cerianthus*	Ayvalik
Eunicella cavolinii (Coral)	*Gorgoniidae*	*Eunicella*	Ayvalik
Ascidiella aspersa (Tunicate)	*Ascidiidae*	*Ascidiella*	Golcuk
Styela clava (Tunicate)	*Styelidae*	*Styela*	Golcuk

Samples Extraction

The samples were cut in small pieces and then extracted by methanol and dried under vacuum. The crude extract was kept at 4ºC for further use. The extract was re-dissolved in HPLC grade methanol (10 mg/mL) and filtered through 0.45 µm membranes.

Antimicrobial activity

The stock solutions of samples extracts were prepared in dimethylsulphoxide (DMSO) at a final concentration of 512 µg/mL and sterilized by using 0.22 µm Millipore Membrane Filter (MA 01730, USA). The strains of Gram-negative bacteria were *Klebsiella pneumonia* (CDC 529), *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853), the strains of Gram-positive bacteria were *Staphylococcus aureus* (JCSC 4744), *Enterococcus faecium* (ATCC 6057), *Streptococcus pneumoniae* (ATCC 6303) and the strains of yeast were *Candida krusei* (ATCC 6303), *Candida parapsilosis* (ATCC 6303). They were employed for the determination of antibacterial and/or antifungal activity of these extracts. The antimicrobial activity was performed by a modified micro-dilution method as described in CLSI M07-A9 standard for bacteria and CLSI M27-A3 standard for yeasts [6, 7]. The tested two fold serial dilutions of the extracts were between 256 and 0.5 µg/mL. The sealed micro-plates were placed in a humid chamber and incubated at 35ºC for 24 and 48 hours for bacteria and yeasts, respectively. The lowest concentration of the extract that completely inhibited the macroscopic growth of the microorganism was accepted as the minimum inhibitory concentration (MIC).

In-vitro cytotoxic activity assay (MTT assay)

Human larynx epidermoid carcinoma cell line (Hep-2) was 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% oxygen at 37°C. Cells were plated in a 96-well-plate with 1×10^5 cells/well of concentration and were incubated for 48 hours. The methanolic extracts (25 - 200 mg/mL) were added to cells in different concentrations. Subsequently, 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 of growth inhibition was calculated using the formula below, 200 µL of cells (Hep-2) were added without extract as control [18]. Doxorubicin was used as standard drug.

$$\% \text{ Cell Inhibition} = [100 - \text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}] \times 100.$$

Tyrosinase inhibitory activity

Four wells designed as A, B, C, and D contained each 180 µL reaction mixture. Well A contained 20 µL of tyrosinase (20 mM), 140 µL of phosphate buffer (pH 6.8), and 20 µL of methanol; well B contained 160 µL of phosphate buffer (pH 6.8) and 20 µL of methanol; well C contained 20 µL of tyrosinase solution (480 units/mL), 140 µL of phosphate buffer (pH 6.8), and 20 µL of sample solution; well D contained 160 µL of phosphate buffer (pH 6.8) and 20 µL of sample solution. These wells were incubated at room temperature for 10 min. Then 20 µL of L-DOPA (0.85 mM) were added and incubated at room temperature for 20 min, then the absorbance of wells was measured at 490 nm [17]. Kojic acid was used as a positive standard.

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

where 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

The antimicrobial activity of the selected species was tested against *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* as Gram negative strains and *Staphylococcus aureus*, *Enterococcus faecium*, *Streptococcus pneumoniae* as Gram positive strains and *Candida krusei*, *Candida parapsilosis* as yeasts. The results are shown in Table II. Among the tested extracts against Gram negative strains, *H. papillosa* and *A. aspersa* extracts

had a higher effect against *K. pneumoniae* (16 µg/mL) and *S. typhimurium* (31 µg/mL) respectively, than the other species. On the other hand *C. caespitosa* and *E. cavolinini* have shown high efficacy against *E. faecium* (0.25 µg/mL) and *S. pneumoniae* (1 µg/mL). *A. aspersa* extract also had high efficacy against Gram positive strains. As it is shown in Table II the antimicrobial activity of the selected marine species against the Gram positive and yeasts are better than their activity against the Gram negative strains.

Table II

Antimicrobial activity of selected marine species

	MIC (µg/mL)							
	Microorganism							
Extracts	*Klebsiella pneumoniae* CDC 529	*Salmonella typhimurium* ATCC 14028	*Pseudomonas aeruginosa* ATCC 27853	*Staphylococcus aureus* JCSC 4744	*Enterococcus faecium* ATCC 6057	*Streptococcus pneumoniae* ATCC 6303	*Candida krusei* ATCC 6258	*Candida parapsilosis* ATCC 22019
	Gram negative			*Gram positive*			*Yeast*	
P. axinellae	< 128	32	32	32	8	32	128	8
H. papillosa	16	64	128	< 128	64	2	32	16
S. clava	< 128	< 128	< 128	< 128	4	32	32	16
C. caespitosa	< 128	< 128	< 128	64	0,25	1	< 128	64
C. membranaceus	< 128	64	32	< 128	64	64	128	64
E. cavolinini	64	64	32	128	0,25	1	4	1
A. aspersa	64	31	< 128	128	1	4	2	2

Cytotoxic activity of selected marine species was measured by MTT assay on Hep-2 human cells. The results are shown in Table III. *C. caespitose* and *E. cavolinini* with IC_{50} = 10.92 and 17.5 (µg/mL) respectively have a better activity than the other samples, while IC_{50} for doxorubicin, the standard drug was 0.361 (µg/mL).

Table III

Cytotoxic activity (MTT) of selected marine species

Extracts	IC_{50} (µg/mL)
P. axinellae	230.14 ± 2.71
H. papillosa	75.86 ± 2.61
S. clava	72.64 ± 2.17
C. caespitosa	10.92 ± 1.35
C. membranaceus	68.88 ± 1.45
E. cavolinini	17.5 ± 1.47
A. aspersa	86.55 ± 3.69
Doxorubicin	0.362 ± 0.76

Table IV

Tyrosinase inhibitory activity of selected marine species

Extracts	IC_{50} (µg/mL)
P. axinellae	97.72 ± 3.46
H. papillosa	94.96 ± 3.25
S. clava	196.40 ± 6.41
C. caespitosa	72.64 ± 0.12
C. membranaceus	53.39 ± 0.31
E. cavolinini	199.52 ± 0.85
A. aspersa	85.11 ± 3.84
Kojic acid	63.09 ± 0.95

The tyrosinase activity of the samples was determined and the IC_{50} was calculated, kojic acid being used as standard (IC_{50} = 63.09 ± 0.95 µg/mL). As shown in Table IV, the methanolic extract of *C. membranaceus* was more effective than the other tested samples (53.39 µg/mL). *C. caespitosa* and *A. aspersa* had shown a significant inhibition with IC_{50} = 72.64 and 85.11 (µg/mL), respectively.

The bacterial resistance to the antibiotics is well-known fact since the past. Therefore antibacterial agents that derived from natural sources have started to play a significant role in the prevention and treatment of infection diseases [3]. In addition, marine species also can be promising sources of new antimicrobial substances [19]. Furthermore, antioxidants are agents which protect the cells against the deleterious effects of reactive oxygen species that cause degenerative diseases. Some marine species are rich sources of potential antioxidant secondary metabolites [25]. As it was published before, two peptides (Styelin A and Styelin B), isolated from *S. clava*, were effective against Gram negative and Gram positive bacterial human pathogens, with minimal inhibitory concentrations below 1.5 µg/mL [16]. The crude extract and fractions of *S. clava* showed anti-proliferative activities, as well as NO release promoting activities, and the petroleum ether fractions showed the strongest effect. The *in vitro* antioxidant (DPPH) activity of the crude extract and fraction also established that ethyl acetate fraction was much stronger than that of the other fractions [14]. However, in our study *S. clava* methanolic extract has shown low activity and cytotoxic activity. Also, among all tested microorganisms, *S. clava* had a significant efficiency against *Enterococcus faecium*.

Novel bioactive compounds and new anticancer agents from marine species on cancer treatments and prevention have increased over the last decade [8, 24]. Steroids isolated from *Eunicella cavolini* showed partial growth inhibitory effects on MCF-7 human

breast cancer cells [13]. In this study, also, the methanolic extract of *E. cavolini* showed a significant cytotoxic activity which proves the results of the study. Furthermore, sesterterpenoids (cladocorans A and B) isolated from *C. caespitosa* showed phospholipase A2 inhibitory activity almost similar to manoalide [20]. According to the obtained results *C. caespitosa* showed the highest cytotoxic activity and it has a significant potential for further studies and isolation of effective compounds. *In vitro* cytotoxic activity of sulphated alkene and alkanes isolated from *H. papillosa* was investigated on WEHI 164 and C6 cell lines. Data showed that the compounds act selectively on fibrosarcoma rather than glioma cells [1]. Two peptides (halocyntin and papillosin) from *H. papillosa* displayed antibacterial activity against Gram-positive and Gram-negative bacteria [9]. In this study, *H. papillosa* showed a dose dependent and high cytotoxic activity and significant tyrosinase inhibitory activity.

Tyrosinase is the key enzyme in the first two steps of melanin biosynthesis, which is formed by a combination of enzymatically catalysed chemical reactions and catalyses the hydroxylation of L-tyrosine to 3,4-dihydroxy phenylalanine (DOPA) and the oxidation of DOPA to dopaquinone [23]. Moreover, marine species which are a rich source of secondary metabolites have attracted great attention in the search of natural tyrosinase inhibitory agents [26].

In this study, the methanolic extracts of selected marine species were investigated for their tyrosinase-inhibitory activity using mushroom tyrosinase. There are few studies about tyrosinase inhibitory activity of marine species and this study is a new one in this filed. In a previous study, on *Ascidiella aspersa*, polysaccharides from *A. aspersa* displayed a significant anti-inflammatory activity without having a significant anti-coagulant activity [27]. In this study, *A. aspersa* showed a moderate tyrosinase inhibitory and cytotoxic activity and significant antimicrobial activity against yeasts and Gram positive bacterial strains.

In previous studies, on *Parazoanthus spp.* collected from India, there were reported hypoglycaemic and spasmolytic activities, the LD_{50} being more than 1000 mg/kg bw mice [2]. In a study, performed by Cachet *et al*. *P. axinella* showed a moderate tyrosinase inhibitory activity [4].

In the literature reviewed no information was found from previous studies about the biological screening of *C. membranaceus*. The obtained results from our study proved that the methanolic extract of *C. membranceus* has a significant tyrosinase inhibitory activity and a moderate cytotoxic activity. Furthermore, there is no tyrosinase inhibitory activity study on these marine species and this study is, to our knowledge, the first on this topic.

Conclusions

In this work, 7 marine species from the Turkey's coasts were screened for their anti-microbial, cytotoxic and tyrosinase-inhibitory activity. The obtained results revealed some active species for isolation of accountable secondary metabolites from these species. Further studies will be focused on the isolation of secondary metabolites from active species. In conclusion, our results demonstrated that the methanolic extract of *C. membranaceus* has showed the highest tyrosinase inhibitory activity, other species also proving tyrosinase inhibitory activities. Therefore, the studied marine species can be used as a source of natural medicines for the treatment of hyper-pigmentation disorders.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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