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Short Communication

In vitro Evaluation of Bioactivity of *Dictyonella incisa* from Turkey

Hajar HEYDARI [1] Bülent GÖZCELİOĞLU [2] Belma KONUKLUGİL [1]

[1] Ankara University, Pharmacy Faculty, Pharmacognosy Department, TR-06100 Tandoğan, Ankara - TURKEY
[2] Scientific and Technological Research Council of Turkey (TÜBİTAK), TR-06420 Bakanlıklar, Ankara - TURKEY

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Abstract

Marine sources are known to produce structurally unique pharmaceutically potent secondary metabolites. During the course of our studies on Turkish marine sponges, we have collected *Dictyonella incisa* from Turkey's coast and methanolic extract was investigated for antioxidant, cytotoxic and antimicrobial activity. Antioxidant activity was evaluated by the superoxide radical scavenging assay, cytotoxic activity was determined against HCT-116 and HEp-2 cell lines by MTT assay. Antimicrobial activity was tested against some Gram positive, Gram negative bacteria and yeasts. Also cholesterol and cholestane isolated and the structure were identified by NMR methods. This is the first work on the bioactivity and secondary metabolites of *D. incisa* collected from Turkish coasts.

Keywords: *Antimicrobial, Antioxidant, Cytotoxic activity, Dictyonella incisa*

Türkiye'deki *Dictyonella incisa*'nın *In vitro* Biyoaktivitesinin Değerlendirilmesi

Öz

Deniz kaynaklarının, yapısal olarak eşsiz farmasötik olarak etkili sekonder metabolitleri ürettiği bilinmektedir. Türkiye kıyılarından toplanan süngerler ile devam eden çalışmalarımızesnasında toplanan *Dictyonella incisa*'nın metanolik ekstraktının antioksidan, sitotoksik ve antimikrobiyal aktivitelerini araştırılmıştır. Antioksidan aktivite süperoksit radikal süpürme testi ile, sitotoksik aktivite HCT-116 hücre hattı ve HEp-2 hücre hattına karşı MTT yöntemi ile, antimikrobiyal aktivite ise bazı Gram pozitif, Gram negatif bakteri ve maya suşlarına karşı test edilmiştir. Ayrıca süngerde bulunan kolesterol ve kolestan NMR yöntemi ile izole edilip yapısı tayin edilmiştir. Bu çalışma, Türkiye kıyılarından toplanan *D. incisa*'nın biyoaktivite ve ikincil metabolitlerini inceleyen ilk çalışmadır.

Anahtar sözcükler: *Antimikrobiyal, Antioksidan, Sitotoksik aktivite, Dictyonella incisa*

INTRODUCTION

Marine species are rich sources of bioactive compounds used in food and pharmaceutical industries [1]. In the last decades, many bioactive compounds have been isolated from various marine species like sponges, tunicates corals and etc. Sponges are spineless animals belonging to phylum [2]. They are great sources of secondary metabolites which have pharmacological properties. Therefore, sponges have shown widely diverse activities like anti-HIV, anti-bacterial, cytotoxicity, anti-fouling properties [3-5]. Sponges appear to be one of the richest phylum in toxicogenic species; there are approximately 15.000 sponge species which 150 of them are living in freshwaters [6]. In search of novel pharmaceutically active substances from sponges, we have engaged in bioactivity screening of sponges. In this regard, *Dictyonella incisa* was selected for screening of antioxidant, antimicrobial and cytotoxicity activities. *Dictyonella incisa* is a sponge species which belongs to *Dictyonellidae* family and *Dictyonella* genus (World Register of Marine Species). Turkey is a country of peninsula surrounded by the Black Sea at the north, the Aegean Sea at the west, and the Mediterranean Sea at the south. Despite its long coastal line, which is 8300 km in total, there have been limited works on its marine prosperity from the view point of isolation of secondary metabolites and bioactivities. Three records were found according to the literature search. One of these is related to anti-inflammatory activity and isolation of fatty acids [7]. The others are about isolation of ketosteroid and sterol [8,9].

İletişim (Correspondence)
+90 312 2033092
belma.konuklugil@gmail.com

In our continuing search for secondary metabolites and bioactivity of Turkish marine organisms, we have reported the isolation of cholesterol and cholestane, also antioxidant, antimicrobial and cytotoxic activities of *D. incisa* from Turkish Sea.

MATERIAL and METHODS

General

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazolium-bromide (MTT) and nitroblue tetrazolium (NBT) were purchased from Sigma Aldrich, sabouraud 4% dextrose agar, muller hinton broth, sabouraud dextrose broth and ethyl acetate was purchased from Merck. McCoy's 5A medium, fetal bovine serum (FBS), streptomycin and glutamine were from PAA (PAsching, Austaria), HCT 116 colon cancer cells and Hep-2 human larynx epidermoid carcinoma cell lines were kindly provided by Bert Vogelstein and Refik Saydam Hygiene Center, Ankara Turkey respectively. Molecular devices Spectra MAX 190 Microplate Reader helps to get absorbance.

Preparation of Extract

Dictyonella incisa was collected from Seferihisar, Turkey in 2014, by a scuba diver and identified by Dr. Gözcelioğlu. A voucher specimen was deposited at the Pharmacognosy Department of Faculty of Pharmacy, Ankara University. The sample (204.4 g) was cutted to small pieces and then extracted by methanol (500 mL). The extract was dried under vacuum and kept at 4°C until uses.

Antimicrobial Activity

The stock solutions of extract were prepared in dimethyl sulphoxide (DMSO) at a final concentration of 512 µg/mL and sterilized by using 0.22 µm Millipore Membrane Filter (MA 01730, USA). Antimicrobial activity of sample was determined against *Staphylococcus aureus* (ATCC 43300), *Staphylococcus epidermidis* (ATCC 12228) and *Bacillus subtilis (*ATCC 6633) as Gram positive strains; *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa (*ATCC 27853), *Klebsiella pneumoniae* (RSKK 574) as Gram negative strains, *Candida albicans* (10231) and *Candida parapsilosis* (ATCC 22019) as yeast strains. Antimicrobial activity was screened according to the EUCAST recommendations with broth microdilution method. Antimicrobial activity was performed by a modified microdilution method as described in CLSI M07-A9 standard for bacteria and CLSI M27-A3 standard for yeasts (CLSI 2008; 2012) The tested two fold serial dilutions of the extract were between 256 and 0.5 µg/mL. The sealed microplates were placed in a humid chamber and incubated at 35°C for 24 and 48 h for bacteria and yeasts, respectively. The lowest concentration of the extract that completely inhibited macroscopic growth of the microorganism was accepted as minimum inhibitory concentration (MIC).

In-Vitro Cytotoxic Activity Assay (MTT Test)

HEp-2 human cells (human larynx epidermoid carcinoma) and HCT 116 colon cancer cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal 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 h incubation methanolic extract (25-200 mg/mL DMSO) of *D. incisa* was added to cell in different concentrations (1000, 500, 250, 125, 62.5, 32.2, 16,1 µg/mL). Subsequently, MTT reagent (0.5 mg/mL in sterile phosphate buffer) was added directly to the wells and incubated for 4 h. The absorbance was measured at 570 nm. The percentage growth inhibition was calculated using following formula, 200 µL of cells (Hep-2) were added without extract as control group. Results was calculated after 24, 48 and 72 h [10].

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

Superoxide Radical Scavenging Activity by Alkaline DMSO Method

Sodium hydroxide was added to air saturated DMSO for generating the superoxide radical. Alkaline DMSO was prepared by 5 mM NaOH in 0.1 mL water to 1 mL DMSO. Ten µL NBT (1 mg/mL) was added to 100 µL alkaline DMSO and 30 µL of extract at different concentrations, to give final volume of 1.4 mL. The absorbance was measured at 560 nm, The experiment was performed in triplicate. Quercetin and Ascorbic acid was used as standards [11].

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

Cholesterol and Cholestane Isolation

The methanol extract of *D. incisa* (8.9 g) was partitioned between distillated water (50 mL) and n-hexane (200 mL). The polar residue was partitioned by chloroforme (250 mL). *n*-hexane and chloroforme fraction was evaporated under the vacuum. The cholesterol was obtained from the *n*-hexane fraction (2.5 g), loaded to the Silica column chromatography and eluted by ethyl asetate and hexane gradiently. The cholestane was obtained from the choloro-form fraction, loaded to the Silica column chromatography and eluted by chloroforme and methanol gradiently. Obtained compounds (white amorphous) were washed with methanol several times then dissolved in choloroform and sent to Nuclear Magnetic Resonance (NMR). The structures of the compounds were identified by ^{1}H NMR.

RESULTS

Dictyonella incisa's extract was tested against Gram positive, Gram negative strains, and yeast. According to the results, extract was shown to have antimicrobial activity against *S. aureus*, *S. epidermidis* (MIC: 62.5 µg/µL) and against *C. albicans* and *C. parapsilosis* (MIC: 1.56

and 0.78 respectively). The results were shown in *Table 1*. Cytotoxicity activity of the extract was established by MTT assay against HCT-116 (colon cancer cells lines) and HEp-2 (human larynx epidermoid carcinoma), according to the results *D. incisa*'s extract was shown to have higher cytotoxicity against HCT-116 than Hep-2 cell lines. The results were shown in *Table 2*.

Antioxidant activity of *D. incisa*'s methanolic extract was determined by SO (Superoxide Radical Scavenging Activity) assay, according to the results it was show dose dependent antioxidant activity (24.62% inhibition in 800 µg/µL) where quercetin and ascorbic acid were used as standard. The results were shown in *Fig.1*.

Cholesterol and cholestane were isolated from *D. incisa,* the structure of these compounds were identified by ^{1}H NMR and compared with literature. Cholesterol and cholestane structure was shown in *Fig. 2* [12,13].

Cholesterol ($CDCl_3$, 400MHz): H_1: δ1.61 (ddd, *J*=13.7, 3.1, 2.5 Hz), H_2:1.76 (dddd, *J*=13.2, 10.2, 2.5, 2.4 Hz), H_3:4.03 (dddd, *J*=3.3,3.2, 2.4, 2.3 Hz), H_4:2.34 (dd, *J*=14.6, 2.3Hz), H_6: 5.31 (dd, *J*=9.1, 5.2 Hz), H_7: 2.1(ddd, *J*=13.1,10.2,9.1 Hz), H_8:1.46(qd, *J*=10.2,3.3 Hz), H_9:1.47 (ddd, *J*=10.2,10.2, 3.3 Hz), H_{11}:1.49 (dddd, *J*=13.1,10.3,10.2, 3.2 Hz), H_{12}:1.58 (ddd, *J*=13.07,3.17, 2.55 Hz), H_{14}:1.6(dddd, *J*=13.7,8.0,4.6 Hz), H_{15}:1.57(dddd, *J*=13.17,3.19,3.09, 2.51 Hz), H_1:1.6(dddd, *J*=13.7,8.0,4.6 Hz), H_{16}:1.63 (dddd, *J*=13.71,8.04,6.79, 4.63 Hz), H_{17}:1.49(ddd, *J*=7.70,6.79,4.58 Hz), H_{18}: 0.87(s,3H), H_{19}:1.22 (s, 3H), H_{20}:1.47 (dddd, *J*=13.16,3.19, 3.09,2.5 Hz), H_{21}:1.04 (d, *J*=6.8 Hz), H_{22}:0.79(dt, *J*=7.3,7.2 Hz), H_{23}:1.23 (quint, *J*=7.18 Hz), H_{24}:0.66 (q, *J*=7.2 Hz), H_{25}:1.47(dddd, *J*=13.2,3.2,3.1, 2.5 Hz), H_{26}, H_{27}:0.73-0.84 (d, *J*=6.6 Hz),OH: 2.63(s, 1H).

Cholestane ($CDCl_3$, 400MHz): H_1, H_4: δ1.53 (dddd, *J*=13.92,6.95, 4.82, 1.73 Hz), H_2, H_3: 1.6 (dddd, *J*=13.92,8.02, 3.22, 1.53 Hz), H_5: 1.25 (m, *J*=10.26 Hz), H_6: 1.75 (dq, *J*=13.05, 2.78 Hz), H_7: 1.53 (dddd, *J*=13,87, 8.0, 4.48, 1.65 Hz), H_8: 1.36 (tdd, *J*=10.25,10.20, 2.79 Hz), H_9: 1.24 (ddd, *J*=10.25, 10.18, 3.28 Hz), H_{11}: 1.5 (dddd, *J*=13.07,10.24, 10.18, 3.18 Hz), H_{12}: 1.67 (ddd, *J*=13.08,3.18, 2.53 Hz), H_{14}: 1.5 (ddd, *J*=10.2,9.75, 4.82 Hz), H_{15}: 1.5 (dddd, *J*=13.92,8.24, 4.18, 1.18 Hz), H_{16}: 1.65 (dddd, *J*=13.75,13.79, 4.65, 1.73 Hz), H_{17}: 1.66 (ddd, *J*=8.25, 4.63, 4.3 Hz), H_{18}: 0.75(s,3H), H_{19}:1.2 (s, 3H), H_{20}: 1.45 (m, *J*=7.28 Hz), H_{21}: 1.04 (m, *J*=6.8 Hz), H_{22}:0.72 (dt, *J*=7.3,7.2 Hz), H_{23}:1.13 (quint, *J*=7.2 Hz), H_{24}:0.68 (q, *J*=7.5 Hz), H_{25}: 1.47 (m, *J*=7.16 Hz), H_{26},H_{27}:0.77-0.88 (d, *J*=6.6 Hz).

Table 1. *Antimicrobial activity of D. incisa's extract (MIC value)*

Extract and Standards	MIC (µg/mL)							
	***S. aureus* ATCC 43300**	***S. epidermidis* ATCC 12228**	***B. subtilis* ATCC 6633**	***E. coli* ATCC 25922**	***P. aeruginosa* ATCC 27853**	***K. pneumoniae* RSKK 574**	***C. albicans* 10231**	***C. parapsilosis* ATCC 22019**
D. incisa	62.5	62.5	not active	not active	not active	not active	500	500
Ciprofloxacin	0.625	0.078	0.078	0.009	0.625	0.039	No determined	No determined
Miconazole	No determined	No determined	No determined	No determined	No determined	No determined	1.56	0.78

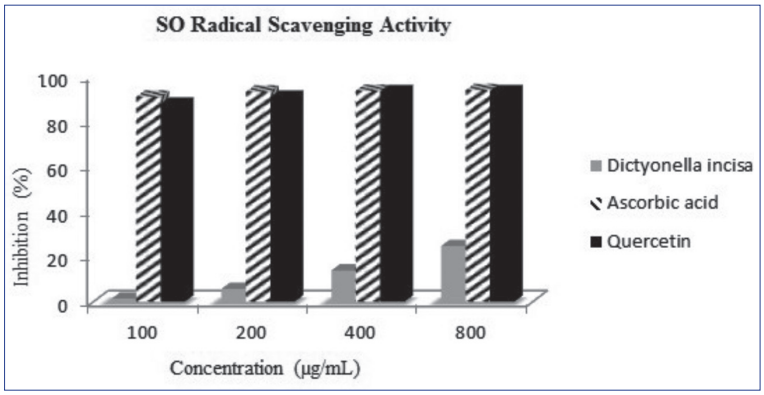

Fig 1. Antioxidant activity of *D. incisa* and standards

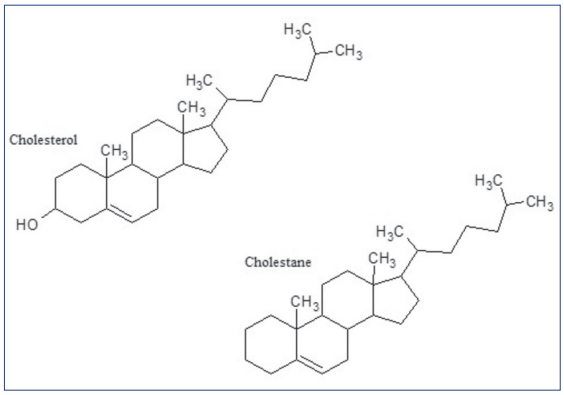

Fig 2. Cholesterol and cholestane structure

Table 2. *Cytotoxicity activity of D. incisa's extract*

Extract and Standards	IC_{50} (µg/mL)	
	HCT-116	Hep-2
D. incisa	229.08±0.52	57.09±0.89
Adriamycin	No determined	0.362±0.12
Camptothecin	102.67±2.9	No determined

DISCUSSION

Among the marine organisms; marine sponges (Phylum Porifera) are the oldest and simplest multicellular animals on earth. A huge number of bioactive compounds have been

isolated from marine sponges. The majority productive marine producers of novel and unique compounds are sponges, with more than 250 new secondary metabolites reported every year. This great potential has revived applications of secondary metabolites as therapeutics and at present, a number of promising compounds are in clinical and preclinical trials such as Discodermolide, Monanchocidin, Renieramycin M, Hetronemin (anticancer), Tsitsikammamine C, Psammaphysin H (antimalarial) Norbatzelladine L (antiviral), (-)-ageloxime D (antifungal), Manolide, Spongidines A-D (antiinflammatory) and Eryloside F (cardiovascular agent) [2-4]. Sponges are one of the most interesting marine organism, they are a rich sources of secondary metabolites with pharmaceutical potential which serve them as a chemical defense against predators [14]. Among 39 anticancer compounds which isolated from marine organism 18 compounds have sponge origin [15], however, antimicrobial activity of sponges and their metabolites have not been proven [16]. Several reports have shown sponges are rich of steroids and fatty acids, (22E)-cholesta-4,6,8(14),22-tetraen-3-one [7], incisterol, (17R)-17-methylincisterol, (17S)-17-Methylincisterol, (17R)-17-Ethylincisterol [8], (9Z,19Z)-3, 6-epoxyhexacosa-3,5,9,19-tetraenoic acid, (SZ, 11 Z, 14Z,17Z)-3, 6-epoxyeicosa-3,5,8,11,14,17-hexaenoic acid, (8Z, I1Z,14Z,17E)-3, 6-epoxyeicosa-3,5,8,11,14,17-hexaenoic acid and (5Z,8Z,11Z,14Z,17E)-eicosa-5,8,11,14,17-pentaenoic acid were isolated from *D. incisa* [9]. According to the results which obtained in this study, *D. incisa* was shown antimicrobial activity against Gram positive (62.5 µg/mL) and yeast strains (500 µg/mL), while in our previous study methanol extract of *D. incisa* was shown 81% inhibition against Methicillin-resistant *Staphylococcus aureus* [17]. *D. incisa*'s extract was shown dose dependent antioxidant activity. Cytotoxicity activity of *D. incisa*'s extract was shown highest activity against HCT-116 cell lines than Hep-2 cell line. Camptothecin was used as standard against HCT-116 cell lines while it was shown IC_{50}: 102.67 µg/mL, the *D.incisa* extract was shown IC_{50}: 229.08 µg/mL. Furthermore, Adriamycin was used as standard drug against Hep-2 cell line (IC_{50}: 0.362±0.12 µg/mL) but the *D. incisa*'s extract was not shown good activity against this cell line.

This study demonstrated that *D. incisa* with significant cytotoxicity activity against colon cancer cell lines is a good candidate for further *in vitro* studies and isolation of responsible secondary metabolites from *D. incisa*. Further and detailed studies are needed.

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