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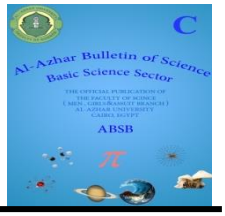

CYTOTOXICITY AND ANTIMICROBIAL ACTIVITIES OF SOME SOFT CORALS INHABITING THE RED SEA, EGYPT.

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ABSTRACT

Cancer and infectious diseases are notoriously known as deleterious health threats for the world, especially in the developing countries. The aim of the present study was to evaluate the cytotoxicity and antimicrobial activities of the methanolic extracts of the soft corals *Nephthea elatensis*, *Heteroxenia fuscescens*, *Ellisella juncea*, *Dendronephthya mollis*, and *Sinularia hirta*, that are native to the Red Sea in Egypt. The cytotoxicity assay was carried out by the enzymatic reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay against lung adenocarcinoma cell line (A549). Moreover, the antimicrobial activity was carried out against 11 human pathogenic bacterial and fungal strains using well-cut diffusion technique, while the Minimum Inhibitory Concentrations (MICs) were determined by microdilution method. *Nephthea elatensis* showed potent cytotoxicity [half maximal inhibitory concentration (IC_{50}) 11.9 ± 1.2 µg/mL]. Also, it exhibited a potent antibacterial activity against *Staphylococcus aureus* (MIC 1.0 µg/mL). *Sinularia hirta* exhibited significant antimicrobial activities against *Salmonella typhimurium* and *S. aureus* (MIC 5.0 and 10.0 µg/mL, respectively). The results recommended *N. elatensis* and *S. hirta* as promising sources for new anticancer and antibiotic natural candidates.

Keywords: Antimicrobial; Cytotoxicity; MTT, *Nephthea elatensis*; *Sinularia hirta*; Soft corals.

1. INTRODUCTION

Cancer and infectious diseases are deleterious health problems that severely impact the developing countries [1]. Cancer is one of the major causes of morbidity and mortality in Mediterranean Sea Region [1]. The progressive increase in the number of incident cases in Egypt is expected to be 331,169 by 2050 [2]. Furthermore, the wide spread of tobacco products could be related to the elevated number of deaths due to lung cancer in 2018, which was around 9.6 million patients in the Middle East and North Africa only [3]. Furthermore, another serious health threat that emerged in the world of the 21 century was the emergence of antibiotic-resistant microorganisms. These pathogens flourished due to the misuse, overuse, and continuous consumption of antibiotics for long periods of time, [4, 5]. Around 700,000 deaths occur worldwide because of drug-resistant pathogens [6].

In light of these increasing rates of morbidity and mortality, the world's interest is more directed towards searching for cheap, safe, and effective alternative antitumor and antimicrobial agents, especially from natural sources [7]. The unique chemical and biological diversity of the marine environments led to the discovery of many bioactive secondary

metabolites that contributed much as therapeutic agents [2]. Among marine organisms, marine invertebrates are the most producer of marine natural products [2]. The Red Sea region is a rich source of biodiversity of marine organisms due to its several distinctive features that provided the biota with rates of endemism and favorable conditions that exceed any other marine areas in the world [8]. Among these peculiar groups come in advanced position the soft corals (Octocorallia, Alcyonacea). This group is among the most abundant organisms of the Red Sea and represent around 40% of all the identified soft coral species worldwide [9]. The crude extracts as well as many of the secondary metabolites of some soft corals exhibited promising biological activities such as neuroprotection, cytotoxicity, anti-inflammatory, antimicrobial, antifouling, and antiprotozoal activities [10,9, 11].

In the context of continued efforts to explore effective marine resources that can provide anticancer and antibacterial activities, *in vitro* evaluation of these activities in methanolic extracts of Red Sea soft corals has been proven to be a very efficient approach (for examples, see [12, 13]. The current study aimed to assess the activites of methanolic extracts of 5 soft coral species that are located in the Egyptian Red Sea, i.e. *Nephthea elatensis* (also synonymized *Litophyton striatum* , see [14]; *Heteroxenia fuscescens, Ellisella juncea, Dendronephthya mollis,* and *Sinularia hirta,* against lung adenocarcinoma cell line, and against 11 pathogenic bacteria and fungi. The results were expected to preliminarily shed light on which of these species can be considered as a source of bioactive, therapeutically active products.

2. MATERIAL AND METHODS

2.1. Cell lines, bacteria, and fungi

The A549 cell lines used in the current work were gained from the Japanese National Institute of Biomedical Innovation, Japan. The media used for biological assessment and their constituents as well as 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and etoposide standard were obtained from Nacalai Tesque, Kyoto, Japan. The cell culture plates were purchased from Franklin Lakes, NJ, USA.

The bacterial isolates were kindly provided from microbiology laboratory, National Institute of Oceanography and Fisheries, Alexandria, Egypt. The yeast and fungal strains were kindly obtained from the Center of Fungi, Assiut University, Assiut, Egypt. Nutrient agar (NA) was used to culture the reference bacterial strains and determine the antibacterial activities of the soft corals' samples [15]. Sabouraud dextrose agar (SDA) [16]and potato dextrose agar (PDA) were used to culture yeasts and fungi [15]. These bacterial species were *Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Bacillus subtilis, Staphylococcus aureus,* and *Vibrio fluvialis.* The fungal species tested were *Candida albicans, Fusarium solani, Aspergillus niger,* and *Rhizoctonia solani.*

2.2. Animal materials

The soft coral species *N. elatensis, H. fuscescens, E. juncea, D. mollis,* and *S. hirta* were collected from the Red Sea on the Hurghada city, Egypt, from a depth of 5 – 12 m in March 2018 via Contained Underwater Breathing Apparatus (SCUBA). The samples were identified as possible to the nearest species according to [17]; Versevldt (1982); and [18]. Voucher specimens were allocated at Department of Zoology, Faculty of science, Al-Azhar University, Assiut Branch, Assiut, Egypt with the following symbols (NE-12, HF-9, EJ-3, DM-1, and SH-7 for *N. elatensis, H. fuscescens, E. juncea, D. mollis,* and *S. hirta;* respectively.

2.3. Extraction and isolation of bioactive metabolites

The fresh collected samples *N. elatensis, H. fuscescens, E. juncea, D. mollis,* and *S. hirta* (20.0, 18.0, 21.0, 22.0, and 17.0 gm wet wt.; respectively) were chopped into small pieces and extracted by maceration separately in methanol (150 mL\ three times). The solvent was distilled off by rotary evaporator at low temperature (50 °C) to obtain a dry residues, which weighed 0.5, 0.4, 0.6, 0.65, and 0.45 gm of the mentioned corals; respectively.

2.4. Cytotoxic assay

The cytotoxic activity toward the A549 cancer cell line was carried out through MTT assay. In brief, the Dulbecco's modified Eagle's medium and a 96-well plate were used for the

cancer cell's culture. Around 99 µL of medium containing 5×10^3 cells and 1 µL of the sample solution was added to each well. After that, the plate was incubated for three days. The cell culture was incubated at 37 °C, in an atmosphere with 5% CO_2. The media were aspirated at the end of the incubation period and 100 µL of MTT solution were added and incubated again for an hour. The solution was aspirated and 100 µL of DMSO were added to dissolve the formed formazan crystals. The absorbance was measured at 540 nm using a VersaMax™ Absorbance Microplate Reader (Molecular Devices, LLC, USA). Etoposide, the standard cytotoxic agent, was used as positive control. For the negative control, DMSO was the used. Each experiment was carried out in triplicate. The following equation was used to calculate the cytotoxic activity:

$$\% \text{ inhibition} = [1- (A_{test} - A_{blank})/(A_{control} - A_{blank})] \times 100$$

where $A_{control}$ is the absorbance of the negative control (DMSO), A_{test} the absorbance of the test wells, and A_{blank} the absorbance of the cell-free wells [19].

2.5. Antimicrobial assay

2.5.1. Antibacterial assay

In sterile-capped test tubes, 15 mL of sterilize nutrient agar for bacteria and Sabouraud dextrose agar for yeast were poured and allowed to cool to 50°C in a water bath. About 100 µL of inoculate (10^8 CFU for bacteria and yeast) were added. The tubes were mixed using a vortex for 15-30 s. Thereafter, each test tube contents were poured onto a sterile 100 mm diameter Petri dish for solidification [20]. The activity was evaluated using well-cut diffusion technique. Wells were punched out using a sterile 7 cm cork-borer in nutrient agar plates containing the tested microorganisms. Each soft coral crude extract was dissolved separately in DMSO to get a final concentration of 500 µg/mL as stock. From the stock solution (100 µg/mL) of each crude extract; appropriate amount was transferred into each well. They were subjected to 4°C incubation for 2 h, and then were later incubated at 37°C for 24 h. The results were obtained by measuring the diameter of inhibition zone three times for each well and expressed in millimeter [21].

2.5.2. Antifungal bioassay

2.5.2.1. By pouring technique

The indicator fungi were evaluated by introducing aliquots of each soft coral crude extract to PDA medium at a concentration of 10% (v/v). One disc from each of the seven fungal growths was located in the centre of a crude extract-PDA medium plate. All plates were incubated at 28 °C until the fungal growth on the control was totally covered. To determine the suppressive effect of crude extract against the indicator fungi, the radius-growth of each indicator fungus was evaluated [22].

2.5.2.2. By well-cut diffusion technique

One disc of the fungal growth was separately put on the top of a plate containing PDA medium. About 100 µg/mL of the soft corals crude extract dissolved in DMSO was transferred into each well. All plates were incubated at 28ºC until the control was completely covered with fungal growth. The results were obtained by measuring the inhibition zone diameter three times for each well and expressed in millimetre [23].

2.5.3. Minimum inhibitory concentration (MIC) experiment

The investigation of MIC proceeded using microdilution method described by [24]. Each crude extract's stock solution was diluted in DMSO to a final concentration of 100; 75; 50; 25; 20; 15; 10 and 5 µg/mL. Then, in a 96-well plate, 100 µl of each concentration of sample was mixed to 100 µl of suspension inoculum of test pathogen, which was adjusted to match the 0.5 McFarland standards. As a sterility control, sterile particular broth medium for each pathogen was employed, and inoculum pathogens were used as a growth control. The MIC was determined in three different ways. The minimum inhibitory concentration (MIC) of an extract is defined as the concentration at which observable growth is suppressed.

3. RESULTS AND DISCUSSION:

3.1. Cytotoxic activity

The assay result (Table 1, Fig. 1) demonstrated that the five soft corals tested against the A549 cell line showed variable cytotoxicities. Among the tested samples, the

methanolic extract of the soft coral *N. elatensis* was the most potently cytotoxic against the respective cell line (IC_{50} 11.9 ± 1.2 µg/mL) in comparison to the standard etoposide (IC_{50} 19.8 ± 0.9 µg/mL) while *H. fuscescens* showed moderate activity (IC_{50} 60.2 ± 5.4 µg/mL). Extracts of *E. juncea* and *D. mollis* exhibited weak activities and considered non-cytotoxic (IC_{50} 178.3 ± 9.2 and 147.4 ± 5.7 µg/mL, respectively). *S. hirta* was inactive under the maximum concentration used (200 µg/mL). The potent activity of the *N. elatensis* may be attributed to its expected high contents of sesquiterpenes, polyhydroxylated sterols, and cembranoid diterpenes which reported to have strong cytotoxic activity and isolated previously from the genus *Nephthea* (*Litophyton*) [25, 26]. For examples, the sesquiterpenoids alismorientol B isolated from *L. arboreum* was reported to have a potent cytotoxic activity against the breast cancer cell line (MCF7) [27]. Also, the cembranoid derivative, 11-acetoxy-15,17-di-hydroxy-2,12-epoxy-(3*E*,7*E*)-1-cembra-3,7-diene showed activities against the MCF-7, Hepatocellular carcinoma (Hep-G2), and HC-T116 cancer cell lines [28]. In addition, the steroids 7*β*-acetoxy-24-methylcholesta-5-24(28)-diene-3,19 diol exhibited cytotoxicity against HeLa cancer cells [28]. Further studies are recommended to isolate the active compounds responsible for the cytotoxic activity of *N. elatensis* against the A549 cell line. Furthermore, our results agreed with the reported moderate cytotoxicity of *H. fuscescens* against the A549 cell line [29].

3.2. Antimicrobial activity

The growing needs for drugs to control new illnesses and to combat resistant strains of microorganisms became more urgent than ever [4]. It is widely accepted that new drugs, especially antibiotics, and that the most propitious source natural products. Accordingly, as a part of our search for new sources of marine bioactive natural products, the second part of our experiments was intended to screen the same five soft corals extracts against 11 human pathogenic microbes. Our screening results showed that the *N. elatensis* had significant antimicrobial activity against the Gram-Positive *S. aureus* with inhibition zone (18.5 ± 2.12 mm/100 µg/mL) while *S. hirta* exhibited strong activity against the Gram-Positive *S. aureus* and Gram-Negative *S. typhimurium* with inhibition zones (17.3 ± 1.4 and 17.5 ± 2.3 mm/100 µg/mL, respectively). The Gram-Positive *S. aureus* was also susceptible to the crude extracts of *H. fuscescens* and *D. mollis* (9.6 ± 0.57 and 10.6 ± 1.1 mm/100 µg/mL, respectively). It's interesting that all tested fungi were not susceptible to any of the five tested soft corals' extracts. This may be due to the different nature of the cell wall of fungi and bacteria [30]. Furthermore, MIC of each active antibacterial extract was determined using microdilution method (see the experimental section). The results (Table 2) showed that the *N. elatensis* was the most potent antimicrobial activity against *S. aureus* with MIC (1.1 µg/mL) followed by *S. hirta* which exhibited strong activity against the Gram-Positive *S. aureus* and Gram-Negative *S. typhimurium* with MIC (10 and 5 µg/mL, respectively).

Table 1: Cytotoxic activities of the crude extracts of selected soft corals from Red Sea against A549 cell line.

Soft corals	IC_{50} µg/ mL
N. elatensis	11.9 ± 1.2
H. fuscescens	60.2 ± 5.4
E. juncea	178.3 ± 9.2
D. mollis	147.4 ± 5.7
S. hirta	>200
Etoposide	19.8 ± 0.9

It was reported that the Gram-Positive strains are more susceptible to marine secondary metabolites [31]. The antibacterial activity of *N. elatensis* may be ascribed to its mentioned potent cytotoxicity (Table 1, Fig. 1). The steroidal secondary metabolites isolated from the genus *Litophyton* such as litosterol (a polyhydroxy steroid isolated from an Okinawan soft coral *L. viridis*) was reported to have potent antibacterial activity, which supports our findings [32]. Among our tested soft coral species; the only one which showed activity against both Gram-Positive and Gram-Negative strains was *S. hirta* which belongs to the genus Sinularia. These results coincided with similar results reported by Khalesi and co-workers [33], who concluded that the genus *Sinularia* produces antibacterial compounds that are stronger than similar compounds obtained from other genera of soft corals. The same study reported that more than 60% of the studied soft

coral species of *Sinularia* contained terpenoid compounds which may be responsible for their broad-spectrum antibacterial activity.

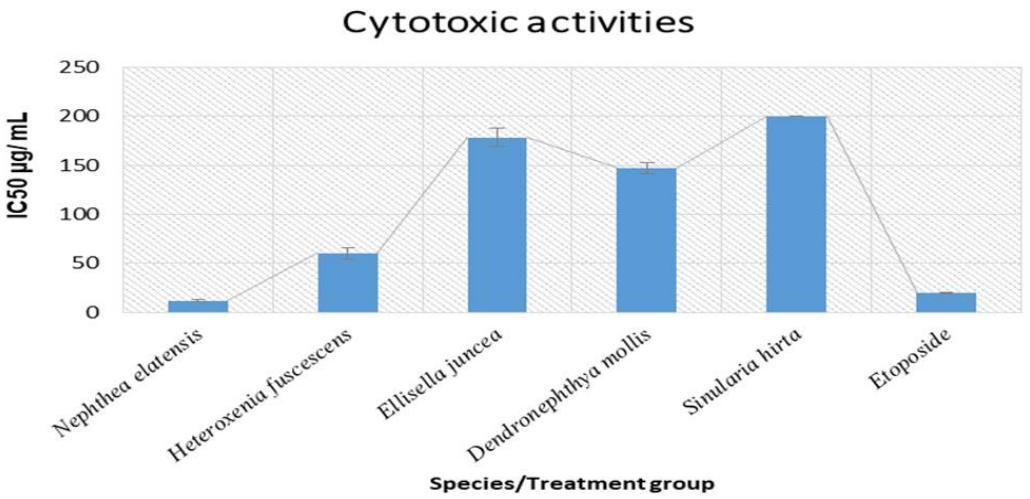

Fig. 1. Cytotoxic activities of the applied soft coral species against the lung adenocarcinoma cell line (A549). Data are represented as Mean IC50±SEM (standard errors of means). Etoposide was applied as a standard cytotoxic agent.

Table 2. Minimum inhibitory concentration (µg/mL) of crude extracts of selected soft corals from Red Sea against microbial strains expressed in the inhibition zone (mm).

No	Microbial strain	*N. elatensis*	*H. fuscescens*	*E. juncea*	*D. mollis*	*S. hirta*
		MIC (µg/mL) / Inhibition Zone (mm) ± SE				
1	*Enterococcus faecalis* 29212	ND	ND	ND	ND	ND
2	*Escherichia coli* 8739	ND	ND	ND	ND	ND
3	*Pseudomonas aeruginosa* 9027	ND	ND	ND	ND	
4	*Salmonella typhimurium*	ND	ND	ND	ND	5 / 5.5 ± 0.86
5	*Bacillus subtilis* 6633	ND	ND	ND	ND	
6	*Staphylococcus aureus* 25923	1 / 3.6 ± 0.57	25 / 4.6 ± 0.57	ND	25 / 4.3 ± 0.57	10 / 4.8 ± 0.76
7	*Vibrio fluvialis*	ND	ND	ND	ND	ND
8	*Candida albicans* ATCC 10237	ND	ND	ND	ND	ND
9	*Fusarium solani*	ND	ND	ND	ND	ND
10	*Aspergillus niger*	ND	ND	ND	ND	ND
11	*Rhizoctonia solani*	ND	ND	ND	ND	ND

4. CONCLUSION

Five selected Red Sea dominant soft corals showed varied cytotoxic and antibacterial activities. *Nephthea elatensis* showed potent cytotoxicity against A549 cell line and antimicrobial activity against *S. aureus*, while *S. hirta* exhibited noticeable antimicrobial activities against *S. typhimurium* and *S. aureus*. Further investigations to discover the effective antibacterial and cytotoxic metabolites from these soft corals are needed, considering the promising results obtained herein in the current study.

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Conflict of Interests

The authors declare that there is no conflict of interest.

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السمية الخلوية والأنشطة المضادة للميكروبات لبعض الشعاب المرجانية الرخوة والمستوطنة للبحر الأحمر، مصر.

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الملخص:

السرطان والأمراض المعدية من المشاكل الصحية المزعجة والمنهكة التي تواجه البلدان النامية والعالم. الهدف من هذه الدراسة هو التقييم المخبري للسمية الخلوية والأنشطة المضادة للميكروبات للمستخلصات الميثانولية للشعاب المرجانية الرخوة *نفثيا إلاتنسيس* و*هيتيروزينيا فوسينس* و *اليسيلا جنكا* و *دندرونفثيا موليس* و *سنيولاريا هيرتا*، والتي تم جمعها من ساحل البحر الأحمر المصري، ضد خط خلايا الرئة السرطانية الغدية A549 ،وضد 11 سلالة بكتيرية وفطرية ممرضة للإنسان, تم إجراء اختبار السمية الخلوية بواسطة بروتوكول مقايسة MTT ، بينما تم تنفيذ النشاط المضاد للميكروبات باستخدام تقنية انتشار الحفر المقطوعة، وتم تحديد MICsبطريقة التخفيف الدقيق. وقد أظهر مستخلص الشعاب المرجانية اللينة من النوع *N. elatensis* سمية خلوية قوية (IC50 1.2 ± 11.9ميكروغرام / مل) , ونشاط مضاد للميكروبات ضد Staphylococcus *aureus* (MIC 1.0ميكروغرام / م)، بينما أظهرت الشعاب المرجانية الرخوة من النوع *S. hirta* أنشطة كبيرة في مضادات الميكروبات ضد *Salmonella typhimurium* و *Staphylococcu aureus*(MIC 5.0, 10.0 ميكروغرام/ مل)، على التوالي .خلصت نتائجنا إلى أن المرجان الرخو *N. elatensis* و *S. hirta* يمكن اعتبارهما مصادر واعدة لمضادات السرطان والمضادات الحيوية الطبيعية المرشحة الجديدة، على التوالي.