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Cytotoxic activity of extracts of demosponges *Haliclona caerulea*, *Axinella sinoxea* and *Ircinia mutans* from Persian Gulf

Melika Nazemi[1]*, Hadi Ghaffari[2], Yazdan Moradi[2], Mohammad Reza Amiran[3] & Soheila Aghaei Dargeri[4]

[1]Persian Gulf and Oman Sea Ecological Research Center, Iranian Fisheries Science Research Institute (IFSRI), Agricultural Research, Education and Extension Organization (AREEO), Bandar Abbas, Iran

[2] Iranian Fisheries Research Institute (IFSRI), Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

3. Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran

[4]Departman of Horticulture, Faculty of Agricultures and Natural Resources, University of Hormozgan, Bandar Abbas, Iran

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Sponges are valuable source of bioactive natural products. Although marine benthic invertebrate communities occur in the Persian Gulf they have been little explored for their biomedicinal potential. Here, we studied the methanol and diethyl ether extracts of sponges *Haliclona caeralea*, *Axinella sinoxea* and *Ircinia mutans* sponges for their cytotoxic activity against human epidermis carcinoma (KB/C152) and T-cell lymphoma cell lines (HUT-78/C185) cell lines. Sponges after collection and identification were extracted with methanol and diethyl ether extract. The cell viability and cytotoxicity induced by the extracts were assessed using XTT and lactate dehydrogenase release assays (LDH leakage). The results indicated that the methanol and diethyl ether extract of *I. mutans* exhibited strong cytotoxicity towards KB and HUT cell lines and diethyl ether extract of *H. caeralea* and *A. sinoxea* had significant cytotoxicity in HUT and KB cells compared to methanol extract. The results indicated that the methanol and diethyl ether extract of *I. mutans* possess significant cytotoxic activity.

Keywords: Anticancer activity, Lactate dehydrogenase assay, LDH leakage, Sponges, XTT assay

Marine invertebrates have been widely studied for their bioactive properties[1]. Marine sponges belonging to the phylum Porifera are rich source of marine natural products including anticancer agents[2-5]. As major sources of secondary metabolites and bioactive compounds with antifungal, anti-inflammatory[2], anticancer[7], antiviral[8] and antioxidant[9] activities these sponges serve as a potential drug candidates[6]. The secondary metabolites with cytotoxic or anti-inflammatory activity attracted many for developing anticancer drugs[1].

In 2018, cancer affected 18.1 million people from 185 countries worldwide in 36 forms and put 9.6 million to death which may increase to 27.5 million new cases and 16.3 million deaths by 2040[10,11]. Holding approximately 60% of the global population, Asia accounts for 48.4% of cancer cases world over, and 57.3% of cancer deaths[10]. While the Eastern Asia leads with 5.6 million cases and 3.4 million deaths, the South Central Asia is reported to have 1.7 million cases and 1.2 million fatalities[11]. In United States, though the mortality rate has dropped by 29% during last two decades, cancer still remains the second most lethal disease[12]. Worldwide, malignant neoplasms is reported to cause 16% of total mortality, next only to cardiovascular diseases — about 1 out of 6 deaths, more than AIDS, TB and malaria combined[11]. While lung, prostate and colorectum cancers are most common among men, breast, cervix uteri and colorectum types are top three in women[10]. Lymphoma, the cancer of immune cells called lymphocytes, may occur at any age but most common in young adults[9]. The two main groups of lymphoma, Hodgkin lymphoma and non-Hodgkin lymphoma, accounts for 0.4 and 2.8% of fresh cases, respectively in 2018. The complexity and severity of cancer has attracted many researchers for better understanding and to develop improved therapies including effective drugs, particularly from natural resources[13-16].

Persian Gulf, though a rich source of marine organisms, information on biomedical properties of the marine fauna remains scanty[17,18]. In this study, we tried to evaluate the cytotoxicity of the methanol and diethyl ether extracts of three marine sponges

*Correspondence:
Phone: +98 9123612456 (Mob.)
E-mail: melikanazemi@yahoo.com

(*Haliclona caerulea*, *Axinella sinoxea* and *Ircinia mutans*) and also studied their anticancer potential on Human epidermis carcinoma (KB/C152) and T-cell lymphoma (HUT-78/C185) cell lines.

Material and Methods

Sampling and identification

Haliclona caerulea and *Axinella sinoxea* (class Demospongiae, order Haplosclerida, Fam. Chalinidae and Axinellidae), and *Ircinia mutans* (Class: Demospongiae, Order: Dictyoceratida, and Family: Irciniidae) (Fig. 1), were collected by scuba diving in July 2012 and 2013 at a depth of 20-25 m from Larak Island and Kish Island in the Persian Gulf. The sponges were transferred to the laboratory under frozen condition. The collected samples were cleaned, identified using scanning electron microscope, skeletal slides and dissociated spicules mounts based on Hooper identification key, and maintained at 18°C[19].

Extraction

The sponge samples (*H. caerulea* 780 g, *A. sinoxea* 478 g and *I. mutans* 843 g, wet wt.) were cut into small pieces (1.0 cm). Extracts of sponge were obtained using two different solvents including: methanol and diethyl ether. After 24 h of exposure in diethyl ether, the extracts were removed and sample after drying extracted with methanol for 72 h. Both extracts concentrated under low pressure at 35-40°C by rotary evaporation. Ether-methanol was formed by adding ether in order to separate the methanol-aqueous extract, then the upper phase was separated by separation funnel[20].

Cell culture

Human epidermis carcinoma (KB/C152) and T-cell lymphoma cell lines (HUT-78/C185) were obtained from Pasteur Institute, Iran. The cells were maintained in DMEM media (Gibco) supplemented with 10% fetal calf serum (Gibco), l-glutamine (2 mM), penicillin G (100 U/mL), streptomycin (100 mg/mL) and incubated in 5% CO_2 at 37ºC.

XTT-based cytotoxicity assay

The cytotoxicity of the compounds was evaluated using cell proliferation XTT assay[21]. The cells cultured in 96-well plate at a density of 50×100 cells per well and allowed to attach for 24 h. After that the cells were treated with various concentrations (1, 2, 4, 10, 50, 100, 200, 300, 400, 800, 1000 and 2000 µL) of the methanol and diethyl ether extracts of *A. sinoxea*, *H. caerulea* and *I. mutans*. Cyclosporine @ 1.0 to 400 µL was used as positive controlfrom. After incubation at 37ºC with 5% CO_2 for 24 and 48 h, 50 µL of prepared XTT mixture (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide) was added to each well. The cells were further incubated for 24 h to lead the production of XTT Formosan. Absorbance was read with the ELISA reader (Bio-TekELx 800) at a test and reference wavelengths of 490 nm and 690 nm, respectively. IC50 values represent cytotoxicity was calculated as follows:

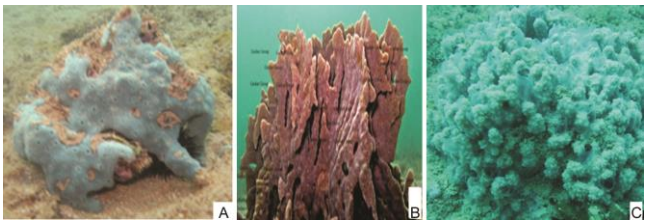

Fig. 1 — (A) *Haliclona caeralea*; (B) *Axinella sinoxea*; and (C) *Ircinia mutans* collected from Larak Island and Kish Island (Photo by Melika nazemi)

$$IC50 = \frac{(\text{average of negative control } OD - \text{average of sample control } OD)}{(\text{average of negative controls } OD)} \times 100$$

Lactate dehydrogenase (LDH) release assay

Lactate dehydrogenase (LDH) is a key marker for cell degeneration and used to measure cell death by a LDH-estimation kit. LDH activity was measured through the oxidation of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD+) at a wavelength of 340 nm. The rate of increase in enzyme activity due to the formation of reduced nicotinamide adenine dinucleotide (NADH) is directly proportional to the LDH activity in the sample. The KB/C152 and HUT-78/C185 cells were plated at a density of 5×104 cells/well in 24-well plates. After 24 h, the cells were treated with different concentrations of (1, 2, 4, 10, 50, 100, 200, 300, 400, 800, 1000 and 2000 µL) of the methanol and diethyl ether extracts of *A. sinoxea*, *H. caerulea* and *I. mutans*. The total LDH was measured by lysis (2% Triton X-100) of untreated cells, which were selected as the total LDH activity. The cells were precipitated by centrifugation at 2500 rpm for 5 min at 4°C. The supernatant (100 µL) was mixed with 900 µL of kit reaction mixture. Cell damage was evaluated by measuring the leakage of intracellular LDH into the medium[22].

Statistical analysis

Each experiment was repeated thrice separately. The parameters of the experiment are expressed as mean ± SD. Statistical evaluation of the data was done using

One-way ANOVA with the level of significance at $P < 0.05$.

Results

The results of the *in vitro* cytotoxic activity of the sponge extracts (methanol and diethyl ether) and cyclosporine (positive control) are shown in the Fig. 2. The IC_{50} values of methanol and diethyl ether extracts of *H. caeralea* in HUT cell were observed to be approximately 2000, respectively. The IC_{50} values of diethyl ether extract of *A. sinoxea* in HUT cell were observed to be approximately 642 µL, but methanol extract was not showed any cytotoxicity activity. Based on Fig. 2, the IC_{50} values of methanol and diethyl ether extracts of *I. mutans* in HUT cell were observed to be approximately 450 and 500 µL, respectively. The data indicated that methanol and diethyl ether extracts of *H. caerulea* and *A. sinoxea* had no cytotoxic activities against KB cells at concentrations 1.0 to 2000 µL.

The result of LDH release assays revealed that the all sponge extracts (methanol and diethyl ether) and cyclosporine (positive control) decreased the percent viability of KB/C152 and HUT-78/C185 cells (Fig. 2 A-C). The result of LDH release showed that methanol and diethyl ether extracts of *H. caeralea* in HUT cell decreased cell viability more than KB cell line also the values indicate that diethyl ether extracts of *A. sinoxea* in HUT had more cytotoxicity than methanol extract. The LDH release of treated HUT and KB cells with methanol and diethyl ether extracts of *I. mutans* were represented almost similar cytotoxicity. The activity induced by the extracts of *I. mutans is* almost similar to the standard drugs cyclosporine. The data indicated that methanol and diethyl ether extracts of *H. caerulea* and *A. sinoxea* had no cytotoxic activities against KB cells and methanol and diethyl ether extracts of *I. mutans* had the best cytotoxicity activity.

Discussion

Marine invertebrates, sponges in particular, are rich sources of secondary metabolites with antibacterial, antimicrobial, antitumor and cytotoxic properties[20,23-28]. In the present study, we evaluated the cytotoxicity of methanol and diethyl ether extracts of sponges *Haliclona caerulea*, *Axinella sinoxea* and *Ircinia mutans* collected from Larak Island and Kish Island in the Persian Gulf. The extracts of *I. mutans* were strongly active on KB and HUT cells. Diethyl ether extract at 300 µL concentration showed significant cytotoxicity against KB cells. Earlier, extract of *I. mutans* collected from Kish Island in the Persian Gulf has been shown to possess antibacterial and antifungal activities[29,30]. Meesala *et al*.[25] have reported antibacterial compounds, variabilin, iricinialactam A and a new imidazole alkaloid isolated from the Arabian marine sponge *Ircinia fusca*. Hydroethanolic extract of *I. strobilina* has been shown to possess inhibitory activity on cultured cell growth and sea urchin eggs division at 500 µL concentration[31].

The results obtained in the present study indicate that the extracts of *H. caeralea* is cytotoxic and

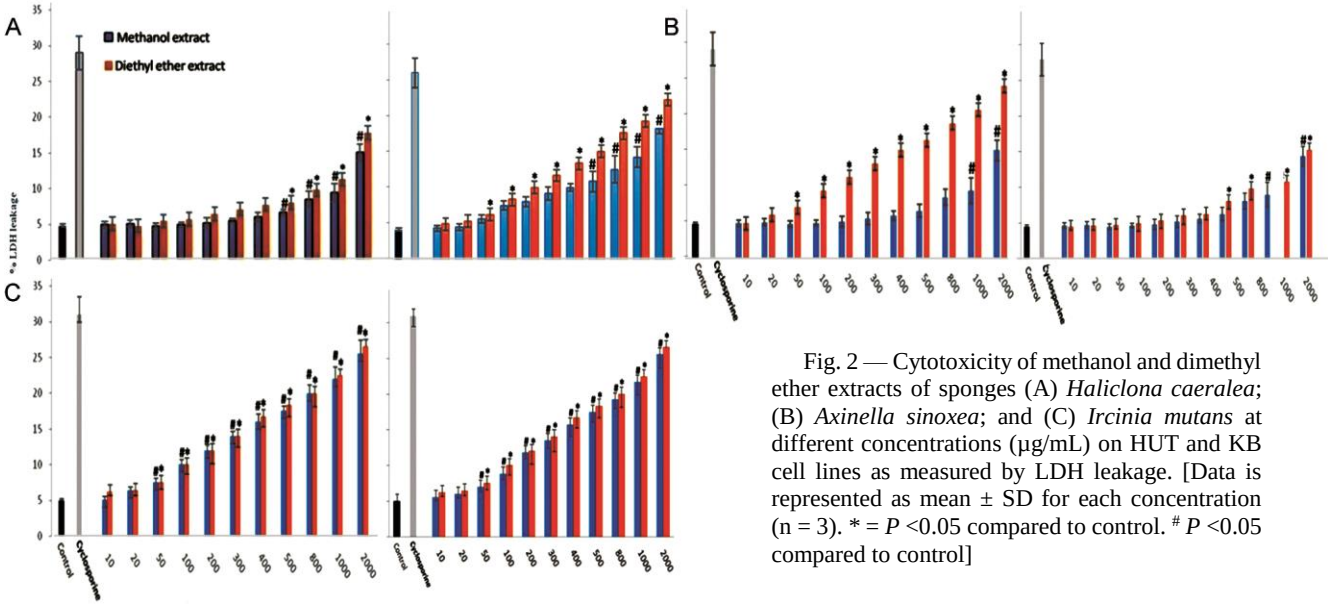

Fig. 2 — Cytotoxicity of methanol and dimethyl ether extracts of sponges (A) *Haliclona caeralea*; (B) *Axinella sinoxea*; and (C) *Ircinia mutans* at different concentrations (µg/mL) on HUT and KB cell lines as measured by LDH leakage. [Data is represented as mean ± SD for each concentration (n = 3). * = $P < 0.05$ compared to control. # $P < 0.05$ compared to control]

inhibited proliferation of a HUT cell line, but didn't exhibit any activity on KB cell line. The type and strength of the extract in terms of concentration may also have an impact on the activity. To show cytotoxic activity, it was necessary to use at least 2000 µL of methanol extract, while for the diethyl ether extract, 556 µL was sufficient. Another study reported ethyl acetate extract from *Haliclona exigua* inhibited MCF7 cell growth, the IC50 of the sponge was approximately 310 µL[32]. The Red Sea sponge *Hyrtios erectus* has been demonstrated to inhibit HUT cell line[18]. Other experiments with the brine shrimp assay for cytotoxicity carried out with dichloromethane extract of the sponge *Haliclona* sp., indicated high bioactivity, with IC50 approximately 1000 µL[29]. Rivera & Uy[33] who studied the extract of *Haliclona* sp. from Qeshm Island in the Persian Gulf have suggested that it can be considered as a source of novel metabolites with antibiotic and antifungal potential.

Our results also indicate that the extracts derived from *A. sinoxea* possessed an infirm activity, among the extracts. While the diethyl ether extract was toxic and inhibited the HUT cell line the KB cell line didn't have any such activity in alignment with our earlier observation with *I. mutans*[34]. Methylene chloride-2-propanol extract of *Axinella* spp. also showed the highest inhibitory activity on PS leukemia and leukemia P388 cells at 2/5 0/21 µL concentration, respectively[35].

Conclusion

In this study *Haliclona caerulea*, *Axinella sinoxea* and *Ircinia mutans* collected from Larak and Kish Island in the Persian Gulf have been shown to possess significant cytotoxic activity. The results indicated that the methanol and diethyl ether extract of *I. mutans*, *H. caeralea* and *A. sinoxea* exhibited cytotoxic activity on cancer cell lines with the least activity on normal cell line so it will be subjected for further investigation for isolation of biological active molecules.

Conflict of Interest

Authors declare that they have no conflict of interests.

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