ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Sponge Mesohyl Induces Anti-Proliferation Activity and Cell Cycle Arrest in Colon Cancer *In-Vitro*.

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ABSTRACT

Colon cancer is the third most dangerous type of cancer that causes death. Anticancer therapy is one of the biggest challenges in medicine. The curative effects of the existing chemotherapeutic drugs are not good enough and they have numerous side effects. Therefore, searching for highly efficient anticancer drugs remains a hot research area. Many species of sponge produce bioactive compounds, which have pharmaceutical value as anticancer drugs. In the present study, comparative study was performed on four species of Red sea marine sponges (Negombata magnifica, Acanthella carteri, Crella spinulata, Hemimycale arabica). The anticancer activity of mesohyl of the four selected species was tested against colon cancer cell line (Caco-2) in vitro. Tumor cell growth was assessed using MTT assay and cell cycle analysis was evaluated using flow cytometry. All the four selected sponge species significantly decreased the proliferation of Caco-2 cells in a dose-dependent manner. Flow cytometry in case of the four treatments indicated that mesohyl caused a cell accumulation in G0/G1 phase, inducing G0/G1 cell cycle arrest, with a corresponding decrease of cells in the S-phase thereby contributing to cell proliferation inhibition.

Keywords: Anticancer, sponge, cytotoxicity, cell cycle, DNA content, mesohyl.

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ISSN: 0975-8585

INTRODUCTION

Colorectal cancer is one of the most common malignant tumors in gastrointestinal track [1]. Surgical resection is the optimal treatment for this kind of cancer, while chemotherapy serves as one of the important adjuvant therapies for its treatment [2]. Although screening modalities for early detection and therapeutic management for human colorectal cancer (CRC) has improved considerably, this disease still remains the second leading cause of cancer-related deaths [3].

Marine sponges are the best source of many bioactive metabolites which can be used as new anticancer drugs. An intriguing group of bioactive peptides are cyclic peptides, which exhibits a wide range of biological activities including cytotoxic activity were found in sponge tissues. Antitumor pharmacological studies were conducted, with marine natural products, in a number of experimental and clinical models [4].

The body of a sponge consists of two cell layers separated by a gelatinous region, mesohyl. Mesohyl is a connective tissue layer between an epithelial-like layer called the pinacoderm and the choanoderm. It is the only layer of the sponge body wall that typically is not bathed with the environmental water. In this sense, mesohyl is the sole internal compartment of the body. As a connective tissue, the mesohyl is composed of a proteinaceous gel-like matrix that contains differentiated and undifferentiated cells [5].

Marine natural products (MNPs) have demonstrated exceptional potency and potential as anticancer therapeutics. The Red Sea sponge Negombata magnifica is a potential source of the cytotoxin, latrunculin B (lat-B). Negombata sponge was shown to produce potent cytotoxic macrolides called latrunculins (e.g. latrunculins A and B) in addition to other cytotoxic compounds. Latrunculins are unique macrolides containing a thiazolidinone moiety. Latrunculin A, latrunculin B, 16-epi-latrunculin B, and latrunculin T were isolated from the Red Sea sponge Negombata magnifica [6]. Acanthella carteri exhibits cytotoxic activity towards NSCLC-N6 human non-small cell lung carcinoma of 9.7 µg/ml [7]. New compounds with anti-tumor activity a class of DNA-intercalating plakinidines were isolated from the marine sponge, Crella spinulata [8]. Recently, Mudit *et al.* [9] characterized guanidine alkaloid compounds derived from Red Sea sponge Hemimycale arabica, that have antitumor, antiviral, antifungal, and anticonvulsant properties.

Deregulated cell cycle is one of the major hallmarks of cancer cells. These cells may lose the ability to regulate the cell cycle and control their rate of proliferation. A rate-limiting step in the cell cycle that is often disturbed in cancer is the progression of cells through the first gap (G1) phase [10].

MATERIALS AND METHODS

Sample collection and preparation

Four marine sponge species, Negombata magnifica (red), Acanthella carteri (orange), Crella spinulata (Pink) and Hemimycale Arabica (Blue) were collected from the Red Sea (Hurghada, Egypt). Sponge specimens were immediately transferred to the laboratory. Healthy fresh sponge specimens were soaked in sterile natural seawater (NSW) supplemented with 25 ppm CuSO4 for 3hr to kill protozoan contaminations and then washed three times with sterile NSW to remove CuSO4. The sponge specimens were soaked in sterile calcium-magnesium free sea water (CMFSW) with antibiotic mixture (streptomycine-penicillin-garamycin) for 1hr. Thereafter, under sterile condition in a laminar flow, the sponge specimen was dissected carefully. The mesohyl was aspirated and collected with a sterile syringe; care should be taken during aspiration to avoid any contamination. Squeezing fresh samples of sponge to get cell suspension may cause bacterial contamination.

Cell line propagation.

Human colon adenocarcinoma cell line (Caco-2), was supplied by Naval American Research Unit–Egypt (NAmRU). Cells were propagated and maintained in RPMI-1640 medium with L-glutamine (Sigma) and supplemented with 10% fetal calf serum (Sigma) for growth and 2% for maintenance medium, and 1% antibiotic mixture (20 units /ml of penicillin G sodium and 20 mg /ml streptomycin sulfate, Gibco).

Treatment of colon adenocarcinoma cell line.

Caco-2 cells at approximately 80 % confluence (i.e., logarithmically growing cells) were selected for trypsinization. The cell suspension in RPMI-1640 culture medium was prepared and then cells were seeded and incubated at 37°C and 5 % CO_2 overnight. Cells were treated with different concentrations of mesohyl of the four sponge species for 48 hr.

Cytotoxicity (MTT assay).

Cytotoxicity against Caco-2 cells was assessed by MTT assay. The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay is widely used as screening method to measure cell viability (cell growth) and proliferation. Briefly, Caco-2 cells at approximately 80% confluence (i.e. logarithmically growing cells) were selected for trypsinization. Cells were seeded in 96-well microplates (3 X 10^3 cells / well) in 100 µl RPMI-1640 culture medium and incubated at 37 °C and 5 % CO_2 overnight. Caco-2 cells were treated by diluted mesohyl with sterile RPMI-1640 media at a concentration of 1, 5, 10 and 50 µl /ml media, and re-incubated at 37°C and 5 % CO_2**.** After 48 h of incubation, cells were washed with sterile phosphate buffer solution (PBS) and 100 µl of the tetrazolium dye (0.5 mg/ml) solution was added to each well. Cells were incubated for an additional 18 h until the purple formazan crystals appeared. The medium was discarded then 100 µl of DMSO was added to dissolve the crystals. The optical density (OD) of solubilized formazan was measured at 570 nm (reference filter 690 nm) using an automatic microplate reader. Results are expressed as mean values in triplicate ± SD of percentage of control. The experiments were done independently three times [11].

Cell cycle analysis

Determination of the cell cycle distribution was performed using flow cytometric DNA content analysis. Briefly, an aliquot of 10^6 cells was fixed in 70 % ethanol and refrigerated. Fixed samples were prepared for analysis by centrifuging the cells to a pellet (1000 X g for 10 min), decanting the ethanol and re-suspending the cells in 1 mL of a DNA staining solution containing 50 µg/ mL propidium iodide (Sigma, St. Louis, MO) and 100 units/mL RNase (Sigma) in PBS containing calcium and magnesium (Invitrogen). Cells remained in the staining solution overnight at 48C. DNA content analysis was performed on a FACS Calibur (Becton-Dickenson, Franklin Lakes, NJ) flow cytometer using 488 nm excitation and fluorescence collection with then propidium iodide filter set. DNA content histograms containing >10^4 cells were collected and had coefficients of variation on the G1-phase peak of <5%. These histograms were analyzed for cell cycle distribution with the MacCycle program (Phoenix Flow Systems, San Diego, CA) using the debris and aggregate elimination options [12].

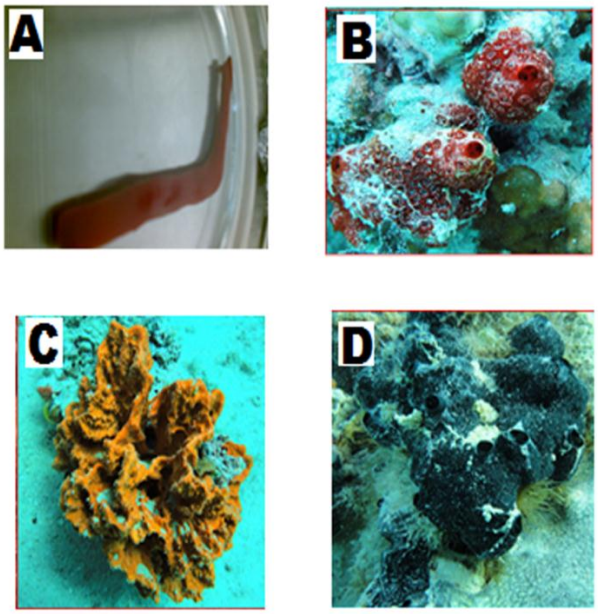
RESULTS AND DISCUSSION

The aim of this work is to estimate the effects of the mesohyl of Red Sea marine sponge Negombata magnifica, Acanthella carteri, Crella spinulata and Hemimycale arabica (Figure 1) on the growth and viability of Caco-2 cells. We examined cell growth and viability using cell growth curve and MTT assay and the cell cycle analysis was performed by flow cytometry (DNA content).

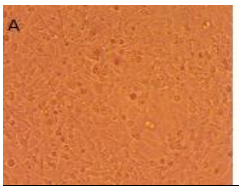
The mesohyl of Hemimycale arabica sponge exhibited a very potent cytotoxicity against Caco-2 cells with IC50 value of 1µl/ ml (Figure 2). The number of cells at G0/G1 phase clearly increased and the population of cells in G2/M phase also increased. In addition, a marked S-phase arrest was observed (Figure 3). In case of the mesohyl of Negombata magnifica sponge, IC50 was 5 µl/ ml (Figure 2). The cell percentage at G0/G1 phase increased and was found to be 61.7% and 10.4% at G2/M phase. S-phase cell percentage decreased to 22.7 % (Figure 3). Crella spinulata exhibited cytotoxicity with IC50 = 8 µl/ ml (Figure 2) and also cells were arrested at G0/G1 phase by a percentage equal to 73 %, while cell percentage at G2/M phase was 10 % and cells at S-phase decreased to 16.6 % (Figure 3). Hemimycale arabica mesohyl exerts cytotoxicity with IC50 = 50 µl/ml (Figure 2) and cells were arrested in both G0/G1 and G2/M phases and the percentage of cells in S-phase decreased to 33.3 % only compared to control (Figure 3).

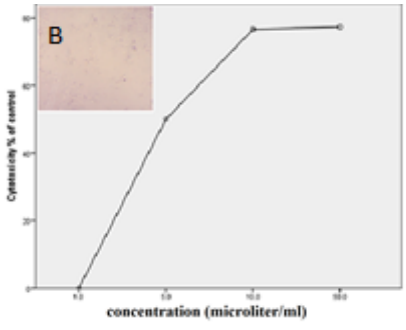
In conclusion, the mesohyl of sponge, in general, is a potent anticancer agent to Caco-2 cells through inhibition of proliferation (cytotoxic effect) and the induction of G0/G1 cell cycle arrest. Furthermore, the anticancer activity is exerted through the decreased cell percentage in S-phase which is a main way for cell growth inhibition.

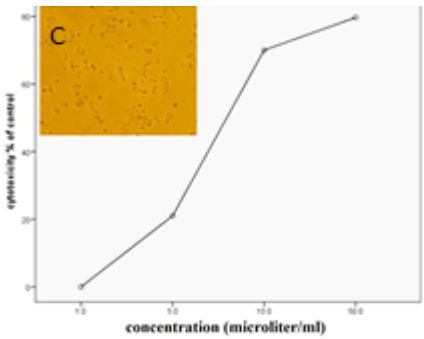
ISSN: 0975-8585



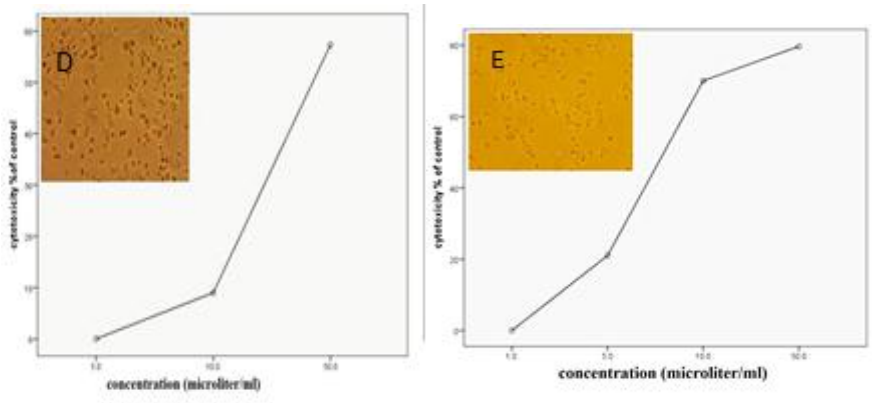
Morphology of selected species of Red Sea Marine sponges. A: Negombata magnifica (red), B: Acanthella carteri (orange), C: Crella spinulata (Pink) and D: Hemimycale Arabica (Blue).



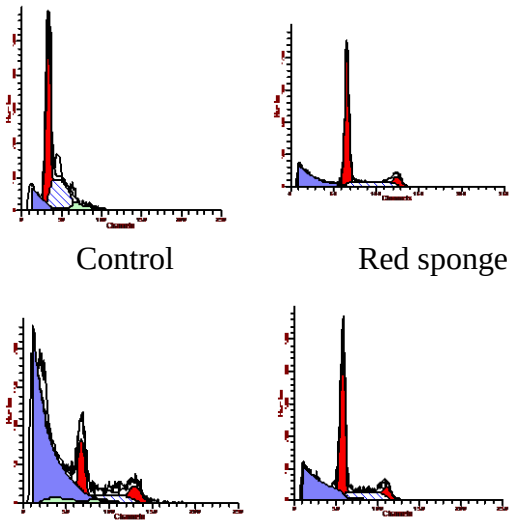


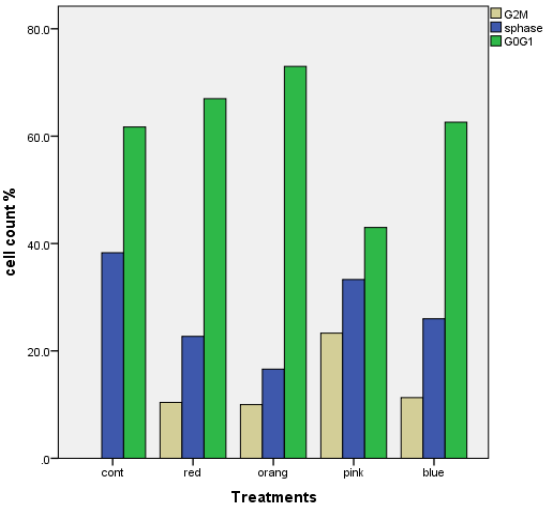


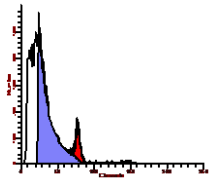
ISSN: 0975-8585



Growth inhibition and viability reduction of Caco-2 cells (A: Control Caco2 cells) by mesohyl of B: Negombata magnifica, C: Acanthella carteri, D: Crella spinulata and E: Hemimycale Arabica sponges.







Analysis of cell cycle distribution of Caco-2 cells treated with mesohyl of Negombata magnifica (red), Acanthella carteri (orange), Crella spinulata (pink) and Hemimycale Arabica (blue) sponges.

ISSN: 0975-8585

STATISTICAL ANALYSIS

Statistical evaluation of the results was done using SPSS version 11. Data were expressed as mean ± SD of percentage of control. One-way analysis of variance (ANOVA) followed by LSD-test was used to assess significant differences which were considered significant at $P<0.001$.

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