

Cytotoxic Effect of Crude Extract of Sea Pen *Virgularia gustaviana* on HeLa and MDA-MB-231 Cancer Cell Lines

Sharareh Sharifi, Pargol Ghavam Mostafavi, Ali Mashinchian Moradi, Mohammad Hadi Givianrad, Hassan Niknejad

Abstract—Marine organisms such as soft coral, sponge, ascidians, and tunicate containing rich source of natural compound have been studied in last decades because of their special chemical compounds with anticancer properties. The aim of this study was to investigate anti-cancer property of ethyl acetate extracted from marine sea pen *Virgularia gustaviana* found from Persian Gulf coastal (Bandar Abbas). The extraction processes were carried out with ethyl acetate for five days. Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were used for qualitative identification of crude extract. The viability of HeLa and MDA-Mb-231 cancer cells was investigated using MTT assay at the concentration of 25, 50, and a 100 µl/ml of ethyl acetate is extracted. The crude extract of *Virgularia gustaviana* demonstrated ten fractions with different Retention factor (Rf) by TLC and Retention time (Rt) evaluated by HPLC. The crude extract dose-dependently decreased cancer cell viability compared to control group. According to the results, the ethyl acetate extracted from *Virgularia gustaviana* inhibits the growth of cancer cells, an effect which needs to be further investigated in the future studies.

Keywords—*Virgularia gustaviana*, Cembrane Diterpene, anti-cancer, HeLa cancer Cell, MDA-Md-231 Cancer cell

I. INTRODUCTION

CANCER is one of the major causes of death in the world despite the increasing advances in prevention and treatment [1]. Since the current treatments for cancer like chemotherapy and radio-therapy are not always effective, and there is a possibility of recurrence due to side effects of current anti-cancer agent, the scientists have focused on natural products as anti-cancer drugs, and among the natural compounds which are extracted from marine organisms [2]. Use of medicinal marine resources in recent years has attracted the attention of many scientists [3]. Ability of these compounds to kill cancer cells and their mechanisms of action have been reported in successive articles [4]. Marine environment has a wide range of condition such as the Arctic

cold water to warm water, high pressure, lack of light and high salinity. Hence, the above mentioned conditions led to the creation of marine compound structures and metabolic pathways with high biotechnological potential [5]. Marine invertebrates have an ability to produce toxic compounds to defend themselves from other marine organisms. These animals released chemical compound into the water. The biodiversity of the marine invertebrate with different chemical products is most likely suitable for research use as clinical target [6]. Marine soft coral is the most general source of cembrane diterpene. Many cembrane-type compounds have been shown to exhibit cytotoxicity against a variety of tumor cells [7].

In this study, we are focused on marine sea pen *Virgularia gustaviana*. Sea pens are the feather-like colony of polyps, this animal's body contains two main parts: the primary part is anchored to soft bed. Rachis is the other part which contains polyps. The aim of this study is the assessment of cell viability of crude extract of sea pen *Virgularia gustaviana* on cervical and breast cancer cells.

II. MATERIALS AND METHODS

A. General Experiments

TLC was carried out on percolated Kieselgel 60 F254 (0.25 mm, Merck), and spots were visualized on UV light. HPTLC was carried out on plate (Silica gel 60 F 254 glass 20*10 cm, Merck, Germany). HPLC was performed using a system comprised of a Cécile pump, a Cécile photodiode array detector, and a Rheodyne injection port. A normal phase column (Hibar 250 × 10 mm, Merck, silica gel 60, 5 µm) was used for HPLC.

B. Animals

Marine sea pen *Virgularia gustaviana* was collected with patrolling the intertidal zone of the estuary Seura in Bandar Abbas. It was frozen immediately after the collection and was moved to the Laboratory of Tissue Engineering Research Center for Nanotechnology of Beheshti University of Medical Sciences, and in -20 °C until the trial was held.

C. Extraction

In order to obtain organic extract, the frozen sea pen was chopped into small pieces (1 kg, wet weight), and all samples were freeze dried and extracted with Ethyl Acetate at room temperature (2L×3). The combined ethyl acetate extracts were filtered by Whatman filter paper (number 1), and the filtered

Sharareh Sharifi is with the Department of Marine Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran (phone: 0098-9128196974, e-mail: sharareh_sharifi63@yahoo.com).

Pargol Ghavam Mostafavi is with the Department of Marine Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.

Ali Mashinchian Moradi and Mohammad Hadi Givianrad are with the Department of Marine Chemistry, Science and Research Branch, Islamic Azad University, Tehran, Iran (e-mail: author@lamar.colostate.edu).

Hassan Niknejad is with the Department of Tissue Engineering and Regenerative Medicine, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical sciences, Teh, Iran (corresponding author, e-mail: h.niknejad@yahoo.com).

solution was extracted using distilled water. Finally, the solvent was removed by evaporation using rotary evaporator to afford 80 g dark orange gummy residue [8].

D. HPLC

Ethyl acetate crude extracts of *Virgularia gustaviana* were studied qualitatively by HPLC. Elution conditions followed by Acetonitrile-Deionized water (pH 3.5 adjusted with phosphoric acid) with 60:40 v/v ratio as mobile phase, and flow rate of 1.5 ml min⁻¹. The highest absorbance was in 220 nm from 70:30 [9].

E. Cell Culture

HeLa and MDA-MB-231 cell line are used to evaluate the cytotoxic effect of the tested extracts. Cells were routinely cultured in RPMI 1640 (Chemi-Con International, Temecula, CA, USA). Media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin [10]. Cells were maintained at 37 °C in humidified air containing 5% CO₂. For sub-culturing, monolayer cells were harvested after trypsin/EDTA treatment at 37 °C. Cells were used when confluence had reached 75%. Test samples were dissolved in DMSO, and then diluted 1000 times for the assay. All experiments were repeated three times, unless mentioned [11].

F. MTT Assay

The cytotoxicity of the ethyl acetate extract of *Virgularia gustaviana* on HeLa and MDA-MB-231 human cancer cells was investigated by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [12]. The HeLa and MDA-MB-231 at density of 5×10^4 per well were seeded in 24-well plate and incubated in incubator with 5% CO₂ at 37 °C, for one day. Then, cells were treated with Ethyl acetate extract with various concentrations 100, 50, 25 µl and incubated at 37 °C for 24h. The cultures without treatment were used as the control group. After 24h, the vitality of the cancer cells, which had been cultured with the Fractions, was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. MTT solution (5 mg of MTT/mL of distilled water) was filter sterilized [10]. Solution was added to growing culture of cancer cells (40 mL in each well) and incubated for 4h at 37 °C. The MTT Formosan crystals were then dissolved with 900 µL DMSO (Sigma-Aldrich) at the ambient temperature. The optical density (OD) was measured at 570 nm with a spectrophotometer (CE7500; Cecil, Cambridge, UK). The blank well containing only medium material was used for zero adjustment [13]. The viable rate was calculated by:
$$\text{Viable rate} = \frac{\text{OD (treated)}}{\text{OD (control)}}$$

G. Statistical Analysis

The data were expressed as mean±standard deviation. The difference between data was assessed using one-way analysis of variance with Tukey post-test. P values ≤0.05 were considered statistically significant

III. RESULT

Sea pens *Virgularia gustaviana* were extracted by ethyl

acetate, and the dark orange extract was yield. For qualitative assessment of extract TLC and HPLC method was used. The mobile phase was used for TLC, Hexane- ethyl acetate at a ratio of 100: 1,100:3. The resulting band on TLC paper is visible under UV lamp where Rf was calculated for each band. According to the results of TLC, 18 compounds were found and they were combined with different Rf. Ethyl acetate extract of mentioned sea pen was characterized using HPLC. The Rt for compound of crude extract is shown in Fig. 2.

The cytotoxic effects of ethyl acetate extract of *Virgularia gustaviana* were evaluated on HeLa and MDA-MB-231 cancer cell lines using MTT assay. The prepared ethyl acetate extract was applied in cell culture media in the amounts of 25, 50, and 100 µl. The viability of both cancer cells was diminished dose-dependently, which is shown on Fig. 2 and Table I.

TABLE I
PERCENTAGE OF HELa AND MDA-MB-231 CELLS ADHESION BY DIFFERENT CONCENTRATIONS OF THE ETHYL ACETATE EXTRACT OF GENUS *Virgularia GUSTAVIANA*

Doses	VIABILITY (%) mean±SEM	
	HeLa	MDA-Mb-231
100	9.00±1.00	18.00±0.57
50	36.00±1.00	49.00±0.57
25	73.00±2.08	82.67±1.45

IV. DISSECTION

The main objective of this study was characterization result of HPLC and TLC analyzed and assessment of capability of organic ethyl acetate extract of *Virgularia gustaviana* collected from Bandar Abbas costal to inhibit growth of cancer cell. Characterization of ethyl acetate extract by HPLC showed that 13 main compounds were eluted by acetonitrile: deionized water as a mobile phase.

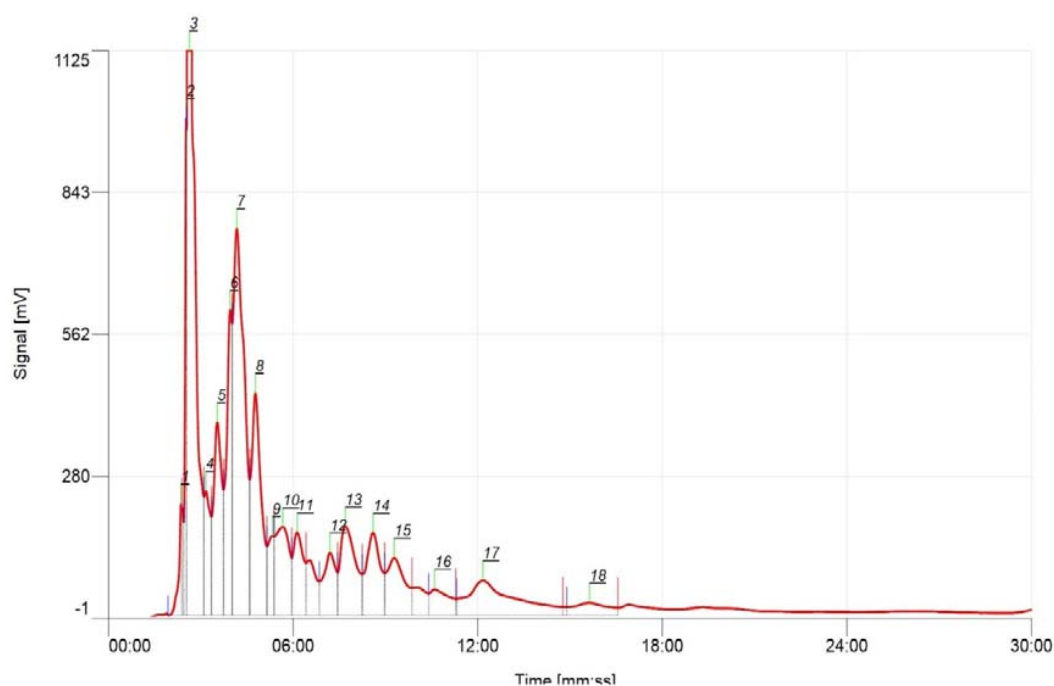
Marine environment is important area of natural compound with potential effect as therapeutic agents [14]. Many cytotoxic compounds were extracted from marine invertebrate such as soft corals, sponge, bryozoan, and etc. [15]. In the past decade, scientists have been focused on anti-cancer properties of marine invertebrate bioactive compounds, for instance pachymatmin from *Pachymatisma johnstonii* genus of marine sponge [16], bryostatins from *Bugula neritina* the marine bryozoan [17], didemnin B from *Trididemnum solidum* a marine tunicate [18]. Marine soft coral which contain coelenterata, octocorallia, alcyonaceae families have been approved as a potential foundation of steroids and diterpenoids. The prostanoids from *C. viridis* have been proposed as anti-tumor agent in some types of human tumors [19]. Australins A-D from ethanoic extract of *C. australis* showed high cytotoxic effect on MCS-7, MDA-MB-231 and HepaG2 cancer cell lines [8]. The ethyl acetate extract of *P. viridis* showed cytotoxic effects towards MCF 7 than for HCF 116 [20]. Treatment of extracts of the genus *Simularia* on SCC25 and HaCaT cells indicated cytotoxic effects [21]. Methanolic extract of the soft coral *Sarcophyton pauciplicatum* showed cytotoxic activity against a panel of eight human cancer cell lines [22]. In last study identification

of fatty acid extracted of sea pen *Virgularia gustaviana* and their anti-bacterial and anti-fungal activity were measured with MIC and MBC methods and the results shown that arachidonic acids of chloroform extract has best activity against *Staphylococcus aureus* on 125 µg/ml dose in MIC method [23]. Also, anti-inflammatory effect of chloroform and hexane extracts of mentioned genus showed strong activity in mice ears even at low doses, which is probably due to 54% arachidonic acid [24]. In this study, the ethyl acetate extract of

marine sea pen *Virgularia gustaviana* presented high cytotoxic effects on HeLa and MDA-MB-231 cancer cell lines. From these hopeful results, ethyl acetates extracted from sea pen show strong cytotoxic effects on human cervical cancer cell line HeLa and Breast cancer cell line MDA-Mb-231. In that purification and identification of any of 18 compounds, assessment of cytotoxic effect of each compound needs more studies.

Run Time [mm:ss]	30:00.0	Sample Rate [points]	12.500	Readings	22500
Sample Type	Unknown	Detector Unit	V (Volts)	Detector Range	1.000
Detector Offset	0.000	Sample Name	cembranoid001	Method Name	Methodcembranoid
Method Location	\cembranoid\	Conc. Factor	1.000	ISTD Conc.	1.000

<Acquired via Channel 1>



No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mVs]	Height [mV]	Quantity
001	***	02:21.2	01:55.9	02:22.9	1430.2	220.1	N/A
002	***	02:31.4	02:26.4	02:32.0	3189.0	984.9	N/A
003	***	02:37.5	02:32.0	03:05.8	23834.0	1125.0	N/A
004	***	03:09.7	03:05.8	03:20.5	3357.9	251.3	N/A
005	***	03:31.8	03:20.5	03:44.0	7123.4	387.0	N/A
006	***	03:57.0	03:44.0	04:01.0	7462.0	610.4	N/A
007	***	04:10.1	04:01.0	04:35.2	19411.0	771.8	N/A
008	***	04:46.3	04:35.2	05:08.8	9914.9	445.4	N/A
009	***	05:19.6	05:08.8	05:22.7	2150.2	161.2	N/A
010	***	05:39.5	05:22.7	05:57.2	5603.1	179.2	N/A
011	***	06:07.8	05:57.2	06:25.1	3912.9	168.7	N/A
012	***	07:11.8	06:51.0	07:26.7	3585.0	128.6	N/A
013	***	07:41.5	07:26.7	08:14.8	6387.4	180.3	N/A
014	***	08:36.0	08:14.8	08:58.5	5374.8	168.0	N/A
015	***	09:16.9	08:58.5	09:51.8	4797.1	118.0	N/A
016	***	10:35.6	10:24.3	11:17.2	2492.3	56.0	N/A
017	***	12:10.1	11:19.0	14:46.1	8435.9	73.3	N/A
018	***	15:38.0	14:53.6	16:33.4	2398.2	29.2	N/A

Fig. 1 HPLC chromatograms of 20 µl injection of Ethyl acetate extract of *Virgularia gustaviana*

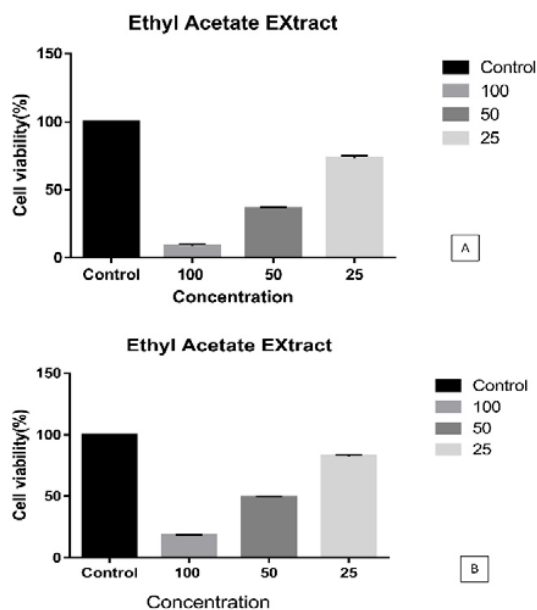


Fig. 2 Viability of HeLa cancer(A) and MDA-Mb-231(B) cells evaluated by MTT assay after 24 h incubation with ethyl acetate extract

V.CONCLUSION

In conclusion, characterization and biological experiments presented that there are 13 different compounds with different Rt on Ethyl acetate extracted of sea pen *Virgularia gustaviana* which showed high cytotoxic effect on HeLa and MDA_Mb-231 cancer cell lines.

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REFERENCES

- [1] L. C. Hu, J. H. Su, M. Y. Chiang, M. C. Lu, T. L. Hwang, Y. H. Chen, W. P. Hu, N. C. Lin, W. H. Wang, L. S. Fang, Y. H. Kuo, P. J. Sung, Flexibilins A-C, new cembrane-type diterpenoids from the Formosan soft coral, *Sinularia flexibilis*, *Marine drugs*, 11 (2013) 1999-2012.
- [2] J. Rocha, L. Peixe, N. C. Gomes, R. Calado, Cnidarians as a source of new marine bioactive compounds--an overview of the last decade and future steps for bioprospecting, *Marine drugs*, 9 (2011) 1860-1886.
- [3] R. Montaser, H. Luesch, Marine natural products: a new wave of drugs? *Future medicinal chemistry*, 3 (2011) 1475-1489.
- [4] J. W. Blunt, B. R. Copp, R. A. Keyzers, M. H. G. Munro, M. R. Prinsep, Marine natural products, *Natural Product Reports*, 33 (2016) 382-431.
- [5] T. E. Adrian, Novel marine-derived anti-cancer agents, *Current pharmaceutical design*, 13 (2007) 3417-3426.
- [6] M. Bayet-Robert, S. Lim, C. Barthomeuf, D. Morvan, Biochemical disorders induced by cytotoxic marine natural products in breast cancer cells as revealed by proton NMR spectroscopy-based metabolomics, *Biochemical pharmacology*, 80 (2010) 1170-1179.
- [7] S. S. Al-Lihaibi, W. M. Alarif, A. Abdel-Lateff, S.-E.N. Ayyad, A. B. Abdel-Naim, F. F. El-Senduny, F. A. Badria, Three new cembranoid-type diterpenes from Red Sea soft coral *Sarcophyton glaucum*: Isolation and antiproliferative activity against HepG2 cells, *European journal of medicinal chemistry*, 81 (2014) 314-322.
- [8] R. Kazlauskas, P. Murphy, R. Wells, P. Schonholzer, J. Coll, Cembranoid constituents from an Australian collection of the soft coral *Sinularia flexibilis*, *Australian Journal of Chemistry*, 31 (1978) 1817-1824.
- [9] S. Bilasy Sel, S. I. Khalifa, S. M. Saleh, S. H. Abou El-Ela, HPLC method for the quantitative determination of sarcophine, a source of cembranoids with cancer chemopreventive activity, *Journal of pharmaceutical and biomedical analysis*, 46 (2008) 784-787.
- [10] H. Niknejad, G. Yazdanpanah, Anticancer effects of human amniotic membrane and its epithelial cells, *Medical hypotheses*, 82 (2014) 488-489.
- [11] J. M. Arif, S. S. Sawant, K. A. El Sayed, M. Kunhi, M. P. Subramanian, Y. M. Siddiqui, D. T. Youssef, K. Al-Hussain, M. N. Al-Ahdal, F. Al-Khodairy, Antiproliferative potential of sarcophine and its semisynthetic sulfur-containing derivatives against human mammary carcinoma cell lines, *Journal of Natural Medicines*, 61 (2007) 154-158.
- [12] H. Niknejad, M. Khayat-Khoei, H. Peirovi, H. Abolghasemi, Human amniotic epithelial cells induce apoptosis of cancer cells: a new anti-tumor therapeutic strategy, *Cytotherapy*, 16 (2014) 33-40.
- [13] H. Niknejad, G. Yazdanpanah, M. Mirasoumi, H. Abolghasemi, H. Peirovi, A. Ahmadiani, Inhibition of HSP90 could be possible mechanism for anti-cancer property of amniotic membrane, *Medical hypotheses*, 81 (2013) 862-865.
- [14] F. Reyes, A. Arda, R. Martin, R. Fernandez, A. Rueda, D. Montalvo, C. Gomez, C. Jimenez, J. Rodriguez, J.M. Sanchez-Puelles, New cytotoxic cembranes from the sea pen *Gyrophyllum sibogae*, *Journal of natural products*, 67 (2004) 1190-1192.
- [15] I. Bhatnagar, S.K. Kim, Marine antitumor drugs: status, shortfalls and strategies, *Marine drugs*, 8 (2010) 2702-2720.
- [16] M. Zidane, P. Pondaven, C. Roussakis, M. T. More, Effects in vitro of pachymatissmin, a glycoprotein from the marine sponge *Pachymatisma johnstonii*, on a non-small-cell bronchopulmonary carcinoma line (NSCLC-N6), *Anticancer Res*, 16 (1996) 2805-2812.
- [17] R. E. Curiel, C. S. Garcia, L. Farooq, M. F. Aguero, I. Espinoza-Delgado, Bryostatin-1 and IL-2 synergize to induce IFN-gamma expression in human peripheral blood T cells: implications for cancer immunotherapy, *Journal of immunology*, 167 (2001) 4828-4837.
- [18] S. Ankisetty, S. I. Khan, B. Avula, D. Gochfeld, I. A. Khan, M. Slattery, Chlorinated didemns from the tunicate *Trididemnum solidum*, *Marine drugs*, 11 (2013) 4478-4486.
- [19] C.-H. Liang, G.-H. Wang, C.-C. Liaw, M.-F. Lee, S.-H. Wang, D.-L. Cheng, T.-H. Chou, Extracts from *Cladiella australis*, *Clavularia viridis* and *Klyxum simplex* (soft corals) are capable of inhibiting the growth of human oral squamous cell carcinoma cells, *Marine drugs*, 6 (2008) 595-606.
- [20] K. Sreejamele, C. Radhakrishnan, Antioxidant and cytotoxic activities of ethyl acetate extract of the Indian green mussel *Perna viridis*, *Asian Journal of Pharmaceutical and Clinical Research*, 6 (2013) 197-201.
- [21] G. H. Wang, T. H. Chou, R. J. Lin, J. H. Sheu, S. H. Wang, C. H. Liang, Cytotoxic Effect of the Genus *Sinularia* Extracts on Human SCC25 and HaCaT Cells, *Journal of toxicology*, 2009 (2009) 634868.
- [22] N. T. Ngoc, N. P. Thao, T. T. Huong, D. C. Thung, N. V. Thanh, N. H. Nam, N. X. Cuong, Y. H. Kim, P. V. Kiem, C. V. Minh, Polyhydroxylated sterols from the soft coral *sarcophyton pauciplicatum*, *Vietnam Journal of Chemistry*, 53 (2015) 18-22.
- [23] S. Sharifi, Fatty Acid Extracts of Sea Pen (*Virgularia gustaviana*) and Their Potential Applications as Antibacterial, Antifungal, and Anti-Inflammatory Agents, *Biotechnology and Bioengineering*, 212 (2016) 10005579.
- [24] S. Sharifi, S. Safaeian, Anti-Inflammatory Effect Of Lipid Extract Of Sea Pen (*Virgularia Gustaviana*) In Mice, *Asian Journal of Pharmaceutical and Clinical Research*, 8 (2015).