

Effect of Ethyl Acetate Extract of Sea Pen *Virgularia Gustaviana* on Viability of Cancer Cells

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ABSTRACT

BACKGROUND AND OBJECTIVE: Due to high cancer incidence rate and become resistant to chemical drugs and their side effects make it necessary to research on new natural compounds. Sea pen with special chemical compounds with anti-cancer effects have been considered in recent years. In this study, extraction of chemical compounds from marine sea pen *Virgularia gustaviana* and their effect on cancer cells were investigated.

METHODS: In this study the ethyl acetate extract of *Virgularia gustaviana* was separated by silica gel column chromatography. The column was washed with N-hexane 100% and N-hexane-ethyl acetate solvent at ratio of 9:1 to 1:9. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) was used for qualitative identification of seven fractions. Viability of HeLa cancer cells was investigated using MTT assay at the concentration of 25, 50 and a 100 µl/ml compounds.

FINDINGS: MTT assay showed that G fraction, dose-dependently decreased cell viability of cells and the most effective concentration was 100 µl (with viability 6.33±2.02% of cancer cells) which was significantly less than control group (p<0.05). Retention time of G fraction in HPLC graph was similar to Cembrane Diterpene isolated from Sarcophyton.

CONCLUSION: The results of the study showed that compounds extracted from *Virgularia gustaviana* inhibit the growth of cancer cells and further research will be required to examine the mechanism of effect.

KEY WORDS: *Hela cancer cells, Virgularia gustaviana, Sea pen, Ethyl acetate, Anti-cancer, Cembrane Diterpene.*

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Introduction

Cancer is a disease caused by increasing activity of Proto-oncogenes or inhibition of genes involved in apoptosis resulting in unchecked proliferation and growth of cells (1). Cancer cells make tumors that in some cases they have the ability to invade and metastasize to other parts of the body. Most types of tumors with surgery, chemotherapy or radiation therapy can be cured. But most metastatic cancers such as breast and bladder cancer cannot be cured with chemotherapy and other methods (2,3).

The biggest obstacle in the way of treating this type of cancer is that they are inherently resistant to chemotherapy or become resistant to the drug during treatment. That's why a lot of efforts to identify new compounds, natural or synthetic anti-cancer effects are applied (4, 5). Of all living forms, marine animals has the largest number of new molecules are discovered in the last 20 years and many of them are biologically effective against human pathogens and of all marine species, benthic marine invertebrate has the highest biological activity against cancer. Therefore, understanding the chemical potentials and discover new natural compounds that have not the side effects of common drugs used in chemotherapy and have significant cytotoxic effects is essential, and the results of such studies can promote the production of new medicines for treatment-resistant cancer (6). In recent years, a large number of discovered marine compounds used by researchers. Many of these compounds are used as anti-cancer drugs on the market today such as cytarabine, which first obtained from *Cryptotethya crypta* sponge species, and approved as an anticancer drug (7), or some other such Ecteinascidin that is a combination extracted from *Halichondria okadai* species Tunicate and commercially is available (8). Life on Earth began in the seas millions of years ago and biological evolution, equipped marine organisms with unique mechanisms to survive in changing conditions such as salinity, temperature, pressure and depth. Overall, only 2 branches of the 28 animal branches do not live at sea and marine species have accounted for almost half of Biodiversity. So marine organisms are a great resource for discovering new drugs that is known as Bioprospecting in medical terms (9).

Marine organisms has unique metabolites and exhibit certain physiological capabilities in marine environments compared with soil organisms have higher potential to produce their bioactive substances

(10). Soft corals (Cnidaria, Anthozoa, Octocoralina) are known as an important source of chemical compounds with higher biological potential that terpenoids have accounted for most of the compounds (11). The most important combination of terpenoids which have been extracted from this group are diterpenes, which will include a huge variety. In terms of ecological, biological compounds that are known in the form of secondary metabolites, play an important role in marine environments (12).

Virgularia gustaviana compounds have anti-cancer properties (13). Lipid compounds derived from this species have antibacterial and anti-inflammatory effects (14) these creatures have 61% terpene. It is debatable whether the diterpenes have new biological activity (15,16). Sea pens are a unique group which are found between the tidal area to depths of 6100 m. This group live in isolated colonies clinging to the bed. Usually Sea pens morphologically have not structures for their defense, thus, derivatives or combinations of these creatures act as a defense mechanism against predators (17, 18).

Researchers have identified and extracted compounds from diterpene from sea pens, that these compounds have cytotoxic effects (11). In the study reported by researchers, they studied the effects of extracts on various cancer cells such as breast cancer, prostate cancer, leukemia, colon cancer, and ..., and consequently, the compounds established cytotoxic effects on cancer cells (19). The aim of this study was to derive new natural compounds found in *Virgularia gustaviana* species and the effect of these compounds on the survival of (Viability) HeLa cancer cells with a view to achieve a new drug application in cancer.

Methods

Sampling: In this study, Sea pens were collected by patrolling the beach in tidal area in khor sora beach and were placed frozen in sub-zero temperatures container with ice and moved to the Research Center of nanotechnology and tissue engineering Shahid Beheshti University of Medical Sciences and were kept at a temperature of -20 C since the experiment began.

Extraction and separation: Samples with wet weighing approximately 500 grams were crushed and were dehydrated by freeze-drying. Dry weight of samples were about 40 grams that was placed on a shaker for extraction with ethyl acetate for 48 hours at

25°C the process was repeated 3 times (20). Extract was filtered by Whatman filter paper score 1 and the solution under the filter was poured in decanter and was extracted with water several times. The organic phase was dehydrated by sodium sulfate. And as much as possible was concentrated using a rotary Evaporator under vacuum at a temperature of 37°C. (21,22). Various compounds present in the extract of the first steps were identified by thin layer chromatography (TLC). Thin Layer Chromatography is a kind of adsorption chromatography which in this method thin plates are used and position of separated components is determined on the screen. The particle on top layer should have a high density and be matched and small. Stationary phase was made of silica (23).

Spots obtained using the various values of the delay parameters (Retention factor) for different combinations were used for the isolation of compounds. In this study, 60 F254 silica gel plates with the mobile phase of n-hexane and ethyl acetate at a ratio of 100: 1 were used. Chromatography column and powdered silica gel of solvent n-hexane, ethyl acetate and n-hexane 100% ratio of 9: 1 to 1: 9 were used, to isolate the compounds from the desired extracts (24).

Seven fractions which were separated by column chromatography was named with the letters A to G, and was examined by thin layer chromatography. With the results of thin layer chromatography for each fraction and calculating Rf values for all compounds, finally 17 compounds were identified. For further study, liquid chromatography (HPLC) was used. The used mobile phase, included water and acetonitrile at a ratio of 70:30 which was used to set the mobile phase PH to 5/3 of phosphoric acid. 0.1 mg of fraction were dissolved in 100 ml of ethyl acetate and then were examined and qualitative identified by injection 20 microliter of fractions to (Cecil CE4200) HPLC with column C18 (Hichrom) and UV detector set at 220 nm and by passing the mobile phase at 5.1 ml per minute in C 25 (25).

Evaluation of cytotoxicity using MTT: MTT is yellow tetrazolium salt soluble in water that is revived and becomes insoluble color combinations Formazan by mitochondrial succinate dehydrogenase in living and active cells that this color dissolved with organic solvents and color intensity at 570 nm is proportional to the cell viability (26). To investigate the anticancer effects of ethyl acetate extract of sea pen *Virgularia gustaviana*, HeLa line cancer was used. Cancer cells

were incubated for 24 hours in the plate 24 in the medium RPMI (chemicon) containing 10% FBS and 1% penicillin-streptomycin at 37°C under 95% air and 5% carbon dioxide and extracts relevant with doses of 25 ml and 50 ml and 100 ml in RPMI and DMSO at a rate of 0.3 percent has been solved and 10% FBS and 1% penicillin-streptomycin were also added to them after removing supernatants of the cancer cells, medium containing the extracts were added to cancer cells (27,28). The experiment was repeated 3 times for each concentration and medium with 0.3% DMSO was used as a control group.

All plates were incubated for 24 hours at 37°C under 95% air and 5% carbon dioxide (29). After 24 hours, the survival rate of cancer cells was determined by MTT assay (30). MTT solution was filtered and sterilized to 5 mg per ml of distilled water, and of this amount 40 ml was added to each well. The mixture was incubated for 4 hours at C 37. Then formazan crystals were dissolved by adding 900 micro liters per well DMSO (Sigma Aldrich). Most formazan absorption is at a wavelength of 570 nm. Using a spectrophotometer (CE7500, Cecil, Uk) obtained absorption and thus the survival rate of cancer cells (26).

Statistical analysis: Results were analyzed using ANOVA (One-way ANOVA) and Tukey's post-test. And $p < 0.05$ was considered significant. Graph pad prism editing 5.04 was used for data analysis.

Results

Sea pen (*Virgularia vianagusta*) extracts were analyzed by thin layer chromatography (TLC) and the resulting band on TLC paper are visible under UV lamps which Rf was calculated for each compound. Results of the Rf for different combinations in each fraction are shown in Table 1.

Cytotoxic effect of fractions of A, B, C, D, E, F, G obtained from extracts of sea pen *Virgularia gustaviana* species collected from the tidal coast at concentrations of 25, 50 and 100 ml on HeLa cancer cells was investigated. The results of HeLa cells treated with fractions of A, C, D, F showed that the survival rate of cancer cells after treatment with these fractions compared to the control group showed significant but not dose-dependent. The survival rate of cancer cells after treatment with G fraction compared to the control group shows that the viability of cancer cells reduces, by increasing concentration, so that the

lowest cell viability was observed at concentrations of 100 micro liters the fraction G in doses of 25, 50 and 100 compared to other fractions have significantly reduced the survival of cancer cells, which was dose dependent. Fraction B, A and F in any of the concentrations had no considerable effect on the survival of cancer cells, compared with the control group (Fig1, table 2).

Table 1. Results of Rf calculated for different combinations in each fraction

Fractions	Compounds	Rf	Fraction characteristics
A	1	0.6	Yellow oily liquid
B	2	0.36	Yellow oily liquid
C	3	0.71	Pale green oily liquid
	4	0.78	
D	5	0.38	Orange oily liquid
	6	0.57	
	7	0.76	
	8	0.83	
E	9	0.19	Colorless liquid
	10	0.26	
	11	0.69	
F	12	0.14	Yellow-orange liquid
	13	0.24	
	14	0.61	
	15	0.76	
G	16	0.58	Bold orange liquid
	17	0.40	

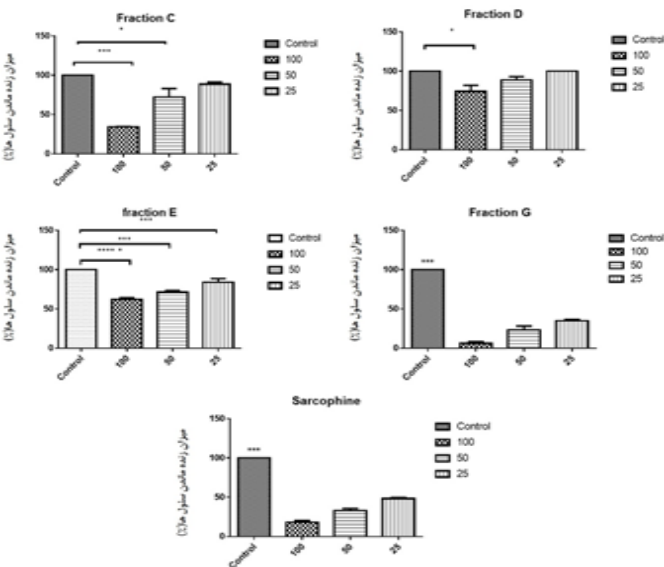


Figure 1. Effect of ethyl acetate extract fractions of sea pen species (*Virgularia gustavina*) and Sarcophine on HeLa cancer cells

Table2. Effect of fractions isolated from extracts of sea pen *Virgularia gustavina* and its effect on HeLa cancer cells results expressed as percentage

Concentration of fractions	100 Mean±SEM	50 Mean±SEM	25 Mean±SEM
C	34.33±0.33	72.33±10.68	86.67±2.06
D	74.33±7.68	89.4±4.16	100
E	62.33±0.02	77.33±1.20	84±2.64
G	6.33±0.02	23.33±4.97	35±1.52
Sarcophine	1.20±18.33	1.53±33	48±0.88

As one of the important compounds isolated from soft coral that has anti-cancer effects is Sarcophine that is a Cembrane Diterpene diterpene with a molecular weight of 316, So, given that both are species of soft corals groups and there is the possibility of similar compounds existence in both species compounds isolated from sea pen *Virgularia gustavina* were compared with Sarcophine. According to the results of thin layer chromatography, it was found, obtained seven fractions have 17 compounds. Since G fraction has spots with similar RF to Sarcophine, high performance liquid chromatography was used to review and identify quality of compounds. G fraction analysis showed that the resulting peak is similar to the peak of Sarcophine with regard to compare retention time and cytotoxicity tests of G fraction and comparing these fractions with Sarcophine compound derived from a soft coral species, the possibility that G fraction has a combination of Cembrane Diterpene diterpene is high that needs additional studies (Fig 2).

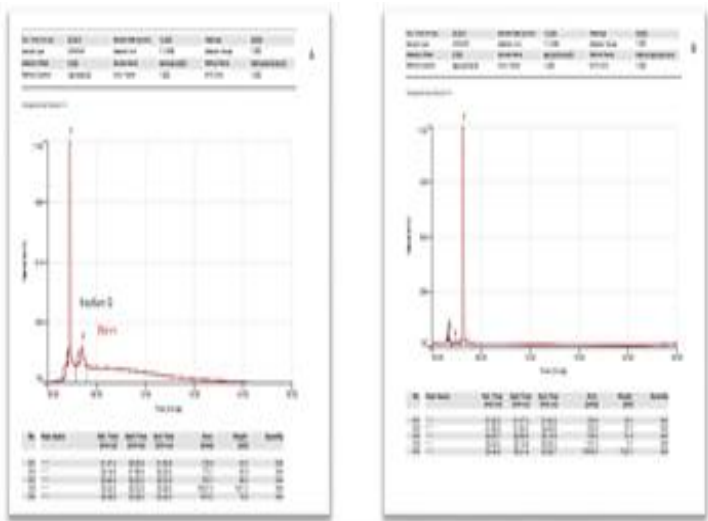


Figure 2. A: Chromatogram of the G fraction injection by HPLC with 3:39 shelf life, B: Chromatogram of the Sarcophine injection by HPLC

Discussion

The results showed that some fractions of the total seven isolated fractions greatly reduce the survival rate of the cells. One of the important points about the results is the compounds in the sea pen *Virgularia gustavina* that is the main factor of the anti-cancer effect. G fraction that was an orange and oily form compound has had more influence than other fractions and at the concentration of 100 micro-liters survival rate of cancer cells treated with the compound reached to about three per cent and this effect was dose dependent.

Can affect more inhibitory effect on cancer cells by realizing the active components of these fractions and purification of these compounds. Many terpene compound extracted from various species of sea pens. In this regard, a kind of Cembrane Diterpene which is derived from extracts of 2-propanol extract of sea pen species *Gyrophyllum sibogae* which has high anti-cancer effects on different cancer lines (19).

Another terpenoids is a combination of A Junceol which is a type of sesquiterpene. The compound extracted from the sea pen species *Virgularia juncea*. The effect of this combination on the survival rate of P-388cancer cells was investigated And showed that Junceol significantly reduced the survival rates of these cells (31).

This species is a species close to the studied species. Also the combination of Klysimplexin B and H, which is part of the Diterpenoids, extracted from *Plexim klyxum* species of the *Alcyonidea* family and is toxic to cancer cells (32). The fractions extracted in this study may have terpene compounds mentioned above but it is likely that the extracted compounds from the sea pen species is a type of Cembrane

Diterpene. According to published studies a substance called Sarcophine of a particular species of soft coral *Sarcophyton glaucum* has a strong influence cytotoxicity was isolated by liquid chromatography (33,34). This substance reduces the survival rate of cancer cells, by the mechanism of induction of apoptosis (35).

As the results showed G fractions, in three doses have stronger effects on HeLa cells in comparison to Sarcophine (anti-cancer compound) both were matched in terms of dose-dependent. Due to the fact that the shelf life of the composition of the G fraction, which was examined by HPLC, is equal to the shelf life of Sarcophine, there is a high probability that this matter is in G fraction. More studies with NMR is necessary. This study showed that the compounds extracted from sea pen species *Virgularia gustavina* which were collected from tidal area of Bandar Abbas, has considerable effects on HeLa cancer cells and the fraction that significantly reduced the viability of HeLa cancer cells and can prevent the proliferation of cancer cells in vitro; was G fraction that due to HPLC studies raises the possibility of Cembrane Diterpene such as Sarcophine in this fraction. According to these results, the study to extract and identify and evaluate mechanism of impact of these compounds on cancer lines is necessary in the future.

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