Original Research Article

PREPARATION OF METHANOLIC CRUDE EXTRACT OF *PADINA GYMNOSPORA* SEAWEED AND THEIR ANTICANCER ACTIVITIES AGAINST CANCER CELL LINE

ABSTRACT

Background: Cancer is a major health problem worldwide and still lacks fully effective treatments. As a result, natural-products-based alternative medicines have been developed. Marine algae are a vital part of the marine environment, with high biodiversity and contain a diverse range of useful chemicals.

Aim: Aim of the study was to assess the anticancer activity of *Padina gymnospora* crude extract against lung cancer cell line

Materials and methods: The Seaweed *P. gymnospora* was evaluated for its anti-cancer activity via MTT assay, further, morphological study of the cells was done to check its efficacy. Finally, the results were analyzed by Student's-t-test using MS-Excel, represented as mean ± SD for triplicates. The results were computed statistically (SPSS/10 Software Package; SPSS Inc., Chicago) using one-way ANOVA. The level of statistical significance was set at $p<0.05$.

Results and Discussion: The results of this study indicate that *P. gymnospora* has significant anticancer activity. At the highest concentration, 500µl, methanolic crude extracts of *P. gymnospora* showed the maximum anticancer activity, where the cell viability was only 16.41$\pm$7.15. The morphological study also revealed that maximum cell death had occurred in the maximum concentration of the methanolic crude extract of *P. gymnospora*.

Conclusion: Despite the widespread use of algae-derived compounds and extracts in the food industry, there are still limited anticancer drugs available in the industry. Thus, it is imperative that new drug discovery programs using seaweeds with a much more mechanistic approach are needed.

Keywords: Anti-cancer, Crude extract; Lung cancer; Seaweed

INTRODUCTION

Cancer is a group of disorders in which cells continue to develop uncontrollably, spread into adjacent tissues, and form tumors. Drug use, infectious organisms, a poor diet, environmental pollutants, inherited genetic mutations, hormones, and immunological disorders are all variables that might cause cancer. These factors can operate together or in sequence to produce cancer. According to the American Cancer Society, 1 685 210 new cancer cases were expected to be diagnosed worldwide in 2016, with 595690 patients in the USA expected to die of cancer, which translates to approximately 1630 people per day.

Cancer is frequently treated using a variety of therapies, depending on the characteristics and stage of the tumor, such as surgery, chemotherapy, radiotherapy, and immunotherapy (1). The goal of treatment in all circumstances is to remove the cells that make up the tumor in order to reduce it without harming healthy cells. Chemotherapy is a popular treatment option. Chemotherapy medications can cause anemia, appetite loss, psychosis, baldness, peripheral neuropathy, and irreversible damage to essential organs, among other side effects. Drug cancer treatment tolerance and side effects of chemotherapy are difficult problems.

Despite decades of research, effective treatment for cancer is still lacking; therefore, there is a need for new compounds with an anticancer activity that is cell-selective with fewer adverse effects, improving the quality of life of patients. Natural products provide a reliable alternative in the search for compounds that can help in the treatment of diseases (2). Over the past few decades, research attention has turned to natural products from marine organisms, mainly because of their large habitat (covering ~70% of the surface of the Earth), (3) high biodiversity (95% of world biodiversity), and the specific conditions under which some species live (e.g., at extremes salinity, pressure, and temperature). The anticancer potential of extracts and chemicals obtained from marine algae is particularly promising among the marine substances examined thus far (3).

Benthic marine algae or seaweeds, especially *Padina gymnospora* species, are plants that live either in marine or brackish water. Marine algae are either unicellular (microalgae) or multicellular (macroalgae) vegetative organisms that vary in size, from 2 m to 30 m, and in their morphology. Hence, the aim of the study was done to evaluate the anticancer activity of *P. gymnospora* methanolic crude extract.

Previous studies show the various pharmacological actions of the plants such as anti-inflammatory activity (4) (5), anti-diabetic activity (6) (7-9), and anticancer activity (10) (11).

Our team has extensive knowledge and research experience that has translated into high-quality publications (12–16),(17-31). This study was another such attempt at decoding the importance of one of the most appreciated seaweed, *P. gymnospora*.

Materials and Methods:

Sample collection and pre-processing the samples:

The *P.gymnospora* seaweed was collected from Thoothukudi coastal area, Tamilnadu showed in figure 1. The sample was washed thoroughly with tap water then shade dried on table tissue paper for 4 weeks and turned into a fine powder using a mortal pistol and represented in figure 1.

Extract preparation: A 25g of dried powdered *Padina gymnospora* seaweed samples were mixed with 100ml of methanol and allowed to place for 24 hours at ambient temperature. Then the mixture was passed through Whatman filter paper (No.4) then the filtrate was centrifuged at 3000 rpm for 10min and further filtered by a 0.45µm syringe microfilter. At last, the solvents are evaporated via a vacuum rotary evaporator (less than 60°C) until samples are obtained in powder form. Then the sample was stored in a shadowy aluminum container at 4ºC for further analysis as shown in figure 1.

MTT Assay: The proliferation of lung cancer cells was assessed by MTT assay (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide) according to Safadi et al. (2003). The lung cancer cells were plated in 48 well plates at a concentration of $2x10^4$ cells/well 24 hours after plating, cells were washed twice with 500µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37ºC. After starvation, cells were treated with *P. gymnospora* methanol extract in different concentrations for 24 hours. At the end of treatment, the medium from control and *P. gymnospora* extract-treated cells were discarded and 200µl of MTT containing DMEM (Dulbecco's Modified Eagle Medium) (0.5 mg/ml) was added to each well. The cells were then incubated for 4h at 37ºC in the CO_2 incubator.

The MTT containing medium was then discarded and the cells were washed with 1x PBS. The crystals were then dissolved by adding 200µl of solubilization solution and this was mixed properly by pipetting up and down. Then the formazan crystals formed were dissolved in dimethylsulfoxide (200µl) and incubated in dark for an hour. The intensity of the colour created was then measured at 570 nm using a Micro ELISA plate reader. The percentage of control cells cultivated in a serum-free medium was used to calculate the number of viable cells. Without any treatment, cell viability in the controlled media was indicated as 100%.

The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells]×100.

Morphology study: Based on the MTT assay we selected the optimal doses of (250µg/ml) were selected for further studies. A phase-contrast microscope was used to examine changes in cell morphology. The cells were plated in six-well plates and given a 24-hour treatment with *Padina gymnospora* methanol extract (250 g/ml for lung cancer cells). At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase-contrast microscope.

Statistical analysis: All data obtained were analyzed by Student's-t-test using MS-Excel, represented as mean ± SD for triplicates. The data were calculated statistically using one-way ANOVA (SPSS/10 Software Package; SPSS Inc., Chicago, IL, USA). The statistical significance level was set as $P>0.05$.

Results and discussion:

Table-1 indicates the cell viability of lung cancer cell lines tested in different concentrations of methanolic crude extract of *P. gymnospora*

Table-1: Cell viability of lung cancer cell line

Drug concentration (µl)	Cell viability (%)
Control	100
100	82.52±8.37
200	61.37±10.16
300	47.68±7.28
400	35.49±10.61
500	26.22±8.25
600	16.41±7.15

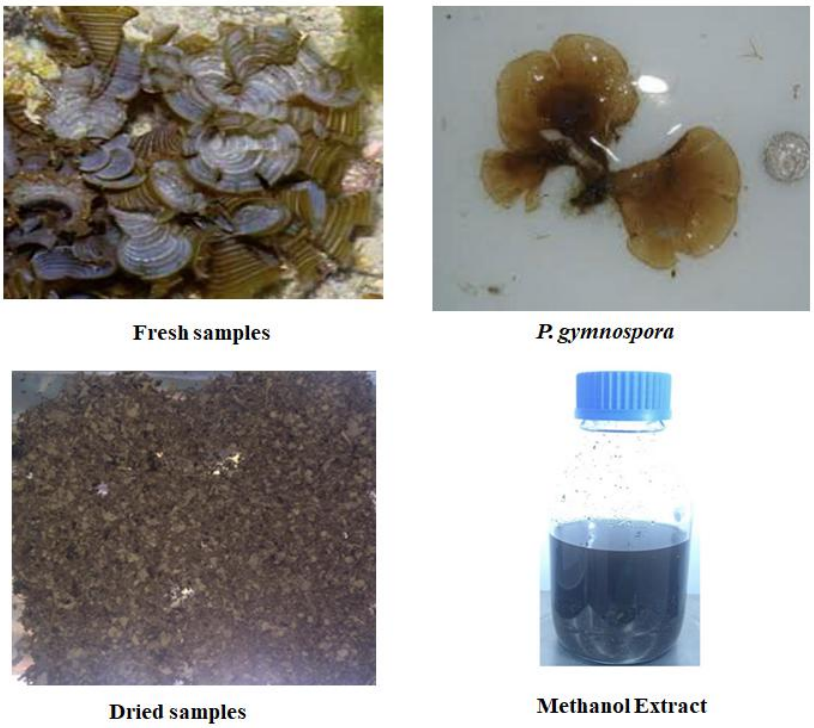

Figure 1: Images of *P. gymnospora* sample and their crude extract preparation

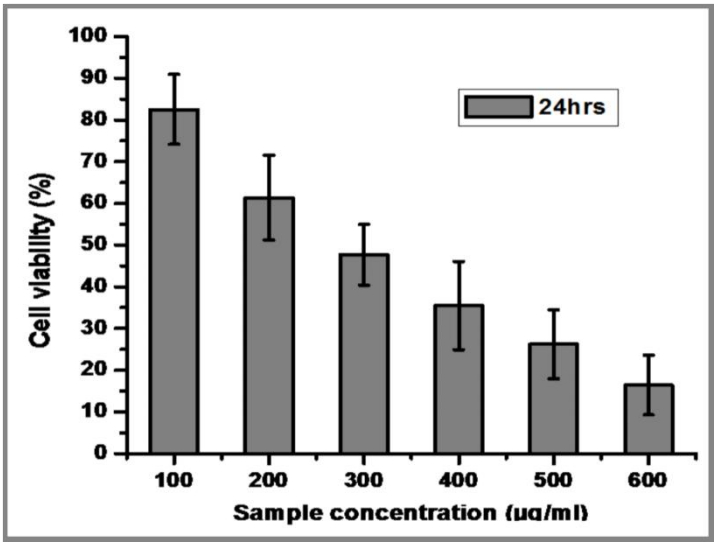

Figure 2: This graph indicates the cell viability of lung cancer cell lines tested in different concentrations of methanolic crude extract of *P. gymnospora*

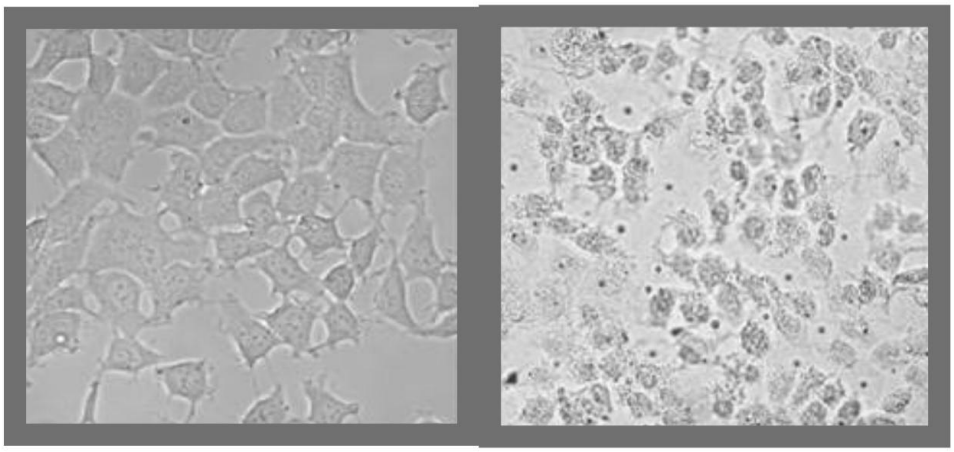

Figure 3: Microscopic image of lung cancer cell lines on being treated with control compared with the microscopic image of lung cancer cell lines on being treated with the highest concentration of the prepared methanolic crude extract of *P .gymnospora.*

Figure 2 indicates the graphical representation of the cell viability of lung cancer cell lines tested in different concentrations of methanolic crude extract of *P. gymnospora* showing concentration-dependent inhibition in cell growth. Figure 3 indicates the cell viability of lung cancer cell lines tested in different concentrations of methanolic crude extract of *P. gymnospora*. According to Figure 3, in 100µl concentration of the methanolic crude extract of *P. gymnospora* showed 82.52% of the cells were viable. In 200µl concentration of the methanolic crude extract of *P. gymnospora* 61.37% of the cells were viable. In 300µl concentration of the methanolic crude extract of *P. gymnospora* 47.68% of the cells were viable and in 400µl concentration of the methanolic crude extract of *P. gymnospora* 35.49% of the cells were viable. Further, in 400µl concentration of the methanolic crude extract of *P. gymnospora* 35.49 % and 500µl concentration of the methanolic crude extract of *P. gymnospora* 26.22% of the cells were viable. In 600µl concentration of the methanolic crude extract of *P. gymnospora* 16.41% of the cells were viable. Figure 3 compared the microscopic image of lung cancer cell lines being treated with control and the microscopic image of lung cancer cell lines being treated with the highest concentration of the prepared methanolic crude extract of *P. gymnospora*.

Over the past few decades, articles in the literature suggested that the anticancer activity of extracts and or compounds isolated from seaweeds has gained interest based on two main factors: (i) the need for new anticancer natural products that are more effective, targeted, and have fewer side effects; and (ii) epidemiological evidence showing diets rich in marine seaweeds reduce the incidence of cancer (32). Similarly, several traditional medicinal

systems, such as Chinese and Japanese approaches, have used seaweeds for centuries to treat neoplasms (32). A European study also has evaluated using both *in-vitro* and *in-vivo* models in the three main groups of seaweeds: *Rhodophyta, Chlorophyta, Phaeophyceae*. Several studies investigating the in vivo anticancer activities of seaweed have focused on compounds from brown seaweeds, especially on leukemia, breast cancer, and Lewis sarcoma models (33). Brown algae are clearly the most investigated, not only for their anticancer properties, but also for their anti-inflammatory, hypoglycemic, anticoagulant, and antioxidant properties, as evidenced by the literature. Hence, the importance of brown seaweed is studied so for by its medicinal properties (34). Solvent extract from seaweeds, such as polysaccharides, fucoidan, phloroglucinol, laminarian, pheophorbide, monoterpenes, and glycoproteins, have been employed in vitro studies to investigate the anticancer activity of seaweeds. These studies have highlighted that the degree of sulfation and composition of polysaccharides from brown seaweeds appear to influence their antitumor activity (35). In addition to that, crude extracts from marine microbes, plants, and animals also showed the potential biological activities in terms of antimicrobial, antioxidant, and cytotoxicity effects, etc (36). However, these studies also pointed out that the low yield obtained from alga- isolated compounds and extracts and the complexity of the compound structures represent a challenge to the use of these compounds in drug discovery. The results obtained have also been heterogeneous, given that anyone compound was able to achieve 100% cell death. However, it has been suggested that seaweed act by increasing the activity of the immune system. The heterogeneity of the experimental design within the studies in literature does not allow us to compare their results, because researchers used different cell types, concentrations, time of treatment, and the parameters evaluated (37). During the 1980s, the US National Cancer Institute (NCI) developed a protocol for the evaluation of the cytotoxicity of compounds with anticancer activity, testing the compounds against eight cell lines derived from the most common human malignancies. In 1990, the NCI introduced the NCI-60 Anti-cancer Drug Screen, which contains 60 different human cell lines against which dose-response curves were drawn up, along with biochemical pathways, and this screen is still in use. In addition, some companies now supply tumor cell panels for testing drugs and extracts. In this context, the use of cancer cell lines in the development and the design of new drugs have several advantages: low cost, repeatability of results, and high throughput. However, these models do not reflect the in vivo activity of a compound or extract (38). As a result, other factors such as absorption, distribution, pro-drug activation, half-life, metabolism, elimination, side effects, and so on must be assessed. Compounds derived from seaweeds have been demonstrated to

target signaling pathways that are resistant to traditional chemotherapy medications. Canadian research revealed that the p53 tumor suppressor protein is not functional in approximately 50% of tumors, causing resistance to chemotherapy. Moreover, triple-negative breast cancer (TNBC), which lacks estrogen, progesterone, and HER2 (Human epidermal growth factor receptor 2) receptors, represents up to 20% of all breast cancers, has a poor prognosis, and cannot be reduced with hormonal or anti-HER2 therapies, which are the standard therapy for breast cancers (39). Hence, seaweed-derived compounds that down-regulate the PI3K/Akt pathway and act downstream of these receptors represent good chemotherapeutic agents against drug-resistant cancers. In addition to cell proliferation, other mechanisms, including the inhibition of angiogenesis, prevention of metastasis, and induction of differentiation, must be evaluated using animal models and specialized approaches, such as murine allografts and xenografts also needs to be studied more extensively as very articles in literature throws light on these concepts. Evaluation of the cell selectivity of anticancer drugs is another important issue, given that anticancer drugs must kill cancer cells without causing extensive damage to non-cancer cells (25) (40-53). Therefore, researchers usually select normal cells (primary cultures and non-cancer cell lines) to evaluate the effect of extracts and compounds. Although some authors have used mouse epidermal JB6 Cl41 cell line, African green monkey kidney Vero cells, among others, these controls might not mimic the effect that drugs will have on7 normal cells because of the interactions within cell lineages and tissues. Furthermore, it is also essential to evaluate compounds against multidrug-resistant phenotype cell lines (e.g., the HCT-15 colon and renal cancer cell lines UO-31 and TK10) (32). Good effects, slight inhibition, and high inhibition have been used by authors to characterize the effects of their compounds, but there have been no measures created to estimate the effectiveness of an extract and/or compound. We believe that total growth inhibition (TGI) is a good metric to utilize when evaluating a compound's effects because it indicates its efficacy.

Conclusion:

From this, the study concluded that *P. gymnospora* has concentration-dependent significant anticancer activity. At the highest concentration, 500µl, methanolic crude extracts of *P. gymnospora* showed the maximum anticancer activity, where the cell viability was only 16.41±7.15. The morphological study also revealed that maximum cell death had occurred in the maximum concentration of the methanolic crude extract of *P. gymnospora*. Based on these results, further studies could be carried out as a search for new compounds from the family of brown algae to develop alternative therapeutic measures against cancer.

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