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Original Article

Evaluation of the Anti-tuberculosis and Cytotoxic Potential of the Seaweed *Padina australis*

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Abstract

Marine seaweeds synthesize different types of compounds with various biological activities, including anti-tuberculosis and anticancer effects. The aim of this study was to evaluate anti tuberculosis of *Padina australis* and cytotoxicity of different fractions of this seaweed. *Padinaaustralis* was collected from Persian Gulf, identified and extracted by maceration with methanol-ethyl acetate. The extract was evaporated and partitioned by Kupchan method to yield Hexane, dichloromethane, Butanol and water partitions. The anti-tuberculosis activity of the crude extract and cytotoxicity of fractions were investigated using GFPMA and MTT methods. The cell survivals of HeLa cell were decreased by increasing the concentration of extracts. The IC50 value of hexane, dichloromethane, butanol and water partitions were 2.0, 20, 19.7, and 182.7µg/ml, respectively. The crude extract was not active against tuberculosis. This study reveals that different partitions of *Padina australis* have cytotoxic activity against cancer cell lines.

Keywords: *Padina*, Persian Gulf, tuberculosis, cytotoxic.

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1. Introduction

Tuberculosis (TB) is an infectious disease that has been identified since the earliest of times. Although chemotherapic agents and vaccines against TB were available even 50 years ago, but a global emergency was declared in 1993 about this disease. The increase of tuberculosis in patients with AIDS disease has caused an additional drug-resistant to Mycobacterium tuberculosis. So the

identification of new sources for discovering novel lead compounds against TB is a critical point of view [1, 2].

In developed countries, cancer is a complex disease that is difficult to cure in most cases. The number of this invasive disease is increasing rapidly while in 2010, 14 million new clinical cases were reported and it is estimated that it will increase to 22 million during the next two decades [3]. Despite rapid research in development of new drugs, there is not any effective treatment for cancer. So, there exists a great demand to search for new chemotherapeutic agents especially from natural sources.

The oceans are a unique reservoir of bioactive natural compounds with characteristic structural and chemical features. [4]. Different marine organisms such as seaweeds, sponges, corals, fungi and ascidians, have been screened for their biologic activities and also potent metabolites [5-6].Seaweeds are one of the marine organisms belong to a group of plants known as algae. They have shown extensive medicinal potential and have been used in traditional medicine since ancient times. They also have been used as human food source by coastal populations. [3]. There are frequent reports on their antibacterial, antifungal, antiviral, anti-inflammatory, antidiabetic, antioxidant, anti-Alzheimer's and cytotoxic activities [8-11]. Until now, more than 2400 marine natural products have been isolated from the algae of different origin that can play an important role in the production of pharmaceutical agents [12]. Seaweeds are divided into the brown, green, and red named Phaeophytes, Chlorophytes and Rhodophytes respectively. Brown algae contain numerous bioactive components such as polyphenols, alkaloids, flavonoids, steroids and terpenoids [13]. Numerous brown algae have shown potent cytotoxic and antimalarial activities and there are several reports about consumption of algae as a preventive agent against several invasive and infective diseases [14-16].

Dictyotaceae is a huge family of brown algae [class Phaeophyceae) with interesting secondary metabolites and biological activities. The genera such as *calcareous, Lobophora, Distromium, Padina* and *Dictyota* are classified in this family [17].

Iran has a rich resource of marines on its coastlines of the Persian Gulf and Oman Sea. Recent report has introduced more than 150 species of marine algae along coastlines of Iranian islands and Hormozgan Province [18]. Despite the high amount of marine algae in this region, there have been only a few studies on the pharmacological properties of these seaweeds. The main objective of the present study is to evaluate the anti-tuberculosis and cytotoxic activity of brown alga *P. australis* collected from Persian Gulf.

2. Materials and Methods

2.1. Authentication of Plant Material

P. australis was collected in autumn 2015 from Bushehr, a Southwest coastline of Persian Gulf. Voucher specimens were made and deposited in the herbarium of the School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences and

were identified by Agricultural and Natural Resources Research Center of Bushehr.

2.2. Preparation of the Extracts

The algae was air –dried in the shade at room temperature and ground to powder with a Philip`s mill and then extracted by maceration with methanol-ethyl acetate (1:1) at room temperature. 10 mg of the extract was subjected to antimalarial test and the residue was evaporated under vacuum and partitioned by Kupchan method to yield Hexane, Dicholoromethane, Butanol and water partitions. The partitions were subjected to cytotoxic test.

2.3. Anti-tuberculosis Assay

M. tuberculosis H37Ra (ATCC 25177) and *M. tuberculosis* H37Rv (ATCC 27294) were purchased from the American Type Culture Collection. pFPV2 (mycobacterial expression vector pMV261 containing red-shifted, high-expression mutant gfp was obtained in Escherichia coli DH12S from Raphael Valdivia, Stanford University, and was cultured overnight in Luria-Bertani broth with kanamycin (30 µg/ ml) [17].

Electroporation and selection of transformants were performed by the method previously described by Cooksey *et al*[18]. The transformants (H37Rv gfp and H37Ra gfp) were cultured in 7H9GC broth with Tween 80 and incubated until observation of turbidity. The cultures were analyzed for fluorescence by a Cytofluor II micro plate fluorometer (PerSeptive Biosystems, Framingham, Mass.) with excitation at 485 nm and emission at 508 nm. The transformants with the highest fluorescent were cultured in 100 ml of 7H9GC with Tween 80 and kanamycin.

Green Fluorescent Protein Micro plate Assay (GFPMA) was used for antimicrobial evaluation. Antimicrobial susceptibility testing was performed in black, clear bottom, 96-well micro plates to decrease background fluorescence. Outer-perimeter wells were filled with sterile water to prevent dehydration. Subsequent twofold dilutions were prepared in 0.1 ml of 7H9GC broth (minus Tween 80). Cultures were diluted in 7H9GC, and 10^5 CFU was added to each test well in a volume of 0.1 ml. Only the wells with drug were used to detect auto fluorescence of the compounds. The other control wells consisted of bacteria only (B wells) and medium only (M wells). After incubation, fluorescence was measured daily for 8 days. The mean for triplicate M wells was used as a background subtraction for all test wells and B wells. Percent inhibition was calculated as 1- (test well fluorescence units/mean fluorescence units of triplicate B wells) × 100 on day 7 of incubation. The lowest drug concentration causing 90% inhibition was considered as MIC.

At day 7 of incubation, 20 µl from each well was dropped onto complete 7H11 agar plates, and the plates were incubated until countable colonies appeared (approximately 14 days).

Antimicrobial susceptibilities were also determined in the BACTEC 460 system as described previously [19]. Briefly, twofold dilutions of antimicrobial agents were

prepared and 50 µl was transported to individual BACTEC vials. The inoculum was made too and diluted in BACTEC 12B medium, and 0.1 ml containing 2×10^6 CFU was delivered to 4 ml of BACTEC 12B medium. Some of the control vials received an inoculum which was further diluted 1:100. The growth index (GI) for each vial was determined until the GI of the 1:100 controls reached at least 30. The GI was then read on the following day, and the GI and daily changes in GI (DGI) were recorded for each drug dilution. The MIC was defined as the lowest drug concentration for which the DGI was less than the DGI of the 1:100 control.

2.4. Cytotoxicity

Cervical cancer cell line, Hela, and human umbilical vein endothelial cells, HUVEC, were obtained from the Pasture Institute of Iran, Tehran. Cells were grown in RPMI-1640 medium supplemented with 10 % (v/v) fetal bovine serum (FBS), penicillin–streptomycin (100 IU/ml and 100 µg/ml, respectively). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were reefed every 2 days to obtain monolayer and at 80% confluence, were sub cultured.

Cytotoxicity of hexane, dichloromethane, butanol and water extracts from *P. australis* were evaluated in HeLa cells as well as HUVEC using by MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay as described previously [1]. Briefly, 180 µl of log-phase cells (5×10^4cells/ml) were seeded in 96 well plates and allowed to attach to the bottom of wells for overnight. Then 20 µl of different concentrations of solvent extracts and were added and incubated for 48 h at 37 °C and 5% CO_2. Every plate had four wells with untreated cells, four wells with cells treated with doxorubicin as the positive control and four wells with cells treated with 1 % (v/v) DMSO as a negative control. To evaluate cell viability, 20 µl of MTT solution [5 mg/ml in PBS) was added to each well which was then incubated at 37 °C and 5% CO_2 for 3 h|. Then, the old medium was removed and 150 µl of DMSO was added to dissolve any formazan crystals formed. Absorbance was then measured at 570 nm by an ELISA plate reader (Awareness Technology Inc., Stat Fax 2100, USA). The percentage of cell viability was calculated using the following equation:

$$\text{Cell viability \%} = 100 \times \frac{\text{absorbance of treated cells} - \text{absorbance of blank}}{\text{absorbance of negative control} - \text{absorbance of blank}}$$

2.5. Statistical Analysis

Each assay was repeated at least three times to ensure reproducibility of results. All data are expressed as the mean ± standard deviation. Significant differences were calculated by analysis of variance (ANOVA) using SPSS version 20 followed by a post Hoc test and differences at $P<0.05$ were considered significant.

Table 1. Antituberculosis activity of *padinaaustralis.*

Screening code	Sample code	Final concentration (µg/ml)	Fluorescence unit at Day 0	Fluorescence unit at Day 7	% Inhibition	Activity	MIC (µg/ml)
Blank	DMSO	0.5% DMSO	39.60	41.10	-	-	-
Negative	TB+ DMSO	0.5% DMSO	111.7	325.30	0	Inactive	-
Positive1	Rifafpicin	0.10	113.51	102.42	105.19	Active	0.0063
		0.05	111.76	101.44	104.83	Active	
		0.025	111.92	101.43	104.91	Active	
		0.0125	109.62	99.02	104.96	Active	
		0.00625	109.41	109.30	100.05	Active	
		0.003125	110.97	155.41	79.19	Inactive	
Positive2	Ofloxacin	3.13	118.34	104.45	106.50	Active	0.391
		1.56	115.63	103.95	105.47	Active	
		0.781	115.62	102.71	106.05	Active	
		0.391	114.55	100.21	106.71	Active	
		0.195	112.56	232.77	43.71	Inactive	
		0.098	112.68	272.03	25.46	Inactive	
Positive3	Streptomycin	5.00	114.34	99.98	106.72	Active	0.625
		2.50	131.59	100.84	105.97	Active	
		1.25	113.33	100.33	106.09	Active	
		0.625	113.39	102.77	104.98	Active	
		0.313	110.06	155.79	78.59	Inactive	
		0.156	109.99	240.85	38.72	Inactive	
Positive4	Isoniazid	0.75	115.29	104.19	105.20	Active	0.0468
		0.375	114.74	104.33	104.88	Active	
		0.1875	115.14	103.44	105.48	Active	
		0.09375	114.17	102.13	105.64	Active	
		0.046875	112.24	100.43	105.53	Active	
		0.0234375	114.41	197.52	61.08	Inactive	
Positive5	Ethambutol	7.50	107.98	100.05	103.71	Active	0.469
		3.75	110.14	100.58	104.48	Active	
		1.875	108.75	100.52	103.86	Active	
		0.938	107.05	99.78	103.41	Active	
		0.469	104.34	97.67	103.12	Active	
		0.234	104.55	146.76	80.23	Inactive	
Sample	*S. boveanum*	50	113.19	341.27	-6.81	Inactive	
		25	114.28	287.26	18.99	Inactive	
		12.5	114.84	264.72	29.81	Inactive	
		6.25	113.80	236.97	42.32	Inactive	
		3.13	114.19	217.74	51.51	Inactive	

3. Results and Discussion

3.1. Anti-tuberculosis Activity

The crude extract of *P.australis* could not inhibit 90% growth in the final concentration of 50 µg/ml (Table 1).

The solution of 0.5% DMSO was used as negative control while rifampicin, streptomycin, isoniazid, ofloxacin and ethambutol were used as the positive ones with MIC of 0.00312- 0.0250 µg/ml, 0.156-0.625 µg/ml, 0.0234- 0.0469 µg/ml, 0.391- 0.781 µg/ml and 0.234- 0.469 µg/ml respectively.

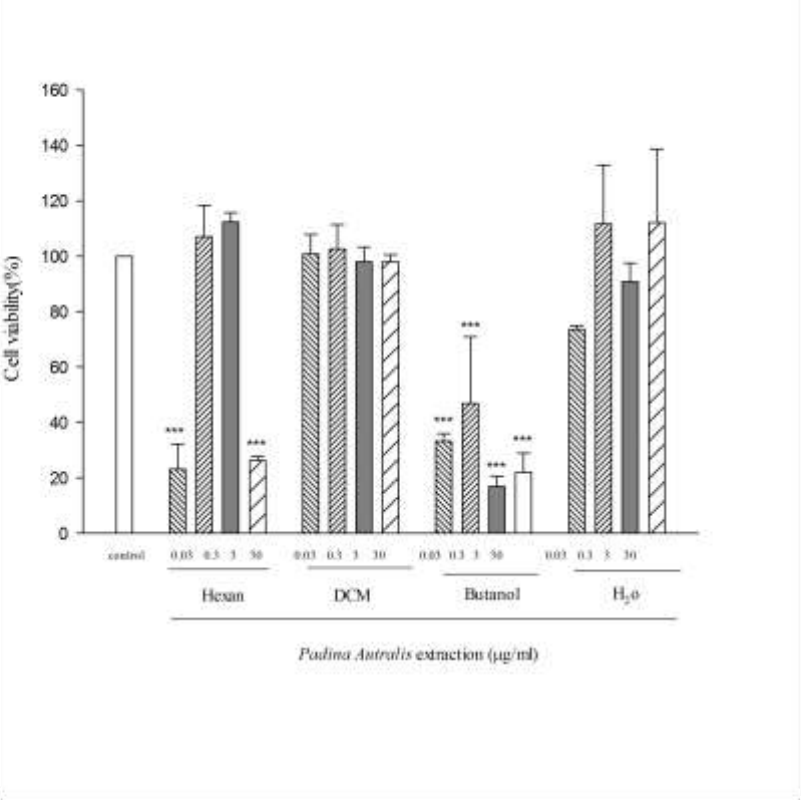

Figure 1. Cytotoxic activity of *P. australis*partitions on HUVEC cell line. Data represent the means ± SEM. separate experiments (significant as compared to control ***p<0.001). Cells viabilities were assessed by MTT assay. Cells were incubated for 72 h.

3.2. Cytotoxicity

The cytotoxic effects of different extracts of *P. australis* were evaluated against a cancerous cell line, HeLa (Figure 1) and a normal cell line, HUVEC (Figure 2) by the MTT assay. The multiple concentration 0.03, 0.3, 3, 30 µg/ml of hexane, dichloromethane, butanol and water extracts from *P.australis* were used. Figure 1 shows the MTT test results for different concentrations of *P. australis* extract partition with different concentrations on the HUVEC cell line after 72 hours (after 24 and 48 hours the cytotoxicity were evaluated too which did not have significant effects, data has not been shown). We observed a significant reduction in cell viability at the doses of 0.03 µg/ml from hexane partition and all doses of butanol partition.

The results for different concentrations from *P. australis* partitions on HeLa cell line, after 72 hours, were shown in figure 2. At the base of this results a significant reduction in cell viability the doses of 3 and 30 µg/ml of DCM partition.

Our preliminary findings show the potential cytotoxic activity of the DCM extracts of the seaweed *P.australis* collected from Persian Gulf against a cervical cancer cell line, HeLa using MTT assay. Other research groups have also investigated the cytotoxic effect of different species of the genus *Padina sp*.

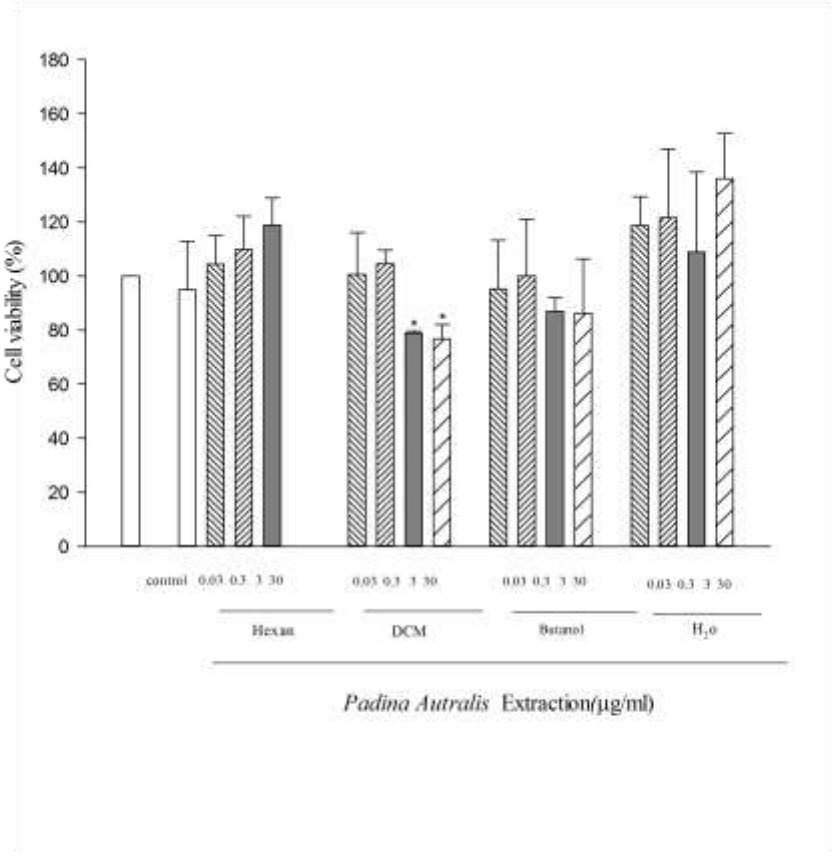

Figure 2. Cytotoxic activity of P. australis partitions on HUVEC cell line. Data represent the means ± SEM. separate experiments (significant as compared to control ***p<0.05).

Tatjana P*et al*. observed that metanolic extract of *P. pavonica* possesses cytotoxic activity with IC50 values 86.45 µg/ml related to HeLa cell and 74.59 µg/ml related to MDA-MB-453 cell.

The extracts did not exert any significant cytotoxicity toward normal human fetal lung fibroblast cells (MRC-5) [21]. Similar to our research , Elica *et al*. reported that chloroform fraction of *P. gymnospora* (8.2±0.4) were active against HEp-2 as well as its ethanol extracts of (15.9±2.8 $\mu g.mL^{-1}$) against the cell NCI-H292.[22].

Mosaddegh*et al*. analyzed cytotoxic activity of some marine algae from the Chabahar coasts of the Oman sea but *P. australis* could not inhibit cell growth with IC50 less than 100 $\mu g.mL^{-1}$[14]. Partitioning pattern of compounds in to different solvents depends mainly on their structure. Non polar compounds will tend to solvents such as hexane, polar compounds are found in butanolic partition while chloroform fraction contains semi polar compounds. We did not investigate fractions phytochemically but it seems that semi polar terpeniods and steroids are the main components of chloroform fraction while non-polar steroids and fatty acids are majorly in hexane partition. Bioassay guided isolation of secondary metabolites in

chloroform and hexane as brown algae represent a rich source of polysaccharides and glycosides, this activity could be connected with these compounds, but also with phenolics detected in examined extract.

Based on the published work [23], some sterols fatty acids, aromatic esters, terpenoids, benzyl alcohol and benzaldehyde may be responsible for the biological activity of *P. pavonia*. Also, several sulfated polysaccharides separated from algae have shown antitumor, anticancer, anti- metastatic activities in mice [24]. In addition, as reported Awad et al. neither cold nor hot aqueous polysaccharide extracted from *P.pavonia* exhibited cytotoxic effect against cultured U251, while hot water extract showed cytotoxic activity against cultured HepG2 *in vitro*. Also, fractions isolated from polysaccharide extracts were cytotoxic against HepG2 and *P.pavonia* showed a cytotoxic activity against KB cells. Oxysterol, hydroperoxy-24 vinyl-24cholesterol was identified as responsible compound for this activity [25, 26].

Screening natural resources for their antimicrobial activity is one of the most common researches in drug discovery and tuberculosis disease is in an urgent need in this area.

Until now, there are different marine compounds isolated from marine organisms with anti-tuberculosis potentials. Massetolide A, a depsipeptide isolated from *Pseudomonas* species cultures from a marine alga displayed MIC values of 5–10 µg/mL .[26].

Pseudopteroxazole and seco-pseudopteroxazole are novel benzoxazole diterpene alkaloids isolated from the gorgonian *Pseudopterogorgia elisabethae*[27, 28]. These compounds exhibited 97 and 66% growth inhibition respectively against *M. tuberculosis* H37Rv at a concentration of 12.5 µg/mL without any cytotoxicity. These researches illustrate the importance of screening marine organisms as a possible unexplored resource of unique anti-tuberculosis structures. Whereas the crude extract of *P.australis* tested in this work was not active against tuberculosis but work on the other seaweeds from Persian Gulf may lead to the isolation and structure elucidation of a number of exciting new pharma-cophores. Although the Persian Gulf of Iran bears a luxuriant and unique treasure of organisms, but there are only limited publications about their pharmaceutical abilities. This was the first report of the anti-tuberculosis activity of *S. boveanum*. Besides, different fractions of this seaweed were tested for their cytotoxic activities. Further work is necessary to isolate the bioactive cytotoxic compounds. We also have some limitations such as lack of other cancer cell lines, lack of plant active ingredients and lack of other normal cell lines to compare with cancer cell lines.

4. Conclusion

We showed for the first time that seaweed *P.australis*, from Persian Gulf, has toxicity against a cervical cancer cell line, HeLa. Other research groups have also investigated the

cytotoxic effect of different species of the genus *Padina sp*.

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