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Screening of *in vitro* cytotoxic activity of brown seaweeds against hepatocellular carcinoma

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ABSTRACT

This study attempts to screen the potential chemotherapeutic agents from three seaweeds extracted with methanol and tested against hepatocellular carcinoma cells (Hep3B) and normal cell line (Vero). Using MTT assay, the extracts were checked for their cytotoxicity at six different concentrations. The study exhibited decrease of the cell viability percentage in dose dependent manner as signified by cell death. *Sargassum wightii* extract shows potential cytotoxic activity with CTC_{50} of 10.02 ± 1.06 and 180.65 ± 2.87µg/ml against Vero cells and Hep3B respectively. The methanol fraction led to cell shrinkage, plasma membrane damage and apoptotic bodies formation showing bioactive components as profound influencing factors for anticancer effects. Further research need to be explored to purify and characterize the methanol extract to identify the potent therapeutic tool against liver cancer.

INTRODUCTION

Cancer is still a life threatening disease, despite the development of medical science and modern therapeutic techniques. Cancer had an effect on approximately 10 million people and death rate tends to 5 million per year in developed countries. Lung, breast, stomach, colorectal and liver cancers are most persistent (Jha *et al.*, 2016). Researchers are engaged in the search of active elements from natural products and one such is seaweeds, which have been repeatedly recognized for production of bioactive substances. Cancer has prospered as a major global problem matching its effects in industrializing nations due to their changes in life style and average increase in life span. As per the recent world cancer report, cases of cancer are expected to increase to 50% i.e. in 2020 it may increases to 15 million (WHO and IARC, 2015).

Cancer is indicated by unrestrained cell division, which has the propensity to permeate into other tissues, by growing directly into adjoining tissues via invasion or by getting implanted to far away site by metastasis via circulatory or lymphatic system (Cragg *et al.*, 2005).

Hepatocellular carcinoma (HCC) is the primary malignancy commonly seen in the liver and accounts for as many as 1 million deaths annually worldwide. In certain areas of the world, HCC has the highest recurrence among internal malignancy and hence the customary reason of cancer death (Ali and Gagan, 2014). Although cancer therapy is often rigorous, debilitating and uncomfortable, many people successfully undergo treatment with a minimal disruption of their normal lives.

The development of pristine perspective to improve screening, diagnosis and treatment of cancer is an area of intensive research spending and has generated numerous innovations that have enhanced the rates of continued existence of cancer patients. Natural products with medicinal importance are of prime interest in the research and development of cancer chemotherapeutic drugs.

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The use of natural products for cancer treatment has been increasing owing to its availability, affordability and relatively lesser side effects when compared to the commercially available chemotherapeutic agents (Manosroi *et al*., 2012; Engel *et al*., 2011). Marine algae are one among the natural resources in the marine ecosystem. They contain various biologically active compounds which have been used as food source, feed for animals and medicine (Faul Kner, 2000).

By now, around 2400 marine bio-products have been obtained from seaweeds (Manilal *et al*., 2009). Current findings proved that seaweeds possesses antiviral (Matsuhiro *et al*., 2005), antibacterial (Xu *et al*., 2003), and antitumoral potentials (Harada *et al*., 1997; Kezia *et al*., 2008) among numerous others.

Marine algae yield a wide range of compounds functioning as chemical defence systems helping in their survival in drastic environments. Algae act as a promising source of bioactive substances that might have pharmaceutical applications due to their biologically active nature (Blunden, 1993).

Animal models have always played a predominant role in both contexts. Although cell culture systems have figured largely in the field of cancer chemotherapy, where the potential value of such systems for cytotoxicity and viability testing is now widely accepted, there is an increase in pressure for a more comprehensive adoption of *in vitro* analysis in safety evaluation (Harada, 1997).

The present study deals to assess the potency of the crude extracts from marine brown algae against HCC by *in vitro* methods and further suggests for the determination and isolation of potent anticancer drug.

METHODOLOGY

Cell lines and culture conditions

In this study, two cell lines were used viz., HCC cells (Hep 3B) and normal fibroblast cells (Vero) which were procured from National Centre for Cell Sciences (NCCS), Pune. The cell lines were developed in Minimal Essential Medium (MEM) supplemented with 2mM glutamine, 1 mM Sodium pyruvate, 0.2 mM essential aminoacids, 2g/L sodium bicarbonate and 10% Fetal Bovine Serum (FBS) (Sigma). Humidified condition with 5% CO_2 was used for culturing the cells.

Collection of algae

Three brown algae *Sargassum wightii, Sargassum duplicatum* and *Sargassum tenerrimum* (Authentication number - S7/09/2012, S3/09/2012, S8/09/2012, respectively) were collected from Mandapam, Tamil Nadu, India (Lat 9.28^o N, Long 79.12^o E). The seaweeds were authenticated by Dr. P. Anantharaman, Associate Professor, Marine Biology, CAS, Chidambaram, Tamil Nadu, India.

Preparation of algal extract

The collected algae were washed with distilled water and air-dried. The crude extracts were obtained from algae by maceration technique using methanol (10g/ 100 ml). The macerated extract was then clarified, filtered and stored at refrigeration condition until use.

Cell viability assay

To detect the cell viability tryphan blue exclusion test was chosen. A density of 1×10^6 cells/well of cancer and normal cells along with different concentrations of extracts were seeded and incubated at 37°C in the presence of 5% CO_2. After 72 hrs, 20µl of medium and 0.2% tryphan blue were mixed, and Neubauer haemocytometer was used to count the live and dead cells (Strober, 1997).

Cell viability (%) = No of live cells/ Total cells × 100

MTT assay

MTT assay was performed using modified method of Mosmann (1983). Cells were added in 96-well tissue culture plate at a concentration of 1×10^6 cells/well and DMSO was used to prepare the stock solutions of the extracts (1mg/ml) and it was then diluted with cell culture medium to obtain the required concentrations (1000, 500, 250, 125, 62.5 and 31.25 µg/ml). Suitable concentrations were added to the cultures and incubated for 72 hrs at 37 °C. Non-treated cells act as control. Incubated cell was then subjected to MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), and concentration was determined by colorimetric assay. The tetrazolium is used to find out cell viability in assays of cytotoxicity and cell proliferation. In metabolically active cells, an insoluble purple formazon product is formed by the reduction of MTT. An ELISA reader (Bio-Rad® 680) was used to read the assay plates at 520 nm. Determination of absorbance and calculation of the corresponding extract concentrations standardizes the cytotoxicity data. The concentration of extract required to kill 50% of cell population (IC_{50}/CTC_{50}) was determined by plotting a dose-response curve.

% Growth Inhibition = (100 - [(OD value of Control/ OD values of Test)] ×100)

Statistical Analysis

The data point was obtained by making at least 5 independent measurements. The results were expressed as mean ± SD. Data were analysed by an analysis of variance and p values set as lower than 0.01 were considered as statistically significant by one way ANOVA.

RESULT

Percentage viability and cell toxic concentration (CTC_{50}) was used to express the cytotoxic activity of the crude extracts (Table 1). The rate of viable normal cells at 1000µg/ml was 80.88 ± 3.43% and the percentage increased up to 99.35 ± 2.86 % when the extract concentration was diluted to 31.25µg/ml. At higher concentration, 52.26 ± 0.53% of viability was observed in cancer cells and the viability increased with decreased concentration. The percentage viability decreases as the concentration increases in

normal cells. The values are compared with the control and p value depicts significant result in all concentrations except 1000 µg/ml in Vero cells tested.

Table 1: Cytotoxic study by Tryphan Blue Dye Exclusion Technique in Vero and Hep 3B.

S. No	Name of the Algae	Concentration µg/ml	Percentage viability (%)	
			Vero cells	Hep 3B
1.	*S. tenerrimum*	1000	72.05 ± 0.34	41.82 ± 1.52
		500	87.00 ± 0.59*	58.33 ± 0.26
		250	89.25 ± 0.42*	60.78 ± 1.35
		125	92.35 ± 0.69*	62.35 ± 0.88
		62.5	93.65 ± 0.71*	66.55 ± 0.81
		31.25	98.50 ± 0.14*	74.10 ± 1.28
2.	*S. duplicatum*	1000	76.56 ± 2.23	44.16 ± 1.05
		500	82.14 ± 2.97*	51.64 ± 1.20
		250	84.68 ± 2.47*	62.56 ± 0.86
		125	88.80 ± 2.32*	70.29 ± 1.41
		62.5	89.10 ± 2.07*	74.40 ± 1.54
		31.25	91.15 ± 2.54*	78.34 ± 1.28
3.	*S. wightii*	1000	80.88 ± 3.43*	52.26 ± 0.53
		500	85.65 ± 3.19*	65.62 ± 0.54
		250	88.35 ± 2.07*	72.69 ± 1.28*
		125	95.25 ± 1.90*	76.98 ± 0.98*
		62.5	98.92 ± 1.66*	80.64 ± 0.73*
		31.25	99.35 ± 2.86*	85.86 ± 0.41*
4.	Cell control	-	100.00	100.00

Values are mean ± S.D., n = 5, $P < 0.01$ = *, when compared to control.

The results were promising in *S. wightii* methanol extract in all doses with a significant value of p< 0.01. In Hep 3B cells concentration less than 250 µg/ml has implicated level of significance with p< 0.01 when compared to control. It is observed crude extract of *S. wightii* is influencing the increase in viability in normal Vero cells and decreased viability in cancer cells when compared to that of *S. tenerrimum* and *S. duplicatum*. Depending on the tryphan blue assay, *S. wightii* methanol extract was selected for advanced studies to determine viability.

Table 2: Result of viability test for Hep 3B cell line of *S. wightii.*

S. No	Extract concentration (µg/ml)	No of dead cells	No of viable cells
1.	1000	1.8×10^6	1.4×10^6
2.	500	1.5×10^6	1.8×10^6
3.	250	0.82×10^6	2.0×10^6
4.	125	0.41×10^6	2.2×10^6
5.	625	0.26×10^6	2.3×10^6
6.	31.25	0.21×10^6	2.4×10^6
7.	15.625	0.18×10^6	2.56×10^6
8.	7.81	0.15×10^6	2.6×10^6
9.	3.90	0.14×10^6	2.72×10^6
10.	1.95	0.12×10^6	2.75×10^6
11.	0.97	0.10×10^6	2.77×10^6
12.	0.48	0.08×10^6	2.82×10^6

In Table 2 one negative control and 11 concentrations of the sample were tested against liver cancer cells Hep 3B. Tryphan blue was added to count the viable and non-viable cells. In negative control, the number of non-viable cells is comparably less and the cell count increased from up to 1.8×10^6 after 72 hours of incubation. Increase in extract concentration enhanced the viable and non-viable cells. Concentrations up to 125µg/ml showed raise in viable cells and decrease in dead cell count. The reduction in the number of viable cells started from the effectual concentration of 250µg/ml. When compared with negative control the initial concentrations of extracts didn't possess anticancer activity against the liver cancer cell line (Table 3).

Table 3: Cytotoxicity Study of *S. wightii* methanol extract by MTT assay In Vero and Hep 3B cell lines.

S. No	Cell line	Concentrations	% Growth Inhibition	CTC_{50} value (µg/ml)
1.	Vero (Normal fibroblast cell)	1000µg/ml	15.20 ± 0.25	10.02 ± 1.06
		500µg/ml	13.11 ± 0.56	
		250µg/ml	12.75 ± 0.29	
		125µg/ml	10.65 ± 0.57	
		62µg/ml	10.25 ± 0.85	
		31µg/ml	10.09 ± 0.68	
		15µg/ml	10.05 ± 0.41	
2.	Vero (Normal fibroblast cell)	Control	0.00	-
3.	Hep 3B (Liver cancer cell)	1000µg/ml	88.32 ± 1.56	180.65± 2.87**
		500µg/ml	74.68 ± 1.40	
		250µg/ml	70.22 ± 1.84	
		125µg/ml	66.91 ± 1.05	
		62µg/ml	65.87 ± 1.81	
		31µg/ml	65.66 ± 1.52	
		15µg/ml	65.65 ± 1.06	
4.	Hep 3B (Liver cancer cell)	Standard - Cisplatin 100 µg/ml	100	28.25 ± 1.02**
		50 µg/ml	95.25 ± 0.65	
		25 µg/ml	90.25 ± 0.58	

Values are mean ± S.D., n = 5, P< 0.01 = **, when compared to standard.

MTT assay an accessible, non-radioactive alternative method is relevant in determining the cell proliferation and cytotoxic concentration. The Vero cells assayed with *S. wightii* extract has imparted 15.20 ± 0.25 to 10.05 ± 0.41% growth inhibition with decrease in concentration and CTC_{50} of 10.02 ± 1.06. In liver cancer cells it is evident at elevated dose was 88.32 ± 1.56 % inhibition and decreased with decrease in concentration up to 62.5 µg/ml and further lower doses maintained a constant percentage inhibition. Hepatocellular carcinoma cells cells were compared with standard silymarin at three doses with CTC_{50} of 28.25 ± 1.02 and statically significant with p value and CTC_{50} 180.65± 2.87.

Methanol extract of *S. wightii* showed 100% cytotoxicity to Hep3B cells (Figure 1) and control showed no cytotoxicity (Figure 2). For Vero cells, methanol extract of *S. wightii* showed less cytotoxicity (Figure 3) and no cytotoxicity was observed in control (Figure 4). Comparable with the other seaweeds extracts taken in the present study, *S. wightii* is considered potent against cancer cells and less toxic to normal cells.

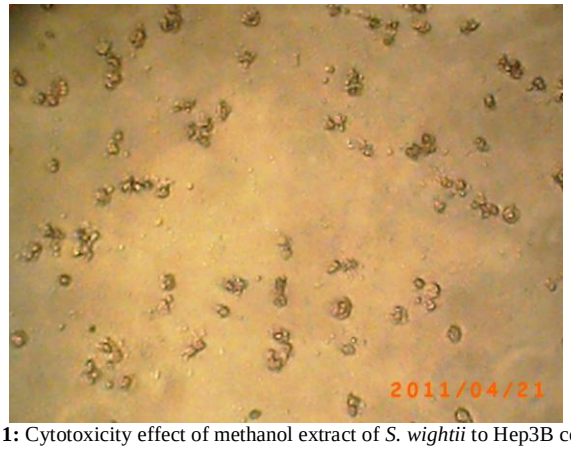

Fig. 1: Cytotoxicity effect of methanol extract of *S. wightii* to Hep3B cells.

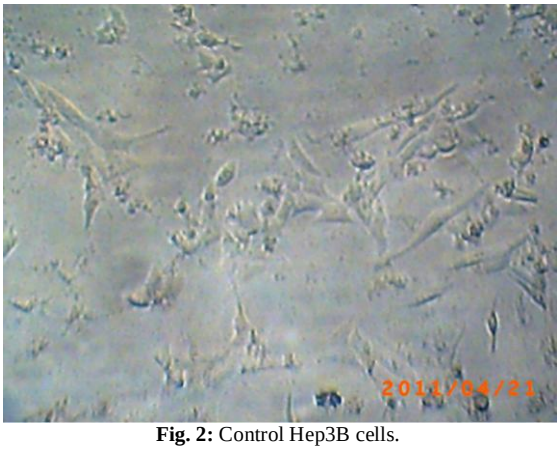

Fig. 2: Control Hep3B cells.

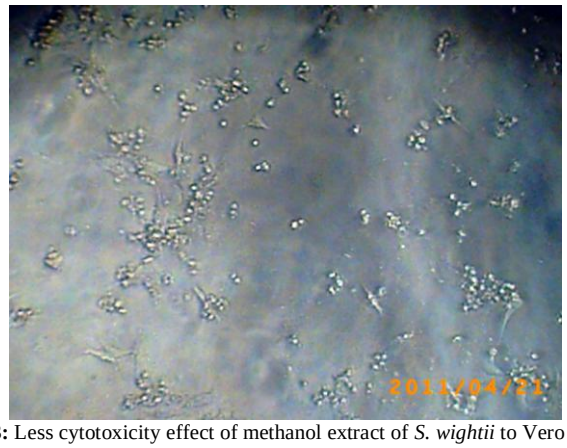

Fig. 3: Less cytotoxicity effect of methanol extract of *S. wightii* to Vero cells.

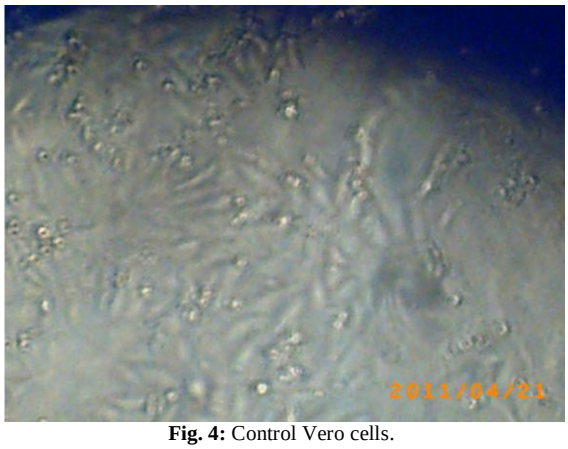

Fig. 4: Control Vero cells.

DISCUSSION

Cancer has a great infliction on the human population for many decades even though several approaches have been in prevention, diagnosis and treatment of the disease. Most of the existing anticancer drugs are toxic to normal cells and causes side effects at various circumstances. The discovery and establishment of a distinct anticancer agent with low side effect and toxicity has become a requisite goal. With this aim, researchers have been concentrating on the discovery of potent novel compounds derived from natural resources with anticancer activity.

The process of drug discovery includes preclinical assessment of large number of drugs for specific and non-specific cytotoxicity against different cells. To screen potential anticancer agents, *in vitro* assays have been a common practice almost since the beginning of cancer chemotherapy in 1946. Natural products have a significant role in cancer therapy today.

These extensive numbers of anticancer agents are either natural (from plants, animals and microorganisms) or their derivatives (Stefania *et al*., 2009). The elevated toxicity of some cancer chemotherapy drugs and their detrimental side effects increase the need for new anti-tumour drugs against incurable tumours (Arnold, 2011). Seaweeds are one among the natural resources in the marine ecosystem that contains numerous biologically active compounds that have been used as a source of food and medicine. Marine seaweeds have been recognized as unique for its structural and functional components when compared to terrestrial organisms. In the progression of natural product drug discovery, it is mandatory to determine the potential of cytotoxicity against human cancer cell lines. The agents which display specific activity on cell lines are recommended for *in vivo* studies. Therefore, it is necessary to screen the agents discovered with the common methods like tryphan blue and MTT assay.

Researchers have reported that colorectal cancer can be effectively treated with marine natural products, which focus ample of biologically active compounds with favourable pharmacological activities (Rye *et al*., 2013). This study was aimed to assess the cytotoxicity of three different seaweeds methanol extracts against Hep3B and Vero cell lines by tryphan blue and MTT assay.

It is observed in the earlier reports ethanol extract of *S. wightii* contains novel polysaccharide which exhibited anti proliferative activity against AGS, HeLa, MCF 7 and PC 12 cell lines. Anti cancer activity in aqueous extract of *S. oligocystum* has proved its effectiveness for treating cancer cell lines, Daudi and K562 with an IC_{50} value of 500 and 400 µg/ml (Faulkner, 2000). The compounds isolated from *Sargassum sp.* are invariably having

pesticidal, antihelmentic, antifungal, antimicrobial, antioxidant, insect repellent, nematicidal and cancer preventive properties (Bluden, 1993). The present study infers that *S. wightii* has potential in inhibiting liver cancer cells. In Vero cells at higher concentration, the viability percentage was moderate and decrease in concentration has increased viability as the toxic level in crude in more at higher concentration. This implies increase in concentration has started inhibiting the normal cells.

Concentration below 500µg/ml has showed drastic decrease in two fold in dead cells. The ranges between 500µg/ml to 250µg/ml have clearly determined the CTC_{50} value as 180.65 ± 2.87µg/ml for cancer cells and 10.02 ± 1.06 µg/ml for normal cells. The methanol extract has induced cell contraction when observed morphologically and evidence the presence of cytotoxic effect. This extract showed comparatively higher regenerative capacity, which is measured with reference to plating efficiency in Vero cell culture, compared to the cancer cells, indicating its affinity to cancer cells. In marine algae, the anticancer activity is one of the essential activities and the cytotoxic properties of the species belong to four structural types (Mayer *et al*., 2003). It was revealed that sulfur-containing compounds such as fuciodans extracted from *Sargassum polycystum* and other brown algae exhibited important roles against some human carcinoma cells (Ly *et al*., 2005). Random screening is effective to have found different marine algae with anti tumour activities. *Sargassum fusiforme* has a discriminatory human cancer cytotoxin and significant activity against Ehrlich carcinoma cells (Ogawa *et al*., 2004). The present work is supported in context with many researchers.

CONCLUSION

A crude natural product extract is in general highly complicated blend of numerous compounds possessing variable chemico physical properties habitually with opposing pharmaceutical properties and removing the compounds might increase the toxic effect on cancer cells and meanwhile no toxicity for normal cells. It is interesting that among the screened one seaweed *S. wightii* methanol extract exhibits effective anticancer activity and seems to have low toxicity on normal cells. Further investigations are required to isolate, characterize and identify the lead compound and it can be implicated as an effective therapeutic tool against liver cancer.

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