RESEARCH
www.commprac.com
ISSN 1462 2815

COMPARATIVE INVESTIGATION OF ANTI-CANCER PROPERTIES IN METHANOL EXTRACTS OF *ULVA LATUCA* AND *SARGASSUM TENERRIMUM*: UTILIZING DIVERSE BIOLOGICAL MODELS

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DOI: 10.5281/zenodo.11082016

Abstract

Seaweeds as a whole or parts are being used pharmacologically worldwide. In the present study, methanol extract of two marine algae, *Ulva lactucaa* (ULME) and *Sargassum tenerrimum* J.Agardh (1848) (STME) collected from, Mandapam Coastal region, Tamil Nadu had been tested for anti cancerous and toxic nature using cancer cell lines (MCF 7, MDA MB 232), *Artemia* sp. (BST), and Zebra fish embryos (Danio rerio, Hamilton, 1822). The anticancer activity was evaluated using MTT assay; Mortality was observed at 12h and 24h for BST assay. The developing zebra fish embryos (ZFE) were incubated with various concentrations of extracts from blastula to Hatching period (extended up to 144h). SWME showed relatively lower activity against those cell lines (54.57 44.54 against MCF 7 and MDA MB 231 cellsthan URME 35.76 and 33.64 µg/ ml respectively). Brine shrimp nauplii were more sensitive to STME (LC_{50} 144.5 and > 3000 µg/ ml) than ULME (165.9, 158.4 µg/ ml on 12h and 24h respectively). No mortality and deformities in the developing embryos were observed in both extracts. But ULME affected hatching percentage at concentrations above 500 µg/ ml. Ten fold decrease was found on 1000 and 2000 µg/ ml than 0 – 500 µg/ ml up to 48h. Both extracts killed *Artemia* nauplii and cancer cells. But, ULME retarded the hatchability of ZFE without killing or teratogenicity. This study also confirms the need of further evaluation, about the identification of active biomolecule, and its selective toxicity on the mammalian cell lines in molecular level.

Keywords: Seaweeds, Brine Shrimp Cytotoxicity, Zebra Fish Embryos, Teratogenicity, Anti Cancer Activity.

INTRODUCTION

In the last decades an increasing interest was addressed towards the natural products, for their potential applications in biotechnology. Marine algae are well- known for their pharmaceutical values, anti-cancer activities either on preventing or curing as traditional medicines (Mayer AMS 2008). Richard (2006) emphasized that natural compounds should be evaluated using high-throughput analysis for the characterization at molecular and cellular level for. To evaluate the toxic influence, a bunch of test batteries has become more common (Castillo et al., 2000; Fochtman et al., 2000 and Lam *et al.,* 2005). Among them, the most used modeling species are brine shrimps (*Artemia* sp. Demaret *et al*., 1995) and *Danio rerio,* Hamilton, 1822 (Zebra fishes) (Rubinstein, 2003; Ponpornpisit *et al.,* 2011). In pharmacological sense, seaweeds are an everlasting source for novel bioactive molecules and help in discovering new active molecules for human welfare (Vasanthi 2011). It has been estimated that there are about 9,000 species of macroalgae broadly classified into three main groups based on their pigmentation *viz*., Phaeophyta, Rhodophyta, and Chlorophyta (or commonly named as brown, red and green algae), respectively (Khan, Rayirath et al. 2009). Many of the seaweeds as part thereof or as whole plant may be used as dietary materials for pharmacological purpose (Sachindra, Airanthi et al. 2010). These dietary substances have been included in clinical trials at various

statuses by many of Asian Universities (Table 1). In previous studies the zebra fish embryos have been used without any chorion treatment for the crude extracts of cyanobacteria (Oberemann et al., 1997), *Ecklonia cava* (Ko et al., 2011) and various marine macro algae (Guinea et al., 2012). Hence, the present study aims to investigate the toxic effects of methanolic extracts of *Ulva rigida* and *Sargassum woefii* collected from Mandapam coastal region, Tamil Nadu on brine shrimp (*A. salina)* nauplii (lethality), human cancer cells (cytotoxicity) and zebra fish embryo (teratogenicity).

Seaweed as dietary substances based clinical trials

Name of the seaweed	Condition	Developer	Clinical status	ID	Reference
Sargassum and other extracts	Asymptomatic Myeloma	Unicorn Pacific Corporation	Phase 2	NCT01096810	http://www.clinicaltrials.gov/ct2/show/NCT01096810?term=sargassum&rank=1
Combination seaweeds with probiotics	To normalize function of intestine	Korea Institute of Planning & Evalution for Technology of Food, Agriculture, Forestry & Fisherie	Recruiting	NCT01651741	http://www.clinicaltrials.gov/ct2/show/NCT01651741?term=seaweeds&rank=1
Not given exactly, but dietary seaweeds	Breast Cancer	University of South Carolina	Completed	NCT01663792	http://www.clinicaltrials.gov/ct2/show/NCT01663792?term=seaweeds&rank=2
Brown Seaweed	Prevention of breast cancer	University of South Carolina	Completed	NCT01204957	http://www.clinicaltrials.gov/ct2/show/NCT01204957?term=seaweeds&rank=3
Polyphenol-enriched Brown Seaweed	Reduction of carbohydrate absorption	InnoVactiv Inc	Completed	NCT00936754	http://www.clinicaltrials.gov/ct2/show/NCT00936754?term=seaweeds&rank=4
Same	Glycemic responses in sucrose absorption	InnoVactiv Inc	Completed	NCT01384110	http://www.clinicaltrials.gov/ct2/show/NCT01384110?term=seaweeds&rank=5
Undaria or Spirulina consumption	Reducing HIV viral load	University of South Carolina	Phase 1 and phase 2 completed	NCT01195077	http://www.clinicaltrials.gov/ct2/show/NCT01195077?term=seaweeds&rank=6

MATERIALS AND METHODS

Collection and extraction of the seaweeds

The seaweed samples were collected from Kovalam, Chennai (12.7925°N 80.2530°E), Tamil Nadu, India. The algae were identified as *Ulva rigida,* Sargassum *woefii*, The collected biomass were kept in ice-cold condition and brought to the laboratory immediately with precaution to avoid any damage. The algal samples were washed thoroughly with running tap water and distilled water to remove all the debris. They were shade dried, powdered and extracted with 80% methanol and evaporated using vacuum. The crude alcoholic extracts were stored at 4^{o}C for further testing.

Bioassay with *Artemia Salina*

The brine shrimp toxicity assay was adopted from Vanhaecke and Persoona (1984), with minor modifications. Briefly, commercially available, *Artemia sp.* was hatched out under standard conditions (with continuous aeration, diluent water (33 ± 0.5‰; pH 8.2 ± 0.2) was used for hatching as well for the test. The pH was adjusted with concentrated 0.1 M hydrochloric acid or 0.1 M sodiumhydroxide. This procedure was earlier standardized for a number of times in order to get accurate hatchability (Data not included) before the actual cpmmencementof expriment. Methanolic extract of *U. rigida* and *S. woefii* was dissolved in dimethylsulfoxide and used for testing at various concentrations (100 – 500 µg/ ml). In each test tube, 3 ml of sterilized diluent was taken and inoculated with twenty-five *Artemia* nauplii in five replicates. The nauplii were kept for 24h covered using black sheet at 28^{o}C and then the number of live *nauplii* was enumerated by using palm lens. The naupllii were considered dead if there is no movement of the appendages are observed within 10s (Carballo et al., 2002). Potassiumdichromate and appropriate concentration of DMSO were used as positive and negative controls in the bioassay.

Lethality concentration determination

The 24 h LC_{50} can be calculated by graphical interpolation. The percentage mortality between 5% and 95% were calculated from the average number of dead nauplii per concentration, and plotted on log- probit paper. A straight line is drawn through the points. The intersection of this line with the 50% mortality determines the 24h LC_{50} value.

Cytotoxicity assay (MTT) with human cancer cell lines cells

MCF 7 and MDA MB 231cells were sub cultured in DMEM media supplemented with 2mM L-glutamine adjusted with 1.5g/L Sodium bicarbonate and 90% fetal calf serum incubated at 37ºc in 5% CO2 incubator. Different concentrations of extracts (10, 20, 50, 100 and 200 µg/ ml) which were filtrated through (20-100µl), were added to the cancer cells and seeded in 96-well microtiter & incubated at 37ºc for 24 hours. At the end of the treatment, 20 µl of MTT [(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] was added to each well & the microtiter plate were incubated for 4hrs at 37ºc. Finally, acidic isopropanal (100 µl) was added to each well, after which optical absorbance was read at 595nm on multi well spectrophotometer plate reader. The percentage viability was calculated as follows:

Cell Viability = Optical density of samples/ Optical density of control X 100

Statistical Analysis

All experiments were repeated three times and data were recorded as mean ± SD (Standard Deviation) using SPSS statistical software. LC_{50} values were determined by linear regression using probit analysis.

RESULTS

Result for cancer cells cytotoxicity

The extracts tested were seemed to have high inhibitory active on estrogen dependent and independent human cancer cell lines MCF 7 and MDA MB 231 respectively than previously reported studies. URME showed IC_{50} value on MCF – 7 cell lines (34.64 µg/ ml) and slightly higher on MDA MB 232 cells (35.76 µg/ ml). SWME showed relatively lower activity against those cell lines (IC_{50} 54.57 and 44.54 µg/ ml against MCF 7 and MDA MB 231 cells respectively) than URME (Figure. 1).

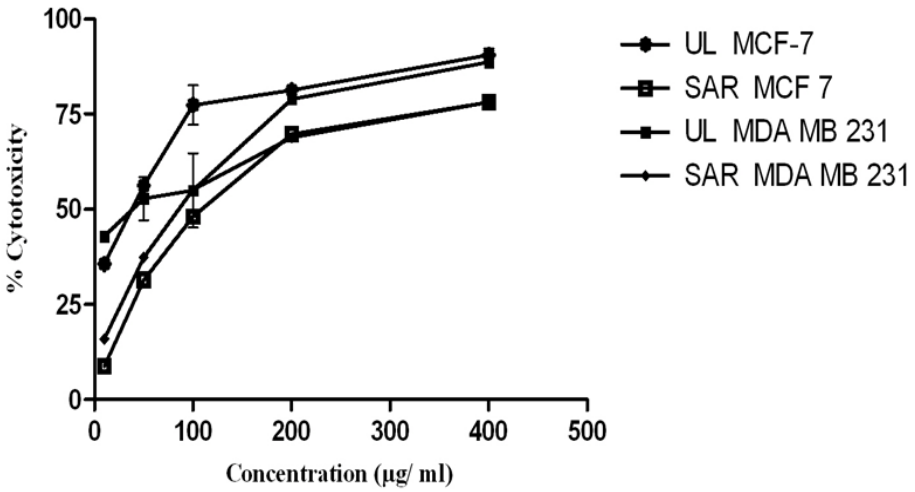

Figure 1: Anti proliferative effect of methanolic extract of *U. latuca* (UL) and *S. tenerrimum* (SAR) against MCF 7 and MDA MB 231 cell lines using MTT assay. SAR showed higher activity than UL. All data expressed as mean of triplicate± SE ($p<0.001$ using One Way ANOVA)**

Cytotoxicity on Brine shimp nauplii

The treated nauplii were examined on transforming to a petri-dish using dissection microscope for clear visualization on 12h and 24h after treatment. They were considered to dead, if no net or appendage movement within 10s (Ajuzie *et al*., 2007). Though, both of the extracts affected severely, no morbidity effect was found on treating either of the extract. Severe mortality of nauplii recorded on all concentrations of SWMR at 12h exposure as dose-dependent manner, whereas, URME showed at 500 µg/ ml, not on lower concentrations (Figure. 2). IC_{50} values for both extracts calculated using probit analysis (Table. 1). A steep increase in mortality on URME was seen between 100 to 200 µg/ ml (17 – 85 µg/ ml). Above 300 µg/ ml, URME showed nauplii mortality on dose- dependent manner. These results showed that the extracts had interrupted in the normal development of the *Artemia* naupili comparatively to the control.

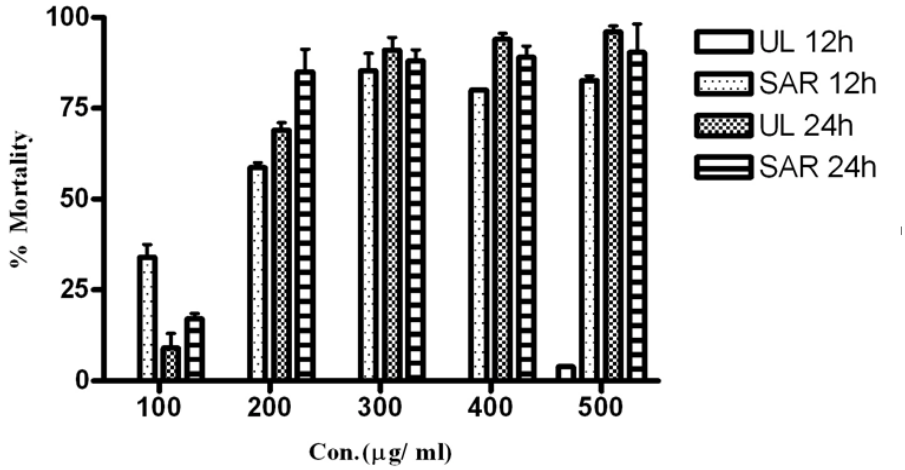

Figure 2: % cytotoxicity of the seaweed extracts against Brine shrimp (Potassiumdichromate was used as positive control, since the data are not met out with extract concentration, it was not shown in figure) *U. latuca* (UL) and *S. tenerrimum* (SAR) (p< 0.01 * using One Way ANOVA)**

Table 2: LC_{50} of the seaweed extracts against Brine shrimp. The brinshrimp were hatched and incubated with various concentrations of seaweed extracts. Mortality % observed at 12h and 24h. LC_{50} was calculated using probit analysis method (n=25 in triplicate).

	LC_{50} (µg/ ml)	
	12h	24h
Potassium dichromate	-	49.54
U. latuca	> 3000	165.9
S. tenerrimum	144.5	158.4

DISCUSSION

The assessment of phytoextracts in various models help scientific community in predicting novel drugs on biochemical or molecular level (Castillo *et al*.,2000; Fochtman *et al*., 2000 and Berry *et al*., 2004). In pharmacological scenario seaweeds remain, as an everlasting source for novel bioactive molecules as huge works had been reported daily (Davis and Vasanthi, 2011). Dietary seaweeds are main responsible in chemoprevention of a broad range of cancers, especially, breast cancer in women population (Kwon *et al*., 2007) due to estrogens and phytoestrogens content (Teas et al., 2009). *Artemia* nauplii has been documented as preliminary screening model for anti cancer activity of marine natural products (Carbello et al., 20….). Zebra fishes and their embryos are mimicking mammalian cells and making an interrelation network with invertebrate, vertebrate and mammalian genome .(Goldsmith, 2004). In the present study *Artemia* nauplii and zebra fish embryos were used to assess the toxicity *U. rigida* and *S. woefii* methanolic extracts which showed anticancer activity against human cancer cells (MCF 7 and MDA MB 232 cell lines). The nauplii and embryos were incubated with various concentration of extracts, since, that the aquatic exposure would be a feasible route of drug administration in aquatic organisms (Murphy and Zon, 2006). The cytotoxicity study against *Artemia*, revealed that the extracts has strong activity towards *Artemia* and both tested cell lines activity (Tab. 1).

The steep increase of % mortality on *A. salina* was seen between 100 and 200 µg/ ml (9.3 and 69.3 µg/ ml) on incubating with URME with dose dependency. In contrast, URME also showed a sudden rise in % inhibition on cancer cells. The difference between the last two concentration is 4.29% which, is nearly 10 times higher between 0 – 500 µg/ ml of extract concentrations, with delaying nearly 48h (two days). The IC_{50} for hatchability inhibition was calculated as 2630.26 µg/ ml using probit analysis. An ideal chemotherapeutic agent should render its toxicity stronger in cancer cells and lesser in normal cells (Oosterveld, Beldman et al. 2002). Targeted drug delivery substantially improves cancer therapy, because the drug can be addressed more effectively to the tumor and less to the normal tissues that results in fewer like-threatening side effects (Mensi *et al*., 2008). isolated molecules from seaweeds controlled the particular gene expression in various cells at various degrees (Chen, Yan et al. 2007);(Mahdi, Falkenberg et al. 2011). Previously, steroidal estrogens have showed cytostatic effects on the zebra fish embryos (Jones et al., 1960). Seaweeds are well- known for containing estrogens and phytoestrogens, this may be a reason to inhibit estrogen dependent (MCF 7) and estrogen independent cell lines (MDA MB 231). SWME showed higher anti cancer activity than URME on both cancer cell lines, This implies that SWME contains some specific molecules that focus on cancer cells and *Artemia* cells, but, not may be on zebra fish. Further studies are required to identify this responsible compound. URME showed toxicity against cancer cells and *Artemia* nauplii also. URME may reduce/ delay the particular protein synthesis or one of the developing stages embryos.

CONCLUSION

The present study aimed to evaluate the toxic nature of anticancer active seaweed methanolic extracts of *U. latuca* (UTME) and *S. tenerrimum* (STME) using cancer cells (MCF 7 and MDA MB 231), brine shrimp and Zebra fish embryos. STME showed higher activity on both cancer cell lines and brine shrimp nauplii than UTME. The mortality observed at 12h for STME; where, UTME showed mortality at 24h only. This study showed STME had selective toxicity towards cancer and brine shrimp cells. Further studies on these extracts would bring a useful “targeted drug” in near future.

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