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Evaluation of Preliminary Cytotoxic Potency of Aqueous and Ethanolic Extracts of *Sargassum wightii* Greville & *Stoechospermum marginatum* (C. Agardh) Kutzing

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Abstract Seaweeds are an abundant reservoir of bioactive compounds with prospective utilization in multidisciplinary domains, particularly in pharmacology. Algal samples were collected from the Southeastern coast of India. Extracts were prepared using water and ethanol as solvents. BSLA can be considered a propitious preliminary toxicity test, as the brine shrimp is acutely responsive to a variety of chemical substances. Trypan blue exclusion method can be described as a straightforward and cost-conscious method for the preliminary cytotoxic examination of natural extracts, using DLA cells and normal spleen cells, in which the degree of cytotoxicity is evaluated concerning the percentage of mortality of cell lines. A dose-dependent increase in the percentage of dead cells demonstrates cytotoxic competence of the extract. Conversely, a higher percentile of live cells reveals the selective cytotoxicity of the extract against cancer cells, which is a favorable attribute of anticancer drugs. The aqueous and ethanolic extracts of *Stoechospermum marginatum*, with an LC_{50} value of 460 and 420 µl/mL, respectively, had the highest cytotoxicity on the brine shrimp larvae compared to aqueous and ethanolic extracts of *Sargassum wightii*. Strikingly, the ethanolic extracts of both algae demonstrated the highest cytotoxic capability against DLA cells, with LC_{50} values of 30.61 and 10.75 µl/mL, while exhibiting comparatively low toxicity towards normal spleen cells. These inferences signify that the ethanolic extracts of *Sargassum wightii* and *Stoechospermum marginatum* possess bioactive compounds with promised anticancer properties and minimal toxicity against normal spleen cells, justifying intensified inspection for the isolation and characterization of the active constituents responsible for the remarked cytotoxic effects.

Keywords *Sargassum wightii*, *Stoechospermum marginatum*, BSLA, Trypan Blue Exclusion Method, DLA Cells, Normal Spleen Cells, Selective Cytotoxicity

1. Introduction

Nature has been functioning as an abundant dispensary of numerous maladies for millions of years. The curative capability of plants has been acknowledged by various traditional medicine practices throughout the World, hinging on the proficiency of their bioactive constituents to battle against illnesses [1]. Over the years, the eagerness to discover the therapeutic potentials of plants has built up remarkable advancement due to the bioactive compounds they own and their tendency to cause fewer side effects compared to artificial alternatives. Of them, marine algae have enticed a wealth of enthusiasm due to their potentially therapeutic attributes [2]. *Sargassum wightii* and *Stoechospermum marginatum* are two species of marine macroalgae, which belong to the family phaeophyceae, are rich with biologically active molecules, and can be showcased as promising novel anticancer drugs [3].

The scourge of cancer continues to be a primary contributor to global mortality with an estimated approximately 10 million fatalities per year [4]. Even though medical science has accomplished substantial advancements in cancer treatment modalities encompassing surgical approaches, chemotherapeutic agents, radiation therapies, etc. Still, the search for anticancer agents with high potential and minimum side effects remains a major challenge [5]. After cardiovascular disease, cancer is the second largest disease responsible for the most number of mortality globally. The increase in the number of scientific investigations on cancer-related illness and mortality rate points out the urgency of innovative remedies [6,7].

On this account, pharmaceutical corporations often showcase a propensity to myopically select synthetic chemical compounds as raw materials for developing anticancer therapeutics, overlooking enormous prospects of an overflowing bounty of bioactive compounds owned by nature. Consequently, a treasure trove of neutraceuticals derived from natural resources remains insufficiently investigated. In the exhaustive elucidation of nature's curative repertoire of counter to progression of cancer cells, assessment of cytotoxic potency of natural drugs functions as a crucial juncture [8].

Currently, a broad scientific community has dedicated its entire life to marine sources to find natural remedies alternative to chemical drugs against various detrimental diseases. It involves both qualitative and quantitative examinations of phytochemicals [9]. Marine macroalgae, colloquially named seaweeds, have recently evolved as a repository of natural compounds revealing a broad spectrum of biological activities, particularly comprising anticancer properties [10]. These marine life forms have been acclaimed as an abundant reservoir of secondary metabolites such as terpenoids, polyphenols, and sulphated polysaccharides. This ensemble of compounds exhibits the potential to combat carcinogenesis through diverse mechanisms encompassing impeding the cell cycle progession, initiation of pcd, and inhibition of angiogenesis [11].

The coastal belt of Indian subcontinent is characterized by a plentitude of two distinct species of brown algae named *Sargassum wightii* and *Stoechospermum marginatum*. *Sargassum wightii* commonly known as Gulf Weed, is native to the Indian Ocean and Western Pacific regions forming massive submarine forests also known as Sargassum beds by flourishing in coastal environments [12]. This particular marine algal species owns a diverse array of beneficial biochemicals having different therapeutic properties including antimicrobial, anti-inflammatory, anticancer, anticoagulant, antioxidant, neuroprotective, antimelanogenic, hypolipidemic and, hepatoprotective activities [13-18].

Concurrently, *Stoechospermum marginatum* is another significant marine algae and seems extensively along tropical and subtropical regions around the world. They are rich source of various bioactive compounds, such as polysaccharides, phlorotannins, pigments, and sterols. The marine environment, distinctively marine macro algae illustrates inimitable and predominantly untapped reservoir of biomolecules having anticancerous properties. If the quest for uncovering novel anticancerous drugs from macro algae becomes a success, it can make a revolution in the area of oncology medicine [19].

The capacity of a compound or extract to trigger cell mortality or suppress their multiplication is termed cytotoxicity. Evaluating the cytotoxic efficacy of natural derivatives is a crucial step in pinpointing promising candidates that could potentially combat cancer [20]. The brine shrimp lethality assay and trypan blue exclusion method using DLA cells are the two most prevalent preliminary cytotoxicity screening assays. In biology, the cytotoxic potential of a substance or compound is typically evaluated through in vitro and in vivo cytotoxicity tests. Between these two, in vitro assays are generally preferred over in vivo assays because of their cost-effectiveness, easily accessible materials, and diminished use of animal experimentation [21].

The brine shrimp lethality assay, employing larvae functions as a biological screening strategy for assessing the toxicity of various compounds. By analyzing the data acquired from the assay, the researchers can examine the lethal effects on these larvae, and they can also identify and isolate the compound of interest, which can complement novel drug development initiatives [22,23,24]. The trypan blue exclusion method is another widely accepted in-vitro model for assessing the cytotoxicity of natural anticancer agents by using Dalton's Lymphoma Ascites (DLA) cells [25]. In this model, the DLA cell line acquired from a spontaneous murine lymphoma is mixed with the trypan blue dye, and using a hematocytometer, viable and non-viable cells were counted. The percentage of mortality is calculated by dividing the number of dead cells by the total number of cells and multiplying by 100 [26,27]. Therefore, the present study has been designed to explore the pharmaceutical potential of two marine macroalgal species, *Sargassum wightii* and *Stoechospermum marginatum*, by highlighting their possible applications in cancer therapy and drug discovery.

2. Materials and Methods

2.1. Collection of Algal Samples

Fresh specimens of *Sargassum wightii* and *Stoechospermum marginatum* were collected from the

South-eastern coast of India. The specimens were thoroughly washed with seawater to remove epiphytes and then exhaustively washed with distilled water to remove salt content and other contaminants. The seaweed materials were subsequently dried in the shade for seven days and then stored for future use after finely ground in an electric mixer [28].

2.2. Extraction

Add 10 g algal powder to 100 ml of ethanol and water. The prepared samples were shaken in an orbital shaker for 7 hours and then centrifuged at 7000 rpm for 15 minutes. The filtrate was concentrated using a rotary evaporator after being filtered with Whatmann's Filter paper. At 4 ℃, the dried crude extracts were kept in airtight bottles. The extracts were dissolved in sterile distilled water and diluted to the required quantities before being tested [29].

2.3. Hatching of Artemia Eggs

Brine shrimp (*Artemia salina*) eggs were hatched in artificial seawater prepared by dissolving 38 g of salt in 1 L of distilled water, mimicking the natural habitat of the respective organism. After the incubation of 24 hours at room temperature, the larvae were collected using a pipette by gathering them to one side of the container with the help of a light source [29].

2.4. Brine Shrimp Assay

Various concentrations of test samples, such as 200, 300, 400, 500, 1000, and 2000 μg/mL were prepared. Potassium dichromate was employed as the positive control, while 0.5% DMSO + seawater without plant extract was the negative control. Ten nauplii were transferred to each tube containing 4.5 ml of brine water and 0.5 ml of the respective test solution (and controls). After 24 hours of incubation under light, the number of surviving larvae and the percentage of mortality were calculated [30]. The percentage of mortality was calculated using the equation:

$$\%\ \text{of Mortality} = \frac{\text{No. of dead nauplii}}{\text{No. of dead nauplii} + \text{No. of live nauplii}} \times 100$$

It is ensured that each treatments, positive, and negative controls were performed for three times [22,23]. The percentage of mortality values was determined as the average of three independent replicates, with standard deviations being provided to exhibit variability. LC_{50} was determined by plotting mean values of the percentage of mortality against concentrations in Microsoft Excel using regression equations. LC_{50} values greater than 1 mg/ml were considered harmless [31].

2.5. *In vitro* Cytotoxic Assay Using DLA Cells

The trypan blue exclusion method enables a short-term study on the in vitro cytotoxicity of *Sargassum wightii* and *Stoechospermum marginatum* on DLA cells. Tumor cells were obtained from the peritoneal cavity of tumor-bearing mice, and washed with phosphate-buffered saline three times. Various concentrations (12.5, 25, 50, 100, 200 μg/mL) of the test sample were prepared by dissolving in DMSO and volume was brought up to 1 ml using PBS. Viable cell suspension was transferred into each test tube. The test tube containing only cell suspension was considered as a control. After the incubation of 3 hours at room temperature, 0.1 ml of 1% trypan blue dye was added to the cell suspension. After the incubation of 3 hours at room temperature, 0.1 ml of 1% trypan blue dye was added to the cell suspension stayed for 3 minutes, and loaded to hemocytometer. The numbers of stained and unstained cells were counted. The percentage of mortality was estimated using the formula:

$$\%\ of\ Mortality = \frac{No. of\ dead\ cells}{No. of\ dead\ cells + No. of\ live\ cells} \times 100$$

Each treatments and control were conducted in triplicates [22,23]. The value, % of mortality was calculated as the mean of three independent replicates, with standard deviation. IC_{50} values were obtained from the best-fit line plotted concentration verses percentage lethality using regression analysis [32].

3. Results and Discussion

3.1. Brine Shrimp Assay

Brine shrimp lethality assay can be utilized as an effective general tool for determining the LC_{50} values of active constituents in a natural extract, offering rapidity, reliability, and cost-effective methods [33]. Table 1 shows the invitro cytotoxic effects of aqueous and ethanolic extracts of *Sargassum wightii* and *Stoechospermum marginatum* towards brine shrimp nauplii at different concentrations. Both algae exhibited an LC_{50} value below 1000 μg/mL. Aqueous and ethanolic extracts of *Stoechospermum marginatum* demonstrate high activity against brine shrimp with an LC_{50} value of 460 and 430 μg/mL, respectively. Ethanolic extract of *Sargassum wightii* was found to be moderately toxic, while *Sargassum wightii* (1000 μg/mL) water extract did not show significant cytotoxic potency characterized by a low LC_{50} value. *Stoechospermum marginatum* shows comparatively higher cytotoxicity than *Sargassum wightii*. Ethanolic extracts have better activity than aqueous extracts. The finding reveals that the percentage of mortality has a positive correlation with the increase in concentration. The reference standard potassium dichromate exhibited an LC_{50}

value of 225 μg/mL, while no mortality was observed in the negative control. As per the findings of Mayer et al. [23], an extract is considered toxic only if its LC_{50} value is lower than or equal to 1000 μg/mL. By Ara et al. [34], cytotoxic potency may be indicative of the presence of anti-tumor compounds. The findings of Sasidharan et al. [29] could be used as a powerful technique to screen the pharmacological potential of various Plant-derived compounds based on the principle that pharmacological responses are triggered by administering compounds at concentrations below their toxic limit. Mclaughlin [35] points out that a substance that exhibits toxicity might demonstrate interesting medicinal effects at lower concentrations. Even though brine shrimp assay is not sufficient to reveal the complete mechanism of action behind the lethality, it is enough to monitor the pharmacological efficacy of a natural extract illustrated by Krishnaraju et al. [36]. As per the opinion of Waghulde et al. [37], the main reason for cytotoxicity is the presence of secondary metabolites.

3.2. *In vitro* Cytotoxic Assay Using DLA Cells

The current investigation uses the trypan blue exclusion method to further investigate the preliminary cytotoxic potential of both algae as a continuation of the brine shrimp lethality assay.

It proved that ethanolic extracts of given algae show better activity when compared to water extracts. Therefore, in the second stage of lethality assessment, only the ethanolic extracts were subjected to trypan blue exclusion assay. Trypan blue is a blue-colored dye that can penetrate dead cells. Live cells possess an intact cell membrane, which excludes the dye. At the same time, the porous cell membrane of dead cells is permeable to the dye, thus becoming a blue color. When observing under a haemogram, the count of dead and live cells could be calculated [38]. Table 2 presents the results of in vitro cytotoxicity assay against DLA cells. The given data demonstrates that the concentration of the test sample and the percentage of cell death are directly proportional. It suggests that when concentration increases, the percentile of mortality also increases at a corresponding rate. Ethanolic extract of *Stoechospermum marginatum* (10.75 μg/mL) exhibited the highest value of IC_{50}. *Sargassum wightii* ethanol extract also displayed significant activity with an IC_{50} value of 39.6 μg/mL. U.S National Cancer Institute developed a criteria to determine the cytotoxicity of a crude extract. According to them, extracts with an IC_{50} value lower than 20 μg/mL can be considered toxic. Others are not [39]. Previous studies have already proved that brown algae possess anti-proliferative properties due to sulfated polysaccharides and fucoidans presented in them [40]. In addition, other polysaccharides, monosaccharides, proteins, phenols, coumarins, fatty acids, and quinines can also be novel sources of anticancer drugs coupled with chemotherapeutic drugs, which need further investigation [41]. The findings of Begum et al. [42], disclosed the role of alcohols, alkenes, and aliphatic functional groups in seaweed extracts as inhibiting agents of cancer cell proliferation. When ethanol is used as a solvent while preparing sample extract, it has additional benefits than water extracts. Ethanol can dissolve polar and non-polar compounds. It includes certain compounds which play significant roles in various biochemical reactions, especially in cytotoxic activities. Ethanol-extracted compounds can readily penetrate cells by breaking the intactness of the cell wall. For this reason, ethanol extracts show better results than aqueous extracts. *Sargassum wightii* is one of the richest sources of a broad spectrum of chemical compounds responsible for anticancerous properties. Abdullah et al. [43] authenticated the biocompatibility of ethanolic extract of *Euphorbia inarticulata* as an anticancer agent on normal and MRC-5 cells. *Sargassum wightii* and *Gracilaria edulis* seem to have notable anticancerous properties with negligible impact on normal cells, paving the way for the discovery of new anticancer drugs of natural origin [44]. Observations of Vaikundamoorthy et al. [45], indicate that polysaccharide fractions isolated from crude polysaccharides of *Sargassum wightii* express anticancer effects by initiating apoptosis of breast cancer cells. According to EL-Rafi et al. [46], high content of phenolic and flavonoid compounds accounts for the lowest IC_{50} value of marine algae against cancer lines. Methanolic extract of brown algae *Nizamuddinia zanardinii* possesses an inhibitory effect on uterine and pancreatic cell lines. The study also proved that the algae contain a notable amount of fucoxanthin [47].

Currently, there is no existing popular and potential anticancer drug developed from *Sargassum wightii* and *Stoechospermum marginatum*, regardless of countless studies conducted on the above algae over the years. The main intention of the present investigation is to reveal the yet uncompromised pharmacological secrets of these two algae from the beginning.

Table 1. Result of brine shrimp lethality assay

Test sample	% of mortality under different concentrations (μg/ml)							LC_{50} (μg/ml)	Toxicity profile
	100	200	300	400	500	1000	2000		
Sargassum wightii Aqueous extract	3.33±3.33	6.66±3.33	23.33±3.33	26.6±3.33	46.6±6.66	50±0	56.66±3.33	1000	Toxic
Sargassum wightii Ethanolic extract	3.3±3.33	6.66±3.33	13.33±3.33	26.6±3.33	53.3±3.33	66.6±6.66	83.33±3.33	660	Toxic
Stoechospermum marginatum Aqueous extract	10±5.77	16.6±3.33	33.3±3.33	36.6±3.33	53.3±3.33	63.3±3.33	83.3±6.66	460	Toxic
Stoechospermum marginatum Ethanolic extract	6.6±3. 33	13.3±3.33	20±3.33	46.6±3.33	56.6±3.33	76.6±3.33	90±6.66	420	Toxic
$K_2Cr_2O_7$	23.3±3.33	40	60±5.77	73±6.66	83±6.66	100±0	100±0	225	Toxic
DMSO+Brine Water	0	0	0	0	0	0	0	0	Non Toxic

% of Mortality values are represented as mean ± standard deviation (SD) from three replicates (n=3).

Table 2. Result of invitro cytotoxicity test using DLA cells

Test sample	% of mortality under different concentrations (μg/ml)					IC_{50} (μg/ml)	Toxicity profile
	12.5	25	50	100	200		
Sargassum wightii Ethanolic extract	15±1.5	32±1.4	58.3±2.8	81.7±2.4	100±0	39.61	Toxic
Stoechospermum marginatum Ethanolic extract	49.5±3.1	68±3.3	78.8±2.1	88.7±1.4	100±0	10.75	Toxic
PBS control	0	0	0	0	0	0	Non toxic

% of Mortality values are represented as mean ± standard deviation (SD) from three replicates (n=3).

4. Conclusions

The outcomes of the present investigation cements the findings of previous studies on the cytotoxic potency of *Sargassum wightii* and *Stoechospermum marginatum*. Both algae possess bioactive compounds with prospective biomedical implementations that have not been efficiently exploited by medical sciences. Expanded research is necessary to identify, isolate, and characterize the compounds responsible for cytotoxicity, elucidate the mechanism of action, and evaluate their selectivity towards normal and cancer cells. This study strongly supports the solid framework of knowledge on the pharmacological efficacy of seaweeds and also lays the foundation for future drug development initiatives.

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Conflict of Interest

The authors state no conflict of interest.

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