

VOLUME 8 ISSUE 1 2022

ISSN 2454-3055



**INTERNATIONAL
JOURNAL OF
ZOOLOGICAL
INVESTIGATIONS**

***Forum for Biological and
Environmental Sciences***

Published by Saran Publications, India



International Journal of Zoological Investigations

Contents available at Journals Home Page: www.ijzi.net

Editor-in-Chief: Prof. Ajai Kumar Srivastav

Published by: Saran Publications, Gorakhpur, India



ISSN: 2454-3055

Phytochemical Profiling and Anticancer Activity of Seaweed *Enteromorpha intestinalis* Extracts Against A375 Cell Line

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Received: 4th December, 2021; **Accepted:** 20th January, 2022; **Published online:** 29th January, 2022

<https://doi.org/10.33745/ijzi.2022.v08i01.022>

Abstract: Nowadays several marine sources like seaweeds are used for anticancer activity against certain types of cancers. Brown algae – *Fucus* spp. and Red algae – *Laminaria* sp. are used to treat breast cancer and colorectal cancer. Green algae- *Ulva fasciata* and Brown algae- *Undaria pinnatifida* are used in treating other cancers. It has been known that consumption of various types of seaweed is responsible for the low incidence of cancer in Asian countries whose inhabitants traditionally consume a high level of seaweeds. Seaweed chemical profiles have demonstrated that they are rich in terpenoids, alkanoids, polyphenols, steroids, pigments and polysaccharides. These metabolites have pharmacological activities including cancer therapy. In this study, the Gutweed- *Enteromorpha intestinalis* was used against A375 cell lines (Skin cancer). The qualitative analysis showed the presence of anticancer properties in this seaweed. GCMS analysis were done for the compound analysis of the seaweed and the highest compound showed the anticancer activity. MTT Assay showed some good control over the skin cancer cells which can be used in future.

Keywords: Seaweed, Gutweed, *Enteromorpha intestinalis*, Marine source, Anticancer activity

Citation: Indumathi R. and Vasuki R.: Phytochemical profiling and anticancer activity of seaweed *Enteromorpha intestinalis* extracts against A375 cell line. Intern. J. Zool. Invest. 8(1): 177-184, 2022.

<https://doi.org/10.33745/ijzi.2022.v08i01.022>

Introduction

Seaweeds or marine macro algae are the group of plants that live either in marine or brackish water environment. Seaweeds (marine algae) are extensively used as functional foods and medicinal herbs and have a long history of use in Asian countries. Like the land plants seaweeds contain photosynthetic pigments and with the help of sunlight and nutrient present in the seawater, they photosynthesize and produce food. Seaweeds are found in the coastal region between high tide and

low tide and in the sub-tidal region up to depth where 0.01% photosynthetic light is available (Salmerón-Manzano *et al.*, 2020). Seaweeds are known as “ecosystem engineers” due to their critical roles in marine environments, where they modulate the supply of resources to other species and alter the physical state of the surrounding environment, including sediments and water flow. The seaweeds are classified into three main groups that is green algae (Chlorophyta), brown

algae (Phaeophyta) and red algae (Rhodophyta).

More than 15,000 seaweed species are found in the oceans of the globe. About 6000 species of seaweeds have been identified and are grouped into different classes (Fonnegra, 2007). Seaweeds grow abundantly along the Indian coast line particularly in the rocky shore regions. Rich seaweed beds occur around the south-east coast of Tamil Nadu particularly in Thoothukudi and Tirunelveli coast. Seaweeds are important for maintaining local biodiversity and create a protective environment for numerous invertebrate species (Joppa *et al.*, 2011). The minerals like sodium, calcium, magnesium, potassium, chlorine, sulphur, phosphorus and micronutrients such as iodine, iron, zinc, copper, selenium, molybdenum, fluoride, manganese, boron, nickel and cobalt are plenty in different species of seaweeds. Apart from that it is a good source of iodine generally highest in brown seaweed. The calcium and protein content varies from species to species but has low-fat content. Generally, green and red seaweeds have high protein content (up to 30%), whereas lower (up to 15%) was found in brown seaweeds.

These seaweeds are used in many maritime countries particularly Asia, Japan, Korea and China as a source of food, industrial applications and for fertilizers. Seaweeds like *Ulva* sp., *Enteromorpha* sp., *Caulerpa* sp., *Codium* sp., *Monostroma* sp., *Sargassum* sp., *Hydroclathrus* sp., *Laminaria* sp., *Undaria* sp., *Macrocystis* sp., *Porphyra* sp., *Gracilaria* sp., *Euclima* sp., *Laurencia* sp. and *Acanthophora* sp. are used in the preparation of soup, salad and curry. Some of the seaweeds are also taken in dried form (Pimm *et al.*, 2014). Seaweeds are used for complementary food to the farm animals such as cattle, poultry and other farm animals. Applying seaweeds/seaweed extracts in modern agriculture leads to increased seed germination rates, improved plant development, elevated defence against pathogens and pests and protection against nutrient deficiency and environmental stresses including salinity, cold or drought. Seaweed builds resistance to disease by

ensuring a complete balance of micronutrients. They also assist in decreasing the rate of mastitis and cow fever (Grover *et al.*, 2002).

Different types of seaweed extracts have been experimentally proved to reduce or to destroy the effectiveness of cancer (Kunle *et al.*, 2012). Seaweeds are rich in bioactive compounds not present in terrestrial plants and food sources. These novel substances such as fucoidan, alginate, fucoxanthin, polyphenols etc., may confer unique health-promoting properties with the potential to be exploited in human health applications including cancer prevention and treatment (Gurib-Fakim, 2006). Several studies have reported that compounds extracted from seaweeds may be effective anticancer agents (Salmerón-Manzano and Manzano-Agugliaro, 2020). Cancer is a group of diseases characterized by uncontrolled cell growth and spread. The incidence of cancers has increased steadily in both developed and developing countries in recent years. Cancer remains one of the main killer diseases of mankind; its treatment and prevention have attracted efforts from all corners of the society. Skin cancer is a major environmental interface for the body, which accidentally or occupationally gets exposed to a number of chemical mutagens and carcinogens. Skin cancer represents a major and growing public health problem (Adoum, 2009). 80% of skin cancers results from basal cell carcinoma (BCC); another 16% are squamous cell carcinomas (SCC), and 4% are melanomas (Tchuifon *et al.*, 2017).

UVB radiation is a complex carcinogen and causes excessive generation of ROS thus resulting in an oxidative stress in the skin (Adoum, 2009). Studies have shown that UVB radiation produces a variety of adverse effects that includes DNA damage, mutations in key regulatory Gene's, inflammation, immunosuppression, photo-sharing and skin cancer. In 2017, out of 5.4 million cases of non-melanoma skin cancer, only 3.3 million people were treated in the US (Tchuifon *et al.*, 2017). According to the recent research on skin cancer trends in Asia, it was found that skin cancer

rates among the fairer-skinned were approximately three times higher than those who generally have darker complexions. In India, skin cancers constitute about 1-2% of all diagnosed cancers. Basal cell carcinoma is the commonest form of skin cancer worldwide, but various studies from India have consistently reported SCC as the most prevalent skin malignancy (Adoum, 2009).

Although complete data of incidence is not available, various cancer registries in India reported cumulative incidence of skin cancer varying from 0.5 to 2 per 100000 population. Melanoma of the skin is the 19th most commonly occurring cancer in men and women. Non-melanoma skin cancer is the 5th most commonly occurring cancer in men and women. Although, the incidence of skin cancers in India is lower as compared to the western world, because of a large population, absolute number of cases is estimated to be significant. In India, death rate of skin cancer is 0.43% and stands in 166th rank (Gurib-Fakim, 2006).

Enteromorpha intestinalis (*Ulva intestinalis*) is a green alga, known as Gutweed and Grass Kelp, in the division Chlorophyta, order Ulvales and family Ulvaceae, and occurs naturally worldwide. *Ulva intestinalis* is a conspicuous bright gray-green seaweed, consisting of inflated irregularly constricted, tubular fronds that grow from a small discoid base. *Enteromorpha* often grows on the rocks of intertidal zones, on the gravel of mud beaches, widely distributed in the oceans of the world. Identification is heavily reliant on cell detail and cell arrangement, in addition to gross morphology, but complicated by the fact that the morphology of a single species can vary in response to environmental conditions. In general, this alga grows as a tube of 1-2 mm length (though it can reach up to 2 cm), composed of irregularly arranged cells as a single layer. The alga blade is smooth at the early stages, while later it is wrinkled and also changes its colour from dark green to light green or yellow green. Branching occurs near the hold fast, which is small and narrow (1mm) (Gurib-Fakim, 2006).

Ulva spp. have been cultivated in many Asian countries, including Japan, Korea, India and Indonesia. In the absence of appropriate bacterially derived signals, germ cells of the genus *Ulva* develop into 'atypical' colonies consisting of undifferentiated cells with abnormal cell walls (Kunle *et al.*, 2012). The nutritional content of *Ulva intestinalis* has been reported as 19.5% protein, 0.3% fat, 58.1% soluble carbohydrates, 6.8% insoluble carbohydrates, and 15.2% ash including essential minerals. The minerals include calcium, phosphorus, iron, sodium, and potassium, at 910, 800, 35, 570, and 3500 mg kg⁻¹ dry weight, respectively (Kunle *et al.*, 2012). In this study, the Gutweed- *Enteromorpha intestinalis* was used against A375 cell lines (Skin cancer). The qualitative analysis showed the presence of anticancer properties in this seaweed. Compound separation using GCMS analysis of this seaweed were done where the highest compound showed the anticancer activity. MTT Assay showed some good control over the skin cancer cells which can be used in future.

Materials and Methods

Collection of samples:

Seaweed material was collected from Rameshwaram, India. The collected sample was stored in plastic bags and transported to the laboratory under ice condition. The samples were initially washed thoroughly with sea water to remove sand and any adhering substance and then washed thoroughly with fresh water to remove salts, and stored at -20 °C until compound extraction.

Extraction of selected seaweed species:

After washing with distilled water for several times, the seaweed sample was again washed with 5% ethanol to remove any epiphytes or any salts. The sample was subjected to air drying under the shade. After drying they were ground by an electrical mixer until they became a powder. Then the powdered sample were stored in a dark place, and subjected to extraction method. Extraction of

powdered seaweed sample was done using aqueous ethanol. Aliquots of 25 g of the powdered seaweed sample were soaked in 250 ml of the solvent for 72 h. Later the soaked sample was filtered, and concentrated under reduced pressure using a rotary evaporator and kept stored at 20 °C (Jones and Kinghorn, 2006).

Phytochemical Analysis of Enteromorpha Intestinalis extract (Jiang et al., 2015):

Test for alkaloids:

Mayer's Test: To the extract, 2 ml of Mayer's reagent was added; formation of reddish brown precipitate indicated the presence of alkaloids.

Test for saponins:

To 1 ml of the extract, 5 ml of water was added and the tube was shaken vigorously. Copious lather formation indicated the presence of saponins.

Test for tannins:

To the extract, ferric chloride was added, formation of a dark blue or greenish black color showed the presence of tannins.

Test for cardiac glycosides:

Keller-Killani test: To 1 ml of the extract added 2 ml of glacial acetic acid containing a drop of FeCl_3 . Equal volume of conc. H_2SO_4 was added from the sides of the tube. A brown colour ring indicated the presence of cardiac glycosides.

Test for flavonoids:

Alkaline reagent test: Extract was treated with 10% NaOH solution; formation of intense yellow colour indicated presence of flavonoid.

Test for phenols:

Lead acetate test: The extract was taken and 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicated the presence of phenolic compounds.

Test for steroids:

1 ml extract was dissolved in 10 ml of chloroform and equal volume of concentrated H_2SO_4 was

added from the side of test tube. The upper layer turned red and H_2SO_4 layer showed yellow with green fluorescence. This indicated the presence of steroid.

Test for terpenoids:

Salkowski test: 5 ml of extract was mixed with 2 ml of chloroform, and concentrated sulphuric acid was carefully added to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

Test for Quinones:

The extracts were treated separately with alcoholic KOH solution. Appearance of colors ranging from red to blue indicated the presence of quinones.

Test for Proteins:

Ninhydrin test: The extract was taken and few drops of freshly prepared Ninhydrin reagent was added and heated. The appearance of pink or purple colour indicated that the presence of proteins, peptides or amino acids.

Thin Layer Chromatography (TLC):

The extract was subjected to thin layer chromatography (TLC) as per conventional method using silica gel 60F254, 5x3 cm (Merck) were cut using TLC cutter. Plate markings were made with soft pencil. Glass capillary tubes were used to spot the extract in TLC plates. Solvent system was tested for the separation of bioactive components. In the TLC chamber the solvent system viz. butanol: acetic acid: water was used. After pre-saturation with mobile phase for 30 min the plate was kept inside the chamber and the elution was performed using above mentioned solvent system. After completion of the elution the plate was dried and subjected to visualize under UV chamber and sprayed using different spray reagents (Sherma and Fried, 2003). R_f values were determined by using following formula:

$R_f = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$

GCMS analysis:

GCMS analysis is a common confirmation test. It is the best used to make an effective chemical analysis. This analysis will provide a representative spectral output of all the compounds that get separated from the sample. The first step of GCMS was started by injecting the sample to the injected port of the Gas chromatography (GC) device. The GC instrument vaporized the sample and then separated and analyzed the various components. Each component ideally produced a specific spectral peak that may be recorded on a paper chart electronically. The time elapsed between elution and injection is called the "retention time". Differentiate between some compounds was identified using the retention time. The peak was measured from the base to the tip of the peak.

MTT assay:

Anticancer Activity studies using MTT:

The MTT system is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The MTT method is simple, accurate and yields reproducible results. The key component is (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT, which is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. This water insoluble formazan can be solubilized using DMSO, acidified isopropanol or other solvents (Pure propanol or ethanol). The resulting purple solution is spectrophotometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material.

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using respective media containing 10%

FBS. To each well of the 96 well microtiter plate, 100 μ l of the diluted cell suspension (50,000 cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 μ l of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37 C for 24h in 5% CO₂ atmosphere. After incubation the test solutions in the wells were discarded and 100 μ l of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The plates were incubated for 4h at 37°C in 5% CO₂ atmosphere. The supernatant was removed and 100 μ l of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC₅₀) values is generated from the dose-response curves for each cell line:

$$\% \text{ Viable cells} = \frac{\text{Total number of viable cells/ml of aliquot}}{\text{Total number of cells /ml of aliquot}} \times 100$$

The half maximal inhibitory concentration (IC₅₀) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or component of a process, i.e. an enzyme, cell, cell receptor or microorganism) by half.

The IC₅₀ of a drug can be determined by constructing a dose-response curve and examining the effect of different concentrations of antagonist on reversing agonist activity. IC₅₀ values can be calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the agonist. IC₅₀ values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve.

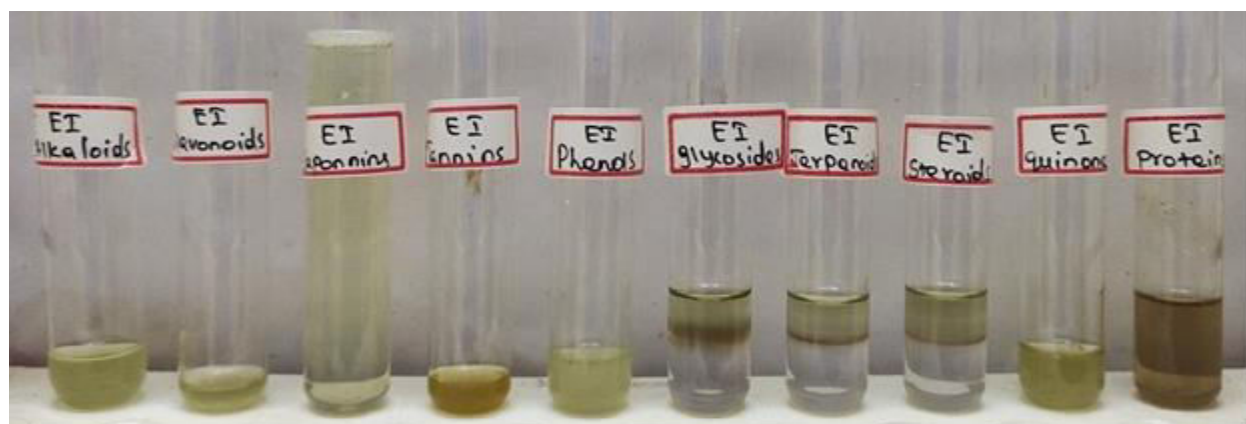


Fig. 1: Phytochemical analysis of *Enteromorpha intestinalis* extracts.

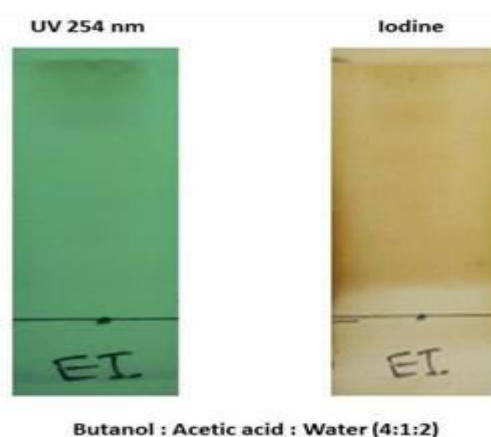


Fig. 2: TLC of *Enteromorpha intestinalis* extract.

Results and Discussion

The phytochemical characteristics of *Enteromorpha intestinalis* tested are summarized in Figure 1 which revealed the presence of medically active phyto-compounds. It was determined that Alkaloids, Saponins, Cardiac glycosides, Flavonoids, Phenols, Steroids, Terpenoids are present and Tannins, Quinones, Proteins are absent. Flavonoids and Phenols are free radical scavengers that prevent oxidative cell damage, and have strong anticancer activities and induce mechanism that affect cancer cells and inhibit tumor invasion. (Pourmorad *et al.*, 2006; Ugwu *et al.*, 2013; Rafat *et al.*, 2008). Saponins are believed to react with the cholesterol rich membranes of cancer cells, thereby limiting their growth and viability (Roa *et al.*, 1995). Hence in this study also, there are flavonoids, phenols and

Saponins present which showed the anticancer activity.

The *Enteromorpha intestinalis* extract was tested for the thin layer chromatography technique (TLC) (Fig. 2). All plates were visualized directly after drying and with the help of UV at 254 nm and 366 nm in UV TLC viewer. The R_f value of the different spots that were observed was calculated. TLC of *Enteromorpha intestinalis* having R_f values of 0.46, 0.63, 0.80, 0.93, respectively (Table 1) when a solvent phase of Butanol: Acetic acid: water (4:1:2) was used.

GC-MS analysis:

The Gas chromatography showed the relative concentrations of various compounds getting eluted as a function of retention time. The Mass spectrometer analyzed the compounds eluted at

Table 1: Thin layer chromatography of *Enteromorpha intestinalis* extract

Extract	Solvent system	Number of spots	Rf value
EI	Butanol: Acetic acid: Water (4:1:2)	04	0.46; 0.63; 0.80; 0.93

Table 2: Anticancer activity of *Enteromorpha intestinalis*

		Concentration Unit: μ G						
EI	BLANK	UNTREATED	3.12	6.25	12.5	25	50	100
Reading 1	0.002	1.258	1.237	1.19	1.065	0.948	0.412	0.153
Reading 2	0.002	1.27	1.252	1.172	1.088	0.931	0.433	0.134
Reading 3	0.004	1.266	1.24	1.181	1.075	0.94	0.413	0.142
Mean	0.0027	1.264666667	1.243	1.181	1.076	0.939666667	0.419333333	0.143
Mean OD-Mean B		1.262	1.240333333	1.178333333	1.073333333	0.937	0.416666667	0.140333
STANDARD DEVIATION		0.006110101	0.007937254	0.009	0.011532563	0.008504901	0.011846237	0.009539
Viability %		100	98.28314844	93.37031167	85.05018489	74.24722662	33.01637612	11.11992
							IC 50 VALUE= 32.62	

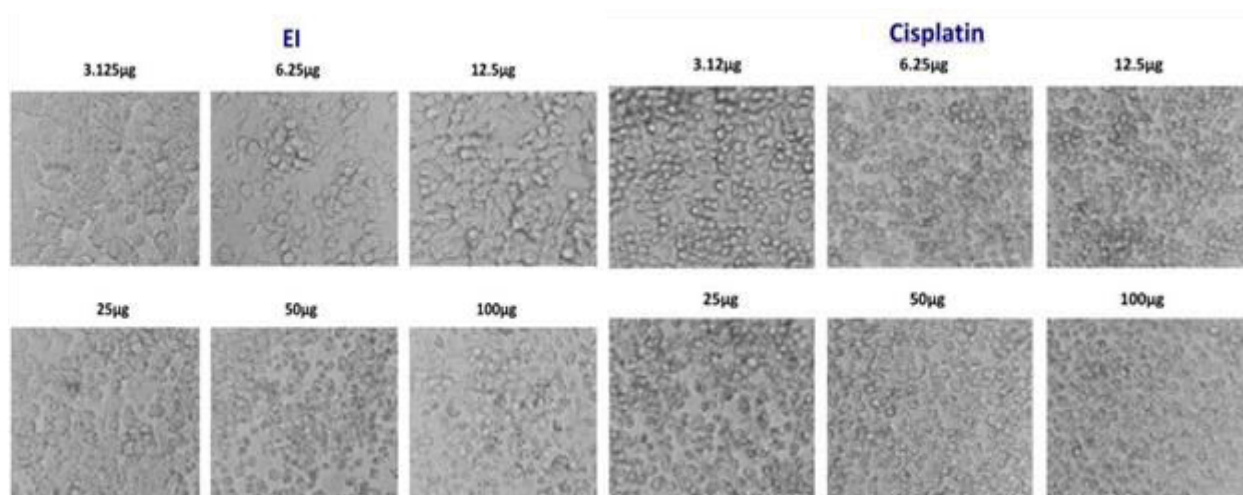


Fig. 3: MTT assay.

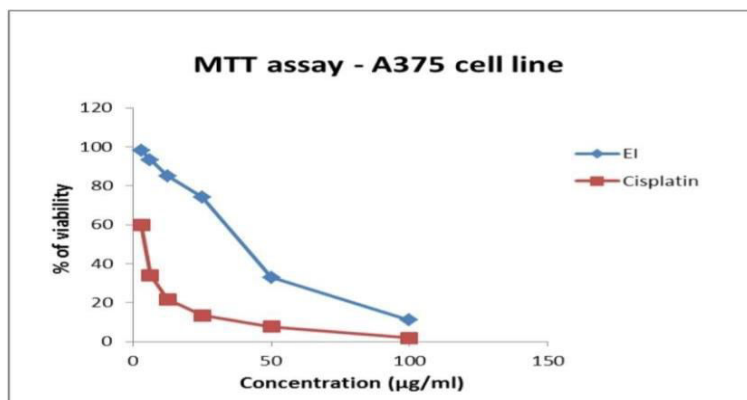


Fig. 4: MTT assay –A375 cell line.

different times to identify the nature and structure of the compounds. 43 components were identified with *Enteromorpha intestinalis* sample. Thus, the identification of bioactive compound in *Enteromorpha intestinalis* was done by GCMS analysis which showed the presence of 43 compounds. Out of these compounds 9,12-Octadecadienic acid (Z, Z), 9-Octodecadinamide showed anticancer activity (Rani *et al.*, 2009; Ponnammam and Manjunath, 2012; Uma *et al.*, 2009). Thus these compounds are present in the sample, which gave good results for the anticancer activity.

MTT assay:

In vitro anticancer activity was determined using A375 cell lines. MTT Assay was done following exposure of cells to various concentration of seaweed (3.12, 6.25, 12.5, 25, 50, 100 µg/ml) for a time span of 24 h (Table 2; Figs. 3, 4).

The IC₅₀ value of the given sample (*Enteromorpha intestinalis*) and standard (Cisplatin) is 32.62 µg/ml and 3.16 µg/ml, respectively. As the results, the IC₅₀ value of the sample (*Enteromorpha intestinalis*) is more than the standard (cisplatin) thus, this seaweed sample could successfully be used in future for the anticancer activity against skin cancer.

Conclusion

In this study, analysis of the *Enteromorpha intestinalis* extract revealed the presence of phytochemicals such as Alkaloids, Saponins, Flavonoids which have strong anticancer activity. TLC of the extract showed high activity. In GCMS analysis, 43 compounds were identified with this *Enteromorpha intestinalis* sample and from those compounds 9,12-octadecadienic acid(Z,Z), 9-octadecadinamide showed anticancer activity. In MTT Assay, the IC₅₀ value of the sample (*Enteromorpha intestinalis*) was more and thus it is concluded that this seaweed sample could successfully be used in future for the anticancer activity against A375 cell lines (skin cancer).

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