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Screening of phytochemicals and *in vitro* anticancer activity of green seaweeds *Ulva lactuca* using A549 cell line

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Abstract

The purpose of this study is to examine the qualitative, quantitative, and *in vitro* anticancer properties of *Ulva lactuca*. To achieve this, we utilized five solvent extracts, and our findings revealed that both methanolic and aqueous extracts contained phytochemical compounds such as alkaloids, phenols, flavonoids, tannins, saponins, terpenoids, steroids, carbohydrates, glycosides, and proteins. However, the ethanol, ethyl acetate, and chloroform extracts of *Ulva lactuca* lacked flavonoids. Moreover, the methanolic extract of *Ulva lactuca* exhibited higher levels of alkaloids, total phenols, terpenoids, steroids, and tannins than the other extracts, as determined by quantitative analysis. Subsequently, we assessed the anticancer properties of the methanolic extract using the lung cancer (A549) cell line and observed potent cytotoxic activity via MTT assay. In light of these results, it is evident that *Ulva lactuca* seaweed extracts hold promise as an effective alternative in cancer treatment.

(Keywords: *Ulva lactuca*, qualitative, methanolic extract, MTT assay, lung cancer (A549) cell line)

Introduction

Marine organisms thrive in intricate environments under harsh conditions and have developed the ability to produce unique secondary metabolites absent in other organisms [1] [2]. Similarly, macroalgae seaweeds, like medicinal plants, contain both organic and inorganic compounds that have medicinal properties beneficial for human health [3]. Seaweeds are a rich source of macronutrients, plant hormones, pigments, amino acids, steroids, alkanes, phenols, terpenoids, halogenated ketones, and cyclic polysulphides that have significant medicinal properties making them a popular ingredient in novel pharmaceuticals [4] [5]. These compounds have been explored for industrial applications, especially in food, pharmaceutical,

and cosmetic industries. Over the past few decades, macroalgae production has significantly increased worldwide, with an annual growth rate of 8% [6] [7]. As a result, an extensive range of macroalgae species is now available for producing various products. Among them, *Ulva lactuca*, a type of green macroalgae, has an interesting chemical composition that is edible and already used as a nutritional supplement and food condiment in Asia, America, and Oceania, as well as traditional Chinese medicine [8]. *Ulva lactuca* thrives in the intertidal zone at all levels and protects harbors up to 10 meters deep. It usually grows in sandy coasts, rocks, and estuaries; abundantly found in the Gulf of Mannar, the first marine biosphere reserve in Southeast Asia [9]. *Ulva lactuca* is composed of polysaccharides (65%), carbohydrates (60%), mineral ash (38%), lipids (3%), and proteins (47%) approximately [10] [11]. It also contains an adequate level of polyphenolic compounds that are proven to confer anti-aging properties [12]. Consuming *Ulva lactuca* is known to prevent obesity, cancer, metabolic syndrome, chronic fatigue, arthritis, autoimmune illness, allergies, and more [13]. Moreover, *Ulva lactuca* contains carotenoids with antioxidant, anti-inflammatory, anti-coagulant, anti-viral, and anti-cancer activities [14]. Its antioxidant properties aid in the prevention and treatment of cancer by reducing cancer cell proliferation, specifically in the lymph and thyroid glands, and decreasing inflammation [15].

In this comprehensive study, we aimed to evaluate the efficacy of five different solvents in extracting the targeted bioactive compounds from *Ulva lactuca*, a type of seaweed. The qualitative analysis of the extracted compounds was conducted, and their effectiveness on total phenols, total terpenoids, total alkaloids, steroids, and tannins was determined quantitatively. From the results, the most effective solvent extracts were selected for further investigation of the *in vitro* anticancer activity of *Ulva lactuca*, especially on the lung cancer (A549) cell line. This study provides valuable insight into the potential of *Ulva lactuca* as a source of bioactive compounds with significant anticancer properties.

Materials and Methods

Collection of seaweed

The mature green seaweed of the *Ulva lactuca* species, collected from the Pamban coast (Gulf of Mannar), Ramanathapuram, Tamil Nadu, India (9.2798° N, 79.2291° E), has been taxonomically authenticated by the Botanical Survey of India (BSI), Coimbatore, Tamil Nadu, India. The species is well-developed and holds significant value.

Preparation and extraction of seaweed

In order to obtain a high-quality seaweed solvent extract, the seaweed samples were initially rinsed with seawater to remove any debris, sand particulate, epiphyte, as well as extraneous substances and it was transferred into sterile bags containing water, brought to the laboratory. Further, it was washed with running tap water, followed by distilled water later the seaweed was dried in a dark state and powdered with a milling machine. The Soxhlet apparatus was then used to extract the sample using various solvents (Methanol, Chloroform, Ethyl Acetate, Hexane, and Aqueous). The thimble containing the seaweed sample was carefully placed in the extractor chamber, and the respective solvent was poured at a 1:10 ratio in a reservoir round bottom flask (60°C) in a heating mantle. We ran 15 refluxes on each sample to

get the good quality solvent extract. Finally, the solvent extract was then condensed in a low temperature, vacuum-filled rotary evaporator, resulting in a precipitate which was collected into a glass jar at a temperature of -20°C and stored for further analysis [16].

Qualitative phytochemical analysis of *Ulva lactuca*

The phytochemical screening of *Ulva lactuca's* five solvent extracts was conducted using the standard methods of Peach and Tracey (1995) and Raaman (2006).The qualitative analysis revealed the presence of phytoconstituents such as alkaloids, phenols, flavonoids, tannins, saponins, terpenoids, steroids, carbohydrates, glycosides, and proteins, indicating the medicinal potential of the seaweed species [17] [18].

Quantitative analysis of *Ulva lactuca*

Determination of alkaloid

The extraction of alkaloids from *Ulva lactuca* was carried out using the Harborne 1998 method, which involved homogenizing 10 mg of seaweed using a mortar and pestle, followed by the addition of 20 ml of methanol: ammonia (68:2). After each addition, the solution was decanted and fresh methanolic ammonia was added. This process was repeated thrice to obtain the extract, which was then evaporated using a flash evaporator. The residues were treated with 1N HCl overnight, and the extracted acidic solution was further treated with 20 ml of CHCl3. The organic layers were pooled, evaporated to dryness, and the weighed fractions were expressed as mg/g. The extraction process was performed with precision and accuracy, ensuring the reliability of the results obtained [19].

Determination of phenol

To determine the total phenolic content of the *Ulva lactuca* sample, we used the Singleton and Rossi (1965) method. We started by grinding 0.5 grams of the sample using a mortar and pestle and combining it with 5 mL of ethanol. After centrifuging the mixture at 2000 rpm for 10 minutes, we collected the supernatant and evaporated the residue. Then, we mixed 0.1 mL of each seaweed extract with 3 mL of distilled water and added 0.5 mL of Folin-Ciocalteau reagent. After allowing the mixture to sit for 3 minutes, we added 2 mL of 20% sodium carbonate solution and boiled the mixture for 1 minute. Finally, the absorbance at 650 nm was measured using a spectrophotometer, and expressed the results in milligrams of gallic acid equivalents (GAEs) per gram of extract [20].

Determination of terpenoids

To determine the concentration of terpenoids in *Ulva lactuca* extract, the Ghorai *et al.,* (2012) method was employed. In this method, 1 mL of the extract was mixed with 3 mL of chloroform and left for 3 minutes. Subsequently, 200 µL of concentrated sulphuric acid (H_2SO_4) was added to the mixture, which was then incubated for 1.5-2 hours in dark conditions at room temperature. This resulted in the formation of reddish-brown precipitate. The supernatant was carefully decanted without disturbing the precipitate. To dissolve the precipitate completely, 3 mL of 95% (v/v) methanol was added, and the mixture was thoroughly mixed until the precipitation dissolved. The concentration of terpenoids was determined by measuring the absorbance at 538 nm using a UV/visible spectrophotometer [21].

Determination of steroids

The quantification of steroids in *Ulva lactuca* extract was conducted. To perform the assay, 0.1 mL of the extract was added to 4.9 mL of ferric chloride precipitating reagent. The mixture was then centrifuged, and 2.5 mL of ferric chloride diluting agent and 4.0 mL of concentrated H_2SO_4 were added to 2.5 mL of the supernatant. After a 30 minute incubation period, the intensity of the colour was measured at 540 nm against a reagent blank. The amount of steroids present in the sample was expressed as mg/dL [22].

Determination of tannins

The Deyab *et al.,* (2016) method was employed to determine the presence of tannins in the *Ulva lactuca* extract. For this test, 0.5 mL of extract was added to a test tube containing 3 mL of distilled water. Subsequently, 5 mL of 35% Na_2CO_3 and 2.5 mL of Folin-Denis reagent were added to the test tube, and the mixture was incubated at room temperature for 30 minutes. Tannic acid solution served as a standard, and distilled water was used as a blank. The absorbance was measured against the reagent blank at 700 nm. Using the standard graph, the amount of tannin present in the sample was calculated [23].

In-vitro anticancer activity of *Ulva lactuca*

Cell culture

The lung cancer cell line (A549) was acquired from the National Centre for Cell Science (NCCS) in Pune and grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum (FBS). The cells were incubated at 37°C, in a 5% CO_2 atmosphere and 95% air with 100% relative humidity. The cultures were maintained by weekly passage, and the medium was changed twice a week.

MTT assay (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide)

For the *in-vitro* cytotoxicity study, methanolic extracts of *U. lactuca* were used. Monolayer cells were separated with EDTA to create single-cell suspensions, and the number of viable cells was measured with a hemocytometer. A 100 µL cell suspension was added into 96-well plates at a density of $1x10^5$ cells/mL and incubated for 24 hours. Varying concentrations of test samples (18.75, 37.5, 75, 150, 300 µg/mL) were added to the plates after 24 hours, with DMSO used for dilution. The plates were incubated for 48 hours, with the control medium without samples in triplicate. After 48 hours, MTT in PBS was added to each well and incubated for 4 hours at 37°C. Formazan crystals were dissolved in 100 µL of DMSO and measured at 570 nm with a microplate reader [24].

Results

Qualitative analysis of *Ulva lactuca*

The analysis of phytochemicals from the extracts of *Ulva lactuca* revealed a diverse range of compounds. The presence of alkaloids was found to be strong in ethanol and ethyl acetate extracts, moderately present in methanolic solvent, while absent in chloroform and aqueous extracts. Phenolic compounds showed strong presence in ethyl acetate extracts, and moderate presence in methanol, chloroform, and aqueous extracts, but absent in ethanol extract.

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Flavonoids were only weakly present in methanolic and aqueous extracts, absent in other solvent extracts. Tannins were strongly present in aqueous and ethyl acetate extracts, and weakly present in other solvent extracts. Aqueous extracts showed strong presence of saponins, while chloroform extracts showed moderate presence, and ethyl acetate extracts did not have any saponins. Terpenoids were found to be strongly present in all the extracts, except for the aqueous extract. Steroids were strongly present in methanolic extracts, and weakly present in other extracts. Carbohydrates were found to be strongly present in all the solvent extracts. Glycosides were moderately present in aqueous and chloroform extracts. Proteins were strongly present in aqueous and ethyl acetate extracts, and moderately present in other extracts.

Table 1: Qualitative analysis of *Ulva lactuca*

Phytoconstituents	Aqueous	Ethanol	Methanol	Ethyl acetate	Chloroform
Alkaloids	+	+++	++	+++	+
Phenols	++	+	++	+++	++
Flavonoids	+	-	+	-	-
Tannins	+++	++	++	+++	++
Saponins	+++	+	+	-	++
Terpenoids	+	+++	+++	+++	+++
Steroids	++	++	+++	++	++
Carbohydrates	+++	+	+++	+++	+++
Glycosides	++	+	+	+	++
Proteins	+++	++	++	+++	++

[-Absent, + Weak, ++ Medium, +++ Strong]

Quantitative analysis of *Ulva lactuca*

The phytochemical analysis of *Ulva lactuca* seaweed revealed that the methanolic extract contained the highest quantity of alkaloids and total phenols, while the aqueous extract contained the least. Terpenoids and steroids were also significantly present in the methanolic extract, while tannins were found in high concentrations in the ethyl acetate extract. The hexane extract had the lowest level of activity. Among the five solvent extracts tested, only the methanolic extract of *Ulva lactuca* exhibited effective bioactive compounds compared to the others.

Table 2: Quantitative analysis of *Ulva lactuca*

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Phytoconstituents	Aqueous extract	Methanol extract	Ethyl acetate extract	Chloroform extract	Hexane extract
Alkaloids (mg AE/g)	1.31 ± 0.12	3.83 ± 0.35	2.80 ± 0.26	2.23 ± 0.37	1.83 ± 0.35
Total phenols (mg GAE/g)	1.50 ± 0.26	2.60 ± 0.20	2.50 ± 0.20	2.33 ± 0.25	1.40 ± 0.20
Terpenoids (mg LE/g)	1.29 ± 0.11	4.33 ± 0.15	3.24 ± 0.15	2.50 ± 0.26	1.46 ± 0.30
Steroids (mg BSE/g)	1.50 ± 0.10	4.63 ± 0.15	1.70 ± 0.20	1.60 ± 0.20	1.53 ± 0.30
Tannins (mg TE/g)	3.20 ± 0.40	3.10 ± 0.29	3.46 ± 0.30	2.06 ± 0.35	1.80± 0.20

In-vitro anticancer activity of *Ulva lactuca*

The effect of the methanolic extract of *Ulva lactuca* on A549 cell proliferation was evaluated. Cell viability was assessed by MTT assay after treating them with various concentrations of the extract (18.75, 37.5, 75, 150, 300 µg/mL). The cell viability decreased in a time-dependent manner, demonstrating a strong selective cell proliferation inhibition of the lung cancer cell line A549 (Figure 1 and 2). The highest cell growth inhibition was 73.2% due to the higher concentration of phenolic, terpenoids, and alkaloid compounds (300µg/mL). This experimental observation showed that the number of non-viable cells increased with increasing concentration of seaweed extract, which demonstrated significant cytotoxic activity.

Figure 1: Graphical representation of *in-vitro* anticancer activity of methanolic extracts of *Ulva lactuca* on lung cancer cell line (A549)

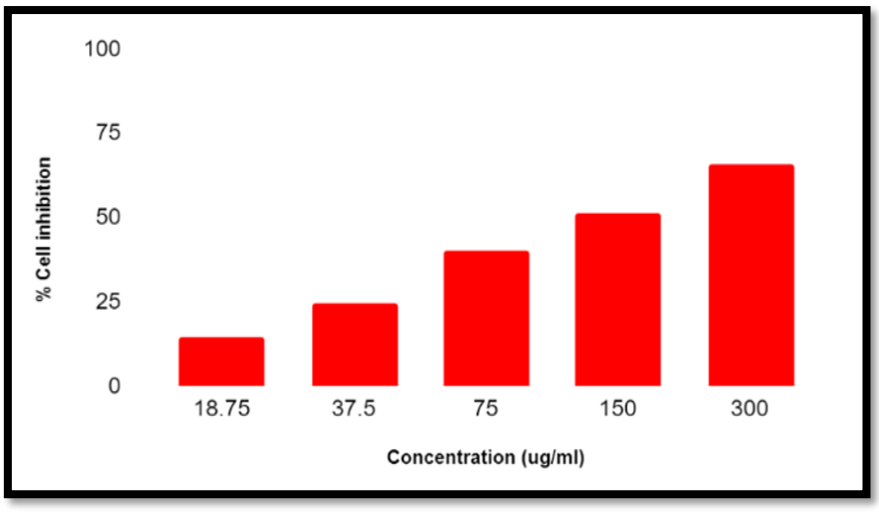

Figure 2: Microscopic observation cytotoxicity activity of *Ulva lactuca* methanolic extract on lung cancer cell line (A549)

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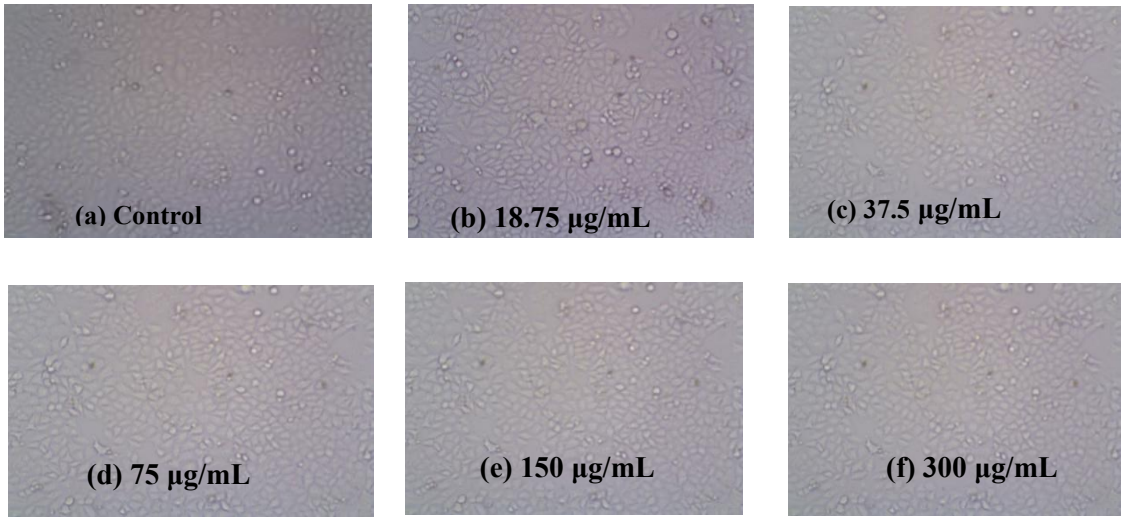

Discussion

The treatment of cancer remains a pressing issue in the present day, as the side-effects of most chemotherapy drugs are significantly high. In response, researchers have undertaken extensive studies to identify bioactive compounds from marine sources that could prove effective against cancer [25]. Among these, seaweeds have emerged as a promising avenue for the development of novel anticancer drugs [26]. Over the past forty years, researchers have isolated numerous bioactive substances from red, blue, and green seaweeds [27]. Green seaweeds, like higher plants, contain Chlorophyll a and b, and research studies have reported on active secondary metabolites extracted from green algae [28]. The use of methanolic extract of *Ulva lactuca* has been demonstrated in several studies, which have proved the presence of phenolic compounds [29]. Similarly, in our present study, we observed that the methanolic extract of *Ulva lactuca* had a significantly higher amount of phenolic compounds compared to other solvent extracts. Green algae contain a higher amount of total phenolic compounds compared to brown and red algae, although higher values have been found in green algae compared to red species [30]. The ethyl acetate extract of green marine algae *Ulva fasciata* contained a higher content of alkaloids than the red algae *Halolactibacillus* species [31].

Another study stated that the methanolic extract of *U. fasciata* contains a larger amount of phenols next to the ethyl acetate extract and lower activity in the aqueous extract, and this study was correlated with our present study [32]. In addition, our study similarly found that the methanolic extract and ethyl acetate extract had higher levels of total terpenoids, alkaloids, steroids, and tannins, while chloroform, hexane, and aqueous extracts of *Ulvaria oxsperma* and *Ulva fasciata* contained the lowest levels of these compounds [33]. Several studies have confirmed that certain seaweed extracts have anti-cancer properties. For example, the ethanolic extract of *Ulvaria oxsperma* effectively reduced the viability of MOLT-3 cells [34]. Additionally, methanolic extracts of *U. fasciata* exhibited cytotoxic activity against lung cancer cells [35]. *Gracilaria corticata* contain polyphenol-rich compounds such as bromophenols and phlorotannins that inhibit *in-vitro* cancer cell proliferation [36]. In the same way, during our present study, we found that the *Ulva lactuca* methanolic extract demonstrated significant cytotoxicity and increased cell viability with increasing concentrations in the A549 cell line, indicating the presence of polyphenolic compounds that may scavenge free radicals and

possess anticancer activity. These polyphenols can eliminate cancer cells by inhibiting cell cycle mechanisms and up-regulating apoptotic pathways through signaling pathways, making them effective anticancer agents [37] [38].

Conclusion

The study found that out of five different solvent extracts of *Ulva lactuca*, only the methanolic extract contained high levels of phenolic, terpenoids and alkaloids compounds. These active compounds have the potential to kill lung cancer cells in the A549 cell line, making the methanolic extract a promising candidate for cancer treatment. However, further characterization studies are needed to identify the specific bioactive compounds in the extract with certainty.

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