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Evaluation of Methanolic Extract of Seaweed *Ulva lactuca against* Resistant Pathogenic Fungal and Microbial Strains

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ABSTRACT

Antibiotic resistance continues to expand for a multitude of reasons, including over-prescription of antibiotics by physicians, non-completion of prescribed antibiotic treatments by patients, increased international travel, and poor hospital hygiene. Ulva lactuca, a gracious food source for humans frequently eaten raw in salads or cooked in soups is known for its widespread therapeutic uses. In present study, antimicrobial and antifungal properties of methanolic extract of Ulva lactuca were evaluated against resistant strains of human pathogenic bacterial and fungal species viz. E.coli, P. aeroginosa, S. aureus, A. fumigates, Rhodotorula sp. and C. albicans by using Kirby-Bauer method.Methanolic crude extract of U. lactuca was characterized using Thin Layer Chromatography, UV Spectroscopy, FTIR Spectroscopy and Mass Spectroscopy. Results confirmed that methanolic crude extract of U. lactuca is a potent antimicrobial and anti fungal agent against resistant strains. This study envisages a detailed investigation for extraction of all active bio ingredients from Ulva and synthesis of their chemical analogue for their future use as potent antimicrobial agents.

KEYWORDS *Ulva* Sp., Sea lettuce, Bioactivity, antibiotic resistance, FTIR Spectroscopy, Mass Spectroscopy.

Introduction

Decades ago, scientists thought antibiotics; the miracle drugs had eradicated infectious agents which were a major health threat those days (Anupurba S. et. al., 2003). Instead, the past two decades have seen an alarming resurgence of infectious diseases and the appearance of new ones. Today, the AIDS virus, tuberculosis, malaria, diarrheal diseases and other infectious diseases pose far greater hazards to human existence than any other creatures. This upsurge of infectious disease is a problem we have unwittingly created for ourselves (Levy S.B., 2002).

The rise of rapid, frequent, and relatively cheap international travel allows diseases to leap from continent to continent. Inadequate sanitation and lack of clean drinking water are another aspect. A third is the "antibiotic paradox", the overuse of the "miracle drugs" to the point that they lose their potency (Salvador et. al., 2007). Whenever antibiotics wage war on microorganisms, a few of the enemy are able to survive the drug. Because microbes are always mutating, some random mutations eventually will protect them against the drug. Too much antibiotic use selects for more resistant mutants (Salvador et. al., 2007). When patients cut short the full course of drugs, the resistant strains have a chance to multiply and spread. In some countries, like India, patients expect and demand antibiotics from doctors, even in situations where they are inappropriate or ineffective. Every time antibiotics are used unnecessarily, they add to the selective pressure we are putting on microbes to evolve resistance. Then, when we really need antibiotics, they are less effective (Salvador et. al., 2007). While drug companies race to develop new antibiotics that kill resistant microbes, scientists are urging patients and doctors to limit antibiotic use. This scenario requires worldwide efforts to look in to new genera of effective antimicrobial and anti fungal agents. In light of growing concerns relating to microbial resistance to antibiotics increasing attention is being given to the role that herbal medicines or sea weeds may play as autonomous antibacterial agents or as adjuvant treatments used to potentiate conventional drugs or to find some novel antibacterial or antifungal compounds against proven antibiotic resistant strains.

Seaweeds are floating or submerged primitive group of marine plants of shallow meadows. Seaweeds are a valuable food source and therefore are ecologically and commercially important. The multiple uses of these plants in food, chemical and textile industries, agriculture, pharmaceuticals and medicine have been well recognized (Sobha, V., et. al., 2008).

Fig. 1. Ulva lactuca, commonly known as sea lettuce, an edible sea weed.

Ulva lactuca, a sea weed commonly known as 'Sea Lettuce' or 'Green Laver', is a vivid green algae abundantly inhabited in sheltered bays or in protected or semi protected areas with limited wave action (Michael N Guiry, 2007). They also thrive in brackish water, particularly in waters with organic enrichment and estuaries. These are usually seen in dense colonial groups living attached to rocks in the middle to low intertidal zone, as deep as 10 meters in calm, protected harbors. They can be quite a nuisance in areas that are nutrient enriched from sewage outfalls, where populations of Ulva may cover large areas of mudflats in the summer (Michael N Guiry, 2007). The shapes of Ulva are quite varied - circular to oval to long and narrow, ranging in size from microscopic to 65 cm. They have fine, silky textures with waved or ruffled margins. The delicate blades of Ulva are usually only 40 microns thick (Michael N Guiry,2007).

Indian coast harbors around 800 seaweed species, of which Ulva lactuca is one of the predominant species. It is a high valued edible marine plant with an enclosed assemblage of Carbohydrates (~44.9%), Proteins (~28.2%), Polyphenols (~18.3%), Ash (~7.5%), and Lipids (~1.2%) (Newman et. al., 2003).

Another distinctive property of Ulva lactuca is that they are rich sources of hydrosoluble and liposoluble vitamins such as Thiamine and Riboflavin, ⊠-carotene and Tocopherols, as well as of long-chain polyunsaturated essential fatty acids of the n-3 family (n-3

LC-PUFA) viz. Eicosapentanoic acid (EPA, 20:5n-3) (Newman et. al., 2003). Ulva is further extracted for a variety of bioactive agents' viz. Glycolipids, Glycoproteins, Terpenes, Steroids, and Polyketide family (Newman et. al., 2003). These bioactive molecules are studied for their antibacterial, antifungal, anti-rheumatic, anti-atherosclerotic, anti-thrombotic, anti-inflammatory, anti-carcinogenic and cell proliferation activities (Kolanjinathan, K. and D. Stella, 2009, Ito, K. and K. Hori, 1989).

Though, Ulva is considered as a widespread therapeutic herbal source of some oriental remedies for some noxious diseases, there is no report available for its efficacy against resistant strains of human pathogenic microbes and fungal species. Consequently, it can be explored for developing some novel therapeutic antimicrobial agents against resistant strains. The present study is aimed at exploring the antimicrobial and antifungal uses of Ulva lactuca. We characterized chemical components of Ulva lactuca by using standard Chromatography and Spectrophotochemical techniques viz. UV-Vis Spectroscopy, FTIR Spectroscopy and Mass Spectroscopy. For evaluating antimicrobial and antifungal effectiveness of Ulva, we challenged its methanolic extract against recognized human pathogenic resistant bacterial and fungal strains. The investigation highlights the importance of Ulva lactuca as a potential source of antimicrobial and antifungal compounds to overcome the constantly magnifying problem of antibiotic resistance. This study further paves the way to isolate the active bioagents of Ulva and its potential use as future antibiotics of herbal origin.

Materials and Methods

Collection and pre-treatment of sample

Fig. 2 . Map shows the Ratnagiri, area of west coast, an inhabitant of at least 100 varieties of sea weeds.

The seaweeds especially Ulva lactuca were collected from the Ratnagiri, Maharashtra located at 16.98° N and 73.3° E on the west coast of India during low tide (17, August, 2016). It is predominantly a coastal line and majorly inhabited by patchy reefs present in intertidal areas at sub-tidal depths. The intertidal area is rich in various algae, particularly Ulva sp. Sampling was mostly done in rocky areas with a few sandy patches. The samples were collected in 250 ml sampling vials (Tarson, Mumbai) and immediately transported in temperature controlled refrigeration system (Blue Star, Mumbai) to the laboratory based at Mumbai. The seaweeds samples were gently rinsed with tap water and then with filtered fresh seawater to remove any traces of salt, sand and epiphytes. Total sea weed sample was analyzed for its species diversification. Approx 96 % of the total sea weed sample was identified as Ulva lactuca using standard characteristic key. A sample specimen slide was deposited in herbarium, University of Mumbai vide voucher no. 267 /Chm-Unr/2016. The material was then dried at room temperature for 6h and than stored in -20 °C until further use.

Preparation of Extract

For extract preparation, air dried sample (100g) of Ulva was pulverized mixer in 100 ml of double distilled water (1:1 w/v). Pulverized sample (150 ml) was extracted with 500 cm^3 of Methanol (Qualigens, India) for 24h in rotatary evaporator at room temperature (Remi, India). The extraction of Ulva sample was further repeated twice with fresh methanol. The final methanolic extract was evaporated under vacuum to dryness. The lyophilized sample obtained after evaporation was stored at -20 °C for further use.

Characterization of Crude Extract

Reagents and chemical

All the chemicals used for crude extract characterization were of AR grade and were procured from Qualigens, India.

Thin Layer Chromatography

For TLC, the methanolic crude extract was reconstituted in solvent and 5µl extract was spotted on silica gel coated alumina backed sheets (Silica gel 60 F_{254}, 0.25 mm thick, Merck). Chromatogram was developed in closed tank saturated with eluent vapors (15% Ethyl Acetate: Hexane; 10% Methanol: Chloroform; BAW). Developed chromatograms were viewed both in daylight and under UV light (254nm) in Gel-Doc (Shimadzu, Japan). The compounds separated on the TLC chromatogram were characterized by their R_f values calculated as the ratio of the distance moved by the solute (compound), to the distance moved by the mobile front (Fried and Sharma, 1999).

UV Spectroscopy

For UV-Vis spectroscopy, the methanolic crude extract was reconstituted in required amount of methanol and their UV spectra were recorded using Shimadzu, Japan UV-2401 PC Spectrophotometer.

FTIR Spectroscopy

The lypholized crude methanolic extract of Ulva was reconstituted with required amount of methanol and their Infrared (IR) spectrum were recorded using Shimadzu Affinity-1 FTIR spectrometer in range of 500-4000 cm^{-1} in a KBr pellet.

Mass spectroscopy

The Mass spectra of crude methanolic extract were obtained using electrospray ionization-time of flight mass spectrometry (ESITOFMS). For this, the crude extract was dissolved in required amount of methanol to prepare dilute solution (approx. 1µg/ml). The prepared solution was directly injected on to ESITOFMS at a potential of 5ev and ESITOFMS spectra were generated for all active bioagents.

Antimicrobial and Antifungal activity

For evaluating antimicrobial efficacy of crude methanolic extract of Ulva against resistant antimicrobial and antifungal strains, requisite assay was performed using Kirby-Bauer method recommended by the Clinical and Laboratory Standards Institute (CSLI) for antimicrobial susceptibility testing (Hun et al. 1994). For this, pure test cultures of resistant strains of human clinical bacterial pathogens viz. Escherichia coli, Pseudomonas aeroginosa, Staphylococcus aureus (MRSA subsp. Rosenbach (ATCC® 33591™) and three resistant strains of known human pathogenic fungus viz. Aspergillus fumigates, Rhodotorula sp. and Candida albicans were procured from Microbiology Department, Smt. CHM College and revived in glycerol for their further use. The glycerol stock cultures of human pathogens was added to sterile Muller Hinton Broth and was incubated on rotory shaker incubator at 28 ± 2°C for 24-48h.

Whatman's filter papers No. 1 were punched for preparing the disc of 6mm diameter. The discs were packed in aluminium foils and sterilized in autoclave (Remi, India) at 121°C, 150psi for 15 min. For antimicrobial essay, 25mg of the dried methanolic extract of Ulva lactuca was reconstituted in 50µl of methanol. 5µl of the reconstituted extract was applied on the sterile disc.

Presynthesized Muller Hinton Agar plates were purchased from Hi-media laboratories Mumbai, for antimicrobial test analysis. The plates were divided into grids and were numbered according to the

extract number. 200µl of the test pathogenic organism was spreaded on the plate and discs loaded with the extract were placed in their corresponding grids with the help of alcohol sterilized forceps. Streptomycin (25µg) was used as the standard antibiotic. The pathogen loaded MH plates were incubated at 37°C for 24h. After incubation, plates were observed for zone of inhibition. The inhibition zones shown by the extracts against the test pathogenic cultures were measured in mm along with the disc diameter.

Results

Thin Layer Chromatography

TLC separation method was used to illustrate the individual primary and secondary metabolites of the crude extract of seaweed Ulva lactuca. TLC severance showed 5 well distinguished bands a, b, c, d, and e were observed (Fig. 3) on TLC plate with Rf values 0.77 (a), 0.54 (b), 0.45(C), 0.34(d) and 0.085(e) respectively. All the bioactive molecules in distinguished 5 spots were scrapped in separate vials and reconstituted in required amount of methanol and stored at -20 °C for our next phase of study.

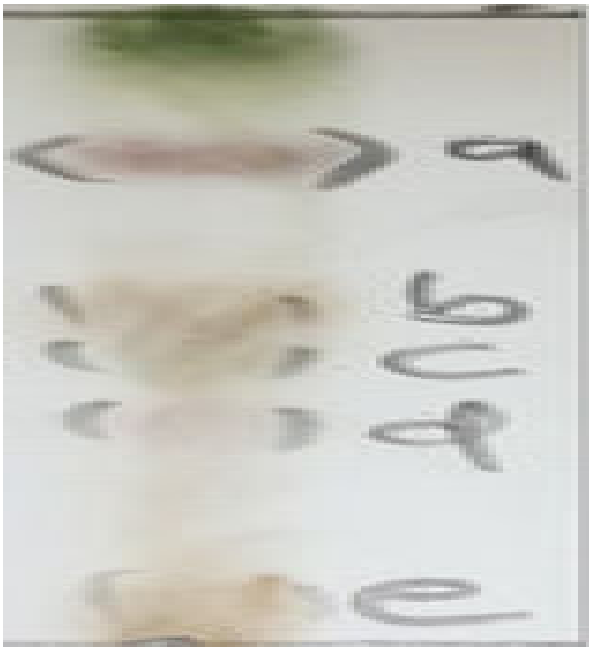

Fig. 3. Thin layer chromatography of Seaweed extract in BAW solvent system.

UV Spectroscopy

In practice UV spectroscopy is limited to conjugated system for the most part and hence, the UV spectrometer can be used in structure elucidation of organic molecules to indicate presence of conjugation in given sample. UV spectra of seaweed extract showed absorption at 665 nm indicates the presence of chlorophyll derivatives, absorption at 409 nm may be due to mixture of carotenoids. Absorption at 345 nm may be due to some UV absorbing compounds such as mycosporine type of amino acids.

FTIR Spectroscopy

The FTIR spectrum was used to identify the functional group of the bioactive components present in crude extract based on the peak values in the region of infrared radiation. The results of FTIR peak values and functional groups are represented in Fig. 4. Peak at 3358 cm-1 indicating the presence of –OH group (alcohols, phenols, acids etc can be present), peak at 2929 and 2852 indicates the presence of –CH group, peak at 1739 indicates the presence of C=O group (esters, ketones, aldehydes), peak at 1614 is broad indicating many peaks combined together compounds with conjugated carbonyl group and C=C absorption are coming together at this region.

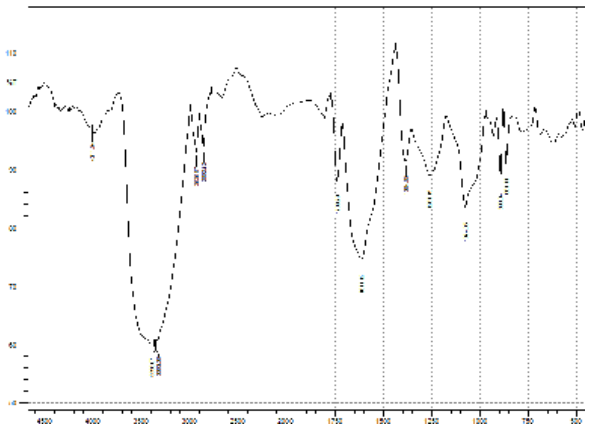

Fig.4. FTIR Spectra of methanolic crude extract of Ulva lactuca an edible sea weed commonly found along west coast of India.

Mass Spectroscopy

The Ulva methanolic crude extract was subjected to tandem MS/MS of the ion at m/z 639.6. The peak is showed in Fig.5 and a daughter ion at m/z243 is visible, which is a characteristic of mono galactosyl diacyl glycerol. Ions reflecting cleavage of C2-C3 bond of glycerol along with the acyl group (in this case acetyl group) as well as the clevage of C1-C2 bond of glycerol are evident from the fragment ions on m/z 567 and 553 respectively. Additional ions are observed due to cleavage of glycosidic bond giving sodiated ion at m/ z 186.This fragmentation is well in agreement with the presence of a major glycolipid in the mixture.

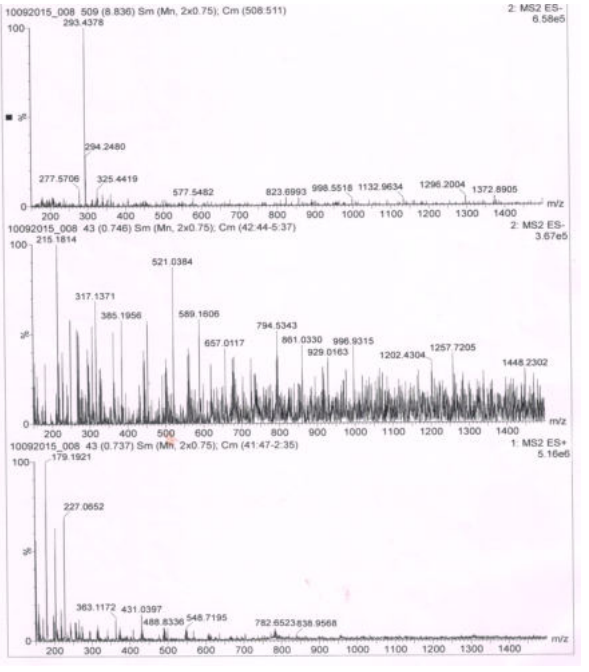

Fig.5. Mass spectra of the crude extract obtained from seaweed.

Antimicrobial activity

Antimicrobial activity was performed in triplicate and the results are summarized in table 1. Methanolic extract of Ulva lactuca was evaluated against resistant strains of all three recognized human pathogenic bacteria species. The crude extract showed significantly strong antimicrobial activity (zone of inhibition, 8.2mm) against Methicillin-resistant Staphylococcus aureus (MRSA) infections compared to standard drug streptomycin (ZOI-4.6mm). For resistant strain of E. coli (ZOI-4.1mm), average antimicrobial activity was observed for crude methanolic extract

compared to standard drug streptomycin (ZOI-4.6mm). For Pseudomonas aeroginosa (ATCC ® 27853™) another resistant human pathogenic bacteria strain, no activity was observed even after 48h of the analysis. Thus crude antimicrobial activity was significant in case MRSA and *E coli.*

Table 1: Antimicrobial activity of the crude extracts of Ulva sp. against known resistant clinical human bacterial species.

Sr. No	Microbial cultures	Zone of inhibition (mm)
1.	*Escherichia coli* (ATCC ® 2592 ™)	4.1
2.	*Pseudomonas aeroginosa* (ATCC ® 27853™)	-
3.	MR Staphylococcus aureus subsp. Rosenbach (ATCC® 33591™)	8.2
4.	*Streptomycin*	4.6

Antifungal activity

Crude methanolic extract of Ulva was evaluated for its antifungal activity. The activity was performed in triplicates against resistant strains of known human pathogenic fungal species. Results are summarized in table 2. Crude extract demonstrated significant antifungal activity against resistant Aspergillus fumigates (ZOI-6.6.mm) strains compared to standard drug Clotrimazole (ZOI-3.1mm). No antifungal activity was observed for resistant strain of fungus Rhodotorula sp. For *Candida albicans* another resistant pathogenic fungus, average antifungal activity was (ZOI-2.8) observed compared to standard drug Clotrimazole (ZOI-3.1mm).

Table 2: Antifungal activity of the methanolic crude extract of Ulva lactuca against known clinical human pathogenic fungal species.

Sr. No	Fungal cultures	Zone of inhibition (mm)
1.	*Aspergillus fumigates* (ATCC ® 46645™)	6.6
2.	*Rhodotorula sp*. (ATCC ® 20837™)	-
3.	*Candida albicans* (ATCC ® 10231™)	2.8
4.	*Clotrimazole ointment*	3.1

Discussion

Herbal medicines continue to serve as important source of drugs for the developing countries like India. Sea weeds as herbal medicines are fast gaining importance. Seaweeds are loaded with an array of inorganic and organic substances which are proven beneficial for human health. These compounds are proven cytostatic, antiviral, anthelmintic, antifungal and antibacterial against human pathogens. In present study an attempt was made to illustrate their antimicrobial and antifungal efficacy against resistant strains of known human pathogenic bacteria and fungus. The metabolic and physiological capacity of sea weeds allows them to survive in complex habitat types and provides a great potential for production of secondary metabolites which are not found in terrestrial environments. Thus, they are among the richest sources of known and novel bioactive compounds (Jha, R.K, 2004).

In the present study, the extraction yield of methanolic extract of U. lactuca was recorded as 67.23%. Our data corroborates with the data of Wang et al. 2009, who demonstrated that there will be considerable variation in extraction yield among different seaweed species such as green, red and brown algae. He also said that methanol extraction yields is always higher than those of other solvent extracts with decrease in polarity which indicated that most of the soluble components in seaweeds were high in polarity. Herrero et. al., 2005, attributed the loss of extraction yield to serial exhaustive extractions involves successive extraction with solvents of increasing polarity from non polar to a more polar solvent in order to ensure that a wide polarity range of compounds could be extracted.

The results from the present research revealed that the strongest antimicrobial activity of methanolic extract was exhibited towards resistant strain of MRSA and E. coli. Manilal et al., 2009, and Rangaiah et al., 2010, reported in their different studies that methanolic extract of Ulva exhibited higher antimicrobial activity towards certain specific human pathogenic strain compared to other bacteria studied. This variation in the results was attributed to the difference in the species used, time and place of sample collection. The antifungal effects of methanolic extract towards resistant strain were comparable to the standard drug Clotrimazole and were found to be active against resistant fungal strain tested.

Crude chemical characterization showed that steroids, fatty acids, esters of fatty acids and other hydrocarbons were recorded in methanolic extract of Ulva which may be responsible effective antifungal or antimicrobial activities. Similar results were obtained by Gonzalez del Val et al. 2001, who used methanol as solvent for extraction of antimicrobial compounds for red, green and brown seaweeds and evaluated their antimicrobial activities.

Plaza et al., 2009, also identified several volatile compounds in ethanol extracts of brown seaweeds Himanthalia elongata. The compounds include fatty acids, alkanes, phenols and compounds such as phytol (2-hexadecen-1-ol, 3, 7, 11, 15-tretramethyl) and neophytadiene. The compounds 1, 2-benzene dicarboxylic acid, bis-(2-ethylhexyl) ester and fatty acids have been evaluated against many microbes as antimicrobial agents (Kavitha et. al., 2009 and Smaoui S. et. al., 2012). Lauric, palmitic, linolenic, oleic, stearic and myristic acids are known to be potential antibacterial and antifungal agents (John Peter et. al., 2013). Similarly in the present study, these compounds were found in methanolic extract of Ulva and may be attributed for their antimicrobial activity against resistant strains. Antimicrobial activity has usually been attributed to long-chain unsaturated fatty acids (C16-C20), including palmitoleic, oleic, linoleic and linolenic acids, while long chain saturated fatty acids, including palmitic and stearic acids were less effective (P. Mac Artain et. al., 2007) In the present study palmitic acid (saturated fatty acid), myristic acid (saturated fatty acid), arachidonic acid (essential polyunsaturated omega 6 fatty acid), were recorded which might be responsible for the highest antifungal activity in the present study.

Further, detailed analysis is required to evaluate spectral composition, effectiveness and potential use of Ulva bioactive compounds for medicinal purposes.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

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Conclusion

The crude extract of Ulva sp. showed significant antimicrobial and antifungal activities against resistant strains of human bacterial and fungal pathogens. This investigation highlights the importance of the Ulva sp. as a source of potent antimicrobial and antifungal compounds and warrants further studies to isolate the active bioagents for their future therapeutic use in the era of antibiotic resistance.

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