EVALUATION OF ANTIBACTERIAL POTENTIAL AND PHYTOCHEMICAL ANALYSIS OF VARIOUS ORGANIC EXTRACTS OF GREEN SEAWEED ULVA LACTUCA

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ABSTRACT

This study was undertaken to explore the antibacterial potentials and phytochemical constituents of green seaweed *Ulva lactuca*. Results of antibacterial activity inferred that, antibacterial activity of organic solvent extracts were in the following order: Acetone > Methanol > Benzene. Amongst the extract tested, acetone extract displayed pronounced antibacterial activity with the inhibitory zones ranged from 3 ± 0.39 to 11 ± 0.09 mm against the test bacterial pathogens. Methanol and benzene extract of *U. lactuca* showed considerably lesser antagonistic activity than the acetone extracts. Phytochemical screening showed presence of alkaloids, tannins, phenols, phytosterols and carbohydrates as the major constituents in the acetone and methanol extracts. The One-way ANOVA for the data on antibacterial activity of methanolic extract of *U. lactuca* as a function of variation between pathogenic bacterial strains was statistically significant

Keywords: Seaweed, Green Alga Phytochemistry and Antibacterial activity

1.0.Introduction

Microorganisms have potential to cause human diseases. Most of the time viruses, bacteria and fungi act as major pathogenic organisms. The discovery of antibiotics in the early 20th century provided an increasingly important tool to combat bacterial diseases (Abeysinghe *et al.*, 2006). However, bacterial resistance to antibiotics has become a worldwide problem in recent years. Indiscriminate use of antibacterial drugs has led to development of many multi-drug resistant (MDR) pathogenic bacterial strains. Development of such newer disease causing pathogens and evolution of existing microorganisms has resulted in severe consequences including increased cost of medicines and mortality of patients (Khan *et al.*, 2009; Mukherjee *et al.*, 2012).

Natural products have historically been a priceless source of therapeutic agents. These products play an important role in the development of new drugs as they possess enormous diversity serving as scaffolds for drug discovery. Plants have been by far the most widely studied source of medicinal compounds (Cragg *et al.*, 1997). The earliest examples of the uses of plants as a source of medicinal products resulted from hundreds of years of trial-and-error experiments on local human populations, which was then assimilated in their culture and passed to their successors. The long history of the therapeutic (ethnomedical) applications has given much fundamental guidance in modern research aimed at elucidating the active compounds contained in a particular medicinal plant. Therapeutic natural products are likely to be safer than

synthetically derived new chemical entities if the active compounds derived from plants previously used for humans use. (Fabricant and Farnsworth, 2001).

Seaweeds are benthic marine macroalgae classified according to their pigmentation into red (*Rhodophyta),* brown (*Phaeophyta)* and green (*Chlorophyta)* occurring along the intertidal and sub-tidal regions of coastal waters of the world. seaweeds living in the sea are constantly contact with these potentially dangerous microbes and they have apparently evolved with chemical defense strategies by synthesizing array of secondary metabolites to defend against the microbial thread (Kubanek, 2003). Thus seaweeds contain rich and varied source of bioactive natural products that exhibit biomedical and antimicrobial properties (Arunkumar *et al*, 2005; Arunkumar and Rengasamy, 2000; Ara, *et al*., 2002).

There are numerous reports from macroalgae derived compounds showing broad range of biological activities such as antiviral, antibiotic, anti-neoplastic, antifouling, antiinflammatory, cytotoxic and antimitotic (Naqvi *et al.*, 1980; Caccamese *et al.*, 1981; Fenical and Paul, 1984; Ballesteros et al, 1992; Bhosale *et al,* 2002). Harder (1917) was the first to observe antimicrobial substances in algae. Seaweeds have caused an emerging interest in the biomedical area due to the presence of potent pharmacologically bioactive substances with wide arrays of potential health benefits (Smit, 2004).

Algae species have been shown to have bactericidal or bacteriostatic substances (Glombitza, I979; Caccamese *et al*., 1980). The antibacterial agents found in the algae include aminoacids, terpenoids, phlorotannins, acrylic acid, phenolic compounds, steroids, halogenated ketones, alkanes, cyclic polysulphides and fatty acids. In large number of marine algae antimicrobial activities are attributed to the presence of acrylic acid., phlorotannins, terpenoids and steroids (Glombitza, 1979 and Arnico *et al*., 1978).

The antimicrobial activities exhibited by the seaweeds are due to the capacity of the seaweeds to synthesize bioactive secondary metabolites (Gonzalezdel Val *et al.,* 2001). There are numerous reports on compounds derived from macroalgae with a broad range of biological activities, such as antibacterial (Oranday *et al*., 2004) antiviral, antitumor and anticoagulant (Athukorala *et al.,* 2006 and Farias *et al.*, 2000). Antibacterial activity of seaweeds varies with season (Moreau *et al.,* 1984). Antibacterial effects of crude methanol extracts obtained from 32 marine macroalgae species (13 Chlorophyceae and 19 Phaeophyceae) harvested from the Atlantic and Mediterranean coast of Morocco were found to be effective against pathogenic bacteria namely *Escherichia coli, Staphylococcus aureus, Klebsiella pnomeuniae* and *Enterococcus faecalis* (Chiheb, 2009). Wide arrays of bioactive compounds found in seaweeds await a major breakthrough for a variety of food/medical applications as natural antioxidants in different food/pharmaceutical products (Cardozo *et al.*, 2007). Thus considering the importance of seaweeds, in the present study an attempt has been made to investigate the *in vitro* antibacterial activity and phytochemical analysis of various organic solvent extracts of *Ulva lactuca* collected from Kanyakumari coast.

2.0.MATERIALS AND METHODS

2.1.Preparation of crude extracts

For the present study, seaweed *Ulva lactuca* was collected from Kanyakumari coast and their taxonomic position was determined based on the standard keys. *Ulva lactuca* is a thin flat green algae growing from a discoid hold fast. For the present study, the seaweed Ulva lactuca was collected from Kanyakumari coast and brought to laboratory in air tight plastic cover. Then the seaweeds were washed in running tap water twice to remove the dirt and adhered particles. After washing, seaweeds were shade dried and powdered. In brief, 100g of *U. lactuca* powder was weighed and subjected to percolation in a series of 300 ml solvent [Methanol, Benzene and Acetone] of varying polarity and stored for 48h. The process was repeated twice; extracts were pooled together and filtered through Whatman No: 1 filter paper. Each filtrate was concentrated to dryness under reduced pressure using a rotary vacuum evaporator. Dried extracts were weighted and stored in screw cap bottles for further study.

2.3.Antimicrobial activity

For the present study, clinical bacterial pathogens such as *Escherchia coli, Enterobacter feacalis, Proteus mirabilis, Pseudomonas fluorescens, Serratia maracescens, Streptococcus mutans, Streptococcus pneumonia, Vibrio vulnificus, V. alginolyticus* and *V. parahaemolyticus* were collected from Department of

Biotechnology, Sri Kaliswari college, Sivakasi.

Antibacterial activity of the crude extracts of *U. lactuca* was tested against test clinical isolates through agar well diffusion method. For this, Muller hinton agar plates (MHA) were prepared separately and overnight culture of test bacterial pathogens were seeded individually over the surface of MHA plates using sterile cotton swabs. Thereafter wells of 6 mm diameter were made over MHA plates using sterile cork borer. The wells were then loaded 500 µg of crude extracts which was prepared in dimethyl sulphoxide. Ciprofloxacin was used as positive control; while DMSO was used as negative control. The plates were then incubated at 37°C for 24h and growth inhibitory activity in terms of zone of inhibition (mm) was measured and recorded. The assay was carried out in triplicates.

2.4.Phytochemical analysis

The phytochemical constituents present in the crude extracts of *U. lactuca* were determined by performing the following tests.

2.4.1.Test for Alkaloids:

A drop of Mayer's reagent was added to a few ml of the filtrates by the side of the test tube. The formation of a creamy or white precipitate indicated the presence of alkaloids.

2.4.2.Test for carbohydrates:

Briefly, 173 g of sodium citrate and 100 g of sodium carbonate were dissolved in 500 ml of water. To this solution, 17.3 g of copper sulphate dissolved in 100 ml of water was added. To 0.5 ml of the herbal extracts, 5 ml of Benedict"s reagent was added and boiled for 5 minutes. The formation of a bluish green colour showed the presence of sugar (Kokate, 1999)

2.4.3.Test for phenols:

Ferric chloride test: The extract was diluted to 5 ml with distilled water. To this a few drops of neutral 5% ferric chloride solution was added. The formation a dark green colour indicated the presence of phenolic compounds (Mace, 1963).

2.4.4.Test for tannins:

About 0.5 mg of extract was boiled in 20 ml of water in test tubes then filtered. A few drops of 0.1 % ferric chloride was added and observed the formation of brownish green or blue black colouration (Segelman *et al.*, 1969).

2.4.5.Test for flavonoids:

A portion of the aqueous extract was added to 5 ml of the dilute ammonia solution, followed by addition of concentrated sulphuric acid. Appearance of yellow colouration indicated the presence of flavonoids (Malick and Singh, 1980).

2.4.6.Test for terpenoids:

5 ml of the extract was mixed with 2 ml of chloroform and concentrated sulphuric acid to form a layer. A reddish brown colouration of the interface showed the presence of terpenoids (Malick and Singh, 1980).

2.4.7.Test for aminoacids:

1 ml of each extract was taken, to that few drops of Millon's reagent was added. A white precipitate indicates the presence of Protein.

2.4.8.Test for steroids:

To 1 ml of each extract, two drops of 10% concentrated sulphuric acid was added and observed for green colour.

2.4.9.Test for anthraquinones:

1 ml of each extract was taken, to that aqueous ammonia was added and observed for change in colour. Pink or violet colour in aqueous layer indicates the presence of anthraquinones.

2.4.10Test for phytosterols:

Libermann-Buchard's test: The extract was mixed with 2 ml of acetic anhydride. To this 1 or 2 drop of concentrated sulphuric acid was added slowly along the sides of the test tubes. An array of colour change showed the presence of phytosterols (Finar, 1986).

2.5.Statistical analysis

The data obtained in the present study were subjected to the following statistical analysis using SPSS 16.0.

3.0. Results and Discussion

3.1. Antibacterial activity of crude extracts of *U. lactuca*

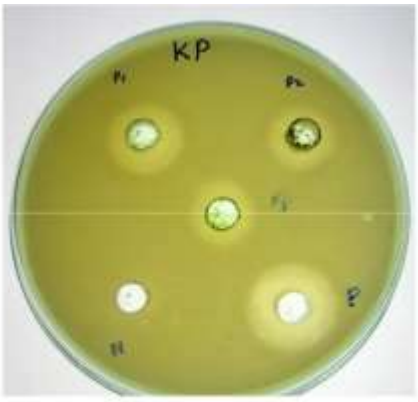
Antibacterial activity of methanolic extract of *U. lactuca* against the test pathogenic bacterial strains inferred that, methanolic extract had notable variation in inhibitory zones ranged between 2 ± 0.16 and 11 ± 0.72 mm. Here, *V. alginolyticus* (11 mm) and *V. parahaemolyticus* (10 mm) were the most susceptible bacterial strains. But, the same extract exhibited moderate antagonistic activity against other tested bacterial strains such as *S. marcescens, E. coli, E. faecalis, P. fluorescens, S. pneumonia, V. vulnificus,* with the inhibitory zones ranged between (2 ± 0.16 to 8 ± 0.68 mm). Amongst the test bacterial strains, *P. mirabilis* and *S. mutans* were found to be resistant to methanolic extract. The One-way ANOVA for the data on antibacterial activity of methanolic extract of *U. lactuca* as a function of variation between pathogenic bacterial strains was statistically significant ($F = 186.4252$; $P < 0.001$) (Figure1.).

Antibacterial activity of benzene extract of seaweed *U. lactuca* against test pathogenic bacterial strains were showed 80% growth inhibition of test bacterial strains with the zone of inhibition ranged from 3 ± 0.16 to 9 ± 0.29 mm. Here, maximum growth inhibitory activity of 9 mm was recorded against *E. faecalis*. On the other hand, minimum antagonistic activity of 3 mm was noticed against *E. coli* and *S. marcescens.* The other test bacterial strains registered moderate growth inhibitory zones ranged between 4 ± 0.20 to 8 ± 0.6 1 mm. However, benzne extract showed no positive influence on antagonistic activity against *P. mirabilis* and *S. mutans.* The One-way ANOVA for the data on antibacterial activity of benzene extract of *U. lactuca* as a function of variation between pathogenic bacterial strains was statistically significant ($F = 260.5132$; $P < 0.0001$).

Antibacterial activity of acetone extract of *U. lactuca* against the bacterial pathogens. As like that of methanol and benzene extract of *U. lactuca,* acetone extract had also showed 80% antagonistic activity. But it showed considerably higher growth inhibitory activity than the other two extracts. Acetone extract exhibited pronounced growth inhibitory against *E. faecalis* (11 mm) and *P. fluorescens* (8 mm). It exhibited moderate growth inhibitory against *V. parahameolyticus, V. alginolyticus, V. vulnificus* and *E. coli* with inhibitory zones ranged between 4 and 6 mm. On the same instance, this extract displayed very least growth inhibitory of 3 mm against *S. marcescens*. The One-way ANOVA for the data on antibacterial activity of acetone extract of *U. lactuca* as a function of variation between pathogenic bacterial strains was statistically more significant ($F = 442.6182$; $P < 0.0001$).

Antibacterial activity of antibiotic ciprofloxacin against pathogenic bacterial strains is recorded prominent growth inhibitory activity than crude extracts. It rendered elevated growth inhibitory activity of 15 ± 0.87 mm against *S. marcescens* and 17 ± 0.56 mm against *V. parahaemolyticus*. Besides, *P. fluorescens, V. vulnificus* and *V. alginolyticus* recorded equal level of growth inhibitory activity of 14 mm. The other tested bacterial pathogens recorded growth inhibitory zones ranged from 11 ± 0.99 to 13 ± 1.10 mm. DMSO which was used as negative control always showed no growth inhibitory activity against the pathogenic bacterial strains tested. The One-way ANOVA test conducted for the data on antibacterial activity of antibiotic ciprofloxacin as a function of variation due to different pathogenic bacterial strains was statistically significant ($F = 68.12347$; $P < 0.0001$).

***Clostridium* sp.** ***Klebsiella* sp.**



P1 – Methanol; P2 – Chloroform; P3 – Methanol: Chlo roform (1:1); P – Azithromycin; N - DMSO

Figure 1. Antibacterial activity crude extracts of *T. populnea* and antibiotic azithromycin against veterinary bacterial pathogens

3.2.Phytochemical analysis of crude extracts of *U. lactuca*

Phytochemical analysis of crude extracts of *U. lactuca* was portrayed in Table.1 Amongst the extract tested, acetone and methanol extract displayed presence of majority of the phytochemical constituent's *viz.* alkaloids, phenols, phytosterols and carbohydrates. But it did not show occurrence of other phytochemicals. The overall results of present study highlighted that crude extracts of seaweed possess excellent antibacterial properties; however further studies on purification and identification of active principles from this plant needs to be addressed for the discovery new drug leads.

Table.1. Phytochemical constituents of crude extracts of *U. lactuca*

Phytochemical constituents	Organic solvent extracts		
	Methanol	Benzene	Acetone
Alkaloids	-	-	+
Phenols	+	+	+
Tannins	+	+	+
Flavonoids	-	-	-
Terpenoids	-	-	-
Steroids	-	-	--
Phytosterols	+	-	-
Carbohydrates	+	-	+

In the present study, the methanolic extract of *U. lactuca* rendered growth inhibitory activity ranged between 3 and 11 mm over the test bacterial pathogens. It showed maximum antagonistic activity against *Vibrio* spp. such as *V. parahaemolyticus* (10 mm) and *V. alginolyticus* (11 mm). The growth of other test bacterial strains such as *P. fluorescens, S. pneumonia* and *V. vulnificus* were also found to be controlled by the impact of methanolic extract. Here, effective growth of inhibition of *Vibrio* spp. suggested that methanolic extract may also contain anti-vibrio compounds. The significant antibacterial activity achieved by the methanolic extract may be due to presence of active antibacterial metabolites within the extract. In accordance to the present study, Selvin et al. (2004) reported that the methanol extract was effective solvent system than acetone, benzene and petroleum ether for marine antimicrobial compound isolation.

Selvi *et al*. (2001) screened around 20 algae using methanol and ethanol along Idinthakarai coast and they reported that *Bacillus subtilis* and *Staphylococcus aureus* species were highly susceptible to most of the algal extract. Followed by methanol extract, benzene extract of *U. lactuca* had also showed noteworthy antibacterial activity against the bacterial pathogens. It rendered maximum bioactivity of *E. faecalis* and *V. parahaemolyticus*. Further observations revealed that benzene extract was least active against *E. coli, S. marcescens* and *S. penumoniae* with the inhibitory zones of about 3-4 mm.

Vallinayagam *et al*. (2009) studied the antibacterial activities of four important seaweeds namely *U. lactuca*, *Padina gymnospora, Sargassum wightii* and *Gracilaria edulis* against bacterial pathogens and inferred that *U. lactuca* had pronounced bioactivity against *S. aureus* and minimum against *P. aeruginosa.* Hydrophobicity is an important characteristic of plant extracts and their components and it enables them to partition the lipid of the bacterial cell membrane and mitochondria, upsetting the cell structures and rendering them more permeable. Death occurs when there is extensive leakage from bacterial cells or the exit of critical molecules and ions. The discrepancy in the effectiveness of the extract against different microorganisms depends upon the chemical composition of the extracts and membrane permeability of the microbes for the chemicals and their metabolism (Jayanthi and Lalitha, 2013).

Seaweed bioactive compounds with antimicrobial properties could be used for defence mechanisms against potential pathogens and as antioxidants. In addition, the interests in seaweed as a rich source of pharmaceutical agents have increased during the last few years (Gupta S, Abu-Ghannam, 2011). There has

been some research, on seaweed extracts that can inhibit human pathogenic microorganism such as bacteria, yeast, and fungi (Priyadarshini *et al*., 2012; Kandhasamy and Arunachalam, 2008; Pesado and Karnam, 1984; Soltani *et al*, 2012 and Dussalt *et al*., 2016). In the present study amongst the extracts tested acetone extract exerted excellent bioactivity than methanol and benzene extracts. It showed higher bioactivity of 11 mm against *E. faecalis* and 6-8 mm against majority of the bacterial pathogens such as *S. pneumonia, V. vulnificus, V. alginolyticus* and *P. fluorescens.* Murugan *et al*. (2007) studied that the acetone extracts of the seaweed *Chaetomorpha antennina* showed antibacterial activity against 70 –80% of the ten h uman pathogenic bacteria and four marine biofilm bacteria. Ravi Kumar *et al*. (2002) studied that the acetone extracts of *Caulerpa cupressoides* showed maximum inhibitory activity against *E .coli* and propanol extracts of *Gracilaria edulis* showed maximum inhibitory effect against *Klebsiella pneumoniae,* acetone extracts of *Padina tetrastromatica* and *Laurencia cruciata* showed maximum inhibitory activity against *Psedomonas aeruginosa*.

The macroalgae are the richest source of noval bioactive compounds (Adikalaraj *et al.*, 2011). The macroalgae are the rich in secondary metabolites which include alkaloid, phenol, saponin, flavanoid, steroid and related active metabolites which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry (Paul, 2011). In the present research an effort was made to study the similarity and differences amongst three species based on phytochemical analysis and bioactivity. Phytochemical tests revealed many similarities and also significant differences. In the present study, qualitative phytochemical analysis of different solvent extracts of *U. lactuca* inferred occurrence of maximum phytochemicals such as alkaloids, phenols, tannins and carbohydrates in acetone extract which is followed by methanol and benzene extract. In general, presence of phytochemicals significantly varies and mainly depends on usage of solvent and also plant parts used for extraction. The presence of various secondary metabolites in the seaweed is a clear indication of their pharmaceutical potential. The secondary metabolites may be useful in containing infection, act as hypolipomic and hypoglycemic agents, reduce blood pressure and regulate cholesterol levels (Krishnamurthy, 2005).

4.0.Conclusion

The present study was undertaken to investigate antibacterial potential and phytochemical constituents of green seaweed *U. lactuca* using solvents of varying polarity. Results on antibacterial activity of various organic solvent extracts of *U. lactuca* revealed that, acetone extract had wide spectral growth inhibitory activity with the zone of inhibition ranged between 3 and 11 mm than methanol and benzene extracts. Antibiotic ciprofloxacin which was used as positive control exhibited markedly pronounced inhibitory zones (9 ± 0.78 to 17 ± 0.74 mm) than the crude extracts. Phytochemical analysis of different solvent extracts of *U. lactuca* showed occurrence of maximum phytochemicals such as alkaloids, phenols, tannins, phytosterols and carbohydrates in acetone and methanol extracts. Further study needs to be addressed to identify unique bio-molecule responsible for the observed antibacterial activity.

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