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PRELIMINARY STUDY ON PHYTOCHEMICAL ANALYSIS, MINERAL COMPOSITION AND ANTIBACTERIAL PROPERTIES OF *Amphiroafragilissima* (LINNAEUS) J V LAMOROUX AND *Ulva Reticulata Forsskal* COLLECTED FROMMANDAPAM COAST, TAMIL NADU

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ABSTRACT

The production of antibacterial compounds from the marine algae is considered to be an indicator of the capacity of the seaweeds to synthesize bioactive secondary metabolites. The present study was carried out to explore the antibacterial activities of different solvents based on its polarity and tested against the Gram positive and Gram negative bacteria. For the phytochemical constituents, in *Amphiroa fragilissima* positive reactions were noted for alkaloids, reducing sugars, flavonoids and saponins. In *Ulva reticulata,* positive reactions were noted for alkaloids, phytosterols, flavanoids and saponins. Antibacterial activity of two seaweeds was compared based on the zone of inhibition. The present study concluded that the selected macroalgae are potential sources of bioactive compounds which have a broad spectrum of antibacterial activity.

INTRODUCTION

The coastal and marine environment offers an extremely rich source of important compounds of structurally novel and biologically active metabolites. It requires inputs from various scientific areas to bring out the marine chemical diversity up to its therapeutic potential [1]. So far, many chemically unique compounds of marine origin, with different biological activities, have been isolated and a number of them are under investigation or being developed as new pharmaceuticals. Among these, seaweeds are a rich source of structurally novel and biologically active metabolites. Algae have been used in traditional medicine for a longtime [2] and some algal substances are known to have bacteriostatic and bactericidal activity [3]. It has been reported that seaweeds contain high levels of minerals, vitamins, essential aminoacids, indigestible carbohydrates and dietary fiber [4]. Several of these bioactive natural products provide vital starting materials for the rational generation of libraries of compounds against infectious diseases, cancer and neurological targets, prepared through semisynthesis and biocatalysis[5]. Naturally occurring antimicrobial agents were reported more than a century ago [6]. Seaweeds from the southwest coast of India are well known for its antibacterial, anticandidal properties [7]. In this context, the objective of this work was to evaluate the phytochemical, mineral composition and antibacterial activity of *Amphiroa fragilissima* and *Ulva reticulate* against *Staphylococcus epidermidis, Bacillus cereus, Micrococcus luteus, Escherichia coli Enterobacter aerogenes*, *Pseudomonas aeruginosa* and preliminary analysis of phytochemicals for further applications.

MATERIALS AND METHODS

Sample collection

The seaweeds, *Amphiroa fragilissima* (Linnaeus) J V Lamoroux and *Ulva reticulata* Forsskal were collected from the Mandapam Coast of TamilNadu (Lattitude 9 °17'N; Longitude79º 11'E) for the present study.

Preparation of seaweed powder and extracts

The shade dried seaweed samples were ground to a coarse powder. One gram of dried seaweed sample was taken into a test tube and 10 ml of solvent was added. The solvents used were aqueous, acetone, methanol, ethanol, ethyl acetate, chloroform ad petroleum ether. The samples were centrifuged at 15,000 rpm for 15 minutes at room temperature. Finally supernatant was collected, filtered and used for further studies.

Phytochemical analysis

The Phytochemical analysis of solvent extracts of *Amphiroa fragilissima* and *Ulva reticulata* were carried out to assess the qualitative determination of chemical compounds. The

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phytochemical compounds such as Tannins[8], Phytosterols [9], Glycosides[8], Alkaloids [8], reducing sugars [10], non-reducing sugars [9], Flavonoids[8], Diterpenes[8], Proteins[8] , Resins[8] and Saponins[8] were determined as per the standard protocol.

Biochemical constituents

***Determination of ash content* (KesavaRao and Indusekar, 1989)**

Two hundred milligrams of the seaweed was dried in a muffle furnace at 400°C and the ash content obtained was weighed[11].

***Estimation of lipid* (Bligh and Dyer, 1959)**

To 200 mg of the sample, 10 ml of chloroform and 5 ml of methanol were added in the ratio 2:1. Then equal volume of distilled water was added and the aqueous phase was separated using a separating funnel. The lipid layer was collected, dried and the lipid content was measured [12].

Estimation of carbohydrate by anthrone method (Hedge and Hofreiter, 1962)

Ten milligrams of sample was ground by adding 2 ml of 10% trichloroacetic acid. It was centrifuged at 3000 rpm for 5 minutes. Supernatant was taken in a test tube and 4 ml of anthrone reagent was added. The mouths of the test tubes were covered with marbles and the test tubes were kept in a boiling water bath for 10 minutes. Tubes were cooled and absorbance was read at 625 nm. Total carbohydrate was extrapolated from the standard graph of glucose [13].

Estimation of Protein by Biuret method (Mansuya *et al.*, 2010)

To 5 mg of dried powder, 1 ml of distilled water was added and treated with 4 ml of Biuret reagent and incubated for 30 minutes at room temperature. Then it was centrifuged for 10 minutes at 4000 rpm and the supernatant was collected. The absorbance was read at 540 nm and the protein content was determined using BSA as the standard [14].

Estimation of Nitrogen by Kjeldahl method (Umbriet *et al.*, 1972)

To 10 mg of the sample, 0.5 ml of concentrated sulphuric acid and a pinch of catalyst were added. They were heated on a digestion rack for 2 hours, until the digest in the flask turned to an apple green colour. They were cooled and 1 ml of distilled water was added to the digested sample. 0.1 ml of aliquot was withdrawn and to this 2 ml of Nessler's reagent and 3 ml of 2 N sodium hydroxide was added. The sample was incubated for 15 minutes and OD was read at 490 nm. The amount of total ammonium nitrogen was calculated by a standard graph prepared with ammonium chloride [15].

Determination of Phenol (Vijayabaskar and Shiyamala, 2011)

One hundred microliter of the crude seaweed extract was mixed with 2 ml of 2% sodium carbonate and allowed to stand for 2 minutes at room temperature in the dark. Absorbance of all the sample solutions was measured at 720 nm using a spectrophotometer. Gallic acid was used as the standard to extrapolate the phenol content [16].

Determination of Flavanoids (Meenakshi *et al.*, 2009)

One milliliter of the seaweed extract was diluted to 25% of the original concentration with methanol and 0.5 ml of FolinCiocalteau's reagent (2N) was added. To this, 3 ml of Na_2CO_3 (200 mg/ml) was added and mixed well. The mixture was vortexed and the reaction was allowed to stand for 15 minutes at room temperature and absorbance was measured at 725 nm in a spectrophotometer. Gallic acid was used as the standard [17].

Determination of Calcium, Sodium and Potassium by Flame Photometer (Manivannan *et al.*, 2008)

To 5 grams of the seaweed powder, a mixture of hydrochloric acid, nitric acid and perchloric acid (HCl, HNO_3, and $HClO_4$) was added in the ratio of 10:5:1 for digestion at 300°C. The digests were filtered and aspirated in a Digital Flame Photometer (Burner Unit 121, Digital Unit 125 and Compressor Unit 122). The obtained values were expressed inmg/g [18].

Atomic Absorption Spectroscopic analysis of Metals (Co, Ni, Fe, Cu, Mg and Zn) (Rizvi and Shameel, 2005)

One gram of the dried seaweed powder was kept for ashing at 500°C for 2 hours and then wetted with 10 drops of distilled water. Thereafter it was dissolved in 3 ml of HNO_3 (1:1) and evaporated gently by keeping on a hot plate at 100 to 120°C. Sample was again ashed at 500°C for 1 hour. It was dissolved in 10 ml HCl (1:1). It was transferred to a volumetric flask and the volume was made upto 5 ml with distilled water and subjected to metal analysis. The concentration of metal ions was determined in Shimadzu AAS 7000[19].

Antibacterial activity

Bacterial cultures

The seven solvents extracts were tested for antibacterial activity against the bacterial strains procured from Microbial Type Culture Collection Centre, Chandigarh. They are *Staphylococcus epidermidis* MTCC 430, *Bacillus cereus* MTCC 2659, *Micrococcus luteus* MTCC 2452, *Escherichia coli* MTCC 443, *Enterobacteraerogenes* MTCC 2822, *Pseudomonas aeruginosa* MTCC 424.

Preparation of broth culture

The test bacterial pathogens were inoculated into the nutrient broth and incubated for 24 hours before start of the experiment.

Screening for antibacterial activity

Antibacterial activity of the seaweed extract was tested by agar-well diffusion method. Petridishes with 20ml of nutrient agar were prepared. Agar plates were surface inoculated uniformly from the overnight broth culture of the tested microorganisms. A 6mm diameter well was cut in the Nutrient agar plates in triplicates. 100µl of the respective extracts of seaweed samples *Amphiroa fragilissima* and *Ulva reticulata* were added to the respective plates and incubated for 24 hours at 37ºC. After an incubation period of 24 hours, the diameter of the inhibition zone was measured in millimeters from the edge of the well to the edge of the inhibition zone as shown in the

figures. The standard antibiotic streptomycin was used as a positive control and DMSO as negative control.

Statistical analysis

The experimental results were done in triplicates and are expressed as Mean ± standard deviation.

RESULTS

Marine organisms are a rich source of structurally novel and biologically active metabolites. Secondary or primary metabolites produced by these organisms may be potential bioactive compounds of interest in the pharmaceutical industry [20].

In the present study, the seaweeds *Amphiroa fragilissima* and *Ulvareticulata* were selected and screened for the presence of phytochemical constituents. The phytochemical constituents such as tannins, phytosterols, glycosides, alkaloids, terpenoids and several other aromatic compounds which are secondary metabolites of seaweeds serve as defense mechanism against predation by many microorganisms, insects and other herbivores. The present study revealed the presence of medicinally active constituents. The phytochemical constituents of the selected seaweeds were investigated and are summarized in Table 1.

The presence of flavanoids, glycosides, phenols, carbohydrates, saponins and tannins of selected seaweeds, could be responsible for the observed antibacterial property. The cold extracts of *Amphiroa fragilissima* showed the presence of tannin, phytosterols, alkaloids, flavanoids, carbohydrates, protein, resins and saponins from the various solvent extracts. Ethanol, ethyl acetate and chloroform extracts of *Amphiroa fragilissima* exhibits maximum phytochemical constituents. Among the various solvent extracts of *Ulva reticulata,* ethyl acetate, aqueous and petroleum ether extract revealed the presence of phytochemicals.

In the elemental analysis of *Amphiroafragilissima* and *Ulvareticulata* the concentration of macronutrients (Na, K, Ca, Mg), micronutrients (Fe, Cu, Co, Ni and Zn) are represented in Table. 2.

Among the macronutrients in *Amphiroa fragilissima* and *Ulva reticulata,* calcium was higher (11.05 mg/l and 3.20 mg/l) and in the micronutrients, iron content was greater (6.56 mg/l and 10.39 mg/l). *Ulvareticulata* had higher content of ash (1.16 ±0.15) and lipid (1.87 ±0.06). Similarly, the content of Carbohydrate, protein, nitrogen, phenol and flavanoid were also higher in the Chlorophycean member *Ulva reticulata* compared to the Rhodophycean member *Amphiroa fragilissima.*

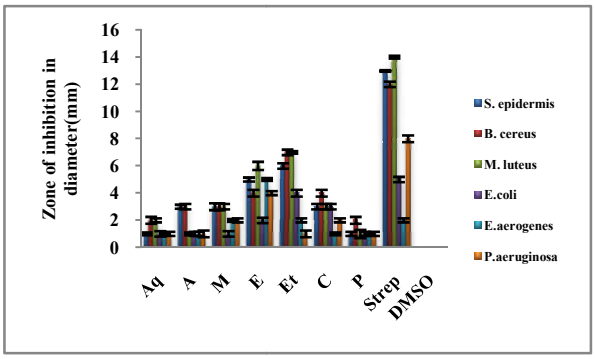

Fig. 1 Antibacterial activity of cold extract of *Amphiroafragilissima*against a few selected pathogens

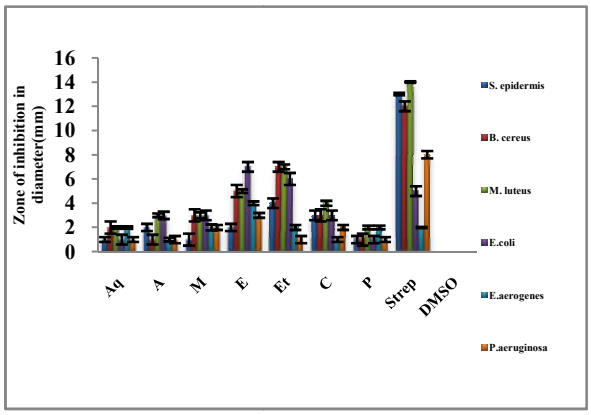

Fig.2 Antibacterial activity of cold extract of *Ulva reticulata*against a few selected pathogens

*All the values are expressed as Mean**±SD**

Table 1 Qualitative analysis of phytochemical constituents in solvent extracts of *Amphiroa fragilissima* and *Ulva reticulata*

Phytoconstituents	Tests	*Amphiroa fragilissima*							*Ulva reticulata*						
		Aq	A	M	E	Et	C	P	Aq	A	M	E	Et	C	P
Tannins	Ferric chloride test	-	-	-	+	-	-	+	+	-	-	-	-	-	+
	Gelatin test	-	-	-	+	-	-	+	+	-	-	-	-	-	+
Phytosterols	Salkowski test-Terpenoids	-	+	-	-	-	+	-	-	+	-	-	-	+	+
Glycosides	Salkowski test- Steroids	-	-	-	+	-	+	-	-	-	-	-	-	+	-
	Keller Killani test	-	+	-	+	+	-	-	+	+	-	-	+	-	-
Alkaloids	Wagner's test	+	-	+	+	+	-	-	+	-	+	+	+	+	+
	Hagner's test	+	-	+	+	+	-	-	+	-	+	+	+	+	+
Carbohydrate	Fehling's test- Reducing sugars	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Benedict's test- Reducing sugars	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Molisch's test- Non Reducing sugars	-	-	+	-	+		-	-	-	-	-	+	-	-
Flavanoids	Alkaline reagent test	+	-	+	-	-	-	+	-	-	-	-	-	-	+
Diterpenes	Copper Acetate test	-	-	-	-	-	-	-	+	+	+	+	+	-	+
Proteins	Xanthoproteic test	-	+	-	-	+	-	-	-	+	-	-	+	-	-
	Biuret test	-	-	-	-	+	-	-	-	-	-	-	+	-	-
Resins	Acetone water test	+	-	-	-	-	-	-	+	+	+	+	+	-	-
Saponins	Foam test	-	-	-	-	+	+	-	+	-	+	+	-	-	-
	Froth test	-	-	-	-	-	-	-	+	-	-	-	-	-	-
Anthraquinone	Borntrager's test	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Note: Aq- Aqueous extract A-Acetone extract M- Methanol extractE-Ethanol extract
Et- Ethyl acetate extract C – Chloroform extract P-Petroleum ether extract

Table 2 Biochemical composition in *Amphiroafragilissima* and *Ulva reticulata*

Seaweed	Ash	Lipid	Carbohydrate	Protein	Nitrogen	Phenol	Flavonoid
A. f	1.02 ± 0.15	1.25±0.06	14.5 ±0.36	16.56 ±1.05	4.23 ± 2.25	33.3 ± 1.52	24.04 ±0.52
U.r	1.16 ±0.15	1.87 ±0.06	83.8 ±2.27	65.03 ± 0.21	59.3 ± 6.42	43.3 ± 1.52	27.59 ± 0.79

Table 3 Elemental composition in *Amphiroa fragilissima* and *Ulva reticulata*

Elements	*A.f*	*U.r*
	Content (mg/L)	
Co	0.0357	0.0454
Cu	0.1667	0.2513
Fe	6.5625	10.3962
Ni	0.0563	0.0845
Mg	1.6815	1.6213
Zn	3.361	1.358
Ca	11.05	3.2
Na	3.25	2.6
K	4.36	1.33

The antibacterial activity of *Amphiroa fragilissima* in different solvent extracts indicated an inhibitory zone with a maximum of (7 mm ± 0.12) in the ethyl acetate extract against *Micrococcus luteus* and *Bacillus cereus* and a minimum of (1 mm ± 0.05) against *Pseudomonas aeruginosa* and *E.coli.* The antibacterial activity of different solvent extracts of *Ulva reticulata,* showed an inhibitory zone with a maximum of (8 mm ± 0.18) in the ethyl acetate extract against *Micrococcus luteus*and a minimum of (1 mm ± 0.06) against *Pseudomonas aeruginosa*. Compared to *Amphiroa fragilissima,* the degree of sensitivity to the extracts of *Ulva reticulata* was higher.

DISCUSSION

The bacterial infection is the most serious global health issue in the 21st century. In recent years, the development of microbial resistance to common antibiotics due to indiscriminate use of commercial antibiotics forced researchers to search for novel antimicrobial substances from various sources. Traditionally, seaweeds have been used in the treatment of various infectious diseases. Many substances obtained from seaweeds have been used for decades in medicine and pharmacotherapy, whereas some of the isolated substances have bacteriostatic and bactericidal properties [21] [22]. Different varieties of marine algae were reported to contain active ingredients that can cure diseases. Nowadays, higher percentage of population prefer to use remedies of natural origin for curing illness as these claimed to produce less side effects [23]. The present study was focused on seven different solvent extracts of *A. fragilissima* and *Ulva reticulata* for the presence of phytochemical substances, elemental analysis and antibacterial activity against Gram -positive and Gram-negative bacteria. Seaweeds are eukaryotic organisms found in salty sea water and synthesises several bioactive compounds which show antimicrobial property [24]. The ash, lipid, protein, carbohydrate, nitrogen, phenol and flavanoid content were greaterin *Ulva reticulata* compared to *Amphiroafragilissima.* This result was in accordance with the results of [25]. Krishnaiah *et al*., (2008). Similarly [26] Parthiban *et al*., (2013), investigated the presence of carbohydrate, protein and lipid content in six edible seaweed samples (*Enteromor phacompressa, E.intestinalis, Dictyotadichotoma, Turbinaria ornata, Gracilariacorticata* and*Hypneamusciformis)*.

He reported that the high protein content was found in brown seaweed *Turbinaria ornata* and low protein in the red seaweed. Both carbohydrate and lipid content were high in green seaweed than in the red seaweed. This result was in accordance with our current research.

From this study, it can be observed that Na/K ratio was below1.0 which reduces the risk of hypertension. Further, it can be observed that the mineral content available in *A.anceps* for human consumption was well within the limits (1.5-10mg). In general, algal product would supplement the daily intake of some trace elements for adults : Fe, 10-18mg; Zn, 15mg; Mn, 2.5-5mg and Cu, 2-3mg in several red edible marine seed vegetables. Similar observation was made in the present investigation, as the Na/K ratio was observed below 1.0 in *Amphiroa fragilissima* and the mineral content present in both the seaweeds were within the limits.

The presence of significant amount of calcium and iron in *Ulva reticulata* may be due to its metabolic system by directly absorbing elements from the sea water. Ca was the major constituent of these algae and formed the bulk of total minerals. Similar observation was made in the present investigation. This was in accordance with the results of Jeyashree *et al*., (2012) [27]. Phenolic and flavanoid compounds are commonly found in plants, including seaweeds, and have been reported to have a wide range of biological activities including antioxidant properties [28].

The seaweeds extracted with seven different solvents showed significant antibacterial activity against the selected pathogens. This study confirmed that the ethyl acetate extracts of *Ulva reticulata* shows better activity compared to *Amphiroa fragilissima.* Most of the secondary metabolites produced by seaweeds have bactericidal or the antibacterial compounds derived from seaweeds consist of diverse groups of bacteriostatic properties terpenols, sterols, polysaccharides, and proteins. Compounds with antibacterial activity have been detected in green, brown and red algae. Discovery of new drugs improves the immune power as well as the control of diseases against the human pathogens. The acetone, ethanol, ethyl acetate and methanol extract were better than the chloroform extract.

Antibacterial activity of red, brown and green algae against both Gram positive and Gram negative bacteria has been established by several scientists [29]. But variation in antibacterial activity may be due to the method of extraction, solvent used in extraction and season at which samples were collected [30]. Several different organic solvents have been used to screen algae for antibacterial activity. Babitha *et al.,* (2016) [31] showed antibacterial activity against Gram - positive and Gram-negative pathogenic strains after successive extraction with chloroform and methanol. [29] Kolanjinathan and Ganesh (2009) indicated that acetone was the best solution for extracting the effective antimicrobial materials from *Sargassum myricystum, Turbinaria conoides, Hypnea*

musiformis, Gracilaria edulis and Halimedia gracilis. Seven different solvents including methanol and ethyl acetate were used for extraction of antibacterial substances from *Codium adherens, Ulva reticulata* and *Halimeda tuna* [32]. In our study, seven different solvent extracts of marine algae collected from the coast of Mandapam, Rameswaram, Tamil Nadu were screened for their antibacterial activities. Antibacterial activities of seaweeds varied with the species from different division. Of the seven solvents tested, ethyl acetate was determined to be the best solvent for isolation of antimicrobial compounds from the tested marine algae followed by ethanol, acetone, methanol and Chloroform (Fig.1 & 2). Among the marine algal extracts tested, some appeared to be specific in their activity against several test bacteria. In this study, the ethanol and ethyl acetate extracts of both the selected species showed high degree of sensitivity towards Gram negative organism *E.coli* compared with the standard antibiotic Streptomycin. This may be important for the development of specific antibiotics, and further work is needed to identify the compounds causing the activity, to evaluate specific antimicrobial activity against pathogenic bacteria causing the human diseases.

CONCLUSION

The results of the present study conclude that the seaweeds are a potential health food in human diet and may be of use to the food industry as a source of ingredients with high nutritional value. The seaweed *Ulva reticulata* have very good antibacterial activity. Further purification of active compounds can be used as a source of antibiotics for the treatment of disease causing pathogens. Similarly, ethyl acetate and ethanol extract of both the selected species can be used for further analysis and study.

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