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Isolation and Characterization of Principle Bioactive Compounds from *Ulva fasciata*

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ABSTRACT: Microorganisms have developed new strategies to evade the action of antibiotics leading to multiple drug resistant bacterial strains, leading to an increasing demand in screening for new therapeutic drugs from natural product. Marine organisms are identified as a potential source of novel bioactive compounds. With this in view the study was undertaken to explore the principle bioactive compounds with antimicrobial activity from green seaweed *Ulva fasciata.* Seaweeds possess bioactive compounds and they have been found to exhibit biological activity against human pathogens. Extraction of the seaweed was carried out using ethanol. The extract was screened for antibacterial and antifungal activity against various bacterial and fungal human pathogens by disc diffusion assay. The bioactive compound present in seaweed was identified using Gas Chromatography and Mass Spectrometry. The ethanolic extract of *U. fasciata* showed wide range of activity against Gram negative bacterial and few fungal pathogens, while the extract was effective only against *Enterococcus faecalis* among the Gram-positive organisms selected for the study. The ethanolic extract of *U. fasiata* was subjected to antibacterial activity guided fractionation which led to the separation of the bioactive compound Cholest–5-en-3-ol. The result of present study revealed that the extracts of the seaweed showed potential antimicrobial activity against the tested pathogens. The presence of the bioactive compound makes the seaweed an effective drug of choice for treating various bacterial infections. Hence it is concluded Cholest-5-en-3-ol isolated from *U. fasciata* can be used as a potential bioactive compound for therapeutic purpose.

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INTRODUCTION

Seaweeds which are also called macro algae are the oldest members of plant kingdom [1]. As a consequence of an increasing demand in screening for new therapeutic drugs from natural product, there is a greater interest towards marine organisms [2]. Several marine organisms produce bioactive metabolites in response to ecological pressures such as competition for space, maintenance of unfolded surfaces, deterrence of predation and the ability to successfully to reproduce [3]. Seaweeds are considered as a source of bioactive compounds that are secondary metabolites having a broad spectrum of biological activities [4]. These secondary metabolites include alkaloids, phenols, flavonoids, saponins, steroids, carotenoids which are extensively used in drug and pharmaceutical industry. The antihelminthic, antiviral, antifungal, antibacterial, cytotoxic, haemolytic and antioxidant activities of the bioactive compounds from seaweeds have been widely reported [5-7]. *U. fasciata* occupies a major share amongst different green algae in the coastal region of southern India [8]. The present study intended to investigate the antimicrobial property of *U. fasciata* and to identify the predominant bioactive compound.

MATERIALS AND METHODS

Collection of seaweed

The seaweed was collected from Thonithurai, Mandapam coast, East of India, Rameswaram with Lat 9º16'N' Long 79º11.3'E by handpicking. The collected seaweed was cleaned

well with seawater to remove all the extraneous matter and later washed thoroughly washed with freshwater, blotted and spread out at room temperature for drying. The seaweed was shade dried and grounded to fine powder. The powdered seaweed was stored in an air tight bag for future use at -20ºC.

Herbarium preparation

The collected fresh specimens were cleaned by washing and removing the adhering particles, epiphytic algae, sand particles, mud and other debris etc. A plastic tray with half filled fresh water was taken and mounting board was placed in the water. The herbarium sheet was placed in the tray on which the specimen was displayed properly with the help of brush to avoid overlapping and hiding of the parts of the specimens. After mounting, the specimens on the herbarium a sheet was slowly tilted the herbarium sheets without disturbing the mounted specimens. The sheets were removed from the tray and spread the specimen properly with the help of forceps. After removing the water from the herbarium, cheese cloth was fully placed on the specimen later another sheet of die blotting paper was placed over the herbarium sheets to remove remaining water from the herbarium. After this process, herbarium was piled one above other and finally placed in between the two sheets of wooden press and tied tightly with a rope. The pressed materials were kept at room temperature for the duration of 24 hours. After 24 hrs the blotting papers were replaced and this process was continued till the specimen free from the moisture content. In such a condition, the cheese cloth is carefully removed from the specimen and then herbarium sheet was labelled [9] (**Figure 1**).

Fig. 1- Herbarium preparation of the seaweed

Identification of seaweed

The seaweed was identified and authenticated by Dr. V. Veeragurunathan, Scientist, Marine Algal Research Station, Central Salt and Marine Research Institute (Council of Scientific and Industrial Research), Mandapam camp - 623519, Ramanathapuram district, Tamilnadu, India (**Table 1**).

Table 1: Identification of seaweed

S. No	Name of species	Type	Class	Sub class	Order	Family
1	*Ulva fasciata*	Green	Chlorophyceae	Ulvophyceae	Ulvales	Ulvaceae

Extraction of seaweed

The dried seaweed material (100 g) was soaked in ethanol (225m1) for a week. The sample was continuously stirred on a magnetic stirrer at a constant speed (1400 rpm) during the period and then filtered. The filtrate was dried on a Rotary vacuum evaporator. The concentrated crude extract was refrigerated until tested [10].

Microbial strains

Bacterial strains used for the assay were as following: *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Salmonella typhi, Streptococcus pyogenes, Enterococcus faecalis, Pseudomonas aeruginosa, Bacillus subtilus* and *Proteus vulgaris*. The fungal strains *Aspergillus niger, Candida albicans, Penicillium* sp., *Cryptococcus neoformans a*nd *Trichophyton* sp., were also subjected to the study. Microbial strains were obtained from Bioline laboratories, Coimbatore and Kovai Medical Center and Hospitals, Coimbatore and identified according to standard procedure prescribed by Bergey's Manual of Determinative Bacteriology [11-12].

Screening of Antibacterial activity using Disc Diffusion method [13]

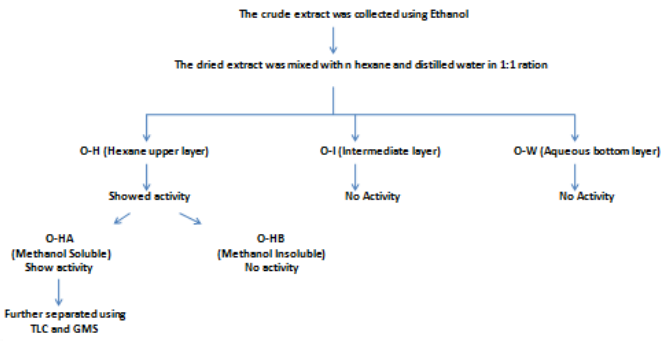
The antimicrobial activity was carried using the disc diffusion method. Sterile disc of 6 mm in diameter was used. The bacterial inoculum was grown in nutrient broth overnight and a lawn of the bacteria was made on to Muller Hinton agar by swabbing. The paper disc of 6 mm in diameter was loaded with 50µl of crude extract and placed onto the bacterial lawn. Ampicillin (10µg/ml) for bacteria, Amphotericin B (100units/disc) for Yeast and Ketakonazole (5µg/ml) for dermatophytes were used as positive control. After the incubation period the plates were measured for the diameter of zone of inhibition.

Antibacterial activity directed isolation of functional compounds [10]

The dried seaweed sample was soaked in absolute ethanol for a week. The sample was continuously stirred on a magnetic stirrer at a constant speed (1400rpm) during the period and then filtered. The filtrate was dried on a rotary evaporator at 40ºC [14]. The dried extract was mixed and shaken thoroughly with equal volume of n-Hexane and distilled water (1:1) in a 1L separating funnel and the contents were left till complete separation of the layers (**Figure 2**). Three different layers were collected separately as fraction 'O-H' (n-Hexane – upper, green layer), fraction 'O-W' (aqueous – lower, yellow layer)

and fraction 'O-I' (Middle solid insoluble mass). The crude ethanolic extract and the fractions O-H, O-I, O-W were screened for their antibacterial activity against *E. faecalis.* The fraction showed antibacterial activity was further partitioned into two portions methanol soluble (O-HA) and methanol insoluble (O-HB). The screening was repeated, the fraction retains the activity was dried and then analysed by TLC-silica using chloroform – acetic acid (9:1) as mobile phase. The developed plate was dried under normal air and the spots were visualized under UV dark chamber of 254nm & 365nm. The separated bands were scratched from the plates and dissolved in methanol – acetone (1:1). The extracts were dried by evaporation and analysed for antibacterial activity.

Figure 2. Flow diagram of Extraction and Fractionation



Characterisation of Functional compound using Gas chromatography-Mass spectrophotometer

The purified fraction was characterized using Gas chromatography-Mass spectrophotometer in DB 35-MS capillary standard non-polar column at temperature between 70^0C - at 6^0C per minute. Sample was run using Helium as carrier gas at the flow rate of 1.0 ml/min.

RESULT AND DISCUSSION

Extraction of active compounds

The crude extract was collected from *U. fasciata* (**Figure 3**) using ethanol and concentrated under rotary evaporator (**Figure 4** and **Figure 5**). The dried material was preserved at 4°C until further use.

Fig.3: *Ulva fasciata*

Fig.4: Crude extract

Fig.5: Rotary evaporator

Separation of extract layers

Three distinct layers were collected separately as fraction 'O-H' (n-Hexane- upper green layer), fraction 'O-W' (aqueous-lower yellow layer) and fraction 'O-I' (middle dark green solid mass insoluble in either of the two layers. The crude extract and the separated fractions O-H, O-I, O-W were screened for their antibacterial activity against *E. faecalis* (**Figure 6**).

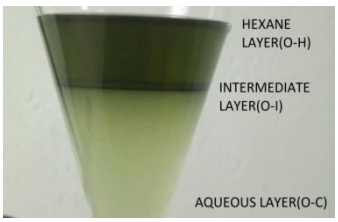


Fig.6: Fractionation using Hexane and Water

Antimicrobial assay

The preliminary screening of antimicrobial activity was performed for the ethanol extract using Disc Diffusion assay. *U. fasciata* showed maximum activity against *E. coli* (15.00±2.11) and minimum activity against *E. faecalis* (08.00±0.69) shown in **Table 2**. *U. fasciata* showed maximum activity against *T. rubrum* (11.00±0.49) and minimum activity against *C. albicans* (09.00±0.82) as shown in **Table 3**. The previous study stated the antibacterial activity of macro algae collected from Egypt against pathogenic Gram-positive and Gram-negative bacteria, and one clinical yeast strain, *C. albicans*. The tested species of Chlorophyta were more potent inhibitors than those from Rhodophyta and Phaeophyta. The findings concluded the extract of *U. fasciata* was the most active due to the phthalate esters derivatives being the active components [15].

Table 2: The antibacterial activity of *Ulva fasciata* tested against the bacterial pathogens

Name of the Seaweeds	Diameter of zone of inhibition (mm)*								
	E. coli	***E. faecalis***	***S. aureus***	***P. aeruginosa***	***S. pyogenes***	***B. cereus***	***P. vulgaris***	***K. pneumonia***	***S. typhi***
U. fasciata	15.00 ± 2.11	08.00 ± 0.58	-	12.00 ± 0.17	-	12.05 ± 0.66	10.00 ± 0.52	12.00 ± 0.39	11.00 ± 0.38

***(or) ± The value indicates the standard Error Mean of experiments done in triplicates.**

-: No detectable zone of inhibition

Table 3: Antifungal activity of *Ulva fasciata* tested against fungal pathogens

Name of the Seaweeds	Diameter of zone of inhibition (mm) *				
	C. albicans	***C. neoformans***	***A. niger***	***P. crysogenum***	***T. rubrum***
U. fasciata	09.00 ± 0.82	-	-	-	11.00 ± 0.49

***(or) ± The value indicates the standard Error Mean of experiments done in triplicates.**

-: No detectable zone of inhibition

The study reported that Gram positive bacteria are more susceptible to *U. fasciata* than the gram negative bacteria [13]. Similar observations, indicating that the more susceptibility of Gram-positive bacteria to the algal extract was due to the differences in their cell wall structure and their composition [16-17]. The resistance of gram negative bacteria towards antibacterial substances is related to the hydrophilic surface of their outer membrane which is rich in lipopolysaccharides molecules, presenting a barrier to the penetration of numerous antibiotic molecules. The membrane is also associated with the enzymes in the periplasmic space which are capable of breaking down the molecules introduced from outside [18]. In the study by Choudhury *et al.*,, ethanolic extract of *U. fasciata* showed maximum activity against Gram negative bacteria than Gram positive bacteria. The study reported that methanol extract of *U. fasciata* showed no activity against *E. aerogenes*[19]. *U. fasciata* has no detectable any antimicrobial activity against the tested microorganisms. The variation in antibacterial activity may be due to the season at which samples were collected [20]. The previous findings reported no antifungal activity was found against *C. albicans* (MY 1055), *S. cerevisae* (W303) and *A. fumigatus* (MF5667) with extracts of 40 species of marine algae except the extract of *C. barbata* (green algae) and *A. taxiformis* (red algae). The present study revealed the effective antifungal activity against *C. albicans* (09.00 ± 0.82) [21].

Purification of the active compounds [22]

The Antibacterial activity spectrum of *U. fasciata* was studied against *E. faecalis* (**Table 4-6**) Among the different layers of the extract tested, the fraction O-H and O-I showed good activity against *E. faecalis* (12mm) and (8mm) respectively. Fraction O-C showed no activity against the tested pathogen. Hence these fractions were purified further. The hexane fraction was collected and washed with absolute methanol. The methanol soluble fraction (O-HA) and methanol insoluble fraction (O-HB) were tested for its antibacterial activity. Fraction O-HA showed effective inhibition on the growth of *E. faecalis* with the zone of diameter was 11mm, whereas no activity was observed in the fraction O-HB. TLC analysis was done to identify the compounds present in the fraction O-HA. Single band (O-HA1) was observed which showed activity against *E. faecalis* (11mm). The material from TLC band was collected and dried. The purified fraction was characterized using Gas chromatography-Mass spectrophotometer and the fraction was identified as Cholest-5-en-3-ol (**Figure 7**). Cholesterol and demosterol have been identified as the major sterols in several Rhodophyta [23]. Brassicasterol in *G. elegans* was the first C_{28} sterol was identified in a red alga [24].

Table 4: Antibacterial activity spectrum of crude extract, Fraction O – H, O –I and O – C against *E. faecalis.*

Fractions	Diameter of zone of inhibition in mm
	E. faecalis
Crude	11.67±0.58
Hexane (O-H)	11.33±0.58
Intermediate (O-I)	8.67±0.58
Aqueous (O-C)	-

-: No detectable zone of inhibition

Table 5: Antibacterial activity spectrum of Methanol extracted fractions (O – HA, O - HB) against *E. faecalis*

Bacterial strain	Diameter of zone of inhibition (mm)*		
	Methanol Soluble (O - HA) 100µg/ml	**Methanol insoluble (O - HB) 100µg/ml**	**Solvent Blank**
E. faecalis	11.6±0.36	-	-

***(or) ± The value indicates the standard Error Mean of experiments done in triplicates.**

-: No detectable zone of inhibition

Table 6: Antibacterial activity spectrum of bands separated under Thin Layer Chromatography

Bacterial strain	Diameter of zone of inhibition (mm)*
	Band 1 on TLC (O –HA1)
E. faecalis	11.82±0.44

***(or) ± The value indicates the standard Error Mean of experiments done in triplicates.**

Identification of Active Metabolites using Gas Chromatography and Mass Spectrometer

GC-MS analysis of the bioactive compound was identified as Cholest- 5- en- 3- ol at the retention time 33.60 with the probability of 34.44 (**Table 7**). The molecular formula of the compound is $C_{27}H_{46}O$ with the molecular weight of 386 (Fig. 7). Seaweeds contain various organic and inorganic substances [25] large amount of vitamins and minerals widely used in agriculture, pharmaceutical, biomedical and nutraceutical industries [26]. They are used as food stuffs in Asia as they contain carotenoids, dietary fibres, proteins, essential fatty acids, vitamins and minerals [27], used as fertilizer suitable for use in organic aquaculture [28]. The phytochemicals from marine algae are extensively used in various industries such as food, confectionary, textile, dairy and paper mostly as gelling, stabilizing, thickening agents [29]. Seaweeds are excellent source of vitamins (A, B, B12, C, D, E), riboflavin, niacin, pantothenic acid and folic acid as well as minerals such as Ca, P, Na, K. Intake of minerals, vitamins and other nutrients help to protect the body against heart disease, cancer and aging process. Antioxidants may have a protective role in preventing the severity of these diseases. Phytochemicals reduces the risk of cancer. Carotenoids and flavonoids are potential antioxidant compound [30-31]. Seaweeds are rich in Iodine which could be used for controlling goitre disease caused by deficiency in Iodine. Seaweeds administration lower blood pressure and reduces cholesterol level in serum.

Seaweed polysaccharides are important dietary fibres which prevent heart disease and colonic inflammation and neoplasm. From the earlier findings [13] stated that among the phytochemicals of *U fasciata*, cardiac glycosides were present in all the extracts except acetone and methanolic extracts. Seaweed or macroalgae provide a great variety of metabolites and natural bioactive compounds with antimicrobial activity such as polysaccharides, polyunsaturated fatty acids, phlorotannins and other phenolic compounds, and carotenoids [32]. Algal lipids content in seaweed ranges from 0.12% to 6.73% (dry weight), and are composed mainly of phospholipids, glycolipids and non-polar glycerolipids (neutral lipids). Sterols are structural components of cell membrane and regulate membrane fluidity and permeability. The main sterols in macroalgae are cholesterol, fucosterol, isofucosterol, clionasterol [33]. Two sterols named as srangosterol and 24-methy cholesta-5,25-dien-3β-ol from *E. binghaniae* and a sterol, sargasterol from *D. indica.* The crystalline compound was identified as Cholesterol was found as a major metabolite isolated from *L. obtusa* extract eluted with hexane:chloroform (1:1) [34].

Table 7. Composition of *U fasciata* ethanol extract as investigated by GC-MS chromatography

S.No	Compounds	CAS Number	R.time	Probability	Mol. Formula	MW (Da)	Area (%)
1.	Borane dibutylamine	42282	4.69	24.18	$C_8H_{22}BN$	143	1.76
2.	1-(2,4-dimethylphenyl)-2-phenylethne	157592	13.72	17.40	$C_{16}H_{14}$	206	0.75
3.	Isoheptadecanol (CAS)	274990	17.24	12.17	$C_{17}H_{36}O$	256	2.07
4.	Isopropyl Tetradecanoate	307612	20.28	84.11	$C_{17}H_{34}O2$	270	8.46
5.	3-[2'-Acetylethyl]-5,7-dichloroindole	270999	23.15	53.17	$C_{12}H_{11}C_{12}NO$	255	2.94
6.	Hexadecanoic acid, Ethyl ester	339813	23.62	23.47	$C_{18}H_{36}O_2$	284	1.19
7.	Ethanol, 2-(9-octadecenyloxy)-, (Z)- (CAS)	400966	25.88	8.14	$C_{20}H_{40}O_2$	312	0.89
8.	Heptadecane, 9-hexyl- (CAS)	425449	29.02	19.16	$C_{23}H_{48}$	324	2.65
9.	Octadecane, 3-ethyl-5-(2-ethylbutyl)- (CAS)	497991	30.26	22.98	$C_{26}H_{54}$	366	4.75
10.	Nonacosane (CAS)	552006	31.48	12.14	$C_{29}H_{60}$	408	4.00
11.	2,6-dimethyl-N-(2-methyl-à-phenylbenzyl)aniline	377351	32.18	26.99	$C_{22}H_{23}N$	301	4.73
12.	Cholest-5-en-3-ol (3á)- (CAS)	526062	33.60	34.44	$C_{27}H_{46}O$	386	32.61
13.	Octadecane, 1-[2-(hexadecyloxy)ethoxy]- (CAS)	631158	34.60	13.97	$C_{36}H_{74}O_2$	538	5.06
14.	7aH-Cyclopenta[a]cyclopropa[f]cycloundecene-2,4,7,7a,10,11- hexol, 1,1a,2,3,4,4a,5,6,7,10,11,11a-dodecahydro-1,1,3,6,9-pentameth yl-, 2,4,7,10,11-pentaacetate	-	36.25	30.75	$C_{30}H_{44}O_{11}$	580	2.27
15.	Nonacosane (CAS)	552006	36.70	15.53	$C_{29}H_{60}$	408	3.87

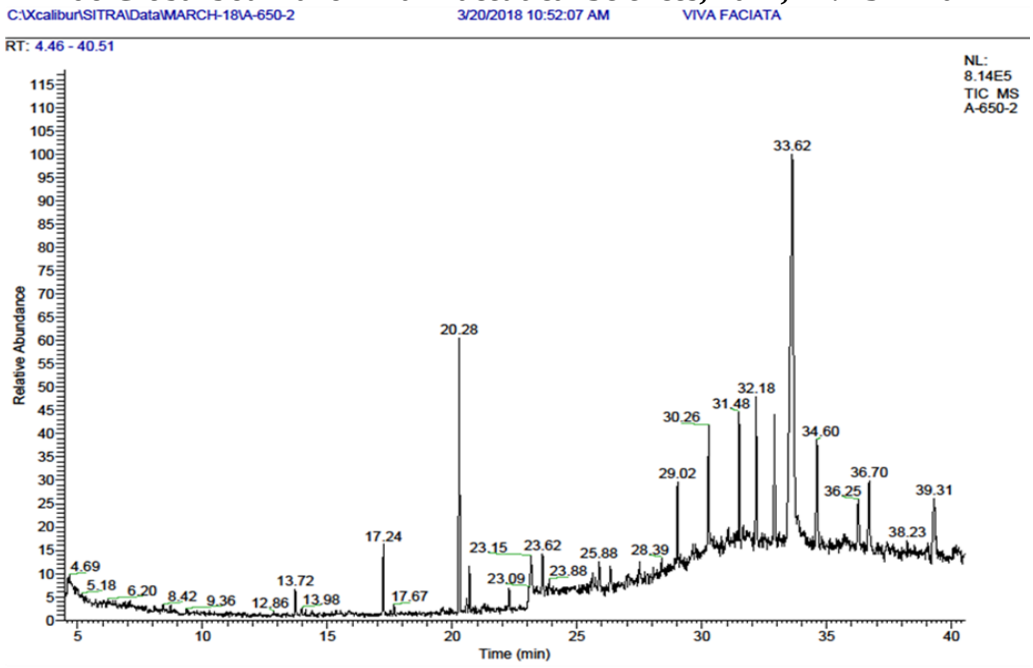


Figure 7: Identification of active metabolites using Gas Chromatography and Mass Spectrophotometer

CONCLUSION

The present study was carried out to isolate and characterize the bioactive compounds from *Ulva fasciata*. The study revealed that the extracts of the seaweed showed potential antimicrobial activity among the tested pathogens. The presence of these bioactive compounds makes the seaweed an effective drug of choice for treating different ailments. Hence the seaweed is considered as a promising drug in future.

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DATA AVAILABILITY

Not declared

ETHICS STATEMENT

The authors have taken all the necessary permissions as per ethical guidelines wherever applicable. The authors will be responsible for all the technical content mentioned in the manuscript. Journal and Publisher will not be responsible for any copyright infringement and plagiarism issue.

CONFLICTS OF INTEREST

The authors have no known conflict of interest.

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AUTHORS' CONTRIBUTION

All the authors were contributed for manuscript preparation, conducting of the experiments, data collection, interpretation and analysis of the result.

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