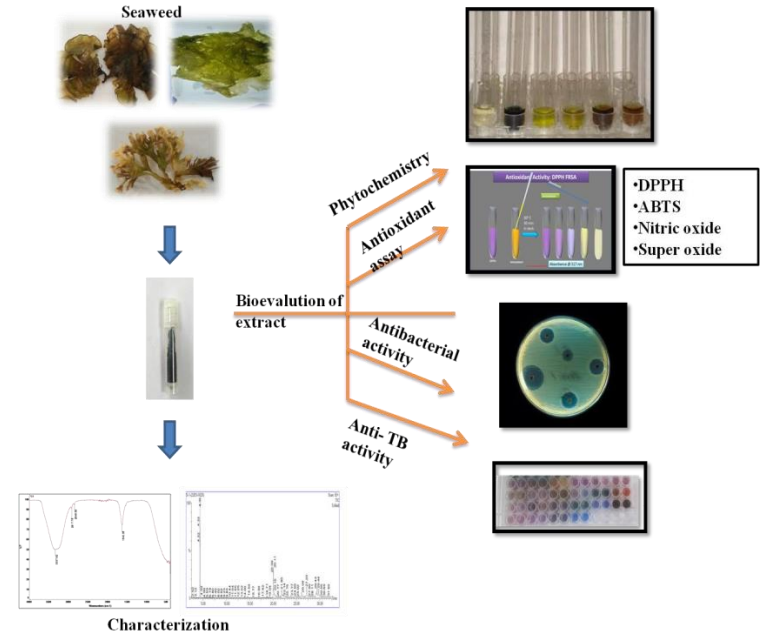
Anti-Tuberculosis Evaluation of Bioactive Compounds from Marine Seaweeds

Mary Shamya ArokiaRajan[1], Rajasekar Thirunavukkarasu[1]*, Mahesh F[2], Kumaran Subaramaniyan[1], Jerrine Joseph[1]

[1]Centre for Drug Discovery and Development, Col. Dr. Jeppiaar Research Park, Sathyabama Institute of Science and Technology, Jeppiaar Nagar, Rajiv Gandhi Road, Chennai-600 119.
[2]Department of Biochemistry, Siddha Central Research Institute, Chennai- 600106.
*Corresponding author's E-mail: microraja09@gmail.com

Graphical abstract



Abstract

Introduction: Marine seaweeds produce a variety of compounds with different biological activities, including anti-tuberculosis and antioxidant properties. The main aim of this study was to investigate the antituberculosis and antibacterial activity of the bioactive compounds present in the different seaweed extracts namely *Padina gymnospora*, *Gracileria edulis*, and *Ulva fasciata* which were collected from Rameshwaram, Mandapam region.

Methods: The seaweeds were extracted by soxhlet apparatus with ethanol and acetone solvent. The antituberculosis, antibacterial, and antioxidant assay of the crude extract were investigated using Microplate Alamar Blue Assay (MABA), DPPH, Superoxide radical scavenging activity, Nitric oxide radical inhibition assay, Total iron reducing power assay, and agar plate diffusion methods.

Results: From the three seaweed extracts, a high amount of total flavonoids was noted in acetone and ethanolic extract of U.fasciata (2.41± 0.01), and Total Phenol content was observed high in acetone and ethanolic extract of P.gymnospora (1.86 ± 0.56) and the elevated amount of Total Tannin content was observed in ethanolic extract of U.fasciata (1.89± 0.02) in mg CAE /g dry wt respectively. whereas acetone and ethanolic extract of P.gymnospora showed satisfactory antibacterial activity against all 5 pathogens which include Pseudomonas aeruginosa (29mm) and Escherichia coli (31mm). And the antimycobacterial activities of all three extracts showed inhibition at 250 µg/ml and 500 µg/ml concentrations which were indicated by the color change after 24 hours of incubation post the addition of dye. Based on the above assays the acetone and ethanolic extract of P.gymnospora were chosen to study the Structural features through the IR spectra and major antioxidant and antimycobacterial compounds were identified using GC-MS analysis. The presence of Lupeol, n –hexadecanoic acid, and oleic acid could be one of the reasons for the antioxidant, antibacterial and anti-TB properties of the extract.

Conclusion: In the future, it can act as a promising antioxidant, anti-TB, and anticancer agent for upcoming applications in pharma industries.

Keywords: antioxidant, antibacterial activity, anti-mycobacterial activity, phenol, seaweed.

Introduction

The marine domain is an exceptional reservoir of bioactive legitimate products (Chia et al., 2015) possessing unique structural aspects that are not found in terrestrial topography (Krishnaveni et al., 2012). It follows that marine creatures have bioactive metabolites that could be used to create novel drugs. Secondary metabolites found in seaweeds have a wide variety of biological effects. Algal metabolites having cytostatic, antiviral, antihelminthic, antifungal, and antibacterial properties have been found in green, brown, and red algae (Chakraborty et al., 2010). Pathogenic bacterial reproduction can be inhibited by bioactive compounds found in some macroalgae (Kolanjinathan et al., 2009). Polyphenols, terpenoids, alkaloids, saponins, tannins, and steroids seem to be just a few of the bioactive constituents that can be found in abundance in seaweeds (Ragunathan et al., 2019). As a photosynthetic organism, marine algae are continually subjected to environmental stresses such as ultraviolet (UV) radiation, oxygen-free radicals, and more. Algae contain a variety of elements that kill bacteria, including amino acids, terpenoids, phlorotannins, acrylic acid, phenolic compounds, steroids, halogenated ketones and alkanes, cyclic polysulfides, and fatty acids(Watson & Cruz-Rivera 2003). Due to the prevalence of new diseases, the rise of multidrug resistance in common pathogens, and the possible application of multidrug-resistant agents in bioweapons, the number of new antimicrobial drugs needed is more than ever (Chew et al., 2008). Mycobacterium is the etiologic agent of tuberculosis (TB) in humans which vitiates about one-third of the global populace, especially those in poorer nations (WHO 2020). It mainly affects the lungs and is one of the most pathogenic diseases caused by bacteria. It is transported from one person to another by air droplets. Around 10 million people have developed TB infection and 1.3 million deaths every year. 90% of the people are adults in India (27%) of the people are suffering from TB (Godreuil et al., 2007). Chemotherapy treatment and many drug combinations (6 to 9 months) are available to treat TB infection but the taken for curing is quite long and this could be the reason for the increasing rate of drug resistant-TB, MDR, and XDR-TB development (Bamuamba et al., 2008). Hence people are focused to develop active compounds to overcome these side effects from natural compounds. Phytomedicines have been shown to effectively treat infectious disorders, and their use has been linked to reduced toxicity in the body from conventional antimicrobial treatments. HIV/AIDS, malaria, TB, sickle cell anemia, diabetes, mental disturbance, and microbiological infections are just some of the disorders that have been helped by herbal treatments derived from these plants. Sulfated polysaccharides are the primary bioactive ingredient in seaweed and have found widespread use in the cosmetics industry due to its antiviral, antioxidant, anticancer, immunomodulatory, antilipidemic, blood coagulation, and other biological applications. Based on the above information we aimed to evaluate the antimycobacterial activity of bioactive compounds present in the three selected seaweed extracts such as *Padina gymnospora* (brown); *Gracileria edulis* (red) and *Ulva fasciata* (green) along with determining their phytochemicals seek out new medicines that show promise as medicinal agents.

Materials and methods

Sample Collection and extract preparation

The seaweeds *Padina gymnospra* (Brown), *Gracileria edulis* (Red), and *Ulva fasciata* (Green) were collected fresh during the rainy season in October 2020 from Mandapam, Rameshwaram, India, and then washed with distilled water. Some seaweed samples were processed as herbarium on the same day of collecting, while other full thalli were recognized by Dr. Anantharaman, CAS marine biology Annamalai University. On absorbent paper, the other part was air-dried at ambient temperature. The dried algae samples were crushed and sieved and stored at –20 ºC. Using a continuous soxhlet extraction method, powdered materials were then exposed to solvent extraction with acetone and ethanol. After straining the mixture through two layers of cheesecloth and then Whatman number 1 filter paper, the resulting filtrate was stored in a clean, airtight bottle.

Qualitative Analysis of Phytochemicals

According to the conventional qualitative protocols established by Evans, three distinct seaweed extracts were analyzed to determine the presence of specific biomolecules (Evans 2009). Alkaloids by Meyer's reagent (Singh et al., 2012); Terpenoids (Wadood, et al., 2013) ; Saponins; Flavonoids; Tannin; Glycosides; Coumarins; cardiac glycosides; Quinones; carbohydrate testing and protein has been tested.

Quantitative Analysis of Phytochemicals

Estimation of Total Phenol content

The Folin-Ciocalteu test was used to assess total phenolic content. Using a PerkinElmer Lambda 25 UV- Visible spectrophotometer, the absorbance was measured at 750 nm. The total phenol concentration was calculated using the Gallic acid standard curve (2-10 g/mL). The extract's total phenol content was determined to be Gallic acid equivalent in mg/g (Janarthanan et al., 2013).

Estimation of Total Flavonoid content

As a means of quantifying total flavonoid content, a colorimetric assay based on aluminum chloride was used. The reaction mixture was allowed to sit at room temperature for 30 minutes before the absorbance at 435 nm was measured. For the calibration curve, quercitin was employed as a standard. The result was reported in mg/g of extract as Quercitin equivalent (Janarthanan et al., 2013 & Okwu 2004).

Estimation of Tannin

Extracts' tannin concentrations were measured spectrophotometrically at 500 nm using a vanillin method. The tannin content of the extracts was determined using a standard curve for catechin x
100 g-1 dried weight methanol solutions (Broadhurst & Jones 1978).

Screening of Antimicrobial Activity

Agar Plate Diffusion Assay

The Medical Microbiology Laboratory, Periyar University, Salem, Tamil Nadu, provided five different bacterial strains: *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*. The density was then compared to 0.5 Mc Farland criteria, and the antibacterial activity was determined (Marudhupand et al., 2013). The agar well diffusion method was used to test the antibacterial activity of various solvent extracts of *Padina gymnospra*, *Gracileria edulis*, and *Ulva fasciata*. 0.1 ml of test organisms was swabbed on agar medium with sterile swabs from the stock broth. Then, using a cork borer, 6 mm diameter wells were drilled into the agar plates. With the use of a sterilized pipette, 100 l of each was put to the appropriate wells. After that, the plates spent 24 hours in a 37℃ incubator. The antibacterial activity of the test was measured and compared on the plates by measuring the zone of inhibition (in mm). The trials were repeated three times, with the mean value determined and tabulated (Magaldi et al., 2004).

Anti-TB activity

Microplate Alamar Blue Assay (MABA)

Mycobacterium smegmatis (ATCC 19420), a non-pathogenic fast-growing mycobacterial strain, was obtained from the CSIR-Institute of Microbial Technology's Microbial Type Culture Collection and Gene Bank in Chandigarh, India. Test inoculum was prepared in 7H9GC-tween broth, adjusted to a no. 1 McFarland tube standard, and diluted at a ratio of 1:10 in the enriched broth; mycobacterial strains were maintained on the Lowenstein Jensen slopes. After that, the plates spent 24 hours in a 37 C incubator. Palomino et al., 2016 and Nguta et al., 2002 suggested that 100 mL of enriched broth be poured into each crude extracts were plated in the first well of a 96-well sterile flat-bottom plate and subsequently diluted by a factor of 2 in each subsequent well.

We normalized the conditions by covering the microtiter plates and incubating them at 37℃. After incubating *M. smegmatis* for 48 hours, 32.5 L of a 1:1 combination of 10% tween 80 and alamar blue solution was added to each well, and the plate was incubated overnight. Color change from blue to pink indicated mycobacterial development, allowing for the determination of the minimum inhibitory concentration (MIC). Tests were conducted using pharmaceutical and crude extract

concentrations of 2g/mL for the positive control Rifamycin (RIF) and 250 and 500g/mL, respectively. All tests were run twice in a BSL-3 laboratory to ensure accuracy.

Characterization using Gas chromatography-mass spectroscopy and FTIR

The crude extract of *Padina gymnospora* was extracted using a (1:1) ratio of ethanol and acetone, and the extracted fraction was analysed using GC-MS. The components were separated using Helium as the carrier gas at a constant flow rate of 1 ml/min on a Clarus 680 GC with a fused silica column packed with Elite-5MS (5 percent biphenyl 95 % dimethylpolysiloxane, 30 m 0.25 mm ID 250m df). The component spectrums were compared to a database of known component spectrums kept in the NIST GC-MS library (2008). The Fourier Transform Infrared Spectrometer was provided by the analytical instrumentation facility. A qualitative and preliminary analysis of the major functional groups was made using an ethanolic extract of the *Padina gymnospora* sample, which was then evaluated and recorded using a piece of IR Affinity-1 equipment
(Shimadzu, Japan).

Statistical analysis

Total phenolics, total flavonoids and anti-TB activity were all determined in triplicates in all trials. The mean and standard deviation are used to express the data (SD). One-way ANOVA and least significant difference (LSD) on mean values were used to examine analysis of variance and significance of difference between means. Data was statistically examined for standard deviation using MS Excel, and a graph was created using Origin Pro3.

Results

Phytochemical Screening

We selected three different seaweeds from the brown, red, and green groups for our investigation.

The first one is *Padina gymnospora*, which belongs to the Phylum Ochrophyta; Order Dictyotales; Family Dictyotaceae; the second is *Gracileria edulis*, which belongs to the Phylum Rhodophyta; Order Gracilariales; Family Gracilariaceae; and the third is *Ulva fasciata*, which belongs to the Phylum Chlorophyta; Order Phytochemicals are non-nutritive plant substances that have no health or plant-protective characteristics. Only 12 phytochemicals were examined for three separate extracts in the above-mentioned seaweed types, and the results are shown in Table 1.

Table 1. Phytochemical content of seaweeds in three different solvents

Phyto Chemicals	Acetone Extract			Ethanol Extract			Acetone And Ethanol Extract		
	P.gymnos pora	***G.edulis***	***U.fasciata***	***P.gymno spora***	***G.edulis***	***U.fasciata***	***P.gymnos pora***	***G.edulis***	***U.fasciata***
Alkaloid	+	-	-	-	+	+	-	+	+
Terpenoids	+	-	-	+	+	-	-	+	-
Saponins	-	+	-	-	+	-	+	+	-
Flavonoids	+	-	-	+	+	+	+	-	-
Tannins	-	-	-	+	+	+	-	+	-
Glycosides	-	-	-	-	-	+	-	-	+
Phenol	-	-	-	-	-	+	+	-	+
Cumarins	_	+	-	-	+	-	-	+	-
Cardiac glycosides	+	-	+	-	-	+	-	-	+
Quinones	-	+	-	-	-	-	-	-	-
Carbhohydra	+	-	-	+	+	+	+	-	+
Tes Protein	-	-	-	-	-	-	-	+	

(+) indicates presence; (-)indicates absence

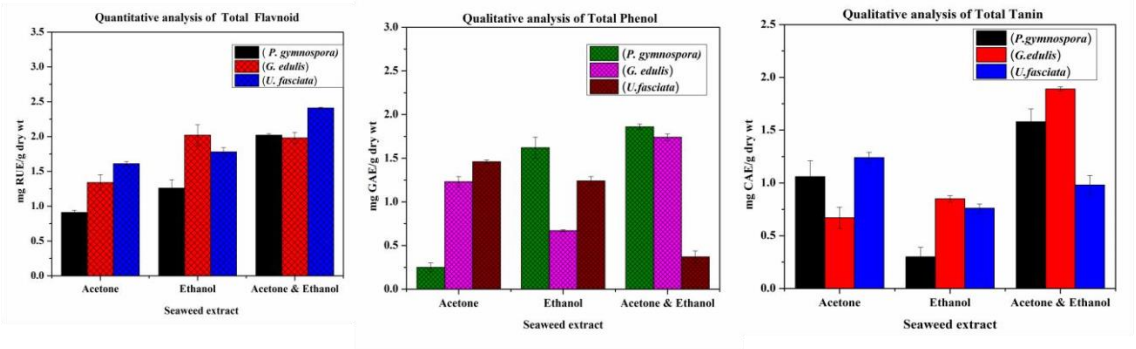
In comparison to the other two solvent extracts, the ethanolic extract of all three seaweed groups contained the most phytochemicals. *P.gymnspora* contains alkaloids, terpenoids, flavonoids, cardiac glycosides, and carbohydrates in its acetone extract. Saponin, cumarin, and quinones are all lacking in *G. edulis*. Only cardiac glycosides were detected in *U. fasciata*. Other phytochemicals are lacking from the ethanolic extract of *P. gymnospora*, including terpenoids, flavonoids, tannins, and carbohydrates. Apart from glycosides, phenol, cardiac glycosides, and quinones, *G.edulis* contains the majority of phytochemicals. Saponin, coumarin, and quinine are lacking from the ethanolic extract of *U.fasciata* terpenoid, although others are present. Finally,

the third group was investigated by combining both solvents. Saponin, phenol, flavonoid, and carbohydrate are all positive in *P.gymnospora*. Where in *G.edulis* other than flavnoid, glycoside, phenol, cardiac glycoside quinone and carbhohydeates are present. Acetone and ethanolic extract of *U.fasciata* hold the presence of alkaloid, glycoside, phenol, cardiac glycoside and carbhohydrate.

Quantitaive Analysis of Total Flavonoid, Phenol and Tanin

From the three seaweed extracts, a high amount of total flavonoid was noted in the acetone and ethanol extract of *U.fasciata* (2.41± 0.01) and a lower amount was detected in the acetone extract of *P. gymnospora* (0.91 ± 0.03) in mg RUE/g dry wt. Where the high amount of Total Phenol content was observed in the acetone & ethanolic extract of *P.gymnospora* (1.86 ± 0.56) and the minimum amount was noted in the acetone extract of *P.gymnospora* (0.25 ± 0.54) in mg GAE/g dry wt. The elevated amount of Total Tanin content was observed in ethanolic extract of *U.fasciata* (1.89± 0.02) and the beneath value was noted in acetone extract of *G. edulis* (0.30± 0.90) in mg CAE /g dry wt respectively. The results of the phytochemical content in different solvent seaweed extracts were depicted in Fig.1

Fig. 1 Quantitative analysis of total Flavonoid, Phenol and Tannin in three different solvents



a. b. c.

Antibacterial activity

Three distinct seaweed extracts were tested for antibacterial activity against gram-positive and negative bacteria, and the results are reported in Table 2.

Table 2. Antibacterial activity of seaweed extract against bacterial pathogens

Bacterial cultures	Acetone extract (mm in dm)			Ethanolic extract (mm in dm)			Acetone & ethanolic extract (mm in dm)		
	P.gymnospora	*G.edulis*	*U.fasciata*	*P.gymnospora*	*G.edulis*	*U.fasciata*	*P.gymnospora*	*G.edulis*	*U.fasciata*
Bacillus subtilis	11	8	6	9	13	10	13	8	12
Staphylococcus aureus	16	5	3	16	-	17	19	-	-
Klebsiella pneumoniae	-	-	-	12	18	19	15	17	21
Pseudomonas aeruginosa	29	26	18	32	31	26	32	27	24
Escherichia coli	31	30	17	32	24	31	34	22	22

The agar well diffusion method was used for the experiment. Acetone seaweed extract showed moderate antibacterial activity against *Pseudomonas aeruginosa* (29mm) and *Escherichia coli* (31mm) in *P.gymnospora*, and negligible activity against *Bacillus subtilis* in *G.edulis*. In acetone extracts of all three seaweeds, there is no zone of inhibition against *Klebsiella pneumoniae*. The ethanol extract of *P.gymnospora* had the greatest action against *E. coli* and *Pseudomonas aeruginosa* (32 mm), and the smallest zone of inhibition against *Bacillus subtilis* (9mm). In acetone and ethanol extracts, the largest zone of inhibition against *Escherichia coli* (34mm) was detected in *P.gymnospora* while the lowest level was reported in *P.gymnospora* (32mm) against *Bacillus subtilis* (13mm).

MABA Results

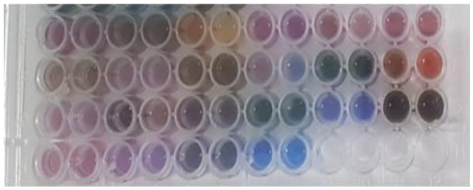
P.gymnospora, *G.edulis*, and *U.fasciata* acetone, ethanolic, and acetone & ethanol extracts were evaluated against *M.smegmatis* in a BSL III cabinet with all precautions. Based on the antibacterial test *Padina* has been taken for MABA activity. The ethanolic,

acetone and acetone & ethanolic extract extract of *P.gymnospora*, were found to have anti-mycobacterial action at lower doses (250 g/ml) and higher concentrations (500 g/ml). The colour change after 24 hours of incubation with the addition of dye was noticed as shown in table 3 and fig. 2.

Table 3. MABA assay against *Mycobacterium smegmatis*

S.no	Reference name	Test concentration (µg/ml)	Result
1	*Padina* gymnospora (PA) acetone extract	250	Inhibition
		500	Inhibition
2	*Padina* gymnospora (PE) ethanol extract	250	Inhibition
		500	Inhibition
3	*Padina gymnospora* (PAE) acetone & ethanol extract	250	Inhibition
		500	Inhibition

Fig. 2 MABA assay against *Mycobacterium smegmatis* in 96 well plate

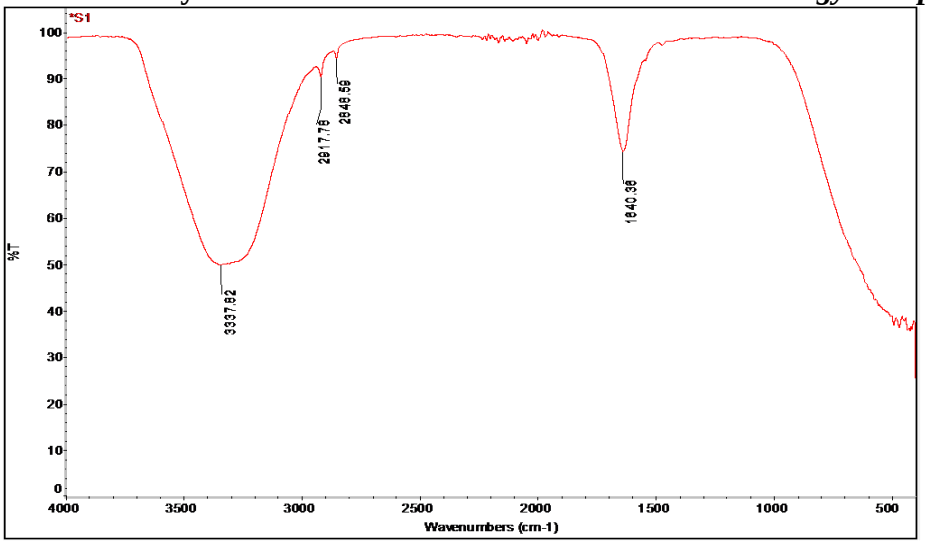


Row 1&2: 250µg/ml concentration along with drug control, growth control and DMSO control
Row 3&4: 500µg/ml concentration along with drug control, growth control and DMSO control

Characterization

The spectrum acquired between 500 and 4000 cm 1 can be used to analyze the structural properties of various compounds, such as glucosidic interactions and functional groups in crude extract. Based on the findings of the preceding tests, the characterization investigation used acetone and ethanolic extracts of *Padina gymnospora*. The chromatogram shows four separate peaks identified by FTIR analysis: 1640, 2848, 2917, and 3337. As shown in fig.3, the asymmetric strength vibration of COO- of uronic acids was assigned to 1640-1680 cm 1 and denotes the existence of alkene group (C=C), 2850-2975 cm 1 denotes the presence of alkane group (C-H), and 3300-3350 cm 1 denotes the presence of amines (N-H) and represents the O-H group.

Fig. 3 FTIR analysis of acetone and ethanolic extract of *Padina gymnospora*



GC-MS analysis revealed the presence of numerous bioactive chemicals; the eluted molecules are listed in Fig.4 and the chromatogram is shown below.

Fig. 4 GCMS analysis of acetone and ethanolic extract of *Padina gymnospora*

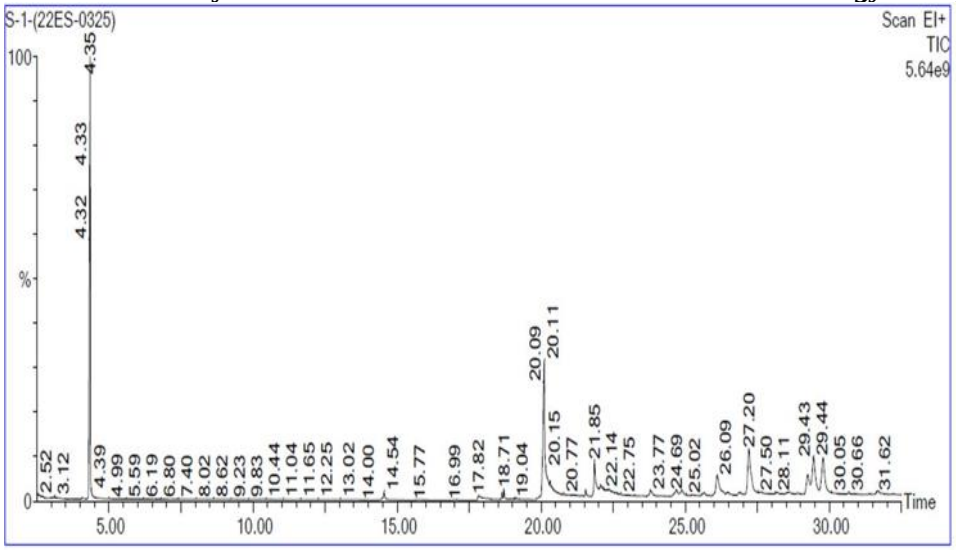
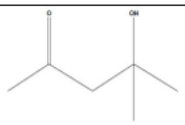
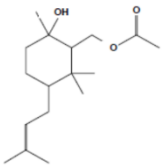
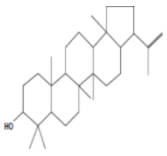
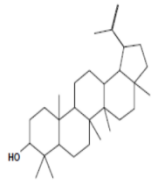
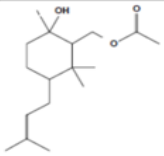


Table 4 . GC-MS Peaks and their compounds

S. no.	RT	Name	Structure	Mol. t g/mol	Peak rea	Mol. formula	IUPAC Name
1.	4.349	2PENTONONE, 4-HYDROXY, 4-METHYL		116	35.835	C6H12O6	4-Hydroxy-4methylpentan-2one
2.	20.105	N -HEXADE CANOIC ACID		256	20.786	C16H32O2	Palmitic Acid
3.	21.846	OLEIC ACID		282	4.372	C18H34O2	(Z)-octadec-9enoic acid
4.	26.108	DIHYDRO-CIS-ALPHA-COPAENE-8-OL		222	5.632	$C_{15}H_{26}O$	2,8-dimethyl-5-propan-2-yltricyclo[4.4.0.02,7]decan-4-ol
5.	27.203	2R-ACETOXYMETHYL-1,3,3-TRIMETHYL-4T (3-METHYL-2-BUTEN-1YL)1T-CYCLOHEXANOL		282	11.688	$C_{17}H_{30}O_3$	6-hydroxy-2,2,6-trimethyl-3-(3-methylbut-2-enyl)cyclohexyl] methyl acetate
6.	29.244	HOP-22(29)-EN-3.BETA.OL		426	4.246	$C_{30}H_{50}O$	3β)-Hop-22(29)-en-3-ol

7.	29.444	LUPEOL		426	9.024	$C_{30}H_{50}O$	1R,3aR,5aR,5bR,7aR,9S,11aR,11bR,13aR,13bR)-3a,5a,5b,8,8,11a-hexamethyl-1-prop-1-en-2-yl-1,2,3,4,5,6,7,7a,9,10,11,11b,12,13,13a,13b-hexadecahydrocyclopenta[a]chrysen-9-ol
8.	29.789	2R-ACETOXYMETHYL-1,3,3-TRIMETHYL-4T (3-METHYL-2-BUTEN-1YL)1T-CYCLOHEXANOL		282	8.418	$C_{17}H_{30}O_3$	6-hydroxy-2,2,6-trimethyl-3-(3-methylbut-2-enyl)cyclohexyl] methyl acetate

Based on the comparison with the library shown in table. 4, a total of 8 distinct compounds were predicted, three of which have good antioxidant and antibacterial activity. Oleic acid is a monounsaturated omega-9 fatty acid found in both animal and plant sources. Oleic acid boosts antioxidants and antipolymerization agents (Lampi & Kamal-Eldin 1998).

Palmitic acid, the most abundant saturated fatty acid found in animals, plants, and bacteria, has anti-inflammatory properties and may help with metabolic health. Lupeol is an anti-inflammatory, anti-microbial, anti-protozoal, anti-proliferative, anti-invasive, anti-angiogenic, and chemopreventive active pentacyclic triterpenoid.

Discussion

A variety of metabolites derived from marine algae contain biological activity. Three seaweeds, *P.gymnspora, G.edulis*, and *U. fasciata*, were examined for phytochemicals, anti-bacterial, and anti-TB effects in this study. Although three different solvents have been employed to screen seaweeds for antibacterial and anti-TB activities, the best solvent for effective seaweed extraction remains a mystery (Yi et al., 2001). In numerous experiments, methanol extract expressed 70% of antibacterial activity (Chakraborty et al., 2015 & Chan et al., 2015]. Secondary metabolites of seaweed such as polyphenols, flavonoids, and alkaloids have shown therapeutic advantages and are considered promising antioxidant sources. The crude methanol extract of *G. edulis* had the highest alkaloid level, even though the ethyl acetate fraction had the highest phenol and flavonoid content. Many researchers have shown that seaweeds extracted with methanol and water have antibacterial activity in vitro (Dayuti, 2018). The antibacterial effectiveness of different seaweed species is dependent on the solvent employed to extract the chemicals (brown, red and green). Cox et al., 2010 found that methanol extract of brown and red seaweed had good activity against gram positive and negative bacteria and fungi, whereas acetone would be an effective solvent for green seaweed, which is consistent with our findings. The antitubercular activity shown in our work is unusual because the exterior cell wall of the Mycobacterium has a lipid rich bilayer made up of mycolic acid high molecular weight fatty acids with 60 to 90

carbon atoms and a basic -hydroxyl—alkyl branching structure (Gupta et al., 2012). The crude extract of *S. boveanum* tested against tuberculosis was not too active, according to Yegdaneh et al., 2021 and this was the first report of *S. boveanum's* anti-tuberculosis activity. This study looked into *Padina gymnospora's* ability to suppress Tb bacteria and clinical pathogens, and the results show that this alga can be used to prevent bacterial infections. According to a previous study by Manilal et al., 2016, *Padina sps* possesses antibacterial activity against clinical infections. Many studies, however, have not focused on their anti-mycobacterial characteristics, which we did in this research report and found to be good. According to Shaikh et al., 1991 GC-MS analytical results, the presence of fatty acids in *Padina sps* could explain their phytochemical properties. This algae sps is a rich source of bioactive compounds with diverse applications, according to the study.

Conclusion

A saltwater plant called seaweed has a lot of bioactive substances. It might play significant roles in a variety of businesses in the future, helping people become more environmentally aware by utilizing natural resources that were previously inaccessible before being discovered in the past ten years. The uniqueness of this study is that other plant extracts have been shown to be effective against the TB pathogen in the same way that we have attempted this seaweed extract. We have unexpectedly detected a positive reaction against the pathogen during an in vitro experiment. The inclusion of lupeol, n-hexadecanoic acid, and oleic acid in the extract could be responsible for its antibacterial and anti-TB activities.

Acknowledgement

The authors are grateful to the Management of Sathyabama Institute of Science and Technology Chennai, Tamilnadu for providing the necessary infrastructure facilities to carry out the research work**.**

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