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In-silico analysis of bioactive compounds extracted from seaweed *Amphiroa anceps* on the pathogenicity of bacteria

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Screening of biochemical compounds in marine algae is a gateway to understand the bioactive principles of antimicrobial activity and may be a significant tool in developing environment friendly herbicides and in drug discovery. In this study, the antibacterial activity of the seaweed *Amphiroa anceps* extracted with methanol and ethanol was determined against clinical isolates namely *Vibrio cholerae* 01 ogawa, *Vibrio cholerae* O139, *Vibrio fluvialis*, *Vibrio parahaemolyticus*, *V. parahaemolyticus* 81, *V. parahaemolyticus* O3:K6, *salmonella enteritidis* type 5, Enterotoxigenic *E. coli*, *Bacillus cereus* by disc diffusion method at different concentrations. Ethanolic extract showed the highest activity against *V. parahaemolyticus*, *V. parahaemolyticus* 81 and *V. cholerae*. The FTIR results showed the presence of major functional groups like amine, hydrogen bonded alcohols, alkanes and aromatic rings. Four major bioactive compounds were selected based on GC-MS data and used as ligands to dock *in-silico* against proteins of pathogenic bacteria that were used in antibiotic studies.

[**Keywords**: *Amphiroa anceps*, Antibacterial activity, Docking, Pathogens, Phytocompounds]

Introduction

Due to increase in demand of the screening for new therapeutic drugs from natural bio based products, there is a more prominent attention towards marine organisms across the globe today. Diversified environments offered by ocean are natural habitats for enormous variety of living organisms and have rich sources of biological and genetic diversities. This has been exploding in the detection of bioactive compounds from natural bio based sources, and there are quite a number of molecules of unique chemicals having greater potential for marine pharmaceutical industries. Numerous marine organisms yield bioactive metabolites due to unfavourable environmental circumstances such as race for space, maintenance of antifouling secretions, deterrence of predation and the capability to effectively replicate[1]. Many studies have shown that, among all marine organisms, marine algae specifically are rich sources of structurally varied bioactive compounds with numerous biological activities and thus attracted researchers to work on marine algae[2-4]. It is identified that marine algae are part of the habitually consumed diet and traditionally used as medicines in Asia, Polynesia and the Pacific Islands. In these regions, particularly in Japan, occurrence of low levels of hormone sensitive cancers like prostate and breast cancer have been attributed to regular consumption of seaweeds[5,6]. The remarkable medicinal properties of seaweeds include antibacterial[7], antifungal[8], antiprotozoal[9], antifertility[10], antiviral[11], anticancer[12,13], anticoagulant, antithrombic[14], antifouling[15], antiinflamatory[16], hypoglycemic[17,18] and antioxidant[19]. Previous experimental evidence on unique phytochemicals of seaweeds with animal models and cell cultures were successfully evolved as new drugs[20,21] and some of them are still waiting for certain clinical trials to form new pharmaceuticals.

Seaweeds ability to produce primary and secondary metabolites[22] that are not found in terrestrial plants certainly creates a great interest among researchers and pharmaceutical industries. Moreover, there is an increase of resistance towards microorganism to antibiotics which are commonly used. Thus, marine organisms such as microalgae are being explored as an efficient alternative source of new drugs which can be used against some of the infectious disease. *Amphiroa* sp. is a large genus articulated coralline red algae and was first described by Lamouroux (1812) and distributed in tropical and subtropical regions and plays important role in coral reef and intertidal region. It has been found that there has been limited number of research reports on the phytochemical and antibiotic potential. Qualitative analysis of methanol, aqueous and isopropanol extracts of *Amphiroa anceps*

by HPLC and UV –Vis studies showed that phenols, tannins, alkaloids, glycosides, steroids, flavonoids and saponin were present in seaweed extracts with varying grades[24]. The primary goal of the study was to analyse the antibacterial activity of different extracts to pandemic pathogenic bacterial isolates and the phytochemical compounds using GC-MS and FTIR.

Bioinformatics and computational techniques simplify the screening of phytocompounds and optimization of ligands by adopting powerful tools like docking analysis for known protein structure and thus have essential role in drug[25] and agrochemical discovery[26]. To explore the phytochemicals which seem to be mainly responsible for the inhibition of certain pathogenic proteins of bacterial isolates, *in-silico* molecular docking studies were also performed in the present study and binding energies has been used to analyse the inhibitory property of certain phytocompounds quickly as a screening technique.

Materials and Methods

Sample collection

The seaweed, *Amphiroa anceps* collected from Andaman and Nicobar Islands, India was completely washed with seawater first and then with distilled water to expel all the extraneous matter such as sand particles, epiphytes, salt content, shells and pebbles and then sample was transported to the laboratory in plastic bags. The samples were blotted and spread out for drying at room temperature for a week. The samples were powdered into fine powder using electrical mixer grinder. The ground samples were then stored in a freezer until use further.

Preparation of the seaweed extract

20 g of powdered seaweed powder and 350 ml of solvents (ethanol and methanol) were placed in Soxhlet apparatus and the process ran for 5 h in respective solvent temperatures and the extracts obtained were filtered using Whatman No. 1 filter paper. Filtrates were evaporated to dryness using vacuum evaporator to reduce the solvent content and the amorphous residues were weighed and stored at 4 °C for further experimental studies.

Disc preparation

The stock of *A. ancepus* extract solution was prepared (100 mg/ml) using the respective solvents and they are used to make three different concentrations (0.25, 0.50 and 1.0 mg/ml). The Whatman filter paper (No.1) discs were sterilized and were impregnated with solvent extracts of *A. anceps* at the above three different concentrations. Solvent and streptomycin standard disc (50 ug/ml), as positive controls, were maintained.

Test pathogenic isolates

The test pathogens used for the antibacterial assay were clinical isolates of *Vibrio cholerae* 01 ogawa, *Vibrio cholerae* O139, *Vibrio fluvialis*, *Vibrio parahaemolyticus*, *Vibrio parahaemolyticus* 81, *Vibrio parahaemolyticus* O3:K6, *Salmonella enteritidis* type 5, Enterotoxigenic *Escherichia coli* and *Bacillus cereus*.

Antibacterial assay

The antibacterial assay was carried out by disc diffusion method of Bauer *et al*.[27]. The ethanol and methanol extracts were tested against the pathogens in three different concentration levels of 1 mg/ml, 0.5 mg/ml and 0.25 mg/ml separately. The bacterial strains were inoculated in nutrient broth and incubated for 18 hours at 37 °C. The medium was poured in the disposable petri dishes. Then using a sterile swab, the 18 hours old cultures were swapped on plates comprising Mueller Hinton agar (Hi-Media). Discs impregnated with seaweed extracts were placed on agar medium and then incubated for 18 – 24 h at 37 °C. Experimental results were obtained by measuring the zones of inhibition around the discs and inhibited zones were determined by the average of triplicates.

Gas Chromatography and Mass Spectrometry (GC-MS) analysis

The extract was analysed using GC-MS by means of gas chromatograph interfaced to a mass spectrometer Clarus 600 system equipped with Elite-1 fused silica capillary column (30 m × 1 µl was composed of 100 % Dimethyl poly siloxane). An electron ionization energy system with ionization energy of 70 eV was used, for GC-MS detection. Mass spectra were taken at 70 eV; a scan interval of 0.5 second and fragments from 50 to 600 Da. Helium gas (99.999 %) was used as the carrier gas, at a constant flow rate of 1ml/min and an injection volume of 1 µl was employed (split ratio of 10:1). The software implemented to analyse chromatograms and mass spectra was a Turbo Mass Ver. 5.4.2. The relative percentage amount of each component was computed by comparing its average peak area to the total areas.

Fourier transform infrared spectroscopy analysis

This technique, FTIR, was employed to obtain the functional groups present in the bioactive compounds of seaweeds. KBr thin disc method was implemented to get the IR spectrum, using Perkin Elmer 2000 infrared spectrometer. The sample was skimmed from 4000 – 400 cm^{-1}, for 32 times to enhance the signal to noise ratio.

Docking of phytochemical compounds with bacterial proteins

From the GC-MS results, four bioactive compounds were selected as ligands (Table 1) for the molecular docking studies. The Protein Data Bank (PDB) formats of the pathogenicity causing proteins (Table 2) were obtained and the docking analysis was done using the HEX protein docking software.

Results

Morphological features of the genus *Amphiroa* sp. were defined elaborately, but the reports on their phytochemical compounds and antibacterial activities were inadequate. To understand their antibacterial activity of *A. anceps* against clinical pathogenic bacteria *viz*., *V. cholerae* 01 ogawa, *V. cholerae* O139, *V. fluvialis*, *V. parahaemolyticus*, *V. parahaemolyticus* 81, *V. parahaemolyticus* O3:K6, *S. enteritidis* type 5, Enterotoxigenic *E.coli* and *B. cereus*, they were tested against the different concentrations (0.25, 0.50, 1.0 mg/ml) of methanolic and ethanolic extracts of seaweed, *A. anceps.* Among the two solvents tested, the ethanolic extract has shown the maximum inhibition at the highest concentration tested except for *V. parahaemolyticus* O3:K6 and *B. cereus* (Fig. 1). Generally, the effect was dose dependent; the methanolic extracts of *A. anceps* expressed the highest zone of inhibition 15, 14 and 11 mm at 1.0, 0.5, and 0.25 mg/ml (Fig. 2) respectively. *B. cereus* showed comparatively less activity than the rest of the gram-negative bacteria for both methanolic and ethanolic extracts. The seaweed extracts were highly active against all *Vibrio* sp. tested followed by *S. enteritidis* type 5, Enterotoxigenic *E. coli*, *V. parahaemolyticus* O3:K6 and *B. cereus.*

The functional groups in *A. anceps* extracts were identified by FTIR analysis using KBr disc method. The results are summarized in Table 3 and the functional groups carboxylic acids, hydrogen bonded alcohols, alkenes, alkanes and aromatic rings were observed. Amines were observed at frequency

Table 1 — Molecular structures of selected phyto-compounds from *Amphiroa anceps* as ligands for *in-silico* docking studies

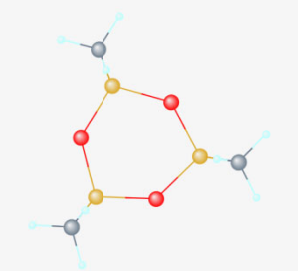
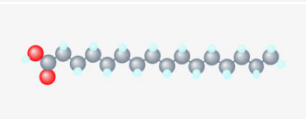
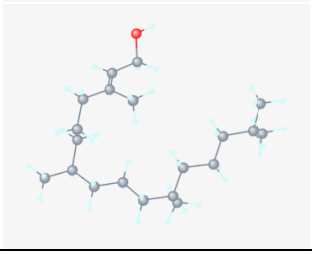
S. No.	Ligands (PubChem ID)	Chemical name	Molecular weight (g/mol)	Chemical structure	Retention time (s)
1	10914	Hexamethylcyclotrisiloxane	222.46		27
2	12401	Nonadecane	268.52		15.48
3	985	N-Hexadecanoic acid	256.42		18.26
4	5366244	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	296.53		17.19

Table 2 — Details of proteins used in *in-silico* Docking studies

S. No.	Protein name (receptors)	PDB ID	Active sites
1	*Escherichia coli* heat labile enterotoxin type IIB B- pentamer *(E. coli)*	1QCB	ARG 51, ALA 52, LYS 53, ASP 54, TYR 55, ASN 58, VAL 59, THR 61, ALA 62, GLU 63, ARG 65, LYS 66, MET 69, ALA 70, LEU 73
2	Cholera toxin *(Vibrio cholerae 0139)*	1XTC	PRO 13, ASP 14, LYS 17, GLN 18, SER 19, GLY 20, ASN 21, ASP 22, LYS 23, ILE 24, PHE 25, GLU 29, TYR 30, PHE 31, ASP 32, ARG 33, GLY 34, LYS 43, ILE 74, ALA 75, TYR 76, LEU 77, THR 78, GLU 79, ALA 80, LYS 81, MET 101, ALA 102, ASN 103, PRO 120, TYR 121, SER 122, TYR 125, TRP 127, HIS 140, ARG 141, ASN 142, ARG 143, GLY 144, TYR 145, ARG 146, ASP 147, GLN 215, VAL 218, LYS 219, ILE 222, PHE 223, GLY 225, TYR 226
3	Crystal structure of Hemolysin component from Bacillus cereus (*Bacillus cereus)*	2NRJ	GLU 24, GLN 27, LYS 28, GLY 30, LEU 31, ALA 33, LYS 34, GLN 197, LEU 198, GLU 199, GLY 202, PHE 203, VAL 205, LYS 207, GLY 208, THR 329, LEU 330, GLU 332, GLY 333, ILE 334, GLU 336, ILE 337
4	SipD from salmonella typhimurium (*Salmonella enteriditis)*	2YM9	HIS 56, GLN 57, GLN 59, GLN 60, LEU 62, GLN 63, SER 64, TRP 135, VAL 138, SER 139, ASN 141, ASP 147, LEU 150, GLY 151, GLU 154, LYS 295, GLU 299, LYS 302, TYR 312, ASN 316, TYR 319, ASP 320, VAL 323, LYS 324, SER 327, SER 328, SER 331, PHE 340, LEU 341, GLN 342, GLY 343
5	Crystal structure of thermostable direct hemolysin (*Vibrio parahaemolyticus)*	3A57	ARG 21, LYS 124, ASN 136, GLU 138, TYR 140, VAL 149, CYS 151, GLU 160, CYS 161, LYS 162, HIS 163, GLN 164, GLN 165

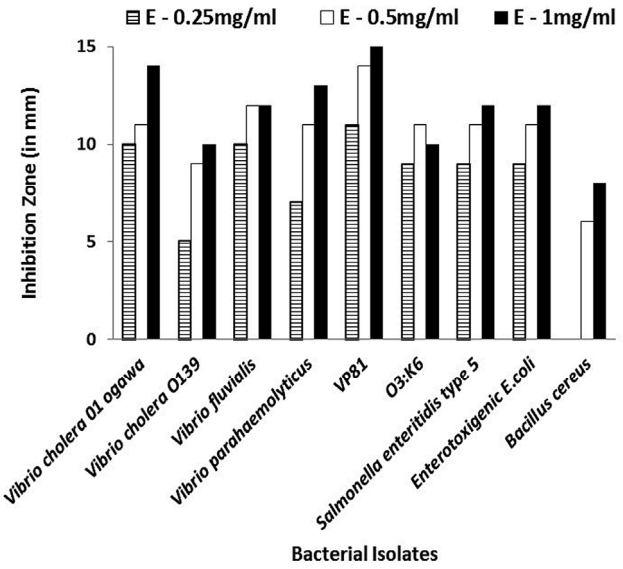


Fig. 1 — Antibacterial activity of ethanolic extract of *Amphiroa anceps* against clinical bacterial isolates

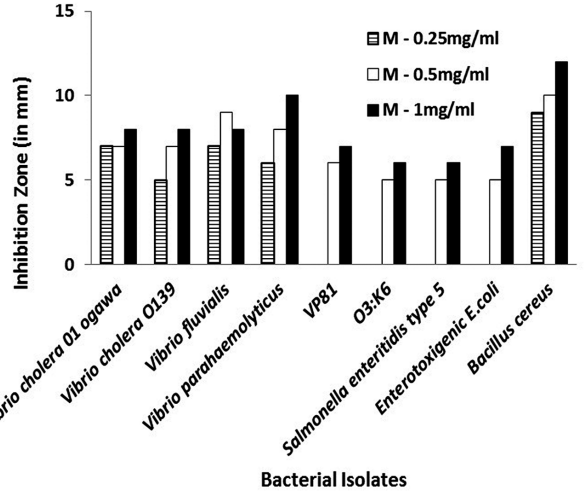


Fig. 2 — Antibacterial activity of methanolic extract of *Amphiroa anceps* against clinical bacterial isolates

3369.64 cm^{-1} peak and carbonyl at 1246.02 cm^{-1}. Alkenes were observed at 1641.42 cm^{-1} frequency and alkanes were observed at 1446.61 cm^{-1} frequency (Fig. 3). Seaweed contains a wide range of significant phytochemicals which might be responsible for their biological activities.

In order to get a descriptive account on the phytochemical compounds in methanolic and ethanolic extracts of *A. anceps*, GC-MS analysis was performed and the chromatograms of the extracts were analysed. The methanolic and ethanolic extracts showed the presence of N-hexadecanoic acid (palmitic acid), 3,7,11,15- Tetramethyl-2- hexadecen-1-ol (phytol), 2,4,4- Trimethyl-3- hydroxymethyl-5a-(3-methyl-but-2-enyl)- cyclohexene, cyclotrisiloxane hexamethyl, eicosanoic acid and some saturated and unsaturated alkane hydrocarbons like nonadecane, hexadecane, octadecane.

Based on the major peaks of GC-MS chromatograms of ethanol and methanol extracts of *A. anceps*, hexamethylcyclotrisiloxane, nonadecane, N-hexadecanoic acid, 3,7,11,15- Tetramethyl-2-hexadecen-1-ol were selected as ligands (Table 1) for *in-silico* analysis. By reviewing the pathogenicity of

Table 3 — FTIR absorption frequencies (cm^{-1}) and functional groups of seaweeds *Amphiroa anceps*

S. No.	IR frequency range (cm^{-1})	Bond	Functional groups	*Amphiroa anceps* IR Frequency (cm^{-1})
1	3300-3500	N-H	Amines	3369.64
2	3200-3600	O-H	Hydrogen bonded alcohols	3251.98
3	1640-1680	C=C	Alkenes	1641.42
4	1350-1470	C-H	Alkanes	1446.61
5	1180-1360	C-N	Carboxylic acids	1246.02
6	675-870	C-H	Aromatic rings	854.47

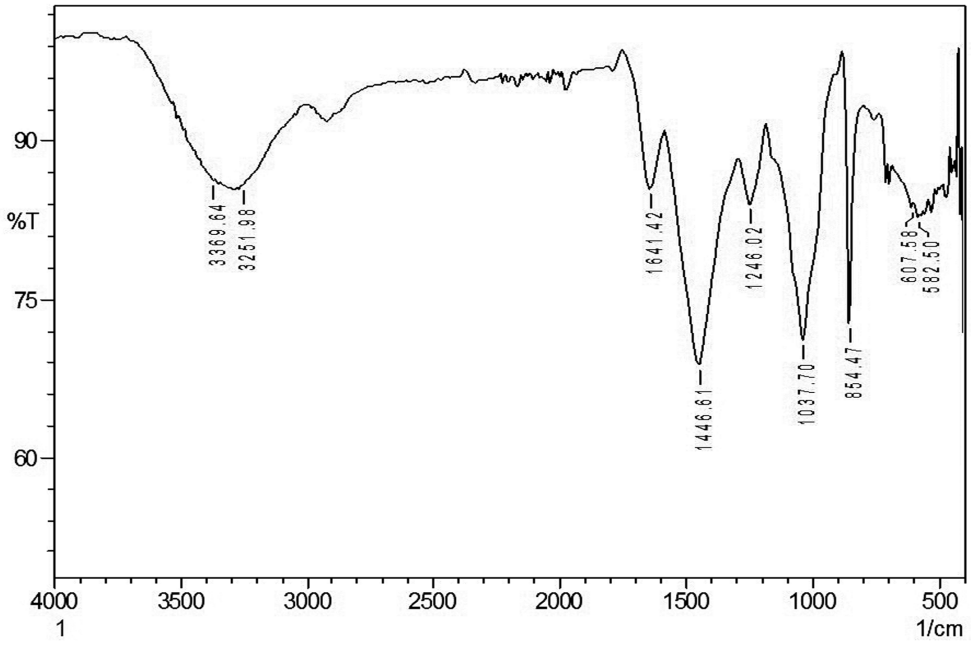


Fig. 3 — Fourier Transform Infrared Spectrum of *Amphiroa anceps*

few bacterial isolates used in antibiotic test, five proteins responsible for their pathogenicity were selected as a receptor for docking study. HEX is the software was used to dock the proteins with ligands to form docked complexes in the present study. The E-value (KJ/mol) is the binding energy liberated when the ligand binds to the receptor. *E. coli* heat labile enterotoxin type IIB B-pentame, Cholera toxin (*V. cholerae* 0139), hemolysin component from *B. cereus*, SipD from *S. typhimurium*, thermostable direct hemolysin (*V. parahaemolyticus*) and the amino acids in their active sites were presented in Table 2. Based on the *in-silico* analysis performed using the proteins from the pathogenic bacteria of interest and the phytochemicals, it was found that the compound 3,7,11,15-Tetramethyl-2- hexadecen-1-ol (phytol) exhibited the maximum binding energy with all the proteins tested.

Figures 4 and 5 depict the docked complexes of proteins 1QCB and 1XTC with 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, respectively and they produced the maximum binding affinity. The

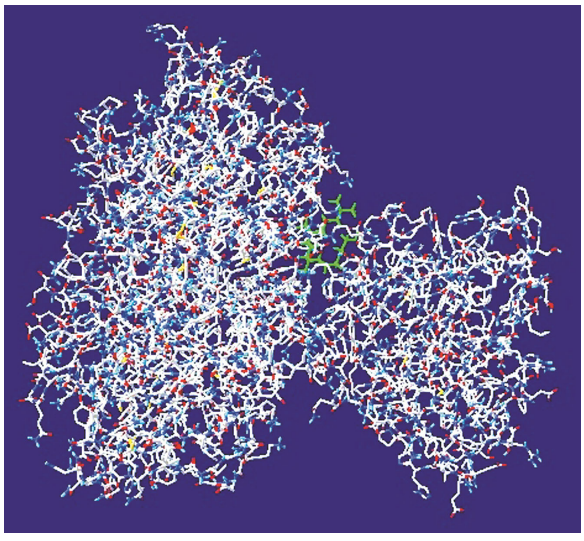


Fig. 4 — Docked complex of receptor 1XTC and ligand 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (centred in green)

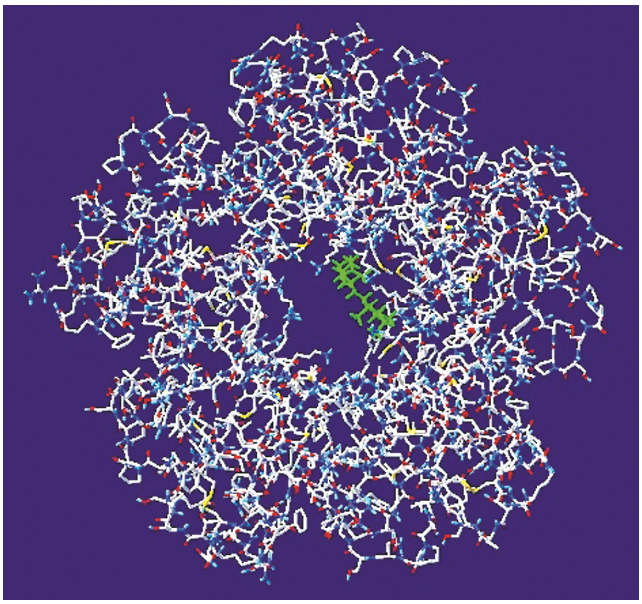


Fig. 5 — Docked complex of receptor 1QCB and ligand 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (centred in green)

compound hexamethylcyclotrisiloxane showed low binding energies when compared to other chosen compounds with the proteins. SipD from *S. typhimurium* (PDB ID: 2YM9) protein showed the least binding energy with all the phytocompounds.

Discussion

Globally there is a marked trend in academic, pharmaceutical, food and cosmetic industries for screening active biocompounds from marine organisms. Phytocompounds isolated from seaweeds were found to play an important role because of their proved nutritive values and folk medicines. In the last four decades, a wide range of studies highlight some interesting ways in which these phytocompounds could make useful natural product that is beneficial to mankind. Among them, the most relevant compounds found in algae are antibiotics, antioxidants and anticancerous biomolecules which are probably the key compounds that have attracted most research due to their fight against various diseases and antibiotic resistant bacteria. Secondary metabolites from red algae of halogenated terpenoids are measured to be hopeful in anticancer[28]research. Halomon and dehydrothyrsiferol extracted from *Laurencia viridis*[29] and *Portieria hornemanii*[30], respectively, have been established to the preclinical phase.

A. anceps, red algae generally spotted in Asia, Australia, New Zealand, South America, Africa, Islands of Pacific Ocean[31] has more biomass at high exposed reefs. Methanol and ethanol extracts of the seaweed exposed to different bacterial pathogenic isolates exhibited the antibacterial activity. The crude extracts of *A. anceps* expressed great potential as antagonist activity in chicken meat associated pathogens, *Vibrio* sp., *Yersinia* sp. and *Streptococcus* sp.[32]. Methanolic extracts of *Gracilaria edulis*, *Spirulina platensis*, *Jania rubens* and few marine algae from Turkey revealed the best antibiotic properties[33]. Antibacterial activity of ethanol extract of *Gelidium acerosa*[34] was strongest and it shows similarity with the present investigation, where ethanol extract has showed better antibiotic and binding activity against pathogenic bacteria and proteins tested respectively. Previous studies on antibacterial activity against certain seaweeds showed better inhibitory activity on gram negative than gram positive bacteria[35,36] which was also evident in the present investigation. In *A. anceps*, the occurrence of phytocompounds like saponins, alkaloids, phenolic groups, steroids, flavonoids, terpenoids and tannins[37] have been described earlier and they might have exhibited their antimicrobial properties against pathogens.

Ethanolic and methanolic extracts of *A. anceps* were analysed by FTIR and GC-MS to confirm the presence of antimicrobial compounds. Analytical results of FTIR highlight the presence of alkanes, amines, aromatic rings, carboxylic acids which are proved to be antimicrobial in many previous studies[38-40]. Volatile compounds identified using GC-MS were hexamethylcyclotrisiloxane, nonadecane, N- hexadecanoic acid and unsaturated and saturated fatty acids of solvent extracts of *A. anceps* and their relative presence might have played a role in the variation of antibacterial activity. The antimicrobial activity was endowed by the presence of long chain unsaturated fatty acids[41] which are evident in both the solvent extracts that might have revealed antibacterial property in the present study.

Molecular modelling and docking is a rational computational technique that is often used as a significant tool to predict protein- protein, protein-nucleic acid and protein - chemical compounds interaction because of the advancement in algorithms. Receptor and ligand docking studies aid in rapid computation and screening of compounds to establish their inhibitory activity. Experimental techniques such as antibacterial activity could be accompanied by

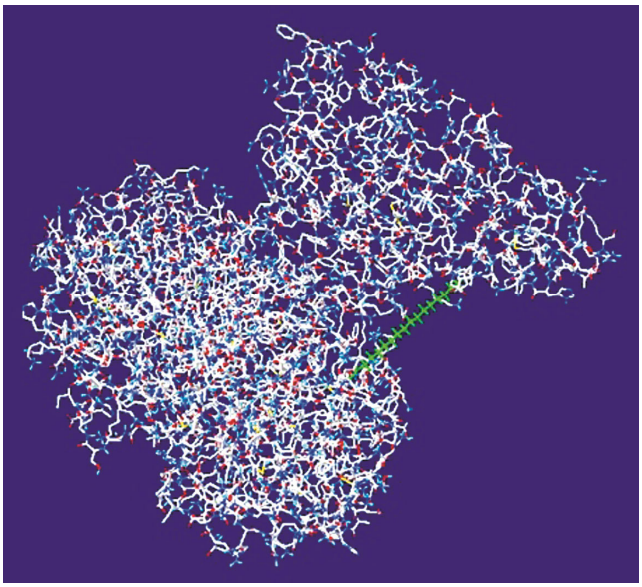


Fig. 6 — Docked complex of receptor 1XTC and ligand N-hexadecanoic acid (centred in green)

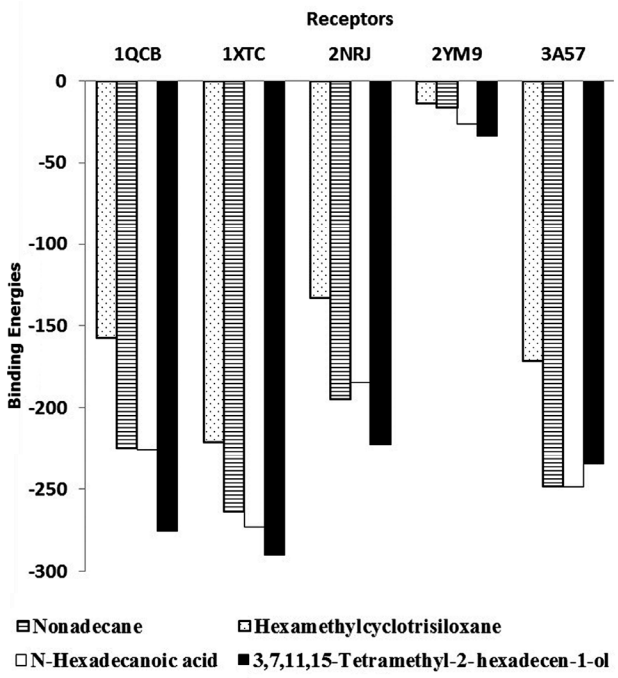


Fig. 7 — E-values of docked complexes of selected proteins and phytocompounds [Proteins: 1QCB - *Escherichia coli* heat labile enterotoxin type IIB B- pentamer, 1XTC - Cholera toxin (*Vibrio cholerae*), 2NRJ - Hemolysin -*Bacillus cereus*, 2YM9 - SipD from *Salmonella typhimurium*, 3A57 - Hemolysin (*Vibrio parahaemolyticus*)]

computational *in-silico* docking methods to identify out the active compound extracted from seaweed.

The pathogenic proteins selected as receptors for docking analysis are virulent pollutants, toxins, contaminants of *E. coli and V. cholerae*, Hemolysin of *V. parahaemolyticus* and *B. cereus* and *Salmonella* sp. SipD protein. Ligands are selected based on the functional group visible in FTIR and GC-MS results hexamethylcyclotrisiloxane (cyclicvolatile methyl siloxan), nonadecane (alkane hydrocarbon), N- hexadecanoic acid (carboxylic acid) and phytol (diterpene alcohol). Phytol and N-hexadeconic acid have high affinity towards cholera toxin (Fig. 6), heamolysin of *V. parahaemolyticus* followed by *E. coli* enterotoxin, suggesting that they may have the ability to inhibit the pathogenicity of proteins tested. Phytol has proved to be antibacterial, cytotoxic, anti-inflammatory[41] and fatty acids isolated from marine algae have expressed antifungal, antimalarial and antibacterial activity[42]. Solvent extracts showed the maximum antibacterial activity against *Vibrio* sp. which can be related to the *in-silico* analysis of bioactive compounds against *Vibrio* sp. and these proteins showed the highest affinity towards all the chemical compounds analysed (Fig. 7). *Salmonella* sp. SipD is the protein, which injects virulence proteins into their hosts and cause infections[42] and it showed very less affinity when compared with all the compounds tested which are of virulent bacterial toxins. In general, these results indicated that *A. anceps* has bioactive compounds that could be used to inhibit the pathogenic activity in bacteria and their growth, and hence they can be further investigated to utilise them as antimicrobial agents and for other therapeutic needs.

Conclusions

The study comprises the screening of methanolic and ethanolic extracts of *A. anceps* to antibacterial activity against few endemic pathogenic isolates and phytochemical constituents using FTIR and GC-MS. The results emphasized the antibacterial activity of seaweed extracts against pathogens and have potentially high impact on *Vibrio* sp. in general. Analytical spectrum and chromatograms revealed the presence of various groups of chemical compounds namely, carboxylic acids, phytol, saturated and unsaturated fatty acids and certain alkanes which facilitate antibiotic activity. *In-silico* docking studies of pathogenic proteins with the phytochemical ligands suggest that phytol has high affinity for *V. cholerae* and *E. coli* toxin. From these results, the inhibitory ability of various phytoconstituents of *A. anceps* towards bacterial isolates of pathogenic *Vibrio* sp., *E. coli* and *B. cereus* were evident.

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Conflict of Interest

The authors report no conflicts of interest.

Ethical Statement

The paper reflects the authors own research and analysis in a truthful and complete manner.

Author Contributions

DM & RC conceptualised the study. DM designed the study, acquired data, performed analysis, interpreted the results and edited the manuscript. RC drafted the manuscript.

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