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Efficiency of solvents in identification of bioactive compounds from selected marine red algae of the Gulf of Mannar, India

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In the present study, three red algae samples, *Acanthophora spicifera, Hypnea musciformis* and *Gelideilla acerosa,* collected from Mandapam Coast, Gulf of Mannar, India, were extracted using polar solvents (Ethanol and Acetone) and non-polar solvents (Hexane and Chloroform). The crude extract of each alga was subjected to GCMS (Agilent 7890B; 5977A) analysis. A total of 288 compounds from all three red algae were identified. Of the 146 compounds identified in *A. spicifera*, 10 compounds have been commonly identified in all three solvents (hexane, ethanol and chloroform); another six compounds in ethanol and chloroform; five compounds in hexane and ethanol and another ten compounds in hexane and chloroform. Totally, 109 compounds have been identified in *H. musciforms*, only one compound, n-Hexadecanoic acid, was reported in all three solvents (hexane, ethanol and chloroform), and another 12 compounds have been commonly identified from ethanol and chloroform extracts. In *G. acerosa*, out of 151 compounds, seven compounds were identified commonly in ethanol and eleven in acetone and chloroform extracts. Overall the present study concludes that 32, 11, and 49 compounds identified were specific for hexane, ethanol and chloroform extracts of *H. musciforms*, respectively; 38, 27, and 42 compounds from ethanol, hexane and chloroform extracts of *A. spicifera*, respectively and exclusively 47, 35, and 35 different compounds in acetone, ethanol and chloroform extracts of *G. acerosa*, respectively. Therefore, the isolation of bioactive compounds is found to be polarity-specific. n-Hexadecanoic acid was the major constituent identified from *A. spicifera* in all three solvent extractions; in *H. musciformis* and in *G. acerosa*, only the acetone extract showed the highest peak area for n-Hexadecanoic acid. Seventy-one compounds identified through this study are yet to be reported in algae of India or elsewhere, and many of them have been found to have no report of biological activities. Therefore, these compounds would be evaluated, which will have a source for new drug candidates for communicable and non-communicable diseases. Further, the antimicrobial properties of crude extracts of all three red algae were tested using human pathogens (bacteria and fungi). The results revealed stronger antifungal activity against *Candida* sp. with hexane and chloroform extracts of *H. musciformis* and ethanol extracts of *G. acerosa,* whereas the antibacterial activity of *H. musciformis* extracts was stronger against all four human pathogenic bacteria than that of the other two red algae tested in this study. Therefore, the purification of compounds specific for antibacterial and antifungal properties from marine red algae may pave the way for new drug leads for the treatment of various human diseases.

Keywords: 2,4,6-Tris(1-methyl-2-(a-methylbenzylamino)-ethen-1-yl)-1,3,5-triazine, Antimicrobial properties, Astaxanthin, Cholesta-4,6-dien-3-ol, (3β), GCMS analysis, Gulf of Mannar, Marine red algae, n-Hexadecanoic acid, γ-Sitosterol

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Introduction

The macro-algae (seaweeds) of marine origin contain major chemical constituents (proteins, carbohydrates and lipids), and also diverse bioactive compounds such as polysaccharides (alginate, agar, and carrageenan), lipids, polyphenols, steroids, glycosides, flavanoids, tannins, saponins, alkaloids, triterpenoids, antheraquinones and cardiac glycosides including pigments[1-5]. The methanol and hexane extracts of red alga *Halymenia* spp., reported alkaloids, flavonoids, tannins, triterpenoids and saponins[6]. Normally, the marine red algae (Rhodophyta) exhibit high nutritional value and are also a rich source of secondary metabolites which have antibacterial, antitumour, antioxidant, antiviral, antifungal properties, etc.[7-11].

The phytochemical compositions of red algal extracts varied depending upon the solvent's polarity;

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Supplementary table is available online only

hence, they played a major role in the isolation of a variety of secondary metabolites with different biological properties[12-13]. Many studies focused on the identification of bioactive compounds using various organic solvents by GCMS[14-22]. The methanol, ethanol, chloroform and water (crude) extracts of red algae *Acanthophora spicifera* and *Portieria hornemannii* have been reported for a variety of bioactive compounds, which showed higher antimicrobial and antioxidant activities[12]. GCMS study reported that the methanol extract of red algae *Gracilaria cervicornis* and *Gracilaria gracilis* had 50 different compounds in each species. The crude extracts have higher antifungal activity against *Macrophomina phaseolina* and *Lasiodiplodia theobromae*[16].

Ninety-one compounds have been identified from red alga *Kappaphycus alvarezii* using polar and non-polar solvents like chloroform, ethanol and distilled water[4]. Another study reported 27 compounds from the methanol extract of *H. valentiae* by GC-MS analysis with major constituents such as Hexadecanoic acid-methyl ester, Hexadecen-1-ol-tetramethyl; 3-Heptadecene,(Z)-,13-Docosenamide,(Z)- and 2-Pentadecanone, 6, 10, 14-trimethyl- Retinoic acid, methyl ester[20]. Similarly, the methanol extract of *Halymenia dilatata* by GCMS analysis reported seven compounds, two of which, namely hydroperoxide and acetyl valeryl, are beneficial for the growth and survival of fish[17]. The ethanol extract of *Gelidium pusillum* of the Mandapam coast reported only seven compounds by GC-MS analysis, out of which two compounds are found to have antimicrobial activity[21].

Red alga *Gracilaria salicornia* extracted with n-hexne, chloroform, ethyl acetate and methanol by graded maceration method showed that the chemical nature of compounds is based on the polarity of solvent used i.e., n-hexane extract was dominated by steroid compounds; chloroform extract by fatty acid compounds; ethyl acetate by ester and alkanes compounds and methanol extract by steroids and esters[13] and this study confirmed that polarity of solvent is found to have impact on compound separation.

Various studies used the crude extracts to test the biological properties of marine macro algae[9,12,23,24]. The red algal extracts have a reducing effect on plasma lipids and obesity. Red algae-derived phytosterols are a promising therapeutic approach for decreasing the intertwined neuro-inflammatory and neurodegenerative processes featuring multiple CNS disorders[25].

The primary biomolecules, minerals, precursors of many vitamins and steroids, as well as the secondary metabolites of pharmaceutical properties from red algae, will be attractive in future to increase the pharmaceutical and medicinal interest, i.e., treating various human diseases[26]. Some studies used different polarity of solvents for the extraction of compounds from algal species, while some other studies have used only a single solvent. Due to a lack of comparative studies on the suitability of organic solvents in extraction and identification of bioactive secondary metabolites from red algae of the Indian Coastal waters, the present study was conducted to investigate the polarity of organic solvents in extraction and identification of secondary metabolites from three red algae. Extracts of *Acanthophora spicifera, Hypnea musciformis* and *Gelideilla acerosa* sampled at Mandapam Coast, Tamil Nadu, India were subjected to Gas Chromatography–Mass Spectrophotometer (GC-MS) and also to test their antimicrobial potential for treatment of various human diseases, including multidrug resistant bacteria (MDRP).

Materials and Methods

Collection and identification

In the present study, three different red algae, *Hypnea musciformis* (Wulfen) (Lamouroux 1813), *Acanthophora spicifera* (Vahl) (Borgesen 1910) and *Gelidiella acerosa* (Forsskal) (Feldmann & Hamel, 1934) were collected from the coastal waters of Mandapam, Gulf of Mannar, Southeast Coast, India during July 2022. The sampled locations were marked using Garmin GPS (N 09° 12568' and E 079° 08634'). The algae were handpicked and separated individually. They were washed with fresh water two to three times to remove the epiphytes, sand and other unnecessary materials. The sampled algae were kept in a sterilised Ziploc bag. These were shade dried for 7 to 10 days in the laboratory. After complete drying, they were powdered using a mixer grinder and kept in a clean and dry Ziploc bag for further analysis. The photographs of algae were taken immediately after collection for identification. The collected red algae were identified based on the taxonomic keys described by Rao[27]. The herbarium of red algae sampled in this study was kept in the Department of Marine Biotechnology, AMET Deemed to be University, Chennai.

Solvent extraction and crude preparation

The powdered sample in each alga species (500 g) was kept in a 1000 ml conical flask and suspended in polar solvents such as ethanol and acetone and non-polar solvents such as hexane, chloroform, and ethyl acetate (Merck AR Grade). They were kept in a horizontal shaker (Remi) at room temperature for two to three days. The extract was filtered using Whatman No.1 filter paper and stored in a clean and dry conical flask. The extraction process was repeated two to three times. The extracts were pooled and stored in a conical flask. Then the extract was concentrated using a rotary vacuum evaporator (Puchi RII, Switzerland) at 40°C. The final concentrated crude extract was individually stored in sterile air-tight sampling vials and kept in a refrigerator until further use[28].

GCMS Analysis

In this study, the volatile compounds in the polar and non-polar solvent extracted crudes of all three red algae were separated and identified using Gas Chromatography- Mass Spectrometry (GCMS) (Agilent Model 7890B with 5977A MSD). The chromatography was performed on a capillary HP-5MS 5% Phenyl Methyl Silox-60-325°C (325°C) 30 m × 250 µm × 0.25 µm. The injection volume of the sample was 1 µL with a 1:1 split ratio. Helium (99.999%) was used as a carrier gas throughout the column. The injector source temperature was 250°C. The initial temperature in the oven was programmed from 50°C hold for 2 min then ramp 3°C/min upto 270°C, hold for 10 min at 270°C[44]. The Mass spectra were taken using an MS detector with a detector temperature of 280°C at a total running time of 10 minutes. The sample data were compared with the NIST MS2011 Library[29].

Antimicrobial Testing

The antimicrobial activity of the human pathogenic microbes (Gram positive bacteria, *Enterococcus faecalis* and *Staphylococcus aureus* 29213; Gram negative bacteria, *Pseudomonas aeruginosa* 15442 and *Escherichia coli* 25922 and fungus *Candida albicans* MTCC-1511) obtained from the Department of Microbiology, Central University of Tamil Nadu, Thiruvarur, Tamil Nadu were tested using slightly modified Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute[30]. For testing antimicrobial properties of ethanol, hexane and chloroform extracts of *Hypnea musciformis* & *Acanthospora spicifera* and ethanol, acetone and chloroform extracts of *Gelidiella acerosa*, the extract was individually diluted in sterile phosphate buffered saline (20 µL) and transferred to a sterile Whatman No-1 filter paper disc. After air drying, the discs were placed on the Mueller Hinton agar (HiMedia) plates and inoculated with each microorganism at an equal distance. The disc soaked with a similar quantity of phosphate buffer was used as the control. Amoxicillin and fucozonel were used as positive controls for bacteria and fungi, respectively. After 24 h of incubation at 37°C for bacteria and 48 h at 25°C for fungus, the zone of inhibition was measured in mm, and the experiment was carried out in duplicate.

Results

In the present study, the crude extracts of three red algae, *Hypnea musciformis*, *Acanthophora spicifera,* and *Gelideilla acerosa* were subjected to GCMS (Agilent 7890B; 5977A) analysis and the relative percentage was calculated based on the peak area normalisation and the compounds were identified with the NIST library.

In the present study, a total of 288 different compounds were identified in all three red algae (Supplementary Table 1). The total compounds identified in *H. musciformis* (ethanol, hexane and chloroform extractions), *A. spicifera* (ethanol, hexane and chloroform extractions) and *G. acerosa* (ethanol, ecetone and chloroform extractions) were 109, 146, and 151, respectively (Table 1). Overall, *G. acerosa* is found to have a maximum number of compounds, followed by *A. spicifera* and *H. musciformis*. Overall, 71 compounds reported in all three red algae have not been reported in the literature earlier (Supplementary Table 1). The GCMS chromatograms of the red algal extracts are presented in Figs. 1-3.

The GCMS analysis of crude extracts of red algae showed variations in compound detection based on the polarity. Of the 109 compounds identified in *H. musciformis*, the ethanol, hexane and chloroform extracts of *H. musciformis* contained 45, 12 and 66 compounds, respectively (Table 1). Of these n-Hexadecanoic acid was reported in all three solvents (both polar and non-polar solvents) and another 12 compounds such as 1) (E)-Dodec-5-en-4-olide, 2) 1-Dodecanol, 3,7,11-trimethyl, 3) 2-Pentadecanone, 6,10,14-trimethyl, 4) 4,8,12,16-Tetramethylheptadecan-4-olide, 5) Cholesta-4,6-dien-3-ol, (3β)-, 6) Dibutyl phthalate 7) Heptadecane, 8)

Table 1 — Total compounds identified, combination of compounds identified in different solvents and compounds identified exclusively for each solvent from the three red algae, Gulf of Mannar, India, using GCMS analysis

Red Algae Species and Solvents Used	Total compounds identified	Common compounds identified				Total common compounds	Exclusive for Solvent
		ECH	EC	EH	CH		
Hypnea musciformis							
Ethanol (E)	45	1	12	0	0	13	32
Hexane (H)	12	1	0	0	0	1	11
Chloroform (C)	66	1	12	0	0	13	53
			109*				
Acanthospora spicifera		ECH	EC	EH	CH		
Ethanol (E)	69	10	7	4	0	21	48
Hexane (H)	50	10	0	4	10	24	26
Chloroform (C)	68	10	7	0	10	27	41
			146*				
Gelidiella acerosa		ECA	EC	EA	CA		
Ethanol (E)	56	6	3	11	0	20	36
Acetone (A)	76	6	0	11	10	27	49
Chloroform (C)	55	6	3	0	10	19	36
			151*				

*: Total compounds identified in each alga.
E: Ethanol; H: Hexane; C: Chloroform; A: Acetone

Octadecane, 3-ethyl-5-(2-ethylbutyl)-, 9) Phytol, 10) Phytol, acetate, 11) Tetradecanoic acid, and 12) Thiophene, 3-methyl-2-pentadecyl were found to be specific for both ethanol as well as chloroform extracts. All 13 compounds are common in ethanol and chloroform extractions (Table 2). The remaining 32, 11 and 53 compounds identified are specific for hexane, ethanol and chloroform, respectively. About 13 compounds identified in *H. musciformis* are yet to be reported in any group of macroalgae (Supplementary Table 1). The present investigation reveals that the total as well as the specific compounds identified in the chloroform extract of *H. musciformis* showed a higher record of compounds than that of ethanol and chloroform extractions.

Likewise, the GCMS analysis of ethanol, hexane, and chloroform extracts of *A. spicifera* had shown a total of 146 different compounds, and subsequently, 69, 50, and 68 compounds were identified in the respective solvent. Ten different compounds such as 1) Tetradecanoic acid, 2) Phytol, 3) Phenol, 2,4-bis(1,1-dimethylethyl)-, 4) n-Hexadecanoic acid, 5) Heptadecane, 6) Cholesta-5,22-dien-3-ol, (3β)-, 7) Cholest-4-ene-3,6-dion, 8) 3,7,11,15-Tetramethyl-2-hexadecen-1-ol, 9) 2-Pentadecanone, 6,10,14-trimethyl, and 10) Cholesterol were common in all three solvent extractions. Similarly, hexane and chloroform extractions also reported another 10 compounds commonly (1) Tetratriacontane, 2) Octadecane, 3-ethyl-5-(2-ethylbutyl)-, 3) Heptadecane, 9-hexyl, 4) Heneicosane, 5) Eicosane, 2-methyl, 6) Eicosane, 7) Dodecane, 2,6,11-trimethyl, 8) Diisooctyl phthalate, 9) 9-Hexadecenoic acid, 9-octadecenyl ester, (Z,Z)- and 10) Hexadecane, 2,6,11,15-tetramethyl) while another seven compounds such as 1) Tetradecane, 2) Phytol, acetate, 3) Pentadecane, 4) Isophytol, 5) Hexadecanoic acid, methyl ester, 6) Hexadecanoic acid, eicosyl ester, and 7) Cetene were common in ethanol (polar solvent) and chloroform (non-polar solvent) extractions. Another four compounds, such as 1) Phthalic acid, di(2-propylpentyl) ester, 2) Cyclotrisiloxane, hexamethyl, 3) Cyclotetrasiloxane, octamethyl, and 4) Cholesta-4,6-dien-3-ol, (3β) were also identified in hexane (polar) and ethanol (non-polar) solvent extractions. Another way of evaluation showed that the compounds exclusively identified in ethanol, hexane and chloroform extractions in *A. spicifera* were 48, 26, and 41, respectively. A total of 31 compounds are commonly detected in the combination of solvents. The ethanol extract of *A. spicifera* showed a maximum number of compounds, followed by chloroform and hexane. Like *H. musciformis*, 34 compounds identified in *A. spicifera* are yet to be reported in marine macro algae (Supplementary Table 1).

The third alga, *G. acerosa*, extracted using both polar (acetone) and non-polar solvents (ethanol and chloroform) showed a total of 151 unique compounds, whereas individually 76, 56, and 55 compounds were detected in the respective solvent (Table 1). Six compounds such as 1) 3,7,11,15-Tetramethyl-2-

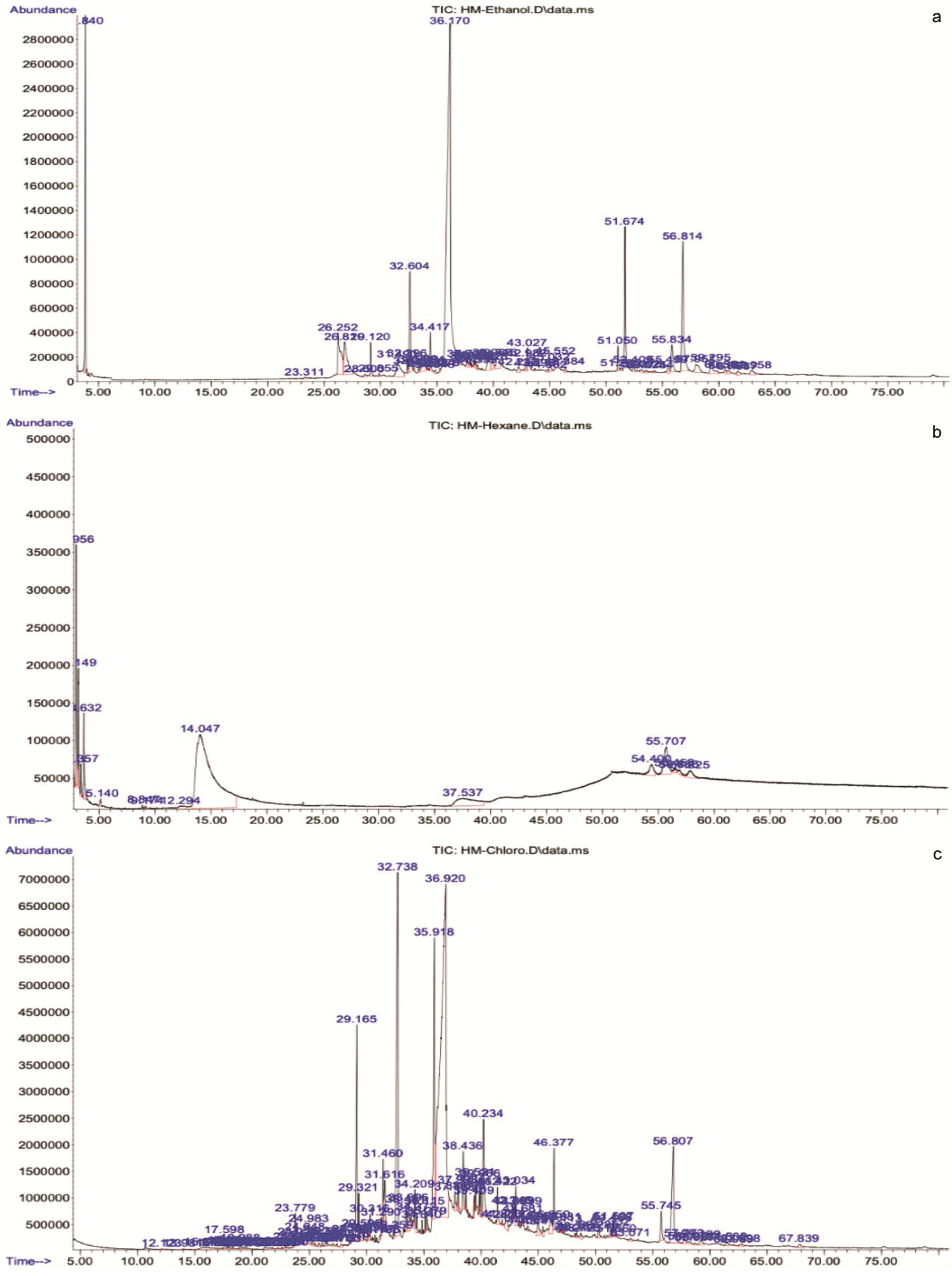

Fig. 1 — GC-MS Chromatogram of a) ethanol, b) hexane, and c) chloroform extracts of red alga, *Hypnea musciformis* of Mandapam Coast, Gulf of Mannar, India.

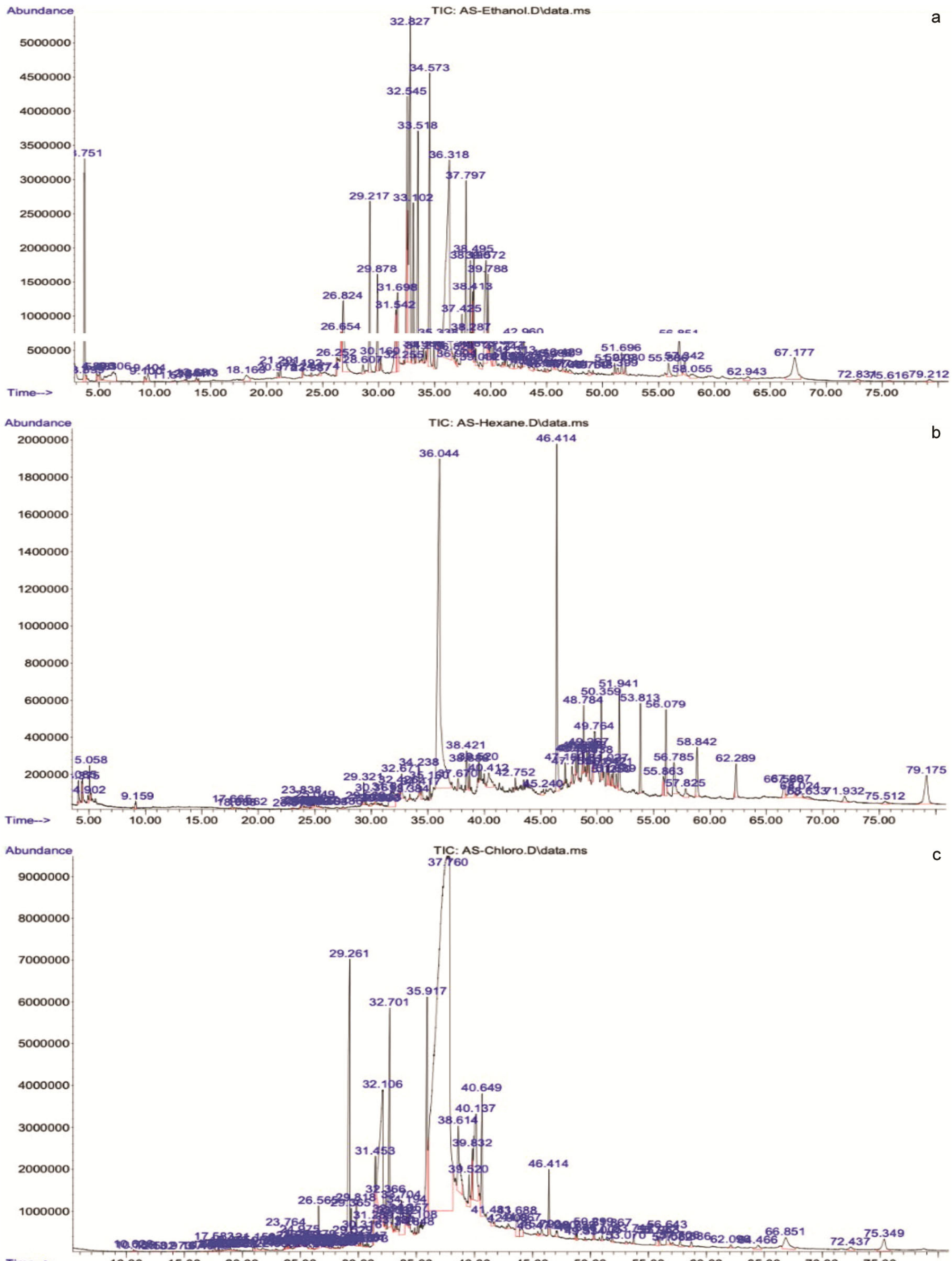

Fig. 2 — GC-MS Chromatogram of a) ethanol, b) hexane, and c) chloroform, extracts of red alga, *Acanthophora spicifera* of Mandapam Coast, Gulf of Mannar, India.

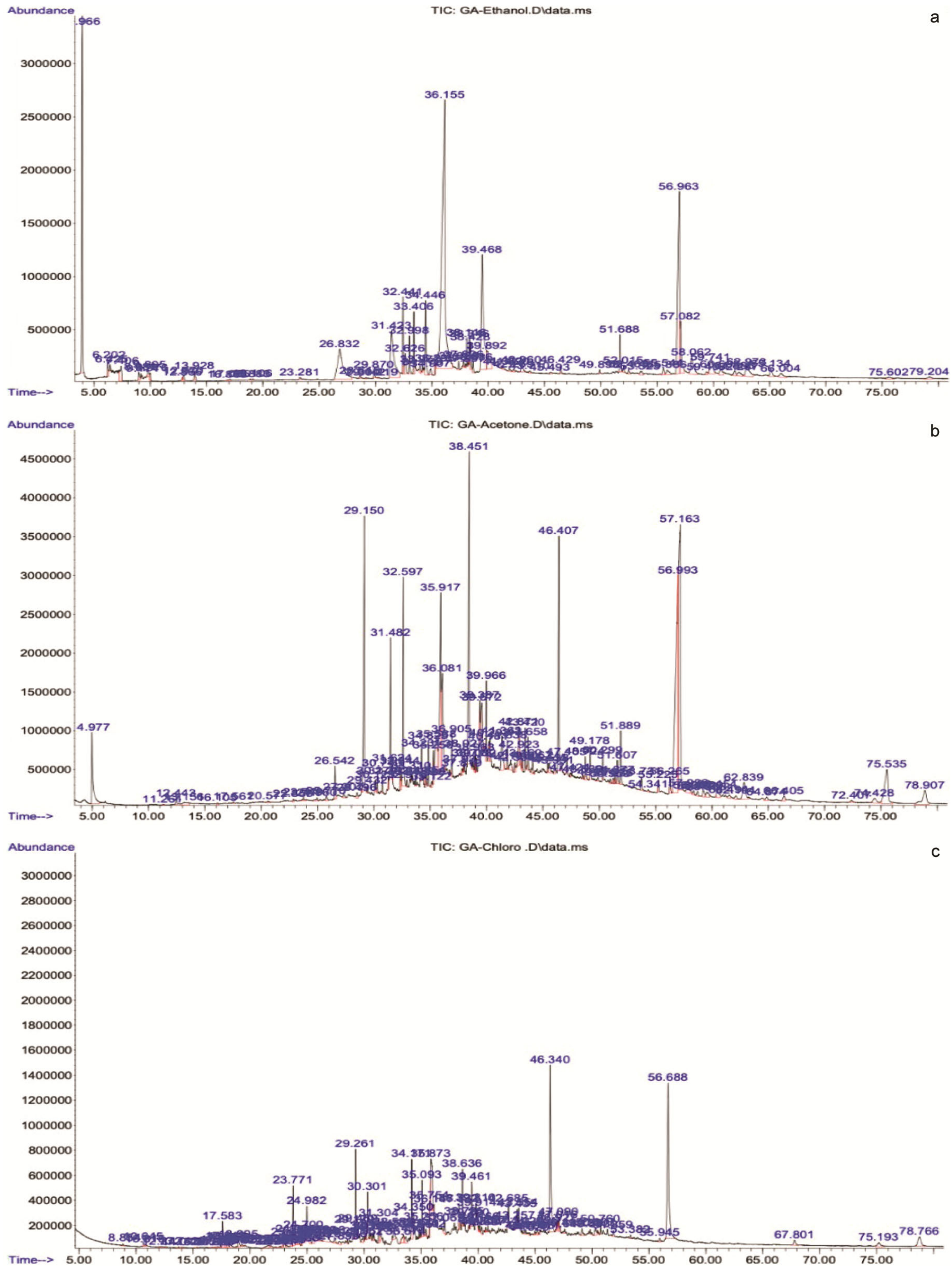

Fig. 3 — GC-MS Chromatogram of a) ethanol, b) acetone, and c) chloroform extracts of red alga, *Gelidiella acerosa*, of Mandapam Coast, Gulf of Mannar, India.

Table 2 — Commonly identified bioactive secondary metabolites from three red algae sampled in the Mandapam Coast, Gulf of Mannar using polar and non-polar solvents

S. No.	*H. musciforms – G. acerosa – A. spicifera*	*H. musciformis – A. spicifera*	*H. musciforms – G. acerosa*	*G. acerosa – A. spicifera*
1	(E)-9-Octadecenoic acid ethyl ester	1-Dodecanol, 3,7,11-trimethyl	2,4,4,6,6,8,8-Heptamethyl-1-nonene	17-Pentatriacontene
2	2-Bromo dodecane	2-Pyrrolidinone, 1-methyl	2H-Pyran, tetrahydro-2-(12-pentadecynyloxy)	1-Hexadecanol, 2-methyl
3	2-Pentadecanone, 6,10,14-trimethyl	Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester	4,8,12,16-Tetramethylheptadecan-4-olide	2,6-Dimethyldecane
4	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Cetene	7-Hexadecenoic acid, methyl ester, (Z)-	4-Aminobenzoic acid, N,O-bis(heptafluorobutyryl)-
5	Cholesta-4,6-dien-3-ol, (3β)	Cholesta-5,22-dien-3-ol, (3β)-	7-Oxo-5-cholesten-3beta-yl benzoate	Benzaldehyde, 2,5-bis [(trimethylsilyl)oxy
6	Cholesterol	Cyclohexane, (1-hexyltetradecyl)-	7-Methyl-Z-tetradecen-1-ol acetate	Benzoic acid, 3,5-dicyclohexyl-4-hydroxy-, methyl ester
7	Cyclotrisiloxane, hexamethyl	Hexadecanamide	Cholest-4-en-3-one	cis-13-Octadecenoic acid
8	Diethyl Phthalate	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Cholesta-4,6-dien-3-one	cis-1-Chloro-9-octadecene
9	Diisooctyl phthalate	Isophytol	Cyclotetrasiloxane, octamethyl	Cyclopentasiloxane, decamethyl
10	Dodecane, 2,6,11-trimethyl	Methyl tetradecanoate	Desmosterol	Didecan-2-yl phthalate
11	Eicosane	Oleic acid, eicosyl ester	Ergosta-5,22-dien-3-ol, (3β,22E,24S)-	Dodecane, 5,8-diethyl
12	Eicosane, 2-methyl	Pentadecanoic acid	Heptadecane, 2,3-dimethyl	Heneicosane
13	Ethanol, 2-(octadecyloxy)-	Silane, dimethyl (docosyloxy)butoxy	Methyl stearate	Heptadecane, 2,6,10, 15-tetramethyl
14	Heptadecan	Silanediol, dimethyl	Nonadecane, 2-methyl	Hexacosyl heptafluorobutyrate
15	Heptadecane, 2-methyl	trans-(2-Docosenyl) succinic acid	Tetracosane	Hexatriacontane
16	Heptadecane, 9-hexyl		Thiophene, 3-methyl-2-pentadecyl	Hydrogen bromide
17	Hexadecane			Octadecane, 2-methyl
18	Hexadecane, 2,6,11,15-tetramethyl			Palmitoleic acid
19	Hexadecanoic acid, ethyl ester			Pentadecane, 2,6, 10-trimethyl
20	Hexadecanoic acid, methyl ester			Pentatriacontane
21	n-Hexadecanoic acid			Tetratriacontane
22	n-Tetracosanol-1			trans-13-Octadecenoic acid, methyl ester
23	Octadecane, 3-ethyl-5-(2-ethylbutyl)			
24	Pentadecan			
25	Phenol, 2,4-bis(1,1-dimethylethyl)-			
26	Phthalic acid, di(2-propylpentyl) ester			
27	Phytol			
28	Phytol, acetate			
29	Tetradecane, 2,6,10-trimethyl			
30	Tetradecanoic acid			
31	γ-Sitosterol			

hexadecen-1-ol, 2) Octadecane, 3-ethyl-5-(2-ethylbutyl)-, 3) Phytol, 4) 17-Pentatriacontene, 5) 2-Pentadecanone, 6,10,14-trimethyl, and 6) Cholesterol were common in all three solvents and another three compounds (1) Cyclopentasiloxane, decamethyl, (2) Cyclotetrasiloxane, octamethyl and (3) Heptadecane, 9-hexyl) were common in ethanol and chloroform solvent extractions. Another eleven different compounds were commonly identified in acetone and ethanol such as (1) 26,26-Dimethyl-5,24(28)-ergostadien-3β-ol, 2) 7-Methyl-Z-tetradecen-1-ol acetate, 3) 7-Oxo-5-cholesten-3beta-yl benzoate, 4) Cholest-4-en-3-one, 5) Cholesta-4,6-dien-3-ol, (3β)-, 6) cis-Vaccenic acid, 7) Hexadecanoic acid, hexadecyl ester, 8) n-Hexadecanoic acid, 9) Phytol, acetate, 10) Thiophene, 3-methyl-2-pentadecyl and 11) trans-13-Octadecenoic acid, methyl ester). Furthermore, 11 more compounds such as 1) Diisooctyl phthalate, 2) Eicosane, 3) Heptadecane, 4) Heptadecane, 2,6,10,15-tetramethyl, 5) Heptadecane, 3-methylor, 2-methyl, 6) Hexadecane, 7) Hexadecane, 2,6,11,15-tetramethyl, 8) Hexatriacontane, 9) Tetracosan, 10) Tetracosane, 11-decyl, and 11) Tetratriacontane were common in acetone as well as chloroform extractions. A total of 31 compounds were identified with a combination of solvents. However, 49, 36, and 36 different compounds were identified exclusively in acetone (polar), ethanol, and chloroform (non-polar) solvent extractions. In *G. acerosa*, the acetone extraction contained a greater number of compounds. The 31 compounds identified in *G. acerosa* are yet to be reported in any group of macroalgae (Supplementary Table 1).

Another approach of comparison shows that of the 288 compounds totally identified from all three red algae, 31 compounds were common to all three red algae (Table 2). Of these, compounds such as 2-Pentadecanone, 6,10,14-trimethyl, 3,7,11,15-Tetramethyl-2-hexadecen-1-ol; Cholesta-4,6-dien-3-ol, (3β); Diethyl Phthalate; Diisooctyl phthalate; Dodecane, 2,6,11-trimethyl; Eicosane; Eicosane 2-methyl; Heptadecane; Hexadecane, 2,6,10,15-tetramethyl; n-Hexadecanoic acid; Phytol and Phytol acetate were found to be in lesser existence than that of other compounds. Among the compounds identified, another 15 compounds were common both in *H. musciformis* and *A. spicifera*; 16 compounds common in both *H. musciformis* and *G. acerosa* and another 22 compounds common in *G. acerosa* and *A. spicifera*. Only one compound, i.e., Cetene, had a higher occurrence in *H. musciformis* and *A. spicifera*. The compounds such as cis-1-Chloro-9-octadecene; Cyclopentasiloxane, decamethyl; Heneicosane; Heptadecane, 2,6,10,15-tetramethyl and Tetratriacontane were detected in higher occurrences in *G. acerosa* and *A. spicifera*, while the compounds commonly identified in *H. musciformis* and *G. acerosa* exhibited lower occurrence than that of other combinations of red algae. The above results indicated that some compounds are specific to solvent and vary from species to species.

Based on the GCMS analysis of *A. spicifera*, it was noticed that the chloroform extract was dominated by n-Hexadecanoic acid with a maximum peak area of 62.819%, followed by Heptadecane (5.551%) and Pentadecaonic acid (5.573%). The highest percentage peak area in ethanol extract was 16.055% for n-Hexadecanoic acid, followed by 10.033% for 2-Pentadecanone, 6,10,14-trimethyl and 6.280% for Hexadecanoic acid, methyl ester, whereas the highest peak area percentage of hexane extract was 35.102% for n-Hexadecanoic acid, followed by 10.443% for Diisooctyl phthalate. This study reveals that n-Hexadecanoic acid was the major constituent in *A. spicifera* which was confirmed in all three solvent extractions.

Similarly, the ethanol extract of *H. musciformis* reported the highest peak area of 42.176% belonging to n-Hexadecanoic acid, followed by cholesterol (8.329%). The hexane extract of *H. musciformis* showed the highest peak area of 64.676% for glycerine, followed by 6.231% and 5.487% for Oleic acid, eicosyl ester and Silanediol, dimethyl compounds, respectively. Similar to ethanol extract, the methanol extract of *H. musciformis* reveals the highest peak area of 40.733% for n-Hexadecanoic acid, followed by 11.648% for 2-Pentadecanone, 6,10,14-trimethyl, 8.172% for Hexadecanoic acid, ethyl ester and 4.906% for Heptadecane.

Likewise, the acetone extract of *G. acerosa* showed the highest peak area of 14.066% for 17-(1,5-Dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11, 12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a] phenanthren-3-ol followed by 11.558% for cholesterol, 8.076% for Phytol, and 6.489% for Heptadecane. The acetone extract of *G. acerosa* reported the highest peak area of 29.295% for n-Hexadecanoic acid, followed by 11.220% for cholesterol, 8.699% for *cis*-Vaccenic acid, 5.607% for Diethyl phthalate and 5.295% for Tetradecanoic acid. The chloroform extract of *G. acerosa* reported the highest peak area of 17.347% for cholesterol, followed by 11.692% for Diisooctyl phthalate and 5.725% for Hexadecanoic acid, ethyl ester.

The above results indicate that n-Hexadecanoic acid was the major constituent identified from *A. spicifera* in all three solvent extractions; in *H. musciformis* and in *G. acerosa*, only the acetone extract showed the highest constituent of n-Hexadecanoic acid. Therefore, the present study has shown that all three red algae have a considerable quantity of n-Hexadecanoic acid.

An interesting observation revealed in the present study is that nearly 16 different types of secondary metabolites are found to have Silica (Si) as a functional group (Supplementary Table 1). A maximum of 10 compounds were reported in *G. acerosa*, followed by *A. spicifera* (9) and *H. musciformis* (4). Most of them have anticancer, antioxidant, antimicrobial and nephroprotective effects while the five compounds such as (1) 2-Amino[1,3]thiazolo[4,5-d]pyrimidine-5,7-diol tritbdms; (2) Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11, 13,13,15,15-hexadecamethyl; (3) Silane,dimethyl (dimethyl(dimethyl (2-isopropylphenoxy)silyloxy) silyloxy)(2-isopropylphenoxy)-;(4) Silane,dimethyl (docosyloxy)butoxy and (5) Stigmasterol are yet to be reported in red algae. Of these five, the compound 2-amino[1,3]thiazolo[4,5-d]pyrimidine-5,7-diol tritbdms (Mol. Formula: C23H46N4O2SSi3 &Mol. Wt. 526.265) was identified only from the acetone extract of *G. acerosa*.

In this study, calcitriol (vitamin D) and a precursor for provitamin-D2, i.e., ergosterol in *G. acerosa* and vitamin E (α-tocopherol) in *A. spicifera* were identified. A carotenoid compound, Rhodopin was also reported in *G. acerosa* at 15.347 minutes of RT with 0.067% peak area. In the present study, an inorganic constituent, Hydrogen bromide, with peak areas of 0.183% and 0.402% was reported in *A. spicifera* and *G. acerosa,* respectively.

The present study tested the antibacterial and antifungal activities of different solvent-extracted red algae, such as *H. musciformis, A. spicifera,* and *G. acerosa*, and presented their zone of inhibition (ZOI) (Table 3). The chloroform extract of *A. spicifera* and ethanol extract of *H. musciformis* showed higher ZOI (7 mm) against *E. faecalis*, while the ethanol extract of *A. spicifera* and *G. acerosa* showed equal ZOI to that of the antibiotic control. The antibacterial activity of chloroform extract of *A. spicifera*, ethanol extract of *H. musciformis* and ethanol and hexane extracts of *G. acerosa* against *Staphylococcus aureus* exhibited higher activity (7 mm) than that of the remaining solvent extracts, while, on comparison, the positive control, Amoxicillin, showed somewhat higher ZOI, i.e., 8 mm. The antibacterial activity of chloroform and ethanol extracts against *Pseudomonas aeruginosa* reported the same ZOI, i.e., 7 mm, when compared with its positive control. Although ethanol extract of *A. spicifera*, ethanol and acetone extracts of *G. acerosa* and hexane extract of *H. musciformis* showed higher ZOI (i.e., 6 mm) than those of other solvent extracts. Comparatively, the ZOI of positive control was somewhat higher, i.e., 7 mm. Similarly, the ZOI of red algal extracts against *Escherichia coli* showed a higher ZOI in the chloroform extract of *G. acerosa* and the hexane extract of *H. musciformis* (7 mm). In comparison, the ZOI of the positive control was slightly higher, i.e., 8 mm.

Further, the antifungal activity of different solvents extracted from red algae had shown that the hexane and chloroform extracts of *H. musciformis* exhibited higher ZOI against the fungus *Candida albicans* (8 mm). In comparison, it was slightly lower when compared with positive control (Fluconazole), while hexane extract of *H. musciformis* and ethanol extract of *G. acerosa* revealed the same ZOI as their positive control (8 mm). The present results showed stronger antifungal activity against *Candida albicans*

Table 3 — Antimicrobial potential of polar and non-polar solvent extraction (crude) of red algae of the Gulf of Mannar, Southeast Coast, India

Red algae / Solvents	Bacteria				Fungus
	Enterococcus faecalis	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Candida albicuns*
	Zone of inhibition (mm)				
Acanthophora spicifera					
Hexane	5.0	6.0	3.0	4.0	4.0
Chloroform	7.0	7.0	4.0	4.0	4.0
Ethanol	6.0	3.0	6.0	5.0	5.0
Gelidiella acerosa					
Chloroform	5.0	4.0	5.0	7.0	4.0
Ethanol	6.0	7.0	6.0	6.0	8.0
Acetone	5.0	6.0	6.0	5.0	6.0
Hypnea musciformis					
Hexane	4.0	7.0	6.0	7.0	8.0
Chloroform	2.0	6.0	7.0	6.0	8.0
Ethanol	7.0	7.0	7.0	6.0	6.0
Fluconazole	-	-	-	-	8.0
Amoxicillin	6.0	8.0	7.0	8.0	

with hexane and chloroform extracts of *H. musciformis* and ethanol extract of *G. acerosa*. In contrast, the antibacterial activity of *H musciformis* extract was stronger against all four human pathogenic bacteria than the other two red algae species tested in this study.

Discussion

Currently, marine macroalgae are the major sources of bioactive secondary metabolites for a variety of pharmaceutical, industrial and food applications[1,2]. The identification of marine bioactive secondary metabolites has been done using polar and non-polar solvent extractions, which confirms the detection of a wide range of compounds at different levels[31]. Some compounds are synthesised at a particular season or found in specific organisms or groups of organisms[29]. The polarity of the solvent is found to play an important role in the extraction of bioactive secondary metabolites from biota. Due to lack of comparative studies on various polarity of solvents as well as different species of red algae, the specificity of solvents in extractions of bioactive metabolites from the three red algae, *H. musciformis*, *A. spicifera* and *G. acerosa* of the Gulf of Mannar, India, was investigated.

The extraction of bioactive compounds from the red algae such as *Kappaphycus alvarezii, Gracilaria cervicornis, Gracilaria corticta, Gracilaria gracilis, Gracilaria edulis, Acanthophora deilei, Halymenia dilatata, Halymenia palmata* and *Hypnea valentiae* of Tamil Nadu coasts, mainly in the Palk Bay and Gulf of Mannar regions, has been reported earlier[4,8,14-18,20,32-34].

They used only a single solvent with a few species or two or three solvents with a single species for the extraction of secondary metabolites and their identification. They reported a few compounds that are not reported in some other solvents in the same species as well as other species[34]. The present results supported the above findings that some compounds are common to polar or non-polar solvents, while some other compounds are in combination with polar and/or non-polar solvents and are also specific to species. Das *et al.*,[4] reported that 94 different bioactive compounds in red alga *Kappaphycus alvarezii* of Thondi coast, India were extracted using chloroform, ethanol and distilled water. The major constituents identified in *Kappaphycus alvarezii* were phenol, decane, dodecane, hexadecane, vanillin, heptadecane, diphenylamine, benzophenone, octadecanoic acid, dotriaconate, benzene, phytol, butanoic acid and 2-hydroxyl-ethyl ether.

Another study conducted by Uma *et al.*,[35] identified 28 different volatile compounds from methanol extract of red alga, *Acanthophora deilei* of Rameswaram coast, India. Of these, 13 compounds have been reported in the present study. Hexadecanoic acid, Methyl ester possessed antibacterial and antifungal activities[36], 9-octadecanoic acid, methyl ester showed anti-inflammatory, anti-androgenic, and anticancer activity[8], and 1,2-Benzene dicarboxylic acid has anti-neurodegenerative and anticancer activities.

Similarly, Anitha *et al.*,[16] reported 50 compounds each in *G. cervicornis* and *G. gracilis*, and most of the bioactive compounds have been reported in the present study. They noticed considerable amounts of phenolic compounds in *G. gracilis* (area - 32.15%) and *G. cervicornis* (area - 38.53%) of the Manalmelkudi coast of Tamil Nadu, India. In *G. gracilis*, the compound, diethyl phthalate, also showed a higher peak area, i.e., 17.88%. The major fatty acid compounds in *G. gracilis* are Hexadecanoic acid, n-Hexadecanoic acid, and Furanacetic acid, and in *G. cervicornis*, Tridecanoic acid, n-Nonadecanoic acid, Cyclopropaneoctanoic acid and Heneicosanoic acid.

Another study carried out by Geetha Devi and Sree Devi Kumari[33] reported 10, 25 and 3 compounds in methanol, chloroform and aqueous extracts of red alga *Gracilaria corticata* of Mandapam coast, India, respectively, using GCMS analysis. As reported by them, Naphthalene was a major compound in the aqueous extract and a lesser amount was also reported in methanol extract, whereas the chloroform extract of *G. corticata* showed a considerable quantity of fatty acid group of compound. They further noticed that the methanol extract of *G. corticata* has antioxidant, anticancer and anti-diabetic properties. In the present study, in the ethanol extract of *A. spicifera*, only one naphthalene group of compounds, i.e., 2-Pentenoic acid, 5-(decahydro-5,5,8a-trimethyl-2-methylene-1-naphthalenyl)-3-methyl-, [1S-[1α(E),4aβ,8aα]]- (area - 0.557%) was identified.

The GCMS analysis of hexane, acetone and methanol extracts of red alga, *Gracilaria corticata* of Mandapam coast, Gulf of Mannar, reported a total of 17 secondary metabolites, i.e., 6 from hexane, 4 from acetone and 7 from methanol extract[19]. Of the 17 compounds, only one compound, 13-Docosenamide, (Z)- was detected in all three solvent extractions.

However, overall, 8 different secondary metabolites have been identified in all three solvent extractions of *G. corticata*. Further, they noticed that the chemical groups such as tannins, saponins and cardiac glycosides were not eluted in non-polar solvent - hexane; terpenoids and flavanoids in the middle-polar solvent-acetone and alkaloids, terpenoids and flavonoids in polar solvent–methanol. El-Din and El-Ahwany[35] reported that the solvent types as well as species have a major role in the screening of phytochemicals. For example, the chemical group saponin was not reported in the methanol, ethanol, acetone and chloroform extracts of *J. rubens*, *C. mediterranea* and *P. capillacea*.

The methanol extract of red alga, *Gracilaria dura* of Mandapam coast, Tamil Nadu, showed a significant amount of terpenoids such as n-hexadecanoic acid, methyl ester; n-hexadecanoic acid; octadecanoic acid and phytol[18]. A study carried out by Arulkumar *et al.*,[8] reported a variety of bioactive secondary metabolites, including sulfurous acid, 2-ethylhexyl isohexyl ester, pentatriacontane, eugenol and phthalic acid from methanol extract of red algae, *G. corticata* and *G. edulis* sampled at Thondi Coast, Palk Bay, India. They also noticed a moderate quantity of phenolic groups in *G. corticata*, while a wide range of flavonoids were found in both red algae. Ragunathan *et al.*,[18] reported 10 bioactive secondary metabolites from the methanol extract of red alga *Gracilaria corticata*. n-Hexadecanoic acid was found to have the highest peak area of 74.198%, whereas the remaining compounds were found to have low occurrence. This is supported by the present results because n-Hexadecanoic acid is a major compound in all three red algae.

The GC-MS analysis of methanol extract of red alga *Halymenia dilatata* collected from the Palk Bay coast, India exhibited a variety of bioactive compounds including Hydroperoxide, 1-methylbutyl; Nonyl trifluoroacetate; Decane 2,2,3-trimethyl; Acetyl valeryl; Dodecyl trifluoroacetate 2-Bromo dodecane and 3 Ethoxy-1,1,1,5,5,5- hexamethyl-3 trimethyl[17]. Uma Maheswari and Reena[37] reported 16 bioactive metabolites from the methanol extract of the red alga, *Halymenia dilatata*, of the Mandapam coast, Tamil Nadu, using GCMS analysis. The majority of them were fatty acids and others were from alkane, acetate, amide, alkenyl, alcohol and steroid groups. Hexadecanoic acid, methyl ester, showed a higher peak area of 11.87%, followed by n-Hexadecanoic acid (8.17%), and the fatty acids possess antibacterial, anti-inflammatory and antifungal activities. In contrast, the present study showed less occurrence of the former compound and higher occurrence of the latter compound in *H. musciformis* and *A. spicifera* of the Gulf of Mannar, India.

GC-MS analysis of ethyl acetate extract of red alga, *Hypnea flagelliformis*, sampled at Tuticorin coast, India, detected 19 compounds with the major peak area of 29.01% for 1,4-Eicosadiene, followed by 14.06% for n-Tetracosanol-1, while n-Hexadecanoic acid showed a lower peak area of 4.39%[38]. n-Tetracosanol-1 reported a wide range of biological activities such as antimicrobial, anticancer, antioxidant, antimalarial, antiplasmodial potentials, peroxidation, enhancing immune functions, inhibition of platelet aggregation, cardiovascular, hepatoprotective and antiproliferative[39]. GCMS detection in methanol extract of *Gracilaria corticata* of Manapad coast, Tamil Nadu, India showed that 1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester, had the highest peak area of 23.15%, possessing a variety of biological activities. The NMR spectral data confirmed the presence of alkyl and aromatic organic compounds as 1,2-benzene dicarboxylic acid, mono (2-ethylhexyl) ester with the molecular formula of $C_{16}H_{22}O_4$[15]. Further, they were confirmed using ^{13}C and ^{1}H NMR.

The methanol extract of red alga, *Hypnea valentiae* sampled at Mandapam coast, Gulf of Mannar region, India reported 30 bioactive compounds including the major compounds *viz*., Hexadecanoic acid-methyl ester; 13-Docosenamide; (Z) and Eicosane (RT, 21.07, 34.46, 16.51; 29.10, 10.27, 10.24%), Cholest-5-en-3-ol; 24-propylidene-, (3á)-; 9-Octadecenoic acid (Z)-; methyl ester (CAS) (RT39.09, 24.38; 9.00%, 8.07%); Hexadecanoic acid (CAS) - Methyl tetradecanoate (RT 21.89, 17.04; 4.74, 3.30%) with different peak area percentages and retention times[20]. In the present investigation, more than 50 compounds (30 in *H. musciformis*, 29 in *A. spicifera* and 30 in *G. acerosa*) are eluted at different retention times with different peak area percentages (Supplementary Table 1).

Deepak *et al.*,[32] reported 20 different types of bioactive compounds with 2-propamine (17.263%) followed by ether, (2-ethyl-1-cyclodecen-1-yl) methyl (6.209%) and Hexadecanoic acid, ethyl ester (5.418%) using GC–MS analysis of methanol extract of red alga *Halymenia palmata* (Hpf-2 fraction) sampled at Mandapam Coast, Tamil Nadu, India. This extract had strong mosquito-larvicidal activity. The methanol extract of four algae, namely, *Padina tertastomatica*, *Sargassum wightii*, *Gracilaria edulis* and *Caulerpa racemosa* showed equal anti-inflammatory effects. Saranya *et al*.,[9] identified 20 compounds from the methanol extract of red alga *Portieria hornemannii* sampled at Kilakarai, Gulf of Mannar coast, India, which showed the highest peak area of 41.88% for n-hexadeconic acid followed by 19.49% as peak area for 9,12, Octadecadienoic acid (Z, Z), which have significant antioxidant and also mosquito-larvicidal activities.

An indole derivative, 1H-Indole-2-carboxylicacid,6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7 tetrahydro-isopropyl ester having insulin sensitising and glucose lowering effects was reported from the ethyl acetate extract of marine red alga *G. edulis*[40], and this displayed promising antiglycation, and hypoglycaemic potential.

The marine macroalgae contain the primary biomolecules and also vitamins, as well as precursors for a variety of vitamins. In this study, the vitamin D (calcitriol) and a precursor for provitamin-D2, i.e., ergosterol, have also been reported in *G. acerosa*. Vitamin E (α-tocopherol) was also detected in *A. spicifera* and another two groups of compounds, like α-Tocopheryl acetate, reported in *G. acerosa*, which supplements skin care products and dietary supplements, as well as (+)-γ-Tocopherol, O-methyl, reported in *A. spicifera*. Vitamin E has diverse biological functions[41]. In this study, erucic acid (cis-13-docosenoic acid/22:1 ω-9; cis-13-docosenoic acid), a monounsaturated omega-9 fatty acid of 22 carbon atoms, was reported in the red alga *H. musciformis* with a peak area of 0.4%, while a significant level of erucic acid was reported in the brown alga, *Dictyota dichotoma*[42]. In the present study, an inorganic constituent, hydrogen bromide, with peak area percentage of 0.183 and 0.402% was reported in *A. spicifera* and *G. acerosa*, respectively.

Glycine, N-[(3α,5β,12α)-3,12-dihydroxy-24-oxocholan-24-yl]- yet to be identified from marine macroalgae, a synonym of Glycodeoxycholic acid reported in *Streptomyces nigra*, *Trypanosoma brucei* and *Caenorhabditis elegans,* was also identified in *A. spicifera* which has agonist activity in human TGR5 expressed in CHO cells by luciferase assay and also cytotoxic against human HET-1A cells[43]. Rhodopin (0.067%), a carotenoid compound in algae, was also identified in *G. acerosa*, which has antioxidant activity[44]. El-Din & El-Ahwany[35] also reported Rhodopin with a peak area of 0.14% in the red alga, *Jaina rubens*, while there was no report of this compound from the other two red algae, *C. mediterranea* and *P. capillacea*.

The marine macroalgae contain a variety of biological activities, including antibacterial and antifungal activities. Earlier, some studies have reported the antibacterial and antifungal properties of crude red algal extracts sampled from Palk Bay and the Gulf of Mannar using different solvents. In the present investigation, the screening of antibacterial and antifungal properties of crude red algal extracts revealed that the higher antibacterial activity was displayed against the human pathogenic bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* in hexane, chloroform and ethanol extracts of red alga *H. musciformis* than that of *A. spicifera* and *G. acerosa*. In contrast, Rajkumar *et al*.,[14] reported that the methanol extract of *Acanthophora spicifera* exhibited the highest antibacterial activity against the pathogenic bacteria *Bacillus cereus* and *Pseudomonas aeruginosa*, and antifungal activity against *Candida albicans* compared to that of chloroform and ethanol extracts[45]. The methanol extracts of red algae *Gracilaria cervicornis* and *Gracilaria gracilis* reported the highest antifungal activity against fungi *Macrophomina phaseolina* (respective ZOI: 19.2 – 20.7 mm) and *Lasiodiplodia theobromae* (respective ZOI: 17.2 – 17.6 mm).

Bahrun and Firdaus[13] reported that hexane and chloroform extracts of red alga *Gracilaria salicornia* showed the highest antibacterial activity against *E. coli*. Likely, the aqueous extract of *Gelidium* sp. was found to have maximum inhibition of the human pathogenic bacteria such as *Escherichia coli*, *Bacillus cereus*, *Bacillus subtilis*, *Vibrio alginolyticus*, *Enterobacter aerogenes* and *Klebsiella pneumonia*e at 25 μL concentration[46]. The 40 mgmL^{-1} methanol extract of red alga, *H. valentiae*, showed higher antibacterial activity against pathogenic bacteria *P. aeruginosa* (5 mm) and *E. faecalis* (2 mm), while less activity was reported against *E. coli* (1 mm)[22].

An investigation by Agarwal *et al*.[21] reported that the ethanol extract of *Gelidium pusillum* showed a significant inhibition against *Aeromonas caviae* compared to that of the aqueous extract. The methanol and toluene extracts of red alga *Corallina elongate* of the west coast of Algeria showed maximum ZOI against *B. subtilus* (16 mm), *E. coli* (12 mm), *Kleibseila* (8 mm), *Staphylococcus* (8 mm)[47]. Shobier *et al.*,[48] reported that the ethanol extract of red alga *C. officinalis* showed higher antibacterial activity against *Staphylococcus aureus* (10 mm), *Pseudomonas aeruginosa* (12 mm), and *Escherichia coli* (10 mm) and antifungal activity against *Candida albicans* (10 mm); however, the ethanolic extract of another red alga *J. rubens* showed the same activity against *Staphylococcus aureus* and *Escherichia coli* (10 mm) and no activity against *C. albicans*. In comparison with the present investigation, the antibacterial and antifungal activities are higher in the ethanolic extracts of *C. officinalis* and *J. rubens*.

Based on the above results, we can conclude that the ethanol extract of *G. acerosa* and *H. musciformis* showed higher antibacterial activity, while the chloroform extract of *G. acerosa* and hexane extract of *H. musciformis* showed higher antifungal activity. Many factors such as period of collection, drying method, growth stages of algae, extraction methodology, species, and solvent used for extraction may influence the compound detection and also the antimicrobial efficacy of marine macro algae[10,11] and also the combination of compounds in the crude extracts influences the antimicrobial properties of red algae[44].

Conclusion

Marine red algae are a major source of a variety of secondary metabolites with different biological activities, including antimicrobial activity. This study concludes that the polarity of solvent plays a significant role in the isolation and detection of secondary metabolites from marine red algae. Even though n-Hexadecanoic acid, also known as palmitic acid (fatty acid group), having antioxidants, hypocholesterolemic, nematicide, and pesticide properties, was the major constituent in *A. spicifera*, which was confirmed in all three solvent extractions. This study confirms that both polar and non-polar solvents extracted crudes showed higher antibacterial property, while the effectiveness of red algal extracts varied depending upon the species as well as the polarity of the solvents and a mixture of compounds in the crude.

Currently, antimicrobial drug-resistant bacteria are highly unsafe and dangerous due to the adaptation against antimicrobial drugs. Therefore, searching for new novel antimicrobial compounds against multidrug-resistant bacteria is being undertaken from different sources, including marine algae. This study confirms 71 compounds as new to the marine macroalgae, which are found to have unreported biological activities and may have potential as new drug leads for various multidrug-resistant bacteria. Hence, further research on purification and identification of bioactive secondary metabolites from red algae of the Gulf of Mannar should be undertaken not only to overcome new multidrug-resistant bacteria but also to treat multiple human diseases.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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