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Isolation and identification of MDR-*Mycobacterium tuberculosis* and screening of partially characterised antimycobacterial compounds from chosen marine micro algae

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ABSTRACT

Objective: To isolate the multiple drug resistance (MDR) *Mycobacterium tuberculosis (M. tuberculosis)* and to screen for the bioactive compounds extracted from marine microalgae. **Methods:** Sixty seven collected samples that were confirmed by 16S rDNA analysis as positive with *M. tuberculosis* infection were subjected to sensitivity test against commercially used front line and second line drugs by absolute concentration method using LJ slants. Seven bacterial isolates numbered I-78, I-101, I-127, I-173, I-202, I-262, I-327 showed resistant for more than 3 drugs were considered as MDR *M. tuberculosis*. Thus the percentage of 10.4 were recorded MDR-TB. Fifteen marine micro algal extracts were screened for antimycobacterial activity and partial characterization of the active principles was done. **Results:** The percentage contribution of marine micro algal species on the extraction of antimycobacterials indicated *Isochrysis galbana (I. galbana)* contain rich bioactive compounds and accounted for 60% inhibition of the total isolates. The percentage contribution of solvents on the extraction of antimicrobials from *I. galbana* showed that the methanol, chloroform, n-Butanol showed maximum of extraction. The purified eluted compounds (R_f 0.43) from TLC plate were chromatographed by gas chromatography. The eluted sterol compounds showed 13 unsaturated sterols with 3 major sterols. **Conclusions:** The present study indicates the presence of unsaturated fatty acids may have the effect on MDR *M. tuberculosis*, indicating a potential natural alternative to antibiotics.

1. Introduction

Over the past decade, there has been a significant increase in the incidence of tuberculosis (TB) worldwide[1]. Thirty-two percent of the world's populations (i.e., 1.86 billion people) are infected with *Mycobacterium tuberculosis (M. tuberculosis)*. Most of these people have latent *M. tuberculosis*. In 1997, there were about 1.87 million deaths, with an average mortality rate of 23%. Nearly 500 000 die from the disease -more than 1 000 per day - one every minute[2]. The incident is high in Africa, with 290 cases per 100 000 populations[3], although the greatest prevalence is in the most populous countries, such as India, Bangladesh and Pakistan. Each year, 636 000 new incident cases(80%) occur in the top 23 TB countries[4]. TB is the second commonest cause of death world wide[5]. In some African countries the mortality rate was as high as 50%[4]. The large majority of the cases and deaths are from the poor nations. Tuberculosis (TB) continues to remain one of the most pressing health problems in India. India is the highest TB burden country in the world, accounting for one fifth of the global incidence - an estimated 1.96 million cases annually[1]. Approximately 2.9 million people die from tuberculosis each year worldwide; about one fifth of them in India alone[1].

Most forms of active TB can be treated with six months of medication. Unfortunately, the very success of the drug treatment of tuberculosis has been the catalyst for the emergence of a new wave of drug resistance. Beginning in 1990, outbreaks of multi drug resistant (MDR) tuberculosis have been reported in hospitals and prisons in the eastern United States[6]. It has been estimated that 3.2% of the world's new cases of TB, in 2000, were MDR-TB, defined as resistant to at least isoniazid and rifampicin[7]. Tuberculosis patients in part of Eastern Europe and Central Asia are 10 times more likely to have MDR-TB than in the rest of the world according to a World Health Organization (WHO)

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report into the deadly infectious disease, China, Ecuador, Israel and South Africa are also identified as key areas. WHO's leading infectious disease experts estimates there are 300 000 new cases per year of MDR-TB worldwide. The association with HIV and increasing MDR-TB appears to be a serious issue, especially for the developing nations. In addition, approximately 12% (226 000) of deaths from TB was attributed to co-infected with *M. tuberculosis* and human immuno deficiency virus (TB-HIV).

Natural products form one avenue in the search for new antituberculosis agents. Nature has continuously provided humankind with a broad and structurally diverse of pharmacologically active compounds that continue to be utilized as highly effective drug to compact a multitude of deadly diseases. Natural products and their derivatives have traditionally been the most common source of drugs, and still represent more than 30% of the current pharmaceutical market[8]. It has long been recognized that natural product structures have the characteristics of high chemical diversity, biochemical specificity, and other molecular properties that make them favorable as lead structures for drug discovery, and which serve to differentiate them from libraries of synthetic and combinational compounds.

The oceans are the source of a large group of structurally unique natural products that are mainly accumulated in marine organisms. Several of these compounds show pronounced pharmacological activities and are interesting candidates for new drugs primarily in the area of cancer treatment. Other compounds are currently being developed as an analgesic or to treat inflammation. Among the many phyla found in the oceans, the best source of pharmacologically active compounds are bacteria (including cyanobacteria), fungi, certain group of algae, sponges, soft corals and gorgonians. Prokaryotic and eukaryotic micro algae produce a wide array of compounds with biological activities. These include antibiotics, algicides, toxins, pharmaceutically active compounds and growth regulators. The micro algal toxins is either important as material for useful drugs or one of the great mysteries in the world of biotoxicology.

Recently, microalge have become targets for screening programmers in search of novel compounds of potential medicinal value[9]. Numerous compounds have been isolated from prokaryotic and eukaryotic microalgae, and have been tested for different types of bioactivity with positive effects. The future role of micro algal compounds in drug discovery is especially in the priority areas for the development of new medicines, namely to fight viral infections and cancer, and to combat infections from antibiotic resistant bacteria and fungi. Discovering new therapeutic molecules is becoming increasingly important as more and more bacteria resistant to the usual antibiotics. Traditionally used asiatic medicines, algae, since the second half of the 20th century, are screened for their biological activities. Thus, antibacterial effects have been noticed in all the algal classes[10-13]. However, most of these antibiotic actions have only been tested against human pathogens and the active molecules were rarely purified. The role of marine algae in the discovery of drugs which could reach the pharmaceutical market has increased notably in recent years, due to substantial improvement in biological screening methods. Only a small percentage of the microbial species have been examined and explored thoroughly for their pharmaceutical potential. Considering these aspects the current study was initiated.

2. Materials and methods

2.1. Collection and identification of MDR M. tuberculosis

The morning sputum samples were collected from 351 patients includes male and female patients from the Kanyakumari District Medical college hospital (formerly Government Chest Disease Hospital) Asaripallam, Tamil Nadu, India in the month of September 2002 with chest symptoms. Sputum samples were collected early morning before meal in a sterile wide mouth screw capped bottle free from antiseptics without contamination of saliva. The samples collected were labeled and transported to the laboratory immediately.

Specific pathogens present in the samples collected from the patients were undergone culture technique and identified based on standard procedures. Initial identification and classification of smears was done by Ziehl-Neelson staining. Smear was prepared from the thick purulent part of the sputum using a new slide. Smear was dried, heat fixed and stained by Ziehl – Neelson technique. The smear was covered with strong carbol fuchsin and gently heated for 7 minutes, without letting the stain boil and become dry. The slide was then decanted, washed with sterile water and decolorized with acid alcohol (3% HCl in 95% ethanol). After washing, the smear was counter stained with Loffler's methylene blue for one minute. The acid-fast bacilli were seen on bright red rods and the smears were classified based on their numbers.

The remaining sputum samples were immediately concentrated by Petroff's method to separate Bacilli from sputum. The sputum was incubated with an equal volume of 4% sodium hydroxide at 37 ℃ with frequent shaking till it becomes clear on an average for 20 minutes. It was then centrifuged at 3 000 rpm for 30 minutes and the sediment was neutralized with 10 N HCl and used for smear and culture technique. The concentrated samples were used for further studies.

The concentrated materials were incubated into two bottles of IUAT – LJ medium and were incubated at 37 ℃ for 3 weeks. Finally, the bottles were examined and the isolated bacterial species were identified based on Bergey's Manual of Determinative Bacteriology[14]. Species confirmation by 16S rDNA analysis

2.2. Sensitivity check by commercial antimycobacterial drugs

Sensitivity test of *M. tuberculosis* against commonly used front line and second line drugs were carried out by absolute concentration method using LJ Slant Kit (Hi media). This was to determine *in vitro* antibacterial susceptibility for commercially available anti tuberculosis drugs like streptomycin, ciprofloxacin, amikacin, ethionamide, isoniazide, ethanbutol, P. aminosaalicilic acid, pyrazinamide and rifambicin. Bacteria that are resistant to antibiotic show growth on the LJ slants which consist of specific anti-tuberculosis drug after incubation at 37 ℃ for 3 weeks. Bacteria which are sensitive show no growth on LJ slants. The isolated organisms showed resistance against more than one antimycobacterial drugs in Absolute Concentration methods were considered as MDR *M. tuberculosis*. The resistant bacteria were isolated and used for further screening producers.

2.3. Culture of marine micro algae

Algal inoculum of *Isocrysis galbana (I. galbana)*, *Nannochloropsis occulata (N. occulata)*, *Dicarteria inorta(D. inorta)* , *Chromulina friebergensis (C. friebergensis)*, *Chlorella marina (C. marina)*, *Tetraselmis gracilis (T. gracilis)* , *Pavlova lutheri(P. lutheri)*, *Chlorella salina*, *Chlorella vulgaris*, *Dunaliella saline*, *Spirulina platensis*, *Platymonas spp*, *Synechocysti salina (S. salina)* , *Nannochloropsis salina (N. salina) and Chlorella ovalis (C. ovalis)* for the present study obtained from Central Marine Fisheries Research Institute (CMFRI) in Tuticorin, Tamil Nadu, India.

For stock culture the autoclaved or heated seawater after cooling was poured to the conical flasks and required nutrients are added. Walne's medium enriched with vitamins is the ideal one to maintain the stock cultures of all the phytoflagellates. About 10 percent of the inoculums in the growing phase were transferred to the culture flasks and the same is placed in front of the tube light (1 000 lux). After 8-10 days. When the maximum exponential phase was reached, light was reduced for further growth. The time required for the maximum cell densities varies depending on the species. Almost all flagellates require 2 weeks for the completion of growth phase before entering into the decline phase. In the stationary phase, the flagellates can be kept for a period of 2 months in the stock culture room, under controlled condition of light and temperature. At the time of maximum exponential phase of growth the colour the turns into dark brown and the cells are found as suspension without movement. The cells of flagellates emerge out for its further growth and multiplication. A minimum of 5 culture flasks were kept for each species as stock culture.

For mass culture filtered seawater was sterilized by autoclaving and after cooling to room temperature; 200 mL was poured into each conical flask. Prior to sterilization, salinity and pH was checked by refractormeter and pH meter respectively. The filtered sterilized seawater is enriched with required quantity of Walne's medium. Then 20 mL of the inoculums in the growing phase was transferred to the culture flask with 200 mL medium. Finally the culture flasks were placed in front of tube lights of 100 lux. The temperature ranged from 28-33 ℃. From the time of inoculation, samples were taken and fixed with the help of formalin for the measurement of cell density. The initial count was calculated before inoculation. After inoculation the cell count was calculated for every 2 days and was tabulated. Cell density was determined with a Neubauer haemocytometer. Duplicates were also made and cell density was recorded for alternative days.

2.4. Preparation of algal extract

Algal cells in exponential growth phase were recovered from culture by batch centrifugation at 3 000 rpm for 10 minutes. The cells were repeatedly washed in haemal saline for three times by centrifugation at low speed and there pellets were dried at 60 ℃ for 24 hours. The resultant algal pellets were equally distributed in 5 tubes and stored in refrigerator till extraction process. The quantity of algal cells was measured. According to the methods of Khan[15] the mehonol extract to be prepared dry algal mass (ratio 1:15g/mL) was extracted by ultra sonication and kept it for 24 hours. After the extraction phase being separated by centrifuged at 10 000 rpm for 15 min, this method was used for the ethanol, n-Butanol, chloroform and water respectively. Finally supernatants were collected and were dried under reduced pressure. All the extracts were preserved at +4 ℃ for further investigation.

2.5. In vitro screening against bacterial isolates with marine micro algal extracts

In vitro screening experiments with algal extract were carried out by absolute concentration method. Each 100 μg of dried algal extracts of different solvent were taken and mixed with 5 mL sterile L-J medium (Hi-media) and poured in the appropriate culture bottles and solidified in slanting position. On the LJ medium slant, the isolates of *M. tuberculosis* were inoculated and incubated at 37 ℃ for more than four weeks, control bottles were maintained along with the test culture bottles. Proper aeration and was given in an aseptic condition through out the incubation. After the incubation, bottles were observed for colony formation. Positive and negative growths were noted and were recorded.

2.6. Tube dilution technique

Colonies were scrapped from a freshly growing (3 to 4 weeks) Lowenstein- Jensen medium slant into 3 mL of middle brooks 7H9 broth containing four to five 3 mm diameter glass beads in a conical tube. The tubes were vortexed vigorously for 3 to 5 min to homogenize the suspension. The large particles were allowed to settle, and the supernatant was adjusted to turbidity equivalent a 0.5 Mc Farland. The increasing concentration of the three algal active principles were mixed with a series of culture tubes contains Middle Brooks 7H9 broth base (with ADC growth suppliment-Hi media) inoculated with the *M. tuberculosis* at 37 ℃ for 4 weeks. After the incubation the growth of the organisms was scanned at 620 nm and the result were recorded. Slides were prepared from each tube for acid fast staining. No organisms other than acid fast bacilli were observed.

2.7. Preliminary biochemical analysis of active principles from chosen extracts

Phytochemical tests were carried on the aqueous ethanolic extracts using standard procedures to identify the constituents as described by Sofowara[16].

2.8. Separation of sterols

The algae are extracted with chloroform-methanol (2:1) and the extracts were filtered and concentrated under reduced pressure, yielding a green viscous residue. The green viscous residue was refluxed for 4 h with 7% methanolic potassium hydroxide. The solvent was removed in vacuum, water is added, and the residue was extracted with ether. Evaporation of ether leaves the NSF which is dissolved in light petroleum and chromatographed on a silica gel column (height 15 cm; diameter 1.5 cm). The stationary phase is a powdered silica gel, which is placed in a vertical glass column. The mixture to be analyzed is loaded on top of this column. The mobile phase is a solvent poured on top of the loaded column. The solvent flows down the column, causing the components of the mixture to distribute between

the powdered adsorbent. Thus the solvent separate the components of the mixture, when the solvent flows out of the bottom of the column, some components elute with early collections and other components elute with late fractions. The compounds are eluted with 40 mL of each of the following concentrations of light petroleum in benzene, 10, 25, 50% (v/v), followed by ether and 25% methanol in ether, and finally with 20% methanol in chloroform. The fractions (15 mL each) are evaporated to dryness.

2.9. Efficacy check with different fractions

The eluted fractions were screened against bacterial isolates by agar diffusion method. 100 μg of evaporated fractions were incorporated in the 5 mL sterile L-J medium (Hi-media) and poured in the appropriate culture bottles and solidified in slanting position. On the slant, the isolates of M. tuberculosis were inoculated and incubated at 37 ℃for more than four weeks, control bottles were maintained along with the test culture bottles.

2.10. Compound characterization preparative thin layer chromatography and GC/MS

Examination of each fraction by qualitative TLC (on silica gel GC-254, developing with light petroleum-ethyl ether -acetic acid, 20:4:1, and spraying with 50% H_2SO_4, and charring at 180 ℃), reveal the presence of sterols at R_f0.43. The sterol fractions are pooled and subjected to preparative thin-layer chromatography (PTLC). The zones of sterols are scraped off the plates, extracted with chloroform-ethyl ether (1:1), and used for further experiments.

Gas chromatography-mass spectrometry(GC/ms) analysis of an active principles was done by A Finnigan 400 quadrupole mass spectrometer interfaced with Finnigan gas chromatograph with a DB - 5 column was used. Temperature programming at 4 ℃/min to 280 ℃. followed by 2 ℃/min to 310 ℃. Em voltage 1500 V, electron energy 70eV; source temperature 240 ℃. The compounds are identified by means of mass spectrometric comparison with standards and by co injection of authentic samples.

3. Results

As a first step towards the development of novel phycochemicals against MDR-TB an intensive study was carried out by taking the samples of the patients with chest symptoms. 361 patients' general proforma were collected includinggeneral symptoms like continuous fever, night sweat, loss of appetite, progressive weight loss, fatigue, weakness, chills, and chest symptoms like prolonged cough, blood tinged sputum, chest pain. The sputum was collected in the morning in sterile disposable cardboard boxes before any meal. The collected 361 samples throughout the month were subjected to direct microscopical examination and culture technique separately based on the result obtained from direct microscopical examination by Ziehl Neelson staining technique.

Sixty seven patients were found TB positive including 30 males and 37 females among the infected ones. The concentration of bacilli in sputum was identified by taking the smear count. Out of 67 patients 49 were identified as 1+ (3 -9 bacilli seen in entire smear), 15 as 2+ (10 or more bacilli seen in the smear), the rest in the 3 patients fell under the category of 3+ (10 or more bacilli seen in single field). The remaining portion of the sputum was used for culture technique after the concentration of sputum by petroff's method. The concentrated materials of 361 patients were inoculated in to two bottles of IUAT LJ medium and were incubated at 37 ℃. As a result colonies were started appearing from the fourth week onwards. It was found that at the end of six months colonies are formed only in 67 samples against 361. The obtained colonies were yellowish, elevated, wrinkled and dried. The remaining samples were confirmed negative.

The presence of *M. tuberculosis* was confirmed by 16S rDNA analysis. From the bacterial isolates the total DNA were extracted by the help of DNA isolation kits (Geni). Isolated DNA was subjected to PCR amplification. The forward primer used was 5′TGGCTCAGATTGAACGCTGGCGGC 3′ and the reverse primer was 5′TACCTTGTTACGACTTCACCCCA 3′. The PCR conditions were maintained in denaturation at 95 ℃ for 30 seconds, primer annealing at 50 ℃ for 30 seconds, polymerization at 71 ℃ for 1 min and final extension at 71 ℃for 10 minutes. These steps were repeated for 30 cycles in a thermocycler. The amplified product was subjected to agarose gel electrophoresis and the amplified 16S rDNA band was observed under UV transilluminator. Finally, amplified product was sequenced by direct sequencing method. The obtained sequence contained 1 500 bp and it was confirmed through BLAST search and multiple sequence alignment algorithms.

Sensitivity test of 67 bacterial isolates against commercially used front line and second line drugs were carried out by absolute concentration method using LJ slants kit- 023 & 024 (Hi media). This was to determine *in vtiro* antibacterial susceptibility for front line antibacterial drugs like isoniazide (0.2 μg/mL), streptomycin (4 μg/mL), ethambutol (2 μg/mL), rifambicin (40 μg/mL), pyrazinamide (200 μg/mL) and second line drugs like kanamycin (30 μg/mL), amikacin (700 μg/mL), ethinomide (20 μg/mL), d-cyloserine (30 μg/mL), clarithomycin (8 μg/mL), ciprofloxacin (12.5 μg/mL), ρ-Amino salicylic acid (2.5 μg/mL), Rifabutin (0.5 μg/mL). The bacterial isolates numbered I-78, I-101, I-127, I-173, I-202, I-262, I-327 showed resistant for more than 3 drugs among front line and for some drugs in second line. These 7 isolates were considered as MDR *M. tuberculosis* (Table 1). Thus the percentage of 10.4 were recorded MDR-TB for the month of September 2002. The screening of antibacterial compounds from chosen marine micro algal extract and partial characterization of active principles behind the antimycobacterial extracts were elucidated with 15 algal inoculums of *I. galbana, N. occulata, D. inorta, C. friebergensis, C. marina, T. gracilis, P. lutheri, C. salina, C. vulgaris, D. saline, C. calcitrans, Platymonas spp, S. salina, N. salina, C. ovalis* were obtained from Central Marine Fisheries Research Institute (CMFRI) at Tuticorin, South India.

The cultures were maintained in laboratory in Walne's medium as stock culture. From the stock culture, they were mass cultured in sterile seawater containing Walne's medium with proper aeration and light. The growth pattern were given in Table 2. Five different polar and non polar solvents were used for extraction of active principles. They were ethanol, n-butanol, chloroform, methanol and water. Each 100 μg of dried algal extract of different solvents were taken and mixed with 5 mL of sterile LJ medium and pored in the appropriate culture bottles. On the slant, the isolates of *M. tuberculosis* were inoculated and incubated at 37 ℃ for more than 4 weeks, control bottles were maintained along with the test culture bottles. The 7 bacterial isolates of *M. tuberculosis* screened for the bioactive compounds

extracted from marine micro algae using different solvents (75 extracts). The percentage contribution of marine micro algal species on the extraction of antimicrobials indicated *Isochrysis galbana* which contain rich bioactive compounds accounted for 50% inhibition of the total isolates, while Chromulina friebergensis and Tetraselmis gracilis account for 17% and 33% seperately. The percentage contribution of solvents on the extraction of antimicrobials from Isochrysis galbana showed that the methanol, chloroform, n-butanol showed maximum of extraction of 33%, 50% and 17%, higher than that of other solvents tested.

The next algae showed the maximum percentage inhibition was *T. gracilis* accounting for 40% of the total isolates. The percentage contribution of solvents on the extraction of antimicrobials from *T. gracilis* showed that the chloroform and methanol showed maximum of extraction than the other solvents. The third alga with rich bioactive compound was *C. friebergenesis* with about 20% inhibition. The percentage contribution of solvents on the extraction of antimicrobials from *C. friebergenesis* showed that the chloroform showed maximum of extraction than the other algae. The rest of the other algae showed no inhibition towards isolates (Table 3).

The tube dilution technique used to determine the concentration dependent growth pattern of 3 algal active principles. The increasing concentration of dried algal extract were mixed in a series of culture tubes contains Middle Brooks 7H9 broth base (with ADC growth supplement -Hi Media) inoculated with the *M. tuberculosis* at 37 ℃ for 4 weeks. After the incubation the growth of the organism was scanned at 620 nm and the result were recorded. The result showed that the minimum inhibitory concentration of *I. galbana* was found to be 50-60 μg against *M. tuberculosis* for *T. gracilis* it was 70-80 μg and for *C. fribergensis* it was 80-90 μg.

Primary phycochemical screening of active three algal showed the presence of sterols, fixed oils and fats etcs (Table 4). The active solvent extracts were then subjected to column chromatography and the collected fractions were subjected to bacterial screening procedures. The NSF of active principle was dissolved in light petroleum and chromatographed on a silica gel column (height 15 cm diameter 1.5 cm) the compounds are eluted with 40 mL of each of the following concentration of light petroleum in benzene 10, 20, 50% (v/v), followed by ether and 25% methanol in chloroform. The fractions (15 mL each) were evaporated to dryness. Examination of each fraction by qualitative TLC (on silica gel GC 254 developing with light petroleum - ethyl ether-acetic acid, 20:4:1, and sprayed with 50% H_2SO_4 and charring at 180 ℃) revealed the presence of sterols at R_f 0.43. The sterol fractions are pooled and subjected to preparative thin layer chromatography (PTLC). The zones of sterols are scraped off the plates and extracted with chloroform-ethyl ether (1:1). Finally compounds were confirmed by gas chromatography.

Table 1

Sensitivity pattern of isolated MDR *M. tuberculosis* against various commercially available anti TB drugs.

Chemotherapeutic Agents	Symbol	Bacterial isolates						
		I-78	I-101	I-127	I-173	I-202	I-262	I-327
Streptomycin	Sm	S	R	R	R	R	R	R
Ciprofloxacin	Cf	S	S	S	S	S	S	S
Amikacin	Ak	R	R	R	R	R	R	R
Ethionamide	Et	S	S	S	S	S	S	S
Isoniazide	I	R	R	R	R	R	R	R
Ethambutol	Eb	S	S	S	S	S	S	S
p-aminosalicilic acid	Ps	S	R	R	R	R	R	R
Pyrazinamide	Pz	S	S	S	S	S	S	S
Rifambicin	R	R	R	R	R	R	R	R

R-Resistant, S-Sensitive.

Table 2

Growth status of marine micro algal species in walney's medium for every two days.

Days	Micro algal species (Number of cells $\times 10^4$)														
	A-1	A-2	A-3	A-4	A-5	A-6	A-7	A-8	A-9	A-10	A-11	A-12	A-13	A-14	A-15
Initial	36	60	40	5	10	12	4	16	19	25	15	4	7	70	65
2nd day	55	130	80	15	27	25	9	49	53	46	48	10	14	130	110
4th day	80	230	170	45	57	56	15	105	103	70	91	21	25	250	199
6th day	145	425	260	65	95	101	22	150	193	100	156	42	50	475	312
8th day	225	640	360	80	125	230	44	200	275	135	196	67	80	645	581
10th day	290	805	460	95	360	333	101	298	370	150	255	98	111	880	700
12th day	480	875	500	120	330	283	145	275	490	175	245	120	126	790	680
14th day	600	880	520	215	301	253	199	265	375	201	230	135	145	710	603
16th day	720	798	540	460	290	233	204	240	250	220	212	150	160	660	589

A-1: *Isocrysis galbana*, A-2: *Nannochloropsis occulata*, A-3: *Dicarteria inorta*, A-4: *Chromulina friebergensis*, A-5: *Chlorella marina* A-6: *Tetraselmis gracilis*, A-7: *Pavlova lutheri*, A-8: *Chlorella salina*, A-9: *Chlorella vulgaris*, A-10: *Dunaliella saline*, A-11: *Chaetoceros calcitrans*, A-12: *Platymonas spp*, A-13: *Synechocysti salina*, A-14: *Nannochloropsis salina*, A-15: *Chlorella ovalis*.

The purified eluted compounds (R_f 0.43) from TLC plate were chromatographed by gas chromatography Hewlette-Packard 5890(series II) with an HP-GC mass selective detector (5971 MSD) (Figure 1). The sterol composition of eluted compound showed thirteen unsaturated sterols with three major sterols such as 24-oxocholesterol acetate (18.9%), Ergost-5-en-3 β-ol (16.2%) and Cholest-5-en-24-1,3-(acetyloxy)-, 3β-ol (11.2%) (Table 5).

Table 3

Sensitivity pattern of MDR *M. tuberculosis* isolates against marine microalgal extracts.

Sl. No	Name of the micro algae	Solvent Used	Bacterial isolates							
			I-78	I-101	I-127	I-173	I-202	I-262	I-327	control
1.	*Tetraselmis gracilis*	Ethanol	-	-	-	-	-	-	-	-
		n-Butanol	-	-	-	-	-	-	-	-
		Chloroform	+	+	+	+	+	+	+	+
		Methanol	+	+	+	+	+	+	+	+
		Water	-	-	-	-	-	-	-	-
2.	*Isochrysis galbana*	Ethanol	-	-	-	-	-	-	-	-
		n-Butanol	+	+	+	+	+	+	+	+
		Chloroform	+	+	+	+	+	+	+	+
		Methanol	+	+	+	+	+	+	+	+
		Water	-	-	-	-	-	-	-	-
3.	*Chromulina friebergenesis*	Ethanol	-	-	-	-	-	-	-	-
		n-Butanol	-	-	-	-	-	-	-	-
		Chloroform	+	+	+	+	+	+	+	+
		Methanol	-	-	-	-	-	-	-	-
		Water	-	-	-	-	-	-	-	-

-: No Inhibition; +: Inhibition

Table 4

Preliminary phytochemical screening of selected crude marine micro algal extracts.

Active principles	Sterols	Terperiods	Carbohydrates	Flavanoids	Proteins	Alkaloids	Glycosides	Tannins	Saponins	Phenols	Fixed oil and fats
MG-2	+	-	+	-	+	-	-	-	-	-	+
MG-6	+	-	+	-	+	-	-	-	+	-	+
MG-10	+	-	+	-	+	-	-	-	-	-	+

(+) Positive, (-) Negative.

Table 5

Natural sterols isolated from *I. galbana*.

Peak No.	Compound	Retention time (min)	Molar mass (Da)	Abundance (%)
1	Unidentified	107.35	428	3.1
2	Unidentified	107.39	428	5.5
3	Cholest-5-en-24-1,3-(acetyloxy)-, 3 β -ol	107.44	426	11.2
4	Ergost-5-en-3 β -ol	112.22	400	16.2
5	(24R)-methylergost-5-en-3 β -ol	112.64	400	3.6
6	5 α -ergosta-7-en-3 β -ol	113.22	400	2.9
7	ergosta-5,7-dien-3 β -ol	114.09	398	6.3
8	ergosta-5,22(E)-dien-3 β -ol	115.20	398	2.6
9	24-oxocholesterol acetate	115.28	386	18.9
10	5 α -ergosta-7,22(z)-dien-3 β -ol	115.39	398	5.3
11	24-methylcholesta-5,22-dien-3 β -ol	115.45	398	9.4
12	ergosta-4,7,22-trien-3 β -ol	117.07	396	4.1
13	ergosta-5,7,22(E)-trien-3 β -ol	117.84	396	4.9
14	24-methylcholest-5-en-3 β -ol	118.74	396	6.3
15	ergosta-5,7,22,24(28)-tetraen-3 β -ol	120.38	394	2.7

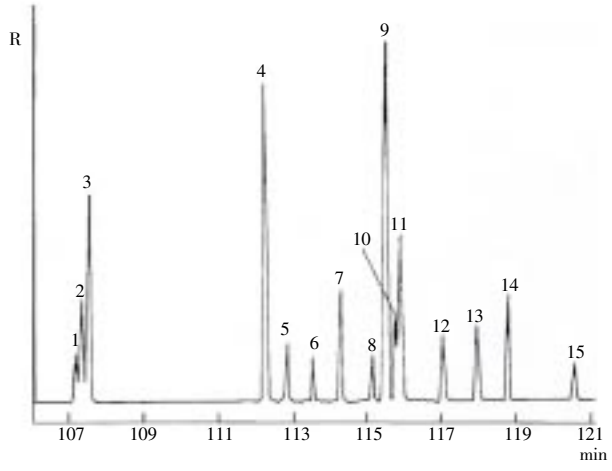

Figure 1. GC-MS chromatogram of natural sterols isolated from *I. galbana*

4. Discussion

The test organisms are based on the choice of their Ubiquitous presence[17]. While screening with commercially available antibiotics shows that the human pathogens were multidrug resistant. In the present study the MDR *M. tuberculosis* were screened for bioactives extracted from marine microalgae with various solvents, it shows *I. galbana* has maximum activity. This is supported by the earlier studies of Lazarus and Valentinbhimba[18], who reported that *I. galbana* has rich bioactive compounds (10.8%). Present study reveals that the crude extracts of *I. galbana* solvent in chloroform showed better antimicrobials than other solvents. This clearly indicates that the strength of active principle depends on the use of suitable solvent. A thin layer chromatography fractionation of the extracts and further GS MS analysis suggested the active principle compound.

The present study indicates the presence of unsaturated fatty acids may have the effect on MDR *M. tuberculosis*, indicating a potential natural alternative to antibiotics. However, the limited number of test organisms owes to a constraint of resource. So the effect of strong bursts of micro algal extracts on human pathogenic bacteria should further be tested on a wide range of test organisms.

Conflict of interest

The authors have no conflicts of interest

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