VIII. SUMMARY

The present study was carried out by the following four major objectives:

- Isolation and identification of MDR *Mycobacterium tuberculosis* from collected specimens.
- Screening of antibacterial compounds from chosen marine micro algal extracts and partial characterization of active principles.
- *In-vivo* acute and sub acute toxicity study of antimycobacterial compounds from marine micro algal extracts in albino mice as animal model.

A sample survey was earned out in the Kanyakumari District medical college hospital of Tamil Nadu, South India with the objective of finding out the prevalence of bacteriologically positive pulmonary tuberculosis among peoples enrolled in the hospital during 2002-2005. In the year 2002 a total of 4292 patients were screened for TB infections out of which 17.26% was TB positive. The percentage incidence of TB during the year 2003 was recorded as 16.2. A very high prevalence of 20.84% was registered in 2004. Similarly for the year 2005 the TB prevalence was recorded as 15.45.

The data of TB incidents of four consecutive years on seasonal basis namely non-monsoon (Jan-May), South-West monsoon (June-Aug), North-East monsoon (Sept-Dec) were recorded. The results showed a highest percentage of 20.22 during South-West monsoon and the least percentage of 15.81 during North-East monsoon. 18.34 percentage of incidence occurred during non-monsoon. In addition, the tabulated data signifies that females were more prone to TB than males. A total 1251 females were recorded TB positive against 899 males.

The prevalence of infected individuals among different age groups during 2002-2005 indicated that the age group of 71-80 was worst affected in the year 2002 (241 infected) followed by the group of 61-70 (212 infected). During the year 2003, the maximum numbers of infected cases were recorded under the age group of 71-80 (103...
infected). In 2004, again the age group of 71-80 stood first with 317 affected cases followed by the age group of 61-70 (213 infected). The number has almost increased four times than 2003 for the age group of 81-90 with 176 being registered positive. The young age group of 30-40 was found least affected (2 cases in 2004 and 5 in 2005) followed by the age group of 41-50 (96 cases in 2004 and 16 cases in 2005). In 2005 maximum number of TB positive were recorded under the age group of 71-80 (117 infected) followed by the group of 61-70 years (61 infected). Thus in total 2150 patients were found AFB positive during 2002-2005.

As a step towards the development of novel phytochemicals against MDR-TB, an intensive study was carried out by taking the samples of the patients with chest symptoms during the month September 2002. Out of 361 patients examined for tuberculosis 146 patients were found to suffer from chest symptoms like prolonged cough and sputum with blood. The collected 361 samples throughout the month was subjected to direct microscopical examination and culture techniques separately. Based on the results obtained 67 patients were found AFB positive. 30 males and 37 females among the infected ones. Thus the percentage contribution among AFB positive includes 44.78% males and 55.22% females.

From the general proforma collected the age limit among AFB positive patient was registered as 42-78 years. 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 of the 3 patients fell under the category of 3+ (10 or more bacilli seen in single field). Thus the percentage contribution of TB patient during the month of September 2002 was 18.56. Sensitivity test of 67 bacterial isolates against commercially used front line and second line drugs were carried out by absolute concentration method. 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 Mycobacterium tuberculosis. Thus the percentage of 10.4 were recorded MDR-TB for the month of September 2002. Finally, the presence of M. tuberculosis was confirmed by 16S rDNA analysis
Screening of antimycobacterial compounds from chosen marine micro algal extracts and identification of active principles were studied. 15 micro algal obtained from CMFRI, Tuticorin, India were maintained in laboratory in Walne’s medium as stock culture. From the shock culture, they were mass cultured in sterile seawater.

The micro algae *Chlorella marina* showed an exponential growth of 720x10^4 cells/ml on the 16th day. *Nannochloropsis oculata* showed the maximum growth of 880x10^4 cell/ml in the 14th day, followed by a decline growth from 16th day onwards. The initial cell count for the marine micro algae *Chromulina friebersgenesis* was 40x10^4 cells/ml. It showed an exponential growth along 2nd, 4th till the 16th day. Finally, the culture of this alga showed the growth of about 540x10^4 cells/ml.

For the micro algae *Tetraselmis gracilis* there was a steady increase in the cell count throughout the fifteen days reaching the final count of 460x10^4 cells/ml on the 16th day. *Isochrysis galbana* showed a steep growth from the initial day (10 x 10^4 cells/ml) to 10th day (360 x 10^4 cells/ml) of the observation, after which a decline was seen till the 16th day. The growth phase of the micro algal species *Dicarteria inorta* almost resembles the *Isochrysis galbana* showing steep increase in cell count till the 10th day after which a decline in growth was observed. *Pavlova lutheri*, the growth was slow and steady until the 8th day. From the 10th day onwards, there was a tremendous increase in the cell count reaching 204 x 10^4 cells/ml on the 16th day.

The growth was almost exponential for the marine algae *Chlorella vulgaris* for the first 10 days followed by a small decline. The culture of *Chlorella ovalis* showed an exponential growth for first 12 days reaching 490 x 10^4 cells/ml on the 12th day after which the cell count was suddenly dropped to about 250 x 10^4 cells/ml on the 16th day.

The cell count of *Chlorella salina* has gradually increased for about 2 weeks reaching 220 x 10^4 cells/ml on the 16th day. The cell growth was exponential for *Tetraselmis suecia* for the first 10 days counting 255 x 10^4 cells/ml on the 10th day. Thereafter the cell count has gradually decreased to 212 x 10^9 cells/ml on the 16th day. The *Dunaliella saline* exhibited steady and gradual increase in cell count of about 67 x 10^4 cells/ml on the 8th day and the final count of 150 x 10^4 on the 16th day.
Synectocytic salina with slow and steady inclination, the cell count has reached about 160 x 10⁴ cells /ml on the final day of the observation. The micro algae Nannochloropsis salina showed a comfortable cell count of 880 x 10⁴ cells/ml on the 10th day followed by a small decline resulting in cell count of 660 x 10⁴ cells/ml on the 16th day. Similarly, culture of Nannochloropsis salina resulted in the cell count of 589 x 10⁴ cells/ml.

The collected 15 different marine micro algal species were screened for antimycobacterial compounds using 5 different solvents namely ethanol, n-butanol, chloroform, methanol and water resulting in 75 extracts. The percentage contribution of marine micro algal species on the extraction of antimicrobials indicated that Isochrysis galbana contains rich bio active compounds than the other algal species. It accounted for 50% inhibition of the total bacterial isolates. Among the 5 solvent used only n-butanol, chloroform and methanol showed inhibition to all the seven bacterial isolates.

Tetraselmis gracilis species showed 33 percentage inhibition of the total bacterial isolates. For this micro algae the extracts obtained from the solvents chloroform and methanol showed maximum inhibition while the other solvents remain inactive. The third algae with rich bioactive compound was Chromulina friebergenesis with about 17% inhibition towards bacterial isolates. Only the Chromulina friebergenesis extract prepared with the solvent chloroform showed inhibition, the other solvents showed no action against the bacterial isolates. The rest of the 12 algae showed no inhibition towards bacterial isolates.

The tube dilution technique was used to determine concentration dependent growth pattern of three algal active principles. Out of the five solvents used for the extraction of active compounds from Isochrysis galbana n-butanol, chloroform and methanol showed a high activity than the other two solvents. The optical density was measured for different concentration of Isochrysis galbana against methanol extract. The minimum inhibitory concentration of Isochrysis galbana against methanol extract was found to be 60 µg. Similarly, the minimum inhibitory concentration of Isochrysis galbana against chloroform extract was calculated as 70µg. Out of the 3 extracts used n-butanol has showed relatively poor performance resulting in minimum inhibitory
concentration of *Isochrysis galbana* to be 80μg. Thus the methanol extracts found to be more effective against bacterial activity of the tested isolates.

Antimycobacterial activity of *Tetraselmis gracilis* showed positive results only for the solvents methanol and chloroform. After the keen observation of the resulted values the minimum inhibitory concentration of *Tetraselmis gracilis* against methanol solvent was found to be 80μg and against the chloroform solvent was registered as 80 - 90 μg.

Finally, the optical density was measured to check the concentration dependent antimycobacterial activity of *Chromulina friebergenesis* against the chloroform extract. From the experiments, it was observed that the concentration Dosage level should be minimum of 90 μg for 100% inhibition. After analyzing all the three micro algal extracts against their active solvents it was concluded that chloroform and methanol were found efficient in extracting the active principles from the subjected crude extracts.

To spot out the compounds present in the crude extracts (MG-2, MG-6 & MG-10) preliminary phytochemical screening were performed qualitatively. All the three extracts showed the presence of sterols, carbohydrates, protein and mixed oils and fats. None of the crude extracts was positive for the phytochemical terperiods, flavonoids, glycosides, flavanoids, alkaloids, tannis and phenols. From our result it is evidenced that the inhibiting activity of the compound found in the microalgae varies with the organic solvent. Chloroform and methanol extract (2:1) of *Isochrysis galbana*, *Tetraselmis gracilis* and *Chromulina friebergenesis* resulted the maximum inhibitory activity against *M. tuberculosis*. On comparison of phytochemical screening and the efficacy of solvent, it was clear that lipids and steroids has played a vital role in inhibiting the bacterial activity on the selected isolates.

The purification of active compound by column chromatography the *Isochrysis galbana* extract consisted of 3 compounds. The extract of *Tetraselmis gracilis* has two fractions after the chromatography elutions. The extract of *Chromulina friebergenesis* gave two fractions after the elution by chromatography.
After the chromatography elution, the fractions were screened against same bacterial isolates. Among the all tested fractions of three micro algae, the results showed that the same fractions of all three algae are effectively controlled the 7 bacterial isolates.

The active fraction principles were further separated by TLC, the lane I of Isochrysis galbana on TLC plate showed 3 compounds, lane 2 of Tetraselmis gracilis active principles spotted on TLC showed 2 compounds and Chromulina frieberggenesis active fraction spotted on TLC plate showed 2 compounds. Among the three bands resulted from the Isochrysis galbana, band II showed the Rf value 0.43. Between two bands resulted from the Tetraselmis gracilis, band II showed the Rf value 0.43 and the two bands resulted from Chromulina frieberggenesis showed the Rf value 0.43. Among the all three lanes, one compound showed the same Rf value at 0.43 which indicates the similar compound in such mobility. The result clearly indicates the presence of sterol compounds in all three micro algal fractions.

The purified eluted compounds (Rf 0.43) from TLC plate were chromatographed by Gas Chromatography Hewlett-Packard 5890 (series II) with an HP-GC mass selective detector (5971B MSD). The sterol composition of eluted compound showed thirteen unsaturated sterol 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%). Three minor sterols such as 24-methylcholesta-5,22-dien-3 β-ol (9.4%), 24-methylcholest-5-en-3β-ol (6.3%) and ergosta-5,7-dien-3β-ol (6.3%) (Fig 3.12). Other identified sterol compounds are ergosta-5,7-dien-3β-ol (6.3%), 5α-ergosta-7,22(z)-dien-3 β-ol (5.3%), ergosta-5,7,22(E)-trien-3β-ol (4.9%), ergosta-4,7,22-trien-3β-ol (4.1%),(24R)-methylergost-5-en-3β-ol (3.6%), 5α-ergosta-7-en-3β-ol (2.9%), ergosta-5,7,22,24(28)-tetraen-3β-ol (2.7%), ergosta-5,22(E)-dien-3β-ol (2.6%) and two unidentified compounds. The present study indicates that the presence of some unsaturated fatty acids may have the inhibitory effect on M. tuberculosis.

Acute and sub acute toxicity study of active principles extracted from the marine microalgae of I. galbana (MG-2), T. gracilis (MG-6), and C. friebergensis (MG-10), was carried out in wister albino mice. The acute toxicity studies were carried on 20-25 gms albino mice with all three samples. Seven groups of albino mice of either sex are selected, each group contain 10 mice. The algal extract was administrated by oral route at
the dose level of 500mg/kg, 1000 mg/kg, 1500 mg/kg, 2000 mg/kg, 2500 mg/kg, 3000 mg/kg and 3500 mg/kg body weight. The results obtained revealed that LD50 value of MG-2 was 3000mg/kg b.wt. The result showed that micro algal (MG-6) is safe in oral administration in albino mice for the dosage levels of 500mg/kg b.wt. However, at 3500mg/kg b.wt stimulation symptoms such as adverse irritability and hyperactivity with mild jumping and twitching during 24th hour of the experiment five animals were found dead. Thus LD50 for the extract MG-6 is 3500mg/kg b.wt. Oral administration of the algal extract MG-10 showed no adverse effects for the dosage level less than 3000mg/kg b.wt. The LD50 value for MG-10 was 3500mg/kg b.wt.

For the sub acute toxicity studies four groups of Albino mice of either sex was selected. Each group containing 6 mice (3 males and 3 females). The algal extracts was administered by oral route at the dosage level of 1% acacia suspension, MG-2 500mg/Kg b. wt, MG-6 700mg/Kg b. wt, and MG-10 700 mg/Kg b. wt. The animals were treated with extracts for 28 days. During this period the average body weight in gram showed a progressive increase in all the groups. This increase in the weight is significantly different (P< 0.05) from that of the control. On comparison of average body weight after algal administration with that of the control it was found that the extract MG-2 and MG-6 were significant (P<0.05 and P<0.05) respectively. The drug MG-10 showed non-significant with the control (P>0.5).

The progressive increase in body weight at doses of 500mg/Kg, 700mg/Kg and 700 mg/Kg of administration of algal extracts (Mg-2, MG-6 & Mg-10) may indicate the improvement of the nutritional status of the animal. The food consumption during the 28 day treatment of algal extracts showed no significant variation (P<0.05) from the control. On comparison of control with other algal extracts for average food consumption after drug administration showed the significant difference of P<0.001 for MG-2, P< 0.01 for MG-6 and P<0.001 for MG-10.

Effect of drugs on organ weight such as heart, liver, spleen and kidney of the tested animal were recorded and studied. All the 3 algal extracts showed a ±0.24 gms, 3.46±0.51 and 4.10±0.13 gms weight changes respectively. The organs spleen and kidney are less affected by the drugs as thin weight change was recorded as 0.42±0.03 and 0.69±0.03 for MG-2, 0.45±0.11 and 0.73± 0.01 for MG-6, 0.56±0.07 and 0.64± 0.07.
Effect of drugs on organ weight of rats. Eg/100g body weight showed the significant difference of P<0.05 from the control. The weight of the liver, Kidney, Heart, and Spleen were unaltered in the experimental groups compared with the control group.

The Histopathological study of the heart of the different groups of rats showed some changes when compared to control. MG-2 administrated group II animals showed very clear polar area of the heart muscle, sample MG-6 administrated group III animals heart section showed the area of necrosis and hemorrhage. Whereas sample MG-10 administrated animals showed early necrotic changes.

The histopathological study of the liver of different groups of rats showed a normal architecture. The treated orally with extract of MG-2, MG-6 and MG-10 for 28 days showed little abnormalities such as ballooning degeneration, vacuolation of hepatocytes. The spleen section of the postmortem animals showed early toxic symptoms like focal necrosis, fibrosis and congestion. The microscopic identification of kidneys of MG-2 orally administrated animals showed the sign of ballooned out tubular epithelium, narrowed lumen. The sign of cloudy swelling of the tubular epithelial cells were found in MG-6 administrated animals. Similar symptoms were seen in MG-10 administrated animals too. There were no significant change in any liver function parameters, such as ALT/SGPT, AST/SGOT, ALKP, ACP and albumin compared to the control group.

Significant differences (P=0.05) in the value of creatinine, total protein, globulin and cholesterol were observed between the test and control groups. Also, there were no significant changes in various haematological parameters such as RBC, ESR and differential count compared to the control group, which indicates that algal extracts may not be toxic and does not affect circulating red cells, hematopoiesis or leucopoiesis. The results obtained in the Haematological picture showed no significant difference in PCV%, HB%, Eosinophilis% and Basophilis%, while there were increase significant changes in WBCs count and monocyte (P=0.001). The histological changes that were demonstrated in the liver, spleen, heart and kidney was however significant.