9. Summary
The urgency of new antibiotic discovery becomes more serious due to lack of effective therapies and the development of resistance of many pathogens. Bacteria can acquire drug resistance in a multitude of ways, thus to solve this problem is not a straightforward matter. A similar situation exists with the need to develop new cancer chemotherapeutic agents in the treatment of many tumors. Fungi, the second most diverse group are still less explored. Marine fungi are known to have novel and biologically active compounds due to their adaptation to a very distinct set of environmental pressures. So they are one of the most significant groups of organisms to be exploited for drug discovery purposes.

Few studies have been reported about bioactivity of fungal metabolites from Gulf of Mannar. However studies on pharmacological activity and cytotoxic activity of fungal metabolites are scanty. Hence, this present work has been attempted to explore bioactive fungal strains from biotic and abiotic sources from Tuticorin and Mandapam coast, Southeast coast of India. The objectives of this study were to isolate fungal strains; to optimize their growth for production of bioactive metabolites to screen antibacterial activity against ten human bacterial pathogens; to identify active strain through molecular identification; to test pharmacological efficiency of active strain through analgesic, anti-inflammatory, CNS stimulant, antipyretic and antiulcer activity; to purify active compounds by chromatographic techniques; to characterize the active compound using, Infrared
Resonance (IR), Mass (MS) spectrometry and Nuclear Magnetic Resonance (NMR) technique and finally to assess their cytotoxic effect.

The associated fungal strains were isolated from seaweeds (*Sargassum wightii, Ulva lactuca, U. reticulata, Acanthaphora spicifera, Gracilaria corticata, G. edulis, Hypnea valentiae, Halimeda tuna, Enteromorpha compressa, Dictyota dichotoma* and *Padina tetrastromatica*) seagrasses (*Halophila ovalis, Thalasia hemprichi* and *Enhalus acoroides*), ascidians (*Phallusia nigra, Aplidium multiplicatum, Polyclinum indicum, Distaplia nathensis* and *Eudistoma viridae*), sponges (*Fasciospongia cavernos, Hyattella cribriformis, Thalysias vulpine, Ircinina sp.* and *Psammaplysilla purpurea*), mangrove rhizosphere sediment (*Avicennia marina*), seawater (1 sample) and sediment (1 sample). Endophytic fungi also were isolated from selected seaweeds such as *Sargassum wightii, Ulva lactuca, Acanthaphora spicifera, Gracilaria corticata* and *Enteromorpha compressa*.

Totally, 288 distinct fungal strains were selected for preliminary screening based on the colony morphology. Antibacterial activity of supernatant from each strain was evaluated using agar well method against 10 human bacterial pathogens (*Staphylococcus aureus, Micrococcus luteus, Streptococcus pyogenes, Bacillus subtilis, Enterobacter faecalis, Escherichia coli, Klebsiella pneumoniae, Shigella sonnei, Salmonella typhimurium* and *Vibrio cholerae*).
Antibacterial activity was observed in 29.2% (84) strains and the highest prevalence of active fungal strains were noticed in sponges (58%) followed in mangrove sediment (30%), seagrasses (26.6%), ascidians (24%), seaweeds (21.8%) and in marine sediment and water (20%). 18 strains showed predominant antibacterial activity; nine from seaweeds (SwI5, SwI8, UL7, UR7, AS4, HV3, DD3, SWEN3 and ULEN4), four from sponges (FC9, TV1, IR3 and PP5), three from mangrove and marine sediment (AMS4, AMS8 and ST7) and one each from sea grasses (TH6) and ascidians (EV1).

Fungal metabolites of these active strains were extracted with various solvents such as hexane, ethyl acetate, chloroform and butanol. Antibacterial potential of fungal crude extracts were evaluated. Ethyl acetate and butanol extracts of all the 18 strains showed good activity. Among the 18 strains, AMS4 from mangrove rhizosphere sediment associated strain showed highest activity against all the 10 human pathogenic bacteria in ethyl acetate extract with inhibition zone ranging from 7 to 15 mm (Vibrio cholerae) followed by butanol, hexane and chloroform extracts. The minimum inhibitory concentration of AMS4 strain was found to be 30±5 µg/disc.

Also the ethyl acetate and butanol extracts of ST7, TV1 and FC9 strains showed wide spectral activity. Hexane and chloroform extracts of ST7 strain showed broad spectral activity against seven pathogens. The chloroform extracts
of EV1 showed activity against 6 pathogens while FC9 and TV1 strains against 5 pathogens. Ethyl acetate extracts of SWI5, DD3, SWEN3, ULEN4, EV1, TH6 and IR3 showed higher wide spectral activity against pathogens. Hexane extract of SWI5, DD3 and chloroform extract of UL7, UR7, TH6 strains did not show any activity against any pathogen.

Based on the morphological characters, the selected 18 active fungal strains were grouped under four genera which are Aspergillus sp., Fusarium sp., Penicillium sp. and Cladosporium sp. The fungal strains SWI8, SWEN3, UL7, AS4, FC9, TV1 and AMS4 were identified as Aspergillus sp.; SWI5, ULEN4, EV1, PP5 and AMS8 strains were Fusarium sp.; UR7, HV3 and TH6 were Penicillium sp. and DD3, IR3 and ST7 were identified as Cladosporium sp. The active fungus AMS4 was identified and ascertained as Aspergillus oryzae (JN86006) by molecular characterization.

The ethyl acetate extract of supernatant of Aspergillus oryzae (AMS4) was evaluated for pharmacological activity such as Central Nervous System (CNS) stimulant, anti-inflammatory, analgesic, antipyretic and anti-ulcer activities. The extract produced significant antipyretic, analgesic and CNS stimulant effect in a dose dependant manner. The anti-inflammatory and anti-ulcer activities were pronounced in extracts.
The anti-inflammatory activity was observed by reduction in paw volume of rats in dose dependent manner with the higher dose showing higher anti-inflammatory activity. Tail-flick behavior in rat indicated analgesic activity of extract which also showed increasing activity with increasing extract concentration. In antipyretic assay, test extract showed almost equal effect at 200 mg/kg as that of standard drug Paracetamol at 45 mg/kg. In CNS stimulant test, the locomotor activity was greatly stimulated by low dose concentrated test extract while higher dose decreased locomotor activity. In anti-ulcer activity, 200 mg/kg of extract significantly reduced the volume of gastric juice, free and total acidity found to be potent against gastric ulcers in rats.

In bio-assay guided column fractionation of crude ethyl acetate extract of A. oryzae, nine fractions were collected. Among them, fraction A3 (50%H: 50%EA) showed wide spectral activity against all human bacterial pathogens. The zone of inhibition ranged between 7 and 15 mm. Fraction A3 (50%H: 50%EA) was further fractionated with hexane, dichloromethane, butanol and ethyl acetate and 13 sub fractions were obtained. The purity of all the fractions was checked by TLC. Among them, fraction B6 (75%DCM: 25%B) showed wide spectral activity which was again fractionated with dichloromethane, dichloroethane and butanol and nine fractions were obtained; fraction C6 (75%DCE: 25%B) exhibited wide spectral activity. The zone of inhibition varied between 4 and 7 mm. The active fraction C6 (75%DCE: 25%B) was finally
fractionated with dichloroethane and butanol and eight fractions were obtained, among them fraction D1 (85%DCE: 15%B) showed wide spectral activity with a zone of inhibition range of 2 to 5 mm. The purity of active fraction D1 was confirmed by TLC profile and HPLC. Solvent system of hexane, ethyl acetate, dichloromethane and butanol was found to be good for separation of active components from crude fungal extracts and it can also be suggested that the activity in non-polar fraction may be due to the presence of active non-polar compounds, while the activity in other fractions due to the presence of multiple compounds with different polarity.

The purified fraction of A. oryzae (85%DCE: 15%B) was subjected to ESI-MS, IR and NMR spectroscopy for structural elucidation of active compound. The molecular weight of compound was found to be 414 (DI) (M$^+$). From the IR value which has led to the presumption that the compound may contain methylene, ketone and hydroxyl functionalities. The NMR spectrum showed that the compound may have CH$_2$, CH$_3$, OH, C-H and aromatic protons functionalities. The elemental data showed the presence of Carbon, Hydrogen, and Hydroxyl group. The obtained data revealed that the isolated compound may belong to Ergosterol. But, the characterization was only indicative and not considered as complete. However, the $^{13}$C NMR would have provided some accuracy to data.
The purified fraction exhibited cytotoxic activity in dosage dependent manner. The increasing extract concentration significantly decreased the cell viability. The GI_{50} (50% inhibition of HeLa cell viability) value of active extract was found to be 1.7 µg/ml while positive control Doxorubicin exhibited GI_{50} value at 0.1 µg/ml. While staining with fluorescent dye propidium iodide, cells treated with fraction of *A. oryzae* at 10 and 30 µg/ml showed more apoptotic cells when compared to untreated cells after 48 hrs of incubation. There were characteristic nuclear fragmentations of nuclei in treated HeLa cells, whereas untreated control cells did not show any nuclear fragmentation. The apoptotic cells displayed the characteristic features of reduced size, intense fluorescence of condensed nuclear chromatin and formation of membrane blebs. In flowcytometry analysis, treated cell lines showed changes in light scatter, a decrease in forward scatter (FSC) as an indication of lower cell size and an increase in side scatter (SSC) as an indication of granularity compared to control cells in dot plots which indicate the characteristic feature of the cells dying of apoptosis in treated cells.

After 24 hrs treatment, the percentage of cells containing apoptosis in sub G0-G1 phase increased from a control value of 1.79% to 1.9 and 1.87% in cells treated with active fraction at 10 and 30 µg respectively which indicates the process of induction of apoptosis. The active fraction exhibited marked arrest of cell cycle in S phase and percentage of apoptosis cells extensively increased from a control value of 34.47% to 35.13 and 40.85% at 10 and 30 µg.
respectively. After 48 hrs treatment, active fraction (10 and 30 µg) showed relatively moderate arrest of cell cycle in S phase and percentage of cells significantly increased from a control value of 36.83% to 40.52 and 37.48%. These results confirmed that purified fraction of A. oryzae induced apoptosis, by the mechanism of DNA strand breaks before S phase accumulation.

Among the 288 fungal strains, A. oryzae showed potent antibacterial, pharmacological and cytotoxic activity. The mechanism of induction of apoptosis could not be carried out in the present study due to lack of facilities. The active compound was identified as ergosterol which is already reported to be one of the main fungal sterols. So, further studies are required to ascertain the structural characteristics of the isolated compound. After confirmative studies, computer modeling can then be used to generate chemical analogues of this antibacterial compound and to examine the structure and functional relationships.

The present study has demonstrated that marine fungi with bioactive metabolites can be expected to provide high quality biological material for antibacterial and anticancer screening programmes. The potential of using metabolic engineering and post genomic approaches to isolate more novel bioactive compounds and to make their possible commercial application is not far off. This study also indicates the possible potential for development as an anti-cancer drug and warrants further scientific investigation.