CHAPTER 6

SUMMARY

In our endeavour to isolate bacterial strains capable of producing bioactive metabolites from the water samples collected from Bay of Bengal, particularly from Andaman seas, India, 700 odd isolates were randomly selected. In the present study, out of 700 bacteria isolated from the water column of Bay of Bengal, 8 isolates were selected based on preliminarily screen employing *Bacillus* spore outgrowth inhibition technique. Further screening with topoisomerase inhibition study in yeast mutant strains resulted in selection of two isolates KP2 and KP8 whose crude extracts also showed inhibition of proliferation of mitogen induced PBMC.

The isolates KP2 and KP8 were identified as *S. arlettae* and *P. maritimus* respectively by biochemical and molecular characterization. *S. arlettae* KP2 is gram positive cocci which grow at 30 °C, pH 6.5-8 and in the presence of 0.5-12 % NaCl and produces pale yellow pigment. It can utilize most of the sugars viz. glucose, lactose, maltose, fructose, dextrose, galactose, raffinose, trehalose, sucrose, L-arabinose, mannitol and ribose but not xylose, sorbitol, mannose, inulin and rhamnose. *P. maritimus* KP8 is gram positive cocci. Colonies are smooth, circular and orange in colour. Growth occurs at 4 °C and 41 °C but not at above 42 °C. Optimal growth temperature is 30 °C. Optimal pH for growth is 6.5–8 and grows in presence of 0.5-12 % of NaCl. It can utilize fructose and ribose but not glucose, mannose, trehalose, sucrose, L-arabinose, lactose, xylose, maltose, dextrose, galactose, raffinose, melibiose, sorbitol, inulin, mannitol and rhamnose.
Among the two isolates, on the basis of secondary screens, *P. maritimus* KP8 was selected for further studies. The crude ethyl acetate extracts of *P. maritimus* KP8 inhibited proliferation of mitogen induced PBMC with an IC\(_{50}\) value of 18 µg ml\(^{-1}\) and was found to be non toxic to normal cells. Our findings indicate that the crude extracts not only suppressed cell proliferation but also down regulated the gene expression of IL-1β and TNF-α in mitogen induced PBMC. In our study, parallel decreases in NO production as well as COX-2 enzyme activities were also observed. The crude extracts also inhibited p38 MAP kinase activity.

The lead molecule was purified from the crude extracts of *P. maritimus* KP8 by bio-guided fractionation using column chromatography and the purity of the compound was ascertained by HPLC. The lead molecule was subjected to NMR, FTIR and mass spectroscopic analysis. Based on the results, the molecular weight of the lead molecule from *P. maritimus* KP8 was found to be 135 with molecular formula C\(_7\)H\(_7\)NO\(_2\) and the structure was established as 1H Benzo[c]isoxazole-3 one.

The lead in comparison with the crude extract was scrutinized for their anti inflammatory potential on various markers of inflammation. Prostaglandin synthesis being elevated in inflammatory conditions, effect of the crude extract and the lead molecule on the enzymes involved in production of PG like Phospholipase A\(_2\) and Cyclooxygenase-2 were studied. 45 % and 50 % inhibition of PLA\(_2\) activity was observed in RBL-2H3 cell line by the crude extract and lead respectively. Both the crude extract and lead showed up to 78% inhibition of COX-2 enzyme activity. To determine whether the inhibition of COX-2 enzyme activity is due to the down regulation of COX-2 gene by the crude extract and pure compound, semi quantitative RT-PCR analysis was performed. As expected, down regulation of mRNA expression of COX-2 genes was detected.
Yet another mediator of inflammation is NO. Since inhibition of NO and or iNOS activity has tremendous therapeutic value, we have studied the effect of crude extract and pure compound from *P. maritimus* KP8 at different doses on NO production in LPS stimulated RAW 264.7 cells. Maximum reduction in NO production occurred at 50 µg ml$^{-1}$ and resulted in down regulation of iNOS gene expression both by crude extract and pure compound post 24 h incubation.

Pro and anti inflammatory cytokines play important role in regulating inflammation. To find out whether the crude extract and pure compound do influence cytokine expression in proliferating lymphocytes, RT-PCR analysis was carried out in the proliferating PBMC in the presence and absence of crude extracts and pure compound. The primary cytokines TNF-α and IL-1β was found to be down regulated post 6 h incubation with the lead molecule. The mRNA levels of other cytokines like IL-4, IL-6 and IFN-γ were also lowered by the lead post 24 h incubation. Interesting part of the result was that our lead not only down regulates the pro inflammatory cytokines but also up regulate the anti inflammatory cytokine IL-10.

Since MAP kinases are involved in cytokine production and enzymes that mediate inflammation, we studied the effect of crude extract and lead from *P. maritimus* KP8 on the signaling molecules like ERK and p38 MAP kinases. Down regulation of both the MAP kinases ERK1/2 & p38 was observed in the presence of the added pure compound; however, the crude ethyl acetate extract inhibited only p38 MAPK. To find our whether the inhibition of proliferation leads to apoptosis, crude ethyl acetate extract and pure compound from *P. maritimus* KP8 were tested for their effect on caspase activation. Caspases- 3 as well as caspase-8 was shown to be up regulated in LPS stimulated PBMC treated with crude extract and pure compound. J774 A.1 cell line, NF-κB activity is critical for its growth, was used to
study the effect of the crude extract and lead for their effect on NF-kB activity. 50% inhibition was observed at 50 µg ml\(^{-1}\).

Since most of the anti inflammatory compounds possess anti cancer potential, we examined our lead: 1H Benzo[c]isoxazole-3 one on various cancer cell lines like HeLa, K562 and HT 29. A dose dependent inhibition was observed in all the cell lines tested proving its ability to inhibit proliferation of cancerous cells. Further the ability of the 1H Benzo[c]isoxazole-3 one to induce apoptosis was also investigated in HT 29 cells. Demonstration of chromatin disintegration and apoptosis body formation by AO/EB staining and DNA fragmentation analysis showed their ability to induce apoptosis of cancer cell. Up-regulation of caspase-3 and caspase-8 were also observed in HT 29 cells treated with 1H Benzo[c]isoxazole-3 one illustrating the caspase mediated apoptosis of HT 29 cells, thus proving their anti cancer potential.

Having known the anti inflammatory and anti cancer potential of the lead molecule, 1H Benzo[c]isoxazole-3 one through laboratory experiments, the docking ability of the molecule in the active sites of COX and p28 kinase study was undertaken.

In this work, a molecular docking simulation study was undertaken to investigate the binding mechanism of 1H- Benzo[c]isoxazol-3-one and its structurally similar membrane impermeable inhibitors such as SC558, celecoxib, Iodoindomethacin and meloxicam to the COX-1 and COX-2 enzymes to enable us to find out whether our lead molecule could serve as an anti inflammatory/ anti cancer molecule.
We determined the favoured conformation of the lead with the docking sites for the inhibition. In this study, the hydrogen bond makes important contributions to the interactions between ligand and enzyme. Val 523 is of great concern in drug discovery of selective COX-2 inhibitor, which is believed to be one of amino acid which forms a hydrophobic pocket in the active site of COX-2. Our lead was also found to have interaction with Val 523 apart from His 90 and Phe 518 present in the active site. Besides hydrogen bonding, van der Waals interactions were also taking part in the stabilization of inhibitor binding with the residues such as Leu 352 and Ser 353.

Docking analysis of 1H- Benzo[c]isoxazol-3-one into active site of p38 kinase has been studied in the present work, to identify the inhibitor binding position and affinity to p38 MAPK using GOLD. Our compound, apart from forming two hydrogen bonds, non-bonded interactions also take part in the stabilization of inhibitor, binding to the residues of the active site such as Ala51, Leu 167, Asp 168, Val 38 and Phe 169. On the basis of H-bonding, van der Waals interactions and docking score, the molecule proved to be good inhibitor of p38 kinase. The docking results are in consistent with the laboratory experimental result, where cellular model studies indicated the inhibition of pro inflammatory enzyme, COX-2 and signaling molecule p38 MAPK under inflammatory conditions. Docking results seem to support the biological data. Our docking analysis and binding comparison of inhibitors of COX and p38 MAPK with our lead in silico, is in agreement with the structures of known inhibitors which are already solved, entailing that the overall position of the ligand is in agreement with standard inhibitors. Both the wet lab and simulation study of the lead molecule holds a promising future for the isolated lead to act as anti cancer and/or anti inflammatory molecule.