11. SUMMARY AND CONCLUSION

Marine environment has been recognized as a dynamic source of bioactive metabolites for marine natural products research. Among the faunal diversity, marine invertebrates are the major cluster of biologically and pharmacologically important groups to produce secondary metabolites for the development of novel drug discovery. Generally, marine invertebrates are soft bodied and/or lead a sedentary lifestyle making a chemical system of defence almost essential for survival. Even though, marine organisms not only responsible for producing the natural products, marine microorganisms are also considered as a potent source of unique bioactive metabolites.

In the present study, the lead bioactive secondary metabolites from bivalve mollusc *D. cuneatus* and associated endophytic bacteria was investigated using the combined approach of isolation and determination for their biological and pharmaceutical activities.

In the case of proximate composition, the amount of moisture, protein, lipid, carbohydrate and ash content was estimated as 77.04 %, 42.05 %, 9.75 %, 14.73 % and 2.59 % in *D. cuneatus*. The minerals content was estimated as Magnesium (0.92 %), Calcium (2.46 %), Chloride (1.75 %), Ammonia (nil) and Sulphate (10.20 %). The amino acid analysis of beach clam exhibited Serine, Glutamic acid, Glycine, Arginine, Alanine and Valine as major amino acids.

The whole body tissue of *D. cuneatus* was extracted with different polar and non-polar solvents including acidic extraction. The crude extracts represented varied amount of protein and carbohydrate content depending upon the various solvent system. The molecular weight has also revealed the number of proteins bands ranging between 99 kDa to 14 kDa. These extracts also show notable haemolytic activity.
Among all the extracts, Ax extract has pronounced antibacterial activity after the purification by Silica column with three steps elution profile increasing ACN proportion (10 %, 40 % and 80 %). The active 40 % SP fraction was further purified in HPLC. Of these four fractions (Fr1- Fr4), Fr1 fraction was found to be more active using MIC assay. The active Fr1 fraction was characterized using FTIR, GCMS and NMR. IR spectra clearly represented peaks with the presence the Benzimidazole formation, Benzimidazole ring analogues, aromatic and C–H stretching frequencies. In the case of GC-MS, three peaks were observed, of which the major peak obtained was detected as benzimidazole derivative at the retention time of 19.48 min and other were of minor peaks. The $^1$H NMR spectral analysis of the fraction Fr1 showed the signals in the region of 7.29–7.67 ppm with 4 protons integral value are assigned to aromatic protons benzimidazole ring. Further, signal which appeared at 8.07 ppm with one proton integral value is assigned to N-H proton. It has been evident that similar $^1$H NMR of benzimidazole at lower field $\delta$ 7.71 (C$_2$-H), 7.67 (C$_4$-H), 7.17 (C$_5$.H), 7.24 (C$_6$.H) and 7.32 ppm (C$_7$.H) respectively. The derivatives are known to play extremely crucial roles in the structure and function of biologically important molecules.

In the case of biological properties, the in vitro antioxidant activities of D. cuneatus against the DPPH radical scavenging activity, the Acm and Ax extracts exhibited maximum scavenging activity of $61.33 \pm 2.54\%$ (100 $\mu$g/ml). In reducing power assay, the higher reducing capacity was observed in Acm (0.39 OD) and Ax extracts (0.22 OD) at the concentration of 500 $\mu$g/ml. In the total antioxidant activity, the maximum activity was observed in the Me and Acm extracts ($2.21 \pm 0.08$ and $1.64 \pm 0.04$ mg/g) of ascorbic acid equivalent at the concentration of 500 $\mu$g/ml. In hydroxyl radical scavenging activity, the Acm and Me extracts showed prominent hydroxyl radical scavenging activity $84.53 \pm 3.42\%$ and $78.43 \pm 3.40\%$ respectively. While in hydrogen peroxide, the maximum scavenging activity

**Summary and Conclusion**
was observed in Me and Aqu extracts (73.74 ± 3.10 % and 68.64 ± 2.73 %). Among them, the Acn extract was found to be more active and it is suitable for further purification process.

The active Acn extract was purified by two steps preliminarily by activated Sephadex G-10 coulumn and HPLC. The partially purified fraction was named as *D. cuneatus* antioxidative peptides (DCAP) and the fractions were numbered consecutively and tested for DPPH radical scavenging activity. Among the 4 fractions, DCAP2 showed higher scavenging activity. The molecular mass of sub-fraction DCAP2 was determined using ESI-MS, the spectrum exhibited the major constituent as molecular ion, m/z 256.8 [M+H]+ whereas the fragmentation spectrum contained major ion at m/z 140 and 118 Da. The active fraction (DCAP2) possessed cytotoxic activity against human osteosarcoma cell line (MG 63) with IC₅₀ values of 112.5 μg/ml.

Furthermore, the endophytic microorganisms associated with the marine bivalve was also investigated since they are presented as a original subject to find novel natural products, which make them a potentially rich and innovative source for new leads. The *D. cuneatus* associated bacterial population was estimated as 3.2 x 10⁶ CFU/ml to 6 X 10⁹ CFU/ml. The three endophytic pigment producing strains selected like EPB CPA3, EPB CAG6 and EPB CAM9 based on the (i) the more number of counts (frequency of culture in plates) and (ii) their vibrant color. The strains were tested for antibiotic susceptibility test against five standard antibiotics (AMP, AZM, CIP, C and TE), among which the strain CPA3 exhibited increased resistance. The pigments from selected strains were extracted with Acetone: Methanol (7:3). The UV-visible absorption spectrum of pigments confirms the presence of carotenoids since carotenoids have maximum absorption at 300 – 600 nm. The three pigments also evidenced that potent antibacterial and antioxidant activity where the highest activity was observed (PPA6).
The presence of carotenoid groups, carotenoid ketone and carotenoid hydroxylates in the pigment extracts was confirmed by HPTLC. The type of carotenoid present in the pigments was identified by comparing with the Rf value of the standards Astaxanthin, \( \beta \)-Carotene and Lyopene. Where, the bands formed for the two pigments PPA3 and PAM9 did not match with any of the three standards, whereas PAG6 exhibited three bands of which, the first band similarly with the standard astaxanthin at the Rf value of 0.30. The partially purified astaxanthin of PAG6 that scraped from HPTLC was validated by comparing the retention time of standard astaxanthin in HPLC. The standard and the sample possessed same retention time of 3.9 min. The partially purified astaxanthin from PAG6 exhibited cytotoxicity effects on human breast cancer cell line (MCF-7) with IC\(_{50}\) value of 9.37 \( \mu \)g/ml. Further, the three pigment producing strains CPA3, CAG6 and CAM9 were identified through their morphological, biochemical characteristics and 16S rRNA sequencing and designated as \( Kocuria \) \( \text{flava} \) PA3, \( Pontibacter korlensis \) AG6 and \( Staphylococcus saprophyticus \) AM9 respectively. The accession number for each strain was assigned by the GeneBank (NCBI, USA) upon submission.

In addition, all the three selected EPB strains were also examined for its antibiotic producing capability. The strains were cultured using flask method was centrifuged and extracted using ethyl acetate. The three samples such as SAP3, SAG6 and SAM9 were used for further assays. The extracts exhibited significant antibacterial activity against the five tested pathogens of which, SAP3 showed pronounced activity. Thus, it was further selected for purification process using column chromatography (Silica gel) eluted with hexane, ethyl acetate and methanol.

Among the partially purified fractions, the fractions Fr3, Fr6 and Fr7 exhibited significant activity was selected and pooled for characterization process. The pooled active fraction was further subjected to HPTLC, FTIR and GCMS analysis. The GCMS spectrum of

---

**Summary and Conclusion**
the active pooled fraction exhibited 11 major compounds by comparing with NIST library. The active pooled fraction produced strain was found to be *K. flava* using molecular techniques.

From the foregoing account, clearly depicts the nature of marine invertebrates which have effectively developed the use of their innate immune system to defend against pathogenic attack by antimicrobial peptides or through the presence of endophytic bacteria. The autonomous aptitude of these marine organisms to produce substances that are biologically active may possibly accumulate, modify, abduct and use toxin of other organisms which emphasise the importance of marine organisms. The marine mollusc *D. cuneatus* is rich source of such potent molecules with novel and unique structural motifs not only by the individual it is also from associated endophytic microorganisms. The bioactive compounds and the pigment extracted were also found to exhibit potential cytotoxic effect against the cancer cell lines, marking the importance of such active molecules since the incidence of cancer increases constantly constituting of enormous challenge with medicines used in chemotherapy treatment provoking secondary toxicity or resistance. Therefore, the diverse relationships exist between organism and their guests microorganisms provoke that the bacterial compounds can eventually be used as a source of new leads for betterment of human welfare.