9. DISCUSSION

This general discussion chapter of the study will focus on the data presented in chapters 4 – 8 of the thesis as well as discussing the results critically comparing the findings from this study compared to others..

Ocean is a valuable reservoir of extremely potent, complex and diverse compounds with wide array of biological properties produced by marine organisms [208]. Until 1970, only 15,000 marine derived natural products were reported. However, due to the advancements in the isolation and characterisation techniques, the exploration of marine bioactives has widened [4]. The marine natural products (MNPs) possess unique and complex structures which are entirely different from the terrestrial forms. The MNPs exhibit a wide range of therapeutic properties including antibacterial, antifungal, antiviral, antitumor, anticancer and antihypertensive [5]. Hence MNPs are utilized as the lead compounds in the discovery and development of novel drugs [209]. The marine algae are a major group of marine plants representing a vast reservoir of potential compounds. The use of marine algae as food and medicine is well documented in the traditional medicine of China and Asia [8]. With the approval of Kainic acid, the first marine based drug the marine environment has drawn the attention of the biomedical industry towards it [10].

Lung carcinoma is the most prevalent and lethal form of cancer which caused 1.7 million deaths in 2015 [24]. The standard treatments include chemotherapy, radiation and surgery which were not found to improve the survival of patients [25]. The survival rate of lung cancer is estimated as 18% after diagnosis [24].

Further, natural products does not induce adverse toxicity in animals and hence are more preferred than their synthetic counterparts. Based on these, the current study was intended to isolate, characterize and analyze the antioxidant, anticancer, antimetastatic and anti-inflammatory potentials of marine red algae *Gelidiella acerosa* under *in silico*, *in vitro* and *in vivo* conditions.

In the current study, *Gelidiella acerosa* was extracted sequentially which resulted in six different algal extracts. These extracts were qualitatively and quantitatively

analyzed for the phytochemicals present in them. The outcomes showed that the algae was a source of various phytocompounds which included the tannins, coumarins , flavonoids, phytosterol, glycoside, oils, alkaloids fats, protein, carbohydrate and terpenoids. The results of the current study coincided with the previous reports in *G.acerosa* [13],[18],[63] thus confirming the algae as a rich source of bioactives.

Further, the major antioxidant and anticancer compounds especially the polyphenols and flavonoids were abundant in the ethyl acetate algal extract (GAE) when compared to the other extracts. Previous studies have reported the abundance of these phytocompounds in red algae [158] which correlated with the current findings.

The separation of the algal extracts by HPLC showed the presence of various phytocompounds in each extract and eight compounds were found to be present in the ethyl acetate extract. These results correlated with the preliminary phytochemical screening which showed that the phytochemicals were abundant in ethyl acetate extract.

The FTIR analysis revealed the various functional groups in the algal extracts that may contribute to the therapeutic efficacy of the algae. The FT-IR analysis of hexane, dichloromethane, ethanol, methanol and water extracts revealed the presence of primary and secondary amines, aldehydes, ketones, alkanes and terpenes, whereas the presence of terpenes, alkenes, coumarins, glycosides and phenolics in ethyl acetate extract coincided with the findings of the qualitative screening. These outcomes of the FT-IR analysis are correlating with similar studies in marine algae [147],[148].

Since, the outcomes of the phytochemical screening revealed the abundance of phytochemicals in the ethyl acetate extract (GAE), the extract was selected for further studies.

Following this, GAE was separated by column chromatography which yielded four compounds. These compounds were analyzed by GC MS and two compounds GAC 1 was identified as Palmitamide of molecular mass 256. 26 2 and GAC2 was identified as a novel compound (2, 3, 4, 7, 8, 9, 10, 11, 12, 13, 14,15,16,17-

tetradecahydro-10-methyl–17-(7 methyloctan-2-yi)-1H-cyclopenta [α] phenanthren -3-ol) of molecular mass 386.35. The other two compounds were identified as mixtures and labelled as GAC 3 and GAC 4.

The isolation and characterization of the algal compounds (GAC 1and GAC 2) was followed by determining their interaction with the proteins in the apoptotic, cell survival and inflammatory pathways under *in silico* conditions.

In the apoptosis pathway the GACs were interacted with caspase 3, caspase 8, Bax, Bcl 2 and Bcl-Xl. GAC1 and GAC 2 interacted with the residues in the catalytic site of caspase 3 thus causing its activation and there by the intrinsic pathway of apoptosis. Following this the interaction of GACs with caspase 8 was analyzed. The results showed that both GAC 1 and GAC 2 interacted with the residues that interrupt the activation of caspase 8. These results coincided with the previous studies where several inhibitors are found to inhibit caspase 8 [169]. Followed by these results, GAC 1 and GAC 2 were interacted with Bax which showed that the algal compounds interacted with the residues in the hydrophobic groove of Bax thereby activating it. These results correlated with the interaction of small molecule 106 which promoted binding of Bax to Mitochondrial Outer Membrane [170]. Similarly, the interaction results of GACs with Bcl2 showed that the compounds interacted with residues (Lys 17 and Glu 42) in the active domain of Bcl 2. The results of the current observation correlated with the interaction of anticancer drugs cisplatin, gefitinib and vinorelbin. These drugs interacted with similar residues in the active site of Bcl2 [155].Further, GACs Hence, the in silico findings showed that the algal compounds inhibited Bcl2 in the same way as the anticancer drugs. As the GACs did not interact with the active site of Bcl- XL, showing that the algal compounds inhibit the activity of Bcl-Xl. These outcomes showed that the GACs interacted and activated caspase3 and Bax and inhibited Bcl2 and Bcl-Xl, thus promoting the activation of apoptosis. These outcomes showed that the GACs interact with the apoptotic proteins in a similar manner as the anticancer drugs.

As GACs interacted with the apoptotic proteins, the next step was to determine the interaction of GACs with the cell survival proteins (PI3K, Akt, GSK3β and PTEN). GAC 2 interacted with the PIK accessory domain of PI3K which is involved

in substrate binding. Hence, the binding of GAC 2 to PI3K interferes with the interaction of PI3K with its substrates which may disrupt its activity. The findings of the current *in silico* analysis revealed that these algal compounds can inhibit the activation of PI3K protein.

Subsequently, the interaction of GACs with Akt was determined. The outcomes showed that, GAC1 interacted with the inhibitor binding site of Akt (Figure 5.20). The binding was specific and the result showed that GAC 1 can act as a potent Akt inhibitor. The findings correlated with the interaction of Akt 1 inhibitors Pyrrolopyrimidine and ipatasertib [161] on the other hand, the compound GAC 2 did not interact with Akt1 as shown by molecular docking studies (Figure 5.21).

Similarly, the interaction of GACs with GSK3β was analyzed. The results showed that compound GAC 1 interacted with the binding pocket of GSK3β. This interaction prevents the binding of phosphorylated substrates to GSK3β, thereby maintains the protein in its active state. The findings there by revealed that the GACs prevent the phosphorylation of GSK3β and hence maintains the protein in the active conformation. These results coincided with the interaction results of GACs with Bax and Bcl 2 where, GACs activate Bax and inhibit Bcl2. The outcomes suggest that GACs induce apoptosis by activating GSK3β which in turn alters the ratio of Bax/Bcl2.

The next protein that was interacted with the GACs included the PTEN. The outcomes showed that GAC 1 interacted with the active site residues of PTEN, which are crucial for its catalytic activity. Thus GAC 1 may promote the activity of PTEN. The results coincided with the interaction of thymoquinone with PTEN [171]. Similarly, GAC 2 interacted with the C2 domain of PTEN that accounts for the tumor suppressor activity of PTEN. These outcomes revealed that the GACs inhibited the activation of the cell survival proteins especially the PI3K and Akt thereby they can regulate the pro survival pathway in cancer.

In the inflammatory pathway, the interaction of GACs with the inflammatory complex lKBα-NFKB-p65-p50 was analyzed. The results showed that GAC1 interacted with the Rel homology domain and GAC 2 interacted with the Rel domain. Since the Rel domain is essential for DNA binding and nuclear

translocation of NFKB, binding of GACs to this domain may interrupt the activation of NFKB.

Followed by the in silico analysis, the therapeutic potentials of *G.acerosa* was investigated under *in vitro* conditions.

Marine natural products are rich sources of antioxidants. Hence the current study investigated the antioxidant activity of GAE and GACs. Among the six algal extracts analyzed, the ethyl acetate extract (GAE) exhibited strong antioxidant activity than the other extracts. Since, polyphenols and flavonoids were shown to be abundant in the ethyl acetate extract they may contribute to the high antioxidant activity of GAE. The results of the current analysis coincided with the previous studies in *G.acerosa* [14], [63] thus confirming the algal extract as a good antioxidant.

Further, the study also investigated the antioxidant efficacy of GACs. The decreased activity of SOD and POX in cancer cells accounts for the oxidative stress. In the present investigation, the treatment of cancer cells with GAE and GACs increased the SOD and POX activities *in vitro*. These results showed that the algal extract and the compounds isolated from it possess good free radical scavenging activity. These results coincided with previous studies [210],[211].These results confirmed that *G.acerosa* is a good source of antioxidants and is capable of enhancing the antioxidant defense mechanism. As natural antioxidants are preferred than synthetic ones due to toxic effects on humans *G.acerosa* can be considered as a source of natural antioxidant.

The efficacy of the algal extract (GAE) to induce cytotoxicity in L132 cells, A549 and HeLa cells were analyzed. The results showed that GAE did not affect the viability of L132 cells at the concentrations used but induced cell death in A549 and HeLa cells. Hence the results showed that GAE does not affect normal cells but has a negative impact on the viability of cancer cells. These findings correlated with previous cytotoxicity assessments of *G.acerosa* [65]. The outcomes suggested that GAE is more effective in inhibiting cancer cell proliferation than the healthy cells. Similarly, the pure (GAC 1, 2, 3 and 4) were analyzed for their cytotoxicity in cancer cell lines (A549 and Hela). The results showed that GACs were more cytotoxic in these cell lines than the crude algal extract (GAE). These

results revealed that the cytotoxicity exhibited by the GACs was concentration dependent and their efficacy was different in different cell lines. These findings suggested that GAE and GACs are cytotoxic against lung and cervical cancer cell lines.

Following the cytotoxicity analysis, the induction of apoptosis were observed by the microscopic studies by staining with DAPI, PI, and Acridine orange. The results showed the fragmented nuclei, translocation of phosphatidyl serine and crescent shaped nuclei in GAE and GACs treated A549 cells.

The microscopic studies were followed by the analysis of the pathways involved in apoptosis. The findings of Real- Time PCR and Western blot revealed the increased expression of caspase 3 and Bax whereas, that of caspase 8, Bcl 2 and Bcl-Xl were suppressed. These outcomes confirmed that both GAE and GACs induced apoptosis by affecting the Bax/Bcl-2 ratio and that apoptosis was mediated through the intrinsic pathway as shown by cleaved caspase 3. These findings correlated with other studies where medicinal plants and algal compounds induced apoptosis through the activation of intrinsic pathway [183],[212].

In the next step the study analyzed the expression of the cell survival pathway in A549 cells. The results showed an activation of GSK3β following GAE and GACs treatment when compared to the untreated cells. Since GSK3β is essential for the expression of Bcl-2 family proteins [213],[214], it plays a crucial role in apoptosis and survival. The results revealed that cells treated with GAE/GACs showed the activation of GSK3β followed by increase in Bax expression, caspase 3 activation and promotion of apoptosis. As the activation of GSK3 is strictly regulated by its immediate upstream targets PI3K and Akt [186],[215],[216], the study further investigated the expression of these pro-survival components.

The findings showed that the treatment with GAE and GACs interfered with the activation of PI3K and Akt. Moreover, the phosphorylation of PI3K and Akt differed among the GACs. The treatment with GAC 1, GAC 3 and GAC 4 decreased the phosphorylation PI3K and Akt which is accompanied by the activation of GSK3β whereas GAC 2 did not alter the phosphorylation of PI3K but inhibited the phosphorylation and activation of Akt. This variation among the GACs revealed

their specific mode of action on the PI3K/Akt/GSK3β pathway. The results of the current study are similar to previous observation, where suppression of PI3K/Akt enhanced apoptosis [187],[196].

Metastasis accounts for the majority of cancer-related deaths and poor prognosis [190]. As GAE and GACs induced apoptosis and inhibited the PI3K pathway, their effect on the process of metastasis was evaluated. The findings of the study revealed that, GAE prevented the invasion of A549 cells into the wounded region and also inhibited the colony formation of A549 cells. Further, the expression of MMP2 and MMP9 were also investigated. The results showed that the expression levels of MMP2 was decreased. In NSCLC population, the expression of MMP2 is an indicator of metastasis [217]. Since treatment with GAE and GACs decreased the expression of MMP2, which are essential for cell invasion and colonization of cancer cells, the antimetastatic potential of *G.acerosa* was revealed. The overall outcomes of the study confirmed that both GAE and GACs inhibited cell survival, cell migration and colonization of lung cancer cells which are the major obstacles in the treatment of cancer.

As inflammation is a key component to tumor progression [199] and chronic inflammation is reported in most of the human cancers [201] the study then investigated the effect of GAE and GACs on inflammation. The study investigated the expression levels of proteins including NFKB, TNF α, IL-1β, IL 10 and PTEN The data showed that GAE and GACs treatment of A549 cells decreased the expression levels of NFKB, IL-1β and TNFα whereas, the expression levels of IL 10 and PTEN was increased. These results showed that GAE and GACs inhibited the activation of NFKB which inhibited the production of proinflammatory cytokines and alternately increased the expression of IL 10. The current findings are correlated with the outcomes of a previous study where the carotenoid fraction from *D.salina* suppressed proinflammatory cytokine levels through the inhibition of NFKB [218]. Similarly, the ethanolic extract of *S.horneri* elicited anti-inflammatory activity by down regulating the expression of proinflammatory cytokines[416] . In case of prostate carcinoma, the suppression of proinflammatory cytokines was reported to inhibit the activation of NFKB [206].

The outcomes of the *in vitro* study revealed that treatment with GAE and GACs decreased the expression levels of proteins (Bcl 2, Bcl-Xl, PI3K, Akt and NFKB) thus promoting apoptosis (increased Bax, cleaved caspase 3, GSK3β), inhibiting metastasis (decreased MMP 2) and inflammation (increased IL-10) in cancer. Further, the findings of the in vitro analysis coincided with the current *in silico* findings. Based on these findings, it was concluded that both GAE and its compounds the GACs have anticancer activity.

In order for newly synthesized compounds or natural products to be subjected to human exposure, the toxicity or adverse effects that the compound may induce has to be evaluated. Hence, the study investigated the toxicity of GAE under in vitro and in vivo conditions. The results revealed that, GAE did not induced any adverse effects under the conditions tested and is safe for animal use. The outcomes correlated with a previous report where *G.acerosa* was reported to protect PBMCS from TDCC induced toxicity [14], [18]. Following this, the anticancer activity of GAE and GACs were determined in tumor models of Zebrafish namely the lung (A549 tumor model), colon (HCT tumor model) and liver (HepG2 tumor model). The results of the study revealed that both GAE and GACs decreased the tumor cell population and extent of angiogenesis in A549 tumors thus affecting the proliferation and survival of tumor cells. These results correlated with the protein expression studies on the apoptotic, cell survival and inflammatory pathways. The analysis of protein levels in the apoptotic pathway revealed that the proapoptotic protein Bax was increased accompanied by decreased levels of Bcl 2 in the GAE and GACs treated A549 tumors when compared to the untreated tumors. Similarly, the expression levels of PI3K and Akt were decreased and that of GSK3β was increased in GAE and GACs treated A549 tumors than the untreated tumors. Also, the levels of the inflammatory regulator NFKB, IL 1β and TNFα were decreased whereas, the levels of IL 10 was increased in the GAE and GACs treated tumors when compared to the untreated tumors. These results clearly showed that GAE and GACs not only induced apoptosis through the regulation of prosurvival pathway in cancer but also regulated the inflammatory response in cancer. Further these findings coincided with the current *in vitro* data

thus confirming the anticancer and anti-inflammatory activities of GAE and its compounds GACs. On the other hand, histological analysis of HCT (colon) tumor induced and HepG2 (liver) tumor induced Zebrafishes showed no alteration in tumor anatomy, tumor cell population after treatment with GAE and GACs. The findings of the histological study revealed that both GAE and GACs are effective only on lung cancer model of Zebrafish.

The outcomes of the study, showed that GAE and GACs can function as good antioxidants with anticancer, antimetastatic and anti-inflammatory properties which can be utilized in the treatment of PI3K related diseases without potential adverse effects.