7. EVALUATION OF THE ANTICANCER AND ANTI-INFLAMMATORY PROPERTIES OF THE RED ALGAL EXTRACT (GAE) AND THE ISOLATED COMPOUNDS (GAC 1-4) UNDER IN VITRO CONDITION.

7.1 Introduction

Molecular research employs large number of techniques for evaluating the efficacy of the compound to be developed as a drug. One such technique widely employed in the Pharma industry is the animal cell culture. The technique is highly preferred as it is cost effective, requires less quantity of compound and takes short duration for analysis. The data presented in chapter 3 revealed that the algal extract from *G.acerosa* contains potential phytochemicals which have antioxidant, anticancer and other properties. The study was aimed to find if the phytochemicals and compounds isolated from the algal extract possess anticancer properties under *in vitro* conditions.

7.2 Methods

As described in chapter 3, the effect of GAE and GACs on cell viability and mechanism of cell death was determined under *in vitro* conditions in A549 (Adenocarcinoma), MCF 7 (Breast cancer), HeLa (Cervical) cancer cell lines and L132 (Lung epithelial) cell line.

7.3 Results

7.3.1 GAE and GACs are cytotoxic under *in vitro* conditions

The current chapter of the thesis analyzed the efficacy of the algal extract (GAE) to induce cytotoxicity in L132 cells and A549 cells. The extract was tested in varying concentrations (100, 500, 1000, 1500 and 2000 µg/ml) and the IC50 was determined by MTT assay. Figure 7.1 shows the GAE treatment in A549 cells at 24 hours duration.
Figure 7.1 Photographs showing the effect of GAE on viability of A549 cells at 24 hours.
(a) The control cells retained their normal morphology whereas, the GAE treated cells (b) showed a loss of morphology and exhibited a rounding of cells.

— Scale bar 20x. n=3.

Following this the effect of GAE on A549 and L132 cell lines was analyzed in a dose dependent manner.

Figure 7.2 Dose dependent (0-2000 µg/ml) activity of GAE on the viability of L132 (lung epithelial cell line), A549 (Adenocarcinoma cell line) and HeLa (Cervical carcinoma cell line) cells.
Similarly the effect of Doxorubicin on viability of A549 cells was also determined.

Figure 7.3 Dose dependent (0-100 µM/ml) activity of Doxorubicin on the viability of A549 (Adenocarcinoma cell line).

The data showed the influence of various concentrations of GAE on viability of L132 cells, A549 and HeLa cells compared to untreated cells. The results showed that GAE can kill both normal and cancer cells but with more lethal effect employing high dosage. The GAE was more effective in killing A549 and HeLa cells compared to L132 normal cells. GAE concentration of 1500µg/ml killed 50% and 51% of the A549 and HeLa cell population (IC50=1500 µg/ml, IC50=51%) whereas, the same concentration induced only 20% cell death in L132 cells. (GAE concentration of 3000µg/ml killed 100% A549 cells).

Following this the effect of GACs (1-4) on A549 cells was analyzed at different concentrations. The IC50 values in A549 cell line were identified as 12.5, 8, 15 and
25 µg/ml for GAC 1, GAC 2, GAC 3 and GAC 4 respectively (Figures 7.4 – 7.7). Similarly, the IC50 values for HeLa cell line were determined as 12.5, 10, 10 and 15 µg/ml for GAC 1, GAC 2, GAC 3 and GAC 4 respectively (Figures 7.8 – 7.11).

Figure 7.4 Dose dependent (0-50 µg/ml) activity of GAC 1 on the viability of A549 (Adenocarcinoma cell line).

Figure 7.5 Dose dependent (0-50 µg/ml) activity of GAC 2 on the viability of A549 (Adenocarcinoma cell line).

Figure 7.6 Dose dependent (0-100 µg/ml) activity of GAC 3 on the viability of A549 (Adenocarcinoma cell line).

Figure 7.7 Dose dependent (0-100 µg/ml) activity of GAC 4 on the viability of A549 (Adenocarcinoma cell line).
Note: In all these experiments, the data represented are mean ±S.D. n=3, * p<0.05 for treated cells compared to untreated cells.

In a similar manner, the effect of GAE on the viability of HeLa cells was also determined. In a similar manner, the anticancer activity of GAE was determined in HeLa cells.

Figure 7.8 Dose dependent (0-50 µg/ml) activity of GAC 1 on the viability of HeLa (Cervical carcinoma cell line). Data represented are mean ±S.D. n=3, * p<0.05.

Figure 7.9 Dose dependent (0-100 µg/ml) activity of GAC 2 on the viability of HeLa (cervical carcinoma cell line). Data represented are mean ±S.D. n=3, * p<0.05.
Since the crude extract (GAE) and its four compounds (GACs 1-4) have demonstrated strong anticancer activity, therefore it was further investigated to identify the mechanism of action, targets, receptors and pathways involved.

### 7.3.2 GAE and GACs induced apoptosis

The GAE treated A549 cells were analyzed for the apoptotic hallmarks by staining with DAPI, Propidium Iodide and Annexin v to observe the cell biological changes under the fluorescent and confocal microscopes.
Figure 7.12. Photograph showing the effect of GAE at 24 hours in A549 cells. Fluorescent imaging with Annexin, Propidium iodide and DAPI revealed the translocation of Phosphatidyl serine to the cell membrane and nuclear fragmentation in GAE treated cells. DIC images revealed loss of structural integrity in GAE treated cells whereas, control cells exhibited intact nuclei and unaltered cell morphology. C - Control, T – treated. Scale bar 20X. n=3

In a similar way, the apoptotic hallmarks were analyzed by DAPI and acridine orange staining in GAC treated A549 cells which revealed the fragmentation of nuclei. These results confirmed the induction of apoptosis by GACs.
Figure 7.13 Photograph showing the effect of GACs at 24 hours in A549 cells. Fluorescent imaging with DAPI and Acridine orange revealed the nuclear fragmentation in GACs treated cells. DIC images revealed loss of structural morphology in GAC treated cells whereas, control cells exhibited intact nuclei and unaltered cell morphology. C - Control, T – treated. Scale bar 20X. n=3.
7.3.3 GAE and GACs activated the intrinsic pathway of apoptosis

Since the apoptotic hall marks were observed, the study was further extended to analyze the pathway through which apoptosis was induced by GAE and GACs. Hence, the study investigated whether the induction of apoptosis was caspase-mediated, and investigated the expression of execution caspase-3, initiation caspase-8, proapoptotic protein Bax and antiapoptotic proteins Bcl 2 and Bcl-Xl by Real-Time PCR and western blot analysis in A549 cells treated with IC\textsubscript{50} values of GAE and GACs for 24 hours.

Figure 7.14 Analysis of expression of gene encoding caspase 3 in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are \(2^{-\Delta\Delta CT}\). n=3,*\(p<0.05\).

Figure 7.15 Analysis of expression of gene encoding caspase 8 in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are \(2^{-\Delta\Delta CT}\). n=3,*\(p<0.05\)
Figure 7.16 Analysis of expression of gene encoding Bax in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are $2^{-\Delta\Delta Ct}$. n=3,*p<0.05

Figure 7.17 Analysis of expression of gene encoding Bcl 2 in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are $2^{-\Delta\Delta Ct}$. n=3,*p<0.05

Figure 7.18 Analysis of expression of gene encoding Bcl-XI in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are $2^{-\Delta\Delta Ct}$. n=3,*p<0.05
Figure 7.19 Effect of GAE and GACs treatments on the expression of proteins involved in apoptosis.
A549 cells were treated with GAE and GAC (1 - 4) for 24 hours and the expression levels of proteins involved in the apoptotic pathway were examined by western blotting. The image is representative of 3 such different experiments.
Figure 7.20 Histogram showing the quantification of Bax in GAE and GACs treated A549 cells. The intensity was analyzed by ImageJ software. Values represented are of mean ±SD. C - Control.

Figure 7.21 Histogram showing the quantification of Bcl 2 in GAE and GACs treated A549 cells. The intensity was analyzed by ImageJ software. Values represented are of mean ±SD. C - Control.

Figure 7.22 Histogram showing the quantification of Bcl - XI in GAE and GACs treated A549 cells. The intensity was analyzed by ImageJ software. Values represented are of mean ±SD. C - Control.

Figure 7.23 Histogram showing the quantification of caspase 3 in GAE and GACs treated A549 cells. The intensity was analyzed by ImageJ software. Values represented are of mean ±SD. C - Control.
In order to confirm the involvement of caspase-3 and 8 in induction of apoptosis, the expression levels of caspase-3 and 8 were analyzed by Real-Time PCR and western blot analysis in GAE and GACs treated cells. The Real-Time PCR data revealed that, the expression levels of caspase-3 mRNA was increased and that of caspase-8 was decreased in GAE and GACs treated cells (Figure 7.14). Further, the results of western blot analysis confirmed the activation of caspase-3 as evidenced by the cleaved forms of caspase-3, however that of caspase-8 was unaltered (Figure 7.19). The data at the transcript and protein level prompted the activation of caspase-3 as compared to caspase-8 in the initiation of apoptosis by GAE. Following this, the role of apoptotic regulators such as, Bax, Bcl2 and Bcl-XL were further evaluated. As demonstrated by the Real-Time PCR results, the expression levels of Bcl2 and Bcl-xL mRNAs were suppressed whereas, that of Bax was increased after exposure to GAE and GACs (Figures 7.15 – 7.18). Similarly, the expression levels of the proteins Bax, Bcl2 and Bcl-XL were determined by western blot (Figures 7.19 – 7.24). The outcomes showed the

Figure 7.24 Histogram showing the quantification of caspase-8 in GAE and GACs treated A549 cells.

The intensity was analyzed by ImageJ software. Values represented are of mean ±SD. n=3, C - Control
increased expression levels of Bax and decreased expression levels of Bcl2 and Bcl-XI which correlated with the transcription results.

As the molecular analysis of apoptosis revealed the involvement of caspase 3 and alteration of apoptotic regulators especially the Bax/Bcl2 ratio, it was logical to investigate the involvement of the GSK3β and its upstream regulators (PI3K and Akt) in GAE and GACs treated cells. Hence, the expression of the genes and proteins in the cell survival pathway PI3K/Akt/GSK3β by Real-Time PCR and Western blot were investigated.

Figure 7.25 Analysis of expression of gene encoding GSK3β in GAE and GACs treated A549 cells by Real-Time PCR.

The values represented are $2^{-\Delta\DeltaCT}$. n=3, *p<0.05 for treated cells compared to untreated cells.
In order to investigate the molecular mechanism that altered the Bax/Bcl-2 ratio, the expression level of GSK3β, was determined in A549 cells treated with GAE and GACs. The Real-Time PCR results (Figure 7.25) showed that the expression of GSK3β was upregulated (2-2.8 fold) after GAE and GACs treatment.

Figure 7.26 Analysis of expression of gene encoding PI3K in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are $2^{\Delta\Delta CT}$. n=3, *p<0.05 for treated cells compared to untreated cells.

Figure 7.27 Analysis of expression of gene encoding Bcl-Xl in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are $2^{\Delta\Delta CT}$. n=3, *p<0.05 for treated cells compared to untreated cells.

In order to investigate the molecular mechanism that altered the Bax/Bcl-2 ratio, the expression level of GSK3β, was determined in A549 cells treated with GAE and GACs. The Real-Time PCR results (Figure 7.25) showed that the expression of GSK3β was upregulated (2 – 2.8 fold) after GAE and GACs treatment. The expression levels of mRNA and protein from cells exposed to GAE and GACs were analyzed. The results revealed that the expression of unphosphorylated
GSK3β was higher than the phosphorylated form. The results revealed that the treatments prevented the phosphorylation and inactivation of GSK3β (Figure 6.28). The quantification of proteins are shown in Figure 7.29.

Figure 7.28 Effect of GAE and GAC (1-4) on the expression of proteins involved in cell survival cascade.

A549 cells were treated with GAE and GACs for 24 hours and the expression levels of proteins involved in cell survival cascade was determined by western blotting. The image is representative of 3 such different experiments.
Figure 7.29 Histogram shows the quantification of GSK3β in GAE and GACs treated A549 cells. Relative expression of proteins were normalized with β actin. The intensity was analyzed by ImageJ software. Values represented are of mean ±SD.

Figure 7.30 Histogram shows the quantification of phospho PI3K in GAE and GACs treated A549. Relative expression of proteins were normalized with β actin. The intensity was analyzed by ImageJ software. Values represented are of mean ±SD.

Figure 7.31 Histogram shows the quantification of phospho Akt in GAE and GACs treated A549 cells. Relative expression of proteins were normalized with β actin. The intensity was analyzed by ImageJ software. Values represented are of mean ±SD. n=3.
The expression levels of PI3K and Akt mRNAs were observed to be down regulated in GAE and GACs treated cells by Real-Time PCR (Figure 6.26 and 6.27). The protein expression results indicated decreased phosphorylation of both PI3K and Akt (Figure 6.28), the immediate the upstream targets of GSK3β, which in turn correlated with the increased expression of GSK3β. These data indicated that GAE and GACs induced apoptosis, activated GSK3β through the inhibition of PI3K/Akt cascade in A549 cells. The quantification of the protein levels are shown in Figure 6.30 and 6.31.

Since, the proteins in the cell survival pathway were down regulated followed by the activation of GSK3β, the study confirmed the induction of apoptosis through the inhibition of PI3K/Akt/GSK3βpathway in A549 cells.

7.3.4 Effect of GAE on cell migration

As the algal extract showed efficacy in regulating PI3K/Akt activation in the current investigation, the invasive potency of GAE was analyzed by the Scratch assay. For determining the effect of GAE on cell migration, the A549 cells were treated with the noncytotoxic dose of GAE and investigated for their potential to migrate into the wounded area.
Figure 7.32 The effect of GAE on inhibition of cell invasion.

A549 cells were treated with GAE (1mg/ml) for a duration of 24 hours. The results showed a decreased migration of cells in the GAE treated wound when compared to the control. n=3. The image is representative of 3 such different experiments.

The findings of the assay revealed that the GAE treated cells were less efficient in migrating to the wound when compared to the untreated cells. Thus, the results showed that GAE treatment (1 mg/ml) decreased the migration of cells. On the other hand, the concentrations less than 1mg/ml were not effective (Figure 7.32). The results of the invasion assay revealed that GAE can inhibit the invasion of A549 cells.
7.3.5 GAE treatment suppressed colonization

As GAE inhibited invasion and proliferation of A549 cells, the efficacy of GAE on colony formation was investigated by the clonogenic assay.

![Image](image.png)

Figure 7.33 The clonogenic assay result shows the inhibition of proliferation of cells after GAE treatment.

(a) Colony formed in control cells after 7 days, (b) magnified colony of control cells, (c) Treated cells after 7 days, (d) treated cells magnified. C-control, T-treated. n=3.

The image is representative of 3 such different experiments.

The results indicated that treatment with GAE (1mg/ml) effectively prevented colony formation of A549 cells, whereas the concentrations below 1mg/ml were not effective (Figure 7.33).

7.3.6 GAE and GACs suppressed MMP2 level

Since GAE inhibited the invasion and colonization of A549 cells, the next step of was to determine the expression of Matrix Metalloproteinases (MMPs) which are involved in the remodeling of extracellular matrix thus contributing to metastasis in cancer. The expression of MMPs especially MMP2 and MMP9 which is related
tumour proliferation, angiogenesis, disease progression and metastasis was analyzed by western blot.

Figure 7.34 Effect of GAE and GACs (1-4) on the expression of proteins involved in metastasis. A549 cells were treated with GAE and GACs for 24 hours and the expression levels of proteins involved in metastasis (MMP2 and MMP9) was determined by western blotting. The image is representative of 3 such different experiments.
The results indicated that treatment with GAE and GACs effectively decreased expression levels of MMP2 (Figure 6.34 – 6.36) rather than MMP9. Thus, the findings confirmed that both GAE and its compounds (GACs) regulated metastasis by inhibiting MMP2 rather than MMP9.

The constant activation of cell survival pathway (PI3K/Akt) is reported to promote invasion in cancer [175], [176]. As GAE showed efficacy in regulating PI3K/Akt activation in the current investigation, the invasive potency of GAE was analyzed by the Scratch assay and colony formation assay. The results revealed that GAE inhibited both cell invasion and colonization. Since, Matrix Metalloproteinases (MMPs) are involved in the remodeling of extracellular matrix and contribute to metastasis in cancer [176]. The expression of MMPs is related to tumour
proliferation, angiogenesis, disease progression and metastasis [177]. Increased expression of MMP2 is correlated with cancer proliferation, tumour size [178] and malignant progression. Similarly, the expression of MMP9 is correlated with the invasion of glioma cells [179]. As GAE inhibited cell migration and colony formation, the expression levels of matrix metalloproteinases which enable the degradation of extracellular matrix was determined. The results showed decreased expression of MMP2 in cells treated with GAE than the control cells (Figure 6.34) whereas, the expression of MMP9 was not significantly affected. Thus, based on these data, the current study confirmed that the anti-invasive potency of GAE and GACs on metastasis and tumour formation were mediated through the inhibition of MMP2 expression.

Similarly, the efficacy of GACs on the expression levels of MMP2 and MMP9 was evaluated. The results showed that GACs inhibited MMPs as GAE. The GACs 1, 2 and 4 inhibited the expression of MMP2 alone and did not affect the expression of MMP9 whereas, the GAC 3 inhibited the expression levels of both MMP2 and MMP9. These results (Figure 6.34) revealed that the mechanism and target of GAE and GACs is MMP2 to regulate metastasis of A549 cells.

7.3.7 GAE and GACs modulated NFkB expression

NFkB mediates the expression of numerous genes with varied functions. As GAE and GACs influenced the expression of Bax, Bcl2 and MMPs which are regulated by NFkB, it was logical to determine the effect of GAE and GACs on NFkB activation. A549 cells were treated with inhibitory concentrations of GAE and GACs and analyzed for the expression of NFkB, IL1β, TNFα and IL 10.
Figure 7.37 Analysis of expression of gene encoding NFKB in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are $2^{\Delta\Delta\text{CT}}$. n=3,*p<0.05

Figure 7.38 Analysis of expression of gene encoding IL 1β in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are $2^{\Delta\Delta\text{CT}}$. n=3,*p<0.05

Figure 7.39 Analysis of expression of gene encoding TNFα in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are $2^{\Delta\Delta\text{CT}}$. n=3,*p<0.05

Figure 7.40 Analysis of expression of gene encoding IL 10 in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are $2^{\Delta\Delta\text{CT}}$. n=3,*p<0.05
Figure 7.41 Analysis of expression of gene encoding PTEN in GAE and GACs treated A549 cells by Real-Time PCR. The values represented are $2^{-\Delta\Delta CT}$. n=3, *p<0.05

Figure 7.42 Effect of GAE and GACs treatment on the expression of proteins involved in inflammation by western blot analysis. A549 cells were treated with GAE and GAC (1 - 4) for 24 hours and the expression levels of proteins involved in the NFKB pathway were determined by western blotting. C - Control. n=3.
Figure 7.43 Histogram shows the quantification of NFKB. Relative expression of protein was normalized to β actin. The intensity was analyzed by ImageJ. Values represented are mean ±SD.

Figure 7.44 Histogram shows the quantification of IL 1β. Relative expression of protein was normalized to β actin. The intensity was analyzed by ImageJ. Values represented are mean ±SD.

Figure 7.45 Histogram shows the quantification of TNF α. Relative expression of protein was normalized to β actin. The intensity was analyzed by ImageJ. Values represented are mean ±SD.

Figure 7.46 Histogram shows the quantification of IL 10. Relative expression of protein was normalized to β actin. The intensity was analyzed by ImageJ. Values represented are mean ±SD.
NFKB is an antiapoptotic factor [180] and the findings of our study also showed decreased expression level of antiapoptotic proteins (Bcl2 and Bcl-XL) together with a decreased expression of MMPs. These results prompted the analysis of NFKB and PTEN activity in G.acerosa treated cells. The expression levels of mRNA and protein from A549 cells treated with GAE and GACs and untreated control cells was analyzed for the expression levels of the active form of NFKB (p-NFKB-p65-S536) and PTEN. The results of Real-Time PCR (Figure 7.37) and western blotting analysis showed a decreased level of p-NFKB-p65-S536 in treated cells when compared to the control, (Figure 7.42) thus suggesting the inhibition of NFKB activation by G.acerosa. Further, the data showed an increased expression of PTEN at both the transcript (Figure 7.41) and protein levels in treated cells (Figure 7.42). The inhibition of NFKB directly correlates with the decreased expression of antiapoptotic proteins (Bcl2 and Bcl-XL) and MMP2.

In the current investigation as the inhibition of NFKB was observed, the expression levels of the proinflammatory cytokines especially TNFα and IL-1β were analyzed. The results showed that the expression of TNF α and IL-1β mRNAs in treated cells was decreased (Figure 7.38 and 7.39). Similarly, the protein samples from GAE, GAC 1 and GAC 4 treated cells exhibited an inhibition of both TNF α and IL-1β,
whereas protein from GAC 2 and GAC 3 treated cells showed an down regulation of TNFα but the expression level of IL-1β remain unaltered (Figure 7.42). These results suggested the differential specificity of GAE and GACs in regulating the proinflammatory cytokines and hence inflammation.

As NFKB-p65 is a central regulator of inflammation, the expression of the anti-inflammatory marker IL-10 was determined by Real-Time PCR and western blot. The results of Real-Time PCR revealed that, the expression of IL-10 mRNA was upregulated (1-1.5 fold) in cells treated with GAE and GACs (Figure 7.40). Similarly, western blot analysis showed that, the expression level of IL-10, the major anti-inflammatory cytokine transcribed by NFKB was higher in the GAE and GACs exposed cells as compared to the control cells (Figure 7.42). The protein levels were quantified and the results are shown in Figure 6.46). These results confirmed the anti-inflammatory activity of GAE and GACs in cancer. Hence, these outcomes revealed that both GAE and GACs regulate NFKB /IL1β/ TNFα/ IL 10/ PTEN expression in adenocarcinoma cells under in vitro conditions.

7.4 Discussion

Cytotoxicity is defined as the ability of chemical compound(s) to induce death or damage to cells. In the current study, the efficacy of the algal extract (GAE) to induce cytotoxicity in L132 cells, A549 and HeLa cells were analyzed (Figure 7.2). The increase in cytotoxicity was observed with increased dosage of the compound. The IC50 value of GAE in A549 and HeLa cell lines were identified as 1500 µg/ml and dosages below 1000 µg/ml did not induce cytotoxicity in either cell lines. Similarly, GAE treatment in L132 cell line was carried out to determine its toxicity in normal cell line. The results showed that GAE did not hamper the viability of L132 cells at the concentrations used. Hence the outcomes of the analysis showed that GAE is not toxic in normal cells but can affect the viability of cancer cells. These findings are in line with earlier cytotoxicity assessments of G.acerosa [65]. This suggests that GAE is more effective in inhibiting cancer cell proliferation than the healthy cells.
The pure compounds isolated from *G.acerosa* (GAC 1, 2, 3 and 4) were utilized to test their cytotoxicity in cancer cell lines (A549 and Hela). The results (Figures 7.4 – 7.11) showed that GACs were more cytotoxic in these cell lines when compared to crude algal extract (GAE). These findings revealed that the cytotoxicity exhibited by GACs was concentration dependent and their efficacy was different in different cell lines.

The overall findings of the cytotoxicity assay revealed that both GAE and its compounds (GACs) were cytotoxic in cancer cells which coincided with the previous reports in *G.acerosa* [16], [65]. These results suggest that GAE and GACs possess cytotoxic activity against lung and cervical cancer cell lines.

### 7.4.1 GAE and GACs induced apoptosis

Apoptosis is a morphologically distinct form of cell death [181]. Later it was recognized as an important step in various cellular mechanisms including embryo development, wound healing, immune response, aging and drug-induced cytotoxicity [33]. Deregulation of apoptosis results in disorders of the immune system, AIDS, neurodegeneration, ischemia and cancer [85]. Cancer is an example for decreased apoptosis characterized by abnormal proliferation of cells which escape apoptosis. Cancer cells utilize numerous mechanisms to overcome or prevent apoptosis. One such mechanism includes the alteration of pro and antiapoptotic protein expression. Hence anticancer therapies aim at inducing apoptosis thereby controlling the proliferation and survival of tumour cells. The evaluation of apoptosis included the analysis of morphological changes, DNA fragmentation, cleavage of caspases and analysis of membrane alterations. In the current study in order to investigate whether the antiproliferative activity exhibited by GAE and GACs was mediated through the induction of apoptosis, the hallmarks of apoptosis were analyzed in A549 cells, treated with GAE and GACs. The fluorescent imaging of GAE treated A549 cells showed fragmented nuclei and irregular nuclear morphology whereas the control cells exhibited intact nuclei (Figure 7.12). These features are reported in cells undergoing apoptosis [182]. The PI staining of A549 cells treated with GAE showed a crescent-shaped nuclei
indicating the alteration of nuclear morphology usually observed in cells undergoing apoptosis. The control cells exhibited unaltered nucleus (Figure 7.12). The results of DAPI and PI staining revealed that GAE promotes cell death in cancer cells through apoptosis. AnnexinV is considered as a non-quantitative probe to detect apoptosis [135]. Annexin staining of A549 cells treated with GAE showed increased fluorescence near the surface of the cells which indicated the translocation of PS from the inner leaflet to the outer layer of plasma membrane whereas the control cells are not stained with AnnexinV (Figure 7.12). The results of AnnexinV staining identified that the GAE treated cells were undergoing early apoptosis.

Similarly, DAPI and acridine orange staining in GAC treated A549 cells revealed the fragmentation of nuclei which confirmed the induction of apoptosis by GACs (Figure 7.13). The overall results of fluorescent and confocal imaging confirmed the induction of apoptosis by GAE and GACs.

7.4.2 GAE and GACs activated the intrinsic pathway of apoptosis

Apoptosis is executed through different pathways, the first which involves the activation of caspase-8 and the second that involves caspase-3. The involvement of caspases in apoptosis is well documented in earlier studies [67]. In the current study, the results of both Real–Time PCR and Western blot showed the activation of caspase 3 rather than caspase 8. Similarly, the expression of Bax was upregulated when compared with Bcl2 and Bcl-XI. These outcomes confirmed the activation of the intrinsic pathway of apoptosis by GAE and GACs. Further, these findings strongly suggested that both GAE and GACs induced apoptosis by altering the Bax/Bcl-2 ratio. These findings are in correlation with other studies where medicinal plants and algal compounds were shown to induce apoptosis through the activation of intrinsic pathway [183], [184]. The effect of G.acerosa on apoptosis is shown in Figures 7.48 and 7.49.
Figure 7.48 Flow diagram showing inhibition of apoptosis in A549 cells by anti-apoptotic proteins. \[\text{\_}\] - indicate inhibition.

Figure 7.49 Flow diagram showing regulation of apoptosis in A549 cell by GAE.
7.4.3 GAE and GACs activated GSK3β

In the current study, the variation of the Bax, Bcl2 protein levels strongly confirmed the regulation of apoptosis by GSK3β in the apoptotic pathway. As GSK3β mediates the expression of Bcl-2 family proteins, [185] it plays a central role in regulating both cell death and survival. Since, the loss of balance between the pro and anti-survival factors causes malignancy, the expression levels of GSK3β in association with them was analyzed. The data showed that exposure to GAE/GACs inhibited the phosphorylation of GSK3β, which in turn caused the up-regulation of Bax expression, activation of caspase-3 and resulted in apoptosis. As PI3K and Akt regulate the activation status of GSK3β [186], the study further investigated the expression of these pro-survival components.

7.4.4 GAE and GACs affected PI3K/Akt expression

In order to investigate the underlying mechanism that activates GSK3β, the expression of its immediate upstream regulators (PI3K and Akt) was analyzed. The activation of the cell survival pathway PI3K/Akt accompanied by the inactivation of GSK3β was reported in majority of Human cancers. The outcomes showed that the phosphorylation of PI3K and Akt varied among the GACs. The GAC 1, GAC 3 and GAC 4 showed a decreased phosphorylation of both PI3K and Akt which led to the activation of GSK3β whereas GAC 2 did not alter the phosphorylation of PI3K but prevented the phosphorylation and activation of Akt. This variation among the GACs revealed their unique mode of action on the PI3K/Akt/GSK3β pathway. The findings of the current research are in correlation with earlier reports, where the inhibition of PI3K/Akt induced apoptosis [187]. As the constant activation of PI3K pathway inactivates GSK3β and inhibits apoptosis, exposure to GAE/GACs down regulated PI3K/Akt expression, activated GSK3β and favoured apoptosis. Moreover, GSK3β serves as a probe for determining the PI3K/Akt activity [185], [186]. The overall findings of the research strongly confirmed the suppression of PI3K and Akt activity which suggested that GAE and GACs can function as PI3K/Akt inhibitors. The results of the current analysis correlated with similar reports on algal polysaccharides which inhibited PI3K/Akt activation in gastric...
The effect of *G. acerosa* on PI3K cascade is shown in Figures 7.50 and 7.51.

Figure 7.50 Flow diagram showing aberrant expression of PI3K/Akt pathway causes prolonged survival in cancer

↑ – indicates up regulation, ↓ - indicates down regulation.
Figure 7.51 Flow diagram showing treatment of A549 cells with GAE decreased expression of PI3K/Akt pathway, activated GSK3β and induced apoptosis. ↓ - indicates decreased expression

7.4.5 GAE inhibited cell migration, colonization through MMP2

Although the antiproliferative efficacy of *G. acerosa* was reported previously [140], the current study is the first to investigate on its antimetastatic activity. The major obstacle in cancer treatment and management is metastasis which involves the degradation of ECM and cell invasion and colonization [190]. The prominent contributors of metastasis in lung, ovary, breast and prostate carcinomas are the Matrix metalloproteinases (MMPs) [191],[192].

MMPs are essential for tumorigenesis, cancer cell proliferation, angiogenesis and migration. Among the MMPs, MMP2 and MMP9 contribute to the disintegration of ECM and thus favour tumour metastasis [193]. In the current study, the findings of cell invasion assay and colonization assay revealed the efficiency of GAE to inhibit cell invasion and uncontrolled proliferation which directly correlated with the
expression levels of MMPS. The findings showed a decrease in the expression of MMP2 when compared to MMP9 in cells treated with GAE/GACs. Based on these outcomes, it was confirmed that G.acerosa exhibited antimetastatic activity through the inhibition of MMP2. As MMP2 was reported to be over expressed in NSCLC, it was considered as a probe for metastasis [194], [195]. Hence, the overall findings confirmed that both GAE and GACs affected cell survival, migration and colony formation, which are the major challenges in the management of cancer.

7.4.6 GAE and GACs affect NFkB expression

In the current investigation, the expression of NFkB and PTEN were analyzed. The outcomes showed a decrease in the expression of NFkB accompanied by an increase expression of PTEN at both the transcript and protein levels. These results prompted that both the GAE and its compounds (GACs) have inhibitory activity on NFkB activation. Further, as NFkB regulates the expression of MMPs, and our results also showed a decrease in the expression of MMP2, the inhibition of NFkB by G.acerosa is confirmed. These findings closely correlate with similar studies where NFkB was reported to regulate apoptosis and cell migration through MMP2 [196], [197], [198]. Based on these outcomes, the activation of tumour suppressor PTEN and inhibition of NFkB by G.acerosa was established.

Inflammation is a major contributor of tumour progression [199]. Chronic inflammation is reported in most of the human cancers [200], [201]. As NFkB regulates the expression of proinflammatory cytokines including IL-1β, IL 6, iNOS, TNFα, IL 10 and COX which regulate the inflammatory response and pathogenesis in cancer [202], [203] the study was extended to determine the expression of these cytokines. The data showed a decreased expression of IL-1β and TNFα whereas, the expression levels of IL 10 was increased in GAE and GAC treated cells. These results showed that GAE and GACs inhibited the activation of NFkB which decreased the production of proinflammatory cytokines and increased the expression of IL 10. The current findings are similar to the outcomes of an earlier study where the carotenoid fraction from D.salina suppressed proinflammatory cytokine levels through the inhibition of NFkB [204]. Similarly, the ethanolic extract
of *S. horneri* elicited anti-inflammatory activity by down regulating the expression of proinflammatory cytokines [205]. In case of prostate carcinoma, the suppression of proinflammatory cytokines was reported to inhibit the activation of NFKB [206]. The effect of *G.acerosa* on NFkB is shown in Figures 7.53 and 7.54.

Figure 7.52 Flow diagram showing activation of NFKB cascade results in inflammation in cancer. ↑ – indicates increased expression.
The outcomes of this chapter of the thesis revealed that the expression levels of Bcl2, Bcl-Xl, PI3K, Akt, NFKB-p65, TNFα and IL-1β were upregulated in (Adenocarcinoma) A549 cells which cause uncontrolled proliferation, prolonged survival, metastasis and inflammation in cancer. Whereas, treatment with GAE and GACs decreased the expression levels of these proteins thus promoting apoptosis (increased Bax, cleaved caspase 3, GSK3β), inhibiting metastasis (decreased MMP 2) and inflammation (increased IL-10) in cancer. Based on these findings, it was concluded that both GAE and its compounds the GACs have anticancer activity. The overall mechanism of *G. acerosa* are shown in Figure 7.54.

Figure 7.53 Flow diagram showing treatment of A549 cells with GAE resulted in decreased expression of NFKB and decreased production of proinflammatory cytokines and increased production of anti-inflammatory marker IL 10.

↑- indicates decreased expression, ↓- indicates increased expression.

### 7.5 Conclusion

The outcomes of this chapter of the thesis revealed that the expression levels of Bcl2, Bcl-Xl, PI3K, Akt, NFKB-p65, TNFα and IL-1β were upregulated in (Adenocarcinoma) A549 cells which cause uncontrolled proliferation, prolonged survival, metastasis and inflammation in cancer. Whereas, treatment with GAE and GACs decreased the expression levels of these proteins thus promoting apoptosis (increased Bax, cleaved caspase 3, GSK3β), inhibiting metastasis (decreased MMP 2) and inflammation (increased IL-10) in cancer. Based on these findings, it was concluded that both GAE and its compounds the GACs have anticancer activity. The overall mechanism of *G. acerosa* are shown in Figure 7.54.
Figure 7.54 Flow diagram showing regulation of PI3K/Akt/GSK3β/NFKB cascade by *G. acerosa* extract (GAE). The treatment of A549 cells with GAE results in the inhibition of PI3K/Akt followed by activation of GSK3β, Bax and caspase3 which induce apoptosis by the intrinsic pathway. Similarly inhibition of NFKB leads to inhibition of pro inflammatory cytokine production (IL 1β, TNF α) and increased production of IL 10 which accounts for the anti-inflammatory response. The treatment with GAE induces apoptosis and decreases inflammation in cancer. 

- indicate down regulation, ↑ - indicate up regulation.