8. EVALUATION OF THE TOXICITY, ANTICANCER AND ANTI-INFLAMMATORY PROPERTIES OF THE RED ALGAL EXTRACT (GAE) AND ISOLATED COMPOUNDS (GAC 1- 4) UNDER *IN VIVO* CONDITIONS IN TUMOUR MODELS OF ZEBRAFISH.

8.1 Introduction

In order for a newly synthesized compound or a natural products to be subjected to human exposure, a strict monitoring of the adverse effects that the compound may induce needs to be evaluated. The evaluation involves the assessment of the cytotoxicity under *in vitro* and *in vivo* conditions. Since GAE induced cytotoxicity in cancer cell lines under *in vitro* conditions, it was logical to investigate its cytotoxicity under *in vivo* conditions. This toxicity analysis was carried out in two stages namely, the acute toxicity testing and the chronic toxicity testing using Zebrafish as animal model.

Toxicity testing involving rodent or non-rodents is an expensive and time consuming process. Zebrafish (*Danio rerio*) has emerged as a popular toxicity assessment model as it is cost effective, easy to maintain, has large fecundity and possess 75% genetic similarity with humans. Hence in the current study the toxicity and anticancer and anti-inflammatory activities of GAE were in Zebrafish.

8.1.1 Acute Toxicity analysis

Acute toxicity analysis evaluates the adverse effects of short-term exposure of chemicals in animals. It is an integral step in assessing the toxic potential of chemicals or compounds as per the regulatory framework of US Environment Protection Agency (EPA 1998a). In acute toxicity testing the animals are exposed to a single dosage or to several doses in a 24 hour time period and observed for a period of 14 days for changes in behaviour, growth and mortality. Acute toxicity enables the determination of the lethal dose or LD 50, the dose which induces death in 50% of the animal group under analysis. It is essential to decide the dosage level for chronic toxicity testing.

Acute toxicity analysis has reduced the use of test animals, pain and distress of the test animals, and provides information about pathogenesis of toxicity. It also provides information whether toxicity induced is spontaneous or delayed and time dependent or independent. It enables to identify the suitable routes of drug administration, bioavailability of the compound and organs targeted by the compound.

8.1.2 Chronic Toxicity analysis

Chronic toxicity analysis is performed to investigate the cumulative adverse effects of frequent daily exposures of test animals to various doses of a chemical for a duration of 12 months (EPA 1998e).

8.2 Methods

As discussed in chapter 3, Zebrafish was utilized to determine the toxicity of GAE under acute and chronic testing protocols. The fishes were fed with GAE mixed with the pellet. The concentrations administered are given in the Table 8.1. A control group which received only the pellet without GAE was maintained. Following this the anticancer activity of GAE and GACs were analyzed in tumour models of Zebrafish.

Table 8.1 Oral admistration set up of GAE in Zebrafish.

Study Group n=6	Concentration of GAE per pellet	GAE fed per day	GAE fed in 14 days (Acute toxicity analysis)	GAE fed in 30 days (Chronic toxicity analysis)
GAE 250µg/ml	83.3 µg	83.3 µg x 3 pellet/day	3500 µg	7500 µg
GAE 500 µg/ml	83.3 µg	83.3 µg x 6 pellet/ day	7000 µg	21000 µg

8.3 Results

8.3.1 GAE is not toxic in animals

Table 8.2 shows the results of chronic toxicity screening of GAE in Zebrafish. Groups of two adult male fishes were housed in 2 liters water tank with continuous aeration, maintained at 14/10 light dark cycles, 26°C for 30 days. The fishes were exposed to different concentrations of GAE (250 and 500 µg/ml) mixed with feed for a duration of 14 days (Acute toxicity analysis) and for 30 days (Chronic toxicity analysis). The major organs were analysed for pathological changes. Both the acute and chronic toxicity analysis did not show any pathological changes in the organs tested at these concentrations.

Table 8.2 Chronic Toxicology Screening of GAE in Zebrafish

Clinical Parameters	Control	Chronic Toxicity 250µg/ml	Chronic Toxicity 500µg/ml
Heart Anatomy	Normal	Normal	Normal
Liver Anatomy	Normal	Normal	Normal
Brain Anatomy	Normal	Normal	Normal
Muscle Anatomy	Normal	Normal	Normal
Liver Pathology	Normal	Normal	Normal
Brain Pathology	Normal	Normal	Normal
Heart Pathology	Normal	Normal	Normal
Skeletal Muscle Pathology	Normal	Normal	Normal

The results of acute and chronic toxicity testing in zebrafish revealed that GAE is not toxic in the concentrations used. The images showing the pathology of the toxicity analysis are shown in Figure 8.1.

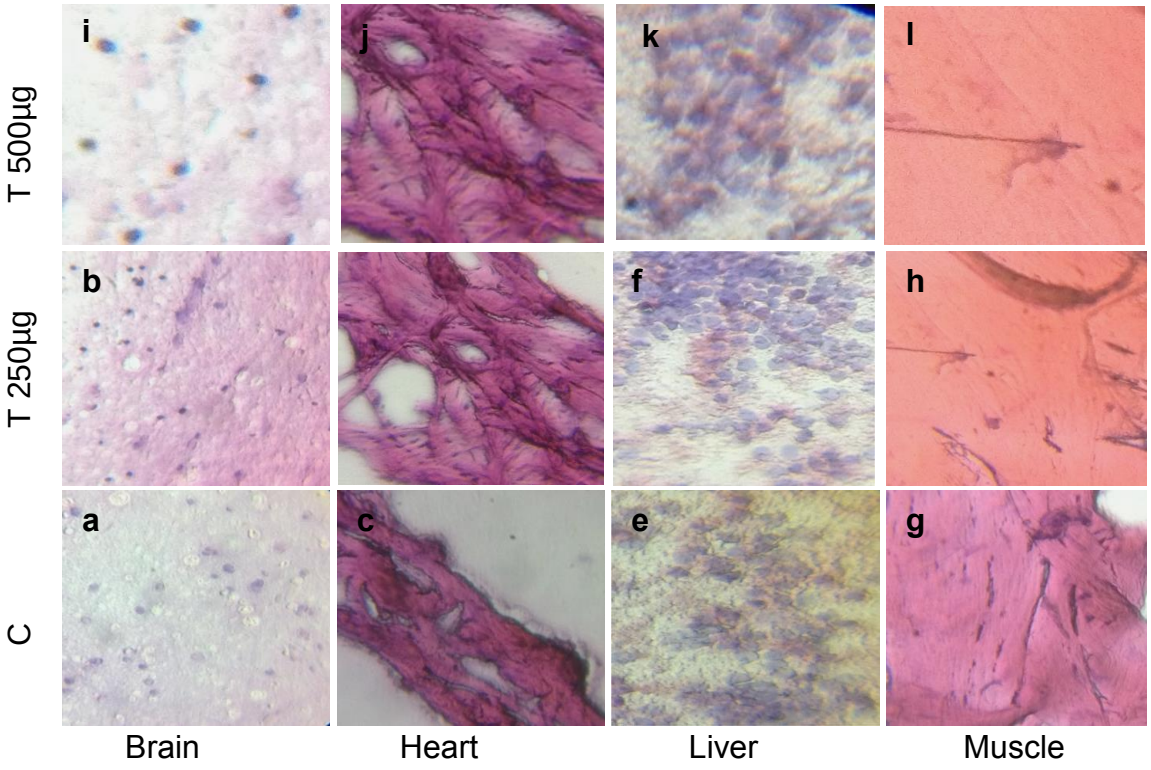

Figure 8.1 *In vivo* chronic toxicity analysis**.** (a, e) shows the unaltered brain pathology of control and GAE treated Zebrafish. (b, f) shows the pathology of heart muscles in control and GAE treated Zebrafish. (c, g) shows the unaltered muscle pathology of control and GAE treated Zebrafish. (d, h) shows the pathology of liver cells in control and GAE treated Zebrafish. The pathology results confirm that GAE is not toxic to these major organs under chronic conditions. C - control, T- treated, n=6

Following this the toxicology analysis of GAE was confirmed through the lab on chip technology. The results are shown in Figure 6.2.

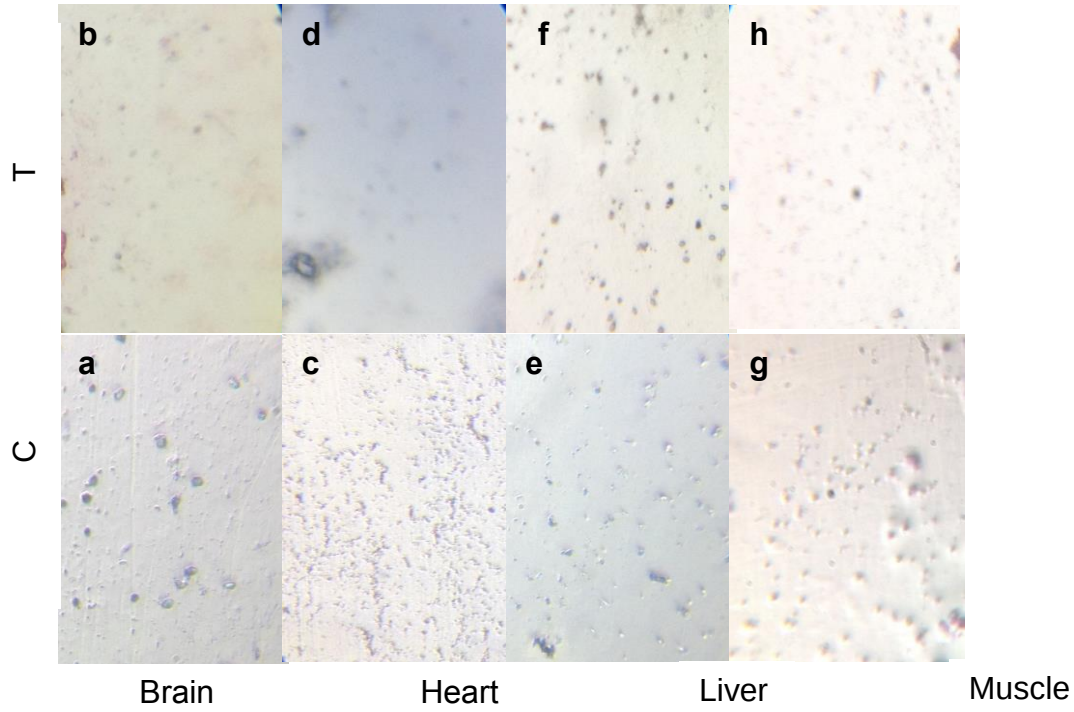

Figure 8.2. Toxicity analysis by using Tissue-Chip technology. The pathology studies of vital organs including brain, heart, liver and muscle were analyzed in both control (a, c, e, g) and in treated (b, d, f, h) tissues. The treatment with algal extract (500µg) did not affect the tissue architecture. The results of tissue chip analysis also support GAE to be safe without toxicity. These tissues were developed and analysed for toxicity in Tissue-Chip Pentagrit, Chennai. C-control, T-treated, n=6.

The outcomes of *in vivo* toxicology screening (both acute and chronic) showed that GAE was safe at the optimum concentrations used for the current analysis. Moreover, the morphology of the brain, liver, heart and muscles were unaltered by GAE treatment (Figure 8.1) (Table 3). Similarly, the *in vitro* toxicity analysis based on the tissue-chip also showed the ability of cells from these major organs to regenerate even at the highest dose used (500 µg) (Figure 8.2). The overall findings of both the *in vitro* and *in vivo* toxicity screening showed that GAE is safe for animal use.

8.3.2 GAE and GACs inhibited lung tumour proliferation *in vivo*

Since, GAE did not induce any adverse effects under *in vivo* conditions (Figures 8.1 and 8.2) the next step in the study was to determine its anticancer and anti-inflammatory properties in tumour model of Zebrafish. As discussed in chapter 3, the (A549) lung tumour model, (HCT) colon cancer tumour model and the (Hep G2) liver cancer tumour models of Zebrafish were utilized for the study. Both GAE and its compounds the GACs (1- 4) were analyzed for their anticancer and anti-inflammatory activities.

The anticancer efficacy of GAE and GACs was investigated under *in vivo* conditions in the tumour model of Zebrafish. The findings were based on the analysis of tumour anatomy, tumour pathology and muscle pathology.

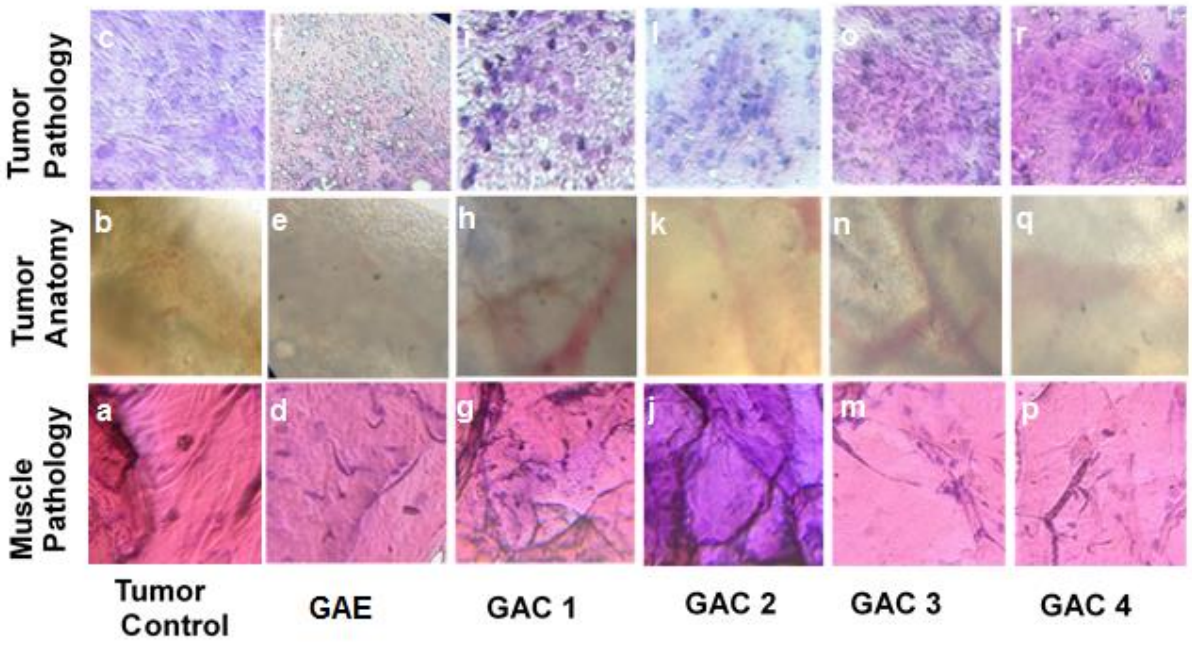

Figure 8.3 *In vivo* analysis of antitumour activity in A549 tumour induced Zebrafish. (a, b, c) Histopathological analysis of muscle and tumour of tumour induced zebrafish. (d, e, f) Histopathological analysis of muscle and tumours from GAE treated tumour induced zebrafish. (g, h, i) Histopathological analysis of muscle and tumours from GAC 1 treated tumour induced zebrafish. (j, k, l) Histopathological analysis of muscle and tumours from GAC 2 treated tumour induced zebrafish. (m, n, o) Histopathological analysis of muscle and tumours from GAC 3 treated tumour induced zebrafish. (p, q, r) Histopathological analysis of muscle and tumours from GAE treated tumour induced zebrafish. n=6

The histological analysis (Figure 8.3) revealed that, the A549 tumour induced fishes demonstrated swollen muscles with altered cell architecture whereas, the GAE and GAC 1, GAC 3, GAC 4 treated fish exhibited normal muscle architecture and lysing tumour cells. Similarly the population of normal cells were observed to

increase in the treated group (60µg) than tumour control. The other dosage groups (15, 30 & 45 µg) did not show any change.

Similarly the anticancer activity of GAE and GACs were analyzed in HCT tumour model of Zebrafish. Figure 8.4 shows the effect of GAE and GACs on HCT tumour model of Zebrafish.

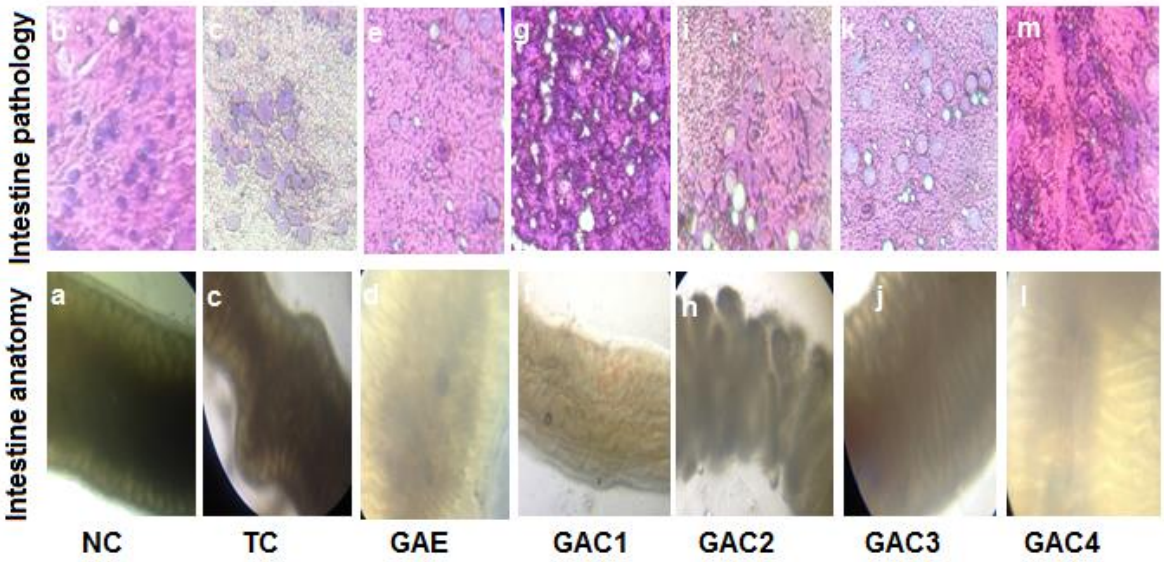

Figure 8.4. *In vivo* analysis of antitumour activity in HCT tumour induced Zebrafish. The tumour induced zebrafish were treated with 60 µg/ml of GEA and GACs for 10 days period. The intestine anatomy and pathology were observed. (c, d) Tumour control showed swollen intestine anatomy and irregular cells, (e – f) GAE and GACs treatment at 60 µg showed reduced swelling. Intestine pathology showed recovery cells in GAE, GAC 1 and GAC 3 treated Zebrafish. (NC – Normal control, TC –Tumour control, n=6).

In a similar way the anticancer activity of GAE and GACs were analyzed in HepG2 tumour induced Zebrafish.

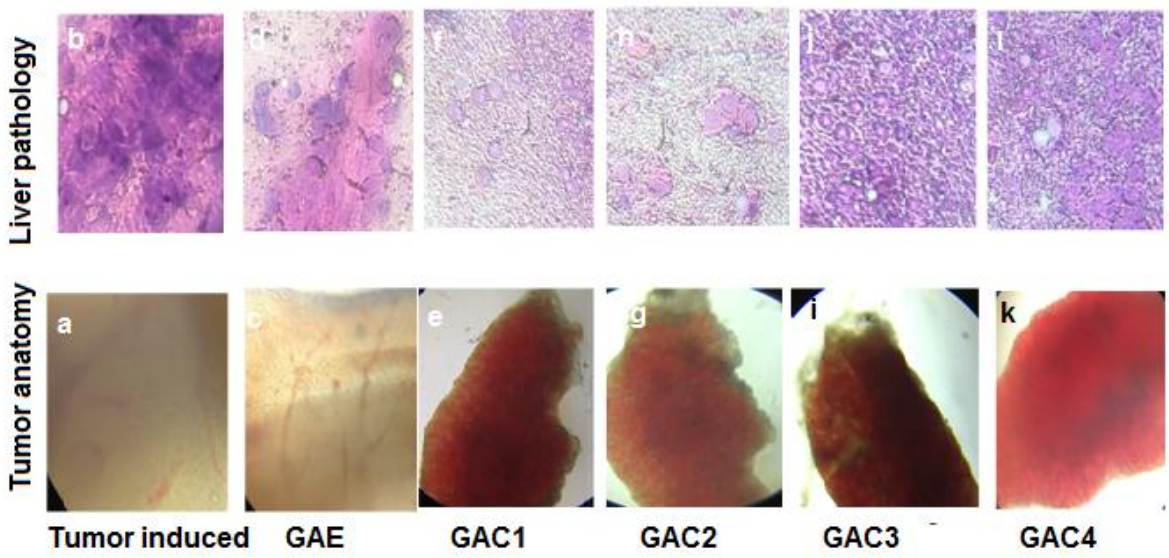

Figure 8.5 *In vivo* analysis of antitumour activity in HepG2 tumour induced Zebrafish. The tumour induced zebrafish were treated with 60 µg/ml of GEA and GACs for 10 days period. The liver anatomy and tumour pathology were analyzed. (a, b) The tumour induced fish showed swollen liver with irregular cells. (c - k)The tumours treated with 60 µg of GAE and GACs showed irregular cells but with swollen liver. The treatment did not induce any changes in the HepG2 tumours. n=6.

The histological analysis of HCT tumour induced (Figure 8.4) and HepG2 tumour induced (Figure 8.5) Zebrafishes did not exhibit any change in tumour anatomy, tumour cell population after treatment with GAE and GACs. The results of the histological study revealed that both GAE and GACs are effective on lung cancer model of Zebrafish when compared to the other cancer models.

8.3.3 GAE and GACs inhibited PI3K/Akt/NFKB pathway

Based on the above findings, protein was isolated from the A549 tumour induced and GAE/GACs treated Zebrafishes and analysed for the expression of proteins involved in apoptosis (Bax, Bcl2), cell survival (PI3K, Akt, GSK3β) and inflammation (NFKB, TNFα, IL-1β and IL-10). The results are shown below.

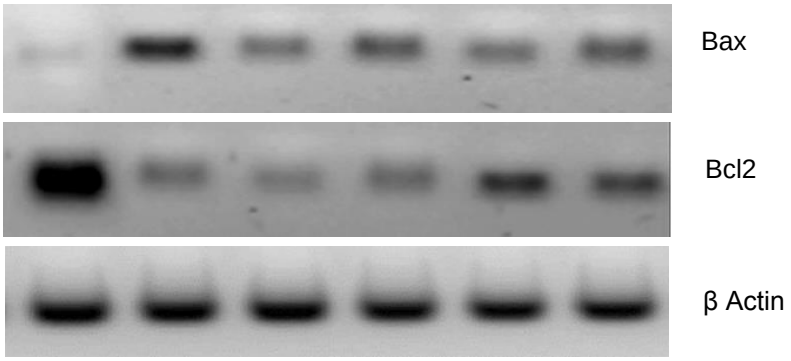

Figure 8.6 Effect of GAE and GACs treatment on the expression of proteins related to apoptosis by western blot analysis. A549 tumour induced Zebrafish were treated with GAE and GAC (1 - 4) and the expression levels of proteins in the apoptotic pathway were examined by western blot. Treatment with GAE and GACs increased the levels of Bax and reduced the levels of anti-apoptotic protein Bcl2 in A549 tumour induced Zebrafish. TI – Tumour induced, GAE & GACs – Tumour induced treated with GAE/ GAC.n=6.

Following the analysis of apoptotic and anti-apoptotic protein expression, the quantification of the same was carried out. The protein levels were measured by Image J and the results were normalized with β actin.

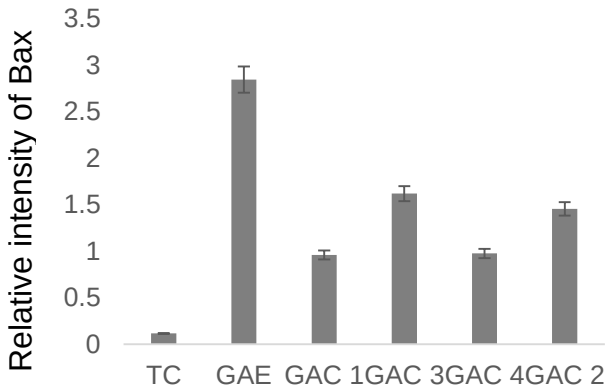

Figure 8.7 Histograms show the quantitative evaluation of Bax in A549 tumour induced Zebrafishes treated with and without GAE and GACs. TC – Tumour induced, GAE & GACs – Tumour induced treated with GAE /

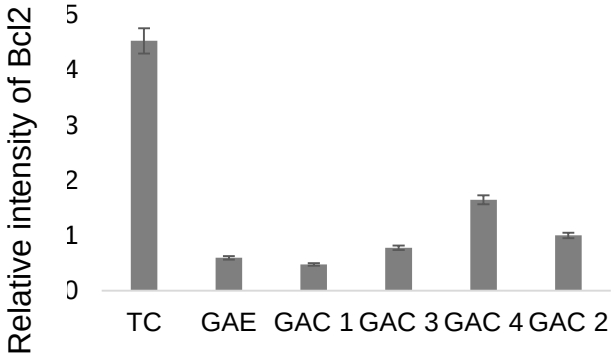

Figure 8.8 Histograms show the quantitative evaluation of Bcl2 in A549 tumour induced Zebrafishes treated with and without GAE and GACs. TC – Tumour induced, GAE & GACs – Tumour induced treated with GAE / GACs.

Following this the proteins in the cell survival pathway was analyzed by western blot.

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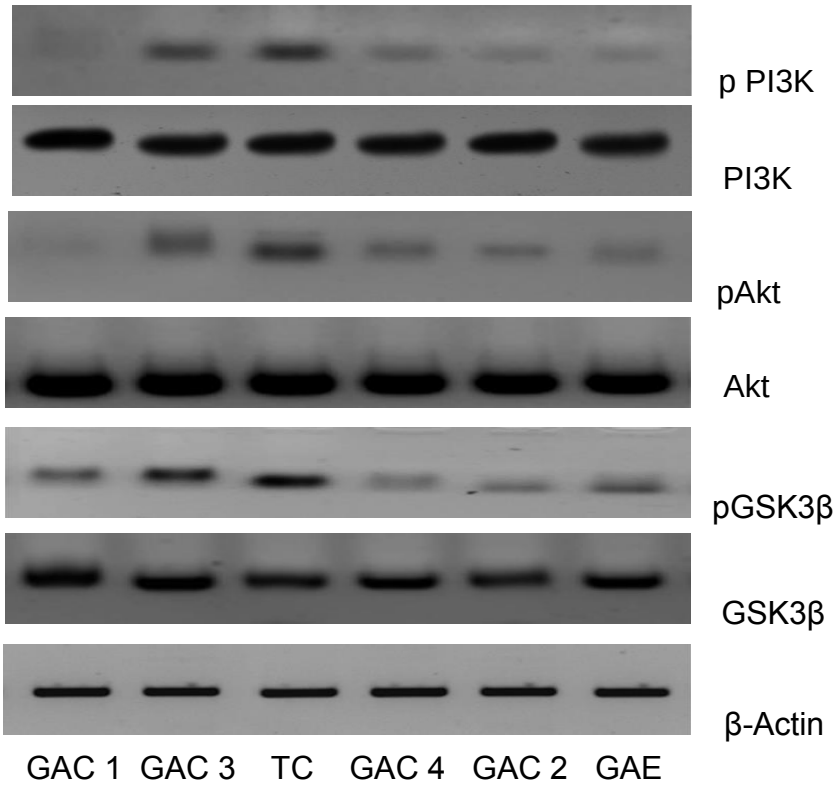

Figure 8.9 Effect of GAE and GACs treatment on the expression of proteins involved in cell survival cascade by western blot analysis. A549 tumour induced Zebrafish were treated with GAE and GAC (1 - 4) and the expression levels of proteins in the cell survival pathway were examined by western blot. The treatment with GAE and GACs decreased the phosphorylation of GSK3β, Akt and PI3K. TC – Tumour induced, GAE & GACs – Tumour induced treated with GAE / GACs.

Following the analysis of protein expression in the prosurvival pathway, the quantification of the same was carried out. The protein levels were measured by Image J and the results were normalized with β actin.

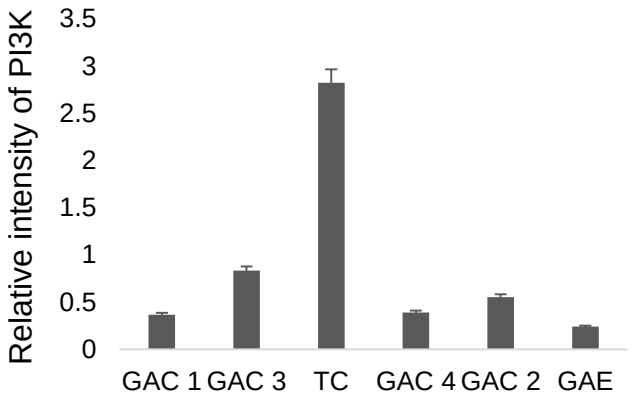

Figure 8.10 Histograms show the quantitative evaluation of PI3K in A549 tumour induced Zebrafishes treated with and without GAE and GACs. TC – Tumour induced, GAE & GACs – Tumour induced treated with GAE / GACs.

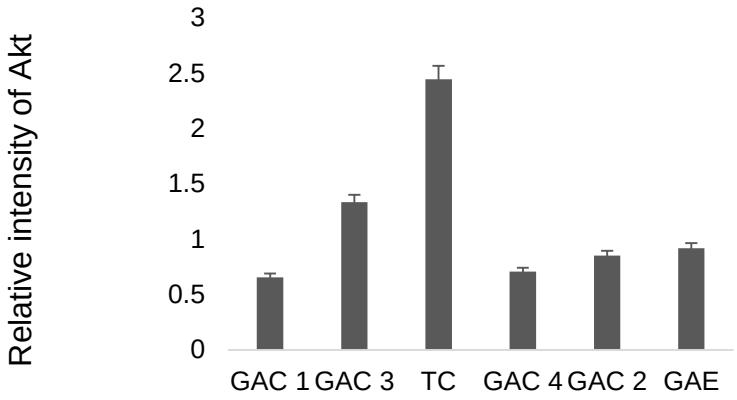

Figure 8.11 Histograms show the quantitative evaluation of Akt in A549 tumour induced Zebrafishes treated with and without GAE and GACs. TC – Tumour induced, GAE & GACs – Tumour induced treated with GAE / GACs.

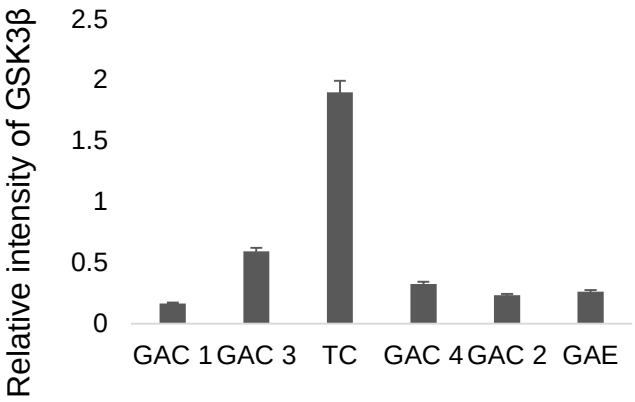

Figure 8.12 Histograms show the quantitative evaluation of GSK3β in A549 tumour induced Zebrafishes treated with and without GAE and GACs. TC – Tumour induced, GAE & GACs – Tumour induced treated with GAE / GACs.

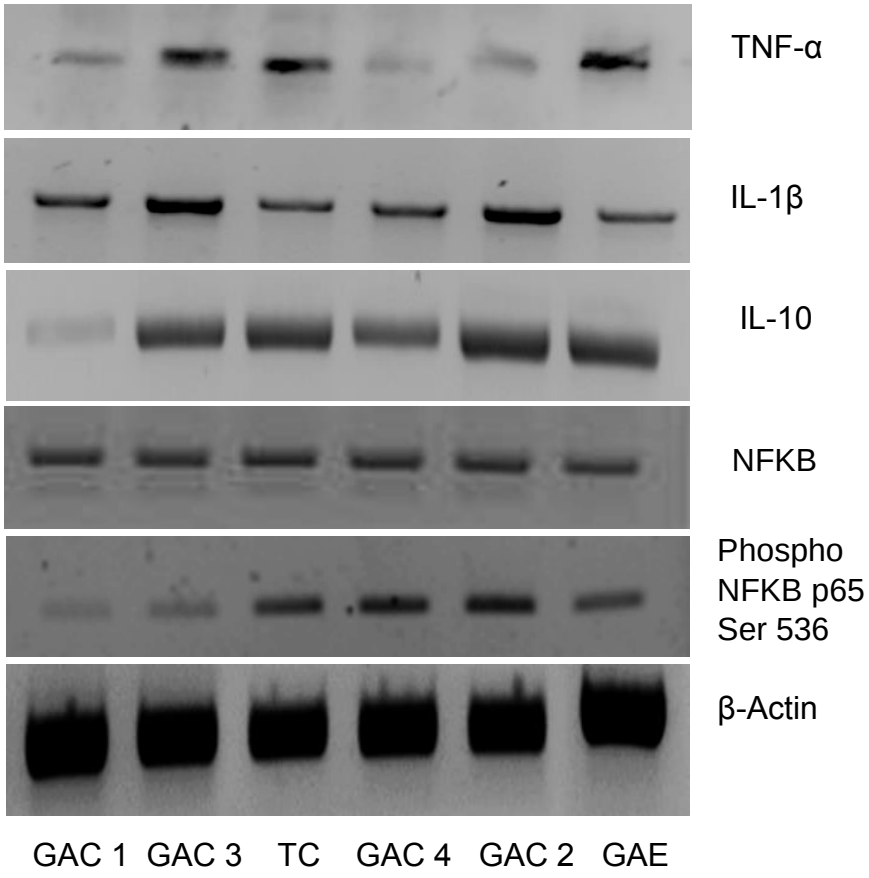

Figure 8.13 Effect of GAE and GACs treatment on the expression of proteins involved in anti-inflammatory cascade by western blot analysis. A549 tumour

induced Zebrafish were treated with GAE and GAC (1 - 4) and the expression levels of proteins in the anti-inflammatory cascade were examined by western blot. The treatment with GAE and GACs decreased the activation of NFKβ and TNF α proteins. TC – Tumour induced, GAE & GACs – Tumour induced treated with GAE/ GACs.

Figures 8.14 – 8.17 show the protein levels in the anti-inflammatory pathway that were measured by Image J and the results were normalized with β actin.

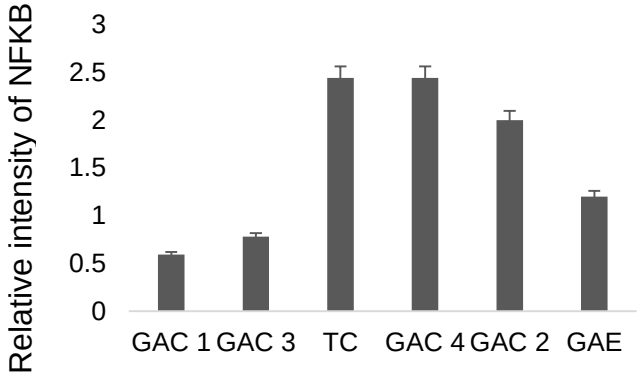

Figure 8.14 Histograms show the quantitative evaluation of phospho NFKB in A549 tumour induced Zebrafishes treated with and without GAE and GACs. TC – Tumour induced, GAE & GACs – Tumour induced treated with GAE / GACs.

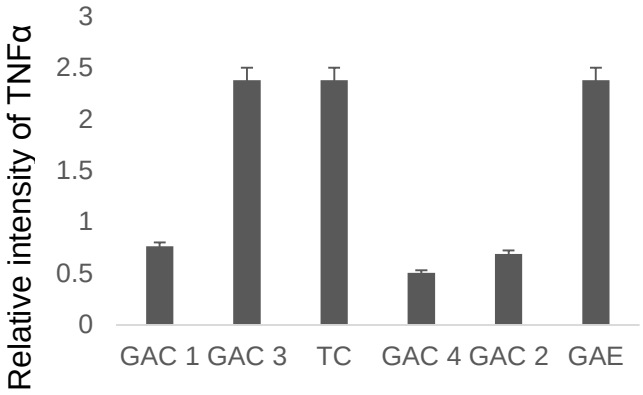

Figure 8.15 Histograms show the quantitative evaluation of TNF α in A549 tumour induced Zebrafishes treated with and without GAE and GACs. TC – Tumour induced, GAE & GACs – Tumour induced treated with GAE / GACs.

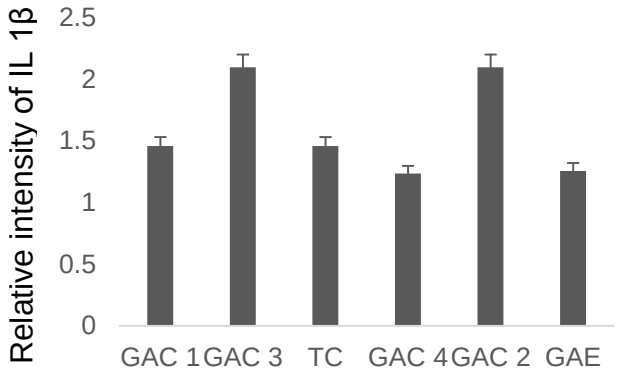

Figure 8.16 Histograms show the quantitative evaluation of IL 1β in A549 tumour induced Zebrafishes treated with and without GAE and GACs. TC – Tumour induced, GAE & GACs – Tumour induced treated with GAE / GACs.

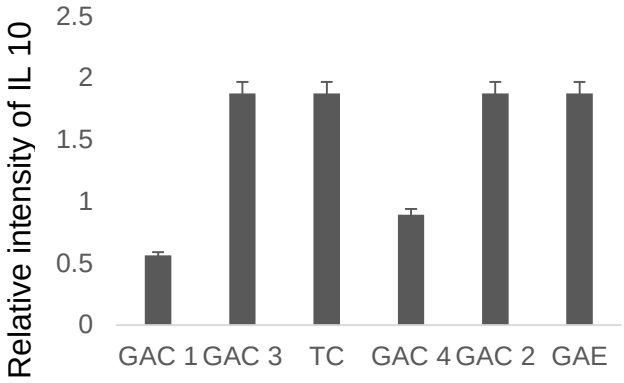

Figure 8.17 Histograms show the quantitative evaluation of IL 10 in A549 tumour induced Zebrafishes treated with and without GAE and GACs. TC – Tumour induced, GAE & GACs – Tumour induced treated with GAE / GACs.

Note that: Data represented are mean ± SD, n=3.

8. 4 Discussion

The chapter 8 of the thesis investigated the cytotoxicity, anticancer and anti-inflammatory properties of GAE and GACs under *in vivo* conditions. Based on FDA regulations, acute and chronic toxicity studies are mandatory for compounds / formulations which are intended for human use. As compounds which were potential under *in vitro* conditions, were toxic under *in vivo* conditions, the current study analyzed GAE for its adverse effects both under *in vitro* (Zebrafish Tissue-chip method) and *in vivo* (Zebrafish) conditions.

The findings of the *in vitro* and *in vivo* toxicology analysis revealed that GAE did not induce adverse side effects in animals. These outcomes coincide with early reports where extracts from G. acerosa were shown to protect human PBMCs and erythrocytes from TDCC induced toxicity [14], [18].The outcomes of the toxicology analysis were followed by the determination of anticancer properties in tumour models of Zebrafish. Three tumour models namely the lung (A549 tumour model), colon (HCT tumour model) and liver (HepG2 tumour model) were investigated in the current research. The results revealed that both GAE and GACs were effective on A549 tumours under *in vivo* conditions. Histopathological analysis of the A549

tumours showed that GAE treatment decreased angiogenesis which prevents tumour spread (Figure 8.3). Similarly, the number of tumour cell population were decreased in the GAE and GACs (1, 3, 4) treated tumours. On the other hand, treatment with GAC 2 did not induce any alteration in the tumour anatomy or tumour cell population. These outcomes showed that GAE and GACs were effective on A549 tumours under *in vivo* conditions. 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 in the GAE and GACs treated tumours when compared to the untreated tumours (Figure 8.6). Similarly the levels of antiapoptotic protein Bcl2 was decreased in GAE and GACs treated tumours. These findings showed that both GAE and GACs were capable of inducing apoptosis which correlated with the decreased tumour cell population as observed in the histopathology studies. Similarly the protein levels in the prosurvival pathway namely the PI3K and Akt were decreased in the GAE and GACs treated tumours whereas the levels of GSK3βwas increased which coincide with the alteration of Bax/Bcl2 ratio (Figure 8.9). Further, the levels of the inflammatory regulator NFKB, IL 1β and TNFα were decreased and that of IL 10 was increased in the GAE and GACs treated tumours when compared to the untreated tumours (Figure 8.13). These results clearly showed that GAE and GACs induce apoptosis through the regulation of prosurvival pathway in cancer but also regulate the inflammatory response in cancer. Further these findings coincided with our *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 tumour induced (Figure 8.4) and HepG2 tumour induced Zebrafishes (Figure 8.5) did not exhibit any change in tumour anatomy, tumour cell population after treatment with GAE and GACs. The results of the histological study revealed that both GAE and GACs are effective only on lung cancer model of Zebrafish.

8.5 Conclusion

The outcomes of the current chapter clearly showed the antitumour efficacy of *G.acerosa*. The findings of the study emphasized that GAE was effective under *in vitro* and *in vivo* conditions and did not induce any adverse side effects. Further, the up-regulation of Protein kinase B or Akt by PI3K contributes to resistance against chemotherapy [207] thus the inhibition or suppression of PI3K is essential in regulating cell proliferation and survival. The current study explored the molecular mechanism by which *G.acerosa* the induced apoptosis and regulated the PI3K pathway in lung cancer. Based on these outcomes, we can conclude that, the marine algae are a source of novel PI3K inhibitors which can be utilized in the effective treatment of cancer.