3. MATERIALS AND METHODS

3.1 Seaweed collection and processing

The marine red alga *G.acerosa* was sampled from the Mandapam coastal region of Tamil Nadu, India. It was identified and authenticated by Dr. Saravanan, Scientist, Central Marine Fisheries Research Institute (CMFRI), Mandapam, Tamil Nadu, India. A voucher specimen of the algae was deposited at CMFRI, Mandapam (accession number: MMM-CMFRI17002). The seaweed was cleaned thoroughly to remove debris, shade dried and milled in an electric grinder.

3.2 Extraction of the alga

The phytochemicals of the algae were extracted sequentially as described previously [126]. 100 g of the dried algal powder was extracted with 300ml of solvent in the following order: hexane, Dichloromethane, ethyl acetate, ethanol, methanol and water. The extraction was carried out for 72 hours in an orbital shaker. The mixture was filtered, and the filtrate was evaporated in a rotary evaporator. The dried crude extracts were utilized for further studies.

3.3 Phytochemical screening

The phytoconstituent composition of the crude extracts was determined based on the standard protocols [127].

Test for Tannins

To 1 ml of the crude extract 3 drops of ferric chloride (0.1%) was added. The formation of bluish black or brownish green precipitate indicated the presence of tannins.

Test for Alkaloids

To 1 ml of the crude extract 3ml of hydrochloric acid (10%) was added and incubated in a water bath for 10 minutes. The contents were filtered and to the filtrate 2-3 drops of ammonia was added followed by 10 drops of Wagner's reagent. The formation of reddish brown precipitate indicated the presence of alkaloids.

Test for flavonoids

(a) Alkaline reagent test

To 1 ml of the crude extract few drops of sodium hydroxide solution (20%) was added. The formation of yellow colour which disappeared upon addition of acid indicated the presence of flavonoids.

(b) Lead acetate test

To 1 ml of the crude extract lead acetate solution (1ml) was added. The formation of white precipitate indicated the presence of flavonoids.

Test for Terpenoids

To 1 ml of crude extract 1ml of chloroform was added followed by concentrated sulphuric acid along the sides of the test tube. Formation of reddish brown colour indicated the presence of terpenoids.

Test for saponins

To 1 ml of the crude extract 3ml of distilled water was added and shaken vigorously for 3 minutes. The formation of froth indicated the presence of saponins.

Test for Phytosterol

1 ml of the crude extract was mixed with 1ml of chloroform and filtered. To the filtrate 1 ml of acetic acid was added and incubated in a water bath for 5 minutes followed by concentrated sulphuric acid along the sides of the test tube. Formation of a brown ring at the junction indicated the presence of phytosterols.

Test for glycosides

2 ml of the crude extract was mixed with 2ml of dilute hydrochloric acid, hydrolyzed and filtered. To 1 ml of the filtrate 1ml of ferric chloride was added and incubated in a water bath for 5 minutes. The mixture was cooled and equal volume of benzene was added and shaken vigorously. The benzene layer was separated and added to ammonia solution. The formation of pink colour indicated the presence of glycosides.

Test for cardiac glycosides

To 2 ml of the crude extract glacial acetic acid (1ml) containing a drop of ferric chloride followed by 1ml of concentrated sulphuric acid was added. The formation of a brown ring at the interface indicated the deoxy sugar characteristics of cardenolides.

Test for oils and fats

A volume of 1-3 drops of the crude extract were added on a filter paper and pressed. The presence of an oily stain on the filter paper indicated the presence of fatty acids.

Test for Polyphenols

To 1 ml of the crude extract a few drops of ferric chloride solution was added. The formation of bluish black colour indicated the presence of polyphenols.

Test for Proteins

1 ml of the crude extract was mixed with 1ml of sodium hydroxide solution (40%) and incubated in water bath for 5 minutes, and few drops of copper sulfate solution (1%) was added. The formation of purple colour indicated the presence of proteins.

Test for Amino acids

To 1 ml of the crude extract few drops of ninhydrin solution was added and incubated in a water bath for 5 minutes. The formation of blue colour indicated the presence of proteins/peptides/amino acids.

Test for Carbohydrates

A volume of 1 ml of the crude extract was mixed with 1ml of α-naphthol and 1 ml of concentrated sulphuric acid was added along the sides of the test tube. The formation of a reddish violet ring at the junction of the two layers indicated the presence of carbohydrates.

Test for Resins

To 1 ml of the crude extract a few drops of acetone and distilled water were added and shaken well. The formation of turbid precipitate indicated the presence of resins.

Test for Coumarins

To 1 ml of the crude extract 1ml of sodium hydroxide solution was added. The appearance of yellow fluorescence indicated the presence of Coumarins.

3.4 Quantification of Polyphenols.

The polyphenol content was analyzed by the Folin-Ciocalteau method [128]. In brief, 100 µg of the crude extracts were mixed with 500 µl of FCP, 150 µl of sodium carbonate (2%) and incubated for 2 hours in dark. The absorbance was measured at 760 nm in a Multimode plate reader (Perkin Elmer). A blank devoid of algal extract served as negative control and gallic acid served as positive control.

3.5 Quantification of Flavonoids

The flavonoid content was determined according to [129]. 100 µg of the crude extracts were added to 100 µl of aluminum chloride (10%) and 100 µl of sodium acetate (1 M) and incubated for 30 minutes. The absorbance was measured at 415 nm in a multimode plate reader (Perkin Elmer). A blank devoid of algal extract served as a negative control. Quercetin served as positive control.

3.6 Characterization of crude extracts

3.6.1 Fourier transform infrared (FT-IR) spectrometry

FT-IR spectrometry was utilized to determine the functional groups present in the crude extracts [130].The crude extracts (10 mg) of *G.acerosa* were mixed individually with 10 mg of KBr and subjected to FT-IR analysis in Jasco FT-IR 4600 series. The IR spectrum generated revealed the possible functional groups present in each crude extract.

3.6.2 High-performance liquid chromatography (HPLC)

The current study employed the reverse phase HPLC which utilizes a nonpolar stationary phase and a polar mobile phase for the separation of components in the crude extracts. The HPLC separation was carried out based on the protocol described previously [131]. HPLC separation was carried out in Shimadzu HPLC 9A series (Japan) equipped with LC 20AD binary gradient pump, RF-fluorescence detector, and SPD- M20A diode array detector. The reverse phase separation was done in C18 G column of 250×4.6 mm of particle size 5 µm with a C18 guard column (3.9×20 mm) of particle size 4 µm maintained at 37°C with a flow rate of 1 ml/minute. The compounds in the crude extracts were separated using Acetonitrile and water in the ratio 30:70. The injection volume was 20 µl, and the monitoring was done by the diode array detector set in the acquisition range of 200-700 nm. The total running time was set for 25 minutes. The retention time (RT) of the compounds was recorded.

3.6.3 Determination of antioxidant activity

The algal extracts were analyzed for their Total Antioxidant Capacity (TAC) [132]. 100 µg/ml of the crude extracts were mixed with 100 µl of 0.1 mM DPPH solution in methanol. The contents were incubated in dark (30 minutes). The absorbance was read at 517 nm in Multimode plate reader (Perkin Elmer). A blank sample devoid of crude extracts served as control and L-ascorbic acid (100µg/ml) was used as the standard. The antioxidant content of the crude extracts was determined using the formula

$$\% \text{ of free radical scavenged} = Ac\text{-}As/Ac \times 100$$

Where Ac represents absorbance of the control and As represents absorbance of the sample. The experiment was carried out in triplicate and the values represent mean ± SD.

3.7 Isolation and characterization and identification of compounds from Crude ethyl acetate extract (GAE)

Crude ethyl acetate extract (hereafter referred as GAE) was subjected to open column chromatography on silica grade II (100X10 mm) column. The column was washed with petroleum ether and filled with dry silica powder. Petroleum ether was passed through the column and the flow rate was adjusted to two to three drops per second. GAE was dissolved in ethyl acetate and layered on the top of the silica column. Petroleum ether and ethyl acetate were mixed in different ratios (98:2, 96:4, 94:6, 92: 8) and used as the mobile phase to isolate the compounds of GAE. Once the mobile phase was added, the top of the column was covered to prevent the evaporation of the mobile phase. The column was eluted by adjusting the mobile phase composition and four different compounds (GAC 1, GAC 2, GAC 3 and GAC 4) were isolated from GAE. The compounds were collected and concentrated in a rotary evaporator and stored at 4°C for further studies.

3.7.1 Characterization of compounds: $4^{1}H$ and ^{13}C Nuclear Magnetic Resonance spectroscopy analysis of GAE compounds.

The Proton NMR (^{1}H NMR) analysis was carried out to identify the possible protons and their positions in GACs. The measurements were performed in Bruker Avance III Fourier transform NMR spectrometer. Deuterated chloroform (CDCl3) was used for the analysis. The temperature was maintained at 45°C and argon gas was passed through alumina column. The traces of solvents were removed from the samples and the chemical shifts of ^{1}H were measured. Similarly the Carbon NMR (^{13}C NMR) of the GACs was also carried out [133].

3.7.2 GC-MS analysis of GAE and GACs

In the current study, GAE and GACs were separated in JOEL GCMATE GC-MS equipped with data system to identify the molecular structure of compounds. The instrument has a resolution of 6000 Daltons with a maximum calibrated mass of 1500 Daltons. It has two source options namely the electron impact (EI) and Chemical Ionization (CI).The mass range varies between 2- 1000 amu with a resolution of 0.1 amu. The ion source temperature was 350°C, transfer line temperature was 100- 400°C and quadrupole temperature was 100-200°C. The ionization energy was 5-200 eV and the ionization current was 1-300 µA. Based on the mass spectrum generated and NIST library search the compounds of GAE were identified.

3.8 *In silico* studies

The compounds isolated and identified from GAE by GC-MS were docked with the target proteins of the current study and their interactions were analyzed. The docking studies included the following steps (a) Preparation of protein (b) Preparation of ligand (c) Molecular docking and (d) Post-docking analysis.

3.8.1 Preparation of Protein

The crystal structures of proteins involved in apoptotic pathway (Bax, Bcl2, Bcl-XL, caspase-3, caspase-8, GSK3β), cell survival pathway (Akt, PI3K) and anti-inflammatory complex (IKBα-NFKB-p65-p50) were obtained from the Protein Data Bank.

The SYBL- X version 1.3 software (Tripos Inc., USA) was used to prepare the target proteins. The preferred protein input format of SYBL is the PDB format. The proteins were prepared by eliminating all the substructures and water followed by addition of hydrogen.

3.8.2 Preparation of ligand

The ligands were prepared based on the ligand preparation protocol of SYBL- X version1.3. The preferred ligand input format of SYBL is the SD file. The structures of Doxorubicin and Dexamethasone were downloaded from PubChem and used as standard compounds for molecular docking.

3.8.3 Molecular docking

The molecular docking of the ligands and the receptor proteins were carried out in the Surflex –Dock module of SYBL [107]. This software generates an automatic ProtoMol representing the binding site of the target protein where the ligand binds [Tripos 2006]. In this program, the ligands are flexible structures whereas the target proteins are rigid structures. For the current work, the ProtoMol- bloat was fixed as 0 and ProtoMol- threshold was fixed as 0.5 Å and the rest of the parameters were fixed as default values. The docking results were visualized in Pymol.

3.8.4 Post-docking analysis

After completion of docking, the SYBL generates a protein – ligand interaction profile based on the energy required and stability of the interaction. Based on the interaction profile, the best 10 confirmation poses were considered and the best pose was selected based on the orientation of the docked conformation and the conformation with the highest/best Consistent score (C score).The docking scores of the ligands were compared with that of the standard drugs used.

3.9 Determination of Toxicity

3.9.1 Acute and chronic toxicity analysis in Zebrafish

Adult Zebrafish were housed in pairs. The Zebrafish were fed orally with varying concentrations of GAE (100,250,500 µg/day) mixed with the fish pellet. The fishes were analyzed for behavioral changes and toxicology screening both acute (14 days) and chronic (30 days). The experiment was carried out in triplicate and repeated thrice. The control group represented the Zebrafish fed with normal fish feed.

3.9.2 Novel Zebrafish Tissue-Chip for toxicology analysis

The novel Zebrafish Tissue-chip was used to screen the efficacy of the GAE. The Lab on-Chip is a new platform that replaces the need for animal models.

The Tissue-Chip (Pentagrit, Chennai) used is a 3D scaffold that enables the cells from different organs to grow simultaneously in a single chip. Adult male Zebrafish were euthanized and the major organs (liver, heart, muscle, brain) were dissected, teased and converted to a single cell suspension. The mixture was centrifuged (9,000 rpm, 15 min). The cells settled to form a pellet which was mixed to form a single cell suspension. Approximately, 5µl of the cells from each organ was loaded to the separate scaffolds provided in the Tissue-Chip. The Cells were cultured in DMEM (without glucose and with 0.01% tetracycline) along with different concentrations of GAE (100,250,500µg/ml) at 37°C for 48 hrs.The tissue developed was removed and stained with Hematoxylin and Eosin stain and observed under the microscope to determine cell viability.

3.10 Determination of anticancer activity under *in vitro* conditions

3.10.1 Cell lines and maintenance

Cytotoxicity studies were carried out in Adenocarcinoma A549, Cervical cancer Hela, Colon cancer HCT-129, Liver cancer HepG2 cell lines obtained from NCCS, Pune, India and normal lung epithelial cell line L132 was received as a gift from Dr. Savithri Shivakumar, Aaranya Biosciences, Chennai. The cells were grown in DMEM supplemented with FBS (10%) and with antibiotic cocktail (GIBCO, USA). The cells were maintained at 37°C at 5% CO2. The cells were subcultured for further studies.

3.10.2 Analysis of cell viability (MTT assay)

The efficacy of GAE and GACs to inhibit the proliferation of tumor cells was determined by the MTT assay [134]. The monolayer of cells was trypsinized to obtain a single cell suspension of $2x10^5$ cells/ml. A volume of 100 µl of cells were loaded to each well of a 96 well microtitre plate. The cells were incubated for 24 hours in a CO2 incubator under appropriate conditions and allowed to proliferate to form a monolayer. The supernatant was removed and 100 µl of GAE (100-2000 µg/ml) /GACs (5-50 µg/ml) was added and incubated for 24 hours in a CO2 incubator. Following incubation, the supernatant was removed and added 20 µl of MTT (5 mg/ml) to the wells. The cells were incubated for 2 hours in a CO2 incubator and observed for the formation of formazan crystals. The dye was removed and 100 µl of isopropanol was added to each well to solubilize the crystals and shaken gently in dark for 20 minutes. The absorbance was read at 490 nm in a multimode plate reader. The cells which were not exposed to GAE/GACs served as control and the blank represented ethyl acetate alone. The cell viability was determined based on the formula

$$\% \text{ cell viability} = \frac{Ac - At}{Ac} \times 100$$

Where,

A_c represents absorbance of untreated cells and

A_t represents absorbance of GAE/GACs treated cells.

The experiment was carried out in triplicate and repeated three times to obtain consistent values. Doxorubicin treatment (100-2000 µg/ml) of A549 cells was used as reference standard. The same procedure was carried out with L132 cells and the efficacy of GAE on the viability of L132 cells was determined.

3.10.3 Analysis of Apoptosis

3.10.3.1 Cell biology studies

The nuclear fragmentation was detected under fluorescent and confocal microscope by using DAPI (4, 6 Diaminido- 2- Phenylindole Dihydrochloride), Propidium iodide (PI) [135] and Annexin V [136].

The monolayer of cells were treated with trypsin to get a single cell suspension. $2x10^5$ cells/ml were subcultured in cell culture plates to become 80% confluent. The cells were treated with the inhibitory concentrations (IC50) of GAE /GACs for 24 hours. The cells were fixed with ice-cold methanol (70%). Later, the cells were stained with DAPI (5 µl) and observed under the inverted fluorescent microscope (Zeiss1.0). The same protocol was utilized for propidium iodide (5 mg/ml) and AnnexinV staining.

3.10.3.2 Flow cytometry analysis

For FACS analysis, $5x10^3$ cells were subcultured in 35 mm Petriplates and exposed to GAE (1.5 mg/ml) for 6 hours. The cells were subjected to trypsinization, stained with DAPI (5 minutes) and analyzed in Beckman Coulter Moflo for apoptosis, changes in cell cycle and DNA content [137]. The cells without GAE treatment was served as control. The same protocol was followed for PI staining and the cells were analyzed in the flow cytometer.

3.10.3.3 Immunoblot analysis of apoptosis and cell survival cascade

Approximately, $5x10^5$ cells were subcultured in 100mm Petriplates and allowed to attain confluence. The cells were treated with GAE/GACs for 24 hours. The cells unexposed to GAE/GACs served as control. The cells were rinsed twice with PBS (ice-cold) followed by lysis with RIPA buffer. The cell contents were centrifuged at 14,000 rpm for 10 minutes. The supernatant was separated and stored (-20°C) for further analysis. The concentration of protein in the supernatant was determined by Lowry's method. The protein was subjected to SDS- PAGE (10%) and the separated proteins were transferred onto a PVDF membrane (BioRad, USA). 5% skim milk (BioRad, USA) was used to block the proteins followed by exposure to primary antibodies against Bax (1:500 Abcam, USA), Bcl-2 (1:500 Abcam, USA), Bcl-XL (1:500 Abcam, USA), Caspase 3 (1:200 Abcam, USA), PI3K (1:500 Abcam, USA), p PI3k (1:1000 Abcam, USA), Akt (1:500 GenetexBio, USA), p Akt (1: 1000 GenetexBio, USA) and β actin (1:1000 1:1000 Abcam, USA). The membrane was incubated with HRP-conjugated form of anti-mouse (1:2500) and anti-rabbit (1:2000) secondary antibodies for 1hour (RT) with constant shaking. The protein bands were viewed using the ECL staining kit (Amersham Pharmacia Biotech, Sweden).

3.11 Determination of antimetastatic activity

Metastasis requires digestion of ECM, followed by migration and colony formation of cells at secondary sites. The antimetastatic activity of GAE was determined based on Scratch assay, clonogenic assay and expression of matrix metalloproteinases (MMP2 and MMP9) by immunoblotting.

3.11.1 Scratch assay

The inhibitory effect of GAE on cell invasion was determined by the wound healing / Scratch assay [138]. Actively dividing A549 cells ($2x10^5$ cells/ml) were subcultured in Petriplates to attain 90% confluence and treated with serum-free DMEM medium for 12 hours. The cells were scratched with a 200 µl micropipette tip and a wound was created. The cells were washed with PBS, and exposed to serum-free DMEM medium comprising varying concentrations of GAE (100-1500 µg/ml). The cells that invaded were fixed with cold methanol (75%) for 30 minutes and treated with ice-cold PBS twice. The cells that migrated the wounded area were imaged at 0 and 24 hours.

3.11.2 Clonogenic assay

The efficiency of single cells to maintain their reproducing potential and proliferate into a colony was determined based on the clonogenic assay [139]. Actively dividing A549 cells ($2x10^5$ cells/ml) were subcultured in 6 well plates to become confluent. The cells were treated with various concentrations of GAE (100 – 1500 µg/ml) for 24 hours. The cells were subjected to trypsinization and a single cell suspension was obtained. Approximately 500 cells, were seeded in a 6 well plate and incubated for 7 days, stained with Trypan blue and observed under the microscope.

3.11.3 Immunoblot analysis of MMP expression

The antimetastatic activity of GAE/GACs was determined based on the expression of MMP2 and MMP9 [138]. Actively dividing A549 cells ($2x10^5$ cells /ml) were subcultured in 6 well plates. The monolayer of cells was treated with GAE/GACs (24 hours). The cells unexposed to GAE/GACs served as control. The total protein was isolated by digestion with RIPA buffer and analyzed for the expression levels of MMP2 and MMP9 in the treated and control groups. Approximately 50µg of total cell protein was separated by SDS-PAGE (10%) and transferred to PVDF membrane by semi-dry transfer method. The nonspecific binding of antibodies was inhibited by blocking with skim milk (5%) with 0.1% Tween 20. The PVDF membrane with transferred proteins was incubated with primary antibodies against MMP2 and MMP9.The antibodies were used at 1:200 dilution, and exposed to HRP conjugated secondary antibody (1:500). The enhanced chemiluminescent kit was used to visualize the immune complex in ChemiDoc. The intensity of the bands was compared between the control and the treated samples.

3.12 Determination of anti-inflammatory activity

3.12.1 Analysis of NFKB activity

The efficacy of GAE and GACs on inflammation was analyzed by western blot. The activation of NFKB is marked by the phosphorylation of NFKB-p65 at Ser 536 which translocates to the nucleus to activate the expression of proinflammatory cytokines and anti-apoptotic proteins (Bcl2 and Bcl-XL). Single suspension of cells ($2x10^5$ cells /ml) was grown in 6 well plates. The monolayer of cells was treated with GAE/GACs for 24 hours. The cells that were not subjected to GAE/GACs treatment served as control. The protein was isolated by RIPA buffer digestion and analyzed for the expression of proteins in the NFKB pathway in the treated and control

groups. Approximately 50 µg of total cell protein was separated by SDS-PAGE (10%), followed by transfer to PVDF membrane through semi-dry transfer. The non - specific binding of antibodies was inhibited by blocking with skim milk (5%) with 0.1% Tween 20. The nitrocellulose membrane was incubated with primary antibodies of NFKB, p-NFKBP65-S536, TNFα and IL-1β.The antibodies were used at 1:200 dilution, followed by exposure to HRP conjugated secondary antibody (1:500). The enhanced chemiluminescent kit was used to visualize the immune complex in ChemiDoc. The intensity of the bands was compared between the control and the treated samples.

3.12.2 Analysis of anti- inflammatory marker

The inhibition of NFKB is the primary mechanism behind the regulation of inflammatory process, the expression level of IL-10 was considered as an anti-inflammatory marker. The expression of IL-10 was analyzed in the control and algal extract treated samples by western blot using the primary antibody (IL-10, 1:500).The immunodetection was performed using the chemiluminescent kit.

3.12.3 Analysis of gene expression by Real-Time PCR

Expression of genes regulating apoptosis, cell survival and inflammation were analyzed by Real-Time PCR.

3.12.3.1 Isolation of RNA

Approximately $2x10^5$ cells/ml were subcultured and exposed to inhibitory concentrations (IC50) of GAE/GACs for 6 hours. The cells treated only with media served as control. Following incubation, the media was removed and 300 µl of TRIzol reagent was added to the cells and the mixture was homogenized. The homogenate was incubated at room temperature (5 minutes), for the dissociation of nucleoprotein complex and added 200µl of chloroform and shaken vigorously.

The contents were left undisturbed (3 minutes), centrifuged at 12,000 rpm (15 minutes,4°C). The mixture was separated into the top aqueous phase, an interphase and a lower phenol phase. The aqueous phase harboring the RNA was transferred to a new tube followed by addition of 500 µl isopropanol and incubated for 10 minutes at room temperature. The contents were centrifuged at 12,000 rpm for 10 minutes (4°C). The total RNA precipitated at the bottom of the tube was washed with 1 ml of 75% ethanol and centrifuged at 7,500 rpm for 5 minutes at 4°C. The supernatant was discarded, the RNA pellet was air dried and suspended in 50 µl of RNase free water and stored at -80°C until use.

3.12.3.2 Synthesis of cDNA

cDNA was synthesized from the isolated RNA using the high capacity cDNA Reverse Transcription kit (Applied Biosystem).

3.12.3.4 Real-Time PCR

The cDNA synthesized was used for Real - Time analysis of gene expression using BioRad SYBR Green PCR Master mix in CFX96 TOUCH BioRad. The program (95°C for 3 min, 40 cycles of 95°C for 15 sec and 55°C for 30 sec) was followed. Melt curve analysis was also carried out. The primers used are shown in Table 3.1.

Table 3.1 List of primers used for Real-Time PCR.

Genes	Primer sequences used
Bcl-2	(F) 5'-CTGCACCTGACGCCCTTCACC-3'
	(R) 3'-CACATGACCCCACCGAACTCAAAGA-5'
Bcl-xl	(F)5'-GATCCCCATGGCAGCAGTAAAGCAAG -3'
	(R 3'-CCCCATCCCGGAAGAGTTCATTCACT-5'
Bax	(F) 5'- GGA CGA ACT GGA CAG TAA CAT GG-3'
	(R 3'- GCAAAGTAGAAAAGGGCGACAAC-5'
Caspase -3	(F) 5-'TTAATAAAGGTATCCATGGAGAACACT-3'
	(R 3'-TTAGTGATAAAAATAGAGTTCTTTTGTGAG-5'
Caspase-8	(F) 5'- GGATGGCCACTGTGAATAACTG-3'
	(R 3'- TCGAGGACATCGCTCTCTCA-5'
PIK3CA	(F) 5'-TGCAAAGAATCAGAACAATGCC-3'
	(R 3'- CACGGAGGCATTCTAAAGTCA-5'
PTEN	(F) 5'- CCAGGACCAGAGGAAACCT-3'
	(R 3'- GCTAGCCTCTGGATTTGA-5'
Akt	(F) 5'- CATCACACCACCTGACCAA-3'
	(R) 3'- CTCAAATGCACCCGAGAAAT-5'
GSK3β	(F) 5'- GGAACTCCAACAAGGGAGCA-3'
	(R) 3'- TTCGGGGTCGGAAGACCTTA-5'
NFKB	(F) 5'-CCAGACCAACAACAACCCCT-3'
	(R) 3'-TCACTCGGCAGATCTTGAGC-5'
TNFα	(F) 5'-CCCAGGGACCTCTCTCTAATCA-3'
	(R) 3'-GCTTGAGGGTTTGCTACAACATG-5'
IL 1β	(F) 5'-AAATACCTGTGGCCTTGGGC-3'
	(R) 3'-TTTGGGATCTACACTCTCCAGCT-5'
IL 10	(F) 5'-CATCGATTTCTTCCCTGTGAA-3'
	(R) 3'-TCTTGGAGCTTATTAAAGGCATTC-5'
β Actin	(F) 5'- TAGAAGCCTCTTCATGGACAAC-3'
	(R 3'- GTATCAGGCATGCAACACAAG-5'

(F: denotes forward primer, R: denotes reverse primer)

The program used included 98°C for 2 minutes, 25 to 40 cycles (98°C) for 10 seconds, 55°C for 5 seconds, and 72°C for 20 seconds. The analysis was done in triplicate and the fold change in gene expression was calculated based on $2^{-\Delta\Delta ct.}$

3.13 Determination of anticancer activity under *in vivo* conditions

Zebrafishes, male weighing approximately 1.5 ± 0.5 g were used to develop tumors [119].The fishes were maintained in three groups namely the normal control (un-induced), tumor induced and tumor treated. Each group harbored 6 fishes each. A549 cells (5 µl) were injected in the muscle region of the fishes twice in 7 days interval and the fishes were maintained in 14/10 hours light and dark cycles, with normal fish feed for a period of 60 days until they developed tumors. The fish were dissected to confirm the formation of tumors. Later, the tumor-induced fishes were fed with feed containing GAE/GACs (15, 30, 45 and 60 µg/day) for a duration of 10 days. Following this, the fishes were dissected and the muscle anatomy, tumor anatomy and pathology were examined. The treatment was given in triplicate with two fishes/group/dosage. The same protocol was followed for inducing tumors in the colon by injecting HCT cells in the intestinal region and Hep G2 cells were used to induce liver tumors in Zebrafish.

The anticancer activity was determined based on the histological studies and the expression of proteins involved in apoptosis, cell survival and inflammation analyzed by Western blotting.

3.14 Statistical analysis

Statistical analysis were performed using SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). Student's t-test was used and the data were expressed as the mean ± SD. Each experiment was repeated at least 3 times. $p<0.05$ was considered to indicate a statistically significant difference.