3. Material and method

3.1 Chemicals and Reagents

Dimethyl sulphoxide (DMSO), diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), 3-4, 5- dimethylthiazol-2, 5 biphenyl tetrazolium bromide) propidium iodide (MTT), 3, 3′-diaminobenzidine (DAB) tablet, carboxymethylcellulose (CMC), paraformaldehyde (PFA) and citrate buffer were procured from Sigma Aldrich. Himedia provided the fetal bovine serum (FBS), HEPES buffer, eosin, haematoxylin, and crystal violet.

Tris-buffered saline (TBS), Tween 20, Enhanced chemiluminescence (ECL), Radioimmunoassay precipitation assay (RIPA) buffer, Bicinchoninic acid assay (BCA) kit, protease/phosphatase inhibitor, bovine serum albumin (BSA), ponchau stripping buffer, Lamillae buffer/dye, Polyvinylidene difluoride (PVDF) membrane was obtained from Bio-Rad. Carbon tetrachloride (CCl_4), xylene, 2-propanol, Tween 80 were obtained from SDFCL. We bought the Matrigel from Corning India and the ABC kit from Vector Laboratory. Hydrogen peroxide (H_2O_2), Triton X 100, methanol, hydrochloric acid (Hcl), sodium azide, magnesium chloride, and sodium hydroxide were purchased from Merck, India. The supplier of isoflurane was Raman and Weil Pvt. Ltd.

Propidium iodide, Annexin V, and FITC were acquired from BD Biosciences. While antibodies against CD31, ERK, p-ERK, and M-actin were acquired from Cell Signaling Technology, antibodies against Ki67 were procured from Abcam.

Sun Pharma Industries Limited provided doxorubicin and sorafenib. We bought the Sorafenib tablet (Soranib^{TM}, Cipla Ltd.) from Maheshwari Medical Agency in Ahmedabad.

Slides with a positive charge were acquired from VWR. Purchasing items from Gibco included sodium pyruvate, Eagle's Minimum Essential Medium (EMEM), penicillin (100 I.U./mL), streptomycin (0.1 mg/mL), 0.05% trypsin/EDTA, and L-glutamine. Rest all of the reagents were of analytical grade.

3.2 Animals

In this study, 180-200 gram body weight male and female wistar rats, aged 6–8 weeks, were used. In the limit test assay, female rats were used to evaluate the safety of the extracts. Male rats were used for chemically induced HCC animal model.

For HepG2 cell line induced (xenograft) animal model, male and female athymic nude mice were used. The mice were of 18-24 gm body weight with 6-8 week age. The rats and mice were received from Sun Pharma Advanced Research Company Limited. The animals were acclimatized for one week before grouping and beginning of the study.

The experimental protocol for conducting animal studies was approved by the Institutional Animal Ethics Committee (IAEC) of Sun Pharma Advanced Research Committee Ltd., IAEC No. 908 in 72th IAEC meeting held on 21 August 2021 (Annexure 8.5). All experimental method was carried out in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate Change, Government of India in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility. These animals were housed in individually ventilated (IVC) cages under controlled conditions. Cages were maintained under constant temperature (18-26°C), humidity (30%-70%) and lighting conditions (12 h light and 12 h dark). Animals received reverse osmosis (RO) water and Harlan rodent standard normal chow diet *ad libitum*.

The human hepatoma cell line HepG2 was received from In-vitro Biology Department, Sun Pharma Advanced Research Company Ltd (SPARCL). The cells were grown in EMEM medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/mL), and penicillin (100 U/mL) and kept at 37°C with 5% CO_2.

3.3 Collection of plant materials

The roots of *A. aspera* and *B. diffusa*, and the entire parts of the plant of *E. littorale* were purchased from Dr. Hitarthini N. Chudasama, Vinayak Ayurved Clinic, Waghodia Road, Vadodara for preparation of different extracts.

3.4 Herbarium preparation and plant identification

For identification purposes, a herbarium of all three plants was prepared and submitted to the BARO Herbarium in charge at the Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda. Dr. P. Nagar, an Associate Professor in the botany department at The MS University of Baroda's faculty of science, verified the authenticity of the plant using specimen numbers K000357272 (*A. aspera*), K000438312 (*E. littorale*), and K001138105 (*B. diffusa*) and issued the certificate on 16 October 2020 (Annexure 8.1).

3.5 Extraction of plant material

To prepare the plant material for various extracts, it was shade dried, pulverized to a coarse powder in a grinder, and defatted using petroleum ether. Plant material (500 gm) in the form of coarse powder was packed into the Soxhlet apparatus (Borosil®) and extracted; the extraction process was continued until the solvent in the siphon tube become colorless. Using Soxhlet equipment, the three plants were extracted individually using petroleum ether, ethyl acetate, alcohol, hydro alcohol and distilled water. All the extracts were concentrated at 40˚C under low pressure using a rotatory evaporator (32). The extracts were collected and kept at -20˚C until needed again in tubes that were appropriately labeled. All the extracts were subjected to proximate analysis. Also the extractive values of the extract were determined.

Alcoholic extracts of all the three-plants was commercially procured from Amsar Pvt Ltd., Indore (Annexure 8.2 to 8.4).

All the extracts (total 15) were subjected to MTT assay as described in section 3.10.1.MTT assay was the primary screening assay for shortlisting of the extracts for further studies. Among all the 15 extracts, alcoholic extracts of three plants showed highest cytotoxicity and better IC_{50} value as compared to other extracts. These three alcoholic extracts were screened in pilot anti-cancer activity employed in HepG2 cells tumor bearing mice post acute toxicity study as per the OECD guideline. Among these three extracts alcoholic extract of *A. aspera* and *B. diffusa* showed better anti-cancer activity as compared to alcoholic extract of *E. littorale*.

So all further in-vitro, in-vivo studies, LCMS analysis of extract and molecular docking was performed with alcoholic extract of *A. aspera* and *B. diffusa.*

3.6 Phytochemical screening

A. aspera, *B. diffusa*, and *E. littorale* alcoholic extracts were subjected to phytochemical analysis using standard laboratory procedures in order to determine the presence of a variety of secondary metabolites, such as phenols, steroid glycosides, alkaloids, flavonoids, saponins, tannins, reducing sugars, protein, anthraquinones, quinones, fat, and fixed oil (186–187).

3.7 Phytochemical analysis by LCMS

The alcoholic extracts of *A. aspera* and *B. diffusa* were subjected to LCMS analysis for qualitative detection of various classes of phytoconstituents. The analysis was conducted

using a Xevo TQD Triple Quadrupole Mass Spectrometry instrument. Following 45 minutes of sonication, extract (1 mg) was centrifuged for 10 minutes at 13,000 rpm, supernatant was collected and filtered using 0.22 µm membrane filter. The filtrate was then separated using, The XBRIDGE BEH AMIDE C18 (150 X 2.1) mm 2.5 µm column. 5 mM ammonium acetate as mobile phase A and acetonitrile as mobile phase B was used in the separation process. The flow rate of 0.3 mL/min for mobile phase, and the column temperature of 35 °C were maintained during the separation process. The gradient elution program used during the elution process was described in Table 3.1 (188).

Table 3.1: Mobile phase and elution program

Time (minute)	5 mM Ammonium Acetate	Acetonitrile
0 to 6	95%	5%
6 to 12	70%	30%
12 to 20	40%	60%
20 to 26	20%	80%
26 to 30	95%	5%

3.8 Molecular docking

Selected phytochemicals (5 compounds) identified by the LCMS method from alcoholic extract of B. *diffusa* and *A. aspera* were used as ligands for the molecular docking analysis.

The two proteins, p53 (tumor suppressor gene; PDB ID: 1TUP) and PI3K alpha (protein involved in proliferation; PDB ID: 5itd). The protein VEGFR 2 (PDB ID: 4ASD) having important role in angiogenesis and neovascularization.

3D structure of all the three protein was downloaded from the Protein Data Bank. Existing ligand and water molecules were removed using Biovia Discovery Studio 2020 visualizer (Version 2020; BIOVIA, Dassault Systèmes, USA). Auto Dock Tool Version 1.5.6 was used for preparation of protein structures, including the steps of “add hydrogens,” “add Kollman charges,” and “exported into a dockable pdb format for molecular docking.” The 3D structures of active compounds of extracts were downloaded from the PubChem database, and all ligands were converted to dockable pdb format using Open Babel 3.1.1.

Using Auto Dock Vina 1.1.2, the bioactive chemicals were molecularly docked on the target proteins. Docking scores were reported in kcal/mol. Finally, molecular interactions between the proteins and ligands were visualized by Biovia Discovery Studio Visualizer (189).

The interaction between receptor VEGFR 2, p53 and PI3K alpha and phytochemicals of both the extracts were anticipated using the Vina score.

There are two modes of docking calculations: XP (Extra Precision) and an SP (Standard Precision) method. Thus concerning the accuracy, the Glide module of XP visualizer was used in this study to estimate the binding affinity.

The software was generated a list of docked poses for each ligand with the docking scores, ligand-protein interactions, and binding modes to identify potential inhibitors for desired proteins as mentioned in Table 3.2 (190).

The three proteins with their PDB ID are as mentioned in Table 3.2

Table 3.2: Docking protein with their PDB ID

Sr. No.	Protein	PDB ID
1	VEGF (VEGFR 2)	4ASD
2	p53	1TUP
3	PI3K alpha	5itd

3.9 Cell culture

HepG2, a cell line that causes liver cancer was grown in EMEM medium that has been supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 U/mL penicillin. All cells were grown in a humidified environment with 5% CO_2 at 37°C. The result of MTT assay was deciding factor for fate of the extract for further screening in in-vitro and in-vivo experiment as shown in the Figure 3.1.

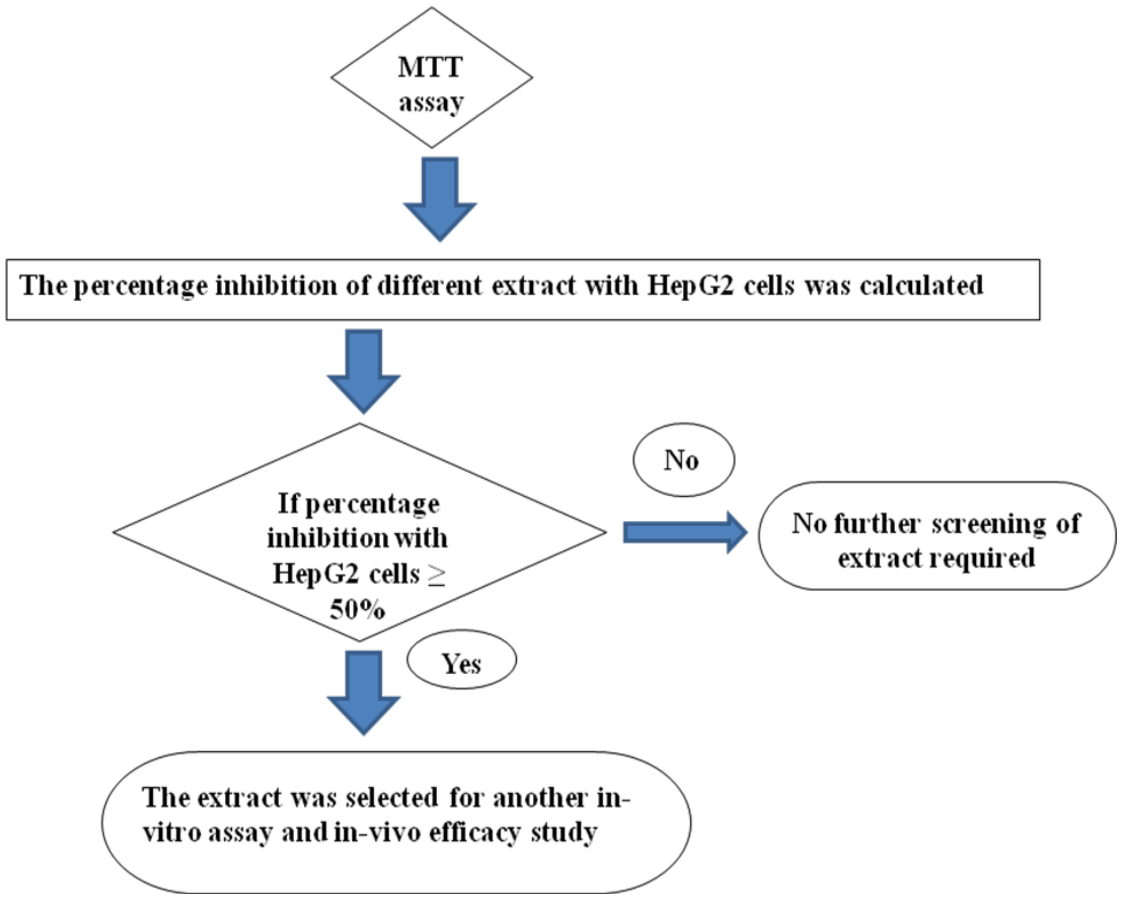


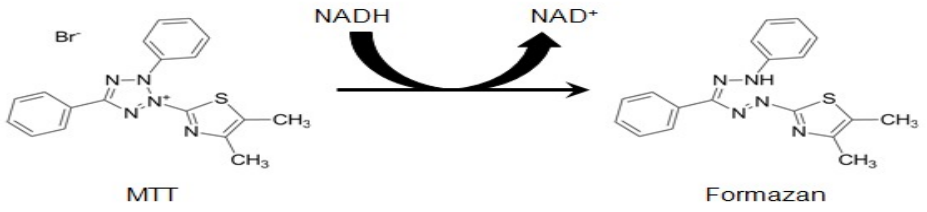
Figure 3.1: Flow chart for selection of extract for in-vitro and in-vivo study based on MTT assay

3.10 In-vitro assay

3.10.1 MTT cell viability assay [33, 192]

Principle

Cell viability is determined by measuring cellular metabolic activity using the MTT test. This colorimetric assay relies on the transformation of purple formazan crystals into a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) by metabolically active cells. The MTT is converted to formazan by the NAD(P)H-dependent oxidoreductase enzymes found in the live cells as shown in Figure 3.2 (191). The insoluble formazan crystals were dissolved using DMSO and the resulting-colored solution is quantified by measuring absorbance at 570 nanometers using a spectrophotometer. The darker the solution, the greater the number of viable, metabolically active cells.



Yellow colour Purple colour

Figure 3.2: Mechanism for conversion of MTT to formazan

Procedure:

MTT was employed in the cytotoxicity assay to measure the viability of the cells. MTT assay is the colorimetric assay which measures the reduction of MTT (yellow) to an insoluble colored (dark purple) formazan product produced by mitochondrial succinate dehydrogenase.

In brief, 96-well plates were seeded with 5×10^3 HepG2 cells for 24 hours. The alcoholic and aqueous extract of all the three plants, *B. diffusa*, *A. aspera* and *E. littorale* were prepared in water. The remaining extracts (hydro-alcoholic, ethyl acetate and petroleum ether) as well as reference standard sorafenib were made in DMSO. Following that, the cells were cultured independently for 48 hours with concentrations of petroleum ether, ethyl acetate, alcohol, hydro alcohol, and aqueous extracts of all three plants ranging from 5 to 640 µg/mL as well as the reference standard sorafenib at doses of 0.156 to 20 µM.

At the end of treatment, each well received 15 µL of MTT (5 mg/mL) and was incubated for an additional 4 hours. The formazan crystals were dissolved by adding 100 µL of DMSO in each well after the supernatant was removed. The absorbance of every sample at 570 nm was measured using a microplate reader. To calculate the viability, the following equation was applied.

Cell viability percentage = [1-(ODt/ODc) x 100%],

Where ODc and ODt represent the mean optical density of wells untreated (control) cells and cells treated with the test compound. The concentration essential to cause fatal effects in 50% of intact cells (IC_{50}) was calculated using a dose-response curve (149, 152)

3.10.2 Wound healing assay (Scratch motility assay) [33, 193]

Principle

The scratch-wound assay measures the basic cell migration parameters such as speed, persistence, and polarity. Cells are grown to confluence and a thin "wound" introduced by scratching with a pipette tip. Cells at the wound edge polarize and migrate into the wound space.

Procedure

For the determination of HepG2 cells migration capacity, the wound healing assay was performed. In brief, 12-well plate was seeded with 10^5 cells per well, and the cells were grown to reach the confluency. Then, cells monolayer was scratched with a sterile 20–200 µL micropipette tip, washed twice with sterile PBS to take away the loose cells or debris. The "wounded" area is photographed immediately after wounding and recorded as a baseline picture. Cells were treated for 48 hours at the IC_{50} concentration of the alcoholic extract of *A. aspera* and *B. diffusa* obtained by MTT assay in order to examine the impact of extracts and standard on the cell migratory capacity. Following treatment, phase contrast image of cells moved inside the scratched area were captured. Using Image J software, the difference between the initial width of the wound and its post-cell migration width was measured. The percentage of wound healing (closure) was computed using the following formula (33, 193).

Wound healing (%) = $((W_0-W_t)/W_0) \times 100$

Where, W_0 denotes the wound area at 0 hours and W_t denotes the wound area following incubation at indicated time.

3.10.3 Colony formation assay[7, 194]:

Principle

The colony formation assay, or clonogenic assay, is designed to measure the ability of a single adherent cell to survive over time and expand into a clonal population. For applications such as cancer drug screening, it is important to distinguish cells that retain this proliferative capacity from those that do not.

Procedure

To investigate antiproliferation capabilities of the extracts on HepG2 cells, colony formation assay was performed. In a 6-well plate, 500 HepG2 cells were seeded per well. Following a 24-hour period, the cells were exposed to sorafenib at their IC_{50} concentration for 48 hours

together with the alcoholic extracts of *A. aspera* and *B. diffusa*. On every 3-4 days the old media was replaced with the new media. Following a 14-day incubation period, the formed colony were rinsed with PBS, fixed for 20 minutes with 4% paraformaldehyde, and then stained for 10 minutes at room temperature with 0.5% crystal violet. Finally, for the quantification, the images were captured in microscope and total number of colonies per well were counted manually by eye (7, 194).

3.10.4 Assay of apoptosis (Annexin V FITC/PI Assay) [193, 7]

Principle

One can quantify the proportion of cells in a population that are actively going through apoptosis using FITC Annexin V. It depends on the ability of cells to lose asymmetry in their membranes during the initial stages of apoptosis. Phospholipid (PS), a membrane phospholipid, is translocated from the inner to the outer layer of the plasma membrane in apoptotic cells, opening it up to the outside environment. A calcium-dependent phospholipid-binding protein with a strong affinity for PS, Annexin V is helpful in recognizing cells that have undergone apoptosis and have exposed PS. A standard flow cytometric viability probe used to differentiate between viable and nonviable cells is propidium iodide (PI). The membranes of dead and injured cells allow PI to get through, whereas the membranes of viable cells with intact membranes do not. Apoptosis is occurring in cells that stain negative for PI and positive for FITC Annexin V. When a cell expresses both FITC Annexin V and PI, it indicates that it is either toward the end of its apoptotic process, going through necrosis, or has already dead. When cells do not exhibit detectable apoptosis and stain negative for both FITC Annexin V and PI, they are still viable.

Procedure

Using Annexin V-FITC and propidium iodide (PI) double staining, apoptosis was investigated and examined using flow cytometry. The assay was carried out in compliance with the manufacturer's instructions. Following a 24-hour incubation period in six-well plates, HepG2 cells (5.5×10^6/mL) were subjected to a 48-hour treatment with sorafenib and alcoholic extracts of *A. aspera* and B. diffusa. After that, the cells were gathered into tubes, cleaned with PBS, and centrifuged for ten minutes at 300 ×g. Following centrifugation, the cells were incubated at room temperature in the dark for 15 minutes with 5 µl of FITC Annexin V. Then, 400 µl of 1X binding buffer and 5 µl of PI (50 µg/ml) were added,

vortexed gently, and immediately processed for flow cytometry analysis to differentiate the necrotic, apoptotic, and intact cells (7, 193).

3.11 Evaluation of the extract in in-vivo animal model

3.11.1 Evaluation of the extracts for safety by acute toxicity study[195]

Acute oral toxicity test was performed as per OECD guideline 423 (196). Nulliparous, non-pregnant, healthy young adult female rats weighing 200–250 grams were used in the study. Female rats were chosen because literature reviews of traditional LD_{50} testing indicate that, in most circumstances, there is minimal variation in sensitivity between the sexes; nevertheless, in those instances when variations are noted, females tend to be marginally more sensitive. Prior to dosage, the animals were housed in their cages for a week to enable them to become used to the lab environment. The humidity and room temperature were kept at 30-70% and 22°C (± 3°C), respectively. There was artificial lighting with a 12-hour light and 12-hour dark cycle.

The animals were given a continuous supply of purified drinking water and commercially available standard pellet chow to eat.

Procedure for limit test

Animals were given alcoholic extracts of *A. aspera, B. diffusa,* and *E. littorale* orally in a single dosage after an overnight fast. The dose volume of extracts was given at 2 mL/kg body weight. Following the period of fasting, the fasted body weight of each animal was determined. The body weight was taken into account while calculating the dose. The extracts at a dose of 2000 mg/kg were given orally, and food was not allowed for an additional three to four hours after the extracts were administered. After then, for a maximum of 14 days, animals were monitored at least twice a day to look on any signs of toxicity, changes in behaviour, or survival.

3.11.2 Development and standardization of chemically induced animal model for HCC

3.11.2.1 Diethyl nitrosamine (DEN) and 2- acetyl amino furorene (2-AAF) induced HCC in rats[30, 141]

For the experiment, male Wistar rats weighing 200–250 g were utilized. Based on their body weight, the animals were randomly divided into two groups (n = 6 per group). The first group (control) was vehicle treated group.

The second group was given a single intraperitoneal injection of DEN at a dose of 200 mg/kg, i.p. After 2 week of DEN administration, 2-AAF was given at 30 mg/kg, p.o. daily for 2 week as shown in Figure 3.3.

DEN was prepared in saline. Formulation of 2-AAF was prepared in 0.5% carboxy methyl cellulose (CMC). Required quantity of 2-AAF was weighed and transferred to mortar then it was triturated with 0.5% CMC with the help of pestle. The 2-AAF was administered at 2 mL/kg dose volume (195,197).

The baseline body weight was measured on day one before DEN administration and every other day throughout the experimental period for all rats in each group. The weight gain was calculated and expressed as a percentage with following formula:

$$\%\,\text{Weight gain} = \frac{(\text{Final weight - Initial weight})}{\text{Initial weight}} \times 100$$

% Weight gain = (Final weight - Initial weight)/Initial weight x100

At the end of the experimental period, animals were fasted overnight; blood was collected from the retro-orbital plexus under the isoflurane anesthesia in non heparinzed tube. The slow CO_2 inhalation was utilized for euthaniztion of animals. After 30 minutes at ambient temperature, the blood was centrifuged for 10 minutes at 3000 rpm and 24°C; the separated serum was stored in a -70°C freezer until analysis. The liver and spleen was quickly isolated, washed with saline, blotted dry on filter paper, and weighed. The rat liver and spleen index were calculated as follows:

Liver index = liver wet weight (g) / body weight (g) × 100%;

Spleen index = spleen wet weight (g) / body weight (g) × 100%.

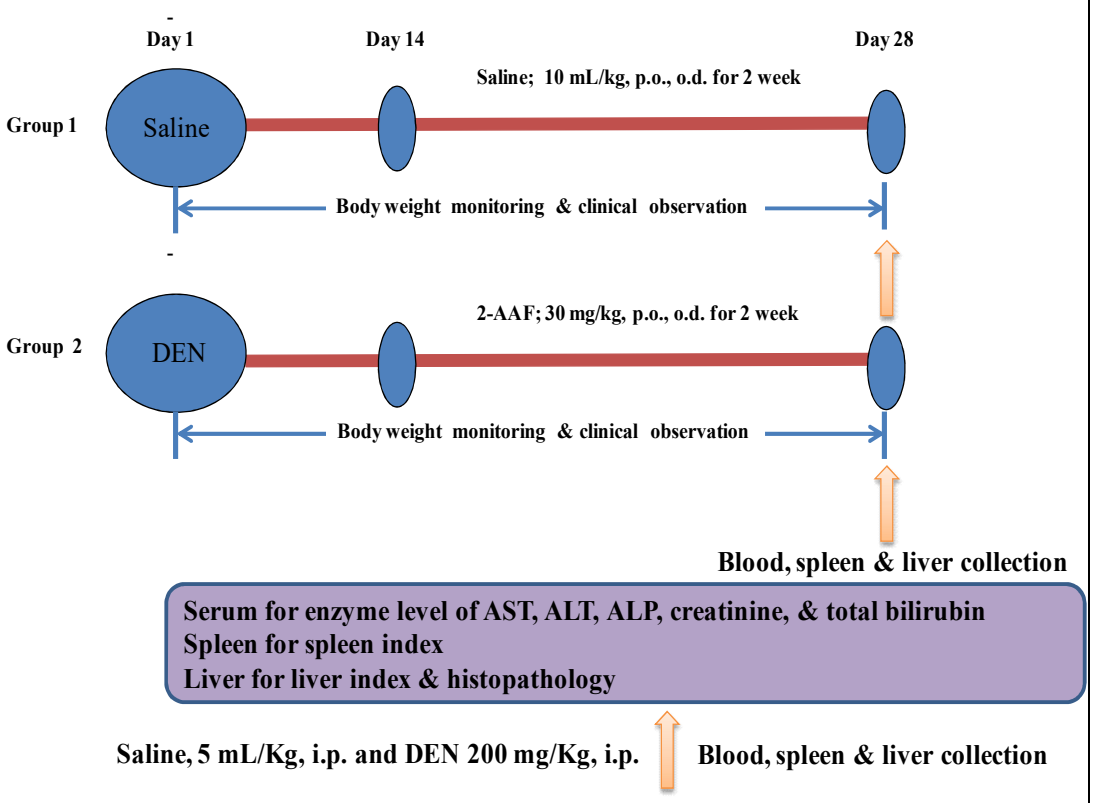


Figure 3.3: Systematic diagram for DEN and 2-AAF induced HCC model in rats

Serum biochemical analysis

The serum was analysed for aspartate transaminase (AST), serum alanine transaminase (ALT), alkaline phosphatase (ALP), creatinine, and total bilirubin in an automated biochemical analyzer (model Daytona, Randox Laboratories Ltd., Antrim, UK) using commercial kits (Randox® Laboratories Ltd., Antrim, UK) according to the manufacturer´s recommendations (198).

Histopathology of liver

At the end of the study, liver from each rat was be excised after dissection. A small piece from liver of each rat of all groups was fixed in 10% neutral buffered formalin for 24 h. Then, all specimens were cleared and embedded in paraffin. A paraffin block of liver tissues were prepared and 5 micron sections taken on slides for staining. The obtained tissue sections were stained by hematoxylin and eosin (H&E) stain. The examination of section was performed under the microscope for the presence of cancerous cells and any other pathological changes to confirm the development of the disease.

3.11.2.2 Diethyl nitrosamine (DEN) and CCl_4 induced HCC in rats[200-202]

For the experiment, male Wistar rats weighing 200–250 g were utilized. Two groups of six animals had been formed by random selection from among the animals. The first group (control) received vehicles used for preparation of DEN and CCl_4. The second group was injected with DEN (200 mg/kg i.p.) to initiate hepatocarcinogenesis. Two week later animals in group 2 were given a single dose of CCl_4 (2 mL/kg i.g) by gavage as 1:1 dilution in corn oil to stimulate liver cells proliferation and regeneration according to previously published protocols. In group 3, two more dose of CCl_4 (2 mL/kg i.g) was given at the interval of one week each of post 1st CCl_4 administration as shown in systematic Figure 3.4 (199-201).

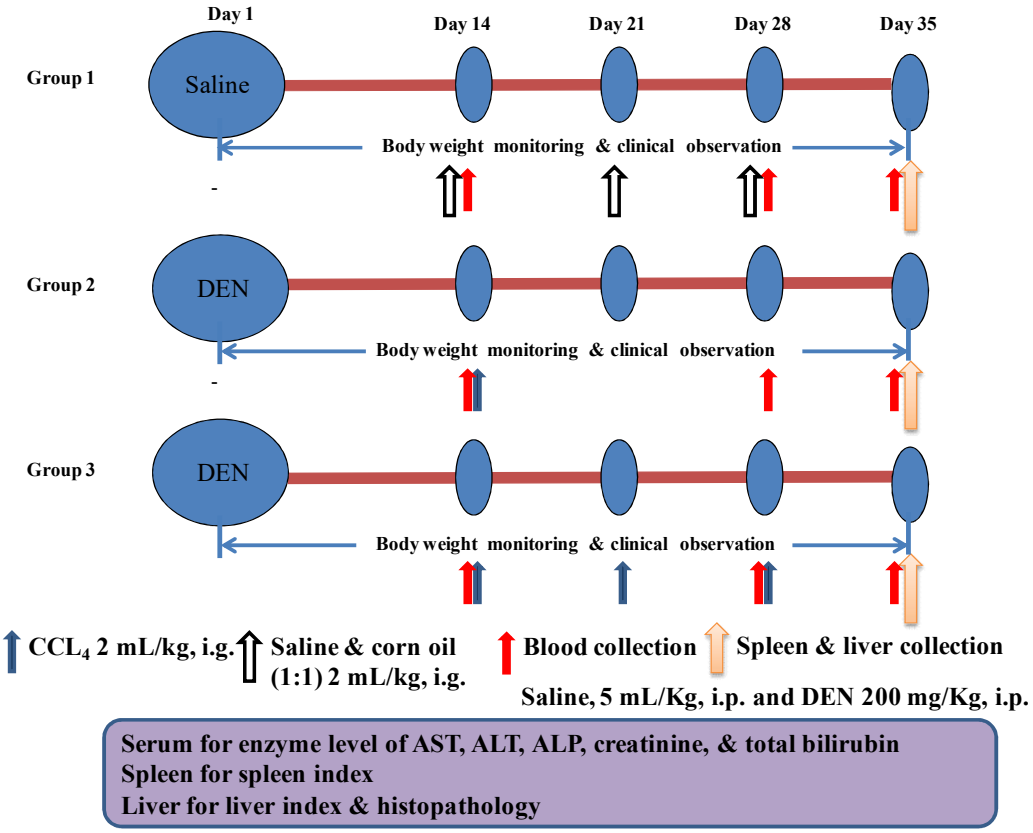


Figure 3.4: Systematic diagram for DEN and CCl_4 induced HCC model in rats

Blood samples were collected by retro-orbital plexuses periodically on day 14 (2nd week post DEN injection) day 28 and day 35 under isoflurane anesthesia into non-heparinized eppendorf tubes. Blood was kept at ambient temperature for 30 minute and then centrifuged at 3000 rpm at 24°C for 10 minute; serum was separated and kept in -70°C freeze until

analysis. At the end of treatment protocol, after 5 weeks rats were fasted overnight and euthanized by a slow CO_2 inhalation and the liver and spleen was quickly isolated, washed with saline, blotted dry on filter paper, and weighed. Percentage change in body weight as compared to day 1, and liver index and spleen index was calculated.

Serum biochemical and tumour markers estimation

Serum was analysed for serum aspartate transaminase (AST), serum alanine transaminase (ALT), alkaline phosphatase (ALP), total protein, total bilirubin, and creatinine in an automated biochemical analyzer (model Daytona, Randox Laboratories Ltd., Antrim, UK) using commercial kits (Randox® Laboratories Ltd., Antrim, UK) according to the manufacturer´s recommendations. Alpha feto protein (AFP) content in serum samples were analysed at pathology laboratory, Dr Udyan Laboratory, Vadodara (197).

Histopathology of liver

At the end of the study, liver from each rat was excised after dissection. A small piece from liver of each rat of all groups was fixed in 10% neutral buffered formalin for 24 h. Then, all specimens were cleared and embedded in paraffin. A paraffin block of liver tissues were prepared and 5 micron sections taken on slides for staining. The obtained tissue sections were stained by hematoxylin and eosin (H&E) stain. The examination of section was performed under the microscope for the presence of cancerous cells and any other pathological changes to confirm the development of the disease.

3.11.3 Development of tumor model with HepG2 cells in nude mice

Male and female nude mice of 6-8 week-old were used for the study. These animals were housed under controlled conditions (temperature 22 ± 2°C, relative humidity 30% to 70%) with a natural light-dark cycle and acclimatized for a minimum of one week prior to execution of the experiment.

The hepatic liver cancer cell line, $HepG_2$ was cultured in EMEM medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/mL), and penicillin (100 U/mL). All cells were fostered at 37°C in a humidified atmosphere containing 5% CO_2. The cells were counted with trypan blue in nuebauers chamber. The cells were diluted in EMEM medium to obtain the required number of cells. Human hepatocellular carcinoma HepG2 cells were inoculated into immunodeficient nude mice to establish the animal model of HCC.

3.11.3.1 Standardization of tumor model with different HepG2 cell concentration

The HepG2 cell at concentration of 5×10^7 cells/mL was collected in sterile falcon tube. Required quantity of cell suspension was further diluted with media to get final cell concentration of 1×10^7 and 5×10^6 cells/mL. Then further cells were mixed at 1:1 volume with ice cold BD-Matrigel (Corning, India) and injected subcutaneously with a volume of 0.2 ml on the right rear flank of nude mouse. So the final inoculated cell concentration was 5, 1, and 0.5 million cells per mouse (202-204). Systematic diagram for cell injection and tumor staging is shown in Figure 3.5.

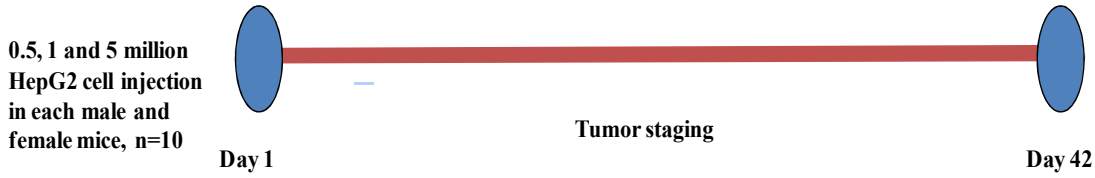


Figure 3.5: Systematic diagram for cell injection and tumor staging

3.11.3.2 Standardization with male vs. female mice species for xenograft model development

To compare the growth pattern of HepG2 cells, cells were inoculated with concentration of 5, 1, and 0.5 million cells per mouse in both male and female mice.

Tumor take rate was calculated with following formula-

Tumor take rate =100 * (No of mice having palpable tumor on respective day/ total no of mice inoculated with the cell line)

Tumor volume measurement

Once tumor were palpable, the long diameter (a), short diameter (b), of tumor was measured in mm^3 with the vernier caliper and tumor volume was calculated with formula (205)-

Tumor volume = $(a \times b^2)/2$

Tumor growth inhibition (TGI) is calculated for each group using the formula:

$TGI\ (\%) = [1-(T_i-T_0)/(V_i-V_0)] \times 100$

T_i and T_0 is the average tumor volume of a treatment group on a given day and first day of treatment respectively. V_i and V_0 is the average tumor volume of the vehicle control group on the same day with Ti, and on the first day of treatment respectively (206).

Percentage change in tumor volume was calculated as follows-

Percentage change in tumor volume= {(Final tumor volume)-(Initial tumor volume)/ (Initial tumor volume)} *100

Body weight percentage was calculated by using the formula:

Body weight change (%) = {(Final body weight) - (Initial body weight)/ (Initial body weight)}*100.

Efficacy of each treatment was further evaluated by comparing the final mean weight of tumors in the drug-treated arm (T) to that of the control arm (C) called as T/C ratio calculated by following formula

T/C ratio= {Weight of tumors in the drug-treated arm (T)/ Weight of tumors in the vehicle-treated arm (T)} x 100

As per the National Cancer Institute (NCI) criteria, T/C ratio with a value of <0.42 considered an active response (207).

Dose selection

The recommended human dose of sorafenib is 400 mg twice daily by oral route. When sorafenib was given to mice at a dose of 30 mg/kg p.o. gives similar exposure as that of clinical study. Sorafenib formulation was prepared by triturating the sorafenib tablet (Soranib™) with 0.4% Tween 80 and 0.5% CMC to form uniform suspension for in vivo experiment (206, 208-210).

In this experiment the aim was to use two doses of sorafenib; one 30 mg/kg which is a clinical dose and second was half of the clinical dose i.e. 15 mg/kg.

3.11.3.3 Evaluation of efficacy of extracts in tumor bearing mice

Study design

Animals were randomized into different group based on the tumor volume when reached a volume of 200-300 mm^3. Systematic diagram for cell injection, dosing & efficacy study of

extracts is shown in Figure 3.6. All the formulations were prepared freshly in 0.4% Tween 80 (w/v) and 0.5% CMC. Required quantity of extract and sorafenib was weighed and transferred in mortar and triturated with Tween 80 (0.4% w/v of formulation) and 0.5% CMC was added slowly with trituration in order to achieve the uniform suspension.

A feeding cannula made of 24 gauge stainless steel was used to administer the formulation. The dosage was given at a rate of 10 mL/kg of body weight.

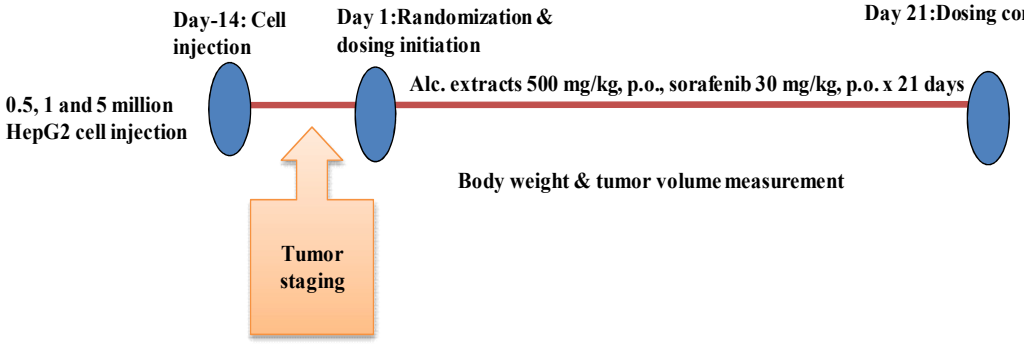


Figure 3.6: Systematic diagram for cell injection, dosing & efficacy study of extracts

Tumor bearing mice were randomized into different groups based on their tumor volume. Group I was disease (vehicle) control group received 0.4% Tween 80 in 0.5% CMC. Group II, III and IV group were treatment group received 500 mg/kg, p.o. extracts of *A. aspera*, *B. diffusa and E. littorale* respectively. Group V was a standard treatment group received sorafenib at 30 mg/kg, p.o. dose. Group VI, VII and VIII were treatment group received extract at low dose (150 mg/kg, p.o.) of *A. aspera, B. diffusa,* and *E. littorale,* respectively.

In literature *A. aspera* extract at 100, 200 and 400 mg/kg dose protected the rats against NDEA and CCl_4-induced carcinogenesis (36). *B. diffusa* and *E. littorale* extract were given at 250 and 500 mg/kg dose in mice for screening of anticancer activity (42, 49). Further *E. littorale* leaves extract was given at 250 mg/kg in hamster for anticancer activity in 7, 12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch carcinogenesis (47). Vishwakarma et. al. had studied the hepatoprotective activity of *E. littorale* extract in CCl_4 induced hepatotoxicity in mice (182).

In ayurvedic traditional system 3-5 gm of powder of the plant was given daily for treatment of different disorders. So considering 5 gm as a human dose and converting this to mice dose, it comes to 600 gm. So based on this, 3 doses 50, 150 and 450 gm were selected. In the literature discussed above 500 mg/kg dose has been reported widely. Based upon the literature and ayruvedic system efficacy study was performed at 150 and 500 mg/kg dose.

All vehicle control mice received 0.4% Tween 80 in 0.5% CMC. Treatment was given for 21 days. Mice tumor dimensions were measured twice weekly with the vernier caliper and body weights were recorded on alternate days starting with the first day of treatment. Mice were observed for body weight change, any clinical sign and tumor volume during the experimental period. Mice having tumor volume greater than the 10% of their body weight were humanely euthanized.

3.11.3.4 Evaluation of efficacy of extracts alone and combination with sorafenib in tumor bearing mice

The HepG2 cells at concentration of 1×10^7 cells/mL were collected in sterile falcon tube. Cells were mixed at 1:1 volume with ice cold BD-Matrigel (Corning, India) and injected subcutaneously with a volume of 0.2 ml on the right rear flank to get 1 million cells per mouse. Details of cell injection, dosing & efficacy study of extract and sorafenib combination is shown in Figure 3.7.

Mice tumor dimension was measured twice weekly with the vernier caliper and body weights were recorded on alternate day starting with the first day of treatment. Mice were observed for body weight change, any clinical sign and tumor volume during the experimental period. Mice having tumor volume greater that the 10% of their body weight were humanely euthanized. Treatments producing >20% lethality and/or 20% net body weight loss were considered ‘‘toxic’’. Systematic diagram for cell injection, dosing & efficacy study of extract and sorafenib combination

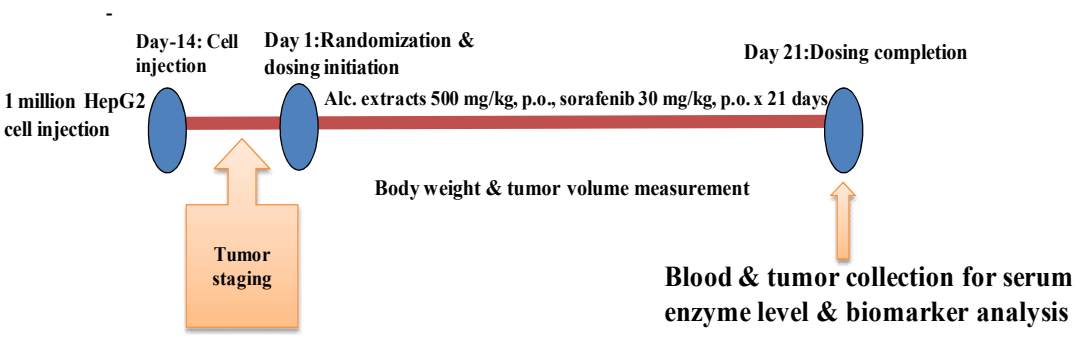


Figure 3.7: Systematic diagram for cell injection, dosing & efficacy study of extract and sorafenib combination

Study design

Animals were randomized based on the tumor volume. Treatment was initiated when tumors reached to a 150-250 mm^3 volume. All the formulations were prepared freshly in 0.4% Tween 80 (w/v) in 0.5% CMC. Required quantity of the extracts and sorafenib was weighed and transferred in mortar and triturated with Tween 80 (0.4% w/v of formulation) and 0.5% CMC was added slowly with trituration to get the uniform suspension.

The formulation was administered with 24 gauge stainless steel feeding cannula. Mice dose was calculated on the basis of body weight and dosed at 10 mL/kg dose volume.

Tumor bearing mice were randomized (n=6 mice/treatment) into different groups based on their tumor volume. Group I was disease (vehicle) control group received 0.4% Tween 80 in 0.5% CMC. Group II, and III were treatment group received 500 mg/kg, p.o. extracts of *A. aspera* and *B. diffusa* respectively. Group IV and VII were a standard treatment group received sorafenib at 30 mg/kg and 15 mg/kg respectively. Group V and IX were combination treatment group received *A. aspera* extract at 500 mg/kg, p.o. and sorafenib at 30 mg/kg and 15 mg/kg, respectively. Group VI and VIII were combination treatment group received *B. diffusa* extract at 500 mg/kg, p.o. and sorafenib at 30 mg/kg and 15 mg/kg respectively. Vehicle control group mice received 0.4% Tween 80 in 0.5% CMC. Treatment was given for 21 days.

On day 21, blood was drawn from the retro-orbital sinus of mice, two hours after the administration of last dose under isoflurane anesthesia. After 30 minutes of room temperature storage, the blood was centrifuged for 10 minutes at 3000 rpm and 24°C. The serum was collected and stored in a -70°C freezer until analysis. Serum was stored to analyze

biochemical parameters. Then, mice were euthanized by a slow CO_2 asphyxia and tumors were isolated. The tumor was weighed, and divided into two halves. For immunohistochemistry (IHC) and histopathology, one portion of the tumor was fixed with 4% paraformaldehyde; the other portion was snap frozen for western blot biomarker analysis. Also, nude mice of each group were dissected, and vital organs were observed visually for any deformity.

Analysis of biochemical factors

To evaluate liver and kidney functions the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, and creatinine (Cr) were determined by using an automated biochemical analyzer (model Daytona, Randox Laboratories Ltd., Antrim, UK) using commercial kits (Randox® Laboratories Ltd., Antrim, UK) according to the manufacturer´s recommendations. (198, 211).

3.11.3.4.1 Western blot assay[210, 211]

On dry ice, the preserved tumor samples were crushed into a powder using a mortar and pestle. Tumor powder ~ 30 mg was filled in chilled eppendorf tubes. All this procedure should be done on dry ice so that the powder should be free flowing. The tumor powder was stored at -70ºC until further use.

Protein estimation

RIPA buffer cocktail was prepared by mixing 5 mL RIPA buffer with 50 µL protease/phophatase inhibitor. To each tube contacting tumor powder 100 µL of cocktail was added and triturated with the pestle. Then, the tubes were placed on rotor at 20 rpm at 4ºC temperature for 30 minute so that the maximum proteins get extracted. Further, each sample was ultrasonicated for 10 second and the cycle was repeated thrice. The tubes were centrifuged at 12000 x g for 7 minute at 4ºC. The supernatant was collected in new tube. The sample was diluted to 1:10 for estimation of protein concentration. For extrapolation and comparison standard sample of bovine serum albumin (BSA) were prepared at 4 mg/mL concentration and further diluted to 3 mg/mL, 2 mg/mL, 1 mg/mL and 0.5 mg/mL concentration. Using the bicinchoninic acid (BCA) test, the protein concentration was determined. To the 96 well plate 5 µL of blank or sample or standard was added, further, 200 µL of $CuSo_4$ + BCA (1:40) was added to it, and incubated at 37ºC for 30 minutes and

absorbance was measured at 562 nm. The protein standard curve was used to calculate the protein concentration.

Preparation of samples for gel loading

Samples (2 mg/mL concentration) were prepared for gel loading with addition of Laemmli dye in it, mixed well and kept at 95ºC for 10 minutes. Samples were stored at -20ºC till further process.

Separation of protein (gel electrophoresis) based on their molecular weight

Ready to use gel (10% SDS-PAGE) was used for loading of samples (40 µg) and ladder to separate the protein as per the molecular weight. The gel was run at 50 V for 5 minute and 100-150 V later on. After completion of run the gel was collected.

Transfer of protein from the gel to the membrane

For transfer of protein from the gel to the nitrocellulose membrane, gel was placed on membrane covered with stacks on both upper and down side in transfer tank of Trans blot instrument. The instrument parameters were set to 2.5V, 25 Ampere for 10 minute. Once the completion of run the membrane was collected and stored in Tris-buffered saline (TBS).untill processed for blocking.

Antibody incubation

The membrane was blocked in 5% BSA for 2 hrs at room temperature and incubated with following specific antibodies: VEGF (1:25), p-ERK (1:50), total-ERK (1:50) and β-actin (1:10,000) overnight at 4°C on shaker at 23-25 rpm speed. The membrane was washed three times in TBST, followed by incubation with the appropriate horseradish peroxidase (HRP) linked secondary antibody (1:10,000) for 1 h at room temperature. Again the membrane was washed with TBST.

Imaging and data analysis

The specific proteins on the blots were developed using enhanced chemiluminescence substrate (ECL) exposure to blot for 1-2 minute and visualized as bands on ChemiDoc® imaging system (209-210). Then, the captured images were analyzed with Image Lab software (Bio-Rad).

3.11.3.4.2 Immunohistochemistry[212, 213]

The freshly dissected tumor was cut into two portions with the help of scalpel bled and one half part was fixed in 4% paraformaldehyde solution for 48 hour. After that, the tissue was rinsed for five minutes under running water.

Preparation of formalin-fixed, paraffin-embedded tissue sections

The tissue were put in plastic cassette in tissue processor and processed for dehydration through 70%, 80%, 95% alcohol, followed with 3 times of 100% alcohol, for 5 min at each solvent. Further, the tissue was cleared twice in xylene and finally in paraffin for 5 min each.

Lastly, the tissue was put within a paraffin block and preserved at room temperature. On a microtome, tissue sections (5 μm) of paraffin-embedded tissue blocks were cut, and floated in a water bath with distilled water at 40°C. The sections were then moved, with the aid of a brush, onto glass slides for immunohistochemistry. Before being used, the slides were let to dry overnight and kept at room temperature.

Antigen retrieval, peroxidase blocking and antibody addition

The immunohistochemical analysis was processed according to standard procedures. Formalin-fixed paraffin-embedded (FFPE) tumor sections were dewaxed in xylene for 2 times, 10 min each. Then the sections were hydrated in gradient alcohol, 100% alcohol, for 2 times, 10 min each, and then transferred once through 95%, 70%, 50% and 30% alcohols, respectively, for 5 min each and lastly in water for 10 minute.

Then the slides were kept for 30 minute with citrate buffer having pH 6.5 at 97°C for antigen retrieval. Then, the citrate buffer was allowed to cool at room temperature for 20- 30 minute and further the slides were washed with PBS for 2-3 times. Then, 3% H_2O_2 was added to the sections to remove endogenous peroxidase and washed with PBS 2-3 times. Immunostained sections were preincubated with normal serum and then incubated with specific primary antibodies against Ki-67 (1:50) or CD31 (1:25) for overnight at 4°C. The sections were washed with PBS (2-3 times) pre and post incubation with the horseradish peroxidase streptavidin biotinylated secondary antibody (Vector Laboratories) for 2 hr followed by diaminobenzidine (DAB, Sigma). For the negative controls, the primary antibody was replaced with normal serum. Staining intensities were determined by measurement of the integrated optical density (IOD) with light microscopy using a computer-based Image

System. The mean value of at least three fields selected at random from each slide (n = 3) in each group is used to express the results. The mean number of vessels ± standard deviation of the mean was used to express the results. For Ki67, percentage positivity was determine from no of cells and positive cells for no. of Ki67 nuclear staining with the help of QuPath software version 0.3.2.

The number of microvessels per field was scored by averaging 5 field counts for each group. Microvessel density (MVD) was calculated for the angiogenesis marker CD31 (212-213).

3.12 Statistical analysis

Mean ± standard deviation (SD) was used to express the data. For the statistical analysis, Graph Pad Prism 5.0 (Graph Pad Software Inc., San Diego, CA, USA) was utilized. All data were analyzed using t test, one or two-way analysis of variance (ANOVA), followed by Bonferroni multiple comparison post hoc test. p value of <0.05 was considered as an indication for statistical significance.