CHAPTER II

MATERIALS AND METHODS

Chapter II: Materials and Methods

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2.1 MATERIALS

2.1.1 *CancerCell line*

Murine cancer cell line - Dalton's Lymphoma Ascites (DLA) cells were maintained in the peritoneal cavity of *Swiss albino* mice.

2.1.2 *Animals*

Swiss albino mice weighing 22-29g were purchased from the Small Animal Breeding Section (SABS), Government Veterinary College, Mannuthy, Thrissur, Kerala. They were kept under standard environments of temperature and humidity in the Centre's Animal House Facility. The animals were provided with standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad-libitum*. All animal experiments in this study were undertaken with the prior certification of the Institutional Animal Ethics Committee (IAEC) and strictly following to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) instituted by the Animal Welfare Division, Government of India.

2.1.3 *Instruments used*

Light microscope	: Labomed, India
Fluorescent microscope	: Olympus, Japan
High speed cooling centrifuge	: Hettich Universal-320R, Germany; Remi, India
Micro-centrifuge	: Eppendorf , India
Hot air oven	: Beston, India
Vacuum oven	: Pathak electrical works, Mumbai
Microwave oven	: Kenwood
Water bath with shaker	: Remi, India
Auto hematology analyser	: Mindray, BC-2800 VET, China
Biochemical analyzer	: Mispa Plus
Deep freezer (-70^{0}C), -200 C	: SANYO ultra low
Deep freezer (-20^{0}C)	: VOLTAS (TATA product)
Refrigerator	: SAMSUNG, South korea
UV-VIS Spectrophotometer	: Thermoscientific, Chemito Spectra Scan (UV2100)
Digital pH meter	: Scientific Tech., India
Electrophoresis unit	: Bangalore Genei, Bangalore

Gel documentation system	: UVI TEC, Cambridge
Thermal cycler	: Applied Biosystems (2720)
StepOnePlus Real Time – PCR	: Applied Biosystems
Microplate Reader	: BioRad, model 680
Laminar air flow cabinet	: Beston, India
CO_2 incubator	: Thermo scientific
Tissue homogenizer	: Remi, India
Ultrasonicator	: Bandelin sonoplus
Weighing balance	: Schimadzu (AX200)
Rotary microtome	: York Scientific Industries Pvt Ltd
Vortex mixer	: Rotex; Remi
MilliQ water unit	: Labconco Water Proplus

2.1.4 ***Chemicals***

RPMI medium	: Hi-Media, Mumbai
Thiobarbituric acid (TBA)	: Sigma-Aldrich, St Louis, USA
DPPH (2,2-diphenyl-1-picrylhydrazyl)	: -do-
Normal melting point agarose	: -do-
Low melting point agarose	: -do-
ABTS [2,2'-azinobis (3-ethylbenzothiazoline -6-sulfonic acid) diammonium salt]	: -do-
Ethidium Bromide	: -do-
Bovine serum albumin	: -do-
Nitroblue tetrazolium (NBT)	: -do-
Riboflavin	: -do-
Reduced glutathione (GSH)	: -do-
5-5' dithiobis-2- nitrobenzoic acid (DTNB)	: -do-
Acridine orange	: -do-
Thiosemicarbazide	: -do-
Ascorbic acid	: -do-
Ferrozine	: -do-
Trichloroacetic acid (TCA)	: -do-
Polyoxyethylene-25-propylene glycol	

stearate (POES)	: -do-
Polyvinyl pyrollidone (PVP)	: -do-
Glycine	: -do-
Sodium nitrite	: -do-
Griess reagent solution	: -do-
Brij 35	: -do-
p-nitrophenyl β-D-glucuronide	: -do-
Sodium carbonate	: -do-
Citric acid	: -do-
Di-Sodium hydrogen phosphate	: -do-
Potassium dihydrogen phosphate	: -do-
o-dianisidine	: -do-
Berberine chloride	: -do-
Guanidinium thiocyanate	: -do-
Sodium citrate	: -do-
N-laurosylsarcosine (Sarkosyl)	: -do-
2-mercaptoethanol	: -do-
Sodium acetate (anhydrous)	: -do-
Glacial acetic acid	: -do-
Phenol (nucleic acid grade)	: -do-
Chloroform	: Merck India Ltd., Mumbai, India
Ferric chloride	: -do-
Ferrous chloride	: -do-
Isoamyl alcohol	: -do-
Isopropanol	: -do-
Ethanol	: -do-
Formaldehyde	: -do-
Hydrochloric acid (HCl)	: -do-
Triton X	: -do-
EDTA	: Sisco Research Ltd (SRL), Mumbai
Tris Buffer	: -do-
Tris-HCl	: -do-
Folin’s reagent	: -do-

Sodium azide	: -do-
Hydroden peroxide	: -do-
Bromophenol blue	: Bangalore Genei, Bangalore, India
Doxorubicin Hydrochloride	: Miracalus Pharma, Mumbai, India
cDNA synthesis reagents	: Genei, Bangalore; Origin, Kerala
PCR reagents	: Genei, Bangalore; Origin, Kerala

2.1.5 *Diagnostic reagent kits*

Serum Urea and Creatinine analysis kit	: Agappe Diagnostics, Kerala
Serum Glutamate pyruvate transaminase (SGPT)	: Agappe Diagnostics, Kerala
Glutamate oxaloacetate transaminase (SGOT)	: Agappe Diagnostics, Kerala
Alkaline phosphatase (ALP)	: Agappe Diagnostics, Kerala
CK-MB	: Agappe Diagnostics, Kerala
CK-NAC	: Agappe Diagnostics, Kerala
Lactate dehydrogenase	: Agappe Diagnostics, Kerala
Total protein	: Agappe Diagnostics, Kerala
Bilirubin	: Agappe Diagnostics, Kerala

2.1.6 *Reagents and stain*

- Phosphate Buffered saline (PBS); pH – 7.4

NaCl	: 8.00g
KCl	: 0.20g
KH_2PO_4	: 0.20g
$Na_2HPO_4.2H_2O$	: 1.44g

These are dissolved in milli-Q water, made up to 1000ml and pH was adjusted to 7.4

- Trypan blue (0.4%)

Trypan blue	: 0.4g
Normal saline (0.9% NaCl)	: 100ml

Trypan blue stain was dissolved in saline and filtered using whatmann No.1 filter paper.

- Comet lysis Buffer; pH - 10

NaCl	: 2.9g
Tris	: 0.12g
EDTA	: 2.9g
Sodium sarcosinate	: 1g

These are dissolved in 100mlmilli-Q water and Triton X-100 (1ml) was added (pH was adjusted to 10).

- Comet electrophoresis Buffer; pH ≥ 13.0

NaOH : 1.2g

EDTA : 0.372g

These are dissolved in milli-Q water and 0.2ml DMSO was added and made up to100ml.

- Reagents for Protein estimation

- Reagent A

Na_2CO_3 : 2g

NaOH : 0.4g

Na-K tartarate : 20mg

These are dissolved in milli-Q water and made up to100ml.

- Reagent B

$CuSO_4$: 50mg (dissolved in 10ml of milli-Q water)

Reagent C : 50ml Reagent A+1ml Reagent B (50:1)

Follin's phenol reagent : Diluted with equal volume of milli-Q water

- Thiobarbituric acid (TBA) reagent

TBA : 93.75mg

TCA : 3.75ml

Concentrated HCl : 0.625ml

EDTA : 56mg

These are dissolved in milli-Q water and made up to 25ml.

- Reagents for Ferrozine assay

- Reagent 1

Thiosemicarbazide : 100mg

Ascorbic acid : 1g

Ferrozine : 100mg

These are dissolved in milli-Q water and made up to 100ml with 0.1mol/L HCl.

- Reagent 2

Thiosemicarbazide : 100mg

Ascorbic acid : 1g

These are dissolved in milli-Q water and made up to 100ml with 0.1mol/L HCl.

- Tris acetate EDTA (TAE) buffer (50X stock solution)

Tris base : 242g

Glacial acetic acid : 57.1ml

500mM EDTA : 100ml

These are dissolved in milli-Q water and made up to 1000ml.

2.2 METHODOLOGY

2.2.1 *Preparation and characterization of Iron-oxide nanoparticles*

The co-precipitation method was used to prepare the iron-oxide nanoparticles (NP). The metal chlorides - ferric chloride and ferrous chloride in 2:1 molar ratio - were prepared in milli-Q water. The pH of the solution was increased to 11-12 by the drop wise addition of 28% ammonia solution, subsequently stirred and heated the slurry at 80°C for 1hr. Polyvinyl pyrrolidone (PVP) was added to the above slurry and provided the same conditions for 1hr and then cooled to RT to settle the particles. The settled particles were washed repeatedly with milli-Q water to remove impurity ions and to reduce pH to physiological range. In order to make the particles more hydrophilic, the surface was modified with polyoxyethylene 25-propylene glycol stearate (POES) [Jayakumar et al., 2009]. Thus obtained NPs were dried in vacuum oven and kept in an air tight bottle. These particles were characterized by XRD and TEM.

2.2.2 *Complexing of magnetic iron-oxide nanoparticle with drugs*

The NPs were complexed with the drug, BBN (1:1) by sonochemical method. The NPs were complexed with BBN, SAN and BBN-SAN to obtain NP-BBN, NP-SAN and NP-BBN-SAN complexes (0.2%; 1:1 ratio) and sonicated for 20min at RT. The NP-DOX and NP-DOX-SAN complexes were prepared using NPs, DOX and DOX-SAN solutions (1%) in the ratio of 10 to 1.

2.2.3 *Characterization of NPs and NP-drug complexes*

The NPs and its drug complexes were characterized by FTIR, XRD, TEMand nanosize analyzer. FTIR spectra were recorded on Perkin Elmer, spectrum 400 FTIR spectrometer using KBr pellet and Nicolet iS10, thermoscientific. TEM images were obtained using a JEOL, JEM-2100 transmission electron microscope. In XRD, all liquid samples were dried on the glass substrate by using heater. For drying the liquid samplesprior to XRD measurements, cleaned substrates (Glass) were mounted on the heater (Heidolph MR 3001 K heater) and slowly poured liquid samples on to it. XRD measurements: The phase

composition ofthe various samples was determined by means of X-ray powder diffraction, using a Siemens Diffractometer 5000. The operating parameters were: monochromatic Cu-Kα radiation with a wavelength of 1.5418 Angstroms (I=40 mA and V=35 kV), 2θ scanning from 25 to 80^{o}, scan step size of 0.002. Phase identification was carried out using the reference database (JCPDS-files) supplied with the equipment. Size distribution pattern of NP-drug complexes were analysed by Malvern nanosize analyzer.

2.2.4 *Determination of loading efficiency*

The loading efficiency of berberine on NPs was determined by spectrophotometric method. In brief, various concentrations of NP-drug complexes were prepared in different ratios via sonochemical method. After the preparation, the free drug BBN was collected by solvent extraction method using isoamyl alcohol as organic solvent. The absorbance of free drug in the extract was measured at 480nm. A standard curve prepared using different concentrations of BBN was used for calculating its concentrations in the samples.

2.2.5 *Drug release study*

The pH triggered drug release under *in vitro* condition was studied by dialysis method(Kapoor et al., 2014). NP-BBN-SAN complexes (1ml) in a dialysis bag were kept in buffer (PBS; 10ml) solution. Berberine released from the nanoparticles diffuses to the outer compartment (buffer) was measured at pre-defined time intervals at 480nm using UV-VIS spectrophotometer. The concentration of BBN released in different buffers of pH 3.5, 6.6 and 7.4 was calculated using the standard curve.

2.2.6 *In vitro analysis*

Dalton's Lymphoma Ascites (DLA) cells were maintained in the peritoneal cavity of mouse. 1×10^{6}DLA cells/ml were suspended in DMEM with 12% foetal bovine serum (FBS) and used for all *in vitro*studies.

2.2.7 *In vivo analysis*

Tumor was developed by transplanting 5×10^{6} DLA cells subcutaneously on the hind limbs of the animals and the drugs were administered orally when the size of the tumor reaches approximately $1cm^{3}$.

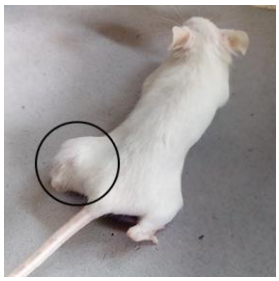


Figure 2.1: Image of animal-bearing solid tumor on the hind limb (in black circle).

2.2.8 *Drug excretion study*

The tumor-bearing animals were administered with NP-BBN and NP-BBN-SAN complexes (20mg/kg) and faeces were collected at pre-determined time intervals. The samples were homogenized in PBS and the drug released was extracted by solvent extraction method using isoamyl alcohol and the absorbance was determined spectrophotometrically. The concentration was calculated by interpolating the values with the standard curve using various BBN concentrations.

2.2.9 *Cytotoxicity study*

The cell viability was estimated by Trypan blue dye exclusion method (Strober, 1997). The live cells with undamaged cell membranes can exclude the stain, while the dead cells cannot. The DLA cells (1×10^6cells/ml) were incubated with the drugs and their complexes with NPs. After incubation, the cells were mixed with 0.4% trypan blue (1:1) and kept for approximately 3min at RT. The number of dead cells (stained) and live cells (not-stained) were counted using hemocytometer.

The percentage of cell death was calculated by using the formula,

$$\text{Percentage of dead cells} = \frac{\text{number of dead cells}}{\text{total number of cells}} \times 100$$

A graph was plotted with percentage mortality of cells against the treatments.

2.2.10 *Apoptosis by dual staining method*

To find out the mechanism behind the cytotoxicity 1×10^6 cells/ml were incubated with the drug and its complexes with NPs. Then these cells were stained with propidium iodide and acridine orange (1:1; 20µg/ml) [Richard et al., 1994]. The cells, after incubation, were mixed with dual stain (10:1) and viewed under fluorescent microscope.

Viable cells with intact nucleiappear in green colour, while the chromatin of non-viable cells gets stained in bright orange colour. The apoptotic cells can be distinguished as it

contains membrane with blebbing and condensed nuclei. The necrotic cells appeared orange with normal nuclei.

The percentage of apoptotic cells was calculated by the formula,

$$\text{Percentage of apoptosis (or) live cells} = \frac{\text{number of apoptotic (or) live cells}}{\text{total number of cells}} \times 100$$

2.2.11 ***Alkalinesingle cell gel electrophoresis***

The extent of cellular DNA damages was assessed by alkaline single cell gel electrophoresis or Comet assay. During electrophoresis, the cells with strand breaks in DNA lose their supercoiling and become free to move toward the anode, resembling a comet. The cells following various treatments were immobilized with 1% low melting agarose on 1% normal agarose-coated slides and allowed to break the membrane in lysis buffer for 90min. Finally, electrophoresis was carried out in alkaline buffer for 20-30min (25V, 300mA). The slides were washed with distilled water following electrophoresis and allowed to get dried. The cells in the slides were stained with propidium iodide (25µg/ml) and photographed under fluorescent microscope (40X magnification) [Sandeep and Nair, 2010; Cerda et al., 1997]. A software, Comet Assay Software Programme (CASP), was used to calculate comet parameters such as tail length, % DNA in tail, tail moment (tail length × % DNA in tail) and olive tail moment (distance between the centre of gravity of the head and the centre of gravity of the tail × % DNA in tail [Końca et al., 2003].

2.2.12 ***Polymerase chain reaction***

The tissues excised (80mg) from the animals were processed for isolating RNA by acid guanidium thiocyanate – phenol – chloroform extraction method [Chomczynski and Sacchi, 1987]. RNA was separated from DNA and contaminants, such as endogenous ribonucleases(RNases) and other proteins, using an acidic solution containing guanidinium thiocyanate (an inhibitor of RNases), sodium acetate, phenol and chloroform. Under acidic pH, the isolated total RNA remains in the upper aqueous phase, while interphase and/or lower organic phase contain most of the DNA and proteins.

The protocol for isolating RNA was as follows,

Homogenization

- Added 750µl solution D to the cells or tissues in a 2 ml centrifuge tube

- Added 75µl sodium acetate, mixed thoroughly by inversion; 750µl water-saturatedPhenol, mixed thoroughly by inversion and 150µl chloroform/Isoamyl alcohol (49:1),
  Shaken vigorously by hand for 10sec
- Cooled on ice for 15min and centrifuged for 20min at 10000g and 4°C.

First Precipitation

- Transferred upper aqueous phase to a clean 2ml centrifuge tube
- Added 1ml of isopropanol (precipitation of RNA); incubated for 1hr at -20°C [PausePoint (PP)] and Centrifuged for 20min at 10000g, 4°C
- Discarded the supernatant (RNA forms a gel like precipitate).

Second precipitation

- Dissolved pellet in 0.3ml solution D and isopropanol.
- Incubated at -20°C for 30min (PP)
- Centrifuged for 10min at 10000g, 4°C; discarded the supernatant.

RNA Wash

- Re-suspended the pellet in 1ml 75% ethanol and vortex for a few seconds (PP: It can be stored at -20°C up to 1 year)
- Incubated for 15min at RT (to dissolve residual guanidium); centrifuged for 5min at10000g, 4°C and discarded the supernatant. Air dried the pellet for 10min at RT.

RNA solubilisation

- Dissolved the pellet in 100-200µl sterile milli-Q water
- Incubated for 10-15min at 60°C (PP: It can stored at -70°C)
- Absorbance at 260nm was measured and quantified the RNA using the formula,
  Concentration of RNA (µg/ml) $=A_{260} \times$ Conversion factor × Dilution factor
  Conversion factor is the concentration of RNA (40µg/ml) when OD at 260nm is 1.
- The isolated RNA was visualized by agarose gel electrophoresis by 1% agarose and visualized using Sybr safe nucleic acid gel stain as the intercalating agent under gel documentation system.

2.2.13 ***Synthesis of cDNA from mRNA***

The isolated RNA was converted to complementary DNA (cDNA) using cloned Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase. Thus generated RNA-DNA

hybrid contains cDNA of the RNA template. RNase H degrades this RNA and separates the single stranded cDNA. The protocol for cDNA synthesis is presented in table 2.1.

Table 2.1: Protocol for cDNA synthesis

Reagents	Stock concentration	Concentration (1X)	Volume(1X)µl
MMLV Reverse Transcriptase (RT)	200U/ µl	10U	0.05
RT Buffer	5X	1X	4.0
Random hexamer	100	0.4	0.16
RNase Inhibitor	40U	2.5U	0.1
dNTPs	10mM	1mM	2.0
DTT	100mM	2mM	0.4
Sterile MilliQ water	-	-	4.29
RNA	-	-	9.0
Total reaction volume	-	-	20.0

The samples were vortex for 2sec and spin it for 10sec at 1000rpm.

Conditions for reverse transcription:37ºC - 60min; 70ºC - 15min; 4ºC - infinity

Finally the synthesized cDNA was kept at 4ºC.

2.2.14 *Polymerase chain reaction*

The synthesized cDNA was used for reverse transcriptase (RT) and Real Time (RTq) PCR to identify the changes in the transcriptional expression of genes using specific primers.

2.2.14.1 *RT-PCR*

Based on specific primers, PCR amplifies a part of DNA contains the gene of interest to a large number of copies with the help of DNA polymerase. The temperature dependent denaturation and annealing is the basic principle of PCR reaction.

***Steps involved in PCR reaction*:**

Denaturation: The double stranded DNA is denatured to single stranded at 94ºC

Annealing: Depending on the base composition and size, oligonucleotide primers anneals to DNA complementary sequences at about 55ºC.

Extension: DNA polymerase at an optimal temperature of 72ºC start to extend the DNA synthesis from the primers to get double stranded DNA. This DNA polymerase, isolated from *Thermus aquaticus*, can withstand in various temperatures and active at higher

temperatures. To get an exponential amplification of the desired gene, this cycle is repeated 25 to 40 times. The protocol for RT-PCR and the primer sequences are presented in table 2.2 and 2.3, respectively.

Table 2.2: Protocol for RT-PCR

Reagents	Stock concentration	Concentration (1X)	Volume(1X) in µl
Taq polymerase	5U/µl	2.5U	0.5
Taq Buffer	10X	1X	2.5
Primer 1 (forward)	50µM	0.4µM	0.2
Primer 2 (reverse)	50µM	0.4µM	0.2
dNTPs	40mM	0.2mM	0.125
Sterile MilliQ water	-	-	19.475
Subtotal	-	-	23.0
RNA	-	-	2.0
Total reaction volume	-	-	25.0

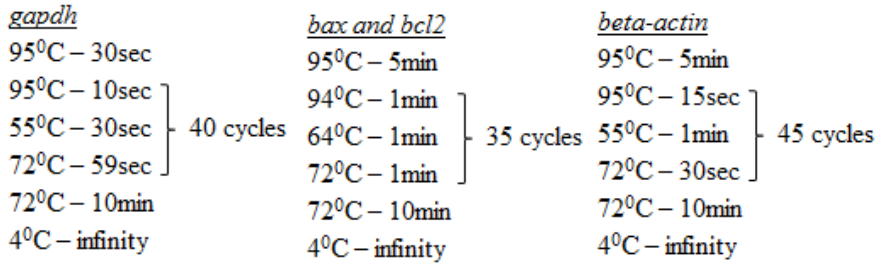
Table 2.3: Primer sequences used for RT-PCR

Gene	Forward primer (5'to3')	Reverse primer (5'to3')	Accession number
bcl2	CTCGTCGCTACCGTCGTGACTTCG	CAGATGCCGGTTCAGGTACTCAGTC	BC068988.1
bax	AAGCTGAGCGAGTGTCTCCGGCG	GCCACAAAGATGGTCACTGCTTGCC	NM_007527.3
gapdh	AAGGGCTCATGACCACAGTC	TGTGAGGGAGATGCTCAGTG	XR_405643.1
β-actin	ATGAGCTGCCTGACGGCCAGGTCATC	TGGTACCACCAGACAGCACTGTGTTG	NM_007393.3

Agarose gel electrophoresis (gel: 2% agarose in 1X TAE buffer; electrophoresis buffer: 1X TAE buffer) of the RT-PCR product was carried out for 1hr at 10V/cm. To visualize these amplicons, the PCR product, an intercalating dye Syber safe was used and analysed in gel

documentation system.Relative expression of genes was calculated based on the expression of house-keeping gene.

Cycling conditions:



2.2.14.2 ***Quantitative PCR (qRT-PCR)***

In qRT-PCR, the fluorochromes are used to detect the amplified products. These dyes have the capability to interact with these amplicons and generate fluorescence. Sensors in qRT-PCR machine detect this fluorescence and the amount of fluorescence is converted to the relative number of copies of amplicons in the mixture. The major attraction is that it is possible to quantify the expression of genes in real time.

Table 2.4: Protocol for qRT-PCR

Reagents	Stock concentration	Concentration (1X)	Volume(1X) in µl
Sybr green master mix	2X	1X	10
Primer 1 (forward)	50µM	0.5µM	0.2
Primer 2 (reverse)	50µM	0.5µM	0.2
Sterile Milli-Q water	-	-	7.6
Subtotal	-	-	18.0
cDNA	-	-	2.0
Total reaction volume	-	-	20.0

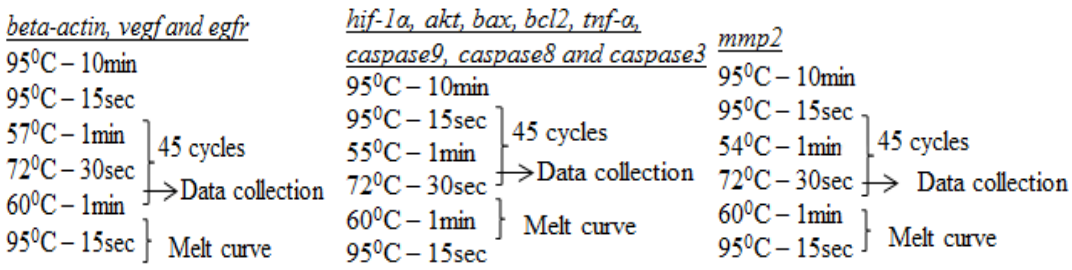
The endpoint for real-time PCR is the threshold cycle (CT) - the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. The amount of amplicon in the reaction is inversely related to the numerical value of the CT (i.e., lower the CT, greater the amount of amplicon). The real-time data is presented either as absolute expression level where the number of RNA is represented as copy number per cell based on a standard graph or as relative expression levels where the data is presented relative to another gene often referred to as an internal control.

The expression of genes involved in hypoxia-associated tumor progression (*hif-1α*, *vegf*, *akt*, *egfr*) and apoptosis (*bax*, *bcl2*, *caspases*) were studied. The primers (table 2.5) were designed using NCBI -PrimerBlast tool. The primer sequences were purchased from Hysel India Private Limited as lyophilised powder which was reconstituted to 50mM using sterile Milli-Q water (It can be stored at -70^{0}C). The protocol is presented in table 2.4.

Table 2.5: Primer sequences used in qRT-PCR

Gene	Forward primer (5'to3')	Reverse primer (5'to3')	Accession number	Reference
β-actin	ATGAGCTGCCTGAC GGCCAGGTCAATC	TGGTACCACCAGA CAGCACTGTGTTG	NM 007393.3	Harris et al., 1992
hif-1α	TCGAAGTAGTGCTG ATCCTG	GGCTGGGAAAAG TTAGGAGT	X95580.1	Wenger et al., 1996
vegf	TGGACATCTAGGAG TACC	CTGTAGGAAGCTC ATTCTCTC	AB086118.1	Maeda et al., 2003
egfr	GGCCATGAACATCA CCTGTA	GCCCAGCACATCC ATAGGTA	AF275367.1	Reiter et al., 2001
mmp2	CCCATGAAGCCTTG TTACC	TCTCGGGACAGAA TCCATAC	NM_008610.3	Bauters et al., 2015
bax	TGCTACAGGGTTTC TCCAG	CACGTCAGCAATC ATCCTCT	NM_007527.3	Broome et al., 1995
bcl2	AGGATTGTGGCCTT CTTTGA	ATGCTGGGGCCAT ATAGTTC	NM_009741.5	Chen et al., 1997
caspase9	TGACATCCTTGTGT CCTACTC	CCAGGAATCTGCT TGTAAGTC	AB019600.1	Fujita et al., 1999
caspase8	GATGTTGGAGGAA GGCAATC	ATTCCAACTCGCT CACTTCT	AJ007749.1	Van de Craen, 1998
caspase3	GAAATTCAAAGGA CGGGTCG	GGACACAATACA CGGGATCT	Y13086.1	Van de Craen et al., 1997
tnf-α	GTGCCTATGTCTCA GCCTCT	TCCACTTGGTGGT TTGTGAG	NM_013693.3	Pasparakis et al., 1996

Cycling conditions:



The relative expression data was used to calculate fold change in the expression of genes in comparison with the control group by comparative CT method [Livak and Schmittgen, 2001; Schmittgen and Livak, 2008].

Fold change = $2^{-\Delta\Delta CT}$, where $2^{-\Delta\Delta CT}$ = [(CT gene of interest - CT internal control) Sample A – (CT gene of interest - CT internal control) Sample B]

The results were presented as histogram which indicates the down regulation and up regulation in the expression of these genes. The expression of house keeping gene, β-actin, was used as positive control.

2.2.15 *Histopathology of tissues*

To visualize the morphological changes after treatment the tissues were excised and fixed in 10% formalin and embedded in paraffin wax for pathological examinations. Sections of 5 micron thickness were prepared and stained with haematoxylin-eosin at Pathology Laboratory, Pushpagiri Institute of Medical Sciences and Research Centre, Tiruvalla, Kerala, India [Culling, 1974].

The Haematoxylin (H) & Eosin (E) staining method includes five basic steps:–

(1) Dehydration: Removal of water from tissues using different grades of alcohol allows thetissues to mix with wax

(2) Clearing: Alcohol in the tissue is replaced by a Xylene that will dissolve the wax with which the tissue is to be impregnate

(3) Wax impregnation: The tissue is transferred into a molten paraffin wax. During this step, the clearing agent is removed from the tissue by diffusion into molten wax and wax in turn enters into tissues

(4) Embedding and sectioning: Filled the mould with molten paraffin wax, fix the tissue in it and cut it properly using an instrument called microtome

(5) Staining: It is done with haematoxylin and eosin. Haematoxylin is basic in nature and takes acidic stain and Eosin is acidic stain that stains the cytoplasm.

(6) Mounted the slides with DPX.

2.2.16 *Serum biochemical analysis*

Blood was collected by cardiac puncture, serum was separated and analysed various biochemical parameters using MISPA PLUS agape diagnostics. Serum markers of cardiac, liver and kidney functions such as Lactate Dehydrogenase (LDH), Creatine Kinase-MB (CK-MB), Creatine Kinase-NAC (CK-NAC), Urea, Creatinine, Albumin, Total protein and Serum Glutamate Pyruvate Transaminase (SGPT) were examined [Weisshaar et al., 1975; Gerhardt and Waldenström, 1979; Kassirer, 1971; Winkelman et al., 1974; Witt and Trendelenburg, 1982; Doumas et al., 1971; Allen et al., 1982].

- ***Serum glutamate oxaloacetate transaminase (SGOT)***

Serum SGOT activity was determined according to the method of Thefeld *et al*., (1974). The transfer of amino group between L-aspartate and α-ketoglutarate to form oxaloacetate and glutamate is catalysed by SGOT (AST). The oxaloacetate thus formed reacts with NADH in the presence of malate dehydrogenase to form NAD which is measured as a decrease in absorbance, proportional to the SGOT activity in the sample. The working reagent was prepared by mixing reagent 1 [Tris buffer (88 mmol/L, pH 7.8), L-Aspartate (260 mmol/L), LDH (1500 U/L), MDH (900U/L)] with reagent 2 [α-Ketoglutarate (12mmol/L, NADH (0.24mmol/L)] in 4:1 volume. 0.1 ml of serum was mixed with 1.00 ml of the working reagent and incubated for 1min at 37^0C. The change in absorbance was measured per minute for 3min at 340 nm.

$$\text{SGOT activity (U/L)} = (\Delta \text{ OD / min}) \times 1745$$

- ***Serum glutamate pyruvate transaminase (SGPT)***

Serum SGPT activity was determined according to the method of Thefeld *et al*., (1974). The transamination reaction is between L-alanine and α-ketoglutarate to form pyruvate and glutamate is catalysed by SGPT. The pyruvate formed reacts with NADH in the presence of lactate dehydrogenase to form NAD which is measured as a decrease in absorbance, proportional to the SGPT (ALT) activity in the sample. 4 volume of Reagent1 [Tris buffer (110 mmol/L, pH 7.5), L-Alanine (660 mmol/L), LDH (1500 U/L)] is mixed with 1 volume of Reagent2 [α-Ketoglutarate (16mmol/L, NADH (0.24mmol/L)] to prepare

working reagent.0.1 ml of serum was mixed with 1.00 ml of the working reagent and incubated for 1min at 37^0C. The change in absorbance was measured per min for 3min at 340 nm.

SGPT activity (U/L) = (Δ OD / min) × 1745

- ***Serum bilirubin***

Serum bilurubin activity was determined according to the method of Walter and Gerard (1970). Sulfanilic acid reacts with sodium nitrite to form diazotized sulfanilic acid. Serum bilurubin react with diazotized sulfanilic acid to form azobilurubin (colored). The absorbance of this coloured product was measured photometrically. 0.05 ml of serum was mixed with 0.02 ml of activator and I ml of bilirubin reagent as test and 0.02 ml of activator mixed alone with I ml of bilirubin reagent was used as blank. Incubated for 5min and measured the absorbance of test against reagent blank at 546nm.

Total Bilurubin = OD of test – OD of reagent blank ×29.

- ***Serum urea***

Serum urea was determined according to the method of Chaney and Marbach (1962). Urea hydrolyzed in the presence of water is converted to ammonia and carbon dioxide catalysed by urease. The ammonia reacts with ketoglutarate and NADH to produce glutamate and NAD, which was measured at 340nm. The optical density is inversely proportional to the concentration of urea. Working reagent was prepared by mixing 4 volume of Reagent 1 [Enzyme concentrate Ureases (> 500K U/L)] with one volume of reagent2 [Buffer (pH 7, 120 mmol/L), Sodium Salicylate (60mmol/L), Sodium nitropruside (5mmol/L)]. 0.01ml of serum was mixed with 1ml of working reagent and 0.01 ml of Urea-B standard concentration (40mg/dl) provided in the kit was added to the working reagent as standard. Mixed well and read the optical density (T1) 30sec after the sample or standard addition. Take second reading (T2) exactly 60sec after the first reading.

$$concentration\ of\ Urea(\frac{mg}{dl}) = \frac{(T1-T2) of\ sample}{(T1-T2)\ of\ standard} \times 50$$

- ***Serum creatinine***

Serum creatinine was determined according to the method of Bonsnes and Taussky (1945). Creatinine in reaction with picric acid forms a yellow-orange compound. The intensity of the color was measured at 520 nm and is proportional to the creatinine concentration. The working reagent was prepared by mixing equal volume of Reagent 1 [picric acid (8.73

mmol/L), Surfactant] with Reagent 2 [Sodium hydroxide (300 mmol/L), Sodium phosphate (25 mmol/L)]. 0.1ml of serum was mixed with 1ml of working reagent and 0.1ml of creatinine standard was also mixed with the working reagent was used as blank. The optical density was read at 60sec after (T1) the sample or standard addition. Exactly after 60 seconds after the first reading the second reading (T2) was taken.

$$Concentration\ of\ creatinine\ (\frac{mg}{dl}) = \frac{(T1-T2) of\ sample}{(T1-T2)\ of\ standard} \times 2$$

- ***Serum lactate dehydrogenase (LDH)***

Serum LDH was determined according to the method of McQueen (1975). The reduction of pyruvate with NADH to form NAD catalysed by the enzyme lactate dehydrogenase was measured as a decrease in absorbance, which is proportional to the LDH activity in the sample. 0.01ml of serum was added to 1ml of working reagent prepared by mixing 4:1 ratio of Reagent 1 [Tris buffer (pH 7.4, 80mmpl/L), Pyruvate (1.6 mmol/L), Sodium Chloride (200 mmol/L)] and reagent 2, [NADH (240µmol/L)] provided in the kit. Mixed well and incubated for 1min at 37^0C·

LDH activity (U/L) = (Δ OD / min) x 16030

- ***Serum CK-MB and CK-NAC***

The levels of serum CK-MB and CK-NAC were determined according to the method of Witt and Trendelenburg (1982). The CK-activity in the samples was measured by theuse of an antibody to CK-M monomer. The activity of CK-MM and half of CK-MB activity was inhibited by the antibody. CK method was then used to quantify the CK-B activity. The CK-MB activity is calculated by multiplying the CK-B activity. To 1ml of working reagent {4 volume of Reagent 1 [Imidazole (pH6.7, 125mmol/L), D-Glucose (25mmol/L), N-Acetyl-L-cysteine (25mmol/L), magnesium acetate (12.5mmol/L), NADP (2.55mmol/L), EDTA (2.025mmol/L), hexokinase (> 6800 U/L)] + 1 volume of reagent 2 [Creatinine phosphate (250.5mmol/L), ADP (15.25mmol/L), AMP (25mmol/L), Diadenosine pentaphosphte (103mmol/L), G-6-PDH (> 8800 U/L)]},0.04ml of serum was added and incubated for 5min at 37^0C.

CK-MB activity (U/L) = (Δ OD / min) x 8254

ATP was produced from creatine phosphate by the catalytic reaction by CK. Glucose, in the presence of ATP, is gets converted to glucose-6-phosphate which in turn reacts with $NADP^+$ catalysed by glucose-6-phosphate dehydrogenase. The absorbance was measured

at 340nm. The working reagent was prepared by mixing 4 volume of Reagent 1 [Immidazole buffer (125mmol/L), D-Glucose (25mmol/L), N-Acetyl-L-cysteine (25 mmol/L), Magnesium acetate (12.5mmol/L), NADP (2.4 mmol/L), EDTA (2.0mmol/L), Hexokinase (>6800 U/L)] with 1 volume of Reagent 2 [Creatine Phosphate (250 mmol/L)]. 0.04ml of sample was mixed with 1ml of working reagent, incubated at 37^0C for 100sec, and measured the change in absorbance per minute (OD/min) during 3min.

Creactine kinase activity (U/L) = (OD/min) x 4127

2.2.17 ***Analysis of antioxidant status in tissues***

The levels of superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx) and lipid peroxidation in tissue homogenates of animals following various treatments were analysed.

Superoxide dismutase assay:The SOD activity was measured according to McCard and Frederich method, 1969. In the presence of EDTA, photoilluminated riboflavin undergo redox reactions and simultaneously reduces O_2 to O_2-radical. The assay is based on the reduction reaction of nitroblue tetrazolium (NBT) to formazan blue in connection with this radical and the absorbance was read at 560nm. This reaction is blocked in the presence of the enzyme SOD in the sample. To the 10µl tissue homogenate 200µlof 6.6mM EDTA prepared in 0.3mM NaCN, 0.1ml of 50mM and 2.64ml of SOD buffer [8.5ml of KH_2PO_4 (9.1g/L) and 91.5ml of Na_2HPO_4 (9.5g/L)] was added. After the addition of 50µl of 2mM riboflavin, and the absorbance was measured at 560nm. The tubes were illuminated with bright lamp for 15min and measured the absorbance again at 560nm. The difference in absorbance before and after illumniation was calculated for each sample. The percentage inhibition was calculated by comparing it with with the absorbance of the control (the samplewith enzyme activity). The results were expressed as U/mg protein for tissue [one unit of enzyme activity is the amount of SOD in the sample required to scavenge 50% of the generated superoxide anion].

Dtermination of reduced Glutathione activity: The GSH in the samples react with dithiobis-2-nitrobenzoic acid (DTNB) and produces yellow coloured product on reduction showed maximum absorption at 412nm [Moron et al., 1979]. To 100 µl of the tissue homogenate, 63µl of 25% TCA and 300µl of 5% TCA was added. These were then subjected to centrifugation for 5min at 3000g. From the supernatant 150µl was taken and

mixed with 350µl of sodium phosphate buffer (0.2M, pH 8.0) and 1.0ml of DTNB (0.6 mM in phosphate buffer). The absorbance of the yellow coloured product thus obtained was measured. The GSH content of the sample was calculated from the standard graph (prepared using 10 to 50 nmoles) of GSH and expressed as nanomoles/mg protein.

Determination of Glutathione peroxidase activity: The GPx assay was carried out based on the method described by Hafeman et al, 1974.

In detail, 25µl of tissue homogenate was incubated with 50µl of 5mM GSH, 250µl of 1.2 mM H_2O_2, 50µl of 25 mM NaN_3 and 875µlof phosphate buffer (1M, pH7.0) at 37^0C for 6min. 1.0ml of 1.67 % H_3PO_4 was used to stop the reaction. This mixture was centrifuged at 3000rpm for 10min. 1.0ml of the supernatant was mixed with 1.0ml 0.4M $Na_2HPO_4$and 1ml of 1mM DTNB (in buffer), incubated for 10min at 37^0C. The yellow coloured reaction product thus obtained was measured at 412nm and expressed as Units/mg protein.

$$GPx\ activity\ (Units\ per\ mg\ Protein) = \frac{OD\ of\ Blank - OD\ of\ sample}{0.001 \times mg\ protein}$$

Blank: sample without the tissue homogenate

One unit of enzyme activity was defined as decrease in log GSH by 0.001/min after subtraction of the decrease in log GSH per minute for the non-enzymatic reaction.

Determination of lipid peroxidation: The levels of lipid peroxides in the samples react with thiobarbituric acid in acetic acid solution and measured as malondialdehyde at 532nm [Janero D.R., 1990]. Briefly, to 1ml of tissue homogenate (100ml tissue homogenate + 900 mL distilled water), 1ml TBA reagent was added [0.375% thiobarbituric acid, 0.025 N HCl, 15% trichloroacetic acid and 6.0 mM EDTA]. The reaction mixture was boiled for 30min, cooled and centrifuged at 10,000g for 10min. The amount of TBAReacting Substance in the supernatant was estimated by measuring the absorption at 532 nm. The lipidperoxidation values are expressed as nanomoles of MDA per mg protein.1, 1, 3, 3-tetraethoxypropane was used as the standard.

Estimation of protein: The protein levels in the samples were analyzed by Lowry's method [Lowry et al., 1951]. The phosphomolybdate and phosphotungstate components of Folin-Ciocalteau reagent get reduced by tyrosine and tryptophan residues of proteins in an alkaline medium to give a bluish purple colour with absorbance at 660 nm. 1ml tissue homogenate (10µl sample + 990µl distilled water)was incubated with 5ml reagent C [50ml

Reagent A (Sodium Carbonate, Sodium hydroxide and Sodium-Potassium tartrate) + 0.1ml Reagent B(Copper Sulphate)] for 10minat RT. Then these samples were again incubated for 30min at RT after the addition of 500µl Follin's Ciocalteaureagent and measured absorbance at 660nm.

2.2.18 *Tumor markers*

Beta-glucuronidase and myeloperoxidase assays were performed in tumor, liver and kidney tissues [Kawai and Anno, 1971; Desser et al., 1972].

The basis of the beta-glucuronidase assay is the formation of p-nitrophenol by beta-D-glucuronidase in reaction with the substrate, p-nitrophenyl β-D-glucuronidase. In brief, 100µl of tissue homogenate (100mg tissue was homogenated in 3ml of 0.1% Brij-35) was incubated with 0.4ml buffered substrate [p-nitro phenyl β-D-glucuronidase in 0.1M acetate buffer] for 30min at 30^0C. The reaction was stopped by the addition of 0.2M Na_2CO_3 and the absorbance was measured at 400nm. The controls for each sample were prepared, by the addition of stopping reagent, prior to incubation. The activity of the enzyme was expressed as Units/mg protein.

The basic reaction catalysed by the enzyme myeloperoxidase is the oxidation of o-dianisidine in the presence of H_2O_2. To the tissue homogenate (100µl), 1ml of Reagent A [6ml of 0.1M citrate phosphate buffer + 60µl of 30% H_2O_2 + 0.5ml of o-dianisidine (10mg/ml of methanol)] was added and incubated for 30min at RT in dark condition. After the incubation, 1ml of 5N HCl was added and the absorbance of the orange red coloured product was measured at 400nm. The controls were obtained by the addition of 5N HCl before the incubation of the samples. The results were expressed as OD/mg protein.

2.2.19 *Estimation of nitric oxide by Griess reaction*

The nitrotriazole derivative Sanazole produces nitric oxide as a product of its reduction. The levelsof nitric oxide in the samples were measured in the form of nitrites.Nitrites in the sample react with Sulfanilamide (sulphonamide) and form a diazonium salt. The addition of azodye (N-alpha–naphthyl-ethylene diamine) to the diazonium salt results in the development of a coloured (pink) product and it can be measured using spectrophotometer. The samples, following the treatment, were treated with 50µl of 10% trichloroacetic acid (TCA) to precipitate proteins. These samples were centrifuged at 8000rpm for 5 to 10min and the supernatant was collected for the assay. Briefly, 100µL of

supernatant was mixed with an equal volume of Greiss Reagent and incubated for 10min at RT. The absorbance was determined at 543nm [Kondakova et al., 2004]. A calibration curve was established using sodium nitrite solution (0.1-1.0µM).

2.2.20 *Direct specific determination of Iron in tissue samples using Ferrozine assay*

The concentration of iron in the tissues was estimated following the administration of NP-drug complexes. In this assay, the iron released from the storage protein was reduced at pH 1.7 by treating with ascorbic acid solution (10g/L prepared in 0.1mol/L HCl). When ferrozine is added to this solution, it forms a complex along with the reduced iron, produces an intensively coloured product at higher pH and measured absorbance at 570nm [Riemer et al., 2004]. The tissues of the animals not treated with the nano-drug complexes (Control) were used as the baseline iron content in the tissues.

2.2.21 *Tumor imaging*

The animals bearing tumor in the hind limb were anesthetized (Ketamine-xylazine) following oral administration of NP-BBN-SAN complexes and imaging was taken using XENGEN IVIS imaging system.

2.2.22 *Statistical analysis*

The results were presented as Mean ± SD (standard deviation) and were analysed by GraphPad PRISM software version 5. Statistical analyses of the results were performed using One- way analysis of Variance (ANOVA) with Tukey-Kramer multiple comparisons test.