2 MATERIAL AND METHODOLOGY

For proposed research work, following protocols are adopted to prove the hypothesis on IGF-IR ablation and neuroblastoma regression. *In vitro* cell culture techniques were utilized to carry out research.

2.1 Molecular Docking:

Picropodophyllotoxin (PPP) is a known inhibitor of the IGF-IR, wherein it binds to the ATP binding domain. As it is a competitive inhibitor of ATP, we have hypothesized that PPP could bind to another surface receptor-tyrosine kinases as well. One such receptor-tyrosine kinase is ALK, whose amplification is a characteristic feature of the NB. To evaluate the binding affinity of the PPP with IGF-IR and ALK, molecular docking was carried out.

2.1.1 Ligand preparation: The 3D structure of Picropodophyllotoxin was obtained from PubChem (PubChem Id: 72435) in SDF format. The ligand in SDF format was retrieved into the workspace of AutoDock tools 1.5.7.

2.1.2 Protein preparation: The 3D structures of protein IGF-IR (PDB Id: 5FXQ) and ALK (PDB Id: 5FTO) were downloaded from RCBS Protein Data Bank and retrieved into the workspace of AutoDock tools 1.5.7 and prepared for docking.

2.1.3 Docking: Molecular docking was performed using AutoDock Vina 1.5.7. Following set of commands were used to perform the molecular docking.

i) To call files:

cd <Path of prepared files of ligand and protein>

ii) To run Autodock Vina and initiate docking:

"<Path of vina.exe> \vina.exe" --receptor <protein name>.pdbqt --ligand <ligand name> --config config.txt --log log.txt --out output.pdbqt

The log file generated after successful docking was utilized to get the binding affinity of the ligand with the selected protein.

2.1.4 Visualization: For 2D visualization of the docked file, BIOVIA Discovery Study Visualizer 2021 was used. For 3D visualization of the docked file, PyMOL 2.5 software was used.

2.2 Cell culture

Human neuroblastoma SH-SY5Y cell line was procured from National centre for cell sciences (NCCS), Pune. Cell culture was maintained in MEM/F-12 medium with 10% FBS and 1X antibiotic. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 level.

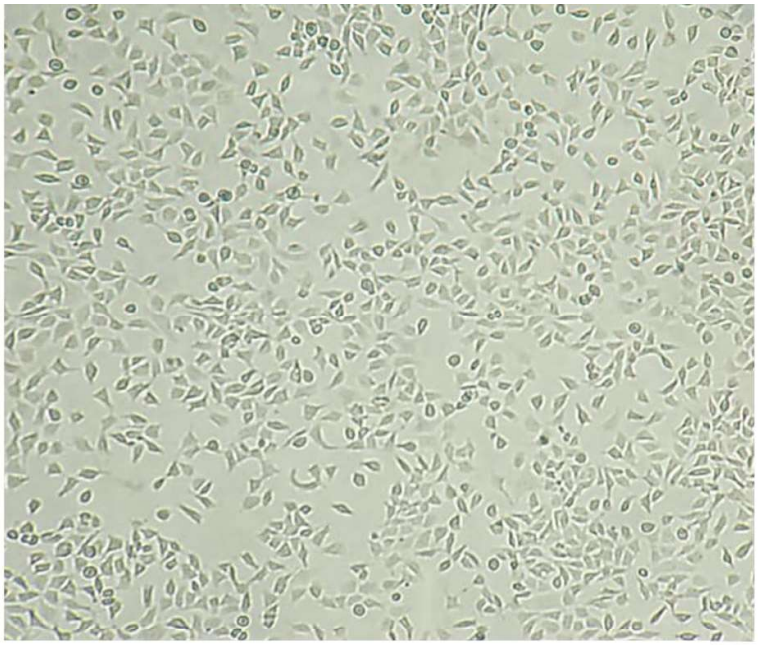

Fig 2.1: Representative image of SH-SY5Y (Neuroblastoma) cell line

2.3 Cytotoxicity Assay

Picropodophyllotoxin (PPP, Sigma-Aldrich) was used to inhibit the IGF-IR signaling in neuroblastoma cell line. To check the cytotoxicity of PPP, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was performed. SH SY5Y cells (6000/well in 96 well plate) were incubated overnight for adhesion. Cells were treated with 5 different concentrations of PPP (0.89, 0.68, 0.48, 0.20 and 0.068 µM) for 24 hours to determine the IC50 value. After 24 hours of treatment, culture medium was removed and 200µl of serum free culture medium with MTT (5mg/ml) was added. Cells were then incubated in dark at 37°C for 4 hours. The MTT-containing medium was discarded, and the purple-colored formazan crystals were dissolved in 200ul DMSO. Absorbance was measured at 540 nm using microplate reader (Tacan, Infinite M200 pro). The IC50 value was calculated using Origin 2021 version software.

% inhibition = 100- (OD of test/OD of control) X 100

2.4 mRNA transcript study

2.4.1 RNA isolation

Neuroblastoma cells were treated with 16 nM concentration of IGF-IR inhibitor, PPP (Value derived from MTT assay) for 24 hours. SH-SY5Y cells were scrapped after treatment period and centrifuged at 6000 RPM for 3 minutes. Cells were agitated in 700µl TRIzol® (RNAiso plus, Takara) for 1 minutes. Cells were centrifuged at 12,000 RPM for 15 minutes at 4°C after adding 300µl of chloroform. Aqueous phase was collected in a new microcentrifuge tube. Equal volume of Isopropanol was added and incubated for 1 hour at -20°C. After incubation, microcentrifuge tube was centrifuged at 12,000 RPM for 10 minutes at 4°C for precipitation of RNA to obtain a pellet. Supernatant was discarded and 200µl of chilled ethanol was added followed by centrifugation at 10,000 RPM for 15 min at 4°C. Ethanol was discarded and the pellet was air dried and dissolved in 20µl DEPC treated water and stored at -20°C until further use.

2.4.2 Quantitative and qualitative check of RNA

The BioSpec-NanoDrop spectrophotometer was used to measure the total RNA concentration and quality at 260/280 ratio. 1 µl RNA sample, isolated from above procedure, and 1 µl DEPC water mix was used for quantification of RNA at 260 nm and 280 nm. A ratio of 2±0.2 was accepted as indicative for purity of the RNA.

RNA quality was assessed using 1.2% agarose gel. Band for RNA quality was observed under Gel documentation system. The integrity of the RNA was checked by the intensity of its two major subunits 28S and 18S bands. A good quality RNA was accepted when 28S band intensity is twice than of the 18S band.

2.4.3. cDNA Synthesis

The Bio-Rad iScript cDNA synthesis kit was used to reverse transcribe 1µg of total RNA into cDNA. 1µg RNA (volume is calculated on the basis of concentration measured in NanoDrop, 4µl iScript reaction mix, 1µl iScript reverse transcriptase, and 14µl nuclease free water made up the reaction mixture. The cDNA reaction was carried out for each sample in a total volume of 20 µl according to instruction given with kit. Priming of reaction mixture was processed at 25°C for 5 minutes in thermal cycler then reverse transcribed at 46°C for 20 minutes. It was then held at 95°C for 1 minute to heat inactivate the enzyme. Samples was stored at -20°C for further experiments.

2.4.4 Primer Designing

RNA primers were designed using Primer3Plus software. The primer length was kept between 18 to 22 bp. Primers were designed to meet the specific requirements for quantitative RT-PCR, with 55-60% GC content and melting temperatures of approximately 55ºC. Primers were also designed to produce an amplicon of approx. 100-200 bases in length. Self-complementary of forward and reverse primer was analyzed. Primers were procured from Xcelris labs limited, Ahmedabad. Below is the list of the primers designed for the proposed study.

Table 2.1: List of the primers used for the study.

Gene	Accession number	Sequence
IGF1R-F	NM_000875.5	TGT TGA TCG TGG GAG GGT TG
IGF1R-R		GCA AGG TCT CTG TGG ACG AA
PI3KCB-F	NM_006219.3	GAC TTT GCG ACA AGA CTG CC
PI3KCB-R		AGG TAT GCA TGG CCT CCT TC
AKT-F	NM_005163.2	GGC AAG GTG ATC CTG GTG AA
AKT-R		CGA CCG CAC ATC ATC TCG TA
BAX-F	NM_138764.5	GCC CTT TTG CTT CAG GGT TT
BAX-R		GGA AAA AGA CCT CTC GGG GG
BCL2-F	NM_000633.3	CTG GTG GGA GCT TGC ATC
BCL2-R		ACA GCC TGC AGC TTT GTT
E-Cadherin-F	NM_004360.5	CGG ACG ATG ATG TGA ACA CC
E-Cadherin-R		CCA CAT TCG TCA CTG CTA CG
*Snail*1-F	NM_005985.4	ACC CCA CAT CCT TCT CAC TG
*Snail*1-R		AGT TCT GGG AGA CAC ATC GG
Nanog-F	NM_001297698.2	GGT GAA GAC CTG GTT CCA GA
Nanog-R		AGG AGG GGA GAG GAA GGA TT
Twist-F	NM_001271893.4	GCT ACA GCA AGA AGT CGA GC
Twist-R		GTC ACT GCT GTC CCT TCT CT
GAPDH-F	NM_001289745.3	GAC AGT CAG CCG CAT CTT CT
GAPDH-R		GCG CCC AAT ACG ACC AAA TC
Tubulin F	NM_006082.3	CTG GCC AAG GTA CAG AGA G
Tubulin R		CAG GGC CAA AAG GAA TGG

CDK8 F	NM_001260.3	CAG CAG GGC AAT AAC CAC AC
CDK8 R		GGT TGG GAT AGG CAG CAT GT
p53 F	NG_017013.2	TGT CCT TCC TGG AGC GAT CT
p53 R		CAA ACC CCT GGT TTA GCA CTT C
ALK F	NM_001353765	CAT CCA CCG AGA CAT TGC TG
ALK R		TCC CAT AGC AGC ACT CCA AA
MYCN F	NM_001293228.2	GGG ACT GTT TCT GCT TCC GA
MYCN R		GCT CGT TCT CAA GCA GCA TC
NFκB F	NM_003998.4	TGG TGG AGT CTG GGA AGG AT
NFκB R		TGA CCT CAC CAT TCC CAA CG

2.4.5. Real Time-PCR Protocol:

qPCR was performed for all quantitative gene expression analyses on cDNA using Power up iTaq universal SYBR Green supermix by Bio-RaD and, 0.1 µM of forward and reverse primer of each respective gene were added. The *GAPDH* gene was used as a reference gene to normalize the relative expression between the samples. Each sample of RNA was analyzed in triplicates. Numbers of cycles (40) were adjusted to obtain amplified cDNA. According to manufacturer's instruction, each reaction mix was prepared with following composition:

Component of kit	**Volume**
2X SYBR Green master-mix	5µl
Forward primer	0.5µl
Reverse primer	0.5µl
cDNA template	1µl
Nuclease-free water	3µl
TOTAL	10µl

The following program was used for amplification of cDNA using Applied Biosystem QuantStudio 5 System Real Time PCR machine:

Temperature	Time	Cycle
95 ºC	20 seconds	
95 ºC	15 seconds	40
60 ºC	1 minute	
Melt-curve analysis		
Temperature	**Time**	
95 ºC	2 seconds	

2.4.6. Data analysis:

CT value of each well was obtained directly from the RT-PCR machine. CT value of above 35 was not considered for the calculation. Normalized CT value calculated by subtracting the CT value of GAPDH, a reference gene, from those of the target genes. Mean of these normalized CT values were plotted.

$$\Delta CT = CT\ target - CT\ endogenous\ control$$

Fold change in expression was calculated by method of Livak & Schmittgen, 2001. For fold change of gene expression in treatment as compared to that of in control was calculated according to formula given as below:

$$\Delta\Delta CT = \Delta CT\ target\ sample - \Delta CT\ control\ sample$$

$2^{-\Delta\Delta CT}$ values were calculated and graph was prepared to analyze the change in fold expression.

$$\textbf{Fold change in the expression} = 2^{-\Delta\Delta C\ T}$$

2.5 RNA interference study (miRNA)

2.5.1 MicroRNA scoring

To select the microRNA (miRNA), for understanding of its control of proposed gene, scoring was performed by miRDB, an online database. Specific microRNA was selected based on score with proposed genes. Another software, miRCancer was used to confirm the expression of miRNA in neuroblastoma tumor. microRNA those are used for study are: a) hsa-miR-9-5p; b) hsa-miR-223-3p; c) hsa-let-7a-5p; d) U6.

Table 2.2: List of miRNAs, their sequence and score with specific gene.

Name	microRNA sequence	Target	Scoring
hsa-miR-223-3p	UGUCAGUUUGUCAAAUACCCCA	IGF-IR	88
		CDK	89
hsa -let 7a-3p	CUAUACAAUCUACUGUCUUUC	PI3K	98
		IGF-IR	80
hsa-miR-9-3p	AUAAAGCUAGAUAACCGAAAGU	CDK	84
		PI3K	88

2.5.2. MicroRNA Primers

microRNA primers are made through www.srnaprimerdb.com/search. U6 was used as internal housekeeping gene.

Table 2.3: List of RT primers for miRNA

Name	RT Primer
hsa-miR-9-5p	*GTCGTATCCAGTGCAGGGT*CCGAGGTATTCGCACTGGATACG ACTCATAC
hsa-miR-223-3p	*GTCGTATCCAGTGCAGGGT*CCGAGGTATTCGCACTGGATACG ACTGGGGT
hsa-let-7a-5p	*GTCGTATCCAGTGCAGGGT*CCGAGGTATTCGCACTGGATACG ACAACTAT

Table 2.4: List of F and R primers for miRNA

Name	qPCR
Reverse primer (R Primer)	GTCGTATCCAGTGCAGGGT
hsa-miR-9-5p (F)	AGCGAGGCTCTTTGGTTATCTAG
hsa-miR-223-3p (F)	AAC GGC TGT CAG TTT GTC AA
hsa-let-7a-5p(F)	AAGCGACCTGAGGTAGTAGGT
U6 (F)	GCTTCGGCAGCACATATACTAAAAT
U6 (R)	CGCTTCACGAATTTGCGTGTCAT

2.5.3. miRNA Isolation

Total RNA was isolated after treatment of PPP for 24 hours. Total RNA sample was applied to Mini Spin column where total RNA binds to the membrane and all contaminants were washed away. High miRNA was eluted in RNase free water.

2.5.4. qPCR of miRNA

The primer mix was prepared for each of the miRNAs and endogenous controls into one mixture called primer pool mix. 10µl of each primer were added together in a sterile tube and made up to 1ml with sterile RNase free TE buffer to make the primer pool. cDNA synthesis was performed using TaqMan miRNA cDNA synthesis kit. miRNA was reverse transcribed for cDNA according to manufacturer protocol. The PCR condition for the reverse transcription included 16°C for 30 minutes, then 42°C for 30 minutes followed by the 5 minutes hold at 85°C. cDNA was used for qPCR study. The following program was used for amplification:

Stage 1(Hold Stage)	Stage 2(PCR Stage)	Stage 3(Melt curve)	Cycles
50.0 °C-02:00 mins	95.0°C-00:15 mins	95.0°C-00:15 mins	40
95.0 °C-10:00 mins	53.0°C-01:00 mins		

2.6 Western blot study for Protein analysis

2.6.1. Reagent Preparation

A. Solubilizing buffer (2X)

- Tris base: 62.5 mM, SDS: 2%, Glycerol 25%, Bromophenol blue 0.01%; pH is 6.5.

B. Running Buffer (10X)

- Tris base: 250 mM, Glycine: 1.92 M, SDS: 1%, pH: 8.3

C. Coomassie stain

- CBB-R250 0.1%, Methanol 40%, Glacial acetic acid 10%

D. RIPA Buffer

- Tris-HCl: 25 mM, NaCl: 150 mM, Triton X-100: 1% SDS 0.1%, Sodium deoxycholate 1%; pH was set to 8. Protease inhibitor (Sigma-Aldrich) was added freshly before use.

E. Transfer buffer

- Tris: 25 mM, Glycine: 192 mM, Methanol: 10%, pH: 8.3.

2.6.2. Protein Isolation

Neuroblastoma cells were incubated overnight for surface attachment. After overnight incubation, cells were treated by IGF-IR inhibitor for 24 hours. Cells were scraped and collected in 1.5ml microcentrifuge tube for protein isolation. Cells were washed with PBS twice and incubated in RIPA lysis buffer on ice for about 30 minutes. The pellet was then sonicated for 1 minute. The contents were centrifuged at 6000 RPM for 15 minutes at 4ºC. The protein content of the

supernatant was estimated by BCA assay kit (Thermo Fischer scientific). The extracted protein was stored at -20°C for western blotting.

2.6.3. Sample Preparation

The protein isolated from cells was heat denatured and run on SDS-PAGE gel prior to Western blotting. The heat denaturation step unwinds the tertiary structure of the protein and facilitates the protein to get resolved in the SDS –PAGE gel based on its molecular weight. The 25 µg of sample was solubilized in solubilizing buffer. Solubilizing buffer contains β-mercaptoethanol, glycerol, bromophenol blue and SDS in 0.5M Tris - HC at pH 6.8. Each of these ingredients- unwinds the complex structure of the protein, provides density to sink into the wells, act as a tracking dye and provides equal negative charge respectively.

2.6.4. SDS-Polyacrylamide Gel Electrophoresis

Bio-Rad electrophoresis is used for SDS-PAGE. 5 ml of 10% separating gel mixture was prepared which contains Acrylamide/ Bis-acrylamide mix (30% w/v, 1.7 ml), 1.5M Tris - HCl (pH 8.8, 1.3 ml), SDS (10% w/v, 50µl) and distilled water (1.9 ml). Freshly prepared APS (10% w/v, 50µl) and TEMED (5µl) was added and the separating gel mixture was immediately poured into the gel cassette assembly. The gel was allowed to polymerize for 30 minutes. 2 ml of 4% stacking gel mixture was prepared that contains Acrylamide/ Bis-acrylamide mix (30% w/v, 340 µl), 0.5M Tris - HCl (pH 6.8, 260 µl), SDS (10% w/v, 20µl) and distilled water (1.37 ml). Freshly prepared APS (10% w/v, 20µl) and TEMED (2µl) was added and the stacking gel mixture was immediately poured above the polymerized separating gel. The stacking gel was allowed to polymerize. 25µg/ 30µl of heat denatured protein samples were loaded into individual wells. Precision Plus Protein ladder (Bio-Rad) was used to analyze the band molecular weight. The loaded proteins were allowed to resolve on 10% SDS-PAGE gel at 120 volts at 4ºC.

2.6.5. Electro Transfer

Western blotting is a technique which uses appropriate antibody to identify specific protein expression. The expression level of the protein was measured against a housekeeping protein β-actin gene expression.

The experiment required transferring the bands from the gel on to the PVDF membrane for antibody treatment. The transfer was made by stacking the gel on the PVDF membrane (Bio-Rad) under electric field of 80 volts for 2 hours. The PVDF membrane with protein transferred was treated with 5 ml of 5% BS in TBS-T buffer (20mM Tris HCl, 150mM NaCl and 0.1% Tween 20) for 1 hour at the room temperature. The blocking solution was used to mask the nonspecific binding of antibodies on the PVDF membrane.

2.6.6. Antibody treatment

The blot was washed with TBS-T for 5 minutes after blocking and the washing was done thrice to remove the excess blocking solution from the blot. The blot was treated with primary antibody (List is given in table 2.5) in 5% BSA in TBS-T according to provided dilution range of manufacturer instruction. The blot was incubated with primary antibody overnight at 4°C. After primary antibody treatment, the blot was washed thrice with TBS-T buffer (5 minutes for each wash) and incubated with HRP conjugated goat anti-rabbit IgG secondary antibody in TBS-T (1:1000 dilution) for 2 hours.

2.6.7. VISUALISATION

After treatment of secondary antibody, blot was washed thrice with TBS-T and incubated with ECL plus chemiluminescence (Bio-Rad). After documentation, the bound antibodies were removed by incubating in 10X reblot solution for 1 hour. Then the blot was incubated for housekeeping β-actin antibody that was followed by the HRP conjugated secondary antibody. The

intensity of the bands was observed in iBright CL100 Chemidoc (Thermo Fischer) and analysed by ImageJ software.

Table 2.5: Details of antibodies for western blot.

Name of antibody	Manufacturer and catalogue number	Dilution
Primary Antibody		
Anti-human Phospho-IGF-I Receptor ß (Tyr1135) (DA7A8) Rabbit mAb	Cell Signaling Technology #3918	1:1000
Anti-human IGF-I Receptor ß (D23H3) XP® Rabbit mAb	Cell Signaling Technology #9750	1:1000
Anti-human Insulin Receptor ß (4B8) Rabbit mAb	Cell Signaling Technology #3025	1:1000
Anti-human ALK/CD246 Rabbit mAb	Invitrogen #51-3900	1:1000
Anti-human ß-Actin (D6A8) Rabbit mAb	Cell Signaling Technology #8457	1:1000
Secondary antibody		
Goat anti-Rabbit IgG secondary antibody, HRP conjugate	Invitrogen #65-6120	1:1000

2.7 Immunocytochemistry (ICC)

Immunocytochemistry is a method utilized for localizing the expression of protein in cells. The protein of interest is conjugated with its primary antibody which then in turn is conjugated with fluorescence tagged secondary antibody. Cells were incubated on poly L-lysine coated coverslip in 6 well plate. After PPP treatment for 24 hours, the cells were washed with cold PBS and

permeabilized with 1% Triton X-100 in PBS for 5 minutes. Blocking was performed for 2 hours in buffer containing 2% BSA in PBS with 0.1 % Tween-20. Cells were washed in chilled PBS and incubated with the primary antibody overnight at 4°C. After incubation period, the cells were washed with PBS and incubated with FITC-conjugated secondary antibody for 1 hour at room temperature in dark. Cells were observed under confocal microscope (Zeiss LSM 710 Confocal Microscope). Further evaluation was performed using ImageJ software, wherein the images were first converted to 8bit, followed by measurement of gray value fluorescent intensity. Measurements were taken from 10 cells each in 10 different fields.

Table 2.6: Details of antibodies used for ICC.

Name of antibody	Manufacturer and catalogue number	Dilution
Primary Antibody		
NF-κB p65 (D14E12) XP® Rabbit mAb	Cell signaling Technology #8242	1:1000
IGF-I Receptor ß (D23H3) XP® Rabbit mAb	Cell Signaling Technology #9750	1:1000
ALK/CD246	Invitrogen #51-3900	1:1000
Secondary antibody		
Alexa Fluor™ 488 goat anti-rabbit antibody	Invitrogen #R37116	1:5

2.8 Scratch assay

Scratch assay was performed to understand the properties of PPP as blocker of cancer cell's invasive/proliferative character. For *in vitro* scratch assay, 6 well plates were used and 50,000 cells were seeded in each well. Once the cells reached around 70% confluence, a scratch was made to form wound.

Scrape cell layer in a straight line using a 1ml pipette tip. Keep tip perpendicular to the bottom of the well. Scratch another line perpendicular to the first line to create a cross in each well. Cells were washed thrice with PBS and measured the gap form in each well. Three wells were used as control and 3 wells were used for treatment. In three wells, PPP treatment was given for 24 hours. After incubation, cells were observed under phase contrast microscope to analyze gap. Percentage of cells migrated from control and treated cells were calculated and final gap width, after 24 hours of incarnation to initial gap width (0 hour) was calculated for statistical analysis to understand the migration of cells inhibited by PPP.

2.9 Cell death analysis

2.9.1 Fluorescent assorted flow cytometry (FACS)

Cell Apoptosis Kit with Annexin V Alexa Fluor 488 & Propidium Iodide (PI, Invitrogen; V13242). was used to measure early apoptosis by detecting phosphatidyl serine (PS) expression and membrane permeability using FACS. Cells were incubated up to 70% confluence in T25 flask. First flask was used as control, second was used as positive control (H_2O_2 treatment) and third was used for PPP treatment. After treatment, cells were harvested in 1.5 ml microcentrifuge tubes and washed twice in PBS. Cells were stained according to manufacturer's instruction for Annexin V / PI staining. Cells were resuspended in 100 µl of 1X annexin-binding buffer, 5 µl of Annexin V & 1 µl of PI for 20 minutes in dark. After the incubation period, 400 µL of 1X annexin-binding buffer was added and the sample vials were kept on ice. Cells were analyzed by FACS for cell

death analysis via measuring the fluorescence emission at 530 nm and > 575 nm (BD FACSCanto™). The optimum concentration for flow cytometry analysis is 2 x 10^6 cells per 200 µL volume. Also, flow cytometry analysis was repeated at least 3 times. For understanding of cell death, typical quadrant (q) analysis was performed. Each quadrant was designated with number (Q1 Unstained; Q2 live; Q3 Necrosis and Q4 apoptotic) and % of each cell in each quadrant was calculated.

2.9.2. Microscopic study for cell morphology

Dual staining of acridine orange (AO)/ ethidium bromide (EtBr) was performed to characterize apoptosis induced by PPP treatment in neuroblastoma cells. Cells were seeded on poly L- lysine coated slide in 6 well plate at a density of approximately 20^6 cells/well and incubated overnight. After adhesion on surface, cells were treated with PPP for 24 hours. After the end of the incubation, cells were washed with PBS and fixed with Methanol. After fixation cells were washed again and stained with AO (20µl)/EtBr (10µl) staining solution (a mixture of stain containing 100 µg/mL AO and 50 µg/mL EtBr) for 10 minutes at room temperature in dark. Cell morphology was examined under a fluorescent microscope (Zeiss Axio Observer A1 Fluorescence Inverted Microscope). Further analysis was done by ImageJ software.

2.10 Statistical analyses:

Statistical analysis: Data are expressed as Mean ± SEM. Data was analyzed on GraphPad prism software using student's t-test. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ and **** $p \leq 0.0001$ were used as criterion for significance when treatment was compared to control.