Chapter 3

Materials & Methods
3. Materials and Methods

3.1 Preparation of water extract of Ashwagandha leaves (ASH-WEX):
ASH-WEX was prepared by suspending 10 g of dry leaf powder in 100 ml of distilled water. It was incubated at 45°C for overnight with slow stirring. The slurry was centrifuged at 10,000 rpm and was then filtered under sterile conditions. The filtrate so obtained was treated as 100% ASH-WEX. It was stored at -20°C in 1 ml aliquots until further use.

3.2 Cell culture and maintenance:
C6 (rat glioma) and IMR-32 (human neuroblastoma) cells were purchased from National Centre for Cell Science (Pune, India). The cells were routinely grown in DMEM supplemented with 10% Fetal bovine serum and 1X PSN mix (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO₂. Undifferentiated cells were sub cultured by trypsinisation (0.1% trypsin) when they reached 70-80% confluency in the split ratio of 1:4.

3.3 Cell culture treatments:
3.3.1 For neurooncology studies
Undifferentiated cultures were different culture dishes depending upon the requirement. C6 cells were seeded at the density of 10⁴ cell/ml and IMR-32 cells were seeded at 2X10⁴ cell/ml seeding density. After 24hrs, when the cells were properly attached, cultures were treated with ASH-WEX (0.1- 2.5% diluted in medium) for 72 h. The medium of control culture was replaced with a fresh one.

3.3.2 For neuroprotection studies
Undifferentiated cultures were subcultured by trypsinization and cultured in 96 and 24 well plates according to the requirement of the experiment. After 24 hrs of seeding, C6 and IMR-32 cells were differentiated for 4 and 6 days, respectively by adding retinoic acid (RA) to the culture medium to a final concentration of 10μM. The medium was changed every two days. RA differentiated cultures were pretreated
with 0.05% and 0.1% ASH-WEX for 24hrs and then exposed to glutamate (0.06 mM - 10 mM) in the presence or absence of ASH-WEX.

3.4 Proliferation and Cytotoxicity assays:

The cell viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test. This method is based on the reduction of the tetrazolium salt MTT into a crystalline blue formazan product by the cellular oxidoreductase. The amount of formazan produced is considered as a reliable representation of viable cell number. After 24 hrs of treatment with glutamate, the culture medium was removed and replaced with fresh culture medium containing MTT (0.5 mg/ml). After 4 h incubation at 37 °C, this solution was removed, and the resulting blue formazan was solubilized in 100μl of DMSO and the optical density was read at 595 nm using microplate reader (Multiskan PLUS, Thermo Scientific). Four replicate wells were used for each analysis for each concentration tested in three independent experiments.

In order to assay the cytotoxicity, Lactate dehydrogenase (LDH) assay was done as described by Abe and Matsuki (2000). Briefly, LDH substrate mixture (1 ml) was prepared as follows; 2.5 mg l-lactate lithium salt and 2.5 mg NAD were dissolved in 0.9 ml of 0.2 M Tris–HCl buffer (pH 8.2) with 0.1% (v/v) Triton X-100, and 0.1 ml of MTT stock solution (2.5 mg/ml) and 1 μl of MPMS stock solution (100mM) were added. 50μl of the culture supernatant was transferred to 96-well culture plates, and mixed with 50 μl of the LDH substrate mixture. The reaction was stopped by adding 100 μl of a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (DMF/SDS, pH 4.7). The absorbance was measured at 570 nm with Multiskan PLUS reader (Thermo Scientific).

3.5 Trypan blue dye exclusion assay:

In order to assay membrane integrity and viability growth curves were obtained using Trypan Blue staining method. Both floating and adherent cells, cultured in 24 well plates, were harvested after trypsinization (trypsin 0.1%) and centrifuged for 5 minutes at 1000 rpm. The cells were suspended in 100 μl Hank’s Solution and stained with Trypan blue at a final concentration of 10% (v/v). Two replicate plates were used for each analysis and three independent experiments were carried out for the same. The viable and non-viable blue cells/ml were determined
counting the number of cells in 10 µl of cell suspension for each experiment in the Neubauer chamber.

3.6 Hoechst 33258 staining:

This stain is specific for nucleus and hence used to study the nuclear morphology. A cell that is undergoing apoptosis demonstrates nuclear condensation and DNA fragmentation, which can be detected by staining with Hoechst staining and fluorescence microscopy. C6 glioma cells were cultured on glass coverslips and then treated with Ashwagandha water extract for 72h. Cells were fixed with 4% Paraformaldehyde and permeabilized with 0.32% PBST. Subsequently Coverslips were washed and exposed to Hoechst 33258 (0.5ug/ml) in the dark at room temperature for 20 minutes. After washes, the antifading reagent was dropped onto the coverslips. The fluorescent images were observed under Nikon E600 fluorescent microscope.

3.7 Chemical standardization of ASH-WEX and nature of active components:

ASH-WEX was subjected to preliminary phytochemical screening for alkaloids, amino acids, anthraquinones, flavonoids, phytosterols, saponins, steroids, tannins, triterpenoids and reducing sugars following the methods of Harborne (1998) and Kokate (2001). It was further subjected to thin layer chromatography (TLC) using chloroform:methanol (1:1) as solvent front. TLC plate was subjected to UV radiation and iodine vapors for observation.

ASH-WEX was also subjected to heat- (95°C), protein- (proteinase K, 1 mg/ml and trypsin, 1 mg/ml), and nucleotide- (DNase I, 100 µg/ml), RNase, 100 µg/ml) -inactivation for 30 minutes each and chilled immediately on ice. The control samples were kept on ice during the treatments. These extracts were then tested for anti-proliferative and differentiation-inducing activities.

3.8 Immunostaining:

All cells, control and treated, were rinsed three times with 0.1M PBS and fixed with Paraformaldehyde (4%) for 30 minutes. Permeabilization was carried out with 0.32% Phosphate buffer saline (PBST) for 15 minutes. Coverslips were thrice washed with 0.1%PBST followed by blocking with 5% NGS (Normal Goat Serum prepared in 0.1% PBST) for 30mins at room temperature. Cells were incubated with anti-GFAP
(1:500, Sigma), anti-HSP70 (1:1000, Sigma), anti NF200 (1:500, Sigma), anti-NCAM (1:500, Sigma), anti-PSA-NCAM (1:250, AbCys), anti-mortalin (1:200), anti-cyclin D1 (1:200), anti-bcl-xl (1:200), anti-Akt-P (1:250) diluted in 0.1% PBST, for 24h at 4°C in humid chamber. Secondary antibody (Alexa Fluor 488, 546, Invitrogen) was applied for 2h at room temperature. Cells were incubated with (DAPI, 1:5000 in 0.1%PBST) for 10 minutes for nuclear staining and then mounted with anti-fading reagent (Fluoromount, Sigma) and observed under the microscope (Nikon A1R Confocal and Nikon E600 fluorescent microscope). Images were captured under (40X and 60X) and were analyzed using image pro-plus software version 4.5.1 from the media cybernetics.

3.9 Protein assay and Western blotting:

Cells grown and treated in 10 cm dishes were rinsed twice with PBS (ice-cold) and harvested with PBS-EDTA (1mM). The cells from 3-5 (10cm) petris of same group were pooled together and centrifuged at 1500rpm for 5mins. Cell pellet was resuspended in 5 volumes of chilled lysis buffer containing 50 mM Tris, 150mM NaCl, 1mM EDTA, 100uM NaVO₄, 1 mM PMSF, 0.5mM DTT, 1% NP-40 and protease inhibitors and centrifuged for 10 min at 10,000 rpm. Protein content in the supernatant was determined by the Bradford method. Each lysate was then diluted in lysis buffer so as to equilibrate the protein content in all the samples. The samples were mixed 1:1 with sample buffer [0.25 M Tris-HCl (pH 6.8), 20% glycerol, 4% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol and 1 mg bromophenol blue] and stored at −20°C.

30 μg of protein was electrophoresed on one-dimensional 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) under standard denaturing conditions. The separated proteins were then blot transferred onto a PVDF membrane (Hybond-P, Amersham Pharmacia Biotech) using the semidry Novablot system (Amersham Pharmacia) at 25 V for 90mins. Subsequently, membranes were blocked for overnight at 4°C with 5% skimmed milk solution in TBS-T buffer (13.3 mM Tris, 0.8%, w/v, NaCl; pH 7.6) containing 0.1% Tween-20 (Sigma) and immediately incubated with mouse anti-GFAP (1:3000), anti-HSP70 (1:5000), anti-NF200 (1:3000), anti-mortalin (1:2500), anti-NCAM (1:2500), anti-PSA-NCAM (1:2000), anti-cyclin D1 (1:2000), anti-bcl-xl (1:2000), anti-Akt-P (1:2500) antibody for overnight at 4 °C. After three washes of 10 minutes each in TBS-T, conjugated
horseradish peroxidase (HRP) anti-mouse IgG secondary antibody (1:5000) was added for 2h. Immunoreactive bands were visualized using ECL Plus western blot detection system (Amersham Biosciences) according to the manufacturer’s instructions and exposed to Hyper film ECL. The films were then developed and the antibody-labeling intensity (relative optical density) was analyzed using Gel documentation system (AlphaEase™, Alpha Innotech Corporation).

In order to account for potential variations in protein estimation and sample loading, expression of each protein was compared to that of α-tubulin in each sample by stripping the blot in 62.5 mM Tris, 2% SDS and 100 mM 2-mercaptoethanol (pH 6.7) for 30 min at 50°C and re-probing with an anti-α-tubulin antibody (1:3000).

3.10 Semi-quantitative RT-PCR:

Total RNA was extracted from cells by the TRI reagent (Sigma) according to manufacturer’s instruction. Equal amounts of RNA were used for cDNA synthesis in 20μl reactions containing 200U M-MLV reverse transcriptase, 4μl 5X first strand buffer (Fermentas), 5 μg of total RNA, 1 mM each of dNTPs (Fermentas), 20 units of ribonuclease inhibitor (Sigma), and 250ng pd(N)₆ random hexamers (Fermentas). 2 μl of cDNA was amplified in a 50 μl PCR reaction mixture containing two units Taq polymerase, 5 μl 10X PCR buffer, 3.0μl of 25mM MgCl₂ (Sigma), 1μl of 10mM dNTP mix (Fermentas), and 20 picomoles of respective primers as listed in Table 1. Cycling conditions comprised of an initial denaturation of 3 min at 94°C followed by 35 cycles of amplification (at 94°C for 40 sec, 55°C for 45 sec and 72°C for 1 min) and final elongation step at 72°C for 10 min. To control the PCR reaction components and the integrity of the RNA, 2 μl of each cDNA sample was amplified separately for β-actin specific primers.

3.11 Gelatinase Zymography:

Gelatinase Zymography is simple, sensitive, quantifiable, and functional assays to analyze MMPs in biological samples which identify MMPs by the degradation of their preferential substrate gelatine and by their molecular weight. Gelatin zymography is mainly used for the detection of the gelatinases, MMP-2 and MMP-9 as gelatin is the specific substrate for these two MMPs. Gelatinase zymography was performed in 10% SDS Polyacrylamide Gel in the presence of 0.1% gelatin under nonreducing conditions. Culture media (20 μl) were mixed with sample
buffer and loaded for SDS-PAGE. Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 1X Renaturing Buffer (Invitrogen) for 30 min at room temperature to remove SDS. The gels were then incubated at 37°C for 48-72 hrs in Developing Buffer (Invitrogen) stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 min and destained. Upon renaturation of the enzyme, the gelatinases digest the gelatin in the gel and give clear bands against an intensely stained background.

3.12 Wound scratch assay:

In order to investigate cell migration capability, C6 and IMR32 cells were grown to confluent monolayer. Monolayer was wounded by scratching the surface with a needle. Following the treatment, the initial wounding and the movement of cells in the scratched area were photographically monitored for 6 h (C6 cells) and 24 hrs (IMR-32 cells) after the treatment. Four different fields from each sample were considered for quantitative estimation of the distance between the borderlines and in each image four different equidistant points were measured in order to better estimate the real width of the wounded area. The migration rate is expressed as percentage of the control, and it was calculated as the proportion of the mean distance between both borderlines caused by scratching, to the distance which remained cell-free after regrowing. Three independent series of experiments were performed in duplicates.

3.13 Cell cycle analysis using Propidium Iodide:

Cells were plated at $2 \times 10^5$ cells/dish in 10 cm diameter dishes, and then grown either in the presence or absence of ASH-WEX and RA. After 72hrs of treatment, the cells were harvested from dishes by collecting trypsinized cells together with floating cells in the medium. For each condition, a volume of the cell suspension corresponding to $2 \times 10^6$ cells was centrifuged and the resultant cell pellet was resuspended in ice-cold PBS (1.0 ml). Cells were fixed in ice-cold 70 % ethanol and stained with propidium iodide. FACS analysis was performed using a BD Accuri C6 flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA). DNA content histograms and cell cycle phase distributions were modelled from at least 20,000 single events by excluding cell aggregates based on scatter plots of fluorescence pulse area versus fluorescence pulse width using FCS Express 4 flow research edition software (De novo software).
3.14 Annexin-FITC apoptosis assay:

To determine the extent of apoptotic and necrotic cell death, cells were stained with annexin V conjugated with FITC and PI using the Annexin V-FITC Apoptosis Detection Kit (Miltneyi Biotech), according to the manufacturer’s protocols. Annexin V has a high affinity for phosphatidylserine exposed on the outer membrane of apoptotic cells, while PI is transported to late-stage apoptotic/necrotic cells with disrupted cell membranes. The cells from control and treated groups were trypsinized, washed with PBS, and resuspended in 1ml of annexin V binding buffer (1X) with addition of 10µl annexin V-FITC. Following incubation (for 15 min in the dark at room temperature) and centrifugation (5 min, 300Xg), 500µl of annexin V binding buffer and 5µl of PI were added to the cell pellet and incubated for further 5 min in the same conditions. Then, viable (annexin V-, PI-negative), early apoptotic (annexin V-positive, PI-negative), late apoptotic (annexin V-, PI-positive) and necrotic (annexin V-negative, PI-positive) cells were detected by flow cytometry (Accuri C6 flow cytometer; Becton–Dickinson) and quantified by FCS Express 4 flow research edition software (De novo software).

3.15 Data Analysis:

The captured images were analyzed using Image Pro-Plus software version 4.5.1 from Media Cybernetics. The extent of GFAP, NF200, HSP70, mortalin immunoreactivity was quantified by the overall density of their respective immunoreactivity each in 5-6 randomly selected fields on each image using the count/size command of the Image Pro-Plus software. 15 different images were used from three different experiments and the data were averaged and expressed as percentage with respect to control.

3.16 Statistical Analysis:

Data was analyzed statistically using Sigma Stat for Windows (version 3.5). The results were analyzed using One-way ANOVA to determine the significance of the mean between the groups. Values of p≤0.05 were considered significant. The means of the data are presented together with the standard error mean (SEM).
3.17 PROTOCOLS:

DIFFERENTIATION OF CULTURES

Undifferentiated cultures

RA (10μM)

4 days for C6 glioma cells
6 days for IMR-32 neuroblastoma cells

Control

ASH-WEX treated

Glutamate challenged

Glutamate + ASH-WEX treated

MTT assay, LDH assay, Immunocytofluorescence studies, Western blotting,

RT-PCR, Gelatin Zymography
IMMUNOCYTOFLUORESCENCE

Washed the coverslips with PBS 3x 5min

↓

Fixed the cells on coverslips with ice cold 4% PFA for 15min

↓

Permeabilised with PBS Triton X-100 (0.32%) 30 min

↓

Washed with PBS Triton X-100 (0.1%) for 15min

↓

Blocked with 5% NGS in PBST (0.1%) for 30min

↓

Incubated with primary antibody in PBST (0.1%) +BSA (1%) at 4°C for 24 h

↓

Washed with PBST (0.1%) 3x 5min

↓

Incubated with fluorescent conjugated secondary antibodies in PBST (0.32%)
for 2 h at RT

↓

Washed with PBST (0.1%) 3 x 5 min

↓

Counterstained with DAPI (1:10000) for 5 mins

↓

Washed with PBST (0.1%) 3 x 5 min

↓

Slides mounted and cover-slipped with anti-fading fluoromount mounting medium
PROTEIN ESTIMATION
(Bradford Assay)

Diluted samples with buffer to an estimated concentration of 1 to 20 mg/ml

↓

Prepared standards containing a range of 2 to 20 μg protein (BSA) to a volume of 100 μl

↓

Added 20μl 0.15M NaCl to each well in multiwell plate.

↓

Added 170-μl dye reagent and incubated for 5 min

↓

Measured the absorbance at 595nm.
WESTERN BLOTTING

Cells were harvested using ice cold PBS-EDTA (1mM)

Spun at 2000rpm for 5 mins at 4°C

Vortexed the cell pellets in 3X RIPA buffer 3X 5mins

Centrifuged the lysates at 10,000rpm for 10min at 4°C

Transferred the supernatant to fresh, chilled eppendorf tubes

Protein content in the supernatant was determined by the Bradford method

Loaded the samples and run the gel at 125V/30mA at 40C

Transferred proteins to PVDF membrane at 25V and constant current of 1mA/cm^2 for 3hrs

Blocked the membrane with 5% skim milk in TBS Tween (0.2%) for 2hrs at RT

Incubated with primary antibody in TBST for overnight at 4°C

Washed 3x 15min with TBST at RT

Incubated with Secondary antibody for 1h at RT

Washed 3x 15min with TBST

Given a final wash with 1x TBS

The blot was developed using ECL-Plus western blot detection system
RT-PCR

TRI reagent was directly added to the monolayer cells
↓
Isolated RNA using TRI Reagent method
↓
Quantified and equalized RNA
↓
Reverse transcribed RNA to cDNA using reverse transcriptase and random primers at 37°C for 1 hour
↓
Specific genes of interest were amplified using thermocycler
↓
Electrophoresed the reaction mixture in 2% agarose gel containing ethidium bromide
↓
Captured the signal using UV transilluminator
↓
Analysis
GELATIN ZYMOGRAPHY

Media was collected from control and treated samples

\[ \downarrow \]

Spun at 10000rpm for 5 mins at 4°C

\[ \downarrow \]

Supernatant was mixed with 2X non-denaturing zymogram buffer

\[ \downarrow \]

Loaded the samples and run the 0.1% gelatin SDS-PAGE at 125V/30mA at 4°C

\[ \downarrow \]

Gel was renatured in 1X renaturing buffer for 30 min on rocker

\[ \downarrow \]

Gel was washed with MilliQ

\[ \downarrow \]

Gel was kept in 1X developing buffer for 48-72hrs

\[ \downarrow \]

Stained with 0.2% staining solution for 1hr

\[ \downarrow \]

Destained with destaining solution until clear bands against dark blue background was observed

\[ \downarrow \]

Zymogram was captured and analysed
CELL CYCLE ANALYSIS

Cells were collected by trypsinization

↓

Cell pellet was resuspended in ice-cold PBS (1.0 ml)

↓

4 ml of chilled absolute ethanol was added gradually while vortexing at top speed

↓

The cells were left in ethanol for 15 minutes at -20°C

↓

The cells were pellet down and supernatant was discarded

↓

5 ml PBS was added at RT

↓

Cells were allowed to rehydrate for 15 mins at RT

↓

The suspension was centrifuged and supernatant discarded

↓

1 ml of PI diluted in staining buffer was added

↓

Incubated for 15 mins at RT

↓

Sample was analysed by flowcytometery in the presence of dye
ANNEXIN-V FITC/PI ASSAY

Cells were collected by trypsinization and washed with PBS

Cell pellet was resuspended in 1 ml of annexin V binding buffer (1X)

Centrifuged at 300g for 10mins

Pellet was resuspended in 100μl binding buffer per 10^6 cells

Added 10μl Annexin V-FITC solution

Incubated in dark for 15 mins

Washed the cells with 1ml binding buffer and centrifuged at 300g for 10mins

Pellet was resuspended in 500μl of 1X binding buffer

1ml of PI diluted in staining buffer was added

Incubated for 15 mins at RT

5μl of PI solution was added immediately prior to analysis by flow cytometry