Chapter 4

Results
4. RESULTS

4.1 Withania somnifera and Neurooncology:

4.1.1 Effect of ASH-WEX on the proliferation of glioma and neuroblastoma cells:

C6 glioma and IMR-32 neuroblastoma cell lines were cultured in the presence of different concentrations of ASH-WEX. Higher concentration of ASH-WEX caused cytotoxicity and cell death. This relationship between concentration of Ashwagandha extracts and their antiproliferative effects on C6 glioma and IMR-32 neuroblastoma cells was investigated by MTT assay and counting the live and dead cells manually using Trypan blue dye exclusion assay (Fig. 1a,b and 2a,b). Cells were treated with ASH-WEX extract at concentrations ranging from 0 to 2% for 72 h and then the percentage of cell viability was analyzed. ASH-WEX inhibited the proliferation of C6 glioma and IMR-32 cells in a dose-dependent manner (Fig. 1a and 2a). The extract decreased the proliferation of C6 glioma cells and IMR-32 cells with an IC50 (concentrations of extracts leading to 50% inhibition of cell growth) of 1.24% and 0.53% respectively (Fig. 1a and 2a). Cytotoxic effects of the extract on C6 glioma and IMR-32 cells were confirmed by Trypan blue dye exclusion assay. With increase in the concentrations of ASH-WEX, the viability and total number of the cells decreased significantly. The data summarized in Fig. 1b and 2b suggests anti-proliferative effect of the extract on C6 cells at 0.1-1% and on IMR-32 at 0.1-0.5% and cytotoxic effect at the higher concentrations tested. At higher concentrations (>1.0% for C6 and 0.5% for IMR-32 cells) cells rounded up and seemed to undergo apoptotic cell death in about 72 h after the treatment (Fig. 1c and 2c). However, at lower concentrations, cells appeared to be growth arrested and showed morphology that appeared similar to the differentiated cells. When treated with ASH-WEX (0.5% and 1.0% for C6 cells, 0.2% and 0.5% for IMR-32 cells), some cells showed bright, fragmented and condensed nuclei, membrane blebbing and apoptotic bodies as seen by Hoechst staining (Fig. 1d, 2d).

Based on this data, we selected two concentrations of ASH-WEX (0.5% and 1.0% for C6 cells and 0.2% and 0.5% for IMR-32 cells) for testing its cell differentiation inducing potential in further studies.
4.1.2 Possible nature of bioactive components of ASH-WEX:

To ascertain the nature of bioactive components in ASH-WEX extract, we inactivated ASH-WEX with heat, proteinase K, Trypsin, DNase and RNase and tested its activities. MTT assays and morphological observations (Fig. 3a,b) showed no significant difference in anti-proliferative or differentiation-induction effects between control and the cells treated with these inactivated ASH-WEX preparations. Furthermore, preliminary screening tests for phytochemicals showed the presence of flavanoids, steroids, tannins, amino acids, saponins, reducing sugars and alkaloids in ASH-WEX (Table 2). The TLC profile generated by methanol:chloroform (1:1) solvent showed the presence of seven spots with Rf values 0.03, 0.20, 0.31, 0.48, 0.70, 0.77 and 0.98 (Fig. 4a,b).

4.1.3 ASH-WEX induced differentiation like phenotypes:

C6 glioma and IMR-32 cells when treated with low doses of ASH-WEX showed differentiated morphology with increased number of processes and enlarged cell size. The cells were growth inhibited and exhibited stellate process formation indicative of a more differentiated astrocytic phenotype in C6 glioma cells (Fig. 5a). IMR32 neuroblastoma cells when treated with 0.2% and 0.5% of ASH-WEX, showed significant morphological changes as compared to control cells (Fig. 6a). The treated cells showed extended and multiple projections. In order to confirm the induction of differentiation, the expression of glial cytoskeleton marker, GFAP was examined in C6 cells. ASH-WEX was observed to significantly enhance the expression of GFAP (Fig. 5a,c). Treated cells were having multiple processes and enlarged cell size as compared to control cells. Increase in GFAP-ir was also supported by Western blotting and RT-PCR results. Mean values for the percentage of GFAP protein to α-tubulin for control and treated cells are illustrated in Fig. 5d and mRNA expression to β-actin ratio in Fig 5e. The expression of GFAP was found to be significantly increased in 0.5% ASH-WEX treatment group which was further enhanced in cultures treated with 1% water extract as compared to control cultures.

To confirm the induction of differentiation in IMR-32 cells, the expression of mature neuronal marker, NF200 immunoreactivity was examined. ASH-WEX treatment lead to increased expression of NF200 (Fig. 6a,b). Increase in NF200 expression in 0.2% and 0.5% treated cells was also confirmed by Western blotting and RT-PCR to ascertain the changes at both translational and transcriptional levels (Fig. 6d,e).
4.1.4 ASH-WEX induced HSPs and senescence marker mortalin expression in glioma and neuroblastoma cells:

Based on the growth arrest induced by ASH-WEX in glioma and neuroblastoma cells, it was further examined whether it causes induction of senescence in these cells. The control and ASH-WEX treated cells were stained for mortalin that has been previously shown to change its subcellular localization from perinuclear to pancytoplasmic upon induction of senescence. ASH-WEX treated cells which attained differentiated morphology also showed pancytoplasmic staining typical of the normal cells. The shift of mortalin staining pattern occurred in more than 80-90% of the cells treated with ASH-WEX (Fig. 7a,c and 8a). The imunoreactivity of mortalin was significantly enhanced in ASH-WEX treated cells with more pronounced increase in higher dose (1.0% in C6 cells and 0.5% in IMR-32 cells). Results are presented in Fig. 7b and 8b. The potential of ASH-WEX to enhance mortalin-ir was also confirmed by Western blotting and RT-PCR results which showed significant increase in mortalin expression both at translational and transcriptional levels (Figure 7d,e and 8c,d).

HSP70 expression was increased with ASH-WEX treatment in both C6 as well as IMR-32 cells (Fig. 9 and 10). There was a significant (p<0.05) increase in immunoreactivity of HSP70 in a dose dependent manner in 0.5 and 1.0% ASH-WEX treated groups in C6 cells (Fig. 9a,b). Similarly, in case of IMR-32 cells, the HSP70 immunoreactivity was minimal in the control cells and there was significant increase with 0.2% and 0.5% ASH-WEX treatment (Fig. 10c,d). Western blot and RT-PCR results further supported these observations showing significant increase in HSP70 expression (Fig. 9c,d and 10c,d).

4.1.5 ASH-WEX leads to G0/G1 cell cycle arrest:

Since it has been reported that cell cycle arrest at the G0/G1 or G2/M boundaries, as well as cytokinetic block, may be indicative of senescence-like alterations and followed by cell death events, we analyzed cell cycle distribution in C6 and IMR-32 cells in our experimental conditions. As regards the G2/M and S phases of the cycle, there was a significant decrease in the median percentage of cells with DNA content corresponding to these phases after treatment with 0.5% ASH-WEX and RA, in comparison with the population of control cells in both the cell lines under investigation (Fig. 11a, 12a). In case of C6 glioma cells, there were only 22.47% cells in S phase in ASH-WEX treated group as compared to 40.12% in control and 27.37% in RA treated
groups. Similarly there were only 26.26% cells in S phase in ASH-WEX treated group as compared to 39.08% in control and 32.45% in RA treated groups in IMR-32 cells. Concurrently, a significant increase in the median percentage of cells classified as G0/G1, according to their DNA content, has been observed as a consequence of exposure to 0.5% ASH-WEX and RA. ASH-WEX treated group showed highest percentage of cells (72.67% in C6 cells and 64.64% in IMR-32 cells) in G0/G1 phase as compared to control (54.27% in C6 cells and 47.47% in IMR-32 cells) and RA treated groups (65.49% in C6 cells and 59.38% in IMR-32 cells). Results are represented as histogram in Fig. 11b and 12b.

To further verify these observations and to resolve the question if the above-described fluctuations in the percentages of cells between cell cycle phases were related to G0/G1 arrest and consequently differentiation, or rather to an elevated rate of cell death in G0/G1, Annexin V-FITC and PI staining was carried out (Fig. 11c, 12c). With 0.5% ASH-WEX treatment, the median values for annexin V-positive/PI-negative (early apoptotic), annexin V-positive/PI-positive (late apoptotic), annexin V-negative/PI-positive (necrotic) cells were 66.86%, 5.79%, 0.01% for C6 glioma cells and 53.10%, 10.93% and 0.01% for IMR-32 neuroblastoma cells, respectively, which were significantly higher than the control group, indicating a shift toward early apoptosis. RA treatment group further showed an increase in number of early apoptotic (71.24% in C6 cells and 61.60% in IMR-32 cells) and late apoptotic (4.12% in C6 and 16.75% in IMR-32) cells, as compared to the control (Fig. 11d, 12d).

4.1.6 ASH-WEX modulated cell cycle, apoptotic and survival markers:

To further look into the possible signaling pathways associated with antiproliferative potential of ASH-WEX, the expression of Cyclin D1, bcl-xl and Akt-P were studied in both the cell lines. 10μM RA treated cells were taken as positive control for differentiation. Only 0.5% ASH-WEX treatment group was further used for these studies as this concentration of ASH-WEX showed more promising results in both the cell lines during the initial studies. Elevated expression of cyclin D1 is associated with high degree of malignancy and rapid cell proliferation. 0.5% ASH-WEX treatment led to significant decrease in expression of Cyclin D1 immunoreactivity in both the cell lines (Fig. 13a, 14a). The expression was significantly low, both at transcriptional as well as translational levels as assessed by Western blotting and RT-PCR respectively (Fig. 13b,c
and 14b,c) and more prominent changes were observed in ASH-WEX treated cells as compared to RA treated group.

Similarly anti-apoptotic marker bcl-xl was significantly decreased upon 0.5% ASH-WEX treatment as assessed by immunocytofluorecence and expression was significantly lower than the RA treated group in C6 (Fig. 13a) as well as in IMR-32 cells (Fig. 14a). Western blotting and RT-PCR results also showed significant downregulation of Bcl-xl expression upon ASH-WEX treatment (Fig. 13b,c and 14b,c).

Akt is another important cell signalling molecule involved in cell proliferation and survival. Akt-P showed differential expression after treatment with ASH-WEX in C6 and IMR-32 cells. In C6 cells there was significant decrease in Akt-P expression in the ASH-WEX treated cells as compared to control cells. Conversely, Akt mRNA levels were increased in 0.5% ASH-WEX treated cells, although not significantly (Fig. 13a,b,c). On the otherhand, phosphorylated Akt expression appeared to be induced in IMR-32 cells upon treatment with ASH-WEX and RA. This marker was localized more into the neurite like projections of the cells in the treated cells (Fig. 14a). The differential pattern of Akt-P expression in C6 glioma and IMR-32 neuroblastoma after ASH-WEX treatment might be due to difference in cell lineage. The increase in Akt-P expression was statistically significant in the ASH-WEX group as shown by Western blotting results (Fig. 14b). The mRNA expression of Akt was also increased in the ASH-WEX treated group (Fig. 14c).

4.1.7 ASH-WEX induced changes in cell adhesion properties of glioma and neuroblastoma cells:

Brain and neural tumors have the characteristics of being highly invasive and metastatic. Thus the expression of onco-developmental and plasticity markers such as neural cell adhesion molecule and its polysialylated form (NCAM and PSA-NCAM) was studied in C6 and IMR-32 cells to investigate the anti-migratory and anti-invasive potential of ASH-WEX. Low NCAM expression has been shown to relate with clinically aggressive cancers. Immunoreactivity of cell adhesion marker NCAM significantly increased in cells treated with ASH-WEX in both the cell types as detected both by immunostaining and Western blot analysis (Fig. 15a). The expression level of NCAM-120 and -140kDa isoforms significantly increased in cells treated with ASH-WEX as detected by Western blot analyses Fig 15b). This increase in expression was also observed at mRNA level as shown by RT-PCR results (Fig. 15c). Untreated control
IMR-32 cells showed least NCAM immunoreactivity, but with 0.2% and 0.5% ASH-WEX treatment, there was a dose dependent increase in NCAM expression (Fig. 16a). Western blot data further supported significant increase in the expression of NCAM isoforms with a prominent band of 140kDa (Fig. 16b). mRNA expression study for total NCAM mRNA by RT-PCR also supported these observations with significant increase in its expression (Fig. 16c).

Keeping in view the relationship between tumor invasivenesss and PSA-NCAM expression which promotes invasion, we also investigated the expression of PSA-NCAM on both cell types after ASH-WEX treatment. PSA-NCAM expression was significantly downregulated upon treatment with ASH-WEX in both the C6 and IMR-32 cells and the expression was minimal in higher ASH-WEX concentration treated group as shown by immunostaining and Western blotting (Fig. 17a,b and 18a,b). In case of C6 cells, although the PSA-NCAM expression was cytoplasmic, but there was decrease with ASH-WEX treatment to a significant extent (Fig. 17a). In the IMR-32 cells, 80-90% cells in control group expressed PSA-NCAM which was downregulated after ASH-WEX treatment. Only 5-10% cells seemed to be stained positive for PSA-NCAM in 0.5% ASH-WEX (Fig. 18a). Expression of polysialyltransferase (PST) enzyme responsible for addition of PSA residues on NCAM was evaluated using RT-PCR in the ASH-WEX and control cells. There was significant decrease in the expression of PST mRNA expression when treated with ASH-WEX as compared to control group (Fig. 17c, 18c).

To evaluate the anti-migratory potential of ASH-WEX in C6 glioma cells and IMR-32 cells, their response to wound scratch healing with and without ASH-WEX was analyzed. The untreated C6 glioma cells were able to invade the scratched area that was fully re-colonized by 6hr, whereas 0.5% and 1.0% water extract treatment strongly reduced the migration rate of the C6 glioma cells. In fact, 6 h after the scratch, very few cells were seen in the scratched area in the 1.0% ASH-WEX treatment group (Fig. 19a). Quantitative analysis also indicated a significant decrease (about 35 to 75%) in the cell migration rate following ASH-WEX treatment (Fig. 19b). Similarly in case of IMR-32 cells untreated IMR32 cells were able to invade the scratched area that was fully re-colonized by 24 hr. 0.2% and 0.5% water extract treatment significantly reduced the migration rate of the IMR-32 neurob.latoma cells. Quantitative analysis indicated a significant decrease (about 27 to 55%) of the cell migration rate following ASH-WEX treatment (Fig. 20a,b)
MMPs play an important role in tumor invasion and metastasis. Zymography is described as simple, sensitive, quantifiable, and functional assays to analyze MMPs and gelatin zymography is mainly used for the detection of the gelatinases, MMP-2 and MMP-9. Therefore, gelatin zymography was performed to assess the activity of MMP2 and MMP9 matrix metalloproteases to strengthen the anti-migratory/invasive properties of ASH-WEX in C6 and IMR-32 cells. MMP2 and MMP9 activities were significantly reduced in the 1.0% treatment group in C6 cells and 0.5% ASH-WEX treatment group in IMR-32 as compared to control (Fig 21a, 22a). The percentage decrease in MMP2 activity was apparently more as compared to MMP-9 and the change was statistically significant (Figure 21a, 22a). Expression of MMP-2 and MMP-9 was also analysed at mRNA level and 0.5% ASH-WEX treatment lead to significant decrease in their expression in C6 and IMR-32 cells as compared to control untreated cells (Fig. 21b, 22b).

4.2 Withania somnifera and Neuroexcitotoxicity:

4.2.1 ASH-WEX attenuated glutamate-induced cytotoxicity:

RA differentiated C6 and IMR-32 cells, were treated with various doses of glutamate (0.06 mM-10 mM) to determine the excitotoxicity of glutamate. Cultures treated with increasing concentration of glutamate for 24 h exhibited cell shrinkage and rounding (Fig. 23a and 24a). MTT assay on glutamate treated cells revealed a decrease in the number of living cells after treatment with increase in dose of glutamate (Fig. 23b and 24b). The extent of glutamate toxicity for RA-differentiated C6 glial and IMR-32 neuronal cells was different; whereas 1mM glutamate exposure induced more than 50% C6 glial cell death, IMR-32 neuronal cells showed similar effect with 0.5 mM glutamate (Fig. 23b,c and 24b,c). Using these doses as glutamate induced excitotoxicity model, ASH-WEX (0.05% and 0.1%) treatment was given to investigate whether it could protect differentiated C6 and IMR-32 cells against glutamate-induced cell death. Pretreatment for 24 hrs with ASH-WEX (0.1%) significantly inhibited the death of C6 and IMR-32 cells exposed to glutamate (Fig. 23d and 24d). Glutamate-induced changes in the cell morphology were partially suppressed by treatment with 0.1% ASH-WEX. Of note, the recovery in the cell morphology was observed only for the low dose (0.5 mM for C6 and 0.25 mM for IMR-32) of glutamate treatment (Fig. 23a and 24a). LDH assay further supported these results; the enzyme activity increased with rise in glutamate dose.
in both the cell types (Fig. 23c,e and 24c,e). Based upon MTT and LDH assay two doses of glutamate - 0.5 mM and 1mM for C6 cells and 0.25 mM and 0.5mM for IMR-32 cells were selected for further experiments.

4.2.2 ASH-WEX abolished glutamate induced changes in the GFAP and NF200 proteins:

Increase in GFAP expression is a sign of astrogliosis, reactive injury, and neurodegeneration in the differentiated glial cells. Cells were exposed to 0.5 mM or 1 mM glutamate (24 h) after the pretreatment with 0.1 % ASH-WEX for 24h. As shown in Fig. 21a, there was a significant increase (p<0.05) in GFAP expression in the C6 cells upon treatment with glutamate which was suppressed with ASH-WEX pre-treatment in the 0.5 mM glutamate treatment group, whereas, in the 1 mM glutamate treatment group 0.1% ASH-WEX was not able to normalize the GFAP expression level (Figure 25a). GFAP mRNA expression was further studied by RT-PCR and a significant increase was observed in GFAP mRNA expression in glutamate treated group. ASH-WEX pre-treatment was able to suppress the upregulation in GFAP mRNA in both low and high dose glutamate groups (Fig. 26b). Single cell quantitative immunocytofluoroscence for GFAP in these groups further revealed dose dependent increase in GFAP expression in the glutamate-exposed as compared to the control cells (Fig. 25c). In cells pretreated with ASH-WEX, the expression level of GFAP was normalized in low dose glutamate group but there was no significant difference in expression of GFAP in high dose group (Fig. 25d).

NF200 is expressed mainly in the differentiated neurons while other two forms are abundant in pre-natal stages. NF200 expression in IMR-32 cells was reduced upon glutamate exposure, whereas, pretreatment with ASH-WEX lead to recovery of glutamate induced decrease in NF200 levels shown by Western blotting (Fig. 26a). These results were supported by RT-PCR that revealed that ASH-WEX pretreatment resulted in recovery of NF200 mRNA in glutamate treated cells. There was 20-35% increase in NF200 mRNA expression in ASH-WEX pretreated groups as compared to their respective 0.25 mM and 0.5 mM glutamate groups (Fig. 26b). NF200 immunostaining confirmed the changes observed in Western blotting and RT-PCR results at single cell level (Fig. 26c) that was further quantitated by intensity analysis (Fig. 26d).
4.2.3 ASH-WEX abolished glutamate-induced increase in HSP70:

HSP70 is a member of the heat shock protein family and serves as a housekeeper in the cell, assisting in the correct folding, trafficking, and degradation of many proteins during normal and stress conditions. We examined HSP70 expression in control and ASH-WEX pretreated cells that were challenged with glutamate. As shown in Fig. 27a and 28a, HSP70 Western blots revealed significant increase after exposure to glutamate in a dose dependent manner in both C6 and IMR-32 cells, respectively, thus suggesting that glutamate treatment evoked stress response in these cells. ASH-WEX treatment both in C6 and IMR-32 cells resulted in a moderate induction of HSP70 expression and led to normalization of increase in HSP70 induced by low dose of glutamate (Fig. 27a, 28a). Upregulation in HSP70 expression (about 60% increase in C6 cells and 40% in IMR-32 cells) induced by higher dose of glutamate was not recovered significantly upon ASH-WEX pre-treatment (Fig. 27a, 28a). The RT-PCR analysis showed increase (p<0.05) in HSP70 mRNA levels at low dose glutamate treatment group in both cell types; the high dose glutamate did not cause higher induction of HSP70 mRNA (Fig. 27b, 28b). Furthermore, the immunocytostaining for HSP70 showed enhanced intensity in glutamate treated groups as compared to control (Fig. 27c, 28c). ASH-WEX pre-treatment resulted in downregulation of HSP70 in low dose glutamate group in both the cell lines; high dose glutamate groups remained unaffected (Fig. 27d, 28d).

4.2.4 ASH-WEX induced NCAM and PSA-NCAM expression to reduce excitotoxic cell death in glutamate challenged cells:

NCAM has a role in cell–cell adhesion, neurite outgrowth, synaptic plasticity, neuroprotection and learning and memory. NCAM expression in control and treated groups was examined. ASH-WEX treatment caused a minor increase in NCAM expression both in C6 and IMR-32 cells (Fig. 29a and 30a). The low dose treatment of glutamate (0.5 mM) led to upregulation of NCAM expression (p<0.05) which was further increased in the high dose glutamate (1 mM) treated cells as seen in the Western blots (Fig. 29a and 30a). ASH-WEX (0.1%) pre-treatment led to normalization of NCAM expression in the low dose glutamate group but its expression remained significantly higher (around 45%) in the high dose treatment group (Fig. 29a and 30a). These changes were also apparent at mRNA level. Lower dose of glutamate (0.5 mM) exposure led to increase in NCAM mRNA level that was normalized by ASH-WEX in C6 cells (Fig. 29b). On the other hand high dose treatment group did not show
normalization of NCAM mRNA in C6 cells when pretreated with ASH-WEX. Furthermore, ASH-WEX did not cause any recovery in NCAM mRNA expression in both low and high dose glutamate groups of IMR-32 cells (Fig. 30b). Immunocytostaining for NCAM was enhanced upon glutamate exposure in case of C6 cells as well as in IMR-32 cells (Fig. 29c and 30c). ASH-WEX pre-treatment induced normalization was evident from NCAM staining. Consistent with the protein and mRNA expression data, NCAM immunocytostaining in IMR-32 cells revealed that ASH-WEX was not able to recover cells from glutamate-induced changes.

The PSA-NCAM is considered as a marker of developing and migrating neurons and of synaptogenesis in the immature vertebrate nervous system. However, it persists in the mature normal brain in some regions which retain a capability for morphofunctional reorganization throughout life. PSA-NCAM expression was further studied in control and treated groups. Glutamate exposure led to an increase in the PSA-NCAM expression by about 25% at low glutamate dose both in C6 and IMR-32 cells which was further enhanced in ASH-WEX pre-treatment group in the IMR-32 cells (Fig. 31a, 32a). The PSA-NCAM was around 15% (p<0.05) higher at high dose glutamate treatment group in C6 cells as compared to control. ASH-WEX pretreated group did not show any significant change (Fig. 31a). On the other hand, there was a dose dependent increase in PSA-NCAM expression in the IMR-32 cells from 15-45% that was further enhanced in the ASH-WEX pretreatment (Fig. 32a). The expression of PST mRNA was examined by RT-PCR and was found to be significantly increased both in glutamate and ASH-WEX treatment groups as compared to control (Fig. 31b and 32b). Immunocytostaining revealed that PSA-NCAM expression was enriched along the projections of the differentiated cells in the control group that was further enhanced by low glutamate treatment both in the C6 and IMR-32 cells. High dose glutamate led to disruption of surface expression of PSA-NCAM both in C6 and IMR-32 cells (Fig. 31c and 32c).

4.2.5 ASH-WEX modulated MMP-2 and 9 expression after glutamate exposure:

MMPs are a family of proteinases that function to cleave virtually all components of the ECM, making them excellent mediators of early inflammatory processes, tissue remodeling and scar formation following a variety of injury types. In particular, the gelatinases, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) degrade common ECM components, as well as the major CNS matrix component, chondroitin sulfate proteoglycans (CSPGs). MMP-2 and MMP-9 have been linked to blood–brain barrier
disruption, inflammation, angiogenesis, remodeling of the ECM and glial scar formation and are associated with extracellular remodeling that occurs in injury and repair processes in the CNS. The expression and activity of MMP-2 and 9 was studied by gelatin zymography. The expression/activity of both these enzymes was increased in glutamate treatment groups as apparent by the area of the white bands. ASH-WEX reduced the enzyme activity significantly upon treatment in low dose glutamate exposed cultures but was unable to induce any significant changes in high dose glutamate group in both the cell lines (Fig. 33 and 34).
Figure 1: Growth curve inhibition and cytotoxicity in C6 glioma cells as assessed by MTT assay (a), Trypan blue dye uptake assay (b). Data are representative of three different experiments done in triplicates and expressed as mean ± S.E.M. Phase contrast images of C6 glioma cells treated with 0.0% (Control), 0.2%, 0.5%, 1.0%, 1.5% and 2.0% ASH-WEX (c). There was significant difference in cell number and morphology in treated groups as compared to the control. ASH-WEX treated cultures showed multiple processes and more of differentiated morphology. 1.5% and 2.0% ASH-WEX treatment group showed cytotoxic effect of ASH-WEX with majority dead cells. Hoechst staining showing nuclear morphology for control and ASH-WEX treated cells (d).
Figure 2: Growth curve inhibition and cytotoxicity in IMR-32 neuroblastoma cells as assessed by MTT assay (a), Trypan blue dye uptake assay (b). Data are representative of three different experiments done in triplicates and expressed as mean ± S.E.M. Phase contrast images of IMR-32 neuroblastoma cells treated with 0.0% (Control), 0.1%, 0.2%, 0.5%, 0.7% and 1.0% ASH-WEX (c). There was significant difference in cell number and morphology in treated groups as compared to the control. ASH-WEX treated cultures showed multiple processes and more of differentiated morphology. 0.7% and 1.0% ASH-WEX treatment group showed cytotoxic effect of ASH-WEX with majority dead cells. Hoechst staining showing nuclear morphology for control and ASH-WEX treated cells (d).
**Figure 3:** Effect of heat and enzymatic treatments on the anti-proliferative activity of ASH-WEX. The ASH-WEX was treated with heat (95°C), Proteinase K (1 mg/ml), Trypsin (1 mg/ml), DNase I (100 μg/ml), RNase (100 μg/ml) for 30 min and its anti-proliferative and differentiation inducing activity at concentrations ranging from 0.1-2.0% was evaluated in C6 glioma cells. Phase contrast images of cells (control, a), and in the presence of heat and enzymatically treated ASH-WEX (c) showed no significant difference in the cell morphology. MTT assay of control and treated ASH-WEX showed similar anti-proliferative activity (b).
(a) Control

(b) MTT Assay

Control ASH-WEX
- Heat
- Proteinase K
- Trypsin
- DNAse
- RNAse

ASH-WEX
- 0.5%
- 1.0%

Heat

Proteinase K

Trypsin

DNAse

RNAse
**Figure 4:** Analysis of ASH-WEX by thin liquid chromatography. Chloroform: methanol (1:1) solvent system revealed seven different spots. These spots were observed under UV light (a) and later visualized using iodine vapors (b).
Figure 5: GFAP expression in response to ASH-WEX treatment. (a) Immunofluorescence detection of glial differentiation marker, GFAP, is shown in control, ASH-WEX (0.5% and 1.0%) treated C6 glioma cultures. The relative intensity measurement of immunofluorescence is shown (b). Significant changes were observed in ASH-WEX treated cells as compared to control cells. (c) Single cell micrographs of GFAP immunofluorescence in control (i-iii) and 0.5% ASH-WEX treated cultures (iv-vi). (d) Representative Western blot hybridization signals for GFAP from control and treated cultures (0.5 % and 1.0 % ASH-WEX treated). (e) Representative RT-PCR results for GFAP mRNA from control and treated cultures (0.5 % and 1.0 % ASH-WEX treated). “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treated groups.
Figure 6: (a) NF200 expression in response to ASH-WEX treatment in control, ASH-WEX (0.2% and 0.5%) treated IMR-32 cultures. The relative intensity measurement of immunofluorescence is shown (b). (c) Representative Western blot hybridization signals for NF200 from control and test samples. (d) Representative RT-PCR results for NF200 mRNA in control and treated cells and their relative densitometry analysis represented by histograms. “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treated groups.
**Figure 7:** Mortalin in response to ASH-WEX treatment. (a) Immunofluorescence detection of mortalin is shown in control, 0.5% and 1.0% ASH-WEX treated C6 glioma cultures. Relative intensity measurement of immunofluorescence is shown in (b). (c) Single cell micrographs of mortalin immunofluorescence in control (i-iii) and 0.5% ASH-WEX treated (iv-vi) cultures. Significant change in mortalin expression in response to ASH-WEX treated cultures compared to control is seen. (d) Representative Western blot hybridization signals for mortalin from control and test samples (0.5 % and 1.0 % ASH-WEX treated cells). (e) Representative RT-PCR results for mortalin mRNA from control and treated cultures (0.5 % and 1.0 % ASH-WEX treated). “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treated groups.
Figure 8: (a) Mortalin expression in response to ASH-WEX treatment in control, ASH-WEX (0.2% and 0.5%) treated IMR-32 cultures. Single cell micrographs of mortalin immunofluorescence in control ASH-WEX treated cultures. The relative intensity measurement of immunofluorescence is shown as histogram for Mortalin (b). Representative Western blot hybridization signals for Mortalin (c) from control and test samples and their relative intensity. Representative RT-PCR results for Mortalin mRNA in control and treated cells and their relative densitometry analysis represented by histograms (d). "*" represents the statistical significant (p<0.05) difference between control and ASH-WEX treated groups.
Figure 9: (a) HSP70 expression in response to ASH-WEX treatment in control, ASH-WEX (0.5% and 1.0%) treated C6 glioma cultures. The relative intensity measurement of immunofluorescence is shown as histogram for HSP70 (b). Representative Western blot hybridization signals for HSP70 (c) from control and test samples and their relative intensity. Representative RT-PCR results for HSP70 mRNA in control and treated cells and their relative densitometry analysis represented by histograms (d). “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treatment groups.
Figure 10: (a) HSP70 expression in response to ASH-WEX treatment in control, ASH-WEX (0.2% and 0.5%) treated IMR-32 neuroblastoma cultures. The relative intensity measurement of immunofluorescence is shown as histogram for HSP70 (b). Representative Western blot hybridization signals for HSP70 (c) from control and test samples and their relative intensity. Representative RT-PCR results for HSP70 mRNA in control and treated cells and their relative densitometry analysis represented by histograms (d). “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treated groups.
**Figure 11:**  (a) ASH-WEX affects the distribution of events in the C6 glioma cell cycle. C6 glioma cells were treated with 0.5% ASH-WEX for 72 h. The evaluation of cell cycle progression was done by DNA staining by propidium iodide. The figure shows representative FACS profiles of the distribution of cells in G0/G1, S, and G2/M phases as analysed by FCS software. (b) Histogram represents percentage distribution of the cells in different phases (G0/G1, S, and G2/M) after ASH-WEX treatment as compared to control. (c) Flow cytometric examination of apoptosis, necrosis and cell viability—the Annexin V/PI assay. Diagrams show four subgroups of cells. Viable (Q1, annexin V-, PI-), early apoptotic (Q2, annexin V+, PI-), late apoptotic (Q3, annexin V+, PI+) and necrotic/damaged (Q4, annexin V-, PI+) are represented in different quadrants. (d) Histogram represents percentage distribution of the cells in different quadrants. “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treated groups.
**Figure 12:** (a) ASH-WEX affects the distribution of events in the IMR-32 cell cycle. IMR-32 cells were treated with 0.5% ASH-WEX for 72 h. The evaluation of cell cycle progression was done by DNA staining by propidium iodide. The figure shows representative FACS profiles of the distribution of cells in G0/G1, S, and G2/M phases as analysed by FCS software. (b) Histogram represents percentage distribution of the cells in different phases (G0/G1, S, and G2/M) after ASH-WEX treatment as compared to control. (c) Flow cytometric examination of apoptosis, necrosis and cell viability—the Annexin V/PI assay. Diagrams show four subgroups of cells. Viable (Q1, annexin V-, PI-), early apoptotic (Q2, annexin V+, PI-), late apoptotic (Q3, annexin V+, PI+) and necrotic (Q4, annexin V-, PI+) are represented in different quadrants. (d) Histogram represents percentage distribution of the cells in different quadrants. “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treated groups.
(a) Control, 0.5% ASH-WEX, RA

(b) Control, 0.5% ASH-WEX, 10μM RA

(c) Control, 0.5% ASH-WEX, RA

(d) Viable, Early Apoptotic, Late Apoptotic, Necrotic

- Q1: Viable cells
- Q2: Early apoptotic cells
- Q3: Late apoptotic cells
- Q4: Necrotic cells
**Figure 13:** (a) Immunofluorescence detection of Cyclin D1, bcl-xl and Akt-P is shown in control, 0.5% ASH-WEX and RA treated C6 cells. Representative Western blot hybridization signals for Cyclin D1, bcl-xl and Akt-P from control and test samples (0.5% ASH-WEX and RA treated cells) (b). mRNA expression analysis for Cyclin D1, bcl-xl and Akt-P was done and densometry results for intensity analysis are represented as histogram (c). “*” represents the statistical significant (p<0.05) difference between control and treatment groups (ASH-WEX and RA).
Figure 14: (a) Immunofluorescence detection of Cyclin D1, bcl-xl and Akt-P is shown in control, 0.5% ASH-WEX and RA treated IMR-32 cells. Representative Western blot hybridization signals for Cyclin D1, bcl-xl and Akt-P from control and test samples (0.5% ASH-WEX and RA treated cells) (b). mRNA expression analysis for Cyclin D1, bcl-xl and Akt-P was done and densometry results for intensity analysis are represented as histogram (c). “*” represents the statistical significant (p<0.05) difference between control and treatment groups (ASH-WEX and RA).
Figure 15: NCAM detection in the presence of ASH-WEX. (a) NCAM expression in response to ASH-WEX treatment in control, ASH-WEX (0.5% and 1.0%) treated C6 glioma cultures. Representative Western blot hybridization signals for NCAM 120 and NCAM 140 (b) from control and treated cells and their relative intensity. Representative RT-PCR results for NCAM mRNA in control and treated cells and their relative densitometry analysis represented by histograms (c). “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treatment groups.
(a)

Control | 0.5% ASH-WEX | 1.0% ASH-WEX

(b)

Control | ASH-WEX 0.5% | 1.0%

NCAM 140

NCAM 120

α-tubulin

(c)

Control | ASH-WEX 0.5% | 1.0%

NCAM

β-actin

Percent change

Percent change

Percent change
Figure 16: NCAM detection in the presence of ASH-WEX treatment (a) NCAM expression in response to ASH-WEX treatment in control, ASH-WEX (0.2% and 0.5%) treated IMR-32 neuroblastoma cells. Representative Western blot hybridization signals for NCAM from control and treated cells and their relative intensity (b). Representative RT-PCR results for NCAM mRNA in control and treated cells and their relative densitometry analysis represented by histograms (c). “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treatment groups.
**Figure a**

Control, 0.2% ASH-WEX, 0.5% ASH-WEX images.

**Figure b**

NCAM 225kDa, 150kDa, 102kDa, and α-tubulin images with graphs showing percent change for C, 0.2%, and 0.5% ASH-WEX.

**Figure c**

NCAM and β-actin images with graphs showing percent change for C, 0.2%, and 0.5% ASH-WEX.
**Figure 17:** (a) PSA-NCAM expression in response to ASH-WEX treatment in control, ASH-WEX (0.5% and 1.0%) treated C6 glioma cultures. Representative Western blot hybridization signals for PSA-NCAM (b) from control and treated cells and their relative intensity. Representative RT-PCR results for PST mRNA in control and treated cells and their relative densitometry analysis represented by histograms (c). “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treatment groups.
Figure 18: (a) PSA-NCAM expression in response to ASH-WEX treatment in control, ASH-WEX (0.2% and 0.5%) treated IMR-32 neuroblastoma cells. Representative Western blot hybridization signals for PSA-NCAM from control and treated cells and their relative intensity (b). Representative RT-PCR results for PST mRNA in control and treated cells and their relative densitometry analysis represented by histograms (c). “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treatment groups.
Figure 19: Representative phase contrast images of control, 0.5% or 1.0% ASH-WEX treated cells, in which motility was analyzed by Wound-scratch test. Images show the starting (0 h after scratch) and the end (6 h after scratch) point of the analysis (a). (b) Graph shows that the rate of C6 glioma migration in response to ASH-WEX treatment in comparison to untreated cells. Data are obtained from a set of scratch test analysis (N = 3) and are expressed as means ± standard error. “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treatment groups.
Figure 20: (a) Representative phase contrast images of control, 0.2% and 0.5% ASH-WEX treated cells, in which motility was analyzed by Wound-scratch test. Images show the starting (0 h after scratch) and the end (24 h after scratch) point of the analysis. (b) Graph shows that the rate of IMR-32 migration in response to ASH-WEX treatment in comparison to untreated cells. Data are obtained from a set of scratch test analysis (N = 3) and are expressed as means ± standard error. “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treatment groups.
Figure 21: Representative MMP zymogram from media of control and treated samples of C6 glioma cells and their densometry analysis is represented as histogram (a). mRNA expression for MMP2 and MMP9 was analyzed by RT-PCR. Relative percentage expression was expressed as histogram (b). “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treatment groups.
Figure 22: Representative MMP zymogram from media of control and treated samples of IMR-32 cells and their densometry analysis is represented as histogram (a). mRNA expression for MMP2 and MMP9 was analyzed by RT-PCR. Relative percentage expression was expressed as histogram (b). “*” represents the statistical significant (p<0.05) difference between control and ASH-WEX treatment groups.
(a) Gelatin Zymogram

(b) 

C | 0.5% ASH-WEX

MMP9

MMP2

β-actin

Percent Change

MMP-9

MMP-2
**Figure 23:** (a) The morphological changes in C6 cells were studied using phase contrast images. Cell viability and toxicity of various concentrations of glutamate was assayed by MTT (b) and LDH (c) assays in RA differentiated C6 cells. (d) histograms represents the relative percentage viability of glutamate and ASH-WEX treated C6 cells, respectively, as compared to the control cells. (e) histograms represents the relative LDH activity when the control and ASH-WEX pretreated cells were exposed to different glutamate concentrations. “*” represents the statistical significant difference between all the treatment groups (glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
(a) C6

(b) MTT assay

(c) LDH assay

(d)

(e) Percent change in LDH activity

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i  Control
ii 0.1% ASH-WEX
iii 0.5 mM Glu
iv 0.5 mM Glu + ASH-WEX
v 1 mM Glu
vi 1 mM Glu + ASH-WEX

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Glutamate
Glutamate + 0.05% ASH-WEX
Glutamate + 0.1% ASH-WEX
Figure 24: (a) The morphological changes in IMR-32 cells were studied using phase contrast images. Cell viability and toxicity of various concentrations of glutamate was assayed by MTT (b) and LDH (c) assays in RA differentiated IMR-32 cells. (d) histograms represents the relative percentage viability of glutamate and ASH-WEX treated IMR-32 cells, respectively, as compared to the control cells. (e) histograms represents the relative LDH activity when the control and ASH-WEX pretreated cells were exposed to different glutamate concentrations. “*” represents the statistical significant difference between all the treatment groups (glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
**IMR-32**

(a) Images showing various conditions.

(b) MTT assay graph showing relative units against glutamate concentration.

(c) LDH assay graph showing relative units against glutamate concentration.

(d) Bar graph showing percent change in viability with different glutamate concentrations and conditions.

(e) Graph showing percent change in LDH activity with different glutamate concentrations and conditions.

- i Control
- ii 0.1% ASH-WEX
- iii 0.25 mM Glu
- iv 0.25 mM Glu + ASH-WEX
- v 0.5 mM Glu
- vi 0.5 mM Glu + ASH-WEX

Legend:
- Black: Glutamate
- Blue: Glutamate + 0.05% ASH-WEX
- Light Blue: Glutamate + 0.1% ASH-WEX
Figure 25: (a) Representative Western blots and their densitometry analysis for GFAP for RA differentiated C6 cells. (b) RT-PCR results for GFAP mRNA in C6 cells, respectively and their relative densitometry analysis was represented by histograms. (c) The expression of GFAP in C6 cells was analysed by immunocytostaining and relative intensity was plotted as histogram (d) as analysed by Image pro-plus software. “*” represents the statistical significant difference between all the treatment groups (ASH-WEX alone, glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
Figure 26: (a) Representative Western blots and their densitometry analysis for NF200 for RA differentiated IMR-32 cells. (b) RT-PCR results for NF200 mRNA in IMR-32 cells, respectively and their relative densitometry analysis was represented by histograms. (c) The expression of NF-200 in IMR-32 cells was analysed by immunocytostaining and relative intensity was plotted as histogram (d) as analysed by Image pro-plus software. “*” represents the statistical significant difference between all the treatment groups (ASH-WEX alone, glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
Figure 27: (a) Representative Western blots and their densitometry analysis for HSP70 in RA differentiated C6 cells. (b) RT-PCR results for HSP70 mRNA in C6 cells and their relative densitometry analysis was represented by histograms. The expression of HSP70 in C6 cells (c) was analysed by immunocytostaining and relative intensity was plotted as histogram (d) as analysed by Image pro-plus software. “*” represents the statistical significant difference between all the treatment groups (ASH-WEX alone, glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
**Figure 1**

(a) Western blot analysis of HSP70 and α-tubulin expression in different conditions. Each bar represents the relative units of HSP70 expression compared to control. * indicates statistically significant differences.

(b) Western blot analysis of HSP70 and β-actin expression in different conditions. Each bar represents the relative units of HSP70 expression compared to control. * indicates statistically significant differences.

(c) Immunofluorescence microscopy images of C6 cells treated with different conditions. 

(i) Control
(ii) 0.1% ASH-WEX
(iii) 0.5 mM Glu
(iv) 0.5 mM Glu + ASH-WEX
(v) 1 mM Glu
(vi) 1 mM Glu + ASH-WEX

(d) Graph showing the relative units of HSP70 expression in different conditions. * indicates statistically significant differences.
Figure 28: (a) Representative Western blots and their densitometry analysis for HSP70 in RA differentiated IMR-32 cells. (b) RT-PCR results for HSP70 mRNA in IMR-32 cells and their relative densitometry analysis was represented by histograms. The expression of HSP70 in IMR-32 cells (c) was analysed by immunocyto staining and relative intensity was plotted as histogram (d) as analysed by Image pro-plus software. “*” represents the statistical significant difference between all the treatment groups (ASH-WEX alone, glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
Figure 29: (a) Representative Western blots and their densitometry analysis for NCAM in RA differentiated C6 cells. (b) RT-PCR results for NCAM mRNA in C6 cells and their relative densometry analysis was represented by histograms. (c) The expression of NCAM in C6 cells was analysed by immunostaining. “*” represents the statistical significant difference between all the treatment groups (ASH-WEX alone, glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
a) NCAM and α-tubulin protein Western blot analysis.

- i: Control
- ii: 0.1% ASH-WEX
- iii: 0.5 mM Glu
- iv: 0.5 mM Glu + ASH-WEX
- v: 1 mM Glu
- vi: 1 mM Glu + ASH-WEX

b) NCAM and β-actin mRNA expression analysis.

- i: Control
- ii: 0.1% ASH-WEX
- iii: 0.5 mM Glu
- iv: 0.5 mM Glu + ASH-WEX
- v: 1 mM Glu
- vi: 1 mM Glu + ASH-WEX

C) C6 cell images:

- i: Control
- ii: 0.1% ASH-WEX
- iii: 0.5 mM Glu
- iv: 0.5 mM Glu + ASH-WEX
- v: 1 mM Glu
- vi: 1 mM Glu + ASH-WEX
Figure 30: (a) Representative Western blots and their densitometry analysis for NCAM in RA differentiated IMR-32 cells. (b) RT-PCR results for NCAM mRNA in IMR-32 cells and their relative densometry analysis was represented by histograms. (c) The expression of NCAM in IMR-32 cells was analysed by immunostaining. “*” represents the statistical significant difference between all the treatment groups (ASH-WEX alone, glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
(a) NCAM and α-tubulin expression levels in different groups.

(b) NCAM and β-actin expression levels in different groups.

(c) Immunofluorescence images of IMR-32 cells in different conditions.
Figure 31: (a) Representative Western blots and their densometry analysis for PSA-NCAM in RA differentiated IMR-32 cells. (b) RT-PCR results for PST mRNA in C6 cells and their relative densometry analysis was represented by histograms. (c) The expression of PSA-NCAM in C6 cells was analysed by immunostaining. “*” represents the statistical significant difference between all the treatment groups (ASH-WEX alone, glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
(a) PSA-NCAM
- Relative units of PSA-NCAM
- i: Control
- ii: 0.1% ASH-WEX
- iii: 0.5 mM Glu
- iv: 0.5 mM Glu + ASH-WEX
- v: 1 mM Glu
- vi: 1 mM Glu + ASH-WEX

(b) PST
- Relative units of PST
- i: Control
- ii: 0.1% ASH-WEX
- iii: 0.5 mM Glu
- iv: 0.5 mM Glu + ASH-WEX
- v: 1 mM Glu
- vi: 1 mM Glu + ASH-WEX

(c) C6
- i: Control
- ii: 0.1% ASH-WEX
- iii: 0.5 mM Glu
- iv: 0.5 mM Glu + ASH-WEX
- v: 1 mM Glu
- vi: 1 mM Glu + ASH-WEX

Scale bar: 20 μm
Figure 32: (a) Representative Western blots and their densometery analysis for PSA-NCAM in RA differentiated C6 cells. (b) RT-PCR results for PST mRNA in IMR-32 cells and their relative densometery analysis was represented by histograms. (c) The expression of PSA-NCAM in IMR-32 cells was analysed by immunostaining. “*” represents the statistical significant difference between all the treatment groups (ASH-WEX alone, glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
**a**

PSA-NCAM

α-tubulin

Relative units of PSA-NCAM

i  Control

ii 0.1% ASH-WEX

iii 0.25 mM Glu

iv 0.25 mM Glu + ASH-WEX

v 0.5 mM Glu

vi 0.5 mM Glu + ASH-WEX

**b**

PST

β-actin

Relative units of PST

i  Control

ii 0.1% ASH-WEX

iii 0.25 mM Glu

iv 0.25 mM Glu + ASH-WEX

v 0.5 mM Glu

vi 0.5 mM Glu + ASH-WEX

**c**

IMR-32
Figure 33: Representative Gelatin Zymograms for MMP 2 & 9 from media obtained from different groups of RA differentiated C6 cells. The zymograms were analysed using spot-denso method in Alpha Ease software and data was represented as histograms. “*” represents the statistical significant difference between all the treatment groups (ASH-WEX alone, glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
C6

MMP-9

MMP-2

Relative Units of MMP-9 activity

Relative Units of MMP-2 activity

i  Control
ii  0.1% ASH-WEX
iii  0.5 mM Glu
iv  0.5 mM Glu + ASH-WEX
v  1 mM Glu
vi  1 mM Glu + ASH-WEX
Figure 34: Representative Gelatin Zymograms for MMP 2 & 9 from media obtained from different groups of RA differentiated IMR-32 cells. The zymograms were analysed using spot-denso method in Alpha Ease software and data was represented as histograms. “*” represents the statistical significant difference between all the treatment groups (ASH-WEX alone, glutamate alone or glutamate + ASH-WEX groups) with respect to control group. “#” represents the statistical difference between “glutamate + ASH-WEX” treated groups with their respective “glutamate” treatment groups. “*” and “#” = p<0.05.
**IMR-32**

**MMP-9**

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- i Control
- ii 0.1% ASH-WEX
- iii 0.25 mM Glu
- iv 0.25 mM Glu + ASH-WEX
- v 0.5 mM Glu
- vi 0.5 mM Glu + ASH-WEX
Table 2: Analysis of phytochemicals in the ASH-WEX

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‘+’ = Presence, ‘-’ = Absence