5. DISCUSSION

5.1 Role of Ashwagandha in Neurooncology:

5.1.1 ASH-WEX showed antiproliferative effects and induced differentiated morphology

Glioma and neuroblastoma are one of the most common primary brain tumors with only limited options for treatment and hence calls for the development of novel therapeutic approaches. In the present study, we provide evidence that the water extract of Ashwagandha leaves has anti-proliferative, neurodifferentiation-inducing and anti-migratory activity for rat C6 glioma cell and human IMR-32 neuroblastoma cells. ASH-WEX has several advantages relating to its feasibility and ease of preparation, use and consumption as an Ayurvedic supplement or medicine. We observed that the treatment with ASH-WEX significantly inhibited the proliferation of C6 glioma and IMR-32 cells with a concentration-dependent decrease in the cell viability as assessed by trypan blue dye exclusion analysis and MTT assay with ASH-WEX, higher concentration (above 1.0% for C6 and >0.5% for IMR-32) being cytotoxic. Growth inhibition of many human tumor cell lines, such as human osteogenic sarcoma, fibrosarcoma, breast carcinoma, lung carcinoma and colon carcinoma and normal cells has been reported with Ashwagandha extracts (Jayaprakasam et al., 2003). The anti-proliferative activity of ASH-WEX in C6 and IMR-32 cells may be the result of inhibition of proliferation and differentiation of tumor cells as verified by MTT assay and phase contrast imaging.

To evaluate the possible nature of bioactive molecules in the ASH-WEX, the control and heat, proteinase K, trypsin, DNase and RNase treated ASH-WEX was tested for anti-proliferative activity using MTT assay. There was no significant difference in the anti-proliferative activity of the extract after different treatments suggesting that the active components are neither heat labile, nor prolineaceous and nor nucleic acid in nature. The possibility of the active molecule being a lipid was also excluded, as ASH-WEX is aqueous in nature. Earlier studies with the root extract of Ashwagandha have characterized water-soluble fractions to contain sitoindosides VII-X and withaferin A (Bhattacharya et al., 1987). Interestingly, withaferin A has been found to be present in the alcoholic extracts of Ashwagandha
leaves (Widodo et al., 2007). Withaferin A has been well studied for its tumor inhibitory potential both in vitro and in vivo studies (Devi et al., 1992; Widodo et al., 2007, 2008). Its presence along with other possible bioactive components in aqueous leaf extract is yet to be established by further studies.

The differentiation inducing potential of Ashwagandha was further evaluated using GFAP as marker. The astrocyte marker, GFAP, is involved as a collaborator in the complex cellular processes controlling astrocytoma cell morphology, differentiation and proliferation (Toda et al., 1999). GFAP was found to be increased in C6 glioma cells following ASH-WEX treatment, as observed by immunostaining and Western blotting results. Earlier studies in which C6 cells transfected with GFAP cDNA have shown significantly reduced tumor growth (Toda et al., 1999). The growth and invasive potential of the antisense GFAP-transfected astrocytoma cells was significantly enhanced (Rutka et al., 1994). Some other studies have also detected marked increase in GFAP and induction of differentiation with various differentiating agents such as neomycin (Cuevas et al., 2004), dimethylformamide (Li et al., 1997), tanshione (Wang et al., 2007) and retinoic acid (Rutka et al., 1988). Our data suggested that the ASH-WEX is able to induce differentiation in C6 glioma cells indicating it to be a potential differentiation-inducing and anti-cancer agent in glioma.

Several recent studies have reported that the induction of cellular differentiation is an attractive therapeutic strategy against glioma cell tumorigenicity (Kawamate et al., 2006). In the present study there was significant upregulation of GFAP expression as well as morphological changes with an apparent increase in number and length of projections in the C6 glioma cells. Several in vitro studies have suggested a strong negative correlation between the level of GFAP expression and the dedifferentiation and transformation of astrocytes. In U-251 human astrocytoma cells, suppression of GFAP expression by stable transfection with an antisense GFAP construct led to decreased cell differentiation and loss of the ability to extend processes in response to addition of neurons (Weinstein et al., 1991). Restoration of GFAP expression reversed the phenotype (Chen and Liem, 1994). In another study, U-251 cells transfected with GFAP antisense cDNA showed increased saturation densities, enhanced proliferative potential, and improved anchorage-independent growth in soft agar (Rutka et al., 1994). In SF-126 human astrocytoma cells, which are normally GFAP negative, overexpression of GFAP slowed proliferation, increased the size of cellular processes, and reduced the number and growth of
colonies in soft agar (Rutka and Smith, 1993). Consistent with these studies, our data suggest that ASH-WEX reduced proliferation and induced differentiation of glioma cells. Stable transfection of rat astrocytoma C6 cells with GFAP cDNA suppressed cell growth and increased the extension of cellular processes (Toda et al., 1994) and reduced tumorigenicity in vivo (Toda et al., 1999). In vitro, a reduced expression of GFAP in some glioblastoma cell lines was also associated with more aggressive and invasive potentials (Murphy et al., 1998; Zhou and Skalli, 2000; Lee et al., 2005). The increase in GFAP expression and number of cell projections is indicative of the differentiated phenotype of these cells in response to ASH-WEX treatment.

In IMR-32 cells, the differentiation phenotype was confirmed by NF200 expression which was significantly increased in ASH-WEX treatment group. NF200 and its phosphorylated is a marker of differentiation of neuronal cells (Elder et al., 1998). In an earlier study, the constituents of alcoholic extract of Ashwagandha have been shown to elevate level of NF200 in neuroblastoma cells along with neurite outgrowth (Kuboyama et al., 2002, 2005). Consistent with these reports the increase in expression of NF200 in the present study may be attributed to differentiation inducing activity of ASH-WEX in the neuroblastoma cells. Induction of differentiation by ASH-WEX may be further explored as a novel approach in the multimodality treatment of gliomas and neuroblastomas.

The differentiation-inducing potential of ASH-WEX was also confirmed by immunostaining pattern of mortalin protein in control and treated cells. Mortalin is distributed in a pancytoplasmatic manner in normal cells, but in immortal cells its localization shifts to the perinuclear zone (Wadhwa et al., 1995). In the transformed cells, the reversion of subcellular distribution of mortalin from perinuclear to pancytoplasmic type has been reported to correlate with induction of senescence by introduction of a single chromosome, chromosome-fragments, and genes or chemicals (Michishita et al., 1999; Nakabayashi et al., 1999). Consistent with these observations, in the present data, the control C6 glioma and IMR-32 cells showed perinuclear localization of the mortalin protein, whereas the ASH-WEX treated cultures showed pancytoplasmic localization in both the cell lines. Also, mortalin carries the function of control of cell proliferation and differentiation (Wadhwa et al., 2002; Shih et al., 2011). Its role in neuroblastoma cell differentiation has been established in another recent study as a favorable prognostic indicator of neuroblastoma differentiation (Hsu et al., 2008). Thus the pancytoplasmic
distribution and enhanced expression of mortalin indicates that ASH-WEX treatment may be inducting cellular senescence and thus confirms the differentiation status of the IMR-32 cells in treated group. Furthermore 0.5% ASH-WEX treated cells showed nuclear localization of mortalin. A recent study has shown that the localization of mortalin is remarkably increased in the nucleus of neuroblatoma cells and coincides with the RA-elicited growth arrest, concomitant with a tight correlation between RA-induced nuclear translocation of mortalin and RA triggered neuronal differentiation (Shih et al., 2011).

A significant upregulation in the level of mortalin expression was observed in the ASH-WEX treatment groups both in C6 and IMR-32 cells. Such increase an mortalin may represent adaptive response to the ASH-WEX treatment, an adaptive response. Mechanism and significance of such increase in mortalin in ASH-WEX treated differentiated cells remains to be elucidated. In light of these reports and our current data showing mortalin induction in ASH-WEX treated differentiated glioma cells, it may be proposed that mortalin has some novel function(s) in the induction or maintenance of neuronal and glial differentiation. Molecular mechanism(s) of these novel functions of mortalin and their significance in disease therapy warrant further studies.

The differentiation of neuronal phenotype is accompanied by the sprouting of dendrites and axonal processes and requires an increase in protein transport. In the present results, HSP70 level was found to be elevated when treated with ASH-WEX in glioma and neuroblastoma cells. Elevated levels of HSP70 in ASH-WEX treated cells may depict differentiated phenotype of the cells. HSP70 is an essential ATP-dependent uncoating enzyme and its induction is important in the neuronal differentiation and neurite extension (Black et al., 1991; Yang et al., 2008). Induction of HSP70 has been reported during hematopoietic and neuronal differentiation (Sistonen et al., 1992; Leppa et al., 1997; Mie et al., 2003) and also heat shock factor mediates cell cycle regulation of HSP70 expression (Kanei-Ishii et al., 1997). HSP70 seems to regulate cellular processes in association with other signalling molecules resulting in differentiation as well as apoptosis. Also potent induction of HSP70 by WT1 and the physical association between these two proteins suggests a role for HSP70 in the cellular differentiation pathway (Maheswaran et al., 1998). Another natural molecule, Celasrol has been shown to induce HSP70 in undifferentiated neuroblastoma cells (Westerheide et al., 2004). HSP70 induced differentiation and
apoptosis in leukaemic cells has also been reported earlier (Sharif-Khatib et al., 2007). Thus the increase in HSP70 due to ASH-WEX may support the differentiation inducing activity of this extract in the C6 and IMR-32 cells.

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

The disruption of normal cell cycle regulation, which is the hallmark of cancer, presents numerous opportunities for targeting checkpoint controls to develop new therapeutic strategies in neuro-oncology. Such strategies include induction of checkpoint arrest leading to cytostasis and ultimately apoptosis, arrest of proliferating cells in stages of the cell cycle which may sensitize them to treatment with other therapeutic agents such as radiation, and targeting of therapies toward specific regulatory components of the cell cycle (Johnson and Walker, 1999). RA is a potent regulator of neuroblastoma cell differentiation (Sidell et al., 1983) and used in number of cancer differentiation based therapeutics. RA and its derivatives activate retinoic acid receptors and retinoid X receptor (RAR-RXR) complexes and induce neural differentiation of NSCs (Guan et al., 2001). We used 10μM RA treated cultures as positive controls for differentiation, to compare the potential of ASH-WEX with conventional RA induced changes in cell proliferation and morphology.

ASH-WEX treatment of C6 and IMR-32 cells resulted in downregulation of cyclin D1 expression at transcriptional as well as translational level. Genetic aberrations and over-expression of the Cyclin D1 gene have been reported for several human neoplasms and neuroblastomas (Donnellan and Chetty, 1998, Molenaar et al., 2008) and elevated expression of cyclin D1 is associated with high degree of malignancy and rapid cell proliferation (Sallinen et al., 1999). In gliomas, control of G1/S transition is likely to be important (Louis, 1997) because of deletion, mutation or amplification of related genes (Ueki et al., 1996; Rollbrocker et al., 1996) and malignant astrocytoma cell lines express higher levels of mRNA and protein of multiple cyclins compared to those of normal cells (Dirks et al., 1997). Moreover, Cyclin D1 overexpression has been reported to prevent differentiation in neuroblastoma (Molenaar et al., 2009). Downregulation/silencing of Cyclin D1 mRNA leads to neuronal outgrowth and differentiation (Molenaar et al., 2010). Recently some studies functionally link neuronal differentiation to cell cycle regulation which frequently involves the G1 cell cycle entry point (McClellan and Slack, 2006; Cesi et al., 2002; Georgopoulou et al., 2008; Sumrejkanchanakij et al., 2010).
Therefore we investigated the phase population in the cell cycle after treatment with ASH-WEX.

Analysis of C6 glioma and IMR-32 neuroblastoma cells after ASH-WEX treatment revealed a strong differentiated phenotype. FACS analysis showed an increase of the G0/G1 fraction at 72 hours after treatment which was even better than RA treated cells under similar conditions. In concordance with the arrest of the cell cycle in G1 phase, ASH-WEX treatment resulted in a reduction in cyclin D1 protein levels. This suggests that neuroblasts differentiate towards a neuronal phenotype after inhibition at the G1 checkpoint. Apart from cell cycle regulation these G1 entry checkpoint regulators have been linked to other signal transduction routes. The involvement of Cyclin D1 in neuronal differentiation processes has been suggested previously (Spinella et al., 1999; Sumrejkanchanakij et al., 2003; McClellan and Slack et al., 2006). It has been shown that Cyclin D1 over-expression itself is a driving event that prevents differentiation in neuroblastoma (Molenaar et al., 2008). This is in line with the findings that growth signaling pathways determine differentiation patterns in non-malignant neuroblasts and influence the differentiation state of neuroblastoma (Molenaar et al., 2010). These signal transduction routes most frequently involve the transcriptional regulation of Cyclin D1 and thus the effect on neuronal differentiation by these signal transduction routes could partly function through Cyclin D1 regulation (Pestell et al., 1999). Inhibition of the G1 regulating genes CDK4 or Cyclin D1 in neuroblastoma cell lines resulted in restoration of the G1 checkpoint and subsequent neuronal differentiation (Molenaar et al., 2008).

In the present study, there was significant downregulation of Cyclin D1 expression both in glioma and neuroblastoma cells. Also cell cycle analysis showed a significant increase in G0/G1 population thereby indicating ASH-WEX mediated cell cycle arrest, at G1 checkpoint, leading to differentiation of cells. In mammalian cells, antimitogenic signals cause arrest in the G1 phase of the cell division cycle. Although growth arrest is the final outcome of antiproliferative signals, the cellular processes initiated by the cell cycle block depend on the nature of the extracellular signals and the cell type receiving them. Differentiation and senescence are two common cellular processes associated with cell cycle withdrawal by antimitogenic factors, and little is known about how the cell cycle engine converts the antiproliferative messages in the different cellular responses of differentiation and senescence (Wright and Shay, 2001; Bringold and Serrano, 2000). Thus there appears to be a good correlation between the
timing and the degree of growth arrest, the kinetics of inhibition of cyclin D-dependent kinase activity, and the choices of NB cells treated with RA with respect to the alternative programs of neuronal differentiation or senescence. Previous reports have established that neuronal differentiation of NB cells triggered by RA fulfills this model (Wainwright et al., 2001). Thus, ASH-WEX treatment led to withdrawal of cell cycle and ultimately differentiation of the cells. The RA treated group served as positive control for differentiation and cell cycle analysis showed that ASH-WEX treatment led to an increase in early apoptotic population better than the RA treated group which shows that the cells are undergoing differentiation and normal cell cycle pathway of apoptosis followed by differentiation.

Ashwagandha alcoholic extract has been earlier reported by our lab to cause cell cycle arrest at G2/M by our lab (Shah et al., 2009). The difference could be due to different nature of extracts and thereby different bioactive molecules and their mode of action. As cell cycle arrest is a prerequisite of differentiation, based on current data, it is reasonable to assign ASH-WEX a key role in regulating cell cycle leading to G0/G1 cell cycle arrest with downregulation of cyclin D1 and consequent differentiation of the IMR-32 cells. Annexin V-FITC/PI staining study further supports this observation as there is increase in early apoptotic cell population which may be due to differentiation inducing ability of ASH-WEX which is finally leading the treated cells to normal apoptotic pathway.

Most of the glioma and neuroblastoma cells are resistant to apoptosis and differentiation. Bcl-xl is widely expressed in glioma and neuroblastoma cells and inhibits chemotherapy-induced apoptosis (Dole et al., 1995; Nagane et al., 1998). Anti-apoptotic functions of bcl-xl are well known. Recent reports on curcumin, andrographolide and cranberry proanthocyanidines have established bcl-xl mediated pro-apoptotic properties of natural compounds (Sukumari-Ramesh et al., 2011; Singh et al., 2012). Therefore, we further investigated whether ASH-WEX also influences the bcl-xl expression in the C6 and IMR-32 cells. ASH-WEX treatment of C6 and IMR-32 cells lead to downregulation of bcl-xl both at transcriptional and translational level, which further supports pro-apoptotic potential of ashwagandha extracts in the cancerous cells as reported earlier (Widodo et al., 2007).

Akt is another important cell signalling molecule involved in cell proliferation and survival. In C6 cells, Akt-P expression was localized in the nucleus and was downregulated with ASH-WEX treatment. The proto-oncogene is activated
in many human cancers, mostly owing to loss of the PTEN tumour suppressor (Luo et al., 2003). In such tumours, Akt becomes enriched at cell membranes where it is activated by phosphorylation. Yet many targets inhibited by phosphorylated Akt are nuclear; it remains unclear how relevant nuclear phosphorylated AKT (pAKT) function is for tumorigenesis. I3K/Akt signaling enhances actin remodeling and formation of membrane protrusions influencing Rac proteins (Scita et al., 2000), and through the activation of p70S6K modulates cell migration and invasion (Qian et al., 2004). Akt is localized at the leading edge of moving cells in actin-rich structures and interacts with actin binding proteins (Wang et al., 2006). The phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway is be the best characterized pathway in the transmission of anti-apoptotic signals for cell survival (Dudek et al., 1997). PI3K pathways regulate several malignant phenotypes including antiapoptosis, cell growth, and proliferation (Sathornsumetee et al., 2007). Activation of PI3K pathway is associated with poor prognosis in glioma patients (Chakravarti et al., 2004). Activated PI3K phosphorylates several downstream effectors including Akt. Akt is a critical mediator of PI3K signaling located at an intersection of multiple pathways involved in cell proliferation, survival, transcription, and metabolic processes (Kennedy et al., 1997; Lawlor and Alessi, 2001). In another study, glioma cell migration and invasion has also been correlated to Akt activation (Zhang et al., 2012). Thus the downregulation of Akt phosphorylation and Akt expression at mRNA levels after treatment with ASH-WEX could possibly indicate the pro-apoptotic and anti-migratory/invasive role of ASH-WEX partially via disrupting Akt/PI3K signalling pathway. Inhibitors of PI3K and Akt have undergone pre-clinical evaluation with encouraging results. Perifosine, an oral Akt inhibitor, is undergoing clinical evaluation for malignant gliomas (Momota et al., 2005; Sathornsumetee et al., 2007). Thus ASH-WEX may prove to be a potential agent against gliomas for its Akt-P inhibitory properties.

Contrary to C6 cells, Akt-P expression was upregulated when IMR-32 cells were treated with ASH-WEX in the IMR-32 cells. It is known that Akt is at a pivotal nodal point in the signaling pathway of almost all RTKs and is activated by the phosphoinositol-3-kinase. It has been reported earlier that in Neuro2a neuroblastoma cells, Akt showed increased phosphorylation after serum withdrawal leading to differentiation (Evangelopoulos et al., 2005). Also estrogen and mevastatin has been known to induce Akt mediated neurite outgrowth leading to differentiation in
neuroblastoma cells (Evangelopoulos et al., 2009; Takahashi et al., 2011). In keeping with this proposal, Perez-Tenorio et al. showed P-Akt to strongly associate with a lower S-phase fraction (Perez-Tenorio et al., 2002). In addition to its vital function in cell survival, a role for PI3K/Akt signalling has also been implicated in neuronal differentiation, and several aspects of neurite outgrowth, including elongation, calibre and branching, are regulated by activated Akt (Huang and Reichardt, 2003). These findings suggest that IMR-32 neuroblastoma cells after treatment with ASH-WEX with relatively high P-Akt levels may remain well-differentiated and exhibit a slower growth rate. P-Akt post translational change is the activated form of Akt and changes in expression of P-Akt at protein level and Akt at mRNA level could be due to post-translational modifications for signalling mechanisms. Based on the previous findings, we propose that the upregulation of Akt-P expression on treatment with ASH-WEX is an indicator of induction of cell differentiation in the IMR-32 cells.

5.1.3 ASH-WEX induced anti-migratory properties

The present data also indicated that the C6 glioma cells that express NCAM on their cell surface, show reduced proliferation in response to ASH-WEX treatment. Western blot of NCAM in ASH-WEX treated C6 glioma cells showed significant increase in the expression of NCAM. NCAM is an important plasticity marker for neurite outgrowth in culture and in axonal guidance and setting up of the neuronal network in vivo (Reichardt et al., 1989; Martini, 1994). NCAM is a developmentally regulated protein and is implicated in a variety of cellular processes, such as cell-cell adhesion, cell migration, neurite outgrowth, and synaptic plasticity. Highly up-regulated expression of NCAM, in cytoplasm as well as on surface, was observed upon 0.5% ASH-WEX treatment in IMR-32 neuroblastoma cells which appeared to be translocated into the growing neurites that developed upon differentiation, thus suggesting their participation of NCAM in neurite outgrowth and adhesion. The data was further supported in the present anti-motility assay study in which ASH-WEX treated cells were least motile, whereas control cells migrated to the scratched area and covered it. Migration of cells in culture can be correlated with in vivo invasive property of the cells.

The anti-proliferative and differentiation inducing activity of ASH-WEX could also be correlated with upregulation of NCAM expression upon treatment as homophilic interaction of NCAM induces signal transduction, resulting in neuronal
differentiation (Kolkova et al., 2000) and inhibition of cell proliferation (Perl et al., 1999, Cavallaro et al., 2001). Several studies have demonstrated the important role of NCAM in tumor migration and metastasis, with an inverse relationship between migratory potential of the cells and NCAM expression. Diminished expression of NCAM was also associated with clinically aggressive colon cancers (Roesler et al., 1997; Huerta et al., 2001), and dissemination of pancreatic beta tumor cells (Perl et al., 1999; Cavallaro et al., 2001). The consequences of drug resistance might preferably be treated by agents which reverse cellular adhesion. In this context, the branched-chain fatty acid valproate has been demonstrated to inhibit tumor cell motility and adhesion by up-regulating NCAM (Walmod et al., 1998; Beecken et al., 2005). The same is true for the differentiation inducing compound trichostatin A (Lampen et al., 2005). A significant increase in NCAM expression level accompanied by blocking cellular adhesion has also been observed when neuroblastoma cells were treated with retinoic acid (Voigt et al., 2000; Singh and Kaur, 2005). The upregulation of NCAM isoforms upon treatment with ASH-WEX in C6 glioma as well as IMR-32 cells might be due to its anti-migratory properties. Prag et al., (2002) showed that the expression NCAM 140 kDa caused a significant reduction in cell motility compared with NCAM-negative cells. Ectopic expression of the cytoplasmic domain of NCAM 140 also inhibited cell motility, presumably via the non-receptor tyrosine kinase with which NCAM 140 interacts. Furthermore they showed that the extracellular part of NCAM acted as a paracrine inhibitor of NCAM-negative cell locomotion through a heterophilic interaction with a cell surface receptor. On the other hand, Edvardsen’s works (Edvardsen et al., 1993, 1994), employing an experimental model, stressed the role of NCAM in the invasiveness process of glioma cells. In line with these studies ASH-WEX induced NCAM expression might be a consequence of differentiation resulting into decreased motility/migration proving it to be a reliable anti-migratory agent.

NCAM undergoes post-translational modification including the addition of PSA chains on its extracellular domain (Horstkorte et al., 2004). Expression of PSA is associated with cellular migration, axon induction and also contacts with their target (Kiss and Rougon, 1997). Although the expression of PSA has been shown to correlate with the progression of certain tumors such as small cell lung carcinomas (Kontogianni et al., 2005), very few studies there have been addressed to determine the role of PSA in gliomas. Suzuki et al., (2005) have detected PSA more frequently
in astrocytoma cells which spread extensively. PSA expression in most cancer cells is correlated with tumor metastasis and associated with tumor differentiation as well as serves as an onco-developmental antigen (Tanaka et al., 2000; Gallagher et al., 2000). In the present study, ASH-WEX treated C6 and IMR-32 cells showed significantly reduced expression of PSA-NCAM which is further supported by Western blotting results. ASH-WEX treatment caused downregulation of polysialylation through inhibition of PST enzyme as suggested by RT-PCR results of PST expression. The decrease in surface expression of PSA-NCAM may be attributed to differentiated phenotype of IMR-32 neuroblastoma cells as PSA-NCAM shows very high expression in embryonic and neonatal brain. Moreover study from our lab reported that high PSA-NCAM expression is retained in brain regions such as hippocampus, hypothalamus, piriform cortex etc, which retain the capacity to undergo neuronal plasticity (Sharma and Kaur, 2005). Neuroblastoma proliferation has been shown to be facilitated by polysialylation of NCAM and surface expression of PSA is regulated at the level of polysialyltransferase transcription (Hildebrandt et al., 1998).

PSA-NCAM expression in cancerous cells appears to facilitate mitosis and metastasis (Fukuda, 1996). Bertozzi’s group showed that feeding cells N-butylnanosamine can inhibit polysialyltransferase activity and suggested that glycosylation efficiency may decrease substantially when the structure of a precursor carbohydrate residue is modified (Mahal et al., 2001; Miyazaki et al., 2008). Regulation of polysialyltransferase expression was suggested to occur at the transcription-initiation level. Studies of the proximal promoter regions revealed possible binding sites for transcription factors MFZ1 and Sp1 (Yoshida et al., 1996; Takashima et al., 1998). Moreover, RA interacts with MFZ1 sites and promotes changes in polysialyltransferase expression after RA-induced differentiation in neuroblastoma cell lines (Seidenfaden and Hildebrandt, 2001). Nakagawa et al., (2002) demonstrated that the cAMP-CREB pathway regulates polysialyltransferase expression in cell culture. In addition, Bruses and Rutishauser (1998) have proposed a calcium-dependent regulatory mechanism for PST enzyme activity. It has also been reported that polysialyltransferase phosphorylation may be involved in regulation of PSA expression (Gallagher et al., 2000). Thus present result compares well with previous results on other PSA-expressing tumors, such as nonsmall-cell lung cancer, in which ST8SiaII/STX mRNA and PSA expression correlates with tumor progression (Tanaka et al., 2000).
To further assess the anti-migratory potential of ASH-WEX in glioma and neuroblastoma cells, MMPs expression was studied by gel zymography. The anti-cancer activity of ASH-WEX is also supported by downregulation of the activity/expression of MMP2 and 9 in the present study as established by the gelatin zymography and RT-PCR results thus indicating its anti-invasive/migratory properties. MMPs play an important role in tumor invasion and metastasis (Ray and Stetler-Stevenson, 1994; Chambers and Matrisian, 1997). MMP-2 and MMP-9 appear to be differentially expressed during development of the rat CNS (Deb and Gottschall, 1996). It has been known that increase in the expression, secretion in the media, and activation of MMP-2 and 9, leads to a more tumorigenic phenotype due to increased MMP-2 mediated invasion (Noujaim et al., 2002; Deryugina and Quigley, 2006). It is also well known that metastatic aggressiveness of the tumor is inversely related to differentiation status of the tumor. Using an in vitro model of cell migration and MMP inhibitors, Ogier et al., (2006) demonstrated a key role for MMP-2 in astrocyte cell morphology and migration; MMP-2 expression was principally associated with actin motility structures such as filipodia and lamellipodia in astrocytes. Furthermore, integrin and MMP-2 were shown to be partially colocalized at the periphery of the astrocyte, highlighting key roles of MMPs in neural cell migration via interactions with cell surface integrins. Downregulation of Akt expression (in particular Akt2) with antisense or dominant negative constructs resulted in inhibition of glioma cell invasion in vitro (Pu et al., 2004) and in vivo (Pu et al., 2006). The expression of matrix metalloproteinases (MMPs)-2 and -9 was inhibited in the rat tumor tissue with reduced Akt2 expression (Pu et al., 2006). Furthermore, the labeling index (LI) for cyclin D1 was often higher within invasive areas than in solid tumor (Cavalla et al., 1998). Cyclin D1 may modulate invasive ability by increasing MMP activity and cell motility, and suggests a novel function of cyclin D1 in the progression of malignant gliomas (Arato-Ohshimaa and Sawa, 1999). It was also reported that the transfection of the NCAM-negative rat glioma cell line C6 with a cDNA encoding 140 kDa NCAM isoform reduced invasiveness, both in vitro and in vivo, through the down regulation of the secretion of MMPs (Owen et al., 1998). Another natural molecule Mangiferin has been reported to suppress MMP-9 expression at the promoter, mRNA, and protein levels and additionally inhibited
MMP-9 enzymatic activity. The matrigel invasion assay showed that mangiferin suppresses the in vitro invasiveness of glioma cells, which appears to be correlated with mangiferin-mediated MMP-9 inhibition (Jung et al., 2012). Thus ASH-WEX seems to inhibit cell migration/motility through multiple pathways suppressing Akt activation, cyclin D1 expression, MMP-2 and MMP-9 activity as well as by inducing NCAM expression and suppressing its polysialylation.

Although Ashwagandha is most commonly used in Indian Ayurvedic medicine, the mechanistic aspects of its effects are still unknown and potential of its bioactive components are yet to be recognized. In the present study, ASH-WEX induced upregulation of GFAP (in C6 cells), NF200 (in IMR-32 cells), HSP70 and mortalin expression may be correlated with the induction of differentiation in these cells. The upregulation of NCAM and downregulation of PSA-NCAM and MMPs may explain the anti-migratory and differentiation inducing properties of ASH-WEX. The decrease in Cyclin D1 and bcl-xl expression and modulation of Akt-P may be resulting in arrest of C6 glioma and IMR32 cell proliferation and their differentiation into mature astrocyte and neuron-like cells, respectively. FACS analysis demonstrated that ASH-WEX caused an arrest of the cell cycle in the G0/G1 phase with a decrease of cell population in synthesis and mitosis phases in C6 and IMR-32 cells. Since most of the antineoplastic drugs in clinical use block the cell cycle in the S or G2/M phases whereas, ASH-WEX blocks the cell cycle in the G1 phase, it may be predicted that a combination of ASH-WEX as adjuvant therapy with currently used chemotherapeutic drugs may improve glioma and neuroblastoma prognosis. ASH-WEX treatment shows decreased glioma and neuroblastoma cell proliferation, cell migration in addition to induction of senescence and cell cycle arrest leading to differentiated phenotype. ASH-WEX appears to affect multiple pathways for its anti-cancer and differentiation inducing role in glioma and neuroblastoma cells instead of targeting a single protein or pathway which needs to be further studied. The current study supports the idea that ASH-WEX may have the potential to reduce the malignancy of neuroblastomas and prove to be suitable as adjunct therapy by its differentiation inducing activity. Thus evaluation and characterization of the water-soluble active components for discovery of potentially safe brain cancer phytoreagent based therapeutic is warranted.
5.2 Role of Ashwagandha in Neuroexcitotoxicity

5.2.1 ASH-WEX attenuates glutamate induced cytotoxicity

The current data reveal that the ASH-WEX ameliorated glutamate induced decrease in cell viability and excitotoxicity in a dose-dependent manner. Glutamate excitotoxicity induced apoptosis/necrosis of cells was also attenuated after treatment with ASH-WEX (0.1%) as evident from LDH assay and phase contrast images of cells. Although Ashwagandha has been reported to improve learning and memory in rats and as a potent neuroprotectant (Rajasankar et al., 2009), but the water soluble Ashwagandha leaf extract has not been evaluated for its cytoprotective potential.

5.2.2 ASH-WEX normalizes GFAP/NF200 and HSP70 expression in low dose glutamate exposed cells

In the present study, enhanced expression of GFAP upon glutamate exposure of RA differentiated C6 cells may be attributed to reactive gliosis and its induction. Upregulation of intermediate filament proteins, in particular GFAP by reactive astrocytes is perhaps the best known hallmark of reactive astrocytes and reactive gliosis. IF upregulation has been found in CNS trauma, hypoxia, around growing tumors, and in many neurodegenerative conditions (Pecky and Nilsson, 2005). Recently it has been demonstrated that a crosstalk between GFAP and glutamate signalling exists and the expression of GFAP is essential to anchor the glutamate transporter GLAST in the astrocyte plasma membrane thus enhancing GLAST-mediated transport (Sullivan et al., 2007). Also GFAP knockout mice exhibit reduced glutamate clearance (Hughes et al., 2004). Thus changes in GFAP gene expression and glutamate homeostasis might mutually influence each other. Glutamate activates the GFAP gene promoter of astrocytes through TGF-β pathway (Romao et al., 2008). Normalization of GFAP expression with low dose of ASH-WEX (0.1%) in glutamate (0.5 mM) treatment group depicted possible cytoprotective effect of the extract in C6 cells.

The expression of NF200 and its phosphorylated form was reduced upon treatment with glutamate as compared to control and corresponding ASH-WEX treatment groups. In an adult neuron, Neurofilaments (NFs) are major cytoskeletal components of neurons and are composed mainly of three different polypeptide subunits: NF-L (68 kDa); NF-M (160 kDa); and NF-H (200 kDa) (Lee and
Cleveland, 1994). Extensive phosphorylation of NFs at the carboxyterminal domains has been considered one of the means by which neurofilaments crosslink and stabilize the axonal cytoskeleton (Nixon and Shea, 1992). Therefore, NF200 degradation and dephosphorylation in the glutamate treatment group may be a sign of loss of neuronal function and eventual neuronal cell death. The ASH-WEX treatment seems to overcome the glutamate induced adverse effects in IMR-32 cells with respect to NF200 expression and its phosphorylation thus proving its neuroprotective potential. Ashwagandha has been reported to protect against stress induced neuronal damage in rats due to its antioxidant properties (Jain et al., 2001). Here we propose that the reversal of glutamate mediated changes in IFs could be partially attributed to its antioxidant mediated neuroprotective properties.

Glutamate exposure lead to increase in HSP70 expression in dose dependent manner which was reduced in low dose glutamate exposed cells treated with ASH-WEX. The HSP70 has been shown to have a neuroprotective role both in animal and cell culture models of neurotoxicity such as ischaemia (Xu et al., 2006), trauma (Brown et al., 1989), seizures (Pietrzik and Behl, 2005) and Alzheimer’s disease (Hamos et al., 1991). HSPs provide a line of defense against misfolded aggregation prone proteins and among the most potent suppressors of neurodegeneration in animal models (Meriin and Sherman, 2005). Neurons may rely on their constitutive levels of HSC70 as a ‘pre-protection' mechanism for defense against protein misfolding and aggregation that is induced by stressful stimuli or associated with neurodegenerative diseases. The expression of HSP70 was also upregulated in ASH-WEX alone treated group, thus suggesting that the ASH-WEX treatment could possibly induce HSP70 expression increasing protective capacity of cells against glutamate toxicity. In earlier studies, certain herbal extracts have been reported to induce HSP70 expression (Yan et al., 2004). Our current results suggest that ASH-WEX treatment mediated induction of HSP70 expression may be one of the mechanisms for its neuroprotective potential against glutamate toxicity. The increase in expression of HSP70 and cell survival in the 1 mM glutamate exposed cells treated with ASH-WEX may be rescuing the cells under stress conditions. Overexpression of HSP70 has been reported to be associated with a decrease in apoptotic cell death and a reduction in matrix metalloproteinases (Yenari et al., 2005).
5.2.3 **ASH-WEX modulated MMP-2 & 9 expression after glutamate exposure**

In the present study, we further observed that MMP-2 and MMP-9 activity is upregulated during glutamate induced damage. Upon ASH-WEX treatment the expression was significantly lowered especially in the low dose glutamate treatment group in C6 and IMR-32 cells. Although MMP-2 is expressed constitutively in normal nerve cells, its expression is upregulated after injury. The temporal pattern of this activation coincides with nerve degeneration and suggests that MMP-2 plays a role in the regenerative process. Also, MMP-9 is detected in the nerve immediately following injury and is most abundant at the site of injury (Taskinen and Roytta, 1997). Another excitotoxic agent Kainic acid (KA) has been shown to induce neuronal degeneration by up regulation of MMPs expression. Similarly glutamate mediated upregulation of MMPs exacerbates neuronal and glial damage (Zhang et al., 1998). Consistently glutamate led to increase in MMPs expression in dose dependent manner. The exact mechanisms that trigger glutamate induced protease synthesis are not clear. It is evident in the present study that ASH-WEX intervention leads to protection of both the cell types, at least in low glutamate treated group, which may be explained by the decrease in expression of MMPs as shown by gelatin zymography.

5.2.4 **ASH-WEX induces NCAM and PSA-NCAM expression to reduce excitotoxic cell death induced by low concentrations of Glutamate**

In the present study, we observed marked increase in NCAM expression in glutamate treated group. ASH-WEX treatment further upregulated NCAM expression besides enhanced cell viability even at high dose of glutamate. Our study is consistent with the earlier report where excitotoxic increase in NCAM has been shown in hippocampal slices (Hoffman et al., 2001). NCAM and PSA-NCAM are important cell surface plasticity markers that play important role in regeneration and repair. NCAM is developmentally down-regulated but has been shown to increase after brain injury and this increase has been linked to potential of brain for regeneration (Sharma and Kaur, 2008). The increase in cell viability could be partially due to enhanced NCAM expression which is a potent neuroprotection conferring target as evident from previous studies (Wu et al., 2001; Sharma and Kaur, 2008; Wu et al., 2010). Even soluble NCAM has been shown to interfere with glutamate-induced cell death in *in vitro* excitotoxicity assays. The growth factor, FGF-2 associated with NCAM
signalling has been described to be neuroprotective against excitotoxicity caused by glutamate (Mattson et al., 1989). Control of PSA-NCAM expression by NMDA receptor activation has been described in several systems, suggesting a functional link between these two proteins. NMDA receptors exhibit a dichotomy of signalling with both toxic and plastic responses. Recent reports from our lab have shown that exposure to subtoxic concentration of NMDA results in a PSA-NCAM mediated neuroprotective state that was measured when these neurons were subsequently challenged with toxic doses of glutamate (Singh and Kaur, 2007; 2009). Constituents of Ashwagandha have been associated with neuritic regeneration and synaptic reconstruction (Kuboyama et al., 2005; Tohda et al., 2005). NCAM and its polysialylated form being important molecules for CNS repair and regeneration, there may be direct association between NCAM and PSA-NCAM expression and ASH-WEX mediated regenerative and protective effects towards normalization and repair, which needs to be explored further.

PSA-NCAM expression was found to be significantly enhanced in response to glutamate induced excitotoxicity in C6 and IMR-32 cells, which may represent a compensatory mechanism to combat stress. In C6 cells, low dose glutamate exposure lead to significant increase in the PSA-NCAM expression level but percent change was less than the high dose treated cells. Moreover, the expression of PST in C6 cells showed dose dependent increase with increase in glutamate treatment groups and its expression was further elevated to significant level in the ASH-WEX treatment groups. In contrast, there was dose dependent increase in PSA-NCAM expression in glutamate treated IMR-32 cells. ASH-WEX treatment leads to further increase in expression of PSA-NCAM as well as PST. These differences could be possibly due to difference in cell type and therefore the differential expression of NCAM and degree of polysialylation on neuronal and glial cells.

Several studies have shown that PSA is a potent target to prevent excitotoxic neuronal cell death during development as well as under pathological conditions, resulting in glutamate release, at least in cases when glutamate is accumulated in the extracellular space at low concentrations. PSA has been proposed to inhibit activation of GluN2B-containing receptors, possibly by steric hindrance of the ligand to access the glutamate binding site at low micromolar concentrations of glutamate (Hammond et al., 2006). PSA has been shown to act as a neuroprotective agent, disconnecting overstimulated synapses to protect the relevant circuits from damage caused by
excess glutamtergic input (Sandi et al., 2001). In another study the upregulation of HSP70 and PSA-NCAM by hyperthermia has been correlated and reported to significantly impact the hippocampal plasticity, permitting induction of the complex molecular cascade responsible for neuroprotection (Ditlevsen et al., 2003; Duveau et al., 2007). In line with these results, it may be proposed that observations of increase in expression of HSP70 and PSA-NCAM upon glutamate treatment protected the cells from excitotoxic cell death. Pharmacological and biochemical analysis of PSA synthesis have suggested calcium dependent PST activity (Bruses and Rutishauser, 1998). Thus the changes in the expression of PSA-NCAM in the present study may possibly be attributed to accumulation of intracellular calcium due to glutamate exposure.

Alteration in PSA-NCAM expression levels on cell surface could also reflect differential delivery of PSA to cell surface as evident by a study in oligodendrocyte precursor cells in which NMDA induced influx of calcium probably enhanced transport of PSA to the cell surface (Wang et al., 1996). PSA inhibits GluN2B-containing receptors at low micromolar concentrations of glutamate found in the extracellular space (Sherwin, 1999; Ueda et al., 2000). It has been shown that PSA inhibits NMDAR currents at lower but not at higher concentrations of glutamate possibly by competing with glutamate in binding to positively charged amino acids. Furthermore, the expression and cleavage of the extracellular domain of NCAM/PSA-NCAM is regulated by metalloproteinase activity resulting in MMP induced proteolysis resulting in neuronal damage (Kalus et al., 2006; Shichi et al., 2011). Thus the decrease in MMP levels upon ASH-WEX treatment could possibly lead to cellular protection against any such damage. The increase in NCAM and PSA-NCAM expression upon glutamate exposure could be protective and regenerative response of the cells towards glutamate induced damage which is further enhanced by ASH-WEX treatment possibly leading to recovery of cells from excitotoxicity. In another study of glutamate-induced excitotoxicity it was revealed that treatment with PSA prevents cell death, whereas removal of neuronal cell surface-expressed PSA promotes cell death (Bouzioukh et al., 2001). Thus, PSA carried by NCAM regulates both synaptic plasticity and viability via modulation of NMDA receptors. The increase in PSA levels seen in the current results may be functionally linked to cell tolerance e.g. protection against glutamate-induced cell death, which is apparent at lower concentration of glutamate only.
Ashwagandha extracts has been widely studied for their neuroprotective properties in animal models and in vitro studies. One of the components of alcoholic extract of leaves, Withaonone has been shown to impart protection against Methoxyacetic acid (MAA) induced toxicity by suppressing the ROS levels, DNA and mitochondrial damage in vitro (Priyandoko et al., 2011). Its bioactive components Sitoindosides VII-X and withaferin A have been shown to modulate brain functions by binding with cholinergic receptors (Schliebs et al., 1997). Modulation of release of three neurotransmitters i.e., acetylcholine, glutamate and serotonin by Ashwagandha in all probability contributes to inhibition of nNOS in extract treated stressed mice (Rajasankar et al., 2009b). The neuroprotective properties of Ashwagandha have been attributed to neurochemical alterations of specific neurotransmitter systems and suppression of glucocorticoid release in chronic stress which could be exploited for treatment of neurodegenerative diseases (Bhatnagar et al., 2009). Ashwagandha root extracts have been shown to impart protection against 6-hydroxydopamine induced rat model and various other animal models for neurological disorders (Kulkarni, 1998; Kulkarni and Dhir, 2008; Sankar et al., 2007). Evidence also indicate that withanolide A, withanoside IV and withanoside VI from the Ashwagandha extract induced significant regeneration of both axons and dendrites, in addition to the reconstruction of pre- and postsynapses in the neurons (Kuboyama et al., 2005). The crude ethanolic extract of Ashwagandha roots has been shown to mitigate the effects of excitotoxicity and oxidative damage in hippocampus and the underlying mechanism could be attributed to its antioxidative properties (Jain et al., 2001; Parihar and Hemmani, 2003; Bhatnagar et al., 2009).

Consistent with these neuroprotective properties of Ashwagandha extracts, present study illustrates the neuromodulatory role of aqueous extract from leaves of Ashwganadha against glutamate induced stress and upregulation of plasticity marker proteins such as HSP70, NCAM and PSA-NCAM may rescue the glial and neuronal cells from glutamate induced cytotoxicity.

The cytoprotective effects observed in this study may also be attributed to the presence of free radical scavenging compounds in the water extract of Ashwagandha. In the present study low level glutamate induced effects were normalized by ASH-WEX but it could only partially revert the cytotoxic effects when challenged with high dose of glutamate. The higher expression of HSP70, NCAM and PSA-NCAM in response to glutamate exposure could be possibly due to cytoprotective response of
cells towards excitotoxicity in the time frame of these experiments. ASH-WEX treatment lead to significant increase in viability in glutamate treated groups implicating its cytoprotective role against cytotoxicity. As elevated levels of glutamate have been implicated in a wide range of neurological diseases thus further research into the molecular mechanism of ASH-WEX mediated neuroprotection and the search for bioactive component(s) in these extracts may prove valuable therapeutic agent to combat neurological disorders.

5.3 Conclusion:

The present study supports the idea that ASH-WEX may have the potential to reduce the malignancy/invasiveness of neuronal cancer type and may prove be an adjunct factor for glioblastoma and neuroblastoma therapy by inducing their differentiation. Since most of the antineoplastic drugs in clinical use block the cell cycle in the S or G2/M phases, whereas, ASH-WEX blocks the cell cycle in the G1 phase, a combination of ASH-WEX with currently used drugs might possibly improve therapies of glioma and neuroblastoma. Overall ASH-WEX treatment shows decreased cell proliferation, cell migration in addition to induction of senescence and cell cycle arrest leading to differentiated phenotype in both glioma and neuroblastoma cells. ASH-WEX appears to affect multiple pathways for its anti-cancer and differentiation inducing role instead of targeting a single protein or pathway which needs to be further studied. Owing to ASH-WEX based low dose, differentiation inducing potential along with involvement of stress chaperones as well as cell cycle and cell adhesion proteins, it may be a potential candidate for adjunct therapy. On the other hand, ASH-WEX treatment lead to significant increase in viability in glutamate treated RA differentiated groups implicating its cytoprotective role against glutamate mediated excitotoxicity. Thus, ASH-WEX has therapeutic potential for inducing differentiation in glioblastoma and neuroblastoma cells and on the other hand preventing the neurodegeneration associated with glutamate-induced excitotoxicity emphasizing the role of Ashwagandha in brain cancers as well as neuroprotection.

Of note ASH-WEX at concentration above 0.2% was observed to possess antiproliferative and differentiation inducing properties (0.5% and 1.0% in C6 cells, 0.2% and 0.5% in IMR-32 cells were used for anti-cancer studies) in glioma and neuroblastoma cells. Neuroprotective properties and selective killing of Ashwagandha alcoholic leaf extract has been reported earlier (Widodo et al., 2007;
Rajasankar et al., 2009). Thus we further proposed to test whether ASH-WEX may protect neuronal and glial cells against increasing concentrations of glutamate as excitotoxicity model. 0.05% and 0.1% ASH-WEX was able to significantly elevate the viability of these glutamate challenged cells. It is suggested that neuroprotective property of ASH-WEX at lower concentration may be due to low dosage of bioactive molecules as well as due to activation of different signaling pathways. At higher (>0.2%) ASH-WEX concentrations, it is deriving the cancerous cells towards differentiation and normal apoptotic cell death but at low concentrations (0.1% ASH-WEX) it may be acting as neurohormetic agent, activating the cellular protective machinery and preparing it against damage caused by glutamate excitotoxicity.

5.4 Future directions:
The evaluation and characterization of the water-soluble active components of ASH-WEX for discovery of potentially safe brain cancer and neuroprotective therapeutic phyto-reagents is warranted. It is predicted that the bioactive molecules in ASH-WEX may be various derivatives of known withanolides or some other novel compounds. Evaluation of their properties may ensure some novel molecules or a formulation with neuroprotective properties which could serve as a valuable adjunct therapeutic agent(s) with existing conventional chemo- and radio-therapeutic modalities. Further research into the molecular mechanism(s) of ASH-WEX mediated neuroprotection and anti-cancer properties may prove it to be a valuable therapeutic tool to combat brain disorders ranging from cancers to neurodegenerative diseases.