6. SUMMARY

As research on modern medicines continues to expand, simultaneously use of botanical medicines is also increasing. It is believed that multi-site mechanisms of action of herbal preparations from crude extracts may offer greater chances for success of treatment where conventional single-site agents have been disappointing. Several bioactive compounds have emerged from research in Ayurvedic herbals. *Withania somnifera* (Ashwagandha) is widely used in the Indian traditional system of medicine, Ayurveda and is considered to be a rasayana herb. Ashwagandha, although, shows great potential as a safe and effective antineoplastic agent, but more research is required to determine if Ashwagandha can duplicate this activity in brain and neural cancers. Furthermore, earlier reports have revealed that Ashwagandha is a potent protective agent in neurodegenerative and neuropsychiatric disorders. Also, its constituents induce significant regeneration of axons and dendrites, in addition to reconstruction of pre- and postsynapse in the neurons, therefore it proves to be an important candidate for treatment of neurodegenerative diseases. The present study was carried out on two aspects (i) **Role of *Withania somnifera* in Neurooncology** and (ii) **Role of *Withania somnifera* in Neuroexcitotoxicity**

I. Role of *Withania somnifera* in Neurooncology

Gliomas and Neuroblastomas are the most common primary neural tumors with only limited options for treatment. The majority of these tumors develop into malignancy and remain incurable inspite of therapies like external beam radiation, surgery and chemotherapy. Hence, there is need for the development of novel therapeutic approaches. The present study was aimed to investigate whether Ashwagandha leaf water extract can be a potential candidate for differentiation based therapy, which is an attractive alternative therapeutic approach possibly leading to the treatment of brain and neural tumors. The use of aqueous leaf extract (ASH-WEX) is ecofriendly as there is no need to uproot the plants as in case of roots and no organic solvents are involved for extraction. C6 glioma and IMR-32 neuroblastoma cell lines which are well established model systems for neurooncology related studies were used in the present investigation.
Objectives and Methodology:

- The anti-proliferative and cytotoxic effects of ASH-WEX on glioblastoma and neuroblastoma cell lines were tested by MTT assay and Dye exclusion test.
- Since the preliminary studies confirmed the anti-proliferative activity of ASH-WEX in *in vitro* model system which was observed to be closely associated with the induction of differentiation in the glioma and neuroblastoma cells, the expression of different differentiation markers such as glial fibrillary acidic protein (GFAP, in glioma), Neurofilament 200 (NF200, in neuroblastoma), were studied. To confirm the cell fate, the senescence marker Mortalin was studied using Western blotting and RT-PCR. Mortalin is distributed in a pancyttoplasmatic manner in normal cells, but in immortal cells its localization shifts to the perinuclear zone.
- Brain tumors are highly malignant and invasive. Thus molecules involved in regulation of cellular migration, metastasis and invasivity were investigated. The cell surface markers neural cell adhesion molecule (NCAM) and its polysialylated form PSA-NCAM are important onco-developmental markers and are involved in invasion and migration of brain tumors. Their expression was studied using immunocytofluorescence and Western blotting and RT-PCR. The *in vitro* anti-migratory/anti-invasive properties of the ASH-WEX were further tested using wound scratch assay and the expression level of extracellular matrix components, matrix metalloproteases (MMP 2 and 9) was investigated by gelatin zymography as MMPs are strongly related to tumor invasion and metastasis.

II. Role of *Withania somnifera* in Neuroexcitotoxicity

Since our initial observations revealed differentiation inducing potential of ASH-WEX at low doses so it was further planned to study whether Ashwagandha extract has the potential to protect glial and neuronal cells against glutamate induced excitotoxicity. As glutamate is the major excitatory neurotransmitter and commonly involved in neurodegenerative disorders, protection against its excitototoxicity may be a beneficial therapeutic intervention in related neuropathological conditions. Retinoic acid (RA) differentiated C6 and IMR-32 cells have been reported as an acceptable model system for primary glial and neuronal, cells respectively.

Objectives and Methodology:
➢ To test whether Ashwagandha extract can protect differentiated C6 glioma and IMR-32 neuroblastsoma cells against glutamate induced toxicity, MTT and LDH assays were performed on RA differentiated cultures.

➢ To get insights into the neuroprotective mechanism of Ashwagandha water extract, expression of markers such as specific interfilaments (IFs) GFAP (glia), NF-200 (neurons), stress protein HSP70 and plasticity markers NCAM and PSA-NCAM were investigated on RA differentiated cultures challenged with glutamate by immunocytofluorescence and Western blotting.

Results and Conclusion:

Withania somnifera and Neurooncology:

There was a concentration-dependent decrease in the cell viability as assessed by MTT assay with ASH-WEX in the C6 glioma and IMR-32 neuroblastoma cell lines. Treatment of C6 glioma and IMR32 neuroblastoma with ASH-WEX (0.5% and 1.0% in case of C6, 0.2% and 0.5% in case of IMR-32 cells) for 72 h significantly inhibited their proliferation which was also depicted by phase contrast images. No significant difference in the anti-proliferative activity of the extract was observed after different enzymatic and denaturing treatments suggesting that the active components are neither heat labile, nor proteinaceous or nucleic acid in nature. The TLC profile generated by methanol:chloroform (1:1) solvent showed the presence of seven distinct spots.

The enhanced expression of GFAP in C6 cells, a marker of glial cell differentiation and NF200 in IMR-32 cells as indicated by immunocytofluorescence, Western blotting and RT-PCR further established the differentiated phenotype of the cells under investigation. The differentiation-inducing potential of ASH-WEX was further confirmed by immunostaining pattern of mortalin in C6 glioma and IMR-32 neuroblastoma cells as control cells showed perinuclear localization of the mortalin, the ASH-WEX treated cultures showed pancytoplasmic localization as well as upregulation in expression of this senescence marker protein.

NCAM expression in ASH-WEX treated C6 and IMR-32 cells was upregulated thus indicating its potential to reduce migration. Western blot and RT-PCR expression data further showed significant increase in the expression of NCAM isoform upon ASH-WEX treatment. NCAM is involved in neuronal differentiation
and inhibition of cell proliferation through heterophilic interactions. Anti-migration activity of ASH-WEX treated cells was observed in wound scratch assay, whereas control cells migrated to the scratched area within 6 h (C6 cells) and 24h (IMR-32) of treatment and covered the scratched area. The anti-cancer activity of ASH-WEX is also supported by downregulation of the activity 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. ASH-WEX significantly reduced the expression of PSA-NCAM in C6 and IMR-32 cells as supported by immunostaining and Western blotting results. Downregulation of polysialylation through inhibition of polysialyltransferase (PST) enzyme as suggested by RT-PCR results further confirm these observations.

The downregulation of cyclin D1 expression upon ASH-WEX treatment also support cell cycle arrest and neural differentiation of C6 and IMR-32 cells. Downregulation of Bcl-xl both at transcriptional and translational level, which further supports pro-apoptotic potential of ASH-WEX extracts in the cancerous cells under investigation. Akt-P expression was downregulated in C6 glioma cells upon ASH-WEX treatment but was upregulated in the IMR-32 cells when treated with ASH-WEX. In addition to its vital function in cell survival, a role for Akt signalling has also been implicated in neuronal differentiation, and several aspects of neurite outgrowth, including elongation and branching, are regulated by activated Akt. The current study supports the idea that ASH-WEX may have the potential to reduce the malignancy of glioblastomas and neuroblastomas and prove to be suitable as adjunct therapy by its differentiation and senescence inducing activity.

*Withania somnifera* and Neuroprotection:

Neuroprotective potential of ASH-WEX was further tested against glutamate induced excitotoxicity in the retinoic acid differentiated C6 glioma and IMR-32 neuroblastoma cells using MTT and LDH assays. The current data revealed ASH-WEX marked amelioration of glutamate induced decrease in cell viability by ASH-WEX in a dose-dependent manner. Moreover, glutamate-induced apoptosis/necrosis was also attenuated after treatment with ASH-WEX as evident from LDH assay and phase contrast images of the cells. Enhanced expression of GFAP upon glutamate exposure of RA differentiated C6 cells may be attributed to reactive gliosis and its induction which was normalized by 0.1% ASH-WEX treatment in low dose
glutamate (500μM) treatment group depicting 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 which may be a sign of loss of neuronal function. The ASH-WEX treatment seems to overcome the glutamate induced adverse effects in IMR-32 cells as inferred from NF200, HSP70, NCAM and PSA-NCAM expression. NCAM and its polysialylated form being important molecules for CNS repair and regeneration, these molecules may be the targets for regenerative and cytoprotective potential of ASH-WEX. Furthermore, PSA has been shown to act as a neuroprotective agent, disconnecting overstimulated synapses to protect the relevant circuits from damage caused by excess glutamatergic input.

MMP-2 and MMP-9 activity was upregulated in glutamate exposed cells, whereas ASH-WEX treatment significantly lowered their expression especially in the low dose glutamate treatment group in C6 and IMR-32 cells. It is evident from the current data that ASH-WEX intervention leads to protection of both the cell types, atleast in low dose glutamate treatment group. Consistent with these neuroprotective properties of ASH-WEX, present study illustrates the neuromodulatory role of ASH-WEX against glutamate induced excitotoxicity by upregulation of plasticity marker proteins. As elevated levels of glutamate have been implicated in a wide range of neurological diseases thus further research into the molecular mechanism(s) of ASH-WEX mediated neuroprotection and the search for bioactive component(s) in these extracts may prove valuable therapeutic agent(s) to combat brain cancers and neurological disorders.