Chapter 1

Introduction
1. INTRODUCTION

The mainstream pharmaceutical research is on its way towards veering from mono-molecular or single target approach to combinations and multiple target strategies (Wermuth, 2004). Perhaps, multi-site mechanisms of action of herbal preparations from the crude extracts may offer greater chances for success where conventional single-site agents have been disappointing. Auspiciously, many of these traditional herbal medicines are now increasingly being appreciated with Western models of integrative health sciences and evidence-based approach both in research and practice. Several bioactive compounds have emerged from research in herbal medicine. Among others include, *Rauwolfia* alkaloids for hypertension, psoralens for vitiligo, *Holarrhena* alkaloids in amoebiasis, guggulsterones from *Commiphora* as hypolipidemic agents, *Mucuna pruriens* for Parkinson's disease, bacosides from *Bacopa monnieri*, antivirals from phyllanthins, withanolides and many other steroidal lactones and their glycosides as immunomodulators (Mishra *et al.*, 2000; Hegde *et al.*, 2003; Malik *et al.*, 2007).

Most of the new chemical entities approved by FDA have been derived directly or modified from natural products (Cragg and Newman, 2001). However, natural products have been often neglected as resources for drug candidates mainly due to the inability of providing hundreds of thousands of compounds for the high-throughput screening systems adopted by pharmaceutical companies. There is need for use of natural products as resources for drug discovery due to safety issues and failure of existing regimens. Moreover, as the recent approaches take advantage of biomimicry (mimicry of biological system) for the drug development, there arises the potential need of natural products. Agents that are safe and can be administered as dietary supplements appear to be most feasible for therapy.

1.1 Neurooncology and therapeutics:

In the arena of experimental neuro-oncology, there remains a need for new and innovative treatment methodologies for the tumors of central nervous system (CNS). Brain tumors remain a neurosurgical and oncological enigma. Despite major advances in the diagnosis and treatment of cancer in recent years, brain tumors are
still rarely curable and most patients diagnosed with them die within a year. Gliomas and neuroblastomas are the most common primary brain tumors with only limited options for treatment. The majority of these tumors develop into malignancy and remain incurable in spite of the therapies like external beam radiation, surgery and chemotherapy and hence call for the development of novel therapeutic approaches. Differentiation therapy focuses on the development and use of specific agents designed to selectively engage the process of terminal differentiation, leading to the eventual elimination of tumorigenic cells and retrieval of normal cellular homeostasis. Extensive in vitro studies of the molecular mechanisms underlying drug-induced maturation has allowed the realization and application of a differentiation-based therapy to the clinic. Rationalization of this mode of therapy has included the combined use of differentiation agents with low-dose chemotherapy to lessen adverse cytotoxicity and to enhance the efficacy of differentiation agents, allowing some success in their application to conditions resistant to conventional therapy. Phytochemicals like cranberry proanthocyanidines, (Singh et al., 2012), Tithonia diversifolia extract (Lee et al., 2011), melanoids from potex (Langner et al., 2011), Carnosic acid, a rosemary phenolic compound (Tsai et al., 2011) has been studied for the apoptosis inducing potential in neuro-oncology.

1.2 Neurodegeneration and Neuroprotection:

Neurodegeneration is defined as progressive loss of neuronal structure and function that ultimately leads to neuronal cell death. It occurs in various diseases affecting the central nervous system (CNS). The loss of specific populations of neurons related to functional neuronal networks determines the clinical presentation of the neurodegenerative disease.

The neurodegenerative molecular pathways are poorly understood largely due to the difficulty in distinguishing primary from secondary events. One important player in neurodegeneration is glutamate, the major excitatory neurotransmitter in particular in the forebrain regions. Glutamate has been known to play an important role in memory, neuronal survival, neuronal differentiation, learning and behaviour at the normal range of concentration (Dingledine et al., 1999; Mattson, 2008, Yang et al., 2011). However, over-expression of glutamate receptor by high concentration of glutamate resulted in neuronal cell death in the CNS, and may be responsible for neuropathological disorders such as Parkinson’s disease, Alzheimer’s disease,
epilepsy, seizures, ischemic stroke and spinal cord trauma (reviewed in Lau and Tymianski, 2010). Thus, neuronal cell protection against neurotoxicity in the glutamate-injured neuronal cells has been one of the research targets to develop drugs for neurodegenerative disorders (Rajendra et al., 2004).

Initial studies with neuroprotective agents were mainly focused on their roles in acute ischemic brain injury, but the research interest has switched its gear to include various other types of chronic neurodegenerative diseases. Based upon the mode of action of underlying mechanisms of neuroprotective agents, they have been grouped into several categories: free radical scavengers (Green and Ashwood, 2005; Yoshida et al., 2006; Hardeland, 2009), anti-excitotoxic agents (Mattson, 2003; Volbracht et al., 2006; Xia et al., 2009), apoptosis inhibitors (Guan et al., 2006; Kooncumchoo et al., 2006; Peng et al., 2008), neurotrophic agents (Semkova and Kriegstein, 1999; Sun et al., 2008, Saragovi et al., 2009), and ion channel modulators (Lysko et al., 1994; Herin et al., 2001; Gribkoff and Winquist, 2005; Leung, 2010). The issue of clinical efficacy of neuroprotective agents is still debating. Nevertheless, the potential of neuroprotective agents to treat neurological disorders is attracting interest because of their potential to alleviate symptoms accompanying most neurological disorders. Diverse natural products with distinct chemical structure for example alkaloids (Zhang et al., 2007), diterpenoids (Koo et al., 2007), isoflavonoids (Schreihofefer, 2009), and phenylpropanoid glycosides (Kim and Kim, 2000), have been reported to have neuroprotective activities. Among them, the majority of naturally-derived neuroprotective agents are polyphenolics such as flavonoids and phenylpropanoid derivatives. 3,6'-disinapoyl sucrose from Radix Polygala (Hu et al., 2012), Grape seed proanthocyanidin extract (Ahn et al., 2011, Narita et al., 2011), Papaya epicarp extract (Guziani et al., 2011), Withanone (Konar et al., 2011), Ginkgo extract (Mdzinarishvili et al., 2012), Cyanidin-3-glucoside from Mulberry (Bhuyian et al., 2011), memantine. (Kutzing et al., 2012) are some of the important phytochemicals which have been studied as potent neuroprotection agent against different neurodegenerative disease models.

Despite the intensive research into excitotoxic mechanisms, very few pharmacologic treatments have been shown to be successful in related neurological disorders. This failure has been recently suggested to be the result of an overly simplistic NMDA-AMPA model of excitotoxicity (Besancon et al., 2008). As such, alternative targets for attenuating excitotoxic injury such as AMPAR and kainate
receptor antagonists, glutamate release blockers, free radical scavengers and antioxidants and nitric oxide synthase inhibitors have recently been the focus of considerable attention (reviewed in Lau and Tymianski, 2010). There are currently few clinical strategies in place, which provide effective neuroprotection and repair, despite an intense global effort over the past decades. One possible explanation for this is that a deeper understanding is required of how endogenous mechanisms act to confer neuroprotection. Recent studies have focused on the possible capacity of natural compounds extracted from fruits, vegetables and beverages to prevent certain age-related neurological disorders. Some beneficial phytochemicals, especially polyphenols such as quercetin, (+)-catechin and resveratrol, display protective abilities in various animal models of neurological disorders. Lonicera japonica, Taraxacum platycarpum, Polygonum aviculare, Gardenia jasminoides, Forsythia viridissima, Lygodium japonicum, Panax notoginseng, Akebia quinata, Anemarrhena asphodeloides and Phellodendron amurense have shown significant neuroprotective activities against glutamate-induced neurotoxicity in primary rat cortical cells (Won and Ma, 2009; Weon et al., 2011). The underlying mechanisms include ischemic preconditioning, antioxidation, anti-inflammation, inhibition of microglia recruitment; in terms of underlying mechanisms, the “preconditioning” or “neurohormetic” pathways seem most attractive (Dorre, 2005; Mattson and Cheng, 2006).

1.3 Withania somnifera - Queen of Ayurveda

Ayurveda is a 5000 year-old system of Indian traditional medicine using natural plant extracts. The basic principle in Ayurveda is the holistic approach towards overall wellness and health rather than to make selective treatment (Deocaris et al., 2008). It has become very popular as it uses reagents and remedies essentially drawn from nature and is both eco- and bio-friendly. Ayurvedic medical system practices the use of dry powder or crude extract, and assignment of bioactivities to a particular compound is not preferred. One of the most prominent therapeutic plants of Ayurveda, Withania somnifera, also known as Ashwagandha, is a member of GRAS (Generally Regarded As Safe) plants and a popular home remedy in the Indian pharmacopoeia. Ashwagandha, the Queen of Ayurveda, is widely used in Ayurveda and is considered to be a rasayana herb, an adaptogen, and is commonly referred to as ‘Indian ginseng’. Owing to wide variety of health promoting effects, it is categorised
along with the world’s most renowned herbal tonics such as ginseng (Panax ginseng), astragalus (Astragalus membranaceus), dang gui (Angelica sinensis), reishi mushroom (Ganoderma lucidum) and South American suma (Pfaffia paniculata) (Kaur et al., 2007). Many pharmacological studies have investigated the properties of Ashwagandha in an attempt to authenticate its use as a multi-purpose medicinal agent. In addition to leaves and roots, other parts of the Ashwagandha plant, including shoots, seeds and berries, have also been used in daily tonics and various home remedy recipes to improve health and longevity. They are a source of unique alkaloids and withanolides that have been shown to act as steroidal hormones and antioxidants with favourable impact on human health. Ashwagandha preparations have been reported to modulate GABAergic and cholinergic neurotransmission and protective in neurodegenerative and neuropsychiatric disorders (Kulkarni et al., 2008; Bhattarai et al., 2010). Its constituents induce significant regeneration of axons and dendrites, in addition to reconstruction of pre- and postsynapse in the neurons, therefore an important candidate for neurodegenerative diseases (Kuboyama et al., 2006; Tohda and Joyashiki, 2009). Despite being under-appreciated in the area of neurooncology, experiments in test tubes and in animal models demonstrated that Ashwagandha plays an anticancer role by inducing apoptosis and cell cycle arrest, enhancing the immune response and inhibition of angiogenesis and metastasis. Interestingly, as Ashwagandha exhibits both anti-oxidant and pro-oxidant activities, it has been reported to sensitize tumors to radiation while presenting itself a radio/chemo-protector for normal cells.

1.4 Rationale of study

The most common primary brain tumors such as gliomas and neuroblastomas have only limited options for treatment and hence call for development of novel therapeutics. These are complicated by their heterogeneity, poor prognosis, blood brain barrier (BBB) and unsuccessful chemotherapy and radiotherapy regimens. Hence there is a strong demand for developing effective and alternate therapeutic strategies and reagents for treatment of brain tumors. Extracts of Ashwagandha have been reported to have inhibitory effects on different types of cancers in animal models and cell lines. However there is very little information on their effect on brain tumors and tumor-derived cell lines. Thus, the present study was designed to look into anti-proliferative effects of Ashwagandha in brain tumor derived cell lines. To
test anti-proliferative effects of ASH-WEX, initial study was performed on rat glioma C6 cells, human glioma cells - YKG1, U118MG and A172 and human neuroblastoma cells TGW and IMR-32 cells. Finally, for further detailed study, the astrocytoma-derived C6 cell line and human neuroblastoma IMR-32 cells were selected for ease of culture. These cell lines have been widely studied and well established in vitro model system. As Ayurveda emphasizes the use of aqueous crude extracts, Ashwagandha leaf water extract (ASH-WEX) was chosen for investigations. More over use of crude leaf water extract is both eco- and bio-friendly as neither there is a need to sacrifice whole plant (as in case of roots) nor any organic solvents are involved in extraction. Moreover the aqueous extract is easy to prepare and convenient as well as safe to use. The current study was planned with following objectives:

- To explore the anti-proliferative and cytotoxic effects of ASH-WEX on neuroblastoma and glioblastoma cell lines by MTT assay and Dye exclusion test
- Since the preliminary studies confirmed the anti-proliferative activity of ASH-WEX in in vitro model system and it was perceived 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) and Neurofilament 200 (NF200, in neuroblastoma) were studied. To further confirm the cell state, the senescence marker Mortalin was studied as this protein is distributed in a pan-cytoplasmatic manner in normal cells, but in immortal cells its localization shifts to the perinuclear zone. Further cell cycle analysis and Annexin-V FITC apoptosis assay was performed to establish the cellular state of the cells after the ASH-WEX treatment
- Since brain tumors are highly malignant and invasive, so the molecules involved in regulation of cellular migration, metastasis and invasivity were also investigated. The cell surface markers Neural cell adhesion molecule (NCAM) and its polysialylated form PSA-NCAM are important oncodevelopmental 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 investigated by wound scratch assay
and the extracellular matrix components, matrix metalloproteinases (MMP 2 and 9) by gelatin zymography as MMPs are strongly associated with tumor invasion and metastasis.

The initial *in vitro* anti-proliferative studies established ASH-WEX as a potent differentiation inducing agent in brain tumor derived cell lines at low doses. Owing to Ashwagandha’s antioxidant and neuroprotective properties, it was further planned to study whether Ashwagandha extract was able to protect neuronal and glial cell lines against neurodegenerative insults. The glutamate excitotoxicity model was chosen for investigation as glutamate is the major excitatory neurotransmitter which is commonly involved in neurodegenerative disorders (stroke, amyotrophic lateral sclerosis, epilepsy, Parkinson's disease etc.). Neuroprotection against glutamate excitotoxicity may be a potential beneficial therapeutic intervention in related neuropathological conditions. Retinoic acid (RA) differentiated C6 glioma and IMR-32 neuroblastoma cells have been widely accepted for *in vitro* studies due to their close resemblance to glial and neuronal cells, respectively (Rabinovskv *et al.*, 1992; Singh *et al.*, 2009). The following parameters were chosen for further study on ASH-WEX:

- To test whether ASH-WEX can protect differentiated C6 glioma and IMR-32 neuroblastoma cells against glutamate induced toxicity, MTT and LDH assays were performed on RA differentiated cultures
- To get insights into the neuroprotective mechanism of ASH-WEX, expression of markers such as cell specific interfilaments (IFs) GFAP (glia), NF-200 (neurons), stress response protein HSP70 and plasticity markers NCAM and PSA-NCAM were investigated on RA differentiated cultures by immunocytofluorescence, Western blotting and RT-PCR
- Further MMP 2 and 9 activity was analysed by gelatinase zymography in response to glutamate challenge and ASH-WEX treatment as overexpression of MMPs has been implicated in CNS related pathologies and injuries