To check the glioprotective effect of Withaferin A on C6 Glioma cell Culture challenged with Kainic Acid

Project report submitted to the Central University of Punjab

For the award of

Master of Science

In

Life sciences with specialization in Animal Sciences

By

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May - 2018

Declaration

I declare that the dissertation entitled **"To check the glioprotective effect of Withaferin A on C6 glioma cell cultures challenged with Kainic acid"** has been prepared by me under the guidance of Dr. Jyoti Parkash, Assistant Professor, Department of Animal Sciences, Central University of Punjab, Bathinda. No part of this thesis / dissertation has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

I certify that Bindu Balaya Dash has prepared his thesis/dissertation entitled **"To check the glioprotective effect of Withaferin A on C6 glioma cell cultures challenged with Kainic acid"** for the award of Master's degree at the Central University of Punjab, under my guidance. He has carried out this work at the Department of Animal Sciences, School of Basic and Applied Sciences, Central University of Punjab.

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(**Bindu Balaya Dash**)

ABSTRACT

Title: To check the glioprotective effect of Withaferin A on C6 glioma cell culture challenged with Kainic acid.

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Key words: Glutamate, Excitotoxicity, Kainic acid, Withaferin A, Glioma

Last many years perception of glial cell function in CNS has been changed. In fact abnormalities in glial cells also contribute to various disorders. Glutamate induced excitotoxicity attributed to various CNS related diseases. Kainic acid is a potent agonist of kainate receptors, a subclass of glutamate receptors, is 30 fold more neurotoxic than glutamate. Natural herbal extracts are attracting researchers for their pharmacologic properties against diseases associated with CNS. Root and leaf extracts of *Withania somnifera,* used since many years to treat several diseases in traditional medicine system. Present study was designed to see the glioprotective potential of Withaferin A, a natural extract from Withania somnifera or Ashwagandha, against Kainic acid induced excitotoxicity in C6 glioma cell line. Pre-treatment of 0.5 µM Withaferin A showed defensive potential against 100 µM and 200 µM concentration of Kainic acid. To check expression of GFAP (well known marker of astrocytes) and NCAM, Immunocytochemistry was performed. Withaferin A treatment helps in normalizing of GFAP (Glial Fibrillary Acidic Protein) and NCAM (Neural Cell Adhesion Molecule) expression in kainic acid exposed cells. Our result suggested that Withaferin A have defensive potential against kainic acid induced excitotoxicity. As a potent glioprotective agent, Withaferin A could be used as therapeutic drug to treat glioblastomas and other neurological disorders.

(Bindu Balaya Dash) **(Dr. Jyoti Parkash)**

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LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1	*Withania somnifera*	WS
2	Kainic acid	KA
3	Withaferin A	WA
4	World Health Organisation	WHO
5	Glutamate Receptors	GluR
6	Ionotropic Glutamate Receptors	iGLURs
7	Metabotropic Glutamate Receptors	mGLURs
8	Ɣ- amino butyric acid	GABA
9	Endoplasmic Reticulum	ER
10	Calcium ion	Ca^{2+}
11	Dimethyl Sulfoxide	DMSO
12	Cyclooxygenase-2	COX-2
13	5-Lipooxygenase	5-LOX
14	Degree Celsius	°C
15	N-methyl-D-aspartate Receptors	NMDA
16	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate Recepotors	AMPA
17	Neural Cell Adhesion Molecule	NCAM

18	Glial fibrillary acidic protein	GFAP
19	Glioblastoma	GBM
20	Phosphate Buffer Saline	PBS
21	Millilitre	Ml
22	Microliter	µl
23	Millimolar	mM
24	Micromolar	µM
25	Dulbecco’s Modified Eagle Medium	DMEM
26	Fetal Bovine Serum	FBS
27	Bovine Serum Albumine	BSA
28	Phosphate Buffered Saline, Triton-X	PBST
29	Fluorescein isothiocyanate	FITC
30	4',6-diamidino-2-phenylindole	DAPI

1. Introduction

Neurodegenerative diseases are one of the major problems in today's world. These kind of diseases affecting millions of people worldwide. Alzheimer's diseases and Parkinson's diseases are most common among them. Neurodegenerative diseases occur when nerve cells in brain and peripheral nervous system lost their function and ultimately they die. But recent researches showed that not only nerve cells, but also glial cells dysfunction also have contribution to neurodegenerative diseases in many extent. Glial cell pathology is increasingly recognised in various neurological disorders like Alzheimer's, Parkinson's, and Autism etc. Another fact is that glial cells are most abundant cell types in central nervous system (CNS). So primary abnormalities in glia may be the reason behind many disorders that were considered as neurodisorder. (Miller *et al.*, 2013).

Glial cells, which are orthodoxically believed to be acted as simply 'glue' of the nervous system, are gaining attention now a days regarding nervous system dysfunction. In neurodisorders, contribution of glia cells and their interaction with neurons, have been started attracting researchers of neuroscientific community. Reactive glial changes like gliosis in astrocytes, microglia activation, occurs in most neurodegenerative disorders. (Eng and Ghirnikar, 1994). Till today there are not many potential therapeutics to encounter glial cell dysfunctions.

There has been a new trend in creating and marketing of drugs based upon medicinal plants. Herbals today are used as potential agents for treatment of various diseases, particularly for which there is no effective cure is available by synthetic drugs. According to WHO, traditional medicines derived from ayurvedic plants are used by approximately 80% of world's population and 21000 plant species have the potential for being used as medicinal plants. It has been estimated that in developed countries like United States, plant drug extracts constitute as much as 25% of total drugs. In developing countries like India and china almost 80% of drugs are plant extracts. Ayurvedic medicine is a traditional medicine system native to India and an entire concept in Ayurveda called **'Vata Vyadhi'** in Sanskrit, associated with nervous system disorder. Many scientific studies have been showed various medicinal plants for strengthening the nervous system and restoration of memory and termed those plants as **'Nervines'** (Rao R.V., *et al*., 2012). Present study emphasises on curative properties of Withaferin A, a steroidal lactone derivative of *Withania somnifera* in neurodegeneration.

Natural extracts from many herbal plants are known for providing protection against neurodegenerative models (Rao R.V. *et al*, 2012). *Withania somnifera*, or commonly known as Ashwagandha has significance role in Ayurvedic medicine system (Mishra L.C. and Singh B.B., 2000). Ashwagandha, a member of **Solanaceae** family, holds a large importance in ayurvedic field as a known nerve tonic, and adaptogen and helps body to adapt stress (Ahmad *et al*., 2017). Medicinal properties of WS roots are primarily attributed to the presence of active multicomponents known as withanolides (Kurapati *et al*. 2013), which have shown antioxidant effect in the brain and are considered to be responsible for its diverse pharmacological properties (Mirjalili *et al*. 2009). In CNS Ashwagandha is known to improve learning and memory (Dar N.J. *et al*., 2015). Due to its importance it perfectly described as **Rasayana** (Dar N.J. *et al*., 2015) in Ayurveda. A rasayana is an herbal product which is used for various pharmacological properties. The roots of plant is mostly used part in Ayurveda, though seeds and flowers are also used.

Table.1: Major chemical constituents of *Withania somnifera* (*Source*: Dar *et al.*, 2015)

Alkaloids	Withanine, withananine, withasomnine, somniferine, tropeltigloate, somniferinine, somninine and nicotine
Steroidal lactones	Withaferin-A, Withanone, withanolide-E, withanolide-F, Withanolides-A, withanolide-G, withanolide-H, withanolide-I,withanolide-J, withanolide-K, withanolideL, withanolide-M
Steroid	Cholesterol, b-sitosterol, stigmasterol, diosgenin, stigmastadien, sitoinosides-VII, sitoinosides-VIII, sitoinosides-IX, sitoinosides X
Salts	Cuscohygrine, anahygrine, tropine, pseudotropine, anaferine
Flavonoids	Kaempferol, quercetin
Nitrogen containing compounds	Withanol, somnisol, and somnitol

This plant is widely distributed in the drier parts of the tropical and subtropical region ranging from Canary Islands, South Africa, Middle East, Sri Lanka, and India to China. However in India it is mostly grown as a medicinal crop. A large number of Withanoloids has already been isolated from *W.somnifera* and also tested for their medicinal properties (Table.1)**. Withaferin A** is one of the most bioactive compound

among them. It was discovered in 1962 by two Israeli chemist, Asher Lavie and David Yarden and they isolated it from *Withania somnifera*. It has a steroid structure as it is a steroidal lactone derived from *W.somnifera*. This phytochemical has considerable pharmacological activity such as anti-cancerous, anti-inflammatory, immunomodulaory, anti-metastasis, anti-carcinogenic properties (Ahmad *et al*., 2017).

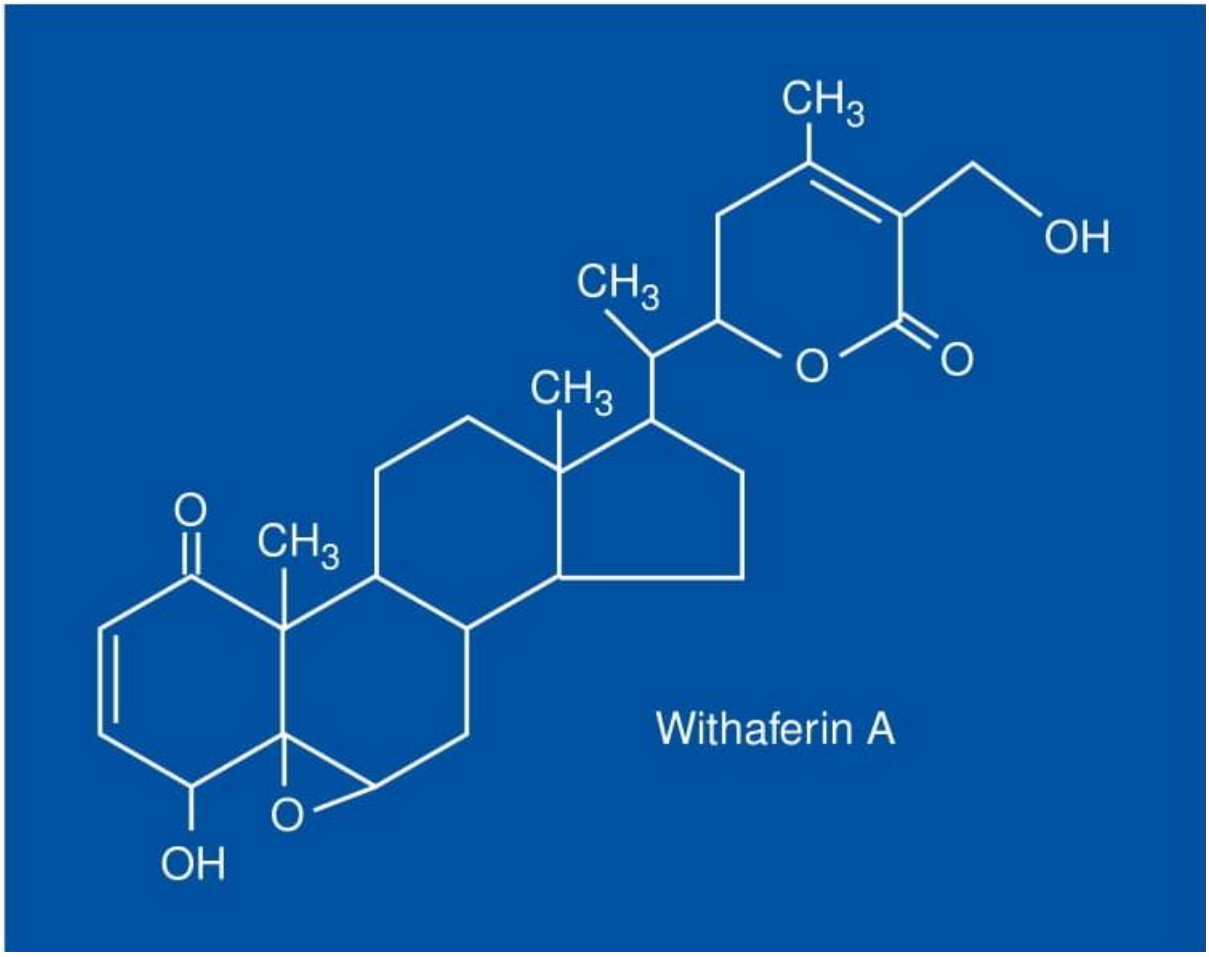

Fig.1: Chemical structure of Withaferin A (*Source:* Mishra L.C., Singh B.B., 2000)

As previously discussed neurodegeneration is gradual deterioration of neurons structure and function. There are several biological phenomenon behind neurodegeneration. In many neurodegenerative disease **excitotoxicity** is major reason of neuron loss. Excitotoxicity occurs when nerve cells are over stimulated by nerve impulses, causing them to be damaged or killed. It is a pathological process that happens when receptors for the excitatory neurotransmitters glutamate is over activated. The concept of excitotoxicity was given by Olney in 1969 (Sairazi N.S.M.

et al., 2015). Glutamate is one of the major excitatory neurotransmitter. It's important for learning, memory, cognitive function (Kataria *et al*., 2012). However Glutamate can be highly toxic to nerve cells if present in excess amount. Excess glutamate can lead to over-excitation of receiving nerve cells eventually causing their death (Kataria *et al*., 2012). Glutamate acts via glutamate receptors which are divided into two classes i.e. ionotropic glutamate receptors (iGLURs) and metabotropic glutamate receptors (mGLURs), based upon their function. The iGLURs mediate fast postsynaptic transmission by activating ion channels. The mGLURs mediate slow postsynaptic trasnsmission by activating G-protein. Now iGLURs is further divided into three classes i.e. N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors, and Kainate receptors (Sairazi *et al*., 2015). In vivo and in vitro experimental model, several convulsants are used to excitotoxicity artificially, but in most of the cases researchers used kainic acid. (Zhang and Zhu, 2011).

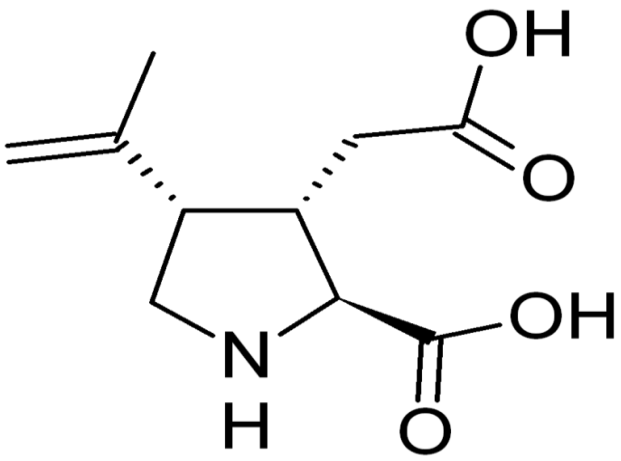

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Fig.2. Structure of Kainic acid

Kainic Acid (2-carboxy-4-(1-methylethenyl)-3-pirrolidiacetic acid) is a very powerful neurotoxin, which kills neurons by means of over-excitation. It is used to study the molecular mechanism behind the neurodegeneration by creating the model of cell damage (Tsai H.L. *et al*., 2014). It is a non-degradable analog of glutamate and 30 fold more in neurotoxicity than glutamate. It is an agonist of Kainate receptors. Kainic acid activates glutamate receptors and over activation of these receptors lead

to depolarization of neuronal cell membrane, which causes influx of Ca^{2+} ions and triggers excitotoxic neuronal cell death (Zhang *et al*., 2011). Administration of KA is known to cause epilepsy seizer syndromes and induce behavioural changes in rodents (Popescu B.O. *et al*., 2002). The complete mechanism of how kainic acid serves as neurotoxin inducing neuronal death is described in Fig.2.

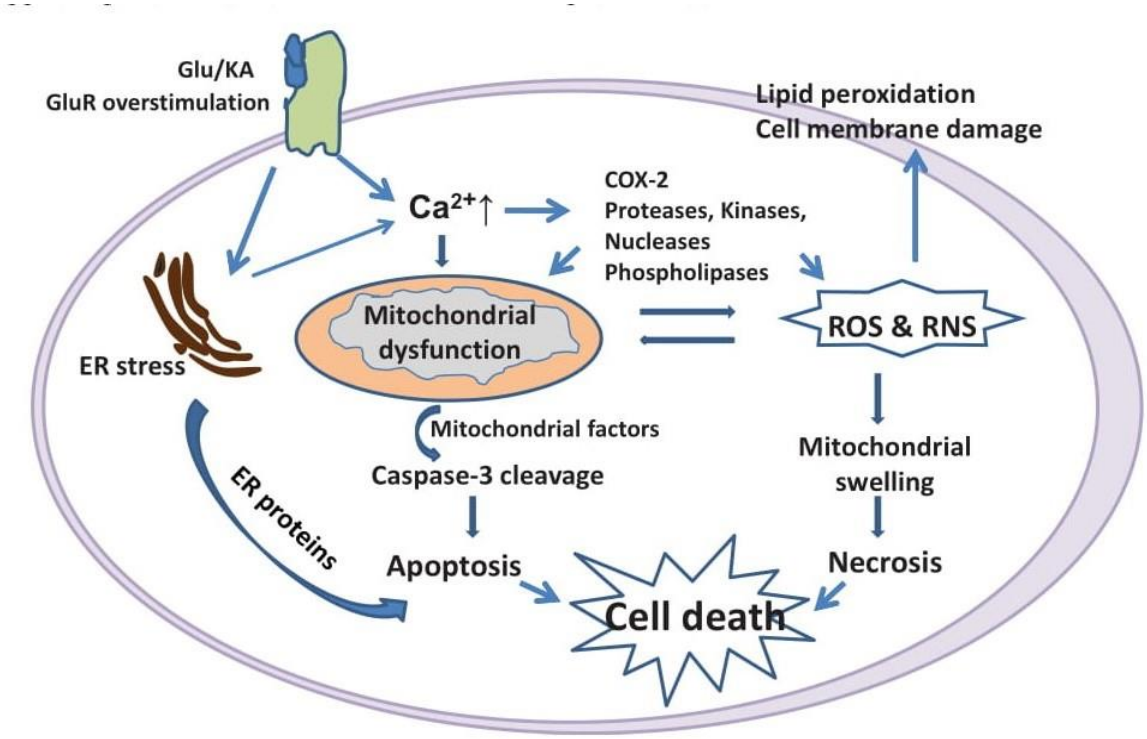

Fig.3: Schematic Overview of KA-Mediated Neuronal Death. (1) By stimulating glutamate receptors (GluR), kainic acid (KA) elicits the increase of intracellular Ca2+, activation of Ca2+-dependent enzyme and production of free radicals; (2) Excessive Ca2+ and free radicals cause mitochondrial dysfunction, release of mitochondrial factors, activation of caspase-3, leading to neuronal apoptosis; (3) KA causes the disintegration of the endoplasmic reticulum (ER) and ER stress with the activation of the ER proteins Bip, Chop, and caspase-12, involved in neuronal apoptosis; (4) Ca2+ overload and excessive free radicals cause directly mitochondrial swelling, leading to neuronal necrosis. COX: cyclooxygenase; ROS: reactive oxygen species; RNS: reactive nitrogen species. (*Source*: Zhang, Zhu, 2011)

There is a relation between Glial activation and neuroinflammation in many neural disorder. An interaction occurs between glial cells (mainly astrocytes and microglia) and neuron cells followed by neuronal injury. Microglia are neuronal macrophages so these cells are important for neuronal survival and post-injury repair. After treatment of kainic acid activation of these glial cells associated with neuronal death (Sairazi, *et al*., 2015).

C6, an established cell line derived from a rat glioma, differentiates into astrocyte-like cells that express the astrocytic marker GFAP under specific culture conditions and have been used to model astrocytes in culture (Mangoura, D *et al.*, 1989).The present study was aimed to address the important question of astroglial plasticity and the underlying mechanisms thereof in response to Kainate receptor activation in C6 glioma cell cultures. In this study we used kainic acid as a neurotoxin in the *in-vitro* model to induce cell death by excitotoxicity in order to test the potential neuroprotectiveness of Withaferin A. The results elucidate the possible cellular and molecular mechanisms of regulation of NCAM expression in astroglial cultures by extracellular signals.

Objectives

1. To study the glioprotective effect of Withaferin A against Kainic Acid induced excitotoxicity on C6 glioma cell lines.

2. To study the protein expression of NCAM (Neural Cell Adhesion Molecule) and GFAP (Glial Fibrillary Acidic Protein).

2. Review of Literature

Glutamate is an amino acid and the major excitatory neurotransmitters in brain. Neurotransmitters are methods of communication between neurons. These may be excitatory or inhibitory. Glutamate and Aspartate are major excitatory neurotransmitters while GABA, glycine are examples of inhibitory types of neurotransmitters (Mark *et al*., 2001). Glutamate is important for learning, memory and cognitive function, and thus plays an important role in mammalian CNS (Sairazi, N.S.M., *et al.*, 2015). However excessive amount of glutamate have toxic effect on neurons and it can cause death to neurons by over excitation. Glutamate acts through glutamate receptors which are mainly of two types i.e. ionotropic and metabotropic. Ionotropic receptors again divided in to 3 subtypes which are, NMDA, AMPA, and Kainate receptors. These subtypes are named for their selective chemical agonists, which resemble glutamate but do not naturally exist in the brain (Mark *et al.*, 2001). Excitotoxicity caused by high amount of glutamate is known as glutamate induced Excitotoxicity and is triggered primarily by intracellular calcium overload arising from overstimulation of NMDA type of glutamate receptors (Zhang, Zhu, 2011).

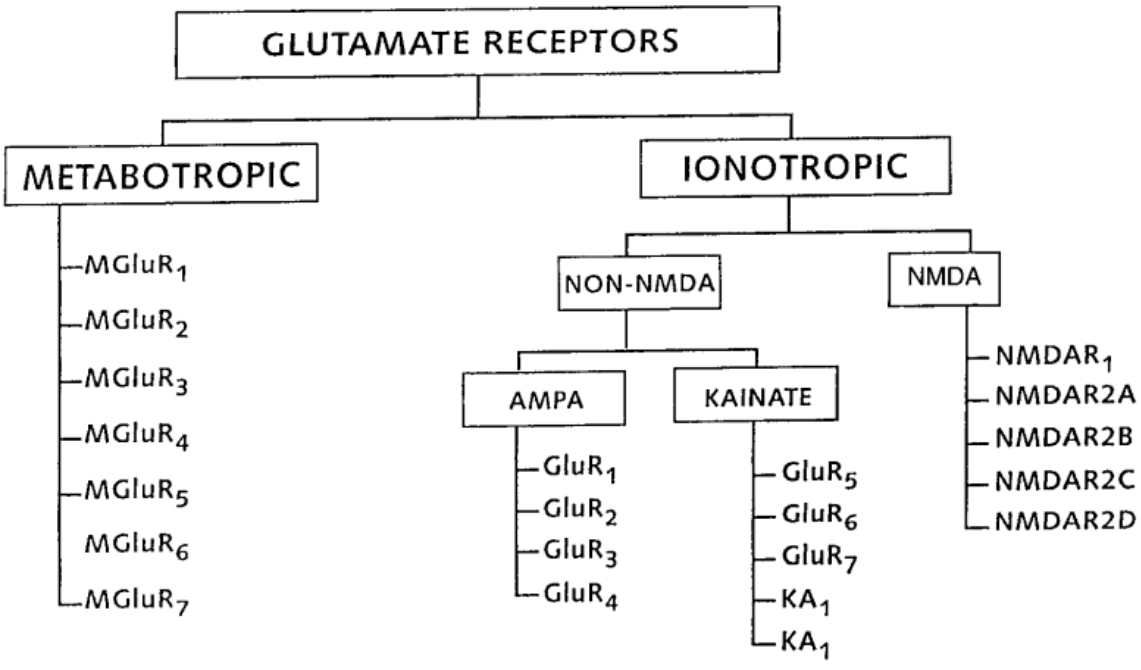

Fig.4: Classification of Glutamate receptors (*Source:* Shaw *et al*., 1997).

Kainic acid is a potent neurotoxin which is an analog of glutamate and it acts by activating kainate subtype of glutamate receptors. It was originally isolated from

seaweed in 1953. There are earlier reports which shown kainic acid induce excitotoxic cell death in immature and adult animals by systemic and intraventricular injection and in primary cultures and in vitro cell lines (Tsai *et al*., 2014). Administration of KA in to rodents showed seizer syndromes in them (Ben-Ari, 1985) (Mulle *et al*., 1998). Hippocampal region is particularly more suspected to KA induced damage as they contain high density of kainate receptors (Darstein *et al*., 2003). In a study by (Gilliams-Francis *et al*., 2003) the intracerebral injection of KA has resulted in DNA damage, PARP-1 activation, and neuronal death. The work suggested that there is a kind of relation between activation of caspase pathways and excitotoxic cell death and the neurons undergo caspase-mediated apoptotic death, involving the DNA fragmentation and cleavage of PARP-1.

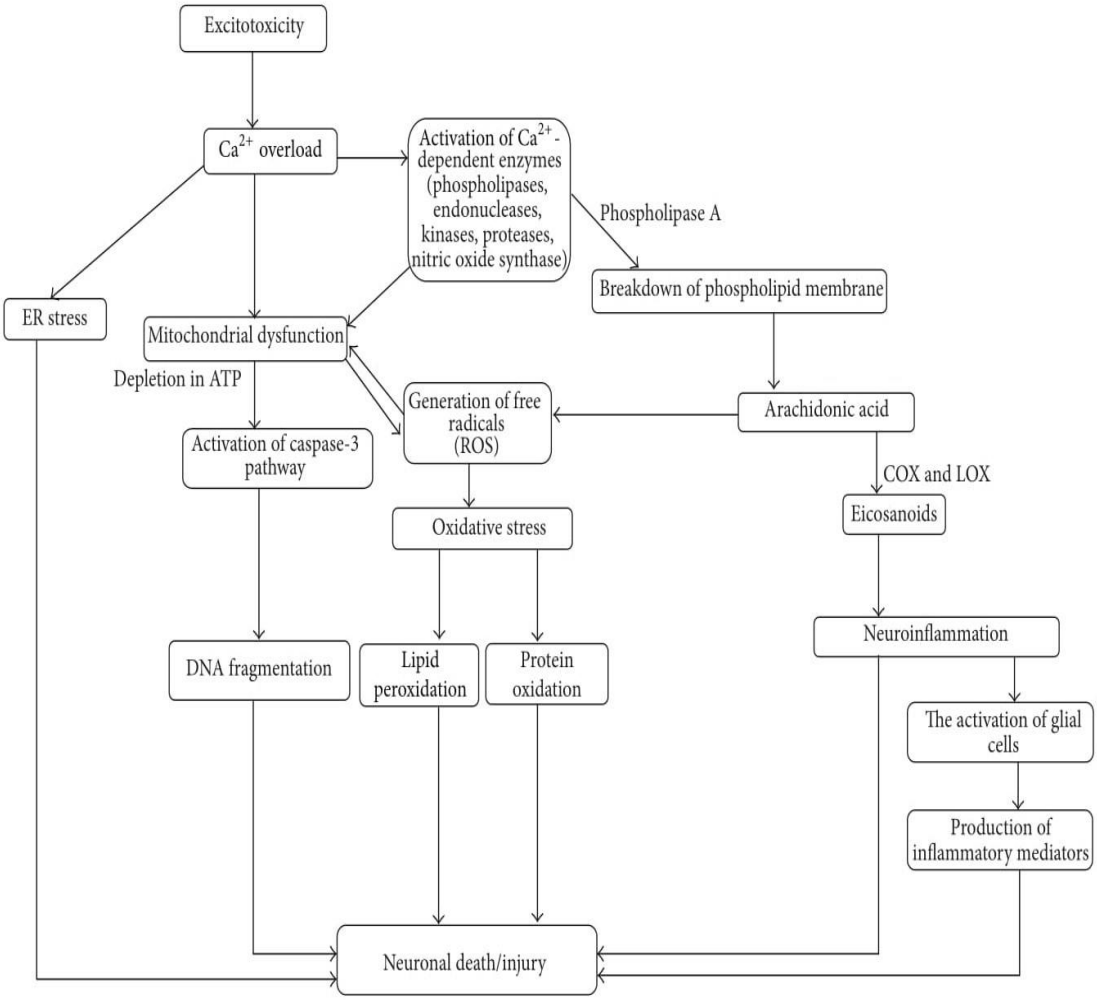

Fig.5: Proposed mechanism of action in KA-induced excitotoxicity (Sairazi *et al*., 2015)

As excitotoxicity is an important process in the pathogenesis of various neurodegenerative diseases and neuroprotection serve as a promising effect for the preventive and therapeutic approaches in treating these neurodegenerative diseases (Kim *et al*., 2011). Neuroprotection slows down the rate of neurodegeneration through the interaction with the pathological processes (Sairazi *et al*., 2015). As oxidative stress is a major event in excitotoxic cell death, anti-oxidants and anti-inflammatory agents can serve as potential candidate for neuroprotection role. The limiting of inflammation via Cyclooxygenase-2 (COX-2) and 5-Lipoxygenase (5-LOX), activities could also decrease inflammatory molecules. Other possible attempt for neuroprotection are to improve function of ER and mitochondria to inhibit ER stress and mitochondria dysfunction. A short summary and illustration of the proposed mechanism of action for the preventive and therapeutic strategies for neurodegenerative diseases are presented in Fig. 6.

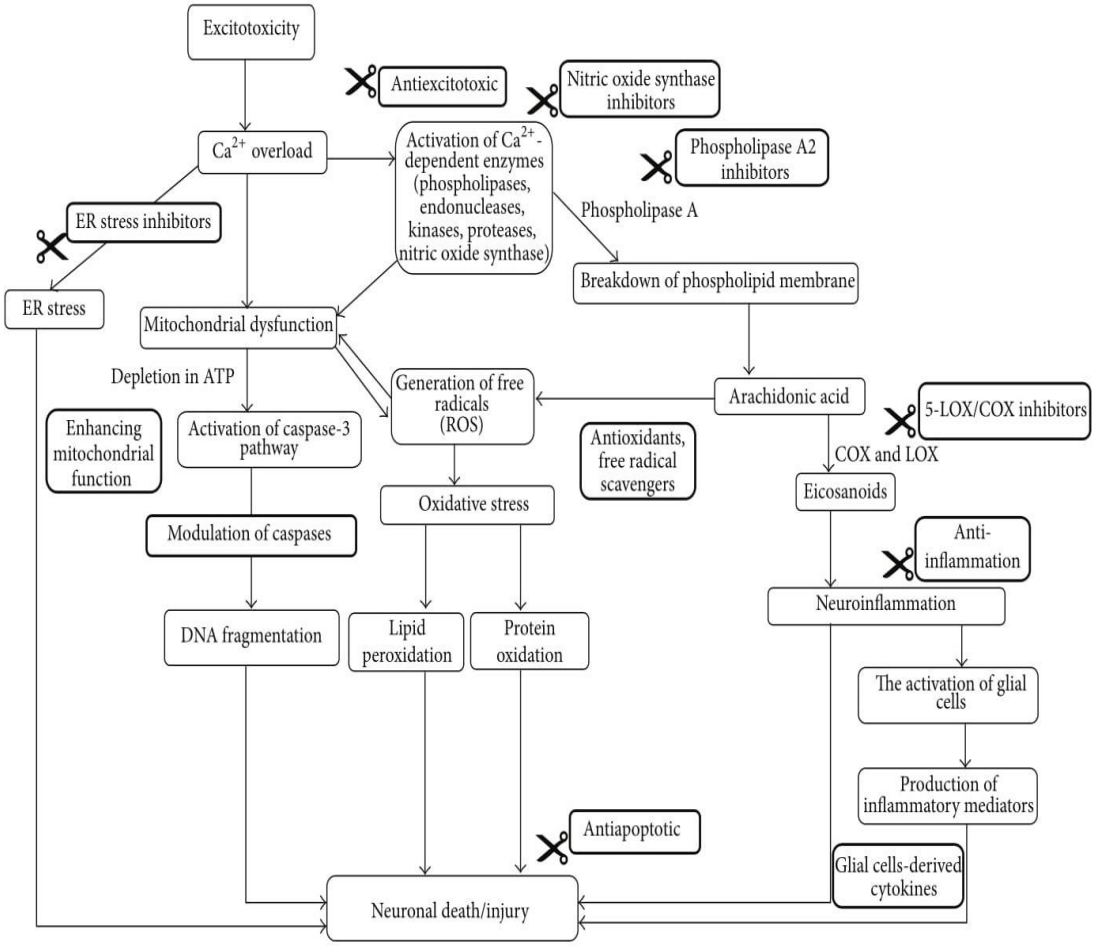

Fig.6: Illustrations of proposed mechanism of preventive and therapeutic treatment approaches in neurodegeneration. (Sairazi *et al*., 2015)

Herbal medicinal plants are used to treat various kinds of diseases and disorders since centuries. Their medicinal values are gradually gaining attention in scientific researches and pharmacological field. In neurological field, natural plant extracts attracting scientists to develop drugs for treatment of nervous system disorders. Reports on effectiveness of herbal extracts against neurological disorders are continuing to increase. Some of the herbs have been classified as brain tonics or rejuvenators in Ayurveda, the traditional Indian medicine system. Withania somnifera or Ashwagandha is an important ayurvedic plant used to treat various kinds of diseases. It has been categorized as Rasayana in Ayurveda, means to increase defence against diseases, arrest aging, revitalize the body in debilitated condition, increase the capability of the individual to resist adverse environmental factors and create a sense of mental wellbeing (Bhatnagar *et al*., 2005). Besides, it has amazing ability to reduce reactive oxygen species, modulate mitochondrial function, regulate apoptosis, and reduce inflammation and enhance endothelial function (Dar *et al*., 2015). The countless therapeutic potentials of *W. somnifera* are related to the presence of alkaloids and lactones, which can be found at various concentrations in plant parts like roots, stems, and leaves and are together contribute to its pharmacological diversity (Rai *et al*., 2015). Among the most recent therapeutic applications of W. somnifera, are the studies in the treatment of various types of cancer (Vyas & Singh, 2014; Winters, 2006), neuroblastoma (Kataria *et al*., 2012), breast cancer (Szarc vel Szic *et al*., 2014; Yang *et al*., 2013), prostate (Roy *et al*., 2013), and myeloid cells (Sinha & Rosenberg, 2013). Ashwagandha was found effective against various rheumatologic condition. This may be due to its anti-inflammatory properties. WS was found to cause considerable reduction in inflammation, when powdered root extract was given to rats (Anbalagan *et al*., 1981). Also in another study water extracts from leaves of WS have protective effect against glutamate induced Excitotoxicity in C6 glioma cells and IMR-32 neuroblastoma cells (Kataria *et al*., 2012). It also effectively counters the toxic effect caused by lead nitrate (Kumar *et al*., 2014).

Withaferin A is a steroidal lactone derivative from Ashwagandha and known for its anti-cancerous effect. Withaferin A ($C_{28}H_{36}O_6$) is one of the Withanolides which are naturally occurring C_{28} – steroidal lactones. It is a highly oxygenated white crystalline compound. The molecular weight of this compound is 470.6 and melting point is

241-245°C. It is a clear colourless solution at 20mg/ml In DMSO. It can be stored at -20° C in liquid form with a stability of up to 3 months. They contain four cycloalkane ring, three cyclohexane rings and one cyclopentane ring. The presence of both an unsaturated lactone in side chain to which an allylic 1° alcohol is attached as well as highly oxygenated rings A and B at other end of the molecule are influential behind its anticancer properties. So its anti-cancer activity is somewhat related to its structure (Dhami *et al*., 2016).

Table.2: Properties of Withaferin A

Name	Withaferin A
Structure	$C_{28}H_{36}O_6$
Mol.Wt.	470.6
Melting point	241-245°C
Solubility	DMSO. It can be dissolved in distilled water too however there is less stability in water.
Storage Temperature	-20°C
Stability	Up to 3 months in DMSO at -20°C. In water stability is only up to 24 hours.

The specific mechanism of WA's anti-tumour action remains elusive, but the development of WA as an anticancer agent has been suggested to provide a potentially novel approach to treat GBM as WA modulates several oncogenic pathways simultaneously. WA induces cell cycle arrest (Kataria *et al*., 2009) and apoptosis in tumor cells (Grogan *et al*., 2011). Another recent study done with WA on GBM stem cells by (Zhang *et al*.,2014) also supports its anti-tumor activity.

GFAP (Glial Fibrillary Acidic Protein) is an intermediate filament protein that is expressed in glial cells (astrocytes) and increased GFAP immunoreactivity (or astrocytes activation) is usually viewed as an index of gliosis or a relatively slow-developing correlate of neural damage (Finch, 2003; Hausmann, 2003). It has been

already an established marker for gliosis. GFAP is consistently up-regulated with age across rodents and humans and is a surrogate marker of aging in the brain, as indicated by unbiased microarray analysis of various brain regions across rat, mouse, and human species (Teter, 2009). If brain or spinal cord cells are injured through trauma or disease, astroglial cells react rapidly and ultimately GFAP expression increased.

The morphological plasticity in the adult mammalian CNS has been correlated with the enhanced expression of the NCAM (Neural Cell Adhesion Molecule), polysialylated form of neural cell adhesion molecule (PSA-NCAM), growth associated protein-43 (GAP-43) and synaptophysin (Seki and Arai, 1993; Aigner *et al*., 1995; Alonso *et al*., 1997; Prevot *et al*., 2000). NCAM is the most extensively studied cell adhesion molecule in CNS (Welzl and Stork, 2003; Kiselyov *et al*., 2005). Cell-cell interactions mediated by NCAM are dynamically regulated during nervous system development (Edelman and Jones, 1998) and also play a critical role in activity dependent synaptic plasticity in adulthood (Fields and Itoh, 1996; Murase and Schuman, 1999; Theodosis *et al*., 1999; Majdoubi *et al*., 2000). NCAM exhibits structural diversity in the form of three major proteins (NCAM-180, -140 and ~120) that are generated from different mRNAs produced by alternative splicing of exons from a single gene composed of 26 exons (Goridis and Brunet, 1992). However at least 20-30 distinct isoforms are generated by alternative splicing and by post-translational modifications (Murray *et al*., 1986; Goridis and Brunet, 1992). In addition to their adhesive properties, NCAM regulates cell migration, neurite outgrowth, fasciculation, synaptogenesis and intracellular signalling, which are closely connected with the activation of secondary messengers (Walsh and Doherty, 1997). NCAM which is also known as CD56 was originally found in nervous system but subsequently found in cells other than neural cells also and belongs to a group of cell adhesion molecules including cadherins, selectins, and integrins. It is an immunoglobin superfamily protein. NCAM regulates cell adhesion and neurite outgrowth by means of homophilic binding and subsequent activation and intracellular signalling through mitogen activated protein kinase (MAPK) pathway (Ditlevsen *et al*., 2008). NCAM is neuronal plasticity marker involved in cell adhesion, migration, and neurite extension (Chuong *et al*., 1984).

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3. Materials and Methods

3. Materials and Methods

3.1. Chemicals

Kainic acid (Mol.Wt. 231.25) and Withaferin A of concentration 106.02 mM was purchased from Sigma-Aldrich.

3.2. Cell culture

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favourable artificial environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been established. The C6 glioma cell line was obtained from Guru Nanak Dev University Amritsar (GNDU), Punjab, India. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 2mM-glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), gentamicin (100g/ml) and 10% (v/v) heat inactivated Fetal Bovine Serum (FBS) (Himedia). Cells were maintained at 37 ˚C in CO_2 incubator in a saturated humidity atmosphere containing 95% air and 5% CO_2.

3.3. Protocol for cell culture

- Cells were taken in T-25 flask and media was discarded.
- Then cells were washed with 1xPBS and PBS was removed.
- Then 1ml trypsin was added to detach cells from surface and incubated in 5% CO_2 for 30-45 seconds.
- Cells were observed under microscope to check whether the cells got detached or not. Then 1ml media was added to culturing flask.
- Cells were transferred in to centrifuge tube and centrifuged at 1000rpm for 5 minutes. Pallet was formed at bottom of the centrifuge tube.
- Supernatant was discarded and 1ml media was added to tube.
- Cells were then transferred to T-25 flask containing pre-warmed fresh media and left for incubation.

3.4. Experimental protocols: C6 Glioma Cell Culture and Its Treatment

C6 glioma cell lines were maintained in T-25 flask regularly on DMEM Medium supplemented with 1xPS, 10% FBS (Sigma) at 37°C and humidified environment

containing 5% CO_2. For treatment C6 cells were seeded in 6 well plate (n=2) and after 24 hours incubation Cultures were treated with 0.5 µM Withaferin A and incubated for 24 hours. After 24 hours of incubation with WA, cells were exposed to two increasing concentration (100µM and 200µM) of kainic acid for 3 hours. Earlier Kainic acid was prepared as a stock of 10mM concentration of volume 1ml, in 2 drops of 1N NaOH and brought up to volume by adding distilled water.

Fig.7. Experimental protocols: C6 Glioma Cell Culture and Its Treatment.

4.5. Immunocytochemistry

After 3 hours of exposure to kainic acid in WA pre-treated cells, were used to perform immunocytochemistry. Immunocytochemistry is a technique used to check expression and location of target protein in the cell via specific combination with antibodies. In this study immunocytochemistry was used to check protein expression of NCAM and GFAP.

4.5.1. Protocol for Immunocytochemistry

- Media was discarded from well plate and washed with chilled 1xPBS two times of 5 minutes each.

- Control and treated cells were fixed with 4% paraformaldehyde for 20 minutes followed by permeabilization with 0.3% PBST.
- After washing cells with 1xPBS for 5 minutes. Blocking was done with 2% BSA.
- After blocking the coverslips were treated with anti-GFAP (1:500, Sigma, Cat. No. G9269), and anti-NCAM (1:500, Sigma) antibodies diluted in 2% BSA and incubated overnight in humid chamber at 4°C.
- Cells were washed three times with 1xPBS for 5 minutes each time.
- After washing cells were treated with secondary antibody i.e. anti-Mouse IgG (Whole Molecule) FITC conjugated,.(Sigma, Cat. No. A9044) for 2 hours at room temperature inside humid chamber.
- Cells were washed three times with 1xPBS for 5 minutes each time.
- Cells were then mounted with mounting medium which contained DAPI.
- The coverslips were then washed extensively and mounted on glass slides with anti-fading medium for quantitative image analysis.
- Finally cells were observed under Confocal Microscope (Olympus FV/1200).

4. Results

4.1. Kainic Acid disturbs morphology of C6 glioma cells as well as reduces cell viability:

To demonstrate the toxicity level of Kainic acid, C6 glioma cell lines were treated with two increasing concentrations (100µM and 200µM) of Kainic acid for 3 hours. By analysing immunocytochemistry images of kainic acid treated cells with control and Withaferin A treated cells it was observed that cells in control and Withaferin A have more confluency and distinct morphology. Kainic acid treatment causes a great disturbance in cell morphology and induces cell death by apoptosis and necrosis. No significant differences was observed in viability of the cells in control and Withaferin A treated group whereas with increasing concentrations of kainic acid number of cells were decreased. Most of the cells in Kainic acid treated group showed altered morphology and bursting of cell membrane showing necrosis. From phase contrast images it was clear that cells were more aggregated in KA treated groups (Fig. 9 (c), (d)) in comparison with controls group (Fig. 9 (a), (b)) shows disturbance in their structure. Cells with 200µM (Fig. 9 (d)) kainic acid has more disturbed morphology than those with 100 µM (Fig. 9 (c)) concentration of kainic acid. Cells were totally burst out in 200 µM kainic acid and also very low number of cells were there. In 100 µM kainic acid treatment there is a change in cell structure though not as much intensity those seen in case of 200 µM kainic acid treatment. Morphological changes in nucleus are clearly distinct as condensation of chromatin and nuclear fragmentation and higher intensity of staining in KA treated groups (Fig. 8 (c), (d)). In Withaferin A treated cells (Fig. 8 (b) and 9 (b)) not much difference in morphology observed as compared to the control.

4.2. Withaferin A minimizes Kainic acid induced cytotoxicity in C6 glioma cells:

To observe glioprotective effect of Withaferin A, we pre-treated C6 cell lines with 0.5 µM concentration of Withaferin A for 24 hours. After 24 hours of incubation, these Withaferin A pre-treated cell cultures were again treated with 100 µM and 200 µM concentrations of kainic acid for 3 hours. Pre-treatment with 0.5 µM Withaferin A significantly inhibits cell death as well as restored normal morphology (Fig.10 (c), (d), and 11 (c) (d)). WA helps in developing resistance in cells against toxic effect of KA and prevents expected morphological changes under influence of KA in cells.

Cells with these combined treatment with Withaferin A and Kainic acid showed typical morphology with less difference from those in control (Fig. 10(a) and 11(a)) and only Withaferin A (Fig. 10b, 11b) treated group. So kainic acid induced changes in morphology were partially suppressed by Withaferin A.

4.3. Withaferin A abolished kainic acid induced changes in the expression of C6 marker proteins.

GFAP is an excellent marker for astrocytes activation, which responds to CNS damage induced by many neurotoxic agents. Immunocytochemistry was performed to see the localization of the GFAP expression. Cells were exposed to 100 µM and 200 µM concentrations of kainic acid (3h) after pre-treatment of 0.5 µM Withaferin A (24h). Only kainic acid treated groups showed considerable increase in GFAP expression (Fig. 12 (b) and (e)) comparing with control and Withaferin A treated groups (Fig. 12 (a), (d)), indicating gliosis. But expression of GFAP seen decreased (Fig. 12 (c), (f)) in groups those were treated with both kainic acid and Withaferin A (pre-treated) Decrease of GFAP expression shows reduced gliosis and injury hence proves protective effect of WA.

4.4. Withaferin A induces NCAM expression to reduce excitotoxic cell death in kainic acid challenged cells:

NCAM is a glycoprotein of immunoglobulin (Ig) superfamily, expressed on the surface of neurons and glia cells. It has a role in cell–cell adhesion, neurite outgrowth, synaptic plasticity, neuroprotection and learning and memory. We examined NCAM expression in control and treated groups. It was found that groups treated with Withaferin A only (Fig.13 (d)), showed a minor increase in NCAM expression compared to control groups. The lower dose treatment of kainic acid (100µM) causes enhanced expression of NCAM (Fig. 13 (b)) and there is further increased immunolabelling for NCAM (Fig. 13 (e)) in higher dose concentration of kainic acid treated group (200µM). But 0.5 µM Withaferin A pre-treatment led to normalization of NCAM expression (Fig. 13 (c), (f)).

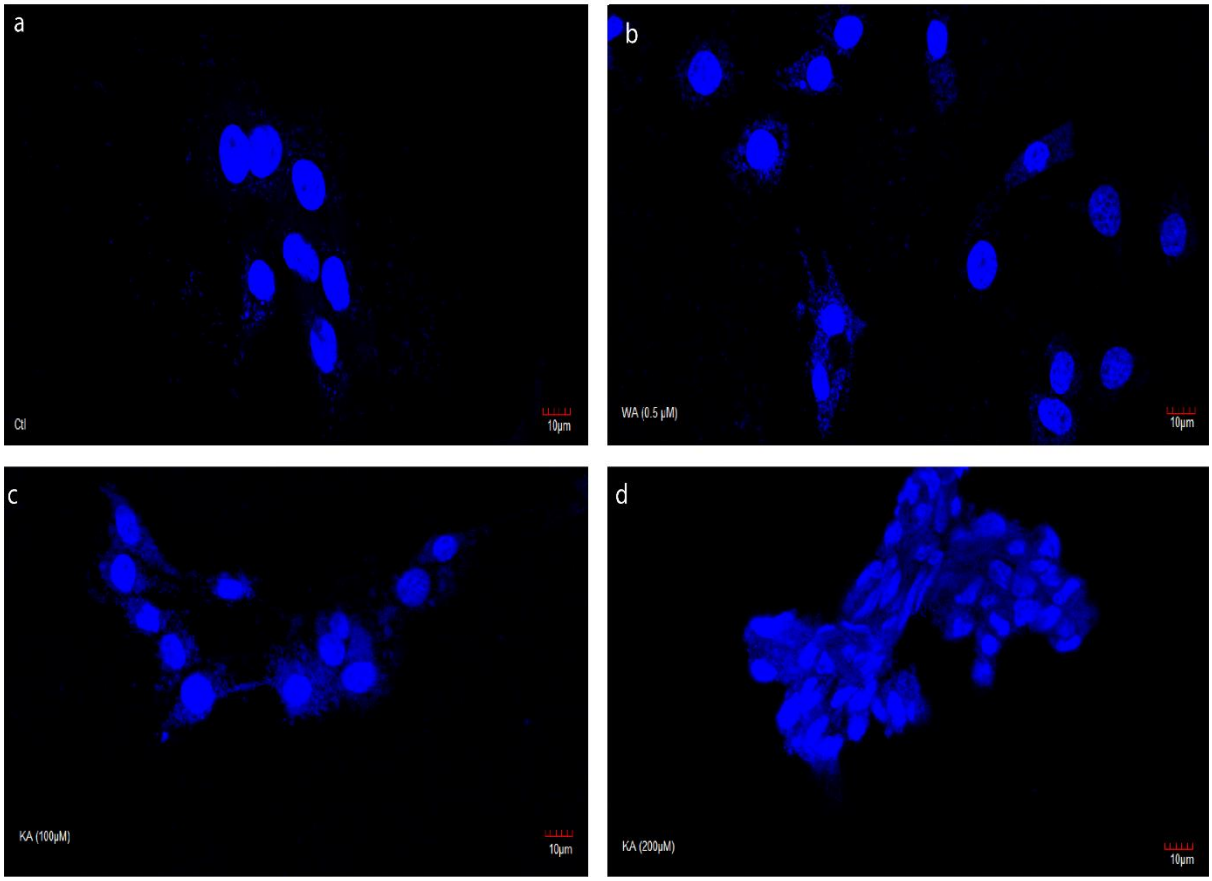

Fig.8: Morphological changes induced by Kainic acid treatments in C6 glioma cells. Morphological changes observed in DAPI staining. Changes in nuclear morphology can be detected by nuclear fragmentation, and high intensity of staining which are clearly seen in KA treated cells (Fig. (c), (d)) compared with Control and WA treated cells (Fig. (a), (b)). Images were captured using Olympus FV/1200 Confocal Microscope. Scale bars, 10µM. (Ctl = Control, WA= Withaferin A, KA= Kainic acid. Concentration of WA is 0.5µM)

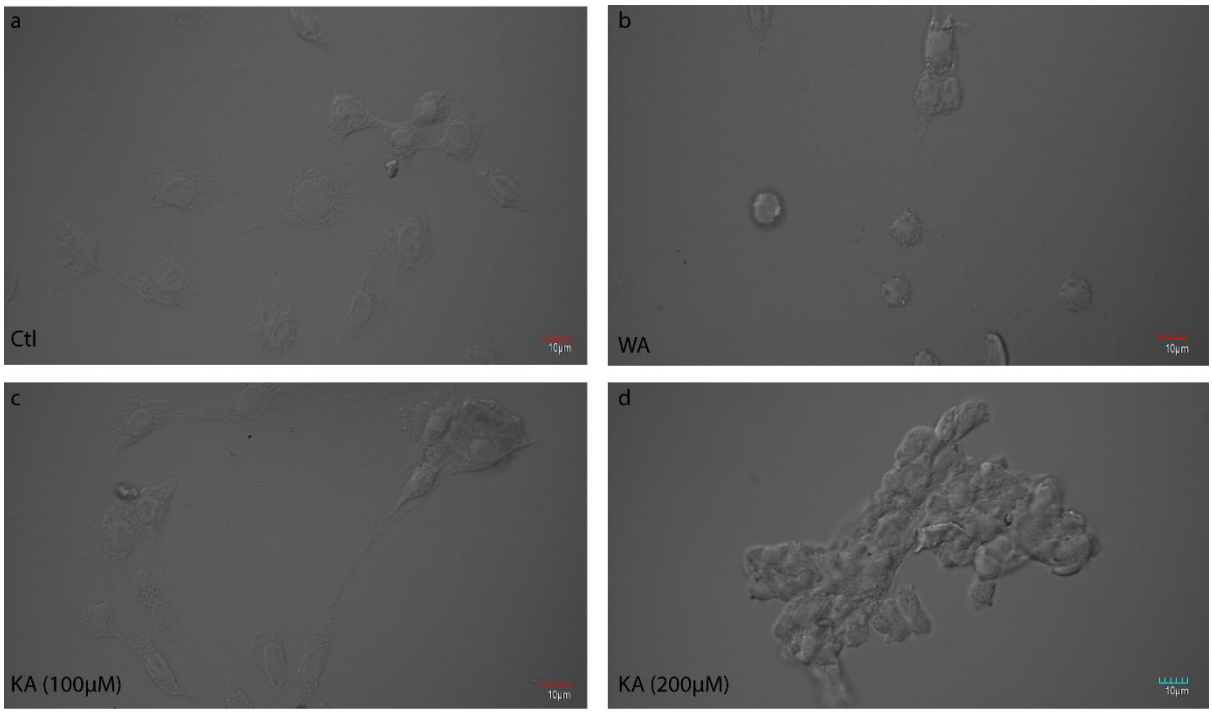

Fig. 9: Phase contrast images of C6 glioma cells treated with different concentrations of KA, were captured using Olympus FV/1200 Confocal Microscope. KA treatment causes a significant changes in morphology of cells by nuclear fragmentation and cells are in more aggregated form (Fig. (c), (d)). Scale bars, 10µm.

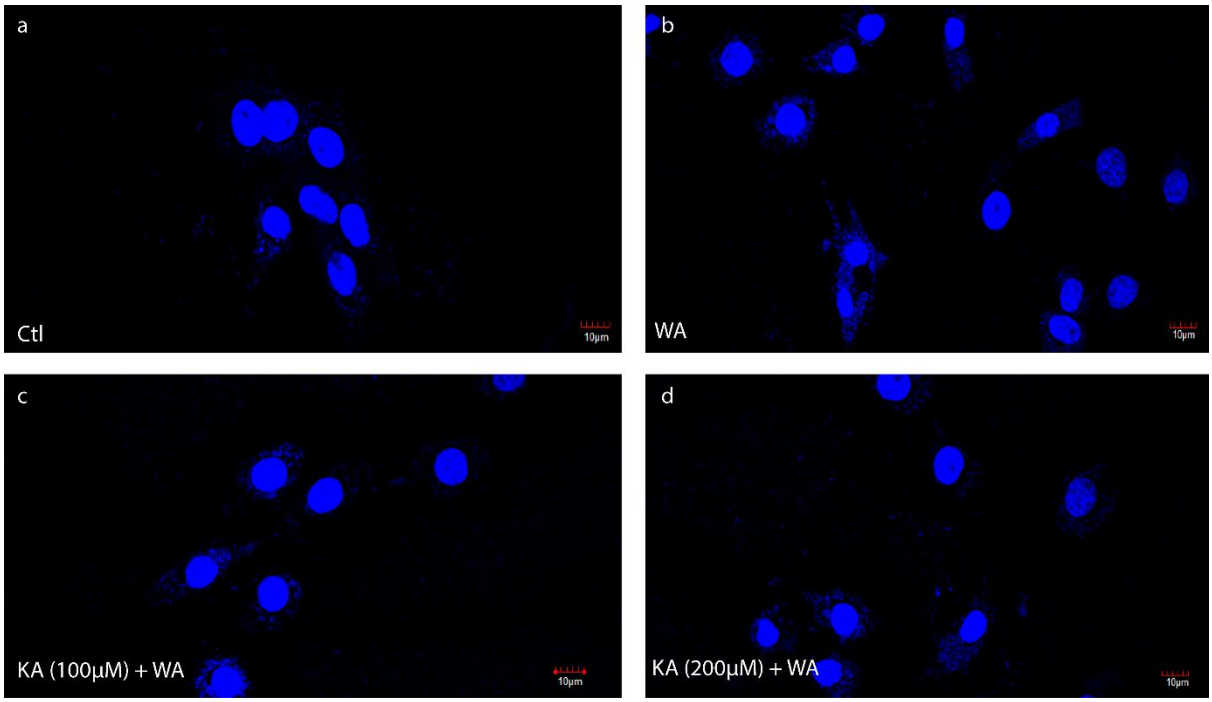

Fig.10: Morphological analysis of C6 glioma cells treated with different concentrations of kainic acid and WA. Cells were grown on coverslips and treated with kainic acid for 3 hours. Cells were then fixed and stained (a - d) with DAPI. All images were captured using Olympus FV/1200 confocal microscope (60X). WA helps in reversing the morphological changes that is supposed to be happened under influence of KA. In Fig. (c), (d) WA helps in developing resistance against KA. Scale bars, 10µM. (Ctl = Control, WA= Withaferin A, KA= Kainic acid. Concentration of WA is 0.5µM)

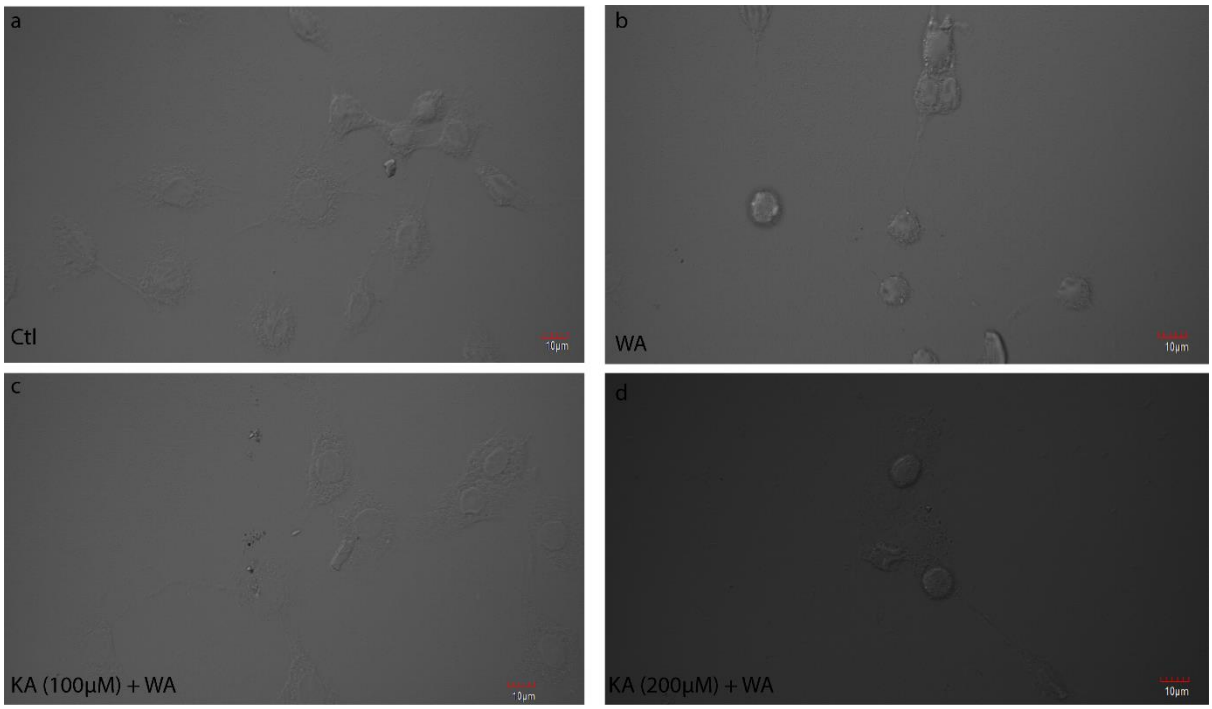

Fig.11: Phase contrast images of C6 glioma cells, treated with different concentrations of KA and WA, were captured using Olympus FV/1200 Confocal Microscope. WA pre-treatment protects the cells from toxic effect of Kainic acid, by maintaining typical cell morphology and structure (Fig. (c), (d)) Scale bars, 10µm.

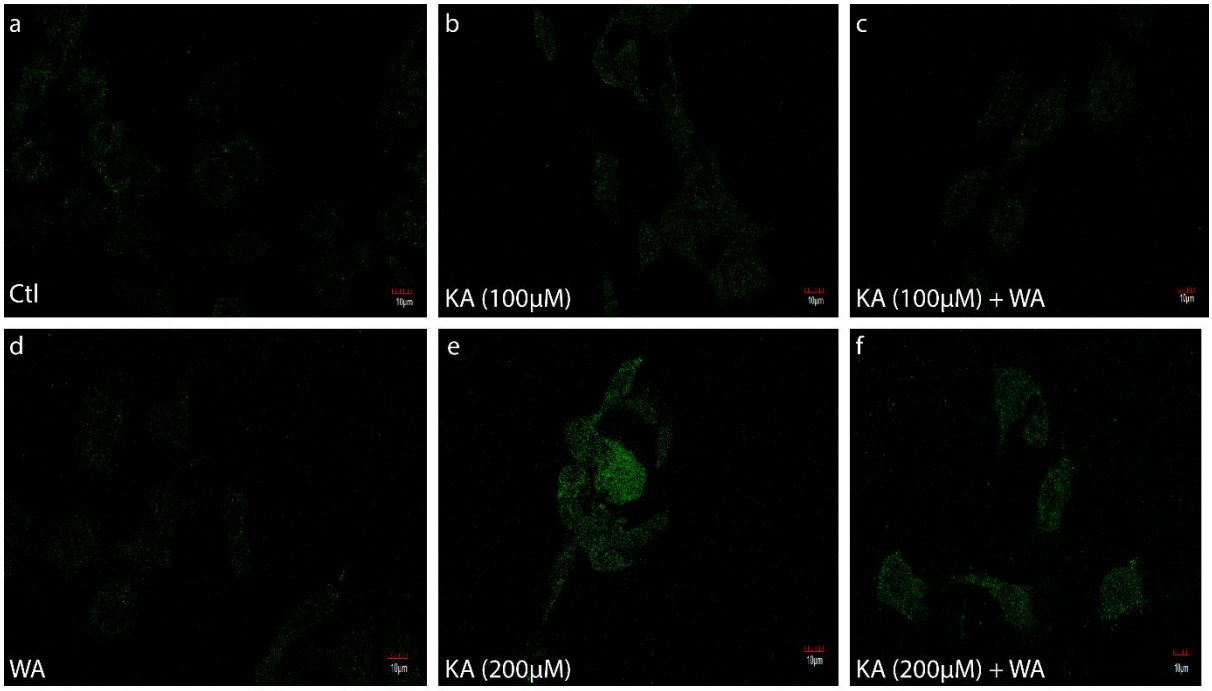

Fig.12: Expression of GFAP after pre-treatment with WA followed by KA treatment. Cells were grown on coverslips and treated with Withaferin A for 24 hours followed by kainic acid for 3 hours. Cells were then fixed and stained (a to f) for GFAP. All images were collected using Olympus FV/1200 confocal microscope. (60X). Protein expression of GFAP was significantly elevated in KA treated cultures that was reverse with WA pre-treatment. In WA treatment alone, GFAP did not show any significant changes (Fig. (d)) as compared to control. In Fig. (c) and (f), WA helps in developing resistance against KA. As it is clear in Fig. (b) and (e), treatment with KA increases GFAP expression which get normalized by WA (Fig. (c) and (f)). (Ctl = Control, WA= Withaferin A, KA= Kainic acid. Concentration of WA is 0.5µM)

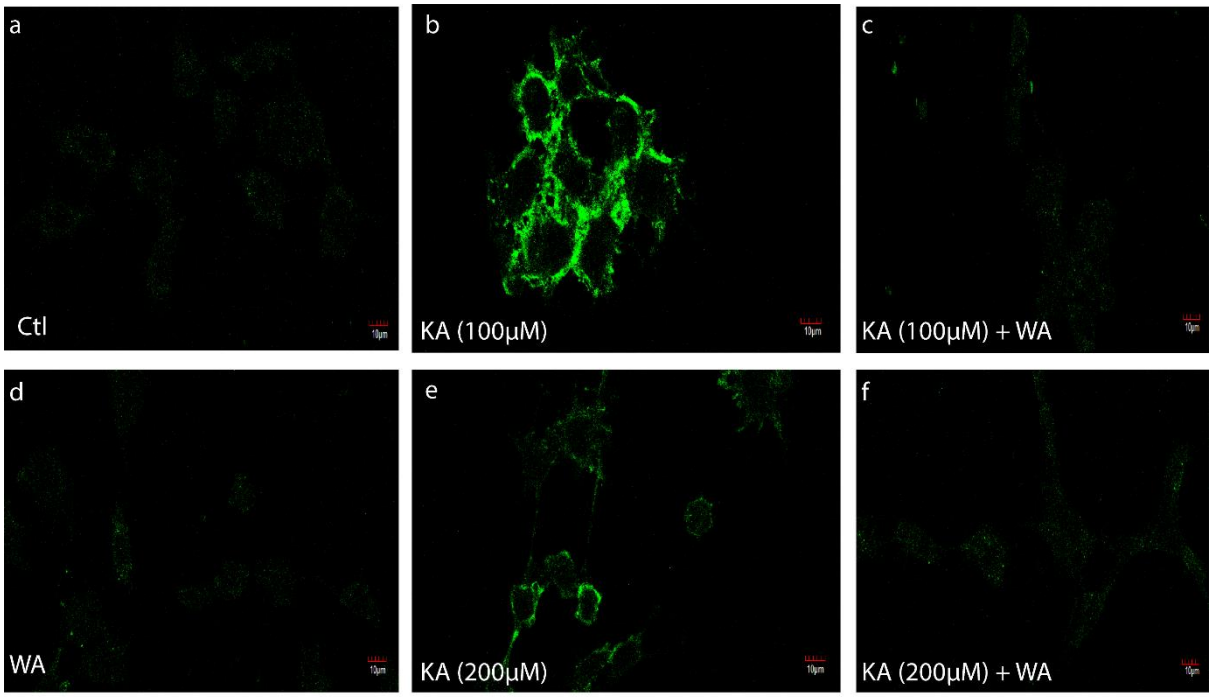

Fig.13: Localization of NCAM in, Kainic Acid-treated ((b) and (e)) and WA +KA treated ((e) and (f)) C6 glioma cultures. Cells were grown on coverslips and treated with WA for 24 hours. Cells were then fixed and stained with DAPI conjugated with FITC for NCAM (green) immunoreactivity. All images were captured using Olympus FV/1200 confocal microscope (60X). Expression of NCAM after treatment of KA and WA. KA causes increase in NCAM expression (Fig. (b) and (e)). WA pre-treatment helps normalizing NCAM as seen in Fig. (c) and (f). In Fig. (d), WA treatment alone causes a minor increase in NCAM. Scale bars, 10µM (Ctl = Control, WA= Withaferin A, KA= Kainic acid. Concentration of WA is 0.5µM)

5. Discussion

Glioma is one of the most common tumour associated with brain and as there is not much effective treatment against this, there is a need of potential therapeutic approaches (Kataria *et al*., 2009). It is well known fact that ionotropic glutamate receptors are involved in death of neurons by means of excitotoxicity. Kainite receptors are one of them upon which kainic acid acts (Popescu B.O. *et al*., 2002). Kainic acid is a potent neurotoxin and cells treated with KA undergone with apoptosis and necrosis. Kainic acid induced neurodegeneration by several mechanism like it activates excitatory pathways and also causes epileptic seizers which is characterized by abnormal brain behaviour (Popescu B.O. *et al*., 2002). It may also kill a cell directly by apoptosis where it binds with its receptors. Many efforts attempted to invent pharmacological treatment against neurodegenerative diseases. Recently the search for natural extracts to counter these disorders has been increasing gradually because of its cost-effective ness and negligible side effects (Sairazi *et al*., 2015).

Ashwagandha is one of the most sought after ayurvedic plant for its various medicinal properties. Above all it is a well-known nerve tonic. Ashwagandha root and leaf extracts has been shown to treat oxidative damage and physiological abnormalities in mouse model of Parkinson's disease. The present study was designed to test advantageous effect of its steroidal extract Withaferin A against KA induced neurotoxicity using in vitro C6 Glioma cells.

Withaferin A (0.5µM) is able to normalize the toxic effect of KA in KA+WA treatment group as evidence of normal cell morphology which was disturbed fiercely in alone KA treated group. This suggests the cytoprotective role of WA. In the present experiment upregulation of GFAP expression was seen in KA treatment. This indicates reactive gliosis induced by KA which was confirmed by increased GFAP. But WA treatment showed a decrease in GFAP expression in cells exposed to both KA and WA. Also previous studies showed association of increased GFAP levels and hypertrophic changes with susceptibility to toxic effect to C6 cells (Kataria *et al*., 2012). Ashwagandha found to antagonize the DNA damage and oxidative stress induced by lead (Kumar *et al*., 2014). Also it has been showed that Ashwagandha extract and its other bioactive component Withanone was able to revert scopolamine induced changes in GFAP expression in neuronal cells (Konar *et al*., 2011). Also normalization of GFAP levels by Ashwagandha in glutamate induced neuron

degeneration in retinoic acid differentiated C6 and IMR-32 cells has been already shown (Kataria *et al*., 2012), which is supporting our results outcome. GFAP is a glial cell marker which is expressed as a hallmark of activated astrocytes and microglia upon gliosis. KA disturbs cell morphology as well as increased GFAP expression suggesting gliosis but the WA treatment revert back cells to normal morphology and provided evidence for its neuroprotective role. Withaferin A also causes differentiation in C6 cells. In another study by (Shah *et al*., 2009), Withaferin A is in combination with Withanone induces differentiation with increased GFAP expression which further strengthen our results.

Cell-cell interactions mediated by NCAM are dynamically regulated during nervous system development (Edelman and Jones, 1998) and also play a critical role in activity dependent synaptic plasticity in adulthood (Fields and Itoh, 1996; Murase and Schuman, 1999; Theodosis *et al*., 1999; Majdoubi *et al*., 2000). NCAM exhibits structural diversity in the form of three major proteins (NCAM-180, -140 and ~120) that are generated from different mRNAs produced by alternative splicing of exons from a single gene composed of 26 exons (Goridis and Brunet, 1992). However at least 20-30 distinct isoforms are generated by alternative splicing and by post-translational modifications (Murray *et al*., 1986; Goridis and Brunet, 1992). In addition to their adhesive properties, NCAM regulates cell migration, neurite outgrowth, fasciculation, synaptogenesis and intracellular signaling, which are closely connected with the activation of secondary messengers (Walsh and Doherty, 1997).

NCAM is an important cell surface plasticity markers that play a significant role in regeneration and repair of neurons. It is important for cell-cell adhesion, neurite outgrowth, and memory. It is expressed in embryonic stage but generally downregulated in adults but has been shown to increase its expression after some brain-injury and its increase is an indication of some kind of neurodegenerative disease (Kataria *et al*., 2012). In the present study we tested NCAM expression in various treated group. We observed an increase in NCAM expression in KA treated group showing toxic effect of KA. WA helps normalizing NCAM levels along with normal morphology as seen in KA + WA treated group, indicating protective effect of WA. Our study is consistent with previous reports where ASH-WEX normalizes

NCAM expression in glutamate induced toxicity in C6 cells (Kataria *et al*., 2012), and another study demonstrate that excitotoxicity increase in NCAM expression in hippocampal region of the brain (Hoffman *et al*., 2001). But our study differs from previous study where NCAM was downregulated in C6 glioma cells after lead nitrate exposure, and Ashwagandha treatment partially increases NCAM expression (Kumar *et al*., 2014).

The neuroprotective effect seen in this study could be attributed to the presence of free radical scavenging properties of WA. In our experiment WA is able to exert its cytoprotective effect against KA in both low dose (100µM) and high dose (200µM) treated groups. Especially in high dose KA treated group, where morphology of cells were damaged in a great extent, but in KA (200µM) +WA (0.5µM) treated group WA brings back the cells to normal morphology, showed the incredible protectiveness of WA. Collectively our study determines the protective role of WA against neuronal loss caused by KA, and further study is needed to evaluate various mechanism of actions of WA against KA induced excitotoxicity.

Conclusion and Future Prospective

Conclusion

Neurodegenerative diseases and nervous system disorders like Alzheimer's disease, Parkinson's disease are increasing now a days and there is not much effective therapies against them. Natural products and herbal extracts known to have immense potential to treat CNS related diseases (Sairazi *et al*., 2015). They are less toxic alternatives to modern medicine and offer much scope for preventing diseases. Ashwagandha is most commonly used plant in Indian Ayurvedic tradition. On the basis of our results we hypothesize that WA is able to counter toxic effect of KA and excitotoxic effect. WA may have potential to reduce malignancy of glioblastoma and neuroblastoma. From our study it is clear WA is efficient even in very low dose and this can make WA a potential candidate for treatment of glioblastomas. Our findings could be valuable to anti-epileptogenic treatment strategies and for development of neuroprotective drugs. Withaferin A is known for its anti-cancerous properties and from our findings its glioprotective property also should be taken in to consideration and more works needs to be done.

Future Prospective

Though present study reveals WA as a potential neuroprotective drug, several limitations still exists. Ashwagandha is used mostly in Ayurveda and more clinical trials should be done. Besides WA its other root and leaf extracts may also have beneficial properties. The mechanism of action of WA is yet to known. Clinical trial are required in order to evaluate optimum doses of WA and duration of treatment, as in large amount WA could be toxic to cells (Kuboyama *et al*., 2014) and causes apoptosis (Shah *et al*., 2009). In a previous study ASH-WEX was not much effective against glutamate induced excitotoxicity when doses of glutamate increased. So curative effect of WA against other neurotoxins besides KA, such as Manganese, Lead, Glutamate, Nitric Oxide etc. should be analysed as toxicity levels of various substances are different and an optimum effective doses should be calculated. It should be great if in vivo studies related to glioprotective effect of WA will be done. Constituents of *Withania somnifera* has been associated with neuritic regeneration and synaptic reconstruction. NCAM being known for its regenerative and repair mechanism in CNS, there may be kind of relation between NCAM expression and

Withania mediated regenerative and protective effects towards normalization and repair which needs further studies to explore.

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