# TO TEST THE ROLE OF *WITHAFERIN A* IN NEUROPROTECTION ON SH-SY5Y CELL LINE CHALLENGED BY KAINIC ACID

Project Report submitted to the Central University of Punjab

For the award of

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**Life Sciences (Specialization in Animal Sciences)**

Department of Animal Sciences
**BY**

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## DECLARATION

I declare that the dissertation entitled "TO TEST THE ROLE OF WITHAFERIN A IN NEUROPROTECTION ON SH-SY5Y CELL LINE CHALLENGED BY KAINIC ACID" has been prepared by me under the guidance of Dr. Jyoti Parkash, Assistant Professor, Department of Animal Sciences School of Basic and Applied Sciences, Central University of Punjab, Bathinda. No part of this dissertation/thesis has formed the basis for the award of any degree or fellowship previously.

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## CERTIFICATE

I certify that Aishwarya Pruseth has prepared her dissertation entitled "TO TEST THE ROLE OF WITHAFERIN A IN NEUROPROTECTION ON SH-SY5Y CELL LINE CHALLENGED BY KAINIC ACID" for the award of M.Sc. Degree of the Central University of Punjab, under my guidance. She has carried out this work at the Department of Animal Sciences, School of Basic and Applied Sciences, Central University of Punjab, Bathinda.

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Aishwarya Pruseth

**ABSTRACT**

**TO TEST THE ROLE OF *WITHAFERIN A* IN NEUROPROTECTION ON SH-SY5Y CELL LINE CHALLENGED BY KAINIC ACID**

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Key worlds : *Withania Somnifera*, Withaferin A, NCAM, Kainic acid, Neurodegenerative diseases.

**Abstract**

*Withania somnifera* (WS) has been used in traditional medicine for a variety of neural disorders. Recently, chronic neurodegenerative conditions have been shown to benefit from treatment with this crude extract. Neurotoxicity induced by Kainic acid causes stroke, head trauma and leads to various neurodegenerative diseases. It is a common pathway for neuronal injury and death. Withaferin A (WA) is the steroidal lactone and phytochemical of medicinal plant WS. These days research on the medicinal plant is the major theme so as to identify the novel, natural and safe phytotherapies has been flourished. WS is a popular ayurvedic plant with an array of medicinal properties including neuroprotection. It also appears to exert a great influence on the cardiopulmonary, endocrine, and central nervous systems. Recently several *in-vitro* and *in-vivo* preclinical studies validating the therapeutic value of newly identified phytochemicals have been launched. The use of medicinal plants and its chemical component can be exploited in the treatment of neurodegenerative diseases like Parkinson, Alzheimer's, Epilepsy and so on. Ayurvedic products are the only promising way to treat these diseases with negligible side effects. In the current study the neuroprotective role WA has been studied on SH-SY5Y neuroblastoma cell line challenged by kainic acid. Cell viability and expression of neuronal cell differentiation marker NCAM was investigated on

Kainic acid challenged SH-SY5Y cells with and without the presence of WA. When the cells are challenged via Kainic acid there is a decrease in cell viability due to the phenomenon of excitotoxicity and this was reversed by WA treatment. NCAM is an important cell surface plasticity marker that have role in repair and regeneration. Potential mechanisms underlying the observed neuroprotection were examined. Additionally, morphological changes were monitored following KA treatment. Upon excitotoxicity with Kainic acid NCAM expression increased but the effect is reverse upon pre-treatment with WA. Altogether, the present study suggests that WA is neuroprotective and provides neuroprotection against KA induced excitotoxicity could have therapeutic potential to target factors involved in neurodegenerative diseases.

Aishwarya Pruseth Dr. Jyoti Prakash

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## LIST OF ABBERVIATIONS

| Sr. No. | Full Form | Abbreviation |
|---|---|---|
| **1.** | 12-O-tetradecanoyl-phorbol-13 acetate | TPA |
| **2.** | 4,6 diamidino-2-phenylindole | DAPI |
| **3.** | 4,6-diamidino-2-phenylindole | DAPI |
| **4.** | Alzheimer’s disease | AD |
| **5.** | Amyotrophic Lateral Sclerosis | ALS |
| **6.** | Bovine Serum Albumin | BSA |
| **7.** | Brain-derived neurotropic factor | BDNF |
| **8.** | Central Nervous System | CNS |
| **9.** | Dimethyl Sulfoxide | DMSO |
| **10.** | Dlubecco’s Modified Eagle’s Medium | DMEM |
| **11.** | Dopamine Transporter | DAT |
| **12.** | Endoplasmic Reticulum | ER |
| **13.** | Fetal Bovine Serum | FBS |
| **14.** | Fibroblast Growth Factor-2 | FGF-2 |
| **15.** | Flouroscein Isothiocyanate | FITC |
| **16.** | Gene protein Couple Receptor | GPCR |
| **17.** | Glutamate Receptors | GluR |
| **18.** | Horseradish Peroxidase | HRP |
| **19.** | Immunoglobulin | Ig |
| **20.** | Inositol Triphosphate | $IP_3$ |
| **21.** | Ionotropic Glutamate Receptor | iGLURs |
| **22.** | Kainic Acid | KA |
| **23.** | Kainic Acid Receptor | KAR |
| **24.** | Metabotropic Glutamate Receptors | mGLRs |
| **25.** | Microtubule- Associated Protein-2 | MAP-2 |
| **26.** | Neural Cell Adhesion Molecule | NCAM |
| **27.** | Neuroblastic Type | N-Type |
| **28.** | Nitric Oxide | NO |
| **29.** | Nitric Oxide Synthase | NOS |

| **30.** | N-Methyl-D-Aspartate | NMDA |
|---|---|---|
| **31.** | N-Methyl-D-Aspartate Receptor | NMDAR |
| **32.** | Non- steroidal anti-inflammatory Drugs | NSAIDs |
| **33.** | Norepinephrine Transporter | NET |
| **34.** | Parkinson disease | PD |
| **35.** | Phosphate Buffer Saline | PBS |
| **36.** | Polysialytransferase | PST |
| **37.** | Polysiayated Neural Cell Adhesion Molecule | PSA-NCAM |
| **38.** | Retinoic Acid | RA |
| **39.** | Sodium Dodecyl Sulfate | SDS-PAGE |
| **40.** | Substrate- adherent Type | S-Type |
| **41.** | Temporal lobe Epilepsy | TLE |
| **42.** | Tyrosine Hydroxylase | TH |
| **43.** | Withaferin A | WA |
| **44.** | *Withania somnifera* | WS |
| **45.** | α-amino-3-hydroxy-5methyl-4-isoxazolepropionic Acid | AMPA |
| **46.** | γ Amino butyric Acid | GABA |

# CHAPTER-1

# INTRODUCTION

*Withania Somnifera* (WS) is a well-known medicinal plant, known for its various medicinal properties. Numerous experiments have been demonstrated on the medicinal properties of the plant and its crude extract. WS belongs to the family *Solanacea*. It is a shrub of 1 meter long and possesses largest number of Withanolides. In Indian mythology it is called as "Rasayana" due to its medicinal qualities and also known as Ashwagandha. It has been traditionally used to treat various health conditions such as depression, stress, anxiety, memory loss etc. There are other medicinal plants which have been into research such as *Curcuma longa, WS, Panax ginseng, Ginkgo bilobae* and so forth have been distinguished for its neuroprotective role in different neurological disorders. The phytochemicals of WS have different functional groups and varying in therapeutic values. Several studies have reported that Withanolides may activate neurite development and can be used for many neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson disease (PD), Epilepsy, traumatic brain injury, Temporal lobe Epilepsy (TLE), Huntington's disease, amyotrophic lateral sclerosis (ALS) and so on (Sangwan and Chaurasiya, 2007). Traditionally Non-steriodal anti-inflammatory drugs (NSAIDs) such as rofecoxib, COX 2 inhibitors, aspirin mefenamic acid, indomethacin and ketoprofen have been used to treat neurological disorders. Natural products are more appreciated in todays' era because the artificial drugs such as NSAIDs possesses certain side effects including dizziness, gastrointestinal problems, headache, nausea etc. Hence natural products are more successful than conventional agents with negligible side effects. The biologically active constituent of WS contains steroidal lactones, saponins, alkaloids, flavonoids, phenols. Among the phytochemicals WS possess Withaferin A (WA) is the most abundant and bioactive compound found in the leaves and root extract.

WA is a steroidal lactone which has been screened for its several medicinal qualities such as anti-oxidant, anti-inflammatory, anti-tumour, anti-stress etc. This phytochemical has been successfully validated for its therapeutic use in cancer (Shi *et al.*, 2015). It is being selected for its drug likeliness, mutagenicity, human intestinal absorption, and blood brain barrier penetration (Kumar and Patnaik, 2016). Research on the curative properties of phytochemicals found in WS has been increasing day by day to find out the safe alternative of modern medicines and synthetic drugs. The *in vivo* and *in vitro* preclinical studies validating its

therapeutic values have been evaluated. (Kataria *et al.*, 2012). The products of medicinal plant especially Withaferin A (WA) has shown beneficial effects in providing neuroprotective against excitotoxicty in the Central Nervous System (CNS). A unified insights into the function of WA is still forthcoming. The molecular mechanism study of anti-cancer properties of the compound has been shown that it targets NF-Kb, Bcl-2, FOXO3A, HSP90, phosphorylated STAT3, and annexinn II27 (Singh *et al.*, 2007, Falsey *et al.*, 2006). Although the neuroprotective neuro-regenerative and anti-cancer properties of *Withania Somnifera* have shown outstanding results, by using the neuroblastoma cells SH-SY5Y. The present study has been designed to study the role of steroidal lactone WA in neuroprotection challenged by neurotoxic dose of Kainic acid (KA) in SH-SY5Y neuroblastoma cell line.

Kainic acid [2-carboxy-4-(1-methylethelyn1)-3-pirrolidiaacetic acid] is a potent neurotoxic which causes excitotoxicity in the neuronal cells. It is the L-analogue of Glutamate a major neurotransmitter in the CNS. Excitotoxicity is the major pathological process of neurodegeneration studied in most of the neurodegenerative diseases (Kaur *et al.*, 2012), which damages the nerve cells by the over stimulation of neurotransmitter such as glutamate or similar substances. The excitotoxicty process was formulated by Olney (1969) and demonstrated that the process mainly triggered by excess glutamate and the prolonged activation of glutamate receptors. There are generally 2 types of glutamate receptors e.g. Ionotropic and metabotropic. This is the major process of most of the neurodegenerative diseases. KA acid activates the ionotropic glutamate receptors NMDA (N-methyl-D-aspartic-acid), AMPA (α-amino-3-hydroxy-5-methyl-isoxazole propionate) and Kainate receptors (KAR). Which causes the neuronal membrane depolarization. It causes the dysregulation of intracellular $Ca^{2+}$ homeostasis which leads to the activation of calcium dependent enzymes such as caspases. These enzymes cleaves the important proteins and also leads to endoplasmic reticulum (ER) fragmentation and ultimately neuronal death (Sairazi *et al.*, 2015, Chang *et al.*, 2015). As these mechanism common in many neurological diseases, targeting these receptors and reduction of neurotoxicity is important in therapeutic use such as drug designing. Natural products are an only promising way for successful treatment in combatting acute and chronic neurodegeneration.

SH-SY5Y neuroblastoma cell line has been used as a model cell line for research purposes and mostly in pathological studies of neuronal degeneration. The cell line has been used widely in experimental neurological studies, including analysis of neuronal differentiation, metabolism, and function related to neurodegenerative processes, neurotoxicity, and neuroprotection. SH-SY-5Y (Neuroblastoma Cell Lines) which is human derived cell lines and is used as a model in various scientific researches. It was isolated from the 4-year-old girl suffering from neuroblastoma as SK-N-SH. It was cloned to SH-SY-Y cell lines and was finally clone to SH-SY-5Y. It has been reported that these cell lines have a saturation density greater than 1 X $10^6$ cells/$cm^2$ and they are also reported to show moderate levels of Dopamine β - hydroxylase activity (Biedler et al., 1978). Cell lines have been used as in-vitro models for neuronal function and differentiation. Differentiated and undifferentiated SH-SY-5Y cell lines both have been used for neuronal-like cells (Tosseti et al., 1998). Undifferentiated cells are defined morphologically by neuroblast-like, non-polarized cell bodies with few truncated process. These cells proliferate continuously and represent the expression of immature neuronal markers. Induction of differentiation by retinoic acid results in inhibition of cell growth and enhanced production of noradrenaline form of SH-SY-5Y. These cell lines show both adherent and suspension properties and both these properties are interconvertible. These cells possess an abnormal chromosome-1 where there is an additional copy of 1q segment referred to as trisomy 1 (Jane et al., 2013). They are relatively easy to grow in culture and used for several experimental purposes. Moreover, they can be induced to differentiate into specific neuronal types with different agents or under different growth conditions (Encinas et al., 2000). The present study was designed to test the hypothesis that WA may confer protection against Kainic acid-induced excitotoxicity. Further the expression of neuroplasticity markers NCAM was supposed to be evaluated to establish their role in WS mediated neuroprotection.

Neural cell adhesion molecule (NCAM) is a transmembrane glycoprotein and calcium independent protein which is involved in cell-cell and cell-matrix interaction. It is generally expressed during neuronal plasticity, neurite growth and synapse formation. It is most abundant in hippocampal excitatory synapse. NCAM expression is high during embryogenesis and decreases during adult and confined to few tissues and cell types. NCAM, a member of the immunoglobulin superfamily

of cell recognition molecules, is widely expressed on axons and dendrite and is a key regulator of neuronal development and growth. Cell-cell interaction mediated by NCAM are dynamically regulated during development of nervous system (Edelman and Jones, 1998) and also play a crucial role in activity dependent regulation synaptic plasticity in adult hood (Fields and Itoh, 1996; Murase and Schuman,1999; Theodosis *et al.*,1999; Majdoubi *et al.*, 2000). The present study was designed to test the hypothesis that NCAM, neuroplasticity marker will be upregulated after exposure to neurotoxic KA to confer protection against the neural degeneration.

**Objectives:** As WA increases defence against diseases, arrest aging, revitalize the body in the debilitated condition, increase the capability of the individual to resist adverse environmental factors and create a sense of mental wellbeing. . NCAM is a member of the immunoglobulin super family of cell recognition molecules, expressed on the axons and dendrites (Rutishauser et al., 1988) and it is a major regulatory molecule in neuronal function and development (Panicker et al., 2004). Both NCAM and PSA-NCAM are neuronal plasticity markers and have an important role in regeneration and repair.  PSA is a dynamically regulated product of post-translational modification of NCAM by enzyme PST. On the basis of this following objectives designed:

1. To study neuroprotective effects of Withaferin A against kainic acid induced excitotoxicity.
2. To study activity dependent regulation of NCAM expression in SH-SY5Y neuroblastoma cells.

# CHAPTER - 2

# REVIEW OF LITERATURE

## 2.1 SH-SY5Y cell line:

Neuro-blastoma cell line SH-SY5Y is derived from parental SK-N-SH cell line. It contains both neuro-blast and epithelial like properties. It. It has a stable karyotype of 47 chromosome, which can be differentiated into more neuron like phenotype. These cell line grow continuously and express mature neuronal marker. SH-SY5Y cells can be differentiated by RA treatment and it takes about 7 days for these cells to grow via RA treatment alone (Preis *et al.,* 1988; Sarkanen *et al*., 2007). Differentiation by retinoic acid (RA) results in inhibition in cell growth and enhanced production of non-adrenalin form of SHSY5Y. As a result of which cells get differentiated into neuronal like properties such as neurite outgrowth and morphological changes. The previous study reported that RA downregulate mRNA and protein. These cell line shows both adherent and suspension properties and both are interconvertible. They are relatively easy to culture and can be manipulated for experimental purposes. The cell line possess an additional copy of large segment of chromosome 1 which is referred to as trisomy-1. Exclusively 20% of the Latest study has been reported that some chemicals such as Nerve growth factor (NGF) and brain- derived nerve growth factor (BDNF) with or without extracellular matrix may further improve neuronal differentiation and sustain these conditions in RA- differentiated SH-SY5Y cell line. Despite the fact that SH-SY5Y doubling time has not been reported precisely, the parental neuro-blast like cells population have a doubling time of approximately 27 hours same as that of sub-clones. These cells show growth saturation density of > $1 \times 10^6$ cells/cm$^2$. These cells show both adherent and floating properties. Adherent cells are removed in the culture medium by the action of trypsin. Cells are grown in a $CO_2$ incubator with 5% $CO_2$ at $37^0$ C (Biedler *et al*., 1978). Research suggest that upon RA differentiation floating cells more frequently adhere and differentiate into "N" type cells as compared to adherent cells present in the culture (Pahlman *et al*.,1984) Upon treatment with differentiation producing agents, SH-SY5Y cells become morphologically identical to primary neurons with long processes. They become uniquely polarized by differentiation rate as the cells are detached from the cell cycle and upregulation of Neuron-Specific Enolase (NSE), which is the principal enolase- isozyme present in neuronal and neuroendocrine tissues (Encianas *et al*., 2000).

Encinas *et al.*, 2000 reported that undifferentiated cells are either S-type (Substrate adherent) without neuron- like phenotype or N-type.

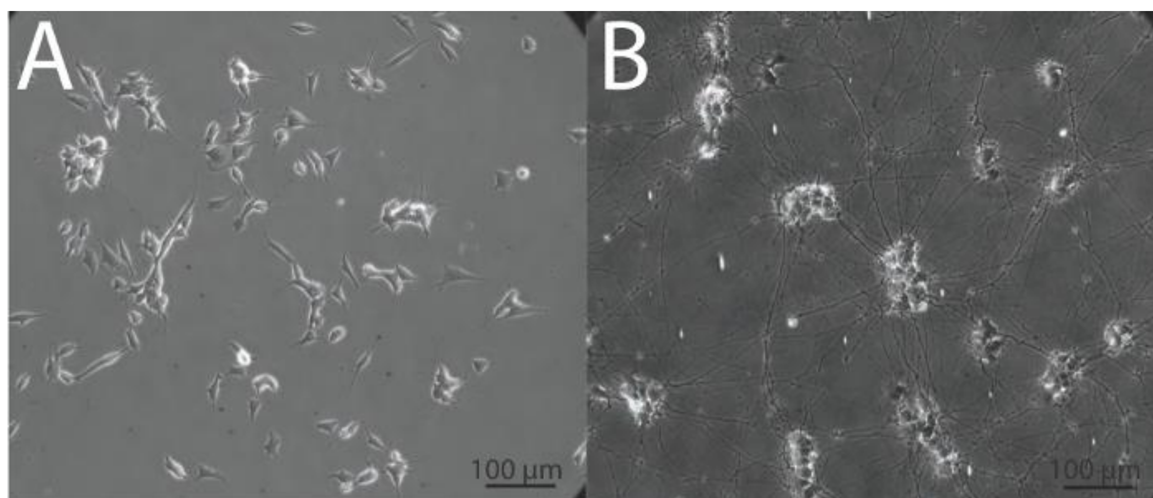



**Figure.1:**

Morphological appearance of undifferentiated and differentiated cell line (A) undifferentiated SH-SY5Y cells have a flat phenotype with a few projections while (B) Differentiated SH-SY5Y neurons have extensive and elongated neurite projections. Images were captured in phase at 20X magnification using epifluorescence microscope.

## 2.2 *Withania Somnifera*:

*Withania Somnifera* (WS) the ayurvedic medicinal plant reported to possess free radical scavenging activity, anti-stress, enhance cellular antioxidant, very effective in increasing stamina, and also in prevention of gastric ulcers (Singh *et al.*, 2011). It shows its antioxidant activity in foot-shock stress induced changes in rat brain frontal cortex and stratium by normalizing the lipid-peroxidisation, superoxide dismutase (SOD) and glutathione peroxidase activity (GPx) (Bhattacharya *et al.*, 2000). Neuroprotective role of WS owes to neurochemical alteration of specific neurotransmitter system. It decreases the glucocorticoid release in chronic stress. The alcoholic and water extract are also used as a natural product and it has shown to have anti-inflammatory properties also act as an adaptogen to improve physical conditions in stress (Singh *et al.*, 2008). It has been reported that the root extract of the medicinal plant have immunomodulatory qualities also (Pratte *et al.*, 2014). The glycol-withanolides of the WS possess antioxidant properties. The crude extract of WS contain 14 different Withanolides which has different functional groups. Namely Withanone, Withaferin A, Withanolide A, Withanolide B, Withanolide D, Withanolide

E, Withaphysalin C, Withaphysalin D, Withaphysalin F, Withaphysalin M, Withaphysalin N, Withaphysalin O, Withacnistin, Beta setosterol, Stigmasterol etc. Among the 14 different constituents Withaferin A is the most abundant.

**2.3 Kainic acid:**

Glutamate analogue kainic acid is generally used in experimental research to produce excitotoxicity and neurodegenerative processes. Kainate receptor acts upon several glutamate receptors to depolarize the neuronal membrane and calcium influx into the neuronal cell and after excessive stimulation it leads to cell death (Schwob *et al.*, 1980; Michaelis., 1998). The kainate receptor is distinct from ionotropic receptors such as NMDA (N-methyl D-aspartate). There are antagonist which act to block Kainate receptor and NMDA inducing excitatory mechanism in neurons. It is reported that by targeting NMDA recepotor kainic acid neurotoxicity can be decreased. The neurotoxic effects of kainate is accompanied by reactive gliosis. In which astro-glia and microglia proliferate in the region affected by kainate and microphages infiltrate the region. (Behan and *et al.*) Kainic acid exerts its neurotoxicity property by binding to kainate receptors which has pre-synaptic modulatory and post synaptic excitatory properties. Dysregulation of Ca2++ initiates the exocitotoxic cascade events. First it causes the fragmentation of ER membrane and ER stress which in turn lead to activation of ER proteins like binding immunoglobulin protein (Bip, also known as glucose regulated protein 78/GRP78), CCAAT/Enhancer binding protein (C/EBP) and CASPASE 12 which are involved in neuronalplasticity. (Sokka et al,. 2007). Second excessive ion influx of ca2++ through ionic channel leads to generation of free radicals also leads to generation of calcium which causes mitochondrial dysfunction that ultimately result in mitochondrial swelling and release of mitochondrial factors and various reactive oxygen and reactive nitrogen species from inner mitochondrial membrane. These factor trigger the release of caspase and protease that causes neuronal death by apoptosis. (Bruce *et al.*, 1995). Third, increase in calcium causes generation of reactive oxygen species Reactive Oxygen Species (ROS). It causes the neuronal destruction and activates the Kainate receptors. It also leads to the release of enzyme such as proteases responsible for breaking down membrane and cytoskeletal proteins, endonucleases and causes DNA fragmentation, kinases, and phospholipase damages the membrane, phosphatases and nitric oxide synthase

(NOS) all of which causes neural inflammation and ultimately death of cell. (Wang *et al.*, 2005)

## 2.3 Glutamate:

### 2.3.1 Role of Glutamate as a neurotransmitter:

Glutamate is one of the major excitatory neurotransmitter present in the central nervous system (CNS). It is stored in vesicles, present at the terminal of nerve cell. Nerve impulses trigger its release from the pre-synaptic nerve terminals and glutamates binds to and activate glutamate receptors. Glutamate generally involves in fast synaptic transmission, neuronal plasticity, out growth and survival, memory, learning and behaviour.

There are two classes of glutamate receptors-

1. Ionotropic Glutamate Receptor
2. Metabotropic Glutamate Receptor (Zahr *et al.*, 2009)

Ionotropic glutamate receptor are mediated by fast synaptic potentials by activating ion channels directly and on the other hand metabotropic glutamate receptors are mediated by slow post synaptic potentials by coupling with G-protein coupled receptors (GPCR) and secondary messenger.(Losher and Lehman, 1999)

3 subtypes of ionotropic receptors are

1. NMDA (N-methyl-D- Aspartic acid)
2. AMPA ( α-amino-3-hydroxy-5-methylisoxazole-4-propionate)
3. Kainate receptors

Both AMPA and Kainate receptors subtype gets activated in response to Kainic acid induced excitotoxicity. They are also termed as non-NMDA receptors.

### 2.3.2 NMDR receptor:

Three subunit families have been identified for NMDR receptors NR1, NR2, NR3 which have different isotopes and splice variants (Cull, Candy *et al.*, 2001). The central function of NMDAR are voltage sensitive block of cations channel by extracellular $Mg^{2+}$ a high permeability to $Ca^{2+}$ and unusually slow “activation/deactivation” kinetics (Cull ,Candy *et al.*, 2001). Excessive activation of

NMDR can cause excitotoxic neuronal death and blockade of NMDARs has been neuroprotective in animal models of both stroke and seizure. (Lee *et al.*, 1999).

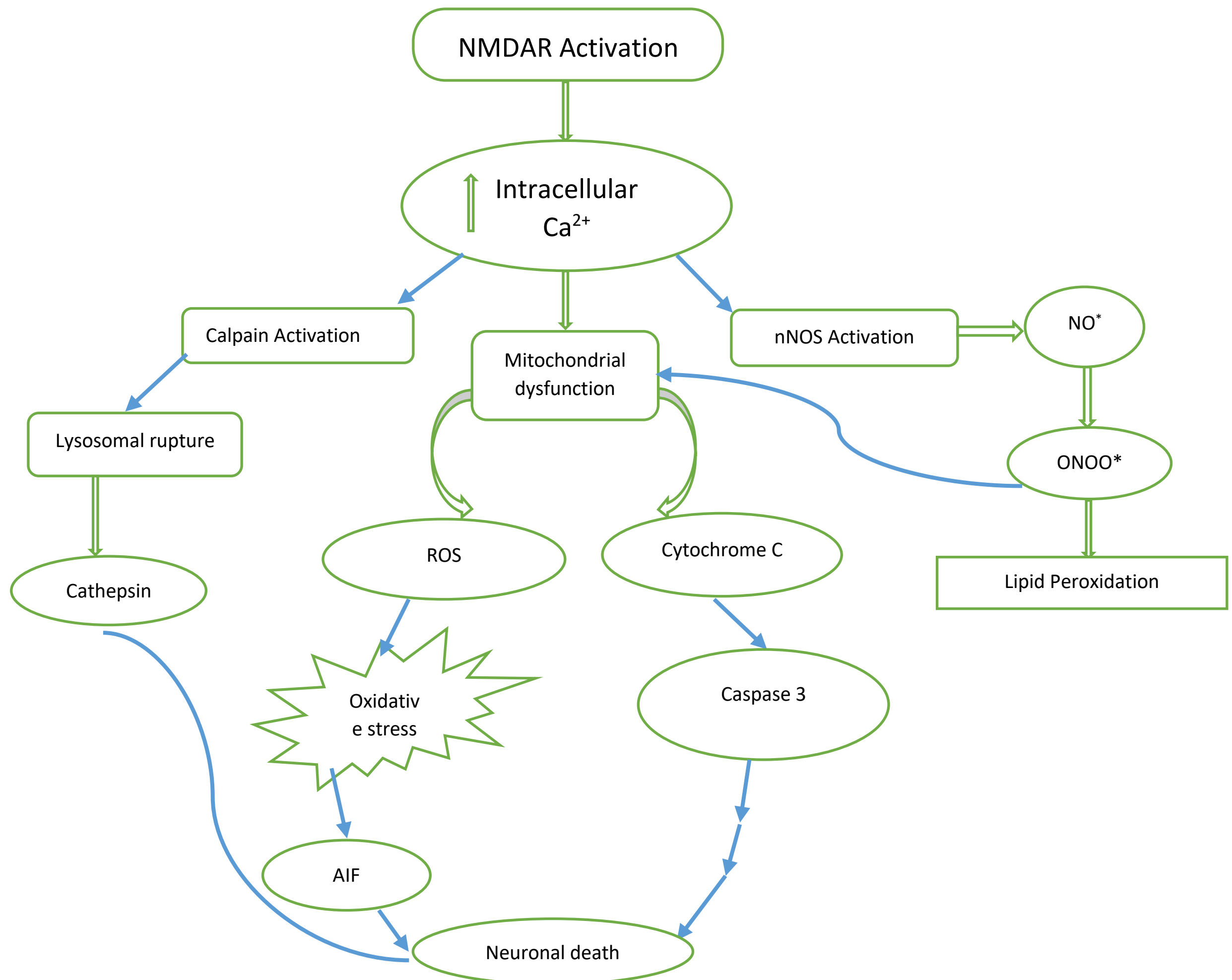



**Figure 2:**

NMDARs mediated excitotoxicity resulting neuronal cell death. Activation of NMDARs leading to intracellular $Ca^{2+}$ overloads resulting activation of calpain, neuronal nitric oxide synthase (nNOS) and mitochondrial oxidative stress. Calpain activation engaging both cathepsins and calpain-dependent cell death. Activation of nNOS leading to peroxynitrite (ONOO_) production that causes lipid peroxidation and mitochondrial dysfunction. Leakage of cytochrome C- an inner mitochondrial membrane protein into the cytosol activates caspase-3 resulting neuronal cell death. Formation of reactive oxygen species (ROS) causes oxidative stress leading to production of apoptosis inducing factors (AIF) which promote apoptotic cell death

### 2.3.3 Kainate/ AMPA receptors:

In the past AMPA and kainate receptor have been considered as only one functional unit because of difficulties in differentiating them, and they were called KA/AMPA receptors (Catarzi *et al.*, 2007). The α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid receptor (AMPA) is a non-NMDA ionotropic transmembrane receptor for glutamate which causes fast synaptic transmission in the CNS. These receptors are present in many parts of the brain and most readily found in the nervous system. Kainate receptors are ionotropic receptors that respond to the neurotransmitter glutamate. They were first classified as the specific receptor type due to their selective activation by the agonist kainite. These receptors are less understood than AMPA and NMDA receptors, and they were distributed less in brain.

## 2.4 Withaferin A (WA)

It is extracted from the root and leaves of medicinal plant *WS.* This can be applied for a wide range of physical and mental health problems. It has been reported that the steroid interact with many proteasomes and also HSP70. Given such targets it is not surprising that it can inhibit neurodegeneration by regulating the receptors such as NMDA, AMPA or Kainate receptors and also by calcium signalling. WA has been studied in murine models for different types of cancer. It is also reported to inhibit gliomas in cellular and murione models (Chang *et al.*, 2016). The potential therapeutic effects of WA were examined in cell proliferation, apoptosis, cell cycle regulation and migration/invasion in AGS (Human gastric adenocarcinoma) cells. The molecular mechanism underlying the antitumor activity of WA has been demonstrated in previous studies. WA inhibits the invasive effects in AGS cells *in vitro*. WA has been reported to inhibit the metastatic behaviour of cancer cells. It targets the matrix metalloproteinases (MMPs) and Vimentin in AGS cells. WA modify the structure of Vimentin by binding with the cysteine residues (Kim *et al.*, 2017). Some of the phytochemicals such as Withaferin A, Anaferin, Beta-Sitosterol, Withanolide A, Withanolide B and Withanolide D showed higher affinity for GluN2B-containing NMDA receptor. Previous studies have reported that steroidal frame work and the antiangiogenic properties makes WA a successful agent to modulate various oncogenes and tumour suppressor genes in *in vivo* condition. It becomes

a suitable anticancer agent because of its bioavailability and less toxicity. It is also reported that WA causes premature senescence by preventing uncontrolled proliferation in malignant cancer cells (Rasool *et al.*, 2017).

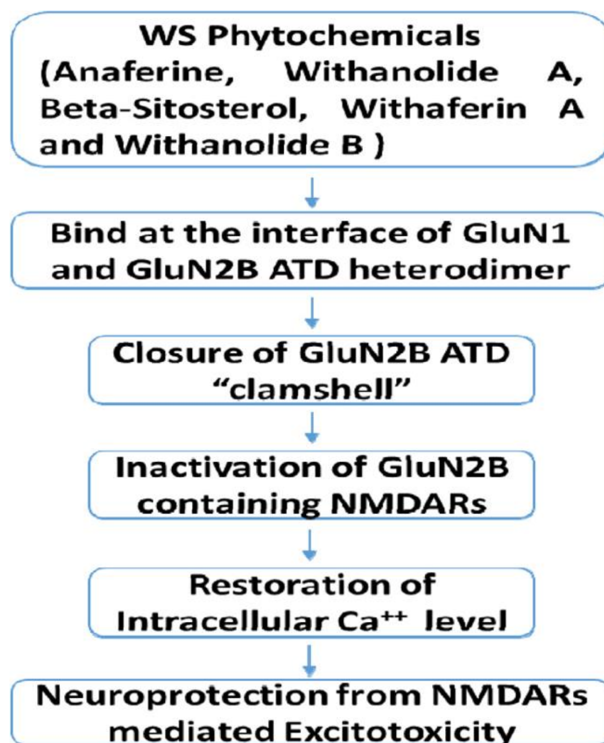



**Figure. 3:**

Proposed mechanism for allosteric inhibition of GluN2B containing NMDA receptors by phytochemicals found in *WS* (Rasool *et al.*, 2017).

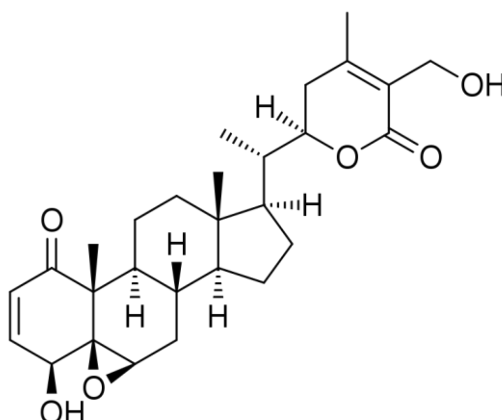



**Fig. 4:** Structure of Withaferin A

# Chapter 3

# MATERIAL AND METHODS

## 3.1 Chemicals:

Withaferin A (Mol. Wt 231.25) and Kainic acid (Mol. Wt 470.6) was purchased from Sigma - Aldrich.

## 3.2 Material required:

- Assembly
- CO2 incubator
- Laminar flow
- DMEM (Gibco)
- 10% FBS
- Antibiotics and Antimycotic solutions
- T-25 flask
- Falcon tubes (15ml)
- Sodium bicarbonate (3.7g)

## 3.3 Thawing Of Cells:

The cells to be cultured were placed in centrifuge tubes along with complete media. The tubes were covered with paraffin film and were centrifuged at 1000-2000 rpm for 5 minutes to remove the DMSO which is a toxic to cells. The supernatant was discarded, and the pellet was stored.

## 3.4 Culturing Of Cells:

The SH-SY5Y neuroblastoma, (passages 6-20) cells were routinely grown in DMEM (Dulbecco's Modifies Eagle's Medium) 20ml F12, Supplemented with 10% Fetal Bovine Serum and 1X antibiotic and antimycotic. Cells were maintained at $37^o$ C in a saturated humidified atmosphere containing 95% air and 5% $CO_2$. After reaching 70-80% confluency cells were seeded onto six well plates for Immunocytochemistry assay.

## 3.5 Coverslip treatment:

Material required- Petri-dish, Coverslip, HCl, distilled water, chloroform, Methanol.

- Coverslips were taken and put in a petri-dish.
- Concentrated HCl was added for 2min.
- HCl was discarded in water.

- The coverslips were cleaned with distilled water for 2 mint.
- Chloroform was added for 2min.
- Chloroform was discarded and washed with distilled water.
- Methanol was added for 2min and then discarded and washed with water.
- The coverslips were allowed to dry in dry oven for overnight.
- Then the coverslip were taken in falcon and then allowed for autoclaving.

### 3.5 Cell Seeding:

- Media was removed from culturing flask and the cells were washed with PBS and then trypsin was added for detaching cells from the flask surface.
- Cells were transferred into centrifuge tubes and allowed for centrifugation at 2000rpm for 2mins.
- Pellet was taken and re-suspended into 1-2ml complete media.
- On paraffin added trypsin blue and cells in 1:1 ratio (10μl:10μl)
- Counted the cells using haemocytometer & calculated average no. of cells in the chambers:

$$\text{Seeding density} = \frac{\text{No. of cells seeded/ml} \times \text{volume of medium}}{\text{Actual count} \times 10^4}$$

### 3.6 Treatment of cells:

The SH-SY5Y cells were treated with Kainic acid and Withaferin A. WA was treated for 24 hours whereas KA treatment for 3 hours as used in the previous study (Tsai and Chang et al., 2014) The cells were also treated with KA (3 hours) after the pre-treatment of WA (For 24 hour time interval).

## Cell treatment

**Figure.5:** Schematic representation of experiment design for different treatment of KA and WA in six well plate.

### 3.7 Immunocytochemistry (ICC):

Immunocytochemistry is the identification of a certain antigen in a histological tissue section or cytological preparation via an antibody specific to the antigen or protein.

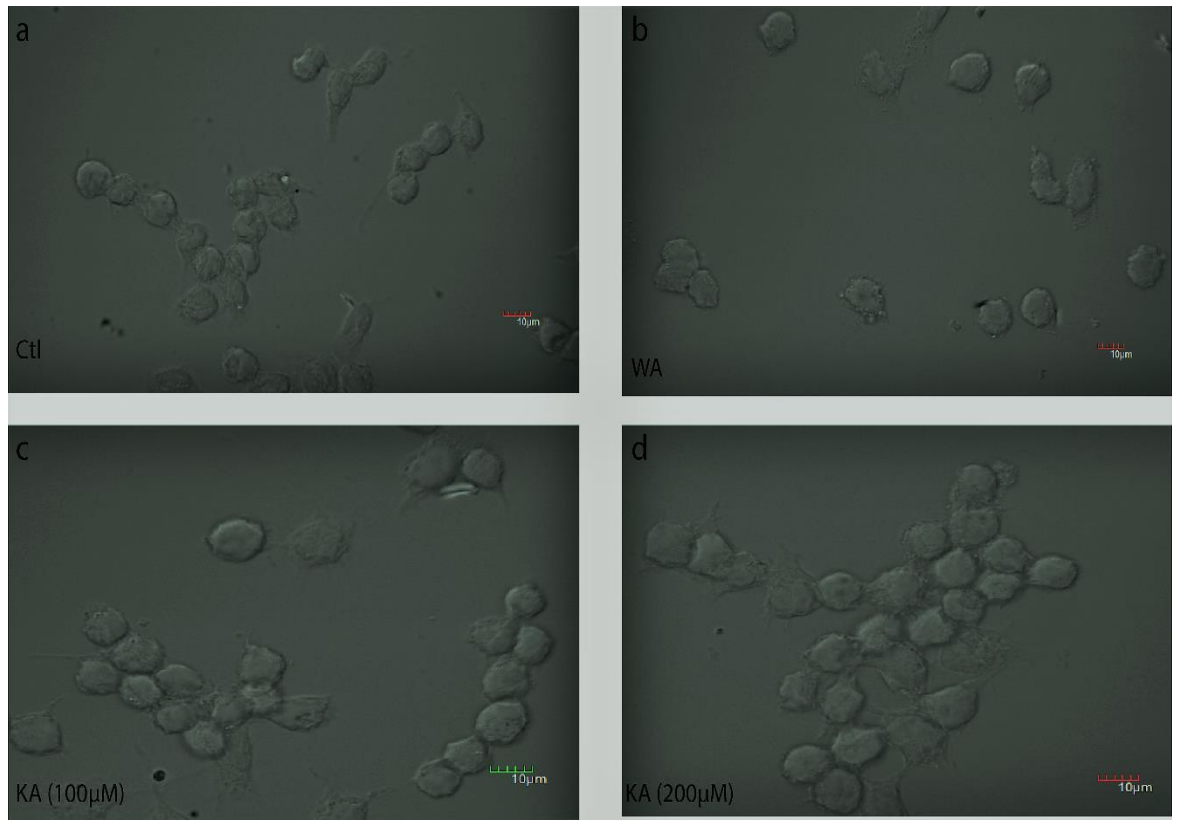
This technique was used to check protein expression of NCAM by treating cells with once Kainic acid and KA+WA (as mentioned above)

- Control and treated cells were fixed by 4% paraformaldehyde followed by permeabilization with 0.3% triton X-100 in PBS.
- Cells were incubated with anti- NCAM (1:500) Cat no. C9672 diluted in 0.1% PBST and 2% BSA solution for 24 hours in a humidified chamber.
- Secondary antibody i.e. anti mouse IgG (whole molecule) FITC conjugated, (1:200; Cat no. A9044) was added for 2 hours at room temperature.
- Cells were incubated in DAPI (1:5000 in 0.1% PBST) for 10 mins for nuclear staining.
- Images were captured at 60X magnification under confocal microscope (Olympus FV/1200).

# Chapter-4

# RESULTS

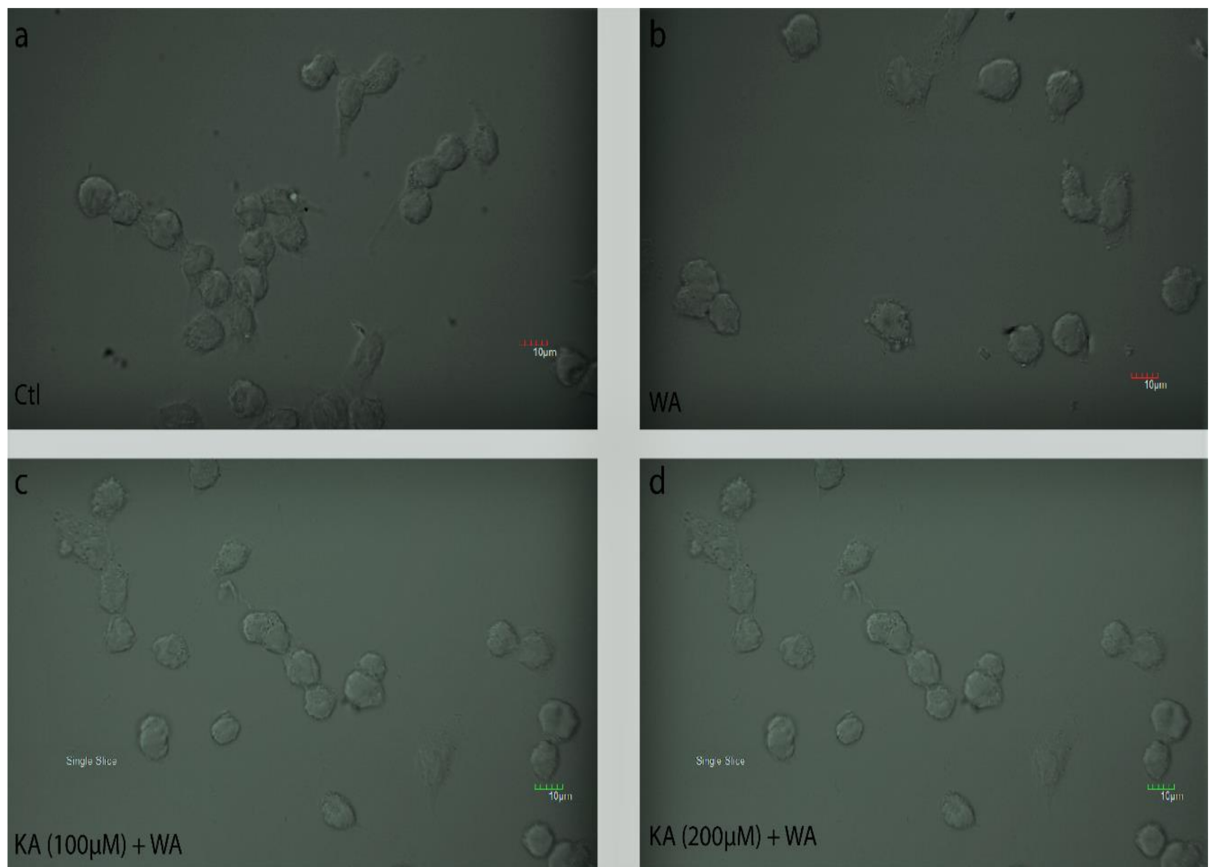
**SH-SY5Y (Neuroblastoma cells)**




**<u>Figure 6:</u>**

Phase contrast images of SH-SY5Y cells treated with different concentration of KA, were captured using Olympus FV/1200 Confocal Microscope. KA treatment causes a significant changes in the nuclear morphology of the cells as a result of nuclear fragmentation. (Fig c and d) scale bars 10µM.

**SH-SY5Y (Neuroblastoma cells)**




**<u>Figure.7</u>:**

Representative phase contrast images taken from Olympus FV/1200 confocal microscope (60X) shows the morphology of SH-SY5Y cells treated with two different concentration of KA (100 µM & 200 µM) and WA (0.5µM). Scale bar, 10µm. a) Control cells, b) WA, c) KA (100µM) + WA d) KA (200µM)

## SH-SY5Y (Neuroblastoma)

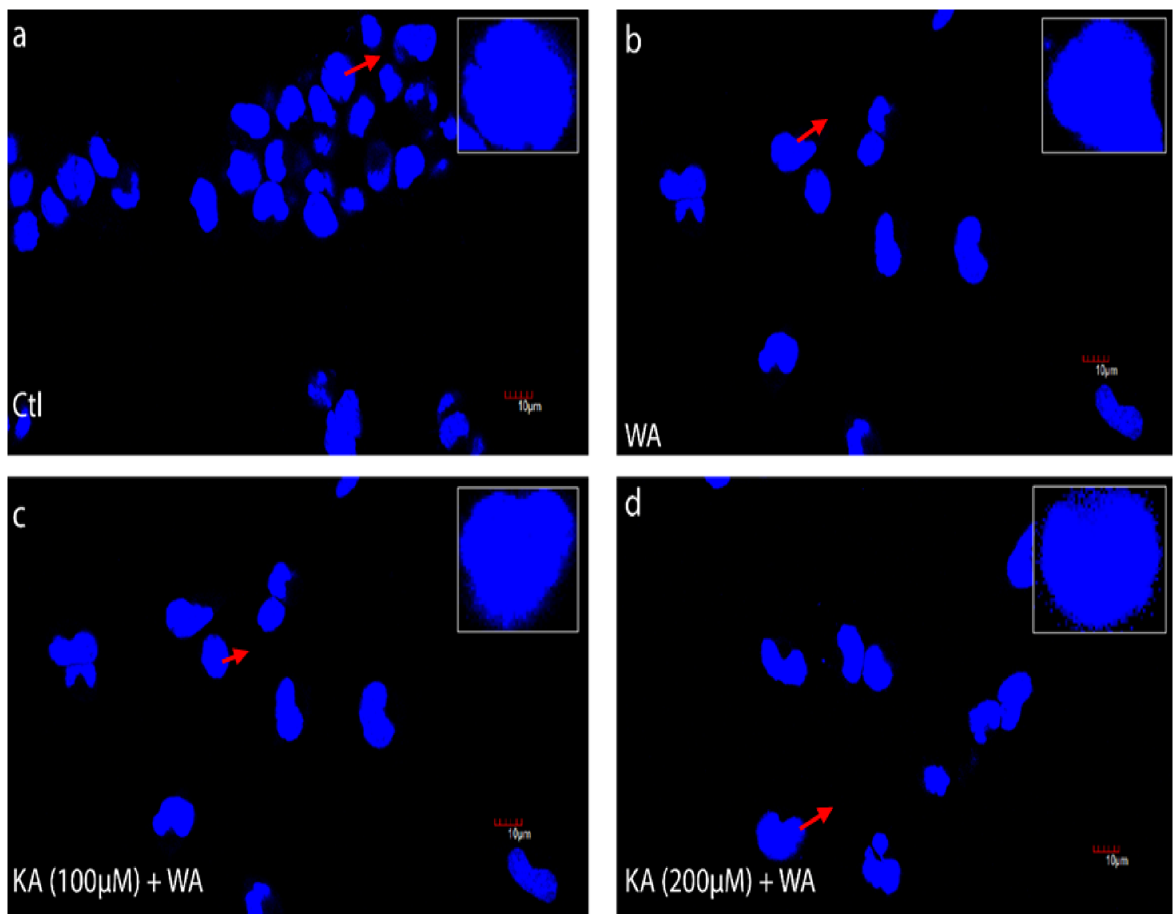



**Figure.8:**

Morphological changes induced by neurotoxic KA in SH-SY5Y neuroblastoma cell. Morphology of the nucleus are changed (Fig. (c), (d)) under toxic effect of KA in KA treated cells in comparison with control and WA treated (Fig. a, b). Nuclear degeneration is clearly distinguished as a result of nuclear fragmentation and chromatin condensation. Images were captured in Olympus FV/1200 confocal microscope (60X). Scale bar, 10µm.

SH-SY5Y neuroblastoma cell line

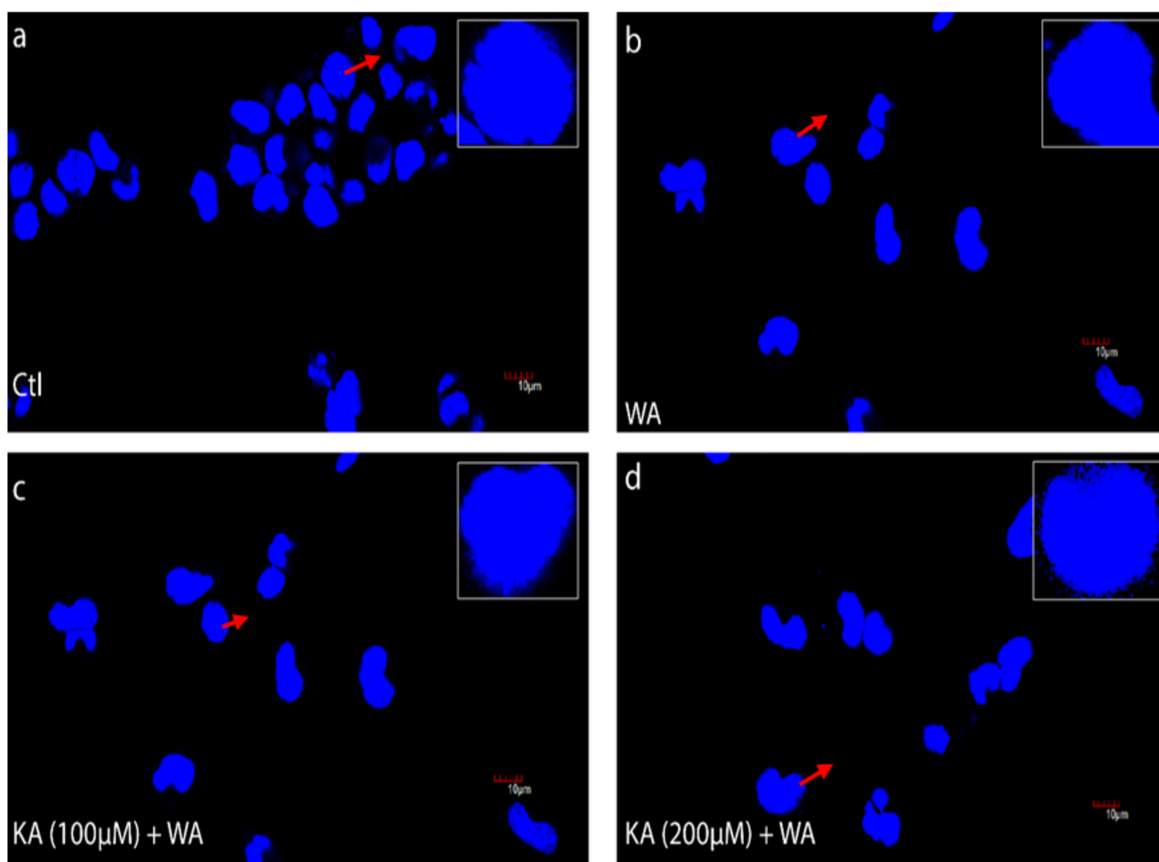



**Figure.9:**

Morphological representation of SH-SY5Y neuroblastoma cell line treated with different concentration of Kainic acid and WA. Cells were treated with Kainic acid for 3 hours and treated with WA for 24 hours. Cells were then fixed with 4% paraformaldehyde and stained with DAPI. All images were captured using Olympus FV/1200 confocal, microscope (60X). WA reverse the morphological changes induced by neurotoxic dose of KA. Scale bars, 10μM (Ctl = Control, WA = Withaferin A, KA= Kainic acid. Concentration of WA is 0.5μM)

## NCAM EXPRESSION (SH-SY5Y neuroblastoma cells)

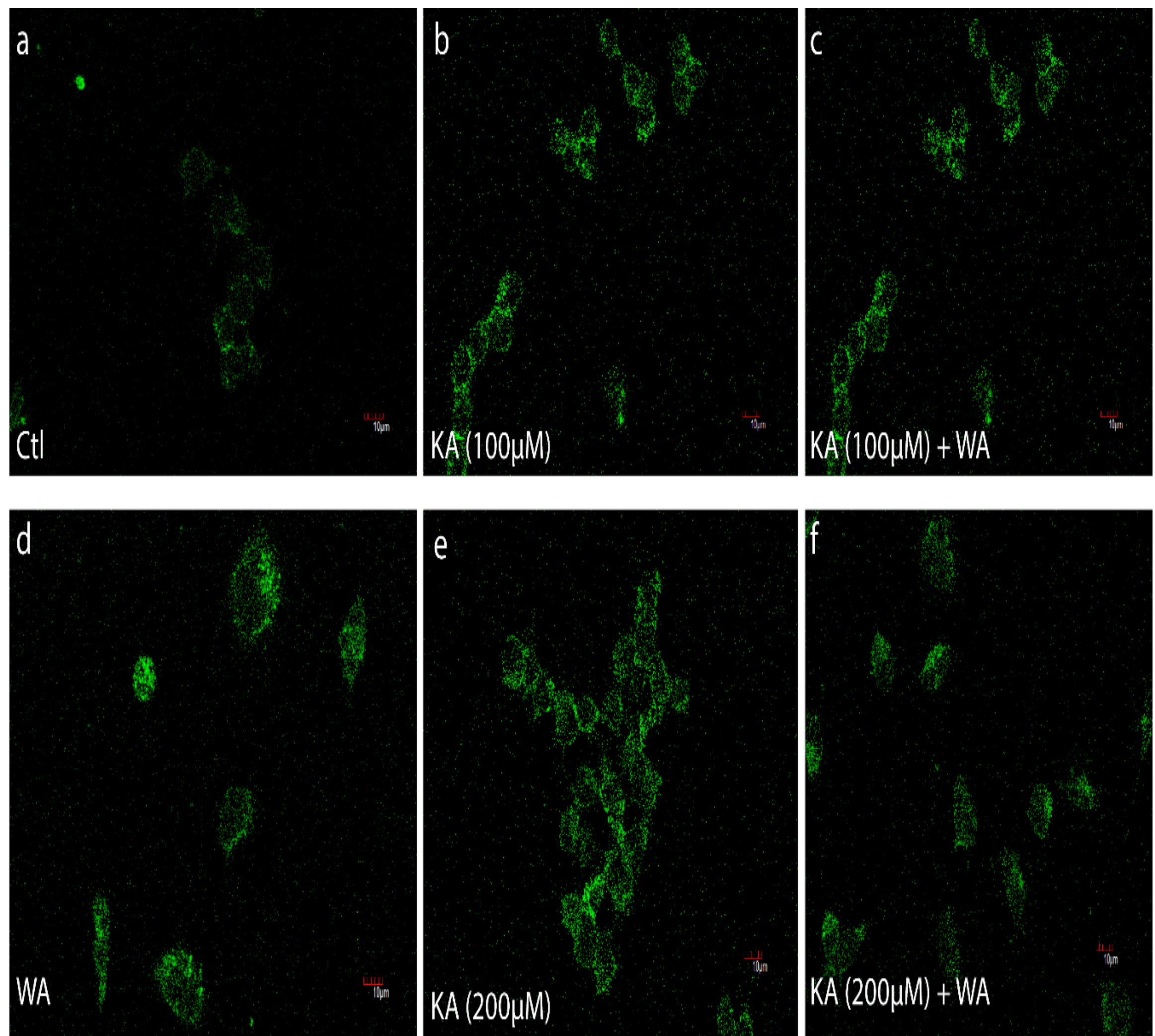



**Figure.10:**

Expression of protein NCAM in SH-SY5Y cell line. The images were captured under confocal microscope to check the expression of NCAM. a) Control cells, b) 0.5μM WA, c) KA (100μM) d) KA (200μM) e) KA (100μM) + WA f) KA (200μM) + WA Scale bar 10 μM.

## 4.1 <u>Kainic acid (KA) induced neurotoxicity</u>

To determine whether Kainic acid causes neuronal death by inducing excitotoxicity in neuroblastoma cells, SH-SY5Y cells were exposed to two different concentration of Kainic acid i.e.it 100µM and 200µM (fig.8 c, d). Cultured cells were treated with KA for 3 hours, exhibited cell shrinkage and apoptosis. Treatment of 100µM KA in SH-SY5Y cells caused severe nuclear damages (fig.8 c). DAPI (4',6- diamidino-2-phenylindole) stain was used to observe the morphological changes of cell nucleus. The degeneration of nucleus was observed as there is sign of condensation of chromatin and nuclear fragmentation. DAPI staining is high at high dose concentration of KA (200µM). Treatment of high dose KA (200µM) induced changes in nuclear shape and also leads to complete disruption of nucleus as compared to control and WA treated cells. Cells exposed to 100µM KA also shows disturbed morphology but its effect was less as compared to 200µM concentration. In 200µM concentration the nucleus exhibited complete disruption of nuclear structure.

## 4.2 <u>Withaferin A protects the cells from Kainic acid induced excitotoxicity:</u>

To study the neuroprotective property of Withaferin A SH-SY5Y neuroblastoma cells, cells were pre-treated with 0.5µM Withaferin A for 24 hours, followed by KA for 3 hours. By using different concentration of KA as mentioned above we investigated that in cells which were exposed to both WA and KA shows less distorted morphology and the morphology of the nucleus resembled to the control cells (fig.9 c, d). WA protected the cells to a large extent from nuclear degeneration induced by Kainic acid. The morphology of cells in combine treatment of WA and KA cells were almost similar to the control groups (fig.9 a, b, c, d).

## 4.3 <u>Withaferin A induced neuroprotective properties in SH-SY5Y neuroblastoma cells challenged by Kainic acid:</u>

Expression of plasticity marker such as neural cell adhesion molecule NCAM was studied in neuroblastoma cell line to investigate their adhesion and migratory characteristics with and without the treatment with WA and KA. NCAM is one of the glycoprotein of immunoglobulin (Ig) superfamily expressed on the surface neuronal cells and is an important neuroplasticity marker. It has role in cell adhesion and homo-philic interaction. The expression of NCAM protein was examined in both control and treated groups. It was found that only WA treated cells shows a minor

increase in NCAM expression whereas exposure with KA increased the expression of the NCAM protein in a dose dependent manner (fig.10 c, d, and e). Immunostaining with FITC (Flouroscien isothiocyanate) revealed that intensity of the NCAM was less in 100µm KA treated groups as compared to 200µm KA treated groups. But cells, pre-treated with WA shows normalized expression of NCAM. Hence WA helps reducing the expression of NCAM proving its protective properties. We also observed that cells were adhered to each other in KA treatment 100µM concentration, the cell clumping was more when the concentration of KA was increased to 200µM concentration (Fig.10 c, d).

# Chapter-5

# DISCUSSION

Previous studies suggests that *Withania Somnifera (*WS) and its phytochemical have neuroprotective and cyto-protective role in many diseases such as neurodegenerative and cancer. Withaferin A has role in inhibition of metastasis and anti-proliferative properties in disease such as gastric ulcer, leukaemia, glioma (Chang *et al.*, 2016). Role of Withaferin A in neuroprotection against the excitotoxicity process induced by Kainic acid was unexplored. Hence we have designed a novel assay to dissect neuroprotective role of WA in excitotoxicity. The present study demonstrates that WA abolished the KA induced morphological changes and prevent the neurotoxicity as an evident of confocal imaging. Although *WS* has been reported to improve learning and memory as a potent neuro-protectant, but the phytochemical WA extracted from both roots and leaves was not evaluated for its neuroprotective effects against KA induced neurotoxicity in SH-SY5Y cell line. Moreover KA induced neurotoxicity was attenuated by WA in a dose dependent manner. It prevents the nuclear degeneration and ultimately prevents apoptosis of cells at a concentration of 100µM and 200µM. However it is reported that WA concentration of more than 3mM induces apoptosis of cells.

NCAM is a glycoprotein of immunoglobulin (Ig) superfamily which is expressed on the surface of neurons. They have a role in cell- cell adhesion, neurite outgrowth, synaptic plasticity, neuroprotection and learning, and memory. NCAM is associated in cell-cell interaction throughout the nervous system (Edelman and Crossin, 1991). WA causes the increase in NCAM expression which causes the reduction in cellular motility (Prag *et al.*, 2002). Recent research suggests that there is a high upregulation of expression of NCAM with 0.5% WA treatment. On the contrary, low NCAM expression is correlated to aggressive cancer and vice-versa (Huerta *et al.*, 2001). Earlier reports have shown that excitotoxic increase in NCAM has been shown in hippocampal slices (Hoffman *et al.*, 2001). The increase in cell viability could be partly due to enhanced NCAM expression which is an important neuroprotection (Wu *et al.*, 2010; Wu *et al.*, 2001). The growth factor, FGF-2 correlated with NCAM signalling has been illustrated to confer neuroprotection against excitotoxicity caused by glutamate (Mattson *et al.*, 1989). The expression of this neural marker was supposed to be observed on Kainic acid-challenged cells with and without the presence of WA. It is expected that low dose treatment of Kainic acid (0.05mM) may lead to upregulation of NCAM expression. As the dose

of Kainic acid increases, there was a further increase in the expression of NCAM. WA pre- treatment may lead to normalization of NCAM expression in the low dose of Kainic acid however its expression remained somewhat higher in the high dose (10mM) treatment group.

Recently it was reported by Gorgan and others (2013) WA halt proliferation of glioblastoma (cytotoxicity effect) via altering Akt/mTOR signalling pathway. NCAM expression was found to be significantly enhanced in response to KA induced cytotoxicity in SH-SY5Y cells, which may represent compensatory mechanism to combat neurotoxicity. The expression was increased in a dose dependent manner with the treatment of KA. Treatment with WA leads to decrease expression of NCAM. Several studies have shown that NCAM is a potent target to prevent excitotoxicity neuronal death during prenatal development and as well as under pathological conditions. Its polysialated form PSA NCAM" reported to inhibit the activation of GluN2B containing receptor, possibly by steric hindrances of the ligand. PSA has been shown as a neuroprotective agent by disconnecting the synapses. The increase in expression of NCAM is upon kainic acid exposure could be protective and regenerative towards neuronal damage. Which is further decreased by treatment of WA when the cells are already protected by the pre-treatment.

*Withania Somnifera (*WS) composed of six different types of water-soluble molecules (Wadhwa *et al.*, 2002) which might be alone or in combination are related with a neuroprotective activity of the extract. Bioactive components of WS such as Sitoindosides VII-X and Withaferin A have been shown to balance brain functions by binding with cholinergic receptors. By controlling the release of three neurotransmitters, i.e., acetylcholine, glutamate, and serotonin by WS in all cases result in inhibition of nNOS in extract treated on stressed mice (Rajasankar *et al.*, 2009). The neuroprotective properties of WS have been contributed to neurochemical alterations of specific neurotransmitter systems and inhibition of glucocorticoid release in chronic stress which could be taken advantage for treatment of neurodegenerative diseases (Bhatnagar *et al.*, 2009). The protective effects seen in this study could be associated with the presence of free radical scavenging compounds present in WS. Upon treatment with low levels of Kainic acid-induced effects were reverted by WS. However, with high levels of dose, there

could be only partial revert of toxic effects. The higher expression of NCAM and PSA-NCAM in response to Kainic acid exposure could be probably due to the cytoprotective effect of cells towards excitotoxicity. WS treatment is expected to cause a remarkable increase in viability of cells in Kainic acid treated groups showing its cytoprotective role against cytotoxicity.

The extracts of *Withania Somnifera* (WS) are widely studied for their neuroprotective properties in animal models and also *in vitro* studies. Consistent with this neuroprotective effects of Withania extract current study illustrates the neuromodulatory role of phytochemical WA against KA induced neuronal abnormalities and upregulation of plasticity marker protein NCAM which may rescue the neuronal cells from degeneration. Previous studies have demonstrated that the immature brain resists neuronal damage from kainic acid whereas mature brain are more prone to neuronal degeneration (Rizzi *et al.*, 2003 and Ravizza *et al.*, 2005). Upregulation of NCAM expression may be attributed to neurodegenerative conditions. It is the best known hallmarks of neuroplasticity. NCAM is developmentally downregulated but shown to increase after brain injury. WA normalizes the NCAM expression by enhanced cell viability.

**Future Perspectives:**

Most of the neurodegenerative disease like Parkinson, Alzheimer's are due to loss of neurons. There is obscure knowledge regarding the causative reagents. PD is an age related disorder. There is no therapy developed yet which can stop the progression of the disease effectively without side effects. Drugs are generally effective in early stage of the disease but possess serious side effects in long term use (Muzamil Ahmad *et al*). In this current study we hypothesized the effects of WA on KA- induced neurotoxicity on SH-SY5Y cells.

It induces stress and upregulate the plasticity marker NCAM which may defend the neural cells from effects of excitotoxicity. NCAM is a crucial cell surface plasticity marker that have a significant function in regeneration and repair. From the above study it is clear that WA reduces the excitotoxicity caused by KA, but the molecular mechanism behind this is still unclear. Exploring this mechanism is of great importance in the treatment of neurodegenerative diseases.

The validation study of phytochemical WA in neuroprotective role could contribute to the development of drugs countering neurodegenerative disorders.

From the previous study, it is clear that WS helps in reducing excitotoxicity, but the mechanism under this pathway remain unclear. Exploring this mechanism is of great importance in the treatment of neurodegenerative diseases. Natural products and plant extracts offer various potential beneficial effects on the CNS, particularly their neuroprotective effect against excitotoxicity. They provide the promising avenue for further research to guard against development and progression of acute and chronic neurodegeneration.

Further efforts required to target simultaneous pathways that underlie the various mechanisms involved to expand the therapeutic yields for various neurodegeneration diseases. As elevated levels of glutamate have been encountered in a wide range of neurological diseases thus further research into the molecular mechanism of *Withania Somnifera (*WS) mediated neuroprotection and the exploration for bioactive component(s) in these extracts may serve as a potent therapeutic agent to deal with neurological disorders.

The possible co-relation between NCAM expression and WA may provide protection against KA excitotoxicity mediated regeneration and also protective effects towards normalization and repair that need to be explored further. The neuroprotective roles of WA have been attributed to the neurochemical alteration of specific neurotransmitter system and suppression of excitotoxicity in neurodegeneration which could be exploited further.

Further research into the molecular mechanism of phytochemicals of WS mediated neuroprotection and the search of bioactive components may prove valuable therapeutic agent to combat neurological disorders. More clinical studies should be conducted to support its therapeutic use. The results discussed in this current study is very promising and suggested that this herb should be studied more extensively to assure these results and reveal other potential therapeutic effects.

The recognition of these neuroplasticity marker, and the integrative use of cellular, molecular and genomic approaches, provides us with the unique opportunity to unravel the basic cell-cell regulatory mechanisms of neuroprotection by WS. Detailed mechanistic study is required to understand the mechanism underlying the

beneficial effects of WA and to explore the optimum dosages and duration of treatment to implement the same in clinical perspectives.

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