Ph.D. Thesis

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WITHANIA SOMNIFERA AND WITHANOLIDE A MEDIATED RESTORATION OF AMPA AND NMDA RECEPTOR FUNCTION IN PILOCARPINE INDUCED TEMPORAL LOBE EPILEPSY

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BY

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CERTIFICATE

This is to certify that the thesis entitled **"*WITHANIA SOMNIFERA* AND WITHANOLIDE A MEDIATED RESTORATION OF AMPA AND NMDA RECEPTOR FUNCTION IN PILOCARPINE INDUCED TEMPORAL LOBE EPILEPSY"** is a bonafide record of the research work carried out by **Mr. Smijin K Soman,** under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology and that no part thereof has been presented for the award of any other degree.

Cochin – 682 022
July 2, 2012 **(C. S. Paulose)**

DECLARATION

I hereby declare that the thesis entitled **"*WITHANIA SOMNIFERA* AND WITHANOLIDE A MEDIATED RESTORATION OF AMPA AND NMDA RECEPTOR FUNCTION IN PILOCARPINE INDUCED TEMPORAL LOBE EPILEPSY"** is based on the original research carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, under the guidance of Prof. C. S. Paulose, Director, Centre for Neuroscience, Department of Biotechnology and no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

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Smijin Soman

Dedicated to my beloved family. . .

ABBREVIATIONS

5-HT	Serotonin
AEDs	Anti epileptic drugs
AHS	Ammon's horn sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ATP	Adenosine triphosphate
Bmax	Maximal binding
BS	Brain stem
BZD	Benzodiazepine
CA	Cornu Ammonis
CAT	Catalase
Ca^{2+}	Calcium
CB	Cerebellum
CC	Cerebral cortex
cAMP	Cylic adenosine monophosphate
CaBPs	Calcium-binding proteins
CBZ	Carbamazepine
CF	Cerebrospinal fluid
CNS	Central Nervous System
CPS	Complex partial seizurres
DA	Dopamine
DBH	Dopamine β hydroxylase
DNA	Deoxyribonucleic acid
DG	Dentate gyrus
E/I	Excitation/inhibition
EAAC-1	Excitatory amino acid carrier 1
EC	Entorhinal Cortex
ECD	Electrochemical detector
EDTA	Ethylene diamine tetra acetic acid
EEG	Electroencephalogram

EPI	Epinephrine
ER	Endoplasmic reticulum
GABA	Gamma amino butyric acid
GABAAR	GABAA receptor
GABABR	GABAB receptor
GABA-T	GABA Transporter
GAD	Glutamic acid decarboxylase
GAD-IR	GAD- immunoreactive
GDPs	Giant Depolarizing Potentials
GEFS+	Generalized Epilepsy with Febrile Seizures Plus
GEPRs	Genetically epilepsy prone rats
GFAP	Glial fibrillary acidic protein
GIRKs	G protein-coupled inwardly rectifying K+ channels
GLAST	Glutamate/aspartate transporter
GLT-1	Glutamate transporter-1
GluR2	Glutamate Receptor-2
GLUT4	Glucose transporter type 4
GPCR	G protein-coupled receptor
HD	Heptahelical domain
HPLC	High performance liquid chromatography
Hsp70	Heat-shock protein 70
ILAE	International League against Epilepsy
i.p.	Intraperitoneally
IPI	Initial Precipitating Injury
IPSCs	Inhibitory post-synaptic currents
KA	Kainate
KCCs	K^+Cl^- co-transporters
Kd	Dissociation constant
Km	Michaelis constant
LDH	Lactate dehydrogenase
LFPs	Local field potential

LGICs	Ligand-gated ion channels
LTD	Long term depression
LTP	Long term potentiation
LTLE	Lateral temporal lobe epilepsy
MDH	Malate dehydrogenase
MF	Mossy fiber
mGlu	Metabotropic glutamate receptors
MPA	3-mercaptoproprionic acid
MR	Magnetic resonance
MRC	Mitochondrial respiratory chain
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRS	Magnetic resonance spectroscopy
MSN	Medium Spiny Neurons
MTLE	Mesial temporal lobe epilepsy
NE	Norepinephrine
NKCC1	Na-K-Cl cotransporter-1
NMDA	N-methyl-D-aspartate
P	Level of significance
P450	Cytochrome P450
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Triton X- 100
PC	Purkinje cell
PF	Parallel fiber
PFC	Prefrontal cortex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
ROM	Reactive oxygen metabolites
SE	*Status Epilepticus*
S.E.M	Standard error of mean
SOD	Superoxide dismutase

SRMS	Spontaneous recurrent motor seizures
SSRIs	Serotonin reuptake inhibitors
TBARS	Thiobarbituric acid reactive substances
T3	Triiodothyronine
T4	Thyroxine
TLE	Temporal lobe epilepsy
TM3	Transmembrane-3
VFT	Venus Flytrap domain
vGAT	Vesicular Transporter for GABA
VTA	Ventral tegmental area
WS	*Withania somnifera*
WA	Withanolide-A

CONTENTS

Cerebral Cortex

Introduction

Epilepsy has afflicted human beings since the dawn of our species and has been recognized since the earliest medical writings. Epilepsy is a disorder that results from the surges in electrical signals inside the brain, causing recurring seizures. An epileptic seizure, occasionally referred to as fits, is defined as a transient symptom of "abnormal excessive or synchronous neuronal activity in the brain". Seizures can vary from the briefest lapses of attention or muscle jerks to severe and prolonged convulsions (i.e. violent and involuntary contractions, or a series of contractions of the muscles) (Panayiotopoulos *et al*., 2012).

In the medieval period, most individuals with epilepsy were thought to be possessed and the word “seizure” is derived from that notion, implying that gods take hold or “seizes” a person at the time a convulsion occurs (Scharfman *et al.*, 2007). Basic concepts surrounding epilepsy in ancient Indian medicine were refined and developed during the Vedic period of 4500–1500 BC. In the Ayurvedic literature of Charaka Samhita (which dates to 400 BC—the oldest existing description of the complete Ayurvedic medical system), epilepsy is described as ‘apasmara’ which means ‘loss of consciousness’. Babylonian tablets emphasize the supernatural nature of epilepsy, with each seizure type associated with the name of a spirit or god. The idea that epilepsy is a supernatural, demonic or spiritual disorder, persisted throughout the medieval ages. Although Hippocrates considered epilepsy to have a natural cause, only in the 19th and 20th centuries rational and scientific notions replaced primitive concepts of the medical Dark Age (Pierce, 2002). Under the leadership of three English neurologists--John Hughlings Jackson, Russell Reynolds and Sir William Richard Gowers, the modern medical era of epilepsy begins.

In this modern era, epilepsy is the most frequent neurodegenerative disease after stroke. It afflicts more than 50 million people worldwide (Strine *et al*., 2005). The estimated number of people with epilepsy in India is 5. 5 million and half a million new cases of epilepsy arises every year. As India has a large

rural population, majority of the patients would be deprived of specialized treatment (Bharucha *et al*., 2003).

The causes of epilepsy vary with geographical location and with age. The aetiology may be multifactorial and is unknown in about two-thirds of patients (Panayiotopoulos, 2005). Aetiology may be divided into epilepsies due to genetic, acquired causes and those due to a combination of both, which contribute to the predisposition of recurrent seizures. Brain trauma, infection, alcohol withdrawal and hypoxia are considered to be major causes. Single gene mutations especially causing chanelopathies, brain malformations and progressive myoclonic epilepsy is also of greater concern (Duncan, 2004).

Cellular alterations and their temporal distribution are best characterized in the hippocampus, particularly in *Status epilepticus* SE models. These include neurodegeneration, neurogenesis, gliosis, invasion of inflammatory cells, axonal sprouting, axonal injury, dendritic plasticity, angiogenesis, changes in extracellular matrix, and alteration in voltage and ligand-gated ion channels in individual neurons. These alterations are accompanied by a variety of molecular changes. As a consequence, several functional impairments in addition to epilepsy can develop, including developmental delay, memory impairment, emotional impairment, behavioral impairment, somatomotor decline, and drug refractoriness (Pitkänen *et al*., 2009). During the entire epileptogenic process, these alterations are subject to modulation by genetic background, developmentally regulated genetic programs, or epigenetic factors.

Temporal lobe epilepsy (TLE) is among the most frequent types of drug resistant epilepsy. In a population of new patients presented with epilepsy, almost 30% of them had seizures originating from the temporal lobe of the brain (O'Brien *et al*., 2012). Individuals affected with TLE typically have comparable clinical description; including an initial precipitating injury such as the SE, head trauma, encephalitis or childhood febrile seizures (Fisher *et al*., 1998; Cendes, 2004). In many individuals with TLE, an initial insult or injury leads to a period of time without evidence of overt seizures and then recurrent seizures begin. Examples include birth trauma, a febrile seizure, or infection such as encephalitis

(Scharfman, 2007). However, the insult may also occur later and a common example is a war injury as an adult that leads to TLE decades later. The fact that these events are injurious, leading to neuronal damage and the fact that many patients with TLE have dramatic neuronal loss upon autopsy, have led many to conclude that damage to neurons is critical to the pathophysiology of TLE (Meldrum, 1999).

Various types of brain insults such as SE, traumatic brain injury, and stroke can trigger the epileptogenic process. Epileptogenesis refers to a process in which an initial brain-damaging insult triggers a cascade of molecular and cellular changes that eventually lead to the occurrence of spontaneous seizures (Löscher *et al*., 2010). An imbalance in the excitatory and inhibitory neurotransmission is the hallmark of Epileptogenesis, leading to the initiation of abnormal neural impulses. Excitatory synapses mature earlier than inhibitory synapses and this, coupled with an increase in the susceptibility of excitatory neurotransmitter receptors, increases the likelihood that an excitation–inhibition imbalance may occur. There are various theories explaining the hyperexcitability of neurons in hippocampus and subsequent formation of Epileptogenesis (Primer & Stafstrom, 2010). The leading hypothesis was that the death of GABAergic inhibitory interneurons resulted in attenuation of inhibition, which in turn led to pathologic hyperexcitability of the remaining principal neuronal populations—pyramidal and dentate granule neurons of the hippocampus (Zhang *et al*., 2006; Sloviter RS *et al*., 1983). Apparently, GABA immunoreactive neurons were more resistant to seizure-induced neuronal death than other hippocampal neurons. This led to the postulation of "dormant basket cell" hypothesis, which suggests that the seizure-induced death of excitatory neurons in the hilus (probably mossy cells) removes a tonic excitatory projection to GABAergic basket cells, the inhibitory neurons in the dentate gyrus, resulting in a disinhibition because basket cells lie dormant when they are not activated by mossy cells (Sloviter *et al*., 2003). An alternative to the "dormant basket cell" hypothesis is the possibility that hyperexcitability of dentate granule cells is a consequence of a pathologic neuronal rearrangement in which excitatory granule cells innervate themselves, resulting in a recurrent excitatory circuit and

another vicious cycle (Whitlock *et al*., 2006).This observation supports the argument that the observed hyperexcitability is not simply a reduction of inhibition but an increase in excitation and thus evidence for the emergence of functional recurrent excitatory synapses (Bromfield *et al*., 2006; Shin and McNamara, 1994)

Glutamate is the principal excitatory neurotransmitter in the brain and as such it plays an inevitable role in the initiation and spread of seizure activity. There is a surge in levels of extracellular glutamate before or during the onset of seizures, which means impaired uptake or enhanced release of this substance leads to seizure initiation. Glutamate exerts its excitatory action via ligand-gated ion channels (NMDA and AMPA receptors) to increase sodium and calcium conductance, and a myriad of reciprocal regulatory interactions that exist between the activation of glutamatergic receptors and other transmitter systems, ion transport, gene activation and receptor modification (Ghasemi and Schachter, 2011). Intracellular recordings in an epileptic focus during "spike discharges" or in a normal cortical neuron during generalized seizure activity reveal a so-called "paroxysmal depolarizing shift" associated with a burst of membrane spikes. This depolarization is analogous to a giant excitatory synaptic potential; its earliest component is due to activation of AMPA receptors and its later component to activation of NMDA receptors (Rogawski, 2011). In knockout or knockdown rodent models, altering glutamate receptor or glutamate transporter expression can induce or suppress epileptic seizures. The flexibility and complexity of these interactions place glutamate-mediated transmission in a pivotal position for modulating the excitatory threshold of pathways involved in seizure generation.

The surge in glutamate content due to alteration of neuronal transmission and neuronal circuit's leads to glutamate mediated excitotoxicity in CNS. Increased extracellular glutamate levels leads to the activation of Ca^{2+} permeable AMPA receptors on myelin sheaths and oligodendrocytes, leaving oligodendrocytes susceptible to Ca^{2+} influxes and subsequent excitotoxicity. One of the damaging results of excess calcium in the cytosol is the initiation of apoptosis through cleaved caspase processing (Paz *et al*., 2011). Another

damaging result of excess calcium in the cytosol is the opening of the mitochondrial permeability transition pore, a pore in the membranes of mitochondria that opens when the organelles absorb too much calcium. Opening of the pore may cause mitochondria to swell and release reactive oxygen species and other proteins that can lead to apoptosis (Pastalkova *et al*., 2006). The pore can also cause mitochondria to release more calcium. In addition, production of adenosine triphosphate (ATP) may be stopped, and ATP synthase may in fact begin hydrolysing ATP instead of producing it. Inadequate ATP production resulting from brain trauma can eliminate electrochemical gradients of certain ions. Glutamate transporters require the maintenance of these ion gradients to remove glutamate from the extracellular space. The loss of ion gradients results not only in the halting of glutamate uptake, but also in the reversal of the transporters. The Na^+-glutamate transporters on neurons and astrocytes can reverse the glutamate transport and start secreting glutamate at a concentration capable of inducing excitotoxicity (Allan, 2004).

Treatment strategies to counter TLE associated neuronal injury is gaining wide acceptance. The present treatment against epileptic seizure is symptomatic in nature. The modern treatment of epilepsy began with potassium bromide. Phenobarbital (PHB), which was used to induce sleep, was found to have anti-epileptic activity and became the drug of choice for many years. In 1968, carbamazepine (CBZ) was approved, initially for the treatment of trigeminal neuralgia; later, in 1974, it was approved for partial seizures (Brodie *et al*., 2010). The effect of anti-epileptic drugs in amplifying epileptic manifestations is well defined. Despite the increasing number and variety of anti-epileptic drugs, more than 30% of epilepsy cases are medically intractable, with TLE having one of the worst prognoses among epileptic disorders (Bancila *et al*., 2004). Moreover, anti-epileptic drugs merely provide symptomatic treatment without having much influence on the course of the disease. Hence, there is a need for the development of new anti- epileptic drugs with fewer adverse effects and higher efficacy. There is growing evidence on the prevalence of oxidative stress in the pathophysiology of TLE (Chuang *et al*., 2009). To get more insight into molecular mechanisms

underlying oxidative stress and its role in TLE, a systematic investigation of various components of antioxidant system together with markers of oxidative protein and lipid is necessary.

Oxidative stress (OS) is emerging as a key factor that not only results from seizures, but may also contribute to Epileptogenesis. It plays a major role in the initiation and progression of epilepsy and therapies aimed at reducing oxidative stress, ameliorate tissue damage and favourably alter the clinical course of the disease. Therefore, antioxidant therapies aimed by reducing OS have received considerable attention in the treatment of epilepsy. Rasayana chikitsa is a specialized section of Ayurveda, which mainly deals with the preservation and promotion of health by bringing equilibrium into the prooxidant/antioxidant homeostasis (Govindarajan *et al*., 2005). *Withania somnifera* (WS) Dunal popularly known as Ashwagandha is widely considered as the Indian ginseng is classified as a rasayana or rejenuvator. WS has antioxidant, anti-stress, anti-inflammatory and adaptogenic properties which could be held responsible for its role in ameliorating neurodegenerative disorders like Epilepsy, Alzheimer's disease and Parkinson's disease (Namikawa *et al*., 2000). Screening active compounds from plants crude extracts leads to discovery of new medicinal drugs, which have efficient protection and treatment role against various diseases including epilepsy. Withanolide-A is a C_{28}-steroidal lactone isolated from WS, which can induce nerve development and improve nervous system function. Withanolide A (WA) is considered to be one of the major active component present in WS responsible for its pharmacological properties.

Pilocarpine induced temporal lobe epilepsy model was used to study the complex molecular, biochemical, physiological and structural changes in the brain that contribute to Epileptogenesis. Understanding the pathophysiogenesis of TLE largely rests on the use of models of *status epilepticus*, as in the case of the pilocarpine model. The main features of TLE are: (i) epileptic foci in the limbic system, (ii) an "initial precipitating injury", (iii) the so-called "latent period" and (iv) the presence of hippocampal sclerosis leading to reorganization of neuronal networks (Fawley *et al*., 2012). Many of these characteristics can be reproduced in

rodents by systemic injection of pilocarpine; in this animal model, SE is followed by a latent period and later by the appearance of spontaneous recurrent seizures (SRSs) (Curia *et al*., 2008).

In the present study the effect of WS and WA treatment on pilocarpine induced temporal lobe epileptic rats is studied. Epileptic seizure severity was analysed after viewing video recordings and evaluated using Racine scale. Epileptic seizures results in alteration in neuronal circuits and connections leading to cognitive deficit. Behavioural alterations were studied using radial arm maze, y maze, Rotarod test, Grid walk test and Narrow beam test. Neuronal degeneration especially in hippocampal region is frequently observed in temporal lobe epileptic patients. In order to evaluate neuronal damage and the effect of WS and WA on neuronal viability, Nissl staining and Topro staining were performed on hippocampal sections. Oxidative stress resulting from excessive free-radical release is implicated in the initiation and progression of epilepsy. Therefore, antioxidant therapies aimed at reducing oxidative stress have received considerable attention in epilepsy treatment. In this study liipid peroxidation was studied using TBARS assay. Superoxide dismutase (SOD) and Catalase (CAT) assay were performed to evaluate the activity of antioxidant enzymes. SOD and Glutathione peroxidase (GPx) gene expression was done to understand gene expression levels. Alteration in glutamate transport and metabolism is often held responsible for increasing epileptic severity. Glutamate content, Glutamate dehydrogenase (GDH) activity, Glutamate decarboxylase (GAD) expression and Glutamate Aspartate transporter (GLAST) expression was studied to analyse alteration in glutamate metabolism and transport. N-Methyl-D-aspartic acid (NMDA) receptor and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor are responsible for synaptic plasticity, actively involving in learning processes like long term potentiation and long term depression. NMDA and AMPA receptor binding parameters were studied using radioreceptor binding assays. NMDA R1, NMDA 2B and AMPA (GluR2) receptor gene expression was studied using Real-time PCR. Immunohistochemistry studies using confocal microscope were carried out to confirm receptor density and gene expression

results. The activation of cell survival pathways is essential for countering stress in epileptic condition. Gene expression profiles of Caspase 8, Bax, and Akt was studied to understand the possible mechanism behind *Withania somnifera* mediated neuroprotection. The present study will enlighten the cellular and molecular mechanism behind *Withania somnifera* mediated anti-epileptic activity leading to considerable therapeutic value.

OBJECTIVES OF THE PRESENT STUDY

1. To induce Temporal lobe epilepsy model in rats using I.P administration of pilocarpine

2. To study anti-epileptic activity of *Withania somnifera* and Withanolide A

3. To investigate the behavioural changes in control and experimental rats using Radial arm maze test, Y maze test, Rotorod test, Grid walk test and Narrow beam test in experimental rats

4. To study neuronal viability using Nissl staining and TOPRO-3 staining in brain sections of control and experimental rats

5. To study antioxidant potential of *Withania somnifera*, Withanolide-A and Carbamazepine using TBARS Assay, SOD Assay, CAT Assay, SOD Gene Expression and GPx Gene Expression in the brain regions – hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats

6. To measure glutamate content in the brain regions – hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats

7. To study the synthesis, transport and metabolism of Glutamate using GDH assay, GLAST expression and GAD expression in the in the brain regions – hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats

8. To study AMPA and NMDA receptors binding parameters in the brain regions – hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats

9. To study NMDA and AMPA receptor subunits expression in the brain regions – hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats using Real-Time PCR.

10. To measure the second messenger IP3 levels in the brain regions – hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats

11. To study the localisation and expression status of NMDA R1, NMDA 2B, AMPA (GluR2) receptor subunits by immunofluorescent specific antibodies in the brain slices of control and experimental rats using Confocal microscope

12. To study activation of anti-apoptotic pathway using Akt, Bax and Caspase 8 gene expression in the brain regions – hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats using Real-Time PCR.

13. To study localisation and expression of Phospho- Akt by specific immunofluorescent antibody in hippocampus of control and experimental rats using confocal microscope.

Literature Review

Temporal lobe epilepsy was defined by the International League against Epilepsy (ILAE) as a condition characterized by recurrent, unprovoked seizures originating from the medial or lateral temporal lobe. The term is used generally to refer to any epilepsy originating in the temporal lobe but can include several different underlying pathologies that cause the seizures (Berg *et al*., 2010). The condition is characterized by two or more recurrent epileptic seizures over a period longer than 24hours, unprovoked by any immediate identified cause. In clinical practice, one divides intractable epilepsy into generalized and partial epilepsy. When the seizures affect consciousness (complex partial seizures) the most common site of seizure onset is the temporal lobe (Fisher *et al*., 2005). Complex partial seizures are defined by the part of the brain from which the seizures arise (Drexel *et al*., 2011). Therefore TLE is defined as epileptic seizures originating in, or primarily involving, temporal lobe structures (Kotsopoulos *et al*., 2002). This is an important distinction when considering the mechanism and further management of these patients, in particular the possibility of surgical treatment of the epilepsy. Two major types of TLE are usually recognized: mesial TLE (MTLE), where the onset of seizures is from the hippocampus, amygdala, or other medial structures in the temporal lobe (Cersósimo *et al*., 2011), and lateral (neocortical) TLE (less than 10% of TLE cases) (Ng WH *et al*., 2010), where seizures arise from the temporal neocortex. Mesial temporal lobe epilepsy is the most common form of human epilepsy, and its pathophysiological substrate is usually hippocampal sclerosis, the most common epileptogenic lesion encountered in patients with epilepsy. The disabling seizures associated with MTLE are typically resistant to antiepileptic drugs. In MTLE, the first seizures often begin at early childhood to cessate after the childhood years. At that time the seizures are usually well controlled with AEDs. However, the seizures start again after a few years in the adolescence or early adulthood and sometimes they become more severe and progress eventually to refractory epilepsy (Engel *et al*., 2001).

Historical Background of Temporal lobe epilepsy

The word epilepsy is derived from the Greek verb "epilavainem", meaning "to be seized" or "to be taken hold of." This reflected an ancient but enduring idea that epilepsy, like other diseases, occurred as the result of actions by gods or evil spirits (Gilman, 2006). The modern era of epilepsy as a neurological disorder arising from brain dysfunction only dates to the end of the 19th century and the contributions of John Hughlings Jackson, Jean-Martin Charcot, and William R. Gowers (York & Steinberg, 2011). Jackson is credited with the first biological definition of epilepsy as "an occasional, an excessive, and a disorderly discharge of nerve tissue." He was also one of the first to propose that the different clinical manifestations of seizures resulted from specific, localized areas of the brain that became corrupted by this abnormal "disorderly discharge" (Iniesta, 2011). The idea that a characteristic type of seizure was associated with the temporal lobe evolved from neuroanatomical developments: distinguishing the unique features of the temporal lobe and its circuitry from other areas of the cerebrum. A second development was the recognition that some seizures seemed clinically intermediate between grand mal and petit mal. That is, they had motor movements that were more complex and less convulsive than those seen with grand mal seizures (e.g., ambulatory automatisms), as well as hallucinatory phenomena (both psychic and sensory) and disturbances in mood, memory, awareness, and other cognitive functions. A third development was neurophysiological: correlating particular electroencephalogram (EEG) patterns with clinical observations of seizure phenomena (Gilman, 2006). As evidence continued to accumulate that the unique clinical and EEG features of psychomotor seizures had their origin in the temporal lobe, the terms temporal lobe epilepsy or temporal lobe seizures supplanted the older terminology.

Epidemiology of Temporal lobe epilepsy

Population studies show that partial seizures account for up to 50% and 60% of incident and prevalent epilepsy cases (Zenteno & Ronquillo, 2011),

respectively, and that complex partial seizures (CPS) are the most frequent single seizure class (Hauser 1997, Williamson *et al*., 1997). Partial epilepsy is often of temporal lobe origin. However, the true prevalence of temporal lobe epilepsy is not known, since not all cases of presumed temporal lobe epilepsy are confirmed by video-electroencephalography and most cases are classified by clinical history and interictal electroencephalogram (EEG) findings alone (Foldvary *et al*., 2000). TLE is considered the most common epileptic syndrome and it is estimated that approximately 80% of patients with partial seizures have temporal lobe epilepsy (Dreifuss 1987, Williamson *et al*. 1987). A worldwide census of epilepsy surgery centre's confirmed that, in surgical centre's, TLE is by far the commonest type of localization-related epilepsy. Of 8,234 operations performed between 1985 and 1990, 66% involved the temporal lobe (Engel & Shewmon, 1993). Moreover, recently recognized incidentally detected mesial temporal sclerosis in otherwise healthy individuals and benign temporal epilepsy indicate that the true epidemiology of TLE is underestimated (Zenteno & Ronquillo, 2011).

Semiology of Temporal lobe epilepsy

A seizure originating in the temporal lobe of the brain may be preceded by an aura or warning symptom (Schaefer & Unnwongse, 2011). Abnormal sensations, hallucinations, déjà vu or recalled memories and intense feeling of emotions are the symptoms which precede TLE seizures (Kaplan & Fisher, 2005). During the seizure, a person may experience motor disturbances, sensory symptoms, or autonomic symptoms (Neppe, 1981). Motor or movement disturbances (called automatisms) may includes rhythmic muscle contractions on one side of the body or face, abnormal mouth behaviors (lip smacking, chewing for no reason, slobbering), abnormal head movements (forced turning of the head or eyes), repetitive movements (such as picking at clothing) (Goldstein & Mellers 2006). Other sensory symptoms may include numbness and tingling; these sensations may start in one area and spread. Autonomic symptoms like abdominal pain or nausea, sweating, flushing, dilated pupils, or rapid heartbeat is also

observed. Depending on whether the victim remains conscious, he or she may not remember having a seizure at all. A period of confusion frequently follows seizures and can last several minutes (Getz *et al*., 2003).

Diagnosis of Temporal lobe epilepsy

The procedures needed for the diagnosis of epilepsy include medical history with information on the possible predisposing events, a detailed description of the seizures and clinical evaluation with special respect paid to the cardiovascular and neurological examination (Seino *et al*., 2006). EEG-recording reveals focal or generalized spikes and slow waves or other epileptic phenomena (Herman *et al*., 2011). Magnetic resonance imaging (MRI) is recommended as the first line imaging method of the brain when seizures are thought to be of focal origin. MRI detects pathologic conditions that cannot be diagnosed with CT (Engel *et al*., 2001).

Prognosis of Temporal lobe epilepsy

The prognosis of epilepsy depends greatly on the underlying cause. In comparison with the general population, morbidity and mortality are increased in persons with temporal lobe epilepsy, due to increased accidents from the episodes of consciousness loss (Liow *et al*., 2007). Epilepsy surgery seems to modify the risk of SUDEP if the patient remains seizure free. About 47-60% of patients become seizure free with medical treatment. After 3 first-line antiepileptic drugs (AEDs) have failed, the chance for seizure freedom is 5-10%. The ILAE now has a formal definition of medically intractable/drug-resistant epilepsy, which defined as after a patient has had an adequate trial with 2 antiepileptic drugs and is still having seizures. Surgery in well-selected patients with refractory temporal lobe epilepsy yields a seizure-free outcome rate of 70-80% (Ko DY *et al*., 1998).

Aetiology of Temporal lobe epilepsy

The most common etiologic factors of epilepsy that can predispose a person to epilepsy are head traumas, neoplasms, degenerative diseases, infections, metabolic diseases, ischemia and hemorrhages (Vinters *et al*., 1993) (Kotagal *et al*., 1999). At present, more and more genetic factors underlying different types of epileptic syndromes are revealed. It is also known that certain brain areas, i.e. temporal and frontal lobes are more susceptible to produce epileptic seizure activity than the other regions (Nair *et al*., 2010). However, there are also patients with unresolved etiology of epilepsy (Hauser *et al*., 1997) (Falconer, 1974). Etiology of epilepsy is also a factor in determining cognitive function and intellectual changes over time. The main distinction is between symptomatic epilepsy which has an identified cause such as stroke or cortical dysplasia and idiopathic epilepsy which has no identified cause other than genetic factors. Lennox *et al*., (1942) recognized that cognitive function was twice as likely to deteriorate in the presence of a known cause of epilepsy even if the idiopathic group had more frequent seizures.

Anatomy of Temporal lobe epilepsy

The temporal lobes are one of the four main lobes or regions of the cerebral cortex. Structures of the limbic system, including the olfactory cortex, amygdala, and the hippocampus are located within the temporal lobes. The temporal lobe is the most epileptogenic region of the brain. In fact, 90% of patients with temporal interictal epileptiform abnormalities on their electroencephalograms (EEGs) have a history of seizures. The temporal onset seizures then need to be divided into medial temporal onset (amygdala, hippocampus, entorhinal cortex and parahippocampal gyrus) and temporal neocortical. Because of the impact of MRI, depth EEG studies are now infrequently performed in cases of TLE with structural abnormality such as HS. Historical data give insights into the areas of onset of temporal seizures. Depth EEG studies indicate that almost 50% of temporal lobe seizures arise from the

hippocampus (Jackson *et al*., 2005). The hippocampus has been implicated in TLE for three main reasons. First, seizure activity can be recorded from the hippocampus (Spencer & Spencer, 1994; Engel, 1995; Bertram, 1997). Second, removal of the affected hippocampus eliminates seizures in 80–90% of TLE patients exhibiting unilateral mesial temporal lobe sclerosis (Falconer *et al*., 1964; Ojemann, 1987). Third, stereotypic neuropathology is found in the hippocampus of TLE patients that is recapitulated in animal models of TLE, termed hippocampal sclerosis (Schwartzkroin & Knowles, 1984; Cavazos & Cross, 2006) Amygdaloid onset seizures are less frequent and may account for approximately 10% of temporal lobe epilepsy (Jackson *et al*., 2005). Neocortical seizures are even less frequent (1-10%). Conversely, regional onset (hippocampus, amygdala, and temporal neocortex) is common in temporal lobe seizures (Gloor *et al*., 1991).

Pathophysiology of Temporal lobe epilepsy

Hippocampal Sclerosis

There is a strong association between seizures and temporal lobe pathology, especially in the hippocampus. By far, however, the most commonly encountered specific type of pathology is hippocampal sclerosis. These include mesial temporal sclerosis, Ammon's horn sclerosis and endfolium sclerosis. The hippocampus is a structure situated along the dorsomedial aspects of the two temporal lobes, lying just posterior to the amygdala. The hippocampal formation can be divided into three regions: the dentate gyrus (or fascia dentata), Ammon's horn (or hippocampus proper), and the subicular complex (Braak *et al*., 1996). The Ammon's horn is further divided into subregions: CA1, CA2, CA3, and CA4 (Strien *et al*., 2009). These regions are differentiated by variations in the size of the pyramidal cells, the primary neuron of the Ammon's horn. The hippocampus receives neocortical, subcortical, limbic, and brainstem afferents from the perforant path and entorhinal cortex to the dentate gyrus (Lace *et al*., 2009). From here the axons of the dentate granule cells, mossy fibers, project to pyramidal neurons in the CA3 region (Sutula & Dudek, 2007) and modified pyramidal cells

of CA4. These modified pyramidal cells of the CA4 region (or mossy cells) are rich in glutamate (an excitatory neurotransmitter) receptors. The CA4 mossy cells give rise to abundant fibers which synapse back to the dentate gyrus creating a feedback circuit between the dentate gyrus and CA3/CA4 region. In 1880 Sommer was the first to describe the pathologic changes of neuronal loss in the hippocampus in patients with epilepsy (Liu *et al*., 1995). In his study, he estimated that approximately 30% of patients with epilepsy have pathologic lesions that affect the Ammon's horn. After studying all available postmortem brain evaluations revealing hippocampal atrophy in patients with epilepsy, he postulated that hippocampal cells are easily destroyed by an insult, and the resulting Ammon's horn sclerosis was the cause of epilepsy.

Mesial temporal lobe sclerosis (MTS) was introduced as a term to encompass sclerosis extending beyond the hippocampus (HS) to involve adjacent medial structures, including the amygdala. Its most common pathological hallmark is asymmetric hippocampal neuron loss (Jardim *et al*., 2012) within the endfolium (hilus and CA3) and CA1, with relative sparing of the dentate granule neurons and CA2 subfield (Mathern *et al*., 1997; Meldrum & Bruton, 1992; Najm *et al*., 2006. Extra-hippocampal neuron loss within cortical regions and amygdala have been reported in some (Du *et al*., 1999; Hudson *et al*., 1993; Pitkanen *et al*., 1998), but not all (Bothwell *et al*., 2001; Dawodu & Thom, 2005 clinical studies. Acutely-incurred damage following *status epilepticus* (SE) in patients is found within hippocampus, amygdala, thalamic nuclei and piriform and entorhinal cortices (DeGiorgio *et al*., 1999; Fujikawa *et al*., 2000). Patients with mesial temporal sclerosis usually have an early brain insult, a febrile convulsion in most cases, and a seizure free interval of variable duration. This is followed by complex partial seizures with stereotypic Semiology (Falip *et al*., 2003). Histological analysis of the hippocampal formation demonstrates a well defined pattern of cell loss and axonal proliferation. This pattern indicates selective cell vulnerability to the excitotoxic process that causes mesial temporal sclerosis, and synaptic and axonal reorganization, which are involved in the pathogenesis of this disorder (Lewis *et*

al., 2003). Epilepsy associated with mesial temporal sclerosis is not a static process, neurons in this region of the temporal lobe are physiologically and biochemically active, participating in the pathophysiology of the disease by facilitating the recurrence of seizures. There are no pathognomonic findings in mesial temporal sclerosis, but its confident diagnosis can be achieved by convergence of different lines of evidence, including clinical, morphological, and functional findings. Complex partial seizures are often resistant to antiepileptic medication, while surgical resection of the epileptic focus provides seizure freedom in a large number of patients (Rein, 1998).

Ammon's horn sclerosis (AHS) is the major neuropathological substrate in patients with temporal lobe epilepsy (TLE). Histopathological hallmarks include segmental loss of pyramidal neurons, granule cell dispersion and reactive gliosis (Blümcke *et al*., 2002). Pathogenetic mechanisms underlying this distinct hippocampal pathology have not yet been identified and it remains to be resolved whether AHS represents the cause or the consequence of chronic seizure activity and pharmacoresistant TLE (Armstrong *et al*., 2005). Whereas the clinical history indicates an early onset in most patients, ie, occurrence of febrile seizures at a young age, surgical treatment is usually carried out at an end stage of the disease. It has, therefore, been difficult to analyse the sequential development of hippocampal pathology in TLE patients. Molecular neuropathological studies focusing on developmental aspects of hippocampal organization revealed 2 intriguing findings in AHS specimens: i) The persistence of Cajal-Retzius cells in AHS patients points towards an early insult and an altered Reelin signaling pathway and ii) increased neurogenesis in and abnormal architectural organization of the dentate granule cell layer can be observed in young patients with early hippocampal seizure onset (Blümcke *et al*., 2002). Endfolium sclerosis is defined by the selective loss of neurons in the endfolium. Severe neuronal loss is limited to the CA4 region. Mild cell loss is seen in the CA3 region, but the neuronal density is maintained in the CA1 and CA2 regions (Iwasaki *et al*., 2009).

Axon Sprouting

In addition to the neuronal loss, the second morphological change induced in the hippocampus by seizures is sprouting of dentate granule cell axons which are commonly referred to as mossy fibres (Parent *et al*., 1997). This occurs in both animal models of epilepsy (Malheiros *et al*., 2012) & (Bausch & Chavkin, 1997) as well as in human epilepsy (Babb *et al*., 1991). Denervation of the inner molecular layer secondary to hilar cell loss is believed to constitute the initial stimulus for sprouting (Rakhade & Jensen, 2009; Tauck & Nadler, 1985). The sprouted mossy fibre axons appear to make synaptic contacts with granule cells and GABAergic basket cells. It has been proposed that seizure induced expression of neurotropic genes which is suggested to underlie the sprouting of axons of the granule cell layer (Sutula *et al*., 1996).

Gliosis

Reactive gliosis occurs in response to injury, including pilocarpine-induced seizures, in the mature central nervous system (CNS). A salient manifestation of reactive gliosis is an increase in glial fibrillary acidic protein (GFAP), a protein subunit of glial intermediate filaments found exclusively in astrocytes in the CNS (Amaducci *et al*., 1981). Glial proliferation characteristically accompanies neuronal loss seen in Ammon's horn sclerosis and after various insults including SE and contributes to epileptogenesis (Pitkänen & Lukasiuk, 2009).

Mossy Fiber Sprouting and Recurrent Excitation

TLE is characterized by several histological aberrations in the hippocampus. In the hippocampal tissues from TLE patients (Zhang and Houser,1999) and experimental animal models (Wenzel *et al*., 2000) frequently observed is abnormal morphology of the axons of granule cells in the dentate gyrus, i.e., sprouting of hippocampal mossy fibers (MFs). MFs normally elongate within the dentate hilus and stratum lucidum and make synaptic connections with

hilar cells and CA3 neurons (Koyama & Ikegaya, 2004). In the TLE hippocampus, however, MF collaterals abnormally grow into the inner molecular layer of the dentate gyrus (Sutula *et al*., 1995; Sutula & Dudek, 2007), in which the sprouted MFs offer excitatory recurrent inputs into dendrites of granule cells (Buckmaster *et al*., 2002; Maglóczky, 2010). This reorganized pathway is generally thought to contribute to hyperexcitability of the hippocampus. Most of the experimental TLE models support a 'recurrent excitation' hypothesis; granule cells elaborate positive feedback MF projections. This recurrent circuit causes granule cells to excite one another and can be the focus of seizure activity (Buckmaster *et al*., 2002). Electron microscopic works indicate that the vast majority of newly formed MF synapses are asymmetric (Cavazos *et al*., 2003) and terminates on dendritic spines of granule cells (Buckmaster *et al*., 2002), suggesting that they are mostly excitatory. In the pilocarpine model, Buckmaster *et al*., (2002) estimated that on average, one MF forms > 500 new synapses, a minority of which (< 25 synapses) contact with GABAergic interneurons. Therefore, the impact on excitatory neurons appears predominant. Interestingly, biocytin labeling has revealed that sprouted MFs make synapses at intervals of 7 μm in the granule cell layer and 3 μm in the molecular layer but do not overlap the dendrites of their original granule cells (Buckmaster *et al*., 2002). Therefore, the newly formed synapses seem to terminate on other granule cells than their parent neurons, indicating the presence of robust control of circuit formation to avoid autapses. Prolonged (usually >30 min) continuous seizures or lack of recovery between discrete seizure for focal, complex partial, absence and other forms of convulsive seizures is termed status epilepticus. Granule cells in the dentate gyrus (DG) normally project MF axons through dentate hilus (DH) to CA3 and make synaptic contacts with hilar cells, CA3 pyramidal cells and various types of interneurons. In TLE, new collaterals arise from the dentate hilus (arrowhead), run across the granule cell layer, project to the inner third molecular layer and contact with dendrites of other granule cells, so-called MF sprouting. Experiments using rats that show status epilepticus have provided direct insights into the function of recurrent excitatory circuits. Focal application of glutamate to the molecular layer or granule cell layer evokes

excitatory postsynaptic currents (EPSCs) (Molnar and Nadler, 1999) or excitatory postsynaptic potentials (EPSPs) (Frotscher *et al*., 2006) in granule cells far apart from the application loci in hippocampal slices from kainate-treated rats, which supports that the recurrent circuits are excitatory. Under pharmacological blockade of inhibitory network by bicuculline, a GABAA receptor antagonist, focal application of glutamate to the granule cell layer evokes trains of EPSPs and burst spike discharges. In kindled rats, the same phenomena are observed 1 week after seizure onsets, but not after 24 hours when no MF sprouting is yet established (Lynch & Sutula, 2000).

Impaired Inhibition

Repeated intense seizures caused an attenuation of gamma-aminobutyric acid (GABA) - mediated inhibition of the granule cells and in the pyramidal cells of the hippocampus (Coulter *et al*., 1996; Maglóczky *et al*., 2005). This change cannot be explained by a selective loss of GABAergic inhibitory interneuron, since the GABA immunoreactive neurons were shown to be more resistant to seizure-induced injury than other hippocampal neurons (Sloviter *et al*., 1987). Preservation of GABAergic cells in surgical specimens from patients with epilepsy was confirmed (Babb *et al*., 1989; Sloviter *et al*., 2003). The neurons among the most sensitive to the seizure-induced neuronal death are the mossy cells in the dentate hilus. These cells receive synaptic input from granule cells *via* collaterals of mossy fibres and from the entorhinal cortex *via* the perforant path. To account for the paradoxical loss of GABA-mediated inhibition with preservation of GABAergic neurons, the dormant basket cell hypothesis (Sloviter *et al*., 1987) suggests that the seizure-induced loss of hilar excitatory neurons removes tonic excitatory projection to GABAergic basket cells, the inhibitory interneuron in the dentate hilus. Being differentiated these cells then lie dormant with the end result being disinhibition (Sloviter *et al*., 1987). Loss of mossy cells which govern lateral inhibition in the dentate area cause functional delimitation of the granule cell layer and result in synchronous multilamellar discharges in

response to excitatory input (Sloviter *et al*., 1994). Therefore, there are 3 premises to this theory: 1) the general preservation of the inhibitory network. 2) the loss of excitatory afferents to GABAergic interneuron, 3) decreased inhibition on principal cells (Bernard *et al*., 1998).

Pilocarpine Model of Temporal Lobe Epilepsy

Pilocarpine is a potent cholinergic agonist originally isolated from the leaflets of Pilocarpus microphyllus. It is commonly used in the treatment of acute glaucoma in humans (Hardman *et al*., 1996). Single systemic high dose (300-400 mg/kg) pilocarpine injection as a novel animal mode1 of TLE was established (Turski *et al*., 1983). The systemic administration of this muscarinic cholinergic agonist produced electroencephalographic and behavioral seizures, accompanied by widespread brain damage similar to that observed in autopsied brains of human epileptics (Haiyun *et al*., 2012). The electroencephalographic findings indicate that one of the most sensitive structures to the convulsant effect of pilocarpine is the hippocampus, while other structures remain unaffected or only slightly affected at early time points following injection. It is generally accepted that the hippocampus is indeed one of the earliest structures affected following pilocarpine treatment. Later studies confirmed that the hippocampus is the earliest structure to be activated according to electroencephalogaphic recordings (Turski *et al*., 1983, 1989). One of the main features of the pilocarpine mode1 that makes it very relevant for comparison to the human epileptic condition is the reproducible occurrence of SRS in rats injected with pilocarpine following a delay or silent period of about 2 weeks (Turski *et al*., 1983, 1989; Cavalheiro *et al*., 1991; Mello *et al*., 1993). Spontaneity is one of the prominent signs of human epilepsy, therefore strengthening the clinical importance of this model (Turski *et al*., 1983; Loscher & Schmidt, 1988). Pilocarpine seizures also provide an opportunity to study the involvement of the cholinergic system in the onset, propagation and pathological consequences of limbic seizures (Clifford *et al*., 1987). Behaviorally, pilocarpine seizures resemble other models of limbic seizures beginning with

facial automatisms, head nodding and progressing to forelimb clonus with rearing and falling. In terms of neuropathology, the cell damage that results from seizures was identical whether they are initiated with a high-dose pilocarpine injection or a lower dose of pilocarpine administered with lithium. Lithium-pilocarpine is an analogous mode1 to pilocarpine injection alone, except that lithium in combination with pilocarpine has been reported to produce a 20-fold shift in the pilocarpine dose-response curve for producing seizures (Clifford *et al*., 1987) thereby permitting the use of a much lower dose of pilocarpine. In terms of cell damage reported at the light microscope level, pilocarpine-induced seizures consistently produce damage in the olfactory nucleus, pyriform cortex, entorhinal cortex, thalamus, amygdala, hippocampus, lateral septum, bed nucleus of stria terminalis, claustrum, substantia nigra and neocortex (Turski *et al*., 1983; Clifford *et al*., 1987; Turski *et al*., 1989). In the hippocampus, the CA3 and CA1 regions are involved and damage has been noted to be greater in ventral as opposed to dorsal hippocampal regions. Interestingly, the highest cholinergic receptor densities are in CA1 and the dentate gyrus, while the region most consistently and severely damaged is CA3 (Clifford *et al*., 1987). This clearly indicates that the spread of seizure activity beyond the initial focus must entail activation of non-cholinergic pathways. Electron microscopic studies indicate the cellular changes include swelling of dendrites, swelling or vacuolar condensation of neuronal cell bodies and marked dilatation of astroglial elements with relative sparing of axonal components (Clifford *et al*., 1987). The neuropathology reported with the pilocarpine mode1 is consistent with prolonged seizures produced by other means (Ben-Ari *et al*., 1985; Kapur *et al*., 1989; Hajnal *et al*., 1997) These findings support that pilocarpine SE model is useful in studying the molecular mechanisms of neuropathology and screening neuroprotectants following cholinergic agonist exposure.

Glutamate exitotoxicity

Glutamate release is closely associated with serious neurological disorders such as epilepsy, stroke, hypoxia, glucose deprivation and brain trauma (Kim *et al*., 2011). In addition to its vital role as a neurotransmitter, glutamate at high levels is excitotoxic to neurons. Increased level of extracellular glutamate following seizures causes over-stimulation of glutamate receptors. Activation of NMDA (N-methyl-d-aspartate), AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), kainate and metabotrobic receptor subtypes by glutamate (Paoletti, 2011), the most ubiquitous cerebral neurotransmitter, leads to an increase in the levels of free intracellular calcium (Coyle and Puttfarcken, 1993; Pivovarova & Andrews, 2011). Such events can cause prolonged depolarization and subsequent ionic imbalance, ATP depletion and increases in intracellular free calcium levels that together culminate in cerebral edema, raised intracranial pressure (ICP), vascular compression and brain herniation, an often fatal complication of severe head injury (Lau & Tymianski, 2010). Thus, understanding of the fundamental mechanisms that lead to raised interstitial glutamate levels and its consequences is crucial. Since the 1950s, a neurotoxic role for glutamate has been considered when Lucas and Newhouse (1957) demonstrated that the systemic injection of L-glutamate into immature mice destroys inner layers of retina, and to a minor extent, in adult rats. It was later shown by Olney (1969) that certain other brain regions were also affected in immature mice, leading to introduction of the term ‘‘excitotoxicity’’ and subsequently, in a wide range of mammals, including primates (Olney, 1990). Overactivation at NMDA receptors triggers an excessive entry of Ca^{2+}, initiating a series of cytoplasmic and nuclear processes that promote neuronal cell death. For instance, Ca^{2+}-activated proteolytic enzymes, like calpains, can degrade essential proteins (Dong *et al*., 2009). Moreover, Ca^{2+}/calmodulin kinase II (CaM-KII) is activated, and a number of enzymes are phosphorylated, which increases their activity. Glutamate increases NO production and superoxide generation by mitochondria resulting in neurologic injury and apoptosis (Arundine & Tymianski,

2003). Glutamate-induced excitotoxicity induces cytoskeletal alterations, excitatory amino acid (EAA) release, impaired EAA uptake, and the production of ROS (Guemez *et al*., 2009). Glutamate also increases DNA binding of the redox-regulated transcription factors, NF-κB and AP-1, in human neuroblastoma cells and increases the expression of the immediate early gene, c-fos, in murine neuronal cells (Griffiths *et al*., 1997). These events occur before glutamate-induced apoptosis or necrosis in several neuronal cell types (Chuang *et al*., 2010; Narkilahti *et al*., 2007; Chuang *et al*., 2007; Bondy and Lee, 1993; Coyle and Puttfarcken, 1993). Not all cell death induced by l-glutamate is necessarily the result of activation of glutamate receptors. For example, in C6 glioma cultures, prolonged exposure to glutamate at elevated concentrations leads to cell death which has been traced to inhibition of cystine uptake through the cystine/glutamate antiporter (Mawatari *et al*., 1996). The effect on the antiporter deprives glial cells of the cysteine needed for the synthesis of one of the major intracellular reducing agents, glutathione (GSH). Increased lipid peroxidation, ATP depletion, and nuclear chromatin condensation are detectable after prolonged exposure to glutamate (Nakatsu *et al*., 2006).

Oxidative stress in epilepsy

The prolonged excitation of neurons during seizures can lead to injury and death resulting from underlying biochemical mechanisms that are not well understood. One plausible mechanism of cell injury involves the formation of excess free radicals (Oliver *et al*., 1990) and (Coyle and Puttfarcken, 1993), leading to abnormal structural alterations of cellular proteins, membrane lipids, DNA and RNA (Nguyen *et al*., 2011). Oxidative stress, which is defined as the over-production of free radicals, can dramatically alter neuronal function and has been related to SE (Chuang *et al*., 2010). It is particularly facilitated in the brain, as the brain contains large quantities of oxidizable lipids and metals, and, moreover, has fewer antioxidant mechanisms than other tissues (Tejada *et al*., 2006). Free radicals are chemical entities characterized by an orbital containing

an unpaired electron. This electron confers on these molecules a strong propensity to react with target molecules by giving or withdrawing one electron from the target molecules to complete their own orbital (Bellissimo *et al*., 2001). Superoxide, a free radical, can be generated in the brain by several mechanisms such as inefficiency of the electron-carrying components of the mitochondrial transport chain, monoamine degradation, xanthine oxidase reaction, and metabolism of arachidonic acid (Chen *et al*., 2011). However, the superoxide produced can be metabolized by superoxide dismutase which is present in both cytosol (copper–zinc-associated isoform) and mitochondria (manganese-associated isoform) (Wu *et al*., 2010). Reactive oxygen species (ROS), such as superoxide, hydroxyl radical, nitric oxide, nitrite, nitrate and H_2O_2, are normally produced in the brain. H_2O_2 is converted into water by catalase and glutathione peroxidase, which involves GSH, a cofactor of this enzyme. GSH is one of the most important agents of the cellular antioxidant defense system (Shin *et al*., 2010). The resulting hydroxyl radical reacts with nonradical molecules, transforming them into secondary free radicals. This reaction takes place during lipid peroxidation and produces hydroperoxides. Lipid peroxidation can impair the function of several membrane transport proteins including Na1/K1 ATPase, Ca21-ATPase (Lu *et al*., 2001) and glutamate transporters (Stark *et al*., 2011). The change in receptor affinity would have arisen from alteration of receptor structural properties due to lipid peroxidation (Guan *et al*., 2008; Wong-ekkabut *et al*., 2007). Lipid peroxidation causes membrane structure alterations that affect membrane fluidity and permeability and membrane protein activity (Blanc *et al*., 1997). In the nervous system, the phenomenon known as excitotoxicity has been related to over-production of free radicals. Accumulating evidence indicates that free radicals, oxidative stress and mitochondrial dysfunction are important factors in the general pathogenesis of epilepsy (Kudin *et al*., 2012; Kann & Kovács, 2007; Lin & Beal, 2006; Patel, 2004). The biological effects of free radicals are controlled in vivo by a wide range of antioxidants such as vitamin E, vitamin C, vitamin A, glutathione and antioxidant enzymes including glutathione reductaseb(GR), glutathione peroxidase (GP), superoxide dismutase (SOD) and

catalase (CT) (Sudha *et al*., 2001). Oxidative injury in the brain is increasingly recognized as a common pathway of cellular injury in many acute neurologic insults including ischemia-reperfusion and epileptiform brain activity, and in more chronic disease states such as Parkinson's or Alzheimer's disease (Beni & Moretti, 1995; Oliver *et al*., 1990; Dexter *et al*., 1994; Sperk, 1994). Oxidative stress is emerging as a key factor that not only results from seizures, but may also contribute to epileptogenesis (Waldbaum & Patel, 2010). It plays a major role in the initiation and progression of epilepsy and therapies aimed at reducing oxidative stress (OS), ameliorate tissue damage and favourably alter the clinical course of the disease (Costello & Delanty, 2004) Therefore, antioxidant therapies aimed by reducing OS have received considerable attention in the treatment of epilepsy (Shin *et al*., 2011).

Role of Neurotransmitters in Temporal lobe epilepsy

Glutamate

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS). Glutamate is found throughout the mammalian brain at high concentration (10 mM) and participates in many metabolic pathways. In addition to its immediate impact as an excitatory amino acid, it has a role in long-term neuronal potentiation, as a proposed molecular substrate for learning and memory (Tzschentke, 2002; Tapiero *et al*., 2002; Attwell, 2000; Meldrum, 2000). Despite the varied primary pathology of epileptic seizures, the mechanisms involved in generating and spreading epileptic discharges converge on a common cellular pathology in which the excitatory glutamatergic system plays a key role. Compelling neurophysiologic, pharmacologic, biochemical and anatomical evidence has been accumulated over the last several decades firmly implicating ionotropic N-methyl-D-aspartate (NMDA), 2 and a-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA)/ kainate and metabotropic glutamate receptor-mediated mechanisms in epileptic seizures. Excitatory glutamatergic mechanisms are involved during both acute, transient, evoked seizures and long-term, adaptive

cellular plasticity associated with epileptogenesis in chronic epilepsy models such as amygdala-kindled rats or rats with spontaneous, recurring seizures after an early episode of induced status epilepticus. Glutamate acts mainly post-synaptically on three families of ionotropic (ligand-gated ion channels) receptors, (Tapiero et al., 2002; Meldrum, 2000) which all possess ion channels that are permeable to cations, although the relative permeability to Na+ and Ca^{2+} varies according to the family and the subunit composition of the receptor (Rossi *et al*., 2000; Meldrum, 2000). Glutamate may also be a potent neurotoxin, and glutamate excitotoxicity has been implicated in the pathogenesis of many devastating human neurological diseases such as stroke, amyotrophic lateral sclerosis and epilepsy (Smith, 2000).

Glutamate is an amino acid and one of a group of amino acid neurotransmitters in the brain, although it is the principal excitatory neurotransmitter. More basically, amino acids consist of a central carbon atom (α carbon) bonded to a carboxyl group (COOH) and an amino group (NH3). A distinctive side chain (R group), which characterizes each amino acid, links to the α carbon. Glutamate consists of the side chain CH_2CH_2COO- (COOH ending γ carboxyl group] for glutamic acid attached to the α carbon, while the closely related glutamine is created from glutamate with ammonia added at the carboxyl group by glutamine synthetase, forming the $CH_2CH_2CONH_2$ side chain R group (Mark *et al*., 2001). Cerebral glutamate is derived solely from endogenous sources; mainly from α ketoglutarate, which is a product of the Krebs cycle.

Glutamate is found throughout the mammalian brain and participates in many metabolic pathways (Attwell, 2000; Petroff, 2002). Glutamine and a-ketoglutarate are thought to be the major precursors of glutamate, which is subsequently packaged into vesicles for future release into the synaptic cleft (Tapiero *et al*., 2002). Glutamine is taken up into the pre-synaptic terminal via an active, Na+ dependent uptake protein. It is then transported to mitochondria, where it is converted via phosphateactivated glutaminase to glutamate and ammonia. α-Ketoglutarate is also actively taken up into the pre-synaptic terminal,

where it is transaminated into glutamate (Daikhin & Yudkoff, 2000). The glutamate in the terminal is then actively taken up into vesicles for future release. Upon release into the cleft, the glutamate either (i) is bound to pre and post-synaptic receptors, (ii) is actively taken back up via a glutamate transporter and repackaged, (iii) diffuses away from the cleft, or (iv) is internalised by glial glutamate transporters (Attwell, 2000). Five different mammalian glutamate transporters have been cloned (Meldrum, 2000; Masson *et al*., 1999). Apart from cells in the retina and cerebellum, which express high levels of tissue-specific transporters, the transporters expressed most commonly throughout the brain are GLAST-1 in glial cells and EAAT3 in neurons (Mitosek *et al*., 2008). Once in glial cells, the glutamate is metabolised via glutamine synthase into glutamine or metabolised into α-ketoglutarate by either glutamate oxaloacetate transaminase or glutamate dehydrogenase. This glutamine and α-ketoglutarate are then actively transported out of the glial cells and back into the pre-synaptic terminals for subsequent re-synthesis of glutamate (Meldrum *et al*., 1999). The extracellular concentration of glutamate is normally very low (Anderson and Swanson, 2000). Glutamate is released from vesicles in pre-synaptic terminals by a Ca^{2+}-dependent mechanism that involves voltage-dependent calcium channels. The glutamate concentration within the vesicle is thought to be approximately 100 mmol/L; release of a single vesicle produces an excitatory post-synaptic potential (EPSP) (Meldrum, 2000). Glutamate may also be ‘‘released’’ by reverse operation of the glutamate transporters. This will occur when the Na+ and K+ gradient across the membrane is reduced during cerebral ischaemia (Meldrum, 2000). The synaptic release of glutamate is controlled by a wide range of pre-synaptic receptors (Anderson and Swanson, 2000). These include not only the Group II and Group III metabotropic glutamate receptors but also cholinergic (nicotinic and muscarinic) receptors, adenosine (A1), j-opioid, c-aminobutyric acid (GABA)B, cholecystokinin and neuropeptide Y (Y2) receptors.

Ionotropic Glutamate receptors

Three classes or families of ionotropic glutamate receptors have been identified in the CNS and were first defined by their pharmacology and subsequently by their molecular biology (Marmiroli & Cavaletti, 2012; Siegel *et al*., 1999). Their names are based upon the pharmacologic agonist that binds to the specific receptor subtype and selectively opens the associated ion channel: the N-methyl-D-aspartate receptor (NMDA), the kainic acid (KA) receptor, and the a-amino-3-hydroxy- 5-methyl-4-isoxazole proprionate receptor (AMPA, or non NMDA receptor) (Tapiero *et al*., 2002; Meldrum, 2000 ; Dingledine *et al*., 1999).

NMDA receptor

It has been accepted that overstimulation of glutamatergic transmission and thereby activation of glutamate receptors may be of significant relevance for its clinical manifestations. Among glutamate receptors, N-methyl-D-aspartate receptors (NMDARs) have been the focus of much basic and clinical research over the past two decades, producing an overwhelming body of evidence that blocking or suppressing NMDARs is effective in the prevention of and, in some cases, reversal of pathology in various models of neurological diseases, including epilepsy (Ghasemi *et al*., 2012). NMDARs are tetrameric structures of seven subunits including at least one copy of an obligatory subunit, NMDA R1, and varying expression of a family of NMDA 2B(NMDA 2BA- D) or NMDA R3 (NR3A-B) subunits, with multiple binding sites including for glutamate, polyamine, Mg^{2+}, and glycine. Pharmacological regulation of the NMDAR depends on effects on unique combinations of subunit-specific binding sites. Both the NMDA R1 and NMDA 2B subunits contribute to the formation of the NMDAR ion channel. The glutamate-binding site is on the NMDA 2B subunits, and the glycine-binding site is located on the NMDA R1 subunits. The glycine (and/or D-serine) co-agonist site must be occupied before glutamate can activate the ion channel. Although NMDAR channels can conduct Na+ and Ca^{2+}, under basal conditions the channel is blocked by Mg^{2+} within the channel pore. The Mg^{2+}

blockade is relieved by cellular depolarization, which has implications for synaptic plasticity, especially long-term potentiation (LTP) (Löscher et al., 1998). Continuous strong stimulation optimally activates NMDARs and plays an important role in LTP. With neurotoxic insults, disruption of energy metabolism diminishes the driving force for the Na+ pump that maintains the resting membrane potential of cells so that neurons become depolarized, relieving the Mg^{2+} block of NMDARs. Excess Ca^{2+} entry then leads to neuronal excitotoxicity and even cell death. Therefore, NMDAR-mediated responses contribute to the later components in paroxysmal depolarizing shifts and provide for much of the Ca^{2+} entry associated with seizure discharges (Chen et al., 2006).

Glutamatergic impulses from the entorhinal cortex constitute the major excitatory input to the hippocampus and a shift in glutamate-mediated excitability may be involved in the pathogenesis of epileptic discharges (Carter *et al*., 2010). NMDA receptor antagonists are potent anti-convulsants in many animal models suggesting a role for these receptors in epileptogenesis (Patrylo *et al*., 1999). It is known that enhancing NMDA receptor mediated excitatory actions (e.g., by lowering the concentration of extracellular Mg^{2+} produces epileptiform activity in experimental models of kindled epilepsy (Chapman, 1998, 2000). It has been postulated that NMDA receptors may change after neuronal damage (Rice and DeLorenzo, 1998). New receptors may be formed that have either less sensitivity to ambient Mg^{2+} or more sensitivity to ambient glycine. Increased excitability could occur within local circuits where the circuitry itself is not very altered (or may occur in addition to circuit alterations) (Meldrum *et al*., 1999). As it is known that the NMDA receptor is subject to modulation by a variety of endogenous agents, including glycine (as a co-agonist with glutamate), polyamines, steroids, neuropeptides (Vezzani *et al*., 2000), pH, the redox state of the receptor, and nitric oxide, there are many chronic alterations in NMDA receptors that could underlie long-term changes in excitability and, thereby, epilepsy. Using non-radioactive situ hybridization methods, Bayer *et al*., (1995) demonstrated that in situ hippocampal specimens of patients with chronic temporal lobe epilepsy showed a

loss of NMDA R1-positive cells that was closely related to the overall neuronal loss in the resected specimen and to Ammon's horn sclerosis. They suggested that loss of NMDAR1 expression may partly reflect pyramidal cell loss (Bayer *et al*., 1995). Further investigation revealed that NMDAR2 subunit mRNA levels were increased in the hippocampus of patients with hippocampal sclerosis (HS) (Mathern *et al*., 1997). In the dentate gyrus, there appears to be an increase in NMDAR2 immunoreactivity that is associated with abnormal mossy fiber sprouting in this region. In chronic temporal lobe seizures are associated with differential changes in hippocampal NMDAR1 and NMDAR2A–D hybridization densities that vary by subfield and physiopathological category (Mathern *et al*., 1996). Using human focal cortical dysplasia specimens obtained during epilepsy surgery, Crino *et al*., (2001) reported that NMDAR2B and NMDAR2C subunit mRNA was increased, and NMDAR2A subunit mRNA was decreased in dysplastic compared with pyramidal and heterotopic neurons.

AMPA receptor

AMPA receptor is a subclass of glutamergic ionotropic receptor. Its name is derived from its ability to be activated by the artificial glutamate analog AMPA or -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid. AMPA receptors are involved in mediating most forms of fast glutamatergic neurotransmission. The cloning of the first AMPA receptor subunit in 1989 enabled the structural analysis of AMPA receptors and a detailed characterization of their physiology and pharmacology (Hollmann *et al*., 1989). It is now well established that there are four AMPA receptor subunits designated GluA1–GluA4 (formerly GluR1–GluR4), each encoded by a separate gene (Lodge, 2009). The subunits have a modular organization (Sobolevsky *et al*., 2009; Mayer, 2006). There is a large extracellular amino terminal domain that is involved in receptor assembly, trafficking and modulation; a ligand-binding domain that serves as the recognition site for agonists (including the natural agonist glutamate) and also represents the binding site for competitive antagonists; a transmembrane domain that forms the

ion channel (consisting of three membrane-spanning hydrophobic domains and one intramembranous reentrant loop); and a short cytoplasmic carboxy-terminal domain that is involved in targeting the receptor to synapses. The peptide segments connecting the ligand-binding domain to the transmembrane domain transmit conformational changes elicited by agonist binding to the transmembrane ion channel domain, allowing agonist binding to gate the channel to the open state; these segments can be considered the "transducing domain" (Szénási *et al*., 2008). This region of the channel is critical to binding of noncompetitive antagonists, which prevent channel gating (Balannik *et al*., 2005). Each subunit consists of approximately 900 amino acids and exhibits 65–75% sequence homology to other subunits. All AMPA receptors are tetrameric combinations of the four subunits. While homomeric receptors are functional, native AMPA receptors are believed to be heteromers. For example, in hippocampal pyramidal cells of mature rats, the most common subunit configurations are GluA1/GluA2 and GluA2/GluA3 (Wenthold *et al*., 1996). GluA2 serves a critical function. Its pre-mRNA undergoes a unique posttranscriptional modification in which coding of a glutamine (Q) in the M2 reentrant loop is changed to that coding for a positively charged arginine (R). When at least one edited GluA2 subunit is present in the tetrameric AMPA receptor, the channel is calcium impermeable; otherwise AMPA receptors are calcium permeable. However, since nearly all GluA2 subunits are edited and the majority of AMPA receptors contain the GluA2 subunit, most AMPA receptors do not flux calcium. However, GluA2-lacking AMPA receptors are common in interneurons and some cortical neurons where their rapid kinetics allows particularly fast synaptic signaling and their calcium permeability mediates novel forms of synaptic plasticity (Isaac *et al*., 2007).

AMPA receptor antagonists, either competitive or non-competitive, are anti-convulsant in rodent models (Rogawski, 2011). Thus, altered function of AMPA receptors could contribute to pro-convulsant or anti-convulsant effects (Meldrum *et al*., 1999). Evidence has accumulated that Ca^{2+}-permeable AMPA receptors may play a role in epileptogenesis and the brain damage occurring

during the prolonged seizures (Rogawski & Donevan, 1999). Because Ca^{2+}-permeable AMPA receptors are predominantly expressed in GABAergic interneurons, it is hypothesized that some forms of epilepsy might be caused by reduced GABA inhibition resulting from Ca^{2+}-permeable AMPA receptor-mediated excitotoxic death of interneurons (Meldrum & Rogawski, 2007).

Glutamate Transporter

Glutamate transporters are expressed in the plasma membrane as well as in mitochondria and synaptic vesicles in glutamatergic neurons (Ozkan and Ueda, 1998; Gegelashvili & Schousboe, 1997). High affinity transporters for l-glutamate as well as l- and d-aspartate have been found on both neurons and astrocytes with Km values ranging from 20 to 90 μM (Drejer *et al*., 1983; Drejer *et al*., 1982). Isolation of a high affinity sodium- and potassium-coupled glutamate transporter greatly aided the cloning of the glutamate transporters (Danbolt *et al*., 1990). To date five plasma membrane glutamate transporters have been cloned and named GLAST (EAAT1) (Tanaka, 1993; Storck *et al*., 1992), GLT-1 (EAAT2) (Pines *et al*., 1992), EAAC1 (EAAT3) (Kanai and Hediger, 1992), EAAT4 (Fairman *et al*., 1995) and EAAT5 (Arriza *et al*., 1997). It is widely believed that GLAST and EAAT2 are primarily localized on astrocytes, whereas EAAT3 is primarily localized postsynaptically on neurons (Danbolt, 2001). Pharmacological approaches such as inhibiting glutamatetransport or causing down regulation have been used in identifying the functional significance of each glutamate transporter subtype in vitro and in vivo. genetic overexpression of EAAT2 in a mouse model of ALS significantly increases the lifespan of the mice, suggesting that elevated expression of EAAT2 can be neuroprotective (Guo *et al*., 2003). The underlying hypothesis for EAAT2-mediated neuroprotection is that glutamate excitotoxicity is limited due to enhanced uptake.

Glutamate dehydrogenase

GDH is important in glutamatergic and GABAergic neurotransmission as it directly regulates the glutamate concentration and indirectly modulates GABA levels by altering the availability of precursors. GDH is potently inhibited by GTP and activated by ADP (Plaitakis & Zaganas, 2001). The amino acid leucine also activates GDH, an effect that is synergistic with ADP (Plaitakis & Zaganas, 2001). While it may not be pharmacologically relevant, it is of considerable interest that GDH is inhibited by antipsychotic drugs such as chlorpromazine and haloperidol (Shemisa & Fahien, 1971). It appears, however, that the potency of these drugs as inhibitors of GDH is quite low relative to blood levels observed in patients treated with chlorpromazine and haloperidol (Couee & Tipton, 1990). It must be kept in mind that, in addition to the ubiquitous GDH1 isozyme, humans express a unique GDH2 isoform (Plaitakis & Zaganas, 2001) that exhibits a higher sensitivity to haloperidol. In this example, haloperidol may affect GDH in humans at clinically relevant blood levels (Plaitakis *et al*., 2011).

GABA

GABA is the major inhibitory neurotransmitter in the CNS (Sivilotti & Nistri, 1991). Inhibitory inter-neurons that make use of GABA as their neurotransmitter are found throughout the brain, but in any region they comprise a wide range of morphological and functional types that participate in different circuits with principal neurons. Thus, in the CA1 area of the rat hippocampus it is possible to distinguish 16 different types of GABAergic interneurons on the basis of their morphology, specific protein content (e.g., calbindin, calretinin, parvalbumin), and pattern of firing in relation to ongoing rhythms and oscillatory firing of pyramidal neurons (Kaila, 1997). Through the mechanism of recurrent inhibitory feedback, GABAergic interneurons in the cortex terminate local sustained burst firing and, through inhibitory surround, limit the lateral spread of seizure activity. Chemical agents that impair GABAergic inhibition are powerful convulsants.

Precise GABAergic synaptic signaling is critical to the accurate transmission of information within neural circuits and even slight disruptions can produce hypersynchronous activity (Chagnac-Amitai & Connors, 1989). Moreover, changes in ambient GABA can alter tonic inhibition and thus the overall synaptic tone of a brain region (Farrant & Nusser, 2005). The mechanisms of GABA synthesis and degradation are well understood. Glutamate is decarboxylated to GABA *via* glutamic acid decarboxylase (GAD). GABA that is released into the synaptic cleft is transported in to both astrocytes and interneurons through specific transporters. Transported GABA can be repackaged for subsequent release in interneuronal terminals while astrocytic GABA is usually metabolized *via* GABA-transaminase (GABA-T). These metabolic cycles are reviewed (Bak *et al*., 2006; Martin & Tobin, 2000). However, there is an increasing recognition that regulating neurotransmitter metabolism provides another avenue for neuromodulation. In terms of the GABAergic system, the anticonvulsant vigabatrin enhances the GABA content of neurons and glia by blocking its degradation, thereby increasing vesicular concentrations (French, 1999) while the expression of the synthetic enzyme, GAD, is enhanced following a seizure (Esclapez & Houser, 1999; Feldblum *et al*., 1990). Moreover, both experimental and modeling studies have shown that modulating the intracellular content determines the degree of vesicular GABA release (Engel *et al*., 2001; Wu *et al*., 2010; Axmacher *et al*., 2004). Liang *et al* (2006) showed that blocking neuronal glutamine uptake reduces evoked inhibitory potentials (IPSCs) in a highly use-dependent fashion, presumably by limiting glutamate availability within interneurons. Similar data were presented by Fricke *et al*., (2007). Studies using isotopically labeled compounds suggest that the bulk of GABA that is released during neurotransmission is freshly synthesized from glutamine rather than transported from the extracellular space (Waagepetersen *et al*., 2001). In addition, there is evidence that GAD and the vesicular transporter for GABA (vGAT) form a protein complex (Jin *et al*., 2003). These data suggest that newly synthesized GABA is preferred substrate for vesicle loading. Consistent with the hypothesis that synaptic inhibition relies on newly synthesized GABA, transported

glutamate is used for GABA synthesis (Mathews & Diamond, 2003) and blocking neuronal glutamate transport is associated with seizure activity *in vivo* (Sepkuty *et al*., 2002). The consequences of this dependence on freshly synthesized GABA, as compared to transported and repackaged transmitter, have not been studied in detail using *in vitro* preparations where better controlled studies of physiological responses can be obtained. There is an extensive literature showing that seizures can be provoked by blocking GABA synthesis with 3-mercaptoproprionic acid (MPA) *in vivo* (Mares *et al*., 1993). These studies were demonstrating the involvement of GABA in the prevention of the overstimulation of neuronal networks.

Acetylcholine

The cholinergic system plays a crucial role in modulating cortical and in particular hippocampal functions including processes such as learning and memory (Ashe & Weimberger, 1991; Dunnett & Fibiger, 1993; Huerta & Lisman, 1993; Shen *et al*., 1994; Winkler *et al*., 1995). Cholinergic actions are involved in the physiopathogenesis of epileptic discharges as suggested by the ability of some cholinergic agents to induce limbic seizures and histopathological changes resembling those seen in patients with temporal lobe epilepsy (Dickson & Alonso 1997; Nagao *et al*., 1996; Liu *et al*., 1994; Turski *et al*., 1989). Cholinergic stimulation of cortical neurons, including those located within the hippocampal formation, results in excitatory effects that are mediated mainly through the activation of muscarinic receptors (McCormick *et al*., 1993).

Cholinergic innervation is present in the subiculum, which is a major synaptic relay station between the hippocampus proper and several limbic structures that are involved in cognitive processes (Lopes da Silva *et al*., 1990 ; Amaral & Witter, 1989). Subicular neurons are also involved in the spread of seizure activity within the limbic system (Lothman *et al*., 1991). To date little is known about the effects of cholinergic agents in the subiculum. The EC is known to be a “gateway” for the bi-directional passage of information in the neocortical

hippocampalneocortical circuit (Silva *et al*., 1990; Witter *et al*., 1989; Van Hoesen, 1982) Lopes da *via* a cascade of cortico-cortical projections, the superficial layers of the EC (II and III) receive an extensive input from polymodal sensory cortices (Jones & Powell, 1970; Van Hoesen & Pandya, 1975) that is then conveyed to the hippocampal formation via the perforant path (Steward & Scoville, 1976). In turn, the hippocampal formation projects back on the deep layers of the Entorhinal Cortex (EC) which provide output paths that reciprocate the input channels (Insausti *et al*., 1997). In addition, the deep layers of the EC also project massively on the EC superficial layers (Kohler, 1986) thereby closing an EC–hippocampal loop. Thus, by virtue of its extensive projection systems, the EC network acts powerfully in the generalization of temporal lobe seizures. The EC is also known to receive a profuse cholinergic input from the basal forebrain that terminates primarily in layers II and V (Gaykema *et al*., 1990), recisely those layers that gate the main hippocampal input and output. It is well known that the cholinergic system promotes cortical activation and the expression of normal population oscillatory dynamics. In the EC, *in vivo* electrophysiological studies have shown that the cholinergic theta rhythm is generated primarily by cells in layer II (Dickson *et al*., 1995). In addition, *in vitro* studies have also shown that muscarinic receptor activation promotes the development of intrinsic oscillations in EC layer II neurons (Klink & Alonso, 1997). On the other hand, some evidence indicates that altered activity of the cholinergic system is relevant to epileptogenesis.

Dopamine

The mammalian prefrontal cortex (PFC) receives a substantial dopaminergic innervation from the midbrain ventral tegmental area (VTA) (Bjorklund & Lindvall 1984). Dopamine (DA) is an endogenous neuromodulator in the cerebral cortex and is believed to be important for normal brain processes (Williams & Goldman-Rakic, 1995). There is strong evidence that alterations in dopamine function play a role in pathogenesis of a number of neuropsychiatric

diseases including epilepsy (Starr *et al*., 1996; Bozzi *et al*., 2000). *In vivo* studies have shown that dopamine increase and decrease spontaneous firing of neocortical neurons (Bradshaw *et al*., 1985). Dopamine favour long-lasting transitions of PFC neurons to a more excitable up state. *In vitro* electrophysiological experiments suggest that dopamine has multiple effects on PFC neurons. Both increases (Ceci *et al*., 1999) and decreases (Geijo-Barrientos & Pastore, 1995) in postsynaptic excitability of pyramidal neurons have been reported following DA D1 receptor activation. In addition, changes in excitability mediated by DA D2 receptors have been reported (Tseng & O'Donnell, 2004). The effects of dopamine on synaptic responses are also complex and species-specific. AMPA receptor mediated excitatory postsynaptic currents (EPSCs) in layer V pyramidal cells are depressed by a DA D1 receptor–mediated effect of dopamine (Law-Tho *et al*., 1994; Seamans *et al*., 2001). NMDA responses have been reported to be both enhanced (Seamans *et al*., 2001) and depressed (Law-Tho *et al*., 1994). EPSCs in layers II/III are enhanced by dopamine in rats (Gonzalez-Islas & Hablitz, 2003) but decreased in primates (Urban *et al*., 2002). The cerebral cortex contains interconnected local and distant networks of excitatory and inhibitory neurons. Stability of activity in such networks depends on the balance between recurrent excitation and inhibition (Shu *et al*., 2003). A shift of the balance toward excitation leads to the generation of epileptiform activity. The presence of massive recurrent excitatory connections that depend on inhibition for regulation has been implicated in the susceptibility of the neocortex and the hippocampus to develop epileptiform activity and seizures (McCormick & Conteras, 2001). Dopamine is known to modulate epileptiform discharges both *in vivo* and *in vitro* (Alam & Starr, 1993). *In vivo* studies in different models of epilepsy have suggested that dopamine have a pro-convulsant effect mediated by DA D_1 receptors and an anti-convulsant effect via DA D_2 receptors (Starr *et al*., 1996). Dopamine-mediated recruitment of neurons in local excitatory circuits and synchronization of activity in these neurons underlie these effects of dopamine in neocortex. Local excitatory neocortical networks are complexes of interconnected pyramidal neurons.

Several anti-epileptic drugs increase extracellular levels of dopamine and/or serotonin in brain areas involved in epileptogenesis. Behavioural and electrocorticographic studies in rats have shown that DA controls hippocampal excitability via opposing actions at DA D1 and DA D2 receptors. Seizure enhancement is presumed to be a specific feature of D1 receptor stimulation, whereas DA D2 receptor stimulation is anticonvulsant (Alam & Starr, 1993). Decreased DA D2 receptor binding in the brainstem were reported in other neurological diseases like diabetes.

Signal transduction through Second Messenger- Inositol 1,4,5-trisphosphate (IP3)

Many biological stimuli, such as neurotransmitters, hormones and growth factors, activate the hydrolysis of phosphatidyl inositol 4,5-bisphosphate (PIP2) in the plasma membrane which is hydrolyzed by phospholipase C (PLC) to produce IP3 and diacylglycerol (DAG). The IP3 mediates Ca^{2+} release from intracellular Ca^{2+} stores by binding to IP3 receptors (IP3R). IP3R are the IP3 gated intracellular Ca^{2+} channels that are mainly present in the endoplasmic reticulum (ER) membrane. The IP3 induced Ca^{2+} signaling plays a crucial role in the control of diverse physiological processes such as contraction, secretion, gene expression and synaptic plasticity (Berridge, 1993). In response to many stimuli such as neurotransmitters, hormones and growth factors, PIP2 in the plasma membrane is hydrolyzed by PLC to produce IP3 and diacylglycerol (DAG). IP3 plays a dominant role as a second messenger molecule for the release of Ca^{2+} from intracellular stores, while DAG activates protein kinase C (PKC).

In mammalian cells, there are three IP3R subtypes- IP3R1, IP3R2 and IP3R3 which are expressed to varying degrees in individual cell types (Taylor *et al.*, 2002; Wojcikiewicz, 1995) and form homotetrameric or heterotetrameric channels (Bosanac *et al*., 2004). In previous studies, a plasmid vector containing full-length rat IP3R3 linked to green fluorescent protein GFP-IP3R3 was constructed and visualized the distribution of GFP-IP3R3 was constructed in

living cells (Morita *et al.*, 2002, 2004). The confocal images obtained in these studies provided strong evidence that IP3Rs are distributed preferentially on the ER network. Furthermore, Morita *et al.*, (2004) demonstrated that the expressed GFP-IP3R3 acts as a functional IP3-induced Ca^{2+} channel. Frequently, IP3Rs are not uniformly distributed over the membrane but rather form discrete clusters (Bootman *et al.*, 1997). The clustered distribution of IP3Rs has been predicted to be important in controlling elementary Ca^{2+} release events, such as Ca^{2+} puffs and blips, which act as triggers to induce the spatiotemporal patterns of global Ca^{2+} signals, such as waves and oscillations (Shuai & Jung, 2003). Tateishi *et al.*, (2005) reported that GFP-IP3R1 expressed in COS-7 cells aggregates into clusters on the ER network after agonist stimulation. They concluded that IP3R clustering is induced by its IP3-induced conformational change to the open state, not by Ca^{2+} release itself, because IP3R1 mutants that do not undergo an IP3 induced conformational change failed to form clusters. However, their results are inconsistent with studies by other groups (MacMillan *et al.*, 2005), which suggested that IP3R clustering is dependent on the continuous elevation of intracellular Ca^{2+} concentration. Thus, the precise mechanism underlying IP3R clustering remains controversial. Studies by Tojyo *et al.*, (2008) have shown that IP3 binding to IP3R, not the increase in Ca^{2+} is absolutely critical for IP3R clustering. They also found that depletion of intracellular Ca^{2+} stores facilitates the generation of agonist-induced IP3R clustering.

Group I mGluRs (mGluR1/5 subtypes) are also demonstrated to mainly affect intracellular Ca^{2+} mobilization (Bordi & Ugolini, 1999). To sequentially facilitate intracellular Ca^{2+} release, group I receptors activate the membrane-bound phospholipase C (PLC), which stimulates phosphoinositide turnover by hydrolyzing PIP2 to IP3 and diacylglycerol. IP3 then causes the release of Ca^{2+} from intracellular Ca^{2+} stores (such as endoplasmic reticulum) by binding to specific IP3 receptors on the membrane of Ca^{2+} stores. Altered Ca^{2+} levels could then engage in the modulation of broad cellular activities.

Present anti-epileptic treatment and challenges

Currently available antiepileptic drugs have limited efficacy, and their negative properties limit their use and cause difficulties in patient management. Antiepileptic drugs can provide only symptomatic relief as these drugs suppress seizures and have no effect on the epileptogenesis, which is a process that converts the normal circuitry of the brain into a hyperexcitable one, often after an injury (Macleod & Appleton, 2007). The long term use of antiepileptic drugs is limited due to their adverse effects, withdrawal symptoms, deleterious interactions with other drugs and economic burden, especially in developing countries (Greenwood, 2000). Despite huge funding, and extensive premarketing testing for the adverse effects of new antiepileptic drugs, they may still show severe side effects after being introduced on to the market (Arroyo, 2001). For example, unexpected visual field defects have been observed in patients taking vigabatrin a few years after its introduction to the market (Hitiris & Brodie, 2006) and in a high number of individuals felbamate unexpectedly caused aplastic anemia and hepatitis, which were not observed during clinical trials with this drug (Bazil & Pedley, 1998). The older antiepileptic drugs exhibit more serious side effects than newer antiepileptic drugs. The main limitation of phenobarbital is its tendency to alter cognition, mood, and behavior (Brodie & Dichter, 1996). With phenytoin and carbamazepine treatment vestibulocerebellar symptoms, such as ataxia, diplopia, nystagmus, and vertigo, are common (Leppik, 2001). Ethosuximide most commonly causes gastro-intestinal symptoms, drowsiness, and headache. Allergic rashes occur in approximately 5% of patients with the use of ethosuximide (Schachter, 2007). Tachyphylaxis is associated with the use of benzodiazepines. Hepatoxicity may result with the use of valproic acid, felbamate, carbamazepine, phenytoin and phenobarbital (Ahmed & Siddiqi, 2006; Bjornsson, 2008) Topiramate has been reported to have deleterious effects on cognition (Bjornsson, 2008). Furthermore, some of the available antiepileptic drugs may even potentiate certain types of seizures for example carbamazepine and vigabatrin have been reported to precipitate or aggravate absence, myoclonic, and complex partial seizures

Gabapentin has been reported to induce absence and myoclonic seizures. Aggravation of myoclonic, tonic-clonic, and absence seizures have also been documented with the use of ethosuximide (Gayatri & Livingston, 2006) Treatment with two or more drugs (polytherapy) may result in drug-drug interactions that may increase the chances of antiepileptic drug toxicity. Pharmacoresistant patients often require treatment with one or more antiepileptic drugs. To further complicate matters most elderly epileptic patients are often prescribed other medications in addition to antiepileptic drugs. Some antiepileptic drugs induce hepatic metabolizing enzymes, e.g. phenytoin, carbamazepine, phenobarbital, and primidone, whereas others inhibit these enzymes, e.g. valproic acid (Tanaka, 1999). Despite the huge funding and development of new antiepileptic drugs some 30% of patients are still pharmacoresistant. Currently there is no drug which can prevent epileptogenesis. The treatment of pharmacoresistant patients usually requires polytherapy, therefore these patients are at increased risk of severe side effects and deleterious drug interactions. Hence, there is a need to understand the mechanism of pharmacoresistance and development of new pharmacoresistant drug with better efficacy and safety profiles than those of older drugs. Any new antiepileptic drug should also be cost effective and display longer duration of action as these properties will improve patient compliance.

Herbal Medicine and epilepsy

Botanicals and herbs have a centuries-old tradition of use by persons with epilepsy, in many cultures around the world. At present, herbal therapies are tried by patients in developing as well as developed countries for control of seizures or adverse effects from antiepileptic drugs (AEDs), or for general health maintenance, usually without the knowledge of physicians who prescribe their AEDs. Well-designed clinical trials of herbal therapies in patients with epilepsy are scarce, and methodological issues prevent any conclusions of their efficacy or safety in this population. Furthermore, some botanicals and herbs may be proconvulsant or may alter AED metabolism. In spite of these limitations, further

preclinical evaluation of botanicals and herbs and their constituent compounds using validated scientific methods is warranted based on numerous anecdotal observations of clinical benefit in patients with epilepsy and published reports showing mechanisms of action relevant to epilepsy or anticonvulsant effects in animal models of epilepsy.

Withania somnifera

Withania somnifera (WS) Dunal also known as ashwagandha or Indian ginseng, has traditionally been used as part of a holistic system of medicine in India known as ayurveda. The use of Ashwagandha in Ayurvedic medicine extends back over 3000 to 4000 years to the teachings of an esteemed sage Punarvasu Atriya. It has been described in the sacred texts of Ayurveda, including the Charaka and Sushruta Samhitas (Bhattacharya *et al*., 2002). In the Ayurvedic medical system, the drug is one of the most well recognised tonic drugs (Venkataraghavan *et al*., 1980). Sushruta, the Indian physician and cofounder of the Ayurvedic system, hailed the root as "rasayana," an alchemical elixir (Muruganandam *et al*., 2002). The species name somnifera means "sleep-bearing" in Latin. Robin Lane Fox, in his biography of Alexander the Great, claims WS has been used in wine in ancient times. According to Anne Van Arsdall, *Withania somnifera* was called apollinaris and also glofwyrt in The Old English Herbarium, and had a legend that Apollo found it first and gave it to the healer Aesculapius. Traditional uses of WS among tribal peoples in Africa include fevers and inflammatory conditionsWS has many significant benefits, but is best known for its powerful adaptogenic properties, meaning that it helps mind and body adapt better to stress (Atal *et al*., 1975).

Taxonomical Classification

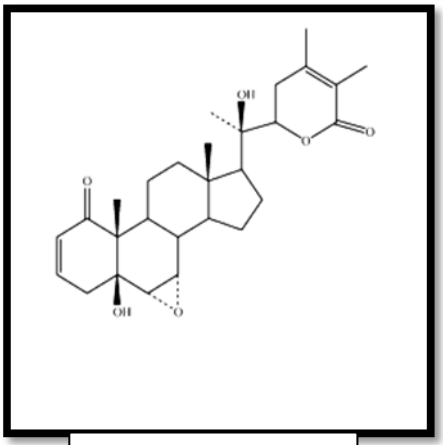
Kingdom: Plantae, Sub-kingdom: Tracheobionta, Super division: Spermatophyta, Division: Angiosperma, Class: Dicotyledons, Order: Tubiflorae, Family: Solanaceae, Genus: Withania, Species: somnifera Dunal.

Plant Description and Distribution

WS is a small, branched, perennial woody shrub that grows usually about 2 feet in height. It has sessile, axillary, greenish or lurid yellow flowers. They are hermaphrodite (has both male and female organs). The fruit is Orange-red berry, smooth, oblong, rounded or somewhat produced at base. It has a more or less tuberous root and the seeds are yellow and scurfy. The fruit is harvested in the late fall and the bright yellow seeds are dried for planting in the following spring. The plant is cultivated as an annual crop and this herb can also be grown with in most home gardens. WS grows abundantly in India (especially Madhya Pradesh), Pakistan, Bangladesh, Sri Lanka and parts of northern Africa. The roots, Bitter leaves and the seeds of the fruits are used in varied purposes.

Constituents

Steroid lactones such as withanolides A-Y, glycowithanolides, dehydrowithanolide-R, withasomniferin-A, withasomi-dienone, withasomniferols A-C, withaferin A, withanone have been isolated from the root and leaf The phytosterols, sitoindosides VII–X and perpetual β-sitosterol were found, alongside the alkaloids ashwagandhine, ashwaghandhinine, cuscohygrine, anahygrine, tropine, pseudotropine, anaferine, isopelletierine, withasomine, visamine, somniferine, somniferinine, withanine, withaninine, pseudowithaninine and solasodine (Williamson, 2002).



Withanolide A

Withania somnifera

Pharmacological properties

WS roots are one of the most highly regarded herbs in Ayurvedic medicine and of similar status of ginseng in traditional Chinese medicine. They are classed among the Rasayanas rejuvenating tonics used for treating age associated decline in cognitive function (Parrotta, 2001).There have been numerous studies regarding the cognitive enhancing activities of WS. Withanoside IV or VI produced dendritic outgrowth in normal cortical neurons of isolated rat cells, whereas axonal outgrowth was observed in the treatment with WA in normal cortical neurons (Tohda *et al*., 2005). Neuritic regeneration or synaptic reconstruction was induced by WA, withanoside IV and VI in amyloid-β (25–35)-induced damaged cortical neurons. In addition, these components also facilitated the reconstruction of post-synaptic and pre-synaptic regions in neurons, where severe synaptic loss had already occurred. WS extract, containing the steroidal substances sitoinodosides VII–X and withaferin A augmented learning acquisition and memory in both young and old rats (Ghosal *et al*., 1989). It enhanced AChE activity in the lateral septum and globus pallidus and decreased it in the vertical diagonal band. Receptor binding on the muscarinic M1 receptor was enhanced in the lateral and medium septum and in the frontal cortices. M2 receptor binding increased in cortical regions. The extract reversed ibotenic acid induced cognitive deficit and reversed the reduction in cholinergic markers, such as acetylcholine

(Schliebs *et al*., 1997). In another study WS treatment significantly downregulated the gene and protein expression of proinflammatory cytokines IL-6, IL-1b, chemokine IL-8, Hsp70 and STAT-2, while a reciprocal upregulation was observed in gene and protein expression of p38 MAPK, PI3K, caspase 6, Cyclin D and c-myc. Furthermore, WS treatment significantly modulated the JAK-STAT pathway which regulates both the apoptosis process as well as the MAP kinase signalling (Aalinkeel *et al*., 2010). In one of the studies, a 2% suspension of ashwagandholine (total alkaloids from the roots of WS) prepared in ten-percent glycol using two percent gum acacia as suspending agent was used to determine acute toxicity. The acute LD50 value was found to be 465 mg/kg (332–651 mg/kg) in rats and 432 mg/kg (229– 626 mg/kg) in mice (Malhotra *et al*., 1965). The extract had no profound effect on central nervous system or autonomic nervous system in doses of up to 250 mg/100 g of mice in toxicity studies. In another long-term study, WS was boiled in water and administered to rats in their daily drinking water for eight months while monitoring body weight, general toxicity, well being, number of pregnancies, litter size, and progeny weight (Sharma *et al*., 1986). The estimated dose received by the animal was 100 mg/kg/day. The liver, spleen, lungs, kidneys, thymus, adrenals, and stomach were examined histopathologically and were all found to be normal. The rats treated with WS showed weight gain as compared to the control group. The offsprings of the group receiving W. somnifera were found to be healthier compared to control group (Sharma *et al*., 1986).

WS preparations have been found to have potential therapeutic role in almost every CNS related disorders. WS modulated GABAergic, cholinergic and oxidative systems. The phytochemicals present in WS are responsible for overcoming the excitotoxicity and oxidative damage (Parihar and Hemnani, 2003; Russo *et al*., 2001). The WS extract inhibited the hydrogen peroxide-induced cytotoxicity and DNA damage in human nonimmortalized fibroblasts (Russo *et al*., 2001). The active principles of WS, sitoindosides VII–X and withaferin A (glycowithanolides), have been extensively tested for antioxidant activity against

the major free-radical scavenging enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) levels of frontal cortex and striatum of the rat brain. Active glycowithanolides of WS (10 or 20 mg/kg., i.p.) when administered once daily for 21 days, an increase in all enzymes was observed, the effect was comparable to those of deprenyl, a known antioxidant (Bhattacharya *et al*., 1997). Withanolides have been found to have calcium antagonistic properties (Choudhary *et al*., 2005). The withanolides inhibited acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities in a concentration-dependent fashion with IC50 values ranging between 29.0 and 85.2 mM for AChE and BChE, respectively. It has been proposed that the cholinesterase inhibitory potential along with calcium antagonistic ability could make the withanolides as possible drug candidates for further study to treat Alzheimer's disease and associated problems (Choudhary *et al*., 2005). Studies have also shown the antiparkinson's like activity of WS, thus possibly modulate dopaminergic system in the brain (Ahmad *et al*., 2005). It is known that immobilization stress for 14 h causes 85% degeneration of the cells (dark cells and pyknotic cells) in the CA(2) and CA(3) subareas of hippocampal region as compared to control rats. Control rats were maintained in completely, nonstressed conditions. Pretreatment with root extract of WS (Stresscom® capsules, Dabur India Ltd.) significantly reduced (80%) the number of degenerating cells in both the areas, demonstrating thereby the neuroprotective effects of plant preparation (Jain *et al*., 2001). EuMil®, a polyherbal medicine consisting of standardized extract of WS, *Oscimum sanctum, Asparagus racemosus and Emblica officinalis* is widely prescribed as antistress formulation in the Indian system of medicine (Bhattacharya *et al*., 2002). WS have profound CNS depressant actions. It has been shown to possess anticonvulsant properties in acute and chronic models of epilepsy (Kulkarni and Verma, 1993; Kulkarni *et al*., 1993). The root extract has antiepileptic activity against pentylenetetrazol (PTZ)-induced kindling in mice (Kulkarni and George, 1996), amygdaloid kindling in rats (Kulkarni and George, 1995), and in *status epilepticus* in rats (Kulkarni *et al*., 1998; Smijin *et al*., 2012).

Materials and Methods

Chemicals used and their sources

Biochemicals

AMPA (α-amino-3-hydroxy-5- methylisoxazole-4-propionic acid), (+) MK-801 [(+) 5 - methyl-10, 11-dihydro-5 H-dibenzocyclohepten-5,10-iminemaleate],ethylene diamine tetra acetic acid (EDTA), Tris HCl, calcium chloride, paraformaldehyde, cresyl violet acetate, atropine methyl bromide, carbamazepine and pilocarpine were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were of analytical grade purchased locally.

Radio chemicals

(+)-[^{3}H] MK-801 (Sp. Activity 27.5 Ci/mmol) was purchased from Perkin Elmer Nen Life and Analytical Sciences, Boston, MA, USA. [3H] AMPA (Sp. Activity 43 Ci/mmol) was purchased from American Radiolabelled Chemicals INC, St Louis, Missouri, USA, [^{3}H] IP3 Biotrak Assay Systems was purchased from G.E Healthcare UK Limited, UK.

Molecular Biology Chemicals

Tri-reagent kit was purchased from Sigma Chemical Co., St. Louis, USA. ABI PRISM High Capacity cDNA Archive kit, Primers and Taqman probes for Real-Time PCR were purchased from Applied Biosystems, Foster City, CA, USA. AMPAR Rn00568544_m1, NMDAR1 (Rn_00433800), NMDA2B (Rn00561352_m1), GLAST (Rn00570130_m1), Bax (Rn 01480160_g1), GAD (Rn00562748_m1), AKt 1 (Rn00583646_m1), Caspase8 (Rn00574069_m1), SOD (Rn 01477289) and GPx (Rn00577994) primers were used for the study.

Confocal Dyes

Rat primary antibody for AMPAR (BD Pharmingen), NMDAR1 (BD Pharmengin), Phospho-Akt (Cell Signalling Technology, USA) secondary antibody of either FITC (Chemicon), Rhodamine dye (Chemicon), Alexa Fluor 488 (Invitrogen), Alexa Fluor 594 (Invitrogen) and CY5 (Chemicon) were used for the immunohistochemistry studies using confocal microscope.

Animals

Adult male Wistar rats of 250-300g body weight were purchased from Kerala Agriculture University, Mannuthy and Amrita Institute of Medical Sciences, Kochi and were used for all the experiments. They were housed in separate cages under 12 hours light and 12 hours dark periods and were maintained on standard food pellets and water *ad libitum*. Adequate measures were also taken to minimize pain and discomfort of the animals. All animal care and procedures were taken in accordance with the Institutional, National Institute of Health guidelines and CPCSEA guidelines.

Plant material

Roots of *Withania somnifera* were provided by Kerala Ayurveda Ltd, Aluva, India. The WS roots were dried, coarsely powdered and 10 g of dry root powder was suspended in 100 ml of distilled water and stirred overnight at 45ºC, followed by filtration under sterile conditions. The filtrate thus obtained was collected and evaporated to dryness followed by lyophilization in Yamato Neocool Lyophilizer. This was used as the crude root extract to study role of *Withania somnifera* crude extract in brain regions of pilocarpine induced temporal lobe epileptic rats. Withanolide A was purchased from Natural Remedies Ltd, Bangalore, India.

Induction of Epilepsy

Experiments were performed on adult male Wistar rats, weighing 250–300 g. They were housed for 1–2 weeks before epilepsy induction. Epilepsy was induced by injecting rats with pilocarpine (350 mg/kg body weight i.p.), preceded by 30 min with atropine methyl bromide (1 mg/kg body weight i.p.) to reduce peripheral pilocarpine effects (Turski *et al*., 1983; Kobayashi *et al*., 2003). Within 20–40 min after the pilocarpine injection, essentially all the animals developed *status epilepticus* (SE). Control animals were given saline injection. Behavioural observation continued for 5 hrs after pilocarpine injection. SE was allowed to continue for 1 hr and then control and experimental animals were treated with diazepam (4 mg/kg body weight i.p.). Animals recovered from this initial treatment within 2–3 days, and were observed for the next 3 weeks. The rats were continuously video monitored for 72 h. The behaviour and seizures were captured with a CCD camera and a Pinnacle PCTV capturing software card. One trained technician, blind to all experimental conditions, viewed all videos. Seizure activity was rated according to Racine Scale using stage 1–5. Stage-1 Facial automatism, Stage-2 Head nodding, Stage-3 Unilateral forelimb clonus, Stage-4 Bilateral forelimb clonus, Stage-5 Rearing, falling and generalized convulsions (Racine, 1972). Seizures were assessed by viewing behavioural postures during observation of the videos. Experimental rats which showed recurrent seizures were used for the further experiments.

Determination of Anti-Epileptic Potential of *Withania somnifera*

Experimental animals were divided into following groups

a) Group 1: Control

b) Group 2: Epileptic

c) Group 3: Epileptic rats treated with *Withania somnifera* (WS)

d) Group 4: Epileptic rats treated with Withanolide A (WA)

e) Group 5: Epileptic rats treated with Carbamazepine (CBZ)

Animal Groups

The rats were initially divided into two groups- Control and Epileptic. The epileptic group was injected with pilocarpine according to the previously established protocols (Reas *et al*., 2008). The control group received saline instead of pilocarpine. The epileptic group showed spontaneous recurrent seizures approximately 20 minutes after pilocarpine injection. Those rats that did not show spontaneous seizures after pilocarpine treatment were excluded from the study group. The rats were singly housed and maintained for 24 days with standard food and water *ad libitum* after pilocarpine treatment. After 21 days the rats were subjected continuous video monitoring for 72 hrs. The behaviour and seizures were observed. Those experimental rats that did not show seizures were excluded from the study group. Experimental rats were divided into five groups: (1) control (C), (2) epileptic (E), (3) epileptic rats treated with WS (E + WS), (4) epileptic rats treated with WA (E + WA) and epileptic rats treated with CBZ (E + CBZ). WS treated rats were given crude extract of *Withania somnifera* orally in the dosage 100 mg/kg body weight/day for 15 days. Withaniolide-A was given orally in the dosage 10µmol /kg body weight/day for 15 days. Carbamazepine- a standard drug used for the treatment of epilepsy was given orally in the dosage 150 mg/kg body weight/day for 15 days.

Tissue Preparation

Control and experimental rats were sacrificed by decapitation. The brain regions (hippocampus, cerebral cortex, cerebellum and brainstem) were dissected out instantly over ice according to the procedure of Heffner *et al*., (1980). The tissues were stored at -80°C for various experiments. All animal care and procedures were in accordance with Institutional and National Institute of Health guidelines.

Nissl staining

The neurons in the hippocampus were visualized by Nissl staining (Montoya *et al*., 2007). The mounted sections were rehydrated in distilled water, and submerged in 0.5% cresyl violet solution for 10 min until the desired depth of staining was achieved. A Histological evaluation was performed using light microscopy.

TOPRO-3 staining

The anaesthetized rats were transcardially perfused with PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. After perfusion the brain was dissected out and fixed in 4% paraformaldehyde for 1 h and then equilibrated with 30% sucrose solution in PBS (0.1 M). 10µm sagittal sections of hippocampus were taken using Cryostat (Leica, CM1510 S). TOPRO-3 stain (diluted 1:1,000 in PBS) was added and kept for 10 min at room temperature. The sections were observed and photographed using confocal imaging system (Leica TCS SP 5) (Matamales *et al.*, 2009).

Behavioural studies

Radial Maze Test

Radial maze behavioural testing was conducted under normal room lighting using an eight armed radial maze elevated 100 cm from the floor. Each arm of the maze (11.5 cm wide) was extended 68.5cm from an octagonally shaped central platform (40 cm across). Black Plexiglas walls (11.5 cm high) were placed for the first 20 cm of each arm to prevent the rat crossing from one arm to another without returning to the central platform. Circular food wells (1.3 cm deep, 3.2 cm diameter) were located 2.5cm from the end of each arm. The maze was centred in an enclosed room where lighting and spatial cues (e.g., posters, door and boxes) remained constant throughout the course of the experiment. Arms were baited by placing one raisin in each food well. Rats were placed on the maze 3 days prior to the start of formal acquisition testing in order to habituate them to the apparatus.

On the first day of habituation, 4 food pellets were scattered along the length of each arm. The rats were then systematically confined to each arm for 1 min to ensure their exposure to the entire maze. On the second day of habituation, the previous day's procedure was repeated except that the animals were not confined to each arm following 5 min of exploration. On the third day, one food pellet was placed in the food well at the end of each arm and a second was placed halfway down each arm. Once the rats were habituated to the maze, testing began. Trials began by placing a single rat in the center of the maze facing away from the experimenter. The trial ended when the rat had obtained all 4 pellets or 5min had elapsed, whichever occurred first. Rats were run until they achieved criterion performance for task acquisition. Criterion was attained when the rat collected 3 out of the 4 food pellets within their first 4 arm entries within a trial (while still completing the trial) with this level of performance being maintained for 5 consecutive criterion performance. The number of trials up to and including the last of these 5 criterion performance formed the "number of trials to criterion" measure. Experimental subjects were tested under blind conditions. The time of testing was consistent from day to day for each subject but testing of the various treatment groups was distributed randomly throughout the day. Performance was recorded during daily behavioural trials according to the terminology in previous studies (Jarrard, 1983; Lopes da Silva *et al.*, 1986). Entry into an unbaited arm was scored as a reference error and re-entry into a baited arm was scored as a working error.

Y-Maze Test

The Y-maze was made of grey wood, covered with black paper and consisted of three arms with an angle of 120 degrees between each of the arms. Each arm was 8 cm width ×30 cm length ×15 cm height. The three identical arms were randomly designated: Start arm, in which the rat started to explore (always open); Novel arm, which was blocked at the 1st trial, but open at the 2nd trial; and the other arm (always open). The maze was placed in a separate room with enough

light. The floor of the maze was covered with sawdust, which was mixed after each individual trial in order to eliminate olfactory stimuli. Visual cues were placed on the walls of the maze. The Y-maze test consisted of two trials separated by an inter-trial interval (ITI). The first trial (training) was of 10 minutes duration and allowed the rat to explore only two arms (start arm and the other arm) of the maze, with the third arm (novel arm) blocked. After a 1 hour ITI (Ma *et al*., 2007), the second trial (retention) was conducted, during which all three arms were accessible and novelty vs. familiarity was analyzed through comparing behaviour in all three arms. For the second trial, the rat was placed back in the maze in the same starting arm, with free access to all three arms for 5 minutes. The time spent in each arm was analyzed. Data was expressed as percentage of performance in all three arms during the five minutes of test (Mathew, *et al*., 2010).

Rotarod Test

Rotarod has been used to evaluate motor coordination by testing the ability of rats to remain on revolving rod (Dunham & Miya, 1957). The apparatus has a horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70 cm long rod was divided into four sections by wooden partitions. The rod was placed at a height of 50 cm to discourage the animals to jump from the rotating rod. The rate of rotation was adjusted in such a manner that it allowed the normal rats to stay on it for five minutes. Each rat was given five trials before the actual reading was taken. The readings were taken at 10, 15 and 25 rpm after 15 days of treatment in all groups of rats.

Grid Walk Test

Deficits in descending motor control were examined by assessing the ability to navigate across a 1 m long runway with irregularly assigned gaps (0.5–5 cm) between round metal bars. Crossing this runway requires that animals accurately place their limbs on the bars. In baseline training and postoperative testing, every animal had to cross the grid for at least three times. The number of

footfalls (errors) was counted in each crossing for 3 minute and a mean error rate was calculated (Chao *et al*., 2012).

Narrow Beam Test

The narrow beam test was performed according to the descriptions of Haydn and Jasmine (1975). A rectangular 1.2-cm wide beam, 1.05m long and elevated 30 cm from the ground was used for the study. After training, normal rats were able to traverse the horizontal beams with less than three footfalls. When occasionally their feet slipped off the beam, they were retrieved and repositioned precisely. The time the rats could remain balanced on the beam was recorded.

Quantification of Glutamate

Glutamate content in the brain regions –hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats were quantified by displacement method using modified procedure of Enna and Snyder, (1976). Tissues were homogenized in 20 volumes of 0.32 M sucrose, 10 mM Tris/HCl and 1 mM MgCl2 buffer, pH 7.4, with a polytron homogenizer. The homogenate was centrifuged twice at 27,000 x g for 15 min. The supernatant was pooled and used for the assay. The incubation mixture contained 1 nM [^{3}H] glutamate with and without glutamate at a concentration range of 10-9 M to 10-4 M. The unknown concentrations were determined from the standard displacement curve using appropriate dilutions and calculated for nmoles/g wt. of the tissue.

Glutamate dehydrogenase Assay

Glutamate dehydrogenase activity was estimated according to the procedure of Balakrishnan *et al*., (2009). Sample extracts were prepared by making a 5% homogenate of the tissue in ice-cold phosphate-buffered saline, pH 7.4. The homogenate was centrifuged at 1000g for 10 minutes to discard the nuclear pellet. The supernatant was centrifuged at 10,000g for 20 minutes, and the enzyme fraction was collected. The reaction mixture in the experimental and reference cuvettes contained triethanolamine buffer, pH 8.0, EDTA, ammonium

acetate and enzyme sample of the appropriate concentration. The reaction mixture of 1 ml volume was assayed at 366 nm in a spectrophotometer by adding different concentrations of α-ketoglutarate and 10 mM NADH. The decrease in optical density due to oxidation of NADH was measured at 15 second intervals of 1 minute at room temperature. The decrease in absorbance was linear during the course of all assays. One unit of enzyme activity was equal to a change in optical density of 0.1 in 100 seconds at 366 nm. Enzyme activity was expressed as specific activity represented by units per milligram of protein.

GLUTAMATE RECEPTOR BINDING STUDIES USING [^{3}H] RADIOLIGANDS IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

NMDA receptor binding studies

The membrane fractions were prepared by a modification of the method described by Hoffman *et al*., (1996). The brain regions - corpus striatum, cerebral cortex, hippocampus, cerebellum and brain stem were homogenized in a 0.32 M sucrose buffer solution containing 10 mM HEPES, 1 mM EDTA buffer, pH 7.0. The homogenate was centrifuged at 1,000 × g for 10 min and the supernatant was centrifuged at 40,000 × g for 1 h. The pellet was re-suspended and homogenized in 10 mM HEPES buffer containing 1.0 mM EDTA, pH 7.0 and centrifuged at 40,000 × g for 1 h. The final pellet was suspended in 10 mM HEPES, 1 mM EDTA buffer, pH 7.0 and stored at -80°C until binding assays were performed. The [^{3}H] MK-801 binding saturation assay was performed in a concentration range of 0.25 to 50 nM at 23°C in an assay medium containing 10 mM HEPES, pH 7.0, 200 - 250 µg of protein, 100 µM glycine and 100 µM glutamate. After 1 h of incubation, the reaction was stopped by filtration through GF/B filters and washed thrice with HEPES buffer pH 7.0. Specific [^{3}H] MK-801 binding was obtained by subtracting nonspecific binding in the presence of 100 µM unlabeled

MK-801 from the total binding. Bound radioactivity was counted with cocktail-T in a Wallac 1409 a liquid scintillation counter

AMPA receptor binding studies

The brain tissue was homogenized in 25 volumes of cold 50 mM Tris-HCl, 10 mM EDTA, pH 7.1, buffer with a Polytron 10,000rpm, 30 s. The pellet was re-suspended in 50 volumes of 50 mM Tris-HC1, pH 7.1, containing 0.04% Triton X-100. The homogenate was incubated for 30 mm at 37°C, then washed three times with 50 mM Tris-HCl, pH 7.1, binding buffer, and centrifuged as above. The final pellet was re-suspended in 50 volumes of binding buffer original wet weight and used as such in the assay. The final concentration of membrane in the assay was 10 mg/ml wet weight. The incubation was performed in the presence of 1, 2.5, 5, 7.5 nM [^{3}H] AMPA respectively; specific activity 43 Ci/mmol]. Nonspecific binding was determined in the presence of 1 mM AMPA. After 1 h of incubation at 4°C, the suspension was filtered Whatman GF/C and washed five times with 3 ml of cold binding buffer. The radioactivity on the filter was measured by liquid scintillation spectrometer. Specific binding was determined by subtracting non-specific binding from the total binding.

Protein determination

Protein was measured by the method of Lowry *et al*., (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein which was read in Spectrophotometer at 660nm.

ANALYSIS OF THE RECEPTOR BINDING DATA

Linear regression analysis for Scatchard plots

The data was analysed according to Scatchard, (1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant

(K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis. The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

GENE EXPRESSION STUDIES IN DIFFERENT BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

Preparation of RNA

RNA was isolated from the different brain regions – hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats using Tri reagent from Sigma Chemical Co., St. Louis, USA.

Isolation of RNA

Tissue (25-50 mg) homogenates were made in 0.5ml Tri Reagent and was centrifuged at 12,000 x g for 10 minutes at 4°C. The clear supernatant was transferred to a fresh tube and it was allowed to stand at room temperature for 5 minutes. 100 µl of chloroform was added to it, mixed vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. The tubes were then centrifuged at 12,000 x g for 15 minutes at 4°C. Three distinct phases appear after centrifugation. The bottom red organic phase contained protein, interphase contained DNA and a colourless upper aqueous phase contained RNA. The upper aqueous phase was transferred to a fresh tube and 250 µl of isopropanol was added and the tubes were allowed to stand at room temperature for 10 minutes. The tubes were centrifuged at 12,000 x g for 10 min at 4ºC. RNA precipitated as a pellet on the sides and bottom of the tube. The supernatants were removed and the RNA pellet was washed with 500µl of 75% ethanol, vortexed and centrifuged at 12,000 x g for 5 min at 4°C. The pellets were semi dried and dissolved in minimum volume of DEPC-treated water. 2µl of RNA was made up to 1ml and absorbance was measured at 260 nm and 280 nm in spectrophotometer (Shimadzu

UV-1700). For pure RNA preparation the ratio of absorbance at 260/280 was ±1.7. The concentration of RNA was calculated as one absorbance $_{260}$ = 42 µg.

cDNA Synthesis

Total cDNA synthesis was performed using ABI PRISM cDNA Archive kit in 0.2ml microfuge tubes. The reaction mixture of 20µl contained 0.2 µg total RNA, 10X RT buffer, 25X dNTP mixture, 10X Random primers, MultiScribe RT (50U/µl) and RNase free water. The cDNA synthesis reactions were carried out at 25°C for 10 minutes and 37°C for 2 hours using an Eppendorf Personal Cycler. The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA designed using Primer Express Software Version (3.0).

Real-Time PCR Assay

Real Time PCR assays were performed in 96-well plates in ABI 7300 Real Time PCR instrument (Applied Biosystems). To detect gene expression, a TaqMan quantitative 2-step reverse transcriptase polymerase chain reaction. (RT-PCR) was used to quantify mRNA levels. First-strand cDNA was synthesized with use of a TaqMan RT reagent kit, as recommended by the manufacturer. PCR analyses were conducted with gene-specific primers and fluorescently labelled TaqMan probe (designed by Applied Biosystems). Endogenous control, β-actin, was labelled with a reporter dye (VIC). All reagents were purchased from Applied Biosystems. The probes for specific gene of interest were labelled with FAM at the 5' end and a quencher (Minor Groove Binding Protein - MGB) at the 3' end. The real-time data were analyzed with Sequence Detection Systems software version 1.7. All reactions were performed in duplicate.

The TaqMan reaction mixture of 20 µl contained 25ng of total RNA-derived cDNAs, 200nM each of the forward primer, reverse primer and TaqMan probes, endogenous control (β-actin) and 12.5µl of TaqMan 2X Universal PCR Master Mix (Applied Biosystems). The volume was made up with RNase free water. Each run contained both negative (no template) and positive controls.

The thermo cycling profile conditions were as follows:

50ºC -- 2 minutes --- Activation

95ºC -- 10 minutes --- Initial Denaturation

95ºC -- 15 seconds --- Denaturation 40 cycles

50ºC -- 30 seconds --- Annealing

60ºC -- 1 minute --- Final Extension

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The ΔΔCT method of relative quantification was used to determine the fold change in expression. This was done by first normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($\Delta CT = CT_{Target} - CT_{\beta\text{-actin}}$). It was further normalized with the control ($\Delta\Delta CT = \Delta CT - CT_{Control}$). The fold change in expression was then obtained ($2^{-\Delta\Delta CT}$).

Determination of SOD Activity

The brain regions were homogenized in 0.1M potassium phosphate buffer, pH 7.8 and centrifuged at 100,000 x g for 60 min at 4°C. The supernatant corresponds to the cytosolic fraction containing CuZn-SOD. The pellets were re - suspended in the buffer, freeze-thawed three times and centrifuged at 100,000 x g for 60 min at 4°C. The supernatant, the particulate fraction containing Mn-SOD, was mixed with the cytosolic fraction to obtain the total enzyme fraction. SOD was analyzed after inhibition by SOD of the pyrogallol autoxidation (Marklund & Marklund, 1974) at pH 8.2 in the presence of EDTA. A 3ml assay mixture contained 0.2 mM pyrogallol, 1 mM EDTA and 50 mM. Tris-HCl buffer. Pyrogallol autoxidation was monitored at 420 nm for 3 min in a spectrophotometer (Shimadzu UV-1700) with or without the enzyme. The

inhibition of pyrogallol oxidation was linear with the activity of the enzyme present. Fifty percent inhibition/mg protein/min was taken as one unit of the enzyme activity.

Determination of Catalase Activity

CAT activity was assayed in the brain regions based on H_2O_2 decomposition monitored at 240nm for 30s (Aebi, 1984). An assay mixture of 500µl contained suitably diluted enzyme protein (100µg) in 50mM phosphate buffer, pH 7.0. The reaction was started by the addition of H_2O_2 (30mM). The decrease in absorbance was monitored and the enzyme activity was expressed as change in absorbance/min/mg protein.

TBARS Assay

As an index of lipid peroxidation the level thiobarbituric acid reactive substances (TBARS) was measured in brain regions- hippocampus, cerebral cortex, cerebellum and brain stem of control and experimental rats. TBARS are products of the oxidative degradation of polyunsaturated fatty acids, in particular malonaldialdehyde (MDA). The reaction mixture contained 0.2ml of hippocampal homogenate (1mg protein), 1.5ml of acetic acid (pH 3.5, 20%), 1.5ml of 0.8% thiobarbituric acid (0.8% w/v) and 0.2ml SDS. The sample was quantitatively analyzed in a spectrophotometer at 532nm (Smijin *et al.*, 2012).

IP3 CONTENT IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS

The brain regions -hippocampus, cerebral cortex, cerebellum and brain stem were homogenised in a polytron homogeniser in 50 mM Tris-HCl buffer, pH.7.4, containing 1 mM EDTA to obtain a 15% homogenate. The homogenate was then centrifuged at 40,000 x g for 15 min and the supernatant was transferred to fresh tubes for IP3 assay using [^{3}H]IP3 Biotrak Assay System kit.

Principle of the assay

The assay was based on competition between [^{3}H]IP3 and unlabelled IP3 in the standard or samples for binding to a binding protein prepared from bovine adrenal cortex. The bound IP3 was then separated from the free IP3 by centrifugation. The free IP3 in the supernatant was then discarded by simple decantation, leaving the bound fraction adhering to the tube. Measurement of the radioactivity in the tube enables the amount of unlabelled IP3 in the sample to be determined.

Assay Protocol

Standards, ranging from 0.19 to 25 pmoles/tube, [^{3}H]IP3 and binding protein were added together and the volume was made up to 100 µl with assay buffer. Samples of appropriate concentration from experiments were used for the assay. The tubes were then vortexed and incubated on ice for 15 minutes and they were centrifuged at 2000 x g for 10 minutes at 4°C. The supernatant was aspirated out and the pellet was resuspended in water and incubated at room temperature for 10 minutes. The tubes were then vortexed and decanted immediately into scintillation vials. The radioactivity in the suspension was determined using liquid scintillation counter.

A standard curve was plotted with %B/Bo on the Y-axis and IP3 concentration (pmoles/tube) on the X-axis of a semi-log graph paper. %B/Bo was calculated as:

$$\frac{\text{(Standard or sample cpm – NSB cpm)}}{\text{(B0 cpm – NSB cpm)}} \times 100$$

NSB- non specific binding and B_0 - zero binding. IP3 concentrations in the samples were determined by interpolation from the plotted standard curve.

PHOSPHO-Akt EXPRESSION IN HIPPOCAMPUS OF CONTROL AND EXPERIMENTAL RATS

Control and experimental rats were deeply anesthetized with ether. The rat was transcardially perfused with Phosphate buffered saline (PBS), pH- 7.4, followed by 4% paraformaldehyde in PBS (Chen *et al*., 2007). After perfusion the brains were dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1 M PBS, pH- 7.0. 20 µm sections were cut using Cryostat (Leica, CM1510 S). The sections were treated with PBS. To block unspecific binding the sections were incubated for 1 hour at room temperature with 5% bovine serum albumin in normal goat serum. Brain slices were incubated overnight at 4°C with either rat primary antibody for Phospho-Akt (diluted in Phosphate buffered saline Triton X- 100 (PBST) at 1: 500 dilution). After overnight incubation, the brain slices were rinsed with PBST. TOPRO-3 stain (diluted 1:1,000 in PBS) was added and kept for 10 min at room temperature. After incubation the brain slices were rinsed with PBST and secondary antibody of FITC (diluted in PBST at 1: 1000 dilution) was added and incubated for 2 hrs. The sections were observed and photographed using confocal imaging system (Leica SP 5).

NMDA R1, NMDA 2B AND AMPA (GluR2) RECEPTOR SUBUNIT EXPRESSION STUDIES IN THE BRAIN REGIONS OF CONTROL AND EXPERIMENTAL RATS USING CONFOCAL MICROSCOPE

Control and experimental rats were deeply anesthetized with ether. The rat was transcardially perfused with Phosphate buffered saline (PBS), pH- 7.4, followed by 4% paraformaldehyde in PBS (Chen *et al*., 2007). After perfusion the brains were dissected and immersion fixed in 4% paraformaldehyde for 1 hr and then equilibrated with 30% sucrose solution in 0.1 M PBS, pH- 7.0. 20 µm

sections were cut using Cryostat (Leica, CM1510 S). The sections were treated with PBS. To block unspecific binding the sections were incubated for 1 hour at room temperature with 5% bovine serum albumin in normal goat serum. Brain slices were incubated overnight at 4°C with either rat primary antibody for NMDA R1 (diluted in Phosphate buffered saline Triton X- 100 (PBST) at 1: 500 dilution), NMDA 2B (diluted in PBST at 1: 500 dilution) and AMPA (GluR2) (diluted in PBST at 1: 500 dilution). After overnight incubation, the brain slices were rinsed with PBST and then incubated with appropriate secondary antibody of either FITC (diluted in PBST at 1: 1000 dilution) or Rhodamine dye (Chemicon, diluted in PBST at 1: 1000 dilution). The sections were observed and photographed using confocal imaging system (Leica SP 5).

Statistics

Statistical evaluations were done by ANOVA using InStat (Ver.2.04a) computer programme. Linear regression Scatchard plots were made using SIGMA PLOT (Ver 2.03). Sigma Plot software (version 2.0, Jandel GmbH, Erkrath, Germany). Competitive binding data were analysed using non-linear regression curve fitting procedure (GraphPad PRISMTM, San Diego, USA). Empower software were used for HPLC analysis. Relative Quantification Software was used for analyzing Real-Time PCR results.

Results

Seizure frequency per 4 hours interval over the 72 hours video recording period of control and experimental rats

In these experiments, pilocarpine (400 mg/kg, i.p.) was injected in adult male Wistar rats, and a progressive evolution of seizures, similar to that classified by Racine (1972) was observed. SE spontaneously remitted 5–6 h after pilocarpine administration and the animals entered post-ictal coma, lasting 1–2 days. Body weight decreased after SE (10–20%), but recovered to pretreatment values after approximately 1 week. The mean Seizure frequency per 4 hours over 72 hours video recording period was calculated. Treatment with CBZ reduced the seizure frequency significantly ($p<0.001$). There was significant ($p<0.001$) reduction of seizure frequency in treatment groups E+WS and E+WA when compared to epileptic group, indicating antiepileptic activity of WS and WA (Figure- 1-4; Table- 1).

Behavioural Studies

Behavioural response of control and experimental rats in Radial Arm Maze test

There was significant increase ($p<0.001$) in the number of trials required to achieve five consecutive criterion performances in the epileptic rats compared to control. The number of trials up to and including the last of these 5 criterion performance formed the "number of trials to criterion" measure. Increased numbers of trials to criterion performance was indicating the learning and memory deficit in epileptic rats. Treatment using WS ($p<0.01$) and WA ($p<0.01$) reversed these changes to near control. There was no significant reversal in CBZ treated group. There was significant increase ($p<0.001$) in the number of reference errors to achieve five consecutive criterion performances in the epileptic rats compared to control. Number of reference errors to achieve five consecutive criterion

performances was significantly decreased in epileptic rats administered with WS ($p<0.001$) and WA ($p<0.001$). CBZ treated group showed no significant reversal. There was significant increase ($p<0.001$) in the number of working errors to achieve five consecutive criterion performances in the epileptic rats compared to control. Number of working errors to achieve five consecutive criterion performances was significantly decreased in epileptic rats administered with WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.01$) (Figure-5-7; Table- 2-4).

Behavioural response of control and experimental rats in Y-Maze test

Number of visits and time spent in the novel arm decreased significantly ($p < 0.001$) in the epileptic group compared to control. Lower percentage of arm visits between the novel arm and the start arm and decreased time spend in the novel arm compared to the other two arms within the epileptic rats showed their decreased exploratory behaviour which have a considerable role in the motor learning. A significant reversal in the number of visit to novel arm was observed in epileptic rats treated with WS ($p < 0.001$) and WA ($p < 0.001$). CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-8; Table-5).

Behavioural response of control and experimental rats on Rotarod test

Rotarod experiment showed a significant down regulation in the retention time on the rotating rod in epileptic rats at 10, 15 and 25 rpm when compared to control. Decreased retention time indicates impairment in motor coordination. Treatment groups significantly reversed the retention time: E+WS ($p<0.01$) E+WA ($p<0.01$) and E+CBZ ($p<0.05$) near to control (Figure-9; Table-6).

Behavioural response of control and experimental rats on grid walk test

There was significant increase ($p<0.001$) in the foot falls in epileptic rats compared to control. Increased footfalls indicate impairment in the ability to integrate sensory input with appropriate motor commands to balance their posture.

Foot falls significantly reversed to near control in epileptic rats administered with WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.05$) (Figure-10; Table-7).

Behavioural response of control and experimental rats on narrow beam test

There was significant decrease in the retention of balance on the narrow beam ($p<0.001$) in epileptic rats compared to control. Balance on the narrow beam significantly reversed to near control in epileptic rats treated with WS ($p<0.001$) and WA ($p<0.001$). CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-11; Table-8).

HIPPOCAMPUS

STUDY OF HISTOPATHOLOGY IN HIPPOCAMPUS OF CONTROL AND EXPERIMENTAL RATS

Nissl Staining in hippocampal sections of control and experimental rats

We analyzed the morphological changes in the hippocampus associated with TLE and effect of WS, WA and CBZ on neuronal survival. In the control animals the Nissl staining of the hippocampal formation and the dentate gyrus showed integrity of all cell layers. In epileptic rats, we observed decreased intensity of Nissl staining and cellular disintegration indicating significant cell loss in all hippocampal fields analyzed, being most evident in the hilus of the hippocampus. In WS and WA treated epileptic rats we observed enhanced staining in the hilus of the hippocampus indicating reduced damage. It is suggested that treatment with WS and WA prevent neuronal death in the hilus of hippocampus CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-12).

TOPRO-3 staining in hippocampus sections of control and experimental rats

Histological analysis of hippocampal section with TOPRO-3 staining showed a significant decrease in the nuclear staining in the epileptic rats. This indicates that there is significant cellular loss in hippocampus of epileptic rats. Treatment with WS and WA resulted in significant increase in nuclear staining indicating in larger number of viable cells in the hilus of the hippocampus CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-12).

STUDY OF ANTIOXIDANT POTENTIAL OF *WITHANIA SOMNIFERA*, WITHANOLIDE-A AND CARBAMAZEPINE USING TBARS ASSAY, SOD ASSAY, CAT ASSAY, SOD GENE EXPRESSION AND GPX GENE EXPRESSION IN HIPPOCAMPUS OF CONTROL AND EXPERIMENTAL RATS

Lipid peroxidation assay in the hippocampus of control and experimental rats

There was a significant increase ($p < 0.001$) in the basal levels of TBARS in the hippocampus of epileptic rats. Lipid peroxidation was markedly increased in hippocampus of the epileptic rats compared with the corresponding values for the control group. Treatment using WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.01$) significantly reversed the levels of TBARS to near control level indicating decreased levels of ROM prerequisite for lipid peroxidation (Figure-13; Table-9).

Superoxide dismutase assay in the hippocampus of control and experimental rats

There was a significant decrease in SOD activity ($p<0.001$) in hippocampus of epileptic rats. Treatment using WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.001$) significantly reversed the activity of SOD enzyme to near control (Figure-14; Table-10).

Catalase assay in the hippocampus of control and experimental rats

There was a significant decrease in CAT activity ($p<0.001$) in hippocampus of epileptic rats. Treatment using WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$) significantly reversed the activity of catalase enzyme near to control (Figure-15; Table-11).

Real time PCR amplification of SOD mRNA from the hippocampus of control and experimental rats

Real time PCR gene expression of SOD showed significant up regulation ($p < 0.001$) in the hippocampus of the epileptic rats compared to the control. This enhanced expression indicates the cellular response to counter increased levels of free radicals during epileptic condition. Treatment using WS ($p < 0.001$) and WA ($p < 0.001$) reversed the changes to near control. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-16; Table-12).

Real time PCR amplification of GPx mRNA from the hippocampus of control and experimental rats

Gene expression of GPx showed significant up regulation ($p < 0.001$) in the hippocampus of the epileptic rats compared to the control. This enhanced expression indicates the cellular response to counter the increased levels of free radicals during epileptic condition. Treatment using WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.01$) reversed the changes to near control (Figure-17; Table-13).

STUDY OF GLUTAMATE SYNTHESIS, TRANSPORT AND METABOLISM USING GLUTAMATE CONTENT, GDH ASSAY, GLAST EXPRESSION AND GAD EXPRESSION IN HIPPOCAMPUS OF CONTROL AND EXPERIMENTAL RATS

Glutamate content in the hippocampus of control and experimental rats

Glutamate content was significantly ($p<0.001$) increased in hippocampus of the epileptic rats compared to the control. Treatment using WS ($p<0.001$) and WA ($p<0.001$) and CBZ ($p<0.01$) reversed the glutamate content to near control (Table-14).

Glutamate dehydrogenase assay in hippocampus of control and experimental rats

Glutamate dehydrogenase kinetic studies indicated that V_{max} significantly increased ($p < 0.001$) in the hippocampus of epileptic rats with no significant change in K_m. Treatment with WS ($p < 0.001$) and WA ($p < 0.001$) significantly reversed the increase in V_{max} to near-control levels, indicating major role of WS and WA in regulating glutamate metabolism. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Table-15).

Real time PCR amplification of GLAST mRNA from the hippocampus of control and experimental rats

Real-time PCR Gene expression of GLAST showed significant down regulation ($p < 0.001$) in the hippocampus of epileptic rats. There was significant reversal in GLAST gene expression in epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.001$) (Figure-18; Table-16)

Real time PCR amplification of GAD mRNA from the hippocampus of control and experimental rats

Real-time PCR Gene expression of GAD showed significant down regulation ($p < 0.001$) in the hippocampus of epileptic rats. In WS ($p < 0.001$) and WA ($p < 0.001$) treated epileptic rats, there was significant reversal and up regulation of GAD gene expression when compared to epileptic and control rats respectively. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-19; Table-17).

NMDA AND AMPA RECEPTOR FUNCTION IN HIPPOCAMPUS OF CONTROL AND EXPERIMENTAL RATS

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the hippocampus of control and experimental rats

Scatchard analysis of NMDA receptors using [^{3}H]MK-801 against MK801 in the hippocampus of epileptic rats showed a significant ($p<0.001$) decrease in B_{max} compared to control rats. This shows decreased NMDA receptor density in the hippocampus of epileptic rats. Significant reversal in the B_{max} was observed in treatment groups: WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.01$). There was no significant change in K_d in all experimental groups of rats (Figure-20; Table-18).

Scatchard analysis of AMPA receptor using [^{3}H]AMPA binding against AMPA in the hippocampus of control and experimental rats

Scatchard analysis of [^{3}H] AMPA against AMPA in the hippocampus showed a significant decrease ($p< 0.001$) in the B_{max} in the epilepsy rats compared to control rats. This result showed decreased AMPA receptor density in the hippocampus of epileptic rats compared to control and the affinity of the AMPA receptor is slightly increased in the epileptic rats. Treatment using WS ($p< 0.001$), WA ($p< 0.001$) and CBZ ($p< 0.05$) significantly reversed the changes in receptor density to near control levels. There was no significant change in Kd in all experimental groups of rats (Figure-21; Table-19).

Real time PCR amplification of NMDA R1 receptor subunit mRNA from the hippocampus of control and experimental rats

Real time PCR gene expression of NMDA R1 receptor subunit showed significant ($p < 0.001$) down regulation in hippocampus of epileptic rats compared to control rats. There was significant reversal in NMDA R1 receptor subunit gene expression in the hippocampus of epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p< 0.05$) (Figure-22; Table-20).

Real time PCR amplification of NMDA 2B receptor subunit mRNA from the hippocampus of control and experimental rats

Real time PCR gene expression of NMDA 2B receptor subunit showed significant ($p < 0.001$) down regulation in hippocampus of epileptic rats compared to control rats. There was significant reversal in NMDA 2B receptor subunit gene expression in the hippocampus of epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p< 0.01$) (Figure-23; Table-21).

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the hippocampus of control and experimental rats

Real time PCR gene expression of GluR2 subunit of AMPA receptor showed significant ($p < 0.001$) down regulation in hippocampus of epileptic rats compared to control rats. There was significant reversal in GluR2 subunit of AMPA receptor gene expression in the Hippocampus of epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p< 0.01$) (Figure-24; Table-22).

NMDA R1 receptor subunit expression in the hippocampus of control and experimental rats using confocal microscope

NMDA R1 subunit specific antibody staining was performed to confirm the receptor and gene expression studies. NMDA R1 subunit specific antibody staining in the hippocampus showed a significant decrease ($p<0.001$) in mean pixel value in the epileptic rats when compared to control. WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.05$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-25).

NMDA 2B receptor subunit expression in the hippocampus of control and experimental rats using confocal microscope

NMDA 2B subunit specific antibody staining was performed to confirm the receptor and gene expression studies. NMDA 2B subunit specific antibody staining in the hippocampus showed a significant decrease ($p<0.001$) in mean

pixel value in the epileptic rats when compared to control. WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.05$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-26).

AMPA (GluR2) receptor subunit expression in the hippocampus of control and experimental rats using confocal microscope

AMPA (GluR2) receptor subunit specific antibody staining was performed to confirm the receptor and gene expression studies. AMPA (GluR2) receptor subunit specific antibody staining in the Hippocampus showed a significant decrease ($p<0.001$) in mean pixel value in the epileptic rats when compared to control. WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.05$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-27).

IP3 content in hippocampus of control and experimental rats

IP3 content was significantly increased ($P < 0.001$) in the hippocampus of epileptic rats when compared to control rats. WS ($P < 0.001$), WA ($P < 0.001$) and CBZ ($P < 0.05$) treatment in epileptic rats significantly reversed the IP3 content to near control (Figure-28; Table-23).

STUDY OF ANTI-APOPTOTIC ACTION OF *WITHANIA SOMNIFERA*, WITHANOLIDE-A AND CARBAMAZEPINE USING Bax GENE EXPRESSION, CASPASE 8 GENE EXPRESSION, Akt GENE EXPRESSION AND PHOSPHO-Akt EXPRESSION IN HIPPOCAMPUS OF CONTROL AND EXPERIMENTAL RATS

Real time PCR amplification of Bax mRNA from the hippocampus of control and experimental rats

Real-time PCR gene expression showed significant ($p < 0.001$) up regulation of Bax in the hippocampus of epileptic rats, indicating activation of apoptotic pathways leading to neuronal death in hippocampus. The treatment with

WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$) significantly reversed Bax gene expression to near control (Figure-29; Table-24).

Real time PCR amplification of Caspase 8 mRNA from the hippocampus of control and experimental rats

Real-time PCR gene expression showed significant ($p < 0.001$) up regulation of caspase 8 in the hippocampus of epileptic rats, indicating activation of apoptotic pathways leading to neuronal death in hippocampus. The treatment with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.01$) significantly reversed caspase 8 gene expression to near control (Figure-30; Table-25).

Real time PCR amplification of Akt-1 mRNA from the hippocampus of control and experimental rats

Real time PCR gene expression of Akt-1 in hippocampus of epileptic rats showed significant ($p < 0.001$) down regulation when compared to control rats. In WS ($p < 0.001$) and WA ($p < 0.001$) treated epileptic rats, there was significant reversal and up regulation of Akt-1 gene expression when compared to epileptic and control rats respectively. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-31; Table-26).

Phospho-Akt expression in the hippocampus of control and experimental rats using confocal microscope

Immunohistochemical localization of Phospho-Akt was performed in hippocampus of epileptic rats to estimate and confirm Akt expression. The phosphorylation status of Akt was analysed using an antibody that recognizes the phosphorylation site of Akt at Ser473. WS and WA treatment in epileptic rats significantly increased ($p<0.001$) the Phospho Akt expression in the hippocampus when compared to epileptic and control rats indicating activation of Akt. There was no significant change in CBZ treated group (Figure-32, 33).

CEREBRAL CORTEX

STUDY OF ANTIOXIDANT POTENTIAL OF *WITHANIA SOMNIFERA*, WITHANOLIDE A AND CARBAMAZEPINE USING TBARS ASSAY, SOD ASSAY, CAT ASSAY, SOD GENE EXPRESSION AND GPX GENE EXPRESSION IN CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

Lipid peroxidation assay in the cerebral cortex of control and experimental rats

There was a significant increase ($p < 0.001$) in the basal levels of TBARS in the cerebral cortex of epileptic rats. Lipid peroxidation was markedly increased in cerebral cortex of the epileptic rats compared with the corresponding values for the control group. Treatment using WS ($p < 0.01$), WA ($p < 0.01$) and CBZ ($p < 0.01$) reversed the levels of TBARS to near control level indicating decreased levels of ROM prerequisite for lipid peroxidation (Figure-34; Table-27).

Superoxide dismutase assay in the cerebral cortex of control and experimental rats

There was a significant decrease in SOD activity ($p<0.001$) in cerebral cortex of epileptic rats. Treatment using WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$) significantly reversed the activity of SOD enzyme near to control (Figure-35; Table-28).

Catalase assay in cerebral cortex of control and experimental animals

There was a significant decrease in CAT activity ($p<0.001$) in cerebral cortex of epileptic rats. Treatment using WS ($p < 0.001$) and WA ($p < 0.001$) reversed the activity of CAT enzyme near to control. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-36; Table-29).

Real time PCR amplification of SOD mRNA from the cerebral cortex of control and experimental rats

Real time PCR gene expression of SOD showed significant up regulation ($p < 0.001$) in the cerebral cortex of the epileptic rats compared to control rats. This enhanced expression indicates the cellular response to counter the increased levels of free radicals during epileptic condition. Treatment using WS ($p < 0.001$) and WA ($p < 0.001$) reversed the changes to near control. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-37; Table-30).

Real time PCR amplification of GPx mRNA from the cerebral cortex of control and experimental rats

Real time PCR gene expression of GPx showed significant up regulation ($p < 0.001$) in the cerebral cortex of the epileptic rats compared to the control. This enhanced expression indicates the cellular response to counter the increased levels of free radicals during epileptic condition. Treatment using WS ($p < 0.01$), WA ($p < 0.01$) and CBZ ($p < 0.05$) significantly reversed the changes to near control (Figure-38; Table-31).

STUDY OF ALTERED GLUTAMATE SYNTHESIS, TRANSPORT AND METABOLISM USING GLUTAMATE CONTENT, GDH ASSAY, GLAST GENE EXPRESSION AND GAD GENE EXPRESSION IN CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

Glutamate content in the cerebral cortex of control and experimental rats

Glutamate content was significantly ($p<0.001$) increased in cerebral cortex of the epileptic rats compared to the control. Treatment using WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.01$) reversed the glutamate content to near control (Table-32).

Glutamate Dehydrogenase Assay in cerebral cortex of control and experimental rats

Glutamate dehydrogenase kinetic studies showed that V_{max} significantly increased ($p < 0.001$) in the cerebral cortex of epileptic rats with no significant change in Km. Treatment with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$) significantly reversed the increase in V_{max} to near-control levels when compared with the epileptic rats, indicating major role of WS and WA in regulating glutamate metabolism (Table-33).

Real time PCR amplification of GLAST mRNA from the cerebral cortex of control and experimental rats

Real-time PCR Gene expression of GLAST showed significant down regulation ($p < 0.001$) in the cerebral cortex of epileptic rats. There was significant reversal in GLAST gene expression in epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$) (Figure-39; Table-34).

Real time PCR amplification of GAD mRNA from the cerebral cortex of control and experimental rats

Real-time PCR Gene expression of GAD showed significant down regulation ($p < 0.001$) in the cerebral cortex of epileptic rats. In WS ($p < 0.001$) and WA ($p < 0.001$) treated epileptic rats, there was significant reversal and up regulation of GAD gene expression when compared to epileptic and control rats respectively. There was no significant reversal in CBZ treated epileptic rats (Figure-40; Table-35)

NMDA AND AMPA RECEPTOR FUNCTION IN CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebral cortex of control and experimental rats

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebral cortex of epileptic rats showed a significant ($p<0.001$) decrease in B_{max} compared to control rats. This shows decreased NMDA receptor density in the cerebral cortex of epileptic rats. Significant reversal in the B_{max} was observed in treatment groups: WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.01$). There was no significant change in K_d in all experimental groups of rats (Figure-41; Table-36).

Scatchard analysis of AMPA receptor using [^{3}H]AMPA binding against AMPA in the cerebral cortex of control and experimental rats

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the cerebral cortex of epileptic rats showed a significant decrease in B_{max} ($p< 0.001$) compared to control rats. This result showed decreased AMPA receptor density in the cerebral cortex of epileptic rats compared to control. Treatment using WS ($p< 0.001$), WA ($p< 0.001$) and CBZ ($p< 0.05$) significantly reversed the changes in receptor binding to near control levels There was no significant change in K_d in all experimental groups of rats (Figure-42; Table-37).

Real time PCR amplification of NMDA R1 receptor subunit mRNA from the cerebral cortex of control and experimental rats

Real-time PCR Gene expression of NMDA R1 receptor subunit showed significant down regulation ($p < 0.001$) in the cerebral cortex of epileptic rats compared to control rats. There was a significant reversal in NMDA R1 receptor subunit gene expression in epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.001$) (Figure-43; Table-38).

Real time PCR amplification of NMDA 2B receptor subunit mRNA from the cerebral cortex of control and experimental rats

Real-time PCR Gene expression of NMDA 2B receptor subunit showed significant down regulation ($p < 0.001$) in the cerebral cortex of epileptic rats compared to control rats. There was a significant reversal in NMDA 2B receptor subunit gene expression in epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.001$) (Figure-44; Table-39).

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the cerebral cortex of control and experimental rats

Real-time PCR gene expression of GluR2 subunit of AMPA receptor subunit showed significant down regulation ($p < 0.001$) in the cerebral cortex of epileptic rats compared to control rats. There was a significant reversal in NMDA 2B receptor subunit gene expression in epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.01$) (Figure-45; Table-40).

NMDA R1 receptor subunit expression in the cerebral cortex of control and experimental rats using confocal microscope

NMDA R1 subunit specific antibody staining was performed to confirm the receptor and gene expression studies. NMDA R1 subunit specific antibody staining in the Cerebral cortex showed a significant decrease ($p<0.001$) in mean pixel value in the epileptic rats when compared to control. WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.05$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-46).

NMDA 2B receptor subunit expression in the cerebral cortex of control and experimental rats confocal microscope

NMDA 2B subunit specific antibody staining was performed to confirm the receptor and gene expression studies. NMDA 2B subunit specific antibody staining in the cerebral cortex showed a significant decrease ($p<0.001$) in mean pixel value in the epileptic rats when compared to control. WS ($p<0.001$), WA

($p<0.001$) and CBZ ($p<0.05$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-47).

AMPA (GluR2) receptor subunit expression in the cerebral cortex of control and experimental rats confocal microscope

AMPA (GluR2) receptor subunit specific antibody staining was performed to confirm the receptor and gene expression studies. AMPA (GluR2) receptor subunit specific antibody staining in the cerebral cortex showed a significant decrease ($p<0.001$) in mean pixel value in the epileptic rats when compared to control. WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.05$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-48).

IP3 content in cerebral cortex of control and experimental rats

IP3 content was significantly increased ($P < 0.001$) in the cerebral cortex of epileptic rats when compared to control rats. WS ($P < 0.001$) and WA ($P < 0.001$) treatment in epileptic rats significantly reversed the IP3 content to near control CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-49; Table-41).

STUDY OF ANTI-APOPTOTIC ACTION OF *WITHANIA SOMNIFERA*, WITHANOLIDE-A AND CARBAMAZEPINE USING Bax GENE EXPRESSION, CASPASE 8 GENE EXPRESSION and Akt GENE EXPRESSION IN CEREBRAL CORTEX OF CONTROL AND EXPERIMENTAL RATS

Real time PCR amplification of Bax mRNA from the cerebral cortex of control and experimental rats

Real-time PCR Gene expression of Bax showed significant up regulation ($p < 0.001$) in the cerebral cortex of epileptic rats compared to control rats. There was a significant reversal in Bax gene expression in epileptic rats treated with WS

($p < 0.001$) and WA ($p < 0.001$). CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-50; Table-42).

Real time PCR amplification of Caspase 8 mRNA from the cerebral cortex of control and experimental rats

Real-time PCR Gene expression of Caspase 8 showed significant up regulation ($p < 0.001$) in the cerebral cortex of epileptic rats compared to control rats. There was a significant reversal in Caspase 8 gene expression in epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$) (Figure-51; Table-43).

Real time PCR amplification of Akt-1 mRNA from the cerebral cortex of control and experimental rats

Real-time PCR Gene expression of Akt-1 showed significant down regulation ($p < 0.001$) in the cerebral cortex of epileptic rats. In WS ($p < 0.001$) and WA ($p < 0.001$) treated epileptic rats, there was significant reversal and up regulation of Akt-1 gene expression when compared to epileptic and control rats respectively. There was no significant change in CBZ treated epileptic rats (Figure-52; Table-44).

CEREBELLUM

STUDY OF ANTIOXIDANT POTENTIAL OF *WITHANIA SOMNIFERA*, WITHANOLIDE-A AND CARBAMAZEPINE USING TBARS ASSAY, SOD ASSAY, CAT ASSAY, SOD GENE EXPRESSION AND GPX GENE EXPRESSION IN CEREBELLUM OF CONTROL AND EXPERIMENTAL RATS

Lipid peroxidation assay in the cerebellum of control and experimental rats

There was a significant increase ($p < 0.001$) in the basal levels of TBARS in the cerebellum of epileptic rats. Lipid peroxidation was markedly increased in cerebellum of the epileptic rats compared with the corresponding values for the control group. Treatment using WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.01$) reversed the levels of TBARS to near control level indicating decreased levels of ROM prerequisite for lipid peroxidation (Figure-53; Table-45).

Superoxide dismutase assay in the cerebellum of control and experimental rats

There was a significant decrease in SOD activity ($p<0.001$) in cerebellum of epileptic rats. Treatment using WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$) significantly reversed the activity of SOD enzyme near to control (Figure-54; Table-46).

Catalase assay in cerebellum of control and experimental animals

There was a significant decrease in CAT activity ($p<0.001$) in cerebellum of epileptic rats. Treatment using WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p<0.05$) significantly reversed the activity of CAT enzyme near to control (Figure-55; Table-47).

Real time PCR amplification of SOD mRNA from the cerebellum of control and experimental rats

Real-time PCR Gene expression of SOD showed significant up regulation ($p < 0.001$) in the cerebellum of epileptic rats compared to control rats. This enhanced expression indicates the cellular response to counter the increased levels of free radicals during epileptic condition There was significant reversal in SOD gene expression treated with WS ($p < 0.001$) and WA ($p < 0.001$). CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-56; Table-48).

Real time PCR amplification of GPx mRNA from the cerebellum of control and experimental rats

Real-time PCR gene expression of GPx showed significant up regulation ($p < 0.001$) in the cerebellum of the epileptic rats compared to the control. This enhanced expression indicates the cellular response to counter the increased levels of free radicals during epileptic condition. Treatment using WS ($p < 0.01$), WA ($p < 0.01$) and CBZ ($p < 0.05$) significantly reversed the SOD gene expression to near control (Figure-57; Table-49).

STUDY OF ALTERED GLUTAMATE SYNTHESIS, TRANSPORT AND METABOLISM USING GLUTAMATE CONTENT, GDH ASSAY, GLAST GENE EXPRESSION AND GAD GENE EXPRESSION IN CEREBELLUM OF CONTROL AND EXPERIMENTAL RATS

Glutamate content in the cerebellum of control and experimental rats

Glutamate content was significantly ($p<0.001$) increased in cerebellum of the epileptic rats compared to the control. Treatment using WS ($p<0.001$) and WA ($p<0.001$) and CBZ ($p<0.001$) significantly reversed these changes to near control (Table-50).

Glutamate Dehydrogenase Assay in Cerebellum of control and experimental rats

Glutamate dehydrogenase kinetic studies indicated that V_{max} significantly increased ($p < 0.001$) in the cerebellum of epileptic rats with no significant change in Km. Treatment with WS ($p < 0.001$) and WA ($p < 0.001$) significantly reversed the increase in V_{max} to near-control levels when compared with the epileptic rats indicating major role of WS and WA in regulating glutamate metabolism. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Table-51).

Real time PCR amplification of GLAST mRNA from cerebellum of control and experimental rats

Real-time PCR Gene expression of GLAST showed significant ($p < 0.001$) down regulation in the cerebellum of epileptic rats. There was a significant reversal in GLAST gene expression in the cerebellum of epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$). (Figure-58; Table-52)

Real time PCR amplification of GAD mRNA from cerebellum of control and experimental rats

Real-time PCR Gene expression of GAD showed significant down regulation ($p < 0.001$) in the cerebellum of epileptic rats. In WS ($p < 0.001$) and WA ($p < 0.001$) treated epileptic rats, there was significant reversal and up regulation of GAD gene expression when compared to epileptic and control rats respectively. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-59; Table-53).

NMDA AND AMPA RECEPTOR FUNCTION IN CEREBELLUM OF CONTROL AND EXPERIMENTAL RATS

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebellum of control and experimental rats

Scatchard analysis of NMDA receptors using [^{3}H]MK-801 against MK801 in the cerebellum of epileptic rats showed a significant ($p<0.001$) decrease in B_{max} compared to control rats. This shows decreased NMDA receptor density in the cerebellum of epileptic rats. Significant reversal in the B_{max} was observed in treatment groups: WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.01$). There was no significant change in K_d in all experimental groups of rats (Figure-60; Table-54).

Scatchard analysis of AMPA receptor using [^{3}H]AMPA binding against AMPA in the cerebellum of control and experimental rats

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the cerebellum showed a significant decrease in the B_{max} ($p< 0.001$ in the epileptic rats when compared to control rats. This result showed decreased AMPA receptor density in the cerebellum of epileptic rats compared to control. Treatment using WS ($p< 0.001$), WA ($p< 0.001$) and CBZ ($p< 0.01$) significantly reversed the changes in receptor density and affinity to near control levels (Figure-61; Table-55).

Real time PCR amplification of NMDA R1 receptor mRNA from the cerebellum of control and experimental rats

Real time PCR gene expression of NMDA R1 receptor subunit showed significant ($p < 0.001$) down regulation in cerebellum of epileptic rats compared to control rats. There was significant reversal in NMDA R1 receptor subunit gene expression in the cerebellum of epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p< 0.01$) (Figure-62; Table-56).

Real time PCR amplification of NMDA 2B receptor mRNA from the cerebellum of control and experimental rats

Real time PCR gene expression of NMDA 2B receptor subunit showed significant ($p < 0.001$) down regulation in cerebellum of epileptic rats compared to control rats. There was significant reversal in NMDA 2B receptor subunit gene expression in the cerebellum of epileptic rats treated with WS ($p < 0.01$), WA ($p < 0.01$) and CBZ ($p< 0.01$) (Figure-63; Table-57).

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from cerebellum of control and experimental rats

Real time PCR gene expression of GluR2 subunit of AMPA receptor showed significant ($p < 0.001$) down regulation in cerebellum of epileptic rats compared to control rats. There was significant reversal in GluR2 subunit of AMPA receptor gene expression in the cerebellum of epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p< 0.01$) (Figure-64; Table-58).

NMDA R1 receptor antibody staining in cerebellum of control and experimental groups of rats using confocal microscope

NMDA R1 subunit specific antibody staining was performed to confirm the receptor and gene expression studies. NMDA R1 subunit specific antibody staining in the cerebellum showed a significant decrease ($p<0.001$) in mean pixel value in the epileptic rats when compared to control. WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.01$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-65).

NMDA 2B receptor antibody staining in cerebellum of control and experimental groups of rats using confocal microscope

NMDA 2B subunit specific antibody staining was performed to confirm the receptor and gene expression studies. NMDA 2B subunit specific antibody staining in the cerebellum showed a significant decrease ($p<0.001$) in mean pixel value in the epileptic rats when compared to control. WS ($p<0.001$), WA

($p<0.001$) and CBZ ($p<0.01$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-66).

AMPA (GluR2) receptor subunit antibody staining in cerebellum of control and experimental groups of rats using confocal microscope

AMPA (GluR2) receptor subunit specific antibody staining was performed to confirm the receptor and gene expression. AMPA (GluR2) receptor subunit specific antibody staining in the cerebellum showed a significant decrease ($p<0.001$) in mean pixel value in the epileptic rats when compared to control. WS ($p<0.01$), WA ($p<0.01$) and CBZ ($p<0.01$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-67).

IP3 content in the cerebellum of control and experimental rats

IP3 content was significantly increased ($P < 0.001$) in the cerebellum of epileptic rats when compared to control rats. WS ($P < 0.001$) and WA ($P < 0.001$) treatment in epileptic rats significantly reversed the IP3 content to near control CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-68; Table-59).

STUDY OF ANTI-APOPTOTIC ACTION OF *WITHANIA SOMNIFERA*, WITHANOLIDE-A AND CARBAMAZEPINE USING Bax GENE EXPRESSION, CASPASE 8 GENE EXPRESSION AND Akt GENE EXPRESSION IN CEREBELLUM OF CONTROL AND EXPERIMENTAL RATS

Real time PCR amplification of Bax mRNA from the cerebellum of control and experimental rats

Real-time PCR gene expression showed significant ($p < 0.001$) up regulation of Bax in the cerebellum of epileptic rats, indicating activation of apoptotic pathways leading to neuronal death in cerebellum. The treatment with

WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$) significantly reversed Bax gene expression to near control (Figure-69; Table-60).

Real time PCR amplification of Caspase 8 mRNA from the cerebellum of control and experimental rats

Real-time PCR gene expression showed significant ($p < 0.001$) up regulation of caspase 8 in the cerebellum of epileptic rats, indicating activation of apoptotic pathways leading to neuronal death in cerebellum. The treatment with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$) significantly reversed caspase 8 gene expression to near control (Figure-70; Table-61).

Real time PCR amplification of Akt-1mRNA from the cerebellum of control and experimental rats

Real-time PCR gene expression showed significant ($p < 0.001$) down regulation of Akt-1 in the cerebellum of epileptic rats. The treatment with WS ($p < 0.001$) and WA ($p < 0.001$) led to significant reversal and up regulation of Akt-1 mRNA when compared to epileptic and control rats respectively, indicating activation of cell survival pathways. The treatment with CBZ did not have a significant change in the gene expression of Akt-1(Figure-71; Table-62).

BRAIN STEM

STUDY OF ANTIOXIDANT POTENTIAL OF *WITHANIA SOMNIFERA*, WITHANOLIDE-A AND CARBAMAZEPINE USING TBARS ASSAY, SOD ASSAY, CAT ASSAY, SOD GENE EXPRESSION AND GPX GENE EXPRESSION IN BRAIN STEM OF CONTROL AND EXPERIMENTAL RATS

Lipid peroxidation assay in the Brain stem of control and experimental rats

There was a significant increase ($p < 0.001$) in the basal levels of TBARS in the brain stem of epileptic rats. Lipid peroxidation was markedly increased in brain stem of the epileptic rats compared with the corresponding values for the control group. Treatment using WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.01$) reversed the levels of TBARS to near control level indicating decreased levels of ROM prerequisite for lipid peroxidation (Figure-72; Table-63).

Superoxide dismutase assay in the Brain stem of control and experimental rats

There was a significant decrease in SOD activity ($p<0.001$) in brain stem of epileptic rats. Treatment using WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$) reversed the activity of SOD enzyme near to control (Figure-73; Table-64).

Catalase assay in Brain stem of control and experimental animals

There was a significant decrease in CAT activity ($p<0.001$) in brain stem of epileptic rats. Treatment using WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p<0.05$) reversed the activity of CAT enzyme near to control (Figure-74; Table-65).

Real time PCR amplification of SOD mRNA from the Brain stem of control and experimental rats

Real-time PCR Gene expression of SOD showed significant up regulation ($p < 0.001$) in the brain stem of epileptic rats compared to control rats. This enhanced expression indicates the cellular response to counter the increased levels of free radicals during epileptic condition There was a significant reversal in SOD gene expression treated with WS ($p < 0.001$) and WA ($p < 0.001$). CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-75; Table-66).

Real time PCR amplification of GPx mRNA from the Brain stem of control and experimental rats

Real-time PCR gene expression of GPx showed significant up regulation ($p < 0.001$) in the Brain stem of the epileptic rats compared to the control. This enhanced expression indicates the cellular response to counter the increased levels of free radicals during epileptic condition. Treatment using WS ($p < 0.01$), WA ($p < 0.01$) and CBZ ($p < 0.05$) significantly reversed the SOD gene expression to near control (Figure-76; Table-67).

STUDY OF ALTERED GLUTAMATE SYNTHESIS, TRANSPORT AND METABOLISM USING GLUTAMATE CONTENT, GDH ASSAY, GLAST GENE EXPRESSION AND GAD GENE EXPRESSION IN BRAIN STEM OF CONTROL AND EXPERIMENTAL RATS

Glutamate content in the Brain stem of control and experimental rats

Glutamate content was significantly ($p<0.001$) increased in brain stem of the epileptic rats compared to the control. Treatment using WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.001$) reverse these changes to near control (Table-68).

Glutamate Dehydrogenase Assay in Brain stem of control and experimental rats

Glutamate dehydrogenase kinetic studies indicated that V_{max} significantly increased ($p < 0.001$) in the brain stem of epileptic rats with no significant change in Km. Treatment with WS ($p < 0.001$) and WA ($p < 0.001$) significantly reversed the increase in V_{max} to near-control levels when compared with the epileptic rats indicating major role of WS and WA in regulating glutamate metabolism. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Table-69).

Real time PCR amplification of GLAST mRNA from Brain stem of control and experimental rats

Real-time PCR Gene expression of GLAST showed significant ($p < 0.001$) down regulation in the brain stem of epileptic rats. There was a significant reversal in GLAST gene expression in the brain stem of epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$). (Figure-77; Table-70)

Real time PCR amplification of GAD mRNA from Brain stem of control and experimental rats

Real-time PCR Gene expression of GAD showed significant down regulation ($p < 0.001$) in the brain stem of epileptic rats. In WS ($p < 0.001$) and WA ($p < 0.001$) treated epileptic rats, there was significant reversal and up regulation of GAD gene expression when compared to epileptic and control rats respectively. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-78; Table-71)

NMDA AND AMPA RECEPTOR FUNCTION IN BRAIN STEM OF CONTROL AND EXPERIMENTAL RATS

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the Brain stem of control and experimental rats

Scatchard analysis of NMDA receptors using [^{3}H]MK-801 against MK801 in the brain stem of epileptic rats showed a significant ($p<0.001$) decrease in B_{max} compared to control rats. This shows decreased NMDA receptor density in the Brain stem of epileptic rats. Significant reversal in the B_{max} was observed in treatment groups: WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.01$). There was no significant change in K_d in all experimental groups of rats (Figure-79; Table-72).

Scatchard analysis of AMPA receptor using [^{3}H]AMPA binding against AMPA in the Brain stem of control and experimental rats

Scatchard analysis of AMPA receptor using [^{3}H] AMPA binding against AMPA in the brain stem showed a significant decrease in the B_{max} ($p< 0.001$) in the epileptic rats when compared to control rats. This result showed decreased AMPA receptor density in the Brain stem of epileptic rats compared to control. Treatment using WS ($p< 0.001$), WA ($p< 0.001$) and CBZ ($p< 0.01$) reversed the changes in receptor density to near control levels (Figure-80; Table-73).

Real time PCR amplification of NMDA R1 receptor mRNA from the Brain stem of control and experimental rats

Real time PCR gene expression of NMDA R1 receptor subunit showed significant ($p < 0.001$) down regulation in brain stem of epileptic rats compared to control rats. There was significant reversal in NMDA R1 receptor subunit gene expression in the brain stem of epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p< 0.01$) (Figure-81; Table-74).

Real time PCR amplification of NMDA 2B receptor mRNA from the Brain stem of control and experimental rats

Real time PCR gene expression of NMDA 2B receptor subunit showed significant ($p < 0.001$) down regulation in brain stem of epileptic rats compared to control rats. There was significant reversal in NMDA 2B receptor subunit gene expression in the brain stem of epileptic rats treated with WS ($p < 0.01$), WA ($p < 0.01$) and CBZ ($p< 0.01$) (Figure-82; Table-75).

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from Brain stem of control and experimental rats

Real time PCR gene expression of GluR2 subunit of AMPA receptor showed significant ($p < 0.001$) down regulation in brain stem of epileptic rats compared to control rats. There was significant reversal in GluR2 subunit of AMPA receptor gene expression in the Brain stem of epileptic rats treated with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p< 0.05$) (Figure-83; Table-76).

NMDA R1 receptor antibody staining in Brain stem of control and experimental groups of rats using confocal microscope

NMDA R1 subunit specific antibody staining was performed to confirm the receptor and gene expression studies. NMDA R1 subunit specific antibody staining in the brain stem showed a significant decrease ($p<0.001$) in mean pixel value in the epileptic rats when compared to control. WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.01$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-84).

NMDA 2B receptor antibody staining in Brain stem of control and experimental groups of rats using confocal microscope

NMDA 2B subunit specific antibody staining was performed to confirm the receptor and gene expression studies. NMDA 2B subunit specific antibody staining in the brain stem showed a significant decrease ($p<0.001$) in mean pixel value in the epileptic rats when compared to control. WS ($p<0.001$), WA

($p<0.001$) and CBZ ($p<0.01$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-85).

AMPA (GluR2) receptor subunit antibody staining in Brain stem of control and experimental groups of rats using confocal microscope

AMPA (GluR2) receptor subunit specific antibody staining was performed to confirm the receptor and gene expression. AMPA (GluR2) receptor subunit specific antibody staining in the Brain stem showed a significant decrease ($p<0.001$) in mean pixel value in the epileptic rats when compared to control. WS ($p<0.001$), WA ($p<0.001$) and CBZ ($p<0.01$) treatment in epileptic rats significantly reversed the mean pixel value to near control (Figure-86).

IP3 content in the Brain stem of control and experimental rats

IP3 content was significantly increased ($P < 0.001$) in the brain stem of epileptic rats when compared to control rats. WS ($P < 0.001$) and WA ($P < 0.001$) treatment in epileptic rats significantly reversed the IP3 content to near control. CBZ treated rats did not showed any significant reversal when compared to epileptic rats (Figure-87; Table-77).

STUDY OF ANTI-APOPTOTIC ACTION OF *WITHANIA SOMNIFERA*, WITHANOLIDE A AND CARBAMAZEPINE USING Bax GENE EXPRESSION, CASPASE 8 GENE EXPRESSION, Akt GENE EXPRESSION AND PHOSPHO-Akt EXPRESSION IN BRAIN STEM OF CONTROL AND EXPERIMENTAL RATS

Real time PCR amplification of Bax mRNA from the Brain stem of control and experimental rats

Real-time PCR gene expression showed significant ($p < 0.001$) up regulation of Bax in the brain stem of epileptic rats, indicating activation of apoptotic pathways leading to neuronal death in brain stem. The treatment with

WS ($p < 0.001$) and WA ($p < 0.001$) significantly reversed Bax gene expression to near control. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-88; Table-78).

Real time PCR amplification of Caspase 8 mRNA from the Brain stem of control and experimental rats

Real-time PCR gene expression showed significant ($p < 0.001$) up regulation of caspase 8 in the Brain stem of epileptic rats, indicating activation of apoptotic pathways leading to neuronal death in Brain stem. The treatment with WS ($p < 0.001$), WA ($p < 0.001$) and CBZ ($p < 0.05$) significantly reversed caspase 8 gene expression to near control (Figure-89; Table-79).

Real time PCR amplification of Akt-1 mRNA from the Brain stem of control and experimental rats

Real time PCR gene expression of AKT-1 in brain stem of epileptic rats showed significant ($p < 0.001$) down regulation when compared to control rats. In WS ($p < 0.001$) and WA ($p < 0.001$) treated epileptic rats, there was significant reversal and up regulation of Akt-1 gene expression when compared to epileptic and control rats respectively. CBZ treated rats did not show any significant reversal when compared to epileptic rats (Figure-90; Table-80).

Figure-1
Seizure frequency per 4 hours interval over the 72 hours video recording period of epileptic rats

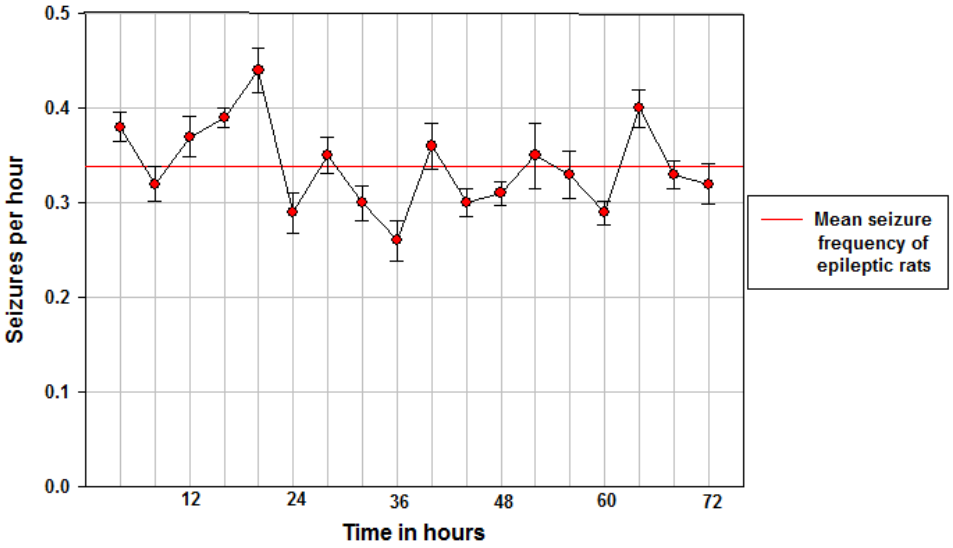


Figure-2
Seizure frequency per 4 hours interval over the 72 hours video recording period of epileptic rats treated with WS

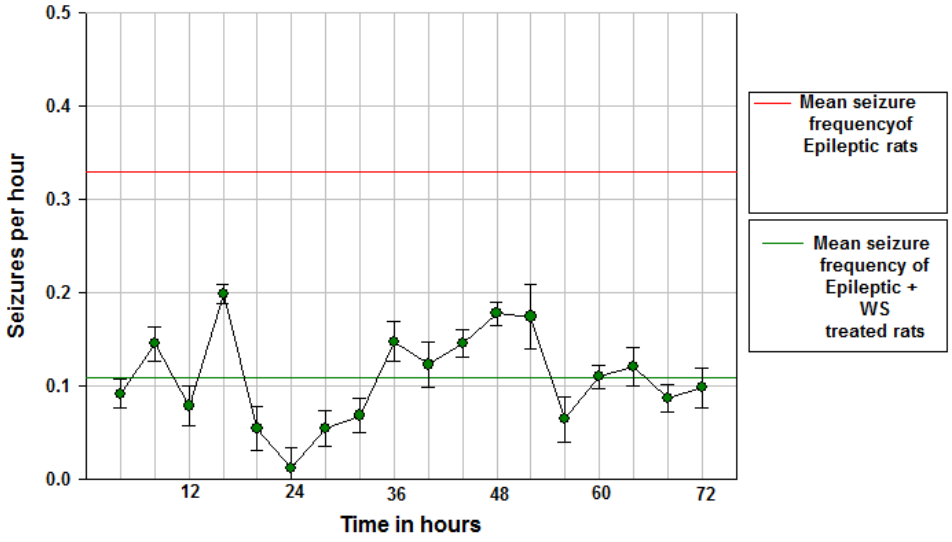


Figure-3

Seizure frequency per 4 hours interval over the 72 hours video recording period of epileptic rats treated with WA

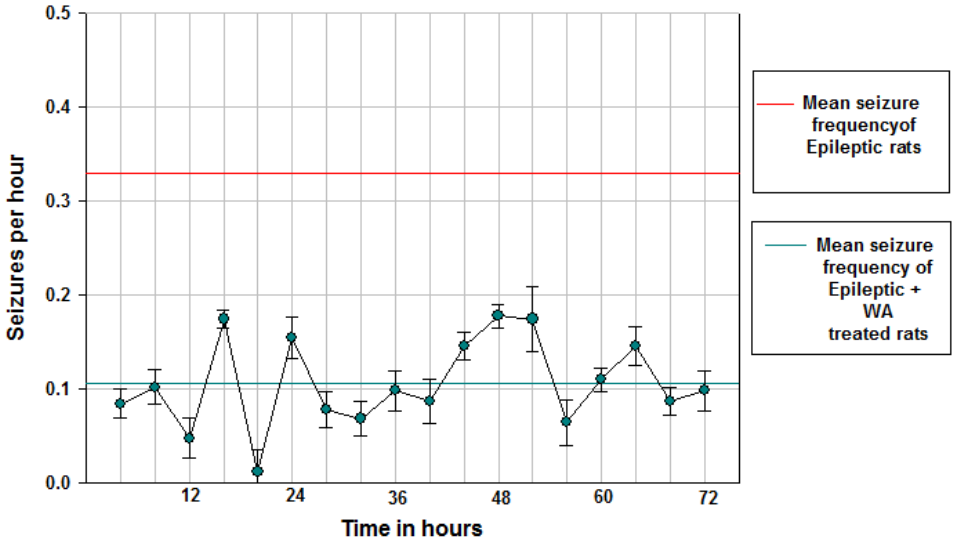


Figure-4

Seizure frequency per 4 hours interval over the 72 hours video recording period of epileptic rats treated with CBZ

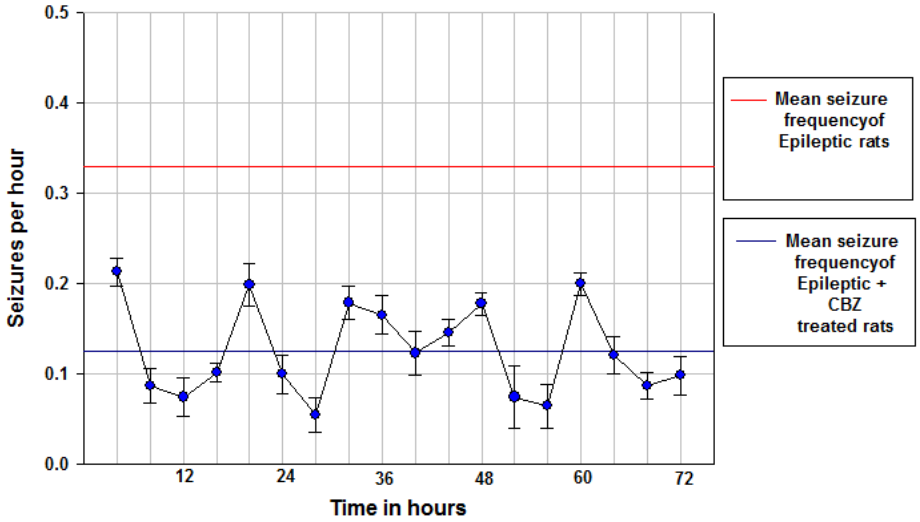


Table-1
Mean seizure frequency per 4 hours interval over the 72 hours video recording period of control and experimental rats

Animal status	Mean seizure frequency/4 hours
Control	**0**
Epileptic	**0.34 ± 0.04** [a]
E+WS	**0.13 ± 0.05** [b, d]
E+WA	**0.12 ±0.06** [b, d]
E+CBZ	**0.11 ± 0.07** [c, d]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-5
Behavioural response of control and experimental rats on criterion performance in radial arm maze test

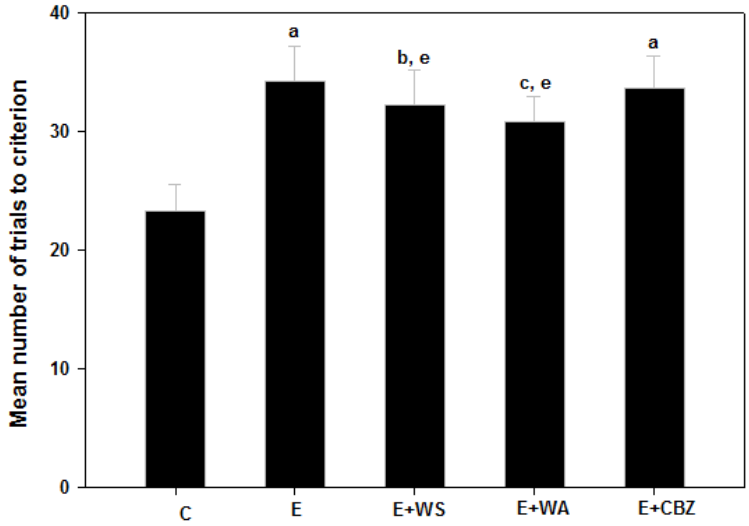


Table-2
Behavioural response of control and experimental rats on criterion performance in radial arm maze test

Animal status	Mean number of trials to criterion
Control	**23.3 ± 2.3**
Epileptic	**34.3 ± 2.9[a]**
E+WS	**32.3 ± 2.4[b, e]**
E+WA	**30.9 ± 2.1[c, e]**
E+CBZ	**33.8± 2.4 [a]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [e] $p < 0.01$ when compared with epileptic group

Figure-6
Behavioural response of control and experimental rats on reference errors in radial arm maze test

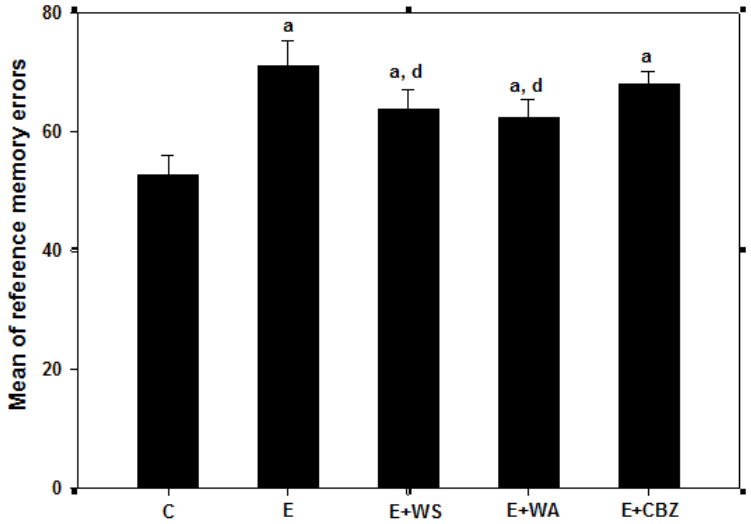


Table-3
Behavioural response of control and experimental rats on reference errors in radial arm maze test

Animal status	Mean of reference memory errors
Control	**52.7 ± 3.3**
Epileptic	**71.2 ± 4.1[a]**
E+WS	**63.8 ± 3.2 [a, d]**
E+WA	**62.4 ± 3.1 [a, d]**
E+CBZ	**68.1± 2.1 [a]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-7
Behavioural response of control and experimental rats on working errors in radial arm maze test

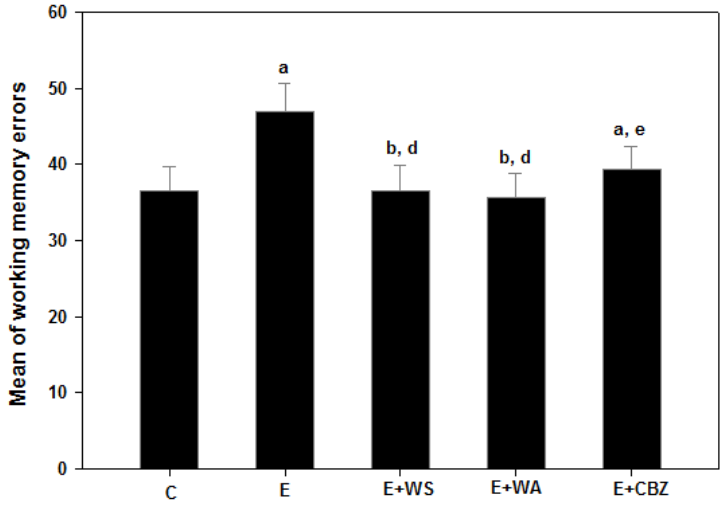


Table-4
Behavioural response of control and experimental rats on working errors in radial arm maze test

Animal status	Mean working errors to criterion
Control	**36.5 ± 3.2**
Epileptic	**46.9 ± 3.8** [a]
E+WS	**36.5 ± 3.4** [b, d]
E+WA	**35.7 ± 3.1** [b, d]
E+CBZ	**42.3 ± 2.8** [a, e]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure-8

Behavioural response of control and experimental rats in Y maze test

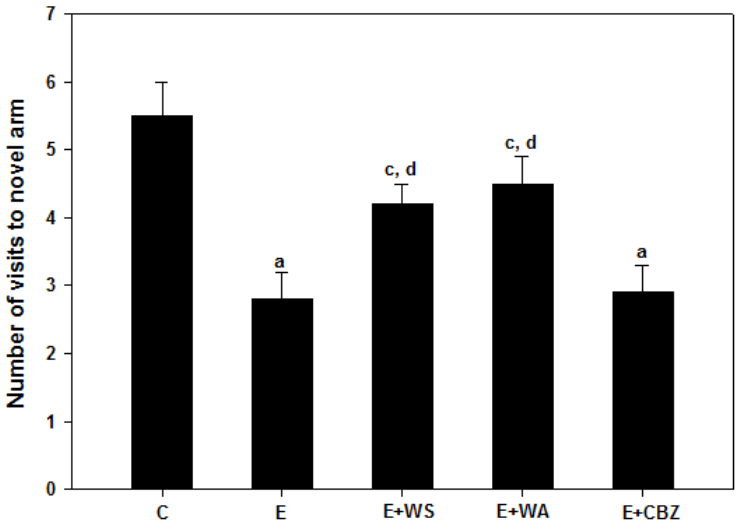


Table-5

Behavioural response of control and experimental rats in Y maze test

Animal status	Number of visits to novel arm
Control	**5.50± 0.5**
Epileptic	**2.80± 0.4** [a]
E+WS	**4.20± 0.3** [c, d]
E+WA	**4.50± 0.4** [c, d]
E+CBZ	**2.90± 0.4** [a]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-9

Behavioural response of control and experimental rats in rotarod test

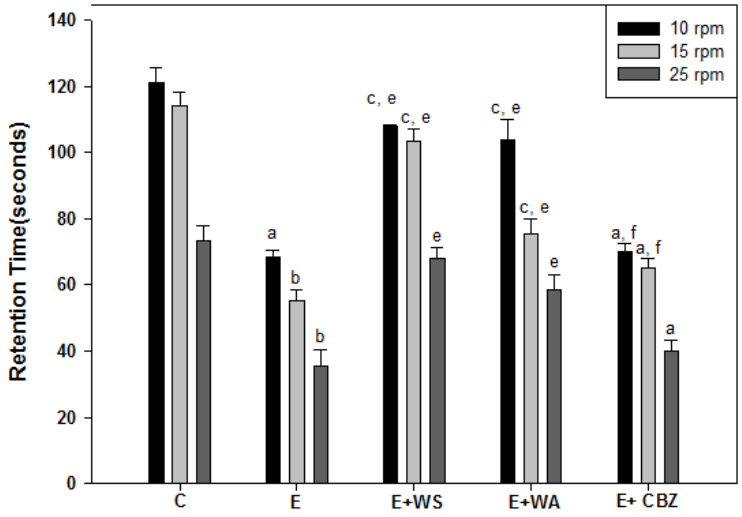


Table -6

Behavioural response of control and experimental rats in rotarod test

Animal status	Retention Time on the Rod (in seconds)		
	10 rpm	15 rpm	25 rpm
Control	121.00 ± 4.65	114.33 ± 3.98	73.34 ± 4.78
Epileptic	68.33 ± 2.40 [a]	55.33 ± 3.38 [b]	35.43 ± 4.85 [b]
E+WS	108.00 ± 0.47 [c, e]	103.45 ± 3.70 [c, e]	68.00 ± 3.36 [e]
E+WA	104.00 ± 6.12 [c, e]	75.45 ± 4.48 [c, e]	58.33 ± 4.95 [e]
E+CBZ	70.20 ± 2.4 [b, f]	65.00 ± 3.2 [b, f]	40.10± 3.1 [b, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group,[b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group,[f] $p < 0.05$ when compared with epileptic group.

Figure-10

Behavioural response of control and experimental rats in grid walk test

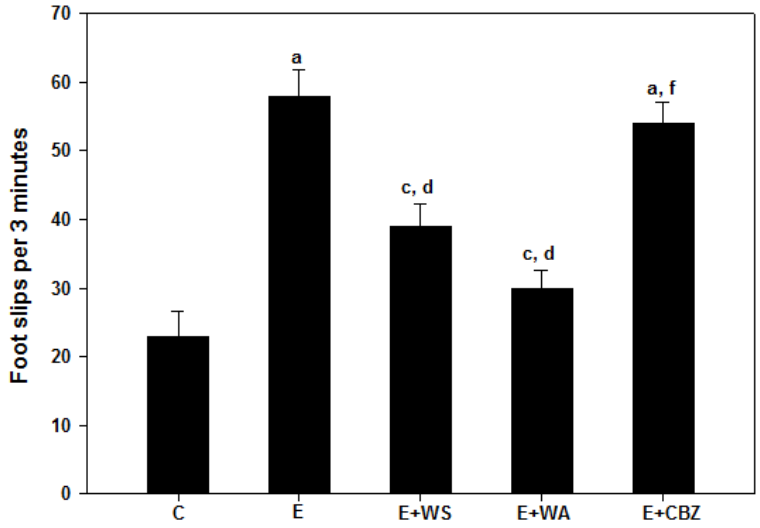


Table- 7

Behavioural response of control and experimental rats in grid walk test

Animal status	Foot slips per 3 minutes
Control	**23.1 ± 3.7**
Epileptic	**58.2 ± 3.9[a]**
E+WS	**39.2 ± 3.2[c, d]**
E+WA	**30.1 ± 2.6[c, d]**
E+CBZ	**54.2 ± 3.2[a. f]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure- 11

Behavioural response of control and experimental rats in narrow beam test

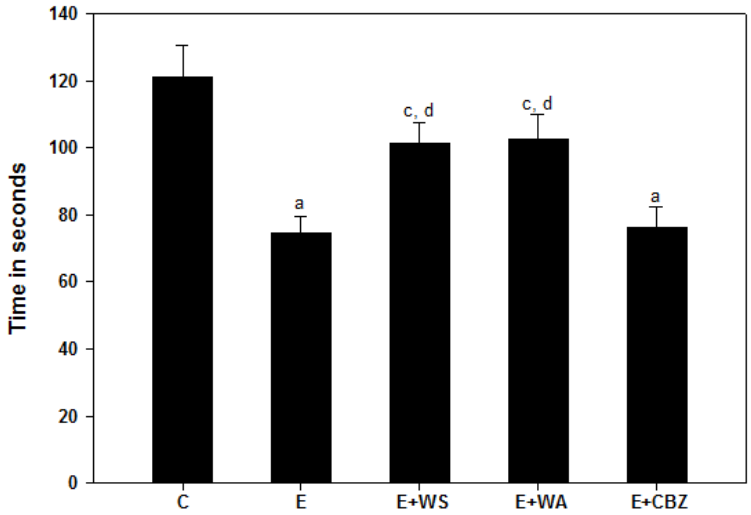


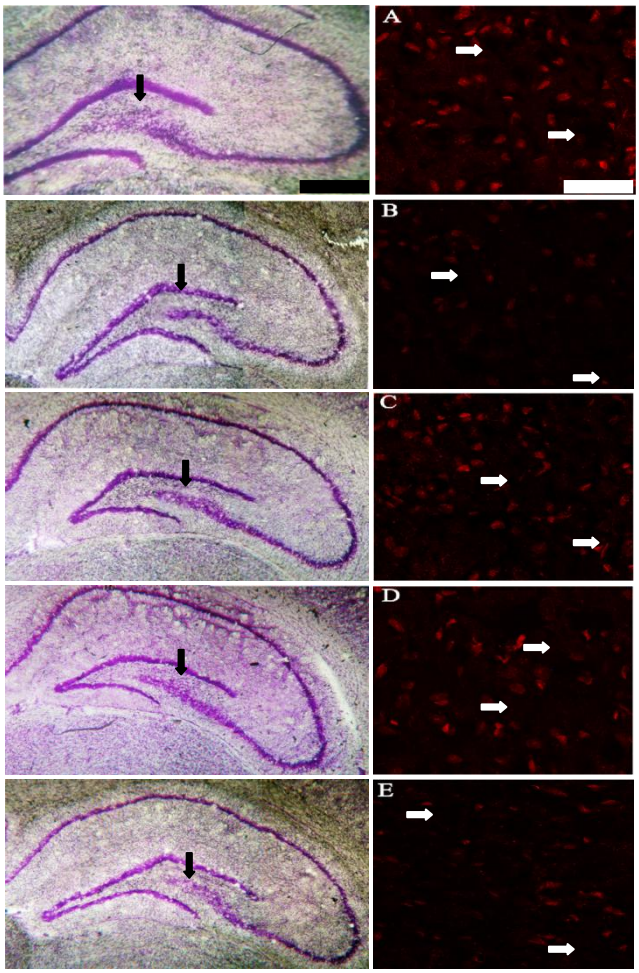
Table- 8

Behavioural response of control and experimental rats in narrow beam test

Animal status	Time in seconds
Control	**121.30± 9.4**
Epileptic	**74.50± 5.1** [a]
E+WS	**101.30± 6.2** [c, d]
E+WA	**102.60± 7.5** [c, d]
E+CBZ	**76.30± 6.3**[a]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-12
Nissl and TOPRO-3 staining in hippocampus of control and experimental rats



Nissl and TOPRO-3 staining of hippocampus in control and experimental rats. (➡) in black shows Nissl stained neurons in hilar region of hippocampus. (➡) in white shows TOPRO-3 stained neurons in the hilar region of hippocampus.A – Control, B – Epileptic, C – Epileptic rats treated with *Withania somnifera* (WS), D – Epileptic rats treated with Withaniolide-A (WA), D – Epileptic rats treated with Carbamazepine (CBZ), Scale bar = 400µm (Nissl stained hippocampal section), 50 µm(TOPRO-3 stained hippocampal section) .

Figure-13
Lipid peroxidation assay in the hippocampus of control and experimental rats

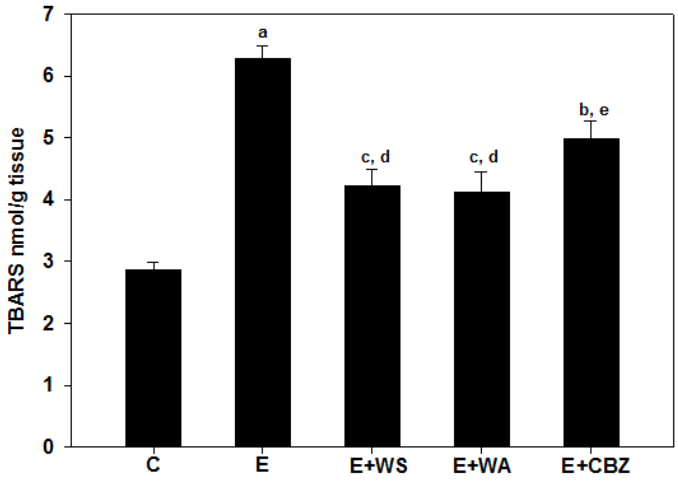


Table-9
Lipid peroxidation assay in the hippocampus of control and experimental rats

Animal Status	TBARS (nmol MDA/mg protein)
Control	**2.87± 0.12**
Epileptic	**6.29± 0.21[a]**
E+WS	**4.21± 0.28 [c, d]**
E+WA	**4.12±0.32 [c, d]**
E+CBZ	**4.98±0.29 [b, e]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure-14
Superoxide dismutase assay in the hippocampus of control and experimental rats

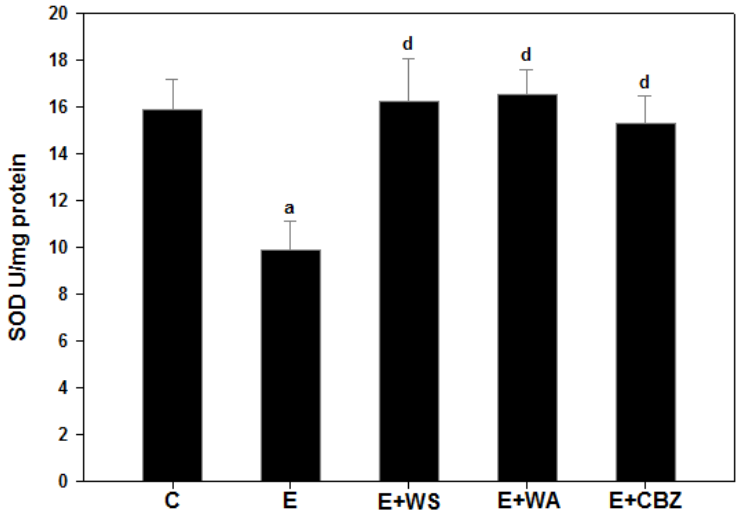


Table-10
Superoxide dismutase assay in the hippocampus of control and experimental rats

Animal Status	SOD activity (unit/mg protein)
Control	**15.90 ± 1.28**
Epileptic	**9.89 ± 1.24** [a]
E+WS	**16.25 ± 1.80** [d]
E+WA	**16.54 ± 1.05** [d]
E+CBZ	**15.29 ± 1.19** [d]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-15

Catalase assay in the hippocampus of control and experimental rats

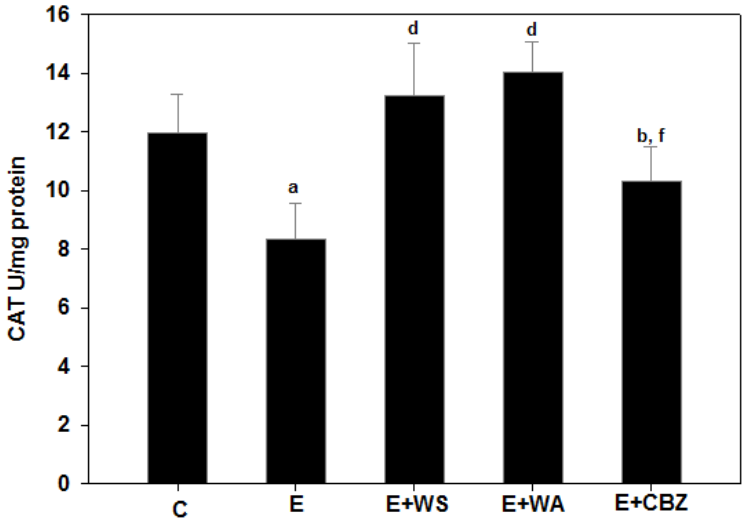


Table-11

Catalase assay in the hippocampus of control and experimental rats

Animal Status	CAT activity (ΔA_{240}/min/mg protein)
Control	**11.98± 1.28**
Epileptic	**8.32± 1.21** [a]
E+WS	**13.23± 1.83** [d]
E+WA	**14.01± 1.25** [d]
E+CBZ	**10.32±1.29** [b, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group

Figure-16
Real time PCR amplification of SOD mRNA from the hippocampus of control and experimental rats

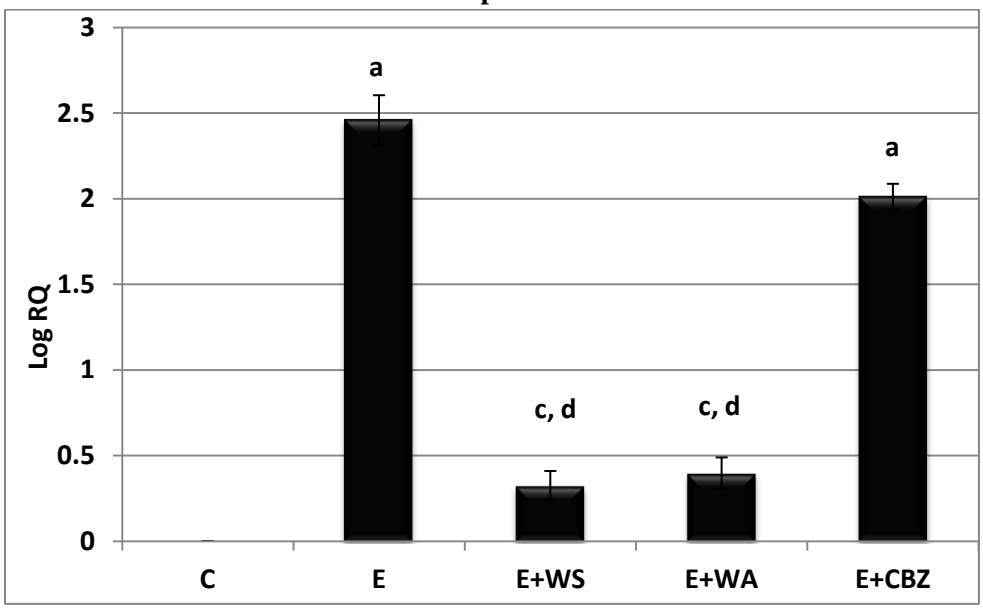


Table-12
Real time PCR amplification of SOD mRNA from the hippocampus of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**2.45 ± 0.14** [a]
E+WS	**0.32 ± 0.08** [c, d]
E+WA	**0.39 ± 0.09** [c, d]
E+CBZ	**2.01 ± 0.07** [a]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-17

Real time PCR amplification of GPx mRNA from the hippocampus of control and experimental rats

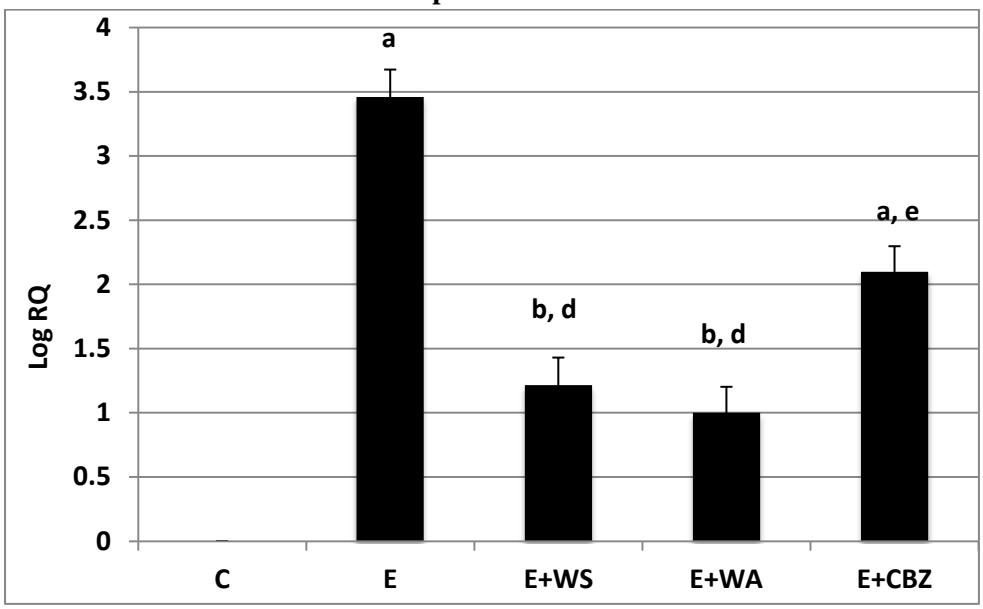


Table-13

Real time PCR amplification of GPx mRNA from the hippocampus of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**3.45 ± 0.21[a]**
E+WS	**1.21 ± 0.22 [b, d]**
E+WA	**1.00 ± 0.20 [b, d]**
E+CBZ	**2.09 ± 0.19 [a,e]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Table- 14
Glutamate content in the hippocampus of control and experimental rats

Animal Status	Glutamate content (nmoles/g wt. of tissue)
Control	**192.41 ± 4.32**
Epileptic	**389.32 ± 7.62** [a]
E+WS	**199.24 ± 5.25** [d]
E+WA	**204.56 ± 3.2** [d]
E +CBZ	**228.45± 4.2** [c, e]

Table- 15
Glutamate dehydrogenase activity in the hippocampus of control and experimental rats

Experimental group	Vmax (mmol/min/mg protein)	Km (mM)
Control	**0.77 ± 0.04**	**0.071 ± 0.09**
Epileptic	**0.92 ± 0.03** [a]	**0.112 ± 0.07**
E+WS	**0.79 ± 0.02** [d]	**0.106 ± 0.08**
E+WA	**0.80 ± 0.01** [d]	**0.105 ± 0.07**
E+CBZ	**0.89 ± 0.02**	**0.108 ± 0.08**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure-18
Real time PCR amplification of GLAST mRNA from the hippocampus of control and experimental rats

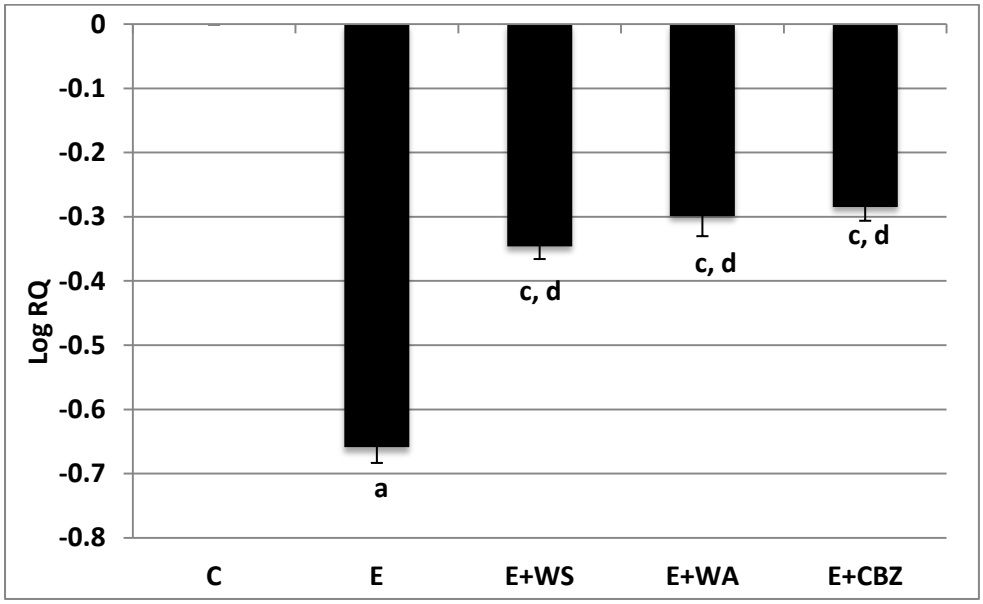


Table-16
Real time PCR amplification of GLAST mRNA from the hippocampus of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.65 ± 0.02** [a]
E+WS	**-0.34 ± 0.01** [c, d]
E+WA	**-0.29 ± 0.03** [c, d]
E+CBZ	**-0.28 ± 0.02** [c, d]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-19

Real time PCR amplification of GAD mRNA from the hippocampus of control and experimental rats

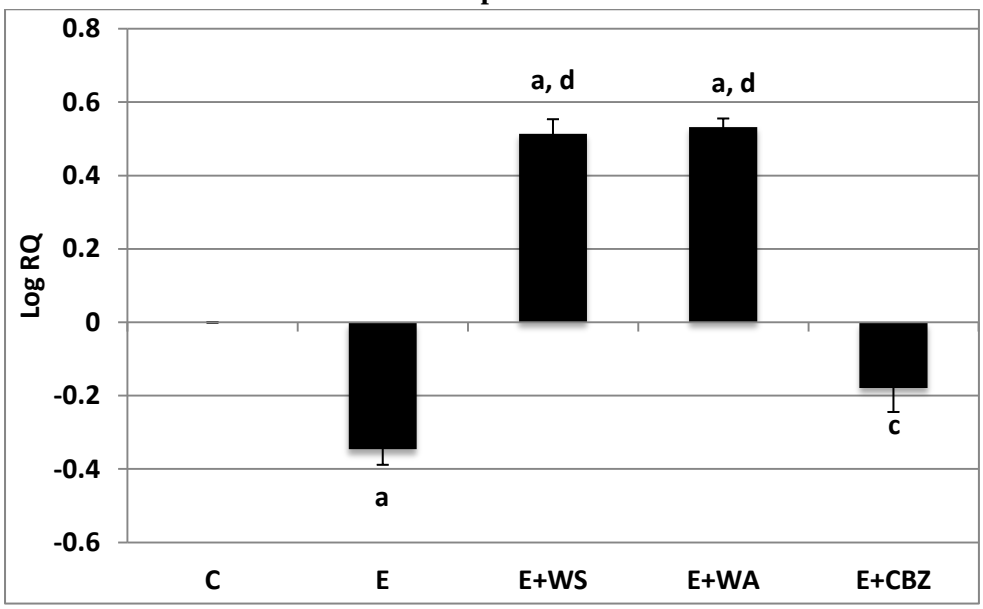


Table-17
Real time PCR amplification of GAD mRNA from the hippocampus of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.34± 0.04** [a]
E+WS	**0.51± 0.03** [a, d]
E+WA	**0.53 ± 0.02** [a, d]
E+CBZ	**-0.17 ± 0.06** [c]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-20

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the hippocampus of control and experimental rats

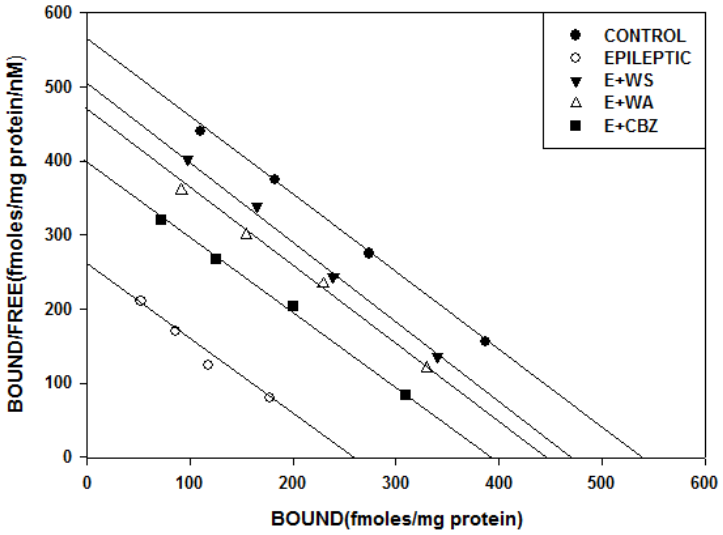


Table-18

Scatchard analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the hippocampus of control and experimental rats

Experimental groups	B_{max} (fmoles/mg protein)	K_d (nM)
Control	**529 ± 14.84**	**0.93 ± 0.08**
Epileptic	**253 ± 22.81** [a]	**1.01 ± 0.09**
E+WS	**469 ± 19.23** [c, d]	**0.92 ± 0.04**
E+WA	**446 ± 17.65** [c, d]	**0.93 ± 0.06**
E+CBZ	**391 ± 22.5** [b, e]	**0.98 ± 0.02**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group). B_{max} – Maximal binding; K_d – Dissociation constant; C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, Epileptic rats treated with carbamazepine; ; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure-21

Scatchard analysis of AMPA receptor using [^{3}H]AMPA binding against AMPA in the hippocampus of control and experimental rats

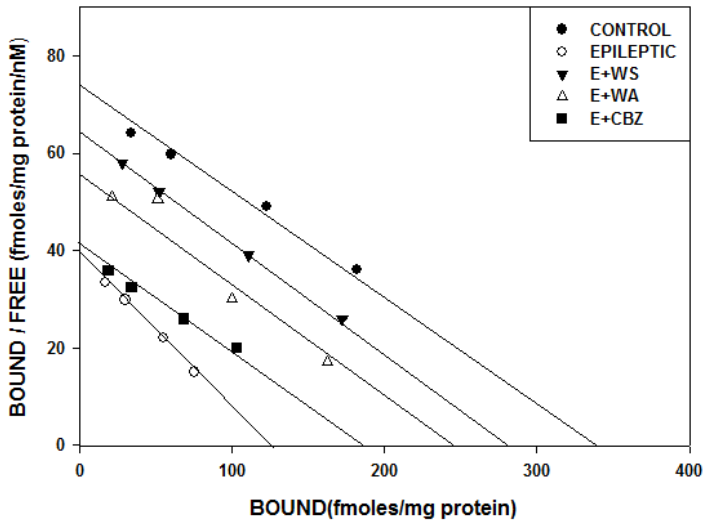


Table-19

Scatchard analysis of AMPA receptor using [^{3}H]AMPA binding against AMPA in the hippocampus of control and experimental rats

Experimental group	Bmax (fmol/mg protein)	Kd (nM)
Control	**338.31 ± 3.09**	**4.61± 0.08**
Epileptic	**124.22 ± 4.52** [a]	**3.23± 0.06**
E+WS	**279.18 ± 2.12** [b, d]	**4.31± 0.08**
E+WA	**244.35 ± 3.16** [b, d]	**4.44± 0.07**
E+CBZ	**182.21 ±3.45** [c, f]	**4.32±0.08**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group). B_{max} – Maximal binding; K_d – Dissociation constant; C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-22

Real time PCR amplification of NMDA R1 receptor subunit mRNA from the hippocampus of control and experimental rats

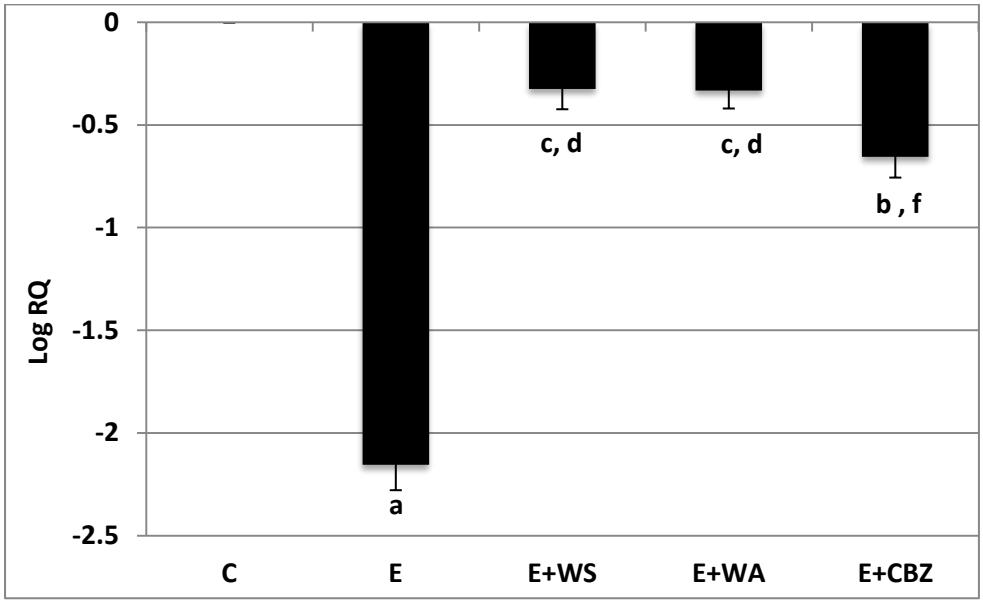


Table-20

Real time PCR amplification of NMDA R1 receptor subunit mRNA from the hippocampus of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-2.15± 0.12** [a]
E+WS	**-0.32± 0.09** [c, d]
E+WA	**-0.33± 0.08** [c,d]
E+CBZ	**-0.65± 0.10** [b, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-23

Real time PCR amplification of NMDA 2B receptor subunit mRNA from the hippocampus of control and experimental rats

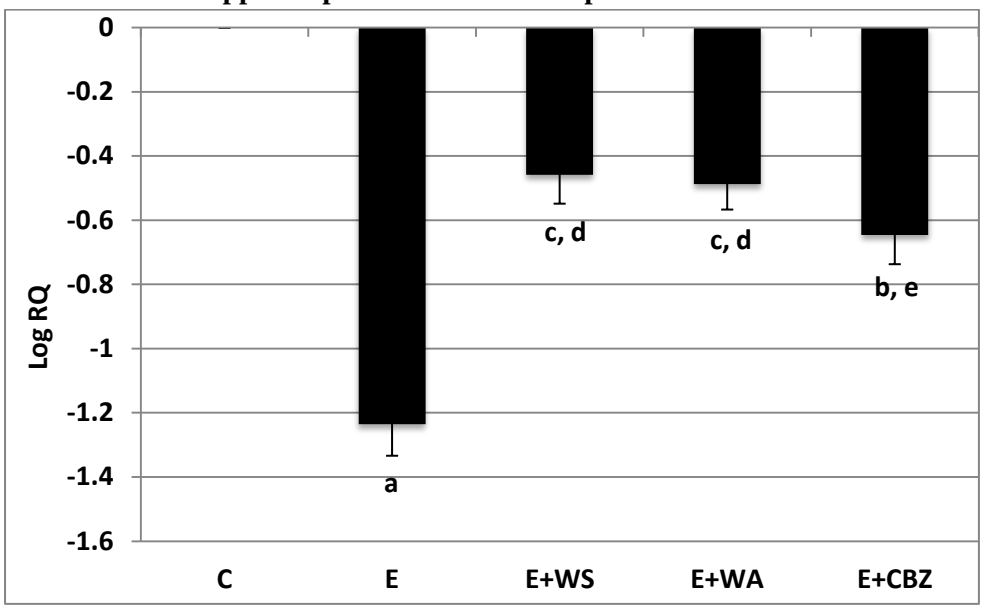


Table-21

Real time PCR amplification of NMDA 2B receptor mRNA from the hippocampus of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-1.23 ± 0.09** [a]
E+WS	**-0.45± 0.08** [c, d]
E+WA	**-0.48± 0.07** [c, d]
E+CBZ	**-0.64 ± 0.09** [b, e]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure-24

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the hippocampus of control and experimental rats

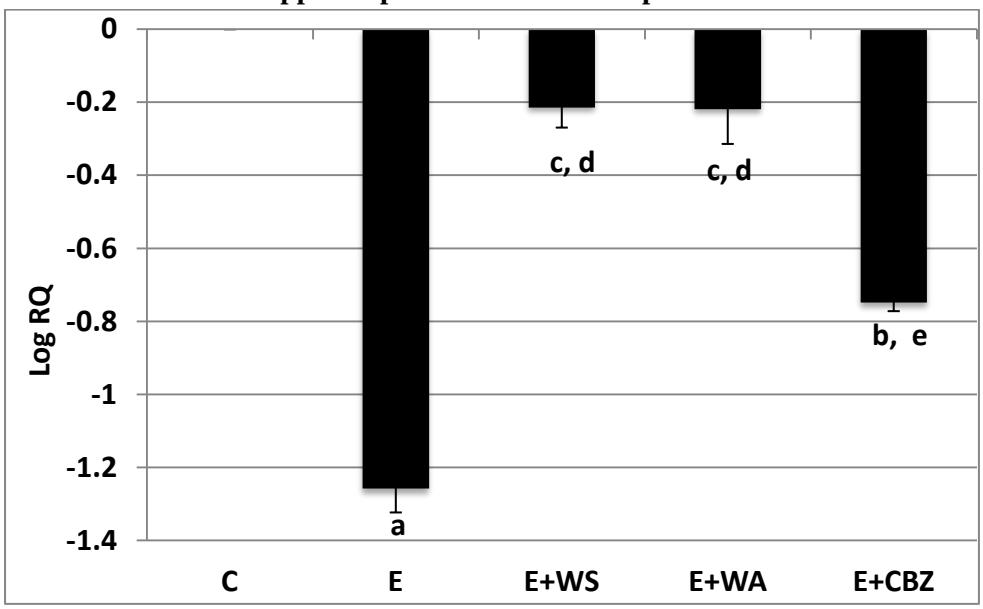


Table-22

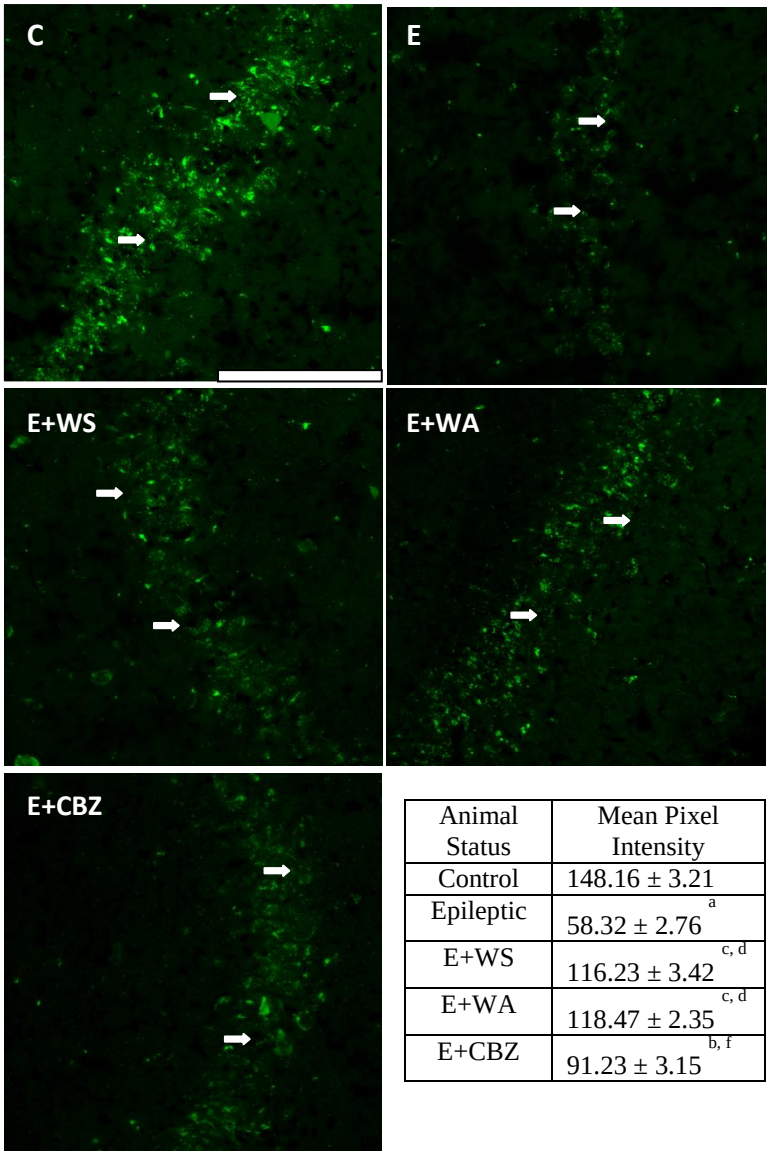
Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the hippocampus of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-1.25 ± 0.06[a]**
E+WS	**-0.21 ± 0.01[c, d]**
E+WA	**-0.21 ± 0.09 [c, d]**
E+CBZ	**-0.74 ± 0.02[b, e]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure-25

NMDA R1 receptor subunit expression in the hippocampus of control and experimental rats

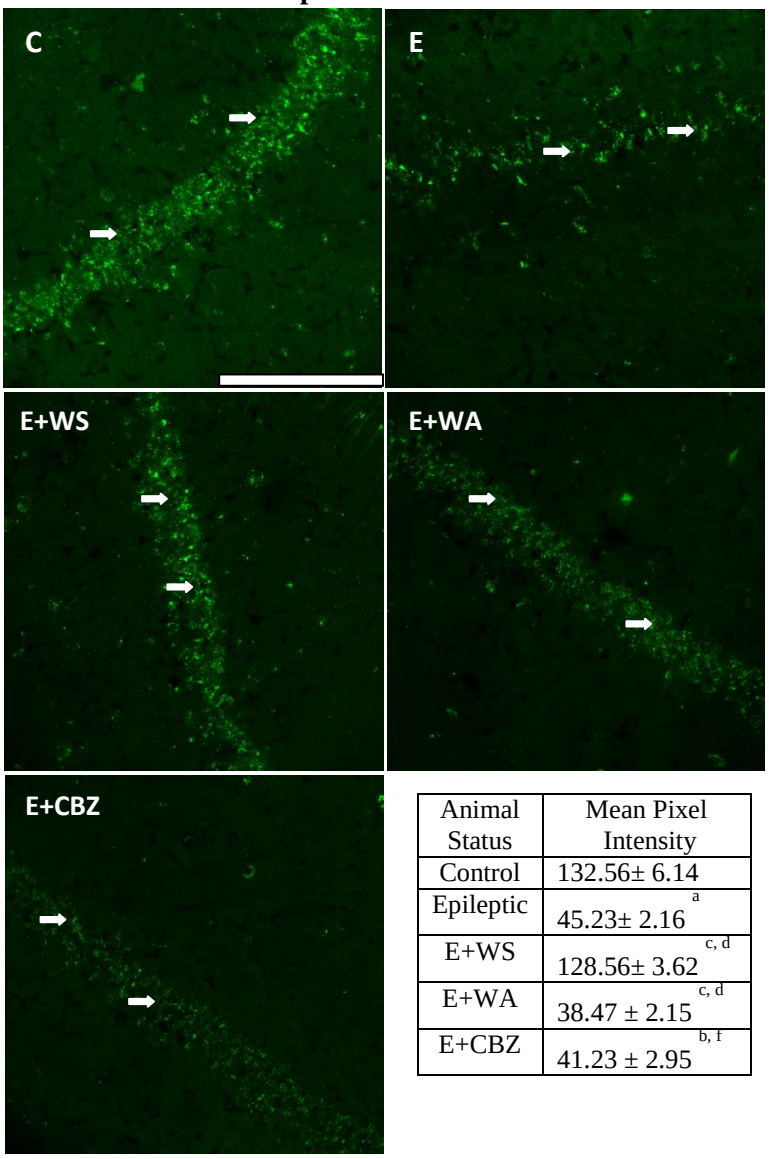


Animal Status	Mean Pixel Intensity
Control	148.16 ± 3.21
Epileptic	58.32 ± 2.76 [a]
E+WS	116.23 ± 3.42 [c, d]
E+WA	118.47 ± 2.35 [c, d]
E+CBZ	91.23 ± 3.15 [b, f]

Confocal image of NMDA R1 receptors in the Hippocampus of C-Control, E-Epileptic, E+WS-Epileptic+*Withania somnifera*, E+WA-Epileptic +Withanolide-A, E+CBZ-Epileptic+Carbamazepinerats using immunofluorescent NMDA R1 receptor specific primary antibody and FITC as secondary antibody;[a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [f] $p<0.05$ when compared to epileptic group; (⟶) in white shows NMDA R1 receptors. Scale bar = 150 µm.

Figure: 26

NMDA 2B receptor subunit expression in the hippocampus of control and experimental rats

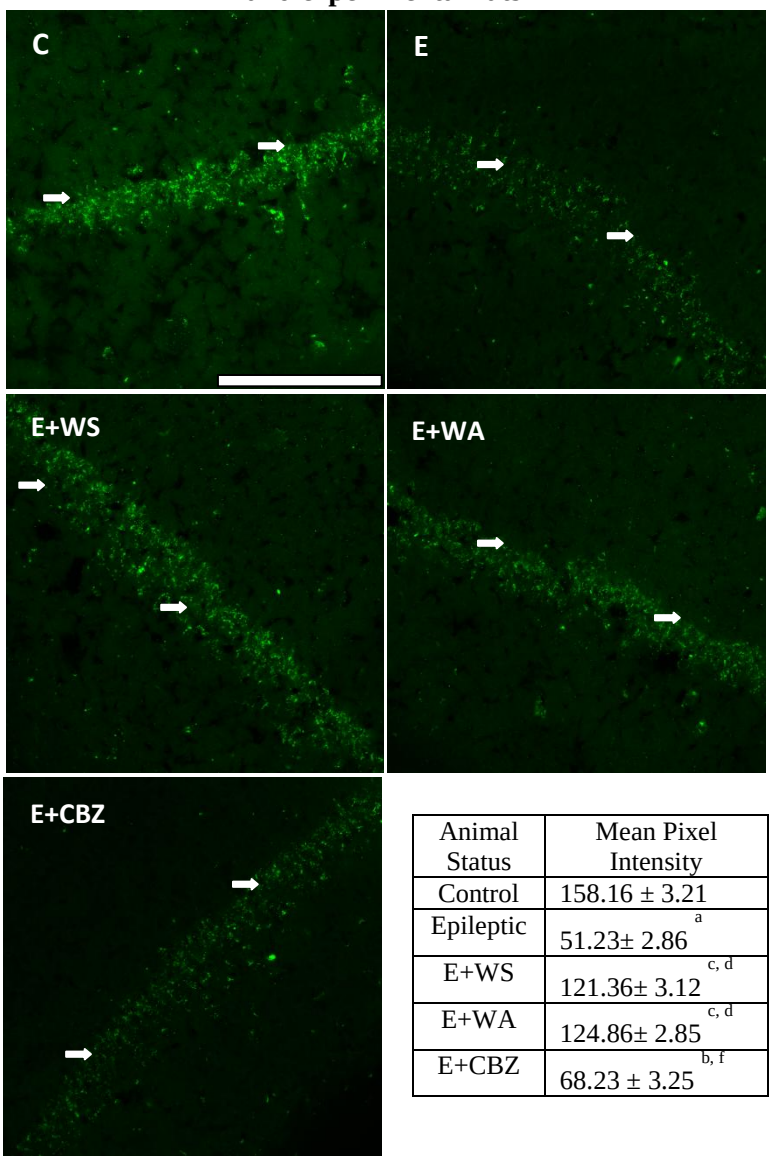


Animal Status	Mean Pixel Intensity
Control	132.56± 6.14
Epileptic	45.23± 2.16 [a]
E+WS	128.56± 3.62 [c, d]
E+WA	38.47 ± 2.15 [c, d]
E+CBZ	41.23 ± 2.95 [b, f]

Confocal image of NMDA 2B receptors in the Hippocampus of C-Control, E-Epileptic, E+WS-Epileptic+*Withania somnifera*, E+WA-Epileptic+Withanolide-A, E+CBZ-Epileptic+Carbamazepine rats using immunofluorescentNMDA 2B receptor specific primary antibody and Alexa Fluor488 as secondary antibody;[a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [f] $p<0.05$ when compared to epileptic group; (⟶) in white shows NMDA 2B receptors. Scale bar = 150 μm.

Figure: 27

AMPA (GluR2) receptor subunit expression in the hippocampus of control and experimental rats



Animal Status	Mean Pixel Intensity
Control	158.16 ± 3.21
Epileptic	51.23± 2.86 [a]
E+WS	121.36± 3.12 [c, d]
E+WA	124.86± 2.85 [c, d]
E+CBZ	68.23 ± 3.25 [b, f]

Confocal image of AMPA receptors in the Hippocampus of C-Control, E-Epileptic, E+WS- Epileptic+ *Withania somnifera*, E+WA-Epileptic + Withanolide-A, E+CBZ-Epileptic + Carbamazepine rats using immunofluorescent AMPA GluR2 receptor subunit specific primary antibody and FITC as secondary antibody;[a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [f] $p<0.05$ when compared to epileptic group; (⟶) in white shows AMPA receptors. Scale bar = 150 μm.

Figure- 28

IP3 content in the hippocampus of control and experimental rats

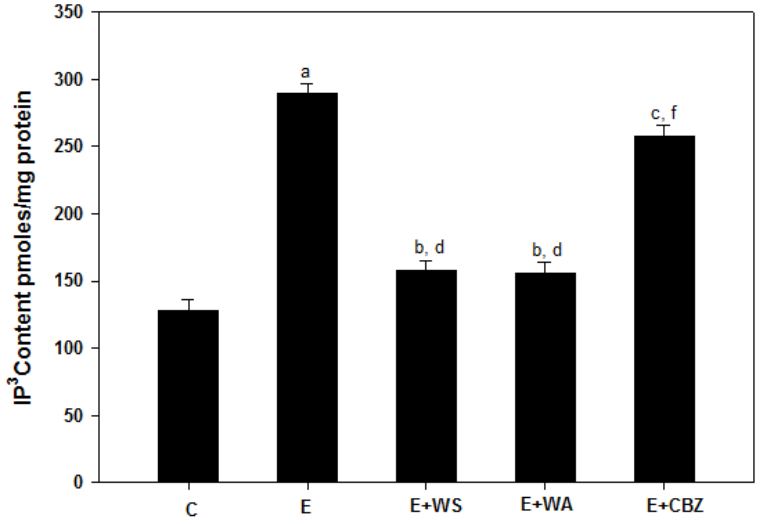


Table- 23

IP3 content in the hippocampus of control and experimental rats

Animal Status	IP3 Content (pmoles/mg protein)
Control	**128.12 ± 8.21**
Epileptic	**289.24 ± 7.56** [a]
E+WS	**158.38 ± 6.81** [b, d]
E+WA	**156.14 ± 8.31** [b, d]
E+CBZ	**258.90 ± 7.90** [c, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group

Figure-29

Real time PCR amplification of Bax mRNA from the hippocampus of control and experimental rats

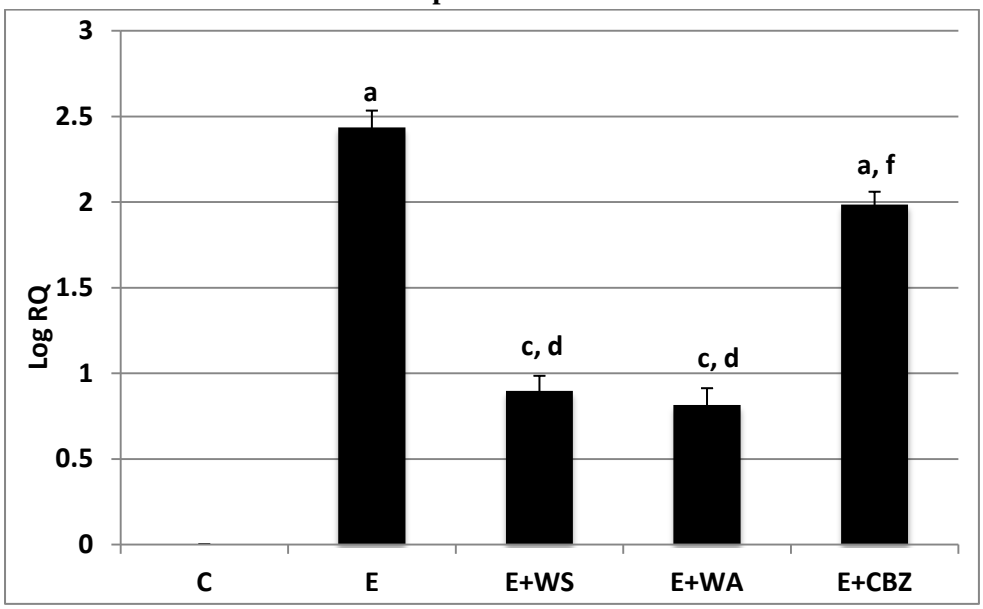


Table-24

Real time PCR amplification of Bax mRNA from the hippocampus of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**2.43 ± 0.09** [a]
E+WS	**0.89 ± 0.08** [c, d]
E+WA	**0.81 ± 0.09** [c, d]
E+CBZ	**1.98 ± 0.07** [a, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure-30

Real time PCR amplification of Caspase 8 mRNA from the hippocampus of control and experimental rats

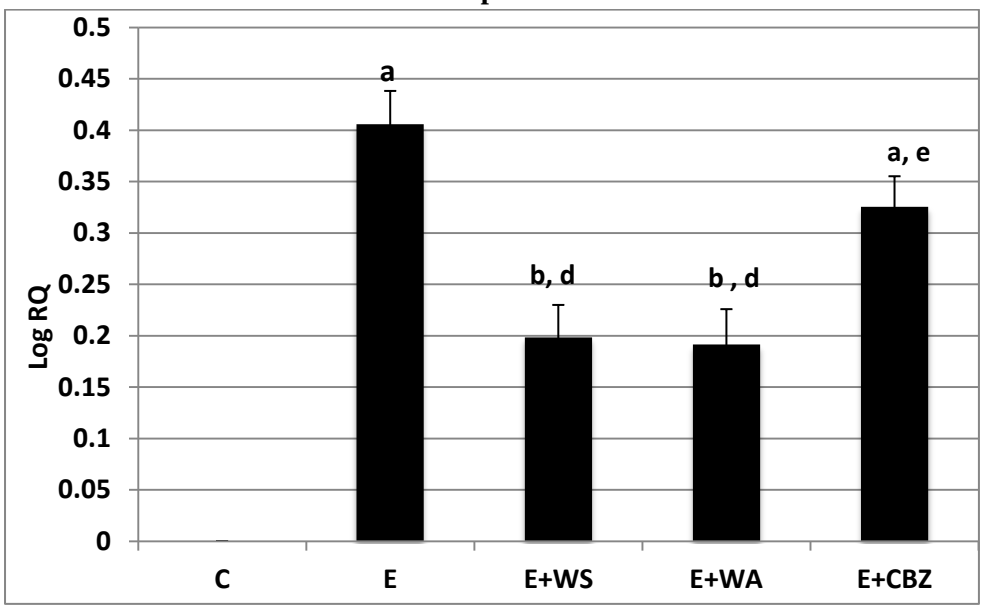


Table-25

Real time PCR amplification of Caspase 8 mRNA from the hippocampus of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**0.40 ± 0.03** [a]
E+WS	**0.19 ± 0.03** [b, d]
E+WA	**0.19 ± 0.03** [b, d]
E+CBZ	**0.32 ± 0.02** [a, e]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure- 31

Real time PCR amplification of Akt-1 mRNA from the hippocampus of control and experimental rats

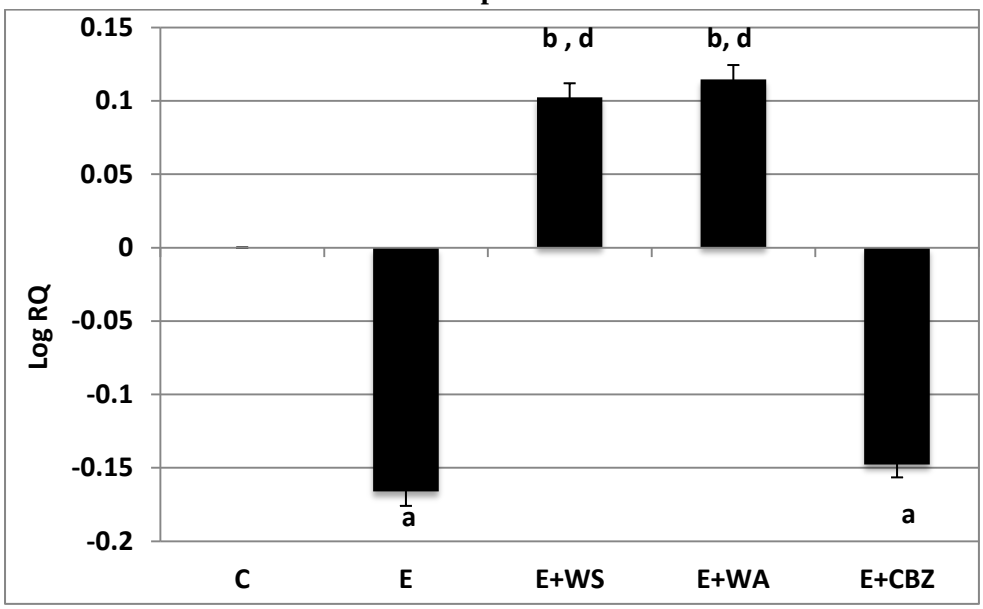


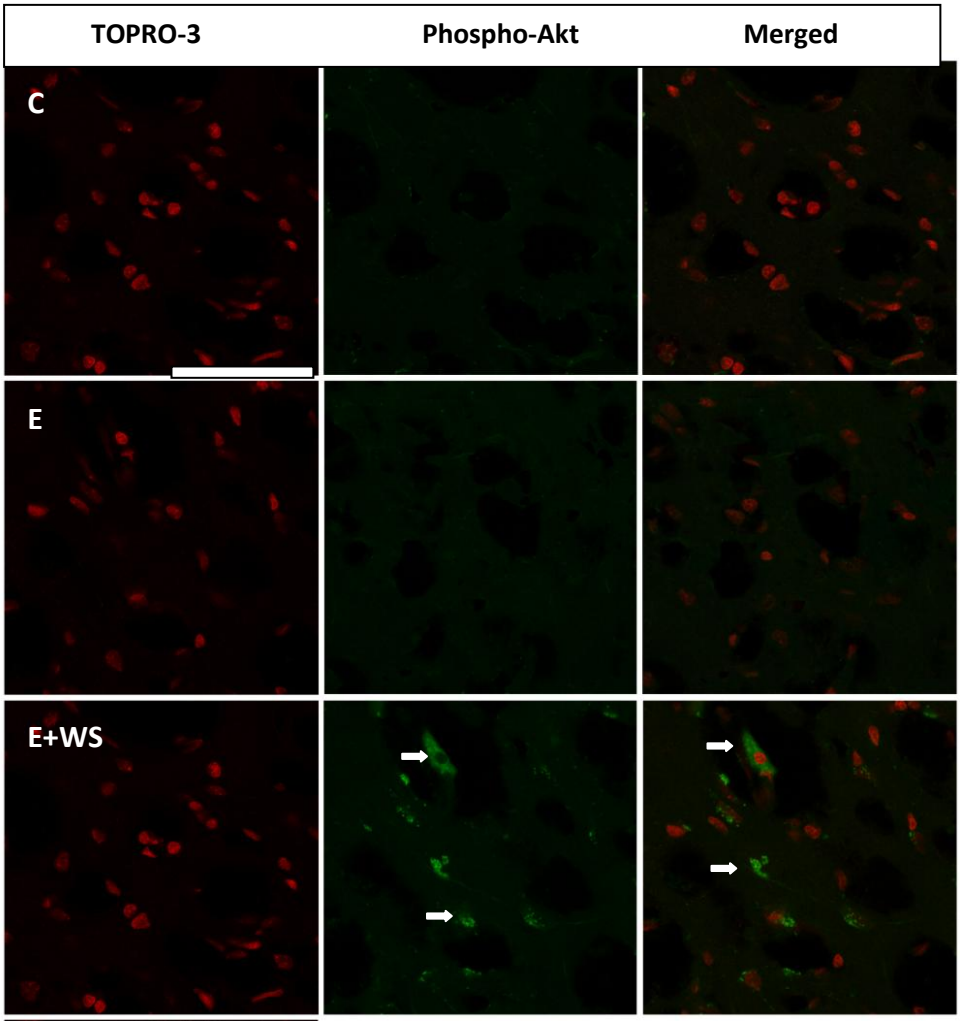
Table-26

Real time PCR amplification of Akt-1 mRNA from the hippocampus of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.16 ±0.09** [a]
E+WS	**0.10 ±0.09** [b, d]
E+WA	**0.11 ±0.08** [b, d]
E+CBZ	**-0.14 ±0.09** [a]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure: 32
Phospho-Akt expression in the hippocampus of control and experimental rats



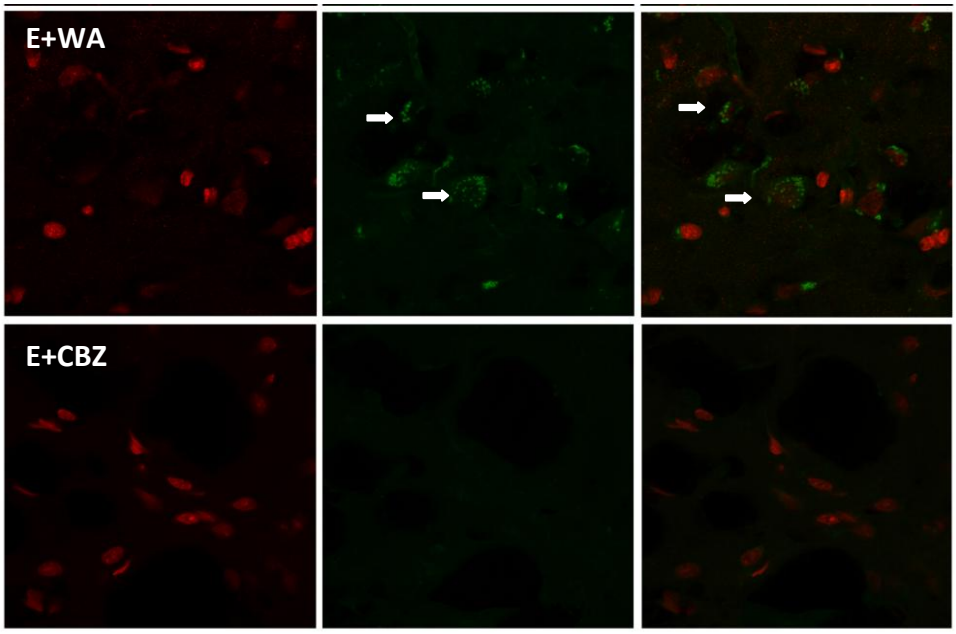
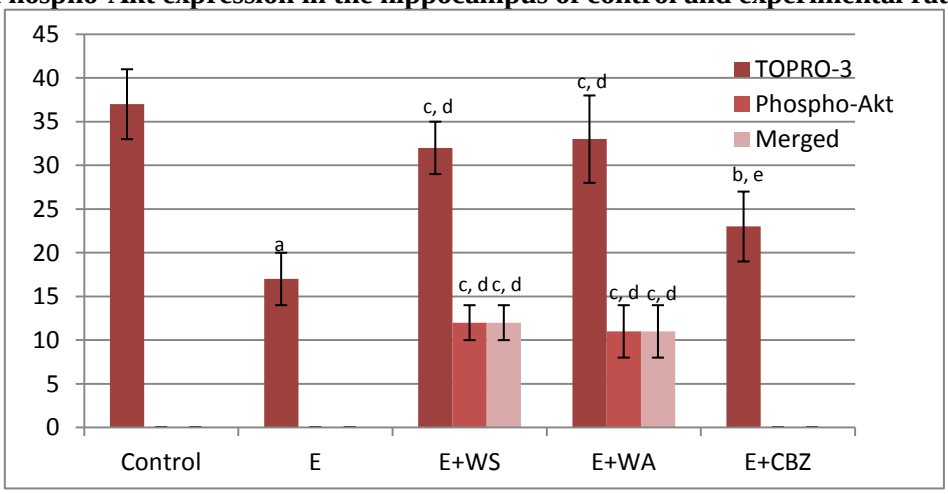


Figure: 33
Phospho-Akt expression in the hippocampus of control and experimental rats



Double immunofluorescent staining for the identification of Phospho-Akt and TOPRO-3 positive cells in the hippocampus ofC-Control, E-Epileptic, E+WS-Epileptic +*Withania somnifera*, E+WA-Epileptic +Withanolide-A, E+CBZ- Epileptic + Carbamazepine. ; [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [e] $p<0.01$ when compared to epileptic group. (⇨) in white shows Phospo-Akt positive cells, Scale bar = 50 µm.

Figure-34

Lipid peroxidation assay in the cerebral cortex of control and experimental rats

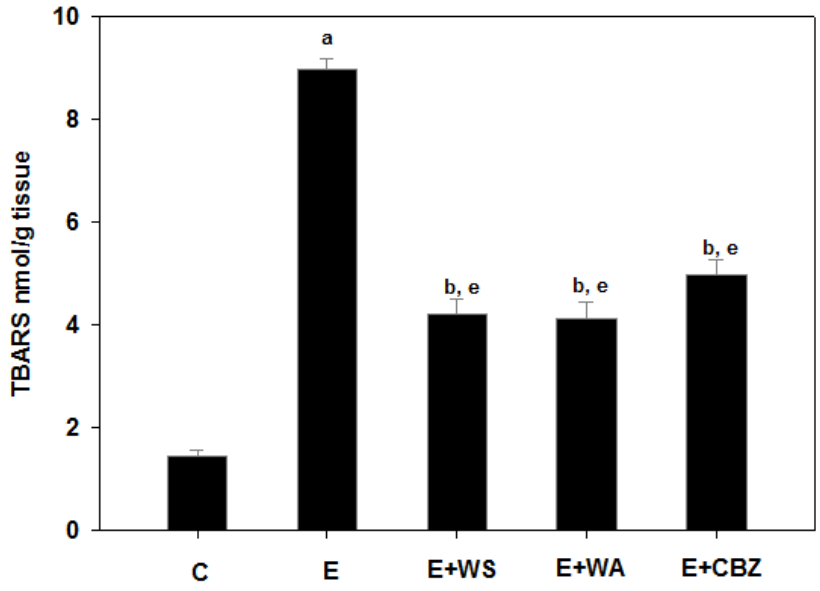


Table-27

Lipid peroxidation assay in the cerebral cortex of control and experimental rats

Animal Status	TBARS (nmol MDA/mg protein)
Control	**1.45 ± 0.12**
Epileptic	**8.98 ± 0.21[a]**
E+WS	**4.21 ± 0.28 [b, e]**
E+WA	**4.12 ± 0.32 [b, e]**
E+CBZ	**4.98 ± 0.29 [b, e]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-35

Superoxide dismutase assay in the cerebral cortex of control and experimental rats

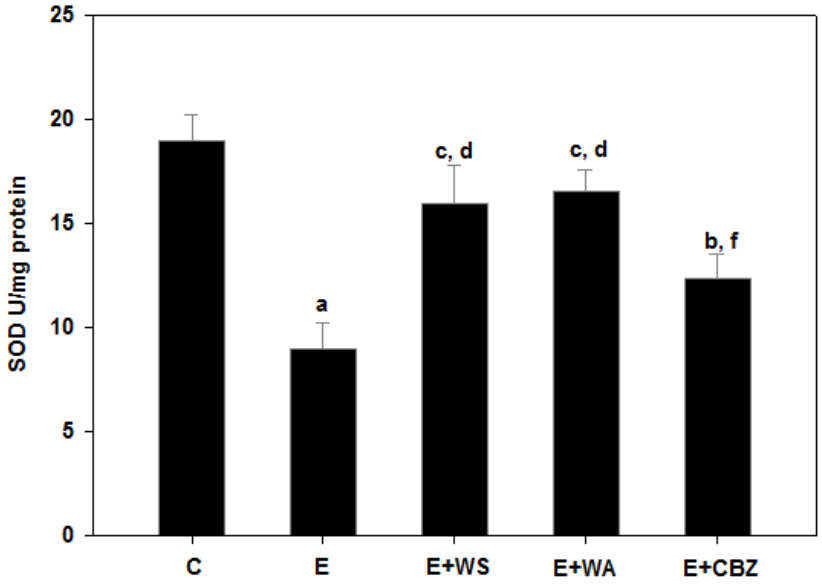


Table-28

Superoxide dismutase assay in the cerebral cortex of control and experimental rats

Animal Status	SOD activity (unit/mg protein)
Control	**18.98± 1.28**
Epileptic	**8.94 ± 1.24** [a]
E+WS	**16.00 ± 1.80** [c, d]
E+WA	**16.54± 1.05** [c, d]
E+CBZ	**12.36± 1.19** [b, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-36
Catalase assay in cerebral cortex of control and experimental animals

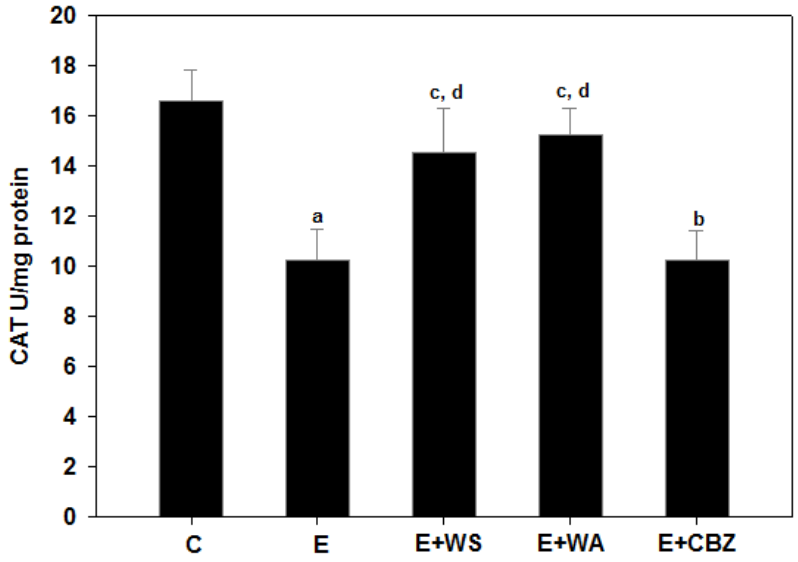


Table-29
Catalase assay in the cerebral cortex of control and experimental rats

Animal Status	CATactivity (ΔA_{240}/min/mg protein)
Control	**16.58 ± 1.28**
Epileptic	**10.23 ± 1.24** [a]
E+WS	**14.52 ± 1.80** [c, d]
E+WA	**15.28 ± 1.05** [c, d]
E+CBZ	**10.23 ± 1.19** [b]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-37
Real time PCR amplification of SOD mRNA from the cerebral cortex of control and experimental rats

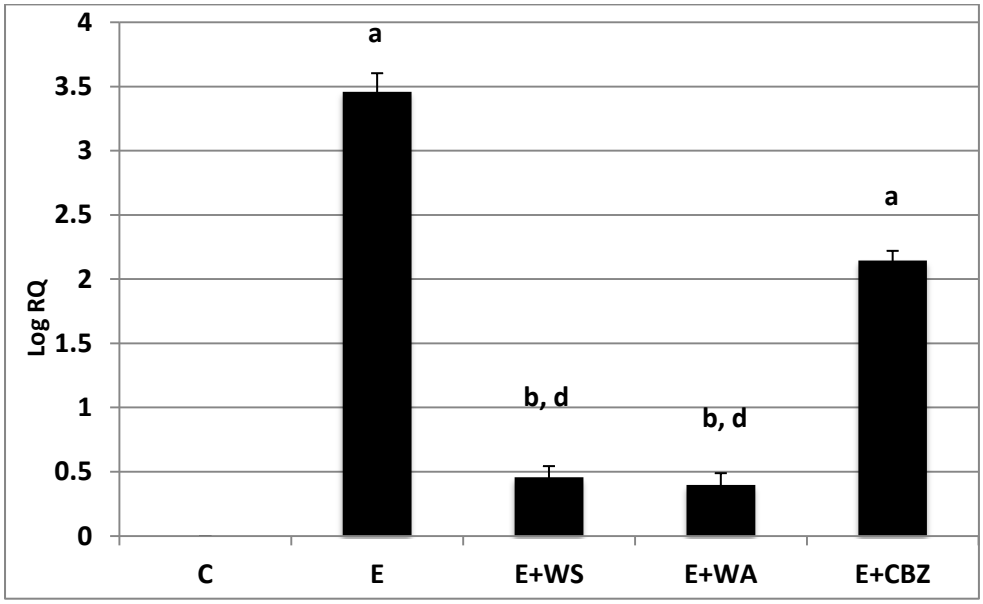


Table-30
Real time PCR amplification of SOD mRNA from the cerebral cortex of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**3.45 ± 0.14[a]**
E+WS	**0.45 ± 0.08 [b, d]**
E+WA	**0.39 ± 0.09 [b, d]**
E+CBZ	**2.14 ± 0.07 [a]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group [d] $p < 0.001$ when compared with epileptic group

Figure-38
Real time PCR amplification of GPx mRNA from the cerebral cortex of control and experimental rats

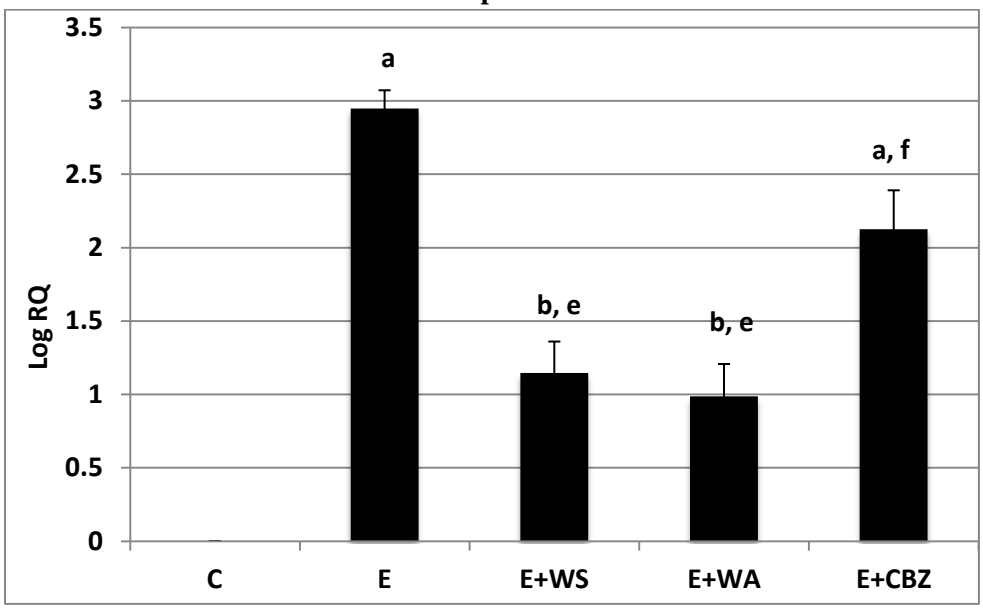


Table-31
Real time PCR amplification of GPx mRNA from the cerebral cortex of control and experimental rats

Animal Status	Log RQ
Control	0
Epileptic	2.94± 0.12[a]
E+WS	1.14± 0.21[b, e]
E+WA	0.98± 0.22[b, e]
E+CBZ	2.12± 0.26 [a, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [e] $p < 0.01$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Table- 32

Glutamate content in the cerebral cortex of control and experimental rats

Animal Status	Glutamate content (nmoles/g wt. of tissue)
Control	**158.41 ± 3.87**
Epileptic	**419.32 ± 6.72** [a]
E +WS	**189.24 ± 5.25** [d]
E +WA	**175.56 ± 3.2** [d]
E +CBZ	**228.45± 4.2** [c, e]

Table- 33

Glutamate dehydrogenase activity in the cerebral cortex of control and experimental rats

Experimental group	Vmax (mmol/min/mg protein)	Km (mM)
Control	**0.701 ± 0.04**	**0.073 ± 0.03**
Epileptic	**0.978 ± 0.03** [a]	**0.101 ± 0.05**
E+WS	**0.787 ± 0.02** [d]	**0.114 ± 0.04**
E+WA	**0765 ± 0.019** [d]	**0.121 ± 0.06**
E+CBZ	**0.892 ±0.02** [f]	**0.103 ± 0.08**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-39
Real time PCR amplification of GLAST mRNA from the cerebral cortex of control and experimental rats

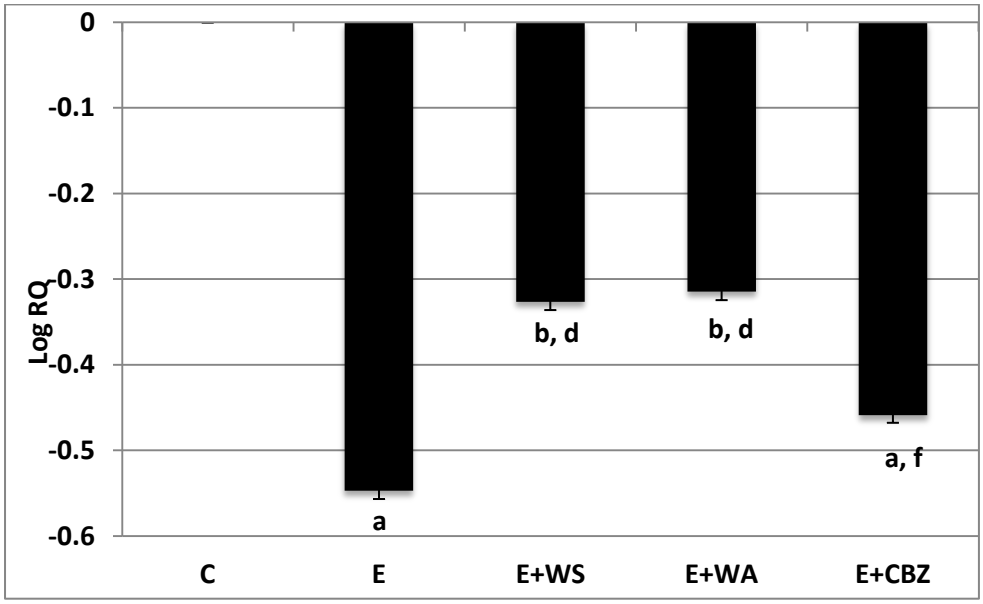


Table -34
Real time PCR amplification of GLAST mRNA from the cerebral cortex of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.542 ± 0.009** [a]
E+WS	**-0.323 ± 0.009** [b, d]
E+WA	**-0.312 ± 0.009** [b, d]
E+CBZ	**-0.458 ± 0.008** [a, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group

Figure-40
Real time PCR amplification of GAD mRNA from the cerebral cortex of control and experimental rats

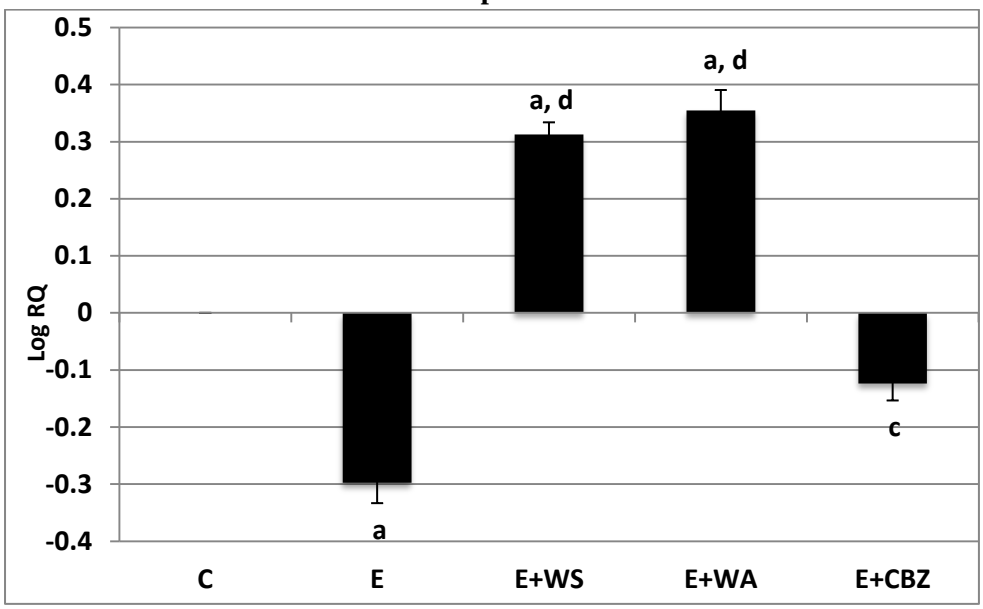


Table -35
Real time PCR amplification of GAD mRNA from the cerebral cortex of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.478 ± 0.003** [a]
E+WS	**0.312 ± 0.002** [a, d]
E+WA	**0.354 ± 0.003** [a, d]
E+CBZ	**-0.123 ± 0.002** [c]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-41
Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebral cortex of control and experimental rats

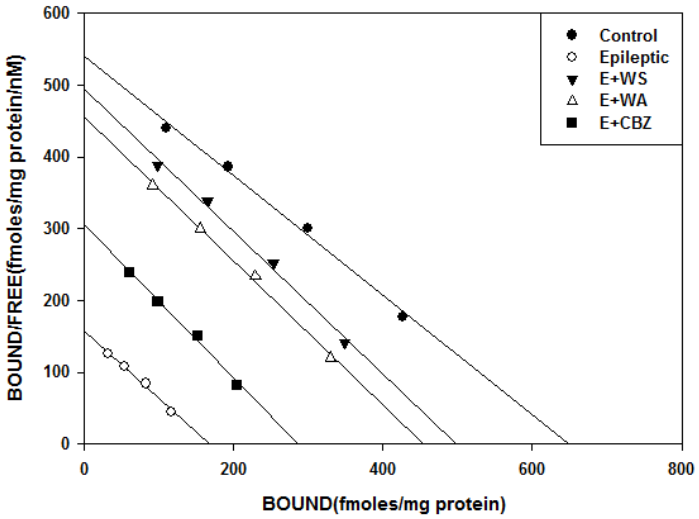


Table-36
Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebral cortex of control and experimental rats

Animal Status	Bmax (fmoles/mg protein)	Kd (nM)
Control	**692.38 ± 32.45**	**1.32 ± 0.10**
Epileptic	**165.61 ± 23.07** [a]	**1.01± 0.05**
E+WS	**497.61 ± 18.05** [c, d]	**1.02 ± 0.04**
E+WA	**453.52 ± 21.16** [c, d]	**1.00 ± 0.01**
E+CBZ	**285.91 ± 32.33** [b, e]	**1.02± 0.15**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group). Bmax – Maximal binding; Kd – Dissociation constant; C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZEpileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure-42

Scatchard analysis of AMPA receptor using [^{3}H]AMPA binding against AMPA in the cerebral cortex of control and experimental rats

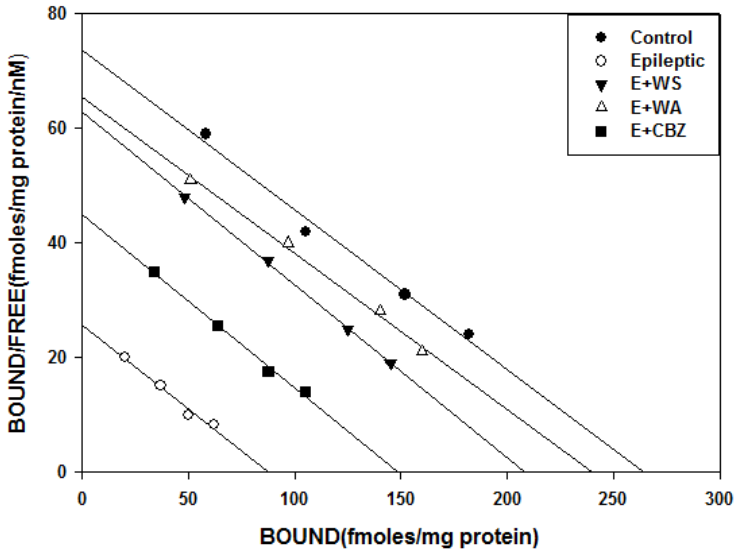


Table-37

Scatchard analysis of AMPA receptor using [^{3}H]AMPA binding against AMPA in the cerebral cortex of control and experimental rats

Animal Status	Bmax (fmoles/mg protein)	Kd (nM)
Control	**264.09 ± 18.45**	**3.51 ± 0.10**
Epileptic	**86.26 ± 11.07** [a]	**3.46 ± 0.09**
E+WS	**239.4 ± 17.05** [d]	**3.61 ± 0.07**
E+WA	**206.9 ± 19.16** [d]	**3.32 ± 0.12**
E+CBZ	**148.1 ± 28.33** [c, f]	**3.31 ± 0.11**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group). Bmax – Maximal binding; Kd – Dissociation constant; C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group

Figure-43

Real time PCR amplification of NMDA R1 receptor subunit mRNA from the cerebral cortex of control and experimental rats

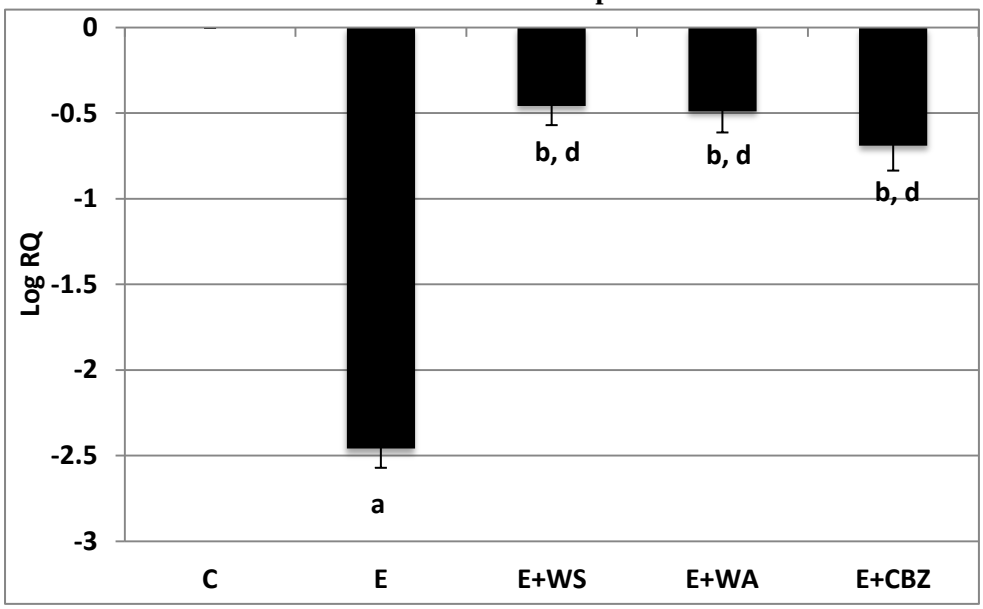


Table-38

Real time PCR amplification of NMDA R1 receptor mRNA from the cerebral cortex of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-2.45 ± 0.13** [a]
E+WS	**-0.45 ±0.10** [b, d]
E+WA	**-0.48 ±0.12** [b, d]
E+CBZ	**-0.68 ±0.14** [b, d]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-44

Real time PCR amplification of NMDA 2B receptor mRNA from the cerebral cortex of control and experimental rats

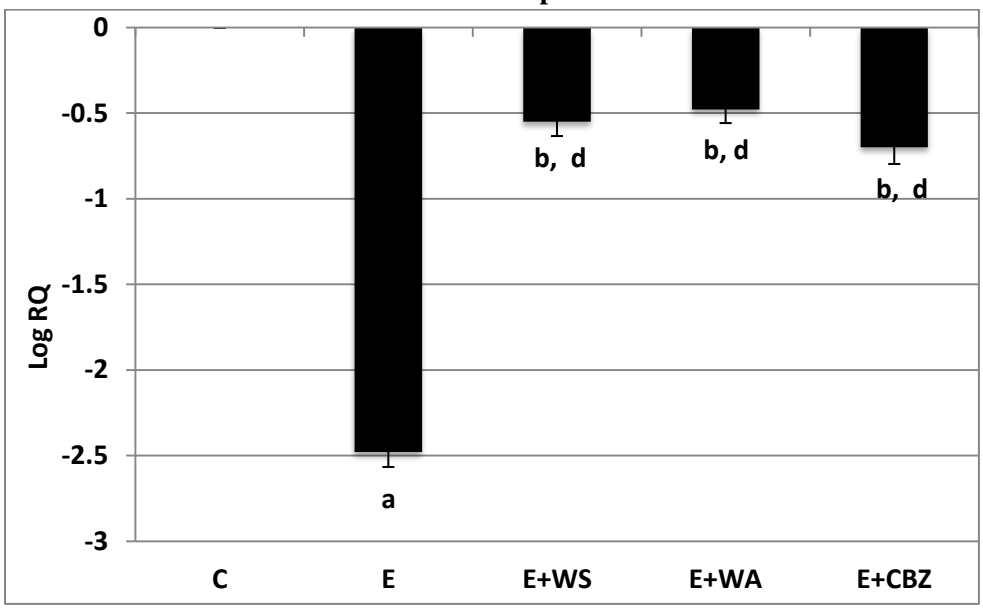


Table-39

Real time PCR amplification of NMDA 2B receptor mRNA from the cerebral cortex of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-2.47 ± 0.08** [a]
E+WS	**-0.54 ± 0.08** [b, d]
E+WA	**-0.47 ± 0.07** [b, d]
E+CBZ	**-0.69 ± 0.09** [b, d]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-45

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the cerebral cortex of control and experimental rats

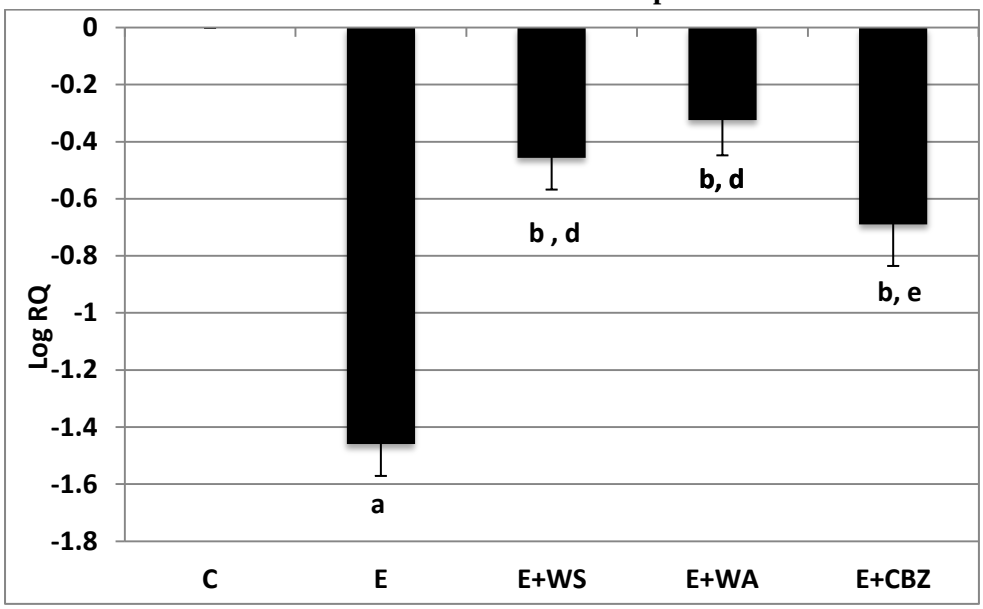


Table-40

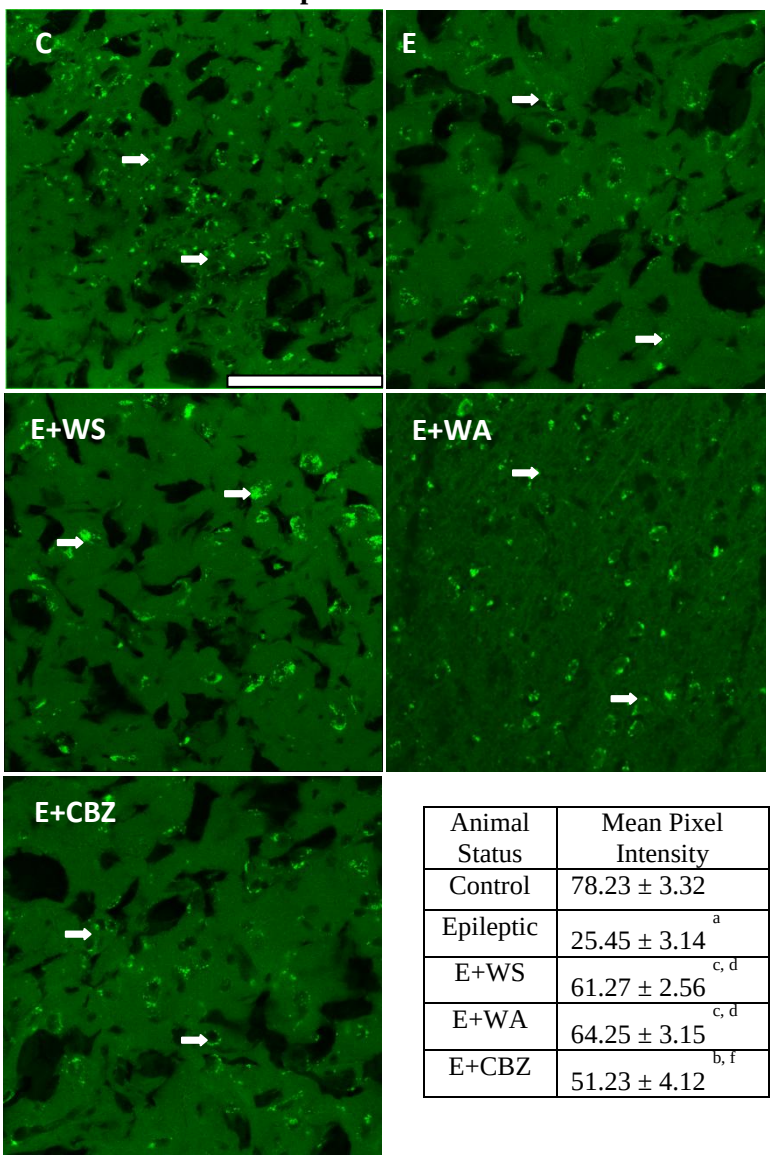
Real time PCR amplification of GluR2 subunit of AMPA receptor from the cerebral cortex of control and experimental rats

Animal Status	Log RQ
Control	0
Epileptic	-1.45 ± 0.11[a]
E+WS	-0.45 ± 0.11 [b, d]
E+WA	-0.32 ± 0.12 [b, d]
E+CBZ	-0.68 ± 0.14 [b, e]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure- 46

NMDA R1 receptor subunit expression in the cerebral cortex of control and experimental rats

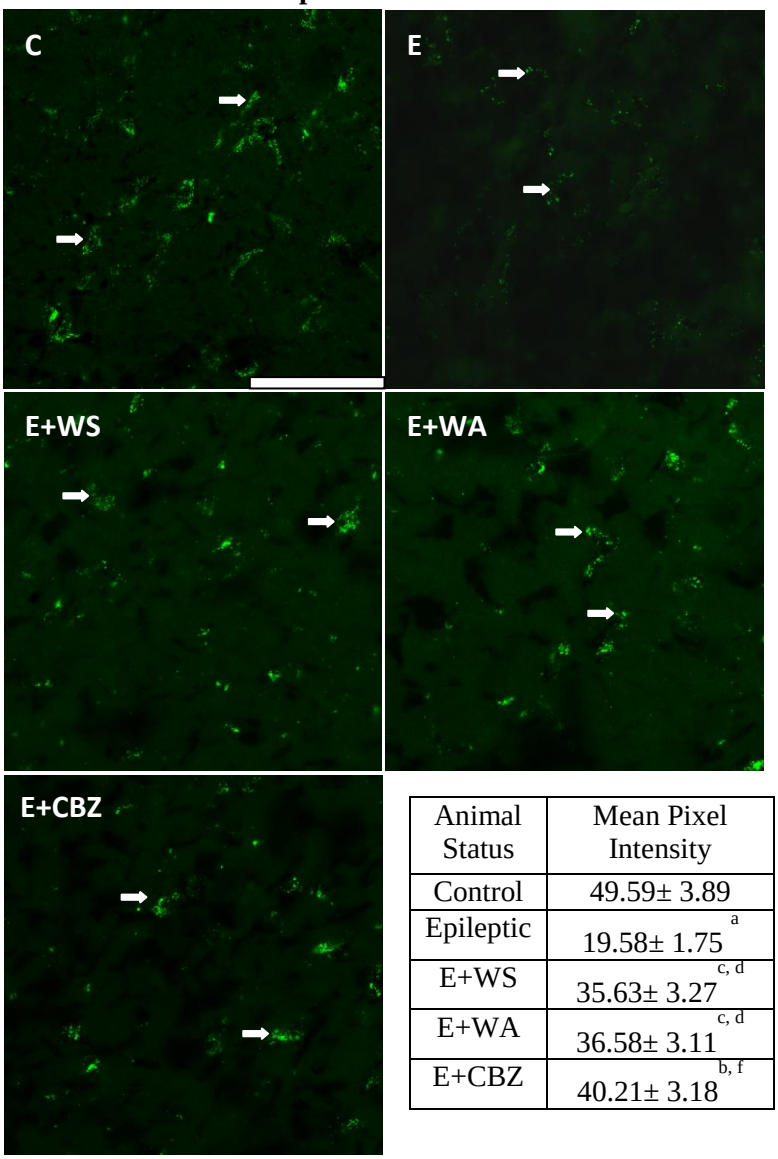


Animal Status	Mean Pixel Intensity
Control	78.23 ± 3.32
Epileptic	25.45 ± 3.14 [a]
E+WS	61.27 ± 2.56 [c, d]
E+WA	64.25 ± 3.15 [c, d]
E+CBZ	51.23 ± 4.12 [b, f]

Confocal image of NMDA R1 receptors in the cerebral cortex of C-Control, E-Epileptic, E+WS-Epileptic+*Withania somnifera*, E+WA-Epileptic+Withanolide-A, E+CBZ-Epileptic+Carbamazepine rats using immunofluorescent NMDA R1 receptor subunit specific primary antibody and FITC as secondary antibody; [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ when compared to epileptic group; (➡) in white shows NMDA receptors. Scale bar = 50 μm.

Figure- 47

NMDA 2B receptor subunit expression in the cerebral cortex of control and experimental rats

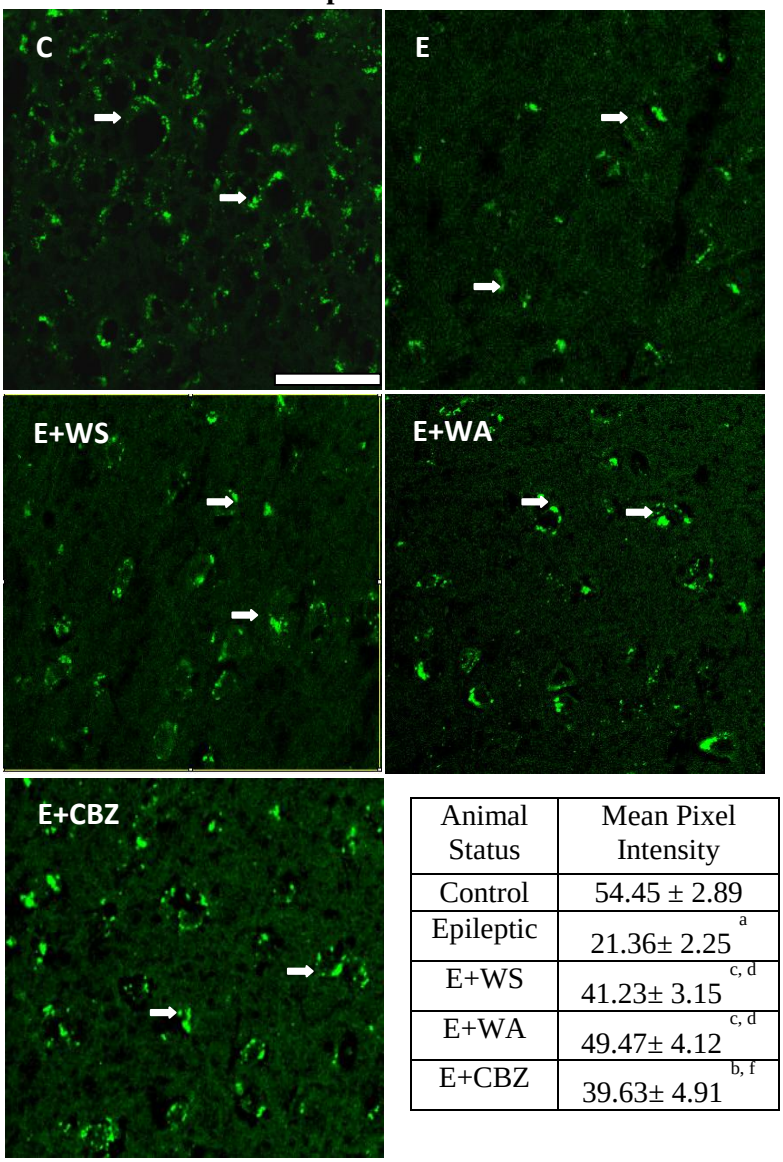


Animal Status	Mean Pixel Intensity
Control	49.59± 3.89
Epileptic	19.58± 1.75 [a]
E+WS	35.63± 3.27 [c, d]
E+WA	36.58± 3.11 [c, d]
E+CBZ	40.21± 3.18 [b, f]

Confocal image of NMDA receptors in the cerebral cortex of C-Control, E-Epileptic, E+WS-Epileptic+*Withania somnifera*, E+WA-Epileptic+Withanolide-A, E+CBZ-Epileptic+Carbamazepine rats using immunofluorescent NMDA R1 receptor subunit specific primary antibody and Alexa Fluor488 as secondary antibody; [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [f] $p<0.05$ when compared to epileptic group; (➡) in white shows NMDA receptors. Scale bar = 50 μm.

Figure- 48

AMPA (GluR2) receptor subunit expression in the cerebral cortex of control and experimental rats



Animal Status	Mean Pixel Intensity
Control	54.45 ± 2.89
Epileptic	21.36± 2.25 [a]
E+WS	41.23± 3.15 [c, d]
E+WA	49.47± 4.12 [c, d]
E+CBZ	39.63± 4.91 [b, f]

Confocal image of AMPA receptors in the cerebral cortex of C-Control, E-Epileptic, E+WS-Epileptic+*Withania somnifera*, E+WA-Epileptic+Withanolide-A, E+CBZ-Epileptic+Carbamazepine rats using immunofluorescentAMPA (GluR2) receptor subunit specific primary antibody and FITC as secondary antibody; [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [f] $p<0.05$ when compared to epileptic group; (➡) in white shows AMPA receptors. Scale bar = 50 μm.

Figure- 49

IP3 content in the cerebral cortex of control and experimental rats

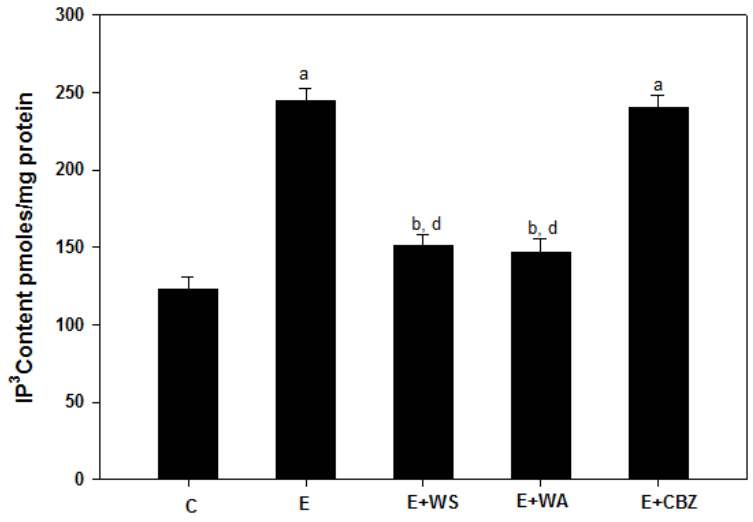


Table- 41

IP3 content in the cerebral cortex of control and experimental rats

Animal Status	IP3 Content (pmoles/mg protein)
Control	**123.11 ± 8.13**
Epileptic	**245.18 ± 7.49** [a]
E+WS	**151.12 ± 6.58** [b, d]
E+WA	**147.41 ± 8.29** [b, d]
E+CBZ	**240.12 ± 7.14** [a]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-50
Real time PCR amplification of Bax mRNA from the cerebral cortex of control and experimental rats

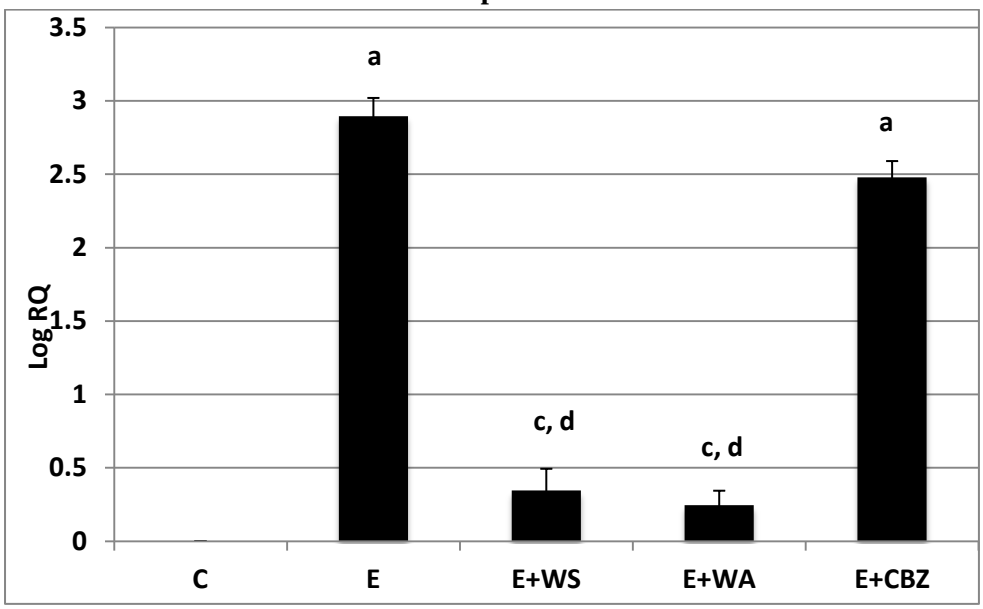


Table-42
Real time PCR amplification of Bax mRNA from the cerebral cortex of control and experimental rats

Animal Status	Log RQ
Control	0
Epileptic	2.8958 ± 0.12 [a]
E+WS	0.3458 ± 0.14 [c, d]
E+WA	0.2457 ± 0.09 [c,d]
E+CBZ	2.4789 ± 0.11 [a]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-51

Real time PCR amplification of Caspase 8 mRNA from the cerebral cortex of control and experimental rats

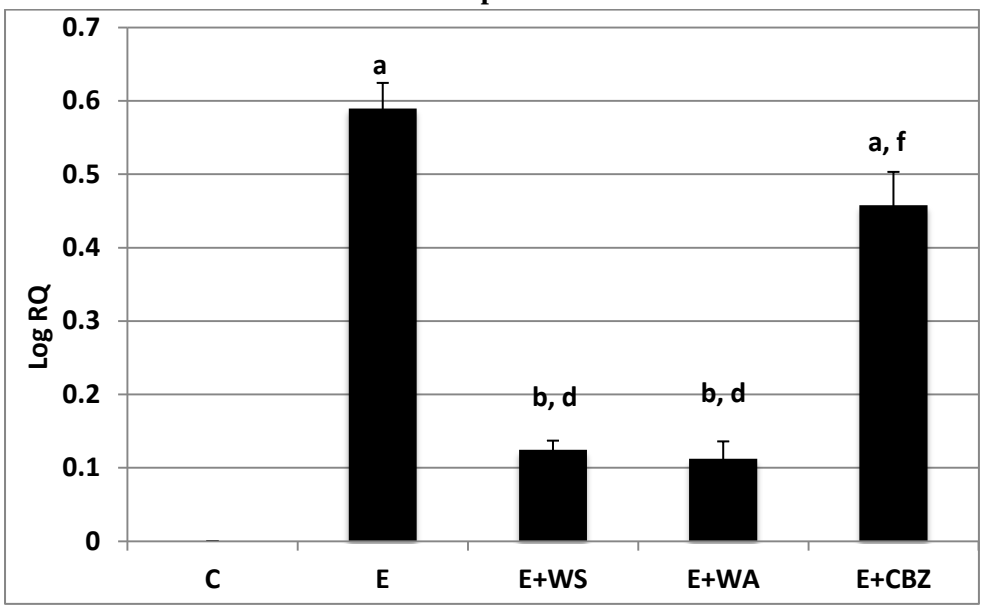


Table-43

Real time PCR amplification of Caspase 8 mRNA from the cerebral cortex of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**0.58± 0.03** [a]
E+WS	**0.12± 0.01** [b, d]
E+WA	**0.11± 0.02** [b, d]
E+CBZ	**0.45± 0.04** [a, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-52
Real time PCR amplification of Akt-1 mRNA from the cerebral cortex of control and experimental rats

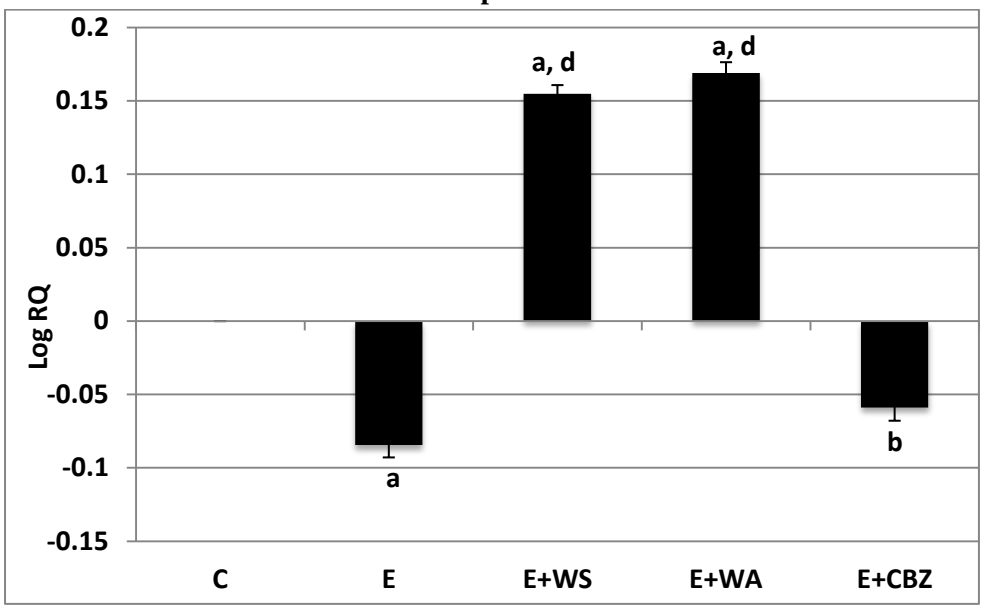


Table-44
Real time PCR amplification of Akt-1 mRNA from the cerebral cortex of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.084 ± 0.008** [a]
E+WS	**0.154 ± 0.006** [a,d]
E+WA	**0.168 ± 0.007** [a,d]
E+CBZ	**-0.058 ± 0.009** [b]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-53

Lipid peroxidation assay in the cerebellum of control and experimental rats

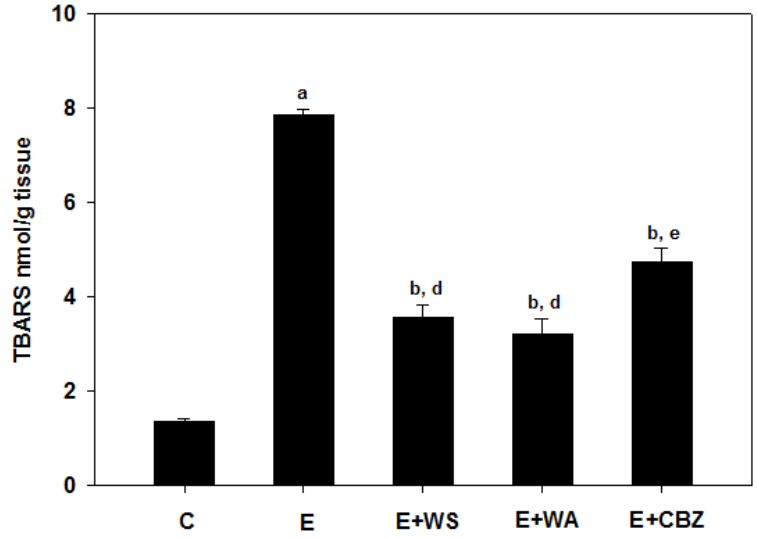


Table-45

Lipid peroxidation assay in the cerebellum of control and experimental rats

Animal Status	TBARS (nmol MDA/mg protein)
Control	**1.36 ± 0.04**
Epileptic	**7.84 ± 0.12** [a]
E+WS	**3.56 ± 0.28** [b, d]
E+WA	**3.21 ± 0.32** [b, d]
E+CBZ	**4.74 ± 0.29** [b, e]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group.

Figure-54
Superoxide dismutase assay in the cerebellum of control and experimental rats

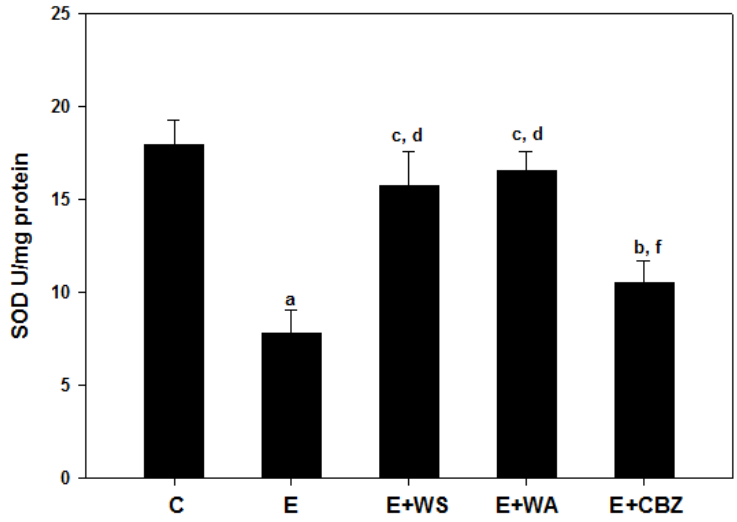


Table-46
Superoxide dismutase assay in the cerebellum of control and experimental rats

Animal Status	SOD activity (unit/mg protein)
Control	**17.98 ± 1.28**
Epileptic	**7.84 ± 1.24** [a]
E+WS	**15.78 ± 1.80** [c, d]
E+WA	**16.54 ± 1.10** [c, d]
E+CBZ	**10.54 ± 1.19** [b, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-55
Catalase assay in cerebellum of control and experimental animals

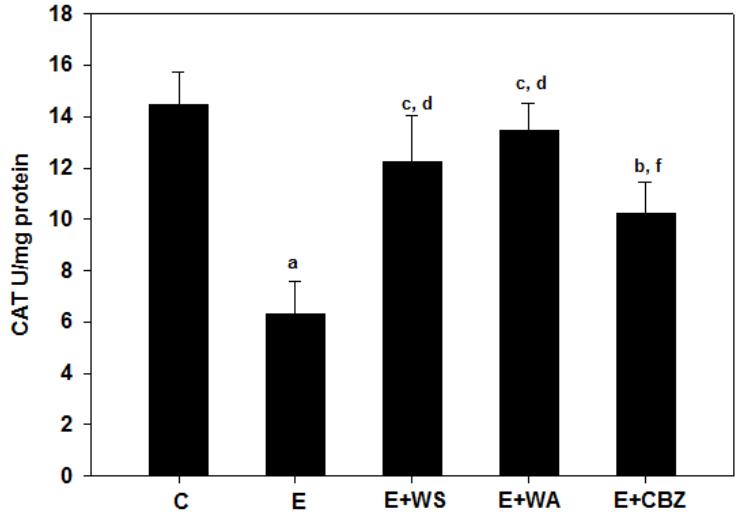


Table-47
Catalase assay in cerebellum of control and experimental animals

Animal Status	CAT activity (ΔA240/min/mg protein)
Control	**14.44 ± 1.28**
Epileptic	**6.32 ± 1.24** [a]
E+WS	**12.25 ± 1.80** [c, d]
E+WA	**13.45 ± 1.15** [c, d]
E+CBZ	**10.23 ± 1.09** [b, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-56
Real time PCR amplification of SOD mRNA from the cerebellum of control and experimental rats

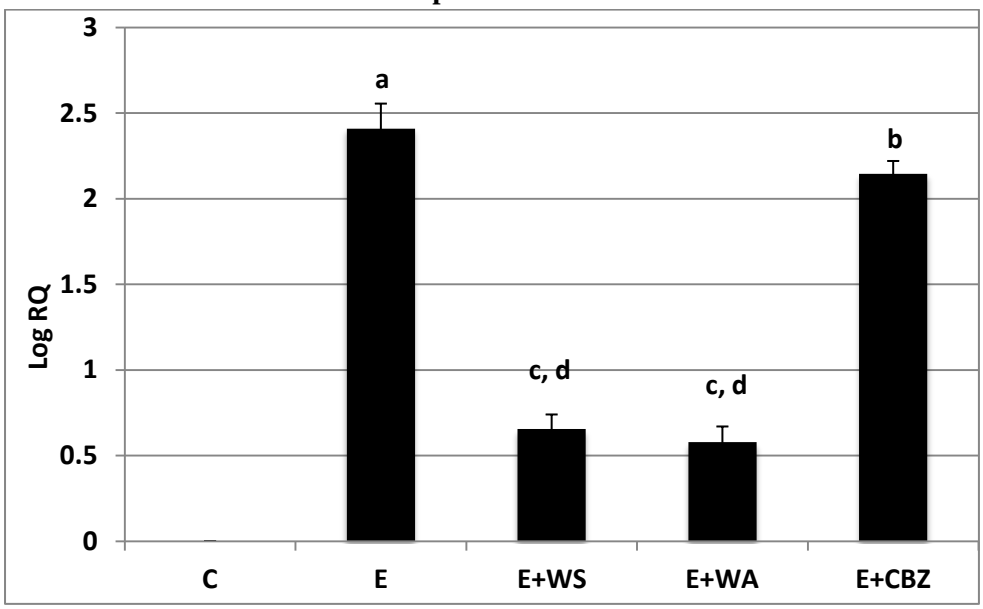


Table-48
Real time PCR amplification of SOD mRNA from cerebellum of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**2.41 ± 0.14[a]**
E+WS	**0.65 ± 0.08 [c, d]**
E+WA	**0.57 ± 0.08 [c, d]**
E+CBZ	**2.14 ± 0.07 [b]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-57
Real time PCR amplification of GPx mRNA from the cerebellum of control and experimental rats

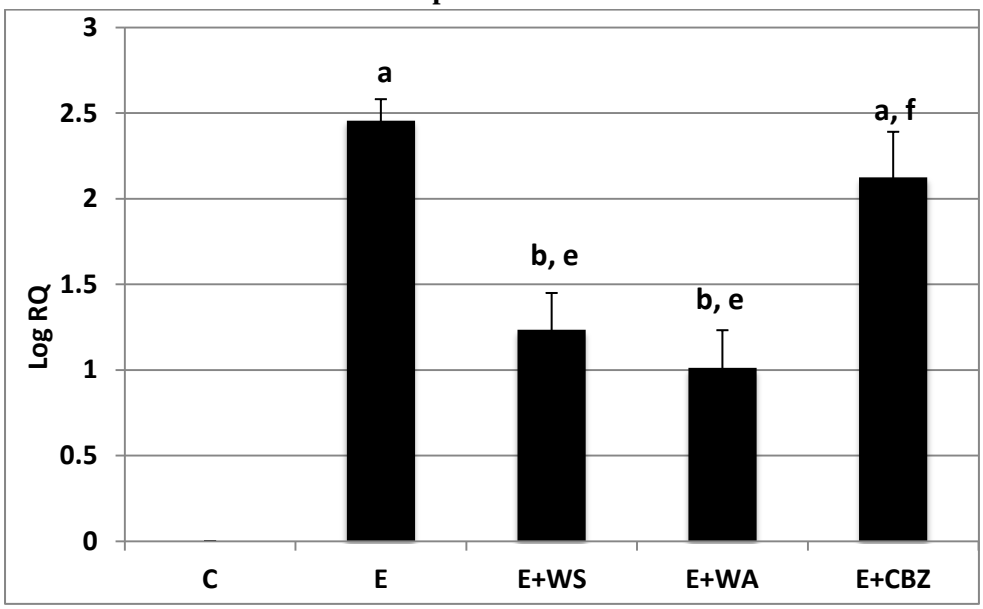


Table-49
Real time PCR amplification of GPx mRNA from cerebellum of control and experimental rats

Animal Status	Log RQ
Control	0
Epileptic	2.45 ± 0.12 [a]
E+WS	1.23 ± 0.21 [b, e]
E+WA	1.01 ± 0.22 [b, e]
E+CBZ	2.12 ± 0.26 [a, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [e] $p < 0.01$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Table- 50
Glutamate content in the cerebellum of control and experimental rats

Animal Status	Glutamate Content (nmoles/g. wt of tissue)
Control	**106.39 ± 12.35**
Epileptic	**288.38 ± 11.56** [a]
E+WS	**122.36 ± 8.23** [c, d]
E+WA	**124.25 ± 12.47** [c, d]
E+CBZ	**130.25 ± 13.39** [c, d]

Table-51
Glutamate dehydrogenase activity in the cerebellum of control and experimental rats

Animal Status	Vmax (mmol/min/mg protein)	Km (mM)
Control	**0.660±0.02**	**0.103 ± 0.02**
Epileptic	**0.731± 0.04** [a]	**0.112 ± 0.01**
E+WS	**0.665± 0.09** [c, d]	**0.107 ± 0.03**
E+WA	**0.612 ± 0.08** [c, d]	**0.108 ± 0.03**
E+CBZ	**0.714± 0.04** [b]	**0.110 ± 0.04**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-58
Real time PCR amplification of GLAST mRNA from cerebellum of control and experimental rats

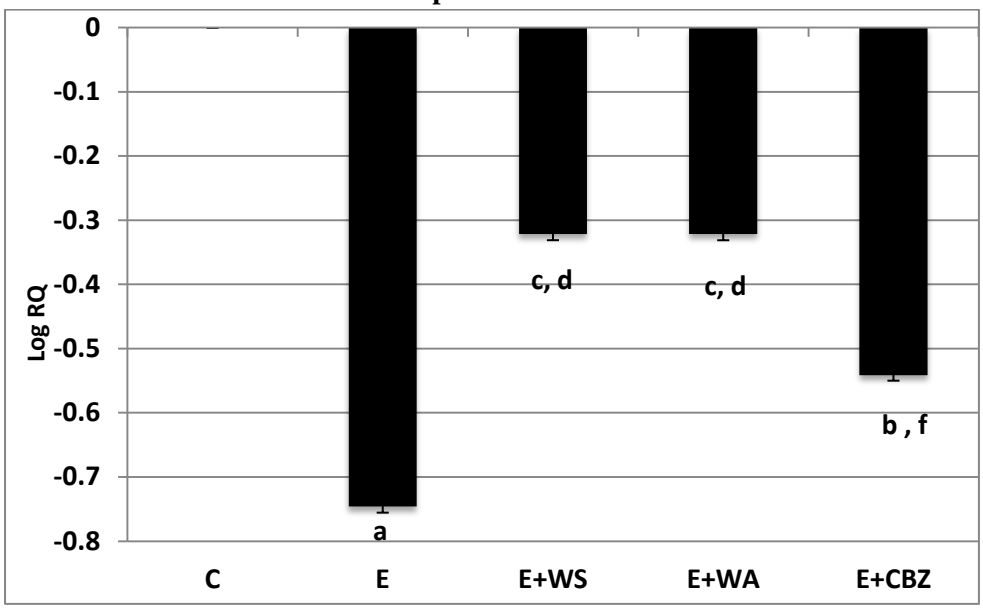


Table-52
Real time PCR amplification of GLAST mRNA from cerebellum of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.7456 ± 0.0009** [a]
E+WS	**-0.3217 ± 0.0008** [c, d]
E+WA	**-0.3214 ± 0.0009** [c, d]
E+CBZ	**-0.5412 ± 0.0087** [b, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-59

Real time PCR amplification of GAD mRNA from cerebellum of control and experimental rats

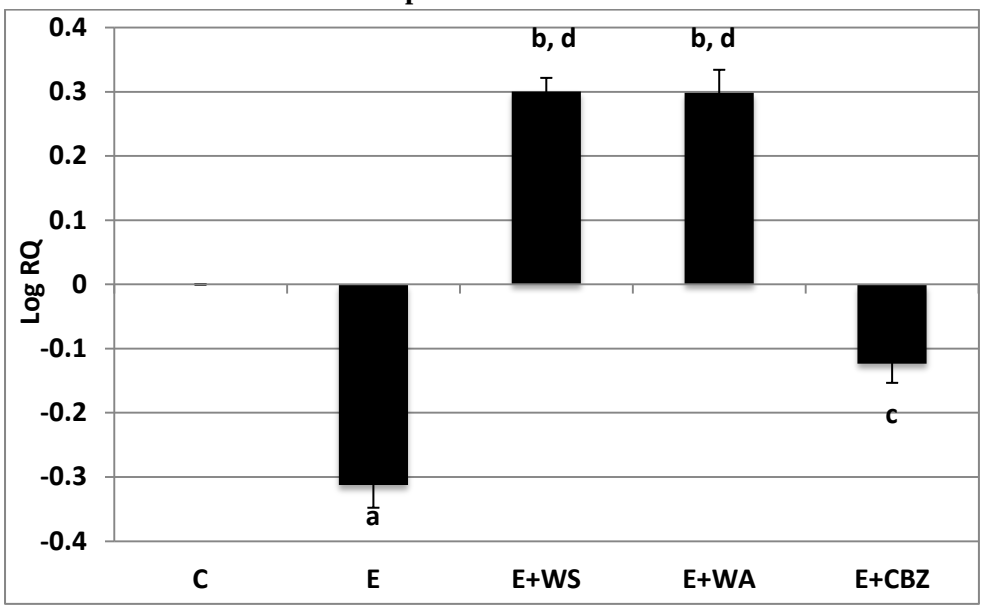


Table-53

Real time PCR amplification of GAD mRNA from cerebellum of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.31 ± 0.03** [a]
E+WS	**0.30 ± 0.01**[b, d]
E+WA	**0.29 ± 0.03**[b, d]
E+CBZ	**-0.12 ± 0.02**[c]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-60

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebellum of control and experimental rats

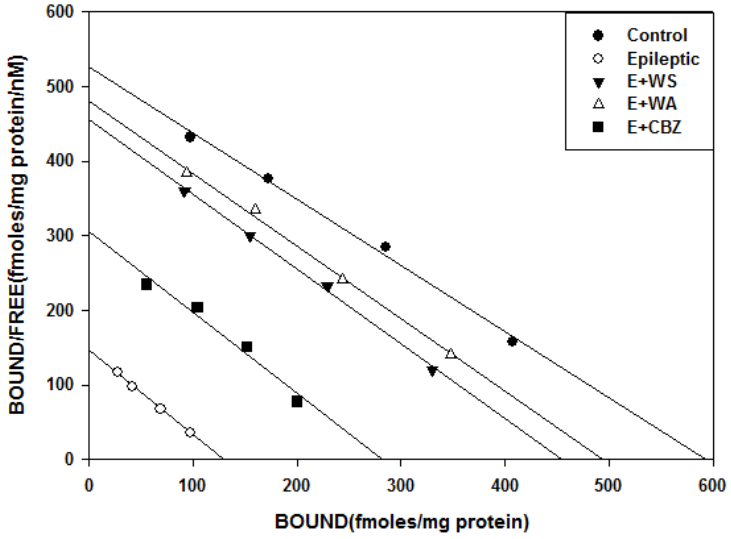


Table -54

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the cerebellum of control and experimental rats

Experimental group	Bmax (fmol/mg protein)	Kd (nM)
C	**592.50± 3.09**	**1.13 ± 0.08**
Epileptic	**126.81± 2.52** [a]	**0.85 ± 0.09**
E+WS	**449.20± 2.12** [c, d]	**0.99 ± 0.06**
E+WA	**490.61± 3.16** [c, d]	**1.03 ± 0.02**
E+CBZ	**280.40± 2.58** [b, e]	**0.93 ± 0.09**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group). B_{max} – Maximal binding; K_d – Dissociation constant; C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure-61
Scatchard analysis of AMPA receptor using [^{3}H]AMPA binding against AMPA in the cerebellum of control and experimental rats

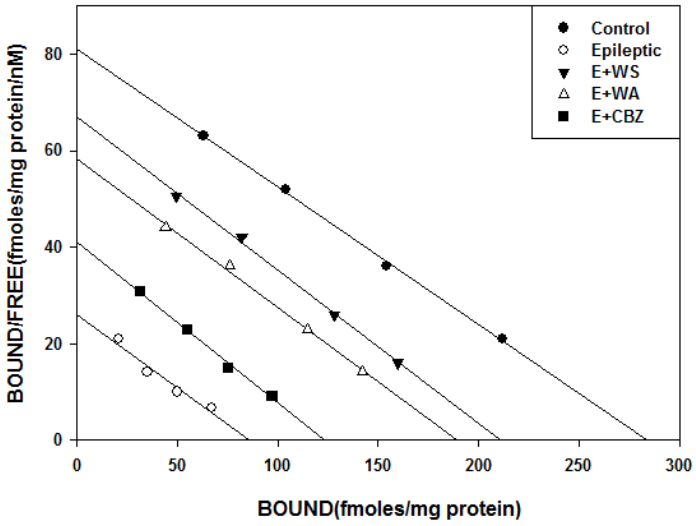


Table-55
Scatchard analysis of AMPA receptor using [3H]AMPA binding against AMPA in the cerebellum of control and experimental rats

Experimental group	Bmax (fmol/mg protein)	Kd (nM)
C	**282.50± 3.09**	**3.50 ± 0.08**
Epileptic	**84.98± 4.52** [a]	**3.48 ± 0.03**
E+WS	**211.30± 2.12** [c, d]	**3.21 ± 0.04**
E+WA	**186.61± 3.16** [c, d]	**3.20 ± 0.06**
E+CBZ	**122.40± 2.58** [b, e]	**3.11± 0.09**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group). B_{max} – Maximal binding; K_d – Dissociation constant; C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure-62
Real time PCR amplification of NMDA R1 receptor mRNA from the cerebellum of control and experimental rats

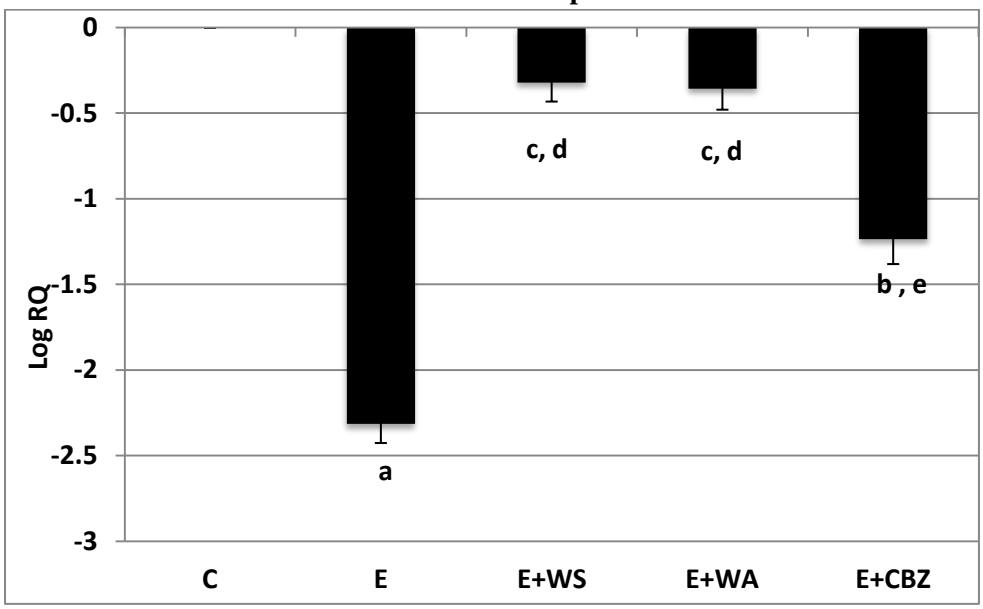


Table-56
Real time PCR amplification of NMDA R1 receptor mRNA from the cerebellum of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-2.31 ± 0.11** [a]
E+WS	**-0.32 ± 0.10** [c, d]
E+WA	**-0.35 ± 0.12** [c, d]
E+CBZ	**-1.23 ±0.14** [b, e]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure-63
Real time PCR amplification of NMDA 2B receptor mRNA from the cerebellum of control and experimental rats

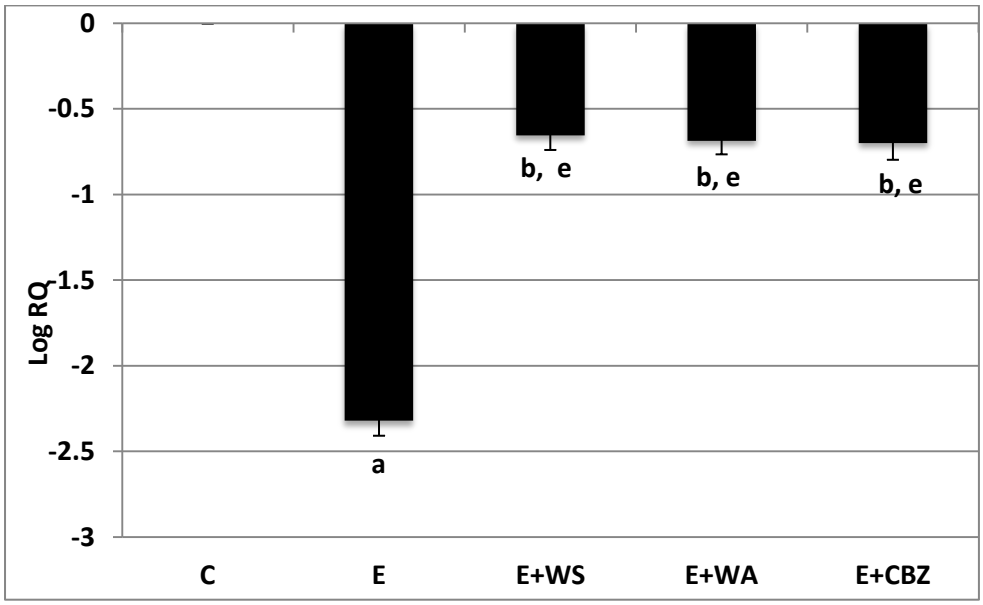


Table-57
Real time PCR amplification of NMDA 2B receptor mRNA from the cerebellum of control and experimental rats

Animal Status	Log RQ
Control	0
Epileptic	-2.32 ± 0.08 [a]
E+WS	-0.65 ± 0.08 [b, e]
E+WA	-0.68 ± 0.07 [b, e]
E+CBZ	-0.69 ± 0.09 [b, e]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [e] $p < 0.01$ when compared with epileptic group

Figure-64

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from cerebellum of control and experimental rats

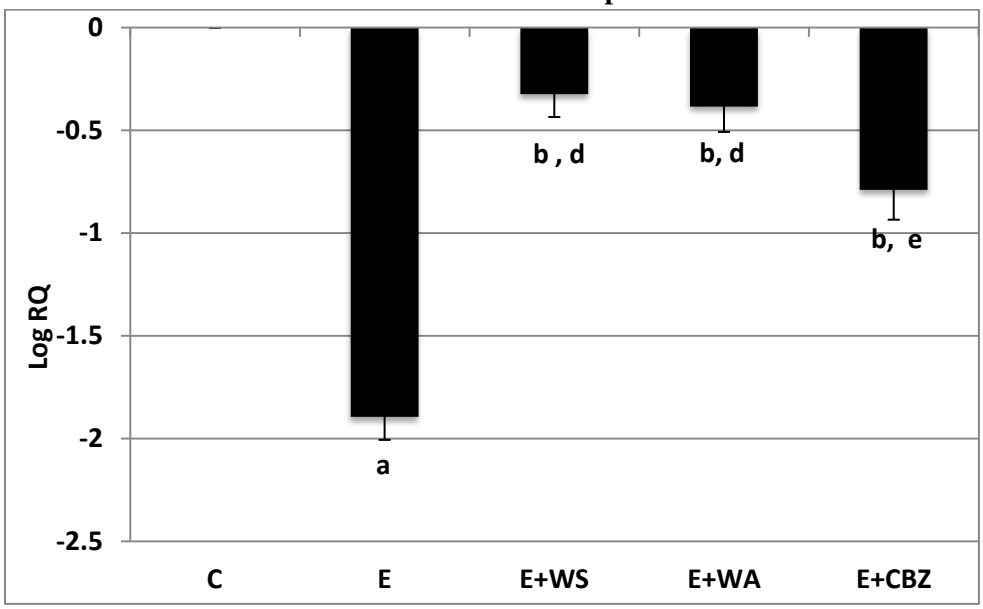


Table-58

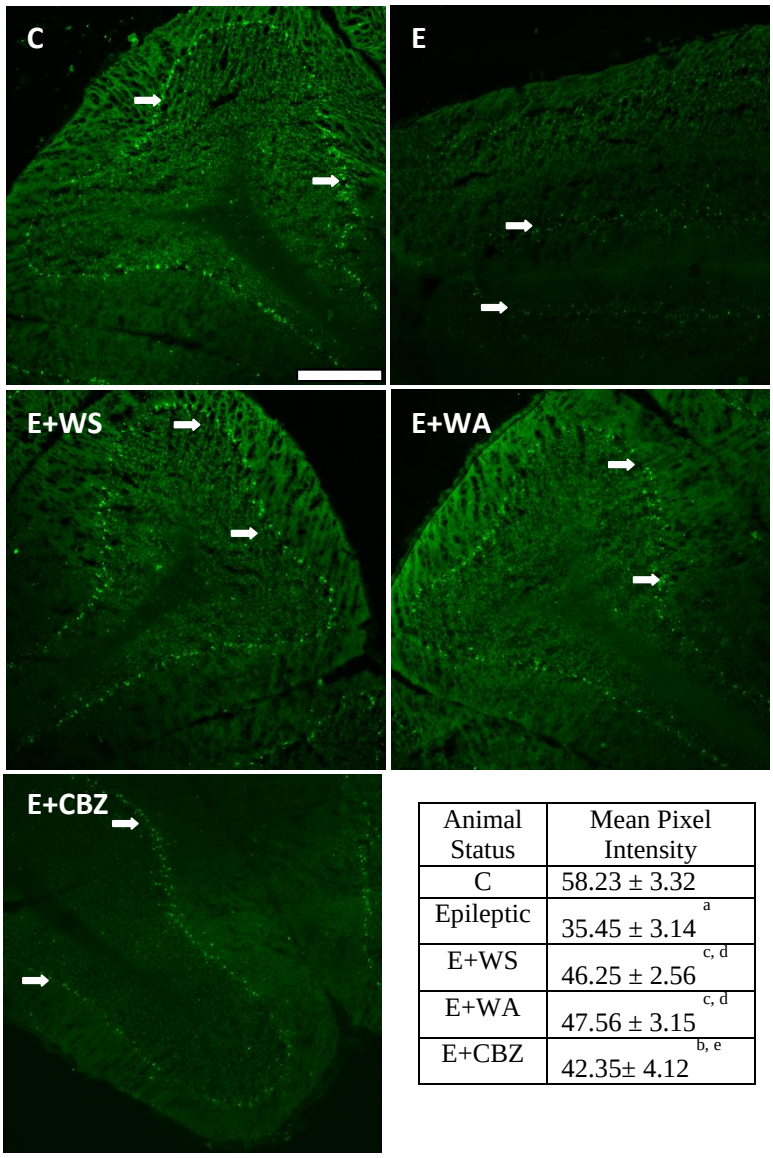
Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from cerebellum of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-1.89 ± 0.11[a]**
E+WS	**-0.32 ± 0.09 [b, d]**
E+WA	**-0.38 ± 0.12 [b, d]**
E+CBZ	**-0.78 ± 0.14 [b, e]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure- 65

NMDA R1 receptor expression in the cerebellum of control and experimental rats

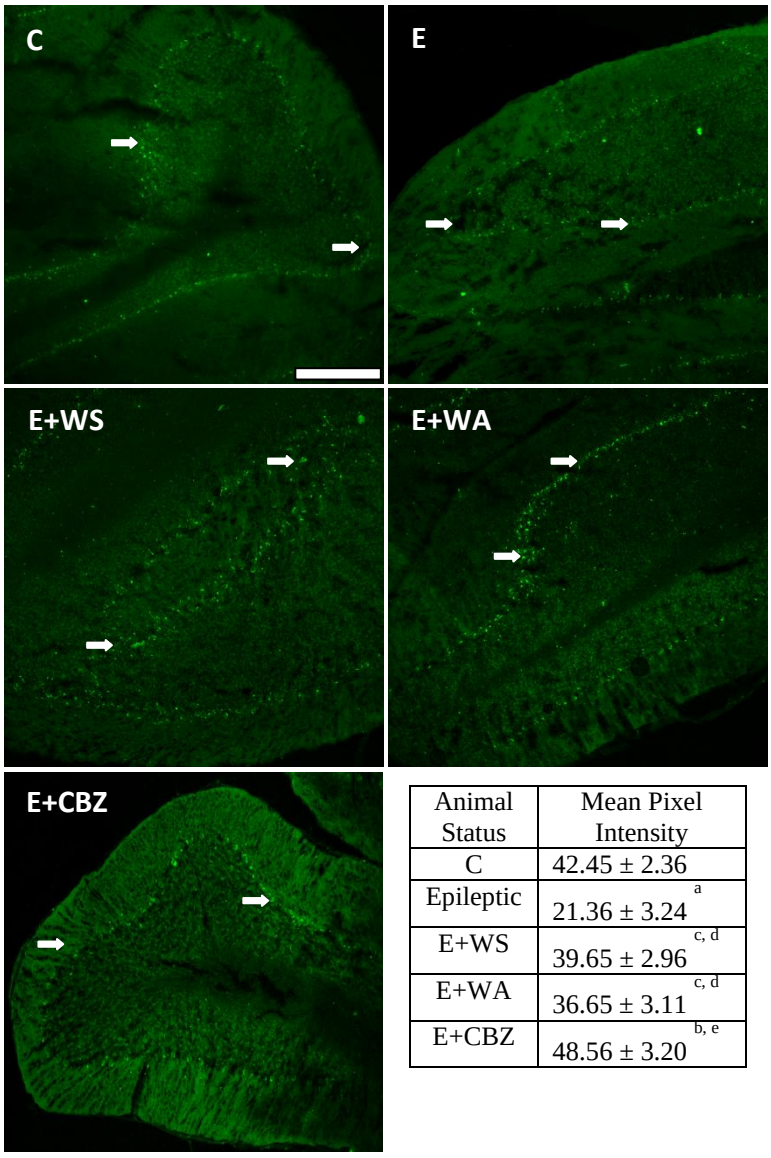


Animal Status	Mean Pixel Intensity
C	58.23 ± 3.32
Epileptic	35.45 ± 3.14 [a]
E+WS	46.25 ± 2.56 [c, d]
E+WA	47.56 ± 3.15 [c, d]
E+CBZ	42.35± 4.12 [b, e]

Confocal image of NMDA R1 receptors in the cerebellum of C-Control, E-Epileptic, E+WS-Epileptic+*Withania somnifera*, E+WA-Epileptic+Withanolide-A, E+CBZ-Epileptic+Carbamazepine rats using immunofluorescent NMDA R1 receptor subunit specific primary antibody and FITC as secondary antibody; [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [e] $p<0.01$ when compared to epileptic group; (➡) in white shows NMDA receptors. Scale bar = 200µm.

Figure- 66

NMDA 2B receptor expression in the cerebellum of control and experimental rats

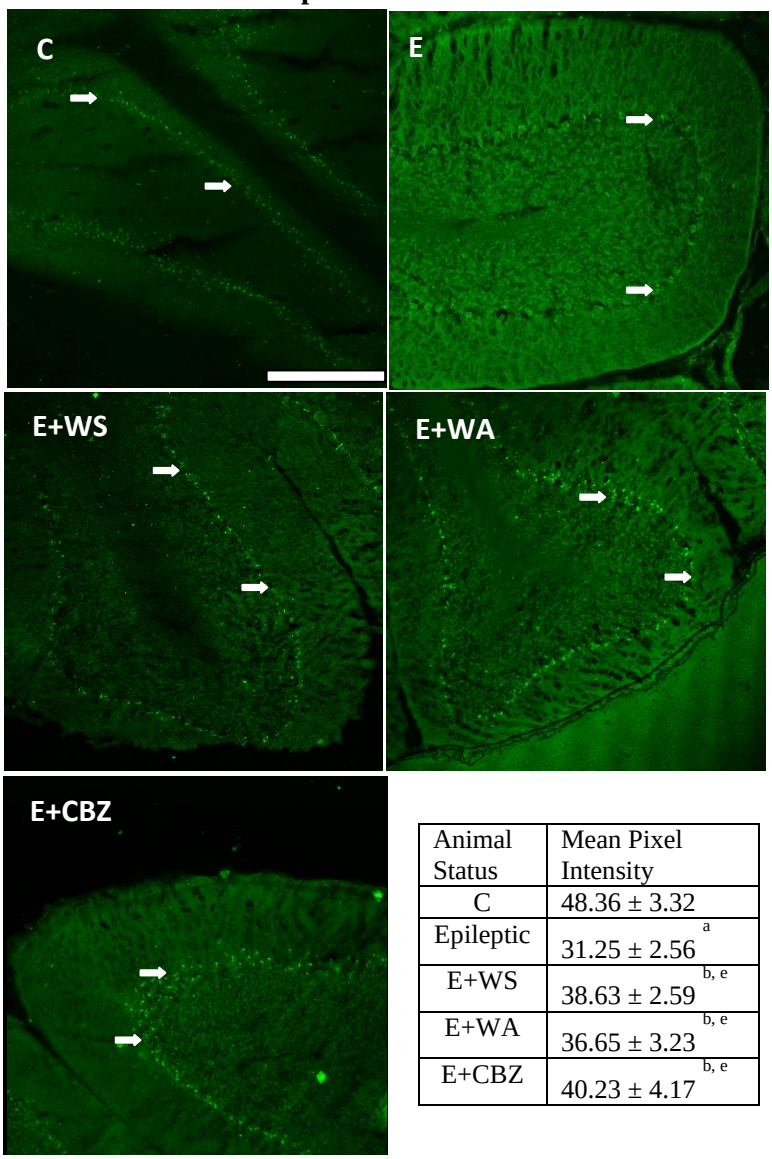


Animal Status	Mean Pixel Intensity
C	42.45 ± 2.36
Epileptic	21.36 ± 3.24 [a]
E+WS	39.65 ± 2.96 [c, d]
E+WA	36.65 ± 3.11 [c, d]
E+CBZ	48.56 ± 3.20 [b, e]

Confocal image of NMDA receptors in the cerebellum of C-Control, E-Epileptic, E+WS-Epileptic + *Withania somnifera*, E+WA-Epileptic + Withanolide-A, E+CBZ- Epileptic + Carbamazepine rats using immunofluorescent NMDA 2B receptor subunit specific primary antibody and FITC as secondary antibody; [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [e] $p<0.01$ when compared to epileptic group; (➡) in white shows NMDA receptors. Scale bar = 200 μm.

Figure- 67

AMPA (GluR2) receptor subunit expression in the cerebellum of control and experimental rats



Animal Status	Mean Pixel Intensity
C	48.36 ± 3.32
Epileptic	31.25 ± 2.56 [a]
E+WS	38.63 ± 2.59 [b, e]
E+WA	36.65 ± 3.23 [b, e]
E+CBZ	40.23 ± 4.17 [b, e]

Confocal image of AMPA receptors in the cerebellum of C-Control, E-Epileptic, E+WS-Epileptic + *Withania somnifera*, E + WA-Epileptic + Withanolide-A, E + CBZ- Epileptic + Carbamazepine rats using immunofluorescent AMPA (GluR2) receptor subunit specific primary antibody and FITC as secondary antibody; [a]$p<0.001$, [b] $p<0.01$ when compared to control group: [e] $p<0.01$, [f] $p<0.05$ when compared to epileptic group; (➡) in white shows AMPA receptors. Scale bar = 200 µm.

Figure- 68

IP3 content in the cerebellum of control and experimental rats

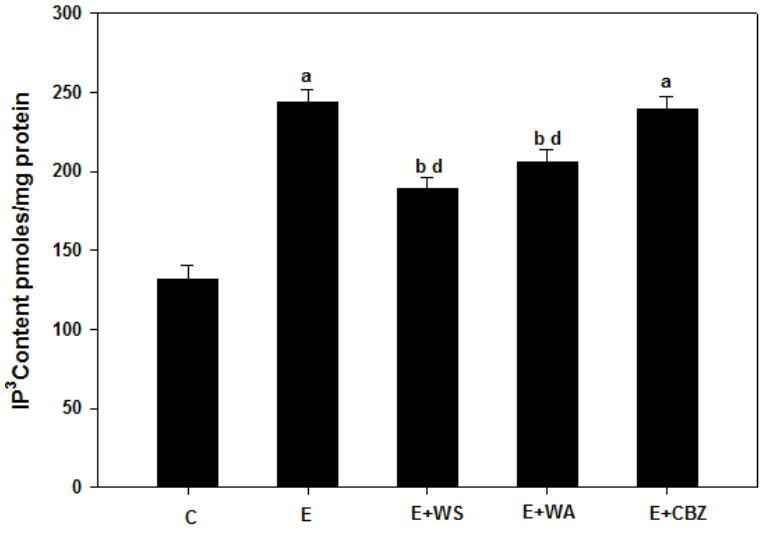


Table- 59

IP3 content in the cerebellum of control and experimental rats

Animal Status	IP3 Content (pmoles/mg protein)
Control	**132.26 ± 7.95**
Epileptic	**243.75 ± 7.56** [a]
E+WS	**189.25 ± 7.12** [b, d]
E+WA	**205.51 ± 8.32** [b, d]
E+CBZ	**239.25 ± 7.91** [a]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-69
Real time PCR amplification of Bax mRNA from the cerebellum of control and experimental rats

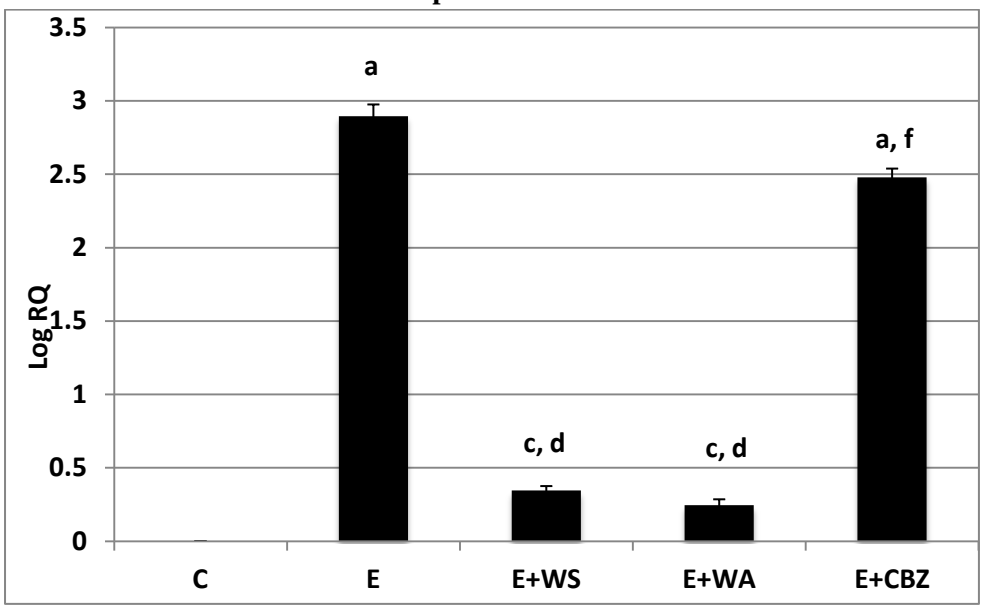


Table-60
Real time PCR amplification of Bax mRNA from the cerebellum of control and experimental rats

Animal Status	Log RQ
Control	0
Epileptic	2.89 ± 0.08 [a]
E+WS	0.34 ± 0.03 [c, d]
E+WA	0.24 ± 0.04 [c, d]
E+CBZ	2.47 ± 0.06 [a, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-70
Real time PCR amplification of Caspase 8 mRNA from the cerebellum of control and experimental rats

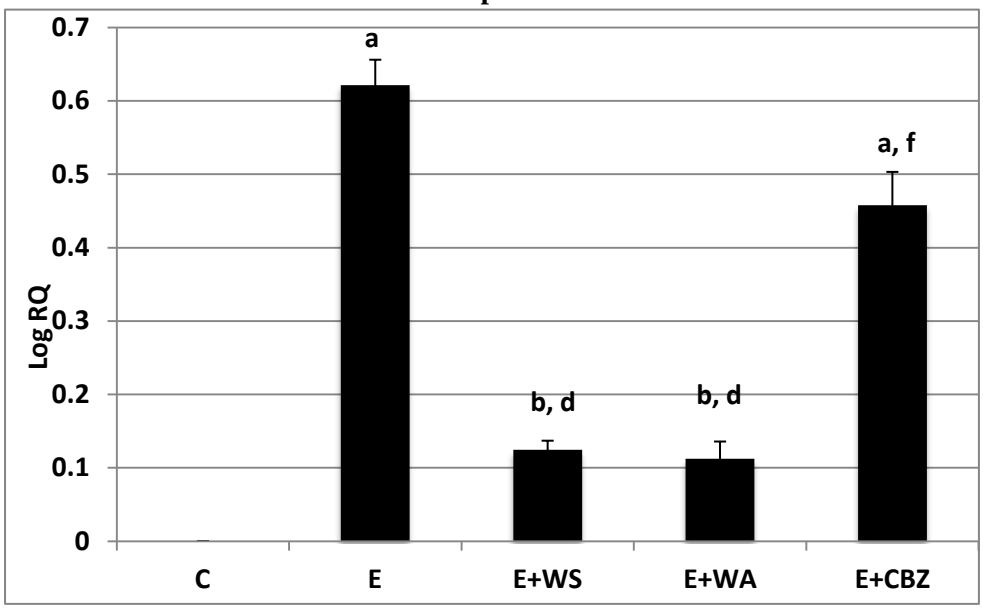


Table-61
Real time PCR amplification of Caspase 8 mRNA from the cerebellum of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**0.62 ± 0.03** [a]
E+WS	**0.12 ± 0.01** [b, d]
E+WA	**0.11 ± 0.02** [b, d]
E+CBZ	**0.45 ± 0.04** [a, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-71
Real time PCR amplification of Akt-1 mRNA from the cerebellum of control and experimental rats

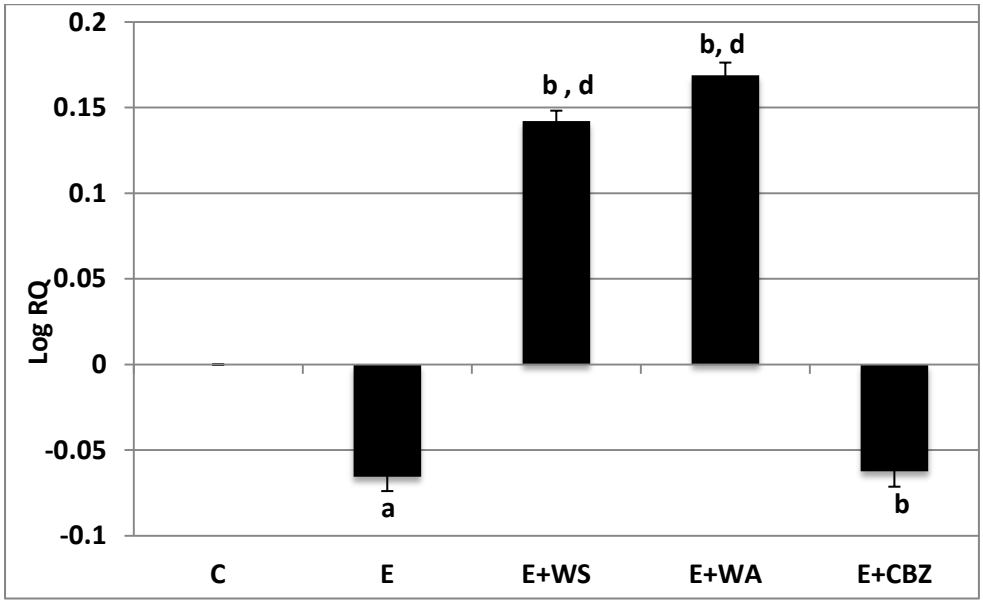


Table-62
Real time PCR amplification of Akt-1 mRNA from the cerebellum of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.065 ± 0.008** [a]
E+WS	**0.142 ± 0.006** [b, d]
E+WA	**0.168 ± 0.007** [b, d]
E+CBZ	**-0.062 ±0.009** [b]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, d $p < 0.001$ when compared with epileptic group

Figure-72

Lipid peroxidation assay in the brain stem of control and experimental rats

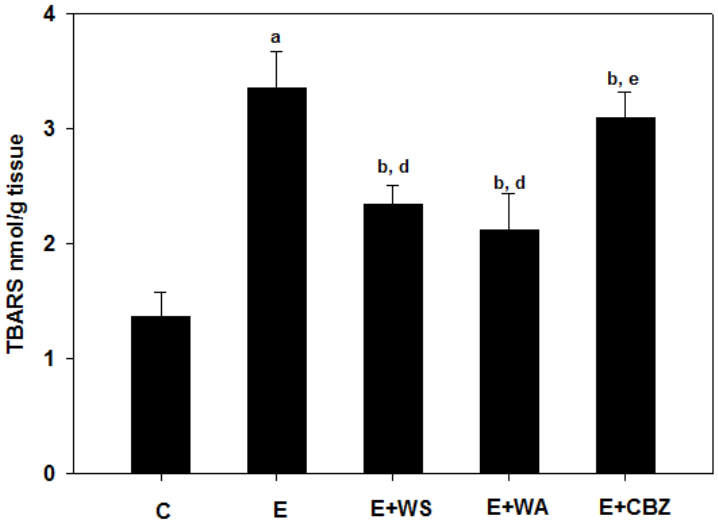


Table-63

Lipid peroxidation assay in the brain stem of control and experimental rats

Animal Status	TBARS (nmol MDA/mg protein)
Control	**1.36 ± 0.21**
Epileptic	**3.35 ± 0.32** [a]
E+WS	**2.34 ± 0.16** [b, d]
E+WA	**2.12 ± 0.31** [b, d]
E+CBZ	**3.10 ± 0.22** [b, e]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure- 73

Superoxide dismutase assay in the brain stem of control and experimental rats

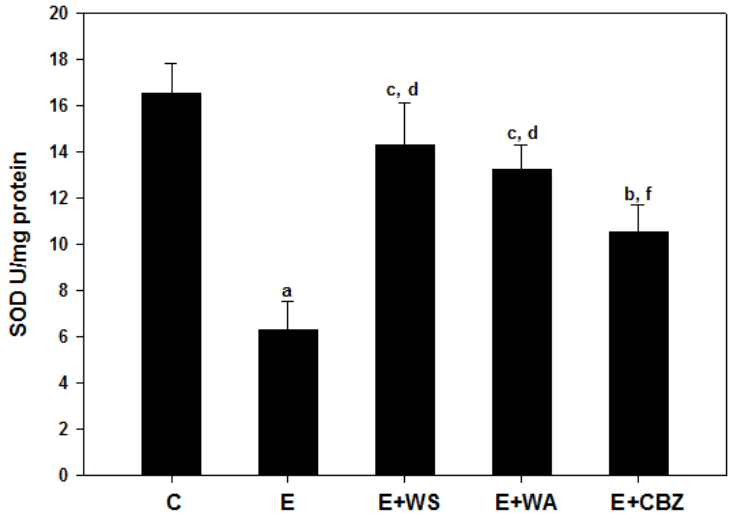


Table-64

Superoxide dismutase assay in the brain stem of control and experimental rats

Animal Status	SOD activity (unit/mg protein)
Control	**16.56 ± 1.28**
Epileptic	**6.32 ± 1.24** [a]
E+WS	**14.32 ± 1.80** [c, d]
E+WA	**13.25 ± 1.05** [c, d]
E+CBZ	**10.54 ± 1.19** [b, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure- 74
Catalase assay in brain stem of control and experimental animals

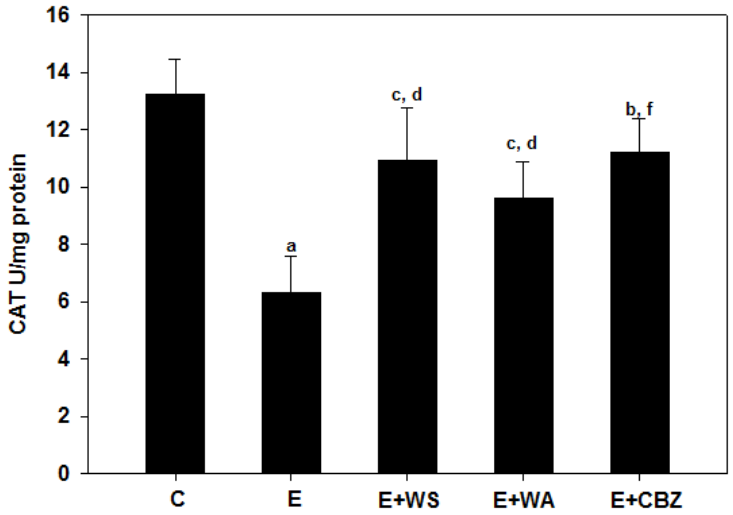


Table-65
Catalase assay in brain stem of control and experimental animals

Animal Status	CAT activity (ΔA_{240}/min/mg protein)
Control	**13.23 ± 1.24**
Epileptic	**6.32 ± 1.28** [a]
E+WS	**10.95 ± 1.09** [c, d]
E+WA	**9.63 ± 1.24** [c, d]
E+CBZ	**11.23 ± 1.14** [b, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-75
Real time PCR amplification of SOD mRNA from the brain stem of control and experimental rats

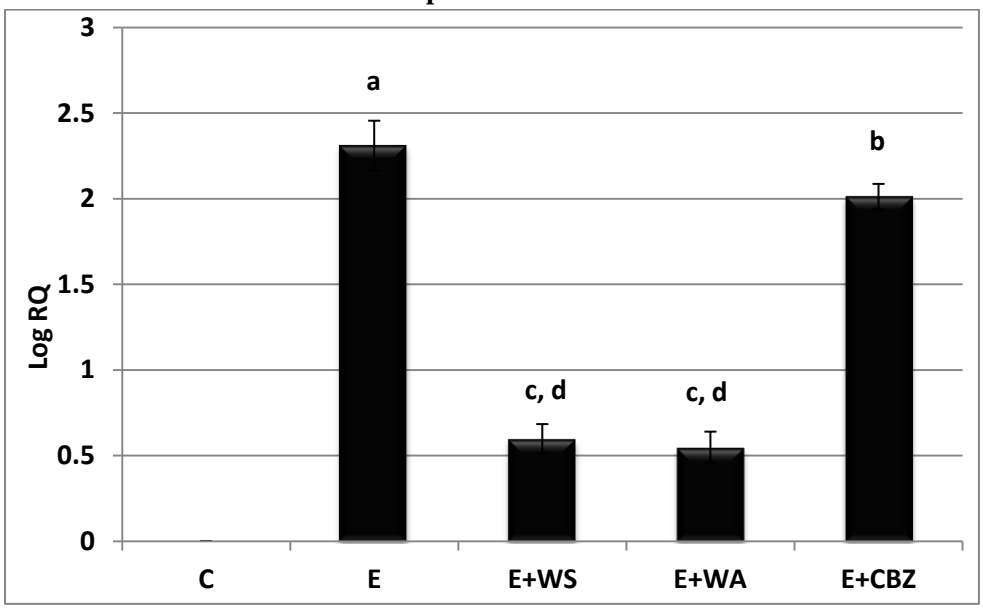


Table-66
Real time PCR amplification of SOD mRNA from the brain stem of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**2.31 ± 0.14** [a]
E+WS	**0.59 ±0.08** [c, d]
E+WA	**0.54 ±0.09** [c, d]
E+CBZ	**2.01 ±0.07** [b]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-76
Real time PCR amplification of GPx mRNA from the brain stem of control and experimental rats

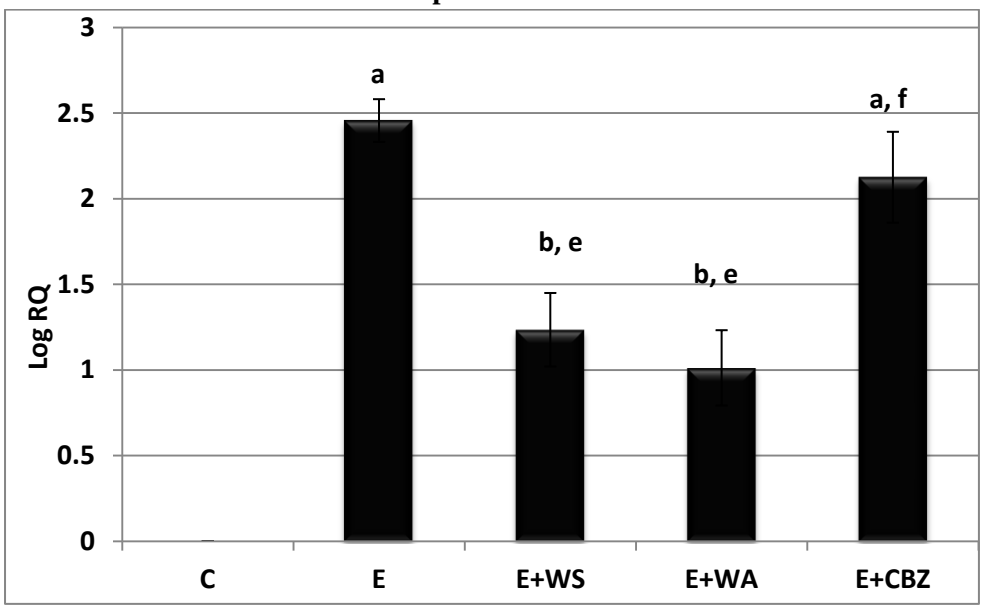


Table-67
Real time PCR amplification of GPx mRNA from the brain stem of control and experimental rats

Animal Status	Log RQ
Control	0
Epileptic	2.45 ± 0.12 [a]
E+WS	1.23 ± 0.21 [b, e]
E+WA	1.01 ± 0.23 [b, e]
E+CBZ	2.12 ± 0.26 [a, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [e] $p < 0.01$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Table- 68
Glutamate content in the brain stem of control and experimental rats

Animal Status	Glutamate Content (nmoles/g. wt of tissue)
Control	**74.32 ± 12.35**
Epileptic	**245.38 ± 11.56** [a]
E+WS	**112.36 ± 8.23** [b, d]
E+WA	**114.25 ± 12.47** [b, d]
E+CBZ	**108.25 ± 13.39** [b, d]

Table-69
Glutamate dehydrogenase activity in the brain stem of control and experimental rats

Animal Status	Vmax (mmol/min/mg protein)	Km (mM)
Control	**0.680±0.02**	**0.103 ± 0.02**
Epileptic	**0.741± 0.04** [a]	**0.112 ± 0.01**
E+WS	**0.695± 0.09** [c, d]	**0.107 ± 0.03**
E+WA	**0.701± 0.08** [c, d]	**0.108 ± 0.03**
E+CBZ	**0.712± 0.04** [b]	**0.110 ± 0.04**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-77
Real time PCR amplification of GLAST mRNA from the brain stem of control and experimental rats

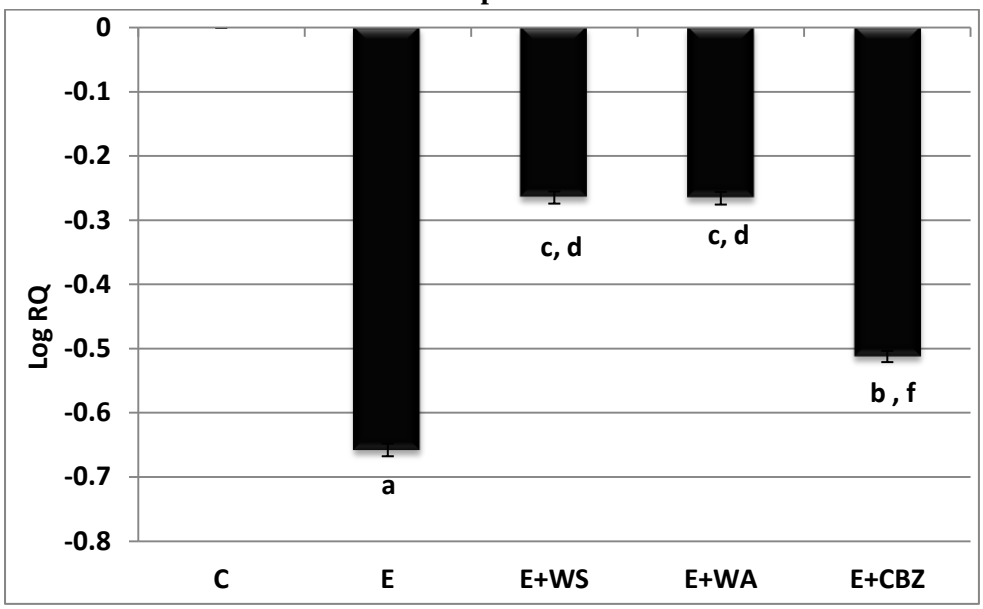


Table-70
Real time PCR amplification of GLAST mRNA from the brain stem of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.65 ± 0.01[a]**
E+WS	**-0.26 ± 0.01 [c, d]**
E+WA	**-0.26 ± 0.02 [c, d]**
E+CBZ	**-0.51 ± 0.01 [b, f]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure-78
Real time PCR amplification of GAD mRNA from the brain stem of control and experimental rats

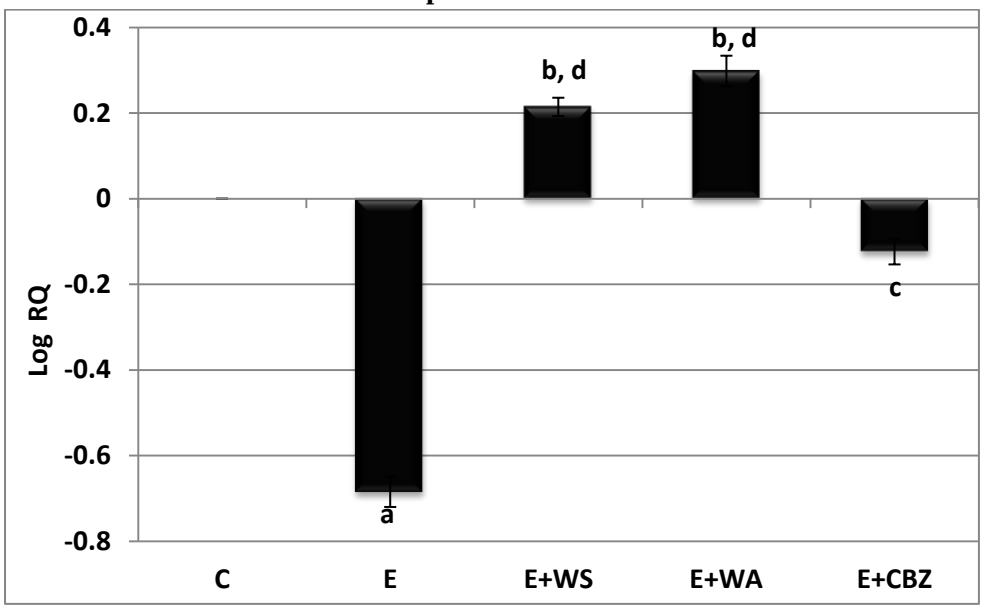


Table-71
Real time PCR amplification of GAD mRNA from the brain stem of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.68 ± 0.03** [a]
E+WS	**0.21 ± 0.02** [b, d]
E+WA	**0.29 ± 0.03** [b, d]
E+CBZ	**-0.12 ± 0.01** [c]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-79

Scatchard Analysis of NMDA receptor using [^{3}H] MK-801 binding against MK-801 in the brain stem of control and experimental rats

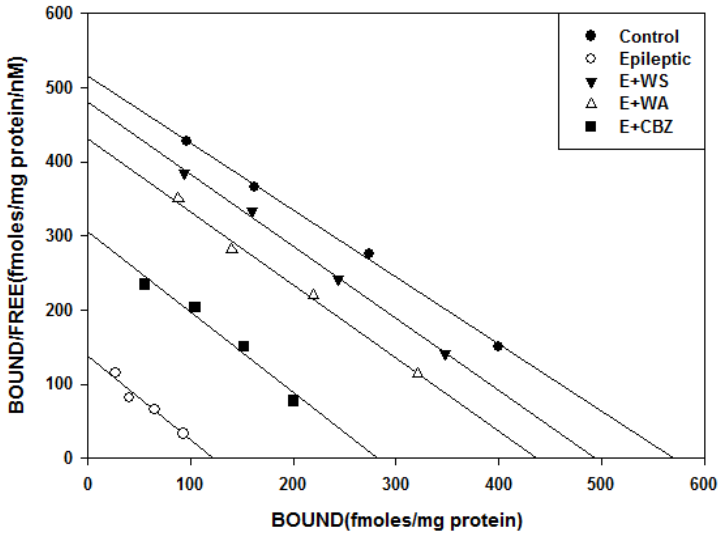


Table-72

Scatchard Analysis of NMDA receptor using [3H] MK-801 binding against MK-801 in the brain stem of control and experimental rats

Experimental groups	Bmax	Kd
Control	**567.51±10.12**	**1.09± 0.02**
Epileptic	**120.40± 9.18** [a]	**0.91 ± 0.08**
E+WS	**493.90±14.56** [c, d]	**1.03 ± 0.09**
E+WA	**437.94± 12.32** [c. d]	**1.02 ± 0.04**
E+CBZ	**277.91± 14.36** [b, e]	**0.93 ± 0.06**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group). B_{max} – Maximal binding; K_d– Dissociation constant; C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ-Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, b $p < 0.001$ when compared with epileptic group, c $p < 0.01$ when compared with control group, d $p < 0.01$ when compared with epileptic group , [e] $p < 0.01$ when compared with epileptic group

Figure- 80

Scatchard analysis of AMPA receptor using [^{3}H]AMPA binding against AMPA in the brain stem of control and experimental rats

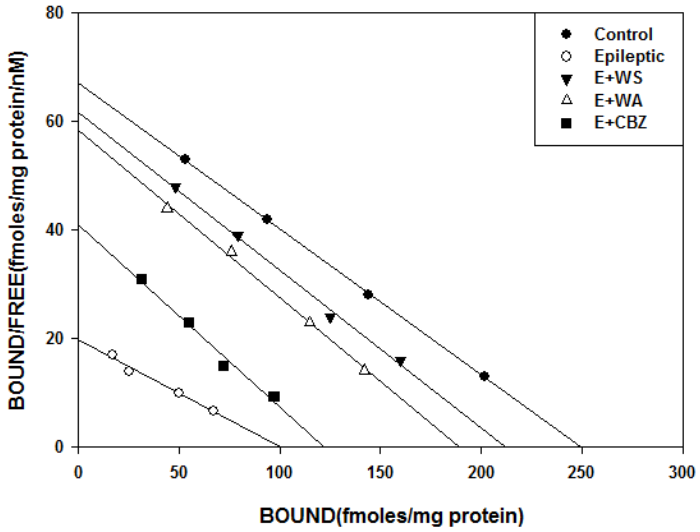


Table- 73

Scatchard analysis of AMPA receptor using [^{3}H]AMPA binding against AMPA in the brain stem of control and experimental rats

Experimental groups	Bmax	Kd
Control	**250.7± 10.8**	**3.75 ± 0.95**
Epileptic	**100.25± 12.36** [a]	**4.85 ± 0.97**
E+WS	**211.94± 13.45** [c, d]	**3.61 ± 0.91**
E+WA	**189.12±12.89** [c, d]	**3.36 ± 0.98**
E+CBZ	**121.32± 14.56** [c, e]	**3.12 ± 0.99**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group). B_{max}– Maximal binding; K_d – Dissociation constant; C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A,E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [c] $p < 0.01$ when compared with control group, [d] $p < 0.01$ when compared with epileptic group , [e] $p < 0.01$ when compared with epileptic group

Figure- 81

Real time PCR amplification of NMDA R1receptor subunit mRNA from the brain stem of control and experimental rats

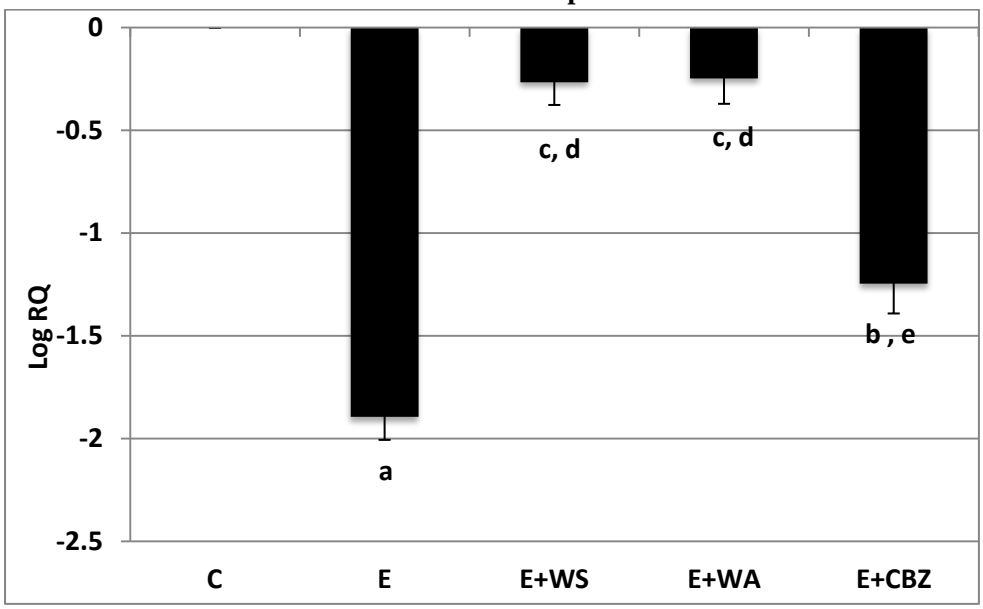


Table-74

Real time PCR amplification of NMDA R1 receptor subunit mRNA from the brain stem of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-1.95 ± 0.08** [a]
E+WS	**-0.23 ±0.09** [c, d]
E+WA	**-0.25 ±0.12** [c, d]
E+CBZ	**-1.45 ±0.14** [b, e]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with Carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group

Figure- 82

Real time PCR amplification of NMDA 2B receptor subunit mRNA from the brain stem of control and experimental rats

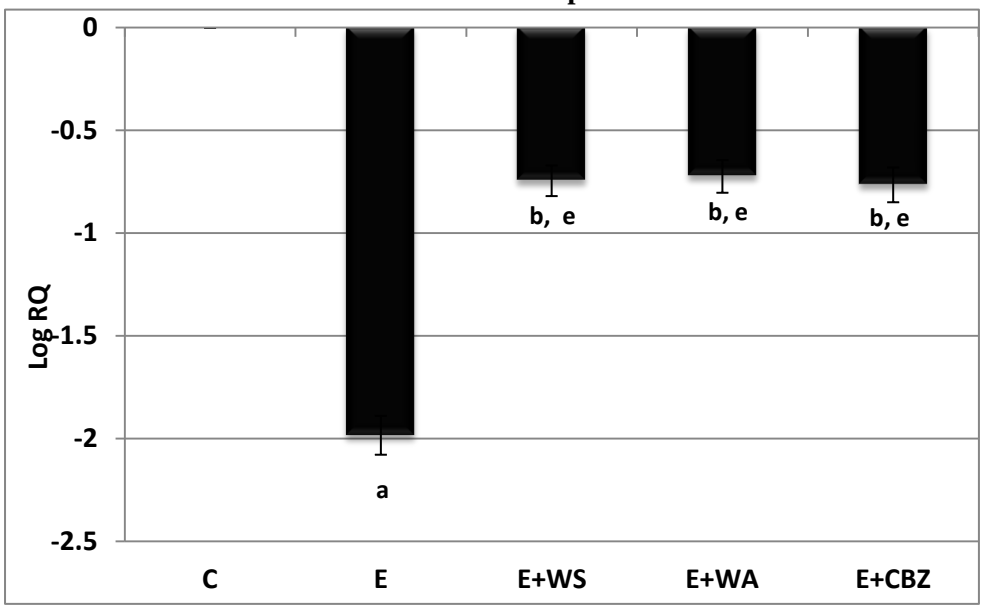


Table-75

Real time PCR amplification of of NMDA 2B receptor subunit mRNA from the brain stem of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-1.98 ± 0.09** [a]
E+WS	**-0.74 ± 0.07** [b, e]
E+WA	**-0.72 ± 0.07** [b, e]
E+CBZ	**-0.76 ± 0.08** [b, e]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [e] $p < 0.01$ when compared with epileptic group

Figure- 83

Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the brain stem of control and experimental rats

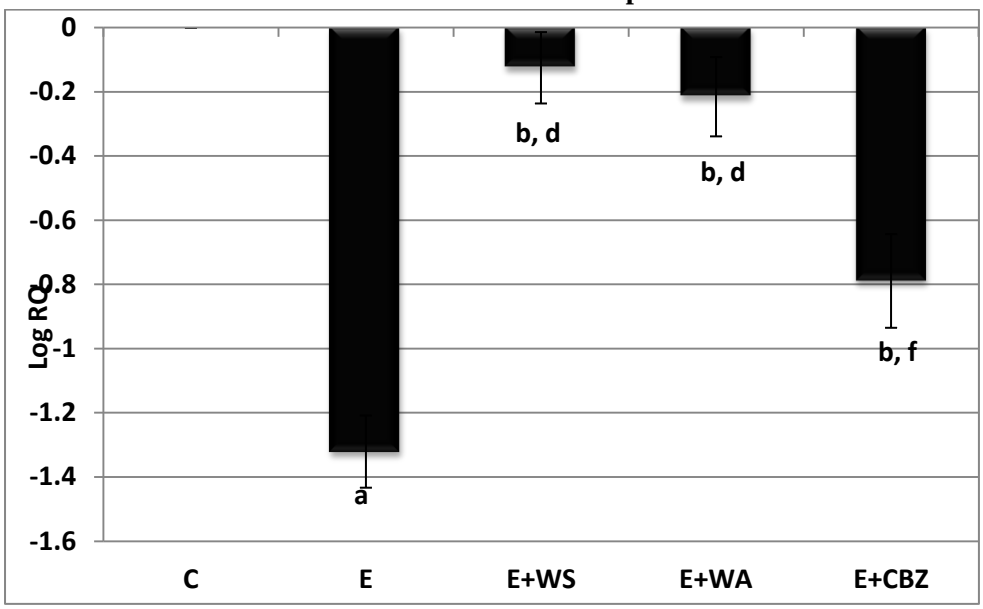


Table-76

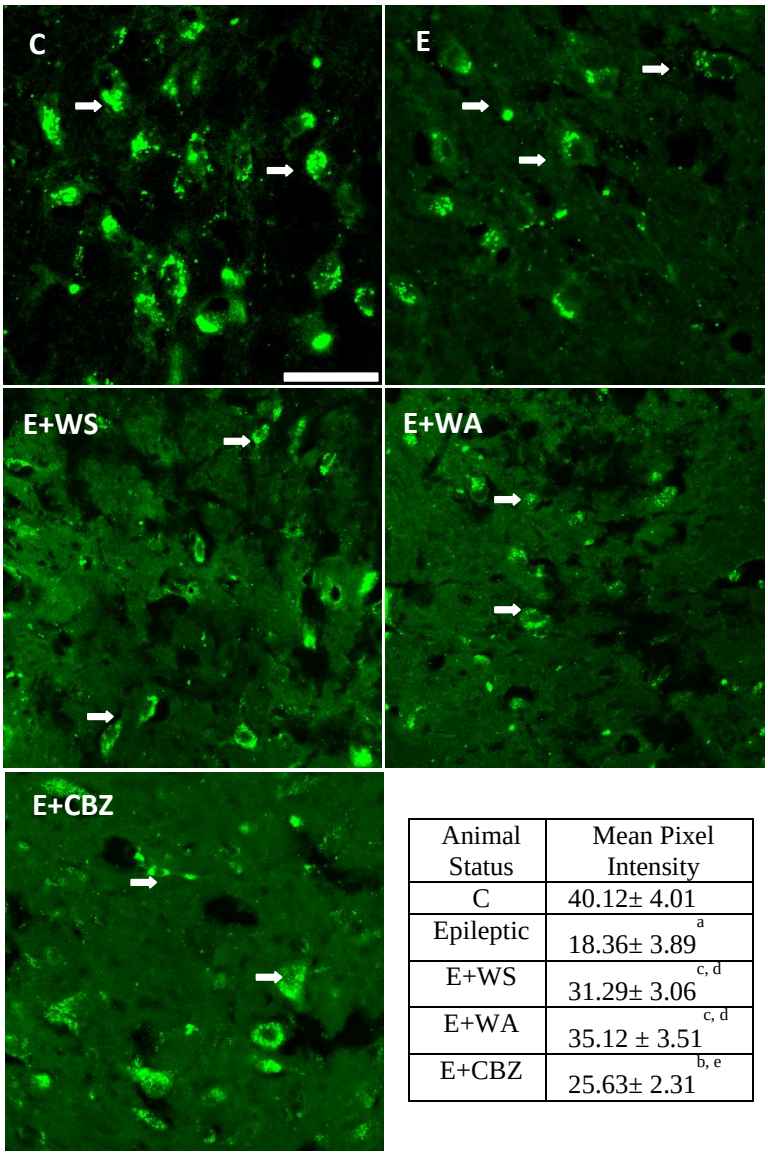
Real time PCR amplification of GluR2 subunit of AMPA receptor mRNA from the brain stem of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-1.41 ± 0.1** [a]
E+WS	**-0.36 ± 0.1** [b, d]
E+WA	**-0.35 ± 0.1** [b, d]
E+CBZ	**-0.78 ± 0.12** [b, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [e] $p < 0.01$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure- 84

NMDA R1 receptor expression in the brain stem of control and experimental rats

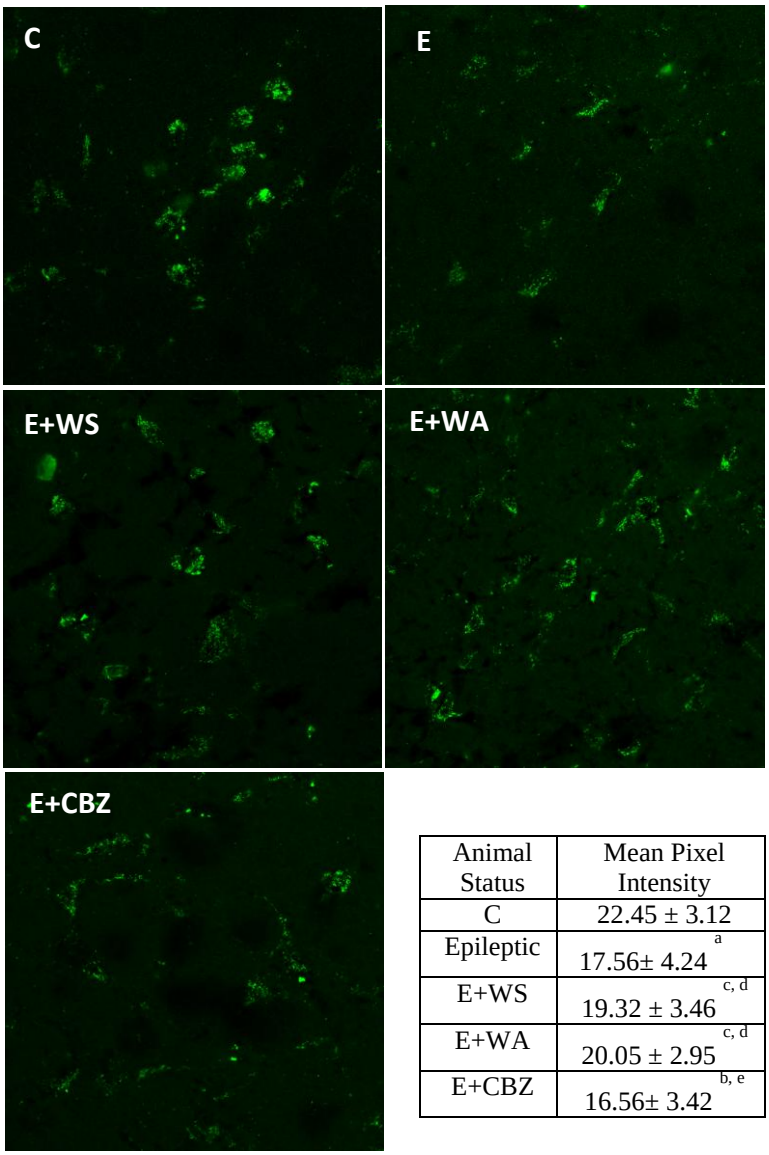


Animal Status	Mean Pixel Intensity
C	40.12± 4.01
Epileptic	18.36± 3.89 [a]
E+WS	31.29± 3.06 [c, d]
E+WA	35.12 ± 3.51 [c, d]
E+CBZ	25.63± 2.31 [b, e]

Confocal image of NMDA receptors in the brain stem of C-Control, E-Epileptic, E+WS-Epileptic+*Withania somnifera*, E+WA-Epileptic+Withanolide-A, E+CBZ-Epileptic+Carbamazepine rats using immunofluorescent NMDA R1 receptor subunit specific primary antibody and FITC as secondary antibody; [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ when compared to epileptic group; (➡) in white shows NMDA receptors. Scale bar = 50 µm.

Figure- 85

NMDA 2B receptor expression in the brain stem of control and experimental rats

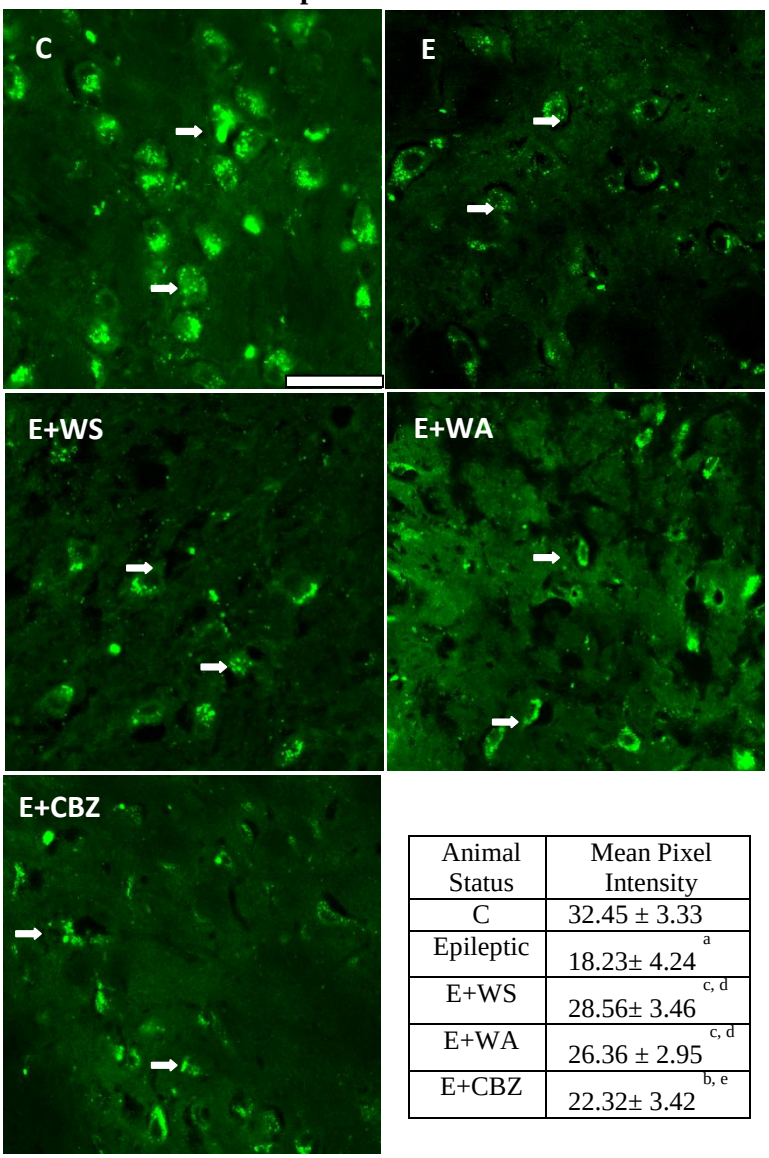


Animal Status	Mean Pixel Intensity
C	22.45 ± 3.12
Epileptic	17.56± 4.24 [a]
E+WS	19.32 ± 3.46 [c, d]
E+WA	20.05 ± 2.95 [c, d]
E+CBZ	16.56± 3.42 [b, e]

Confocal image of NMDA receptors in the brain stem of C-Control, E-Epileptic, E+WS-Epileptic+*Withania somnifera*, E+WA-Epileptic+Withanolide-A, E+CBZ-Epileptic+Carbamazepine rats using immunofluorescent NMDA 2B receptor subunit specific primary antibody and FITC as secondary antibody; [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [e] $p<0.01$, [f] $p<0.05$ when compared to epileptic group; (➡) in white shows NMDA receptors. Scale bar = 50 μm.

Figure- 86

AMPA (GluR2) receptor subunit expression in the brain stem of control and experimental rats



Animal Status	Mean Pixel Intensity
C	32.45 ± 3.33
Epileptic	18.23± 4.24 [a]
E+WS	28.56± 3.46 [c, d]
E+WA	26.36 ± 2.95 [c, d]
E+CBZ	22.32± 3.42 [b, e]

Confocal image of AMPA receptors in the brain stem of C-Control, E-Epileptic, E+WS-Epileptic + *Withania somnifera*, E+WA-Epileptic + Withanolide-A, E+CBZ- Epileptic + Carbamazepine rats using immunofluorescent AMPA (GluR2) receptor subunit specific primary antibody and FITC as secondary antibody; [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$ when compared to control group: [d] $p<0.001$, [e] $p<0.01$ when compared to epileptic group; (➡) in white shows AMPA receptors. Scale bar = 50 µm.

Figure- 87

IP3 content in the brain stem of control and experimental rats

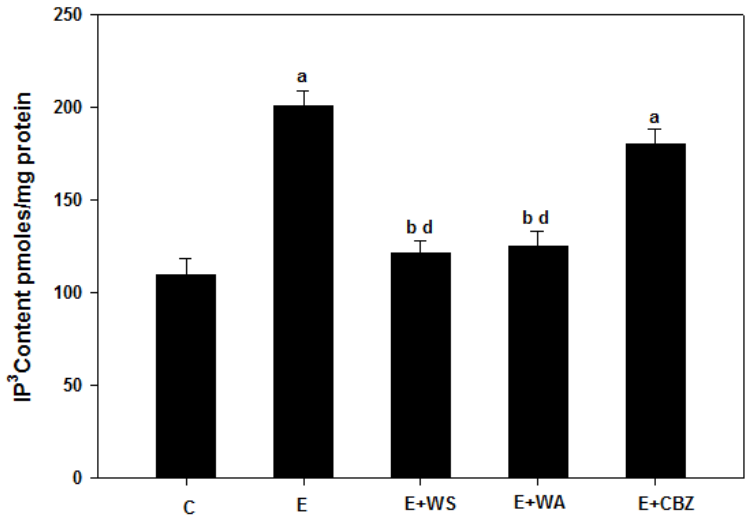


Table- 77

IP3 content in the brain stem of control and experimental rats

Animal Status	IP3 Content (pmoles/mg protein)
Control	**110.32 ± 11.3**
Epileptic	**201.22 ± 11.24** [a]
E+WS	**121.36 ± 10.96** [b, d]
E+WA	**125.65 ± 12.23** [b, d]
E+CBZ	**180.23 ± 10.56** [a]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-88
Real time PCR amplification of Bax mRNA from the brain stem of control and experimental rats

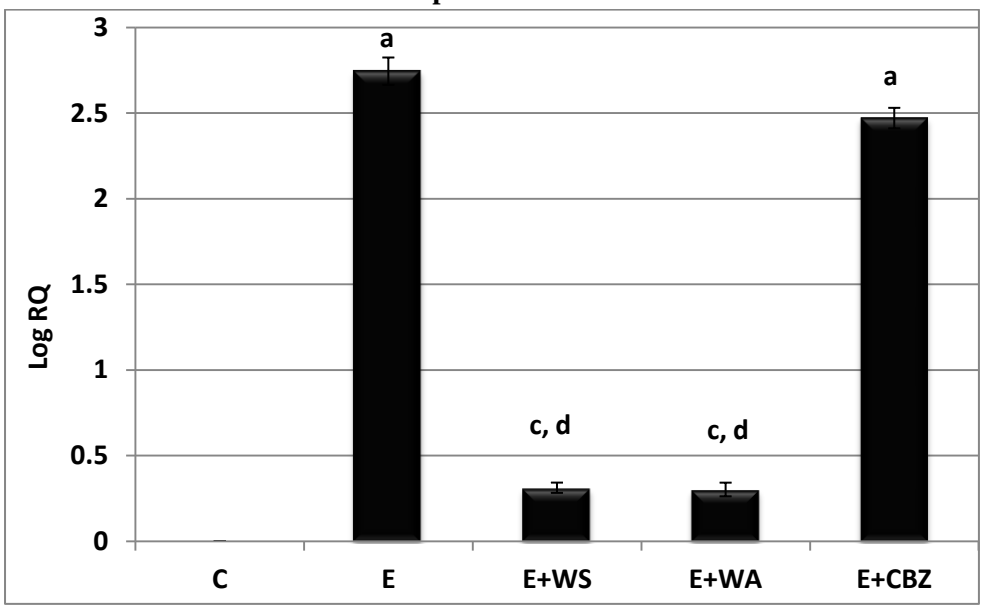


Table-78
Real time PCR amplification of Bax mRNA from the brain stem of control and experimental rats

Animal Status	Log RQ
Control	0
Epileptic	2.74 ± 0.08 [a]
E+WS	0.31 ± 0.03 [c, d]
E+WA	0.30 ± 0.04 [c, d]
E+CBZ	2.47 ± 0.06 [a]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [c] $p < 0.05$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Figure-89

Real time PCR amplification of Caspase 8 mRNA from the brain stem of control and experimental rats

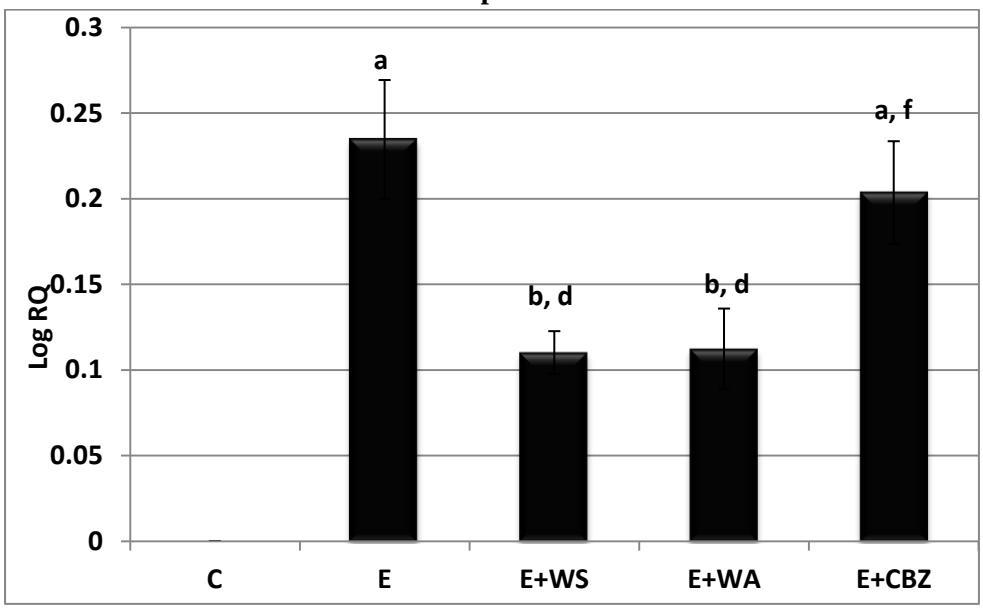


Table-79

Real time PCR amplification of Caspase 8 mRNA from the brain stem of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**0.2345 ± 0.03** [a]
E+WS	**0.1102 ± 0.01** [b, d]
E+WA	**0.1123 ± 0.02** [b, d]
E+CBZ	**0.2036 ± 0.03** [a, f]

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group, [f] $p < 0.05$ when compared with epileptic group.

Figure- 90

Real time PCR amplification of Akt-1 mRNA from the brain stem of control and experimental rats

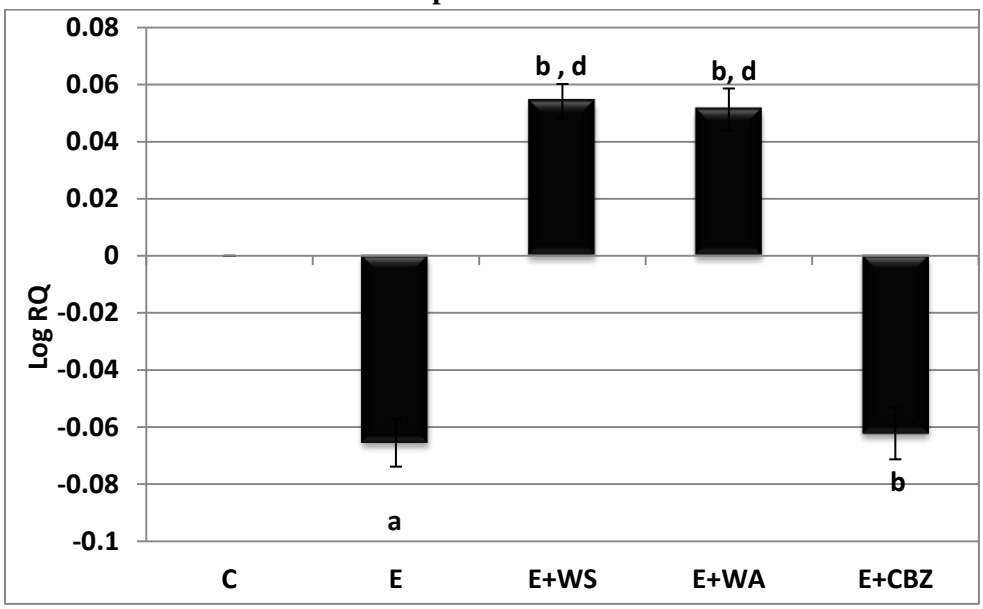


Table-80

Real time PCR amplification of Akt-1 mRNA from the brain stem of control and experimental rats

Animal Status	Log RQ
Control	**0**
Epileptic	**-0.06 ± 0.01[a]**
E+WS	**0.05 ± 0.02[b, d]**
E+WA	**0.05 ± 0.01[b, d]**
E+CBZ	**-0.06 ± 0.03[b]**

Values are means ± SEM of four to six separate experiments (n = 6–8rats per group); C-Control, E-Epileptic, E+WS-Epileptic rats treated with *Withania somnifera*, E+WA-Epileptic rats treated with Withaniolide-A, E+CBZ- Epileptic rats treated with carbamazepine; [a] $p < 0.001$ when compared with control group, [b] $p < 0.01$ when compared with control group, [d] $p < 0.001$ when compared with epileptic group

Discussion

Seizure latency

One of the most important manifestations of pilocarpine administration is the occurrence of seizures and/or *status epilepticus.* In the present study intraperitoneal injection of Pilocarpine produced seizures in rats. The effects of anti-epileptic therapy can be assessed only through evaluation of the patient's seizure frequency (Tomson *et al*., 2007). The treatment with *Withania somnifera*, Withanolide A and Carbamezepine reduced the number of seizures per hour compared to the epileptic rat groups. The severity of the seizures in the treated rat groups was also decreased. Increase in the seizure onset latency and decrease in the duration of seizures of various anti-epileptic drugs were reported earlier (Eric *et al*., 2002). This study suggests that WS and WA is capable of reducing seizure frequency and seizure severity. These results are suggestive evidence of the ability of the WS and WA in reducing the spontaneous seizures, which highlights their anti-epileptic property.

Behavioural Deficits in Epileptic Rats

There are severe behavioural consequences of spontaneous and recurrent seizures. In the present study we used Radial arm maze and Y maze tests to study the effect of epileptic seizures on spatial memory and learning capabilities and to evaluate the extent of recovery post treatment with WS, WA and CBZ. We also used Rotorod test, grid walk test and narrow beam test to analyse the deficits in motor learning and coordination and to evaluate the effect of WS, WA and CBZ administration.

Seizures induce permanent cellular alterations in hippocampal pathways that have been implicated in memory; it was of interest to determine if there are long-lasting impairments in a spatial memory task in rats. In the present study Radial arm maze (RAM) was used to evaluate behavioural deficiency ensuing

excitotoxic insult in TLE. It has been used in a variety of configurations to assess the neurobehavioral bases for learning and memory, the adverse effects of toxic chemicals and the beneficial effects of novel therapeutic treatments (Mitchell *et al*., 2002). There was a significant difference in between the control and the epileptic group in the number of trials required to achieve the criterion performance. Although, there was not much significant difference in the number of trials required to achieve the criterion performance in between the epileptic and treatment groups including E+WS, E+WA and E+CBZ. The difference in the epileptic and treatment group developed when repetitions on consecutive days were performed. The epileptic rats had a significant disability in repeating the criterion performance on consecutive days. This difference in performance indicates deficit in the ability to store and retrieve recently acquired information that was used in preceding performance in radial arm maze tasks. There has been deficit observed which are expressed as reference memory errors and working memory errors. In the present study we observe a significant deficit in the form of working memory errors which represent short term memory. Apparently in the treatment groups, especially E+WS and E+WA, there was a significant reduction in working memory errors indicating restoration of neuronal function. There was less restoratory effect observed in CBZ treated groups, showing the inability of the anticonvulsant drug in countering memory loss.

The neurodegeneration observed in the hippocampus impairs recognition and spatial memory in epileptic rats by disrupting the ability of hippocampal place cells to recognize the position of the animal (Shatskikh *et al*., 2006; Zhou *et al*., 2007). The key-role of temporomesial and neocortical structures for memory in TLE has been demonstrated by a variety of functional and volumetric imaging studies, invasive electroencephalographic studies, correlations of human hippocampal cell counts and LTP to memory performance. Memory impairment in lateralized TLE tends to be material-specific, i.e. left TLE is associated with verbal, right TLE with visual memory impairment. Neocortical temporal and mesial hippocampal structures are differentially involved in episodic memory, i.e.

the mesial structures are more non-specifically involved in consolidation retrieval and neocortical structures are more involved in material specific processing of the contents (Helmstaedter, 2001; Elger *et al*. 2004). We have used Y Maze test to analyze the alteration in recognition and spatial memory associated with hippocampal degeneration (Murugesan, 2005). The performance of epileptic rats in Y-maze test was impaired. The number of trials to attain five consecutive criterion performances increased significantly in the epileptic rats. Increased numbers of trials to criterion performance indicated the learning and memory deficit in epileptic rats. Interestingly, treatment using WS and WA considerably reduced the number of trials to attain the criterion performance indicating the restoration of spatial memory. This supports the previous reports indicating the role of WS in the reconstruction of neuronal networks and the reestablishment of cognitive functions (Kuboyama *et al*., 2005). Although the signal transduction mechanisms of WA remained unknown, one possibility was that WA stimulated signal cascades similar to β-estradiol. WA has a steroidal structure (Kuboyama *et al*., 2002). b-Estradiol is an endogenous factor that induces neurite arborization via extracellular signal-regulated kinase (ERK) (Dominguez *et al*., 2004), and enhances synaptophysin expression via membrane ER and p44 MAP kinase (Yokomaku *et al*., 2003). It is also reported that β-estradiol enhances PSD-95 transcription via the PI3-K following Akt pathway (Akama & McEwen, 2003). Behavioural deficits in epileptic rats have been mainly attributed to the rearrangement of neuronal circuits and neuronal loss in the hippocampus. Experimental studies in rodents have implicated the hippocampus in spatial learning and memory (Jarrard, 1993). Hippocampal pyramidal cells in rats discharge at specific locations in the environment (Muller et al., 1987), and maintain their receptive field in the absence of spatial and visual cues (Quirk *et al*., 1990). These observations have suggested that the hippocampus performs spatial computations by means of a cognitive map (O'Keefe and Nadel, 1978; O'Keefe, 1990). There have been also reports of alterations in neurotransmitter receptors including GABA and glutamate leading to

dysfunction in synaptogenic processes like long term potentiation and long term depression (Reas *et al*., 2009; Mathew *et al*., 2010).

In order to understand the changes in motor learning abilities rotorod test, grid walk test and Narrow beam test were performed in experimental rats. Among several behavioural tests that measure motor performance, the rotarod is a suitable test for evaluation of cerebellar deficits in rodents (Caston *et al*., 1995; Lalonde *et al*., 1995). The motor performance on the rotarod can be influenced by several factors, such as motor coordination, learning and cardiopulmonary endurance. The control rats leaned forward on the rod while stretching the head downward. In contrast, epileptic rats remained on the rod's vertex and were therefore easily pushed backward with increasing speed. Epileptic rats showed lower fall off time from the rotating rod when compared to control. The acquisition of a successful motor strategy with training was reflected in the treatment groups including E+WS and E+WA. The beam walk and grid walk test were demonstrated to detect pure motor incapacities. An increased number of foot slips in beam and grid walk test and decreased time spent in narrow beam test was observed in epileptic rats when compared to control, suggesting impairment in their ability to integrate sensory input with appropriate motor commands to balance their posture. At the same time, they had to adjust their limb movements on the metallic rod which is indicative of cerebellar dysfunction. The deficit observed through shows impaired motor activity suggesting cerebellar dysfunction. Taken together, the combination of three motor tasks including rotarod test, grid walk test and Narrow beam test involve extensive motor coordination and provided an overview on altered motor activity and motor learning behaviour in the rats. The treatment with WS and WA showed an improved motor performance in rotarod, narrow beam and grid walk tests compared to epileptic rats. This indicates reduced cellular stress and restored neuronal function, resulting in lowering their time for spatial recognition, enhanced motor learning and thus helping to maintain their posture during movement on the rod. This result is in agreement with the previous reports

indicating the role of WS in the reconstruction of neuronal networks (Kuboyama *et al*., 2005) and the reestablishment of cognitive functions.

Histopathological Changes in Hippocampus

Neuropsychological impairment is an important co-morbidity of chronic epilepsy (Elger *et al*., 2004). For instance, TLE with hippocampal sclerosis is often associated with memory impairment (Motamedi & Meador, 2003). All hippocampal regions show neuronal loss and gliosis to varying degrees, although the extent of cell loss may vary. In the present study we evaluated the neuronal loss in the hippocampal region using Nissl staining and TOPRO-3 staining. Previous studies by Balta *et al*., 2004 have shown that Nissl staining can be used to quantify experimental brain infarctions. Neuronal loss in the hilar region was the most consistent finding in the hippocampal formation. Hilar neurons die (Babb *et al*., 1984; Mouritzen Dam, 1980) and granule cell axons sprout into areas of the dentate gyrus where they normally are not found (Franck *et al*. 1995; Isokawa *et al*., 1993; Babb *et al*., 1991; Houser *et al*., 1990; Sutula *et al*., 1989; Lanerolle *et al*., 1989). Both hilar neuron loss and axon reorganization could affect functional characteristics of the dentate gyrus network. Neuron loss in the hilus of the dentate gyrus and granule cell axon reorganization have been proposed as etiologic factors in human temporal lobe epilepsy. Earlier studies reveal that hilar neuron loss vacates postsynaptic sites on granule cell dendrites, thereby triggering (or permitting) the formation of excitatory recurrent collaterals (Babb *et al*., 1991; Buckmaster *et al*., 1996; Okazaki *et al*., 1995; Represa *et al*., 1993; Cavazos & Sutula ,1990; Nadler *et al*., 1980). Excitatory recurrent collaterals could produce positive feedback, having an epileptogenic effect, TOPRO-3 staining was performed in the hippocampal sections to understand the extent of viability of cells in the hilar region. Histological analysis of Hippocampal section with TOPRO-3 staining showed a significant decrease in the nuclear staining in the epileptic rats. The results from Nissl staining and TOPRO-3 staining reveal extensive cellular loss in the hilar region of the hippocampus. Treatment with WS

and WA has resulted in enhanced Nissl staining in the hilar region indicating increased density of viable neurons in hilus of the dendate gyrus, asserting the role of WS and WA in protecting neurons from further cellular degeneration. Although CBZ treated group showed more number of viable neurons when compared to epileptic group, there was less number of viable neurons when compared to WS and WA treated groups indicating deficient action of CBZ in countering cellular damage.

Antioxidant potential of *Withania somnifera* in hippocampus

Brain injury resulting from seizures is a dynamic process that comprises multiple factors contributing to neuronal cell death. These involve genetic factors, excitotoxicity-induced mitochondrial dysfunction, altered cytokine levels, and oxidative stress (Ferriero, 2005). The relationship between free radical and scavenger enzymes has been found in the epileptic phenomena and reactive oxygen species have been implicated in seizure-induced neurodegeneration (Freitas *et al*., 2004). Using the epilepsy model obtained by systemic administration of pilocarpine in rats, lipid peroxidation, superoxide dismutase (SOD) and catalase (CAT) activities were investigated in the hippocampus of rats during chronic period. Lipid peroxidation assay is a measure of damage caused by free radicals produced as a consequence of recurring seizures. As an index of lipid peroxidation we used the formation of TBARS, which is widely adopted as a sensitive method for measurement of lipid peroxidation (Felipe *et al*., 2000). There was a significant rise in lipid peroxidation level in hippocampus of epileptic rats. These data are reflected by increase in TBARS concentrations which is related to its intermediate free radicals formation during seizures. The enhanced oxidative stress condition results in a series of changes in the cellular structure and function (Chuang *et al*., 2010). Oxidative stress can drastically affect membrane properties through lipid peroxidation, which is one of the most biologically relevant free radicals reactions. If unopposed with an effective local antioxidative defence system, peroxidative injury to phospholipids would lead to the severe cell

damage (Nguyen *et al*., 2011). Phytochemicals are well known potent free radical scavengers and it has also been reported that the root extract of WS tends to reverse the changes in lipid peroxidation and damage to cells (Dhuley *et al*., 1998). The treatment with WS and WA has considerably decreased TBARS concentration indicating reduced generation of free radicals suggesting WS as a potent antioxidant. CBZ treatment in epileptic rats did not reverse the levels of TBARS when compared to treatment groups E+WS and E+WA. This suggests that present anticonvulsive drugs especially carbamazepine is inefficient in countering enhanced oxidative stress.

The biological effects of free radicals are controlled *in vivo* by a wide range of antioxidants such as glutathione reductase (GR), glutathione peroxidase (GP), superoxide dismutase (SOD) and catalase (CAT). In the present study investigations into the activity of SOD and CAT was carried out in order to analyse the modifications in antioxidant activity in hippocampus of TLE rats. Oxidative stress exacerbates the TLE condition by severely altering the antioxidant system (Oliver *et al*., 1990). We observed a significant decrease in SOD and catalase activity in the hippocampus of pilocarpine induced epileptic rats compared to control rats. An alteration in the activity of antioxidant system including enzymes like SOD and Catalase has been reported and explained to be due to oxidative deactivation of antioxidant enzymes (Halliwell & Gutteridge, 1999; Shin *et al*., 2006). Previous investigators have reported enhanced SOD and CAT activity after treatment with glycowithanolides of WS, comparable to deprenyl an antioxidant (Mishra *et al*., 2000). In the present study the treatment with WS and WA in epileptic rats has resulted in increased activity of SOD and Catalase indicating supplemented antioxidant system. In contrast to enzyme activities there was a significant up regulation observed in SOD and GPx mRNA expression in epileptic rats which indicates post translational modification of antioxidant enzymes. On one hand, the observed reduced activity along with antigenically increased expression may be consistent with inactivation of excess protein that has been synthesized under conditions of high oxidative stress (Omar

et al., 1999). Increased protein oxidation coupled with enzyme inactivation is observed. Alternatively, the increased immunoreactivity reflects a redistribution phenomenon as the enzymes become more concentrated at the sites of increased oxidative stress, despite an overall reduction in their activity.

Altered Glutamate neurotransmission in hippocampus

Glutamate is the primary excitatory neurotransmitter in the central nervous system. It plays a key role in the initiation of seizures and has a predominant role in Epileptogenesis. Glutamate is widely distributed in the CNS and the spinal cord, being the areas of higher concentration the cerebral cortex, the hippocampus and the cerebellum (Wikinski & Acosta, 1995). The hippocampus and parahippocampal gyri play an integral part in the generation of seizures in mesial temporal lobe epilepsy and it is thus crucial to understand the propagation of excitation through these structures. The entorhinal cortex provides the major excitatory input to the hippocampus through the perforant path, which targets neurons in the fascia dentate and in the CA1-3 regions. The axonal tracts that form the perforant path split into two anatomically and functionally distinct pathways, the medial (MPP) and the lateral (LPP) perforant path, which travel along the middle and the outer third of stratum lacunosum-moleculare, respectively and target different sections of the granule cell dendritic tree (HjorthSimonsen and Jeune, 1972; Steward ,1976; Witter, 1993). The changes in the concentration of glutamate have been associated with a number of neurological disorders, including neurodegenerative diseases like Parkinson's disease, cerebrovascular diseases and epilepsy (Marmiroli & Cavaletti, 2012). In the present study glutamate content was significantly increased in the hippocampus of epileptic rats. Previous investigators have reported enhanced levels of glutamate in hippocampus of TLE patients (Cavus *et al*., 2005). In the same time the treatment with WS and WA has reduced the glutamate concentration to basal levels. In the CBZ treated group the glutamate level remained higher similar to epileptic rats. Somewhat unexpectedly, CBZ did not alter the stimulated increase in the excitatory amino acid. Ahmad *et*

al., (2005) have reported the inability of the anticonvulsant drug carbamazepine in reducing hippocampal glutamate level. Chronic exposure to high glutamate has been related to neurotoxicity and cell loss leading to hippocampal atrophy (Cid *et al*., 2003; Tanaka *et al*., 1997; Olney *et al*., 1986). Experiments in cultured hippocampal neurons have demonstrated that pilocarpine, acting through muscarinic receptors, caused an imbalance between excitatory and inhibitory transmission resulting in the generation of SE (Priel & Albuquerque, 2002). In addition, *in vivo* microdialysis studies have revealed that pilocarpine induces an elevation in glutamate levels in the hippocampus following the appearance of seizures (Smolders *et al*., 1997). Substantial evidence now supports the suggestion that, following initiation by Muscarinic M1 receptors, seizures are maintained by NMDA receptor activation (Smolders *et al*., 1997; Nagao *et al*., 1996).

The mechanisms involved in the synthesis, release, reuptake and metabolism of the excitatory neurotransmitter glutamate is of prime importance in seizure control. An increase in basal glutamate levels led to investigation into the synthesis, transport and metabolism of the glutamate. Glutamate dehrdrogenase (GDH) activity was studied, which revealed an enhanced activity of the enzyme in the hippocampus of epileptic rats. GDH catalyzes the reaction between glutamate, α Keto glutarate and ammonia using NAD^+ or $NADP^+$ as the co-enzyme (McKenna *et al*., 2006). GDH is important in glutamatergic and GABAergic neurotransmission as it directly regulates the glutamate concentration and indirectly modulates GABA levels by altering the availability of precursors. GDH is potently inhibited by GTP and activated by ADP (Plaitakis & Zaganas, 2001). The treatment with WS, WA and CBZ significantly reversed the activity of GDH to physiological levels. The treatment with WS, WA and CBZ resulted in reduced seizure frequency and severity. This implies reduced requirement consumption of energy in the form of ATP. The decreased activity of GDH after treatment with WS, WA and CBZ is suggested to be due to reduced metabolic cellular status.

The conversion of glutamate to GABA is a very necessary step in glutamate metabolism, specifically in periods of seizures. GABA is synthesized by decarboxylation of glutamate by GAD (Walls *et al*., 2011). In the present study, the Real Time gene expression analysis of Glutamate decarboxylase (GAD) was done in the hippocampus of epileptic rats. There was a significant up regulation of GAD mRNA observed. Increased GDH and decreased GAD are indicative of the accumulation of glutamate in the rat hippocampus (Reas *et al*., 2008). A possible functional implication for the increased GAD mRNA levels could be a mechanism to reduce neuronal hyperexcitability, synchronization, and/or the spread of seizure (Neder *et al*., 2002). In the pilocarpine model of chronic limbic seizures, Esclapez & Houser (1999) found an up regulation of GAD65 and GAD67 mRNAs in the rat hippocampal formation using immunohistochemistry. They proposed that the observed increase in GAD mRNAs and protein expression in GABA neurons throughout the rat hippocampal formation are activity-dependent and may be an indication that remaining GABA neurons could be highly active. In the present study treatment with WS and WA resulted in significant increase in GAD mRNA. Previous investigators have reported GABA enhancing effects of *Withania somnifera*. From our results it is ascertained that WS has critical role in conversion of glutamate to GABA.

Glutamate transporters are expressed in the plasma membrane as well as in mitochondria and synaptic vesicles in glutamatergic neurons (Ozkan and Ueda, 1998; Gegelashvili & Schousboe, 1997; Sluse, 1996). It is widely believed that EAAT1 or GLAST and EAAT 2 are primarily localized on astrocytes, whereas EAAT3 is primarily localized postsynaptically on neurons (Danbolt, 2001). The mRNA expression of GLAST was done in the hippocampus of experimental rats. There was a significant down regulation in the gene expression of GLAST mRNA in the hippocampus of epileptic rats. In a study using fully kindled rats, Akbar *et al*., (1997) found that the hippocampal expression of glial glutamate transporters GLT-1 and GLAST was unchanged. Miller *et al*., (1997) observed that kindling of rats caused diminished production of GLAST within the piriform cortex and

amygdala. However, spontaneous seizures are rarely observed in fully kindled rats. In contrast to kindling, Kainic acid (KA) induces acute seizures that cause increased hippocampal GLAST mRNA formation mainly in the CA3 area of the hippocampus (Nonaka *et al*., 1998). Furthermore, Simantov *et al*., (1999) found a modest increase in the expression of GLT-1 early after intraperitoneal administration of KA. But these studies failed to evaluate epileptic animals following the establishment of chronic seizures. In this study, Pilocarpine administered animals showed chronic recurrent seizures over the 40-day post-injection period, and at the end of this period they showed lower GLAST mRNA levels than controls. These changes in GLAST expression probably resulted from astroglia proliferation, a characteristic finding in the hippocampus of animals with pilocarpine induced epilepsy (Gorter *et al*., 2002). Mathern *et al*., (1999) found similar changes in expression of GLAST in human temporal lobe tissue obtained from patients with epilepsy. In the present study the treatment with WS, WA and CBZ reversed the GLAST expression. This permanent decrease in GLAST expression, which was observed in rats that experienced spontaneous seizure activity, could lead to normal glutamate levels in the hippocampus.

The enhanced glutamate concentration results in activated or desensitized, altering neuronal excitability (Herman & Jahr, 2007). It has been accepted that overstimulation of glutamatergic transmission and thereby activation of glutamate receptors is of significant relevance for its clinical manifestations (Urbanska *et al*., 1998). Among glutamate receptors, N-methyl-D-aspartate receptors (NMDARs) have been the focus of much basic and clinical research over the past two decades, producing an overwhelming body of evidence that blocking or suppressing NMDARs is effective in the prevention of and, in some cases, reversal of pathology in various models of neurological diseases, including epilepsy (Ghasemi & Schachter, 2011). NMDA receptor complex, consists of a membrane-spanning channel, which is highly permeable to both Na^{+}-K^{+} and Ca^{2+} in a voltage-dependent manner and possesses several regulatory sites, including glycine, Zn^{2+}, polyamine, and phencyclidine binding sites, all of which

allosterically affect glutamate mediated channel opening (Watkins & Krogsgaard, 1990; Flores-Soto *et al*., 2012). Considering the fact that NMDAR activity plays a major role in neuronal excitation in the CNS, this study has evaluated the possible alterations of NMDARs in epilepsy, using a variety of methods such as assessment of binding affinities, subunit gene expression and immunohistochemistry. Glutamatergic NMDA receptor binding studies in the hippocampus of the epileptic rats showed a significant decrease in the Bmax when compared to control, moreover there was a slight but significant change in affinity. The decreased receptor binding in the hippocampus is suggested to be due to the hyper excitability by the glutamate receptors in the initiation of seizures (Reas *et al*., 2008). The change in receptor affinity would have arisen from alteration of receptor structural properties due to change in membrane structure after lipid peroxidation (Smijin *et al*., 2012). The results obtained from gene expression analysis of NMDA R1 and NMDA 2B was similar to the receptor binding data; there was a consistent down regulation of NMDA R1 and NMDA 2B mRNA in the hippocampus of epileptic rats. There was a substantial loss of NMDA R1+ and NMDA 2B+ cells of the hippocampal area in the Immunohistochemical analysis. Previous investigators have indicated that hippocampal NMDA R1 and NMDA 2B mRNA levels change as rats progress from the latent to chronic seizure phase in the pilocarpine model of spontaneous limbic epilepsy and that NMDA R1subunit alterations correlated with mossy fiber sprouting (Mathern *et al*., 1998). In the KA-induced model of limbic seizures in rats, KA-induced seizures decrease NMDA R1 mRNA levels in CA1 and CA3 pyramidal cells (Lason *et al*., 1997). Previous investigators have also observed that the binding of [^{3}H]MK-801 is not changed at 3 h after pilocarpine injection, whereas it is decreased in stratum lucidum at 3 and 24 h after drug injection (Lason *et al*., 1997). Using non-radioactive *in situ* hybridization methods another study demonstrated that hippocampal specimens of patients with chronic temporal lobe epilepsy showed a loss of NMDA R1-positive cells that was closely related to the overall neuronal loss in the resected specimen and to Ammon's horn sclerosis. They suggested that loss of NMDA R1 expression may partly reflect pyramidal cell loss (Bayer *et al*.,

1995). In clinical cases also patients with hippocampal sclerosis, by contrast, showed decreased NMDA 2A hybridization densities per CA2/3 pyramidal neuron compared with non- hippocampal sclerosis and autopsy cases (Ghasemi *et al*., 2011). In the present study the treatment with WS and WA has reversed the receptor binding, gene expression and receptor localisation to near control levels. Active glycowithanolides of WS (10 or 20 mg/kg intraperitoneally) were given once daily for 21 days to groups of six rats. Dose-related increases in all enzymes were observed; the increases comparable to those seen with deprenyl (a known antioxidant) administration (2 g/kg/ day Intraperitoneally). This implies that WS does have an antioxidant effect in the brain which may be responsible for its diverse pharmacological properties (Bhattacharya *et al*., 1997). In another study administration of WS extract (100 mg/kg) prevented an increase in lipid peroxidation (Dhuley, 1998). It is being suggested that increased levels of free radicals resulted in altered NMDA receptor function and antioxidant activity of *Withania somnifera* restored altered antioxidant capacity. There is increased state of oxidative stress during seizures and results in enhanced production on Nitric oxide (NO). NO can down-regulate NMDA receptor-mediated function (Khaldi *et al*., 2002; Dawson *et al*., 1993; Chandler *et al*., 1993). This property is due to nitrosylation of the cysteine residue at position 399 in the N-terminus of the NMDA 2B subunit (Choi *et al*., 2002). Ca^{2+}dependent inactivation of NMDARs has also been reported by previous investigators, it provides an important feedback inhibition of Ca^{2+} influx, preventing excessive Ca^{2+} entry that can lead to neurodegeneration and excitotoxicity (Coyle and Puttfarcken *et al*., Ehlers *et al*., 1996). Moreover Reactive oxygen metabolites affect binding of ligands to membrane receptors and also coupling of receptors to G-proteins and effector enzymes. Peroxidation of membrane lipids leads to a lowered receptor density and also will alter the viscosity of the plasma membrane, which affects receptor coupling. Reactive oxygen species may also interact with thiol/disulfide moieties on receptor proteins or on other factors in the receptor system, which is responsible for alterations in receptor binding or coupling. Moreover, lipid

peroxidation is associated with the phospholipase A2 pathway, which might indirectly affect receptor function (Vliet & Bast, 1992). Although more investigation is needed to specify the role of each NMDAR subunit in pathophysiologic aspects of epilepsy, targeting specific NMDAR subunits will provide new insights into the control of seizures.

Release of glutamate from the presynaptic neuron and its binding to AMPA receptors of the postsynaptic neuron leads to cations influx into the cells, but also causes the receptor to desensitize thus preventing excitotoxic processes. In our study we elucidate the mechanism behind AMPA receptor mediated neurotoxicity in pilocarpine based TLE model. The AMPA type glutamate receptor is one of the major ionotropic receptor which is involved in the epileptogenic mechanisms (Wolfgang, 1998). AMPA receptor contributes to the early, fast component of the excitatory postsynaptic potential (Wisden & Seeburg, 1993). AMPA receptors have been shown to play a significant role in the appearance of epileptiform burst discharges in the hippocampus (Meldrum, 1999). Altered trafficking of AMPA receptors is responsible for memory deficit (Sarantis *et al*., 2012). In the present study receptor binding study of [^{3}H] AMPA in the hippocampus of the epileptic rat showed significant decrease in Bmax and Kd compared to control. GluR2 AMPA receptor subunit mRNA expression was also down regulated in the hippocampus of the epileptic rats compared to control. Considerable evidence implicates a role for down-regulation of the AMPA receptor subunit glutamate receptor 2 (GluR2) in the neurodegeneration associated with severe limbic seizures (Grooms *et al*., 2000; Pellegrini *et al*., 1997). AMPA receptors containing GluR2 are relatively Ca^{2+} impermeable (Huang *et al*., 2002), and down-regulation of this subunit could lead to formation of Ca^{2+} permeable receptors and influx of toxic amounts of Ca^{2+} in response to endogenous glutamate (Lerma, 1998; Cossart *et al*., 1996; Bennett *et al*., 1996). In conclusion, we have extended the evidence for GluR2 down regulation in hippocampal neurons vulnerable to degeneration after status epilepticus induced by pilocarpine injection. GluR2 protein as well as mRNA is decreased before cell death as

indicated by immunohistochemistry, gene expression and receptor binding studies, respectively. Decrease in GluR2 protein is expected to lead to assembly of Ca^{2+} permeable AMPA receptors lacking GluR2. The newly formed receptors could lead to cell death by permitting excessive Ca^{2+} and possibly Zn^{2+} influx in response to endogenous glutamate. Apart from that in this study itself we have reported increased levels of IP3 in hippocampus of epileptic rats. The increased levels of IP3 causes enhanced Ca^{2+} levels resulting in activation of protein kinase C leading to series of events culminating in internalisation of AMPA receptors. Long-lasting and activity-dependent changes in synaptic strength long-term pottentiation (LTP) and long-term depression (LTD) are associated with changes in the phosphorylation and cellular distribution of AMPA receptor, and are thought to underlie learning and memory formation (Morris, 2006; Pastalkova *et al*., 2006; Whitlock *et al*., 2006; Rumpel *et al*., 2005). The altered activity and expression of AMPA receptor is proposed to be one of the contributing factors responsible for the TLE associated cognitive deficit. This cognitive deficit was ameliorated after the treatment with WS and WA which is evident from the results of Y-maze test. The treatment with WS and WA is suggested to have modulated the activity and expression of AMPA receptor. WS and WA treatment reversed the changes in AMPA receptor binding and gene expression binding near to control. Immunohistochemistry studies using confocal microscope confirmed the results of binding parameters and gene expression.

Neuroprotective role of *Withania somnifera* in hippocampus

Seizure is a major form of acute brain injury that could lead to changes in gene expression, receptor composition or synaptic functions, along with activation of late cell death pathways (Sano *et al*., 2012; Liou *et al*., 2003; Macdonald and Kapur, 1999).The most prominent histopathological finding in drug-refractory temporal lobe epilepsy associated with mesial temporal sclerosis (MTS) is selective neuronal loss of varying severity in the hippocampus (Proper *et al*., 2000; Babb *et al*., 1988). The mechanism of neuronal death in MTS is unknown.

Recent evidence from experimental studies suggests that apoptosis may be involved in neuronal death after recurrent seizures (Chuang *et al*., 2010; Narkilahti *et al*., 2007; Chuang *et al*., 2007). Several markers of apoptotic cell death have been detected in rodent models of epilepsy induced by kainic acid, pilocarpine or repetitive perforant pathway stimulation (Roux *et al*., 1999). Moreover, demonstration of nuclear expression of the cell-cycle protein, cyclin B, associated with increased Bax expression in hippocampal neurons of patients with MTS, suggests apoptosis as a way of cell death in this region (Nagy & Esiri, 1998). Factors such as the variation in duration and severity of seizures, metabolic disturbances, bioenergic failure during or after seizures and age or genetic-specific factors contribute to determining the eventual pathway of cell death. Clinical and epidemiological studies suggest that despite optimal anti-epileptic drug therapy, patients with chronic epilepsy undergo progressive brain atrophy that is accompanied by long-term behavioural changes and cognitive decline (Sutula, 2004; Cendes, 2005). One of the decisive steps of the apoptotic cascade is permeabilization of the outer mitochondrial membrane (Crompton, 2000), which leads to the release of cytochrome c from the intermediate space, followed by the activation of a caspase-dependent cascade of apoptotic signalling. In the present study there was substantial cell loss visualised in the hippocampus using TOPRO-3 staining and Nissl staining of the hippocampus. Hilar cell loss was significant in nature and studies were conducted to understand the mechanism of cell death. In accordance with results from previous investigators there was a significant up regulation of Bax and Caspase 8 mRNA in the hippocampus of epileptic rats. The increased mRNA expression of Bax and Caspase 8 has confirmed neurodegeneration in the hippocampus through apoptotic pathway. SE-induced up-regulation of genes associated with apoptosis, such as caspase-3, p53, and Bax, have also been detected in adult rats (Hunsberger *et al*., 2005; Henshall *et al*., 2000; Gillardon *et al*., 1995; Sakhi *et al*., 1994). WS and WA treatment reversed these changes near to control which led us to investigate the possible role of anti apoptotic mechanisms activated after administration of WS and WA in TLE rats. This was studied using Akt or serine threonine kinase which is a member of an

anti-apoptotic cascade of neurons (Endo *et al*., 2006). The constitutively active Akt-overexpressing neurons could survive potential cellular distresses (Namikawa *et al*., 2000). The results of our study led us to a possible explanation for the anti-apoptotic effects of WS extract. Akt is part of Pi3k/Akt pathway which is activated during acute stress conditions for possible survival of cells (Sabbatini *et al*., 1999) and is an effector protein of AMPA activation (Bozzi and Borrelli., 2006). In our study there was a significant down regulation of Akt mRNA expression in the hippocampus of epileptic rats. The decreased Akt indicated the impaired anti-apoptotic system of the cell, which led to neuronal death and subsequently resulting in cognitive deficit. Treatment with WS and WA has significantly up regulated the Akt expression compared to epileptic and control rats. The positive modulation of AMPA receptor has resulted in constitutively active Akt-over expressing neurons which led to activation of cell's anti-apoptotic mechanisms leading to neuroprotection.

Role of Extrahippocampal structures in TLE

Cerebral cortex

TLE is the most common form of medically intractable partial epilepsy in adults and surgery has proved to be effective in the majority of patients. Mesial temporal sclerosis (MTS) is found in about 70% of these cases (Babb *et al*., 1987; Wolf *et al*., 1993; Pasquier *et al*., 1996) and its presence, highly associated with a past history of febrile seizures and with EEG lateralization of the epileptogenic region, is predictive of an excellent postoperative outcome. These findings have led to the definition of the mesial-temporal lobe epilepsy (MTLE) syndrome (Wieser *et al*., 1993; Cendes *et al*., 1997; Engel *et al*., 1997a). This term should be restricted to patients with the typical clinical presentation, MRI evidence of MTS, anterior and mid-inferomedial temporal ictal and interictal discharges on scalp EEG, and additional evidence of temporal lobe dysfunction from functional imaging and neuropsychology consistent with pathology on the same side. In such well selected cases, one can expect 70–80% of patients to become seizure-free

after surgery (Arruda *et al*., 1996; Garcia *et al*., 1994). The choice of whether to perform an anterior temporal lobectomy or a selective amygdalohippocampectomy varies among surgical teams. However, this concept of MTLE does not imply that the onset of seizures is always and exclusively confined to the sole sclerotic hippocampus. This point is illustrated by several studies using intracerebral electrodes (Kahane *et al*., 2001; Isnard *et al*., 2000; Spanedda *et al*., 1997; Munari *et al*., 1994), as well as by increasing evidence of extrahippocampal histological (Pitkanen *et al*., 1998) and morphological (Kuzniecky *et al*., 1987) abnormalities. These can involve other limbic structures, as well as paralimbic and temporal neocortical areas. Thus, the epileptogenic zone extends beyond the atrophic mesial temporal structures, which may explain some failures or long-term relapses of selective mesial temporal lobe (MTL) resections (Berkovic *et al*., 1995). Among extrahippocampal areas possibly involved in the genesis of MTL seizures, several studies have focused on the temporal pole (TP), a paralimbic area strongly connected with the amygdala, the hippocampus, the parahippocampal gyrus, the cingulate gyrus, the orbitofrontal cortex and the insula (Chabardès *et al*., 2005).

It is currently hypothesized that some pathological processes (such as SE), which increase glutamate release, activate a higher number of glutamatergic receptors for a critical period of time, leading to neuronal necrosis by elevating Ca^{2+} and activating potentially destructive Ca^{+2} dependent enzymes (Fujikawa *et al*., 1994). The increase of these Ca^{+2} dependent enzyme activity can induce an oxidative stress which has been implicated in a variety of acute and chronic neurologic conditions, including SE (Walz *et al*., 2000). Nevertheless, it is not well established if the reactive oxygen species (ROS) play a role in pilocarpine-induced seizures. The increase in ROS levels can also be responsible for neuropathology induced by SE (Rong *et al*., 1999). The SE also activates the ROS scavenging enzymes, such as superoxide dismutase (SOD) and catalase, indicating a cellular response to increased ROS (Ferrer *et al*., 2000). SE induces ROS production-mediated protein oxidation as measured by tyrosine nitration (Rong *et al*., 1999), as well as lipid peroxidation as indicated by malondialdehyde (Bruce &

Baudry, 1995). That being so, it is important to investigate lipid peroxidation levels, superoxide dismutase and catalase function during the chronic phase of seizures induced by pilocarpine. In the present study there was an increased level of TBARS in the cerebral cortex of temporal lobe epileptic rats. The enhanced TBARS level is an indicator of lipid peroxidation due to increased state of oxidative stress during chronic seizures. Glutamate exitotoxicity is held responsible for production of enhanced free radicals and alteration in Ca^{2+} homeostasis. There have been reports of enhanced lipid peroxidation after status epilepticus (Freitas *et al*., 2004), this study confirms the maintenance of enhanced TBARS levels in the cerebral cortex of epileptic rats in chronic phase of the disease. Lipid peroxidation in a tissue is an index of irreversible biological damage of the cell membrane phospholipid, which in turn leads to inhibition of most of the sulphydryl and some nonsulphydryl enzymes (Gilbert & Sawas, 1983). Lipid peroxidation can be induced by many chemicals and in many tissue injuries, and has been suggested as a possible mechanism for the neurotoxic effects of convulsive process (Walz *et al*., 2000; Sawas & Gilbert, 1985). Cerebral cortex plays a key role in memory, attention, perceptual awareness, thought, language, mood and consciousness (Mathew *et al*., 2011). The enhanced levels of lipid peroxidation suggest, impairment of cerebral neuronal function leading to considerable behavioural deficit. The treatment with WS and WA has resulted in decreased TBARS level indicating reduced state of oxidative stress. The advent of free radicals is accompanied by activation of antioxidant mechanisms of the cell. In the present study the activity of SOD and CAT was measured in the cerebral cortex of the experimental rats. There was a decreased activity of SOD and CAT observed in the cortex. There have been previous reports stating deactivation of antioxidant enzymes including SOD and CAT in the advent of high oxidative stress (Pigeolet *et al*., 1990). This could lead to an irreversible autocatalytic process in which the production rate of the oxidants will continuously increase, leading to cell death. Mitochondria are the primary site of reactive oxygen species (ROS) production and are uniquely vulnerable to oxidative damage. Oxidative

stress induced mitochondrial dysfunction is also held responsible for alteration of antioxidant status. In contrast to enzyme activities there was a significant up regulation observed in SOD and GPx mRNA expression in epileptic rats which indicates post translational modification of antioxidant enzyme. The enhanced expression is associated with recruitment of more enzymes for countering free radical surge and is also suggested to be due to non availability of active forms of antioxidant enzymes. The treatment with WS and WA ameliorated the altered antioxidant system. This is in agreement with results from previous investigators suggesting *Withania somnifera* as powerful antioxidants. Studies conducted on rats' brains showed the herb produced an increase in the levels of three natural antioxidants- superoxide dismutase, catalase and glutathione peroxidise (Dhuley, 1997).

The involvement of glutamate in epileptogenesis has been implicated in whole animals, slice and tissue culture models of epilepsy. Excitatory effects of amino acids on neurones were first reported by Curtis *et al*., (1959), who described the depolarising effect of glutamate on spinal neurones of the rat. Glutamate is the most abundant excitatory neurotransmitter in the mammalian CNS, accounting for perhaps one- third of all rapid excitatory synapses in the CNS (Cotman *et al*., 1987; Watkins and Evans, 1981). The principal input and output pathways to the brain use glutamate as a neurotransmitter, as do numerous excitatory local circuits in the cortex, hippocampus, cerebellum, and many other brain regions (Salt & Herrling, 1991; Cotman *et al*., 1987). In the present study there was an increase in glutamate content observed in the cerebral cortex. Previous investigators have reported that extrahippocampal volume abnormalities were bilateral and occurred in both temporal and extra-temporal cortical regions in TLE, whereas hippocampal deficits were related to the side of the epileptogenic focus (Marsh *et al*., 1997; Ferrari-Marinho *et al*., 2012). These data suggest that brain abnormalities in TLE are not limited to the epileptogenic region. The enhanced excitatory amino acid levels leading to glutamate excitotoxicity in cortical neurons gives evidence that subtle changes within neocortical regions may

be an additional risk factor for poor cognitive status of epileptic subjects. The processes by which glutamate is synthesized, released, removed from the synaptic and extra synaptic cleft and metabolized are all tightly regulated and have for many years been targets for drug discovery against neurodegenerative disorders. In the present study GDH activity was assayed in the cerebral cortex of epileptic rats. There was an increase in the activity of the enzyme indicating enhanced metabolism of glutamate. The expression of glutamate decarboxylase mRNA was down regulated in cortex of epileptic rats. Decreased GABAergic inhibition has been suggested as one cause of hyperexcitability. The treatment with WS and WA reversed the glutamate content, GDH activity to near control. On the other hand, increased expression of glutamic acid decarboxylase, the rate-limiting enzyme of GABA synthesis, has been found in treatment groups E+WS and E+WA. WS has been previously described by investigators to have GABA enhancing properties. It is proposed that enhanced expression of GAD could be the cause for enhanced inhibitory function. Increased glutamate concentration has been found in epileptogenic foci and may induce local over-excitation and cytotoxicity; one of the proposed mechanisms involves reduced extra-cellular clearance of glutamate by excitatory amino acid transporters. In the present study the GLAST mRNA was significantly down regulated. The down regulation of GLAST mRNA is an adaptive response to neuronal death or it may be a causative event contributing to neuronal death (Sarac *et al*., 2009). It has been also reported that H_2O_2, ROS and peroxynitrite can inhibit glutamate uptake through their oxidant action (Volterra *et al*., 1994; Trotti *et al*., 1996). It is also thought that oxidative processes can result in the multimerization of GLAST (Trotti *et al*., 1998), but few, if any, studies have investigated whether this can lead to down-regulation of GLAST expression. In another study the enhanced expression of SOD is correlated with down regulated expression of GLAST protein (Tortarolo *et al*., 2004). The treatment with WS and WA reversed the expression of GLAST to near control levels. This study suggests that increased GLAST protein expression, which enhances glutamate uptake function, is a potential therapeutic approach for treating epilepsy.

Considerable evidence suggests that abnormalities of specific neurotransmitter systems play a role in epilepsy. In temporal lobe epilepsy, excitatory amino acid receptors in the hippocampus and temporal lobe contribute to both increased excitability and vulnerability to excitotoxic damage (Geddes *et al*., 1990). Based on the electrophysiological and pharmacological characteristics, ionotropic glutamate receptors are usually classified into three subtypes: N-methyl-d-aspartate (NMDA), kainate, and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA). Native AMPA and NMDA ionotropic receptors are mostly heteromeric compositions of several subunits. Furthermore, based on recombinant molecular studies, subtle alterations in the composition of glutamate receptor subunits can alter the receptor's pharmacology and channel characteristics (Monaghan & Wenthold, 1997). Hence, it has been hypothesized that small changes in glutamate receptor subunit composition and/or concentrations could lead to alterations in excitatory neurotransmission and possibly contributes to seizure generation and propagation (McNamara, 1994) and (Mathern *et al*., 1997). The present study demonstrates differences in NMDA and AMPA receptor function and subunit expression as determined by Receptor binding assays, RT-PCR and immunohistochemistry in cerebral cortex of experimental rats. There was a substantial decrease in NMDA and AMPA receptor binding in the cerebral cortex of epileptic rats as compared to control. There was a down regulation of NMDA R1 and NMDA 2B mRNA expression. The decrease in NMDA receptor function in the cortex is suggested to be due to the free radical mediated blocking of NMDA receptor function. Previous investigators have reported reduced [^{3}H]MK-801 binding to NMDA receptors in cerebral cortex (Oguro *et al*., 1990). Based on the electro clinical-pathologic finding, it has been hypothesized that in Hippocampal sclerosis (HS) patients, the damaged hippocampus is a necessary factor in generating mesial limbic seizures. By comparison, in non-HS cases the hippocampus may be the passive recipient of seizures, or may amplify and propagate seizures originating from nearby cortical lesions. In the present study there was a significant downregulation of Glur2 subunit of AMPA receptor. Considerable evidence implicates a role for down-regulation of the AMPA

receptor subunit glutamate receptor 2 (GluR2) in the neurodegeneration associated with severe limbic seizures (Pellegrini *et al*., 1997; Grooms *et al*., 2000). AMPA receptors containing GluR2 are relatively Ca^{2+} impermeable (Huang *et al*., 2002), and down-regulation of this subunit may lead to formation of Ca^{2+} permeable receptors and influx of toxic amounts of Ca^{2+} in response to endogenous glutamate. In the present study there was an up regulation of Bax and Caspase 8 mRNA in cerebral cortex of epileptic rats. This indicates activation of programmed cell death cascades in cortex of epileptic rats. The cortical lesion observed in TLE patients is an evidence of propagation of excitotoxic transmission to cortical areas. The treatment with WS and WA resulted in a significant up regulation of Akt-1 mRNA in the cortex which is supposed to be the reason for activation of cell survival pathways and delivering neuroprotection. In the presence of survival factors, the PI3K–Akt/ Serum and glucocorticoid-inducible kinase (SGK) is activated. Akt and SGK prevent the execution of apoptosis at several levels, in both transcription-dependent and independent manners. Akt and SGK phosphorylate and inhibit the transcription factor FoxO, FOX (Forkhead box) proteins are a family of transcription factors that play important roles in regulating the expression of genes involved in cell growth, proliferation, differentiation, and longevity (Tuteja *et al.*, 2007). Akt indirectly inhibits p53, thereby preventing the expression of their target death genes (Yamaguchi *et al*., 2001). Akt also indirectly activates NF-κB, leading to the expression of survival genes, such as A1, Bcl-xL and IAPs. In addition, Akt acts at a step before cytochrome c release, preventing the association of the pro-apoptotic family member BAD with Bcl-xL, which allows Bcl-xL to promote cell survival. Furthermore, Akt may act at a step subsequent to cytochrome c release, possibly by phosphorylating caspase 9, APAF1 or the inhibitors of apoptosis proteins (IAPs) (Brunet *et al.*, 2001).

Cerebellum

An epileptic seizure involves widespread network interactions between cortical and subcortical structures. Traditionally, epileptic seizures have been thought of as cerebrocortical phenomena, but there have been reports of seizures that were thought to originate within cerebellar structures (Norden & Blumenfeld, 2002). These frequent episodes of seizures contribute to characteristic cognitive deficits in Temporal lobe epilepsy (TLE) including impaired motor learning and coordination (Groticke *et al*., 2007). Motor learning is a function of the brain for acquiring new repertoires of movements and skills to perform them through practice and it involves many areas of the brain (Ito, 2000). At subcortical level, cerebellum is the major brain structure involved in motor learning (Dow & Moruzzi, 1958). Its major functions range from motor and sensory timing to calibration of movements and reflexes indicating its significance in motor learning (Popa *et al*., 2012). The significant role of cerebellum in motor regulation is demonstrated by the ataxia developed following cerebellar disorders in animals and humans (Hartell, 1996). Cerebellar atrophy has been reported in neuropathological investigations of institutionalized epilepsy patients with profound loss of Purkinje cells, granule cell damage and associated Bergmann's gliosis (Gessaga & Urich, 1985). Previous studies have also established that rhythmic output from the cerebellum contributes to the maintenance of generalized seizures implying the role of cerebellum in epileptic manifestations (Norden & Blumenfeld, 2002). In the present study the effect of *Withania somnifera* and Withanolide A on altered cerebellar NMDA and AMPA receptor function and its correlation with impaired motor learning was investigated in pilocarpine model of TLE. There is increased glutamate content observed in cerebellum of epileptic rats leading to glutamate associated toxicity. It has been accepted that overstimulation of glutamatergic transmission and thereby alterations of glutamate receptors holds significant relevance in the aetiology of TLE (Urbanska *et al*., 1998). Our results show increased mRNA expression of glutamate aspartate transporter (GLAST) in cerebellum of epileptic rats. Enhanced

glutamate exposure produces neuronal injury characterized by prolonged reversible membrane depolarization, decreased membrane input resistance, loss of synaptic potentials and neuronal swelling (Sun *et al*., 2011). The increased expression of GLAST mRNA represents a preventive or compensatory response to increased glutamate content. The treatment with WS and WA has resulted in physiological expression of GLAST mRNA and decreased glutamate content indicating regulation of glutamergic neurotransmission. We also report an increase in intracellular IP3 content in cerebellum of epileptic rats. Inositol phosphates are known to regulate AMPA receptor trafficking, intracellular Ca^{2+} homeostasis, particularly the release of stored Ca^{2+} via IP3 receptors (Miyazaki, 1995). This leads to excess Ca^{2+}release from IP3-sensitive leading to neuronal damage. The treatment with WS and WA has resulted in reversal of enhanced IP3 content. Cerebellum is the brain region which is mainly responsible for motor coordination and motor learning. One of the major mechanism which is a hallmark of cerebellar synaptic plasticity is long-term depression (LTD). LTD is modification to synaptic strength, expressed as AMPA receptor responsiveness to glutamate, and is currently the best molecular correlate of learning and memory. The modulation of AMPA receptor numbers in postsynaptic membranes might also provide a powerful mechanism to modulate synaptic strength. Indeed, a large body of evidence supporting this hypothesis has emerged over the last decade (Malinow and Malenka 2002; Sheng and Lee, 2001). LTP and LTD forms of Synaptic plasticity can be affected after AMPA receptor alterations leading to cognitive deficit (Victor *et al*., 2007). AMPA receptors mediate mass excitatory synaptic transmission in the central nervous system (Paz *et al*., 2011) and hold significant role, as they are prone to desensitisation suggesting them as a relevant drug target (Rogawski, 2011). The effect of seizures leading to alterations in glutamate receptor activity and expression is a hallmark of TLE (Ghasemi and Schachter, 2011). Receptor Binding study of [^{3}H] AMPA in the cerebellum of the epileptic rat showed significant decrease in B_{max} compared to control. There was no significant change in K_d. AMPA receptor mRNA was down regulated in the

cerebellum of the epileptic rats compared to control. The decreased receptor binding in the cerebellum is suggested to be due to the hyperexcitability caused by glutamate release in the initiation of seizures. Apart from that in this study itself we have reported increased levels of IP3 in cerebellum of epileptic rats. The increased levels of IP3 causes enhanced Ca^{2+} levels resulting in activation of protein kinase C leading to series of events culminating in internalisation of AMPA receptors. Long-lasting and activity-dependent changes in long-term depression, LTD is associated with changes in the phosphorylation and cellular distribution of AMPA receptor, and is thought to underlie learning and memory formation (Morris, 2006; Pastalkova *et al*., 2006; Rumpel *et al*., 2005). The altered activity and expression of AMPA receptor is proposed to be one of the contributing factors responsible for the TLE associated motor learning deficit. This cognitive deficit was ameliorated after the treatment with WS and WA which is evident from the results of rotarod, grid walk and Narrow beam tests. The treatment with WS and WA is suggested to have modulated the activity and expression of AMPA receptor. WS and WA treatment reversed the changes in AMPA receptor binding and gene expression binding near to control. Immunohistochemistry studies using a confocal microscope confirmed the results of binding parameters and gene expression. The present study demonstrates that alteration in cerebellar AMPA receptor function could severely alter motor learning in TLE rats. The treatment with WS and WA restores motor learning deficit in epileptic rats. The result of the present study also shows that WS and WA modulate AMPA receptor function and ameliorates motor learning deficit.

In the nervous system, the phenomena denominated excitotoxicity have been related to over production of free radicals by the tissues and probably, due to a neuronal hyperactivity and/or an excitotoxicity, might induce an increase (Smijin *et al*., 2012) of free radical levels during pilocarpine-induced seizure and SE (Freitas *et al*., 2004; Simonié *et al*., 2000). In the present study the effect of seizure on antioxidant parameters were analysed. SOD and CAT assay was performed in cerebellum of experimental rats. There was a decreased activity of

SOD and CAT observed in the cerebellum. This is possibly due to inactivation of the enzyme due to enhanced levels of free radicals. This impaired activity of the antioxidant enzymes results in a state of oxidative stress. The expression profiles of SOD and GPx mRNA was contrary to the activity. There was an enhanced expression of SOD and GPx mRNA in the cerebellum of epileptic rats. This expression profile is the result of enhanced need of antioxidant enzymes in the system. The overall peroxidation activity in cerebellum due to enhanced oxidative stress was determined by employing the thiobarbituric acid-reactive substances (TBARS) assay, a measure of lipid peroxidation. There was an enhanced TBARS level in the cerebellum of epileptic rats. It has been reported earlier that increased generation of free radicals or reduced activity of antioxidative defence mechanisms can cause epilepsy and in addition, increases the risk of seizure recurrence. (Hamed *et al*., 2004; Maertens *et al*., 1995) On the other hand, studies in animal models showed the seizures per se may result in free radical production and oxidative damage to lipids and DNA (Chen *et al*., 2010). In the present study, the treatment with WS and WA significantly reduced the altered SOD, CAT, GPx and lipid peroxidation to control levels. The treatment with *Withania somnifera* is capacitive of reducing glutamate exitotoxicity (Smijin *et al*., 2012) leading to reduced state of oxidative stress. Oxidative stress is directly responsible for NMDA receptor function as the product of increased oxidative stress, Nitric oxide can down-regulate NMDA receptor-mediated function (Dawson *et al*., 1993; Chandler *et al*., 1993; Khaldi *et al*., 2002). In the present study NMDA receptor function was significantly impaired, indicated by reduced receptor binding, NMDA R1 and NMDA 2B subunit expression and receptor localisation studies. Long-term changes in synaptic transmission are thought to play an important role in brain learning and computation. Long-term potentiation (LTP) has been observed following high-frequency stimulation of glutamatergic synapses in the hippocampus and neocortex (Kirkwood & Bear 1996; Bliss & Collingridge 1993; Johnston *et al*., 1992). A common form of LTP is that involving N-methyl-D-aspartate (NMDA) receptors. Several studies have shown that NMDA receptors

are activated at the mossy fiber–granule cell (mf–GrC) relay in the cerebellum (Takahashi *et al*., 1996; Ebralidize *et al*., 1996; Kadotani *et al*., 1996; D'Angelo *et al*., 1993; Silver *et al*., 1992). Thus the alteration in NMDA receptor function is suggested to cause severe behavioural deficit keeping in mind the crucial role of NMDA receptor in synaptic functions. The treatment with WS and WA has resulted in reversal of receptor binding to physiological levels, which can be one of the possible reasons for enhanced behavioural attributes in experimental groups E+WS and E+WA. The expression of NMDA R1 and NMDA 2B receptor subunits also significantly reversed to near control. The receptor function was confirmed through receptor localisation studies using confocal microscopy.

Cerebellar atrophy is a prominent co morbidity associated with temporal lobe epilepsy. In the present study there was an increased expression of Bax and Caspase 8 mRNA, which indicated activation of apoptotic pathways in cerebellum. The relationship between chronic epilepsy and cerebellar atrophy has been recognized for quite some time (Bouchet & Cazauvieilh, 1825; Spielmeyer, 1930; Scholz, 1951). Margierison and Corsellis (1966) reported that 45% of 55 patients with chronic temporal lobe epilepsy (TLE) demonstrated injury to the cerebellum, ranging from gross atrophy to gliosis and loss of Purkinje and granular cells in neuropathologic specimens. The treatment with WS and WA resulted in reversed expression profiles of Bax and Caspase 8 mRNA. Though *Withania somnifera* has antioxidant action, the blockade of apoptotic pathway was an exemplarily property of the extract. Further studies revealed upregulation of Akt mRNA after treatment with WS and WA. Akt is part of various cell survival pathways including Pi3K/Akt pathway. *Withania somnifera* extracts have previously reported to have enhaced Pi3k expression in parkinsonian rats. The anti-apoptotic activity of WS has been shown previously by investigators in rat model of stroke (Mohanty *et al*., 2008). In the presence of survival factors, the PI3K–Akt/SGK pathway is activated. Akt and SGK prevent the execution of apoptosis at several levels, in both transcription-dependent and independent manners. Akt and SGK phosphorylate and inhibit the transcription factor FOXO,

and Akt indirectly inhibits p53, thereby preventing the expression of their target death genes. Akt also indirectly activates NF-κB, leading to the expression of survival genes, such as A1, Bcl-xL and IAPs. In addition, Akt acts at a step before cytochrome c release, preventing the association of the pro-apoptotic family member BAD with Bcl-xL, which allows Bcl-xL to promote cell survival. Furthermore, Akt may act at a step subsequent to cytochrome c release, possibly by phosphorylating caspase 9, APAF1 or the IAPs (Brunet *et al*., 2001).

Brain stem

Evidence supporting the role of the brain stem in human epilepsy is found in the infantile spasm, or West syndrome, literature (Go *et al.*, 2012). Infantile spasms are brief seizures consisting of symmetrical flexion, extension, or mixed jerks of axial or limb musculature. They occur primarily in children between the ages of 4 and 12 months and are associated with a classic interictal EEG abnormality known as hypsarrhythmia. Because infantile spasms are relatively symmetrical and are often associated with sleep disturbances, investigators have suggested that the brain stem may be involved in seizure generation (Mackay *et al*., 2004). Indeed, brain stem activation was demonstrated by PET scanning in 21 of 44 patients with infantile spasms (Chugani *et al*., 1992). Another study revealed abnormal brain stem evoked potentials in patients with infantile spasms as well as a high likelihood (6 of 10 patients) of brain stem atrophy on MRI (Miyazaki *et al*., 1993). Immunohistochemical experiments performed on deceased infantile spasm patients demonstrated decreased numbers of brain stem catecholaminergic neurons (Itoh *et al*., 2001). However, it is difficult to determine whether the reduced neuron count reflects the aetiology of infantile spasms or the consequence of multiple seizures.

Neurological functions located in the brain stem include those necessary for survival. The brain stem is the pathway for all fiber tracts passing up and down from peripheral nerves and spinal cord to the highest parts of the brain. In the present study NMDA and AMPA receptor binding studies were performed in the

brain stem of epileptic rats. The treatment with WS and WA reversed the receptor density to control levels. There was a decreased binding indicating decreased receptor density in the brain stem. Generalized seizures involve abnormal electrical activity in all of the cerebral cortex simultaneously. Therefore, it is presumed that the triggers and signals for these seizures are arising in the brain stem (Glötzner, 1979). There was an increase in glutamate content in the brain stem. The cortical brain stem glutamate projection descends from layer 5 pyramidal neurons in the prefrontal cortex (PFC) to brainstem neurotransmitter centers, including the raphe (5HT), the locus coeruleus (norepinephrine), and the ventral tegmental area and substantia nigra (DA). This projection mainly regulates neurotransmitter release in the brainstem. Like normal cerebral function, epileptic seizures involve widespread network interactions between cortical and subcortical structures. Although the cortex is often emphasized as the site of seizure origin, accumulating evidence points to a crucial role for subcortical structures in behavioural manifestations, propagation, and, in some cases, initiation of epileptic seizures. The state of glutamate exitotoxicity led to increase in oxidative stress leading to increased lipid peroxidation. There was also decreased activity of SOD and CAT in the brain stem. This could be the possibly due to alteration of enzyme activity due to oxidative inactivation. The gene expression of SOD and GPx was up regulated indicating enhanced need of antioxidant enzymes. This increased state of oxidative stress led to neuronal death which is indicated by enhanced expression of Caspase 8. The treatment with WS and WA resulted in restored antioxidant status leading to neuroprotection.

In conclusion, carbamazepine treatment faced significant challenges in facing temporal lobe epilepsy associated molecular and cellular alterations. The present study ascertain the role of herbal medicine in the form of *Withania somnifera* in regulating altered cellular, molecular and behavioural changes in temporal lobe epileptic rats.

Summary

1) Pilocarpine induced temporal- lobe- epileptic rats were used as a model to study the alterations of NMDA and AMPA receptor and their functional regulation by *Withania somnifera* and Withanolide A

2) Antiepileptic activity of root extract of *Withania somnifera,* Withanolide A and Carbamazepine were evaluated for seizure frequency over 72 hour's video recording.

3) Behavioural tests were done using radial arm maze, Y-maze, rotarod test, grid walk test, and narrow beam test to assess the motor learning and memory in epileptic rats. Epileptic rats showed impaired behavioural response. *Withania somnifera,* Withanolide A and Carbamazepine treatment to epileptic rats restored the altered behavioural deficit.

4) Histopathological studies in the hippocampus of temporal lobe epileptic rats were carried out using Nissl staining and TOPR0-3 staining. Neurodegeneration was observed in the hippocampus of epileptic rats. *Withania somnifera* and Withanolide A treatment to epileptic rats ameliorated the cellular damage. Carbamazepine treatment showed no significant reversal to control.

5) Lipid peroxidation is an outcome of excessive free radical generation. TBARS assay was performed to estimate lipid peroxidation. Lipid peroxidation was markedly increased in hippocampus, cerebral cortex, cerebellum and brain stem of epileptic rats. Treatment with *Withania*

somnifera, Withanolide-A and Carbamazepine reversed the alteration to near control.

6) Oxidative stress exacerbates temporal lobe epilepsy condition by severely altering the antioxidant system. The extent of oxidative damage was assessed by studying the antioxidant enzyme activities of SOD and CAT and gene expression of SOD and GPx in hippocampus, cerebral cortex, cerebellum and brain stem of epileptic rats. Epileptic rats showed decreased free radical scavenging capability. Concurrently, treatment with *Withania somnifera* and Withanolide A reversed the changes to near control. There was no significant reversal in Carbamazepine treated rats.

7) Glutamate content increased in the hippocampus, cerebral cortex, cerebellum and brain stem of epileptic rats. The treatment with *Withania somnifera*, Withanolide-A and Carbamazepine reversed these changes to near control.

8) Glutamate dehydrogenase activity in hippocampus, cerebral cortex, cerebellum and brain stem showed a significant increase in epileptic rats. The treatment with *Withania somnifera*, Withanolide-A and Carbamazepine reversed the changes to near control.

9) NMDA receptor functional status was analysed by Scatchard analysis using [^{3}H] MK801. The NMDA receptors in hippocampus, cerebral cortex, cerebellum and brain stem of epileptic rats were decreased compared to control with no significant change in the K_d. The treatment with *Withania somnifera*, Withanolide-A and Carbamazepine functionally reversed the alteration in NMDA receptor to near control.

10) AMPA receptor functional status was analysed by Scatchard analysis using [^{3}H] AMPA. The AMPA receptors in hippocampus, cerebral cortex, cerebellum and brain stem of epileptic rats were decreased compared to control with no significant change in the K_d. The treatment with *Withania somnifera,* Withanolide-A and Carbamazepine reversed the changes in AMPA receptor function to near control.

11) Glutamate mediates its action through its receptor subunits – NMDA R1, NMDA 2B, AMPA (GluR2). NMDA and AMPA receptor binding parameters were confirmed by studying the mRNA status of the corresponding receptor using Real-Time PCR. There was a significant down regulation in NMDA R1, NMDA 2B and AMPA (GluR2) mRNA expression, indicating alteration of receptor function in temporal lobe epilepsy. The treatment with *Withania somnifera,* Withanolide-A and Carbamazepine reversed these changes to near control.

12) To prevent glutamate mediated excitotoxic effects, it should be cleared from the extracellular space by the glutamate transporters. The gene expression of GLAST glutamate transporter was studied in control and experimental rats. GLAST showed decreased expression in hippocampus, cerebral cortex, cerebellum and brain stem of epileptic rats. The results showed impaired reuptake of extracellular glutamate formed in the diseased condition. The treatment with *Withania somnifera,* Withanolide-A and Carbamazepine reversed these changes to near control.

13) The mechanisms involved in the metabolism of the excitatory neurotransmitter glutamate are of prime importance in seizure control. Real time PCR gene expression analysis of Glutamate decarboxylase (GAD) was done in hippocampus, cerebral cortex, cerebellum and brain stem of epileptic rats. There was a significant down regulation of GAD

mRNA observed in epileptic rats. Increased glutamate dehydrogenase and decreased glutamate decarboxylase are indicative of the accumulation of glutamate in the rat hippocampus. The treatment with *Withania somnifera* and Withanolide-A resulted in significant up regulation of GAD mRNA indicating increased GABA conversion. There was no significant reversal in Carbamazepine treatment rats.

14) The differential expression of NMDA R1, NMDA 2B and AMPA (GluR2) receptor subunits in hippocampus, cerebral cortex, cerebellum and brain stem of epileptic rats was observed from the Real Time PCR. It was confirmed by Immunohistochemical studies using confocal microscope with specific antibodies in the brain slices. The treatment with *Withania somnifera,* Withanolide-A and Carbamazepine reversed the mean pixel value to near control.

15) Second messenger IP3 was increased significantly in hippocampus, cerebral cortex, cerebellum and brain stem of epileptic rats. The increased levels of IP3 causes enhanced Ca^{2+} levels leading to neurotoxicity. The treatment with *Withania somnifera,* Withanolide-A and Carbamazepine resulted in significant reversal to near control.

16) Increased expression of pro apoptotic factors, Caspase-8 and Bax was observed in hippocampus, cerebral cortex, cerebellum and brain stem of epileptic rats. The treatment with *Withania somnifera,* Withanolide-A and Carbamazepine resulted in significant reversal to near control.

17) A significant down regulation of anti-apoptotic factor Akt-1 was observed in hippocampus, cerebral cortex, cerebellum and brain stem of epileptic rats. The treatment with *Withania somnifera* and Withanolide-A resulted in enhanced expression of Akt-1. Double immunofluorescent staining for the identification of Phospho-Akt was performed in

hippocampus of experimental rats. The treatment with *Withania somnifera* and Withanolide A resulted in activation of Akt, lending neuroprotective effect in epileptic rats.

The present study focuses on the antiepileptic activity of *Withania somnifera* in pilocarpine induced temporal lobe epileptic rats. The increased state of oxidative stress is observed in the form of enhanced lipid peroxidation, decreased SOD and CAT activity and up regulated SOD and GPx expression leading to free radical mediated cellular damage visualized through histopathological studies. There was alteration in glutamate synthesis, transport and reuptake in the form of enhanced activity of GDH, down regulation of GAD gene expression and down regulation of GLAST gene expression leading to excitotoxic concentrations of glutamate. The enhanced state of oxidative stress and concurrent alteration in glutamate concentration lead to modified NMDA and AMPA receptor function. The extent of cellular damage visualized was due to activation of apoptotic pathway evident from enhanced Bax and Caspase-8 gene expression. There was also enhanced expression of Akt in WS and WA treated rats. Thus the treatment with WS and WA reversed the alteration at cellular and molecular level observed in epileptic rats suggesting anti-epileptic and neuroprotective role of *Withania somnifera* and Withanolide A.

Conclusion

The onset of spontaneous seizures triggers a cascade of molecular and cellular events that eventually leads to neuronal injury and cognitive decline. The present study investigated the effect of *Withania somnifera* (WS) root extract and Withanolide A (WA) in restoring behavioural deficit by inhibiting oxidative stress induced alteration in glutamergic neurotransmission. The subdued performance in behavioural tests shows impaired motor coordination and memory. Histopathological investigations revealed significant neuronal loss in hippocampus of epileptic rats indicating glutamate mediated excitotoxicity. The treatment with WS and WA restored behavioural deficit and ameliorated neuronal loss. An altered redox homeostasis leading to oxidative stress is a hallmark of TLE. The antioxidant potential was afflicted in epileptic rats, evident from altered activity of SOD and CAT, down regulation of SOD and GPX expression and enhanced lipid peroxidation. The antioxidant property of WS and WA restored altered antioxidant capacity. Alteration in GDH activity and down regulation of GLAST expression resulted in enhanced glutamate content in the brain regions. The metabolism of glutamate was altered in the form of down regulated GAD expression. The alteration in synthesis, transport and metabolism resulted in further increase of the glutamate concentration at the synapse leading to glutamate mediated excitotoxicity. The decreased NMDA and AMPA receptor binding and down regulated NMDA R1, NMDA 2B and AMPA (GluR2) mRNA expression indicated altered glutamergic receptor function. The treatment with WS and WA reversed altered glutamergic receptor function, synthesis, transport and metabolism. The enhanced levels of second messenger IP3 responsible for Ca^{2+} mediated toxicity was reversed after treatment with WS and WA. Neurotoxic concentration of glutamate resulted in up regulation of pro apoptotic factors Bax and Caspase 8 and down regulation of anti apoptotic factor Akt resulting in neuronal death. The treatment with WS and WA resulted in activation of Akt and down regulation of Bax and caspase 8 leading to blocking of apoptotic pathway. The treatment with WS and WA resulted in reduced seizure frequency and amelioration of associated alterations suggesting the therapeutic role of *Withania somnifera* in temporal lobe epilepsy.

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Publications

1. **Smijin Soman**, Korah P K, Jayanarayanan S, Jobin Mathew, *C. S. Paulose. Oxidative stress induced NMDA receptor alteration leads to spatial memory deficits in Temporal lobe epilepsy: ameliorative effects of *Withania somnifera* and Withanolide A. Neurochem Res. 2012 [PMID: 22700086]

2. Mathew J, **Smijin Soman**, Sadanandan J, Paulose CS. Decreased GABA receptor in the striatum and spatial recognition memory deficit in epileptic rats: effect of Bacopa monnieri and bacoside-A. J Ethnopharmacol. 2010 Jul 20;130(2):255-61. [PMID: 20451596]

3. Anju TR, **Smijin Soman**, Chinthu R, Paulose CS. Decreased cholinergic function in the cerebral cortex of hypoxic neonatal rats: Role of glucose, oxygen and epinephrine resuscitation. Respir Physiol Neurobiol. 2012 Jan 15;180(1):8-13. [PMID: 21907834]

4. Anju TR, **Smijin Soman,** Korah PK, Paulose CS. Cortical 5HT 2A receptor function under hypoxia in neonatal rats: role of glucose, oxygen, and epinephrine resuscitation. J Mol Neurosci. 2011 Mar;43(3):350-7. [PMID: 20857344]

5. Peeyush Kumar T, Antony S, **Smijin Soman**, Kuruvilla KP, George N, Paulose CS. Role of curcumin in the prevention of cholinergic mediated cortical dysfunctions in streptozotocin-induced diabetic rats. Mol Cell Endocrinol. 2011 Jan 1;331(1):1-10. [PMID: 20637830]

6. Nandhu M S, Naijil George, **Smijin Soman**, Jayanarayanan S and C. S. Paulose. Opioid system functional regulation in neurological disease management. Journal of Neuroscience Research 2010.[PMID: 20734417]

7. Sherin A, Anu J, Peeyush KT, **Smijin Soman**, Anitha M, Roshni BT, Paulose CS Cholinergic and GABAergic receptor functional deficit in the hippocampus of insulin-induced hypoglycemic and streptozotocin-induced diabetic rats. Neuroscience. 2011 Dec 3. [PMID: 22155651]

Manuscripts Submitted/ In Press

1. **Smijin Soman**, Jayanarayanan S, Anju TR, Peeyush KT, Paulose CS. AMPA receptor modulation by *Withania somnifera* and Withaniolide-A in hippocampus of pilocarpine induced temporal lobe epilepsy: regulation of glutamate mediatedexcitotoxicity through Akt activation. Neuroscience (under review).

2. **Smijin Soman,** Jobin Mathew, Nandhu MS, Korah.P.K, *C. S. Paulose. Hippocampal oxidative damage in pilocarpine induced temporal lobe epilepsy: Neuroprotective effects of *Withania somnifera* and Withaniolide-A. Phytomedicine (communicated).

3. **Smijin Soman**, Anju TR, Sherin Antony, Jayanarayanan S, *C. S. Paulose. Impaired motor learning attributed to altered AMPA receptor function in cerebellum of Temporal lobe epileptic rats: ameliorating effects of *Withania somnifera* and Withanolide A. Pharmacology Biochemistry and Behaviour (under review).

4. Korah P Kuruvilla, **Smijin Soman**, Jayanarayanan S, C S Paulose. Serotonergic dysregulation in corpus striatum of 6-Hydroxydopamine-induced Parkinsonian rats: antagonism by co-mitogenic 5-HT and GABA along with bone marrow cells. Brain Research. (Under review).

5. Jayanarayan S, **Smijin Soman**, Peeyush KT, Anju TR, Cs Paulose. AMPA receptor modulation in Streptozotocin induced Diabetic rats. Neurprotective effects of Curcumin, Molecular and cellular endocrinology (Under review).

Abstracts/ Scientific Presentations

1. **Smijin Soman**, Jobin Mathew, C. S. Paulose, Decreased Gabab Receptor in the Cerebral Cortex of the Epileptic Rats: Theraputic Application of Bacopa Monnieri, Annual Meeting of Society for Biotechnologists, India (SBTI -2009).

2. **Smijin Soman,** Jes Paul, Nandhu. M. S, Anju TR and C S Paulose. Oxidative Stress mediated apoptosis leading to neuronal damage in the corpus striatum of 6-hydroxydopamine lesioned Parkinson's rats: Neuroprotection by Serotonin, GABA and bone marrow cells supplementation. 5th Congress of FAONS & XXVIII Annual Meeting of Indian Academy of Neurosciences, Lucknow (November 2010).

3. Jobin Mathew, **Smijin Soman** & C.S.Paulose. Deceased GABAA receptor functional regulation in the brain stem of the epileptic rats effects of Bacopa Monnieri and Bacoside A. International Conference on Neurosciences updates & ISN, APSN, IBRO & SNCI school, Cochin (December 2009).

4. Pretty Mary Abraham, Jayanarayanan S, **Smijin Soman** & C S Paulose. Oxidative stress effects Glutamate receptor functional regulation in cerebral cortex of Streptozotocin induced diabetic rats: Neuroprotective effect of pyridoxine and Aegle marmelose. International Conference on Neurosciences updates & ISN, APSN, IBRO & SNCI school, Cochin (December 2009).

5. Korah P Kuruvilla, Jes Paul, Nandhu M. S., **Smijin Soman** and C. S. Paulose. Altered 5HT2A receptor and 5HTT gene expression in the corpus striatum of unilateral 6-hydroxydopamine-induced Parkinsonian rats:

Effect of serotonin, gamma amino butyric acid and bone marrow cell supplementation. The ISN/APSN School 2010 and The 10th Biennial Meeting of the Asia-Pacific Society for Neurochemistry (APSN) 2010, Mahidol University, Thailand (October 2010).

6. Chinthu Romeo, Anitha. M, Jayanarayanan. S, Korah. P. Kuruvilla, **Smijin Soman**, C.S. Paulose. Enhanced malate dehydrogenase, glutamate dehydrogenase, arginase and cholesterol in herbal formulation treated rats: A molecular study. UGC sponsored state level seminar on modern methods in herbal drug development, Kerala. (July28-29, 2010)

Honours and Awards

- Won IBS Award - Medical Biotechnology / application of software technologies in medicine at Annual Meeting of Society for Biotechnologists, India (SBTI -2009).