NEUROPROTECTIVE EFFECT OF SELECTED PLANTS AND THEIR FORMULATION AGAINST INDUCED NEURODEGENERATION

THESIS

Submitted to

The Tamilnadu Dr. M. G. R. Medical University,

Guindy, Chennai-600032, Tamilnadu, India.

In partial fulfillment of the requirement for the award of the degree of

DOCTOR OF PHILOSOPHY

(Faculty of Pharmacy)

Submitted By

C. RONALD DARWIN, M.Pharm.

Under the guidance of

Dr.C.VIJAYA, M. Pharm., Ph. D

PROFESSOR & DEAN (PG)
DEPARTMENT OF PHARMACEUTICS
ULTRA COLLEGE OF PHARMACY
MADURAI - 625020

SEPTEMBER 2014

DECLARATION

I hereby declare that the thesis entitled "**NEUROPROTECTIVE EFFECT OF SELECTED PLANTS AND THEIR FORMULATION AGAINST INDUCED NEURODEGENERATION**" submitted by me to The Tamilnadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment of the requirements for the award of Degree of Doctor of Philosophy in Pharmacy, is the result of my original and independent research work carried out at Department of Pharmaceutics, Ultra College of Pharmacy, Madurai during the period from October 2009 to September 2014 under the supervision and guidance of **Dr.C.Vijaya, M.Pharm., Ph.D.,** Professor and Dean (PG), Ultra College of Pharmacy, Madurai. The thesis or any part thereof has not formed the basis for the award of any degree, diploma, associateship, fellowship, or any other similar title, of this or any other University, previously.

Place: Madurai **Mr. C. RONALD DARWIN**

Date:

ULTRA COLLEGE OF PHARMACY,
4/235, COLLEGE ROAD,
THASILDAR NAGAR,
MADURAI - 20.

CERTIFICATE

This is to certify that the thesis entitled **"NEUROPROTECTIVE EFFECT OF SELECTED PLANTS AND THEIR FORMULATION AGAINST INDUCED NEURODEGENERATION"** is the research work done and submitted in partial fulfillment of the requirement for the award of degree of **Doctor of Philosophy in Pharmacy** of The Tamil Nadu Dr. M.G.R Medical University, Chennai. The work was done by **Mr. C. RONALD DARWIN** in the Department of Pharmaceutics, Ultra College of Pharmacy, Madurai under my guidance and supervision during the period from October 2009 to September 2014.

Place: Madurai
Date:

Dr. C.Vijaya, M.Pharm., Ph. D.,
Professor and Dean (PG),
Ultra College of Pharmacy,
4/235, College Road, Thasildar Nagar,
Madurai - 20.

ULTRA COLLEGE OF PHARMACY,
4/235, COLLEGE ROAD,
THASILDAR NAGAR,
MADURAI - 20.

CERTIFICATE

This is to certify that the thesis entitled **"NEUROPROTECTIVE EFFECT OF SELECTED PLANTS AND THEIR FORMULATION AGAINST INDUCED NEURODEGENERATION"** is the research work done and submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy in Pharmacy** of The Tamil Nadu Dr. M.G.R Medical University, Chennai. The work was done by **Mr. C. RONALD DARWIN** and was guided and supervised by **Dr.C.Vijaya, M.Pharm., Ph.D.,** Professor and Dean (PG), Ultra College of Pharmacy, Madurai, during the period from October 2009 to September 2014.

Place: Madurai
Date:

Dr. A. BABU THANDAPANI, M. Pharm.,Ph.D
Principal
Ultra College of Pharmacy,
Madurai – 20.

ACKNOWLEDGEMENT

First of all, I am thankful to the Almighty God for bestowing me with good knowledge, health, happiness and giving me strength for completing this project work.

My endeavor has been made possible only by the able guidance of my most respected and erudite guide **Dr.C.Vijaya, M.Pharm., Ph.D.,** Professor and Dean (P.G Programme), Ultra College of Pharmacy. I would like to thank her for always being available for me as a guide and supervisor. She was always a source of strength and she gave me the motivation to do things, which were beyond my capabilities. I shall always be indebted to her for the tremendous support and encouragement rendered to me during the course of this work, which made my task interesting and simple.

I take pleasure to express my thanks to the most Respected Chairman **Prof. K.R. Arumugam, M.Pharm,** for giving me an opportunity to work on this project.

I also thank **Dr. A. Babu Thandapani, M.Pharm., Ph.D.,** Principal, Ultra College of Pharmacy for his support and encouragement through the course of work.

I am extremely thankful to my Doctoral committee members **Dr. V. Vaidhyalingam**, **M.Pharm., PhD.,** Director of K. K. College of Pharmacy and **Dr. K.G Lalitha, M.Pharm.,PhD.,** Professor Department of Pharmaceutical Chemistry, Ultra College of Pharmacy for their valuable suggestions, encouragement and support to complete this work efficiently.

I take pride to thank **Dr. A. Meena**, **M.Pharm.,PhD.,** Principal, K.K. College of Pharmacy for the continuous source of encouragement, inspiration, constructive suggestions and her immense support for the successful completion of this project work.

I am deeply thankful to **Prof. Dr. Jayaraman**, Director, National Institute of Herbal Science, Chennai for giving identification and authentification of plant during the course of this work.

My immense thanks to **Dr. JamilaRose**, Associate Professor of Pathology, Government Chengalpattu Medical College, Chengalpattu for helping in the histopathological studies of my research work.

I express my grateful thanks to **Prof. Govindhan**, and **Dr. Vijayakumar**, Department of Biochemistry, Madras Medical College, Chennai for their help and assistance in the biochemical analytical studies.

I would like to remember my mentors **Dr.P.S.S Ramkumar, M.Pharm., Ph.D., Prof. G. Pratheesh, M. Pharm., (Ph.D), Prof. Sivanand, M. Pharm., (Ph.D), Mr.K.Senthilkumar, M. Pharm., Dr. P. Muralidharan, M. Pharm., Ph.D** and **Dr. D Jebasingh, M. Pharm., Ph.D** for their constant encouragement.

I wish to express my gratitude and my sincere thanks to **Prof. S. Kanagaraj**, **Dr. K. SenthilKumaran, Dr. K. Sujith, Mrs. Jamuna Mary**, **Mrs. RajaRajeswari, Mr. Joseph, Mrs. Ezhil and Mr. Jayaraman**, K. K. College of Pharmacy for all their support and concern.

I wish to extent my sincere thanks to my friends and relation **Mr. Kamaraj, Mr. Khalidhas, Mr. Mani, Mr. Bakthasingh** and **Dr. Jepa Chandra Mohan** for their priceless help and support

I wish to extent my sincere thanks to all the **Teaching** and **Non-teaching staff members** of K. K. College of Pharmacy for their help and support to make this work successful.

I thank my mother, brothers, sisters and in-laws who have stood as pillars of support in all my endeavors, whose love and care is always there with me during all my difficult times. They are the source of my inspiration always wishing the best for me from the core of their heart.

A special thanks to **my wife A.D.Sheena** and **my daughter Shauna Ronald** towards their co-operation, sacrifices, compromises, and affection that made me to have firm grip over this target. I express my sincere thanks to one and all and also to those whom I might have missed to mention, who gave constant encouragement and help throughout my educational career.

Last but not least, my thanks to the number of lab animals sacrificed for the completion of my research work.

TABLE OF CONTENTS

ABBREVIATIONS

6-OHDA	6- hydroxy Dopamine
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AMPA	α- amino-3-hydroxyl-5-methyl-4-isoxazolepropionoc acid
APP	Amyloid precursor protein
Aβ	Alzheimer amyloid beta
cAMP	Cyclic adenosine monophospate
ChAT	Cholineacetyl-transferase
CNS	Central nervous system
DA	Dopamine
DPPH	1,1-Diphenyl, 2-picryl hydrazyl
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylene diammine tetraacetate
HAEE	Hydroethanolic extract of *Echinops echinatus*
HAAS	Hydroethanolic extract of *Annona squamosa*
GABA	Gamma amino-butyric acid
GPx	Glutathione peroxidase
GRD	Glutathione reducate
GSH	Reduced glutathione
5-HT	5- Hydroxytryptamine
i.p	Intraperitoneal
MDA	Malondialdehyde
NA	Noradrenaline
NADPH	Nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl- D- aspartate
NO	Nitric oxide

OECD	Organization of economic cooperation development
OH	Hydroxyl radical
p.o	Per oral
PCD	Programmed cell death
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDL	Step down latency
SDS	Sodium dodecyl sulphate
SN	Substantia nigra
SOD	Super oxide dismutase
TBA	Thiobarbituric acid
U.V	Ultra violet
WHO	World health organization
PD	Parkinson's disease
et al	Others
DNA	Deoxyribonucleic acid
mRNA	messenger RNA
SNpc	Substantia nigra pars compacta
OS	Oxidative stress

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

I. INTRODUCTION

1.1 NEURODEGENERATION

Neurodegeneration is defined as the progressive loss of structure and function of neurons in the central nervous system. Neurodegenerative diseases have a devastating impact on millions of individuals and their families. Left unchecked, the prevalence of these diseases will grow at an alarming rate (Goodman 1990). There is an urgent need to accelerate the search for effective treatments and cures. Many neurodegenerative diseases including Parkinson's, Alzheimer's, and Huntington's occur as a result of neurodegenerative processes. WHO also assures that by 2030, as many as 1 in 5 among us will suffer from neurodegenerative disease. If left unchecked few years from now, more than 12 million will suffer from neurodegenerative diseases. Finding treatments and cures for neurodegenerative diseases is a goal of increasing urgency (Scholtissen B 2006).

1.1.1 Common characters of neurodegenerative diseases

The progressivity of neuronal death has also led to speculation on the very long phase of the diseases, which asks key questions concerning early diagnosis and treatment; a stage at which the clinical signs of the disease are not expressed. It is thus claimed that the earlier the treatment of the disease, the better it would be for limitation of neuronal destruction and clinical expression. Such a consideration emphasizes the search for early events of the diseases and critical putative biomarkers as well as stimulating research programs to promote therapeutic approaches focused at the etiological level leading to true neuroprotection against the diseases (Hampel *et al*., 2010).

1.1.2 Neurons

Neurons are the building blocks of the nervous system which includes the brain and the spinal cord. Neurons normally will not reproduce or replace themselves, so when they become damaged or die they cannot be replaced by the body. Neurodegenerative diseases are incurable and debilitating conditions that result in progressive degeneration and death of nerve cells. This causes problems with movement called ataxias and mental functioning called dementias **(**Dauer W *et al.*, 2003**).**

1.2 NEURODEGENERATIVE DISESASES

Neurodegenerative diseases including Parkinson's, Alzheimer's, and Huntington's occur as a result of neurodegenerative processes.

1.2.1 Alzheimer's disease

The most frightening and devastating of all neurological disorders is a dementia that occurs in the elderly. The most common cause of this illness is Alzheimer's disease (AD). The earliest symptoms of AD include forgetfulness, disorientation: to time or place, and difficulty with concentration, calculation, language, and judgment (Ashes *et al.,* 2007).

1.2.2 Parkinson's disease

Parkinsonism describes a syndrome of which Parkinson's disease is the main cause. Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons of substantia nigra pars compact in the ventral midbrain. The loss of dopaminergic neurons, leads to the reduction of dopamine being released into the striatum. This results in bradykinesia, resting tremor, rigidity and difficulty in initiating movements. Defective nucleoli apparently cause oxidative stress in cells. This can lead to massive cell damage and may be a key prerequisite for the typical nerve damage of Parkinson's disease *(*Rieker *et al.,* 2011).

1.2.3 Amylotropic Lateral Sclerosis Huntington's disease

Huntington' disease (HD) is now considered one of the most common hereditary brain disorders. It progresses slowly over a 10 to 20 year period and eventually robs the affected individual of the ability to walk, talk, think and reason. HD usually appears between the ages of 30 and 50. It affects both the basal ganglia, which control coordination, and the brain cortex, which serves as the centre for thought, perception, and memory (Ashes C *et al.,* 2007).

1.3 CAUSES OF NEURODEGENERATION

Protein misfolding

Several neurodegenerative diseases are classified as proteopathies as they are associated with the aggregation of misfolded proteins (Morales R *et al.,* 2009).

Alpha-synuclein can aggregate to form insoluble fibrils in pathological conditions characterized by Lewy bodies, such as Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy (Baba M 1998).

Tau hyperphosphorylated tau protein is the main component of neurofibrillary tangles in Alzheimer's disease (Mandelkow E 2007).

Beta amyloid is the major component of senile plaques in Alzheimer's disease.

Protein degradation pathways

Parkinson’s disease and Huntington’s disease are both late-onset and associated with the accumulation of intracellular toxic proteins. Diseases caused by the aggregation of proteins are known as proteinopathies **(**Warrick JM 1998).

Programmed cell death

Programmed cell death (PCD) is death of a cell in any form, mediated by an intracellular program. There are, however, situations in which these mediated pathways are artificially stimulated due to injury or disease. Caspases (cysteine-aspartic acid proteases) cleave at very specific amino acid residues. There are two types of caspases: initiators and effectors. Initiator caspases cleave inactive forms of effector caspases. This activates the effectors which in turn cleave other proteins resulting in apoptotic initiation.

1.4 NEURODEGENERATION AND PARKINSONS DISEASE

Parkinson's disease is one among the neurodegenerative disorder which affects at least 6.5 million people worldwide, irrespective of gender, social, ethnic, economic or geographic boundaries. Symptoms of the disorder such as tremor, rigidity and bradikinesia develop when about 3/4 of dopaminergic cells are lost in the substantia nigra (McGees *et al.*, 2004).

Depression and hallucinations are common, and dementia eventually occurs in 20% of patients. At this time, there is no treatment to delay or stop the progression of PD. Rather, the medications currently available aim more towards the alleviation of these symptoms. New surgical strategies may reversibly switch on the functionally damaged circuits through the electrical stimulation of deep brain structures, but

although deep brain stimulation is a major advance, it is not suitable for all patients (Oliveri *et al*., 2001).

It remains therefore necessary to generate new safe medicines by using preclinical models. Selective neurotoxic disruption of dopaminergic pathways can be reproduced by injection of 6-hydroxydopamine (6-OHDA) or MPTP (1-methyl-4-phenyl-1,2,3,6-tertahydropyridine) whereas depleting drugs and oxidative-damaging chemicals may also reproduce specific features of PD in rodents. Unlike MPTP, 6-OHDA lesions cause massive irreversible neuronal loss, and can be uni- or bilateral. (Annett LE *et al., 1997*)

The 6-OHDA lesion model is reliable, leads to robust motor deficits, and is the most widely used after 40 years of research in rats. The loss of dopaminergic input occurs within days, and the functional impairments can be monitored over post-operative weeks and months by rating animal rotations induced by dopaminergic agents (Scholtissen *et al*., 2006).

Parkinson's disease is a progressive disorder of movement that occurs mainly in the elderly. The most important characteristic of PD (Parkinson's disease) is degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SNc). Damage of this region result in movement disorders and the most parkinsonian symptoms are tremor, rigidity, and bradykinesia (Rang *et al*., 2007).

1.5 INTRODUCTION OF PARKINSON'S DISEASE

Parkinsonism describes a syndrome of which Parkinson's disease is the main cause. Parkinson's disease (PD) is a chronic neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons of substantia nigra pars compacta in the ventral midbrain. The loss of dopaminergic neurons, leads to the reduction of dopamine being released into the striatum. These processes are then responsible for the clinical features of PD including bradykinesia, resting tremor, rigidity and difficulty in initiating movements (Speciale SG *et al.,* 2002). Mutations in the α-synuclein or Parkin gene have been associated with familial PD (Abbas N *et al.,* 1999).

The prevalence of Parkinson's disease in industrialized countries is estimated at 0·3% of the general population and about 1% of the population older than age 60

years (Rajput AH et al., 1992). People of all ethnic origins can be affected, and men are slightly more prone to the disorder (Baldereschi M *et al* 2000).

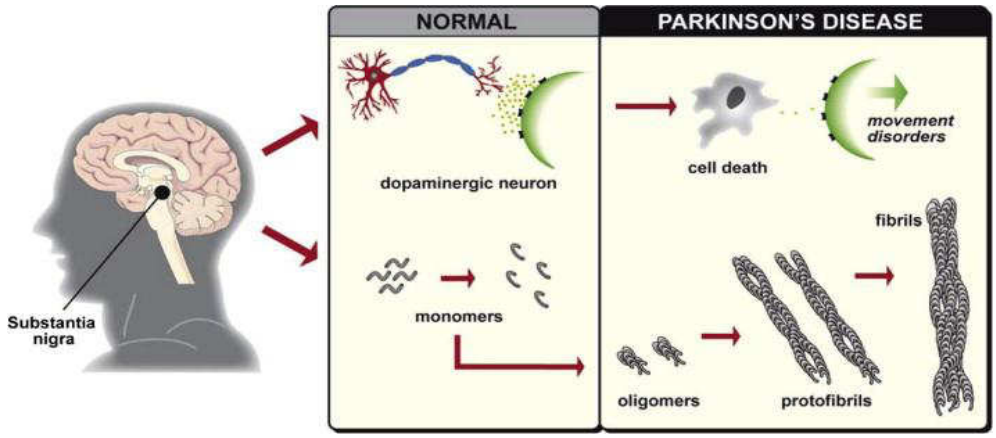


Figure 1. Positron-emission tomographic scan of the brain showing the difference in fluorodopa (FDOPA) levels between those with and without Parkinson's disease.

The Dopaminergic pathways functions are divided into three parts

- ❖ Motor control [Nigrostriatal pathways]
- ❖ Behavioral effects [Mesolimbic / mesocortical pathways]
- ❖ Endocrine control [Tubero infundibular / tubero hypophyseal pathways]

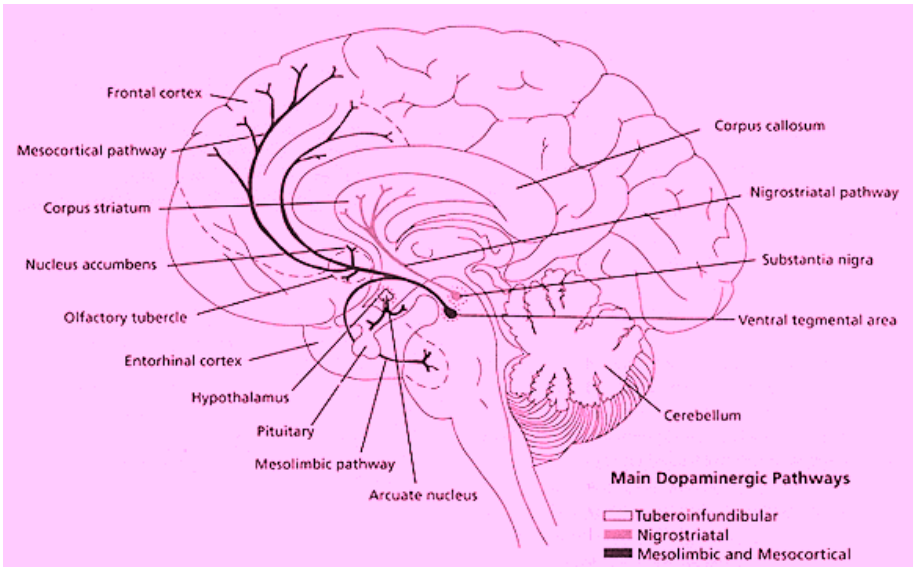


Figure 2. Dopaminergic Pathways in brain

1.5.1 Nigrostriatal pathway in brain

The substantia nigra is a part of extrapyramidal system which ends in the striatum. It is the source of dopaminergic neurons. This each neuron makes thousands of synaptic contacts within the striatum and thereby modulates the activity of a large number of cells. From substantia nigra these dopaminergic projections fire tonically,

rather than in response to specific muscular movements or sensory input. The neostriatum is connected to the substantia nigra by neurons that secrete the inhibitory transmitter GABA at their termini in the substantia nigra. In turn, cells of the substantia nigra send neurons back to the striatum, secreting the inhibitory transmitter dopamine at their termini. This mutual inhibitory pathway normally maintains a degree of inhibition of the two separate areas. Nerve fibers from the cerebral cortex and thalamus secrete Ach in the neostriatum, causing excitatory effects that initiate and regulate gross intentional movements of the body *(*Goodman 1990).

In Parkinson's disease, destruction of cells in the substantia nigra results in the degeneration of neurons responsible for secreting dopamine in the neostriatum. Thus the normal modulating inhibitory influence of dopamine on cholinergic neurons in the neostriatum is significantly diminished, resulting in overproduction or a relative over activity of acetylcholine by the stimulatory neurons. This triggers a chain of abnormal signaling, resulting in loss of the control of muscle movements resulting in the Parkinsonism (Goodman 1990).

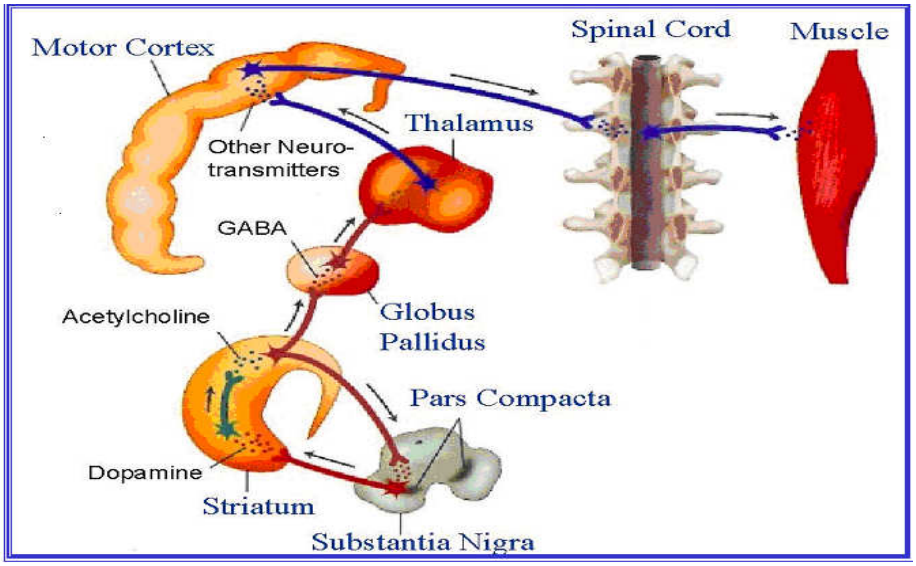


Figure 3. Nigrostriatal pathway in brain

1.5.1 Mesolimbic pathways

In these pathways cell bodies arise from midbrain(mesencephalon) to end in the nucleus accumbens, which is situated cranial to the corpus striatum and is part of limbic system.

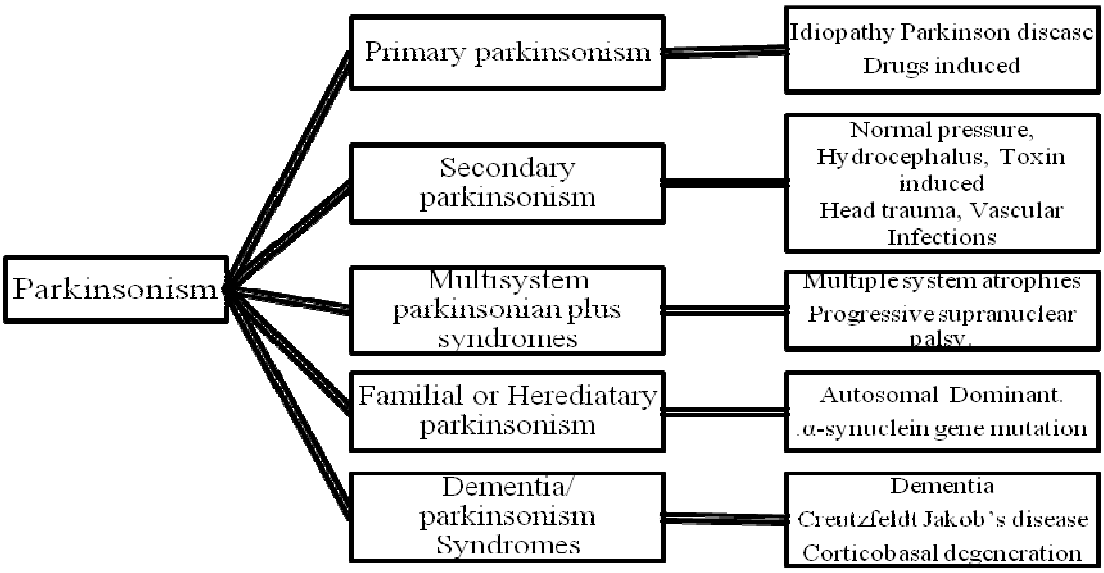
1.5.3 Tubero Infundibular pathways

The cell bodies of these pathways arise from the arcuate nucleus of the hypothalamus to end in the lactotrophs (prolactin secreting cells of the anterior pituitary (Goodman 1990).

1.6 CLASSIFICATION

The causes of Parkinsonism are still mysterious. But based on the etiology it is divided into five types. Majority of cases majorly falls under idiopathic type, in this type the cause is unknown; it is commonly referred as Parkinson disease (PD). Secondary Parkinsonism's, multiple Parkinson plus syndromes and hereditary parkinsonism are less forms of parkinsonism.

Table: 1 Classification of Parkinsonism



1.6.1 Primary Parkinsonism

Idiopathic Parkinson's disease is a neurodegenerative disorder, forms the major subgroup of patients with Parkinsonism, which cannot be cured. The cause of this type is still mysterious. The onset of Parkinson's disease is gradually slow and progressive and also varies with individuals. Characterized by the presence of Lewy bodies in the pigmented neurons of the substantia nigra and loss of neurons from substantia nigra. Besides motor symptoms, cognitive and affective symptoms have been reported. In 65% to 70% patients have asymmetric tremor that too mostly in upper extreme, as the time passes it progresses to other part along with difficulty in

gait, bilateral bradykinesia. Freezing occurs in advanced cases. Postural instability and falls are considered as late feature (Paulson HL & Stern MB 1997).

1.6.2 Secondary Parkinsonism

Unlike the idiopathic Parkinson's disease secondary Parkinsonism can be cured. It can be caused by drugs, arteriosclerosis, tumors, chemicals, trauma, and some photographic dyes and also by various infections (Warrell & Weatherall 2007).

- **Drug induced Parkinsonism** has been the second most common form of Parkinsonism. Drugs that deplete central stores of dopamine such as reserpine, methyldopa and that antagonize central dopamine receptors such as chlorpromazine, haloperidol are major types of Parkinsonism inducing agents. Its symptoms and conditions such as progressive supranuclear palsy(PSP), multiple system atrophy(MSA), dementia with Lewy bodies(DLB), corticobasal degeneration(CBD) and frontotemporal dementia (FTD) are commonly indistinguishable from the PD. They are generally described as symmetrical and PD as asymmetrical. In 30% of drug induced patients asymmetry signs and symptoms are found. Clinical manifestations of drug induced patients vary from PD. The current use of the medicines by the patients showing the signs and symptoms of Parkinsonism are enquired properly and if drug induced Parkinsonism is suspected then the discontinuation of that drug will result in the improvement within 3 months and complete resolution can take place within 1 year (Sethi KD *et al.*, 1990).
- In some cases, Normal pressure hydrocephalus is associated with Parkinson's syndrome. NPH is a neurological condition characterized by pathological dilation of the ventricular system of the brain i.e. the spaces containing CSF in brain is dilated; this is due to the obstruction in flow or absorption of CSF. The parkinsonian symptoms in NPH is due the destruction of basal ganglia, leads to the vascular insufficiency to the nigrostraitum and develop the symptoms which are well documented in Parkinson's syndrome (BowerTH *et al.*, 2003).
- Head trauma can lead to the development of Parkinson's syndrome. Persons suffered with head injuries are more prone to Parkinson's disease when compare with the persons who did not met head injuries. The exact cause is not known for head trauma but it may be due to the death of brain cells during injury or in normal aging, entry of certain poisons or proteins into bloodstream

due to disruption of BBB or production of certain proteins which would not have been developed by brain cells, which starts a cascade leading to cell death (Tolosa ES & Santamaria J 1984).

- Vascular Parkinsonism can be a possible cause of Parkinsonism, also known as “arteriosclerotic parkinsonism”. 3-6% of total Parkinsonism cases fall under vascular Parkinsonism. It results from the lacunars infractions in basal ganglia, frontal lobe infracts, and deep diffusion of ischemic lesions of sub cortical white matter (Critchley M 1929).
- Infections such as influenza virus, Coxsackie virus, Japanese encephalitis B, St. Louis and West Nile viruses and HIV can cause secondary form of Parkinsonism. Among them influenza is main factor, As there are little evidences that virus infection can cause Parkinsonism. They induce by causing cell death by entering into brain, but more than that they induce cytokine storm in the brain. The sequence of cytokine induction lasts for long time and exceeds the presence of the initiating factor (Shinya A *et al.,* 2003).

Multisystem Parkinson plus syndrome

These are uncommon and usually characterized by the presence of parkinsonian motor features along with other psychiatric, neurologic and autonomic abnormalities. Variants such as multiple system atropies, progressive supranuclear palsy and corticobasal degeneration. Generally these atypical forms are unresponsive but at the best transient they give response to anti-Parkinson therapy (Rajput A 2001).

- Multiple system atropies (MSA) is a neurodegenerative disorder, with undetermined etiology. It generally manifests as a combination of Parkinson’s disease, pyramidal signs and symptoms, cerebellar dysfunction and autonomic dysfunction. All the subtypes of MSA can have parkinsonian features; among them one subtype is much difficult to differentiate from PD. In MSA levodopa therapy are difficult to interpret because of their variability. MSA patients are non or poorly levodopa responsive. The clinical studies documented that 40%levodopa efficacy is possible or probable in MSA cases (Kumlesh K *et al.,* 2003).
- Progressive supranuclear palsy patient’s shows symmetric onset of Parkinsonism. They show poor response to dopaminergic treatment.

Characterized by supranuclear gaze palsy, especially downgaze, blepharospam and eyelid opening apraxia (Williams DR *et al.,* 2005).

Dementia/parkinsonism syndromes

- Dementia is the vital loss of memory for a short time. Emotional symptoms and mental slowness were considered to be manifestations of the Parkinsonian syndrome. The relationship that favors between the Dementia and Parkinson's syndrome is the degree of motor impairment and also for various anterior brain symptoms (Rinne JO *et al* 1999).

Familial or hereditary Parkinsonism

- Autosomal dominant is the condition in which faulty gene on one chromosome has impact on the gene of partner chromosome and stops its working. The presence of intracellular aggregates called Lewy bodies are observed, this lewy bodies are nothing but an abnormal accumulation of proteins. Autosomal dominant Parkinsonism can be occurred due to the mutations of two proteins that are α-synuclein (PARK1) and Ub carboxyl-terminal hydrolase (UCH-L1). The α-synuclein gene is thought to produce monomeric Ub by cleaving polymeric Ub chains and/or releasing Ub from small adducts. Mutation in UCH-L1 could result in the reduction of its catalytic activity, which may lead to the mismanagement of Ub recycling (Cotzias *et al.,* 1969).

Corticobasal degeneration, one of neurodegenerative disease, generally occurs in the late middle age. Characterized by neuronal loss, tau deposition in grey and white neocortex, brainstem and basal ganglia. The signs are cortical sensory loss, extrapyramidal symptoms that includes tremor, bradykinesia, myoclonus, dystonia and alien limb phenomenon. Impaired cortical and basal ganglia function in corticobasal degeneration can manifests the parkinson's syndrome(Albin, R.L *et al.,* 1992).

1.7 PATHOPHYSIOLOGY

The primary deficit in PD is a loss of the dopaminergic neurons in the substantia nigra pars compacta which provides dopaminergic innervations to the striatum i.e. caudate and putamen. The present understanding of the pathophysiology

of PD traced to neurochemical investigations which demonstrates that striatal dopamine content is reduced to 80%, which leads to the loss of neurons from substantia nigra (Mink, J.W *et al.,* 1993).

Many factors have been speculated to operate in the mechanism of cell death of the nigrostriatal dopaminergic neurons in PD, including oxidative stress, mitochondrial dysfuntion, Excitotoxicity, exogenous and endogenous toxins, apoptosis, abnormal protein processing, inflammation and genetic causes (Morens DM *et al.,1996*)

Neural Mechanism of Parkinsonism

Various effort has been made to understanding how the loss of dopaminergic input to the neurons of the neostriatum gives rise to the clinical features of PD. The basal ganglia is a modulatory side loop which regulates the flow of information from the cerebral cortex to the motor neurons of the spinal cord (Ikonomidou C *et al.,* 1996).

The neostriatum is the principal input structure of the basal ganglia which receives excitatory glutamatergic input from various areas of cerebral cortex. Many neurons in the striatum are projection neurons, which innervate other basal ganglia structures. The neurons within the striatum are connected through interneurons. This striatal interneuron use Acetylcholine and neuropeptides as transmitters. Projection neurons express predominantly D_1 or D_2 dopamine receptors, as well as interneuron. Outflow from the striatum occurs by two routes. One is direct pathway and other is indirect pathway. (Ody I *et al.,* 1995)

The direct pathway composed of neurons in the striatum that project directly to the output stages of the basal ganglia i.e. from the striatum to the substantia nigra pars reticulata (SNpr) and globus pallidus interna (GPi), that uses the transmitter Gama aminobutyric acid (GABA), which relay to the ventroanterior and ventrolateral thalamus and provides excitatory input to the cortex. In this pathway neurotransmitter for both the links is GABA, which is inhibitory; hence the net effect of stimulation of the direct pathway in striatum is to increase the excitatory outflow from the thalamus to the cortex (Wenning *et al.,* 1994)

The indirect pathway is composed of neurons in straitum that project to the globus pallidus externa (GPe) and the subthalamic nucleus (STN) to the SNpr and GPi (SNpr and GPi output structures of the basal ganglia). It consists of two inhibitory GABAergic links and one excitatory glutamatergic projection (Glu). The net effect of stimulating the indirect pathway in striatum is to reduce the excitatory outflow from the thalamus to cerebral cortex (Weber CA *et al., 2006*)

The substantia nigra pars compacta (SNpc) provides dopaminergic innervations to the neurons in striatum, give rise to both the direct and indirect pathways, and regulates activity of these two paths. The SNpr and GPi provide feedback to the cerebral cortex through the ventroanterior and ventrolateral nuclei of the thalamus.

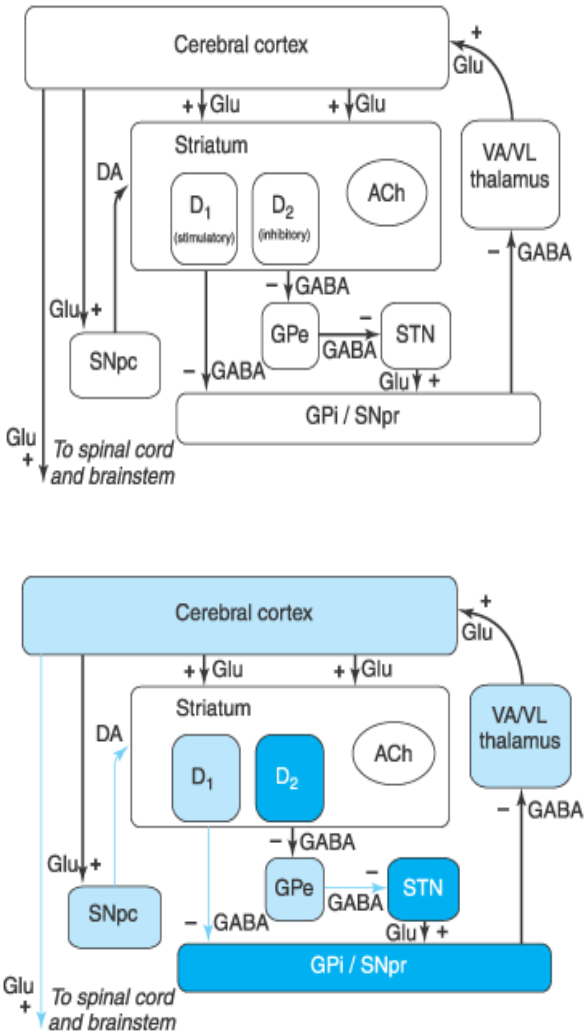


Figure 4. The Basal Ganglion of normal person & the Basal ganglia in Parkinson's disease

In parkinsonian state the neuronal activity in the GPi/SNr output nuclei of the basal ganglia increases, which leads to excessive inhibition of thalamo-cortical and brainstem motor systems. It assumes that reduced activation of dopamine receptors due to the dopamine deficiency, results in decreased inhibition of striatal neurons of the indirect pathway and reduced excitation of striatal neurons of the direct pathway (Beso J.A 2000).

Reduced inhibition from the indirect pathway leads to inhibition of the STN, over inhibition of the GPe and increased excitation of GPi/SNr neurons, whereas reduced excitation from the direct pathway causes a reduction in influence of inhibitory activity on the GPi/SNr. The net result of this is excessive activation of basal ganglia output neurons along with the excessive inhibition of motor systems, leading to parkinsonian motor features. Some evidence supports the use of this model in the Parkinsonian state following dopaminergic lesions. Expression of D_2 receptor and preproenkephalin mRNA increases in striatal neurons of the indirect pathway, whereas expression of mRNA encoding the D_1 receptor, substance P and dimorphic decreases in neurons of the direct pathway (Gerfen RC 1991).

There are many limitations of this model of basal ganglia function. In recent work it was shown that the anatomical connections are more complex. Even the pathways involved not use only one neurotransmitter but several neurotransmitters, such as the neuropeptides substance P and dynorphin are found predominantly in striatal neurons of direct pathway and in straital neurons of indirect pathway enkephalin are expressed. First, it was suggested that to restore the balance of the system through stimulation of dopamine receptors, through the effect of actions at both D_1 and D_2 receptors must be considered. It was then explained that replacement of dopamine is not the only approach to the treatment of PD. Drugs that inhibit cholinergic receptors have been used for treatment of Parkinsonism, it seems that their effect is mediated at striatal projection neurons, which receives cholinergic input from striatal cholinergic interneurons (Parent A & Cicchetti F 1998).

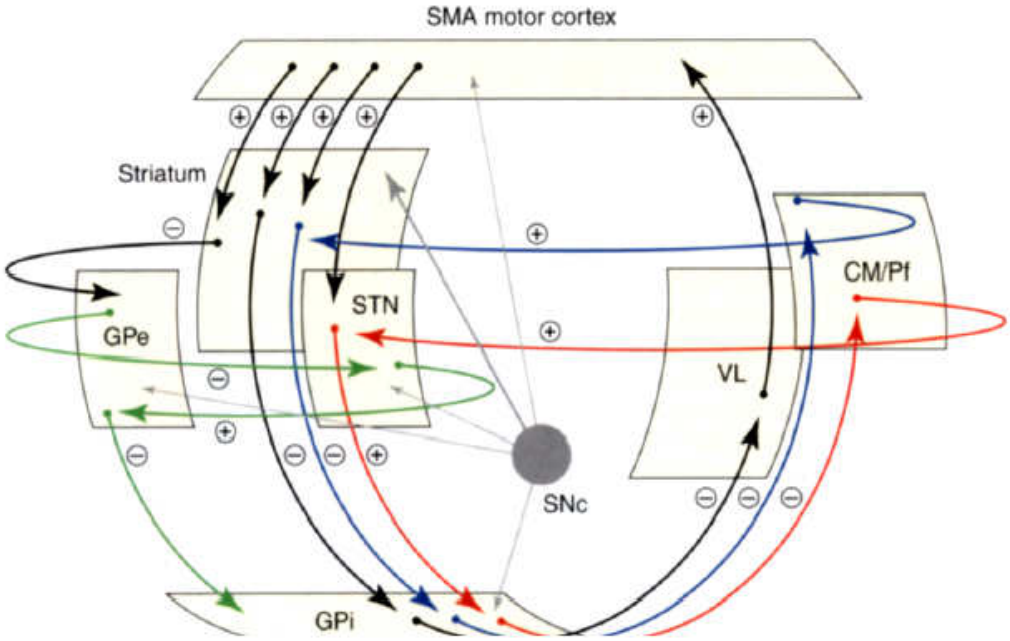


Figure 5. A modern view of the motor circuitry of the basal ganglia

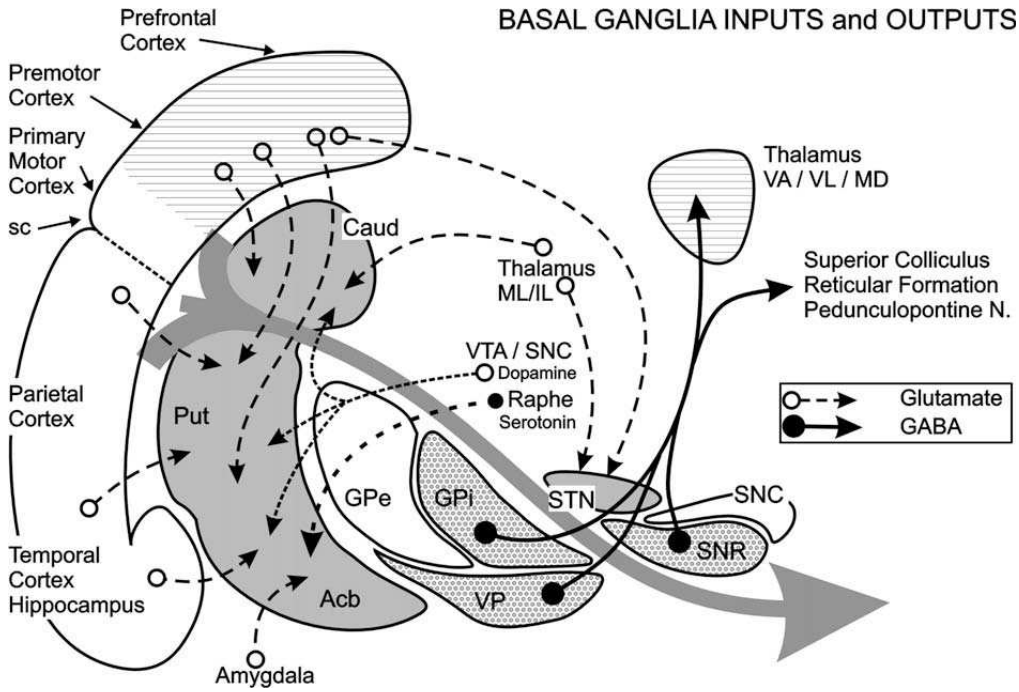


Figure 6. Schematic, semi-sagittal representation of the cerebral cortex

The different nuclei of the Basal Ganglia (BG), and the thalamus, illustrating the most important afferent and efferent connections of the BG.

Oxidative stress

Oxidative damage is believed to play a role in brain aging and neurodegenerative disorders, such as Parkinson's disease. In substantia nigra part of Parkinson's disease brains shows signs of oxidative stress, including, excess accumulation of iron and lipid peroxidation, damaged proteins and DNA, depletion of glutathione (GSH) and reduced complex I activity. In general neurons are more exposed to the toxic byproducts of oxygen metabolism than other cells, because for energy production they depend primarily on oxidative phosphorylation (Jenner *et al.,* 1996).

One more factor is that when compared to other tissues the brain is deficient in antioxidant molecules and free radical scavenging enzymes, which makes neurons vulnerable to oxidative stress. When H_2O_2 is produced during the metabolism of dopamine by monoamine oxidase (MAO), dopaminergic neurons, are subjected to additional oxidative stress. H_2O_2 react with transition metals and forms the highly reactive hydroxyl radical (OH·) which are known to cause damage to lipids, proteins and DNA (Brian C. Kramer *et al.,* 2002).

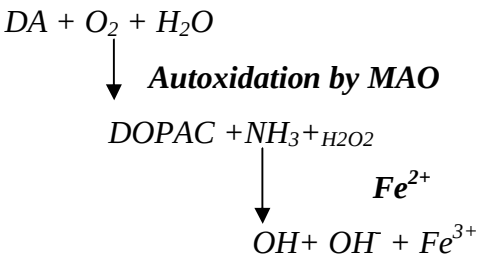


Figure 7. Autoxidation processes in the basal ganglia

Mitochondrial dysfunction

The mitochondrium is the basic site which generates energy supply for the cell. It is regulated by five respiratory chain complexes. Complex I controls the transfer of single electron from NADPH to coenzyme and transfer of two protons to the mitochondrial inter-membrane space, which are then used by complex V to synthesize ATP from ADP. A defect in the mitochondrial complex I lead to cell degeneration in Parkinson's disease through decreased ATP synthesis and contribute to cell death (Pisa 1998).

The disarrangement in complex I cause α-synuclein aggregation, which contributes to the demise of Dopaminergic neurons. Mitochondrial DNA and nuclear DNA encodes mitochondrial complex I, which is encoded, is defective in multiple tissues in PD individuals, thought that defect in complex I may be genetic, arising from mitochondrial DNA. Thus, small mutations in mitochondrial genes encoding complex I may be responsible for PD. Mitochondrial dysfunction is not exclusively be related to Dopaminergic neurons of the SNpc but maybe systematic, as mitochondrial dysfunction in PD is also observed in the striatum and other tissues (Von Bohlen & Halbach *et al., 2004).*

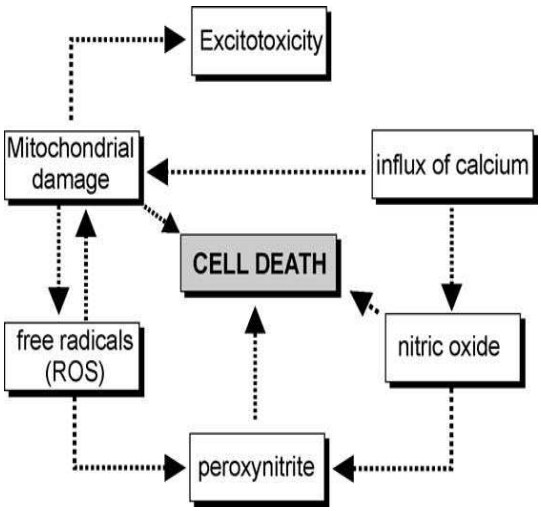


Figure 8. Excitotoxicity, mitochondrial dysfunction and free radicals contributes to cell death

Excitotoxicity

Excess glutamate causes the damage which leads to the changes in the permeability of cells to calcium by acting on and through *N*-methyl-d-aspartate (NMDA) receptors, which considered being involved in neurodegeneration. Glutamate-induced excitotoxicity is thought to play a role in PD. Over activation of glutamate receptors results in excessive rises in cytoplasmic Ca^{2+} which are assumed to underlie the fundamental processes ultimately leading to neuronal death. The calcium-dependent NMDA neurotoxicity is based on two mechanisms as follows: (i) excessive nitricoxide formation and (ii) mitochondrial dysfunction (I konomidou C *et al.,* 1996).

The excessive influx of calcium causes an activation of nitric oxide (NO) synthase that converts l-arginine to citrulline and NO. Excess NO is responsible for glutamate neurotoxicity. Commonly most of the neurotoxic actions of NO are

mediated by peroxynitrite, which obtained from the reaction of NO and superoxide anion *(*Mody I *et al.*, 1995).

The extensive calcium influx accompanies NMDA-receptor activation is get accumulated by the intracellular mitochondria, with effects on mitochondrial membrane potential, ATP synthesis, glycolysis, ROS generation, leading to failures in cytoplasmic calcium homeostasis and thus causes cell death Dopaminergic nigrostriatal neurons have numerous glutamate receptors and receive an extensive glutamatergic innervations from the cortex and the subthalamic nucleus . As the substantia nigra receives rich glutamatergic inputs, it has been assumed that glutamate-induced excitotoxicity may be involved in cell death in PD (Nicholls DG *et al.*, 1998)

1.8 EXOGENEOUS AND ENDOGENEOUS TOXINS

6-Hydroxy-dopamine (6-OHDA)

6-OHDA was the first chemical substance which was found to show a specific neurotoxin effects on central monoaminergic neurons. It uses the same catecholaminergic transport system as nor epinephrine and DA and thereby causes specific degeneration of catecholaminergic neurons. It was found that it is toxic to the mitochondrial complex I and induces the generation of ROS. As it is unable to cross the blood brain barrier, it has to apply directly in substantia nigra or striatum within the 12hr post 6-OHDA injection in nigra; it causes the neuron death in the SNpc followed by striatal fiber degeneration. And when it is injected Intrastriatal it lead to a retrograde-induced cell death in the SNpc and an approximately 70% loss of striatal DA (Ungerstedt U 1968).

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

MPTP is a bypass product, obtained from the chemical synthesis of a meperidine analog with potent heroin-like properties. It is highly lipophilic and readily crosses the blood– brain barrier and mediate toxicity by converting to the 1-methyl-4-phenyl-2,3-dihydropyridium ion (MPP+) by monoamine oxidase B. MPP+ must be transported into Dopaminergic neurons by neurotransmitter transporters to develop its toxicity and once it enters neuron, it is assume that it acts by inhibiting the electron–transport system of the mitochondrial complex I, resulting in cellular energy failure and the formation of superoxide anions .The selective toxicity of MPP+ for

Dopaminergic neurons is due to its high affinity to the dopamine transporter DAT. Depending on the dose, model and route of application of application, MPTP can produce an irreversible and severe Parkinsonian syndrome that replicates nearly all features of PD (Anthony HK, Tsang *et al*., 1792).

MANEB

Maneb is a fungicide, used as chemical for agriculture, of which Manganese ethylene-bis-dithiocarbamate (Mn-EBDC) is the major active element. It is known to damage the Dopaminergic system and induce neurologic alterations in humans, such as postural tremor, cerebellar signs, and bradykinesia. Even its chronic exposure leads to permanent Parkinsonism. When it administered along with the MPTP, it potentiates the MPTP toxicity. Injection of Mn-EBDC directly into lateral ventricles leads to the selective Dopaminergic neurodegeneration (Betarbet, *R et al.,* 2002).

Paraquat (1, 1-dimethyl-4,4-bipyridinium)

Paraquat get metabolized to paraquat radical via complex I of the electron–transport-chain in mitochondria, leads to its inhibition and accelerates lipid peroxidation. As it can penetrate the blood–brain barrier, it can be applied systemically. After intracerebroventricular or intracerebral injection it shows its neurotoxic effects leading to loss of Dopaminergic neuron in the substantia nigra and reduction in the density of striatal TH-ir fibers. Continuous exposure to low levels of paraquat might lead to vulnerability of Dopaminergic neurons in the nigrostriatal system and potentiate neurodegeneration. Its exposure increases brain α-synuclein levels, accompany aggregate formation. Its systemic administration leads to cell losses not only in substantia nigra but also in piriform cortex (Betarbet R *et al*., 2002).

Rotenone

Rotenone is a pesticide, obtained from natural source. It is highly lipophilic and does not depend on dopamine transporter for cellular entry and moreover is not sequestered into synaptic terminal. It was found as mitochondrial toxin which selectively inhibits the mitochondrial complex I, Inhibition of complex I results in the degeneration of highly selective nigrostriatal Dopaminergic (involving caspase-3-mediated apoptosis) and α-synuclein-positive cytoplasmic aggregates in nigral neurons. Even it was found that it causes an increased striatal DA turn-over and reduced TH-ir in the caudate putamen. Infusion of rotenone does not only induce loss

of striatal dopaminergic fibers but also other types of striatal fibers, like serotoninergic fibers (Betarbet R *et al.*, 2002).

Apoptosis

From the long time Apoptosis has been implicated in the process of neurodegeneration in PD pathogenesis. Such as activation of caspase 3 has been studied in the brain tissues, expression of X-linked inhibitor in PD patients. Redox sensitive substrates, caspase 3 and substrates for post-translational oxidative modification are some of the components that initiate apoptosis. Such as it is known that S-nitrosylation can modulate apoptosis by acting on a number of important apoptotic proteins (Virginie Lickera *et al.,* 2009).

Abnormal protein processing

Aggregation of soluble proteins into insoluble complexes and inclusions is found as one of the cause for neurodegenerative conditions, including PD. The one of the mechanism for protein aggregation is when proteins are exposed to hydrophobic segments, which are prone to form inappropriate intermolecular binding, when they are unfolded, partially folded or misfolded. It was found that protein aggregation occurs between similar hydrophobic proteins or distinct proteins sharing a common aggregation-promoting motif. Under physiological conditions, when the production of abnormal polypeptides exceeds the capacity of the UPS, it got sequestrated into microtubule-dependent aggresomes and under non-physiological conditions, soluble monomers and oligomers of native or non-native polypeptides may self-assemble, polymerize and coalesce into giant intracellular inclusions, such as LBs in PD. The UPS is a major pathway which mediates the degradation of abnormal cellular proteins; it has been proposed that its dysfunction may be a key fmay be an important factor in the pathogenesis of PD *(*Saunders-Pullman R *et al.*, 2003).

1.9 CAUSES

Even though the main cause for the Parkinsonism is not to be known but number of the risk factors are clearly evident. It includes

Non-Genetic risk factors

Sex

Males are more prone to Parkinson's syndrome then females. This may be because men having greater exposure to other risk factors as toxins and head trauma. Many studies have prompted the hypothesis that female sex hormones have protection against neuronal cell death. In certain studies it was found that treatment of estrogen to postmenopausal period women with PD shows improvement in motor movements (Tsang KL *et al.,* 2000).

Advancing age

Even though there are occasional cases registered in which disease being developed young adult but it commonly manifests from middle to late years of life. Its prevalence increases after the age of 50 years and accelerates between 65 and 90 years. Only 5-10% of patients have symptoms before the 40years of age *(*Moghal S *et al* 1994).

Environmental factors

The chemicals used in agricultural purpose such as Herbicides, pesticides, fungicides etc, may play a role as a risk factor. The PD prevalence increase in people who have occupational exposure to herbicides and insecticides. Even chronic exposure to farming, pesticides, well- water use and rural living increases the risk of PD. Certain laboratories investigations implicated that an array of agricultural chemicals such as paraquat, rotenone, parathione are capable of targeting and damaging the SNpc. Studies revealed that prenatal exposure to the pesticide maneb produces selective, permanent alterations of the nigrostriatal dopaminergic system. Iron is also one of the causative factor, its deposition may cause synuclein aggregation (Gerlach M *et al.,* 2006).

Dietary factors

The risk of PD is also associated with various food groups and specific nutrients they can cause either high or low risk of PD. The intakes of many nutrients are highly correlated and specific associations and therefore not easily identified. Few population-based cohort studies have been done (Chen H *et al.*, 2002).

Antioxidants

In nutritional epidemiology mainly antioxidants were focused, which is presumed as central role of oxidative stress in the pathogenesis of PD. Antioxidants, such as vitamins E and C, are supposed to protect cells against oxidative damage by neutralizing free radicals(De Rijk MC *et al.*,1997)

Fat and fatty acids

The relation between dietary fat and PD is not clear. High lipid containing diet could increase the amount of oxygen radicals by lipid peroxidation and thereby increase the risk of PD (Farooqui AA *et al.*, 1998).

Dietary iron

Iron may forms the free radicals and increased levels of iron were found in the substantia nigra of PD patients. Few studies found a positive association between iron intake and PD but few found no association (Lai BC 2002).

Inflammation

The role of inflammation in PD is still unknown. In PD patients up regulation of cytokines in the brains and cerebrospinal fluid were found, and activated glial cells have been observed in post-mortem material. It may be due to either immune response or a consequence of neurodegeneration (Chen H *et al.*, 2003).

PD and cancer

Some studies showed a low incidence of common types of cancers in people with PD. The initial hypothesis was that this might have resulted from the inverse association between smoking and PD but it did not hold, because a low incidence has been described for both smoking-related and non-smoking related cancers *(*West AB *et al.*, 2005)

Causative genes

Monogenetic causes do not have primary role in PD. In several studies it was found that positive family history has been associated with a high risk of PD, but in many cases the mode of inheritance is not clear. In a study of almost 20,000 male twins a significant effect of genetic factors was found, but predominantly in PD with onset before age 50 years. Since 1997, many families have been identified with Parkinsonism with Mendelian inheritance, and now it is estimated that 10% of PD cases are caused by monogenetic forms (Hardy J *et al.,* 2003).

Susceptibility genes

Sporadic form of PD is generally thought as a result of complex interactions between environmental and genetic factors. Various studies have been carried out on candidate genes that were hypothesised to contribute to the risk of sporadic PD. Very commonly studied candidate genes include genes involved in dopamine metabolism, mitochondrial metabolism, detoxification, other neurodegenerative diseases, and familial PD and genes associated with risk factors for PD(Tan EK *et al.*, 2000 & Huang X *et al.*, 2004).

1.10 SYMPTOMS

Parkinsonism mainly has three cardinal motor symptoms: grouped under the acronym TRAP: Tremor at rest, Rigidity, Akinesia (or bradykinesia) and Postural instability. Classic features of PD include flexed posture and freezing (motor blocks) is an asymmetrical onset of motor symptoms. Depending on the diverse profiles and lifestyles of those affected by PD, motor and non-motor impairments should be evaluated in the context of each patient's needs and goals (Jankovic *et al.*, 2007).

Motor symptoms

- Tremor, bradykinesia, rigidity, postural instability
- Hypomimia, dysarthria, dysphagia, sialorrhoea
- Decreased arm swing, shuffling gait, festination difficulty arising from chair, turning in bed
- Micrographia, cutting food, feeding, hygiene, slow activities of daily living
- Glabellar reflex, blepharospasm, dystonia, striatal deformity, scoliosis, camptocormia

- Fatigue
- Masked faces (a mask-like face also known as hypomania), with infrequent blinking
- Impairment in fine motor dexterity and motor coordination

Non-motor symptoms

- Cognitive
  - ❖ Bradyphrenia, impaired attention and executive function, visuospatial deficits, dementia, language problems.
- Psychiatric
  - ❖ Depression, anxiety, sleep disturbances, sexual dysfunction.
- Craniofacial
  - ❖ Hypomimia, impaired accommodation, sialorrhea and dysphagia, olfactory hypo function, dysarthria.
- Autonomic
  - ❖ Orthostatic hypotension, impaired gastrointestinalmotility, bladder dysfunction, sexual dysfunction, abnormal thermoregulation and increased sweating.
- Skin
  - ❖ Sensory Cramps, paresthesia, pain, numbness, tingling, Seborrhoea.
- Musculoskeletal
  - ❖ Scoliosis, peripheral oedema

Diagnosis (Stoessl AJ & Rivest J 1999)

Diagnosis of Parkinson's disease is little difficult. No specific blood tests or diagnostic procedure or laboratory test are currently available. Because of this Parkinsonism is diagnosed on the basis of symptoms and results obtained from physical and neurological examinations. Moreover it is difficult to diagnose on basis of early symptoms and also in aged peoples as they have some same problems as in Parkinson’s disease such as loss of balance, slow movements, muscle stiffness, and stooped posture.

It is mainly diagnosed clinically and this clinical diagnosis includes normal ageing, essential tremor, drug-induced Parkinsonism, the Parkinson-plus syndromes, vascular Parkinsonism, and normal pressure hydrocephalus. Less common entities with Parkinsonism DOPA-responsive dystonia, juvenile-onset Huntington's disease, pallidopontonigral degeneration (Bower TH 2003).

In atypical cases neuroimaging and laboratory test are necessary. Neuroimaging tests such as

- Computed Tomography (CT)
- Magnetic Resonance Imaging (MRI)
- Electroencephalograms (EEGs)
- Positron Emission Tomography (PET)
- Single-Photon Emission Computed Tomography (SPECT)

Laboratory Tests include blood tests, such as

- Complete Blood Count (CBC)
- Urinanalysis and blood glucose testing
- EKG to help evaluate the heart

which are commonly perform to look for a structural disorder that may be the cause of the disease.

Table 2. Differential diagnosis of PD

Common Misdiagnoses	Distinguishing features
Essential tremor (ET)	Tremor (action/postural) is the only or predominant feature; no response to PD drugs
Progressive supranuclear palsy (PSP)	Supranuclear down gaze palsy; square-wave jerks; upright posture;gait instability; dysphagia
Multiple system atrophy (MSA)	Autonomic disturbance, cerebellar signs, relative absence of tremor; gait instability; dysphagia
Corticobasal degeneration (CBD)	Limb apraxia; normal cortical sensory is disturbed; early dementia
Diffuse Lewy body dementia (LBD)	Early dementia; psychosis; agitation
Alzheimer's disease	Dementia is the primary symptom
Drug-induced parkinsonism	Exposure to dopamine-blocking drugs; lack of rest tremor and asymmetry
Vascular parkinsonism	Imaging History of chronic hypertension; unilateral.

1.11 **TREATMENT** (Finkel, Richard & Rang H.P)

Drug classes for treatment of antiparkinsonism

I. Drugs affecting brain dopaminergic system
   1. Dopamine Precursor
      Levodopa/ L-dopa
   2. Peripheral Decarboxylase inhibitor
      Carbidopa (Parcopa orally disintegrating tablet)
      Benserazide
   3. Dopamine agonists
      Apomorphine (Apokyn)
      Bromocriptine (Parlodel)
      Cabergoline (Not approved in the US)
   4. COMT Inhibitors
      Entacapone (Comtan, Stalevo)
      Tolcapone (Tasmar)
   5. MAO-B Inhibitors
      Rasagiline (Azilect)
      Selegiline
      Dopamine facilator
      Amantadine (Symmetrel, generics)

II. Drugs affecting brain cholinergic system
   1. Central Anticholinergics
      Trihexyphenidyl (Artane)
      Procyclidine
   2. Antihistaminics
      Orphenadrine
      Promethazine

Levodopa is the first line treatment for parkinsonism, is a metabolic precursor of dopamine that is decarboxylated to dopamine within the presynaptic terminals of dopaminergic neurons in the striatum, responsible for the therapeutic effectiveness of the drug in Parkinsons'disease, Peak concentrations of the levodopa in plasma is between 0.5 and 2 hours after an oral dose with the half-life of 1 to 3 hours. It is combined with a peripheral dopa decarboxylase inhibitor, either carbidopa or

benserazide, which diminishes the peripheral side effects and also combined with plus dopa decarboxylase inhibitor entacapone (inhibitor of COMBT) to inhibit its degradation. About 80% of patients show initial improvement with levodopa, particularly of rigidity, hypokinesia, tremor and bradykinesia, and about 20% are restored virtually to normal motor function

Selegiline is a MAO inhibitor that is selective for MAO-B, Inhibition of MAO-B protects dopamine from intraneuronal degradation, thus decreases the metabolism of dopamine and has been found to increase dopamine levels in the brain and was initially used as an adjunct to levodopa.

Dopamine Receptor Agonists Bromocriptine, an ergot derivative, and few newer, nonergot drugs, ropinirole, pramipexole, rotigotine and Apo morphine. **Bromocriptine** inhibits the release of prolactin from the anterior pituitary gland, its duration of action is longer (plasma half-life 6-8 hours) than that of levodopa. Newer dopamine receptor agonists include lisuride, pergolide, ropinirole**,** carbergoline and pramipexole. They are longer acting than levodopa and need to be given only once or twice daily, with fewer tendencies to cause dyskinesia and on-off effects. Apomorphine*s are* available in injectable and transdermal delivery systems respectively, meant to be used for the acute management of the hypomobility phenomenon, alleviate the motor deficits in both levodopa patients.

Amantadine have many possible mechanisms for its action includes increased dopamine release, inhibition of amine uptake, or a direct action on dopamine receptors and inhibiting the N-methyl-D-aspartate (NMDA) type of glutamate receptors.

Acetylcholine antagonists Benztropine, trihexyphenidyl, procyclidine and biperiden interfere with this inhibitory effect on dopaminergic nerve terminals, suppression of which compensates for a lack of dopamine by muscarinic acetylcholine receptors.

1.12 NEUROTOXIC PROFILE OF 6- OH DOPAMINE

The noradrenergic analog 6-OHDA has been introduced as catecholaminergic neurotoxins (Jonsson G 1983). These neurotoxin compounds especially 6-OHDA, have remained extensively used for both *in vitro* and *in vivo* investigations. Because of practical considerations, in living animals 6-OHDA has been used essentially in small animals such as rodents (Roeling TA *et al.,* 1995).

The neurotoxin 6-OHDA shares some structural similarities with dopamine and nor epinephrine, exhibiting a high affinity for several catecholaminergic plasma membrane transporters such as the dopamine transporter and nor epinephrine transporters. Consequently, 6-OHDA can enter both dopaminergic and noradrenergic neurons and inflict damage to the catecholaminergic pathways of both the peripheral and the central nervous systems. (Roeling TA *et al.,* 1995)

Mode of action

With respect to its mode of action, it is understood that 6-OHDA destroys catecholaminergic structures by a combined effect of reactive oxygen species (ROS) and quinones. 6-OHDA, once dissolved in an aerobic and alkaline milieu, readily oxidizes, yielding hydrogen peroxide (H_2O_2) and *para*-quinone. Although the chemical reaction that underlies 6-OHDA-induced neurotoxicity appears straightforward, it is in fact a remarkably complicated reaction that does not occur as a spontaneous oxidation by molecular oxygen *(*Cohen G *et al.,* 1984).

Route of administration

Like other parkinsonian neurotoxins 6-OHDA can't be administrated by systemic injection, this route of administration will not produce the desired nigrostriatal lesion and also 6-OHDA poorly crosses the blood-brain barrier. To circumvent this problem, 6-OHDA has to be injected directly into the brain either free-hand or by stereotaxic means. (Daniel Alvarez-Fischer.*et al.,* 2008)

1.13 PLANTS AS MEDICINE

The exploitation of plants for medicines has a long and interesting history, since at one time all drugs were obtained from natural sources. During the last few decades, there has been a resurgence of interest in plants as source of medicines and of novel molecules for use in the elucidation of physiological phenomena. Plants have been mostly exploited from the day when humans realized their effectiveness and versatility as medicines. There is a genuine expectation in developing countries that their drug problems can be alleviated through a sensible scientific exploitation of medicinal plants some of which have been used for generations by indigenous populations. Then there is the worldwide green revolution that is reflected in the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs (Gupta SS 1994).

Further underlying this upsurge of interests in plants is the fact that many important drugs in use were derived from plants. Plants have also yielded molecules, which are extremely valuable tools in the medicine. Some scientists thus expect that the plant kingdom holds the key to the understanding of complex human pathology and the cure of man's perplexing diseases (Huxley A *et al.*, 1992).

Traditional systems followed in ancient civilization have been passed on through generations. Systems like Ayurveda, Siddha, Chinese system, Tibetan system indigenous African medicine utilized plant source to the maximum.

The initial optimism, engendered by the idea that a sophisticated understanding of plants by our traditional healers would pave the way to predictable drug development has not been realized. Therefore laboratories around the world are engaged in the screening of plants for biological activity with the therapeutic potential. One major criterion for the selection of a plant for such study is traditional healer's claim for its therapeutic usefulness.

The number of species of higher plants on the planet is estimated between 370,000 and 500,000. All higher plants elaborate chemical secondary metabolites that are of potential medical interest. Therefore, the determination of the criteria for selecting plants for phytotherapeutic investigation is perhaps as important an exercise

as the investigation itself. Selection is mainly based on traditional usage, chemical composition and screening for a specific biological activity.

Medicinal plants and Neurodegeneration

Traditional healers make use of a plethora of plants. The families most often represented are Fabaceae (15 species), Asteraceae (13 species) and Lamiaceae (9 species). These plants come from 26 different families. The number of plants used for dementia and age-related mental problems are lower with only 15 species from 7 families recorded, which could be due to a previous demographic situation, where traditional healers less frequently had very old patients. Amaryllidaceae is the best-represented family with six species. Mental health problems constitute a serious problem. Not surprisingly, a large number, well over 300 species, of plants are used by traditional healers in their treatment of these ailments (Sobiecki *et al.*, 2002).

Studies on plants used to treat Parkinson's disease

In a study of 20 traditionally used plants which were screened for MAO inhibition and specific MAO-B inhibition activity, the non-polar extracts of *Ruta graveolens* L. (Stafford et al., 2007). *Schotia brachypetala* Sond., *Mentha aquatica* L. and *Gasteria croucheri* (Hook.f.) Baker also exhibited good MAO-B inhibition activity. Naringenin, a flavonoid was later isolated from a 70% ethanol extract of *Mentha aquatic*, The antiparkinsonian effect of *Withana somnifera* has been reviewed elsewhere (Kulkarni & Dhir 2008; Gupta & Rana 2007). In short, *Withana somnifera* significantly inhibited haloperidol or reserpine induced catalepsy; reversed all parameter of oxidative stress in the 6-hydroxydopamine model in rats and reduced reserpine-induced vacuous chewing movements and tongue protrusions in animal models of tardive dyskinesia (Kulkarni & Dhir, 2008; Gupta & Rana, 2007).

AIM AND OBJECTIVE

II. AIM AND OBJECTIVE

AIM

The aim of the present study was to evaluate the neuroprotective effect of hydroethanolic extract of roots of *Annona squamosa* Linn., and hydroethanolic extract of aerial parts of *Echinops echinatus* Roxb., on 6 - OH dopamine induced neurodegeneration in Wistar albino rats and to evaluate the *in vitro* and *in vivo* antioxidant acitivity of both the extracts.

OBJECTIVE

The objectives of the present research are as follows:

- To prepare and standardize the hydroethanolic extracts of roots of *Annona squamosa* Linn. Aerial parts of *Echinops echinatus* Roxb.
- To establish the safety of the extracts by carrying out acute and sub acute toxicity studies and investigating the behavioral changes in Wistar albino rats.
- To evaluate the antioxidant potential of the extracts by *in vitro* studies
- To investigate the neuroprotective effect of the extracts in 6-OH dopamine injected Wistar albino rats by following behavioral and biochemical changes
- To study the effect of extract on the levels of neurotransmitters, enzymatic and non enzymatic antioxidants in 6-OH dopamine injected Wistar albino rats.

REVIEW OF LITERATURE

III. REVIEW OF LITERATURE

Chien-Min Lin et *al.*, (2010) found that Crude ethanolic extract (CEE) and total acetate fraction (TAF) of Plumbago scandens (1000 mg/kg, i.p.) decrease locomotor activity, the presence of catalepsy and palpebral ptosis, thus acts against Parkinsonism.

Mi Sun Ju *et al.*, (2010) reported the protective effects of standardized ethanolic extract of *Thuja Orientalis* leave in SH-SY5Y cells. Pretreatment with doses of 0.1–100 lg/ml in 6-OHDA induced neurotoxicity repressed the neuronal cell death, inhibited excess ROS and NO production and high radical scavenging activity, blocked the cytochrome c release, and caspase-3 activation, suppressed the increased level of ERK phosphorylation and anti-mitochondrial-mediated apoptosis.

Christopher A. Lieu et al., (2010) evaluated Dopaminergic anti-parkinsonian medications, such as levodopa (LD) cause drug-induced dyskinesias (DID) in majority of patients with Parkinson's disease (PD).

Shane Grealish et al., (2010) evaluated the behavioral and neurodegenerative changes induced by intranigral 6-hydroxydopamine lesions in a mouse model of Parkinson's disease.

Mei-Hsien Lee *et al.*, (2010) investigated the Neurocytoprotective effects of Pueraria thomsonii bioactive constituents ie daidzein and genistein in 6-OHDA induced apoptosis in differentiated PC12 cells. daidzein and genistein at concentrations of 50 µM and 100µM inhibited caspase-8 and partially inhibited caspase-3 activation, providing a protective mechanism against 6-OHDA-induced cytotoxicity in NGF-differentiated PC12 cells.

Christopher A *et al.*, (2010) reported reputed anti-parkinsonism activity (6-OHDA lessioned rats) of Mucuna pruriens, a legume extensively used in Ayurveda to treat PD. They compared the behavioral effects of chronic parenteral administration of a water extract of M. Pruriens seed powder (MPE). It was found that animals that received LD and BZ or dose-dependent drug induced dyskinesia. LD and BZ at low dose of 2 mg/kg did not provide significant alleviation of Parkinsonism. It was also found that MPE without additives administered chronically provided long-term anti-parkinsonian benefits without causing DID and MPE alone provided significantly more behavioral benefit when compared to the equivalent dose of synthetic LD alone without BZ.

Jin Sup Shima *et al.*, (2009) provided the scientific basis to support the traditional use of the *Uncaria rhynchophylla* in Parkinson's disease. *Uncaria rhynchophylla* possess the neuroprotective activity against 6-OHDA toxicity in PC12 cells. In in-vitro PC12 cells, URE significantly reduced neuronal cell death, increased GSH Levels (74.55±1.57%), attenuated ROS and inhibited the activation of caspase-3 in dose dependant manner induced by 6-OHDA. In in-vivo low dose of extract decreased the number of APO induced rotations by attenuating super sensitivity mediated by a selective irreversible MAO-B Inhibitor of URE, in the striatum and protect DA neurons.

Muzamil Ahmad *et al.*, (2009) studied neuroprotective effects of Ethanolic extract of Nardostachys Jatamansi in a 6-OHDA model of Parkinson's disease. Extract significantly and dose-dependently inhibit marked increase in drug-induced rotations and deficits in locomotor activity and muscular coordination which is a reliable marker for nigrostriatal dopamine depletion. Increased D2 receptor population in

striatum, increased activities of SOD, CAT and GSH significantly restored by pretreatment with Jatamansi by GSH -enhancing or antioxidant effect in 6-OHDA lesioned rats and increased TH-IR fiber density by pretreatment clearly signifies the dose-dependent increase in the number of surviving neurons and confirming the anti-Parkinson effects of Ethanolic extract of Nardostachys Jatamansi.

Mi Sun Ju *et al.*, (2009) reported investigated the effects of extract of *Cassiae semen* (COE) against neurotoxicity in vitro and in vivo PD models. COE attenuated the cell damage induced by administration of 100 µM 6-hydroxydopamine (6- OHDA) stress in MTT assay in PC12 cells, and inhibited the overproduction of ROS, mitochondrial membrane depolarization, glutathione depletion and caspase-3 activation at 0.1–10 µg/ml. Moreover it showed radical scavenging activity in the DPPH and ABTS assays, also protected DA cells against toxicities induced by 10 µM 6-OHDA and 10 µM MPTP in mesencephalic dopaminergic (DA) culture. Oral administration of 85% ethanolic extract of *Cassiae semen (seed of Cassia obtusifolia*) for 15 days significantly inhibit the movement impairment and the loss of DA neurons at dose of 50mg/kg.

Pinna A *et al.*, (2009) found that of *Mucuna pruriens* extract at a dose 16 mg/kg (containing 2 mg/kg of L-DOPA) and 48mg/kg (containing 6 mg/kg of L-DOPA) in 6-OHDA induced parkinsonism consistently antagonized the deficit in latency of step initiation, MP extract acutely induced a significantly higher contralateral turning, at dose of 48 mg/kg (containing 6 mg/kg of L-DOPA), suggested a significant antagonistic activity on both motor and sensory-motor deficits.

Iryna Ziabreva *et al.*, (2006) evaluated the spectrum of Lewy body disease cognitive impairment occurs in PD with dementia (PDD) and dementia with Lewy bodies (DLB).

Muzamil Ahmad *et al.*, (2005) reported beneficial effects of Standard crude Extract of *Ginkgo Biloba* in 6-OHDA induced Parkinsonian rats. The pre-treatment with EGb (50, 100, and 150 mg/kg body weight) for 3 weeks appreciably produce decrease in drug induced rotation and a significant restoration of striatal DA and its metabolites, it is a potent inhibitor of MAO which prevent the degradation of DA and increase its availability, The locomotor deficits were restored, causes increase in the content of GSH and decrease in the extent of lipidperoxidation. Ginkgo biloba appears to act via antioxidant, free radical scavenging, MAO-B-inhibiting, and DA-enhancing mechanisms that rescue the compromised cells within the dopaminergic lesions.

Abdullah Shafique Ahmad *et al.*, (2005) investigated neuromodulatory effects of crocetin (Active constituent of Crocus sativus, an important ingredient of diet in India) in a 6-OHDA rat model of Parkinsonism. In this animals are pre-treated with crocetin for 7 days and on day 8 they are subjected to unilateral intrastriatal injection of 10 µg 6-OHDA. On day 23 of post-injection locomotion and rotation were observed, and after 4 weeks, activity of antioxidant enzymes, dopamine (DA) content and its metabolites were estimated in striatum, whereas glutathione (GSH) content and Thiobarbituric Acid Reactive Substance (TBARS) were estimated in substantia nigra. Crocetin exhibit its therapeutic activity by protecting the levels of GSH, dopamine and activity of antioxidant enzymes. This study revealed that crocetin has therapeutic activity in preventing Parkinsonism.

Heh-In Im et *al.*, (2005) evaluated the effect of baicalein on 6-OHDA induced neurotoxicity. Baicalein was administered by intraperitoneal route 30 min before and 90 min after intracerebroventricularly injection of 6-OHDA Animals received further injection of bacalein for 3 consecutive days. It was found that high dose of baicalein effectively improved muscular strength, locomotor activity and prevented the reduction of striatal DA levels and TH contents in the subtantia nigra and striatum. Lipid peroxidation level was also decreased after 3 and 7 days of 6-OHDA injection. From their results they concluded that biaceline effectively prevents the 6-OHDA-induced dopaminergic dysfunction through an antioxidative action.

Christina L *et al.*, (2004) investigated the effects of bone morphogenetic protein-7 (BMP-7), also called as osteogenic protein-1, on the progression of a striatal 6-OHDA lesion. Previously it was found that BMP-7 has neuroprotective activity against neuronal damage. Intraventricular dose of BMP-7 after 1 week of straital 6-OHDA lesions did not showed any significant effect on locomotor activity but showed significant increase in the density of tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta, in the lesioned hemisphere and also non-lesioned hemisphere. In the contralateral, non-lesioned hemisphere, a significant increase in the DA concentration was noted. Like other intraventricularly administered neurotrophic factors, BMP-7 was not associated with an increase in the sensitivity to pain. These results suggested that BMP-7 act as a dopaminotrophic agent without unwanted side effects and may be a useful in the treatment of Parkinson's disease.

Thoenen H *et al.*, (1968) evaluated chemical sympathectomy by selective destruction of adrenergic nerve endings with 6-hydroxydopamine.

Anton AH & Saybe DF (1964) studied the distribution of dopamine and DOPA in various animals and a method for their determination in diverse biological material.

LITERATURE REVIEW OF ANNONA SQUAMOSA LINN

Mona Agrawal *et al.*, (2012) conducted phytochemical and HPTLC studies of various extracts of *Annona squamosa*. The leaves of the plant *Annona squamosa* were collected, powdered and extracted successively with different solvents. The extracts were subjected to preliminary phytochemical screening, which revealed the presence of alkaloids, flavonoids, carbohydrates, saponins, tannins, and steroids. The TLC and HPTLC techniques were used for qualitative determination of possible number of components in the various extracts. Solvent systems for all the extracts were optimized in order to get maximum separation on plate. Presence of various phytochemicals was confirmed by the use of different spraying reagents.

Saleem Mohamed T.S *et al.*, (2010) evaluated *Annona squamosa* for its hepatoprotective effect in isoniazid and rifampicin induced hepatotoxic model in albino Wistar rats. There was a significant decrease in total bilirubin accompanied by significant increase in the level of total protein and also significant decrease in ALP, AST, and ALT in treatment group as compared to the hepatotoxic group. In the histopathological study, treatment group showed minimal inflammation with moderate portal triaditis and their lobular architecture was normal, the protective effect was evaluated in diethylnitrosamine induced hepatotoxicity. This study revealed that the extracts of *Annona squamosa* exerted hepatoprotective effect and the plant extract could be an effective remedial for chemical- induced hepatic damage.

Singh Sanjiv *et al.*, (2010) studied the effect of Polyherbal formulation of *Annona squamosa* on blood glucose, plasma insulin, tissue lipid profile, and lipidperoxidation in streptozotocin induced diabetic rats. Aqueous extract of Polyherbal formulation of *Annona squamosa* was administered orally (200 mg/kg

body weight) for 30 days. The different doses of Polyherbal formulation on blood glucose and plasma insulin in diabetic rats were studied and the levels of lipid peroxides and tissue lipids were also estimated in streptozotocin induced diabetic rats. The effects were compared with tolbutamide. Treatment with Polyherbal formulation and tolbutamide resulted in a significant reduction of blood glucose and increase in plasma insulin. Treatment also resulted in a significant decrease in tissue lipids and lipid peroxide formation. The decreased lipid peroxides and tissue lipids clearly showed the antihyperlipidemic and antiperoxidative effect of Polyherbal formulation apart from its anti diabetic effect.

Magadula J *et al.*, (2009) evaluated the antibacterial screening by agar cup method indicates that highest zone of inhibition was shown by the methanol extract followed by petroleum ether and aqueous extracts for *Annona squamosa* leaf. Extracts of *Annona squamosa* inhibited the growth of all test strains except Salmonella typhimurium. Aqueous extracts showed less activity than methanol extracts The antibacterial action of the extracts is more pronounced on Gram-positive than on Gramnegative bacteria. Staphylococcus aureus and Vibrio Alginolyticus were the most sensitive bacterial strains in the present experiments. *Annona squamosa* had strong antibacterial activity against these bacterial stains.

Mujeeb Mohd *et al.*, (2009) evaluated anti diabetic activity of *Annona squamosa* root extract in STZ induced hyperglycemia in rats. STZ induced diabetes mellitus and insulin deficiency lead to increased blood glucose level. When *Annona squamosa* root extract was administered to diabetic rats, hypoglycemia was observed after 2 hrs, with the maximum effect being seen at 6 h. From the results it is assumed that the root extract could be responsible for stimulation of insulin release and

observed restoration of blood glucose level. Further, the observed decreased blood glucose lowering effect of the extract in STZ- induced diabetic rats could also possibly be due to increased.

Paramjit Grover *et al.*, (2009) conducted in vivo assessment of genotoxic effects of *Annona squamosa* seed extract in rats. Seed extract of *Annona squamosa* was prepared and found to be a promising pesticide. In order to establish the inherent toxicity and non-target safety required for registration and marketing of pesticides, toxicological studies are conducted. The genotoxicity potential was evaluated in rats with 75, 150 and 300 mg/kg *Annona squamosa* by the comet assay in leucocytes, micronucleus and chromosomal aberration tests in bone marrow. Also study has been conducted at the dose level of 300 mg/kg of extract on lipid peroxidation, reduced glutathione level and glutathione S transferase activity in liver, lungs, brain, kidneys, heart and spleen of treated rats. The comet assay showed a statistically significant dose related increase in DNA migration. The micronucleus and chromosomal aberration tests revealed a significant induction in frequency of micronuclei and chromosomal aberrations at 150 and 300 mg/kg. Annona squamosa treatment significantly enhanced lipid peroxidation, decreased glutathione and glutathione S transferase levels revealing the oxidative stress condition. Our results warrant careful use of *Annona squamosa* seed extract as a biopesticide till more tests are carried out.

Marta MC *et al.*, (2008) Annona squamosa seeds extracts showed anthelmintic activity against *Haemonchus Contortus*, the main nematode of sheep and goat in Northeastern Brazil. A compound 1 was isolated from ethyl acetate extract and inhibited the egg hatching of *H. Contortus* at 25 mg ml^{-1}. The structure of was

determined as a C_{37} trihydroxy adjacent bistetrahydrofuranacetogenin based on spectroscopic analysis.

Rajesh Kumar Gupta *et al.*, (2008) evaluated in vivo evaluation of anti-oxidant and anti-lipidimic potential of Annona squamosa aqueous extract in Type 2 diabetic models. The plant material was extracted with boiling water for 2 h. Albino Wistar rats were divided into four groups. Diabetes was induced by streptozotocin injection at a dose of 50 mg/kg. Animals of treated groups were given the dose of 350 mg/kg of the extract. The results shows that the water extract of Annona squamosa leaves possessed antioxidant activity as shown by increased activities of scavenging enzymes, Catalase (CAT), Superoxide Dismutase (SOD), Reduced Glutathione (GSH), Glutathione Reductase (GR) and Glutathione-Stransferase (GST) and decrease in malondialdehyde levels present in various tissues. Administration of the extract also improved the lipid profile of the treated groups indicating thereby that the high levels of triglyceride and total cholesterol associated with diabetes can also be significantly managed with the extract.

Baskar R *et al.*, (2007) evaluated the ethanolic extract of *Annona squamosa Linn* leaves for hypoglycemic activity in normal rats. The dose of 350 mg/kg body weight reduced the fasting blood glucose level by 6.0% within 1 h, whereas, the peak blood glucose at 1 h during glucose tolerance test was reduced by 17.1% in normal rats. Treatment of alloxan-induced diabetic rabbits for 15 days with a dose of 350 mg/kg of extract reduces fasting blood glucose by 52.7% and urine sugar by 75%. The dose of 350 mg/kg body weight of ethanolic extract in 10-day treatment of a group of STZ-diabetic rats produced 73.3% fall in FBG level and no sugar was observed in fasting urine. An aqueous extract of *A. Squamosa* leaves found to lower considerable

fasting plasma glucose level in streptozotocin nicotinamide induced type 2 diabetic rats. The findings of the study support the ant diabetic claims of *Annona squamosa*.

Panda S & Kar A *et al.*, (2007) evaluated the methanolic extract of seeds of *Annona squamosa Linn.*, in the regulation of hyperthyroidism in mouse model. Hyperthyroidism produced by L-Thyroxine (L-T4) administration (0.5 mg/kg/d for 12 days, i.p.), which increased the levels of serum triiodothyronine (T3) and thyroxine (T4), activity of hepatic G-6-Phospatase, 5'-monodeiodinase (5'DI) and peroxidation (LPO) with a parallel decrease in superoxide dismutase (SOD) and catalase (CAT) activities. However, simultaneous administration of the Annona seed extract (200 mg/kg) to L-T4 induced hyperthyroid animals for 10 days, reversed all these effects indicating their potential in the regulation of hyperthyroidism. Further, the seed extract did not increase, but decreased the hepatic LPO suggesting its safe and antiperoxidative nature.

Roberta Roesler *et al.*, (2007) evaluated the antioxidant activity of *Annona squamosa* major components by electrospray ionization mass spectrometry. The polar components of *Annona squamosa* pulp peel and seeds ethanolic extracts were investigated by direct infusion Electrospray Ionization Mass Spectrometry (ESI-MS) both in the negative ion mode. Characteristic ESI mass spectra with many diagnostic ions were obtained for the extracts, serving for fast and reliable information. The technique provided information of component structures revealing the presence of important bioactive components widely reported as potent antioxidants such as ascorbic acid, caffeic acid, quinic acid, ferulic acid, xanthoxylin, rutin, caffeoyltartaric acid, caffeoyl glucose and quercetin +hexose+ pentose. This is the first report on the

composition by ESI-MS of araticum peel and seed ethanolic extracts demonstrating excellent antioxidant activity.

Morita H *et al.,* (2006) Isolated cyclic octapeptide, cyclosquamosin B, isolated from the seeds of *A. Squamosa Linn* showed a vasorelaxant effect on rat aorta. It showed a slow relaxation activity against norepinephrine (NE)- induced contractions of rat aorta with/without endothelium. It showed inhibition effect on vasoconstriction of depolarized aorta with high concentration potassium, but moderately inhibition effect on NE-induced contraction in the presence of nicardipine. These results showed that the vasorelaxant effect by cyclosquamosin B might be attributed mainly to inhibition of calcium influx from extra cellular space through voltage dependent calcium channels.

Mukhlesur Rahman M *et al.,* (2005) reported Antimicrobial and cytotoxic constituents from the seeds of *Annona squamosa*. Annotemoyin-1, Annotemoyin-2, squamocin and cholesteryl glucopyranoside were isolated from the seeds of Annona squamosa. These compounds and plant extracts showed remarkable antimicrobial and cytotoxic activities

Rajesh Kumar Gupta *et al.,* (2005) evaluated hypoglycemic and anti-diabetic effect of ethanolic extract of leaves of *Annona squamosa L*. in experimental animals. The ethanolic extract of *Annona squamosa* leaves was administered orally at different doses to normal as well as Streptozotocin (STZ)-induced diabetic rats and alloxan-induced diabetic rabbits. The dose of 350 mg/kg body weight (bw) reduced the fasting blood glucose (FBG) level by 6.0% within 1 h, whereas, the peak blood glucose at 1 h during glucose tolerance test (GTT) was reduced by 17.1% in normal rats. The same dose of ethanolic extract reduced FBG by 26.8% and improved

glucose tolerance by 38.5 and 40.6% at 1 and 2 h, respectively, during GTT in alloxan-induced diabetic rabbits. In STZ-diabetic rats, a fall of 13.0% in FBG and an improvement in glucose tolerance by 37.2 and 60.6% at 1 and 2 h, respectively, was observed during GTT. The dose of 350 mg/kg bw of ethanolic extract in 10-day treatment of a group of STZ-diabetic rats produced 73.3% fall in FBG level and no sugar was observed in fasting urine. Treatment of severely-diabetic rabbits for 15 days with a dose of 350 mg/kg of extract reduce FBG by 52.7% and urine sugar by 75%. It brought about fall in the level of total cholesterol (TC) by 49.3% with increase of 30.3% in high-density lipoprotein (HDL) and decrease of 71.9 and 28.7% in low-density lipoprotein (LDL) and triglycerides (TG) levels, respectively.

Annie Shirwaikar *et al.*, (2004) isolated anti diabetic activity of aqueous leaf extract of *Annona squamosa* in streptozotocin–nicotinamide type 2 diabetic rats. Diabetes mellitus was induced with streptozotocin–nicotinamide and graded doses of the aqueous leaf extracts were then administered in drinking water to normal and experimental diabetic rats for 12 days. Fasting plasma glucose levels, serum insulin levels, serum lipid profiles and changes in body weight were evaluated in normal rats while liver glycogen levels and pancreatic TBARS levels were evaluated additionally in diabetic rats. The diabetic groups treated with the aqueous leaf extract were compared with standard glibenclamide. The findings of the study support the antidiabetic claims of Annona squamosa.

Khar Ashok *et al.*, (2004) evaluated anti cancer activity of *Annona squamosa*. The effect of aqueous and organic extracts from defatted seeds of *A. squamosa* was studied on a rat histiocytic tumour cell line AK-5. Both the extracts caused significant apoptoic tumour cell death with enhance caspase-3 activity down regulation of

antiapoptotic genes Bcl-2 and Bclxi and enhance the generation of intracellular ROS, which correlated well with the decreased levels of intracellular GSH. In addition DNA fragmentation and annexin – V staining confirmed that the extracts induced apoptosis in tumour cells through the oxidative stress. Aqueous extracts of *A. squamosa* seeds possessed significant antitumor activity in vivo against AD-5 tumor.

Damasceno DC *et al.*, (2002) evaluated the effects of *Annona squamosa* extract on early pregnancy in rats. *Annona squamosa* is said to show varied medicinal effects, including insecticide, antiovulatory and abortifacient. The purpose of present study was to investigate if *A. Squamosa* seed aqueous extract, in doses higher than that popularly used to provoke abortion, interferes with reproductive performance, and to correlate the ingestion of this extract with possible alterations in rat embryonic implantation. Doses of 300 mg/kg (Treated Group I, n = 17) and 600 mg/kg (Treated Group II, n = 12) body wt. were administered by gavages, during days 1 to 5 of pregnancy (preimplantation period). The control group (n = 13) received water in the same manner, during the same period for comparison with experimental groups. The animals were euthanized on day 10 of pregnancy. Treatment of dams during the preimplantation period showed no signs of toxicity, and no alteration in the corpora lutea, implantations and embryo in terms of development numbers. The percentage of preimplantation and postimplantation losses in treated groups I and II did not differ from those of control. Treatment with aqueous extract of *A. squamosa* seeds caused no morphological change in the endometrium. The absence of morphological alterations in uterine epithelial cells in treated groups I and II permitted a viable embryonic implantation, as verified by the number of embryos in development at day 10 of pregnancy. Thus, *A. squamosa* seed aqueous extract did not interfere with the reproductive performance of pregnant rats.

Morita H *et al.*, (2002) the ent-kaurane diterpenoids, which are isolated from stem of *A. squamosa Linn* are investigated for anti-platelet activity. The ent-kaurane diterpenoids 'ent-Kaur-16-en-19-oic acid' and '16alpha-hydro- 19-al-ent-kauran-17-oic acid' showed complete inhibitory effects on rabbit platelet aggregation at 200 microM.

Dash G K *et al.*, (2001) the seed extract of *A. squamosa Linn* was investigated for post coitus anti-fertility activity. The seed extract of *Annona squamosa Linn.*, shows anti-implantation and abortifacient activities.

Mishar A *et al.*, (1979) a diterpenoid 16 β, 17-dihydroxy-entkauran- 19-oic acid was isolated from *Annona squamosa* Linn., and investigated for their activity against HIV virus. The 16β, 17-dihydroxy-ent-kauran-19- oic acid showed significant activity against HIV replication in H9 lymphocyte cells.

LITERATURE REVIEW OF *ECHINOPS ECHINATUS* ROXB

Sofia Eram *et al.*, (2013) carried out Experimental evaluation of *Echinops echinatus* Roxb., as an effective hepatoprotective. Ethanolic extract of aerial parts of *Echinops echinatus* Roxb., were evaluated on CCl_4 induced liver damage in Rabbits, the extract at the dose level of 500 mg/kg is found to posses significant protection.

Manish Agrawal *et al.*, (2012) evaluated the protective effects of *Echinops echinatus* Roxb., on testosterone-induced prostatic hyperplasia in rats. *Echinops echinatus* Roxb., extracts attenuated the increase in the prostatic/body weight ratio induced by testosterone. Butanolic fraction of ethanolic extract at the dose level of 50 and 100 mg/kg exhibited the best activity.

Amish J. Patel *et al.*, (2011) performed Comparative diuretic activity of root and aerial part methanolic extracts of *Echinops echinatus* Roxb. Methanolic extracts of roots and aerial parts of *Echinops echinatus* Roxb. were subjected to diuretic potential in albino rats methanolic extracts at the dose of 250 and 500 mg/kg body weight shows a significant increase in the urine volume and electrolyte excretion when compared to control.

Leena S & Sitaram K (2010) discussed traditional uses of plants as cooling agents by the tribal and traditional communities of dang region in Rajasthan, India. *Echinops echinatus* Roxb., paste smeared on soles and palms to treat heatstroke.

Rudrappa JN & Mohmoud R (2010) studied Free radical scavenging activity of *Echinops echinatus* Roxb., Root. Extracts of *Echinops echinatus* Roxb, roots were evaluated for radical scavenging activities using different in vitro models

like scavenging of 2, 2 diphenyl-1-picrylhydrazyl (DPPH) radical, nitric oxide radical and superoxide anion.

Singh S *et al.*, (2006) isolated Flavonoids from *Echinops echinatus* Roxb., A new isoflavone glycoside, echinoside (7), together with 7-hydroxyisoflavone, kaempferol-4'-methylether, kaempferol-7-methyl ether, myrecetin-3-O-alpha-L-rhamnoside, kaempferol and kaempferol-3-O-alpha-L-rhamnoside, has been isolated from the whole plant of *Echinops echinatus* Roxb.

Padashetty SA & Mishra SH (2005) described anti-fertility activity of *Echinops echinatus* Roxb., *roots* on male rats. The present study was undertaken to evaluate the effect of terpenoidal fraction prepared from the petroleum ether extract of the roots of *Echinops echinatus* Roxb., on male reproductive parameters.

Khan MA *et al.*, (2000) discussed Ethnobotany and taxonomic studies of *Echinops echinatus* Roxb. (Untkatara) from Potohar region of Pakistan. The ethno botanical information was verified by cross-checking with the people of different localities. The plant is diuretic, alterative, aphrodisiac and nervine tonic. It is also recommended in hysteria, dyspepsia, jaundice and scrofula.

Singh B *et al.*, (1999) described anti-inflammatory activity of ethanol extract of *Echinops echinatus* Roxb., *whole* plant. The extract effectively inhibited the acute inflammation induced in rats by carrageenan, formaldehyde and adjuvant and the chronic arthritis induced by formaldehyde and adjuvant.

Singh UP *et al.*, (1998) investigated antifungal activity of some new flavones and flavone glycosides of *Echinops echinatus* Roxb., Four phenolic compounds, viz., apigenin, apigenin-7-O-glucoside, echinacin, and echinaticin, were isolated from the

whole plant of *Echinops echinatus* Roxb., the latter two compounds were isolated for the first time. Echinacin, which was highly effective at 150 μg mL−1, is considered the most promising of these compounds and its use as a control measure against *Alternaria* blight of pigeon pea under field conditions has been suggested.

Bupinder Sing *et al.*, (1989) investigated Anti-inflammatory activity of taraxasterol acetate from *Echinops echinatus* Roxb., in rats and mice. Taraxasterol acetate, a triterpenoid found in several plant species, demonstrated anti-inflammatory activity in albino rats against carrageenan, formaldehyde and adjuvant induced inflammations in doses between 10 and 100 mg/kg.

Chaudhuri *et al.*, (1987) isolated Echinozolinone, an alkaloid from *Echinops echinatus* Roxb. In addition to echinopsine and echinopsidine, a new alkaloid, echinozolinone, has been identified in *Echinops echinatus* as 3(2-hydroxyethyl)-4(3H)-quinazolinone from its spectral data.

SCOPE AND PLAN OF WORK

IV. SCOPE AND PLAN OF WORK

SCOPE OF WORK

Parkinsonism is one of the commonest neurodegenerative diseases, which is characterized by a selective and progressive degeneration of dopaminergic neurons of substantia nigra pars compacta in the ventral midbrain. The loss of dopaminergic neurons, leads to the reduction of dopamine being released into the striatum. These processes are then responsible for the clinical features of parkinsons disease including bradykinesia, resting tremor, rigidity, and difficulty in initiating movements which might ultimately induce programmed cell death (Speciale *SG.*, 2002), Although the etiology of Parkinsonism remains unknown, recent studies have suggested that oxidative stress (OS), apoptosis as a result of mitochondrial defects, neuroinflammation may play important roles in its pathogenesis.

The routine therapy with Levodopa and Carbidopa itself may induce dyskinesias (DID) in majority of patients with Parkinson's disease (PD) (Christopher A. Lieu et al., 2010). Thus the search for newer antiparkinsonism drugs has a rational basis. Plant drugs give leads in many instances of drug discovery as they possess principles to treat a disease with the simulataneous input of nutrients and neurotonics. The roots of the plant *Annona squamosa* Linn., has a botanical claim as nervine tonic (Warrier PK 1994). The ariel parts of *Echinops echinatus* Roxb., was also selecterd based on the ethnobotanical claim as nervine tonic (Warrier PK 1994).

Nowadays much attention has been focused on stress and mental strain causing CNS disorders by oxidative damage and by many other causes. The fact that natural antioxidant derived from the plant reduce the risk of many diseases including

degenerative diseases like Parkinsonism has been supported by several studies (Stah et al., 2002).

The present research was undertaken to evaluate the neuroprotective effect of the hydroethanolic extracts of roots of *Annona squamosa* Linn., and aerial parts of *Echinops echinatus* Roxb in 6-OH dopamine induced neurodegeneration in Wistar albino rats and to investigate the antioxidant potential of the same extracts in combating the oxidative stress produced by 6-OH dopamine.

PLAN OF THE WORK

The plan of the work is given below

Collection and authentification of roots of *Annona squamosa* Linn., & *Echinops echinatus* Roxb..

i. **Preparation of hydroethanolic extracts by cold maceration method and preliminary phytochemical screening of extracts.**

ii. **Identification and determination of phytoconstituents of extracts.**

   a. TLC and HPTLC studies on the extracts
   b. Determination of total phenolic content.
   c. Determination of total flavonoid content.
   d. Determination of total alkaloid content

iii. ***In vitro* antioxidant studies on the extracts.**

   a. Determination of DPPH radical scavenging activity.
   b. Determination of nitric oxide scavenging activity.
   c. Determination of hydroxyl radical scavenging activity.
   d. Determination of reducing power.

iv. **Toxicological investigation of the extracts.**

   a. Acute toxicity study.
   b. Sub acute toxicity.

v. **Neuropharmacological studies**

   a. Preliminary neuropharmacological studies of the plant extracts.
   b. Evaluation of neuroprotection after ICV injection with 6-OH Dopamine.
      a. Open field test
      b. Gait analysis

c. Force Swim-test

d. Beam walking test

e. Grid test

f. Pole test

g. Rota rod test

c. Estimation of biochemical parameters in rat brain

a. Estimation of Total protein

b. Assay for Thiobarbituric Acid Reactive Substance.

c. Assay for reduced glutathione content

d. Determination of glutathione reductase activity

e. Determination of glutathione peroxidase

f. Determination of superoxide dismutase activity

g. Determination of catalase activity

d. Estimation of neurotransmitters

a. Acetyl Cholinesterase

b. Monoamine Oxidase

c. Dopamine

d. Glutamate

e. Histopathology of Brain

MATERIALS & METHODS

V. MATERIALS AND METHODS

5.1 PLANT MATERIALS

The roots of *Annona squamosa* were collected from Chennai and whole plant material for the plant *Echinops echinatus* were collected from the forest regions of Tirupathi. Both the plants were authenticated by Prof. P. Jayaraman, Director, National Institute of Herbal Science, Chennai. The Ref. no: are PARC/2009/421 and PARC/-2010/043 respectively.

PLANT I

Scientific name : *Annona squamosa* Linn.

Family : Annonaceae

Figure 9. Plant profile of *Annona squamosa* Linn

Botanical Classification

Kingdom	:	Plantae – Plants
Division	:	Magnoliophyta – Flowering plants
Class	:	Magnoliopsida – Dicotyledons
Sub class	:	Magnoliidae
Order	:	Magnoliales
Genus	:	*Annona* L. – annona
Species	:	*Annona squamosa* L. sugar apple

Figure 10. *Annona squamosa* Linn., root

Vernacular Names

English	:	Custard Apple, Sugar apple
Sanskrit	:	Sitaphala
Tamil	:	Sitapalam
Hindi	:	Sitaphal
Malayalam	:	Sitapalam
Telugu	:	Sitapandu

Medicinal uses

The bark and leaves contain annonaine, an alkaloid. In tropical America, a decoction of the leaves is used as a cold remedy and to clarify urine. A bark decoction is used to stop diarrhea, while the root is used in the treatment of dysentery. Roots are also used as nervine stimulant

Habitat

A native to South America and the West Indies; now cultivated throughout India.

PLANT II

Scientific name	:	***Echinops echinatus* Roxb.**
Family	:	Asteraceae

Figure 11. Plant profile of *Echinops echinatus* Roxb

Botanical Classification

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Echinops
Sub class	:	Tracheobionta
Order	:	Yet to know
Genus	:	Echinops
Species	:	Echinatus

Vernacular Names

English	:	Indian Globe Thistle
Sanskrit	:	Brahmadanda
Tamil	:	Kutiraippijan
Hindi	:	Utakatira, Oontkateli, Gokhru
Kannada	:	Brahmadande
Telugu	:	Brahmadandi

Habitat

India - Andhra Pradesh, Bihar, Himachal Pradesh, Jammu and Kashmir, Karnataka, Madhya Pradesh, Maharashtra, Rajasthan, Tamil Nadu, Uttar Pradesh, West Bengal

Figure 12. Dried whole plant of *Echinops echinatus* Roxb

Traditional Uses

The rural population of Gujarat uses the suspension of root bark powder for the treatment of diabetes. Chattisgarh people use this herb in different ways both internally and externally for the treatment of sexual disorders. The patients suffering from respiratory troubles, particularly asthma, are advised to inhale the fumes obtained by burning the leaves & roots of *E. Echinatus* in order to get quick and permanent relief. The root is abortifacient aphrodisiac. The seeds are sweet and aphrodisiac. The plant is bitter, stomachic, antipyretic, analgesic, increases the appetite stimulates the liver, useful in brain disease, used in ophthalmia, chronic fever, pains in the joints, inflammations, the root is aphrodisiac.

5.2 EXPERIMENTAL ANIMALS

Albino Wistar rats of either sex approximately same age group were used after being acclimatized for a week at laboratory conditions. They were provided standard rodent pellet diet (Lipton India) and water *ad libitum*. The animals had free access to food and water and maintained under 12:12 hr light and dark cycle. All experiments were carried out during day time from 09.00 to 17.00 hr. The protocol was approved by Institutional Animal Ethical Committee and care of the animals was taken as per guidelines of committee for the purpose of control and supervision in experiments on animals (CPCSEA), representative of Animal Welfare, Government of India.

The sanction of animal was done by IAEC Ultra college of Pharmacy, Madurai with reference no. IAEC Ref. No. KKCP/2012/011 & UCP/IAEC/2013/071.

Figure 13. Oral administration to Wistar Albino Rats

5.3 NEUROTOXIN 6-OH DOPAMINE

The 6-OH Dopamine model has also been used successfully to demonstrate the importance of dopamine stimulation. The neurotoxin 6-OH Dopamine shares some structural similarities with dopamine and norepinephrine, exhibiting a high affinity for several catecholaminergic plasma membrane transporters such as the dopamine transporter and norepinephrine transporters. The dose level of 6-OH dopamine is 12µg/kg

5.4 CHEMICALS

Table 3. List of chemicals used in the experiment

S.No	Chemicals	Source
1	5,5'-Dithio-bis-2-nitrobenzoic acid(DTNB)	Loba chemicals Ltd., Mumbai.
2	Acetic acid	Paxmy Fine Chemicals – India
3	Acetyl thiocholine iodide	S.d.fine chemicals Ltd.,Mumbai
4	Adenosine 5' diphosphate	S.d.fine chemicals Ltd.,Mumbai
5	Chloroform	S.d.fine chemicals Ltd., Mumbai
6	Disodium dihydrogen phosphate	S.d.fine chemicals Ltd.,Mumbai
7	Disodium hydrogen phosphate	S.d.fine chemicals Ltd., Mumbai
8	Ethyl Acetate	S.d.fine chemicals Ltd.,Mumbai
9	Ethylene diamine tetra acetic acid (EDTA) Disodium Salt	Rolex lab reagent, Mumbai.
10	$FeCl_3$ reagent	S.d.fine chemicals Ltd., Mumbai
11	Glutamate dehydrogenase	S.d.fine chemicals Ltd., Mumbai
12	Glycine	S.d.fine chemicals Ltd., Mumbai
13	Hydrogen peroxide	Qualigens fine chemicals Ltd., Mumbai
14	Hydrazine hydrate	S.d.fine chemicals Ltd..Mumbai
15	Iodine	S.d.fine chemicals Ltd.,Mumbai
16	Methanol (analytical grade)	S.d.fine chemicals Ltd., Mumbai
17	n-butanol	S.d.fine chemicals Ltd.,Mumbai
18	NAD (Nicotinamide adenine dinucleotide)	S.d.fine chemicals Ltd., Mumbai
19	$NADPH_2$	S.d.fine chemicals Ltd., Mumbai
20	Oxidised gluthathione	Qualigens fine chemicals Ltd., Mumbai.
21	Potassium dihydrogen phosphate	S.d.fine chemicals Ltd., Mumbai
22	Pthalyl dialdehyde	S.d.fine chemicals Ltd., Mumbai
23	Perchloric acid	Loba chemicals Ltd., Mumbai.
24	Pyrogallol	S.d.fine chemicals Ltd., Mumbai
25	Reduced Glutathione (GSH)	S.d.fine chemicals Ltd., Mumbai
26	Sodium dodecyl sulphate(SDS)	S.d.fine chemicals Ltd., Mumbai
27	Sodium Acetate	S.d.fine chemicals Ltd., Mumbai
28	Sodium hydrogen carbonate	S.d.fine chemicals Ltd., Mumbai
29	Thionyl chloride	S.d.fine chemicals Ltd., Mumbai
30	Trichloro acetic acid	Ranbaxy laboratories Ltd. Chemical Division, New Delhi.
31	Tris HCl	S.d.fine chemicals Ltd., Mumbai
32	Thiobarbituric acid	Rolex laboratories reagent, Mumbai
33	6-OH Dopamine	Sigma- Aldrich

5.5 METHODS

5.5.1 Extraction and Identification of Phytoconstituents

Extraction of plant material (Harbone 1973)

The air dried Roots of *Annona squamosa* Linn., and whole plant of *Echinops echinatus* Roxb., 500gms each were coarse powdered and extracted with 50% alcohol. The crude extract was further filtered and evaporated by the aid of rotary evaporator. The final mass is weighed and preserved for further use.

5.5.2 Preliminary phytochemical screening

Preliminary phytoconstituents present in the hydroethanolic extract of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., plants were identified based on the chemical test (Kokate CK1994).

1. Test for alkaloids

Treated with dilute Hydrochloric acid and filtered. The filtrate was treated with various alkaloidal agents.

a) Mayer's–Test

Treated with Mayer's reagent and cream colour indicates the presence of alkaloid.

b) Dragendroff's–Test

When little amount of the sample was treated with the Dragendroff's reagent, the presence of reddish brown precipitate reveals the presence of alkaloid.

c) Hager's–Test

Treated with the Hager's reagent and presence of yellow colour precipitate indicates the presence of alkaloid.

d) Wagner's–Test

Treated with the Wagner's reagent, the appearance of brown colour precipitate indicates the presence of alkaloid.

2. Test for carbohydrates

The extracts were treated with 3ml of alpha–Napthol in alcohol and to the sides of the test tube concentrated sulphuric acid was added carefully. Formation of violet colour ring at the junction of two liquids shows the presence of carbohydrates.

a) Fehling's–Test

The extracts were treated with Fehling's solution A and B and heated. Presence of reddish brown colour precipitate indicates the presence of reducing sugars.

b) Benedict's–Test

The extracts were treated with Benedict's reagent and heated and presence of reddish orange colour precipitate indicates the presence of reducing sugars.

c) Barfoed's–Test

The extracts were treated with Barfoed's reagent and heated. Appearance of reddish orange colour precipitate indicates the presence of non reducing sugars.

3. Test for proteins

a) Biuret's–test

When the extracts were treated with copper sulphate solution, followed by the addition of sodium hydroxide solution, appearance of violet colour indicates the presence of proteins

b) Millon's–Test

When the extract was treated with Millon's reagent, appearance of pink colour indicates the presence of proteins.

4. Test for steroids

a) Libermann Burchard Test

When the extracts were treated with concentrated sulphuric acid, few drops of glacial acetic acid, followed by the addition of acetic anhydride, appearance of green colour indicates the presence of steroids.

5. Test for sterols

When the extracts were treated with 5% potassium hydroxide solution, appearance of pink colour indicates the presence of sterols.

6. Test for phenols

When the extracts were treated with neutral ferric chloride solution, appearance of violet colour indicates the presence of phenols. When the extracts

were treated with 10% sodium chloride solution, the appearance of cream colour indicates the presence of phenols.

7. Test for tannins

a) When the extracts were treated with 10% lead acetate solution, appearance of white precipitate indicates the presence of tannins.

b) When the extracts were treated with aqueous bromine solution, appearance of white precipitate indicates the presence of tannins.

8. Test for flavanoids

a) 5ml of the extract solution was hydrolyzed with 10 % v/v sulphuric acid and cooled. Then, it was extracted with diethyl ether and divided into three portions in three separate test tubes. 1 ml of diluted sodium carbonate, 1 ml of 0.1N sodium hydroxide, and 1ml of strong ammonia solution were added to the first, second and third test tubes respectively. In each test tube, development of yellow color demonstrated the presence of flavonoids.

b) **Shinoda's test**

The extract was dissolved in alcohol, to that one piece of magnesium followed by concentrated HCl was added drop wise and heated. Appearance of magenta color shows the presence of flavonoids.

9. Test for gums and mucilage

The extracts were treated with 25ml of absolute alcohol, and then solution was filtered. The filtrate was examined for its swelling properties.

10. Test for glycosides

When a pinch of the extracts were dissolved in the glacial acetic acid and few drops of ferric chloride solution was added, followed by the addition of concentrated sulphuric acid, formation of red ring at the junction of two liquids indicates the presence of glycosides.

11. Test for saponins

Foam test

1ml of the extracts are diluted to 20 ml with distilled water and shaken well in a test tube. The formation of foam in the upper part of the test tube indicates the presence of saponins.

12. Test for terpenes

When the extracts were treated with tin and thionyl Chloride, appearance of pink colour indicates the presence of terpenes.

5.6 TLC and HPTLC of hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb

Dissolve 500 mg of the sample in ethanol, filtered and made up to 15 ml. The solution was applied on Merck Aluminium plate pre-coated with Silica gel 60 F of 0.2 mm thickness. The plate was developed in different solvent system Toluene: Methanol: Ethyl acetate: Formic acid (2.5:1.0:5:1.5) for *Annona squamosa* Linn., and Toluene: Methanol: Ethyl acetate: Formic acid (3:0.5:5:1.5) for Echinops *echinatus* Roxb. The Rf values was calculated. The plate was then scanned at 254 nm using Deuterium lamp in Camag HPTLC instrument provided with CAMAG software.

5.7.1 Determination of total phenolic content

The total phenolic contents of the extracts were determined with gallic acid as a positive standard. Samples (100 µl) were mixed with 2 ml 2% sodium carbonate and incubated at 25°C for 2 minutes. 1:1 (v/v) Folin-Ciocalteu's phenol reagent was added after incubation and the contents were mixed vigorously. The mixture was allowed to stand at 25°C for 30 minutes and the absorbance was determined at 720 nm. The above said procedure was performed with standard gallic acid solutions and a standard curve was obtained. The total polyphenolic contents of the extract were expressed in terms of gallic acid equivalents of the extract.

5.7.2 Determination of total flavonoid content

The total flavonoid content was estimated using quercetin as a positive standard and expressed in terms of quercetin equivalents in mg/g of extract. To the tubes containing extract, sodium nitrite (150 µl, 5% w/v) was added. The contents were uniformly mixed and allowed to stand for 5 min at ambient temperature, then 1.5 ml of 10% (w/v) aluminum chloride were added and the mixture was allowed to stand for another 6 minutes. To this 1 ml 1 M sodium hydroxide was added. After 10 minutes, the absorbance was measured at 510 nm.

5.7.3 Determination of total alkaloid content

The total alkaloid content of the extracts was determined. The extract was dissolved in 2 N HCl and then filtered. The pH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. One ml of this solution was added to a separating funnel and then 5 ml of Bromocresol green solution along with 5 ml of phosphate buffer were added. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extracts were collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm.

5.8 *IN VITRO* ANTIOXIDANT STUDIES OF THE EXTRACTS

1, 1-Diphenyl, 2-Picrylhydrazyl (DPPH) radical scavenging activity (Bang *et al*., 2001)

DPPH scavenging activity was measured by spectrophotometric method. 0.1 mM solution of DPPH was prepared in ethanol. To this solution, 3 ml of hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., solution was added at different concentration (25-800 µg/ml). Equal amount of distilled water was added to the control. The mixture was shaken well and incubated at room temperature for 30 min. The absorbance was read at 517 nm using a spectrophotometer.

Nitric oxide (NO) scavenging *(Garrat* et al*., 1964)*

Nitric oxide scavenging activity was measured by using a spectrophotometer. Sodium nitroprusside (5 mM) in phosphate buffered saline was mixed with different concentrations of hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., (25-800 µg/ml) dissolved in distilled water and incubated at 25 ºC for 30 min. A control without test compound but with equivalent amount of distilled water was taken. After 30 min, 1.5 ml of the incubation solution were removed and diluted with 1.5 ml of Griess reagent (1% w/v sulphanilamide, 2% v/v phosphoric acid, and 0.1% w/v napthyl ethylene diamine dihyrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with napthylethylene diamine was measured at 546 nm.

Hydroxyl Radical Scavenging Activity (Ohkawa *et al*., 1979)

The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe^{3+}/ascorbate /EDTA/ H_2O_2 system (Fenton reaction). The hydroxyl radicals attack deoxyribose that eventually results in TBARS formation. The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbic acid (0.1 mM), KH_2PO_4-KOH (20 mM, pH 7.4) and various concentrations (25 - 800 µg/ml) of the extract in a final volume of 1 ml. The reaction mixture was incubated for 1 hr at 37^0 C. Deoxyribose degradation was measured as TBARS and percentage inhibition was calculated.

The percentage reduction was calculated by comparison with the control using the below formula:

$$\text{Inhibition (\%)} = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100$$

Determination of reducing power (Meir et *al*., 1995)

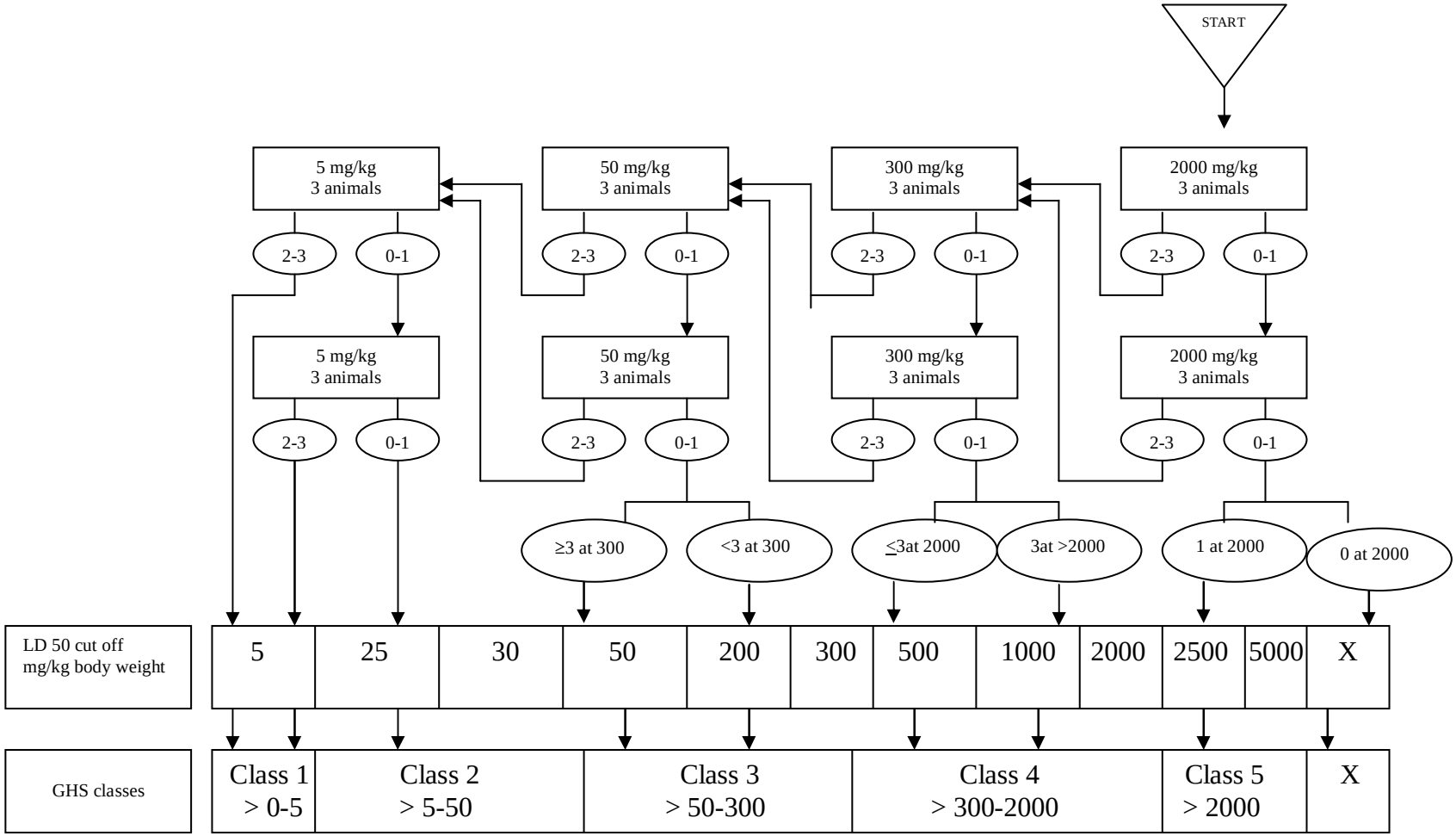
10 mg of hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3 Fe (CN)_6]$ (2.5 ml, 1% w/v). The mixture was incubated at 50 ºC for 20 min. A portion (2.5 ml) of trichloroacetic acid (10% w/v) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride, (0.5 ml, 0.1% w/v). The absorbance was measured at 700 nm. Increased absorbance of the reaction mixture signifies increased reducing power.

5.9 TOXICITY STUDY

5.9.1 Acute oral toxicity study (OECD 423)

For carrying out oral toxicity study OECD guidelines 423 was followed. It is a stepwise procedure with three animals of a single sex per step. Depending on the mortality and/or, 50, 300, 2000mg/kg body weight) and the results allow a substance to be ranked morbidity of the animals a few steps may be necessary to judge the toxicity of the test substance. This procedure has advantage over other methods because of minimal usage of animals while allowing for acceptable data.

The method uses defined doses (5and classified according to the globally harmonized system. The starting dose for hydroethanolic extract of *Annona squamosa* Linn and *Echinops echinatus* was 2000mg/kg bodyweight (p.o). The dose was administered to the rats which were fasted overnight with water ad libitum and observed for signs of toxicity. The same dose was once again tried with another three rats and were observed for 72 hours for symptoms like change in skin colour, salivation, diarrhea, sleep, tremors, convulsions and also respiratory, autonomic and CNS effects.



*0,1,2,3: Number of moribund or dead animals at each step *GHS: Globally Harmonized classified system (mg/kg b.w) *X-Un classified

Figure 14. Flow Chart for acute toxic class method (OECD guidelines 423) at starting dose of 2000 mg/kg body weight/p.o

5.9.2 Sub acute toxicity study (OECD 407)

For carrying out sub acute oral toxicity study OECD guidelines 407 -Repeated Dose 28-Day oral toxicity study in rodents was followed. The duration of the Study was 28 days. Rat were administered with at the dose level of 200 mg/kg. Each group consists of ten animals (five animals/sex/group). The drug was administered orally once daily for 28 days. On 29^{th} day the animals was anaesthetized and blood was collected by retro orbital puncture. Hematological parameters were evaluated. Serum was separated and biochemical parameters were estimated. Animals were sacrificed and organs were removed and weighed. The organs were kept in 10% formalin and used for histo-pathological analysis.

Change in body weight

The change in body weight was observed for a time period of 28 days at an interval of 5 days. Body weight gains were determined from the final and initial body weights.

Organ weight

After sacrifice, organs were quickly excised and weighed and relative organ weights were computed.

Hematological studies

The following hematological parameters were estimated by standard procedures.

Blood samples were drawn by cardiac puncture and haematological parameters were analyzed by autoanalyzer.

i. Total R.B.C. count
ii. Total W.B.C. Count
iii. Differential leukocyte count
iv. Haemoglobin (Hb) concentration

Biochemical studies

Blood samples were drawn by cardiac puncture. Blood from three animals was pooled for serum separation. Each serum sample was analyzed by auto analyzer.

i. Aspartate Aminotransferase (ASAT)
ii. Alanine Aminotransferase (ALAT)

iii. Alkaline Phosphatase (ALP)
iv. Total Bilirubin (TB)
v. Direct Bilirubin (DB)
vi. Urea
vii. Creatinine
viii. Total protein.

Histopathological study

After blood collection rats were sacrificed for tissue studies. The internal organs like liver, kidney, lungs, brain, heart and spleen were isolated and blotted free of blood, weighed immediately to determine relative organs weights and observed for gross lesions. Histological examination was performed on the tissue preserved in 10% buffered formalin solution with particular emphasis on those which showed gross pathological changes.

5.10 PRELIMINARY NEUROPHARMACOLOGICAL SCREENING

Several of the following tests were performed on each rat prior to 6-OH Dopamine administration. Provided the animals were administered 100 & 200 mg/kg (p.o) of *Echinops echinatus* Roxb., *and Annona squamosa* Linn., suspended in 1% carboxy methylcellulose. All the evaluations were carried out between 8 am 11pm.

General behavior test (Dixit and Varma (1976) and Murugesan et al. (1999)

a. **Spontaneous activity**

These were evaluated by placing a rat in a bell jar. It usually shows a moderate degree of inquisitive behavior.

b. Sound responses

Rats normally utter no sound, so that vocalization may point to a noxious stimulus.

c. Touch responses

It was noted when the animal was touched with a forceps (or) pencil at various parts (i.e. on the side of the neck, on the abdomen and on the groin).

d. Pain response

This response was graded when a small artery clamp was attached to the base of tail.

e. Latency to groom

The latency from the time the animal was placed into a test cage until it began grooming was also measured with a stop watch.

f. Locomotion

Each rat was placed into actophotometer (40 cm×20 cm×20 cm) with a plexiglass door and the total number of times an infrared beam was disrupted in 10 min was measured by an automated counter.

g. Postural measures

Head position was measured. The rat was observed individually in an identical cage to the home cage. The position of the head was noted every second for 60 s in triplicate. Deviations $>10^{\circ}$ and the direction (left, right or neutral) were noted. The net ipsilateral–contralateral bias was calculated for each 180s test period.

h. Righting reflex

Rats were treated with the test compounds on the test day. After 15, 30 and 60 minutes, each rat was placed gently on its back on an undulated surface made of white iron and kept at 30^{o}C. If the animal remained on its back for 30 seconds, it was considered as a loss of righting reflex.

i. Pinna reflex

The reflex is examined by touching the centre of pinna with a hair or other fine instrument. The unaffected rat withdraws from the irritating hair.

j. **Grip strength**

The grip strength test is used to assess muscular strength or neuromuscular function in rodents. It was measured by allowing the animal to grasp a pencil in the horizontal position and noting the time taken by the animal to drop the pencil on the table.

5.11 EVALUATION OF NEUROPROTECTION

Animal Grouping

Wistar Albino rats of body weight 150-180g were randomized into 6 groups. Each group have 6 animals (n= 6 per group). The groups and treatment are designated as follows.

Group –I	:	Control (0.9 % saline, p.o)
Group –II	:	6-OHDA (12 µg 6-hydroxydopamine /2µl in 0.1% ascorbic acid-saline into left striatum.
Group –III	:	6-OHDA + low dose (100mg/kg) of ASHE
Group –IV	:	6-OHDA + high dose (200mg/kg) of ASHE
Group –V	:	6-OHDA + low dose (100mg/kg) of EEHE
Group –VI	:	6-OHDA+ high dose (200mg/kg) of EEHE
Group –VII	:	6-OHDA+ 100mg/kg of formulation

The animals were grouped into individual cages and treated with 100 and 200 mg/kg of ASHE and EEHE orally (p.o). The treatment was continued for a period of 21 days.

On the 22nd day animals were anaesthetized by intraperitoneal (i.p) injection of sodium thiopental (40 mg/kg), and additional anesthetics were given when necessary. After being deeply anaesthetized (loss of corneal and toe pad reflexes) rats were mounted in position. The scalp was shaved, swabbed with iodine and a central incision made to expose the skull.

The injections were made manually, with the help of a Hamilton syringe, through the burr holes made on the skull surface in both groups. The injection rate was 1.0µl/min, and the needle was kept in place for an additional 1min before being slowly retracted. The experiments were performed in accordance with the guidelines of the Animal Ethics Committee. The treatment with ASHE and EEHE was continued further for a period of 7 days.

5.12 NEUROPHARMACOLOGICAL PROFILE

i. Catalepsy test (Schmidt WJ 1989)

The tests for catalepsy were conducted on a horizontal bar (10 cm above the table surface) by gently placing both forepaws on the bar by gently placing the animal on the grid and measuring the time until at least one paw was actively displaced (descent latency). A cut-off time of 180 s was used, i.e. the trial was terminated when the animals did not move a paw within that time. Each animal was tested three times on both the bar and grid alternatively and an average of three values was taken as descent latency time.

ii. Open field test (Cryan JF 1986)

The open field apparatus was made of a gray wooden box (50 x 50 x 50 cm) and the results in the open field test were determined automatically. A camera was used to detect the numbers of rearing. The distance of movement (total locomotors activity (cm)) of rat was recorded for the open field experiments, each animal was placed in the center of the open field and allowed to freely explore the apparatus for 5 min. Total locomotor activity and numbers of rearing were recorded automatically. Each rat was used only once.

iii. Gait analysis (Ungerstedt U 1970)

To measure the gait animals were trained to walk through a narrow alley leading into their home cage. Once trained paper was placed along the alley floor and each animal fore limbs and hind limbs were brushed with non toxic paint. Animals were kept at the beginning of the alley. As they walked throught that alley into their home cage, they left their paw prints on the paper. By measuring the distance between paw prints stride length was determined.

iv. Force Swim-test (Alcaro A 2002)

Force swim test was carried out in water tubs (40cm length×25cmwidth×16cm height). Water was kept at the depth of 12cm and the temperature was maintained at 27±2 $^{\circ}$C. The animals were wiped dry immediately after the experiment using a dry towel and returned to cages kept at 27±2 $^{\circ}$C.

v. Beam walking test (Goldstein LB 1990)

Motor c o-ordination and balance were assessed by measuring the ability of the rat to transverse a narrow beam to reach dark goal box in beam walking test. The beams consisted of stationary wooden narrow flat beam (L 100cm × W_1cm) placed at a height of 100cm from the floor. The animals were trained

in beam for ten trials with one minute interval. During experiment animals were monitored and rewarded with food pellets in the goal box. Time taken to transverse the beam from start box to goal box and number of stepping errors were measured.

vi. Grid test (Hua Y 2002**)**

The grid apparatus consisted of a horizontal mesh (total size 12cm^2, openings 0.5cm^2) mounted 20cm above a hard surface, thus discouraging falling, but not leading to the injury in case of falling. The apparatus consists of a 3 inch wall that made up of any opaque sturdy material. Rat were lifted by their tail and slowly placed in the centre of the horizontal grid and supported until they grabbed the grid with both their fore and hind paws. The grid was then inverted so that the rats were hanging upside down for 30seconds and the maximum hanging time was measured.

vii. Pole test (Reddy DS 1799)

Animal was placed head upward on the top of a vertical rough surfaced pole (diameter 1cm and height 55cm). Each rat was habituated to the apparatus on the day prior to testing, then allowed to descend five times. The total time taken by the animal to turn completely downward and climb down to the floor wsa recorded with the maximum duration of 120 seconds. Even if the rat descended part away and fell the rest of the way, the behavior was scored until it reached to the floor. When the rat was not able to turn downward and instead dropped from the pole, TLA was taken as 120 seconds because of maximal severity.

viii. Rota rod test (Kulkarni 2003)

Rota rod unit consists of a rotating spindle (diameter 7.3cm)d individual compartments for each rat with varying rotational speeds. Initially animals were trained for four training sessions on consecutive days (each constituted by a maximum of 10 trails) to achieve the maximal performance. The animals were exposed on a rotating rod at 10 rpm at 5 min interval. Average retention time spent on rod was calculated.

5.13 BRAIN BIOCHEMICAL STUDIES

Tissue preparation for antioxidant enzymes (Paxinos and Watson, 1982).

After the animals were sacrificed and their brains were removed quickly for harvesting striatum and substantia nigra by cutting coronal sections of 1.0-mm thickness, using a rat brain matrix according to the rat brain atlas for enzymatic assays, striatum was homogenized (10% w/v) in 0.01M phosphate buffer (pH 7.0) and centrifuged at 10,500 rpm for 20min at 4°C to obtain post mitochondrial supernatant (PMS), while homogenized substantia nigra 10% w/v was used for the estimation of TBARS and GSH.

i. **Estimation of Total protein in brain** (Lowry et al., 1951)

About 0.2 ml of homogenized brain sample was made up to 1ml using distilled water. The test tube with 1 ml distilled water serves as blank. Add 4.5 ml of Reagent I and incubate for 10 minutes. After incubation add 0.5 ml of reagent II and incubate for 30 minutes Measure the absorbance at 660 nm and plot the standard graph. Estimate the amount of protein present in the given sample from the standard graph

ii. **Assay for Thiobarbituric Acid Reactive Substance.** (Utley et al. (1967)

The method of was modified for the estimation of lipid peroxidation. Briefly, 0.2ml homogenate was pipetted in Eppendorf tube and incubated at 37±1°C in a metabolic water bath shaker for 60min at 120 strokes up and down; another 0.2ml was pipetted in an Eppendorf tube and placed at 0°C incubation. After 1h of incubation, 0.4ml of 5% TCA and 0.4 ml of 0.67% TBA was added in both samples (i.e., 0°C and 37°C). The reaction mixture from the vial was transferred to the tube and centrifuged at 3500×g for 15min. The supernatant was transferred to another tube and placed in a boiling water bath for 10min. Thereafter, the test tubes were cooled and the absorbance of the color was read at 535nm. The rate of lipid peroxidation expressed as nmol of thiobarbituric acid reactive substance formed/min/mg protein.

iii. **Assay for reduced glutathione content** (Jollow et al. (1974).

Reduced GSH was determined by the method of 0.2ml of homogenate was precipitated with 0.2ml of sulfosalicylic acid (4%). The sample was kept at 4°C for at least 1h and then subjected to centrifugation at 1200×g for 15min at 4°C. The assay mixture contained 0.1ml of filtered aliquot, 1.7ml phosphate

buffer (0.1M, pH 7.4), and 0.2ml DTNB (4mg/1ml of phosphate buffer, 0.1M, pH 7.4) in a total volume of 2.0ml. The yellow color developed and was read immediately at 412nm. The results are expressed as n mol GSH formed/g tissue.

iv. **Determination of glutathione reductase activity** (Carlberg and Mannervik 1975)

The reaction mixture containing 1 ml of phosphate buffer, 0.5 ml of EDTA, 0.5 ml of oxidized glutathione and 0.2 ml of NADPH was made up to 3 ml with water. After the addition of 0.1 ml of suitably diluted tissue, the change in optical density at 340 nm was monitored for 2 minutes at 30 sec intervals. The activity of GRD is expressed as n moles of NADPH oxidized / minute / mg protein.

v. Determination of glutathione peroxidase activity (Mohandas et al., 1984).

GPx activity was measured according to the procedure of Mohandas et al. (1984). The reaction mixture consisted of 0.05M phosphate buffer (pH 7.0), 1.0mM EDTA, 1.0mM sodium azide, 1.4U of 0.1ml GR, 1.0mM GSH, 0.2mM NADPH, 0.25mM H_2O_2, and 0.1ml of PMS in a final volume of 2.0ml. The disappearance of NADPH at 340nm was recorded at room temperature. The enzyme activity was calculated as n mol NADPH oxidized/min/mg protein.

vi. Determination of superoxide dismutase activity (Beauchamp and Fridovich 1971)

To 50 µl of the suspension, 75 mM of Tris-HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of pyrogallol were added. An increase in absorbance was recorded at 420 nm for 3 minutes by spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance per minutes at 420 nm. The activity of SOD is expressed as units/mg protein.

vii. Determination of catalase activity (Coliborne 1985).

Catalase activity (CAT) was assayed by the method of Briefly, the assay mixture consisted of 0.05M phosphate buffer (pH 7.0), 0.019M H_2O_2, and 0.05ml PMS in a total volume of 3.0ml. Changes in absorbance were recorded at 240nm. Catalase activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein.

5.14 NEUROTRANSMITTER ESTIMATION

Estimation of Brain Neurotransmitters

i. **Acetyl Cholinesterase in Rats brain (***Ellman et al., 1961)*

Acetylcholine esterase activity was assayed by modified method of Brains from the experimental and control rats were washed with cold sodium phosphate buffer (0.2M, pH 8), homogenized in buffer and centrifuged at 10,000 RPM at 4ºC. The supernatant was used as the enzyme source (AChE). 0.5 ml of the cloudy supernatant liquid was pipetted out in to 25ml volumetric flask and dilution was made with a freshly prepared DTNB solution (10 mg dithiobios 2 nitro benzoic acid in 100 ml of Sorenson phosphare buffer, pH 8). From the volumetric flask two 4 ml portions were pipetted out in to two test tubes. In to one of the tubes 2 drops of eserine solution was added. 1 ml of substrate solution was added to the test tube containing eserine solution and was used for zero setting the colorimeter. The resulting yellow colour is due to the reduction of DTNB by certain substances in the brain homogenate and due to non enzymatic hydrolysis of the substrate. After having calibrated the instrument, change in absorbance per min of the sample was read at 430 nm

ii. Monoamine Oxidase (McEwen, 1977).

The MAO was estimated by the method of Charles and McEwen, (1977). 250 µl of the homogenate was added to 250 µl of serotonin and 250 µl of buffer. The reaction tube was placed at 37°C for 20 minutes and the reaction was arrested by the addition of 200 µl of 1M HCl. The reaction product was extracted with 5 ml of cyclohexane. The organic phase was measured at 242 nm using a spectrophotometer. Blank samples were prepared by adding 1M HCl (200 µl) prior to reaction and the reaction was carried out. The MAO A and B activity was expressed in nmoles/ mg protein.

iii. Dopamine (Yamamoto M *et al.,* 2000)

Weighed a specific quantity of tissue and was homogenized in 3 ml HCl butanol in a cool environment. The sample was then centrifuged for 10 minutes at 2000 rpm. 0.8 ml of supernatant phase was removed and added to an eppendorf reagent tube containing 2 ml of heptane and 0.25 ml 0.1 M HCl. After 10 minutes, shake the tube and centrifuged under same conditions to separate two phases. Upper organic phase was discarded and the aqueous phase was used for dopamine assay. To 0.02ml of the HCl phase, 0.005 ml of

0.4 M HCl and 0.01ml EDTA/ Sodium acetate buffer (pH 6.9) were added, followed by 0.01 ml iodine solution for oxidation. The reaction was stopped after 2 min by the addition of 0.1ml sodium thiosulphate in 5 M sodium hydroxide. 10 M acetic acid was added 1.5 min later. The solution was then heated to 100^{o}C for 6 min. When the sample again reaches room temperature, excitation and emission spectra were read (330 to 375 nm) in a spectrofluorimeter. Compared the tissue values (fluorescence of tissue extract minus fluorescence of tissue blank) with an internal reagent standard (fluorescence of internal reagent standard minus fluorescence of internal reagent blank). Tissue blanks for the assay were prepared by adding the reagents of the oxidation step in reversed order (sodium thiosulphate before iodine). Internal reagent standards were obtained by adding 0.005 ml distilled water and 0.1ml HCl butanol to 20 ng of dopamine standard.

iv. Glutamate (Nayebi *et al.*,2010)

To 1.0 ml of the supernatant from brain homogenate was evaporated to dryness at 70°C in an oven and the residue is reconstituted in 100 ml of distilled water. Standard solutions of glutamate at a concentration of 2mM along with the sample are spotted on Whatman No. 1 chromatography paper using a micropipette. It was placed on a chamber containing butanol: acetic acid: water (12: 3: 5 v/v) as solvent. When the solvent front reached the top of the paper, it was removed and dried. A second run is performed similarly, after which the papers are dried sprayed with ninhydrin reagent and placed in an oven at 100°C for 4 minutes. The portions which carry glutamate corresponding with the standard are cut and eluted with 0.005% $CuSo_4$ in 75% ethanol. Their absorbance is read against blank at 515 nm in spectrophotometer.

5.15 HISTOPATHOLOGICAL SECTIONING STAINING

The rats from each group were anesthesized by intra peritoneal injection of Ketamine and Xylazine. The brain was carefully removed without any injury after opening the skull. The collected brain was washed with ice cold normal saline and fixed in 10% formal saline (10 ml of formaldehyde in 90 ml of physiological saline). Paraffin embedded sections were taken 100 µm thickness and processed in alcohol – xylene series and stained with Haematoxyli- Eosin dye. The sections were examined microscopically for histopathological changes in the striatum.

5.16 PREPARATION OF PLANT EXTRACTS IN A FORMULATION

The plant hydroetnanolic extracts of roots of *Annona squamosa* Linn., and aerial parts of Echinops echinatus Roxb., were made as a formulation. Equal quantity of the extracts (1:1) were mixed with each other and made as a formulation. The extracts were suspended in sodium carboxy methyl cellulose and suspended to Group-VII which contains 6 wistar albino rats. After pre treatment with 6-OH Dopamine post treatment with the formulation was continued. All the animals were subjected to behavoiural, biochemical and neurotransmitter estimation. The treated groups were statistically compared with that of the 6-OH Dopamine treated group.

STATISTICAL ANALYSIS

The statistical analysis was carried by one way ANOVA followed by Dunnet's "t" test. p values < 0.05 (95% confidence limit) was considered statistically significant.

RESULTS AND ANALYSIS

VI. RESULTS AND ANALYSIS

6.1 The percentage yield of hydroethanolic extracts of roots of *Annona squamosa* Linn., was 12% and ariel parts of *Echinops echinatus* Roxb., was 16%.

6.2 Preliminary phytochemical tests

Table:- 4 shows the phytochemical analysis of Hydroethanolic extract of *Annona squamosa* Linn., are shown in revealed the presence of alkaloid, glycoside, sterols, phenols, tannins, flavanoids, terpenes, saponins, flavonoids, tannins, carbohydrates, proteins, phenolic compounds, and phytosterols. Hydroethanolic extract *Echinops echinatus* Roxb., showed the presence of phytoconstituents such as alkaloids, carbohydrates, flavonoids, tannins, steroids, sterols and saponins.

6.3 TLC and HPTLC

In Table:- 5 and 6 shows the separate TLC of hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., showed 17 and 15 spots respectively the R_f. values were also recorded. In HPTLC analysis resolution of spots in shows the presence of various active principles in the extracts.

6.4 Determination of Total phenolic, Total Flavonoid and Total Ascorbic acid content

Total phenolic content detected in hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., found to be 151.42 ± 0.52 and 119.63 ± 0.87 mg/g respectively.

Total flavonoids content of hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., was found to be 29.65 ± 0.83 and 18.43 ± 0.12 mg quercetin equivalents /g plant extract.

Total ascorbic acid content detected for hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., was found to be 0.22 ± 0.07 and 0.17 ± 0.03mg ascorbic acid /g plant extract.

Table 4. Preliminary Phytochemical Screening of Hydroethanolic extract of *Annona squamosa* and *Echinops echinatus*

S.No	Constituents		ASHE	EEHE
1	Alkaloids	Mayer's Test	+ve	+ve
		Dragendroff's Test	+ve	+ve
		Hager's Test	+ve	+ve
		Wagner's Test	+ve	+ve
2	Carbohydrates	Fehling's Test	-ve	+ve
		Benedict's Test	+ve	+ve
		Barfoed's Test	+ve	+ve
3	Proteins	Biuret's test	+ve	-ve
		Millon's Test	-ve	-ve
4	Steroids	Libermann Burchard Test	-ve	+ve
5	Sterols		-ve	+ve
6	Phenols		+ve	+ve
7	Tannins		+ve	+ve
8	Flavonoids		+ve	+ve
		Shinoda's test	+ve	+ve
9	Gums and Mucilage		-ve	-ve
10	Glycosides		+ve	-ve
11	Saponins	Foam test	+ve	+ve
12	Terpenes		-ve	-ve

-ve- indicate the absence of compound

+ve- indicate the presence of compound

Solvent system: - Toluene: Methanol: Ethyl acetate: Formic acid.

Figure 15. Thin Layer Chromatography of hydroethanolic extract of *Annona squamosa* Linn

Table 5. Thin Layer Chromatography R_f values of hydroethanolic extract *Annona squamosa* Linn

Extract	Solvent system	Number of spots		Rf value
Hydroethanolic extract of Annona squamosa	Toluene: Methanol: Ethyl acetate: Formic acid (2.5:1.0:5:1.5)	17	1	0.03
			2	0.08
			3	0.11
			4	0.13
			5	0.15
			6	0.42
			7	0.43
			8	0.47
			9	0.51
			10	0.52
			11	0.54
			12	0.55
			13	0.56
			14	0.74
			15	0.80
			16	0.83
			17	0.85

Solvent system: - Toluene: Methanol: Ethyl acetate: Formic acid (3:0.5:5:1.5)

Figure 16. Thin Layer Chromatography of hydroethanolic extract of *Echinops echinatus* Roxb

Table 6. Thin Layer Chromatography R_f values of hydroethanolic extract *Echinops echinatus*

Extract	Solvent system	Number of spots		Rf value
Hydroethanolic extract of Annona squamosa	Toluene: Methanol: Ethyl acetate: Formic acid (3:0.5:5:1.5)	15	1	0.01
			2	0.02
			3	0.10
			4	0.12
			5	0.18
			6	0.23
			7	0.26
			8	0.36
			9	0.44
			10	0.52
			11	0.60
			12	0.62
			13	0.71
			14	0.77
			15	0.83

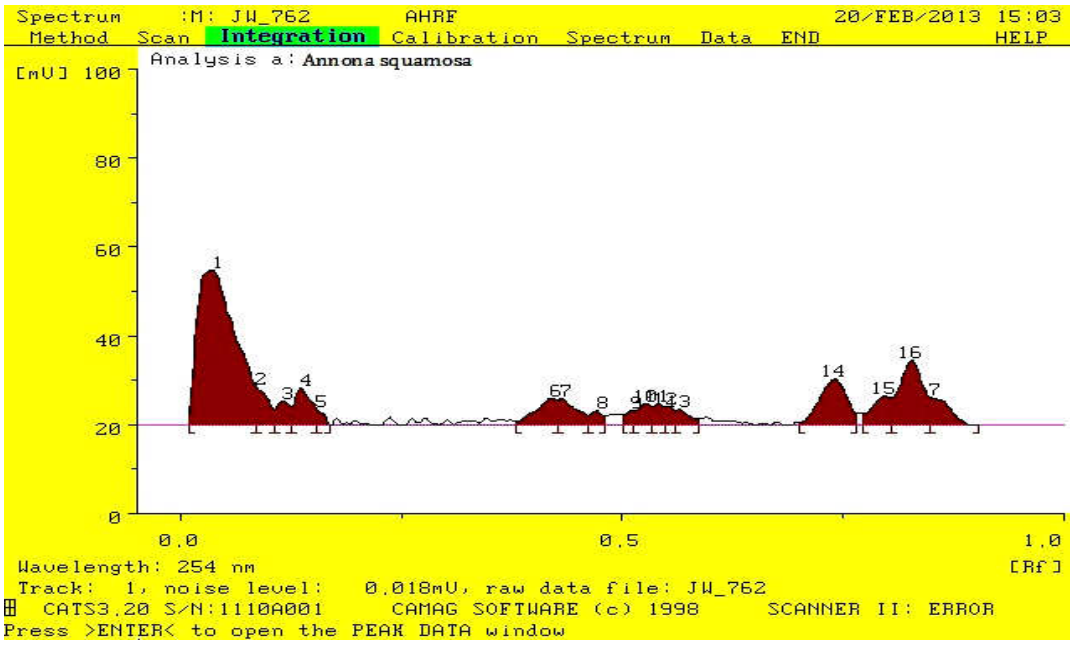


Figure 17. HPTLC of hydroethanolic extract of *Annona squamosa* Linn

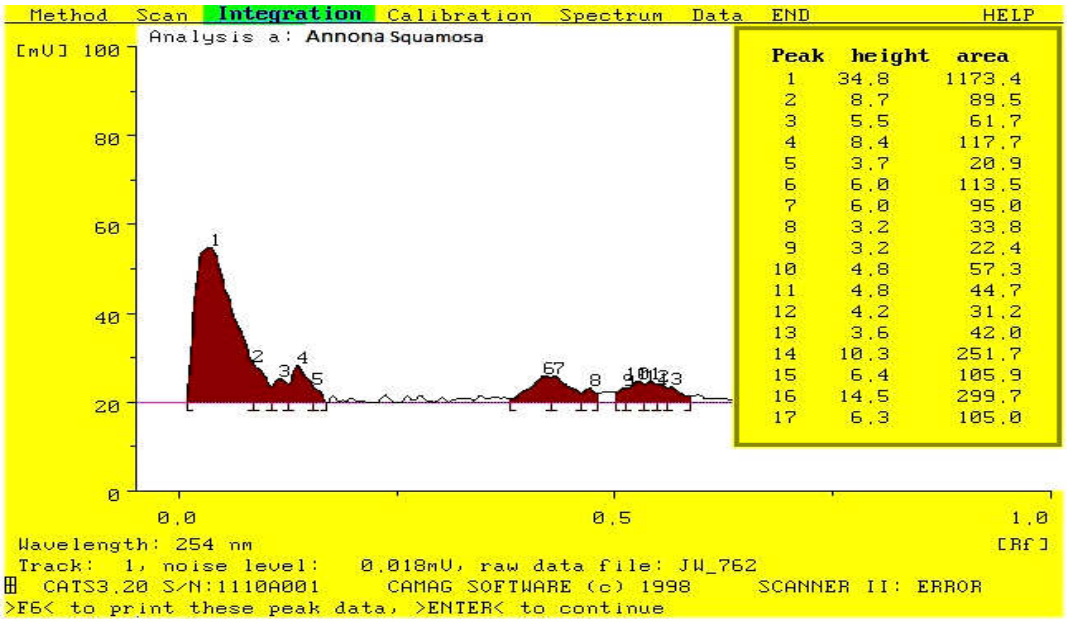


Figure 18. HPTLC of hydroethanolic extract of *Annona squamosa* Linn

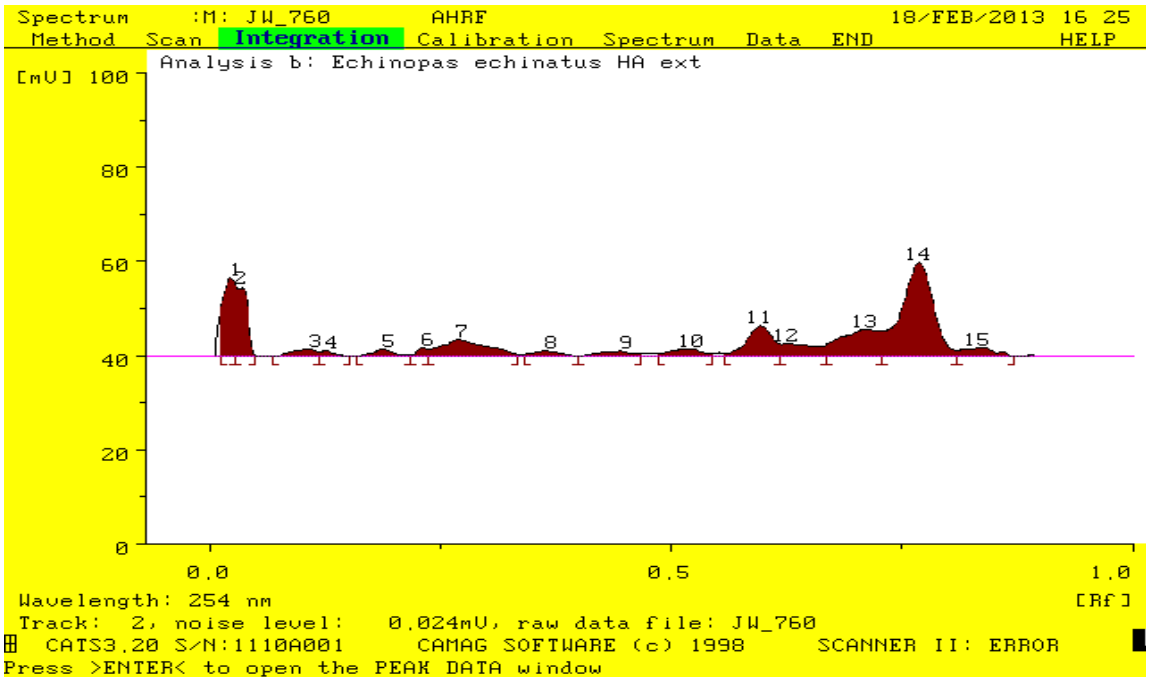


Figure 19. HPTLC of hydroethanolic extract of *Echinops echinatus* Roxb

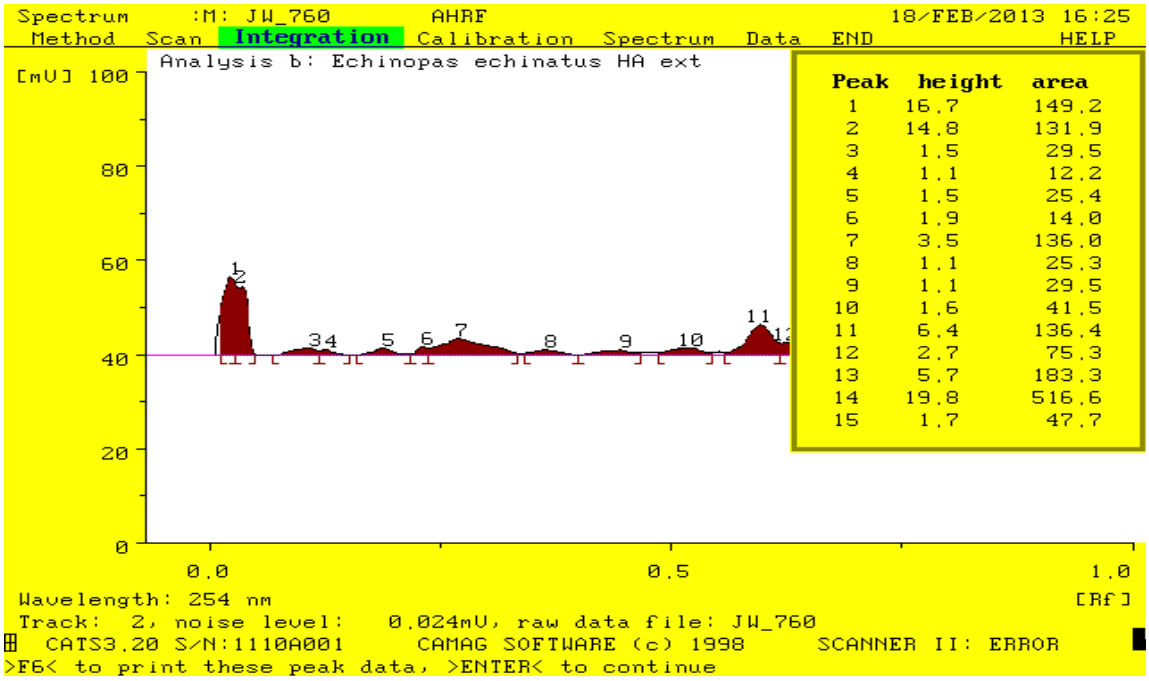


Figure 20. *HPTLC* of hydroethanolic extract of *Echinops echinatus* Roxb.

6.5 In-vitro antioxidant studies of the extracts

Hydroethanolic extract of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., exhibited a significant dose dependent inhibition of *DPPH* activity as shown in the Table: 7. The maximum inhibition was found at the dose level of 800µg/ml. The IC_{50} values are 350.57±3.002 and 380.50±2.6 respectively.

Nitric oxide scavenging activity of hydroethanolic extract of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., scavenging activity in a dose dependant manner. The IC_{50} values are 366.57 ±5.08 and 332.56± 2.16, when compared with standard vitamin C

Both the extracts were capable of scavenging hydroxyl radical and hydrogen peroxide in a dose dependent manner. The IC_{50} values are 376.17 ±7.37 and 419.57 ±1.85 when compared with standard mannitol

Reducing power of increased with increased concentration of test compound. The maximum absorption was found at the 500µg/ml. Oxidative stress can lead to peroxidation of cellular lipids and Lipid peroxidation(LPO) has been implicated in the pathogenesis of number of diseases including neurodegenerative disorders.

Table 7. Free radical scavenging activity of hydroethanolic extracts *Annona squamosa* Linn., and *Echinops echinatus* Roxb., by DPPH Reduction

Concentration (µg)	25	50	100	200	400	800	IC_{50} Value (µg)
Hydroethanolic extracts *Annona squamosa* Linn	8.08 ±0.32	20.11 ±0.43	30.25 ±0.28	42.98 ±0.193	52.62 ±0.326	65.16 ±0.273	350.57 ± 3.002
Hydroethanolic extracts *Echinops echinatus* Roxb.,	6.51 ± 0.092	18.91 ± 0.23	28.19 ± 0.67	40.59 ± 0.10	51.87 ± 0.31	63.4 ±0.21	380.50 ±2.62
Vitamin-C	11.15 ± 0.27	23.8 ±0.33	30.82 ±0.28	44.42 ±0.273	58.45 ±0.28	70.45 ±0.286	275.02 ±0.684

Values are mean ± SEM of 6 replicates

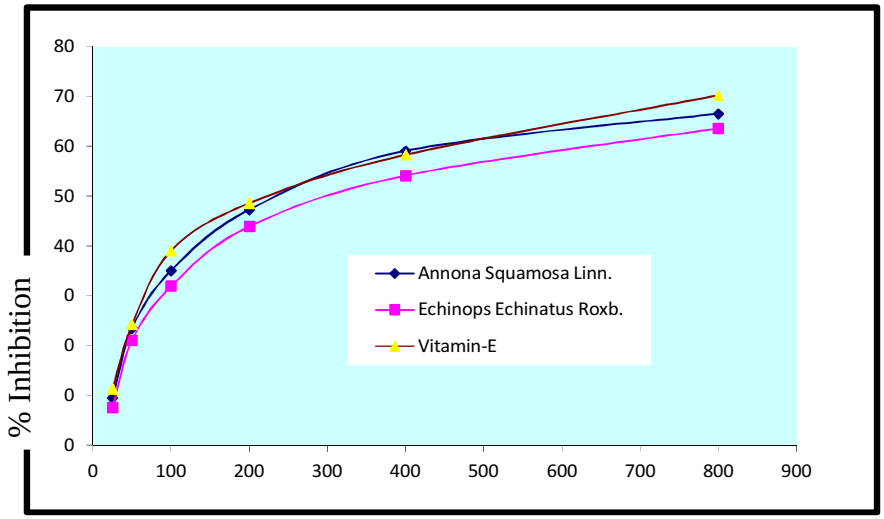


Figure 21. Free radical scavenging activity of hydroethanolic extracts *Annona squamosa* Linn., and *Echinops echinatus* Roxb., by DPPH Reduction

Table 8. Determination of nitric oxide scavenging activity of hydroethanolic extracts *Annona squamosa* Linn., and *Echinops echinatus* Roxb

Concentration (µg)	25	50	100	200	400	800	IC_{50} Value (µg)
Hydroethanolic extracts *Annona squamosa* Linn	9.54 ±0.41	23.53 ±0.41	35.08 ±0.21	47.31 ±.21	59.12 ±0.44	66.57 ± 0.43	366.57 ±5.08
Hydroethanolic extracts *Echinops echinatus* Roxb.,	7.62 ±0.24	21.1 ±0.29	31.98 ±0.10	43.96 ±0.23	54.18 ±0.26	63.64 ±0.10	332.56 ± 2.16
Standard Vitamin-C	11.38 ±0.36	24.28 ±0.44	39.11 ±0.53	48.65 ±0.3	58.37 ±0.50	70.26 ±0.302	262.52 ± 1.46

Values are mean ± SEM of 6 replicates

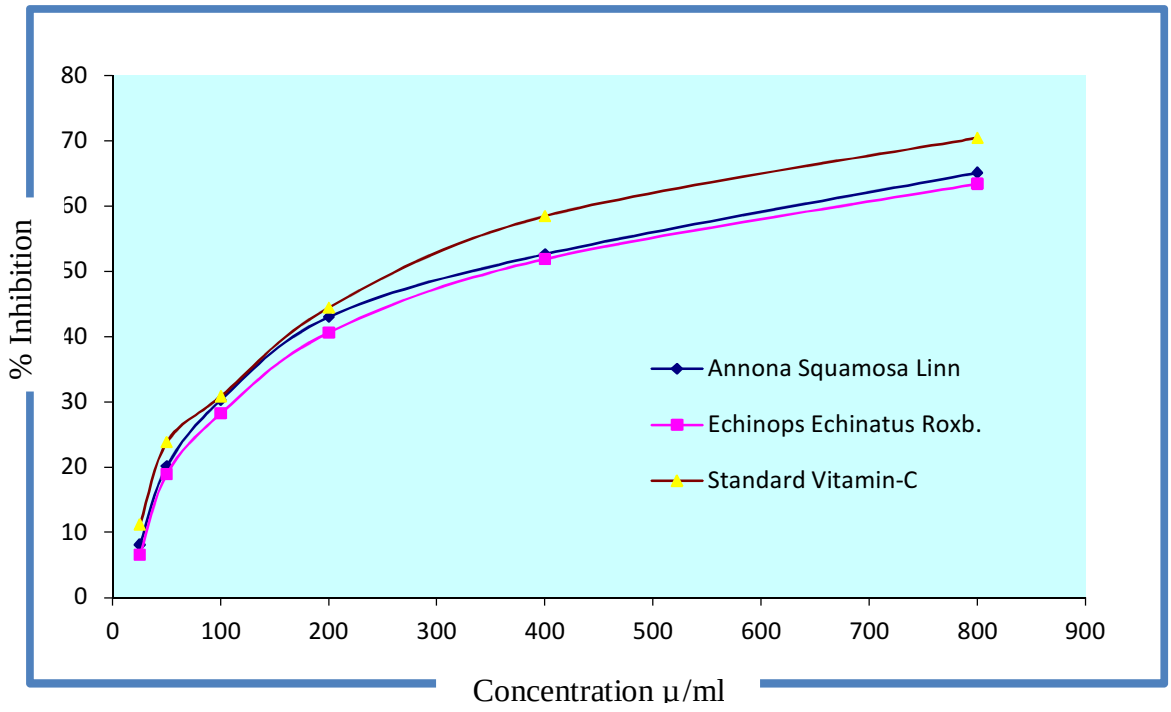


Figure 22. Determination of nitric oxide scavenging activity of hydroethanolic extracts *Annona squamosa* Linn., and *Echinops echinatus* Roxb.,

Table 9. Determination of Hydroxly Radical Scavening activity of hydroethanolic extracts *Annona squamosa* Linn., and *Echinops echinatus* Roxb.

Concentration (µg)	25	50	100	200	400	800	IC_{50} Value (µg)
Hydroethanolic extracts *Annona squamosa* Linn	8.76 ±0.59	17.35 ±0.455	29.89 ±0.89	41.17 ±0.42	51.32 ±0.27	65.63 ±0.61	376.17 ±7.37
Hydroethanolic extracts *Echinops echinatus* Roxb.,	6.35 ± 0.32	13.74 ±0.61	25.25 ±0.785	35.73 ±0.749	48.37 ±0.86	60.47 ±0.45	419.57 ±1.85
Mannitol	11.167 ± 0.95	25.25 ±0.76	34.22 ±0.74	47.13 ±0.74	58.07 ±0.95	70.78 ±1.12	218.01 ±3.08

Values are Mean ± SEM of 6 replicates.

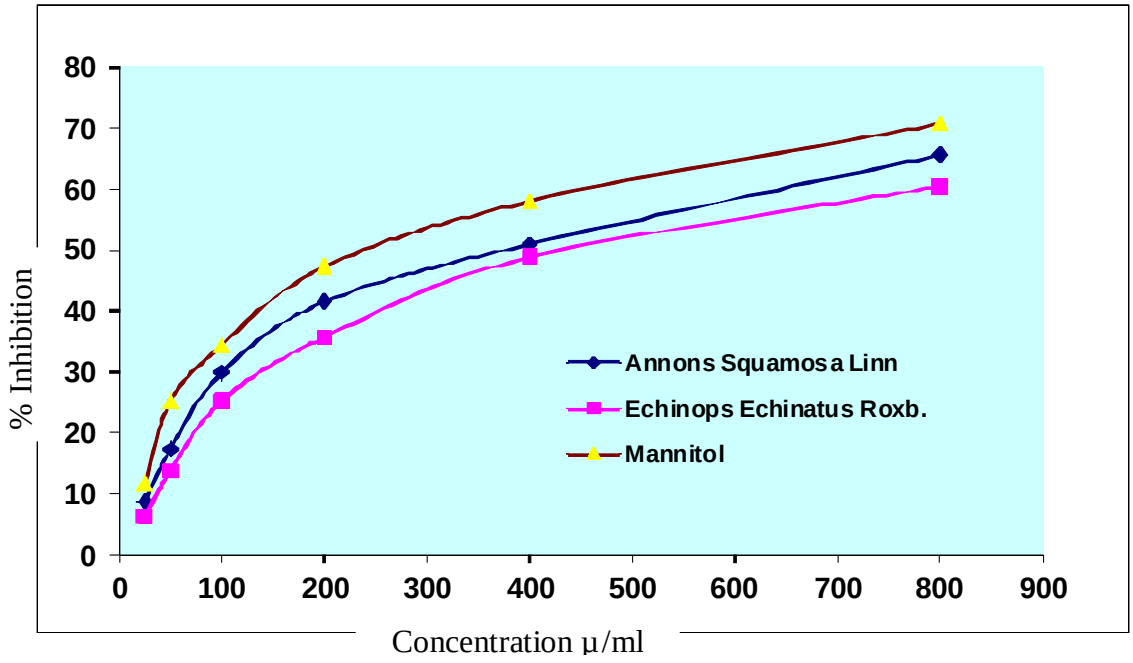


Figure 23. Determination of Hydroxly Radical Scavening activity of hydroethanolic extracts *Annona squamosa* Linn., and *Echinops echinatus* Roxb.

Table 10. Determination of reducing power of hydroethanolic extracts *Annona squamosa* Linn., and *Echinops echinatus* Roxb.

Concentration (µg/ml)	125	250	375	500
Hydroethanolic extracts *Annona squamosa* Linn	0.295 ±0.0037	0.520 ± 0.0022	0.672 ± 0.0037	0.934 ±0.003
Hydroethanolic extracts *Echinops echinatus* Roxb.,	0.284 ± 0.027	0.504 ± 0.029	0.646 ±0.039	0.923 ± 0.045
Standard BHT	0.323 ± 0.021	0.556 ± 0.027	0.721 ±0.024	1.061 ± 0.006

BHT : Butylated hydroxy toluene

Values are Mean ± SEM of 6 parallel measurements.

$p<0.01$ when compared with control

Spectrophotometric deduction of the $Fe^{3+} – Fe^{2+}$ transformation.

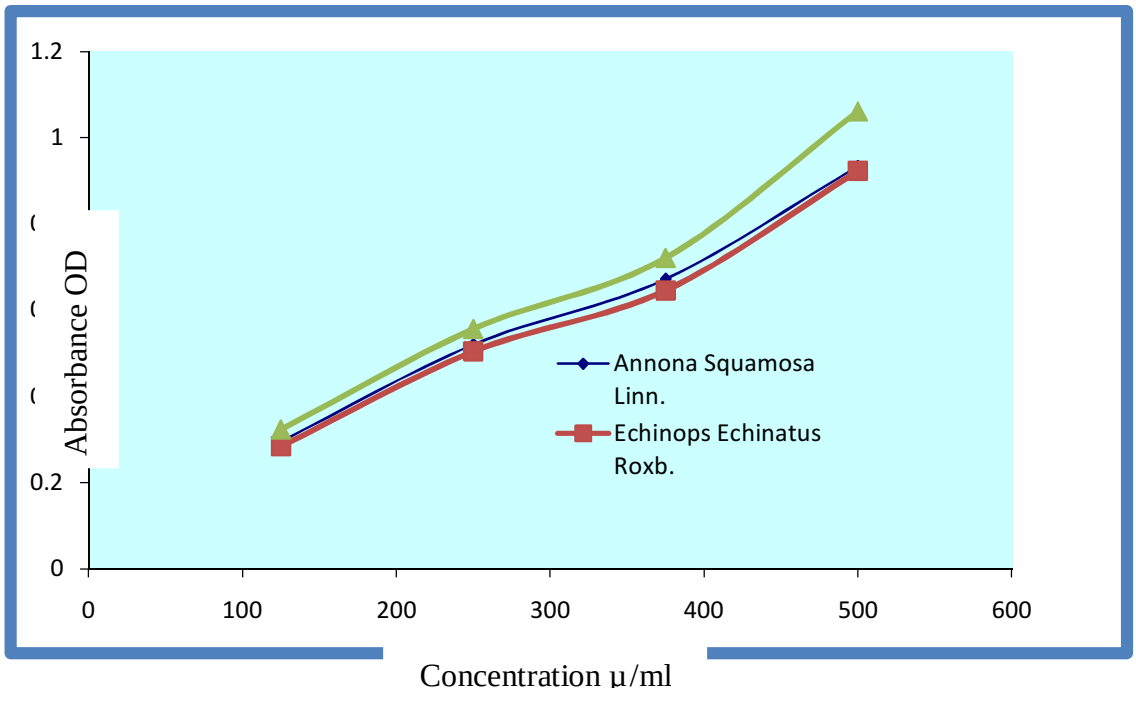


Figure 24. Determination of reducing power of hydroethanolic extracts *Annona squamosa* Linn., and *Echinops echinatus* Roxb.,

6.6 ACUTE TOXICITY STUDY

Acute toxicity study based on OECD guidelines 423 showed the non-toxic nature of hydroethanolic extract *Annona squamosa* Linn., and Hydroethanolic extract *Echinops echinatus* Roxb., *in* rats. Table 11 & 12 shows the observations made during the study.

6.7 SUB ACUTE TOXICITY

In the Sub acute toxicity studies hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., was administered at the dose level of 200mg/kg(p.o), for 28 days. No signs of toxicity or mortality were observed during the experimental period. Changes in body weight shown in Table:13 shows no significant change with the control group. Physical observation has not shown any change in behavioral abnormality at any point in the present study.

6.6.1 Relative organ weight

Table 14 shows the results of relative organ weight of rats treated with hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb. The observation have not shown any evidence of drug-related toxicity. There were no significant difference between controls and extract treated groups in organ weight.

6.6.2 Hematological parameters

Table: 15 shows the Hematological parameters such as red blood cell count, total white blood cell count, hemoglobin, hematocrit, platelet count and differential leukocyte count remained within the physiological range in both control and treated groups for hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., during the experimental period.

6.6.3 Biochemical parameters

Table 16 shows the biochemical parameters on Sub acute oral administration of hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb. repeated administration did not show any significant changes in biochemical parameters such as creatinine, urea, triglycerides, total cholesterol, total protein, albumin, AST, ALT, ALP and total bilirubin in serum when compared to control groups. There were no statistically significant difference in the hematological and serum biochemical parameters analyzed and are found to be within normal limits.

6.6.4 Histological examination

The histological examination of various organs was performed in control, hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., treated groups. The histology of treated organs were compared with the untreated control and are represented in fig.25 to 34. All the sampling tissue sections were within the normal limits and revealed normal architecture on comparison with control groups. No alterations were seen in the microscopic examination of internal organ and there were no degenerative or infiltrative lesion observed in the extract treated groups. Pathological examination of tissues indicated that there were no detectable abnormalities

ACUTE TOXICITY STUDY

Table 11. Acute toxicity dose 2000 mg/kg (p.o) hydroethanolic extracts *Annona squamosa* Linn., and *Echinops echinatus* Roxb.

Parameters observed	Ist hr		IInd hr		IIIrd hr		IVth hr	
	AS	EE	HS	EE	AS	EE	AS	EE
Piloerection	-	-	-	-	-	-	-	-
Edema	-	-	-	-	-	-	-	-
Urine stains	-	-	-	-	-	-	-	-
Alopecia	-	-	-	-	-	-	-	-
Loss of writing reflex	-	-	-	-	-	-	-	-
Circling	-	-	-	-	-	-	-	-
Nasal sniffing	+	+	+	+	+	+	+	+
Lacrimation	-	-	-	-	-	-	-	-
Seizures	-	-	-	-	-	-	-	-
Righting reflex	+	+	+	+	+	+	+	+
Grip strength	+	+	+	+	+	+	+	+
Eye closure at touch	+	+	+	+	+	+	+	+
Rearing	+	+	+	+	+	+	+	+
Straub tail	-	-	-	-	+	-	-	-

Table 12. The 14 days observation after acute oral administration of hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., at the dose of 2000mg/kg(p.o)

Parameters observed	Day 1		Day-2		Day-3		Day-4		Day-5		Day-6		Day-7		Day-8		Day-9		Day-10		Day-11		Day-12		Day-13		Day-14	
	AS	EE	AS	EE	AS	EE	AS	EE	AS	EE	AS	EE	AS	EE	AS	EE	AS	EE	AS	EE	AS	EE	AS	EE	AS	EE	AS	EE
Piloerection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Edema	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urine stains	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alopecia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Loss of writing reflex	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Circling	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nasal sniffing	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lacrimation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Seizures	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Righting reflex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Grip strength	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Eye closure at touch	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rearing	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Straub tail	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 13. Change in body weights of rats in control, hydroethanolic extract of *Annona squamosa Linn., and Echinops echinatus Roxb.,* sub acute toxicity studies

Group	Initial	5day	10 day	15 day	20 day	25 day	28 day
Control	158 ±5.71	160 ±5.23	161 ±3.67	162 ±4.82	163 ±3.56	164 ±3.59	165 ±3.24
Annona squamosa Linn. 200mg/kg	150 ±3.61	146 ±6.52	148 ±3.52	149 ±2.9	151 ±3.67	153 ±3.61	154 ±6.81 ^{ns}
Echinops echinatus Roxb. 200mg/kg	156 ±5.71	154 ±5.23	155 ±3.67	156 ±3.98	157 ±8.89	159 ±6.72	161 ±2.81 ^{ns}

Data are expressed as mean ± SEM (n=10)
ns: represents non significance

Table 14. Relative organ weights (g/100g) of control, hydroethanolic extracts of Annona squamosa Linn., and Echinops echinatus Roxb., on sub acute toxicity studies

Organs	Control		AS (200 mg/kg)		EE (200 mg/kg)	
	Female	Male	Female	Male	Female	Male
Liver	2.66 ±0.02	2.69 ±0.06	2.65 ±0.15 ^{ns}	2.63 ±0.03 ^{ns}	2.65 ±0.15 ^{ns}	2.63 ±0.03 ^{ns}
Kidney	0.62 ±0.03	0.60 ±0.12	0.63 ±0.02 ^{ns}	0.61 ±0.12 ^{ns}	0.63 ±0.02 ^{ns}	0.61 ±0.12 ^{ns}
Brain	0.53 ±0.10	0.49 ±0.14	0.52 ±0.10 ^{ns}	0.55 ±0.16 ^{ns}	0.52 ±0.10 ^{ns}	0.55 ±0.16 ^{ns}
Lungs	0.45 ±0.03	0.44 ±0.01	0.45 ±0.05 ^{ns}	0.49 ±0.02 ^{ns}	0.45 ±0.05 ^{ns}	0.49 ±0.02 ^{ns}
Heart	0.35 ±0.02	0.37 ±0.01	0.38 ±0.02 ^{ns}	0.32 ±0.01 ^{ns}	0.38 ±0.02 ^{ns}	0.32 ±0.01 ^{ns}
Spleen	0.22± 0.09	0.22 ±0.07	0.17 ±0.06 ^{ns}	0.23 ±0.05 ^{ns}	0.17 ±0.06 ^{ns}	0.23 ±0.05 ^{ns}

Data are expressed as mean ± SEM (n=10)
ns : represents non significance

Table 15. Hematological parameters of rats in control, hydroethanolic extract of *Annona squamosa (AS) and Echinops echinatus (EE)* sub acute toxicity studies

Hematological parameter	Control (Vehicle)		AS (200 mg/kg)		EE (200 mg/kg)	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**
Total R.B.C. count ($\times 10^6$/ mm^3)	09.09 ± 1.46	08.13 ± 1.66	8.89 ± 2.05 *ns*	8.43 ±1.78 *ns*	09.09 ± 1.46 *ns*	08.13 ± 1.66 *ns*
Total W.B.C. Count ($\times 10^3/mm^3$)	13.68 ± 1.97	09.58 ± 1.45	11.29 ± 1.88 *ns*	12.63 ±1.26 *ns*	13.68 ± 1.97 *ns*	09.58 ± 1.45 *ns*
Haemoglobin (Hb) (g/dl)	15.82 ± 1.94	13.79 ± 1.27	17.62 ± 0.72 *ns*	16.12 ±1.33 *ns*	15.82 ± 1.94 *ns*	13.79 ± 1.27 *ns*
Hematocrit (%)	42.54 ± 1.36	44.95 ±1.49	40.12 ±3.06 *ns*	41.27 ±2.47 *ns*	42.54 ± 1.36 *ns*	44.95 ±1.49 *ns*
Platelets ($\times 10^3/mm^3$)	652.34 ±12.34	961.75 ±16.64	843.35 ±15.67 *ns*	893.74 ±15.35 *ns*	652.34 ±12.34 *ns*	961.75 ±16.64 *ns*
Neutrophils (%)	17.79 ±2.03	20.94 ±3.11	10.06 ±2.75 *ns*	14.12 ±2.72 *ns*	17.79 ±2.03 *ns*	20.94 ±3.11 *ns*
Lymphocytes (%)	82.43 ±3.43	77.56 ±2.45	79 ±4.55 *ns*	82.04 ±3.52 *ns*	82.43 ±3.43 *ns*	77.56 ±2.45 *ns*
Eosinophil (%)	2.38 ±0.43	1.82 ±0.75	1.64 ±0.25 *ns*	1.42 ±0,64 *ns*	2.38 ±0.43 *ns*	1.82 ±0.75 *ns*
Monocyte (%)	3.10 ±0.13	0.00 ±0.00	1.50 ±0.03 *ns*	0.00 ±0.00 *ns*	3.10 ±0.13 *ns*	0.00 ±0.00 *ns*
Basophil (%)	0.00 ±0.00	1.01 ±0.01	0.00 ±0.00 *ns*	0.00 ±0.00 *ns*	0.00 ±0.00 *ns*	1.01 ±0.01 *ns*

Data are expressed as mean ± SEM (n=10)
ns : represents non significance

Table 16. Biochemical parameters of rats in control, hydroethanolic extract of *Annona squamosa (AS) and Echinops echinatus (EE)* sub acute toxicity studies

Biochemical parameter	Control		AS (200 mg/kg)		EE (200 mg/kg)	
	Female	**Male**	**Female**	**Male**	**Female**	**Male**
Creatinine (mg/dl)	0.64 ±0.01	0.61 ±0.05	0.64 ±0.01*ns*	0.61 ±0.05 *ns*	0.57 ± 0.07 *ns*	0.43 ±0.03
Urea (mg/dl)	15.17 ±1.45	18.12 ±2.53	15.17 ±1.45*ns*	18.12 ±2.53 *ns*	17.14 ± 1.52 *ns*	20.75 ±1.31 *ns*
Triglycerides (mg/dl)	54.21 ±9.03	49.24 ±5.32	54.21 ±9.03 *ns*	49.24 ±5.32 *ns*	52.83 ± 4.92 *ns*	47.25 ±7.34 *ns*
Total Cholesterol(mg/dl)	58.25 ±3.95	52.17 ±3.76	58.25 ±3.95 *ns*	52.17 ±3.76 *ns*	49.33 ± 2.03 *ns*	43.00 ±2.46 *ns*
Total protein (g/dl)	3.57 ±0.14	4.91 ±0.94	3.57 ±0.14 ns	4.91 ±0.94 *ns*	7.23 ± 0.24 *ns*	5.11 ±0.23 *ns*
Albumin (g/dl)	4.27 ±0.19	3.25 ±0.12	4.27 ±0.19 *ns*	3.25 ±0.12 *ns*	3.64 ± 0.05 *ns*	4.29 ±0.03 *ns*
AST (IU/L)	119.6 ±28.8	112.71 ±23.84	119.6 ±28.8 *ns*	112.71 ±23.84	124.01 ± 17.6 *ns*	138.54 ±19.4 *ns*
ALT (IU/L)	63.47 ±5.61	72.45 ±4.02	63.47 ±5.61 *ns*	72.45 ±4.02 *ns*	61.47 ± 3.19 *ns*	71.33 ±6.19 *ns*
ALP (IU/L)	97.54 ±12.76	104.13 ±13.52	97.54 ±12.76*ns*	104.13 ±13.52 *ns*	114.3 ± 12.0 *ns*	108.4 ±15.32 *ns*
T. Bilirubin (mg/dl)	0.31 ±0.09	0.29 ±0.03	0.31 ±0.09 *ns*	0.29 ±0.03 *ns*	0.23 ± 0.05 *ns*	0.27 ±0.12 *ns*

Data are expressed as mean ± SEM (n=10)
ns: represents non significance

HISTOPATHOLOGY OF TOXICITY STUDIES

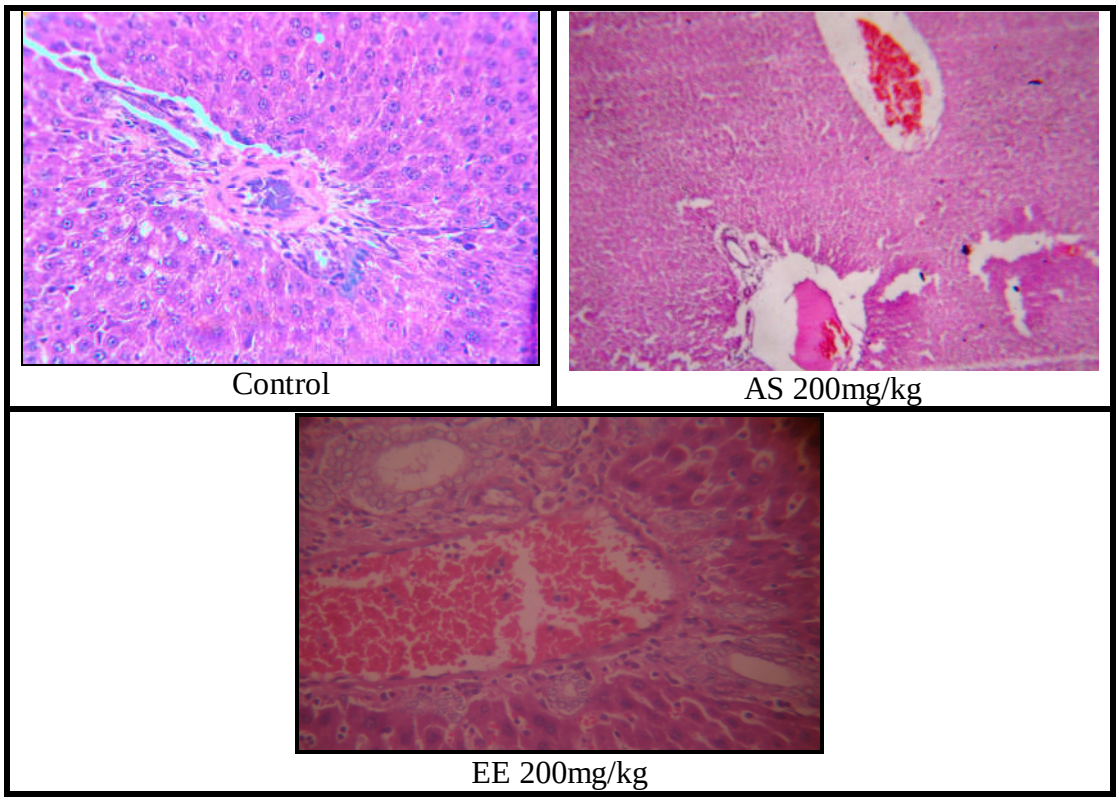


Figure 25. Histopathology of rat liver on treatment with hydroethanolic extract of *Annona squamosa* Linn. (200mg/kg) and *Echinops echinatus* Roxb. (200mg/kg)

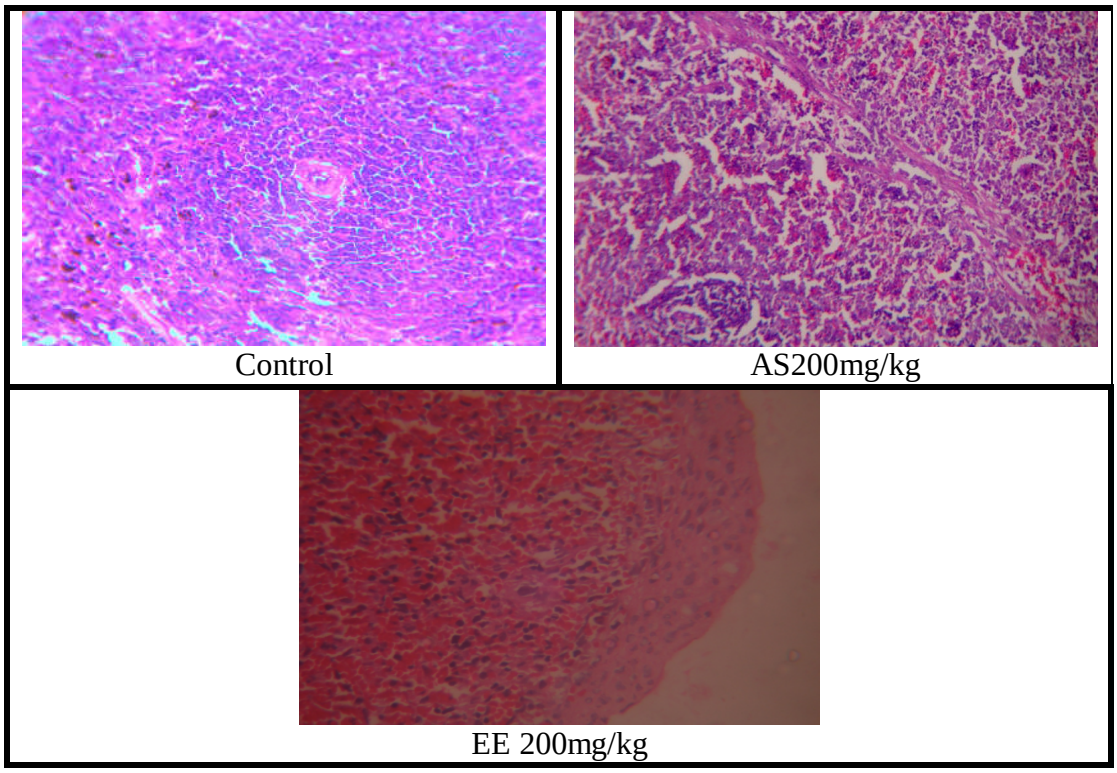


Figure 26. Histopathology of rat spleen on treatment with hydroethanolic extract of *Annona squamosa* Linn. (200mg/kg) and *Echinops echinatus* Roxb. (200mg/kg)

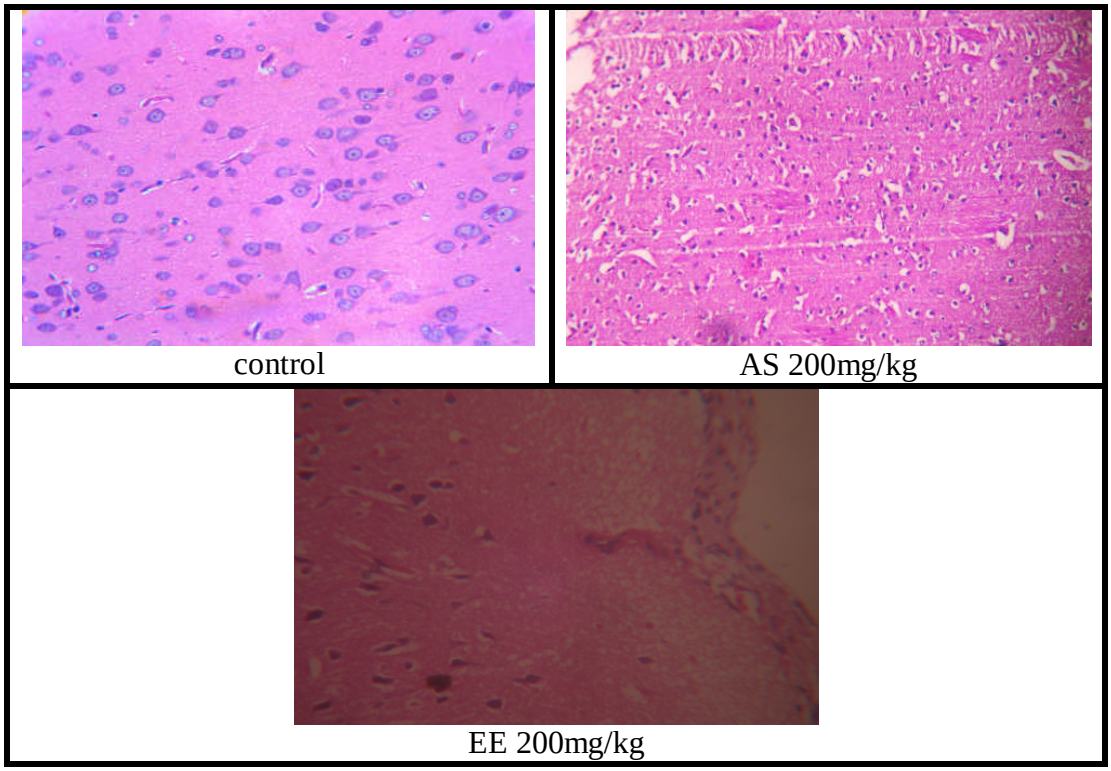


Figure 27. Histopathology of rat brain on treatment with hydroethanolic extract of *Annona squamosa* Linn. (200mg/kg) and *Echinops echinatus* Roxb. (200mg/kg)

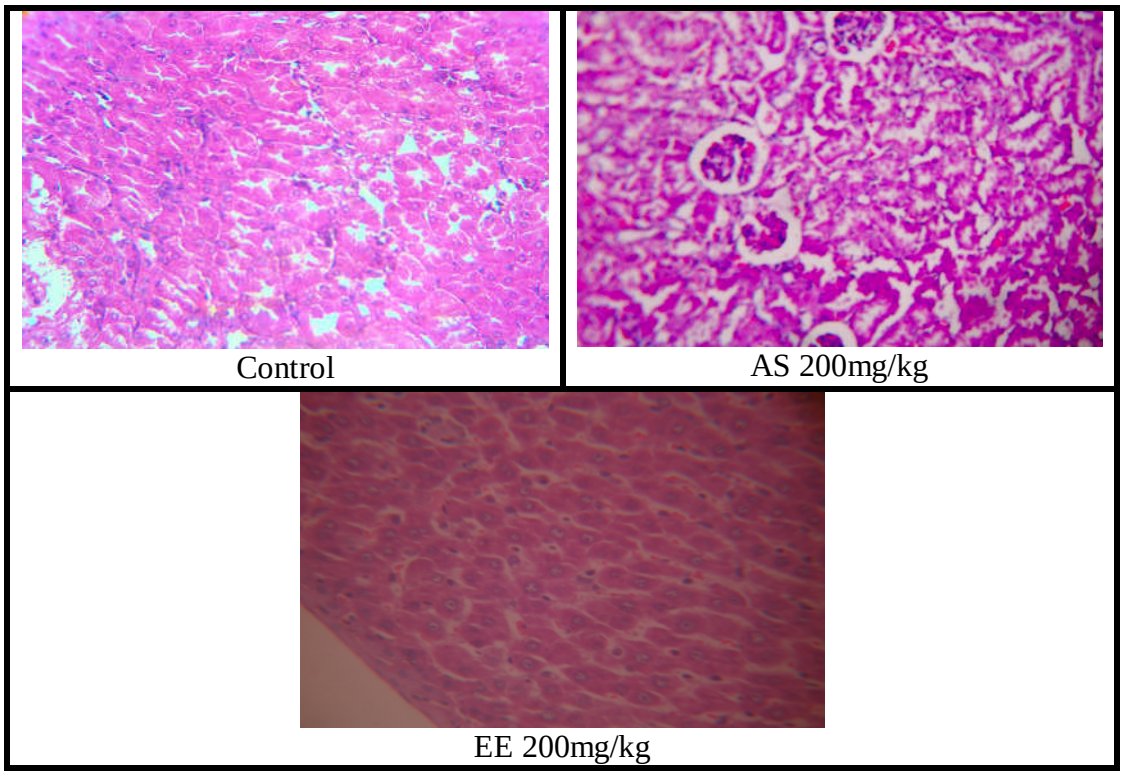


Figure 28. Histopathology of rat kidney on treatment with hydroethanolic extract of *Annona squamosa* Linn. (200mg/kg) and *Echinops echinatus* Roxb. (200mg/kg)

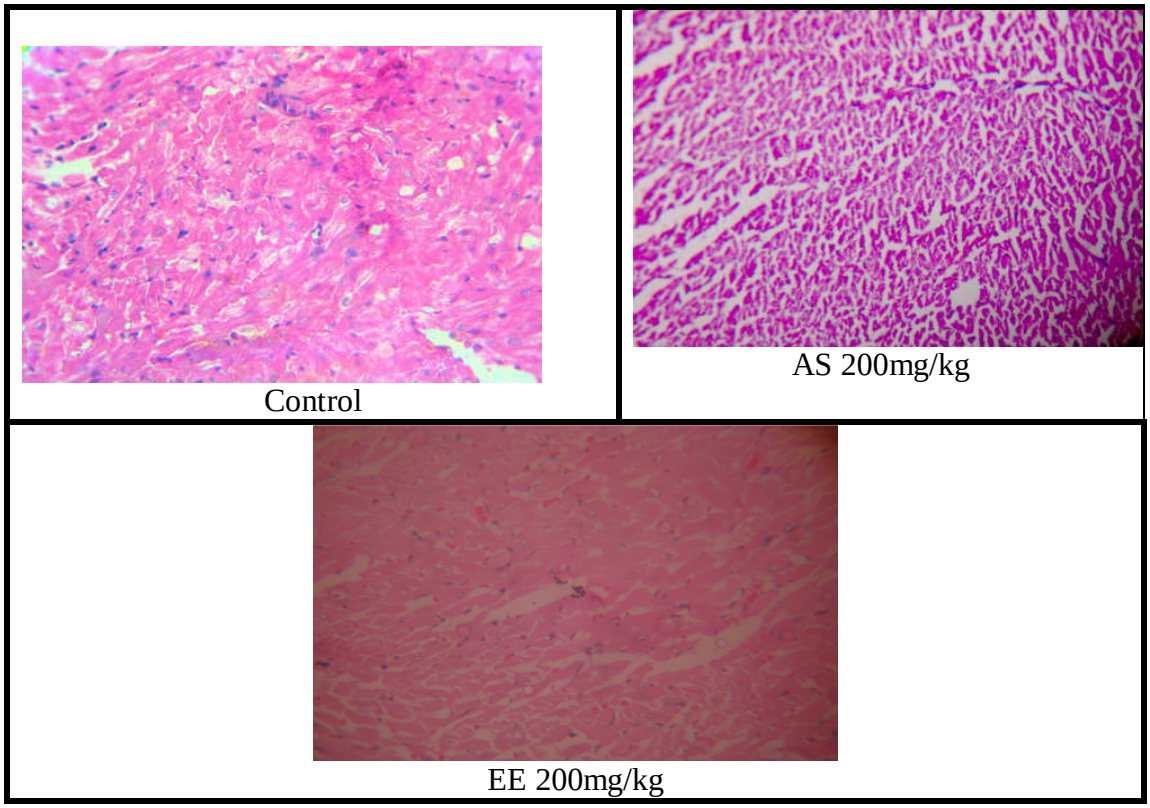


Fig.29 Histopathology of rat heart on treatment with hydroethanolic extract of *Annona squamosa* Linn. (200mg/kg) and *Echinops echinatus* Roxb. (200mg/kg)

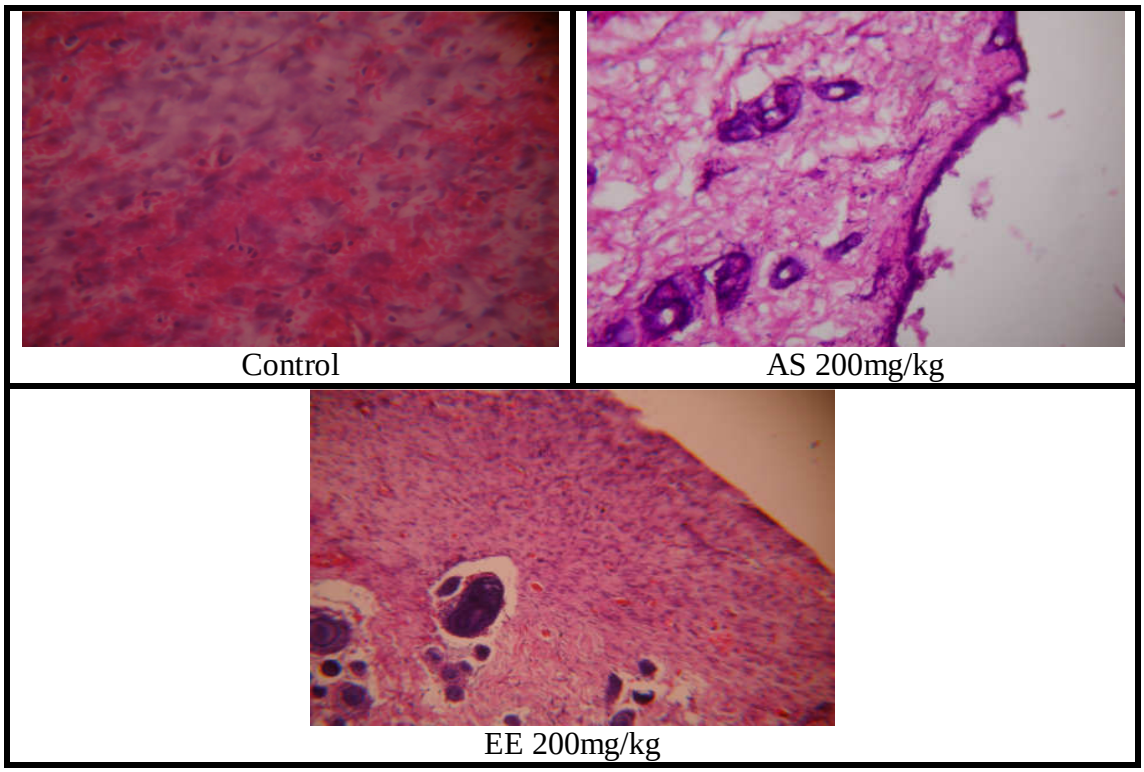


Figure 30. Histopathology of rat skin on treatment with hydroethanolic extract of *Annona squamosa* Linn. (200mg/kg) and *Echinops echinatus* Roxb. (200mg/kg)

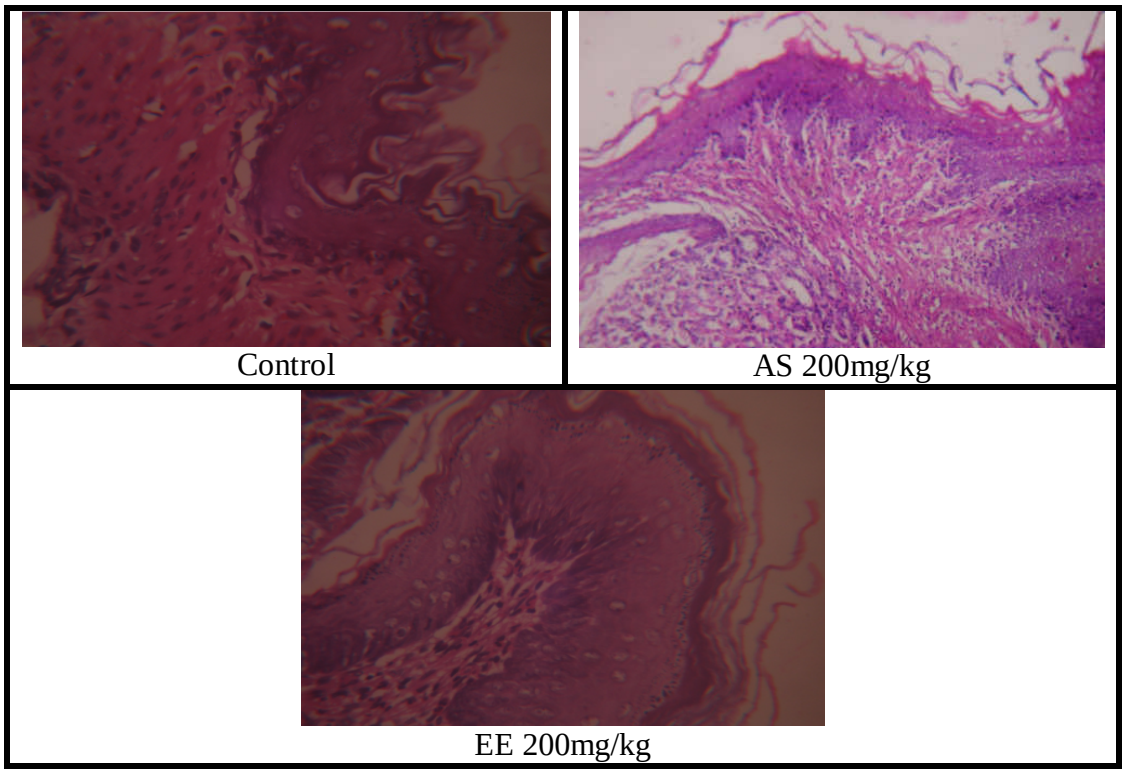


Figure 32. Histopathology of rat stomach on treatment with hydroethanolic extract of *Annona squamosa* Linn. (200mg/kg) and *Echinops echinatus* Roxb. (200mg/kg)

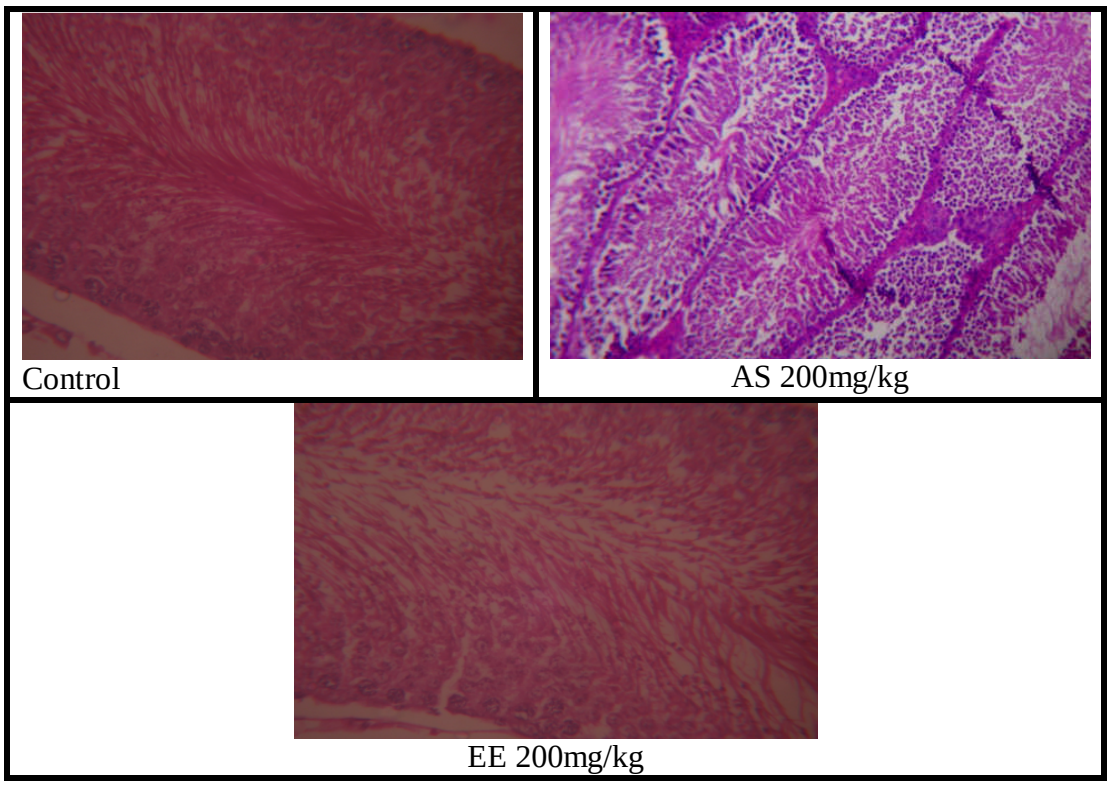


Fig 32. Histopathology of rat testis on treatment with hydroethanolic extract of *Annona squamosa* Linn. (200mg/kg) and *Echinops echinatus* Roxb. (200mg/kg)

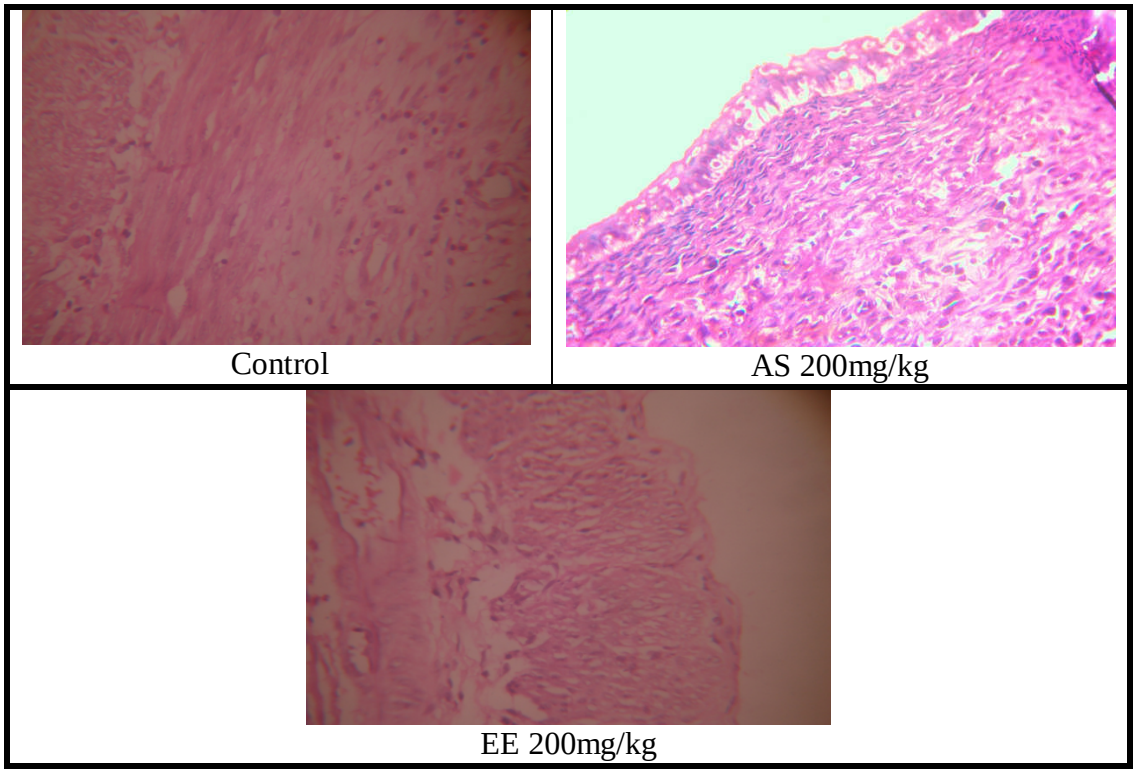


Figure 33. Histopathology of rat ovary on treatment with hydroethanolic extract of Annona squamosa Linn. (200mg/kg) and Echinops echinatus Roxb. (200mg/kg)

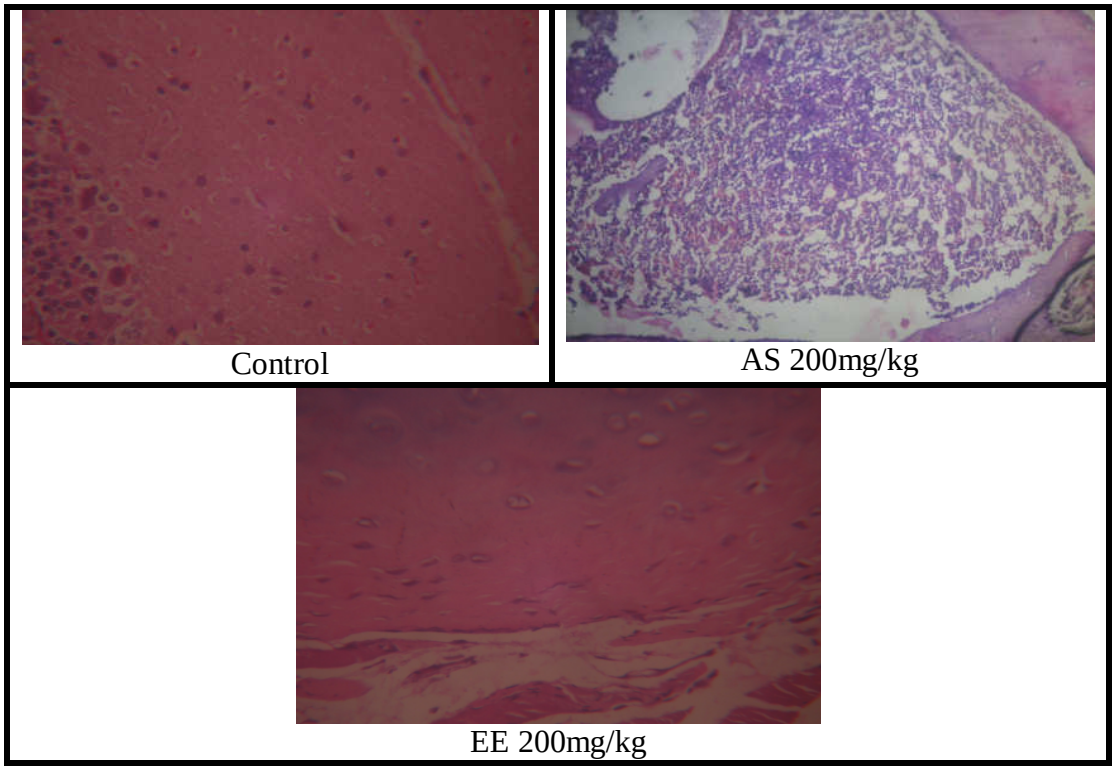


Figure 34. Histopathology of rat bone on treatment with hydroethanolic extract of *Annona squamosa* Linn. (200mg/kg) and *Echinops echinatus* Roxb. (200mg/kg)

6.8 NEUROPHARMACOLOGICAL SCREENING

A. General behavior studies

General behavioral profile studies of rats treated with AS and EE are tabulated in Table 17 did not show any difference in their general behavior. Spontaneous activity, awareness and alertness, Sound responses, Touch responses, Pain response, Latency to groom, Sensory tests normal grooming, motor activity and grip strength were normal. The animals showed no signs of depression during the observation period. General behavior studies suggest that both hydroethanolic extracts does not possess any neurotoxicity.

B. Preliminary neuropharmacological evaluations

i. Catalepsy

Table 18 and fig.35 shows the catalepsy evaluation. The control group catalepsy was not at all observed. When the 6-OHDA injected group compared with control group, it showed a significant ($p<0.001$) rise in cataleptic rigor. Group treated with 100 & 200 mg/kg dose of hydroethanolic extract of *Echinops echinatus* Roxb., showed significant ($p<0.01$) decrease in cataleptic rigor, and treatment with 100mg/kg of Annona squamosa showed significant ($p<0.01$) fall in cataleptic rigor at the dose of 200mg/kg the cataleptic rigor has reduced significantly ($p<0.001$) when compared with 6-OHDA treated group. The formulation has produced significant improvement in the motor coordination ($p<0.001$).

ii. Open field spontaneous activity (Locomotor)

The open field test is a paradigm used for evaluating the effect of drugs on gross general behavior and is used to measure the level of nervous excitability. Table: 19 and Figure 36 shows the results The significant ($p<0.001$) decrease in the number of square crossed, grooming and rearing (Locomotor) in the 6-OHDA injected animals were observed. With the treatment of 100 & 200mg/kg dose of hydroethanolic extract of *Echinops echinatus* showed the significant ($p<0.01$) increase in the no. of sq crossed, at the same dose animals showed the significant ($p<0.001$) increase in the grooming, but little less significant ($p<0.05$) increase in rearing. With the treatment of 100 & 200mg/kg dose of hydroethanolic extract of *Annona squamosa* Linn., animals showed the significant ($p<0.01$) increase in all the three parameters like

number of square crossed, grooming and rearing. The extracts in the formulation of has produced significant improvement in the number of square crossed ($p<0.001$). The observed increase in the square crossing could be with increased motor activity.

iii.Gait

Table: 20 shows the result of Gait analysis. The stride length and fore paw distance width in 6-OHDA treated animals was significantly ($p<0.001$) decreased when compared with the control group while hind paw distance width in 6-OHDA treated animals was significantly ($p<0.001$) increased when compared with the control group. With the treatment of 100mg/kg dose of hydroethanolic extract of *Echinops echinatus* Linn., animals showed the significant ($p<0.01$) increase in stride length and fore paw stance width and significant ($p<0.05$) decrease in hind paw stance width. With the treatment of 200mg/kg dose of hydroethanolic extract of *Annona squamosa*, animals showed the significant ($p<0.001$) decrease in increase in stride length and fore paw stance width and significant ($p<0.01$) decrease in hind paw stance width as compared with the 6-OHDA treated group.

iv.Force swim test (motor impairment)

Table: 21 shows the effect of *squamosa* Linn., and *Echinops echinatus* Roxb., on forced swim test. When 6-OHDA injected group compared with control group, it showed a significant ($p<0.001$) fall in swim time. Group treated *Annona squamosa* Linn., and *Echinops echinatus* Roxb.,200mg/kg dose of showed significant ($p<0.001$) improvement in swim time, and treatment with 200mg/kg of HAEAS showed significant ($p<0.001$) elevation in swim time, when compared with 6-OHDA treated group.

v.Beam walking (Motor co-ordination and Balance)

The significant ($p<0.001$) elevation in the number of foot errors and time taken to travel was observed in 6-OHDA treated animals as shown in the Table: 22. With the treatment of 100 & 200 mg/kg dose of Hydroethanolic extract of *Echinops echinatus* showed the significant ($p<0.01$) decrease in number of foot errors and time taken to travel. With the treatment of 200mg/kg dose of hydroethanolic extract of *Annona squamosa* Linn., animals showed the significant ($p<0.001$) decrease in number of foot errors and time taken to travel as compared with the 6-OHDA treated group.

vi.Grid test (Muscular strength and catching reflex)

Table 23 shows the effect of drugs on the hang time in 6-OHDA injected group compared with control group, has shown a significant ($p<0.001$) fall in hanging time. Group treated with 100mg/kg dose of hydroethanolic extract of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., showed significant ($p<0.01$) improvement in hanging time, and treatment with 200mg/kg of hydroethanolic extract of *Annona squamosa* Linn., *Echinops echinatus* Roxb., and formulation of extracts showed significant ($p<0.001$) elevation in hanging time, when compared with 6-OHDA treated group.

vii.Pole test (Locomotor)

The significant ($p<0.001$) elevation in the time taken by the animal to turn downward and time taken to reach floor was observed in 6-OHDA treated animal's data shown in Table: 24. With the treatment of with 100 & 200mg/kg dose of hydroethanolic extract of *Echinops echinatus* animals showed the significant ($p<0.01$) decrease time in taken to turn downward, at the same dose animals showed the little significant ($p<0.05$) decrease in time taken to reach floor. Whereas, with the treatment of 100 & 200mg/kg dose of hydroethanolic extract of *Annona squamosa*, animals showed the significant ($p<0.01$) decrease in time taken to turn downward and significant ($p<0.001$) decrease in time taken to reach floor as compared with the 6-OHDA treated group.

viii.Rota rod test (Motor co-ordination)

Table 25 shows the results for motor coordination by rota rod test. The motor co-ordination in the control group was significantly ($p<0.001$) high when compared with the 6-OHDA treated animals. Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., improves the motor coordination with a significance of ($p<0.01$) at the dose level of 200mg/kg. The extracts in the formulation has produced significant improvement in the motor coordination ($p<0.001$). The enhancement of dopamine content restored might have altered and muscle coordination.

GENERAL BEHAVIOR TEST

Table 17. Effect of hydroethanolic extract of Annona squamosa Linn., Echinops echinatus Roxb., and formulation on general behavioral studies in rats

Observation	AS (100mg)	AS (200mg)	EE (100mg)	EE (200mg)
Spontaneous activity	normal	normal	normal	normal
Sound response	normal	normal	normal	normal
Touch response	normal	normal	normal	normal
Pain response	normal	normal	normal	normal
Latency to groom	normal	normal	normal	normal
Locomotion	normal	normal	normal	normal
Postural measure	normal	normal	normal	normal
Righiting reflex	normal	normal	normal	normal
Pinna reflex	normal	normal	normal	normal
Grip strength	normal	normal	normal	normal

EVALUATION OF NEUROPROTECTION AFTER ICV INJECTION WITH 6-OH DOPAMINE

Table 18. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on catalepsy test

Groups	Treatment	Latency time
Group-I	Control(vehicle, p.o)	0.0 ± 0.0
Group-II	Negative Control (6-OHDA)	136.54 ± 9.08 ^{a**}
Group-III	6-OHDA + low dose (100mg/kg) of AS	13.83 ± 1.47^{b**}
Group-IV	6-OHDA + high dose (200mg/kg) of AS	4.83 ± 1.62^{b*}
Group-V	6-OHDA + low dose (100mg/kg) of EE	18.50 ± 2.65 ^{b***}
Group-VI	6-OHDA + high dose (200mg/kg) of EE	8.16 ± 1.92 ^{b*}
Group-VII	6-OHDA + 200mg/kg of formulation	3.66±1.838 ^{b***}

- ★ Comparisons were made between

  Control Vs Negative control; Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

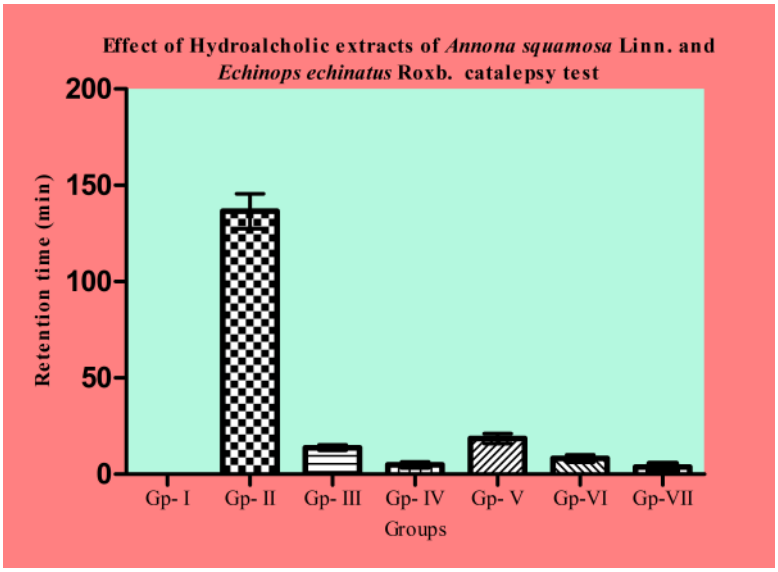


Figure 35. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on catalepsy test

Table 19. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., Open field test

Groups	Treatment	No. of Sq. crossed	Rearing	Grooming
Group-I	Control(vehicle, p.o)	22.66±1.35	10.33±2.12	7.83±1.19
Group-II	Negative Control (6-OHDA)	6.16 ±0.70 ^{a***}	4.86±0.74 ^{a***}	2.50±1.08 ^{a***}
Group-III	6-OHDA + low dose (100mg/kg) of AS	10.16±1.07 ^{b*}	9.02±2.25 ^{b*}	3.83±1.49 ^{b*}
Group-IV	6-OHDA + high dose (200mg/kg) of AS	11.33±1.54 ^{b**}	9.00±1.67 ^{b**}	6.00±1.26 ^{b**}
Group-V	6-OHDA + low dose (100mg/kg) of EE	8.01±1.36 ^{b*}	6.33±1.43 ^{b*}	5.00±0.77 ^{b*}
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	13.33±1.35 ^{b**}	8.16±1.57 ^{b**}	6.33±1.28 ^{b**}
Group-VII	6-OHDA+ 200mg/kg of formulation	16.66 ±1.35 ^{b***}	10.50±1.56 ^{b***}	7.83±1.72 ^{b***}

- ★ Comparisons were made between
  ^{a}Control Vs Negative control ; ^{b}Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

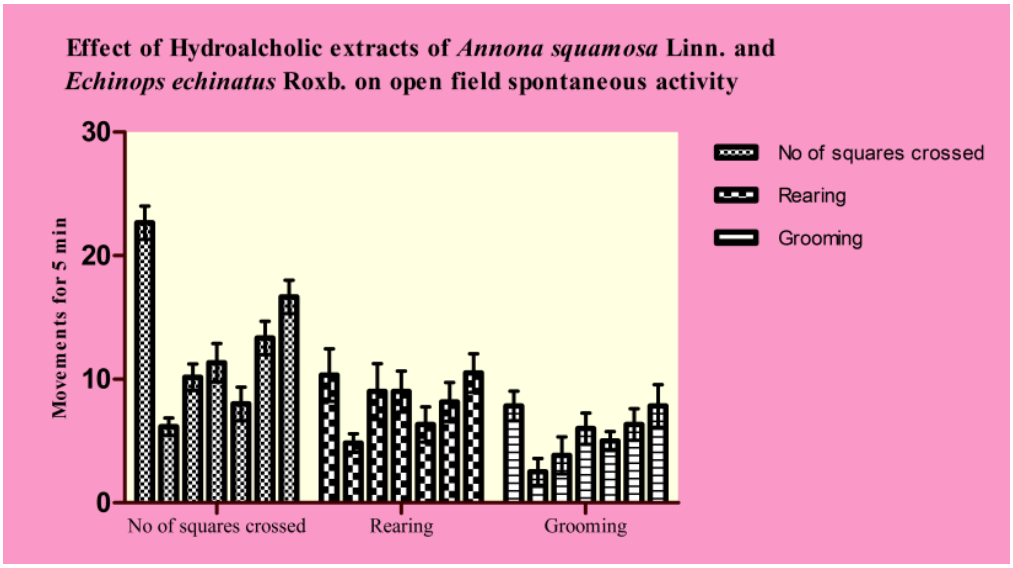


Figure 36. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., Open field test

Groups	Treatment	Stride length (cm)	Forepaw distance width (cm)	Hindpaw distance width (cm)
Group-I	Control(vehicle, p.o)	7.66 ±0.88	1.58 ±0.14	2.28 ±0.06
Group-II	Negative Control (6-OHDA)	2.66 ±0.21	1.28 ±0.09	3.43 ±0.11
Group-III	6-OHDA + low dose (100mg/kg) of AS	2.16 ±0.40	1.66 ±0.08	3.18 ±0.12
Group-IV	6-OHDA + high dose (200mg/kg) of AS	3.33 ±0.76	1.66 ±0.08	2.87 ±0.05
Group-V	6-OHDA + low dose (100mg/kg) of EE	1.83 ±0.16	1.80 ±0.06	3.24 ±0.08
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	4.00 ±0.51	1.73 ±0.09	2.36 ±0.13
Group-VII	6-OHDA+ 200mg/kg of formulation	5.83 ±0.30	1.86 ±0.11	2.17 ±0.15

- ★ Comparisons were made between
  [a]Control Vs Negative control ; [b]Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

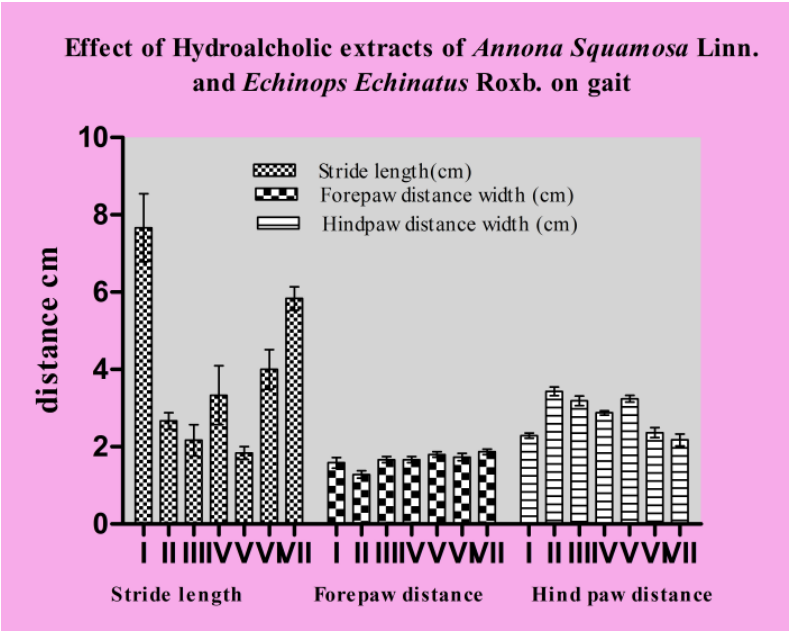


Figure 37. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Gait

Table 21 Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Force Swim

Groups	Treatment	Time in seconds
Group-I	Control(vehicle, p.o)	93.66 ± 7.65
Group-II	Negative Control (6-OHDA)	22.33 ± 5.38[a***]
Group-III	6-OHDA + low dose (100mg/kg) of AS	60.83 ± 4.13[b***]
Group-IV	6-OHDA + high dose (200mg/kg) of AS	64.59 ± 2.88[b***]
Group-V	6-OHDA + low dose (100mg/kg) of EE	62.83 ± 4.32 [b***]
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	64.52 ±3..33[b***]
Group-VII	6-OHDA+ 200mg/kg of formulation	79.66 ± 13.96[b***]

- ★ Comparisons were made between
  [a]Control Vs Negative control ; [b]Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

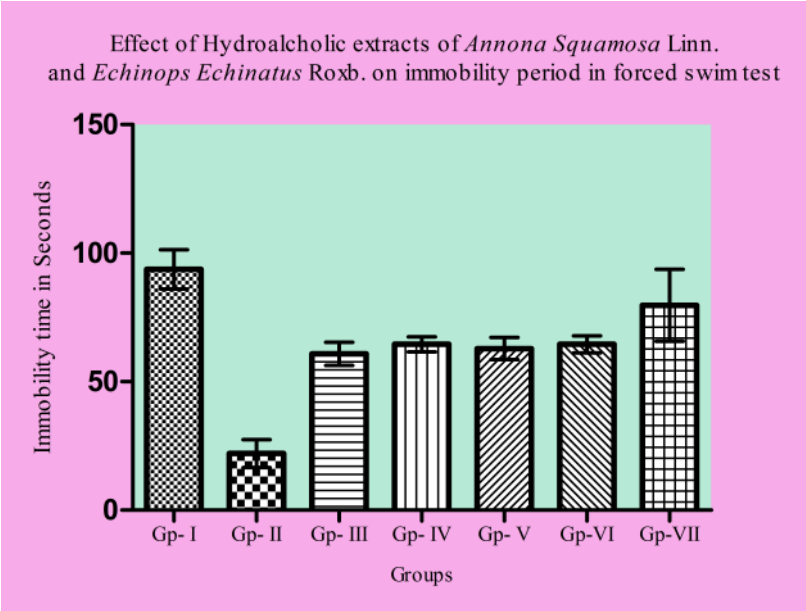


Figure 38. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Force Swim

Table: 22 Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Beam walking test

Groups	Treatment	Time taken to travel (Sec)	No. of foot errors
Group-I	Control(vehicle, p.o)	10.83±1.95	0.16± 0.16
Group-II	Negative Control (6-OHDA)	26.83±1.13	4.83±1.13
Group-III	6-OHDA + low dose (100mg/kg) of AS	20.67±0.76	4.33±0.61
Group-IV	6-OHDA + high dose (200mg/kg) of AS	12.83±1.24	2.50±0.56
Group-V	6-OHDA + low dose (100mg/kg) of EE	10.33±0.80	4.16±1.37
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	11.83±0.83	1.33±0.42
Group-VII	6-OHDA+ 200mg/kg of formulation	11.83±2.27	1.00± 0.36

- ★ Comparisons were made between
  [a]Control Vs Negative control ; [b]Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

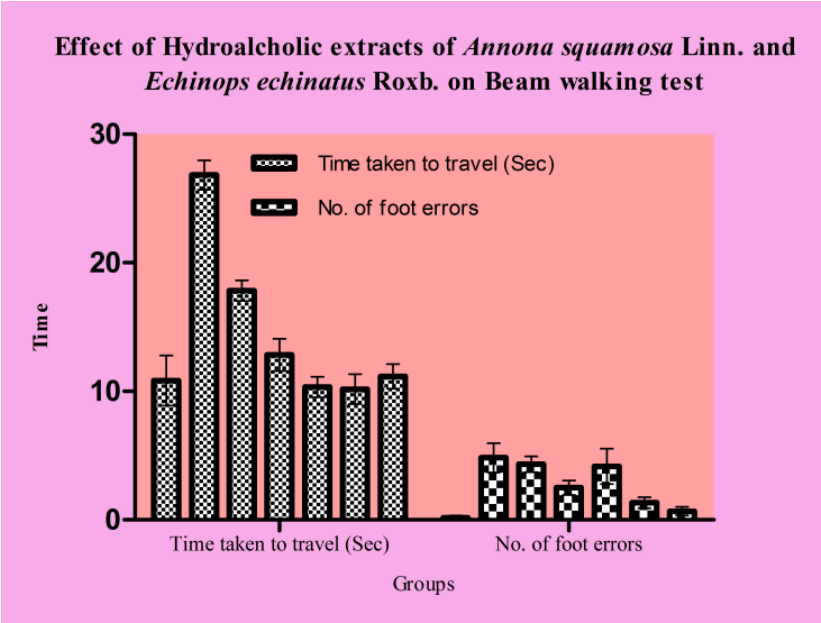


Figure 39. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Beam walking test

Table 23. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Grid test (Hang time test)

Groups	Treatment	Hanging time (sec)
Group-I	Control(vehicle, p.o)	93.83 ± 4.61
Group-II	Negative Control (6-OHDA)	3.33 ± 1.25[a***]
Group-III	6-OHDA + low dose (100mg/kg) of AS	9.01± 2.95[a**]
Group-IV	6-OHDA + high dose (200mg/kg) of AS	23.83 ± 2.651[b***]
Group-V	6-OHDA + low dose (100mg/kg) of EE	14.83± 3.75[b**]
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	40.83 ± 9.26 [b***]
Group-VII	6-OHDA+ 200mg/kg of formulation	45.66 ± 9.20 [b***]

- ★ Comparisons were made between
  [a]Control Vs Negative control ; [b]Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

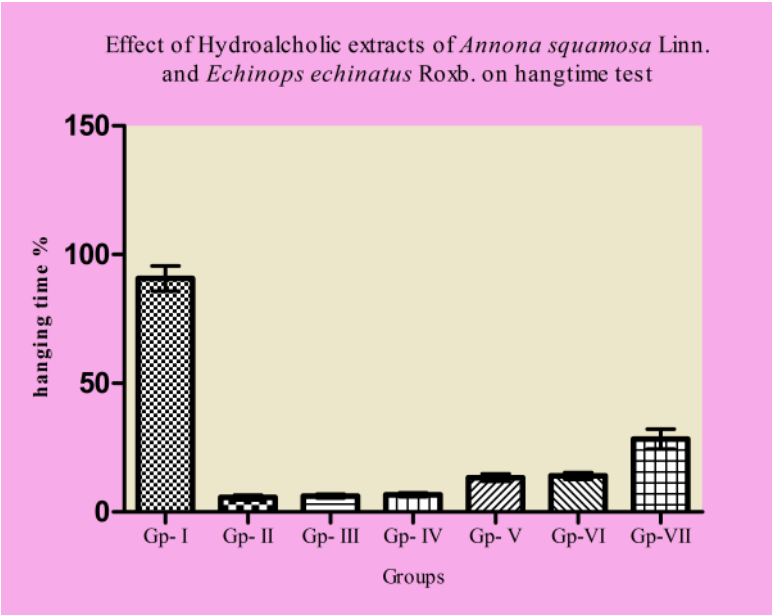


Figure 40. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Grid test (Hang time test)

Table: 24 Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Pole test

Groups	Treatment	Time taken to turn down	Time taken to reach the floor
Group-I	Control(vehicle, p.o)	3.16±0.07	2.83±0.16
Group-II	Negative Control (6-OHDA)	10.16±1.07 [a***]	9.83±0.14 [a***]
Group-III	6-OHDA + low dose (100mg/kg) of AS	5.00±1.54 [b*]	8.67±0.49 [b*]
Group-IV	6-OHDA + high dose (200mg/kg) of AS	4.16±1.27 [b**]	6.83±0.94 [b*]
Group-V	6-OHDA + low dose (100mg/kg) of EE	4.17± 0.65 [b*]	7.67±1.94 [b*]
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	3.83±1.01 [b**]	6.33±0.76 [b**]
Group-VII	6-OHDA+ 200mg/kg of formulation	3.67±1.54 [b***]	4.50±0.56 [b**]

- ★ Comparisons were made between
  [a]Control Vs Negative control ; [b]Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

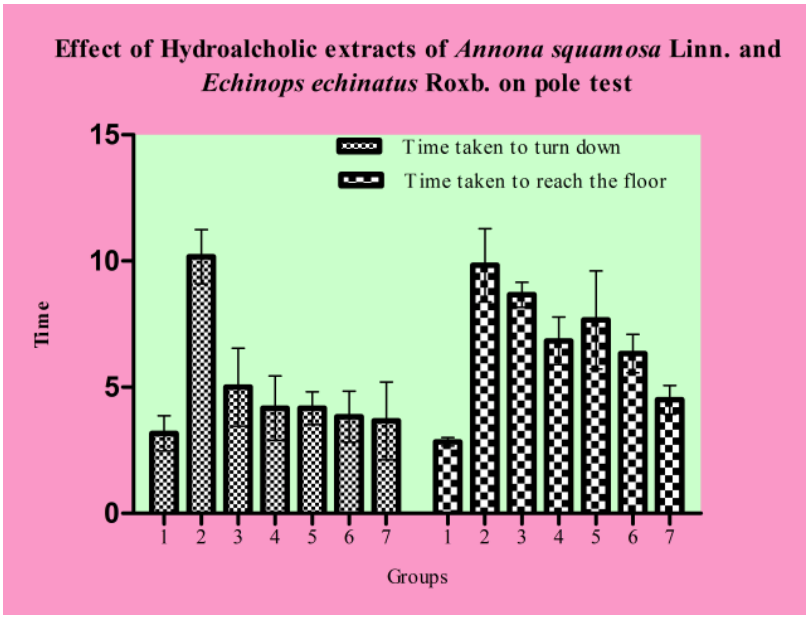


Figure 41. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Pole test

Table 25. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Rota Rod test

Groups	Treatment	Retention time (Sec)
Group-I	Control(vehicle, p.o)	317.2 ± 40.30
Group-II	Negative Control (6-OHDA)	11.17 ±1.49***
Group-III	6-OHDA + low dose (100mg/kg) of AS	65.33±4.70[b]***
Group-IV	6-OHDA + high dose (200mg/kg) of AS	134.70 ± 26.96[b]***
Group-V	6-OHDA + low dose (100mg/kg) of EE	75.17± 6.01[b]***
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	141.2 ± 20.75[b]***
Group-VII	6-OHDA+ 200mg/kg of formulation	206.34 ±34.61 [b]***

- ★ Comparisons were made between

  [a]Control Vs Negative control ; [b]Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

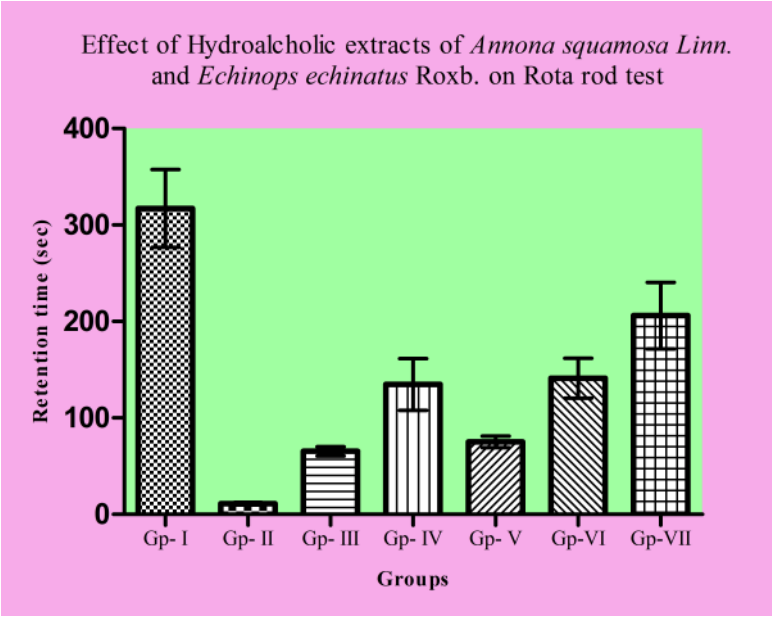


Figure 42. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Rota Rod test

6.9. BIOCHEMICAL STUDIES

a. Thiobarbituric Acid Reactive Substance

Table: 27 show the level of TBARS in the different treatment group. The content of TBARS in the substantia nigra was significantly elevated ($p< 0.001$) in the 6-OH group, as compared to the control group. The increases in the generation of TBARS were significantly and dose-dependently reduced ($p< 0.001$) in 200mg/kg hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb. High degree of significance was observed with the formulation of both extracts ($p<0.001$) as compared to 6-OH group the group.

b. Reduced Glutathione (GSH)

GSH content in the substantia nigra was significantly reduced ($p< 0.001$) in the 6-OH Dopamine group, as compared to the control group. The decrease in GSH content was significantly and dose dependently restored ($p<0.001$) for 200mg hydroethanolic extracts of *Annona squamosa* Linn., ($p<0.001$) for 100 & 200mg/kg of *Echinops echinatus* Roxb., and ($p<0.01$) hydroethanolic extracts of *Annona squamosa* Linn.) treatment groups as compared to 6-Oh Dopamine treated group. High degree of significance was observed with the formulation of extracts ($p<0.001$) as compared to 6-OH group the group.

c. Glutathione reeducates activity

The content of glutathione reeducates in striatum was significantly decreased ($p< 0.001$) in the 6-OH group, as compared to the control group. The activity was significantly restored ($p< 0.001$) in 100, 200mg/kg hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb. High degree of significance was also observed with the the formulation of extracts ($p<0.001$) as compared to 6-OH group the group.

d. Glutathione peroxidase

The content of Glutathione peroxidase in the striatum was significantly reduced ($p< 0.001$) in the 6-OH group, as compared to the control group. The decrease in the generation of Glutathione peroxidase were significantly and dose-dependently increased ($p< 0.001$) in 100 & 200mg/kg hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb. High degree of significance was also observed with the formulation of extracts ($p<0.001$) as compared to 6-OH group the group.

e. Superoxide Dismutase

The activity of SOD in striatum was significantly low ($p<0.001$) in the 6- OH Dopamine treated group when compared to the control group. The decrease in SOD activity was restored significantly and dose-dependently in the groups treated with Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., 100 and 200 mg/kg. High degree of significance was observed with the the formulation of extracts ($p<0.001$).

f. catalase

The content of catalase in striatum was significantly decreased ($p< 0.001$) in the 6-OH group, as compared to the control group. The activity was significantly restored ($p< 0.001$) in 100, 200mg/kg hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb. High degree of significance was also observed with the formulation of extracts ($p<0.001$) as compared to 6-OH group the group.

BIOCHEMICAL STUDIES

Table 26. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Total Protein

Groups	Treatment	Total Protein (mg/gm of tissue)
Group-I	Control(vehicle, p.o)	1.933 ± 0.14
Group-II	Negative Control (6-OHDA)	0.633 ± 0.55[a]***
Group-III	6-OHDA + low dose (100mg/kg) of AS	1.463 ± 0.743[b]**
Group-IV	6-OHDA + high dose (200mg/kg) of AS	1.463 ± 0.743[b]**
Group-V	6-OHDA + low dose (100mg/kg) of EE	1.463 ± 0.743[b]**
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	1.543 ±0.106[b]***
Group-VII	6-OHDA+ 200mg/kg of formulation	1.463 ± 0.743[b]**

- Comparisons were made between

  [a]Control Vs Negative control; [b]Negative control Vs Treatment groups
- Values are expressed as mean ± SEM of 6 animals.
- Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

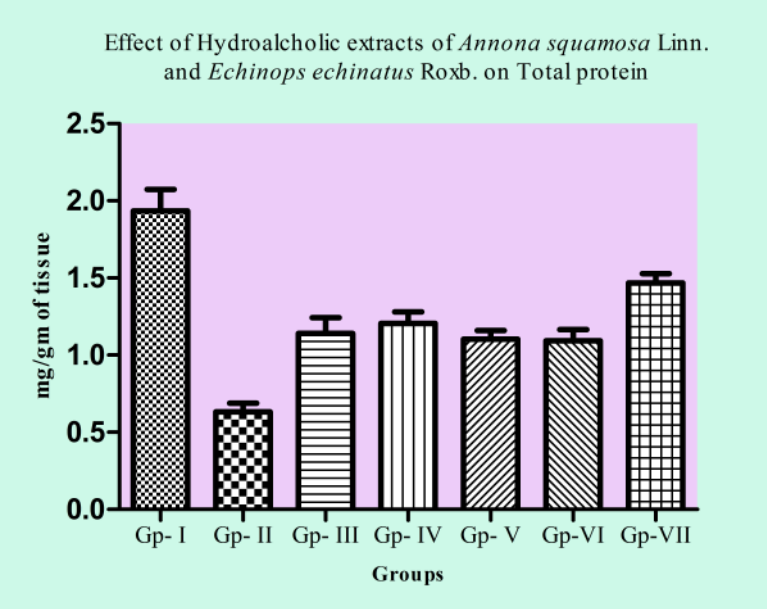


Figure 43. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Total Protein

Table 27. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Thiobarbituric Acid Reactive Substance

Groups	**Treatment**	n mols TBARS/ min/ mg protein
Group-I	Control(vehicle, p.o)	6.00±1.09
Group-II	Negative Control (6-OHDA)	31.50± 1.40[a]***
Group-III	6-OHDA + low dose (100mg/kg) of AS	22.67±1.25[b]***
Group-IV	6-OHDA + high dose (200mg/kg) of AS	17.33±1.05[b]***
Group-V	6-OHDA + low dose (100mg/kg) of EE	21.67±0.95[b]***
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	18.17±0.60[b]***
Group-VII	6-OHDA+ 200mg/kg of formulation	13.00±0.93 [b]***

- Comparisons were made between

  [a]Control Vs Negative control; [b]Negative control Vs Treatment groups
- Values are expressed as mean ± SEM of 6 animals.
- Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

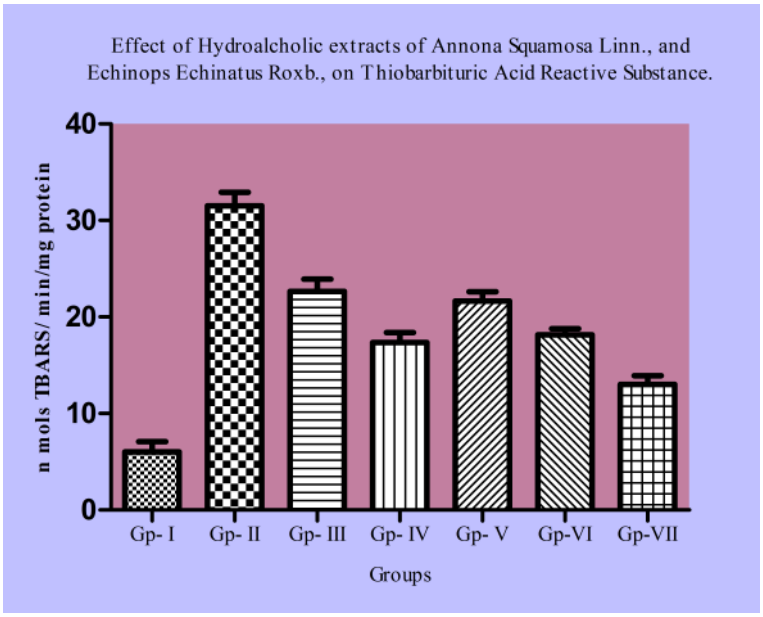


Figure 44. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Thiobarbituric Acid Reactive Substance

Table 28. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Reduced Glutathione (GSH)

Groups	Treatment	n moles GSH/ g tissue
Group-I	Control(vehicle, p.o)	22.50±1.43
Group-II	Negative Control (6-OHDA)	5.50±0.76[a]***
Group-III	6-OHDA + low dose (100mg/kg) of AS	14.00±1.48 [b]**
Group-IV	6-OHDA + high dose (200mg/kg) of AS	20.67±1.68[b]***
Group-V	6-OHDA + low dose (100mg/kg) of EE	14.83±1.49 [b]***
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	22.14±1.80[b]***
Group-VII	6-OHDA+ 200mg/kg of formulation	24.01±1.63 [b]***

- Comparisons were made between

  [a]Control Vs Negative control; [b]Negative control Vs Treatment groups
- Values are expressed as mean ± SEM of 6 animals.
- Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

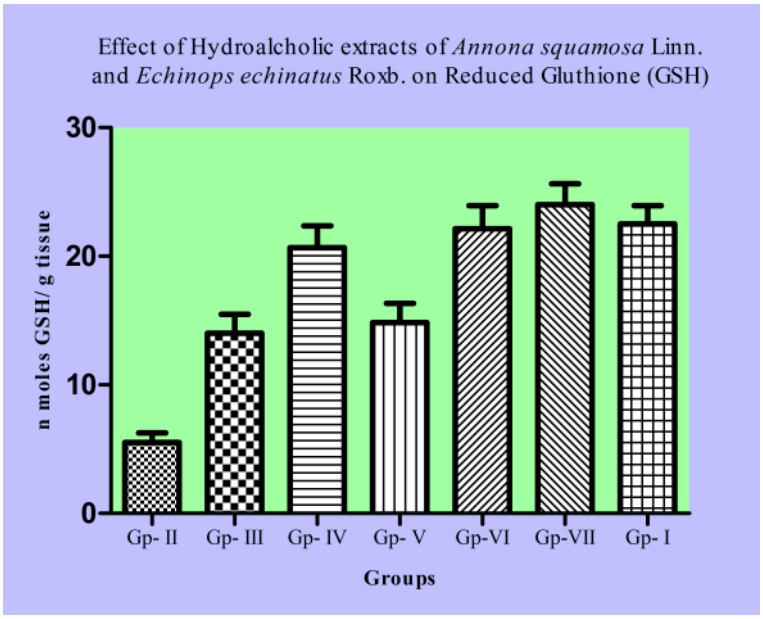


Figure 45. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Reduced Glutathione (GSH)

Table 29. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on glutathione reeducates activity

Groups	Treatment	n mol NADPH oxidized/ min/mg protein
Group-I	Control(vehicle, p.o)	25.67±1.11
Group-II	Negative Control (6-OHDA)	4.83±0.60[a]***
Group-III	6-OHDA + low dose (100mg/kg) of AS	9.01±0.84 [b]*
Group-IV	6-OHDA + high dose (200mg/kg) of AS	11.33±0.88[b]***
Group-V	6-OHDA + low dose (100mg/kg) of EE	9.17±0.54 [b]*
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	10.58±1.22[b]**
Group-VII	6-OHDA+ 200mg/kg of formulation	14.17±1.13 [b]***

- Comparisons were made between

  [a]Control Vs Negative control; [b]Negative control Vs Treatment groups
- Values are expressed as mean ± SEM of 6 animals.
- Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

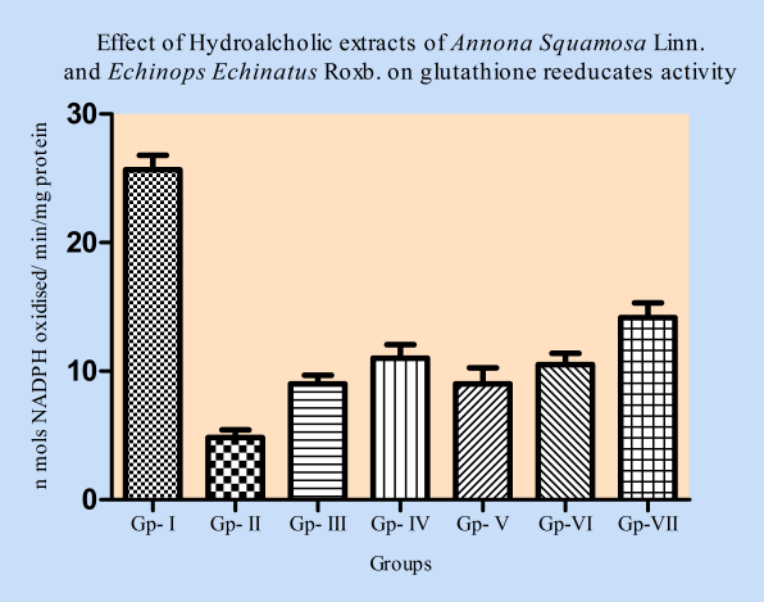


Figure 46. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on glutathione reeducates activity

Table 30. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Glutathione peroxidase

Groups	Treatment	n mols NADPH oxidised/ min/mg protein
Group-I	Control(vehicle, p.o)	11.01±0.93
Group-II	Negative Control (6-OHDA)	1.66±0.33[a]***
Group-III	6-OHDA + low dose (100mg/kg) of AS	4.33±0.55 [b]*
Group-IV	6-OHDA + high dose (200mg/kg) of AS	6.33±0.61[b]***
Group-V	6-OHDA + low dose (100mg/kg) of EE	4.33±0.42 [b]*
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	6.83±0.74[b]***
Group-VII	6-OHDA+ 200mg/kg of formulation	7.66±0.88 [b]***

- Comparisons were made between

  [a]Control Vs Negative control; [b]Negative control Vs Treatment groups
- Values are expressed as mean ± SEM of 6 animals.
- Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

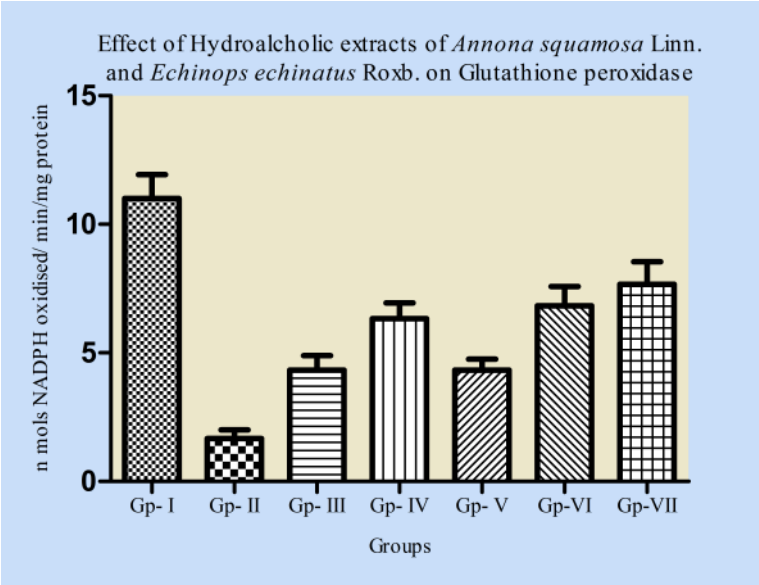


Figure 47. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Glutathione peroxidase

Table 31. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on SOD

Groups	Treatment	U/ mg of proteins
Group-I	Control(vehicle, p.o)	6.91±1.01
Group-II	Negative Control (6-OHDA)	1.31±0.24[a]***
Group-III	6-OHDA + low dose (100mg/kg) of AS	4.01±0.51 [b]*
Group-IV	6-OHDA + high dose (200mg/kg) of AS	5.03±0.73[b]***
Group-V	6-OHDA + low dose (100mg/kg) of EE	4.03±0.36 [b]*
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	5.12±0.51[b]***
Group-VII	6-OHDA+ 200mg/kg of formulation	5.25±0.54 [b]***

- Comparisons were made between

  [a]Control Vs Negative control; [b]Negative control Vs Treatment groups
- Values are expressed as mean ± SEM of 6 animals.
- Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

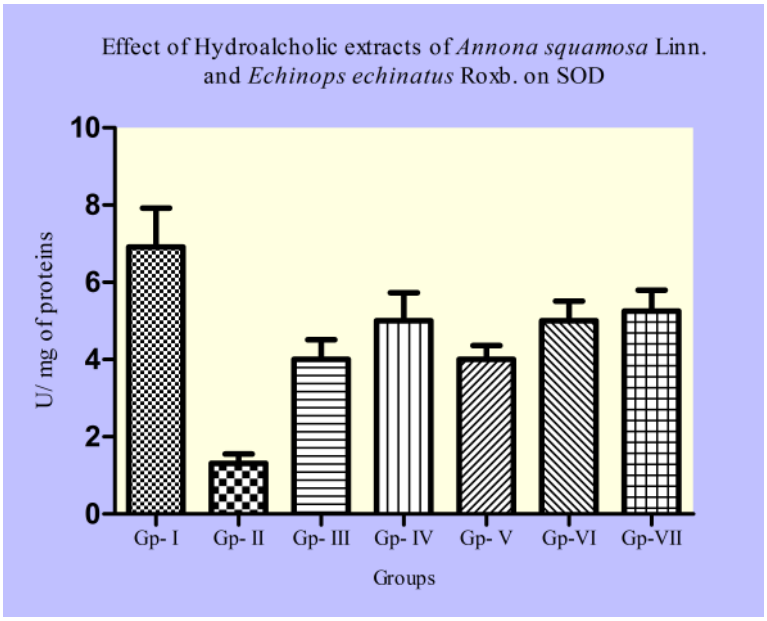


Figure 48. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on SOD

Table: 32 Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on catalase

Groups	Treatment	nmol H_2O_2 consumed/ min /mg protein
Group-I	Control(vehicle, p.o)	7.91±0.98
Group-II	Negative Control (6-OHDA)	1.46±0.40[a]***
Group-III	6-OHDA + low dose (100mg/kg) of AS	3.25±1.12 [b]*
Group-IV	6-OHDA + high dose (200mg/kg) of AS	4.50±0.32[b]**
Group-V	6-OHDA + low dose (100mg/kg) of EE	3.33±0.61 [b]*
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	4.50±0.76[b]**
Group-VII	6-OHDA+ 200mg/kg of formulation	5.33±0.42 [b]***

- Comparisons were made between

  [a]Control Vs Negative control; [b]Negative control Vs Treatment groups
- Values are expressed as mean ± SEM of 6 animals.
- Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

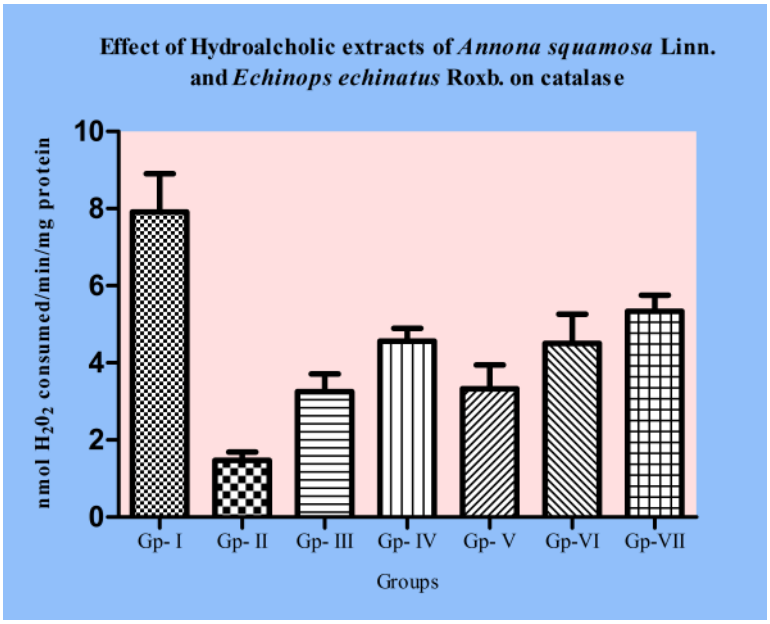


Figure49. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on catalase

6.10. ESTIMATION OF BRAIN NEUROTRANSMITTERS

a) Acetylcholinesterase activity

Table:33 shows the Acetylcholinesterase activity and its was found to be significantly ($p<0.001$) decreased in 6-OHDA treated animals when compared to control group. Provided hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., has shown a significant ($p<0.001$) increase in acetylcholinesterase at the dose level of 200mg/kg and a significance ($p<0.01$) increase at the dose level of 100mg/kg. High degree of significance was observed with the formulation of extracts ($p<0.001$).

b) Monoamino oxidase

Table: 34 show the levels of monoamino oxidase. The levels in 6-OHDA treated group was found to be significantly ($p<0.01$) high in the control group. Pretreatment with hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., at the dose of 100mg/kg and 200mg/kg ameliorate the effect of 6-OH Dopamine on Monoamino oxidase in treated groups. Hydroethanolic extract of *Annona squamosa* at dose 100mg/kg and 200mg/kg treatment significantly ($p<0.001$) decreased the monoamino oxidase and attenuated the effect of 6-OHDA. The progressive reduction was observed as the dose increases.

c) Dopamine

Table: 35 represent the level of dopamine. When compared to the control group, 6-OHDA treated group showed significant ($p<0.001$) decrease in the dopamine level in striatum. The hydroethanolic extract of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., has significantly attenuated the effect of 6-OHDA in the treated groups. The hydroethanolic extract of *Annona squamosa* at the dose of 100mg/kg administration showed significant ($p<0.01$) improvement in dopamine content, whereas 200mg/kg & the formulation of extracts significantly ($p<0.001$) increased the dopamine content in striatum and attenuated the effect of 6-OHDA and reduce the signs of Parkinsonism.

d) Glutamate

Table 36 shows the Glutamate content in control animals was significantly ($p<0.001$) elevated than that of the 6-OHDA injected group. The low and high dose of hydroethanolic extract of *Echinops echinatus* showed little significance ($p<0.05$) in attenuating the effect of 6-OHDA on level of

glutamate in treated animals, whereas the hydroethanolic extract of *Annona squamosa* showed a significant ($p<0.001$) reduction in glutamate content in treated group and ameliorated the effect of 6-OHDA.

6.11 Histopathology

Histopathology of striatum of 6-OH Dopamine shows architectural disturbance and fat body inclusions. The treatment with hydroethanolic extract of *Annona squamosa* Linn., *Echinops echinatus* Roxb., and the formulation of extracts has restored the architecture as an evidence of neuroprotection.

NEUROTRANSMITTER ESTIMATION

Table 33. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Acetylcholinesterase

Groups	Treatment	Acetylcholinesterase level(m moles)
Group-I	Control(vehicle, p.o)	55.33 ± 2.91
Group-II	Negative Control (6-OHDA)	39.33 ± 2.37 [a]**
Group-III	6-OHDA + low dose (100mg/kg) of AS	83.67 ± 6.53 [b]***
Group-IV	6-OHDA + high dose (200mg/kg) of AS	116.8 ± 7.05 [b]***
Group-V	6-OHDA + low dose (100mg/kg) of EE	83.5 ± 7.97 [b]**
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	109.8 ±3.36 [b]***
Group-VII	6-OHDA+ 200mg/kg of formulation	139.2±6.01 [b]***

- ★ Comparisons were made between
  [a]Control Vs Negative control ; [b]Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

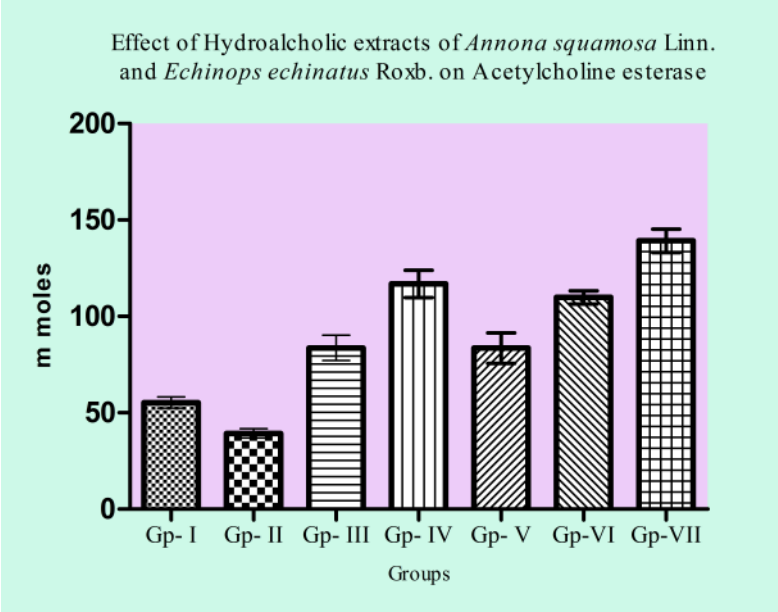


Figure 50. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Acetylcholinesterase

Table 34. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Monoamino Oxidase

Groups	Treatment	nmoles/min/ mg
Group-I	Control(vehicle, p.o)	5550 ±212.7
Group-II	Negative Control (6-OHDA)	7453 ±132.8 [a]**
Group-III	6-OHDA + low dose (100mg/kg) of AS	5399 ±112.7 [b]**
Group-IV	6-OHDA + high dose (200mg/kg) of AS	4795 ± 73.4[b]**
Group-V	6-OHDA + low dose (100mg/kg) of EE	5036 ± 217.5 [b]**
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	4969 ± 123.1[b]**
Group-VII	6-OHDA+ 100mg/kg of formulation	4831 ± 168.5 [b]**

- ★ Comparisons were made between
  [a]Control Vs Negative control ; [b]Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

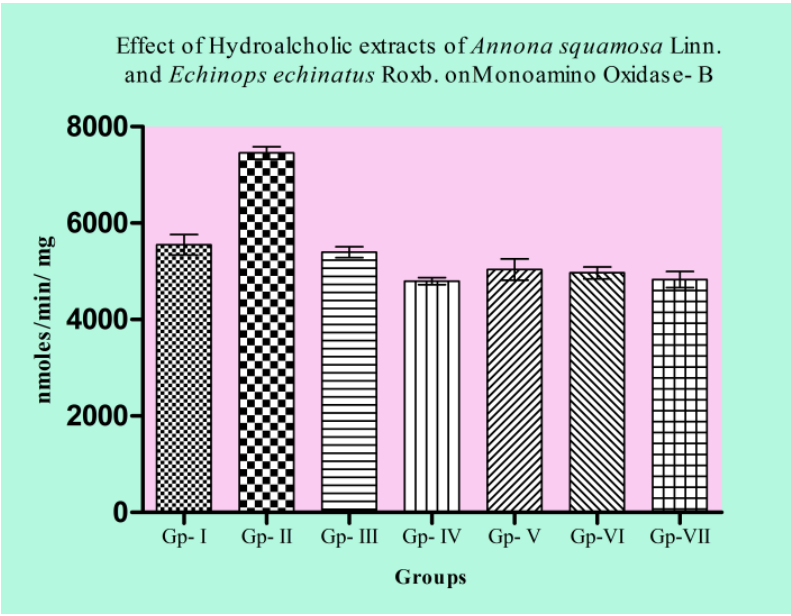


Figure 51. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Monoamino Oxidase

Table 35. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Dopamine

Groups	Treatment	Dopamine (ng/mg tissue)
Group-I	Control(vehicle, p.o)	675.5 ± 10.42
Group-II	Negative Control (6-OHDA)	371.8 ±11.96 [a]***
Group-III	6-OHDA + low dose (100mg/kg) of AS	422.5± 11.39[b]*
Group-IV	6-OHDA + high dose (200mg/kg) of AS	454.9 ± 20.76[b]***
Group-V	6-OHDA + low dose (100mg/kg) of EE	431.2 ±7.80 [b]*
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	524.7 ± 9.034[b]***
Group-VII	6-OHDA+ 100mg/kg of formulation	45.66 ± 9.204 [b]***

- ★ Comparisons were made between
  [a]Control Vs Negative control ; [b]Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

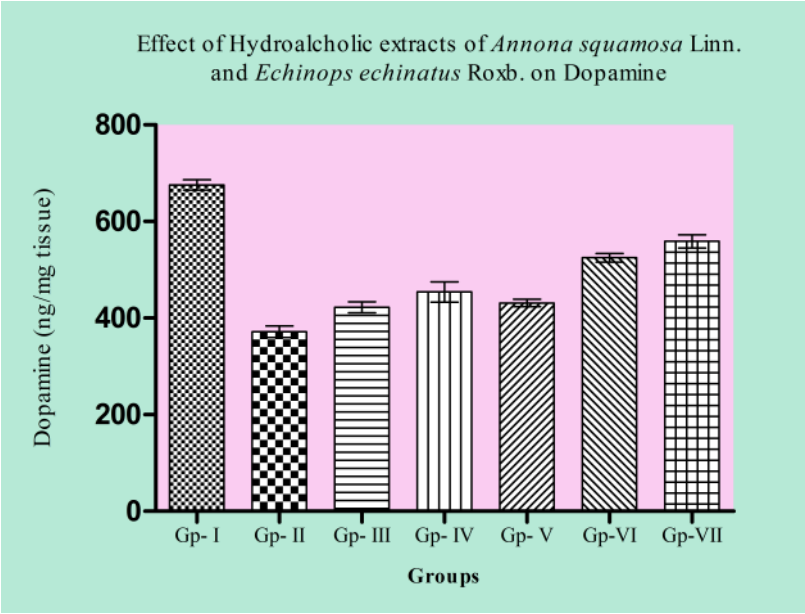


Figure 52. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Dopamine

Table 36. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Glutamate

Groups	Treatment	Glutamate (n moles/gm tissue)
Group-I	Control(vehicle, p.o)	70158 ± 441.2
Group-II	Negative Control (6-OHDA)	87143 ± 433.8 [a***]
Group-III	6-OHDA + low dose (100mg/kg) of AS	82650 ± 704.5[b*]
Group-IV	6-OHDA + high dose (200mg/kg) of AS	81850 ± 569.8 [b*]
Group-V	6-OHDA + low dose (100mg/kg) of EE	80726 ± 551.2 [b**]
Group-VI	6-OHDA+ high dose (200mg/kg) of EE	82893 ± 822.0[b]**
Group-VII	6-OHDA+ 200mg/kg of formulation	79943 ± 999.5[b]**

- ★ Comparisons were made between
  [a]Control Vs Negative control ; [b]Negative control Vs Treatment groups
- ★ Values are expressed as mean ± SEM of 6 animals.
- ★ Symbols represent statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001.
- ★ Statistical Significance test for comparison was done by one way ANOVA followed by Dunnet's't' test.

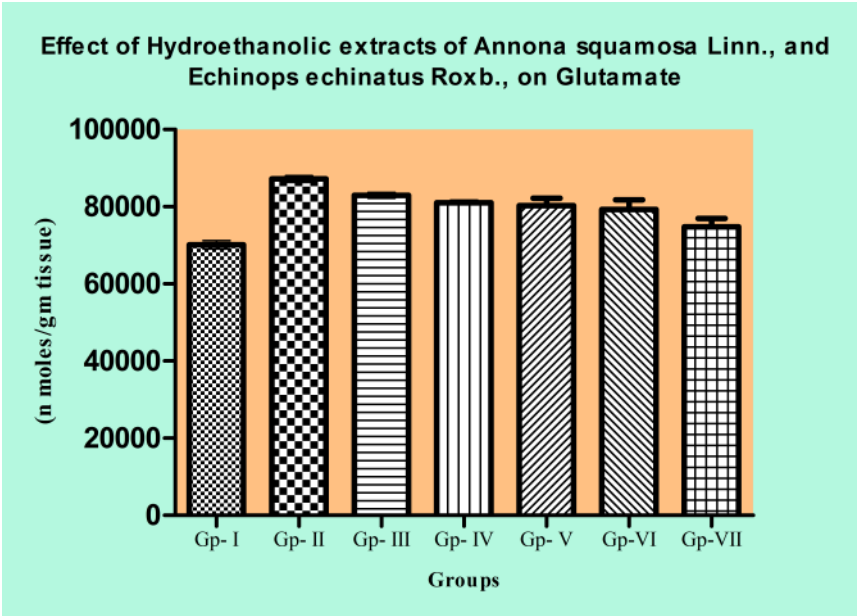


Figure 53. Effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., on Glutamate

HISTOPATHOLOGY

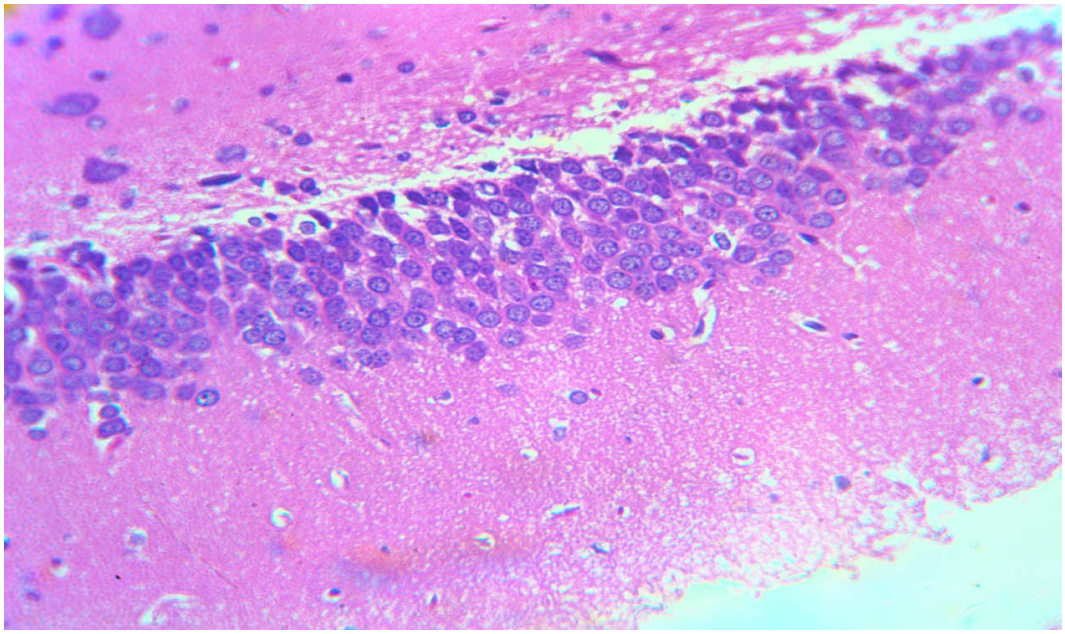


Figure. 54 Photomicrograph shows the section of striatum in rat brain which was identified using hematoxylin-eosin at 10X magnification in Group I (Control group)

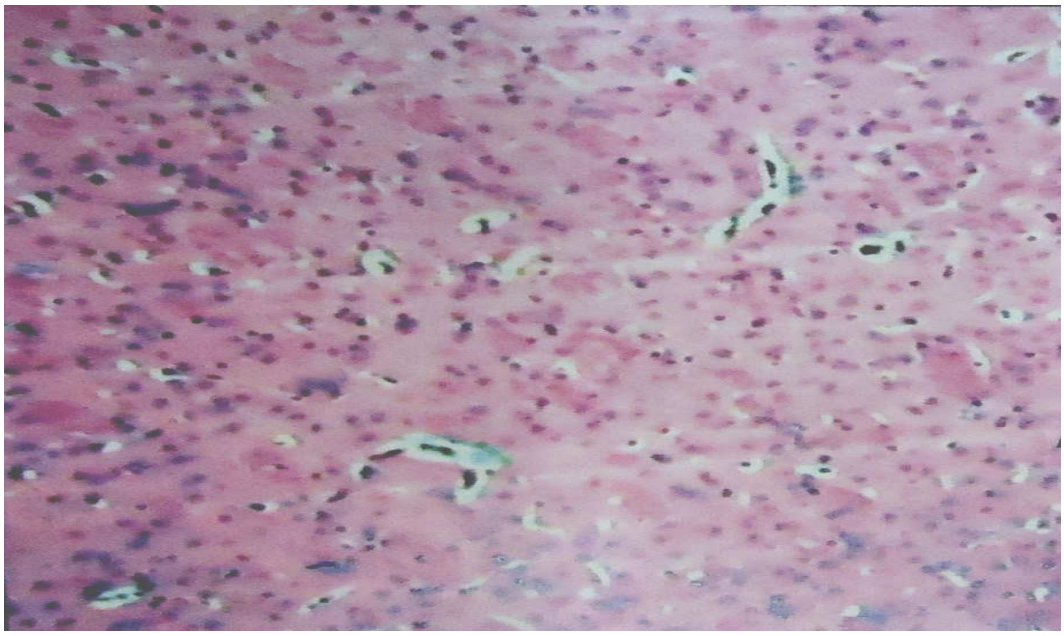


Figure 55. Photomicrograph shows the section of striatum region in rat brain which was identified using hematoxylin-eosin at 10X magnification in Group II (negative Control group)

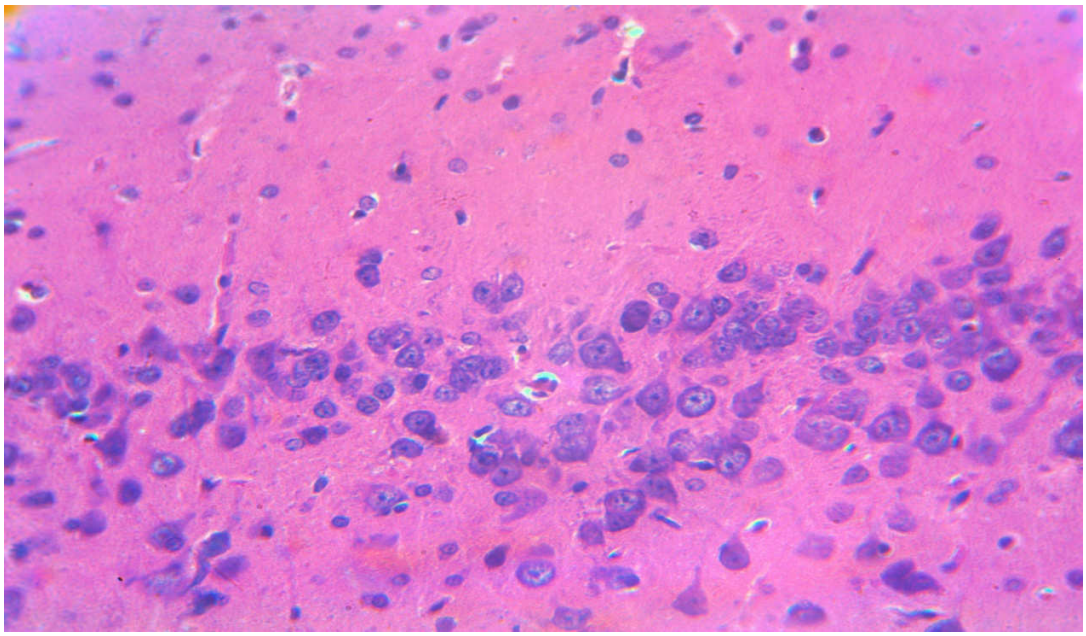


Figure 56. Photomicrograph shows the section of striatum region in rat brain which was identified using hematoxylin-eosin at 10X magnification in Group III (AS low dose 100mg/kg)

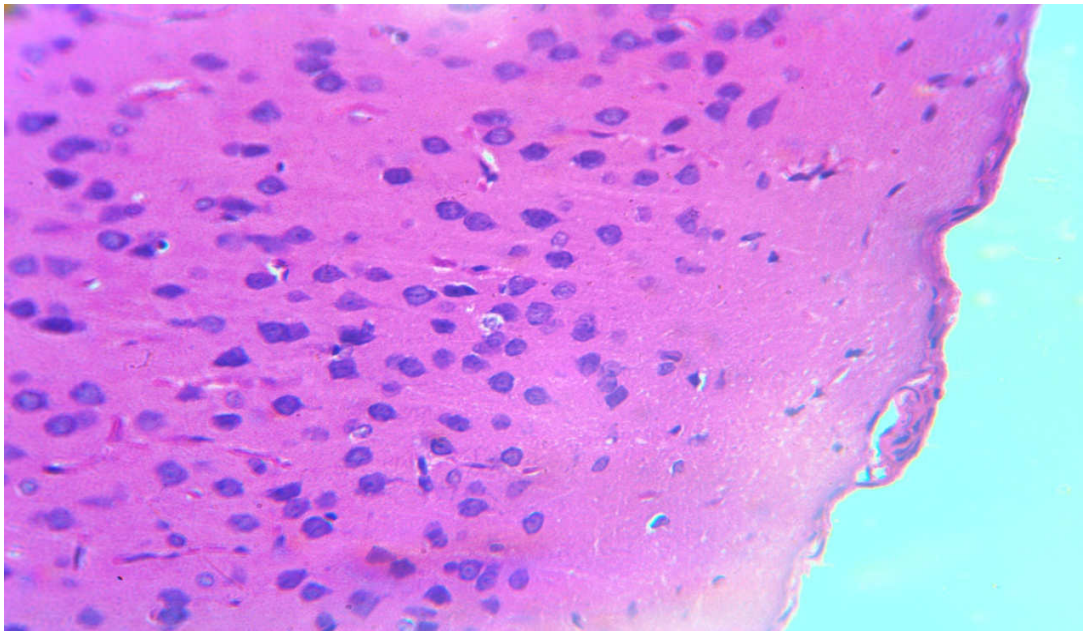


Figure 57. Photomicrograph shows the section of striatum region in rat brain which was identified using hematoxylin-eosin at 10X magnification in Group IV (AS high dose 200mg/kg)

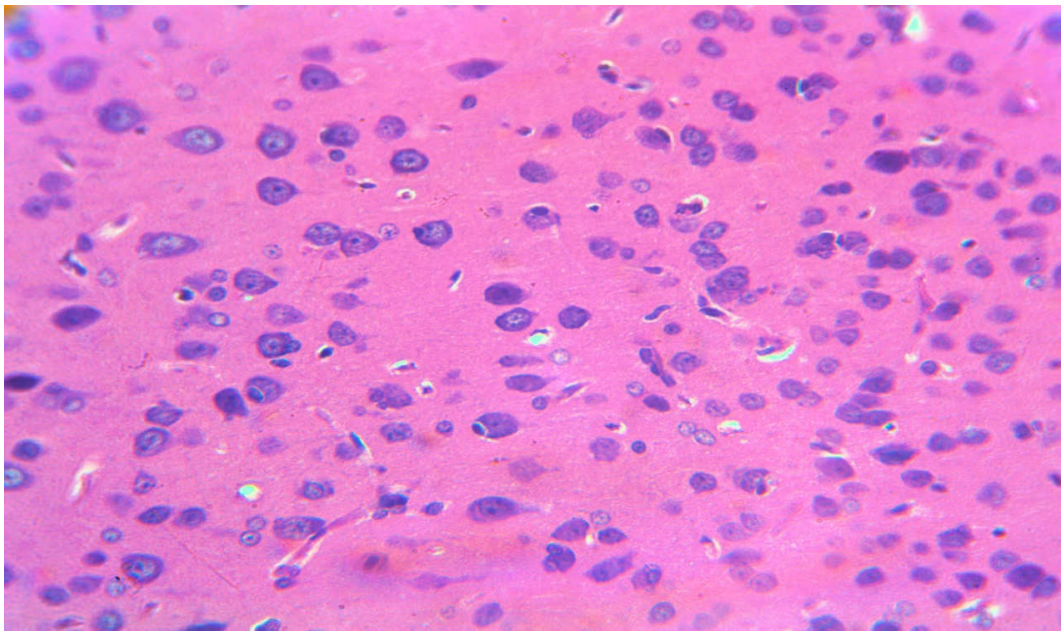


Figure 58. Photomicrograph shows the section of striatum region in rat brain which was identified using hematoxylin-eosin at 10X magnification in Group V (EE low dose 100mg/kg)

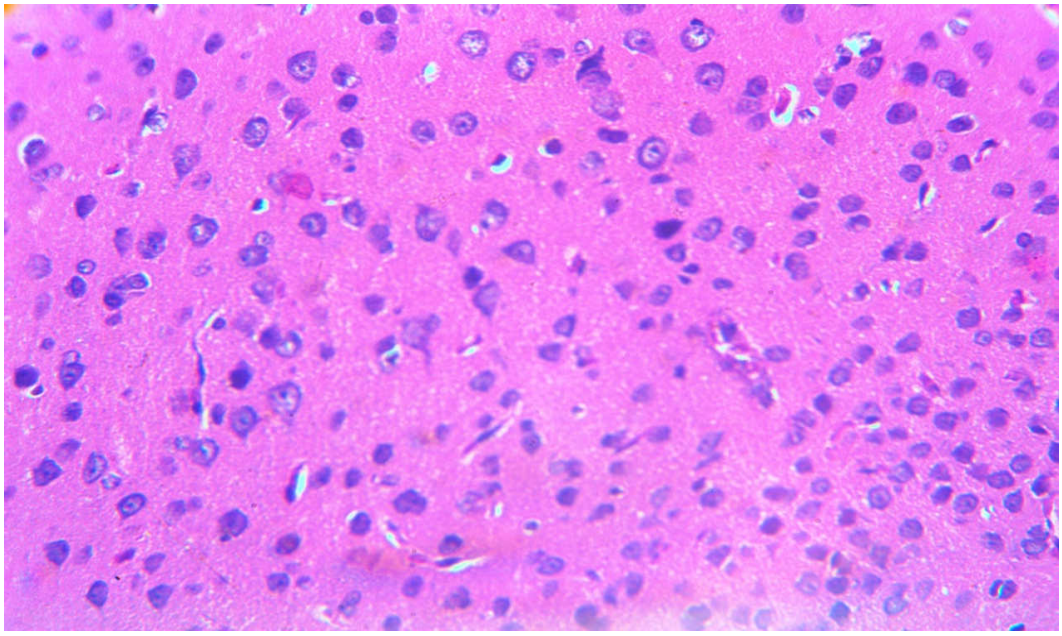


Figure 59. Photomicrograph shows the section of striatum region in rat brain which was identified using hematoxylin-eosin at 10X magnification in Group VI (EE high dose 200mg/kg)

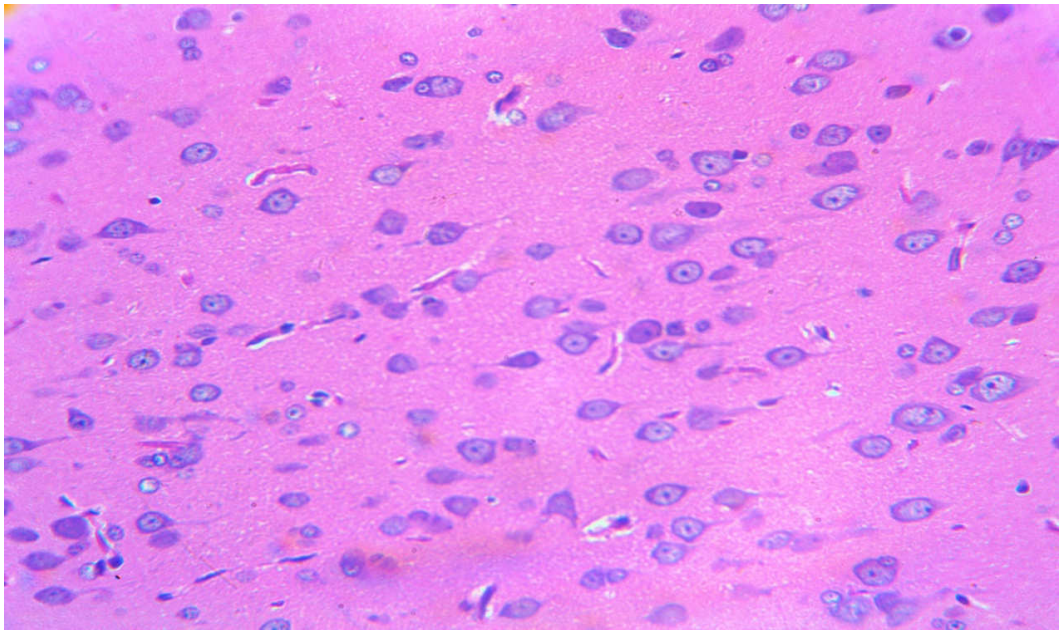


Figure 60. Photomicrograph shows the section of striatum region in rat brain which was identified using hematoxylin-eosin at 10X magnification in Group VII (100mg/kg of formulation)

DISCUSSION

VII. DISCUSSION

Plant based drugs are preferred for human mental illness over synthetic pharmaceuticals because of various side effects. Herbal treatment not only advances persevering compliance but furthermore there are possibilities of enhancing the bioavailability of numerous pharmaceuticals. Active constituents extracted from exact parts of plant origin is beneficial in treatment of mental illness sources have proved to be beneficial (Luchsinger JA *et al.,* 2007).

Herbal remedies requires an influential and deep evaluation of their efficacies and safety due to their growing use all over the world .Thus all the natural products used in therapeutics must be submitted to efficacy and safety tests by the same methods used for new synthetic drugs (Eisenberg DM 1998).

The restoration of dopamine content in the striatum after the treatment with 6-OH Dopamine is the approach to prevent the progression of Parkinsonism (Prabhu *et al.,* 1994).

Evidence of oxidative stress in PD Postmortem investigations has consistently shown that oxidative stress is a hallmark of not only of healthy neurons but of diseased human nigral tissue. Carbonyl modifications, which are indicative of protein oxidation, are increased 2-fold in the SN compared to the basal ganglia and prefrontal cortex of normal subjects (Floor and Wetzel, 1998).

Hydroxyl radical is a principle contributor for tissue damage. H_2O_2 can generate hydroxyl radical via fenton reaction. In addition H_2O_2 can easily cross the cell membrane and exerts an injurious effect of tissue through a number of different mechanisms, such as perturbing intracellular Ca^{2+} monostat, increased intracellular ATP inducing DNA damage and inducing aptotosis. Hydrogen peroxide though not very reactive, but it can occasionally be toxic to cell because it can give rise to hydroxyl radical in the cells (Hu C 2007).

Thus the removal of H_2O_2 is very important for antioxidant defense in cell or food systems. H_2O_2 can cross membranes and may oxidize a number of compounds. The formation of hydroxyl radical from fenton reaction was determined using 2,

deoxy -D-ribose degradation. Studies with extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., have revealed significant hydroxyl scavenging activity in *in-vitro*. Hydroethanolic extracts of roots of *Annona squamosa* Linn., and aerial parts of *Echinops echinatus* Roxb., was capable of scavenging hydroxyl radical in a dose dependent manner (Arora A *et al., 2002*).

Nitric oxide is a potent regulator of many physiological processes and regulation of cell mediated toxicity. It is a diffusible free radical which as a vital roles as an effector molecules in diverse biological systems including neuronal messenger, vasodilation, antimicrobial and antitumour activities. Scavengers of nitric oxide compete with oxygen leading to the reduced production of nitric oxide. (Gibanananda and Sayed, 2002)

Excess production of nitric oxide is associated with several diseases like adjuvant arthritis, neurodegenerative disorders and cancer (Ialenti et al., 1993). Sodium nitroprusside serves as a chief source for NO radicals. The absorbance of the chromophore produced during diazotization of the nitrite with sulphanilamide and further coupling with napthylethylene diamine was measured at 546 nm. The chromophore formation is not complete in the presence of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb.,, as it scavenges the NO thus produced from the sodium nitroprusside.

Hydroethanolic extract of *Annona Squamosa* Linn., and *Echinops Echinatus* Roxb., was found to scavenge the NO free radical dose dependently. The scavenging activity of hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., increases in dose dependent manner. Our finding suggests that the phenolic compounds present in the extract might be responsible for nitric oxide scavenging effect (Govindarajan R *et al.,* 2003)

Reducing power of Hydroethanolic extracts of *Annona squamosa* Linn. and *Echinops echinatus* Roxb., increased with increased concentration of test compound. The reducing capacity of a compound may provide a significant indication of its potential antioxidant activity. The reducing ability is usually affiliated with the presence of reductones, which breaks the free radical chain by donating a hydrogen

atom. The extract had reductive ability which increased with increasing concentrations of the extract. (Meir S *et al.*, 1995).

DPPH is a relatively stable free radical which when encounters proton donors such as antioxidants, it gets quenched and the absorbance decreases (Wu et al., 2003).

Oxidative stress can lead to peroxidation of cellular lipids and lipid peroxidation (LPO) has been implicated in the pathogenesis of number of diseases including neurodegenerative disorders It is well found that bioenzymes are very much susceptible to LPO which is regarded to be the crucial part of many toxic as well as degenerative process. Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., inhibits the rate of lipid peroxidation by a reduction in the red color complex formed reflecting its anti lipid per oxidative potential.

The extract showed a significant protection against Fe^{2+}/ascorbic acid induced lipid peroxidation that could be caused by absence of ferryl-perferyl complex. It is generally assumed that ability of the plant phenolic compounds such as flavanoids to chelate ions in the LPO system is very important for their antioxidant property. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups. Total amount of phenolic, flavonoid and ascorbic acid compounds in extract was investigated. Recent reports reveal alkaloids can be used as good source of natural antioxidants (Fadila MB *et al* 2007).

In the present study four methods were used for evaluation of antioxidant activity. The first three methods were for direct measurement of radical scavenging activity and the fourth method evaluated the reducing power. Hydroethanolic extract of *Annona Squamosa* Linn., and *Echinops Echinatus* Roxb., demonstrated good radical quenching activity. The high antioxidant potential of the hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., may due to their phytochemical constituents.

In the present study no mortality was observed during acute toxicity evaluation in the various doses administered. No signs or symptoms of toxicity were observed. The results of the study reveal that Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., should be regarded practically as non toxic**.** Results from the change in body weight of treated groups when compared to

control rats also suggest that at repeated administration of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., had no effect on the normal growth of rats as change in body weight have been used as an indicator of adverse effect of drugs and chemicals. Both the extracts have not produced any toxic symptoms or mortality up to the dose level of 2000mg/kg in rats and hence the drugs were considered safe for further pharmacological screening. According to (Loomis & Hayes 1996) classification chemical substance within the range of 1–5g/kg is considered as practically low-toxic.

The hematological system is the one of the most sensitive targets for toxic compounds and important index of physiological and pathological states in man and animals. In the present study all the hematological parameters remained under the reference range for the rats in both drugs treated and control groups. A similar absence of toxic effects was observed in biochemical parameters. There were no significant effect on the levels of AST, ALT, ALP, bilirubin, urea and creatinine, which are good indicators of liver and kidney functions. This was further confirmed by histological assessment of these organs. There was no treatment related biological significance on adverse effect of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., extract on the biochemical parameters in rats.

Results of histological analysis of internal organs revealed that there were no treatment related histopathological changes. All the findings were consistent with normal background lesions in clinically normal rats of age and strain used in this study. On oral administration of hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., at a dose of 200mg/kg has not produced any significant alteration in the hematological, biochemical parameters and histological observation in albino Wistar rats. This study provides valuable data on toxicity profile of Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., roots, which could stand as assurance for medicinal use of this plant for long-term treatment.

General behavior studies suggest that hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., extract does not possess any activity. The general behavior study suggest that the hydroethanolic extracts has no symptoms of either depression or stimulation.

Dopamine depletion is considered a cardinal feature in the causation of PD in humans or in animal models of the disease (Carder et al., 1989; Bloem et al., 1990). 6-OHDA produces produced behavioral, biochemical, and pathological changes typical of PD and caused a marked depletion of striatal dopamine content. (Sachs and Jonsson, 1975; Bloem et al., 1990). These toxic effects are related to the formation of various oxidants and free radicals (Cohen et al., 1976).

The neuroprotective effects of hydroalcholic extracts of Roots of *Annona squamosa* Linn and *Echinops echinatus* Roxb., in a 6-OHDA model of Parkinson's disease model was evaluated in the present study.

The protection of dopamine content by hydroalcholic extracts of Roots of Annona Squamosa Linn and Echinops Echinatus Roxb., by might have restored the alterations in motor coordination behavior evaluated by method like catalepsy test, open field test, pole test, grid, gait and forced swim test (Salim et al., 2003).

Oxidative stress refers to the cytologic consequences of a mismatch between the production of free radicals and the ability of the cell to defend against them. This can happen when the production of free radicals increases, scavenging of free radicals or repair of oxidatively modified macromolecules decreases, or both. This imbalance results in a build-up of oxidatively modified molecules. (Halliwell and Gutteridge, 1985; Freeman and Crapo, 1982)

A defect in one or more of the naturally occurring antioxidant defenses could lead to neurodegeneration in PD (Jenner and Olanow, 1996). That can cause cellular dysfunction, and for neurons, it is lethal. Oxidant stress has been implicated in PD because of the coalition of the four biochemical features of the dopaminergic neurons in the substantia nigra, i.e., monoamine oxidase-B activity (Cohen, 1983; Oreland, 1991), autooxidation of dopamine (Graham, 1979; Halliwell and Gutteridge, 1985), accumulation of iron (Youdim and Lavie, 1994), and neuromelanin (Hirsch, 1993).

The extent of lipid peroxidation was estimated by measuring the levels of thiobarbituric acid, a product of lipid peroxidation. Lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acids and its occurrence in biological membranes causes impaired membrane function, impaired structural

integrity, decreased fluidity and inactivation of number of membrane bound enzymes.(Pillai *et al.*, 2007) There is substantial evidence of oxidative damage in the brains of PD patients. Increased levels of the lipid peroxidation product, thiobarbituric acid have been found in the substantia nigra of PD patients (Halliwell et al., 1985).

A reduction in GSH might impair H_2O_2 clearance and promote hydroxyl radical formation and, hence, oxidative stress. In that all antioxidant defenses are interrelated (Sun, 1990), the disturbance in one might derange the balance in all. The depletion in GSH content and enhancement of lipid peroxidation leads to the degeneration of nigrostriatal neurons and, consequently, leads to reduction in the content of catecholamines. The marked restoration of lipid peroxidation as an enhancement of GSH content by pretreatment with hydroethanolic extract of *Annona squamosa* and *Echinops echinatus* is observed in a (Salim et al., 2003) and that of (Tripathi et al.1996).

GSH is the primary low-molecular weight thiol in the cytoplasm and is a major reserve for cysteine. GSH, in conjunction with the reductant NADPH, can reduce lipid peroxides, free radicals, and H2O2. GSH is converted to oxidized glutathione, which is reconverted to GSH by GR, while GST biotransforms the xenobiotics via the mercapturic acid pathway. Because GSH is involved in the detoxification of H2O2, reductions in GSH content could result from increased concentrations of H2O2 and, in the presence of metals, the highly reactive hydroxy radical. Mytilineou et al. (1998). Buthionine sulphoximine (BSO), which is a selective inhibitor of α-glutamylcysteine synthetase (a key enzyme in the synthesis of GSH), induces a reduction in GSH content and is toxic to cultured dopaminergic neurons. GSH depletion enhances the neurodegeneration that is observed when rodents are treated with other toxins, such as 6-OHDA (Pileblad et al., 1989; Wüllner et al., 1996).

Most H_2O_2 in the brain is removed by GPx, which uses it to oxidize GSH (Beckman et al., 1990). Our present study, shows that a restoration in the content of GSH and in the activities of its dependent enzymes might be due to the GSH-enhancing or antioxidant effects of hydroalcholic extract of roots of Annona squamosa Linn., and Echinops echinatus Roxb.

Superoxide dismutase enzymes (SOD) act as an antioxidant and protect cellular components from being oxidized by reactive oxygen species (ROS). Superoxide anion is known to denature enzymes, oxidize lipids and fragment DNA. SOD is an enzyme which acts as a catalyst in the process of dismutation of superoxide into oxygen and hydrogen peroxide. It is therefore a critical antioxidant defense which is present in nearly all cells which are exposed to oxygen (Bowler *et al.,* 1992). It can help neutralize free radicals, and in doing so may limit or stop some of the damage they cause (Gralla *et al.,* 1992). Rotenone treated group showed a decrease in the level of SOD in the brain of animals, thus indicative of production of oxidative stress.

Catalase is an enzymatic antioxidant and helps in neutralizing the toxic effects of hydrogen peroxide. Hydrogen peroxide is not reactive enough to cause a chain of lipid peroxidation reactions, but its combination with superoxide radical produces hydroxyl radical, which is highly reactive and thus initiates lipid oxidation reactions (Dorval *et al., 2003*)

Catalase converts hydrogen peroxide to water and nonreactive oxygen species, thus preventing generation of hydroxyl radical and protecting the cells from oxidative damage. Oxidative stress results in decrease in catalase level. A significant decrease in the level of catalase was observed in hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb.

Our studies show an increase in lipid peroxidation and a decrease in GSH and the activities of related enzymes and of CAT and SOD, observations which are very well supported (Perumal et al., 1992; Kumar et al., 1995).

These toxic effects might be related to the low levels of GSH and diminished activities of antioxidant enzymes, as is clearly shown by the present study. GSH is the primary low-molecular weight thiol in the cytoplasm and is a major reserve for cysteine. GSH, in conjunction with the reductant NADPH, can reduce lipid peroxides, free radicals, and H2O2. GSH is converted to oxidized glutathione, which is reconverted to GSH by GR, while GST biotransforms the xenobiotics via the mercapturic acid pathway. Because GSH is involved in the detoxification of H2O2, reductions in GSH content could result from increased concentrations of H2O2 and, in the presence of metals, the highly reactive hydroxy radical. Mytilineou et al. (1998)

demonstrated that buthionine sulphoximine (BSO), which is a selective inhibitor of α-glutamylcysteine synthetase (a key enzyme in the synthesis of GSH), induces a reduction in GSH content and is toxic to cultured dopaminergic neurons. GSH depletion enhances the neurodegeneration that is observed when rodents are treated with other toxins, such as 6-OHDA (Pileblad et al., 1989; Wüllner et al., 1996).

Parkinsonism was partially protected by the application of antioxidants (Cadet et al., 1989; Perumal et al., 1992; Zafar et al., 2003a,b; Ahmad et al., 2005a,b). Drugs that enhance the availability of dopamine or prevent its breakdown afford protection against PD in humans or in animal models (Parkinson Study Group, 1989; Myllyla et al., 1992).

Brain level of dopamine increase after post treatment would suggest the significance restoration of lost dopaminergic neuron; this could be considered as the index of neuroprotection. (Kumar et al.,1995).

In our study, hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., have both dopamine-enhancing properties and antioxidant potential might have afforded protection. (Tripathi et al., 1996 & Salim et al., 2003). In conclusion, I anticipate that Annona squamosa and Echinops echinatus, having shown anti-parkinsonian properties would open new vistas in the drug treatment of Parkinson’s disease.

SUMMARY AND CONCLUSION

VII. SUMMARY AND CONCLUSION

Drugs from plant source are considered to be safe and efficacious. Hydroethanolic extract *Annona squamosa* Linn., and Hydroethanolic extract *Echinops echinatus* Roxb., have been extensively explored for medicinal values. Even now these plants are been used by the tribal population for various ailments such as diabetes, sexual dysfunction, asthma, and wound. The scientific validation of these plants will open vistas is the drug discovery process.

- The presence of various phytoconstituents in the plant extract was identified by preliminary phytochemical screening. TLC and HPTLC analysis confirmed the presence of various phytoconstituents.
- Total phenolic, flavanoid, ascorbic content in hydroethanolic extract *Annona squamosa* Linn., and Hydroethanolic extract *Echinops echinatus* Roxb., was quantified.
- In vitro antioxidant evaluation was done by DPPH, nitric oxide scavenging, hydroxyl radical scavenging and reducing power. Hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., has shown antioxidant property which was comparable to that of the standard.
- Safety concerns of these plants were evaluated by acute toxicity hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., at the dose level of 2000mg/kg has not produced any signs of behavioral toxicity and mortality.
- In sub acute toxicity testing therapeutic dose (200mg/kg) of hydroethanolic extracts of *Annona squamosa* Linn., and *Echinops echinatus* Roxb., was administered for 28 days. The results of these studies demonstrated the safety of the plants through behavioral, hematological, biochemical and histopathology observations.
- The general behavior evaluation has not shown any change in the behavior of animals would suggest that hydroethanolic extracts of *Annona squamosa* Linn., *Echinops echinatus* Roxb., and the formulation does not possess any neurotoxicity.

- Neuropharmacological screening of hydroethanolic extracts of *Annona squamosa* Linn., *Echinops echinatus* Roxb., and the formulation were done after 6-OH DA administration.
- In catalepsy test hydroethanolic extracts of *Annona squamosa* Linn., *Echinops echinatus* Roxb., and the formulation has produced a significant fall in the cataleptic rigor which is identified as the index of muscle coordination. The improvement in the catalepsy may be mediated through dopaminergic action.
- In open field test the number of square crossed was improved by hydroethanolic extracts of *Annona squamosa* Linn., *Echinops echinatus* Roxb., and the formulation. When compared both of the plants in the formulation has produced significant effect.
- Gait analysis, forced swim test, beam walking test and rota rod test results were compared with 6- OH dopamine treated group. These observations demonstrated a recovery from neurodegeneration induced by 6-OHDA. In all the evaluation the hydroethanolic extracts of *Annona squamosa* Linn., *Echinops echinatus* Roxb., and the formulation has shown neuroprotection in a dose dependent manner.
- The biochemical studies on the rats brain was carried out by evaluating TBARS, in substantia nigra the hydroethanolic extracts of *Annona squamosa* Linn., *Echinops echinatus* Roxb., and the formulation has shown a significant reduction in the level of TBARS.
- GSH in substantia nigra was significantly restored in a dose depend manner when compared with 6- OH Dopamine treated group.
- Glutathione reeducates, Glutathione peroxidase, SOD, catalase in striatum was significantly restored by hydroethanolic extracts of *Annona squamosa* Linn., *Echinops echinatus* Roxb., and the formulation. A high degree of significant was observed in the formulation.
- In the brain neurotransmitter estimation acetylcholine esterase activity was found to be significantly increased which would suggest the cholinesterase activity of the plant.

- ❖ Further, reduction in the MAO after treatment with hydroethanolic extracts of *Annona squamosa* Linn., *Echinops echinatus* Roxb., and the formulation was observed which would suggest the MAO Inhibitory action.
- ❖ Brain level of dopamine increase after post treatment would suggest the significance restoration of lost dopaminergic neuron; this could be considered as the index of neuroprotection.
- ❖ Histopathology studies have shown the restored architecture as an evidence of neuroprotection.
- ❖ Exploration of the possible mode of action was done based on the observation the plant extracts and the formulation would act through enhancing dopaminergic pathways.

In the present study all the results and analysis confirm the neuroprotection status of both the plants. Hydroethanolic extracts of *Annona squamosa* Linn., *Echinops echinatus* Roxb., has produced significant neuroprotection at the dose level of 200mg/kg. As a comparison the formulation of both the plant at the dose level of 200mg/kg has a better neuroprotection against 6-OH Dopamine induced neurodegeneration.

In conclusion the plants *Annona squamosa* Linn., and *Echinops echinatus* Roxb., will prove to be useful in the treatment of Parkinsonism and associated neurodegenerative disorders. Further studies are to be warranted in future.

BIBLIOGRAPHY

IX. BIBLIOGRAPHY

Abbas N, Lucking CB, Ricard S, Durr A, Brice A. A wide variety of mutations in the parkin gene are responsible for autosomal recessive Parkinsonism in Europe. French Parkinson's disease genetics study group and the European consortium on genetic susceptibility in Parkinson's disease. Human Molecular Genetics. 1999; 8(4): 567–74.

Abdullah Shafique Ahmad , Mubeen Ahmad Ansari, Muzamil Ahmad , Sofiyan Saleem Seema Yousuf, Md Nasrul Hoda, Fakhrul Islam, Neuroprotection by crocetin in a hemi-parkinsonian rat model. Pharmacology, Biochemistry and Behavior. 2005; 81: 805 – 813.

Abdullah Shafique Ahmad, Mubeen Ahmad Ansari, Muzamil Ahmad, Sofiyan Saleem Seema Yousuf, Md Nasrul Hoda, Fakhrul Islam, Neuroprotection by crocetin in a hemi-parkinsonian rat model. Pharmacology, Biochemistry and Behavior 2005; 81: 805 – 813.

Adzu B, Amos S, Muazzam I, Inyang US, Gamaniel KS. Neuropharmacological screening of Diospyros mespiliformis in mice. Journal of Ethnopharmacology. 2002; 83:139-143.

Akaikea H, Katsukia T, Kumea T, Maedab. Reactive oxygen species in NMDA receptor-mediated glutamate Neurotoxicity. Parkinsonism and Related Disorders. 1999; 5: 203–207.

Akerud P, Canals JM, Snyder EY, Arenas E. Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson's Disease. J. Neurosci. 200; 2: 8108-8118.

Albin RL, Reiner A, Anderson KD. Preferential loss of striato-external pallidal projection neurons in presymptomatic Huntington's disease. Ann. Neurol. 1992; 31:425-430.

Alvarez-Fischer D, Henze C, Strenzke C, Westrich J, Ferger B, Höglinger GU, Oertel WH, Hartmann A. Characterization of the striatal 6-OHDA model of Parkinson's disease in wild type and a-synuclein-deleted mice. Exp. Neurol. 2008;10:182-193.

Annett LE, Torres EM, Clarke DJ, Ishida Y, Barker RA, Ridley RM. Survival of nigral grafts within the striatum of marmosets with 6-OHDA lesions depends critically on donor embryo age. Cell Transplant. 1997; 6:557–569.

Annie Shirwaikar, Rajendran K, Dinesh Kumar C, Ramgopal Bodla., Antidiabetic activity of aqueous leaf extracts of Annona squamosa in streptozotocin–nicotinamide type 2 diabetic rats. Journal of Ethnopharmacology. 2004; 91:171–175.

Anthony HK, Tsang. Kenny KK, Chung. Oxidative and nitrosative stress in Parkinson's disease. Biochimica Biophysica Act 1792; 2009: 643–650.

Arora A, Sairam RK ,Srivastava G C.Oxidative stress and anti oxidative system in plants. *Curr Sci* 2002; 82: 1227-1238.

Baba M, Nakajo S, Tu PH. Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. Am J Pathol. 1998;152:879–884.

Baldereschi M, Di Carlo A, Rocca WA. Parkinson's disease and parkinsonism in a longitudinal study: two-fold higher incidence in men. Neurology. 2000; 55: 1358–63.

Bang YH, young HL, Jai S, Young SL. Antioxidant benzoylated flavon -3ol glycoside from Celastrus Orbiculantus. 2001; 64: 82-84.

Beauchamp C, Fridovich I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 1971; 44: 276–87.

Beckman JS, Beckman TW, Chen J. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A 1990;87: 1620–1625

Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K,Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. J Neurol Sci 1973; 20:415–455.

Beso JA. Pathophysiology of levodopa-induced dyskinesias in Parkinson's disease: problems with the current model. Ann. Neurol. 2000; 47 (1): 22-34.

Betarbet R, Sherer TB, Greenamyre JT. Animal models of Parkinson's disease. Bioassays. 2002; 24:308–318.

Bloem BR, Irwin I, Buruma OJS, Haan J, Roos RAC, Tetrud JW, et al. The MPTP model: versatile contributions to the treatment of idiopathic Parkinson's disease. J Neurol Sci 1990;97:273–93.

Bower TH. Head trauma preceding Parkinson's disease: A case control study. *Neurology*. 2003; 60: 1610-1615.

Bowler C, Van Montagu M, Inze D. Superoxide dismutase and stress tolerance. Annu Rev Plant Physiol Plant Mol Biol 1992; 4(3): 86–116.

Brian C. Kramer, Jocelyn A, Catherine Mytilineou. Glutathione depletion and oxidative stress. Parkinsonism and Related Disorders. 2002; 8: 385–387.

Brighina L, Frigerio R, Schneider NK. (alpha)-Synuclein, pesticides, and Parkinson disease. A case-control study. Neurology. 2008;12:128-131.

Carder RK, Jackson D, Morris HJ, Lund RD, Zigmond MJ. Dopamine released from mesencephalic transplants restores modulation of striatal acetylcholine release after neonatal 6-OHDA: an in vitro analysis. Exp Neurol 1989; 105:251–9.

Carlberg I, Mannervik B. Glutathione reductase levels in rat brain. J Biol Chem. 1975; 250:5475-80.

Carter RJ, Lione LA, Humby T, Mangiarini L, Mahal A, Bates GP, Dunnett SB, Morton AJ. Characterization of progressive motor deficits in mice transgenic for the human Huntington's disease mutation. J. Neurosci.1999; 19: 3248– 3257.

Chaudhuri PK . Echinozolinone an alkaloid from *Echinops echinatus.* Phytochemistry. *1987;* 26(2): 587-589.

Chavan MJ, Wakte PS, Shinde DB. Analgesic and anti inflammatory activity of Caryophyllene oxide from Annona squamosa L. bark. Phytomedicine. 2010; 17: 149–151.

Chen H, Zhang SM, Hernan MA, Willett WC, Ascherio A. Diet and Parkinson's disease: a potential role of dairy products in men. Ann Neurol 2002; 52: 793–801.

Chen H, Zhang SM, Hernan MA. Nonsteroidal antiinflammatory drugs and the risk of Parkinson disease. Arch Neurol 2003; 60: 1059–64.

Chien-Min Lin, Rong-Dih Lin, Shui-Tein Chen, Yi-Pei Lin, Wen-Ta Chiu, Jia-Wei Lin, Feng-Lin Hsu. Neurocytoprotective effects of the bioactive constituents of Pueraria thomsonii in 6-hydroxydopamine (6-OHDA)-treated nerve growth factor (NGF)-differentiated PC12 cells. Phytochemistry. 2010; 83:139-143.

Christina L Zucha, Daniel Davidb, Livia Ujhelyib, John L Hudsonb, Greg A Gerhardtc, Paul L Kapland, Paula C Bickford. Beneficial effects of intraventricularly administered BMP-7 following a striatal 6-hydroxydopamine lesion. Brain Research. 2004; 10(10): 10–16.

Christopher A Lieu, Allen R Kunselman, Bala V Manyam, Kala Venkiteswaran, Thyagarajan Subramanian. A water extract of Mucuna pruriens provides long-term amelioration of parkinsonism with reduced risk for dyskinesias Parkinsonism and Related Disorders. 2010; 14: 1-8.

Christopher A Lieu, Allen R Kunselman, Bala V Manyama, Kala Venkiteswaran, Thyagarajan Subramanian. A water extract of Mucuna pruriens provides long-term amelioration of parkinsonism with reduced risk for dyskinesias. Parkinsonism and Related Disorders. 2010; 16: 458-465.

Cohen C. The pathobiology of Parkinson's disease: biochemical aspects of dopamine neuron senescence. J Neural Transm 1983; 19:89-103.

Cohen G, Heikkila RE. The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. J. Biol. Chem. 1974; 249: 2447–2452.

Cohen G. Oxy-radical toxicity in catecholamine neurons. Neurotoxicology 1984; 5:77– 82.

Coliborne A. Catalase activity. In: Green Wald RA, editor. CRC handbook of methods for oxygen radical research. Florida: CRC Press; 1985. p. 283–4.

Cotzias GC, Papavasiliou PS, Gellene R. Modification of Parkinsonism: Chronic treatment with L-DOPA. New Engl. J. Med. 1969;280:337-345.

Cotzias, G.C., Papavasiliou, P.S., and Gellene, R. Modification of Parkinsonism: Chronic treatment with L-DOPA. *New Engl. J. Med.*, 1969, *280*:337-345.

Critchley M. Arteriosclerotic Parkinsonism. *Brain* 1929; 5 2: 23–83.

Crofts HS, Dalley JW, Collins P, Van Denderen JC, Everitt BJ, Robbins TW, Differential effects of 6-OHDA lesions of the frontal cortex and caudate nucleus on the ability to acquire an attentional set. Cereb Cortex. 2001; 11:1015–1026.

Cryan JF, Markou A, Lucki I. Assessing antidepressant activity in rodents: recent developments and future needs. Trends Pharmacol Sci. 2002; 23:238–245.

D'Hooge R, Hartmann D, Manil J, Colin F, Gieselmann V, De Deyn PP, Neuromotor alterations and cerebellar deficits in aged aryl-sulfatase A-deficient transgenic mice. Neurosci. Lett. 1999; 273: 93– 96.

Damasceno DC, Volpato GT, Sartori TCF, Rodrigues PF, Perin EA, Calderon, Rudge. Effects of Annona squamosa extract on early pregnancy in rats Phytomedicine 9; 2002: 667–672.

Dandiya PC, Collumbine H. Studies on Acorus calamus: some Pharmacological actions of the volatile oil. Journal of Pharmacology and Experimental Therapy. 1959; 125: 353-359.

Daniel Alvarez-Fischer , Carmen Henze , Corinna Strenzke , Jan Westrich , Boris Ferger ,Günter U. Hoglinger, Wolfgang H. Oertel , Andreas Hartmann. Characterization of the striatal 6-OHDA model of Parkinson's disease in wild type and α-synuclein-deleted mice. Experimental Neurology. 2008; 210: 182–193.

Dauer W, Przedborski S. Parkinson's disease: Mechanisms and models. Neuron 2003;39:889–909.

De Rijk MC, Breteler MM, Den Breeijen JH. Dietary antioxidants and Parkinson disease: The Rotterdam Study. Arch Neurol. 1997; 54: 762–65.

De Rijk MC, Launer LJ, Berger K. Prevalence of Parkinson's disease in Europe: a collaborative study of population-based cohorts. Neurology 2000; 54 (11): 21–23.

Demirkiran M, Bozdemir H, Sarica Y. Vascular Parkinsonism: a distinct, heterogeneous clinical entity. Acta Neurol Scand 2001; 104: 63–67.

Dixit VK, Varma KC. Effects of essential oil of leaves of Blumea lacera DC on central nervous system. Indian Journal of Pharmacology. 1976; 18:7 -11.

Dorr M, Steinberg H, Tomkiewicz M. Persistence of dose related behavior in mice. Nature 1971;231: 121–123.

Dorval J, Hontela A. Role of glutathione redox cycle and catalase in defense against oxidative stress induced by endosulfan in adrenocortical cells of rainbow trout Toxicol Appl Pharmaco 2003; 192 :191–200.

Ecobichon DJ, The basis of toxicity testing, CRC press, Newyork, 2nd edition, 1997; 43-60.

Eisenberg DM, Davis RB , Ettener SL. Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. *JAMA* 1998; 280: 1569-1575.

Eisenberg DM, Davis RB , Ettener SL. Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. *JAMA* 1998; 280: 1569-1575.

Ellman GL, Courtney KD, Ander Jr. V, Featherstone RM. A new and rapid colorimetric determination of Acetylcholinesterase activity. Biochem Pharmacol. 1961; 7: 88-95.

Engber TM. Levodopa replacement therapy alters enzyme activities in striatum and neuropeptide content in striatal output regions of 6-hydroxydopamine lesioned rats. Brain Res. 1991;552: 113-118.

Fadila MB, Sabiha K, Khalida B, Mohamed C et al. Antioxidant activities of alkaloid extracts of two Algerian species of Fumaria: *Fumaria capreolata* and *Fumaria bastardii*. *Rec Nat Prod* 2007;1:28-35.

Farooqui AA, Horrocks LA. Lipid peroxides in the free radical pathophysiology of brain diseases. Cell Mol Neurobiol 1998; 18: 599-608.

Finkel, Richard, Clark, Michelle A, Cubeddu, Luigi X, Lippincott's Illustrated Reviews: Pharmacology, 4th Edition, Pg no.156-163.

Firestone JA, Smith-Weller T, Franklin G, Swanson P, Longstreth Jr WT, Checkoway H. Pesticides and risk of Parkinson disease: a population-based case-control study. Arch Neurol 2005; 62:91-5.

Foleya P, Gerlacha M, Youdimb M B H, Riederer P. MAO-B inhibitors: multiple roles in the therapy of neurodegenerative disorders. Parkinsonism and Related Disorders. 2000; 6: 25–47.

Freeman BA, Crapo JD. Biology of disease: free radicals and tissue injury. Lab Invest 1982; 47:412–26.

Fujimori H, Cobb D. Central nervous system depressant activity of MA 1337,3-[3, 4-m-clorophenyl-1-piperazyl), propyl]-2-4 (1H,3H)quinozolinedione hydrochloride. The Journal of Pharmacology and Experimental Therapeutics. 1965; 148: 151–157.

Gamboa ET, Wolf A, Yahr MD, Harter DH, Duffy PE, Barden HJ, Hsu KC. Influenza virus antigen in post encephalitic Parkinsonism brain. Detection by immunofluorescence, *Arch. Neurol.* 1974; 31: 228–232.

Garrat DC. The quantitative analysis of drugs Happman and Hall Japan 3 (1986); 456-458.

Gerfen RC. Dopamine differentially regulates dynorphin, substance P, and enkephalin in striatal neurons: in situ hybridization histochemical analysis. J. Neumsci. 1991; 11:1016-1031.

Gerlach M, Double KL, Youdin MB, Riederer P. Potential sources of increased iron in the substantia nigra of parkinsonian patients. J Neural Transm Suppl 2006; 70:123-42.

Gerlach M, Double KL, Youdin MB, Riederer P. Potential sources of increased iron in the substantia nigra of parkinsonian patients. J Neural Transm Suppl. 2006; 70:123-42.

Ghosh A, Roy A, Liu X, Kordower JH, Mufson EJ, Hartley DM, Ghosh S, Mosley RL, Gendelman HE, Pahan K. Selective inhibition of NF-kappa B activation prevents dopaminergic neuronal loss in a mouse model of Parkinson's Disease.2007;104, 18754-18759.

Gibanananda, R., Sayed, A.H., 2002. Oxidants, antioxidants and carcinogenesis. Ind. J. Exp. Biol. 40, 1213–1232.

Goldstein LB. and Davis JN, Beam-walking in rats: studies towards developing an animal model of functional recovery after brain injury. J Neurosci Methods.1990;31(2):101-107.

Goodman A, Gilman, Rail TW, Nies AS, Taylor P, Editors. The Pharmacological Basis of Therapeutics. Pergamon Press 1990; 34: 383–435.

Govindarajan R, Vijayakumar M, Rawat AKS, Mehrotra S. Free radical scavenging potential of picrorhiza kurroam Royle ex Benth. *Indian J Exp* 2003; 41:875-879.

Graham DG. On the origin and significance of neuromelanin. Arch Pathol Lab Med 1979;103: 359–62.

Gralla EB, Kosman D J. Molecular genetics of superoxide dismutases in yeasts and related fungi. Adv Genet 1992; 30: 251 -19.

Gulrana Khuwaja, Mohd. Moshahid Khan, Tauheed Ishrat, Ajmal Ahmad Syed Shadab Raza, Mohammad Ashafaq, Hayate Javed, M. Badruzzaman Khan, Andleeb Khan, Kumar Vaibhav, Mohammed M. Safhi, Fakhrul Islam. Neuroprotective effects of curcumin on 6-hydroxydopamine-induced Parkinsonism in rats: Behavioral,

neurochemical and immunohistochemical studies. Brain Research. 2011; 368: 254-263.

Gupta SS. Prospects and perspectives of natural plant products in medicine. Indian J Pharmacol 1994; 26:1–12.

Haass C, Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol. 2007; 8(2):101–12.

HaiJun Yang, Ning Zhang, Xiang Li, Liwei He, Jianwei Chen. New nonadjacent bis-THF ring acetogenins from the seeds of Annona squamosa. Fitoterapia 2009; 80: 177–181.

Halliwell B, Gutteridge J. Oxygen radicals and the nervous system**.** Trends Neurosci 1985; 8: 22-29.

Halliwell B, Gutteridge JMC. Oxygen radicals and the nervous system. Trends Neurosci 1985; 8: 22–9.

Hannah S, Rushton R, Tinson C. Modification of the effects of an amphetamine-barbiturate mixture by the past experience of rats. Nature 1961;2: 533–535.

Haobam R, Sindhu KM, Chandra G. Swim-test as a function of motor impairmentin MPTP model of Parkinson's disease: a compariative study in two mouse strains. Behavioural Brain Research. 2005; 163: 159–167.

Harbone J.B. phytochemical methods. Chapman and hall, London 1st edition. 1973; 60-63.

Hardy J, Cookson MR, Singleton A. Genes and Parkinsonism. Lancet Neurol 2003; 2: 221–28.

Heh-In Im1, Wan Seok Joo, Eunjoo Nam, Eun-sun Lee, Yu-jin Hwang, and Yong Sik Kim. Baicalein Prevents 6-Hydroxydopamine-Induced Dopaminergic Dysfunction and Lipid Peroxidation in Mice. J Pharmacol Sci. 2005; 98: 185 – 189.

Henderson JM, Annett LE, Ryan LJ, Chiang W, Hidaka S, Torres EM, Subthalamic nucleus lesions induce deficits as well as benefits in the hemiparkinsonian rat. Eur J Neurosci 1999; 11: 2749–57.

Henderson JM, Watson SH. Convulsive and postural effects of lesioning the mid-susbstantia nigra pars reticulata in naive and 6-hydroxydopamine lesioned rats. Brain Res Bull 2003; 60:179– 85.

Hirsch EC, Brandel JP, Galle P, Javoy-Agid F, Agid Y. Iron and aluminum increase in the substantia nigra of patients with Parkinson's disease: an X-ray microanalysis. J. Neurochem. 1991;56: 446– 451.

Hu C, Kitts DD.Studies on the antioxidant activity of *Echinacea* root extract. *J Agric Food Chem* 2007; 48: 1466-1472.

Hua Y, Schallert T, Keep RF, Wu J, Hoff JT, Xi G. Behavioral tests after intracerebral hemorrhage in the rat. Stroke. 2002; 33:2478-2484.

Huang X, Chen PC, Poole C. APOE-epsilon2 allele associated with higher prevalence of sporadic Parkinson disease. Neurology 2004; 62: 2198–202.

Huxley. A. The New RHS Dictionary of Gardening. 1992. MacMillan Press 1992 ISBN 0-333-47494-5.

Ikonomidou C, Turski L. Neurodegenerative disorders: clues from glutamate and energy metabolism. Crit. Rev. Neurobiol. 1996; 10: 239-263.

Imam SZ, Ali SF. Selenium an antioxidant attenuates methamphetamine indiced dopaminergic toxicity and peroxynitrile generation. Brain Res. 2000; 855: 186–191.

Irwin S. Comprehensive observational assessment: A systematic, quantitative procedure for assessing the behavioral and physiologic state of the mouse. Psychopharmacologia. 1968; 13: 222–257.

Irwin, S., 1964. Animal and clinical Pharmacologic techniques in drug evaluation. Year Book Medical Publishers, Philadelphia, pp. 36–38.

Iryna Ziabreva, Clive G. Ballard, Dag Aarsland, Jan-Petter Larsen, Ian G. McKeith, Robert H. Perry, Elaine K. Perry. Lewy body disease: Thalamic cholinergic activity related to dementia and parkinsonism Neurobiology of Aging. 2006; 27 : 433–438.

Jankovic J. Pathophysiology and assessment of parkinsonian symptoms and signs. In Handbook of Parkinson's disease. New York: Taylor and Francis Group, LLC, 2007:79–104.

Jaswanth A, Ramanathan P, Ruckmani K. Evaluation of mosquitocidal activity of Annona squamosa leaves against filarial vector mosquito. Indian J Exp Biol. 2002; 40(3):363-5.

Jaswinder S. Bains. Christopher A. Shaw. Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. Brain Research Reviews 1997; 25: 335–358.

Jellinger KA. The role of iron in neurodegeneration: prospects for pharmacotherapy of Parkinson's disease. Drugs Aging 1999; 14: 114-40.

Jenner P, Olanow CW. Oxidative stress and the pathogenesis of Parkinson's disease. Neurology 1996; 47(6 Suppl. 3):161–70.

Jeon BS, Jackson-Lewis V, Burke RE. 6-Hydroxydopamine lesion of the rat substantia nigra: time course and morphology of cell death. Neurodegeneration.1995; 4: 131–137.

Jin Sup Shima, Hyo Geun Kima, Mi Sun Jua, Jin Gyu Choia, Seo Young Jeong, Myung Sook Oha. Effects of the hook of Uncaria rhynchophylla on neurotoxicity in the 6-hydroxydopamine model of Parkinson's disease. Journal of Ethnopharmacology 2009; 126: 361–365.

Johnson CC, Gorell JM, Rybicki BA, Sanders K, Peterson EL. Adult nutrient intake as a risk factor for Parkinson's disease. Int J Epidemiol 1999; 28: 1102–09.

Jollow D, Mitchell JR, Zampaglione N, Gillette JR. Bromobenzeneinduced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenzene oxide as the hepatotoxic metabolite. Pharmacology 1974; 11:151–169.

Jollow DJ, Mitchell JR, Zampagloine N, Gillete JR. Bromobenzene-induced liver necrosis: protective role of glutathione and evidence for 3, 4 bromobenzeneoxide as the hepatotoxic intermediate. Pharmacology 1974; 11:151-69.

Jonsson G. Chemical lesioning techniques: monoamine neurotoxins. In: Handbook of chemical neuroanatomy. Methods in chemical neuroanatomy. Ed 1, Vol 1, pp 463–507. Amsterdam: Elsevier Science Publishers B.V., 1983.

Khan MA, Niazi HA, Khalid S (2000), Ethnobotany and taxonomic studies of Echinops echinatus Roxb. (Untkatara) from Potohar region of Pakistan, Hamdard Medicus, 43(3), 61-64.

Khar Ashok , Pardhasaradhi BVV, Reddy Madhurima, Ali Mubarak A, Kumari Leela A. Differential cytotoxic effects of Annona squamosa seed extracts on human tumour cell lines: Role of reactive oxygen species and glutathione. J. Biosci. 2005; 30(2):237- 244.

Khar Ashok, Pardhasaradhi B V V, Reddy Madhurima, Ali Mubarak A, Kumari Leela A. Antitumour activity of Annona squamosa seed extracts is through the generation of free radical s and induction of apoptosis. Indian journal of Biochemistry and Biophysics. 2004; 41: 167-172.

Kokate CK. Practical Pharmacognosy, vallabha prakashan, delhi, 4th edition. 1994; 107-111.

Kong LD, Christopher HK, Chengb RX Tan. Inhibition of MAO A and B by some plant-derived alkaloids, phenols and anthraquinone. Journal of Ethnopharmacology 2004; 91: 351–355.

Kostic V, Przedborski S, Flaster E, Sternic N. Early development of levodopa-induced dyskinesias and response fluctuations in young-onset Parkinson’s disease. Neurology 1991; 41: 202–205.

Kuhn WL, Maanen FV. Central nervous system effects of thalidomide. Journal of Pharmacology.1961;134: 60–68.

Kulkarni SK, Hand Book of Experimental Pharmacology, 3rd ed., Vallabh Prakashan, New Delhi, 2009, 123-124.

Kumlesh K. Dev, Herman van der Putten, Bernd Sommer, Giorgio Rovelli. Part I: Parkin-associated proteins and Parkinson's disease. Neuropharmacology 2003; 45: 1–13.

Lai BC, Marion SA, Teschke K, Tsui JK. Occupational and environmental risk factors for Parkinson's disease. Parkinsonism Relat Disord 2002; 8: 297–309.

Lee CS, Samii A, Sossi V, et al. In vivo positron emission tomographic evidence for compensatory changes in presynaptic dopaminergic nerve terminals in Parkinson's disease. Ann Neurol. 2000; 47: 493–503.

Logroscino G, Marder K, Cote L, Tang MX, Shea S, Mayeux R. Dietary lipids and antioxidants in Parkinson's disease: a population based, case-control study. Ann Neurol 1996; 39: 89–94.

Loomis, T.A., Hayes, A.W., 1996. Loomis's Essentials of Toxicology, 4th ed. Academic Press, California, pp. 208-245.

Lowry OH, Rosebrough NJ, Farr WL, Randall RJ. Protein measurements with the folin phenol reagent. J BioChem . 1951; 193; 265-275.

Luchsinger JA, Tang MX, Miller J, Green R et al. Relation of higher folate intake to lower risk of Alzheimer's disease in elderly. *Arch Neurol* 2007; 64(1):86-92.

Luchsinger JA, Tang MX, Miller J, Green R et al. Relation of higher folate intake to lower risk of Alzheimer's disease in elderly. *Arch Neurol* 2007; 64(1):86-92.

Lucki I. The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. Behav Pharmacol. 1997; 8: 523–532.

Lucki I. The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. Behav Pharmacol. 1997; 8:523–532.

Mandel RJ, Randall PK. Quantification of lesion-induced Dopaminergic supersensitivity using the rotational model in mouse. Brain Res. 1985; 330:358 –363.

Mandelkow E, Von Bergen M, Biernat J, Mandelkow EM. Structural Principles of tau and the paired helical filaments of Alzheimer's disease. Brain Pathol. 2007; 17:83-90.

Marcel Dekker, Houghton PJ, Howes MJ. Natural products and derivatives affecting neurotransmission relevant to Alzheimer's and Parkinson's disease. Neurosignals. 2005; 14: 6–22.

Maria Nonfon, Folker Lieb, Heinrich Moeschler and Detlef Wendisch. Four annonins from annona squamosa. Phytochemtstry 1990; 29(6): 1951-1954.

Marklund S and Marklund G. Involvement of the superoxide anion radical in the autooxidation of pyragallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974; 47: 469.

Marta MC, Souza, Claudia ML, Bevilaqa, Selene M Morais, Cicero TC. Anthelmintic acetogenin from Annona squamosa L. seeds. Annuals of the Brazilian academy of sciences. 2008; 80(2): 271-277.

Matsumura K, Kabuto H, Makino H, Ogawa N. Pole test is a useful method for evaluating the mouse movement disorder caused by striatal dopamine depletion. J Neurosci Methods. 1997; 73:45-48.

Matthew D Walker, Katherine Dinelle, Rick Kornelsen, Anna Lee, Matthew J Farrer, Jon Stoessl A, Vesna Sossi. Measuring dopaminergic function in the 6-OHDA-lesioned rat: a comparison of PET and Microdialysis EJNMMI Research 2013; 3:69-80.

McGeer PL, McGeer EG. Inflammation and degeneration in Parkinson's disease. Parkinsonism Relat Disord. 2004; 10 (1): 3–7.

Meir S, Kanner J, Abiri B, Hadas SP. Determination and involvement of aqueous reducing compounds in oxidative systems of various senescing leaves. J Agr Food Chem.1995; 43: 13-18.

Meir S, Kanner J,Akiri B, Hadas SP. Determination and involvement of aqueous reducing compounds in oxidative defense system of various *scenescing* leaves. *J Agric Food Chem* 1995; 43: 1813-1817.

Mi Sun Ju, Hyo Geun Kim, Jin Gyu Choi, Jong Hoon Ryu, Jinyoung Hur, Youn Jung Kim, Myung Sook Oha. Cassiae semen a seed of Cassia obtusifolia has neuroprotective effects in Parkinson's disease models. Food and Chemical Toxicology. 2010; 48: 2037–2044.

Mi Sun Ju, Pyeongjae Lee, Hyo Geun Kim, Ki Yong Lee, Jinyoung Hur, Seung-Hun Cho, Sang Hyun Sung, Myung Sook Oha. Protective effects of standardized Thuja orientalis leaves against 6-hydroxydopamine-induced neurotoxicity in SH-SY5Y cells. Toxicology in Vitro 2010; 24: 759–765.

Mink JW, Thach WT. Basal ganglia intrinsic circuits and their role in behavior. Curr. Opin. Neurobiol. 1993; 3:950-957.

Mink JW. The basal ganglia: focused selection and inhibition of competing motor programs. Progress in Neurobiology, 1996; 50: 381-425.

Miodrag, Das TK, Shepherd RJ. Normal pressure hydrocephalus presenting as Parkinson's Syndrome. *Postgraduate Medical Journal* 1987; 63: 113-115.

Mody I, MacDonald JF. NMDA receptor-dependent excitotoxicity: the role of intracellular Ca2+ release. Trends Pharmacol. Sci. 1995; 16: 356–359.

Moghal S, Rajput AH, DArcy C, Rajput R. Prevalence of movement disorders in elderly community residents. Neuroepidemiology 1994; 13:175-8.

Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller D. Differential distribution of glutathione and glutathione related enzymes in rabbit kidneys: possible implication in analgesic neuropathy. Cancer Res. 1984; 4:5086–91.

Mona Agrawal, Yogesh Agrawal, Prakash Itankar, Arun Patil, Jayshree Vyas, Amruta Kelkar. Phytochemical and HPTLC Studies of Various Extracts of Annona squamosa (Annonaceae). International Journal of PharmTech Research. 2012; 4:364-368.

Morales R, Green KM, Soto C. Cross currents in protein misfolding disorders: interactions and therapy. CNS Neurol Disord Drug Targets. 2009; 8(5):363–71.

Morens DM, Grandinetti A, Waslien CI, Park CB, Ross GW, and White LR. Case-control study of idiopathic Parkinson's disease and dietary vitamin E intake. Neurology 1996; 46: 1270–74.

Morita H, Toru Iizuka, Chee-Yan Choo, Kit-Lam Chan, Koichi Takeyad Junichi Kobayashie. Vasorelaxant activity of cyclic peptide, cyclosquamosin B from Annona squamosa. Bioorganic & Medicinal Chemistry Letters. 2006 ;16 : 4609–4611.

Mukhlesur Rahman M, Shahnaj Parvin, Ekramul Haque M, Ekramul Islam M, Mohammad A, Mosaddik. Antimicrobial and cytotoxic constituents from the seeds of Annona squamosa. Fitoterapia 2005; 76: 484–489.

Muralikrishnan D, Mohanakumar KP. Neuroprotection by bromocriptine against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine induced neurotoxicity in mice. FASEB J. 1998; 12:905-12.

Murugesan T, Gosh L, Das J, Pal M, Saha BP. CNS Activity of Jussiaea suffruticosa Linn. Extract in rats and mice. Journal of Pharmacy and Pharmacology. 1999;5: 663-666.

Muzamil Ahmad, Seema Yousuf, M. Badruzzaman Khan , Md. Nasrul Hoda , Abdullah Shafique Ahmad, Mubeen Ahmad Ansari , Tauheed Ishrat , Ashok Kumar Agrawal , Fakhrul Islam. Attenuation by Nardostachys jatamansi of 6-hydroxydopamine-induced Parkinsonism in rats: behavioral, neurochemical, and immunohistochemical studies. Pharmacology, Biochemistry and Behavior. 2006; 83: 150–160.

Muzamil Ahmad, Sofiyan Saleem, Abdullah Shafique Ahmad,Seema Yousuf, Mubeen Ahmad Ansari, M Badruzzaman. Ginkgo biloba affords dose-dependent protection against 6-hydroxydopamine-induced Parkinsonism in rats: Neurobehavioural, Neurochemical and Immunohistochemical evidences. Journal of Neurochemistry. 2005; 93: 94–104.

Mytilineou C, Leonardi EK, Radcliffe P, Heinonen EH, Han SK. Deprenyl and desmethylselegiline protect mesencephalic neurons from toxicity induced by glutathione depletion. J Pharmacol Exp Ther 1998;284: 700–6.

Nayebi MA, Reyhani-Rad S, Saberian M, Azimzadeh S and Samini M. Buspirone improves 6-hydroxydopamine-induced catalepsy through stimulation of nigral 5-HT1A receptors in rat, Pharmacological Reports 2010; 62(2): 258-264.

Nayebi MA, Sheidaei M. Buspirone improves haloperidol-induced Parkinson's disease in mice through 5-HT1A receptors. Daru, 2010; 18: 41-45.

Nicholls DG, Budd SL. Neuronal excitotoxicity: the role of mitochondria. Biofactors. 1998; 8: 287–299.

Nicolaas I. Bohnena,, Roger L., Albinb. The cholinergic system and Parkinson disease. Behavioural Brain Research 2010; 18: 41-45.

OECD, 2001. Organization for Economic Cooperation and Development. Guideline for Testing of Chemicals. Acute Oral Toxicity Acute Toxic Classic method (423).17th December 2007.

OECD, 2008. Guideline for the testing of chemicals. Repeated dose 28-day oral toxicity study in rodents (407). 3rd October 2008.

Ohawa H, Ohisi N, Yagi K. Assay of lipid peroxidation in animal tissue by thiobarbutric acid reaction. Anal Biochemistry .1986; 44: 307-315.

Ohkawa H, Ohisi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-358.

Olanow CW, Tatton WG. Etiology and pathogenesis of Parkinson's disease. Annu. Rev. Neurosci. 1999; 22: 123–144.

Oliveri RL, Zappia M, Annesi G, Bosco D, Quattrone A. The parkin gene is not involved in late-onset Parkinson's disease. Neurology 2001; 57(2): 359–62.

Omaye ST, Turnbull JD and Sauberlich HE. Selected methods for the determination of ascorbic acid in animal cells, tissues and fluids. Methods Enzymol. 1979; 62:3-11.

Padashetty SA, Mishra SH. Effect of terpenoidal fraction of *Echinops echinatus* roots on reproductive parameters of male rats, Journal of Natural Medicines. 2005; 61(4): 452-457.

Panda S, Kar A. Annona squamosa seed extract in the regulation of hyperthyroidism and lipid-peroxidation in mice: Possible involvement of quercetin. Phytomedicine 2007; 14:799–805.

Panda S, Kar A. Antidiabetic and antioxidative effects of Annona squamosa leaves are possibly mediated through quercetin-3-O-glucoside. Biofactors 2007; 31(3):201-10.

Paramjit Grover, Singh SP, Prabhakar PV, Utkarsh A. Reddy, Balasubramanyam A, Mahboob M, Rahman M F, Sunil Misra. In vivo assessment of genotoxic effects of Annona squamosa seed extract in rats Food and Chemical Toxicology. 2009; 47:1964–1971.

Parent A, and Cicchetti F. The current model of basal ganglia organization under scrutiny. Mov. Disord. 1998; 13:199-202.

Park M, Ross GW, Petrovitch H. Consumption of milk and calcium in midlife and the future risk of Parkinson disease. Neurology 2005; 64: 1047–51.

Paulson HL, Stern MB. Clinical manifestations of Parkinson's disease. In: Watts RL, Koller WC, eds. Movement Disorders: Neurological Principles and Practice. New York: McGraw-Hill, 1997:183–199.

Penney J. and Young A.M. Striatal inhomogeneities and basal ganglia function. Mov. Disord. 1986; 1: 3-15.

Perry TL, Godin DV, Hansen S. Parkinson's disease: a disorder due to nigral glutathione deficiency? Neuroscience Letters 1982; 33: 305–310.

Perumal AS, Gopal VB, Tordzro WK, Cooper TB, Cadet JL. Vitamin E attenuates the toxic effects of 6-hydroxydopamine on free radical scavenging systems in rat brain. Brain Res Bull 1992;29: 699–701.

Pileblad E, Magnusson T, Fornstedt B. Reduction of brain glutathione by Lbuthionine sulfoximine potentiates the dopamine depleting action of 6- hydroxydopamine in rat striatum. J Neurochem 1989;52: 978–80.

Pillai A, Parikh V, Terry AV, Mahadik SP. Long term antipsychotic treatments and crossover studies in rats: differential effects of typical and atypical agents on the expression of antioxidant enzymes and membrane lipid peroxidation in rat brain. J Psychiatr Res. 2007; 41: 372–386.

Pinna S, Pontis N, Schintu N, Simola S, Kasture M, Morelli. Assessment of symptomatic and neuroprotective efficacy of Mucuna pruriens seed extract in rodent model of Parkinson's disease. Parkinsonism and Related Disorders. 2009; 15: 129–139.

Pisa M. Regional specialization of motor functions in the rat striatum: implications for the treatment of Parkinsonism, Prog. Neuro. Pharmacol. Biol.Psychiatry.1998. 12: 217–224.

Prabhu VM, Karandh SK, Rao A, Vidya PM, Sudhakar K. Effect of Nardostachys jatamansi on biogenic amines and inhibitory amino acids on rat brain. Planta Med 1994; 60:114–217.

Rajesh Kumar Gupta , Achyut Narayan Kesari , Sandhya Diwakar, Ameetabh Tyagia, Vibha Tandona, Ramesh Chandraa, Geeta Watal. In vivo evaluation of anti-oxidant and anti-lipidimic potential of Annona squamosa aqueous extract in Type 2 diabetic models. Journal of Ethaopharmacology 2008; 118: 21-25.

Rajput A, Rajput AH. Progressive supranuclear palsy: clinical features, pathophysiology and management. Drugs Aging 2001;18: 913–25.

Rajput A. Frequency and cause of Parkinson's disease. Can J Neurol Sci 1992; 19 (1): 103–07.

Raju TR, Kutty BM, Sathyaprabha TN, Shankarnarayana Rao BS. Brain and behavior. National Institute of Mental Health and neurosciences, Bangalore. 2004;1: 134-141.

Rang H.P, Dale M.M, Ritter J.M. and Flower R.J.. Rang and Dale's Pharmacology, 6th edition, pg no. 517-521.

Reddy DS, Kulkami, SK. The nootropic and antiamnesic effects of neurosteroids on aging and dizocilpine-induced learning impairment. Brain Research I 999; 1:215- 229.

Richard A. Helms, David J. Quan, Eric T. Herfindal, Dick R. Gourley. Textbok of therapeutics, drugs and disease management. 8th edition; page no. 1646-1649.

Rieker C, Engblom D, Kreiner G, Domanskyi D, Schober A, Stotz S, Neumann M, Yuan X, Grummt I, Schutz G, Parlato R. Nucleolar Disruption in Dopaminergic Neurons Leads to Oxidative Damage and Parkinsonism through Repression of Mammalian Target of Rapamycin Signaling. Journal of Neuroscience. 2011; 31 (2): 453.

Rinne JO, Lee MS, Thompson PD, Marsden CD. Corticobasal degeneration: a clinical study of 36 cases. *Brain* 1994; 117: 1183–96.

Rita de Cassia Seffrin, Ikkei Shikano, Yasmin Akhtar, Murray B. Isman. Effects of crude seed extracts of Annona atemoya and Annona squamosa L. against the cabbage looper, Trichoplusia in the laboratory and greenhouse. Crop Protection 2010; 29: 20–24.

Roeling TA, Docter GJ, Voorn P, Melchers BP, Wolters EC, Groenewegen HJ. Effects of unilateral 6-hydroxydopamine lesions on neuropeptide immunoreactivity in the basal ganglia of the common marmoset, Callithrix jacchus, a quantitative immunohistochemical analysis. J Chem Neuroanat 1995; 9:155–164.

Roesler, Rodrigo R. Catharino, Luciana G. Malta, Marcos N. Eberlin, Glaucia Pastore. Antioxidant activity of Annona crassiflora: Characterization of major components by electrospray ionization mass spectrometry. Food Chemistry 2007; 104 :1048–1054.

Rotruck JT, Pope AL, Ganther HE. Swanson AB. Selenium: Biochemical roles as a component of glutathione peroxidese. Science 1973; 179: 588-590.

Rozas G, Liste I, Guerra HJ, Labandesia JL. An automated rotarod method for quantitative drug-free evaluation of overall motor deficits in rat models of Parkinsonism. Brain Res Protocols. 1995; 245:151-4.

Rudzik, A.D., Hester, J.B., Tang, A.H., Stray, R.N., Friss, W. The Benzodiazepines. In: Garrattini, S. , Mussinii, E., Randall, L.O. (Eds.). Raven Press, New York. 1973. 285–297.

Rushton R, Steinberg H, Tinson C. Modification of the effects of an amphetamine-barbiturate mixture by the past experience of rats. Nature 1961;192: 533-535.

Ruxandra Iancu, Paul Mohapel, Patrik Brundin, Gesine Paul. Behavioral characterization of a unilateral 6-OHDA-lesion model of Parkinson's disease in mice. Behavioural Brain Research. 2005; 162: 1-10.

Saleem Mohamed TS, Chetty Madhusudana C, Ramkanth S, Rajan VST, Kumar Mahesh K, K Gauthaman. "Hepatoprotective herbs- A Review." Int. J.Res. Pharm.Sci. 2010; 1: 1-5.

Salim S, Ahmad M, Sayeed I, Ahmad AS, Islam F. Protective effect of Nardostachys jatamansi in rat cerebral ischemia. Pharmacol Biochem Behav 2003; 74(2): 481–6.

Saunders-Pullman R. Estrogens and Parkinson disease: neuroprotective, symptomatic, neither, or both? Endocrine 2003; 21: 81–87.

Schallert T, Hall S. "Disengage" sensorimotor deficit following apparent recovery from unilateral dopamine depletion. Behav Brain Res 1988; 30: 15–24.

Schleimer SB, Johnston GAR, Henderson JM. Novel oral drug administration in an animal model of neuroleptic therapy. J Neurosci Methods, Schmidt WJ, Bubser M. Anticataleptic effects of the N-methyl-daspartate antagonist MK-801 in rats. Pharmacol Biochem Behav 1989; 32:621–3.

Schlumpf M, Lichtensteiger W, Langemann H. A flurometric micro method for the simultaneous determination of serotonin, noradrenaline and dopamine in milligram amounts of brain tissue. Biochem Pharmacol 1974; 23: 2337-2446.

Scholtissen B, Verhey FRJ, Steinbusch HWM, Leentjens AFG, Serotonergic mechanisms in Parkinson's disease: Opposing results from preclinical and clinical data. Journal of Neural Transmission, 2006; 113: 59-73.

Schurr A, Livine A. A differential inhibition of mitochondrial monoamino oxidase from brain by hasish components. Biochemical Pharmacology 1976; 25:1201-1203.

Sethi KD, Zamrini EY. Asymmetry in clinical features of drug-induced parkinsonism. J Neuropsych Clin Neurosci 1990; 2:64–66.

Shah ZA, Vohora SB. Antioxidant/restorative effects of calcined gold preparations used in Indian systems of medicine against global and focal models of ischaemia. Pharmacol Toxicol 2002; 90(5):254–9.

Shane Grealish, Bengt Mattsson, Peter Draxler and Anders Bjorklund. Characterisation of behavioural and neurodegenerative changes induced by intranigral 6-hydroxydopamine lesions in a mouse model of Parkinson's disease. European Journal of Neuroscience 2010; 31: 2266–2278.

Shanker KS, Kanjilal S, Rao BV, Kishore KH, Misra S, Prasad RB. Isolation and antimicrobial evaluation of isomeric hydroxy ketones in leaf cuticular waxes of Annona squamosa. Phytochem Anal. 2007; 18(1):7-12.

Shinya A, Suto A, Kawakami M, Sakamoto H, Umemura T, Kawaoka Y, Kasai N, Ito T. Neurovirulence of H7N7 influenza -A virus: brain stem encephalitis accompanied with aspiration pneumonia in mice. *Arch. Virol.* 2005;150:1653–1660.

Sing B, Ram SN, Pandey VB, Joshi VK, Gambhir SS. Studies on antiinflammatory activity of taraxasterol acetate from *Echinops echinatus* in rats and mice, Phytotherapy Research, 1991;5(3): 103–106.

Singh B, Gambhir SS, Pandey VB, Joshi VK. Anti-inflammatory activity of Echinops echinatus. J Ethnopharmacol. 1999; 25(2): 189-199.

Singh Sanjiv, Manvi FV, Nanjwade Basavraj, Nema Kumar Rajesh. Antihyperlipidemic Screening of polyherbal formulation of Annona squamosa and Nigella sativa. International Journal of Toxicological and Pharmacological Research. 2010; 2(1): 1-5.

Singh UP, Pandey VB, Singh KN and Singh RDN. Antifungal activity of some new flavones and flavone glycosides of *Echinops echinatus*, Canadian Journal of Botany.1998; 66 (9): 1901-1903.

Sinha AK, Colorimetric assay of Catalase. Anal Biochem 1972; 47: 389-394.

Sofia Eram, Mahmood Ahmad, Shafia Arshad. Experimental evaluation of *Echinops echinatus* as an effective hepatoprotective. Academic Journals .2013; 8(39):1919-1923

Sofia Eram, Mahmood Ahmad, Shafia Arshad. Experimental evaluation of *Echinops echinatus* as an effective hepatoprotective. Academic Journals .2013; 8(39):1919-1923

Speciale SG. MPTP: insights into parkinsonian neurodegeneration. Neurotoxicol Teratol 2002; 24: 607-20.

Stah A, Moberg P, Ytterberg J, Panfilow O, Brockenhus Von Lowenhein H, Nilsson F. Isolation and identification of a novel mitochondrial metalloprotease that degrades targeting presequences in plants. Journal of Biological Chemistry. 2002; 277: 41931-41939.

Stoessl AJ, Rivest J. Differential diagnosis of Parkinsonism. Can J Neurol Sci 1999; 26 (2): 1–4.

Tan EK, Khajavi M, Thornby JI, Nagamitsu S, Jankovic J, Ashizawa T. Variability and validity of polymorphism association studies in Parkinson's disease. Neurology 2000; 55: 533–38.

Tillerson JL, Michael Caudle W, Reverson ME, Miller GW. Detection of behavioural impairments correlated to neurochemical deficits in mice treated with

moderate dose of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine. Exp Neurol. 2002;178:80-90.

Tolosa ES, Santamaria J. Parkinsonism and basal ganglia infarcts. Neurology 1984; 4: 1516–8.

Tsang KL, Ho SL, Lo SK. Estrogen improves motor disability in parkinsonian postmenopausal women with motor fluctuations. Neurology 2000; 54: 2292–98.

Ungerstedt U, Arbuthnott GW. Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. Brain Res 1970; 24: 485–93.

Ungerstedt, U. 6-hydroxy-dopamine induced degeneration of central monoamine neurons. Eur. J. Pharmacol. 1968;5: 107-110.

Ungerstedt, U. 6-Hydroxydopamine-induced degeneration of the nigrostriatal dopamine pathway: The turning syndrome. Pharmacology & Therapeutics. Part B: General and Systematic Pharmacology.1976; 2: 37-40.

Utley HC, Bernheim F, Hochslein P. Effect of sulfhydryl reagent on peroxidation in microsome. Arch Biochem Biophys. 1967; 260: 521–31.

Virginie Lickera, Enikö Kövarib, Denis F. Hochstrasserc, Pierre R. Burkharda,d, Proteomics in human Parkinson's disease research. journal of proteomics 2009; 73: 1 0 – 2 9

Von Bohlen und Halbach, A. Schober , K. Krieglstein. Genes, proteins, and neurotoxins involved in Parkinson’s disease. Progress in Neurobiology. 2004; 73: 151–177.

Wallina C, Jennersjo AK, Grane Rusb. Prevalence of dementia and regional brain syndromes in long-standing Parkinson’s disease. Parkinsonism and Related Disorders. 1999; 5: 103–110.

Warrell DA and Weatherall DJ. *Oxford textbook of medicine* vol.2, 2nd edition. 2007:P. 1035.

Warrick JM, Paulson HL, Gray-Board GL, et al. Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in Drosophila. Cell. 1998; 93(6):939–49.

Warrier PK, Nambiar VPK, Ramankutty C, Indian medicinal plants: A compendium of 500 species. 1994; 3: 63-69.

Weber CA, Michael Ernst. Antioxidants, Supplements, and Parkinson's disease. The Annals of Pharmacotherapy. 2006; 40: 935-938.

Wenning GD, Ben-Shlomo Y, Magalhaes M, Daniel SE, Quinn NP. Clinical features and natural history of multiple system atrophy: an analysis of 100 cases. Brain 1994; 117: 835–45.

West AB, Dawson VL, Dawson TM. To die or grow: Parkinson’s disease and cancer. Trends Neurosci 2005; 28: 348–52.

Wielosz S, Kleinrok Z. Lithium induced head twitches in rats. J Pharm Pharmacol 1979;31:410– 4.

Williams DR, De Silva R, Paviour DC, Pittman A, Watt HC, Kilford L. Characteristic’s two distinct clinical phenotypes in pathologically proven progressive supranuclear palsy: Richardson's syndrome and PSP-parkinsonism. Brain 2005;128: 1247-58.

Williams DR, de Silva R, Paviour DC, Pittman A, Watt HC, Kilford L, et al. Characteristics two distinct clinical phenotypes in pathologically proven progressive supranuclear palsy: Richardson's syndrome and PSP-parkinsonism. *Brain* 2005;128: 1247-58.

Wu HC, Chen HM, Shiau CY. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (Scomber austriasicus). Food Res. Int. 2003; 36: 949–957.

Yamamoto M, Koshimura K, Kawaguchi M, Sohmiya M, Murakami Y, Kato Y. Stimulating effect of erythropoietin on the release of dopamine and acetylcholine from the rat brain slice. Neurosci Lett 2000; 292:131-133.

Yamamoto Y, Takahashi K. Glutathione peroxidase isolated from plasma, reduces phospholipid hydroperoxide. Archives of Biochemistry and Biophysics 1993; 305:541–545.

Rajput A, Rajput AH. Progressive supranuclear palsy: clinical features, pathophysiology and management. *Drugs Aging* 2001; **18:** 913–25

Youdim MBH, Lavie L. Selective MAO-A and B inhibitors, radical scavenger, and nitric oxide synthase in Parkinson's disease. Life Sci 1994; 55: 2077–82.

ANNEXURES

K. K. COLLEGE OF PHARMACY

(Approved by AICTE, PCI & Government of Tamilnadu and Affiliated to the Tamilnadu Dr. MGR Medical University)

1/161, Sankaralinganar Road, • Gerugambakkam, • Chennai - 600 122
Phone : (044) 32546162, Tele/Fax : 23821272

APPROVAL CERTIFICATE

This is to certify that the project title "**Evaluation of Neuroprotective effect of *Annona squamosa* Linn. on 6-OH Dopamine induced neurodegeneration**" has been approved by IAEC and the details are furnished under

Approval No.	Species	Breakup sex wise	Weight	No's approved
KKCP/2012/011	Swister Rat	30 Female& 30 Male	150-200 gm	60
Sixty animals only				

Chairman IAEC
(Prof. A Meena)

CPCSEA Nominee
Dr. C. Kathirvelan

Veterinary officer

Members

Certificate

This is certify that the project title **"Evaluation of Neuroprotective effect of Echinops Echinatus Roxb. and a polyherbal"** has been approved by the IAEC.

Name of Chairman/ Member Secretary IAEC:

Name of CPCSEA nominee:

Prof. Sripathi Kandula
School of Biology
M. K. University
MADURAI-625021

Signature with date

Chairman/ Member Secretary of IAEC:

CPCSEA nominee:

11/01/2013

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)

Ultra College of Pharmacy, Madura, Tamil Nadu, India

Institutional Animal Ethics Committee

CERTIFICATE

Title of the Project: Evaluation of Neuroprotective effect of Echinops Echinatus Roxb. And Polyherbal formulation

Proposal number : UCP/IAEC/2013/071

Date first Received : 15.06.13

Date received after modification (if any): -

Date received after Second modification: -

Approval Date :11.07.13

Animals :81 Wistar Albino rats

Number of animals sanctioned: 81 Animals

Sex of animals: Either sex

Date:

Place: Madurai

For IAEC

Ultra College of Pharmacy, Madurai

NATIONAL INSTITUTE OF HERBAL SCIENCE

Plant Anatomy Research Centre : PARC

Prof.P. Jayaraman, Ph.D
Director
(Retd,Professor,PresidencyCollege Chennai-5).

 # 4, 2nd Street, Sakthi Nagar,
West Tambaram, Chennai-600 045.
Ph:044-22263236, 9444385098
E.mail:herbalparc@yahoo.com

AUTHENTIFICATION CERTIFICATE

Based upon the organoleptic / macroscopic / microscopic examination of Sample, it is certified that the specimen given by C. Ronald Darwin Asst Prof. K.K. College of Pharmacy is identified as below:

Binomial: Annona Squamosa Linn.

Family: Annonaceae

Synonym(s): Annona

Regional names: San - Sitaphala, Tamil - Sitapalam, Hindi - Sitaphal, Malayalam - Sitapalam

Reg.No of the certificate: PARC/2009/421

References : Nair, N.C & Henry, A.N. Flora of Tamilnadu, India 1 : .1983.

Henry, A.N. *et al.* Ibid. — II : — .1987.

Ibid. — III : — .1989.

Date: 08/09/09

(Prof.P.JAYARAMAN)

Prof. P. Jayaraman, Ph.D.
Director
NATIONAL INSTITUTE OF HERBAL SCIENCE
No.4-II Street, Sakthi Nagar, W. Tambaram, Chennai-45.
Ph : 044 - 22263236, Cell : 9444385098.
E-mail : herbalparc@yahoo.com

NATIONAL INSTITUTE OF HERBAL SCIENCE

Plant Anatomy Research Centre : PARC

Prof.P. Jayaraman, Ph.D
Director
(Retd,Professor,PresidencyCollege Chennai-5).

 # 4, 2nd Street, Sakthi Nagar,
West Tambaram, Chennai-600 045.
Ph:044-22263236, 9444385098
E.mail:herbalparc@yahoo.com

AUTHENTIFICATION CERTIFICATE

Based upon the organoleptic / macroscopic / microscopic examination of Sample, it is certified that the specimen given by C. Ronald Darwin Asst prof. K.K. College of pharmacy is identified as below:

Binomial: Echinops echinatus Roxb.

Family: Asteraceae

Synonym(s): Echinatus

Regional names: Tamil - kuttimaippizhan, Sanskrit - Brahmadandala, Hindi - Utaramitira

Reg.No of the certificate: PARC/2010/043

References : Nair, N.C & Henry, A.N. Flora of Tamilnadu, India 1 : .1983.
Henry, A.N. *et al.* Ibid. II : .1987.
Ibid. III : .1989.

Date:

(Prof.P.JAYARAMAN)

Prof. P. Jayaraman, Ph.D.
Director
NATIONAL INSTITUTE OF HERBAL SCIENCE
No.4-II Street, Sakthi Nagar, W. Tambaram, Chennai-45.
Ph : 044 - 22263236, Cell : 9444385098.
E-mail : herbalparc@yahoo.com