EVALUATION OF ANTIPARKINSON'S ACTIVITY OF *Uncaria rhynchophylla, Mentha aquatica* and *Banisteriopsis caapi*- PLANTS WITH MONOAMINE OXIDASE-B (MAO-B) INHIBITION POTENTIAL

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By

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Biswajit Pal

Dedicated to my Pharmacology teacher

And

To the innocent rats

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LIST OF ABBREVIATION

%	Percentage
µ	Micron
µm	Micrometer
^{0}C	Degree Celsius
6-OHDA	6-hydroxydopamine
AADC	Aromatic amino–acid decarboxylase
AAS	Atomic absorption spectroscopy
ACN	Acetonitrile
AD	Alzheimer´s disease
AIF	Apoptosis inducing factor
AIDS	Acquired immune deficiency syndrome
AMPT	Alpha-methyl para-tyrosine (an antagonist of tyrosine hydroxylase)
ANOVA	Analysis of Variance
AP	anterior-posterior (used for stereotaxic measurement)
ATP	Adenine triphosphate
BBB	Blood brain barrier
BG	Basal ganglia
BSA	Bovine albumin serum
bw	Body weight
Bcl_2	B-cell Lymphoma-2
CAT	Catalase
Ca_v	Voltage gated calcium channel
CMC	Carboxymethylcellulose
CNS	Central nervous system
COMT	Catechol-O methyltransferase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DA	Dopamine
DAB	Diaminobenzidine
DAT	Dopamine transporter
DMSO	Dimethyl sulphoxide

DNA	Deoxy Ribonucleic Acid
DOPAC	3,4-Dihydroxyphenylacetic Acetic (dopamine metabolite)
DV	Dorsal-Ventral (axis used in stereotaxic measurement)
EDTA	Ethylenediamine Tetra Acetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
EBC	Aqueous extract of *Banisteriopsis caapi*
EEE	Mixed plants extract
EMA	Ethanol extract of *Mentha aquatica* L.
EUR	Aqueous extract of *Uncaria rhynchophylla*
FBS	Fetal Bovine Serum
g	Gram
GABA	Gamma-Amino Butyric Acid
GAD	Glutamic Acid Decarboxylase
GDNF	Glial Cell Line-Derived Neurotrophic Factor
Glu	Glutamate
GRD	Glutathione Reductase
h	Hours
H_2O_2	Hydrogen peroxide
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HVA	Homo-vanillic Acid (dopamine metabolite)
IAEC	Institutional Animal Ethical Commitee
IAP	Inhibitor of Apoptosis Protein
IC_{50}	Inhibitory Concentration 50%
IP	Intraperitoneal
IR	Infrared
KBr	Potassium Bromide
L	Liter
L-DOPA	Levo-Dihydroxy Phenyl Alaine
LPS	Lipopolysaccharide
M	Molar

MAO A	Mono Amino Oxidase A
MAO B	Mono Amino Oxidase B
Mg	Microgram
mg	Milligram
ML	Medial-Lateral (used for stereotaxic measurement)
mL	Milliliter
mM	Millimolar
mm	Millimetre
MPP^+	1-Methyl-4-Phenylpyridinium
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
N	Normality
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NE	Norepinephrine
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve Growth Factor
nm	Nanometer
NO	Nitric Oxide
OECD	Organization for Economic Co-operation and Development
P	Probability
PD	Parkinson's Disease
PKC	Protein Kinase C
Rf	Refrective index
RPM	Revolutions per minute
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SEM	Standard Error Of Mean
Sirt-1	Sirtuin-1
SN	Substantia Nigra
SNpc	Substantia Nigra Pars Compacta
SOD	Superoxide Dismutase

STR	Striatum
TCA	Tri Carboxylic Acid
TH	Tyrosine Hydroxylase
THH	Tetrahydroharmine
TLC	Thin Layer Chromatography
TNF–α	Tumor Necrosis Factor- α
UV	Ultraviolet
VTA	Ventral Tagmental Area
µg	Microgram
µl	Micriliter
µM	Micromolar

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INTRODUCTION

INTRODUCTION

Parkinson's disease [1- 6]

Parkinson's disease (PD) is a neurodegenerative disorder caused by the progressive loss of mesencephalic dopaminergic neurons in the substantia nigra innervating the striatum. It was first described by neurologist James Parkinson in 1817 that he called ''Shaking Palsy'', or ''paralysis agitans''. The causes are unknown although risk factors in the genetic and toxic domain are being discovered. An important pathophysiological feature in PD is the loss of part of the dopaminergic neurons in the substantia nigra (SN) resulting in a specific dysorganisation of the complicated basal ganglia (BG) circuits. The relay functions at the level of the striatum e.g., are out of balance leading to disturbed subcorticocortical interactions. Parkinson's disease (PD) is the second most common neurodegenerative disease, primarily affecting people of ages over 55 years (approximately 1.5% to 2.0%), although young adults and even children can also be affected. Research on the pathogenesis of PD has rapidly advanced due to the development of animal models. Through the use of these models, the striatal dopamine deficiency could be associated with the motor symptoms of PD, and levodopa (dihydroxyphenylalanine or L-dopa) was first applied to compensate striatal dopamine losses. L-Dopa treatment still remains the standard of PD therapies. Unfortunately, long-time use of L-dopa results in dyskinesia (involuntary movements). Moreover, the specific etiology of PD is still unknown. Thus, the development of animal models is essential for better understanding pathogenesis and progression of PD and testing therapeutic agents for the treatment of PD patients.

Definition [7]

Parkinsonism is a clinical syndrome consisting of four cardinal features: bradykinesia (slowness and poverty of movement), muscular rigidity, resting tremor (which usually abates during voluntary movement), and an impairment of postural balance leading to disturbances of gait and falling.

Pathophysiology [8-10]

Pathologically, PD is characterized by severe loss of substantia nigra (SN) dopaminergic neurons, visible in brain sections as depigmentation of the substantia nigra in the midbrain. It is estimated that approximately 60% to 70% of the SN

dopamine cells are lost by the time a patient first presents for clinical evaluation, diagnosis, and treatment. Neuroimaging reveals a typically asymmetric loss of dopamine terminals in the striatum, which progresses over time leading to further clinical deterioration.

Since the early 1980s and the discovery of a potent neurotoxin (MPTP-MPP+), a byproduct of illicit drug synthesis, the environment has figured prominently in proposed etiologies for Parkinson's disease. After the original description of this environmental "insult" to the dopamine-producing cells of the substantia nigra, a number of other environmental neurotoxins have been described, which have led to the Parkinsonian state. These discoveries have led to the suggestion that Parkinson's disease may arise as a combined consequence of the ongoing aging process coupled with environmental exposure(s) that accelerate the process of nigral cell death. The unusual clustering of individuals who later developed Parkinson's Disease (including Michael J. Fox), in a Canadian recording studio, emphasizes the possible relation of environment to disease development.

The third component of the puzzle is the possibility that some individuals may have a predetermined genetic susceptibility to these environmental "insults." While Parkinson's disease has been observed to occur throughout the world and in virtually all ethnic groups, there is a very low incidence among Asians and African patients as opposed to white patients. This observation suggests that genetic factors may possibly have an important role in disease production. Other evidence involves twin studies which initially failed to show a high concordance rate among monozygotic twins but are now being reconsidered in light of new evidence. In addition, family history appears to be a very strong predictor, after age, for development of the disease. Recently, a number of families in Greece and Italy with a very high penetrance of Parkinson's disease were shown to have a mutation on chromosome 4 for the alpha-synuclein gene. This is a presynaptic protein of unknown function but with the potential, upon further study of this muted gene, to provide insights into the pathogenesis of this form of autosomal dominant Parkinson's disease. Another gene abnormality on the long arm of chromosome 6 has been identified in patients with a peculiar autosomal recessive form of young onset disease. The protein product of this gene is named Parkin and seems to promote the degradation of certain neuronal proteins. It is closely related to the ubiquitin family of proteins involved in several

neurodegenerative disease states. Research continues at a very high level to identify susceptibility genes and shed additional light on the genetics of Parkinson's disease.

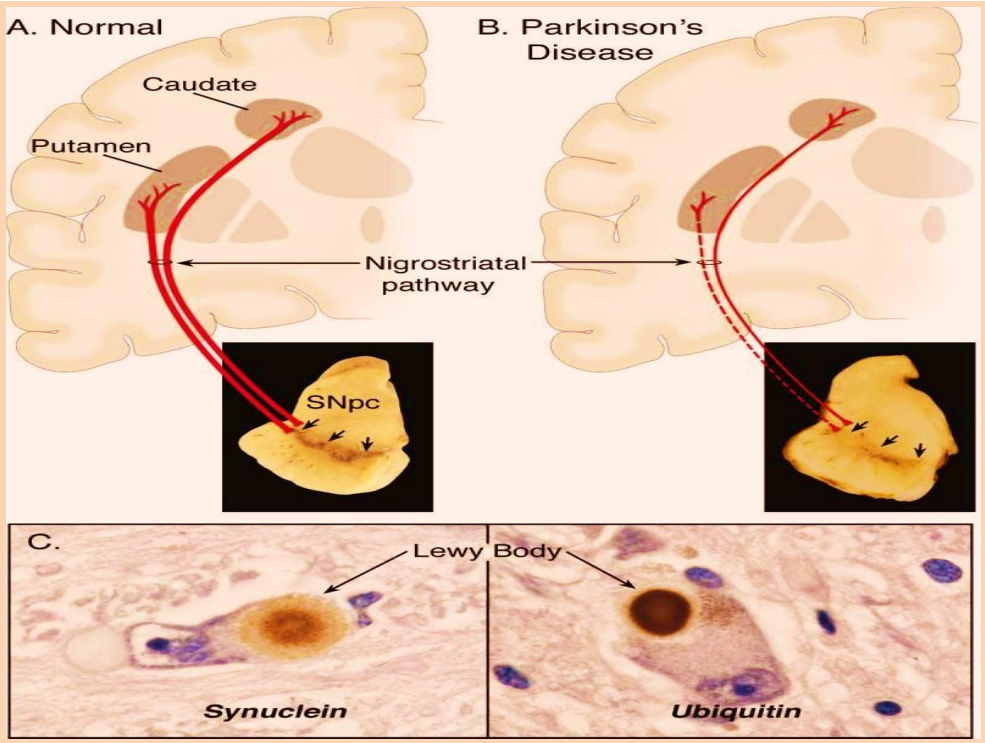


Figure 1: Neuropathology of Parkinson's disease

(A) Normal nigrostriatal pathway (in red). It is composed of Dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta (SNpc; see arrows). These neurons project (thick solid red lines) to the basal ganglia and synapse in the striatum (i.e. putamen and caudate nucleus).

(B) Diseased nigrostriatal pathway (in red). In Parkinson's disease, the nigrostriatal pathway degenerates. There is a marked loss of Dopaminergic neurons that project to the putamen (dashed line) and a much more modest loss of those that project to the caudate (thin red solid line).

(C) Immunohistochemical labeling of intraneuronal inclusions, termed Lewy bodies, in a SNpc Dopaminergic neuron. Immunostaining with an antibody against -synuclein reveals a Lewy body (black arrow) with an intensely immunoreactive central zone surrounded by a faintly immunoreactive peripheral zone (left photograph). Conversely, Immunostaining with an antibody against ubiquitin yeilds more diffuse immunoreactivity within the Lewy body (right photograph).

Monoamine oxidase (MAO-A and MAO-B) involvement in PD [11-16]

MAO exists as two isoforms with different substrate selectivities and different distributions in brain and between species. In man, dopamine is largely metabolised by MAO-B although it can also be a substrate for MAO-A. However, dopaminergic neurones in the striatum contain relatively little MAO-B but the A-isoform is present. Rather MAO-B is found extensively in glial cells and localised to the outer mitochondrial wall. Under normal physiological conditions, dopamine released into the synapse by impulse flow is rapidly 'inactivated' by the high affinity reuptake process that constitutes the dopamine transporter. In PD, the number of presynaptic terminals in the striatum is extensively depleted and now MAO-B in surrounding glial cells becomes a major focus for dopamine metabolism. This provides a targeted and disease specific mechanism through which dopamine degradation can be inhibited by the use of selective MAO-B inhibitors. Selegiline and rasagiline are selective MAO-B inhibitors that irreversible inhibit the enzyme by the covalent binding of the propargylamine moiety to the active site in the mitochondrial membrane. The long lasting inhibition of MAO-B is held responsible for the symptomatic improvement in motor symptoms occurring in monotherapy in early PD and as adjunct therapy to L-dopa and/or dopamine agonist treatment in midand late-stage illness. However, it has been implied that both drugs may also alter the rate of progression of PD through the DATATOP study and subsequent investigations of selegiline and the TEMPO and ADAGIO studies of rasagiline. This raises the question of how such effects might be mediated. Initially, the inhibition of the metabolism of dopamine by MAO-B was thought to lower oxidative stress by preventing the formation of toxic oxygen free radical species. But subsequently the emphasis has evolved to support an action of both selegiline and rasagiline in preventing apoptotic processes leading to cell death through effects at the level of mitochondria. Recent interest has centred on rasagiline's actions based on the finding of improved clinical scores with early drug use in the ADAGIO study and the preclinical evidence to support such an effect will now be explored in greater detail.

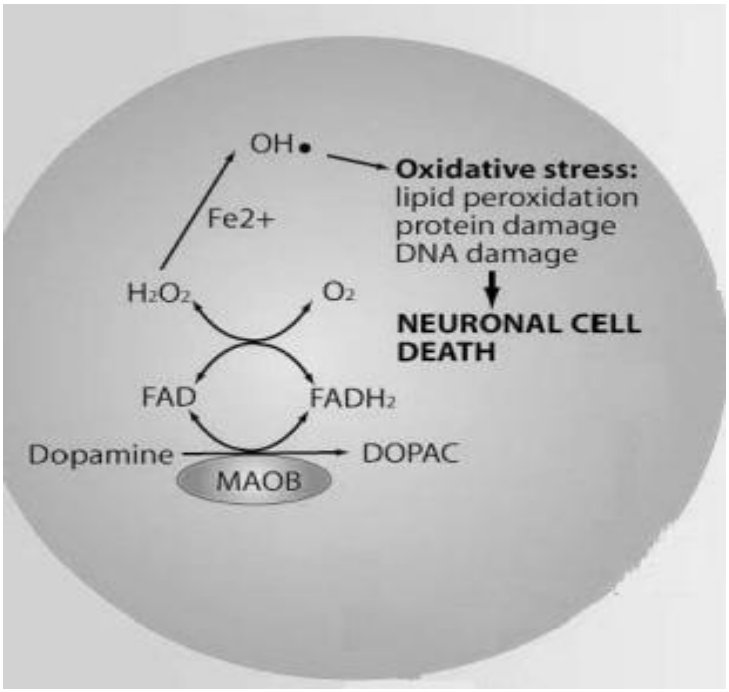


Figure 2: Role of MAO-B in PD; MAO-B plays a major role in the catabolism of neurotransmitters such as DA, serotonin and norepinephrine, leading to hydrogen peroxide formation which contributes to oxidative stress and neuronal cell death

Mitochondria involvement in PD [17-20]

Mitochondria are involved in the regulation of apoptosis. Opening of the MTP and mitochondrial depolarisation seem to be part of the initiation of apoptosis. Consequently, cytochrome-*c* and the apoptosis inducing factors (AIFs) can exit mitochondria and activate proapoptotic proteins close to the outer mitochondrial membrane. Antiapoptotic proteins, such as Bcl-2, are also localised close to the outer mitochondrial membrane which makes mitochondria the location for proand antiapoptotic events. MPP+ opens the MTP and leads to cytochrome c release.

Oxidative stress is caused by an imbalance between the generation of ROS and their enzymatic or non-enzymatic detoxification rate. Oxidative stress plays an important role in brain aging, neurodegenerative diseases and has long been associated with the death of dopaminergic neurons, due to production of toxic species through autoxidation and formation of neuromelanin. The iron and copper levels in SN are high and it contributes to autoxidation of dopamine and its metabolites, leading to production of ROS. Oxidative damage of lipids, proteins and DNA as well as decreased level of an important antioxidant, reduced glutathione, have been detected PD patients'

autopsy brain. Oxidative damage induces SYN aggregation and impairs proteosomal ubiquitination and degradation of proteins, inducing LBs formation, one of PD's characteristic hallmarks. Mitochondrial activity can also be affected by environmental factors that possibly contribute to PD pathogenesis. The ability of the ROS generator, paraquat, to cause specific dopaminergic lesions strongly suggests involvement of oxidative stress in PD. Oxidative stress in PD is not restricted to the brain area alone: a variety of oxidative damage markers, in the serum and CSF of PD patients, suggest a systemic DNA/RNA oxidation.

Oxidative stress in the central nervous system (CNS) comes not only from mitochondria-generated ROS, but also from microglia. Classic microglial activation is a source of intracellular and extracellular ROS, which can induce neuronal damage. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a membrane bound enzyme complex that catalyses the production of superoxide from oxygen, and is implicated as both the primary source of microglial-derived extracellular ROS and a mechanism of pro- inflammatory signalling in microglia. NADPH-oxidase is up regulated in the SN of PD patients.

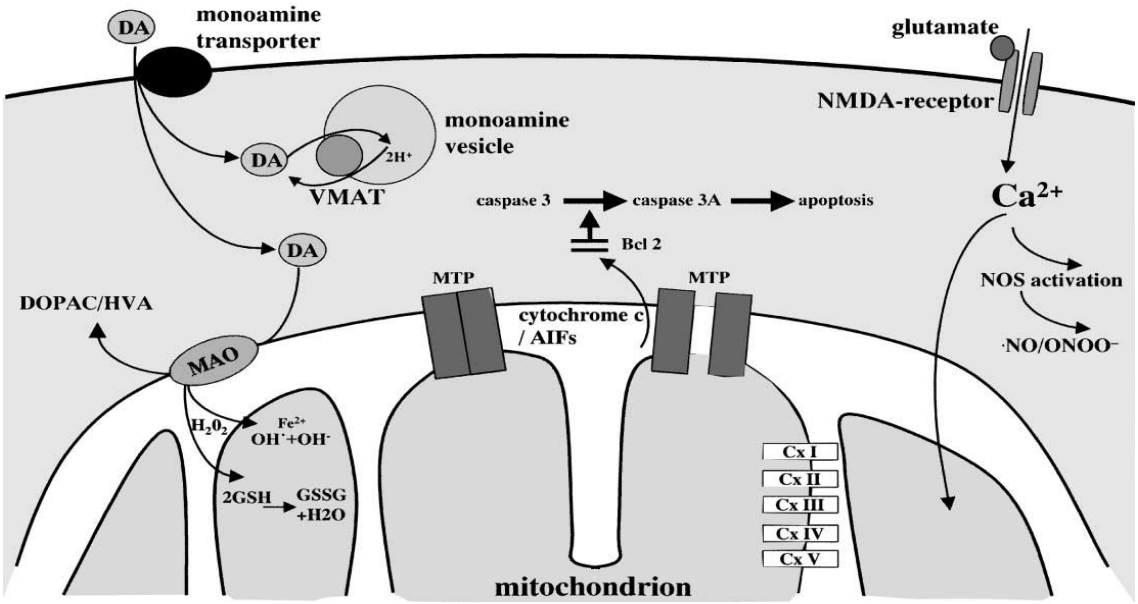


Figure 3: A summary of potential pathways for mitochondrial involvement in Parkinson's disease. Impaired complex I activity may compromise mitochondrial function in dopaminergic neurons endogenously. Mitochondria are involved in DA metabolism. DA can be compartmentalised by vesicular associated monoamine transporters (VMAT) into monoamine storage vesicles. MAO B is localised to the outer

mitochondrial membrane and converts DA into DOPAC and homovanillic acid (HVA). H2O2 generated in this process is detoxified in the mitochondrial matrix. Cytochrome *c* and AIFs can be released by an incompletely understood mechanism when the MTP opens. They can activate proapoptotic proteins at the outer membrane initiating apoptosis which is inhibited by Bcl 2. Mitochondria serve as cellular Ca2+ buffer. Increased cytoplasmic Ca2+ concentrations lead to the activation of NOS and, subsequently, nitric oxide (NO) and peroxynitrite (ONOO−) generation. Increased Ca2+ influx may occur through the NMDA receptor when the ATP and magnesium dependent blockade is lifted leading to increased excitotoxicity.

Role of cellular Calcium on Parkinsonism [21-23]

In a recent epidemiological study of the potential effect of use of Ca^{2+} channel blockers on the evolution of Parkinson's disease, about two-thirds of the patients were treated with diltiazem and verapamil. No relation between use of such drugs and the incidence of Parkinson's disease was found. However, neither verapamil nor dilitazem are potent blockers of the Cav1.3 channels that underlie pacemaking in SNc dopamine neurons. Other nominal Ca^{2+} channel blockers flunarizine and cinnarizine worsen the symptoms of Parkinson's, but this effect is attributable to their antagonism of dopamine receptors, not their block of Ca^{2+} channels. By contrast, retrospective examination of patients treated for hypertension with dihydropyridines the class of drug used to induce reversion of the SNc dopamine neuron pacemaking and neuroprotection in mice revealed a lower than expected incidence of Parkinson's disease. In moving forward, there are several issues that need to be considered. One is the choice of drug. In the absence of a selective Cav1.3 Ca^{2+} channel antagonist, dihydropyridines such as nimodipine offer the best therapeutic options. Dihydropyridines are more selective blockers of L-type channels than are other Ca^{2+} channel blockers approved for human use, and have good brain bioavailability. However, most members of this drug class, including nimodipine and nifedipine, are more potent blockers of Cav1.2 than of Cav1.3 channels.

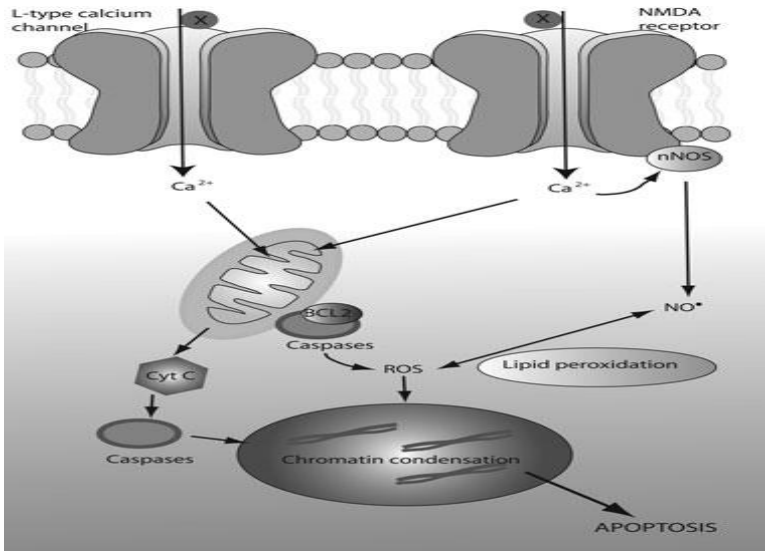


Figure 4: L-type calcium channel antagonist and a NMDA receptor/ion channel antagonist. By modulation of Ca2+ entry during a cerebral ischemic incident, or in chronic Ca2+ overload situations, neuronal cell death might be prevented.

Role of Iron in Parkinsonism [24,25]

Abnormal levels of brain iron have been reported in Parkinsonism, which is characterized by degeneration of dopaminergic (DA) neurons. An animal model of Parkinsonism was used to clarify the contribution of the loss on nigrostriatal DAergic neurons to abnormal iron accumulation. In rats 6-OHDA induced unilateral DA depletion, brain iron deposition and its day-to-day stability can be studied in vivo using magnetic resonance imaging (MRI) scans and Perls'-DAB histochemical stain. The uptake and transport of iron by intrinsic cells of the striatum may very, and this variability may have been exaggerated by the destruction of DAergic nigrostriatal neurons, which are known to modulate the activity of the intrinsic cells.

Role of neuroinflammation in the pathogenesis of PD [26,27]

Glial cells, such as astrocytes or microglia, play important role in CNS homeostasis, mediating immune responses and reducing oxidative stress. When CNS regional homeostasis is disturbed, glial cells release cytokines to re- establish the balance and repair damaged cells. Such a response is natural and beneficial for neurons, however repeated microglia and astrocyte activation evoke chronic inflammatory stress, leading to increased production of ROS and severe neuronal damage. Several cell, animal and human studies indicate involvement of neuroinflammation in the pathogenesis of PD.

Current treatments

Parkinson's Medications [28]

Drug Classes

I. Drugs affecting brain dopaminergic system

1. ***Dopamine precursor***

Levodopa/ L-DOPA (Sinemet®, generics,)

1. ***Peripheral decarboxylase inhibitor***

Carbidopa (Parcopa® orally disintegrating tablet), Benserazide

2. ***Dopamine agonists***

Apomorphine (Apokyn®), Bromocriptine (Parlodel®), Cabergoline (Not approved in the US), Lisuride (Not approved in the US), Pergolide (Permax®; withdrawn from US market March 2007), Pramipexole (Mirapex®), Ropinirole (Requip®) Rotigotine (Neupro® patch)

4. ***COMT inhibitors***

Entacapone (Comtan®, Stalevo®), Tolcapone (Tasmar®)

5. ***MAO-B inhibitors***

Rasagiline (Azilect®)

Selegiline (Eldepryl® orally swallowed pill, Zelapar® orally disintegrating tablet)

6. ***Dopamine facilator***

Amantadine (Symmetrel®, generics)

II. Drugs affecting brain cholinergic system

1. ***Central*** *anticholinergics*

Trihexyphenidyl (Artane®), Procyclidine, Benztropine, Ethopropazine

2. ***Antihistaminics***

Orphenadrine, Promethazine

Levodopa (L-DOPA)

A gold standard medication with a major drawback in the therapy [29-32]

The most effective Parkinson's drug is levodopa, it passes into the brain and is converted to dopamine. Levodopa is combined with carbidopa to create the combination drug Sinemet. The carbidopa protects from premature conversion to dopamine outside the brain; hence prevents nausea. In Europe, levodopa is combined with a similar substance, benserazide, and is marketed as Madopar.

In prolonged levodopa therapy, the apparent buffering capacity is lost and the patient's motor state may fluctuate dramatically with each dose of the drug. A common problem is the development of wearing off phenomenon: each dose of levodopa affectively improves mobility for a period of time, about 1 or 2 hours, but rigidity and akinesia return rapidly at the end of dosing interval. Increasing the dose and frequency of administration can improve this situation, but this often is limited by the development of dyskinesias, excessive and abnormal involuntary movements. Dyskinesias are observed most often when the plasma levodopa concentration is high although in some individuals dyskinesia or dystonia may be triggered when he level is rising or falling. These movements can be as uncomfortable and disabling as the rigidity and akinesia of PD. In the later stages of PD, patients may fluctuate rapidly between being "off", having no beneficial effects from their medications and being "on" but with disabling dyskinesias, a situation called on/off phenomenon.

Metabolism of L-DOPA and Dopamine [33]

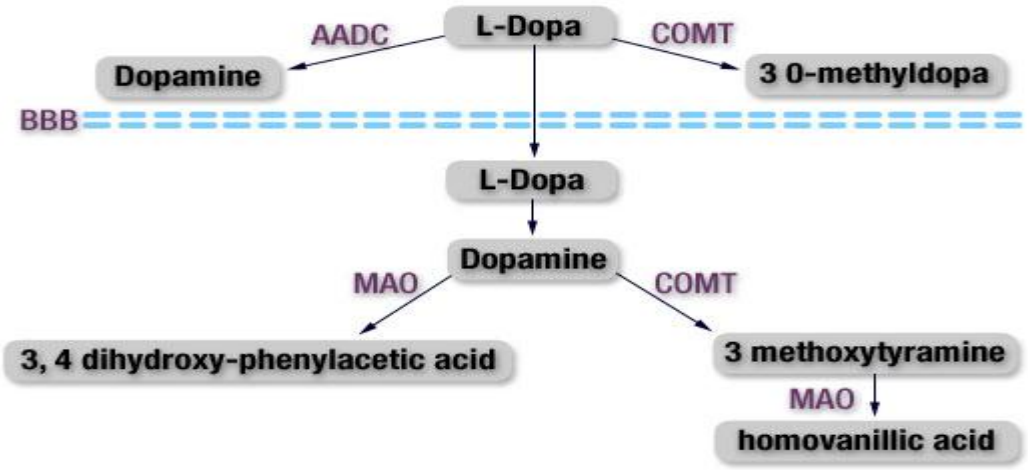


Figure 5: Metaboism of L-Dopa by various enzymetic reaction; AADC (aromatic amino–acid decarboxylase) , COMT (catechol-O-methyltransferase) and MAO (monoamine oxidase).

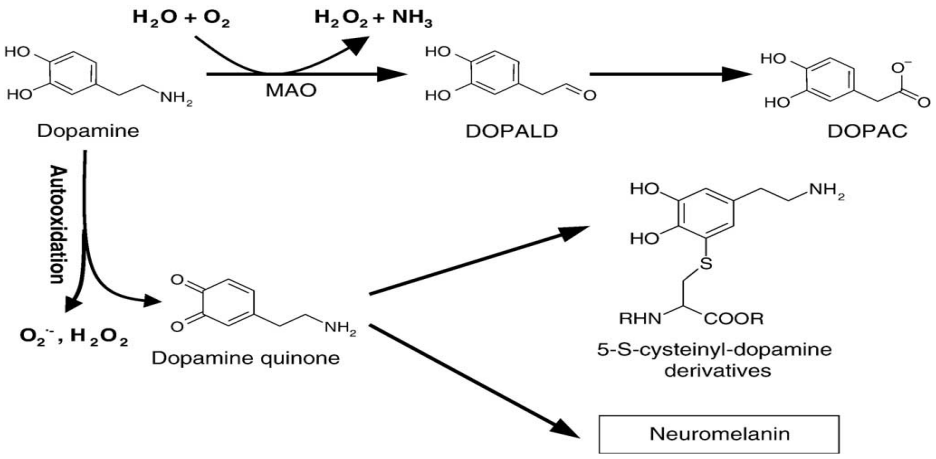


Figure 6: Metabolism of Dopamine, Oxidation of dopamine by MAO or by auto oxidation leads to the production of H_2O_2, which when converted to OH radicals can lead to the oxidization of proteins, lipids, and nucleosides. Auto oxidation of dopamine also leads to the generation of dopamine quinone, which may covalently bind to proteins or further be converted to neuromelanin. The modification of biomolecules by OH or dopamine quinone may exert toxic effects on dopaminergic neurons.

The possible role of antioxidants in Parkinsonism [34-37]

Oxidative stress is strongly associated with PD and supported by post-mortem analysis showing that SN cell degeneration can be induced by oxidative stress. Increased ROS production is substantiated by dysfunction of the mitochondrial complex I, a major hallmark of PD. Elevated iron levels are reported in PD patients' midbrain and it has been shown that iron chelation is effective in delaying progress of PD *in-vivo*. Use of free radical scavengers, such as vitamin A, C and E, would be able to neutralize excess free radicals and thereby prevent oxidative stress. The first clinical trial of neuroprotection in PD was on the most active form of vitamin E, α-tocopherol. This study failed to show any beneficial effect of the vitamin E supplement, but later meta-analysis suggested a protective effect of vitamin E-rich diets. Furthermore, a combination of vitamin E and C did showed some beneficial effect in patients with early PD, whereas vitamin E alone did not. Based on this trial it is recommended to use antioxidant supplements for three population groups: people with high PD risk, early PD stage patients, and patients who are treated by Levodopa alone or in combination with MAO- B inhibitors.

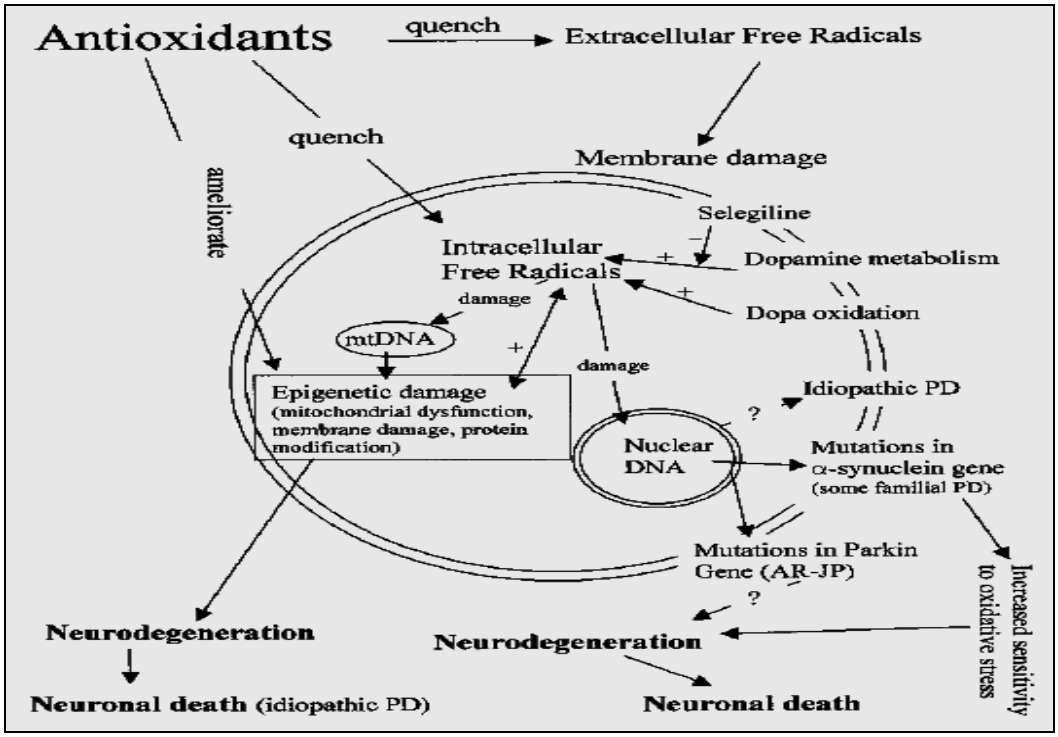


Figure 7: A diagrammatic view of free radical involvement as a common intermediary risk factor for many neurotoxic events in the pathogenesis of PD. mtDNA= mitochondrial DNA, AR-JP= Autosomal Recessive Juvenile Parkinsonism, (+) = increases, (-) = decreases, ? = Evidence lacking for point mutations or altered gene expression.

Experimental Parkinsonism Model

6-Hydroxydopamine lesioned rats [38-41]

6-OHDA is a neurotoxin, which, when directly injected into the medial forebrain bundle, striatum or SN, induces nigrostriatal DA neuronal degeneration. Although 6-OHDA leads to clear apoptosis of nigrostriatal dopaminergic cells, evidence indicates that the toxic effects of 6-OHDA are in part mediated through the activation of microglia. Direct administration of 6-OHDA into the SN of mice activates microglia and increases the number of activated microglia in the SN with the subsequent loss of dopaminergic neurons after 1 week. Furthermore, 6-OHDA-lesioned rats have been demonstrated to have increased levels of TNF-a in both SN and striatum. The effects of striatal injection of 6-OHDA in rats was followed using PET scanning of presynaptic DA transporters following administration of transport site specific ligands. These authors observed progressive striatal neurodegeneration, whereas at the same time, an increase in activated microglia in the striatum and nigra occurred, which was

initially focal but by 4 weeks had become widespread. This led the authors to conclude that neuroinflammation is a significant factor in the 6-OHDA neurodegenerative process.

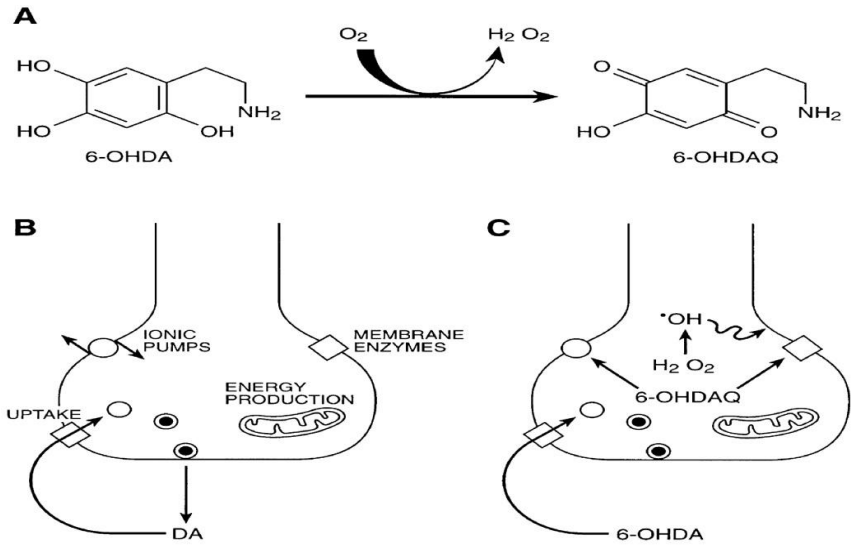


Figure 8: Chemical structure and mechanisms of action of the neurotoxin 6-hydroxydopamine (6-OHDA). (A) This catecholamine derivative can be oxidized into 6-hydroxydopamine quinine, or 6-OHDAQ, with the production of hydrogen peroxyde (H_2O_2). Although H_2O_2 does not contain unpaired electrons, it can interact with transition metals, such as Fe^{2+}, leading to the formation of the highly reactive hydroxyl radical or *OH, that can start membrane lipid peroxydation and cause eventual membrane damage, including increased permeability to ions and water (Cadet and Brannock, 1998). (B) Dopaminergic nerve endings, or terminal varicosities, have an uptake carrier, or DA transporter, that constitutes the main physiological mechanism of DA inactivation. In addition, DA terminals contain mitochondria for energy production, ionic pumps, membrane transporters and other essential enzymes. (C) The neurotoxin 6-OHDA is taken up by the catecholamine transporters, in this case, by the DA transporter or uptake carrier, and will oxidize within the cytoplasm of the terminal leading to the production of 6-OHDAQ that can affect mitochondrial enzymes as well as other proteins that are required to maintain normal cellular metabolism. Also, the inevitable production of H_2O_2 and its interactions with metals will have additional toxic effects, in particular on membrane permeability.

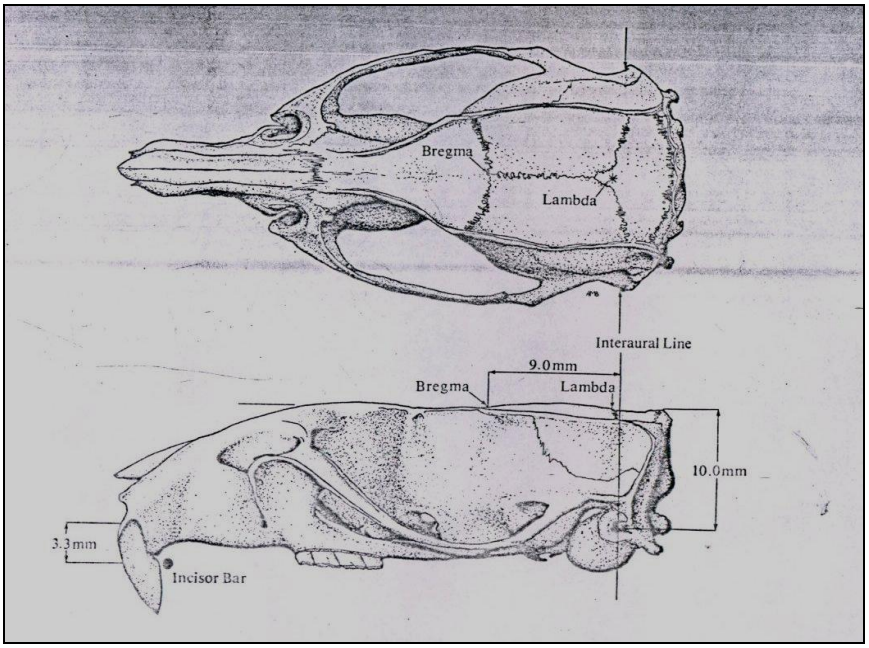


Figure 9: The diagram indicates the skull sutures used to locate the stereotaxic co-ordinates.

1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine [42-44]

MPTP is a neurotoxin that induces Parkinsonian features in humans, rodents and non-human primates, and has been demonstrated to cause rapid and selective DA neurotoxicity. MPTP is taken up by DA neurons causing oxidative mitochondrial damage, which results in neuronal death. To this end, MPTP induced degeneration shows many similarities to PD, although the lack of Lewy bodies displayed after MPTP toxicity indicates differences. With regard to neuroinflammation, MPTP-induced neurotoxicity is linked to microglial activation. This is clearly demonstrated when microglia are added to neuronal cultures leading to a greatly increased level of MPTP-induced DA toxicity. An increased expression of both MHC class 1 and II antigens and upregulation of iNOS have been demonstrated in the striatum and SNc of mice given MPTP as well as increases in proinflammatory cytokines such as IL-1b and IL-6.

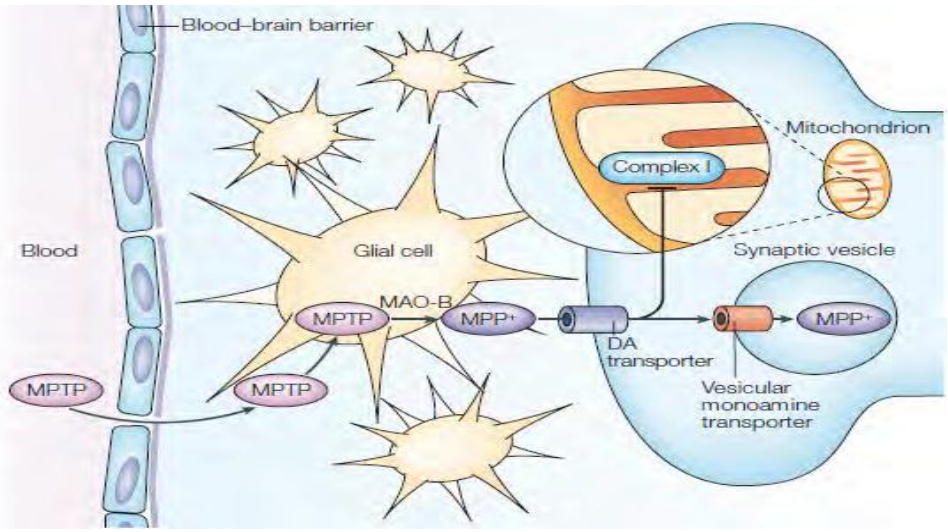


Figure 10: The MPTP model of Parkinson's disease. After systemic administration, MPTP crosses the blood-brain barrier. Once in the brain, MPTP is converted to MPP+ by MAO-B within glial cells and released. Thereafter, MPP+ is selectively concentrated into dopaminergic neurons via the dopamine transporter (DAT). Inside neurons, MPP+ can bind to the vesicular monoamine transporter (VMAT), which translocates MPP+ into synaptosomal vesicles or it can be concentrated within the mitochondria, blocking complex I, thus enhancing production of reactive oxygen species.

Tremorine and Oxotremorine Antagonism [45]

The muscarinic agonist's tremorine and oxotremorine induce parkinsonism-like signs such as tremor, ataxia, spasticity, salivation, lacrimation and hypothermia. These signs are antagonized by anticholinergic drugs. The oxotremorine antagonism has been proven to be a reliable method for testing central anticholinergic activity. The overt isomorphism between the animal model and the symptoms of Parkinson's disease recommend this test for screening of anti-Parkinson drugs. However, the model measures only central anticholinergic activity.

Reserpine Antagonism [45]

Reserpine induces depletion of central catecholamine stores. The sedative effect can be observed in mice shortly after injection, followed by signs of eyelid ptosis, hypokinesia, rigidity, catatonia, and immobility. These phenomena can be antagonized by dopamine agonists. Locomotor activity and grooming scores of drug treated animals are compared with controls treated with reserpine and vehicle only by analysis of variance.

PLANT PROFILE

Uncaria rhynchophylla [46- 49]

Figure 11: *Uncaria rhynchophylla* plant

Scientific classification

Kingdom: Plantae

Order: Gentianales

Family: Rubiaceae

Genus: *Uncaria*

Species: *rhynchophylla*

Binomial name: *Uncaria rhynchophylla*

Vernacular Name(s)

Cat's Claw, Gambir Vine, Gou Teng, Una de Gato, Asen'yaku, or Pale Catechu.

General Information

- Plant parts used: Hooks/Stem
- Storage: Keep in a cool, dry place protected from moisture and heat
- Method of analysis: Gravimetry, HPTLC/HPLC

Constituents

Stems and leaves of *Uncaria rhynchophylla* contain a variety of indole alkaloids. The total alkaloid content is about 0.2 %, in which rhynchophylline (Rhy) is

28 %-50 %, isorhynchophylline (Isorhy) is 15 %. The other trace components include catechin, hirsutine, hirsuteine, corynantheine, dihydrocorynantheine, isocorynoxeine, akuammigine, geissoschijine, and methylethe.

Specifications

Uncaria rhynchophylla is an evergreen woody vine. The Cat's Claw bush can grow to up to 10 meter. Branchlets are quadrangular, brown, clean and hairless. Leaf axils have binate or solitary hook, which bents down, 1.7 to 2cm long, and with pointed tip. Leaves are opposite and petiolate. Leaf blade is ovate, ovate-oblong or elliptic, 5 to 12cm long, and 3 to 7cm wide. Inflorescence is solitary, axillary capitulum or apical raceme, in the diameter of 2 to 2.5cm. Peduncle is slender and 2 to 5cm long. Flowers are yellow and corolla is connate. Capsule is obovate or oval, sparsely pubescent, and with persistent calyx. And both ends of seeds are winged.

Distribution and habitat

Uncaria rhynchophylla is widely distributed in tropical regions, including Southeast Asia, Africa, and South America.

Modern use in herbal medicine

Uncaria rhynchophylla a common herb frequently used in the practice of Traditional Chinese medicine (TCM), it is sweet and bitter in flavor and slightly cold in properties. It was described as having the ability to remove heat, check hyper function of the liver, and relieve dizziness, tremor, and convulsion. The herb is also used to treat headache, dizziness resulting from hypertension, and convulsive disorders, such as epilepsy. *Uncaria rhynchophylla* has also been found to inhibit tumor necrosis factor-alfa (TNF-alfa) and nitric oxide production in mouse microglial cells in vitro. The neuroprotective effects of *Uncaria rhynchophylla* following transient global ischemia have also been evaluated. The herb Cat's Claw has been used in Peru and Europe since the early 1990s as a treatment for cancer and AIDS, as well as for other diseases that target the immune system.

The phytochemical catechin present in *Uncaria rhynchophylla* showed a potent in vitro inhibitory activity against human brain monoamine oxidase (MAO)-B.

Mentha aquatica [50-53]

Figure 12: *Mentha aquatica* plant

Scientific classification

Kingdom: Plantae

Order: Lamiales

Family: Lamiaceae

Genus: *Mentha*

Species: *aquatica*

Binomial name: *Mentha aquatica* L.

Vernacular Name(s)

Water Mint, Menthe acuatique, Wasser-Minze.

General Information

Plant parts used: Stem/Leaf/Flower

Storage: Keep in a cool, dry place protected from moisture and heat

Method of analysis: Gravimetry, HPTLC/HPLC

Distribution and habitat

Mentha aquatica is native to much of Europe, northern Africa and western Asia. It has been introduced to North and South America, Australia and some Atlantic islands.

Constituents

The chemical components include (S)-naringenin, oxygenated monoterpenes (+)-pulegone and (+)-menthofuran, viridiflorol. The volatile oil of *Mentha aquatica* main constituents are: menthofuran (51.27%), limonene (12.06%), izomenthone (8.11%), beta – cis – ocimene (7.92%), ledol (3.01%). The phytochemical (S)-naringenin showed a potent in vitro inhibitory activity against human brain monoamine oxidase (MAO)-B enzyme.

Specifications

Water mint is a herbaceous rhizomatous perennial plant growing to 90 cm tall. The stems are square in cross section, green or purple, and variably hairy to almost hairless. The rhizomes are wide-spreading, fleshy, and bear fibrous roots. The leaves are ovate to ovate-lanceolate, 2 to 6 cm long and 1 to 4 cm broad, green (sometimes purplish), opposite, toothed, and vary from hairy to nearly hairless. The flowers of the water mint are tiny, densely crowded, purple, tubular, pinkish to lilac in colour and form a terminal hemispherical inflorescence; flowering is from mid to late summer. Water mint is pollinated by insects, and also spreads by underground rhizomes, like other species of mint. All parts of the plant have a distinctly minty smell.

Modern use in herbal medicine

The dried leaves of *Mentha aquatica* together with those of *Tagetes minuta* L. are burned and the smoke inhaled by the Venda people of South Africa to treat mental illnesses. It is also used in South African traditional medicine as treatment against colds, respiratory problems and protection against and removal of ‘curses’ and ‘evil spirits’. The symptoms of such ‘curses’ often resemble a depressed state accompanied by lethargy. It is used as a stimulant, emetic, astringent and for difficult menstruation. In a previous screening of plants used in traditional medicine in southern Africa for depression-like conditions.

For MAO-inhibitory activity, a 70% ethanol extract of *Mentha aquatica* had high activity.

Banisteriopsis caapi [54-57]

Figure 13: *Banisteriopsis caapi* plant

Scientific classification

Kingdom: Plantae

Order: Malpighiales

Family: Malpighiaceae

Genus: *Banisteriopsis*

Species: *caapi*

Binomial name: *Banisteriopsis caapi*

Vernacular Name(s)

Ayahuasca, Hoasca, Caapi Vine or yage.

General Information

Plant parts used: Stem/Branch/Leaf

Storage: Keep in a cool, dry place protected from moisture and heat

Method of analysis: Gravimetry, HPTLC/HPLC

Distribution and habitat

Banisteriopsis caapi is native to much of Southern America, Northern South America (Venezuela), Brazil and Western South America (Bolivia, Colombia, Ecuador, Peru).

Constituents

The chemical components include beta-carboline alkaloids harmine- 0.31-8.43%, harmaline- 0.03-0.83% and tetrahydroharmine (THH)- 0.05-2.94% as the principal monoamino oxidase (MAO) inhibitors, together with harmol, harmine N-oxide, harmic acid methyl ester, harmalinic acid, harmic amide, acetylnorharmine, and ketotetrahydronorharmine. The stems contain 0.11-0.83% beta-carbolines, with harmine and tetrahydroharmine as the major components. In addition, pyrrolidines, shihunine and (S)-(+)-dihydroshihunine, and terpenoids stigmasterol, beta-sitosterol, ursolic acid, oleanolic acid and nerolidol were also reported.

Specifications

Banisteriopsis caapi is a shrub or extensive liana in tropical forests. The bark is usually chocolate-brown and smooth. Leaves are opposite, oval-shaped, green, marginally entire, 8-18 cm long, 3.5-8 cm wide, upper surface glaborous. The inflorescence is axillary or terminal cymose panicles. Flowers are 12-14 mm in diameter 2.5-3 mm long, 1.5 mm wide with pink petals, 5-7 mm long, 4-5 mm wide and 10 stamens. The fruit is a samara nut, 5 mm long. This liana is maily propagated vegetatively because it rarely blossoms and sets seed. The fan-like shaped seeds are green when fresh, but turn brown with age.

Modern use in herbal medicine

Banisteriopsis caapi is used to prepare Ayahuasca, a decoction that has a long history of entheogenic uses as a medicine and "plant teacher" among the indigenous peoples of the Amazon Rainforest. Ayahuasca is nowadays used by some religious groups in Brazil to treat alcoholism. Moreover, the alkaloids of *Banisteriopsis caapi* had medicinal properties, such as antimicrobial, anthelmintic and vasorelaxant effects, in addition to sociopsychotherapeutic, ethnopsychiatric and rehabilitative functions.

The beta-carboline alkaloids harmine and harmaline present in *Banisteriopsis caapi* act as MAO inhibitors, and has neuroprotective activity.

AIMS AND OBJECTIVES OF THE WORK

AIM OF THE WORK

- To investigate whether monoamine oxidase-B (MAO-B) inhibitory potential plants *(Uncaria rhynchophylla, Mentha aquatica* and *Banisteriopsis caapi)* protects neuronal cells against degenaration in 6-OHDA rat models, and to analyse the murine preclinical therapeutic efficacy of test drugs in attaining postural stability after completion of treatment.
- To explore the beneficial effects of *Uncaria rhynchophylla, Mentha aquatica* and *Banisteriopsis caapi* in reducing neurodegenaration by controlling iron induced neurotoxicity, retaining dopamine concentrations and lowered oxidative stress in experimental PD.
- To study the effectiveness of *Uncaria rhynchophylla, Mentha aquatica* and *Banisteriopsis caapi* for neuroprotection in PD model through controlling MAO-B associated pathways of metabolism.

OBJECTIVE OF THE WORK

The main pathological correlate of the clinical and pharmacological characteristic of Parkinson's disease is the death of dopanergic neurons in substantia nigra. The cause and exact mode of death of these cells are yet unknown or unresolved. Some of the changes which occurs are; alteration in the brain ion level, mitochondrial function, complex-1 deficiency and reduction in free radical scavenging appears to be some components of disease progression in PD.[58]

The effectiveness of L-DOPA in Parkinson's treatment is remaining obscure and most of the PD patients experienced an intolerable adverse effects and the disease condition seemed to be progressed. Even though L-DOPA is considered to be a gold standard drug for the treatment of Parkinsonism, but during its metabolism it converts to dopamine quinones (amidoquinones and paraquinones) in the neurons and these are highly reactive oxygen species and produces death of surviving dopaminergic neurons. Despite the advent of drugs like dopaminergic agonist, dopamine facilitators and various enzyme inhibitors, virtually all patients develop an unexpected level of disability and mortality rate.[59]

So, by considering the above quoted subject matters, we tried to identify new anti-Parkinsonian drugs from plants like *Uncaria rhynchophylla*,[60,61] *Mentha aquatica*[62,63] and *Banisteriopsis caapi* [64,65] which are showing monoamine oxidase-B (MAO-B) inhibition, neuroprotection and anti-oxidant activity and to find out the possible actions of these plants for alleviating or preventing the neurodegeneration and mitochondrial dysfunction for the treatment of Parkinson's disease.

Monoamine oxidase (MAO), a flavin-containing enzyme, is widely distributed in both the central and peripheral nervous systems [66] and plays a central role in the control of substrate availability and activity. MAO catalyzes the oxidation of a variety of amine-containing neurotransmitters to yield the corresponding aldehyde, hydrogen peroxide (H_2O_2), and ammonia.[67] MAO exists in two forms, MAO-A and MAO-B, which are distinguished on the basis of different pharmacological and biochemical characteristics. MAO is a key enzyme in catecholamine metabolism, and increased catecholamine metabolism seen in aging has been extensively studied. The control on MAO activity may alleviate symptoms and slow the progression of neurodegenerative

disorders. In humans, MAO-B activity increases with age [68] and is especially elevated in certain neurodegenerative diseases.[69] Therefore, inhibition of MAO-B activity may improve the quality of life of the elderly and it is used as part of the treatment for Parkinson's patients.

The major hallmarks of the Parkinsonism such as deposition of ubiquiten, Alpha amyloid plaques, neuroinflammation, mitochondrial dysfunction, nigral iron deposition etc., will be studied and the therapeutic benefit of plants having monoamine oxidase B (MAO-B) inhibition activity will be validated in 6-OHDA model of Parkinson's disease. The pathological interventions during the disease development and after the occurrence of disease will be explored during the study periods. The ultimate aim of our research will be identifying a potential therapeutic herbal drug for the treatment of Parkinson's disease without unacceptable clinical troubles.

REVIEW OF LITERATURE

REVIEW LITERATURE

1. Alexi et al., (2000) [2] have described about the mechanisms of neuronal cell death in neurodegeneration. Metabolic compromise, excitotoxicity, and oxidative stress cause neuronal cell death that is both necrotic and apoptotic in nature. Strategies to rescue or protect injured neurons usually involve promoting neuronal growth and function or interfering with neurotoxic processes. Considerable research has been done on testing a large array of neuroprotective agents using animal models which mimic these disorders. They reviewed neuroprotective strategies which have been found to successfully ameliorate the neurodegeneration associated with Parkinson's and Huntington's diseases. First, they have given an overview of the mechanisms of cell death and the background of Parkinson's and Huntington's diseases. Then they elaborated on a range of neuroprotective strategies, including neurotrophic factors, anti-excitotoxins, antioxidants, bioenergetic supplements, anti-apoptotics, immunosuppressants, and cell transplantation techniques.

2. Brannan et al., (1998) [101] tested the circling response to L-DOPA and apomorphine administration in rats with unilateral 6-hydroxydopamine 6-OHDA lesions of the substantia nigra. Rats demonstrated a progressively diminished circling response when L-DOPA–carbidopa was repeatedly administered at 120 min intervals. This decreasing response was not present when apomorphine was administered under the same conditions. They also perfused L-DOPA directly into the striatum in vivo of rats with an ipsilateral 6-OHDA nigrotomy at 60 min intervals and monitored striatal dopamine levels with the technique of brain microdialysis. Dopamine formation increased from the first to the fifth trial. This may be secondary to the decrease in uptake sites which accompanies the loss of striatal dopamine nerve terminals. They postulated that the continued presence of dopamine at striatal receptor sites conditions a short-term loss of dopamine receptor sensitivity and a consequent decreased circling response.

3. Bharath et al., (2007) [24] investigated the integrating glutathione metabolism and mitochondria dysfunction with implications for Parkinson’s disease. They observed that the SN of early PD patients exhibits significant depletion of cellular glutathione (GSH). GSH, a tripeptide present both in neurons and non-neuronal cells is the major antioxidant and redox modulator in the brain. GSH depletion in the SN is the earliest

known indicator of oxidative stress in presymptomatic PD, preceding both decreases in CI activity and dopamine levels. They showed that mitochondrial dysfunction significantly affects glutathione synthesis thereby increasing the oxidative damage and further exacerbating the toxicities of these mitochondrial agents resulting in neurodegeneration. Rat dopaminergic neuronal cell culture and *in vitro* experiments using mouse brain mitochondria were employed to validate important features of the model. They demonstrated the interdependence of mitochondrial function with GSH metabolism in relation to neurodegeneration in PD.

4. Bartels et al., (2009) [3] explained that an important pathophysiological feature in PD. It was the loss of part of the dopaminergic neurons in the substantia nigra (SN) resulting in a specific dysorganisation of the complicated basal ganglia (BG) circuits. At a functional level this was shown by timing and scaling problems when performing movements and clinically that translated into initiation problems, bradykinesia and others. Dysarthria can of course be an important problem. These basic disturbances of motor organisation can be copied into the cognitive domain.

5. Cleren et al., (2005) [112] have treated the mice with celastrol before and after injections of MPTP, a dopaminergic neurotoxin. A 48% loss of dopaminergic neurons induced by MPTP in the substantia nigra pars compacta was significantly attenuated by celastrol treatment. Moreover, celastrol treatment significantly reduced the depletion in dopamine concentration induced by MPTP. Similarly, celastrol significantly decreased the striatal lesion volume induced by 3-nitropropionic acid, a neurotoxin used to model HD in rats.

6. Deumens et al., (2002) [92] evaluated various 6-OHDA rat models of Parkinson's disease. They concluded that, on independent of the site of injection, the 6-OHDA induced DA depletion appears to be a valuable model to investigate PD symptomalogy and to gain more insight in to the possible pathological mechanisms of this neurodegenerative disease. They also opinioned that the choice of using a bilateral model or a unilateral model depends on the aim of the experiment and one should be aware of the consequences of the choice for a certain 6-OHDA model.

7. Dauer et al., (2003) [93] described the disease mechanisms and various animal models involved in Parkinson's disease. They discussed about the 6-OHDA lesioned rat model

that it is the first animal model associated with SNpc dopaminergic neuronal death. 6-OHDA induced toxicity is relatively selective for monoaminergic neurons, resulting from preferential uptake by DA and nor adrenergic transporters. Since it does not cross blood brain barrier, it must be administered by local stereotaxic injection in to substantia nigra, median fore bundleor striatum to target the nigrostriatal dopaminergic pathway. The unilateral lesion by 6-OHDA can be quantitatively assayed; thus a notable advantage of this model is the ability to assess the anti-PD properties of new drugs.

8. Erez et al., (1969) [86] had developed and described the thin layer chromatography method for the standardization of naringenin in different solvent system. This method involved separation of compounds by TLC using 1-butanol: acetic acid: water 40:11:29 v/v as a mobile phase. The chromatogram was scanned at 288 nm. The Rf value of standard naringenin was reported as 0.90.

9. Eriksen (2004) [39] suggested that gene mutation in mitochondria, protein handling pathways, protein aggregation and reactive oxygen species are key contributions to pathology. Dr J.L. Eriksen and Dr L. Petrucelli have contributed greatly to our current understanding of molecular aspects of Parkinson's disease.

10. Foley et al., (2000) [12] have explained the multiple roles of MAO-B inhibitors in the therapy of neurodegenerative disorders. Monoamine oxidases play a central role in catecholamine catabolism in the central nervous system. This study explains the biochemical and pharmacological properties of inhibitors of the monoamine oxidase type B. Monoamine is an integral protein of the outer mitochondrial membrane, and catalyses the following overall oxidative deamination reaction:

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2$$

Two MAO isozymes are distinguished on the basis of their substrate preferences and sensitivity to inhibition by the MAO inhibitor, clorgyline. The various pharmacological actions of MAO-B inhibitors such as inhibition of MAO-B, interaction with tyramine, monoamine transport systems and dopamine release, neurochemical effects, antioxidative effects, protection from neurotoxins, neurorescuing effects and altered protein expression were described and the study revealed that blocking of MAO-B could further help in the neuroprotection.

11. Fujikawa et al., (2005) [111] showed that, *Acanthopanax senticosus* Harms (ASH) offered protection against PD and its related depressive behaviors in rats MPTP. The extract from the stem bark of ASH was prepared with hot water. The pole test and catalepsy test were used to evaluate the effects of ASH administration on bradykinesia and depressive behaviors in the PD model of rats. Treatment with ASH for 2 weeks resulted in prophylactic effects on MPTP-induced Parkinsonian bradykinesia and catalepsy. Immunohistochemistical analysis using tyrosine hydroxylase (TH) antibody showed that ASH provided cytoprotective effects against MPTP-induced loss of dopamine cells. These results have suggested the possibilities of using ASH for the prevention of nigral degenerative disorders, e.g., PD with depression.

12. Hall et al., (1992) [114] used an animal model of Parkinsonism to clarify the contribution of the loss of nigrostriatal Dopaminergic neurons to abnormal iron accumulations. In rats with 6-hydroxydopamine induced unilateral DA depletion, brain iron deposition and its day-to-day stability was studied in vivo using T_2-weighted magnetic resonance imaging (MRI) scans taken on 4 consecutive days beginning 1-2 months post-surgery and post-mortem by Perls'-DAB histochemicat stain. Unilateral DA depletion (Parkinsonism model) produced large day-to-day fluctuations in T_2 relaxation time in the striatum. The T_2 relaxation time in Sham control rats was relatively minor. The uptake and transport of iron by intrinsic cells of the striatum may vary, and this variability may have been exaggerated by the destruction of DAergic nigrostriatal neurons, which are known to modulate the activity of the intrinsic cells. Inconsistent reports of increased or decreased iron in Parkinsonism may reflect, in part, single time-point measures of widely fluctuating iron.

13. Hou et al., (2005) [60] have studied the MAO-B inhibition properties from the extract of hook of *Uncaria rhynchophylla*, a traditional Chinese herbal drug that generally used to treat convulsive disorders. In this study, the fractionation and purification of *Uncaria rhynchophylla* extracts using a bioguided assay isolated two known compounds, catechin and epicatechin. The compounds inhibited MAO-B, as measured by an assay of rat brain MAO-B separated by electrophoresis on a 7.5% native polyacrylamide gel and inhibition occurred in a dose-dependent manner, as measured by the fluorescence method. This suggested that these two compounds, isolated here for the first time from

Uncaria rhynchophylla, might be able to protect against neurodegeneration *in vitro*, and, therefore, the molecular mechanism deserves further study.

14. Jenner et al., (2012) [11] have studied the role of MAO-B in Parkinson's disease. The cause of cell death in Parkinson's disease (PD) remains unclear. Monoamine oxidase B (MAO-B) located in the mitochondrial wall play a significant role in dopamine degradation in PD. MAO-B inhibitors, such as selegiline and rasagiline offer dopamine stability and required concentration in mid brain and corrects motor instabilities of PD.

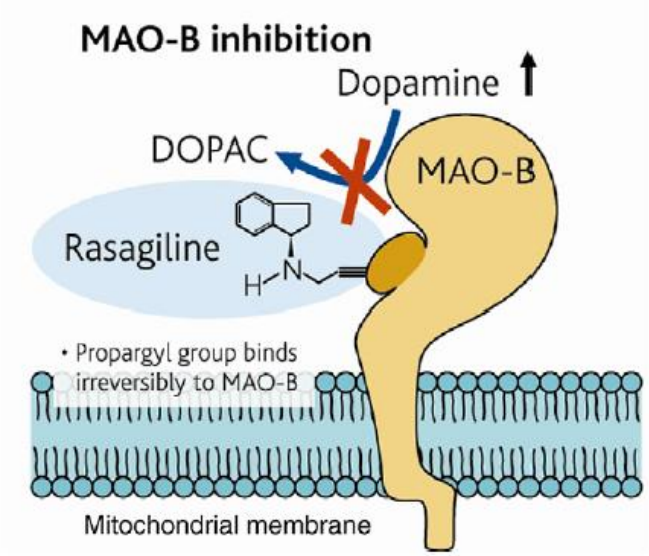


Figure 14: Structure, mechanism of action of rasagiline. Attaching via its propargyl domain, rasagiline irreversibly inhibits MAO-B by covalently binding to the flavin adenine dinucleotide (FAD) moiety of the MAO-B enzyme positioned in the outer membrane of the mitochondria within neuronal cells. As the FAD moiety is required for MAO-B to metabolise dopamine to dihydroxphenlyacetic acid (DOPAC), by blocking this site, rasagiline is able to inhibit the metabolism of dopamine by MAO-B, raising the level of dopamine at the synapse. Rasagiline is metabolised to aminoindan, its major metabolite, which possesses weak MAO inhibitory activity but exerts neuroprotective actions.

15. Kedar et al., (1999) [35] presented that epidemiologic studies have failed to identify specific environmental, dietary or lifestyle risk factors for PD except for toxic exposure to manganese, meperidine (Demerol®, the "designer drug" version of which often

contains a toxic byproduct of the synthesis, 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine [MPTP]), and some herbicides and pesticides. The search for genetic risk factors such as mutation, over expression or under expression of nuclear genes in DA neurons in idiopathic PD has not been successful as yet. Polymorphism in certain genes appears to be a risk factor, but there was no direct evidence for the causal relationship between polymorphism and increased risk of PD. In familial PD, mutation in the α-synuclein gene is associated with the disease, but a direct role of this gene in degeneration of DA neurons remains to be established. Although mutations in the Parkin gene has been associated with autosomal recessive juvenile PD, the role of this gene mutation in causing degeneration of DA neurons has not been defined. They have reported that in hereditary PD, a mutation in the α-synuclein gene may increase the sensitivity of DA neurons to neurotoxins. Although the nature of neurotoxins that cause degeneration in DA neurons in PD is not well understood, oxidative stress is one of the intermediary risk factors that could initiate and/or promote degeneration of DA neurons. Therefore, supplementation with antioxidants may prevent or reduce the rate of progression of this disease. Supplementation with multiple antioxidants at appropriate doses is essential because various types of free radicals are produced, antioxidants vary in their ability to quench different free radicals and cellular environments vary with respect to their lipid and aqueous phases. Since L-dopa is known to produce free radicals during its normal metabolism, the combination of L-dopa with high levels of multiple antioxidants may improve the efficacy of L-dopa therapy.

16. Kumar et al., (2007) [88] described the high performance thin layer chromatography method for the standardization of harmine. This method involved separation of compounds by TLC on precoated silica gel 60F 254 as an adsorbent, toluene: ethyl acetate: methanol (60:20:20 v/v) were used as a mobile phase. The chromatogram was scanned at 324 nm in UV reflectance mode. The Rf value of standard harmine was reported as 0.35.

17. Krishnakumar et al., (2009) [103] investigated the neuroprotective role of *B. monnieri* through the upregulation of 5-HT_{2C} receptor in epileptic rats. They have shown changes in the 5-HT_{2C} receptors binding and gene expression in the cerebellum of control, epileptic and *Bacopa monnieri* treated epileptic rats. There were a

significant down regulation of the 5-HT content, 5-HT_{2C} gene expression and 5-HT_{2C} receptor binding with an increased affinity. Carbamazepine and *B. monnieri* treatments to epileptic rats reversed the down regulated 5-HT content, 5-HT_{2C} receptor binding and gene expression to near control level. Rotarod test confirmed the motor dysfunction and recovery by *B. monnieri* treatment.

18. Lee et al., (1996) [115] investigated the effects of increased Iron in the substantia nigra of 6-OHDA induced Parkinsonian rats by nuclear microscopic study. The rats were administered with four µl of 0.2% 6-OHDA dissolved in 0.02% ascorbic acid solution was then stereotaxically injected into the right SN at AP - 4 mm, ML – 1 ram, DV -7.5 rnm from bregma, according to the atlas of Paxinos and Watson. Control rats received 4 txl of 0.02% ascorbic acid into the right SN. The results suggested that the 6-OHDA induced dopainergic cell death may be related to increased iron.

19. Lopez et al., (2010) [62] have studied the neuroprotective and neurochemical properties of various mint extracts such as *Mentha aquatica, Mentha longifolia, Mentha pulegium, Mentha suaveolens and Mentha piperita*. The methanol extracts of the plants were tested for protective effects against hydrogen-peroxide-induced toxicity in PC12 cells, antioxidant activity and neurochemical properties (MAO-A inhibition, AChE inhibition and affinity to the GABAA receptor). The study revealed *Mentha piperita and Mentha aquatica* produced significant protection of the PC12 cells against oxidative stress. All the plants exhibited antioxidant and MAO-A inhibitory activities. The *Mentha aquatica* showed the highest affinity to the GABAA-receptor assay. Results demonstrate that mints might have effect on the CNS.

20. Maharaj et al., (2006) [121] assessed the ability of the non-narcotic analgesics, acetaminophen and acetylsalicylic acid to prevent any diliterious effects of the potent neurotoxin, MPP+, on mitochondrial function and superoxide anion generation, in vivo. Acetylsalicylic acid and acetaminophen prevented the MPP+-induced inhibition of the electron transport chain and complex I activity. In addition, acetylsalicylic acid and acetaminophen significantly attenuated the MPP+-induced superoxide anion generation. Furthermore the results provide novel data explaining the ability of these agents to prevent MPP+-induced mitochondrial dysfunction and subsequent reactive oxygen species generation. While these findings suggest the usefulness of non-narcotic

analgesics in neuroprotective therapy in neurodegenerative diseases, acetylsalicylic acid appears to be a potential candidate in prophylactic as well as in adjuvant therapy in Parkinson's disease.

21. Mukherjee et al., (2007) [118] investigated the neuroprotective effect of the biflavone rich fraction from *Araucaria bidwillii* Hook (ABH) (Family: Araucariaceae) in ischemic reperfusion (I/R) induced oxidative stress. The I/R was induced by occluding bilateral common carotid arteries (BCCAO) for 30 min, followed by 24 h reperfusion. BCCAO caused significant depletion in superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and significant increase in lipid peroxidation (LPO) in various brain regions. The neurological deficit and sensory motor function were also decreased significantly by BCCAO group as compared to sham group animals. All the alteration induced by cerebral ischemia was significantly attenuated by 7 days' pretreatment with biflavone fraction (BFR) at the dose of 100 and 200 mg/kg, comparable to that given by Vitamin E (200 mg/kg). Consistent with neurobehavioral deficits, pretreatment with biflavones at higher doses significantly reduced ischemia-induced neuronal loss of the brain. In conclusion the biflavone rich fraction from *A. bidwillii* was found to protect rat brain against I/R induced oxidative stress, and attributable to its antioxidant properties.

22. Mritunjay et al., (2011) [84] have developed and described a simple and reproducible high performance thin layer chromatography method for the standardization of catechin. This method involved separation of compounds by TLC on precoated silica gel 60F 254 as an adsorbent, ethyl acetate: glacial acetic acid: formic acid: water 100:11:11:25 v/v was used as a mobile phase and scanned by fluorescence at 254 nm. The Rf value of standard catechin was reported as 0.46.

23. Nissinen (1984) [98] had developed and described a simple and reproducible high performance liquid chromatography method for the determination of monoamine oxidase B. He described High-performance liquid chromatography (HPLC) as an accurate and a sensitive method to assay many of the catecholamine-metabolizing enzymes. In this assay MAO-B activity was determined using benzylamine as substrate, deproteinization with perchloric acid and detection of the product benzaldehyde at 254 nm. The Chromatographic conditions was 5 µl

Ultrasphere-ODS column (4.5x150 mm); mobile phase, 40% methanol in 50 mM sodium phosphate buffer, pH 3.2, containing 1 mM sodium heptanesulphonic acid; flow-rate, 1.5 ml/min; detection at 254 nm; sensitivity, 0.01 a.u.f.s., injection volume, 20 µl. By this method it was possible to determine very low quantity of MAO-B enzyme from rat brain tissue.

24. Orth et al., (2002) [17] reviewed the mitochondrial dysfunction, oxidative stress, and reduced levels of synaptic transmission which are early stages of many neurodegenerative diseases. Many genes associated with neurodegenerative diseases are now known to regulate either mitochondrial function, redox state, or the exocytosis of neurotransmitters. Mitochondria are the primary source of reactive oxygen species and ATP and control apoptosis. Mitochondria are concentrated in synapses and significant alterations to synaptic mitochondrial localization, number, morphology, or function can be detrimental to synaptic transmission. Mitochondrial by-products are capable of regulating various steps of neurotransmission and mitochondrial dysfunction and oxidative stress occur in the early stages of many neurodegenerative diseases.

25. Olsen et al., (2008) [53] have isolated the active compound naringenin from ethanol extract of *Mentha aquatica* and it was further studied for MAO-B inhibition. The extract was obtained by using 70% of ethanol and naringenin was isolated from the extract by bioassay guided fractionation and preparative TLC. The structure of the compound was determined by 1H, 13C and 13C- NMR and optical rotation. Six extracts of varying polarity of *Mentha aquatica* were tested in a photometric peroxidase linked MAO bioassay. The 70% ethanol extract had highest inhibitory activity. The content of naringenin in *Mentha aquatica* might explain its use in traditional medicine for neurodegenaration-like conditions.

26. Richard et al., (2005) [117] reported the role of minocycline's direct antioxidant properties in neuroprotection, they determined potencies for minocycline, other tetracycline antibiotics, and reference antioxidant compounds using a panel of in vitro radical scavenging assays. Data from *in-vitro* rat brain homogenate lipid peroxidation and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays showed that minocycline, in contrast to tetracycline, is an effective antioxidant with radical scavenging potency similar to vitamin E. Our findings suggest that the direct

antioxidant activity of minocycline may contribute to its neuroprotective effects in some cell-based assays and animal models of neuronal injury.

27. Raja et al., (2007) [119] investigated the antioxidant activity of *Cytisus scoparius* L. (Family: Leguminosae) on CCl_4 (carbon tetrachloride) treated oxidative stress in Wistar albino rats. CCl_4 injection induced oxidative stress by a significant rise in serum glutamate oxaloacetate transaminases (SGOT), serum glutamate pyruvate transaminases (SGPT), lactate dehydrogenase (LDH) and thiobarbituric acid reactive substances (TBARS) along with reduction of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione-*s*transferase (GST) and glutathione reductase (GRD). Pretreatment of rats with different doses of plant extract (250 and 500 mg/kg) significantly lowered SGOT, SGPT, LDH and TBARS levels against CCl_4 treated rats. GSH and hepatic enzymes like SOD, CAT, GPx, GRD, and GST were significantly increased by treatment with the plant extract, against CCl_4 treated rats. The activity of extract at the dose of 500 mg/kg was comparable to the standard drug, silymarin (25 mg/kg). Based on these results, it was observed that *Cytisus scoparius* extract protected liver from oxidative stress induced by CCl_4 in rats and thus helped in evaluation of the traditional claim on this plant.

28. Schwarz et al., (2003) [57] have isolated two active ingredients harmine and harmaline from the extract of *Banisteriopsis caapi*. Further study revealed that tests for MAO inhibition using liver homogenate showed that extract and harmaline showed a concentration-dependent inhibition of MAO A and MAO B activity. The extract at 2.5 mg/ml caused a highly significant increase in release of dopamine from rat striatal slices, as did 200 mM harmine and 6 mM harmaline. In both these experiments, the amount of harmine present could not account for the total activity of the extract. The ability of harmine and harmaline to stimulate dopamine release is a novel finding. These results give some basis to the reputed usefulness of *Banisteriopsis caapi* stem extract in the treatment of PD.

29. Sudhakar et al., (2004) [125] investigated the role of calcium in the pathophysiology of essential hypertension. They measured serum calcium levels in 117 subjects with essential hypertension and 77 first-degree relatives. The serum calcium levels were estimated by atomic absorption spectrophotometer (AAS) according to the method of

Zettner and Seligson. The results showed that serum calcium levels were significantly decreased in both males and females with essential hypertension and their first-degree relatives when compared with the normotensive controls.

30. Shim et al., (2009) [61] have investigated the effects of the hook of *Uncaria rhynchophylla* on neurotoxicity in the 6-hydroxydopamine model. The aqueous extract of *Uncaria rhynchophylla* was studied for various in-vivo and in-vitro models of PD, such as the cell viability, anti-oxidative activity, and anti-apoptotic activity was assessed. They had also investigated the behavioral recovery and dopaminergic neuron protection of *Uncaria rhynchophylla*. The results revealed that *Uncaria rhynchophylla* significantly reduced cell death and the generation of ROS, and it lowered dopaminergic neuronal loss in substantia nigra pars compacta.

31. Surendran et al., (2009) [102] investigated the effects of *Withania somnifera* root extract on catecholamines and physiological abnormalities seen in PD model mouse. Mouse were treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine(MPTP) for 4 days to show biochemical and physiochemical abnormalities similar to patients with PD. PD mice were treated with 100 mg/kg body weight for 7 or 28 days. Catecholomaines like Homovanillic acid, Dopamine, glutathione and glutathione peroxidise and lipid peroxidation marker (TBARS) were analysed in the treated and untreated mouse striatum. Mouse treated with MPTP showed reduced levels of DA, DOPAC, HVA, GSH and GPx and induced thiobarbituric acid reactive substance (TBARS) level compared to the control. Physiological abnormalities were seen in the mouse as determined by hang test and rotarod test. Oral treatment of PD mouse with root extract (100 mg/kg body weight) for 7 days or 28 days increased DA, DOPAC and HVA levels and normalized TBARS levels in the corpus striatum of the PD mouse. The 7 days *Withania somnifera* treated mice showed improved motor function as determined by hang test and rotarod test. Treatment with *Withania somnifera* for 28 days increased GSH and GPx levels in the striatum compared to the *Withania somnifera* untreated PD mouse striatum. These data suggest that *Withania somnifera* is a potential drug in treating catecholamines, oxidative damage and physiological abnormalities seen in the PD mouse.

32. Samoylenko et al., (2010) [65] have investigated the monoamine oxidases (MAO) inhibitory and antioxidant activities of aqueous extract of *Banisteriopsis caapi*, which were relevant to the prevention of neurological disorders, including Parkinsonism. The various alkaloid was isolated from aqueous extract of *Banisteriopsis caapi* stems was standardized. The isolates harmine and harmaline were simultaneously tested *in vitro* for inhibition of human MAOs and antioxidant activity. The study revealed that *Banisteriopsis caapi* stem extract could be a future treatment of Parkinsonism, including other neurodegenerative disorders.

33. Wang et al., (2010) [64] have develop the process for preparing standardized extracts of *Banisteriopsis caapi* to achieve high potency for inhibition of human monoamine oxidases (MAO) and antioxidant properties. The aqueous extracts prepared from different parts of the plant collected from different geographical locations and seasons were analyzed by HPLC for principal bioactive markers. The extracts were simultaneously tested in vitro for inhibition of human MAOs and antioxidant activity for analysis of correlation between phytochemical composition of the extracts and bioactivities. The *Banisteriopsis caapi* extracts and standardized compositions were tested in vitro for inhibition of recombinant preparations of human MAO-A and MAO-B. In vitro cell-based assays were employed for evaluation of antioxidant property and mammalian cell cytotoxicity of these preparations. *Banisteriopsis caapi*, HPLC analysis revealed that most of the dominant chemical and bioactive markers were present in high concentrations in dried bark of large branch. The correlation between potency of MAO inhibition and antioxidant activity with the content of the main active constituents of the aqueous *Banisteriopsis caapi* extracts and standardized compositions was established.

34. Zazpe et al., (2006) [106] have investigated the effect of 5-HT1A agonists on tacrine-induced tremulous jaw movements in rats, a putative model of Parkinsonian tremor. The study showed that the high doses of 5-HT1A agonists induced catalepsy in rodents and, like clozapine, the compound reversed the haloperidol-induced catalepsy in rats. These results show that 5-HT1A receptors play a role in the regulation of tacrine-induced tremulous jaw movements in rats and suggest that their activation by novel antipsychotics may not only reduce the extrapyramidal side effects, but also be effective in the treatment of Parkinsonian tremor.

35. Zhang et al., (2007) [94] have studied about the major side effect (dyskinesia) of long L-DOPA treatment. They assessed the effects of SKF83959 on L-DOPA induced dyskinesia (LID) in a unilateral 6-hydroxydopamine (6-OHDA) lesioned rat model of PD. The results indicated that chronic L-DOPA (6 mg/kg) induced a progressive dyskinesia-like behavior in PD rats, whereas SKF83959 (0.5 mg/kg) elicited significantly less severe dyskinesia while exerts its anti-Parkinsonian action effectively. Application of D_1 receptor, but not D_2, α or 5-HT receptor antagonist attenuated SKF83959-induced dyskinesia, indicating that a D_1 receptor-mediated events, assumedly via PI-linked D_1 receptor. Interestingly, chronic co-administration of SKF83959 significantly reduced LID at no expanse of reduction in the anti-Parkinsonian potency in PD rats.

SCOPE AND PLAN OF THE WORK

SCOPE OF THE WORK

Parkinson's disease (PD) is of the major morbid condition which affects the entire CNS and particularly originated from mid brain region.[70] There is no convincing therapeutic strategy to slow or prevent the progression of the disease. The complexity of disease pathology, poor understanding of cellular mechanisms are the greatest challenge in the treatment of PD. The overall duration of PD prognosis is in the age of 15 to 20 years.[71] The long and slow course of neuropathological changes offers an impotent opportunity: to arrest the pathological changes before it ends to complete neurodegeneration.

From ancient we have been depending on herbal and natural remedies to correct the ailments of human society. Our planet earth is blessed with numerous plants and herbal species; which can be used as food supplements, nutrients, vitamin and certainly medicines too. In such an opinion we have reviewed numerous scientific information to accrue knowledge on herbal drugs which could offer either protective or curative actions in PD. Furthermore there is strong evidence of PD treatment by using certain herbal such as *Mucuna pruriens*[72,73], *Withania somnifera*[74, 75], *Tinospora cordifolia*[76,77] and *Ginkgo biloba*[78,79] etc. From the literature review we came to know the effectiveness of MAO-B inhibitors [80-82] in reducing and correcting PD pathology. The depletion of certain catecholamines such as dopamine, noradrenaline, adrenaline by MAO-B enzyme action [83] resulted in oxidative damage in the mid brain cortical region. So inhibition of MAO-B could protect the broader regions of CNS which have vital functions in the human. The plants *Uncaria rhynchophylla, Mentha aquatica* and *Banisteriopsis caapi* are identified to be portent inhibitors of MAO-B. Further, the information acquired is suggestive of safety and tolerability of these plants in animals as well as ancient human society. In this view we decided to conduct a prospective pharmacological analysis of these three plants in animal models of human PD. This research work prompted us to find a novel and a natural drug candidate to treat PD and the successive attempt will be made to explore the pharmacological properties and action of these herbals in suitable animal models. The herbal based drugs could be of less toxic, biocombatable, immune viable and efficient in human system. If we success in this attempt to find a therapeutic candidate from a naturals resource, this shall be a new therapeutic approach for treating PD.

PLAN OF WORK

Phase –I Scientific data collection

1. Review of literature for experimental Parkinson's disease and various drug targets in PD.
2. Construction of research hypothesis and identification of suitable herbal drug molecules for experimental evaluation.
3. Collection and update of drug data with their special emphasis on CNS dopaminergic system.

Phase –II

1. Selection of suitable animal models and neurotoxins.
2. Collection of herbal drugs, authentication, their extraction and characterization.
3. Selection of suitable dose, route of administration and fixing of treatment period.

Phase –III

1. Induction of PD in animals and drug treatment.
2. Evaluation of Pharmacological and Biochemical parameters for anti-Parkinson's activities.

Phase – IV

1. Documentation of results.
2. Evaluation of statistical significance of the results by using a computer aided program and reporting of research finding.

MATERIALS AND METHODS

MATERIALS AND METHODS

Animals

Healthy, adult Wistar rats of both sexes (180-220g) were obtained from the Central animal house facility from Padmavathi College of Pharmacy, Dharmapuri, Tamilnadu. The animals were kept in a well ventilated room and the animals had exposed to 12 hrs day and night cycle with a temperature between 20 ± 3^0C. The animals were housed in large spacious, hygienic polypropylene cages during the course of the experimental period. The animals were fed with water and rat feed adlibitum. All experiments were performed after obtaining prior approval from CPCSEA and IAEC. The animals were housed in suitable environmental conditions.

Approval no: 1143/ac/07/CPCSEA/PCP/IAEC/PhD/132/12

Chemicals

The chemicals which were used for the present study were procured from Sd-Fine Chemicals Mumbai, Sigma Aldrich USA, Loba chemie Mumbai, Merck chemicals Mumbai.

Collection and authentication of plant material

Uncaria rhynchophylla, Mentha aquatica and *Banisteriopsis caapi* were collected from local vender at Coimbatore district, Tamilnadu, India. The collected plants were authentified by Dr. S Rajan, Field Botanist, Survey of Medicinal Plants & Collection Unit, Central Council for Research in Homoeopathy, Dept. of AYUSH, The Nilgiris, Tamilnadu. Herbarium accession no: PCP/ P Cog/ 144, PCP/ P Cog/ 145**,** PCP/ P Cog/ 146 for *Uncaria rhynchophylla, Mentha aquatica* and *Banisteriopsis caapi* respectively.

Method of preparation of plants extract

Aqueous extract of *Uncaria rhynchophylla* (EUR) [49]

The dried stems and hooks of *Uncaria rhynchophylla* were cut in to small pieces and powdered carefully by using mechanical blender. The dried coarse powdered crude plant (500g) was transferred to a round bottom flask and 5 liter of distilled water was added to the round bottom flask and soaked for 1 h. This was then boiled for 3 h. The extract so obtained was filtered then concentrated to 1/6th of the total volume on a water bath. Freeze dryer was used for drying the extract. Percentage

yield of the aqueous extract was found to be; 12.4% w/w. Final extract was stored in a refrigerator at 4-6ºC.

Ethanol extract of *Mentha aquatica* (EMA) [53]

The dried aerial parts of *Mentha aquatica* were cut in to small pieces and powdered carefully by using mechanical blender. Soxhlet extraction method was used for obtaining alcoholic extract. 500g powder was extracted with 1 liter of 70% ethanol. The extract so obtained was filtered. Rotary vacuum evaporation (155 mbar pressure, 46^{o}C temp) and water bath evaporation method was applied to evaporate the extra amount of ethanol from the plant extract. Percentage yield of ethanol extract was found to be; 15.3%w/w. Final extract was stored in a refrigerator at 4ºC.

Aqueous extract of *Banisteriopsis caapi* (EBC) [57]

The dried stems of *Banisteriopsis caapi* was cut in to small pieces and powdered carefully by using mechanical blender. The dried coarse powdered crude plant (500g) was transferred to a round bottom flask and 5 liter of distilled water was added to the round bottom flask and soaked for 1 h. This was then boiled for 3 h. The extract so obtained was filtered then concentrated to 1/6th of the total volume on a water bath. Freeze dryer was used for drying the extract. Percentage yield of the aqueous extract was found to be; 21.2% w/w. Final extract was stored in a refrigerator at 4ºC.

Method of preparation of combined plants extract (EEE)

Ten gram each of EUR, EMA and EBC at a ratio of 1:1:1 was mixed and dissolved in 100 ml of 70% ethanol. The ethanol was used to prepare a uniform extract mixture and the extract mixture so obtained was then concentrated on a water bath to make ethanol free. The crude form of extract mixture was freeze dried and used for the study. The so obtained powder was stored in a refrigerator at 4-6ºC.

HPTLC Standardization of EUR [84,85]

HPTLC standardization of EUR was carried out by using catechin (Sigma Aldrich USA) as a marker constituent. The EUR extract were dried under reduced pressure at temperature 4ºC by using rota-evaporator and 10 mg of extract was further re-dissolved to 1 ml with absolute ethanol, which were used for quantitative estimation.

A CAMAG HPTLC system equipped with a sample applicator Linomat-IV, twin rough plate development chamber, CAMAG TLC scanner and integration software CATS 4.0 was used. An aluminium plate (4.0X20 cm) precoated with silica gel 60F 254 (E. Merck) was used as an adsorbent, ethyl acetate: glacial acetic acid: formic acid: water 100:11:11:25 v/v was used as a mobile phase. The solvent was allowed to run up to 75.00 mm and the chromatograms were scanned by fluorescence at 254 nm. A 0.5mg/ml solution of catechin- reference standard was prepared in absolute ethanol as a stock solution. The five standard levels (0.5, 1, 2, 3, 4 µg) of standard catechin were used for precalibration curve, for which 1, 2, 3, 4 and 5 µl of standard solution was applied on a TLC plate. The test solution was shaken well and 15 µl was also applied on a TLC plate along with 5 µl of standard catechin. The plates were developed up to 75.00 mm under chamber saturation condition. After air drying the solvent, the plate was scanned at 254 nm. The amount of catechin present was determined using the calibration curve plotted between concentration and area of standard. The regression equation was found to be, Y = 1 + 5.608*X with correlation coefficient of 0.9911. The content of catechin in EUR extract was quantified and percentage was calculated. By this method the Rf value of catechin in sample was about 0.45. The content of catechin was found to be 0.724 % w/w in sample.

HPTLC Standardization of EMA [86,87]

HPTLC standardization of EMA was carried out by using naringenin (Sigma Aldrich USA) as a marker constituent. The EMA extract were dried under reduced pressure at temperature 4ºC by using rota-evaporator and 10 mg of extract was further re-dissolved in 1 ml of absolute methanol, which were used for quantitative estimation.

A CAMAG HPTLC system equipped with a sample applicator Linomat IV, twin rough plate development chamber, CAMAG TLC scanner and integration software CATS 4.0 was used. An aluminium plate (4.0X20 cm) precoated with silica gel 60F 254 (E. Merck) was used as an adsorbent, 1-butanol: acetic acid: water 40:11:29 v/v was used as a mobile phase. The solvent was allowed to run up to 75.00 mm and the chromatograms were scanned at 288 nm. A 0.5mg/ml solution of naringenin - reference standard was prepared in absolute methanol as a stock solution. The five standard levels (0.5, 1, 2, 3, 4 µg) of standard naringenin were used for precalibration curve, for which 1, 2, 3, 4 and 5 µl of standard solution was applied on a TLC plate. The test solution was shaken well and 15 µl was also applied on a TLC

plate along with 5 µl of standard naringenin. The plates were developed up to 75.00 mm under chamber saturation condition. After air drying the solvent, the plate was scanned at 288 nm. The amount of naringenin present was determined using the calibration curve plotted between concentration and area of standard. The regression equation was found to be, Y = 0.2 + 2.433*X with correlation coefficient of 0.9332. The content of naringenin in EMA extract was quantified and percentage was calculated. By this method the Rf value of naringenin was about 0.89. The content of naringenin in sample was found to be 0.2735 % w/w in sample.

HPTLC Standardization of EBC [88,89]

HPTLC standardization of EBC was carried out by using harmine (Sigma Aldrich USA) as a marker constituent. The EBC extract were dried under reduced pressure at temperature 4ºC by using rota-evaporator and 10 mg of extract was further re-dissolved to 1 ml with absolute methanol, which were used for quantitative estimation.

A CAMAG HPTLC system equipped with a sample applicator Linomat IV, twin rough plate development chamber, CAMAG TLC scanner and integration software CATS 4.0 was used. An aluminium plate (4.0X20 cm) precoated with silica gel 60F 254 (E. Merck) was used as an adsorbent, toluene: ethyl acetate: methanol (60:20:20 v/v) were used as a mobile phase. The solvent was allowed to run up to 75.00 mm and the chromatograms were scanned at 324 nm. A 0.5mg/ml solution of harmine - reference standard was prepared in absolute methanol as a stock solution. The five standard levels (0.5, 1, 2, 3, 4 µg) of standard harmine were used for precalibration curve, for which 1, 2, 3, 4 and 5 µl of standard solution was applied on a TLC plate. The test solution was shaken well and 15 µl was also applied on a TLC plate along with 5 µl of standard harmine. The plates were developed up to 75.00 mm under chamber saturation condition. After air drying the solvent, the plate was scanned at 324 nm in UV reflectance mode. The amount of harmine present was determined using the calibration curve plotted between concentration and area of standard. The regression equation was found to be, Y = 2 + 9.608*X with correlation coefficient of 1.143. The content of harmine in EBC extract was quantified and percentage was calculated. By this method the Rf value of harmine in sample was about 0.35. The content of harmine was found to be 0.2631 % w/w in sample.

Figure 15: Saturation of HPTLC plate

Acute toxicity study of EUR, EMA, EBC and EEE (As per OECD guide lines number 425) [90]

Female Wistar rats of weight (180-220g) were taken for the study and kept for overnight fasting. Next day, body weight was taken and EUR, EMA, EBC and EEE were administered orally at a dose of 2000mg/kg in 0.3% CMC. Then the animals were observed for mortality and morbidity at 0, 1/2, 1, 2, 4, 6, 8, 12, and 24 hours. Feed was given to the animals after 4 hours of dosing and body weight was checked 6 hours after dosing. Morbidity like convulsions, tremors, grip strength and pupil dilatation were observed. The animals were observed twice daily for 14 days and body weight was taken. The same experiment was repeated once again on 3 rats (preferably female) as there was no observable clinical toxicity for the animals on the phase I study. From this 1/10th of 2000 mg per kilogram body weight was selected for further study for EUR, EMA, EBC and EEE respectively.

OECD guidelines for testing of chemicals are periodically reviewed in light of scientific progress or changing assessment practices. It is carried out for determining the LD_{50} of various chemicals. The test procedure described in this guideline is of value in minimizing the number of animals required to estimate the accurate oral toxicity of chemicals and for this purpose four dose levels were selected and six female rats per group were taken for study.

Grouping of animals

Animals were divided into seven groups of either sex; six rats in each group.

Group I : Sham Control

Group II : 6-OHDA Control

Group III : 6-OHDA +L-DOPA (Standard) (6 mg/kg orally)

Group IV : 6-OHDA+ EUR (200 mg/kg orally)

Group V : 6-OHDA+ EMA (200 mg/kg orally)

Group VI : 6-OHDA+ EBC (200 mg/kg orally)

Group VII : 6-OHDA+ EEE (200 mg/kg orally)

Induction of Parkinsonism by 6-OHDA [91-97]

On the zero day desipramine (Sigma, St. Louis, MO) (25 mg/kg, IP) was administered 30 min before surgery, to protect noradrenaline containing terminals from the effects of 6-OHDA (Sigma). All animals were anaesthetised with ketamine (100mg/kg ip), xylaxine (15mg/Kg im) and were fixed in a stereotaxic apparatus (USA). After the skull was exposed, a burr hole was drilled for the accommodation of needle. The needle was inserted into the substantia nigra with the following coordinates: anterior/ posterior: -4.8 mm; medial/lateral: -2.2 mm; ventral/dorsal: -7.2 mm−3.5mm from bregma and injection of 6-OHDA (20 µg of 6-OHDA hydrobromide in 4µl 0.9% saline with 0.02 µg/ml ascorbic acid) was then made over 5 min and the needle was left in place for a further 5 min. Then the skull was secured with stainless metallic screws and the wound area was covered by dental cement. Each rat was housed individually following the surgical procedure.

Lesion verification: quantification of circling behaviour [92-94]

After 48 hours of surgery, the animals were tested for circling behaviour. Circling behaviour was induced by subcutaneous injection of 0.5 mg/kg apomorphine. The animals were observed for 10 minutes period for counting circling behaviour. During observation period the animals were not disturbed. Only animals showing at least 7 turns/ min were included in this study.

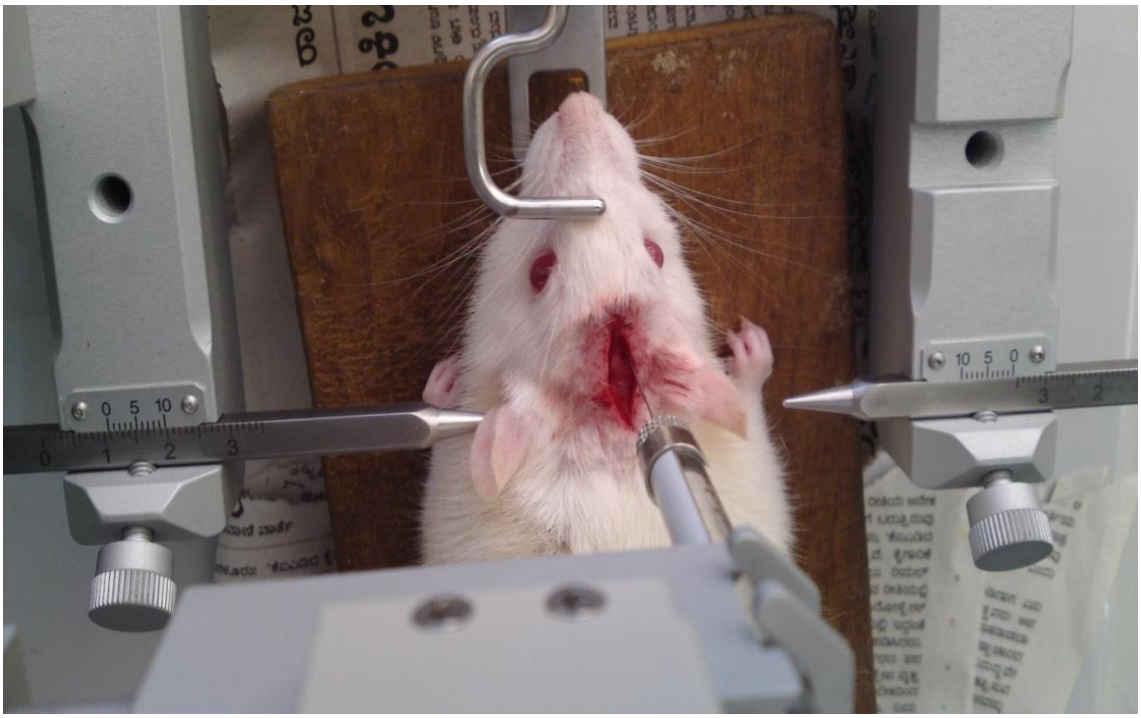


Figure 16: Diagram illustrates the stereotaxic surgery for unilateral injection of 6-OHDA at Pharmacology laboratory, Padmavathi College of Pharmacy, Dharmapuri.

Figure 17: The rat after stereotaxic surgery at animal house Padmavathi College of Pharmacy, Dharmapuri.

Treatment

EUR, EMA, EBC and EEE were given orally at a dose of 200 mg/kg after 48 hrs of induction for sixty days. L-DOPA was given orally at a dose of 6 mg/kg as standard drug.[38,39] Treatment drugs were suspended in 0.3% CMC solution and control animals were treated with 0.3% CMC in saline.

PARAMETERS EVALUATED

The following parameters were evaluated, after the 60th day of treatment.

A. PHARMACOLOGICAL EVALUATION

1. Measurement of Monoamine Oxidase Activity [98]

Principal

High-performance liquid chromatography (HPLC) is an accurate and a sensitive method to assay many of the catecholamine-metabolizing enzymes. In this assay MAO-B activity was determined using benzylamine as substrate, deproteinization with perchloric acid and detection of the product benzaldehyde at 254 nm.

Reagents

- Benzylamine hydrochloride
- Benzaldehyde
- Clorgyline
- Methanol, HPLC grade
- Sodium heptanes sulphonic acid

All other reagents were of analytical grade and purchased from commercial sources.

Procedure

Sample preparation

Rats weighing about 200 g were sacrificed and the brains were quickly removed and homogenized with four volumes of cold 0.9% potassium chloride. Homogenates were kept in small aliquots at -20°C until assayed. The protein concentrations of tissue homogenates were measured by Lowry's method.

Assay

The enzyme incubation mixture contained the following components in a total volume of 0.5 ml: 0.35 ml of 0.2 M sodium phosphate buffer pH 7.2, 0.1 ml of brain

homogenate and 0.05 ml of 2 mM benzylamine. The mixture was incubated for 30 min at 37°C except for the study of time course. The reaction was stopped by the addition of 50 µl of 4 M perchloric acid. Protein was removed by centrifugation. A 20 µl aliquot was injected into the liquid chromatogram.

Chromatographic conditions

5 µl Ultrasphere-ODS column (4.5x150 mm); mobile phase, 40% methanol in 50 mM sodium phosphate buffer, pH 3.2, containing 1 mM sodium heptanesulphonic acid; flow-rate, 1.5 ml/min; detection at 254 nm; sensitivity, 0.01 a.u.f.s., injection volume, 20 µl.

Chromatography

The modular liquid chromatographic system (Shimadzu, Kyoto, Japan) consisted of a 150x 4.6 mm, 5 µm Ultrasphere-ODS column fitted with a 45x4.6 mm precolumn (Beckman Instruments, Fullerton, CA, U.S.A.). The eluted components were detected by ultraviolet (UV) detector absorption at 254 nm. The elution was carried out isocratically at ambient temperature using 40% methanol containing 50 mM sodium phosphate and 1 mM heptanesulphonic acid. The pH was adjusted to 3.2 with sodium hydroxide. The flow rate was 1.5 ml/min. The enzyme activity was calculated as nmoles benzaldehyde formed per min per mg protein.

The calibration curve indicates a linear relationship between the peak height and the amount of benzaldehyde from 0.2 to 20 nmol/ml. The detection limit with signal-to-noise ratio of 5:1 was 150 pmol/ml, making it possible to detect very low MAO-B activities.

The rate of aldehyde formation expressed as growing height of the benzaldehyde peak on the liquid chromatogram showed a linear relationship $Y=8.23X + 0.53$, $r = 0.921$ ($n = 6$), with up to 60 min of incubation time. The standard error between data points was less than 3.0%.

2. Lesion verification: Quantification of circling behavior [99-101]

At the end of the treatment period the animals were tested for circling behavior. Circling behavior was induced by 0.25 mg/kg apomorphine (s.c.) respectively. The animals were observed for 10 minutes period for counting circling behavior. During observational period the animals were not disturbed. The numbers of full and counter

clock wise turns were observed for ten minutes among different groups. The total numbers of circle for ten minutes were recorded.

3. Rotarod (Grip strength) [102-104]

The main symptom of the Parkinsonism disease is muscle rigidity. The loss of muscle grip is an indication of muscle rigidity. This effect can be easily studied in animals by using rotarod apparatus. Rotarod has been used to evaluate muscle grip strength by testing the ability of rats to remain on revolving rod. The apparatus has a horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70 cm long rod was divided into four sections by wooden partitions. First rotarod apparatus was turned on then selected 20 rpm as an appropriate speed. Each rat was given five trials before the actual reading was taken. The animal was placed individually one by one on the rotating rod. The 'fall of time' was noted when animal falls from the rotating rod and then the fall off time of animals were compared in treated group.

4. Catalepsy test (Fore limb placing test) [105-108]

The major clinical symptom of Parkinson's disease includes difficulty to move and change the posture (akinesia and rigidity) and tremors. So by this parameter we could observe the severity of catatonia as fallowed

Stage I- Rat moves normally when placed on table= (Score- 0)

Stage II- Rat moves only when touched/pushed= (Score- 0.5)

Stage III- Rat placed on the table with front paws set at least on a 3 cm high block fails to correct the posture in 10 sec= (Score- 0.5 for each paw total score-1)

Stage IV- Rat fails to remove when front paws are placed alternately on 9 cm block= (score-1 for each paw total Score- 2)

Thus for a single rat maximum possible score would be 3.5 revealing total catatonia.

B. BIOCHEMICAL EVALUATION

5. Estimation of total protein by Lowry's method [109,110]

Principle

The blue colour developed by the reduction of the phosphomolybdic phosphotungstic compounds in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry's method.

Chemicals and reagents

- 50 ml of 2% sodium carbonate in 50 ml 0.1 N sodium hydroxide (Reagent A)
- 0.5% copper sulphate ($CuSO_4.5H_2O$) in 1% potassium sodium tartrate (Reagent B)
- Alkaline Copper solution: Mix 50 ml of A and 1 ml of B prior to use (Reagent C)
- Folin-Ciocalteau Reagent solution- Dilute equal volume of commercial reagent with distilled water (Reagent D)
- Protein solution (Stock solution 1mg/ml)- Weigh accurately 50 mg of bovine serum albumin (Fraction V) and dissolved in distilled water and make up to 50 ml in standard flask.
- Working standard- Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask. One ml of this solution contains 200 µg proteins.

Procedure

Extraction of protein from sample- Extraction was carried out with buffer used for the enzyme assay. 500 mg of the brain sample was weighed and homogenized with 5-10 ml of the buffer. Then homogenize was centrifuged and the supernatant was used for the protein estimation.

Estimation of protein

- Different dilutions of BSA solutions are prepared by mixing stock BSA solution (1 mg/ml) and water in the series of test tubes as given in the table. The final volume in each test tube was 5 ml, and then final BSA concentration was 0.05 to 1 mg/ml.

- From these different dilutions pipette out 0.2 ml protein solution to different test tubes and added 2 ml of alkaline copper sulphate reagent (analytical reagent). Mixed the solution well.
- These solutions were incubated at room temperature for 10 minutes.
- Then added 0.2 ml of reagent Folin-Ciocalteau solution to each tube mixed well and incubated for 30 minute. Blue colour was developed.
- Measured the absorbance by colorimeter at 660 nm.
- Drawn the standard graph and calculated the amount of protein in the sample.

Table 1: Standard dilution for estimation of protein by Lowry's method

Sr. No.	BSA (ml)	Water (ml)	Sample concentration (mg/ml)	Sample volume (ml)	Alkaline $CuSo_4$ (ml)	Lowry's reagent (ml)
1	0.25	4.75	0.05	0.2	2.0	0.2
2	0.5	4.5	0.1	0.2	2.0	0.2
3	1	4	0.2	0.2	2.0	0.2
4	2	3	0.4	0.2	2.0	0.2
5	3	2	0.6	0.2	2.0	0.2
6	4	1	0.8	0.2	2.0	0.2
7	5	0	1.0	0.2	2.0	0.2

Table 2: Absorbance of standard dilution

Sr. No.	Sample concentration (mg/ml)	Optical density at 660 nm
1	0.05	0.016
2	0.1	0.036
3	0.2	0.058
4	0.4	0.116
5	0.6	0.178
6	0.8	0.226
7	1.0	0.298

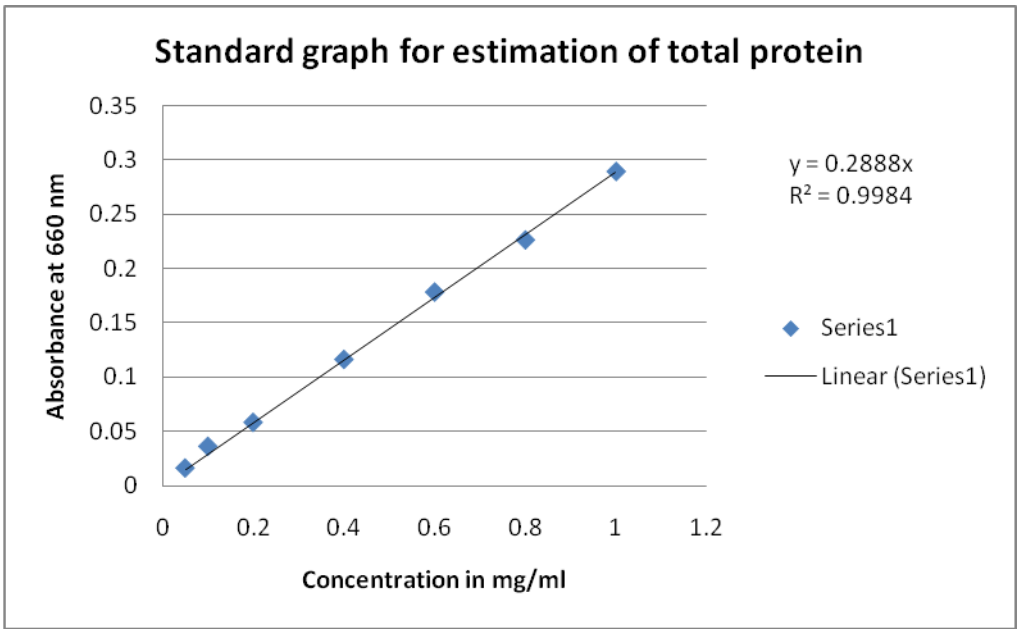


Figure 18: Standard graph for estimation of total protein

6. HPLC measurement of dopamine and metabolites [111-113]

Chemicals and reagent

- 0.1 M Perchloric acid
- 50 mM Ammonium phosphate
- 25mM Hexane sulfonic acid
- Acetonitrile (ACN).

Chromatographic conditions

- **Mobile phase:** 1) 50 mM Ammonium phosphate pH 4.6
  2) 25mM Hexane sullphonic acid pH 4.04
  3) ACN (Acetonitrile)

  pH is adjusted with orthophosphoric acid and triethyl amine.
- **Mobile phase ratio:** 60:10:30
- **Column:** 150mm **x** 4.6mm diameter
  S/n: 07-8494
  PRINCETON SPHERE C_{18} 100A° 5µ
- **Flow rate:** 0.5mL/minute

- **Standard preparation**

  0.1 mg of standard dopamine is dissolved in water and the further dilutions (100 ng/ml) was made with mobile phase.
- **Internal standard:** Paracetamol
- **Dilutions**

  The first dilution is made with water and the second or all the further dilutions are made with mobile phase.

Procedure for estimation dopamine by HPLC

Dopamine content was analyzed according to the previously described method with some modifications. Dissected striata were immediately frozen on dry ice and stored at -80°C. Striatal tissues were sonicated in 0.1 M of perchloric acid (about 100 µL/mg tissue) containing paracetamol (100 µg/ml, lemda max-257) as the internal standard. The supernatant fluids were taken for measurements of levels of dopamine by HPLC. Briefly, 20 µL supernatant fluid was isocratically eluted through an 4.6-mm C18 column with a mobile phase containing 50 mM Ammonium phosphate pH 4.6, 25mM Hexane sullphonic acid pH 4.04, 5% acetonitrile and detected by a UV detector at 254 nanometer. The flow rate was 0.5 ml/min. Concentrations of DA was expressed as nanograms per milligram of protein. The protein concentrations of tissue homogenates were measured by Lowry's method.

7. Localization of iron in substantia nigra [114-116]

(Perl's diaminobenzidene (Perl's-DAB) method)

Chemicals and reagent

- Phosphate-buffer
- Formalin
- Paraffin wax
- 7% potassium ferricyanide
- 7% potassium ferrocyanide
- 3% hydrochloric acid (HCL)
- 3,3'-diaminobenzidine (DAB) 0.75 mg/ml
- 0.015% Hydrogen peroxide (H_2O_2).

Procedure

Brain tissues were stained for ferric ion using the perl's-DAB method. The isolated and partially frozen brains were cut horizontally to get 30-40 µm sections on a vibratome and then mounted on a glass slide. The sections were immersed in 2% potassium ferrocyanide and 2% hydrochloric acid for thirty minutes at room temperature and then rinsed with deionized water for five minutes. The perl's reaction was intensified by placing the tissue in 0.5% diaminobenzidine (DAB) in cold phosphate buffer (pH 7.4) for fifteen minutes. Next, 2ml of 1% hydrogen peroxide was added for every 200 ml of DAB solution. The sections remained in the solution for twenty five minutes. Following DAB treatment the sections were rinsed in deionized water for fifteen minutes. Counter stained the sections by using thionine solution. The slides of the section were made using a motic microscope (model Motic images plus 2.0), under the magnification of 40x equipped with camera.

Image analysis

Perl's stained sections were analyzed by densitometry with an IBM AT computer using Jandel scientific image analysis software (JAWA ^{TM}) with a grey scale range of 256 grey levels. The images were digitized with aid of high resolution video monitor. The substantia nigra was traced and the values for each region were obtained. Iron-assosiated regions appeared as dark areas on perl's DAB stained sections.

The perl's DAB stained sections also showed somewhat variable staining intensities while the relationship between intensity of stain was fluctuating ones. Therefore direct comparison of the densitometry measurements across stained sections would not be appropriate. To solve this problem, for each animal the densitometry values of each brain areas in the perl's-DAB stained section was converted to a ratio according to the following equation.

$$\textbf{Iron asymmetry ratio} = \frac{\textbf{Ipsilateral densitometry value}}{\textbf{Contralateral densitometry value}}$$

Iron asymmetry ratio allowed for comparison of different groups to find out the variations in brain iron content. The mean perl's-DAB substantia nigra asymmetry ratio for the control and treated groups were compared statistically by nonparametric repeated measures ANOVA followed by Dunnet's multiple comparisions test (a brain iron ratio significantly different from 1.00 would indicate an asymmetry).

8. Lipid peroxidation assay [117]

Chemicals and reagents

- 8.1% Sodium dodecyl sulphate (SDS)
- 20% Acetic acid solution (pH 3.5 with 1N NaOH/ 0.1 HCL)
- 0.8% aqueous solution of thiobarbituric acid (pH 7.4 with 1N NaOH/ 0.1 HCL solution)
- Mixture of n-butanol-pyridine (15:1)

Procedure

Lipid peroxidation in rat brain homogenate was carried out essentially as described earlier. Rat forebrain (stored at -80ºC for less than 8 days) was homogenized in 20 mM Tris-HCl, pH 7.4 (10 ml) at 4ºC using a Polytron homogenizer. The homogenate was centrifuged at 1000 g for 10 min at 4°C, and the supernatant collected. Then acetic acid 1.5 ml (20%; pH 3.5), 1.5 ml of thiobarbituric acid (0.8%) and 0.2 ml of sodium dodecyl sulphate (8.1%) were added to 0.1 ml of supernatant and heated at 100 ºC for 60 min. Mixture was cooled and 5 ml of *n*-butanol-pyridine (15:1) mixture, 1 ml of distilled water was added and vortexed vigorously. After centrifugation at 1200×*g* for 10 min, the organic layer was separated and absorbance was measured at 532 nm using Elisa plate reader. Malonyldialdehyde (MDA) is an end product of lipid peroxidation, which reacts with thiobarbituric acid to form pink chromogen–thiobarbituric acid reactive substance.

9. Estimation of catalase (CAT) [118]

Chemicals and reagents

- Potassium phosphate buffer (65 mM, pH 7.8)
- H_2O_2 7.5 mM
- Sucrose 0.3199 M

Procedure

Catalase measurement was carried out by the ability of CAT to oxidize hydrogen peroxide (H_2O_2). 2.25 ml of potassium phosphate buffer (65 mM, pH 7.8) and 100 µl of the brain homogenate were incubated at 25 ºC for 30 min. A 650 µl H_2O_2 (7.5 mM) was added to the brain homogenate to initiate the reaction. The change in absorption was measured at 240 nm for 2–3 min and the results were expressed as CAT µmol/min mg of protein.

10. Estimation of Superoxide dismutase assay (SOD) [119]

Chemicals and reagents

- Sodium pyrophosphate buffer (pH 8.3; 0.052 M)
- Phenazine methosulphate (186 µM)
- Nitro blue tetrazolium (300 µM)
- NADH (750 µM)
- Glacial acetic acid
- n-butanol

Procedure

SOD activity was analyzed by the method described earlier. Assay mixture contained 0.1 ml of supernatant, 1.2 ml of sodium pyrophosphate buffer (pH 8.3; 0.052 M), 0.1 ml of phenazine methosulphate (186 µm), 0.3 ml of nitro blue tetrazolium (300 µM), 0.2 ml of NADH (750 µM). Reaction was started by addition of NADH. After incubation at 30 ºC for 90 s, the reaction was stopped by addition of 0.1 ml of glacial acetic acid. Reaction mixture was stirred vigorously with 4.0 ml of *n*-butanol. Colour intensity of the chromogen in the butanol was measured spectrophotometrically at 560 nm and concentration of SOD was expressed as U/mg of protein.

11. Analysis of GSH/ Glutathion reductase [96,120]

Chemicals and reagents

- Perchloric acid 0.2 M
- 0.01% EDTA (Ethylenediamine tetra acetic acid)
- Reduced nicotinamide adenine dinucleotide phosphate (NADPH) 0.3 mM
- 5,5-dithiobis-2-nitrobenzoic acid (DTNB) 6 mM
- Glutathione reductase 25 units/ml
- Phosphate buffer, pH 7.5

Procedure

GSH was measured enzymatically by the method described by Owen. The striata were homogenized in ice-cold perchloric acid (0.2 M) containing 0.01% EDTA. The homogenate was centrifuged at 10,000 rpm at 4°C for 10 min. The enzymatic reaction was started by adding 200 µl of clear supernatant in a spectrophotometric cuvette containing 500 µl of 0.3 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), 100 µl of 6 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and 10 µl of 25

units/ml glutathione reductase (all the above three reagents were freshly prepared in phosphate buffer at pH 7.5). The absorbance was measured over a period of 3 min at 412 nm at 30°C. The GSH level was determined by comparing the change of absorbance (ΔA) of test solution with the ΔA of standard GSH.

12. Isolation of mitochondria [121-124]

Chemicals and reagents

- Sucrose 70 mM
- Mannitol 210 mM
- Tris HCl 5 mM
- EDTA 1 mM (Ethylenediamine tetra acetic acid)

Procedure

Tissue was homogenized with a Dounce tissue grinder in mitochondrial isolation buffer (70 mM sucrose, 210 mM mannitol, 5 mM Tris HCl, 1 mM EDTA; pH 7.4) and suspensions were centrifuged at 800 g, 4°C, for 10 min. The supernatant fluids were centrifuged at 13000 g, 4°C, for 10 min, and the pellets were washed with mitochondrial isolation buffer and centrifuged at 13000 g, 4°C, for 10 min to obtain the crude mitochondrial fraction.

13. Complex I activity assay [121-124]

Chemicals and reagent

- Potassium dihydrogen orthophosphate (KH_2PO_4) 25 mM
- Magnesium chloride ($MgCl_2$) 5 mM
- Ubiquinone 65 µM
- Reduced nicotinamide adenine dinucleotide (NADH) 130 µM
- Antimycin A 2 µg/ml
- Defatted bovine serum albumin (BSA) 2.5 mg/ml
- Rotenone 2 µg/ml.

Procedure

NADH: ubiquinone oxidoreducase (Complex I) activity was measured in the SN as described in the literature. Brain mitochondria, isolated as above, were lysed by freeze–thawing in hypotonic buffer (25 mM KH_2PO_4, 5 mM $MgCl_2$, pH 7.4). The reaction was initiated by the addition of 50 µg mitochondria to the assay buffer

[hypotonic buffer containing 65 µM ubiquinone, 130 µM NADH, 2 µg/ml antimycin A and 2.5 mg/ml defatted bovine serum albumin (BSA)]. The oxidation of NADH by Complex I was monitored spectrophotometrically at 340 nm for 2 min at 30°C. The activity was monitored for a further 2 min following the addition of rotenone (2µg/ml). The difference between the rate of oxidation before and after the addition of rotenone was used to calculate Complex I activity.

14. Estimation of mid brain Calcium [125-126]

Chemicals and Reagents:

- **Phosphate buffer solution**
- **0.1 M Perchloric acid**
- **10 mg/ml Standard Calcium solution**

Procedure

Wistar rats were sacrificed by excess anaesthesia and brain samples were obtained. Homogenates of brain samples were prepared. Tissues (0.4 g) were diced, added to ice-cold PBS solution (40ml) and homogenized with an Omni 5000 homogenizer over ice for 5 min. The homogenate was centrifuged (3000 rpm- 5 min) and the supernatant was separated and stored at -80 ^{0}C before AAS analysis.

Supernatant (2ml) was diluted with 0.1 M Perchloric acid (0.1ml), mixed well and then centrifuged at 3500 rpm for 10 minutes. The concentration of calcium present in the supernatant was determined by atomic absorption spectroscopy. The standards of different Ca concentrations (i.e., 1, 1.5, 2 and 2.5 µg/ml) were prepared from stock standard. The standards and samples were read against the blank solution. The absorbance of samples, standards and blank were noted. The concentration of calcium in the brain was calculated by reading from the standard curve.

Table 3: Absorbance of standard dilution for estimation of calcium level in brain by AAS

S. NO	Concentration (µg/ml)	Absorbance
1	1.0	0.0228
2	1.5	0.0316
3	2.0	0.0405
4	2.5	0.0492

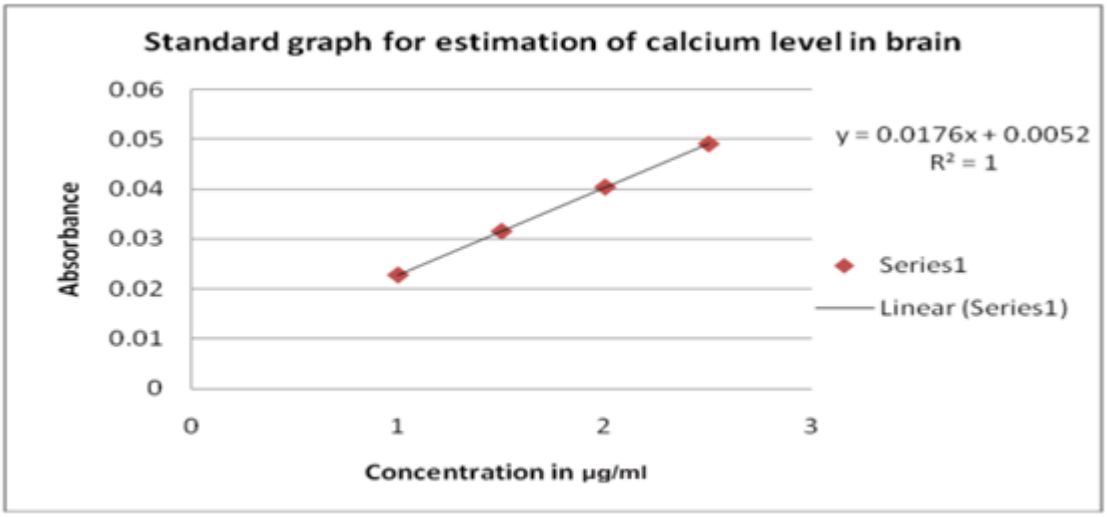


Figure 19: Standard graph for estimation of calcium level in brain

Statistical analysis

The collected data were subjected to appropriate statistical tests like one-way ANOVA (Analysis of Variance) followed by Bonferroni multiple comparisions test. P values of less than 0.05 were considered significant. The analysis was carried using GraphPad Prism software version 5.

RESULTS AND ANALYSIS

RESULTS AND ANALYSIS

Table 4, Figure 20 and 21: HPTLC Standardization of EUR using catechin as a standard

HPTLC standardization of EUR was performed using catechin as a standard marker. From the chromatogram the presence of catechin in the sample was confirmed. The Rf value of catechin in sample was found to be; 0.45 and the amount of catechin present in the sample was found to be; 0.724 % w/w.

Table 5, Figure 22 and 23: HPTLC Standardization of EMA using naringenin as a standard

HPTLC standardization of EMA was performed using naringenin as a standard marker. From the chromatogram the presence of naringenin in the sample was confirmed. The Rf value of naringenin in sample was found to be; 0.89 and the amount of naringenin present in the sample was found to be; 0.273 % w/w.

Table 6, Figure 24 and 25: HPTLC Standardization of EBC using harmine as a standard

HPTLC standardization of EBC was performed using harmine as a standard marker. From the chromatogram the presence of harmine in the sample was confirmed. The Rf value of harmine in sample was found to be; 0.35 and the amount of harmine present in the sample was found to be; 0.263 % w/w.

Figure 20: HPTLC chromatogram for standard catechin

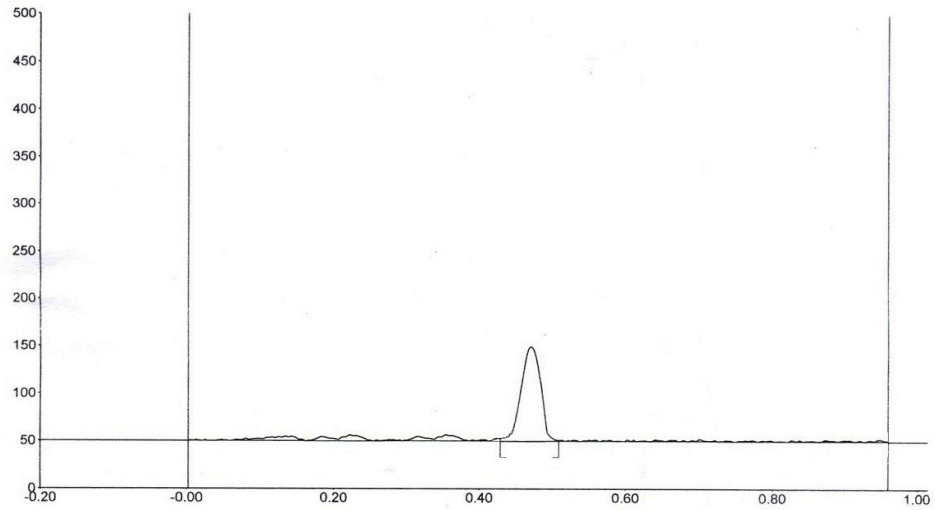


Figure 21: HPTLC chromatogram for EUR

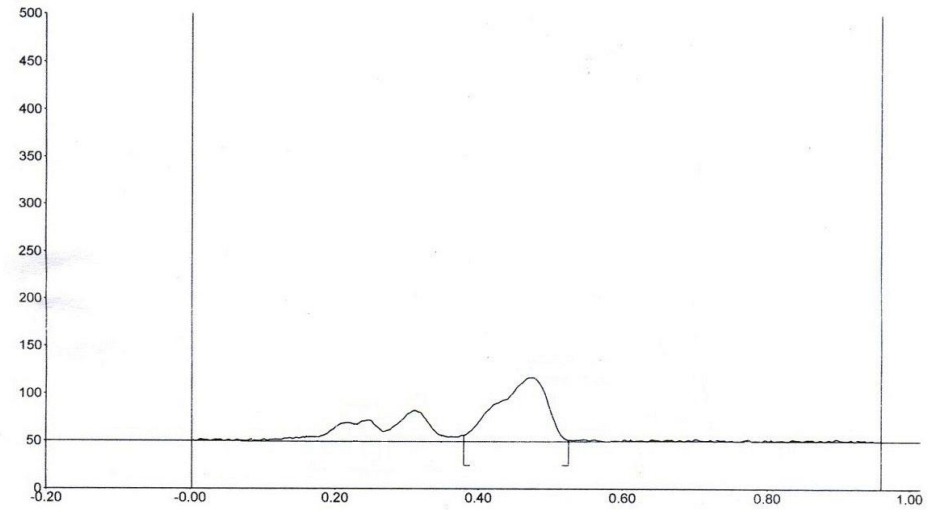


Table 4: Rf value and peak area of catechin in standard and EUR

Assigned substance	Rf Value	Peak Area
Catechin (standard)	0.46	4804.24
EUR (sample)	0.45	6538.11

Figure 22: HPTLC chromatogram for standard naringenin

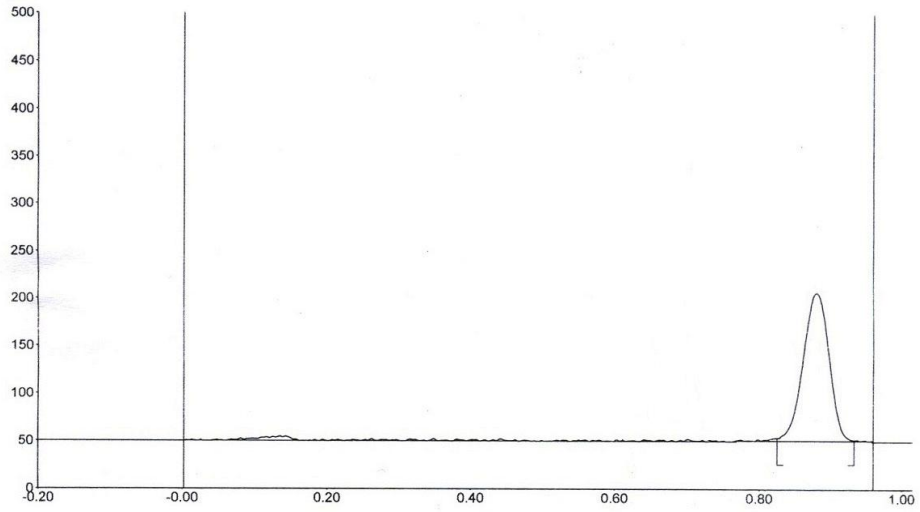


Figure 23: HPTLC chromatogram for EMA

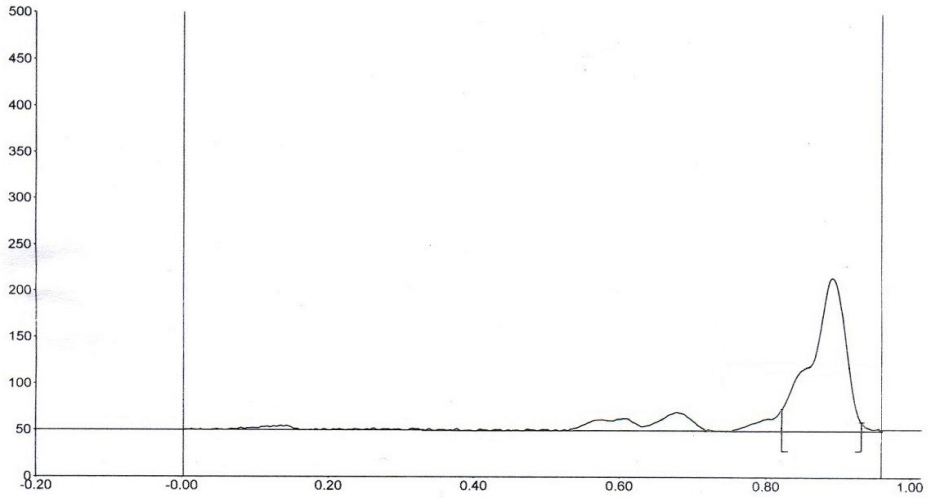


Table 5: Rf value and peak area of naringenin in standard and EMA

Assigned substance	Rf Value	Peak Area
Naringenin (standard)	0.90	5487.42
EMA (sample)	0.89	6843.34

Figure 24: HPTLC chromatogram for standard harmine

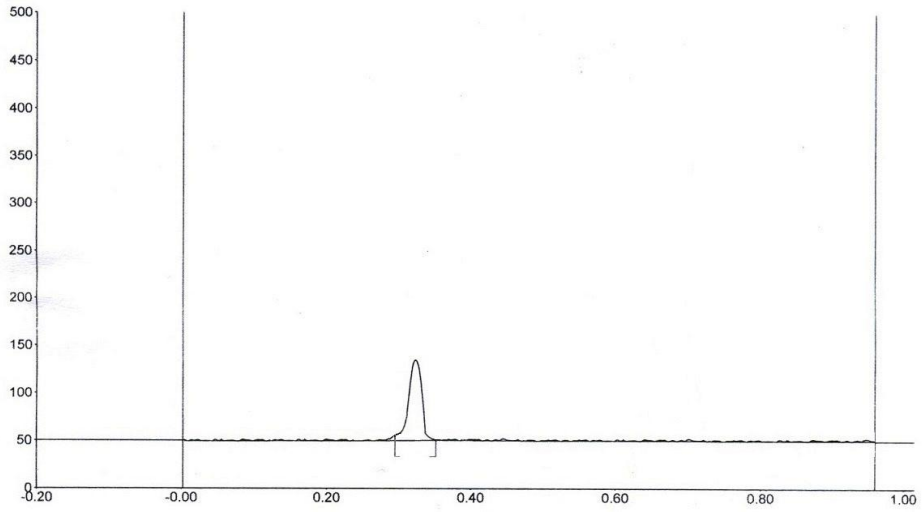


Figure 25: HPTLC chromatogram for EBC

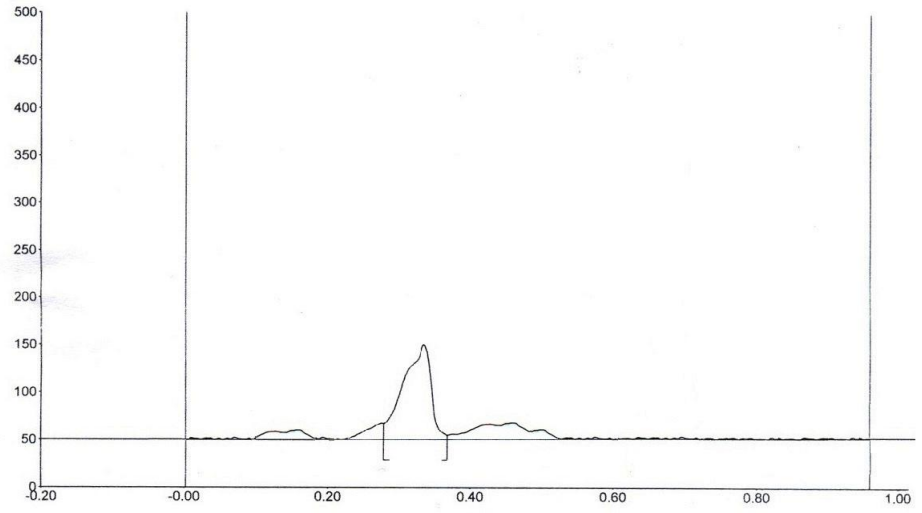


Table 6: Rf value and peak area of harmine in standard and EBC

Assigned substance	Rf Value	Peak Area
Harmine (standard)	0.34	2953.48
EBC (sample)	0.35	4632.24

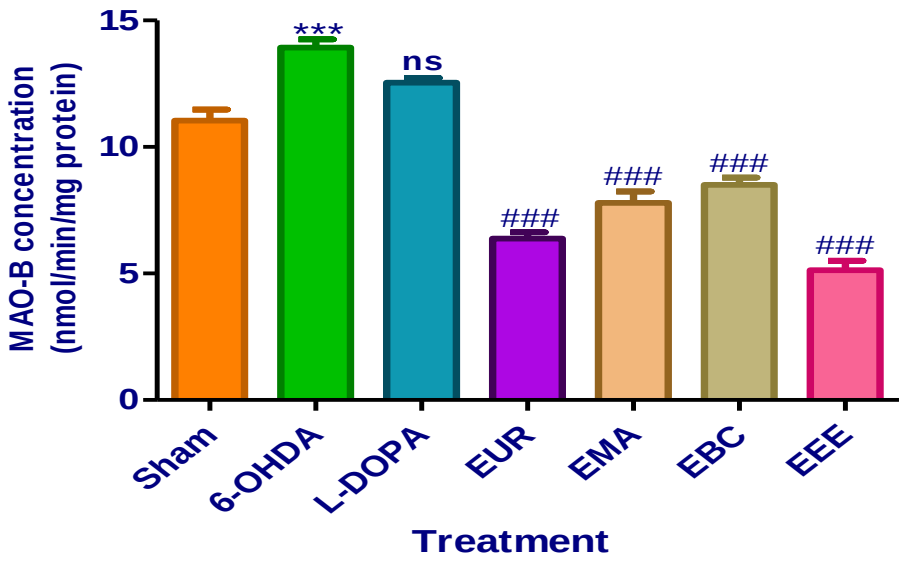
Table 7 and Figure 26: Effect of EUR, EMA, EBC and EEE on monoamine oxidase-B levels in rats

MAO-B has been evaluated on sixtieth day after successive experimental period. The sham control animals were considered to be the reference standard for other experimental animals in this study. When compared with sham control the 6-OHDA control had pronounced ($P<0.001$) increased MAO-B activity, which was at par highly elevated than normal animals. The L-DOPA treatment also elevated MAO-B levels as same as that of 6-OHDA control. The treatment with the EUR significantly ($P<0.001$) reduced MAO-B activity which was near equal to sham controls groups. The treatment with EMA showed a high significant ($P<0.001$) reduction in MAO-B activity when compared with 6-OHDA control. The treatment with EBC also provided a significant ($P<0.001$) reduction in MAO-B when compared with 6-OHDA control. The three test drugs together were tested for its additive or synergestic effect. The combined formulation EEE showed a pronounced decrease in MAO-B activity to near normal as that of sham control. These results indicates the better efficacy of test drugs to control MAO-B activity, and the possible mode of action of test drugs will be discussed in later in the discussion part.

Table 7: Effect of EUR, EMA, EBC and EEE on monoamine oxidase-B levels in rat brain

Sr. No.	Treatment Group	MAO-B concentration (nmol/min/mg protein)
1	Sham Control	11.05 ± 0.438
2	6-OHDA control	13.93 ± 0.327***
3	6-OHDA+ L-DOPA treated group	12.55 ± 0.179^{ns}
4	6-OHDA+ EUR treated group	6.382 ± 0.251$^{###}$
5	6-OHDA+ EMA treated group	7.795 ± 0.444$^{###}$
6	6-OHDA+ EBC treated group	8.508 ± 0.280$^{###}$
7	6-OHDA+ EEE treated group	5.125 ± 0.385$^{###}$

Figure 26: Effect of EUR, EMA, EBC and EEE on monoamine oxidase-B levels in rat brain



Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; $^{###}$P<0.001 and ^{ns}P>0.05 (non-significant) when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

Figure 27: HPLC chromatogram obtained from MAO-B preparation for sham control group

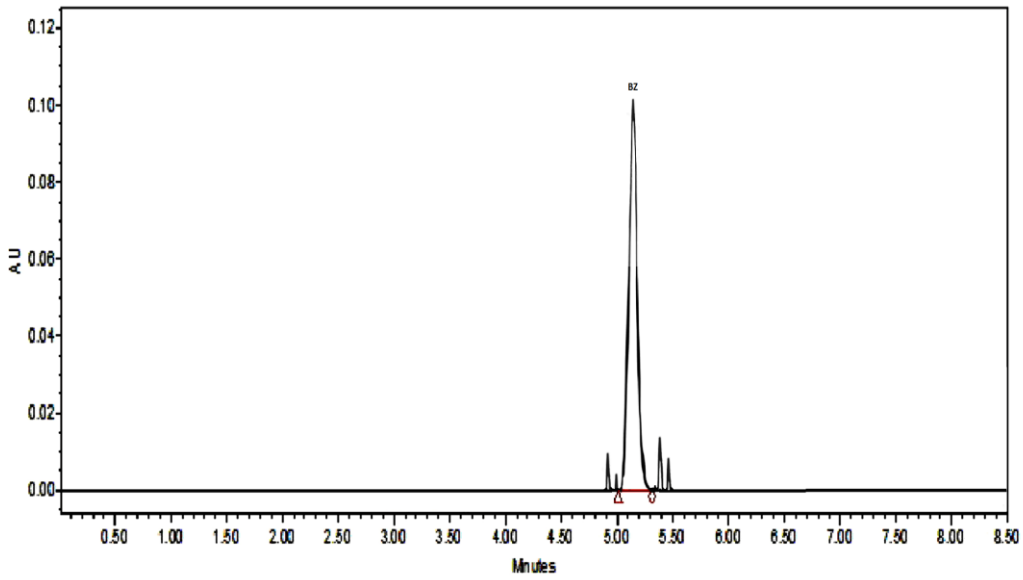


Figure 28: HPLC chromatogram obtained from MAO-B preparation for 6-OHDA control group

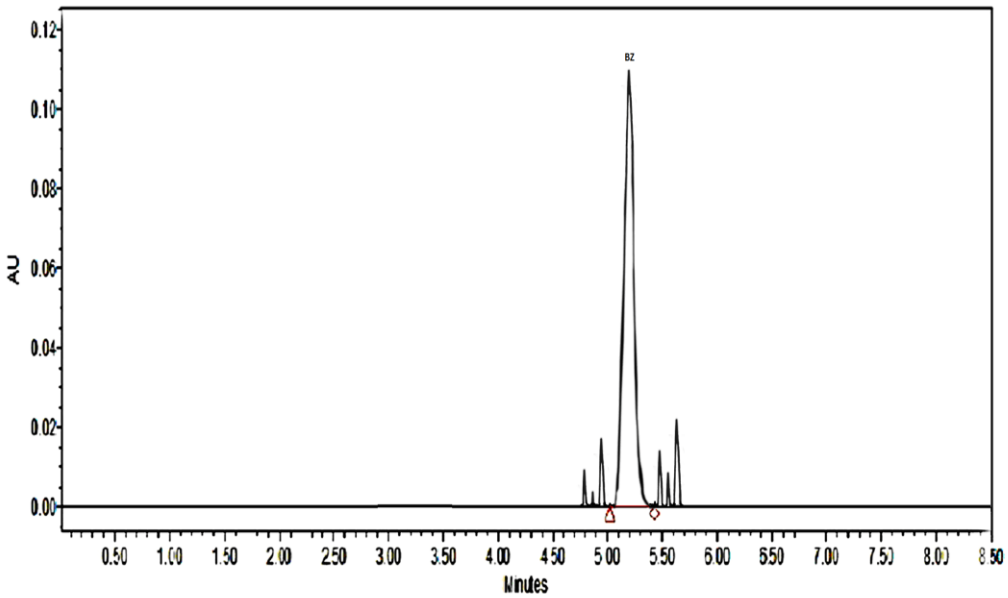


Figure 29: HPLC chromatogram obtained from MAO-B preparation for L-DOPA treated group

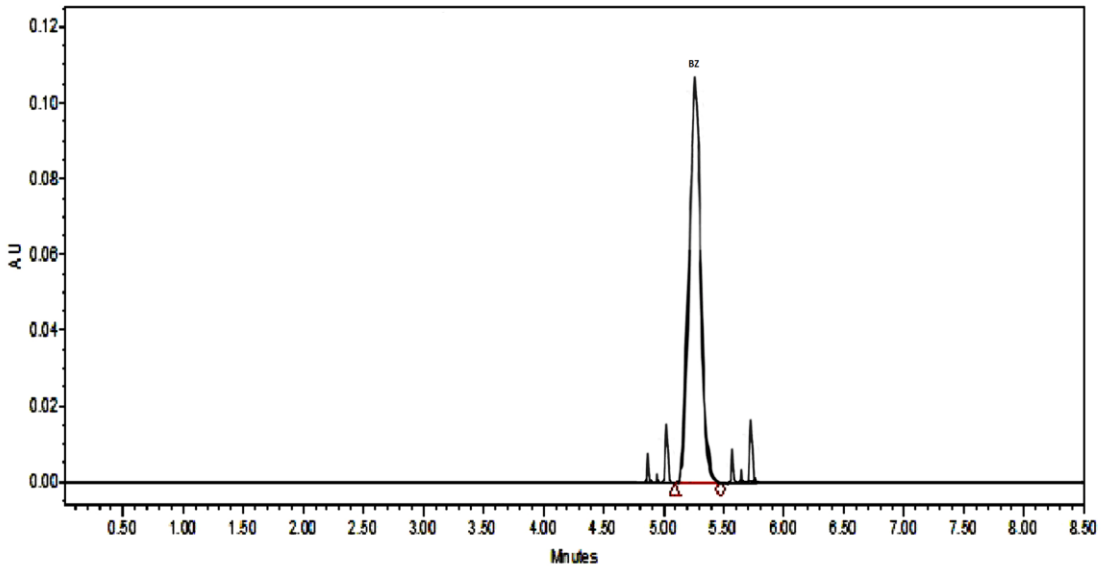


Figure 30: HPLC chromatogram obtained from MAO-B preparation for EUR treated group

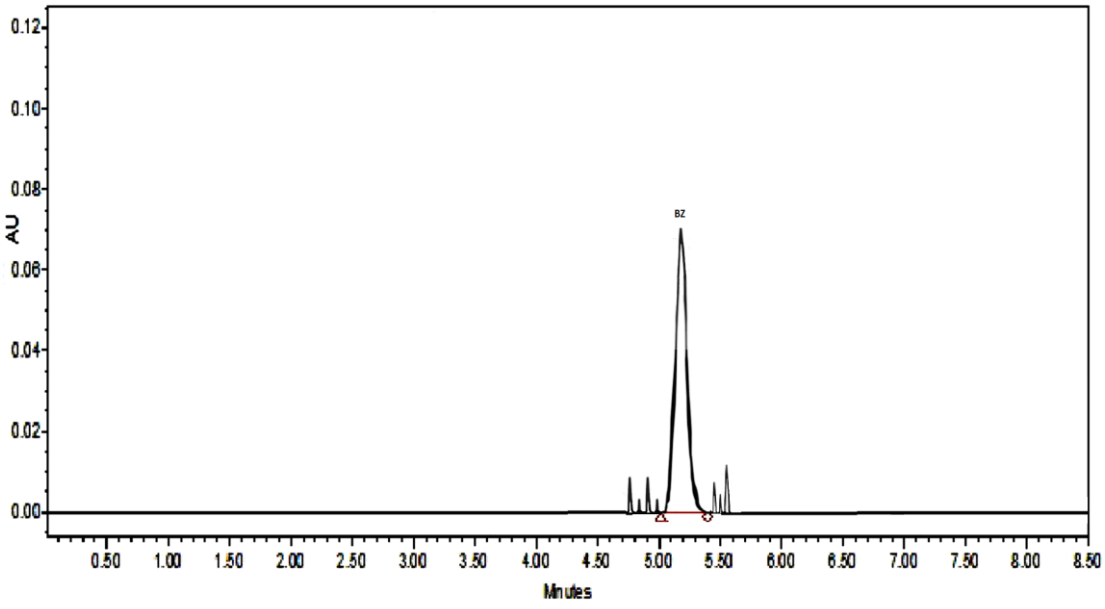


Figure 31: HPLC chromatogram obtained from MAO-B preparation for EMA treated group

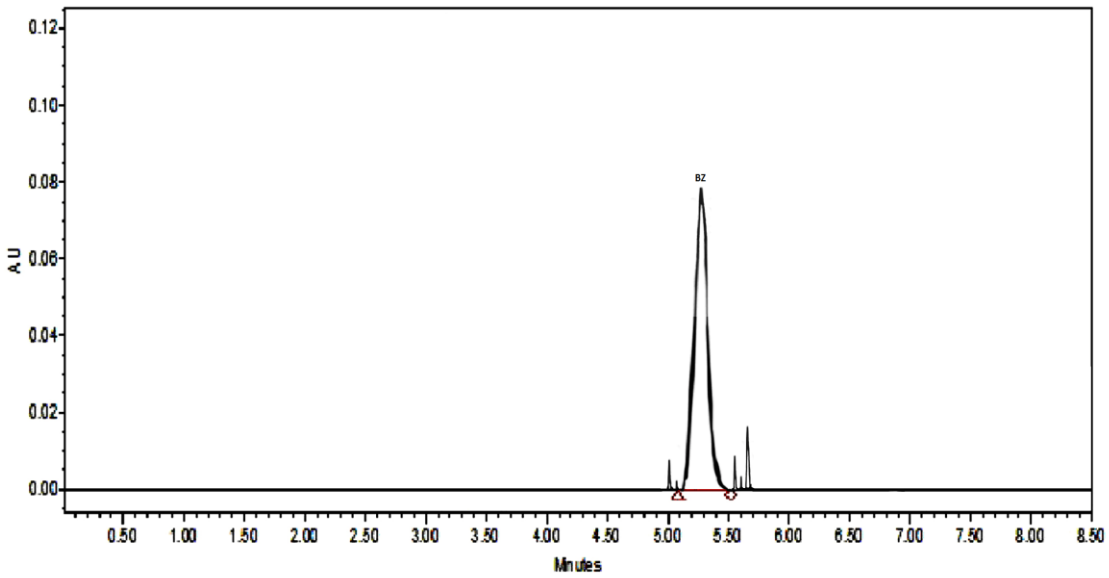


Figure 32: HPLC chromatogram obtained from MAO-B preparation for EBC treated group

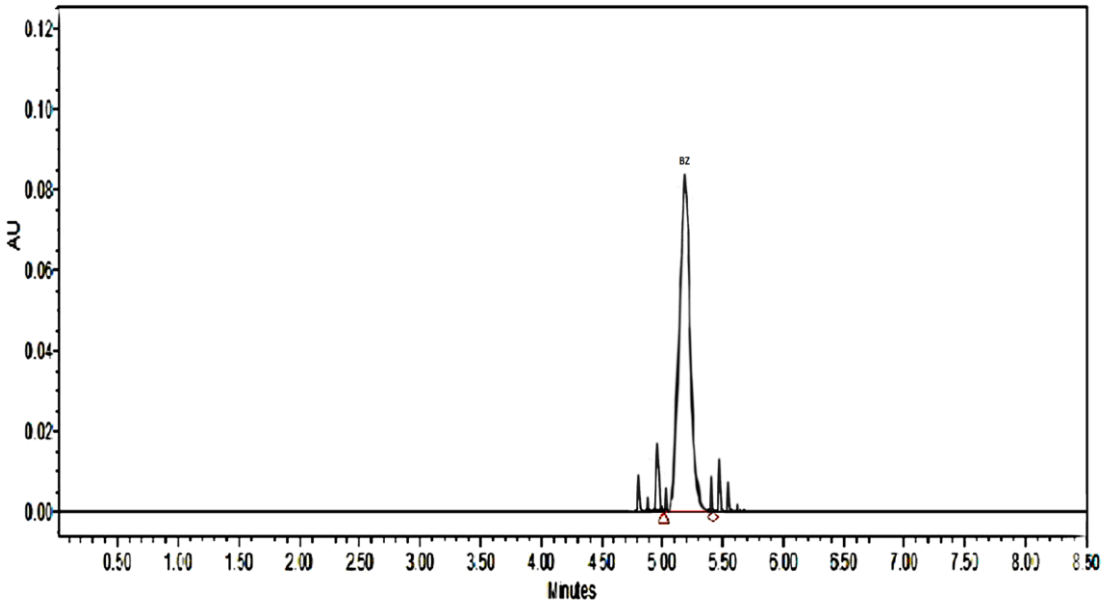


Figure 33: HPLC chromatogram obtained from MAO-B preparation for EEE treated group

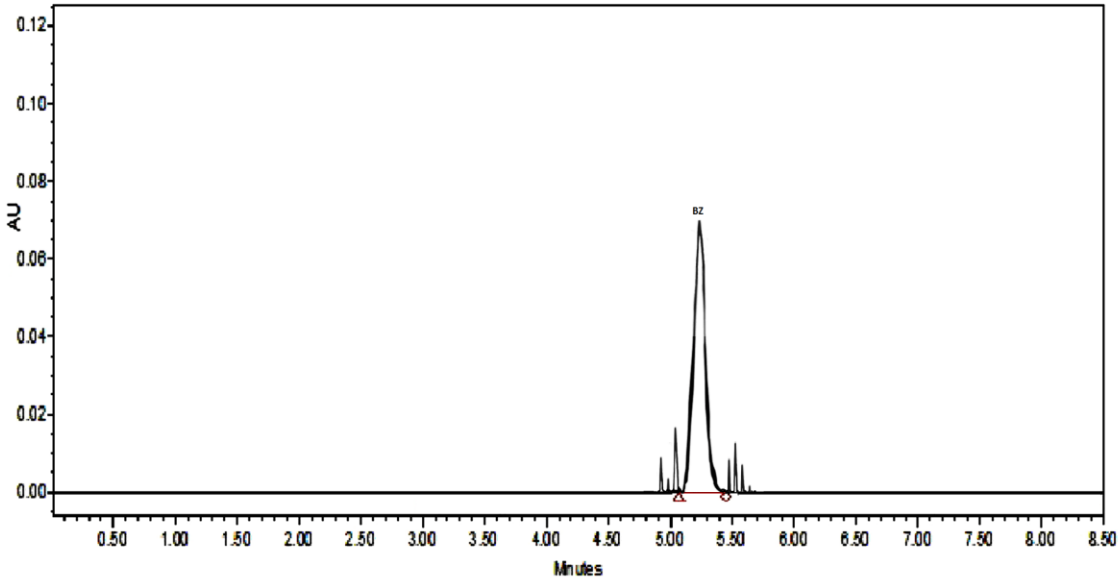


Table 8: HPLC chromatogram data obtained from MAO-B preparations

Sr. No.	Treatment Group	Rf value	Peak Height	Peak Area
1	Sham Control	5.2	177	1286
2	6-OHDA control	5.2	205	1513
3	6-OHDA+ L-DOPA treated group	5.3	186	1431
4	6-OHDA+ EUR treated group	5.3	151	844
5	6-OHDA+ EMA treated group	5.2	155	967
6	6-OHDA+ EBC treated group	5.2	162	1034
7	6-OHDA+ EEE treated group	5.3	148	813

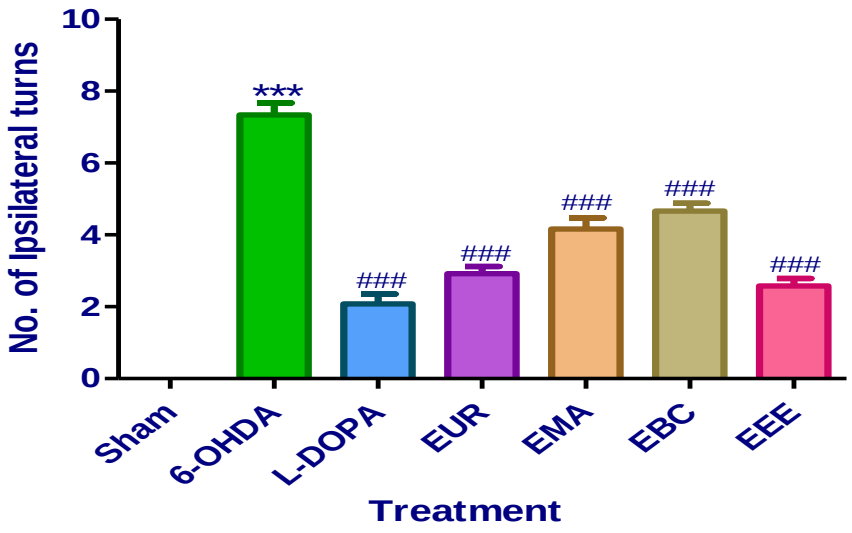
Table 9 and Figure 34: Effect of EUR, EMA, EBC and EEE on quantification of circling behaviour in rats

The circling behaviour of the treated animals has been evaluated on sixtieth day after successive experimental period. In general, there will not be circling behaviour in sham operated animal due to its intactness of lesion. When compared with sham control, the 6-OHDA control group significantly ($P<0.001$) increased the number of Ipsilateral turns in ten minutes which was significantly high than normal animals. In the L-DOPA treated groups, there were reduced numbers of Ipsilateral turns when compared with 6-OHDA control. The treatment with the EUR significantly ($P<0.001$) reduced the number of Ipsilateral turns during the ten minutes were near equal to sham controls groups. The treatment with EMA showed a high significant ($P<0.001$) reduction in Ipsilateral turns when compared with 6-OHDA control. The treatment with EBC provided a significant reduction ($P<0.001$) in Ipsilateral turns when compared with 6-OHDA control. The combined form of three extracts, EEE showed a significant decrease ($P<0.001$) in Ipsilateral turns to near normal as that of sham control. These results indicated the better efficacy of test drugs to control Ipsilateral turns. The possible mechanism of action of test drugs will be discussed in the discussion chapter.

Table 9: Effect of EUR, EMA, EBC and EEE on quantification of circling behaviour in rats

Sr. No.	Treatment Group	Number of Ipsilateral turns in 10 minutes
1	Sham Control	00
2	6-OHDA control	7.333 ± 0.333***
3	6-OHDA+ L-DOPA treated group	2.083 ± 0.271###
4	6-OHDA+ EUR treated group	2.50 ± 0.223###
5	6-OHDA+ EMA treated group	4.167 ± 0.307###
6	6-OHDA+ EBC treated group	4.667 ± 0.211###
7	6-OHDA+ EEE treated group	2.583 ± 0.201###

Figure 34: Effect of EUR, EMA, EBC and EEE on quantification of circling behaviour in rats



Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; ###P<0.001 when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

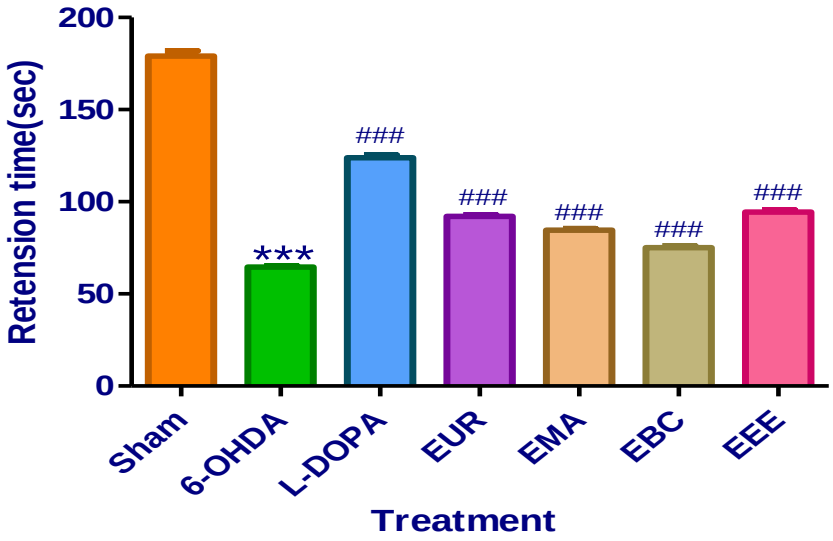
Table 10 and Figure 35: Effect of EUR, EMA, EBC and EEE on rotarod performance in rats

The grip strength of the treated animals has been evaluated on sixtieth day after successive experimental period by rotarod apparatus. The sham control animals were considered to be the comparison group for other experimental groups in this study. When compared with sham control the retention time in 6-OHDA control had significantly ($P<0.001$) decreased. It is probably due to the lesion induced bradykinesia in 6-OHDA groups. In the L-DOPA treated groups, there was better retention time when compared with 6-OHDA control. The treatment with the EUR group significantly ($P<0.001$) increased the retention time which was equal to that of sham controls groups. The treatment with EMA showed a high significant ($P<0.001$) increase in retention time when compared with 6-OHDA control. The treatment with EBC also provided a significant ($P<0.001$) elevation in retention time when compared with 6-OHDA control. The combined test drug formulation EEE showed a pronounced ($P<0.001$) increase in retention time to near normal as that of sham control. These results indicate the better efficacy of test drugs to reduce bradykinesia, and the possible mode of action of test drugs will be discussed in later in the discussion part.

Table 10: Effect of EUR, EMA, EBC and EEE on rotarod performance in rats

Sr. No.	Treatment Group	Retention Time (Sec)
1	Sham Control	179.0 ± 2.887
2	6-OHDA control	64.50 ± 0.763***
3	6-OHDA+ L-DOPA treated group	123.8 ± 1.641###
4	6-OHDA+ EUR treated group	92.0 ± 0.930###
5	6-OHDA+ EMA treated group	84.50 ± 0.957###
6	6-OHDA+ EBC treated group	75.17 ± 0.872###
7	6-OHDA+ EEE treated group	94.33 ± 1.202###

Figure 35: Effect of EUR, EMA, EBC and EEE on rotarod performance in rats



Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; ###P<0.001 when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

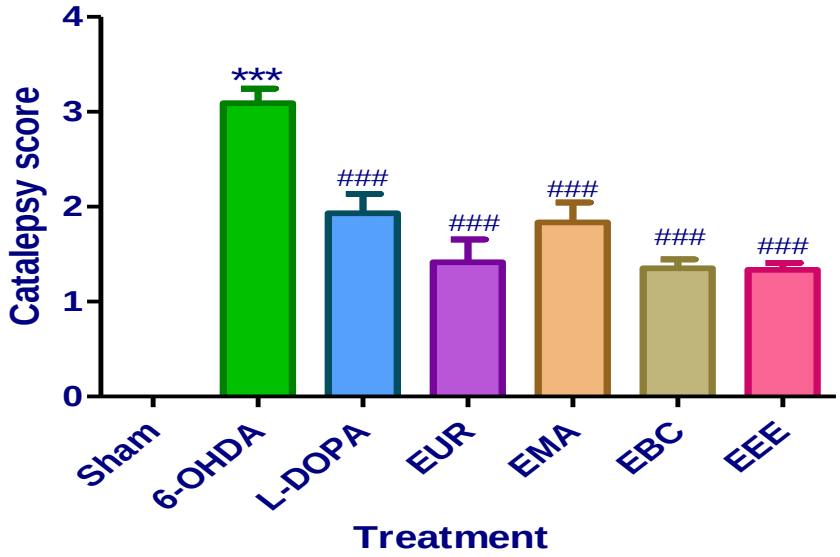
Table 11 and Figure 36: Effect of EUR, EMA, EBC and EEE on Catalepsy test (Fore limb placing test)

The catalepsy tests have been performed on sixtieth day after successive experimental period. When compared with sham control the 6-OHDA control had significant ($P<0.001$) increase in the catalepsy score which was at par highly elevated than normal animals. In the L-DOPA treated group, there were reduced catalepsy score when compared with 6-OHDA control. But, when compared with L-DOPA groups, there was an observable reduction in catalepsy in the test drug treated animal. The treatment with the EUR significantly ($P<0.001$) reduced the catalepsy score which was near equal to sham controls groups. The treatment with EMA showed a significant ($P<0.001$) reduction in catalepsy score when compared with 6-OHDA control. The treatment with EBC also provided a significant ($P<0.001$) reduction in catalepsy score when compared with 6-OHDA control. The three test drugs together were tested for its additive or synergestic effect. The combined formulation EEE showed a pronounced ($P<0.001$) decrease in catalepsy score to near normal as that of sham control. These results indicates the better efficacy of test drugs to control catalepsy, and the possible mode of action of test drugs will be discussed in later in the discussion part.

Table 11: Effect of EUR, EMA, EBC and EEE on Catalepsy test (Fore limb placing test)

Sr. No.	Treatment Group	Catalepsy score
1	Sham Control	00
2	6-OHDA control	3.092 ± 0.153***
3	6-OHDA+ L-DOPA treated group	1.933± 0.202###
4	6-OHDA+ EUR treated group	1.417 ± 0.238###
5	6-OHDA+ EMA treated group	1.833 ± 0.218###
6	6-OHDA+ EBC treated group	1.350 ± 0.095###
7	6-OHDA+ EEE treated group	1.335 ± 0.745###

Figure 36: Effect of EUR, EMA, EBC and EEE on Catalepsy test (Fore limb placing test)



Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; ###P<0.001 when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

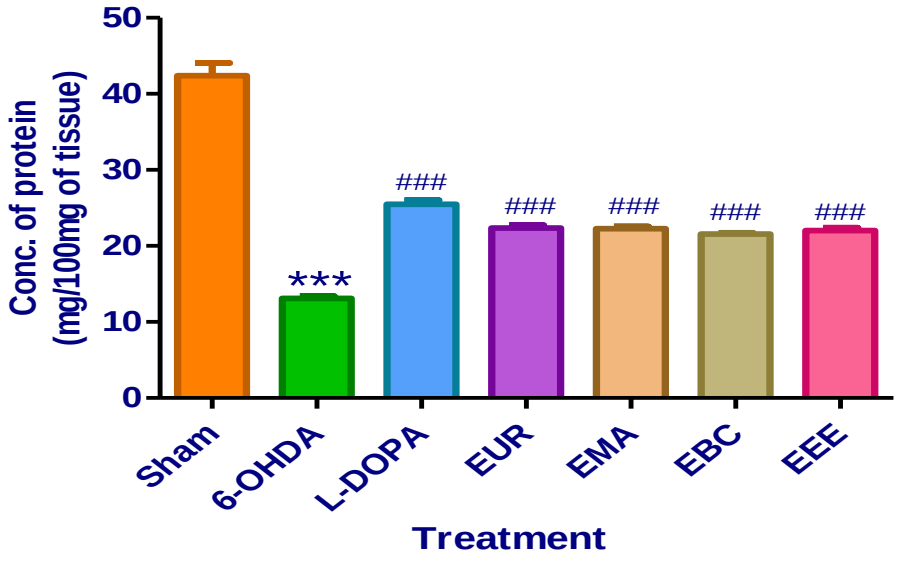
Table 12 and Figure 37: Effect EUR, EMA, EBC and EEE on total protein concentrations in rat brain

The total protein concentration in rat brain tissue has been evaluated on sixtieth day after successive experimental period by Lowry's method. The sham control animals were considered to be the comparison controls for other experimental in this study. When compared with sham control, the 6-OHDA control had significant ($P<0.001$) low concentration of tissue protein. L-DOPA treatment didn't alter the protein level significantly. The treatment with the EUR significantly ($P<0.001$) increased the concentration of tissue protein which was more over equal to sham control groups. The treatment with EMA showed a high significant ($P<0.001$) increase in concentration of tissue protein when compared with 6-OHDA control. The treatment with EBC also provided a significant ($P<0.001$) elevation in concentration of tissue protein when compared with 6-OHDA control. The three test drugs together were tested for its additive or synergestic effect. The combined formulation EEE showed a pronounced ($P<0.001$) increase in concentration of tissue protein near normal as that of sham control. These results indicates the better efficacy of test drugs to improvement in concentration of brain tissue protein, and the possible mode of action of test drugs will be discussed in the discussion part.

Table 12: Effect EUR, EMA, EBC and EEE on total protein concentrations in rat brain

Sr. No.	Treatment Group	Concentration of protein (mg/100mg of tissue)
1	Sham Control	42.39 ± 1.682
2	6-OHDA control	13.09 ± 0.305***
3	6-OHDA+ L-DOPA treated group	25.49 ± 0.53###
4	6-OHDA+ EUR treated group	22.37 ± 0.373###
5	6-OHDA+ EMA treated group	22.25 ± 0.303###
6	6-OHDA+ EBC treated group	21.57 ± 0.180###
7	6-OHDA+ EEE treated group	22.04 ± 0.368###

Figure 37: Effect EUR, EMA, EBC and EEE on total protein concentrations in rat brain



Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; ###P<0.001 when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

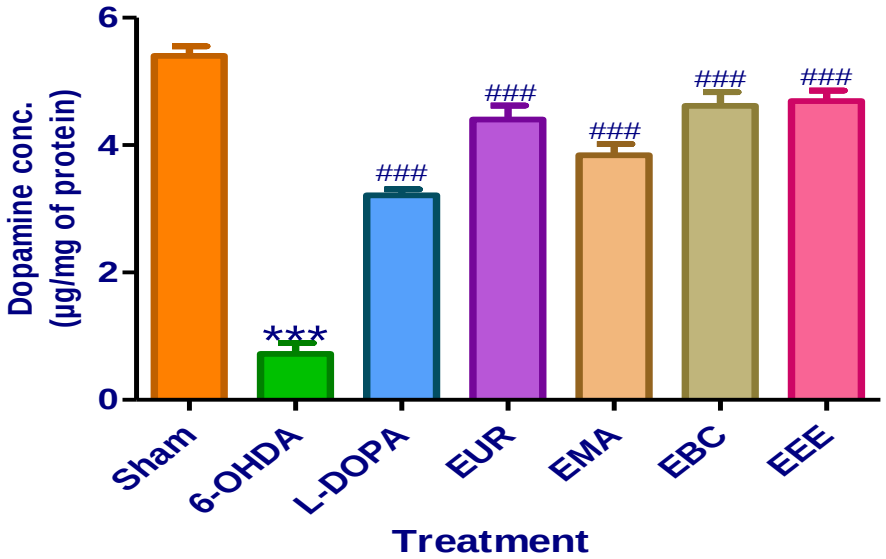
Table 13 and Figure 38: Effect of EUR, EMA, EBC and EEE on concentrations of dopamine level in rat brain

The dopamine concentration in striatal region was measured by HPLC method on sixtieth day after successive experimental period. The sham control animals were considered to be the reference standard for other experimental in this study. When compared with sham control the 6-OHDA control had significantly ($P<0.001$) reduced dopamine concentration. The L-DOPA treated rats had high level of dopamine at the end of the study period. The treatment with the EUR significantly ($P<0.001$) retained the concentration of dopamine, which was near equal to sham controls groups. The treatment with EMA showed a high significant ($P<0.001$) increase in concentration of dopamine when compared with 6-OHDA control. The treatment with EBC also provided a significant ($P<0.001$) elevation in concentration of dopamine when compared with 6-OHDA control. The three test drugs together were tested for its additive or synergestic effect. The combined formulation EEE showed a pronounced ($P<0.001$) increase in concentration of dopamine near normal as that of sham control. These results indicates the better efficacy of test drugs in improving the level of dopamine, and the possible mode of action of test drugs will be discussed in the discussion part.

Table 13: Effect of EUR, EMA, EBC and EEE on concentrations of dopamine level in rat brain

Sr. No.	Treatment Group	Dopamine concentration (ng/mg of protein)
1	Sham Control	5.402 ± 0.151
2	6-OHDA control	0.720 ± 0.170***
3	6-OHDA+ L-DOPA treated group	3.213 ± 0.094$^{###}$
4	6-OHDA+ EUR treated group	4.405 ± 0.218$^{###}$
5	6-OHDA+ EMA treated group	3.842 ± 0.175$^{###}$
6	6-OHDA+ EBC treated group	4.617 ± 0.214$^{###}$
7	6-OHDA+ EEE treated group	4.692 ± 0.165$^{###}$

Figure 38: Effect of EUR, EMA, EBC and EEE on concentrations of dopamine level in rat brain



Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; $^{###}$P<0.001 when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

Figure 39: HPLC chromatogram for standard mixture of Dopamine and internal standard (0.5ml of drug and 1ml of IS)

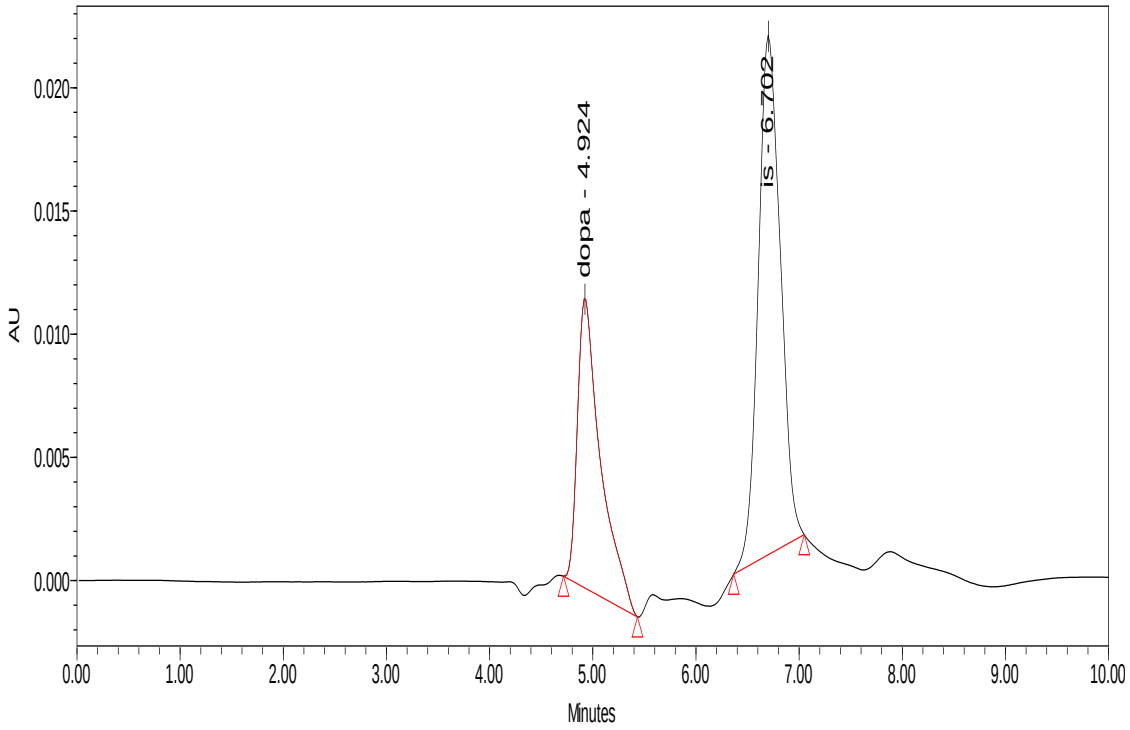


Table 14: Peak area of standard mixture of Dopamine and Internal Standard

Sr. No.	Name	Retention Time	Peak Type	Peak Area
1	INTERNAL STANDARD	6.702	Found	375849
2	DOPAMINE	4.924	Found	177148

Figure 40: HPLC chromatogram of Dopamine from brain homogenate of sham control group

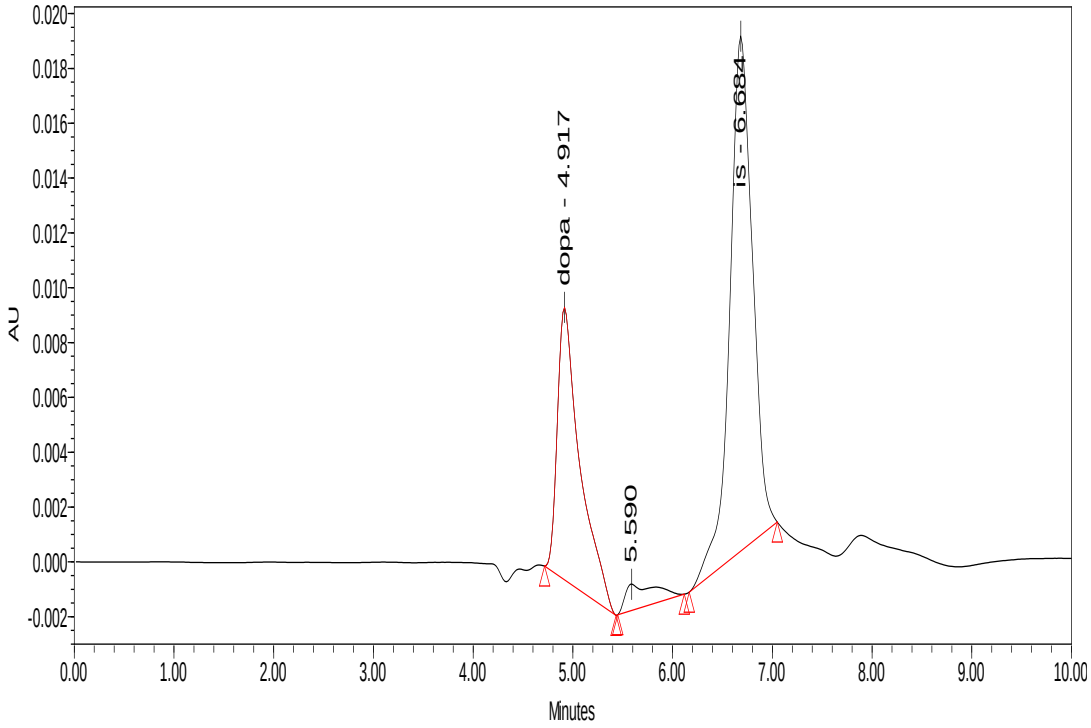


Table 15: Peak area of Dopamine from brain homogenate of sham control group

Sr. No.	Name	Retention Time	Peak Type	Peak Area
1	INTERNAL STANDARD	6.604	Found	314723
2	DOPAMINE	4.917	Found	10491

Figure 41: HPLC chromatogram of Dopamine from brain homogenate of 6-OHDA control group

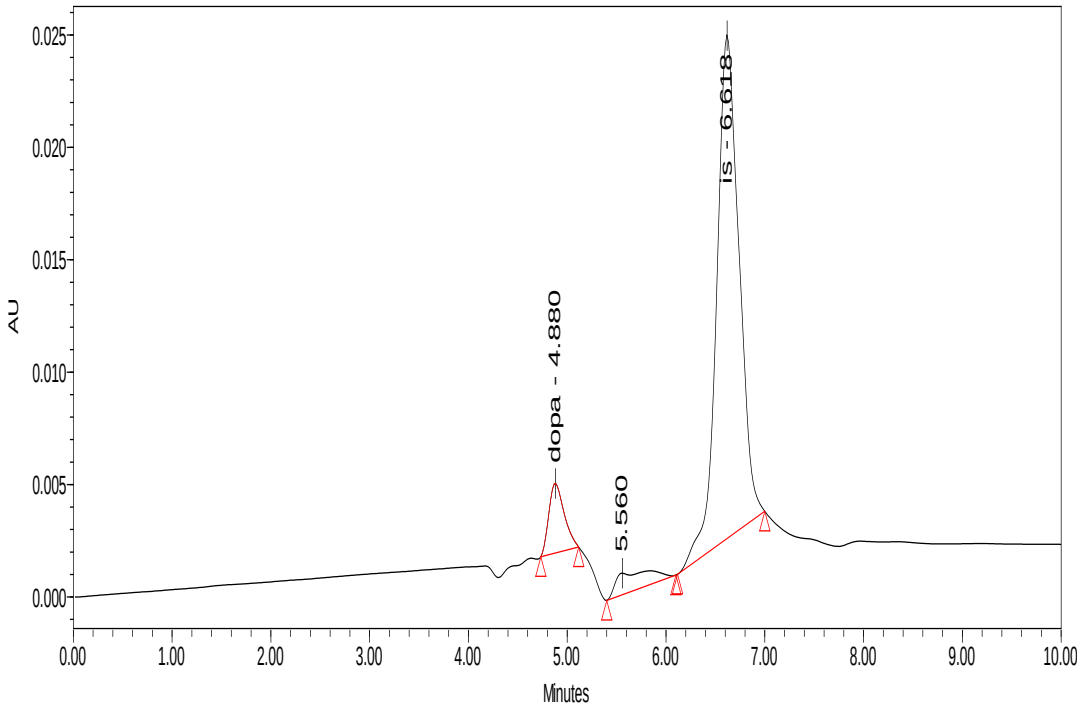


Table 16: Peak area of Dopamine from brain homogenate of 6-OHDA control group

Sr. No.	Name	Retention Time	Peak Type	Peak Area
1	INTERNAL STANDARD	6.018	Found	354038
2	DOPAMINE	4.880	Found	1058

Figure 42: HPLC chromatogram of Dopamine from brain homogenate of L-DOPA treated group

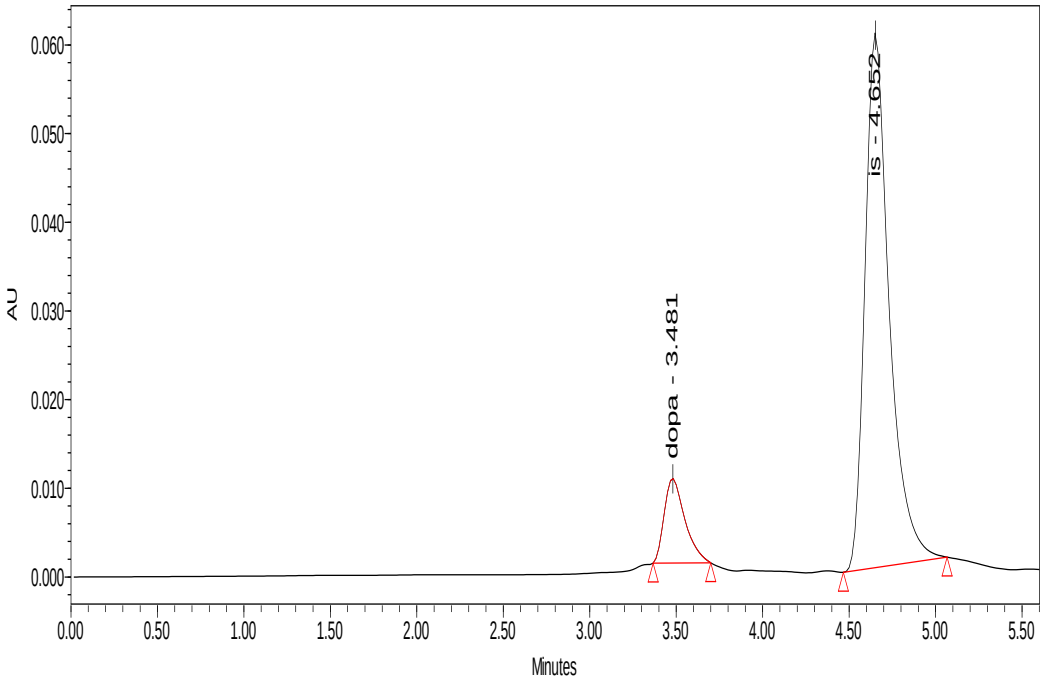


Table 17: Peak area of Dopamine from brain homogenate of L-DOPA treated group

Sr. No.	Name	Retention Time	Peak Type	Peak Area
1	INTERNAL STANDARD	4.052	Found	358291
2	DOPAMINE	3.481	Found	4490

Figure 43: HPLC chromatogram of Dopamine from brain homogenate of EUR treated group

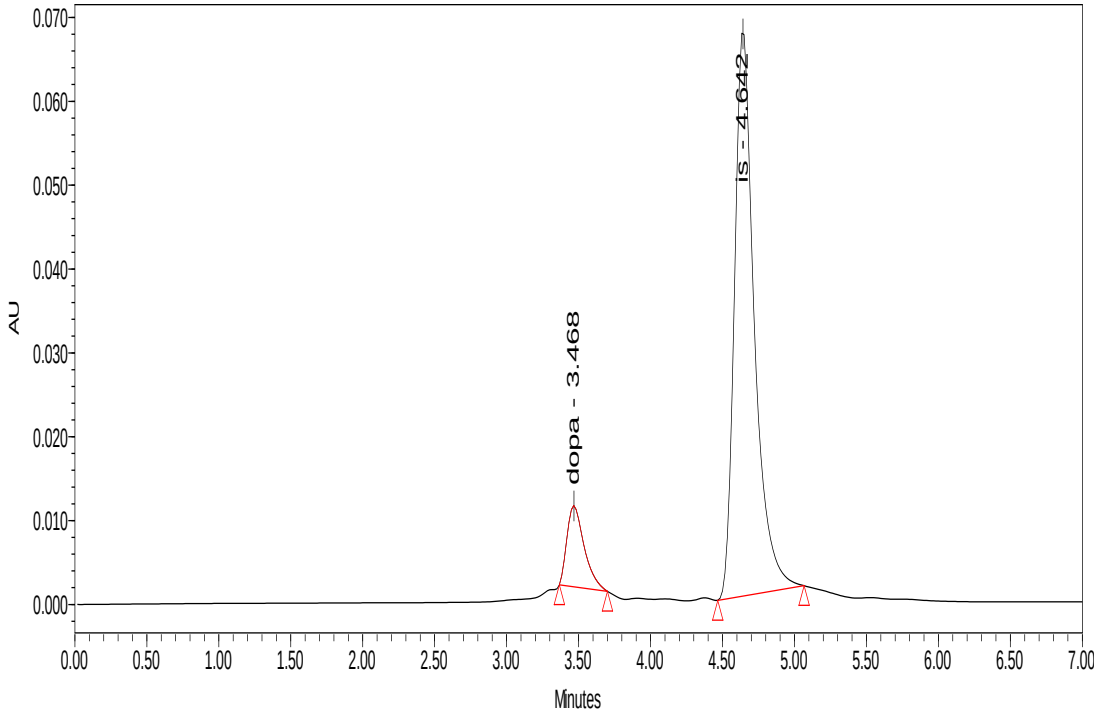


Table 18: Peak area of Dopamine from brain homogenate of EUR treated group

Sr. No.	Name	Retention Time	Peak Type	Peak Area
1	INTERNAL STANDARD	4.642	Found	362901
2	DOPAMINE	3.468	Found	7509

Figure 44: HPLC chromatogram of Dopamine from brain homogenate of EMA treated group

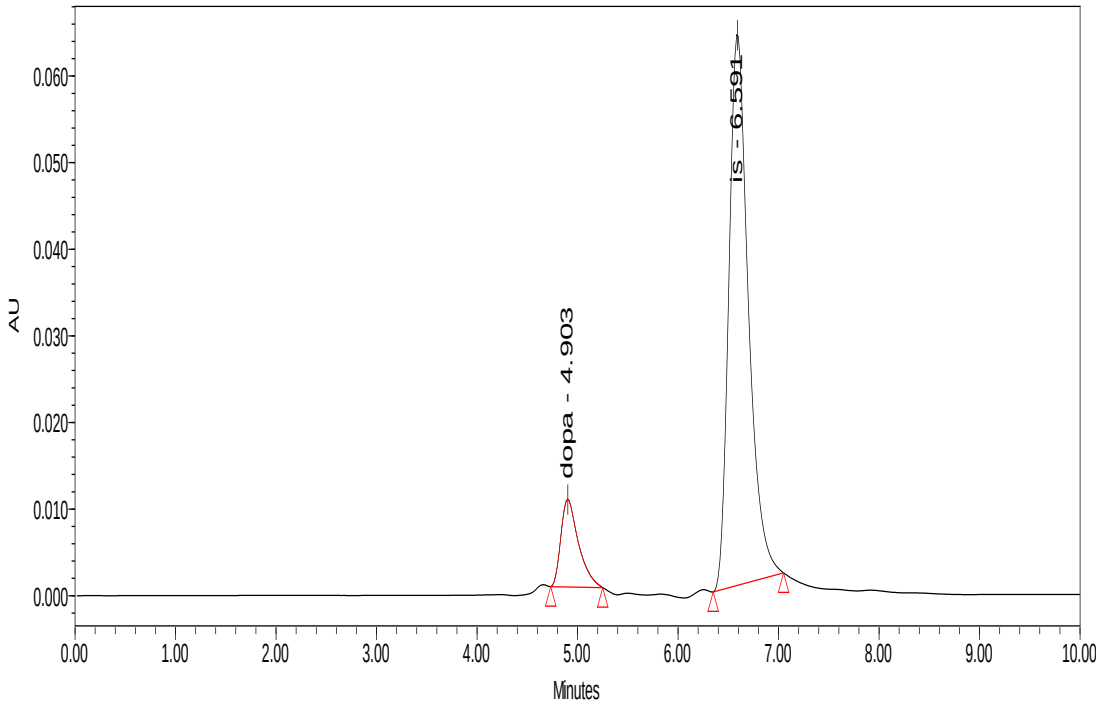


Table 19: Peak area of Dopamine from brain homogenate of EMA treated group

Sr. No.	Name	Retention Time	Peak Type	Peak Area
1	INTERNAL STANDARD	6.591	Found	369135
2	DOPAMINE	4.903	Found	6176

Figure 45: HPLC chromatogram of Dopamine from brain homogenate of EBC treated group

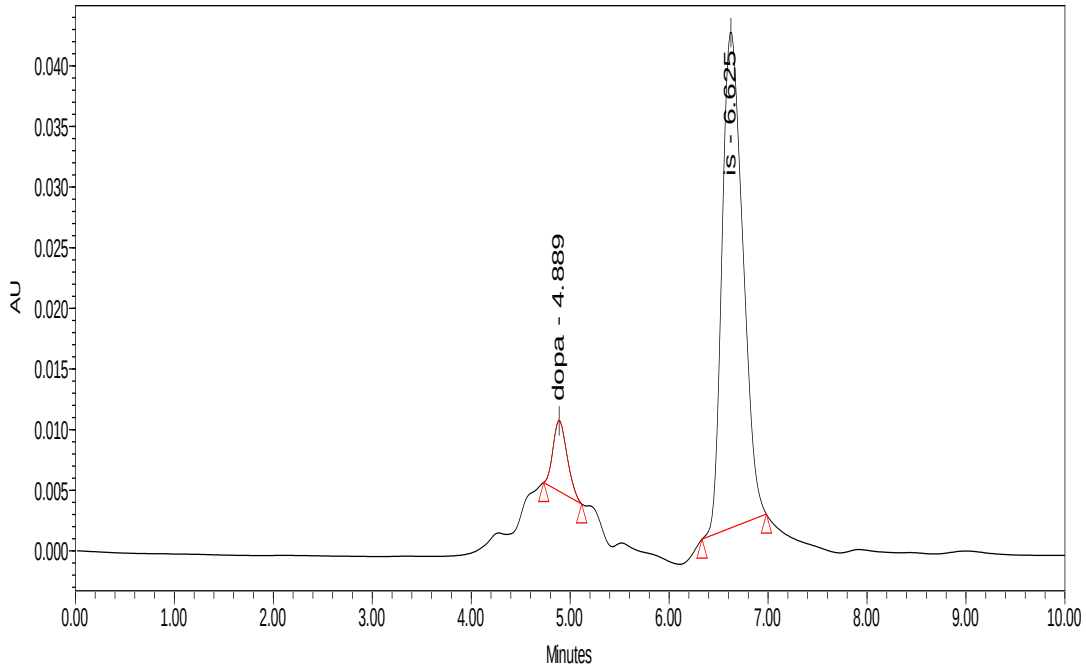


Table 20: Peak area of Dopamine from brain homogenate of EBC treated group

Sr. No.	Name	Retention Time	Peak Type	Peak Area
1	INTERNAL STANDARD	6.025	Found	351943
2	DOPAMINE	4.889	Found	7811

Figure 46: HPLC chromatogram of Dopamine from brain homogenate of EEE treated group

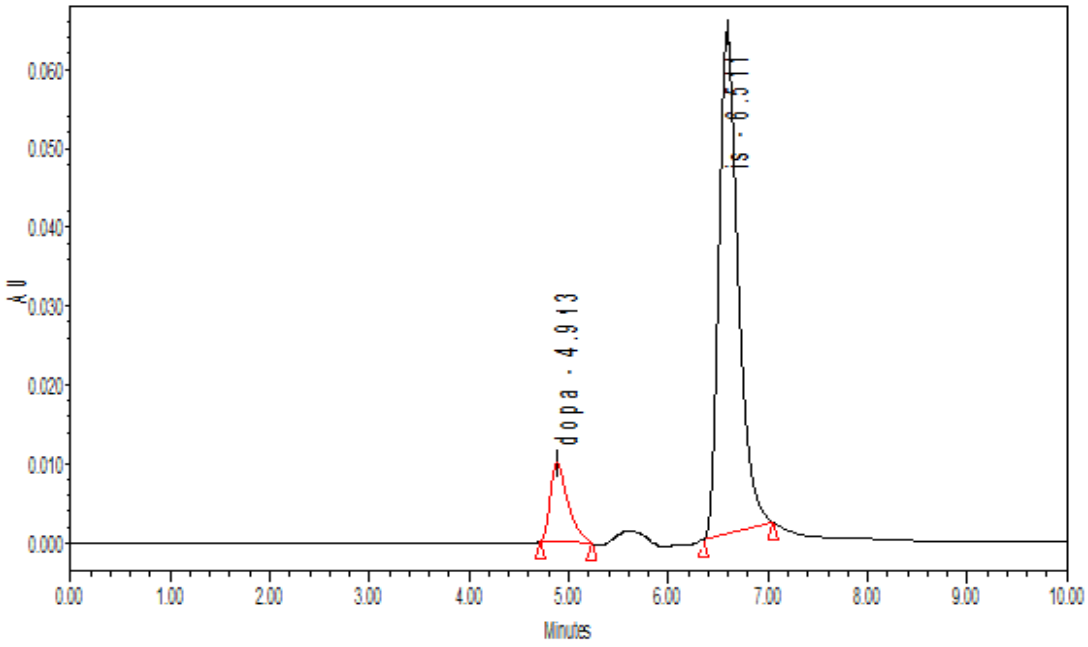


Table 21: Peak area of Dopamine from brain homogenate of EEE treated group

Sr. No.	Name	Retention Time	Peak Type	Peak Area
1	INTERNAL STANDARD	6.511	Found	35428
2	DOPAMINE	4.913	Found	8622

Table 22 and Figure 47: Effect of EUR, EMA, EBC and EEE on localization of iron in substantia nigra (Perl's DAB iron asymmetry) in rat brain

The mean Perl's DAB substantia nigra iron asymmetry ratio for the treatment and sham control groups were compared with population mean of 1.00 using one way ANOVA. The sham control animals showed an iron asymmetry ratio of (1.024 ± 0.052). The 6-OHDA animals showed an iron asymmetry ratio of (1.800 ± 0.025). The levodopa treated animals showed an iron asymmetry ratio of (1.732 ± 0.025).

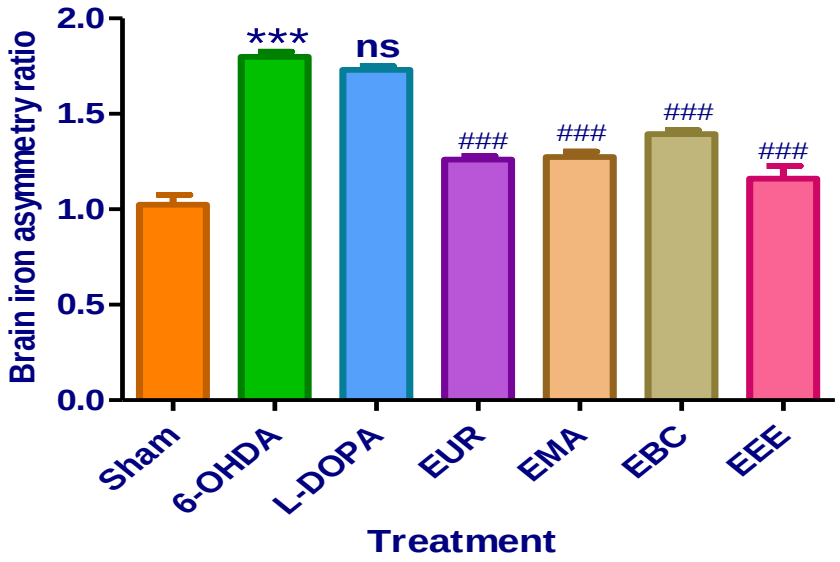
Verification of Brain Degeneration

Micro projector examination of Perl's DAB stained sections showed a darker area in substantia nigra near to the mid brain region. Iron reaction was less or insignificant for vehicle control animals. But for 6-OHDA control and levodopa groups it was higher. The treatment with EUR, EMA, EBC and EEE significantly ($p<0.001$) reduced iron reaction at substantia nigra when compared with 6-OHDA control.

Table 22: Effect of EUR, EMA, EBC and EEE on localization of iron in substantia nigra (Perl's DAB iron asymmetry) in rat brain

Sr. No.	Treatment Group	Brain iron asymmetry ratio
1	Sham Control	1.024 ± 0.052
2	6-OHDA control	1.800 ± 0.025***
3	6-OHDA+ L-DOPA treated group	1.732 ± 0.025^{ns}
4	6-OHDA+ EUR treated group	1.262 ± 0.043$^{###}$
5	6-OHDA+ EMA treated group	1.275 ± 0.028$^{###}$
6	6-OHDA+ EBC treated group	1.395 ± 0.019$^{###}$
7	6-OHDA+ EEE treated group	1.162 ± 0.066$^{###}$

Figure 47: Effect of EUR, EMA, EBC and EEE on localization of iron in substantia nigra (Perl's DAB iron asymmetry) in rat brain



Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; $^{###}$P<0.001 and ^{ns}P>0.05 (non-significant) when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

Perl's-DAB brain iron asymmetry

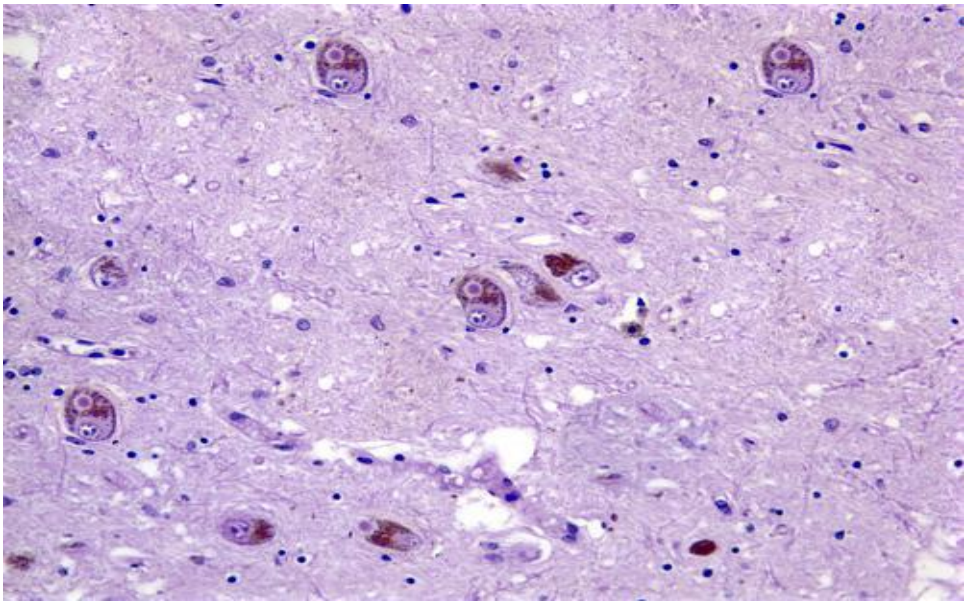


Figure 48: Image shows iron degeneration of substantia nigra in sham control rats

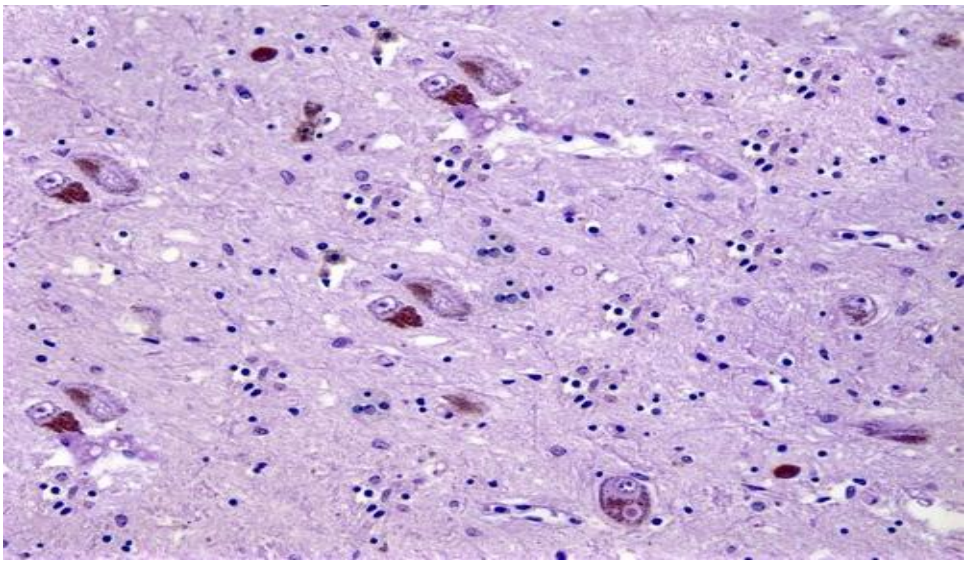


Figure 49: Image shows iron degeneration of substantia nigra in 6-OHDA control rats

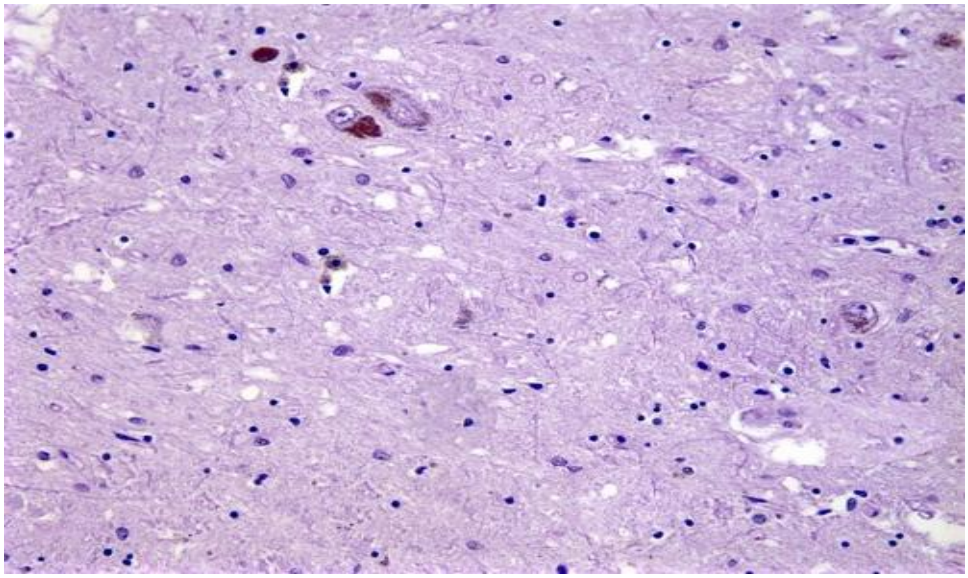


Figure 50: Image shows iron degeneration of substantia nigra in levodopa treated rats

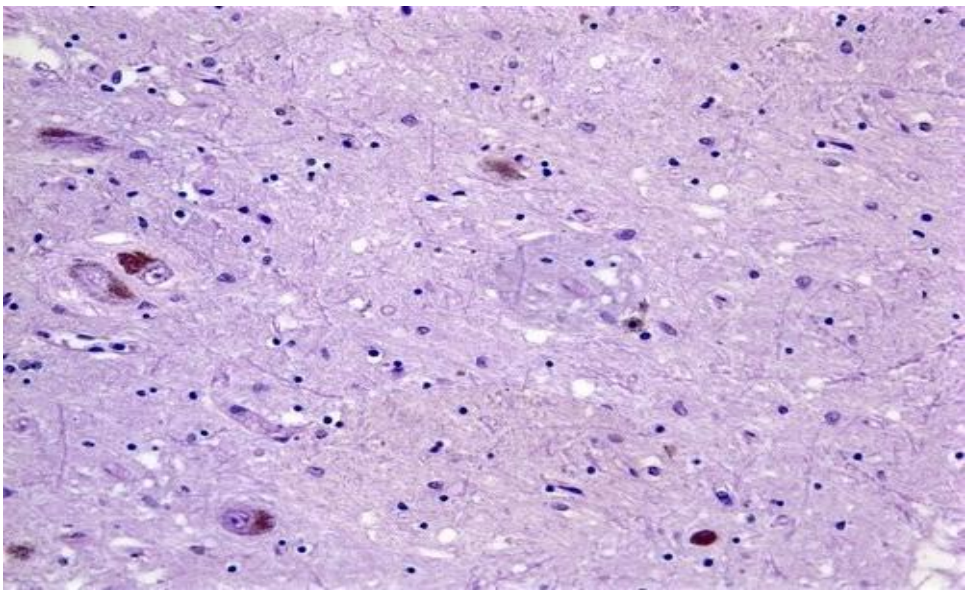


Figure 51: Image shows iron degeneration of substantia nigra in EUR treated rats

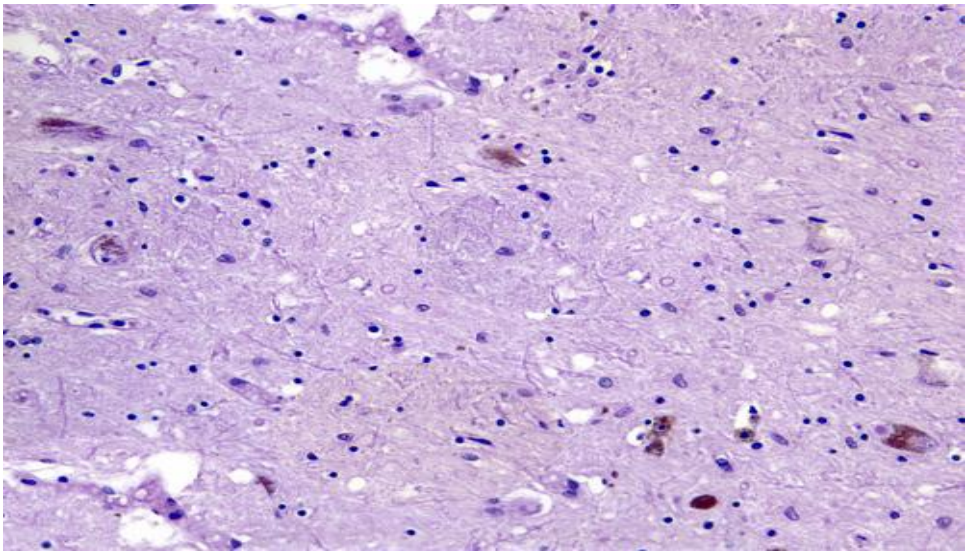


Figure 52: Image shows iron degeneration of substantia nigra in EMA treated rats

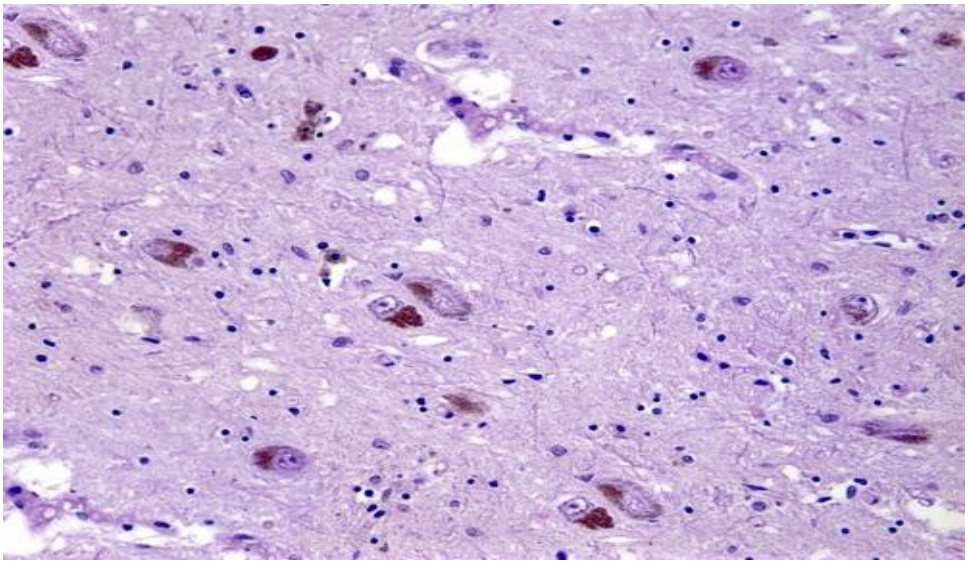


Figure 53: Image shows iron degeneration of substantia nigra in EBC treated rats

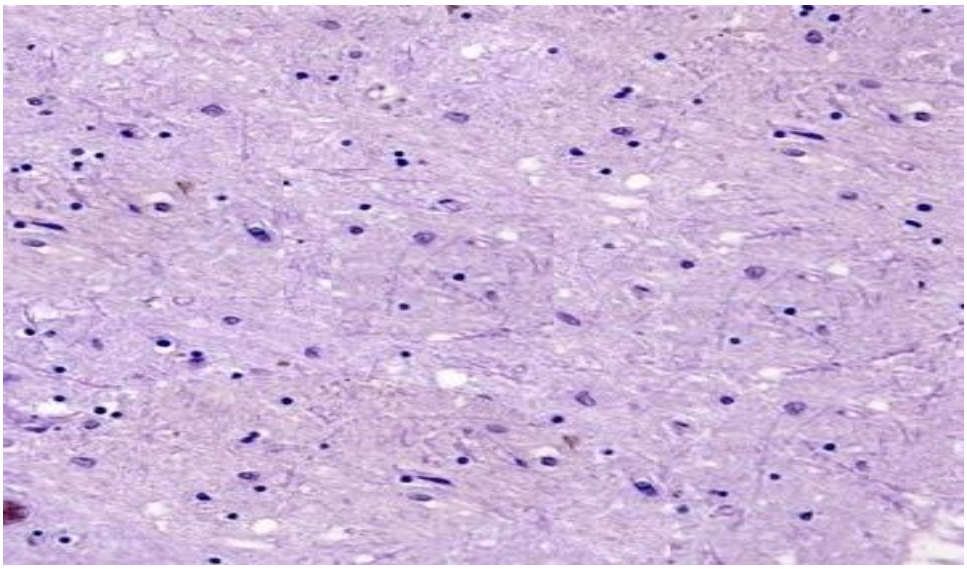


Figure 54: Image shows iron degeneration of substantia nigra in EEE treated rats

Table 23 and Figure 55: Effect of EUR, EMA, EBC and EEE on lipid peroxidation level in rats

The lipid peroxidation level in rat brain tissues have been analyzed on sixtieth day after successive experimental period. The sham control animals were compared with other experimental groups of this study. When compared with sham control the 6-OHDA and L-DOPA control had pronounced ($P<0.001$) increased in lipid peroxidation, which was at par highly elevated than normal animals. The treatment with the EUR significantly ($P<0.001$) reduced lipid peroxidation which was near equal to sham controls groups. The treatment with EMA showed a significant ($P<0.001$) reduction in lipid peroxidation when compared with 6-OHDA control. The treatment with EBC also provided a significant ($P<0.001$) reduction in lipid peroxidation when compared with 6-OHDA control. The three test drugs together were tested for its additive or synergestic effect. The combined formulation EEE showed a pronounced ($P<0.001$) decrease in lipid peroxidation to near normal as that of sham control. These results indicated the better efficacy of test drugs to control lipid peroxidation, and the possible mode of action of test drugs will be discussed in the discussion part.

Table 23 and Figure 56: Effect of EUR, EMA, EBC and EEE on catalase (CAT) levels in rats

The catalase activity has been evaluated on sixtieth day after successive experimental period. The sham control animals were considered to be the reference standard for other experimental anumals in this study. When compared with sham control the 6-OHDA and L-DOPA control had significantly ($P<0.001$) decreased in catalase activity. The treatment with the EUR significantly ($P<0.001$) increased the catalase activity was near equal to sham controls groups. The treatment with EMA showed a high significant ($P<0.001$) increase in catalase activity when compared with 6-OHDA control. The treatment with EBC also provided a significant ($P<0.001$) elevation of catalase activity when compared with 6-OHDA control. The three test drugs together were tested for its additive or synergestic effect. The combined formulation EEE showed a pronounced increase ($P<0.001$) in catalase activity near normal to that of sham control. These results indicates the better efficacy of test drugs to retain the catalase activity, and the possible mode of action of test drugs will be discussed in the discussion part.

Table 23 and Figure 57: Effect of EUR, EMA, EBC and EEE on superoxide dismutase (SOD) levels in rats

The superoxide dismutase level was measured on sixtieth day after successive experimental period. The sham control animals were considered to be the reference standard for other experimental groups in this study. When compared with sham control the 6-OHDA and L-DOPA control had significant (P<0.001) reduction in SOD level than that of other normal animals. The treatment with the EUR significantly (P<0.001) increased the concentration of SOD level and was near equal to sham controls groups. The treatment with EMA showed a high significant (P<0.001) increase in concentration of SOD level when compared with 6-OHDA control. The treatment with EBC also provided a significant (P<0.001) elevation in concentration SOD level when compared with 6-OHDA control. The three test drugs together were tested for its additive or synergestic effect. The combined formulation EEE showed a pronounced (P<0.001) increase in concentration of SOD level near normal as that of sham control. These results indicates the better efficacy of test drugs to improvement in concentration of SOD level, and the possible mode of action of test drugs will be discussed in the discussion part.

Table 23 and Figure 58: Effect of EUR, EMA, EBC and EEE on glutathione (GSH) level in rats

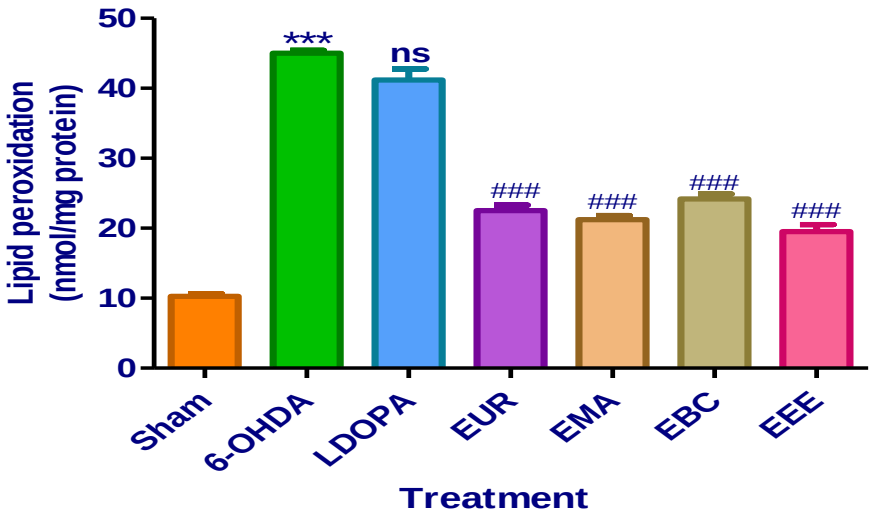
The glutathione reductase level was measured on sixtieth day after successive experimental period. The sham control animals were considered to be the reference standard for other experimental in this study. When compared with sham control the 6-OHDA and L-DOPA control had significant (P<0.001) reduction in GSH concentration. The treatment with the EUR significantly (P<0.001) increased the concentration of dopamine was near equal to sham controls groups. The treatment with EMA showed a high significant (P<0.001) increase in concentration of GSH when compared with 6-OHDA control. The treatment with EBC also provided a significant (P<0.001) elevation in concentration GSH when compared with 6-OHDA control. The three test drugs together were tested for its additive or synergestic effect. The combined formulation EEE showed a pronounced (P<0.001) increase in concentration of GSH near normal as that of sham control. These results indicates the better efficacy of test drugs to improve the concentration of GSH, and the possible mode of action of test drugs will be discussed in the discussion part.

Table 23: Effect of EUR, EMA, EBC and EEE on anti oxidant parameters from rat brain homogenate

Sr. No.	Treatment Group	Lipid peroxidation (nmol/mg protein)	Catalase (µmol/min mg protein)	Superoxide dismutase (U/ml)	Glutathione reductase (nmol/mg of protein)
1	Sham Control	10.27 ± 0.373	0.447 ± 0.014	132.80 ± 2.674	0.915 ± 0.012
2	6-OHDA control	45.04 ± 0.3861***	0.124 ± 0.017***	58.17 ± 1.743***	0.417 ± 0.021***
3	6-OHDA+ L-DOPA treated group	41.19 ± 1.565^{ns}	0.159 ± 0.014^{ns}	66.55 ± 3.690^{ns}	0.504 ± 0.025^{ns}
4	6-OHDA+ EUR treated group	22.52 ± 0.809$^{###}$	0.318 ± 0.005$^{###}$	89.14 ± 1.842$^{###}$	0.641 ± 0.011$^{###}$
5	6-OHDA+ EMA treated group	21.24 ± 0.571$^{###}$	0.284 ± 0.011$^{###}$	94.50 ± 2.467$^{###}$	0.713 ± 0.035$^{###}$
6	6-OHDA+ EBC treated group	24.19 ± 0.708$^{###}$	0.264 ± 0.019$^{###}$	89.20 ± 1.517$^{###}$	0.619 ± 0.030$^{###}$
7	6-OHDA+ EEE treated group	19.53 ± 0.971$^{###}$	0.3611 ± 0.005$^{###}$	103.0 ± 2.306$^{###}$	0.802 ± 0.021$^{###}$

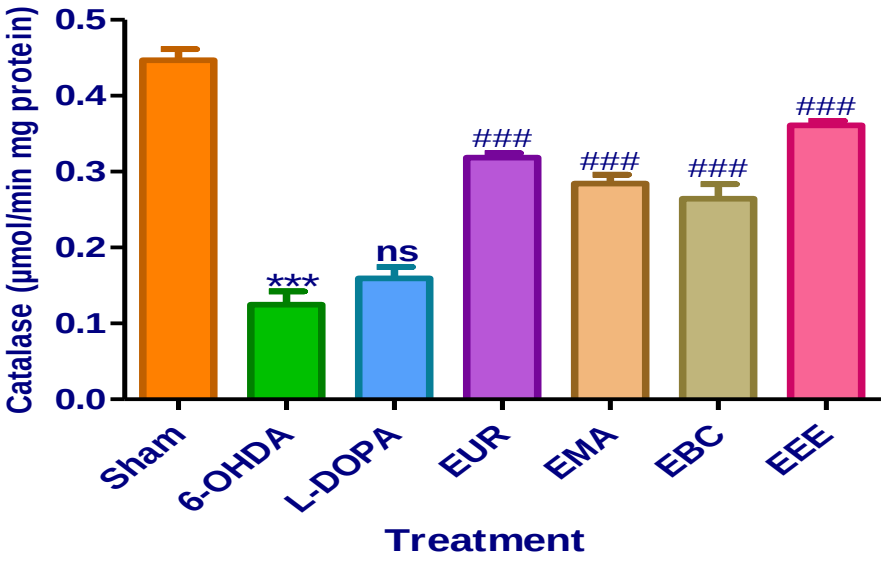
Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; $^{###}$P<0.001 and ^{ns}P>0.05 when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

Figure 55: Effect of EUR, EMA, EBC and EEE on lipid peroxidation level in rats



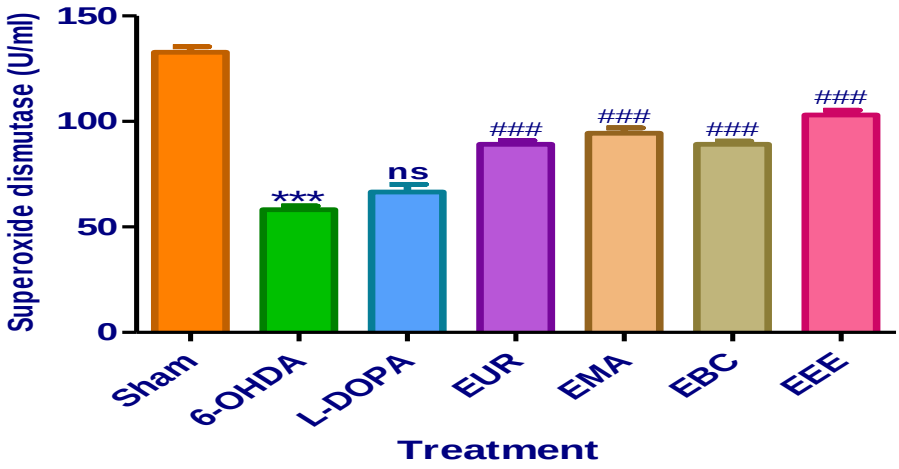
Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; ###P<0.001 and nsP>0.05 when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

Figure 56: Effect of EUR, EMA, EBC and EEE on catalase (CAT) levels in rats



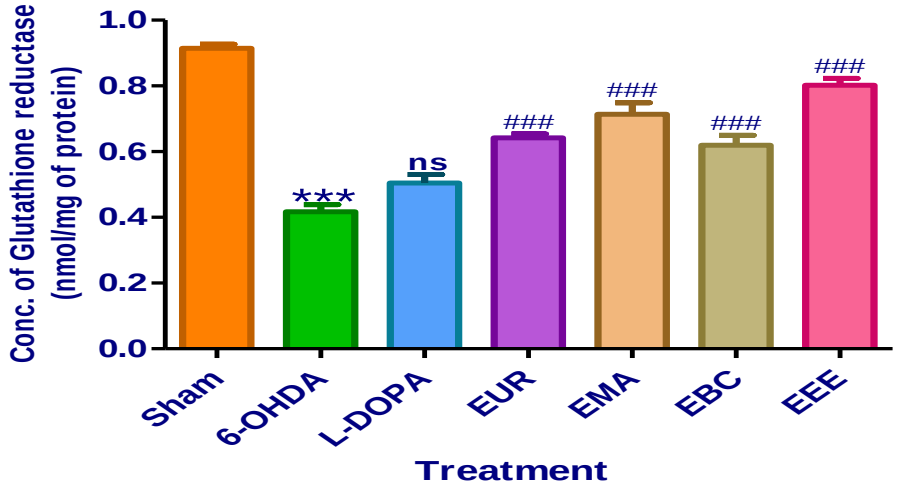
Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; ###P<0.001 and nsP>0.05 when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

Figure 57: Effect of EUR, EMA, EBC and EEE on superoxide dismutase (SOD) levels in rats



Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; ###P<0.001 and nsP>0.05 when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

Figure 58: Effect of EUR, EMA, EBC and EEE on glutathione (GSH) level in rats



Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; ###P<0.001 and nsP>0.05 when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

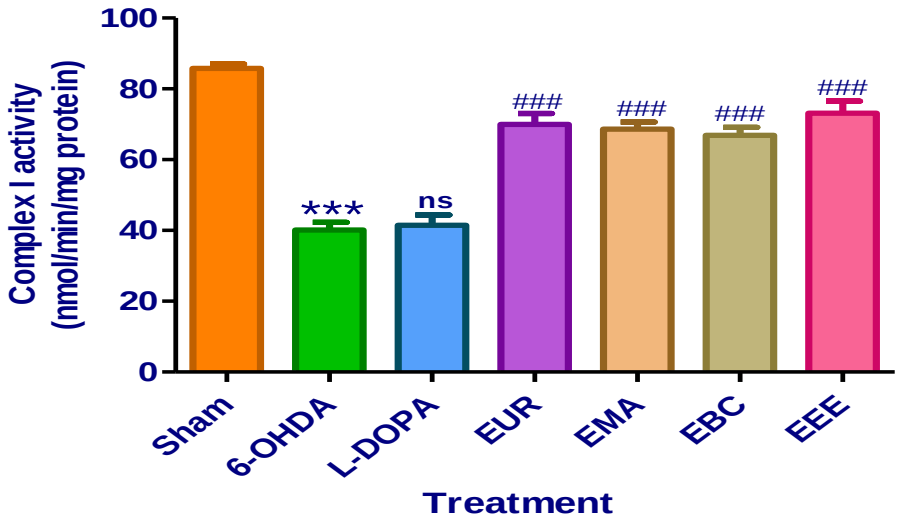
Table 24 and Figure 59: Effect of EUR, EMA, EBC and EEE on mitochondrial Complex I activity in rat brain

The complex I activity was estimated from mitochondrial fractions isolated on sixtieth day after successive experimental period. The sham control animals were considered to be the reference standard for other experimental groups in this study. When compared with sham control the 6-OHDA and L-DOPA control had significantly ($P<0.001$) reduced complex I activity. The treatment with EUR significantly ($P<0.001$) increased the complex I activity, and was near equal to sham controls groups. The treatment with EMA showed a high significant ($P<0.001$) increase in complex I activity when compared with 6-OHDA control. The treatment with EBC also provided a significant ($P<0.001$) elevation in complex I activity when compared with 6-OHDA control. The three test drugs together were tested for its additive or synergestic effect. The combined formulation EEE showed a pronounced ($P<0.001$) increase in complex I activity near normal as that of sham control. These results indicates the better efficacy of test drugs to improve complex I activity, and the possible mode of action of test drugs will be discussed in the discussion part.

Table 24: Effect of EUR, EMA, EBC and EEE on mitochondrial Complex I activity in rat brain

Sr. No.	Treatment Group	Complex I activity (nmol/min/mg protein)
1	Sham Control	85.78 ± 1.300
2	6-OHDA control	40.09 ± 2.235***
3	6-OHDA+ L-DOPA treated group	41.43 ± 2.946^{ns}
4	6-OHDA+ EUR treated group	69.96 ± 3.109$^{###}$
5	6-OHDA+ EMA treated group	68.60 ± 2.019$^{###}$
6	6-OHDA+ EBC treated group	66.90 ± 2.227$^{###}$
7	6-OHDA+ EEE treated group	73.16 ± 3.356$^{###}$

Figure 59: Effect of EUR, EMA, EBC and EEE on mitochondrial Complex I activity in rat brain



Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; $^{###}$P<0.001 and ^{ns}P>0.05 (non-significant) when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

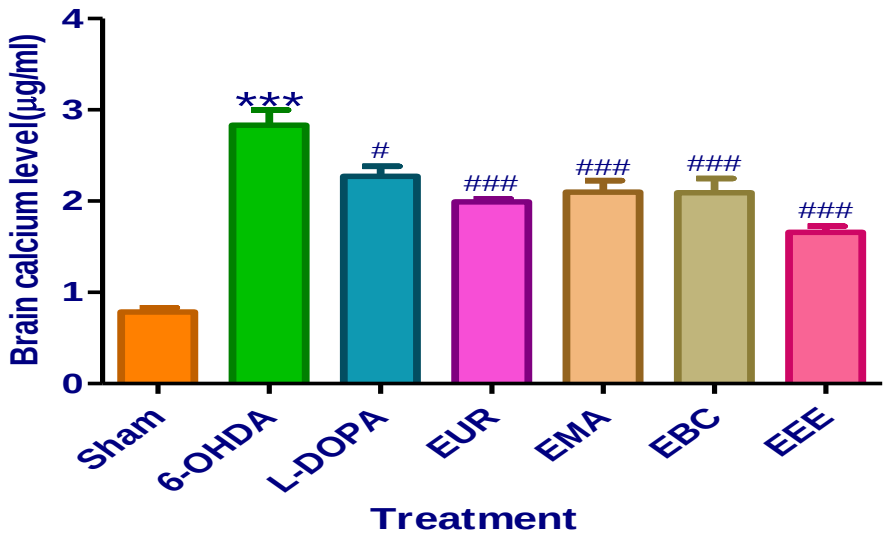
Table 25 and Figure 60: Effect of EUR, EMA, EBC and EEE on rat brain mitochondrial calcium level by AAS

The brain mitochondrial calcium concentration in striatal region was measured on sixtieth day after successive experimental period. The sham control animals were considered to be the reference standard for other experimental animal of this study. When compared with sham control, the 6-OHDA and L-DOPA control had pronounced ($P<0.001$) increased in calcium concentration, which was at par highly elevated than normal animals. The treatment with the EUR significantly ($P<0.001$) reduced calcium concentration which was near equal to that of sham controls groups. The treatment with EMA showed a high significant ($P<0.001$) reduction in calcium concentration when compared with 6-OHDA control. The treatment with EBC also provided a significant ($P<0.001$) reduction in calcium concentration when compared with 6-OHDA control. The three test drugs together were tested for its additive or synergestic effect. The combined formulation EEE showed a pronounced decrease ($P<0.001$) in calcium concentration to near normal as that of sham control. These results indicate the better efficacy of test drugs to control calcium concentration, and the possible mode of action of test drugs will be discussed in the discussion part.

Table 25: Effect of EUR, EMA, EBC and EEE on rat brain mitochondrial calcium level

Sr. No.	Treatment Group	brain calcium level (µg/ml)
1	Sham Control	0.7855 ± 0.047
2	6-OHDA control	2.833 ± 0.166***
3	6-OHDA+ L-DOPA treated group	2.272 ± 0.109$^{\#}$
4	6-OHDA+ EUR treated group	1.992 ± 0.032$^{\#\#\#}$
5	6-OHDA+ EMA treated group	2.098 ± 0.125$^{\#\#\#}$
6	6-OHDA+ EBC treated group	2.092 ± 0.156$^{\#\#\#}$
7	6-OHDA+ EEE treated group	1.660 ± 0.063$^{\#\#\#}$

Figure 60: Effect of EUR, EMA, EBC and EEE on rat brain mitochondrial calcium level



Values are mean ± SEM; n=6 in each group. ***P<0.001 when compared with sham control group; $^{\#\#\#}$P<0.001 and $^{\#}$P<0.05 when compared with 6-OHDA control; One-way ANOVA followed by Bonferroni multiple comparisons test.

DISCUSSION

DISCUSSION

The efficacy of *Uncaria rhynchophylla*, *Mentha aquatica* and *Banisteriopsis caapi* in 6-OHDA induced PD has not been well established. In our study, we have demonstrated the anti-Parkinson's activity of not only the above mentioned three plant extract but also evaluated the combined effects of all three extracts.

In this study, first we have demonstrated the Apomorphne induced circling behavior. Apomorphne is a mixed (D_1 and D_2) dopamine receptor agonist that does not share transport or metabolic pathways with L-DOPA and presumably acts by direct stimulation of dopamine receptor.[100]

In our study, the circling controversial to the lesion side following the administration of L-DOPA or dopamine agonist result from stimulation of dopamine receptor rendered supersensitive by partial denervation.[127] The lesioned rats showed a greater level of circling behavior and other treatment groups might be replenishing dopamine or already protected dopaminergic nurons in mid brain (SNpc). Further it could presumably suggest the confirmation of nigral lesion in all the treatment groups. Anyhow, the significant levels in comparing the degree of lesioning is not mandatory in our study, because post treatment lesion verification is the major focus here.

In rotarod, animals walk on a rotating drum is widely used to assess motor status in laboratory rats. The motor cordination performance is measured by the duration that an animal stays up on the drum as a function of drum speed. This task used to provide rich source of information about qualitative aspects of walking movements.[128] In this experiment we have demonstrated the impairment in the motor function and coordination in Parkinson's rats after 6-OHDA lesion. Parkinson's rats showed short fall off time from the rotating rod when compare to control, suggesting impairment in their ability to integrate sensory input with appropriate motor commands to balance their posture. Lack of motor coordination and maintenance of normal limb posture has been reported in PD condition.[125] The treatment with EUR, EMA, EBC and EEE in rats increased the fall off time when compare to 6-OHDA control rats. This could be the effect of test drugs in mid brain dopaminergic neurons to exert a dopamine agonism or dopamine facilitation. The striatal neurons are highly vulnerable to neurotoxin induced death, the prevention of such death could offer a better somatic balance and this may be

the probable cause of these test drugs in reversing postural imbalance. The experimental evaluation revealed the efficacy of EUR, EMA, EBC and EEE for increasing muscle co-ordination and thus, it could be the possible regenerative or preventive actions on neurotoxin induced cell death offered by the test drugs.

In our study conditions, EUR, EMA, EBC and EEE reversed the catalepsy induced by 6-OHDA induction. The pyramidal dopamine facilitatory actions may be the possible action of those test drugs.

The turnover of dopamine in nigral cells plays a major role in controlling motor function. In our study, we reported that EUR, EMA, EBC and EEE caused a pronounced increase in dopamine levels in mid brain regions of 6-OHDA rats and it could be a result of protection of dopaminergic neurons by these drugs. . Levodopa-treated rats were slower than test drugs treated rats, and also showed increased level of catalepsy, which may have possessed mild as well as unilateral lesion-induced postural bias impairing balance. Levodopa likely exacerbated asymmetry in the dopaminergic system in unilaterally lesioned rats as has been reported in other studies.[129] The duration of treatment was for 60 days and it might be a longer duration for rats. The beneficial roles of these drugs in retaining dopamine levels demonstrated the protection of nigral neuron by test drugs. L-DOPA treatment hiked the dopamine levels and it is easily demonstrated by its high concentration after treatment for 60 days. In the test drugs group it was slight decreasing in numerical values of dopamine and it insisted us to further investigate nigral pathology. With this light we assumed that some other factor may contribute for the protection of nigral cells apart from retaining the dopamine levels.

Oxidative stress and oxidative damage to critical biomolecule based on the important process mediating cell death in PD.[130] The Lipid peroxidation assay gives the idea of both intrinsic and extrinsic cellular protective mechanisms of a test compound. This also given an idea of what extent the test drugs can be of lipophilic and penetrability into CNS.[131,132] The studies showed an increase in lipid peroxidation for 6-OHDA control group and L-DOPA treated groups. The lipid peroxidation was much reduced after treatment with test extracts. In the EUR, EMA, EBC and EEE treated groups a decrease in lipid peroxidation observed and the activities of superoxide dismutase and catalase were also reduced, but increased in all other treatment groups.

Reduction in GSH might impair H_2O_2 clearance and promote OH radical formation and produces oxidative stress.[133] The all antioxidant defense mechanisms were related and disturbance on one might damage the balance in all. The depletion in glutathione reductase content and enhancement of lipid peroxidation leads to the degeneration of nigrostriatal neurons and consequently leads to reduction in the content of catecholamine.

In our study, we have estimated GSH to demonstrate whether the drug treatment could elevate or suppress GSH level intracellularly in mid brain region. With respect to this objective, our finding showed that EUR, EMA, EBC and EEE treatment could maintain the normal range of GSH in brain tissue. This given us a knowledge of possible role of GSH in protecting the mitochondrial activities and reduce in-vivo oxidative stress in neuron.

Mitochondria also play a major role in activating apoptosis and oxidative damage in many human pathologies, including neurodegenerative diseases, ischemia–reperfusion injury, aging, and other inflammatory diseases.[134,135] This chronic oxidative damage impairs mitochondrial ATP synthesis and calcium homeostasis and induces the mitochondrial permeability transition, [136] leading to apoptotic or necrotic cell death. Mitochondria play a central role in regulating apoptosis through enhanced cytochrome-c (cyt-c) release that results in the activation of caspases and subsequent cell death.[137] Our further investigation, revealed a reduced level of complex-I activity and relevant reduction in anti-oxidant enzymes in untreated groups demonstrated the possible role of EUR, EMA, EBC and EEE in neuroprotection in PD. Because chronic, generalized mitochondrial deficits have been found in sporadic PD [138,139] and the increasing GSH could protect the nigral neurons.

The unilateral nigral lesion produced pronounced levels of Iron accumulation. In our experimental study, the iron estimation was carried out by both perl's DAB as well as Bleomycin assay. The pathogenic feature of Parkinson's disease is the destruction of the pigmented substantia nigra, particularly the pars compacta (SNc). The cause of dopaminergic cell death in the SN is still unknown. Recent studies on the pathogenesis of PD have centred on the involvement of environmental and endogenous toxins. Oxidative stress and altered iron content are also thought to be important factors.[140,141]

Iron is a transition metal which can accept or donate an electron to promote redox reactions.[142] Fe^{2+} reacts with the hydrogen peroxide generated in the catabolism of dopamine to form free radicals. Many studies have shown evidence for abnormally high concentrations of iron within the substantia nigra of Parkinsonian patients.[143,144] The decreased levels of the iron binding protein, ferritin, in parkinsonian brain, suggesting increased levels of free iron.[145] The neuronal inorganic calcium is a major contributor in inducing iron binding protein oxidation. Thus oxidation of transferritin will leads to increased iron deposition in SN. Inhibition of calcium overload can prevent the above explained sequence of reactions and reduces iron induced neurodegenaration. So, we came to know that test drugs treatment effectively reduced iron accumulation in midbrain and pronouncedly controlled neurodegenaration in 6-OHDA rat models. The brain iron asymmetry data clearly demonstrate the degeneration of transferrin and subsequent deposition of iron in nigral cells. The 6-OHDA increased iron deposition was significantly lowered by EUR, EMA, EBC and EEE. This pharmacological action explained overall protection of test drugs in retarding iron deposition. However the EUR, EMA, EBC and EEE could reduce iron deposition, and increase mitochondrial activity is a remarkable question. To answer for this question, we have assessed the levels of intracellular calcium in all treatment groups. From that result, it was clear that the EUR, EMA, EBC and EEE significantly reduced intracellular calcium levels and consequent reduction in iron deposition and retain the mitochondrial activity.

Disruption of intracellular calcium homeostasis has been implicated in many neurological insults, such as hypoxia-ischemia, exposure to neurotoxin, and withdrawal of trophic factors from the neurons. Many neurodegenerative diseases such as Parkinson's disease, Huntington's disease and Alzheimer's disease have been associated with impaired mitochondrial energy production.[146] Previous studies have shown that in both primates and mice, DHP antagonism of L-type calcium channels protects the cell bodies of SNc dopaminergic neurons against MPTP, another commonly used dopaminergic neurotoxin. And also it has shown that Ca^{2+} entry through L-type channels elevates mitochondrial oxidant stress in SNc DA neurons. The oxidant stress appears to be a consequence of the metabolic burden created by the need to remove Ca^{2+} from the cytosolic space by adenosine triphosphate dependent mechanisms. This basal oxidant stress should elevate the sensitivity of SNc DA

neurons to mitochondrial toxins. Hence, antagonizing L-type calcium channel should be neuroprotective in toxin-based PD models.[147] Hence, our study indicated that, the capability of test extracts may be due to its action on any of calcium channel types and contributed reduction in calcium induced neurotoxicity.

So these issues addressed the role of calcium in other factors viz, iron deposition and mitochondrial impairment. This fact further answered with the levodopa treated groups, where iron deposition was more and mitochondrial complex-I activity was reduced when compared with the test drug treated groups. This supports the earlier reports such as, L-DOPA treatment may leads to iron degeneration and impairs complex-I activity.[148]

The Monoamine oxidase system is highly vulnerable to dopamine metabolism. There is lots of indirect experimental methods to quantify the MAO-B activity, one such is the iron induced neurodegeneration. Instead of measuring the direct metabolites of Dopamine such as Dihydroxy phenyl acetic acid (DOPAC), we measured neuronal iron deposition, which is an oxidative catabolic product of hydrogen peroxide, and departed from ferritin. This gave us an idea of MAO-B activity and possible action of test drugs in controlling this enzyme. The inhibition of MAO-B caused by these extracts are one of the possible mode of action, in the sense, dopamine induced toxicities might be reduced by this enzyme inhibition. The mechanism of action can be correlated with the improvement in mitochondrial functions and low levels of iron degeneration in mid brain regions. Further we identified the possible anti-Parkinson's activity of the test extracts by measuring anti-oxidant enzymes and we found a valid result of anti-oxidant property offered by test drugs. These findings suggested a possible MAO-B associated anti-oxidant mechanism through reduced or controlled production of amido-quinones and para-quinones. These research finding are much promising information to consider the test drugs as anti-Parkinson's agent.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

The study revealed the anti-Parkinson's activity of three herbal extracts (that *Uncaria rhynchophylla, Mentha aquatica and Banisteriopsis caapi and its combination*) in 6-OHDA induced Parkinson's models. In the standard experimental conditions, all the three extracts showed significant anti-parkinson's activity. The postural and neuro-humoral defects were normalised by the all test drugs.

The systematic pharmacological analysis revealed that *Uncaria rhynchophylla, Mentha aquatica and Banisteriopsis caapi*- plants could be a anti-Parkinson's remedy, and earlier these three plants were ethanopharmacologically proven for its anti oxidant, anti ulcer, antiseptic, antispasmodic, anti diabetic, immunostimulent, anti cancer and CNS activities. The evaluation of anti Parkinson's activities of these plants might be leading to a new drug molecule or herbal moiety which can ameliorate the anti-Parkinson's drug toxicities or can be an anti Parkinson's drug in future. The extracts showed significant anti-Parkinson's activity in 6-OHDA lesioned rat models, but the lead identification was not carried out in this study. The estimated parameters were closely relevant to clinical Parkinsonism and the drug treatment protected the diseased brain of rat. The time constrains to complete this research and nature of experiment made us to restrict experiments specific to pharmacology. Our invention on these herbal drugs shall be an information to further carryout the lead identification for preclinical pharmacology, and we propose suitable lead optimization from these extracts. And we appreciate further detailed molecular studies with these leads in anti-Parkinson's pharmacology and toxicology. From these findings we suggest that, these drug molecules could be a future drug of choice for the treatment of clinical Parkinsonism.

APPENDIX

APPENDIX-I

Diagnosis criteria for Parkinson's disease proposed by the UK Parkinson's Disease Society Brain Bank.[149]

UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria

Step 1 Diagnosis of Parkinsonian syndrome

- Bradykinesia (slowness of initiation of voluntary movement with progressive reduction in speed and amplitude of repetitive actions)
- And at least one of the following:
  - muscular rigidity
  - 4–6 Hz rest tremor
  - postural instability not caused by primary visual, vestibular, cerebellar, or proprioceptive dysfunction.

Step 2 Exclusion criteria for Parkinson's disease

- History of repeated strokes with stepwise progression of parkinsonian features
- History of repeated head injury
- History of definite encephalitis
- Oculogyric crises
- Neuroleptic treatment at onset of symptoms
- More than one affected relative
- Sustained remission
- Strictly unilateral features after 3 years
- Supranuclear gaze palsy
- Cerebellar signs
- Early severe autonomic involvement
- Early severe dementia with disturbances of memory, language, and praxis
- Babinski sign
- Presence of cerebral tumour or communicating hydrocephalus on CT scan
- Negative response to large doses of levodopa (if malabsorption excluded)
- MPTP exposure

Step 3 Supportive prospective positive criteria for Parkinson's disease

(Three or more required for diagnosis of definite Parkinson's disease)

- Unilateral onset
- Rest tremor present
- Progressive disorder
- Persistent asymmetry affecting side of onset most
- Excellent response (70–100%) to levodopa
- Severe levodopa-induced chorea
- Levodopa response for 5 years or more
- Clinical course of 10 years or more

APPENDIX-II

Animal used in this study: Wistar Rats [150]

The Wistar rat is an outbred albino rat. These stocks were developed at the Wistar Institute in 1906 for use in biological and medical research, and is notably the first rat developed to serve as a model organism at a time when laboratories primarily used the common house mouse (Mus musculus). More than half of all laboratory rat strains are descended from the original colony established by physiologist Henry Donaldson, scientific administrator Milton J. Greenman, and genetic researcher/ embryologist Helen Dean King.

The Wistar rat is currently one of the most popular rats used for laboratory research. It is characterized by its wide head, long ears, and having a tail length that is always less than its body length. The Sprague Dawley rat and Long-Evans rats were developed from Wistar rats. Wistar rats are more active than others like Sprague Dawley rats. The Spontaneously hypertensive rat and the Lewis rat are other well-known stocks developed from Wistar rats.

Software used in this study

1. CAMAG TLC scanner integrated with software CATS 4.0
   Available at:
   http://www.camag.com/en/tlc_hptlc/products/evaluation_detection/tlc_scanner_4.cfm
2. Jandel scientific image analysis software (JAWA ^{TM}) with a grey scale range of 256 grey levels.
   Available at:
   http://www.mediacy.com/index.aspx?page=IP_Premier&gclid=CJ6ppKH9jsYCFQFxvAodMXsAqA
3. GraphPad Prism software version 5
   Available at:
   http://www.graphpad.com/scientific-software/prism/

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