EFFECT OF *Cymbopogon flexuosus* (LEMONGRASS OIL) AGAINST ROTENONE-INDUCED PARKINSONISM IN RATS

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CONTENTS

INTRODUCTION

Parkinson's disease (PD) is the most common neurodegenerative disease of unknown etiology and characterized by motor symptoms of tremor, rigidity, bradykinesia, and postural instability. Parkinson's disease is characterized by an abnormal basal ganglia activity. Non-motor comorbidities, such as cognitive impairments (the comorbidity of anxiety and depression in Parkinson's disease) are likely the result of an intricate interplay of multi-system degenerations and neurotransmitter deficiencies extending beyond the loss of dopaminergic nigral neurons [1].

The crude prevalence rate of PD in European countries has been found to range from 65.6 per 100,000 to 12,500 per 100,000, and the incidence from 5 per 100,000 to 346 per 100,000. In Asian countries, the crude prevalence rates seem to be lower and range from 15 per 100,000 to 328 per 100,000 [2].

Several studies report data on the epidemiology of PD. However, methodological differences between studies make direct comparison of prevalence estimates difficult. It is generally accepted that the prevalence of the disease range from 1 to 2 per 1000 in unselected populations and that the disease affects 1% of the population above 60 years. The annual incidence per 100,000 inhabitants ranges from less than 10 to more than 20. Incidence studies may be affected by under-diagnosing of PD, especially among the most elderly[3].

Etiology of PD

- Environmental Factors
- Genetic Factors

Environmental Factors

The specific etiology of Parkinson's disease (PD) is not known. Epidemiologic studies indicate that a number of factors may increase the risk of developing PD. These include,

- Exposure to well water
- Pesticides
- Herbicides
- Industrial chemicals
- Wood pulp mills
- Farming
- Living in a rural environment.

A number of exogenous toxins have been associated with the development of parkinsonism, including trace metals, cyanide, lacquer thinner, organic solvents, carbon monoxide, and carbon disulfide. There has also been interest in the possible role of endogenous toxins such as tetrahydroisoquinolines and beta-carbolines. However, no specific toxin has been found in the brain of PD patients, and in many instances the Parkinsonism seen in association with toxins is not that of typical Lewy body PD.

Genetic Factors

There has been considerable interest in the potential role of genetic factors in the etiology of PD. Approximately 5–10% of PD patients have a familial form of parkinsonism with an autosomal-dominant pattern of inheritance. Large pedigrees have been identified where members in different generations suffer from PD. This suggests that genetic factors are important in young-onset patients but are not likely to play a major role in patients with sporadic PD.

There has been an extensive search for a mutation in the mitochondrial genome, based on the finding of a defect in mitochondrial complex I in the substantia nigra pars compacta (SNpc) of PD patients. Complex-I is composed of 41 subunits, 7 of which are encoded by mitochondrial DNA (mtDNA) [4].

Pathogenesis of PD

PD suggests two major hypotheses regarding the pathogenesis of the disease.

One hypothesis posits that misfolding and aggregation of proteins are instrumental in the death of SNpc dopaminergic neurons, while the other proposes that the culprit is mitochondrial dysfunction and the consequent oxidative stress, including toxic oxidized DA species. The abnormal deposition of protein in brain tissue is a feature of several age-related neurodegenerative diseases, including PD. Although the composition and location (i.e., intra- or extracellular) of protein aggregates differ from disease to disease, this common feature suggests that protein deposition per se, or some related event, is toxic to neurons. Aggregated or soluble misfolded proteins could be neurotoxic through a variety of mechanisms. Protein aggregates could directly cause damage, perhaps by deforming the cell or interfering with intracellular trafficking in neurons [5].

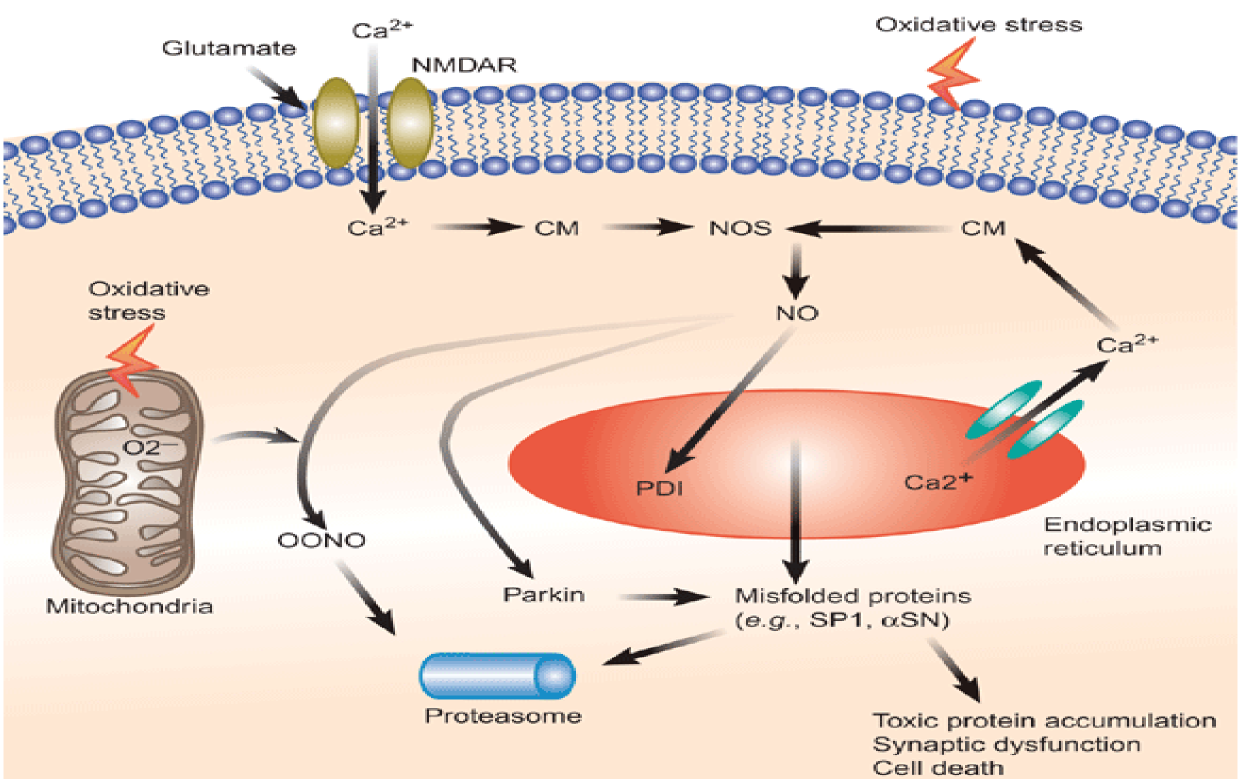
Mechanisms of Neurodegeneration

- Oxidative stress in Parkinson disease
- Altered mitochondrial function in PD
- Altered proteolysis in PD—proteasomal and lysosomal
- Inflammatory change
- Excitotoxic mechanisms

Oxidative stress in Parkinson disease:

Oxidative stress remains a cornerstone of the concepts underlying the loss of dopaminergic neurons in PD.

Fig. No.1: Oxidative Stress and Parkinson's disease

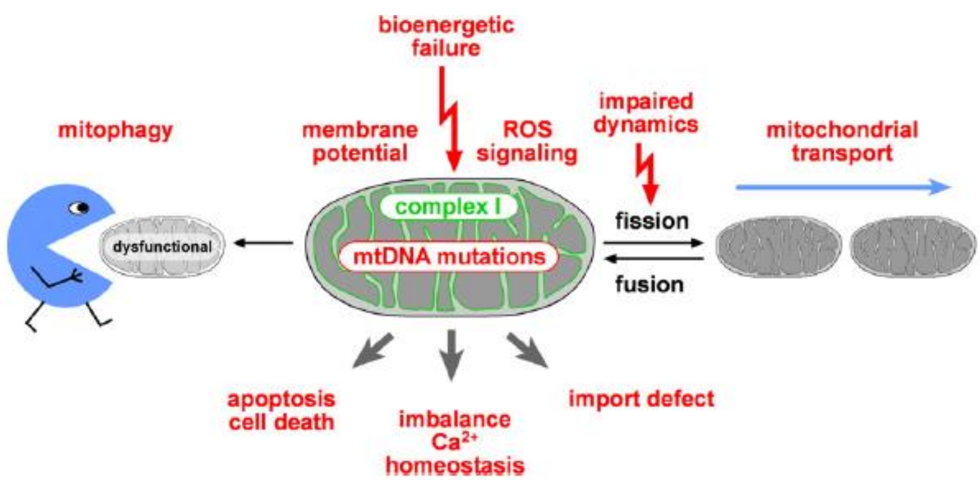


Since the 1980s there has been an exponential growth in publications that implicate the formation of reactive oxygen species as a final step in neuronal death of whatever origin. Increased free radical generation is almost impossible to detect, the potential inducers of oxidative stress and markers of its effects make a substantial case for its occurrence. Enhanced lipid peroxidation (malondialdehyde, lipid hydroperoxides, 4-hydroxynonenal, and advanced glycation end products), protein oxidation (protein carbonyls), and DNA oxidation (8-hydroxyguanosine) in the SNpc in PD and, perhaps, more generally throughout the body, all point in the same direction.

Altered mitochondrial function in PD:

Mitochondrial involvement in cell death in PD has returned to center stage and provides part of a unifying concept of how neuronal loss occurs in both sporadic and inherited disease. The discovery of the neurotoxicity of MPTP through its metabolite MPP^+ identified a role for the inhibition of complex I in pathogenesis that is shared by other substances toxic to dopaminergic neurons, including rotenone and annonacin.

Fig.No.2: Mitochondrial alterations associated with PD



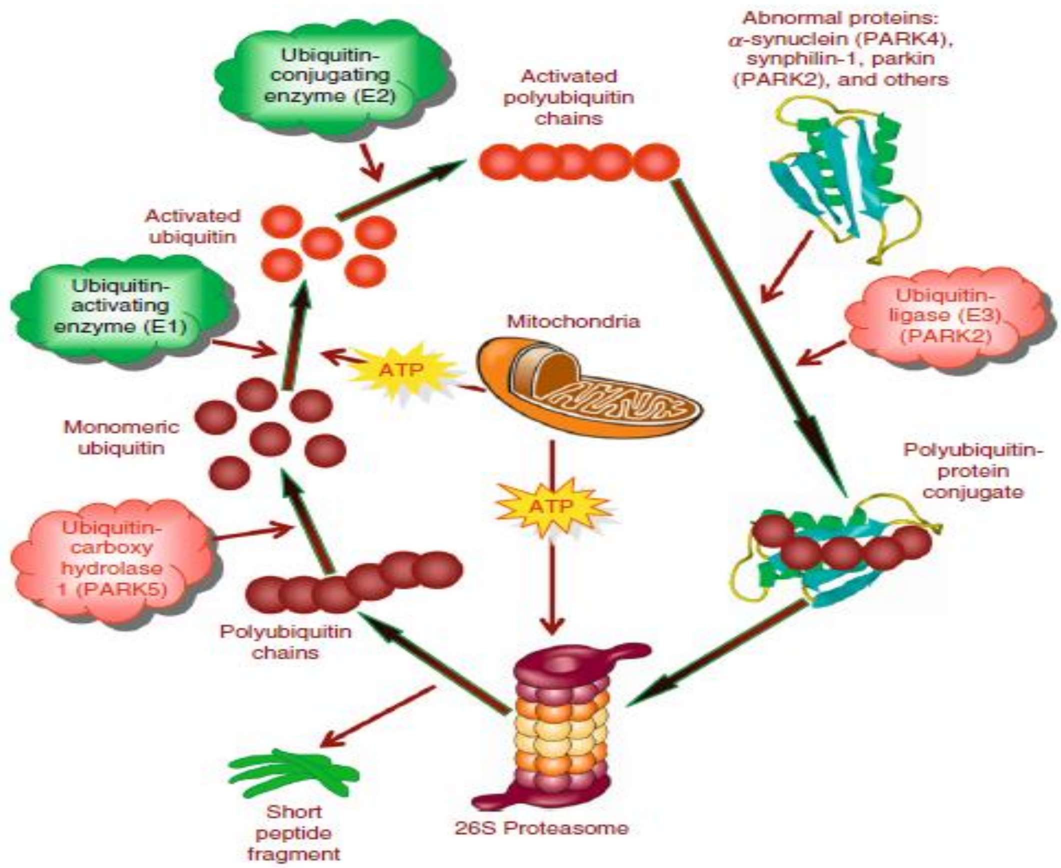
Complex-I is the main gateway for electrons to enter the respiratory chain. It consists of 14 central and up to 32 accessory subunits, which form an L-shaped complex with a membrane arm and a peripheral arm protruding into the mitochondrial matrix. Complex-I catalyze the electron transfer from NADH (derived from the tri-carboxylic acid cycle) to ubiquinone involving a flavin mononucleotide (FMN) and seven iron–sulfur clusters. The mitochondria are an important source of reactive oxygen species (ROS). Increased formation of mitochondrial ROS and/or defective ROS removal by mitochondrial defence systems results in oxidative damage to mtDNA, proteins and lipids and perturbs redox signaling pathways.

The cause and the consequences of complex I deficiency in PD are not well understood. It has been suggested that mutations in complex I genes in the mitochondrial or nuclear genome can account for a dysfunction in complex I activity, assembly and/or stability. Many studies on complex I inhibition and ROS formation have been performed with rotenone, an inhibitor of complex I that binds in proximity to the quinone binding site. When pyruvate or glutamate plus malate are used as substrates to generate NADH and induce forward electron transport, rotenone blocks proton pumping and increases superoxide generation.

Altered proteolysis in PD—proteasomal and lysosomal:

The presence of multiple proteins in Lewy bodies, most notably α-synuclein, led to the idea that the catabolism of unwanted, damaged, or mutated proteins might be disrupted in PD, leading to cellular aggregation and neuronal death. This led to investigation of the roles of the ubiquitin–proteasome system (UPS) and lysosomes in pathogenesis in PD. Examination of the 26S proteasomein PD revealed selective changes in its catalytic activity and composition in the SNc that were associated, perhaps wrongly, with impaired degradation of α-synuclein [6].

Fig.No.3: The ubiquitin–proteasome system: genes involved in PD pathogenesis



During proteolytic stress, here defined as a state in which the levels of unwanted proteins exceed the capacity for clearance due to increased protein production and/or inadequate proteolysis, undegraded proteins readily aggregate and become refractory to normal proteasomal activities.

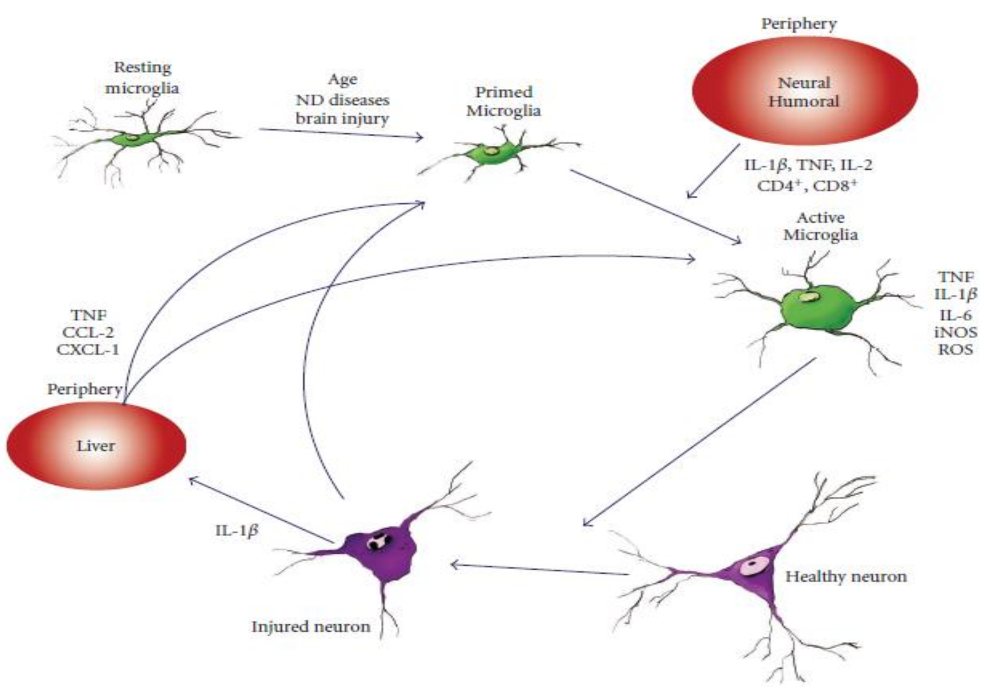
In some cases of familial PD, mutations in the genes encoding α-synuclein, parkin and ubiquitin C-terminal hydrolase L1, are associated with protein accumulation and Lewy body formation in the SNc, locus ceruleus and cerebral cortex. In sporadic PD, the levels of oxidatively damaged proteins and protein aggregation are elevated in the SNc and in other brain regions.

Further, α-synuclein is a major component of Lewy bodies and, because of its normal predominance in presynaptic nerve terminals, this suggests retrograde transport as a means of accumulation to form inclusions. Consistent with this hypothesis, a recent study has shown that α-synuclein is transported along with other proteins to form aggresomes in cell lines.

Inflammatory change:

The concept of inflammatory change in the brain in PD started with the description of activated HLA-positive microglia in SNc. Subsequently, alterations in the cytokines interleukin-1α (IL-1α), IL-1β, and tumor necrosis factor-α (TNF-α) were found in the brain and cerebrospinal fluid, and postmortem studies showed inducible nitric oxide (NO) synthase to be present in activated microglia.

Fig.No.4: Relationship between peripheral inflammation and neuronal loss in PD



Inducible NO synthase is a source of NO, which in turn can react with superoxide from glial or neuronal sources to form the highly reactive peroxy-nitrite. This can nitrate proteins and other biomolecules, and 3- nitro-tyrosine adducts are found in the SNpc in PD.

Microglia activation and inflammatory change were thought to be a consequence of neuronal destruction but there is evidence for a more general systemic inflammatory reaction in PD, suggesting it to be a primary cause of neuronal loss in some cases. In addition, peripheral inflammation may enhance the adverse effects of inflammatory change occurring in the substantia nigra.

Excitotoxic mechanisms:

Excitotoxicity is always included in the list of pathogenic mechanisms that are thought to contribute to cell death in PD. However, the degree of direct evidence for this is small. The major contributor is the over activity of the sub-thalamic nucleus (STN), which releases glutamate and which innervates the substantia nigra pars compacta and the internal segment of the globus pallidus.

In addition, there is evidence of altered glutamatergic input from the corticostriatal pathway. Excitotoxicity might also arise from the dysfunction of mitochondria that occurs in PD. However, experimental studies show that excitotoxins can cause destruction of nigral dopaminergic neurons and that this process can be blocked by various classes of glutamate antagonists.

The scenario would be that as PD develops with the onset of neuronal loss in the SNpc, the increased activity of the glutamatergic input from the STN acts to amplify cell death. Over activity of the glutamatergic output pathways from the STN to the globus pallidus also contributes to the evolution of some of the clinical symptoms of PD.

Indeed, deep brain stimulation (DBS) can correct the over activity of the STN, producing a clinical benefit in PD. Perhaps importantly, recent investigations utilizing DBS in PD have associated it with a lack of progression of motor symptoms [6].

ANIMAL MODELS OF PD

Starting in the late nineteenth century, neuroscientists began generating lesions using surgical, thermal, electrolytic, and toxicant means for scientific study of the effect of central nervous system injury on brain function. In addition to identifying anatomical regions of interest, scientists using early lesioning techniques began to recognize the behavioral similarities to known human neurological disorders and began to recognize the value of animal models.

In general, however, the overall utility of an animal model for a particular disease is often dependent on how closely the model replicates all or part of the human condition. In PD and related parkinsonian disorders, there now exist a variety of animal models, including new invertebrate models, each of which makes a unique contribution to our understanding of the human condition. These models have been developed in a wide variety of species (i.e., mouse, rat, cat, and non-human primate and many other species including *Drosophila*) using a variety of techniques including:

1. Surgical lesioning

2. Administration of pharmacological agents

3. Administration of neurotoxicants

4. Development of transgenic animals [7].

Models of PD

1. Pharmacological-Induced Models of Parkinson's disease
    - Reserpine
    - α-Methyl-para-Tyrosine (AMPT)
2. Neurotoxicant-Induced Models of Parkinson's Disease
    - 6-Hydroxydopamine (6-OHDA)
    - MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)
    - Methamphetamine
    - Rotenone
    - Intranigral Infusion of Iron
    - 1-Benzyl-1,2,3,4-tetrahydroisoquinoline
    - Lipopolysaccharide
    - Quinolinic Acid Lesioning
3. Genetic Models of Parkinson's Disease
    - Spontaneous Rodent Models for Parkinson's Disease
    - Transgenic Mouse Models

        (a) α-Synuclein

        (b) β-Synuclein

        (c) Parkin

        (d) UCH-1
    - Invertebrate Models with α-Synuclein and Parkin

Toxin-Induced Models of Parkinson's disease

Among the various accepted experimental models of PD, neurotoxins have remained the most popular tools to produce selective neuronal death in both *in vitro* and *in vivo* systems. Neurotoxins reviewed here are thought to kill dopaminergic neurons; they all produce specific clinical or neuropathological abnormalities that make them different from each other [8]. Among all neurotoxins, rotenone is selectively used in many studies due to the selective inhibition of complex-I mitochondrial electron transport chain (ETC).

Rotenone neurotoxin

Rotenone is a naturally occurring complex ketone pesticide derived from the roots of *Lonchocarpus* species. It is highly lipophilic and can rapidly cross cellular membranes without the aid of transporters, including the BBB. Rotenone is a strong inhibitor of complex I, which is located at the inner mitochondrial membrane and protrudes into the matrix. Rotenone model of PD, which substantiated involvement of pesticide exposure and systemic complex I dysfunction in PD etiology.

In addition to being a common pesticide, rotenone is a high-affinity inhibitor of complex I of the mitochondrial ETC. Although rotenone caused uniform complex I inhibition throughout the brain, rotenone-treated rats demonstrated many characteristics of PD, including selective nigrostriatal dopaminergic degeneration, formation of ubiquitin and α-synuclein-positive nigral inclusions, and motor deficits [9].

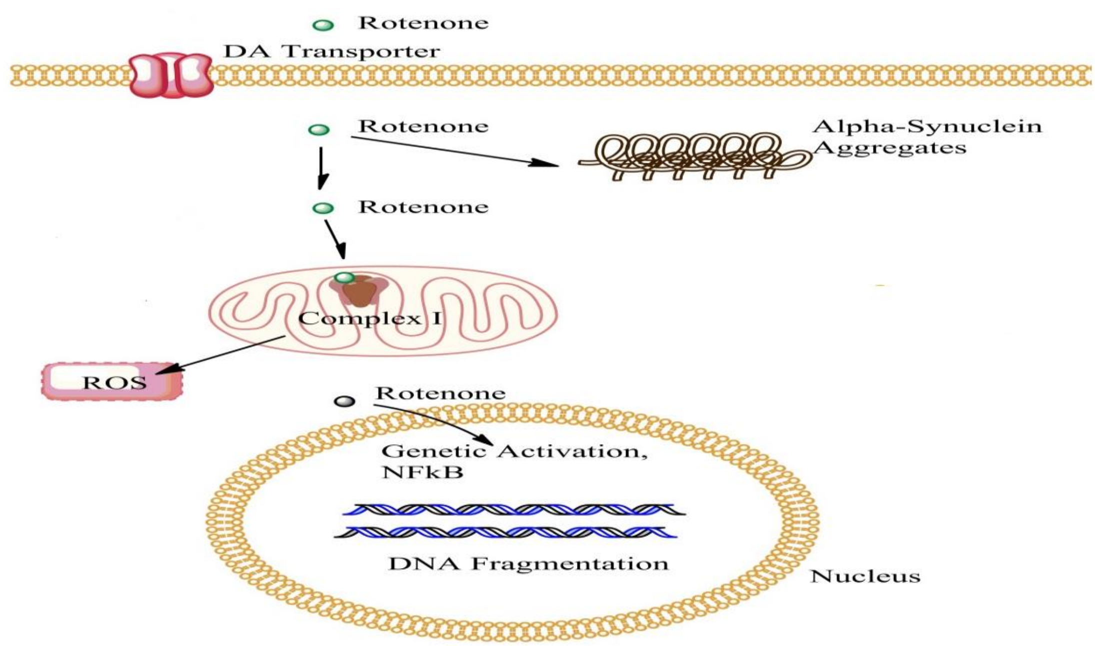
Mechanism of rotenone

Rotenone is a powerful selective mitochondrial complex I inhibitor, which can cause oxidative stress and lead to selective degeneration of striatal–nigral dopaminergic neurons [10]. Besides complex I inhibition, nitrative stress, increased nitric oxide (NO) and malondialdehyde (MDA) levels, aggregation of alpha-synuclein (SNCA) and polyubiquitin, activation of astrocytes and microglial cells, inflammatory reaction, glutamate excitotoxicity, and neuron apoptosis are involved in the mechanisms of rotenone-evoked Parkinsonism.

Oxidative stress

In a variety of cellular sources and cultured brain slices, complex I inhibition, overgeneration of reactive oxygen and reactive nitrogen species, shifting respiration to a more anaerobic state and lipid peroxidation are reportedly potent contributors to rotenone neurotoxicity [11].

Fig.No.5: Rotenone neurotoxicity – Oxidative stress



Behavioral and Pathological Features of rotenone model

1) Parkinsonism (bradykinesia, fixed posture, and rigidity)

2) Good response to L-DOPA and dopamine agonists

3) Loss of Tyrosine hydroxylase neurons and DA-content in nigrostriatal region

4) α-Synuclein-positive inclusions, resemblance to true Lewy body

5) Loss of myenteric neurons

Advantages

- Easily crosses the BBB
- Inhibits electron transport Chain (ETC) complex I
- Upregulation of NADPH oxidase
- Microglial activation
- Highly lipophilic – crosses the blood brain barrier
- It will also inhibit proteasomal activity [12].

CURRENT TREATMENTS FOR PD [13]

Classification of drugs

1. Drugs which replace DA

   Levodopa

   (a) peripheral dopa decarboxylase inhibitors.

   Carbidopa, benserazide

2. Inhibitors of dopamine metabolizing enzymes

   (a) drugs that inhibits catechol-o-methyl transferase

   Tolcapone, Entacapone

(b) Drugs that inhibits Monoamine oxidase B

Selegiline, Rasalgine

3. Drugs that mimic DA (DA agonists)

(a) D_2 agonist and Partial D_1 antagonist

Bromocriptine, Lisuride

(b) Both D_1 and D_2 agonists

Pergolide

(c) Selective D_2 agonists

Praipexzole, Ropinirole

4. Muscarinic acetylcholine receptor antagonists

Benzatropine, Trihexyphenidyl, Diphenhydramine

5. Drugs that release DA

Amantadine

<u>Levodopa</u>

Levodopa (L-dopa), the metabolic precursor of dopamine, is the single most effective agent in the treatment of PD. Both its therapeutic effect and adverse effects result from the decarboxylation of levodopa to dopamine by dopa-decarboxyase. Dopamine cannot pass through Blood-brain barrier, but L-dopa can. The passage of L-dopa across BBB is an active process, mediated by a carrier specific for aromatic amino acids.

The dopamine produced is responsible for the therapeutic effectiveness of the drug in PD. The decarboxylation of L-dopa to DA in brain, takes place within the presynaptic terminals of dopaminergic neurons in the striatum.

In practice, L-dopa is almost always administered with peripheral dopa decarboxylase inhibitors (e.g.) carbidopa, benserazide, that do not penetrate into the CNS. These drugs inhibit the decarboxylation of L-dopa in periphery and increase the amount of L-dopa crossing BBB up to 10-fold.

L-dopa produces dramatic effects on all the signs and symptoms of PD. The degree of improvement in tremor, rigidity and bradykinesia is nearly complete in early phase of treatment. Some dopamine released will be stored in vesicles and that the improvement is uniform. Some symptoms like dysphagia, cognitive decline, etc. are not improved.

The adverse effects of L-dopa are mainly due to its conversion to dopamine in the peripheral mainly including the alteration of blood pressure, ionotropic effect on heart, inhibition of prolactin secretion, nausea, vomiting, anorexia, postural hypotension, palpitation, arrhythmia, etc. Chronic adverse effects may include hallucinations, insomnia, restlessness, delusions, etc.

But the severe effect of chronic use of L-dopa in PD patients include dyskinesia and on/off phenomena. This is largely due to inability of the dopaminergic neurons to reuptake, store and later release the dopamine formed from administered L-dopa. Also it is important to note that the dopamine, thus converted from L-dopa, undergoes oxidative metabolism further generates large quantities of free radicals which accelerate the neuronal death due to oxidative stress.

Inhibitors of dopamine metabolizing enzymes

Catechol-o-methyl transferase and monoamine oxidase B are the enzymes mainly involved in the metabolism of dopamine. Hence inhibition of these enzymes helps in increasing the available concentration of dopamine leading to alleviation of symptoms in PD. Tolcapone and entacapone are durgs that inhibit Catechol-o-methyl transferase and drugs like selegeline, rasalgeline, *etc* inhibit monoamine oxidase B. Even though these drugs are slightly better in producing side effects when compared to L-dopa, but do produce effects like hepatotoxicity, anxiety, insomnia, etc.

Drugs that mimic DA (DA agonists)

Drugs that mimic the effects of dopamine at the dopaminergic receptors are a good alternatives to L-dopa. They directly activate the receptor without any enzyme mediation for activation. They also do not depend on the functional ability of the dopaminergic nerves in the nigrostriatum i.e., ability to store and release dopamine. There is no free radical formation which would further degenerate the nerves. But chronic use of these agents too is limited by the appearance of side effects like cardio vascular disorders, somnolence, nausea, etc.

Muscarinic acetylcholine receptor antagonists

In a normal individual there exists a fine balance between the stimulatory cholinergic and inhibitory dopaminergic systems in the nigrostriatum. Degeneration of dopaminergic neurons results in loss of balance and cholinergic system contributing the symptoms of PD like tremors, etc. Hence muscarinic antagonists are used to reduce the cholinergic stimulation and thereby bringing back the balance. The drugs include trihexyphenidyl, benztropine, diphenhydramine, etc. Most troublesome side effects like sedation, mental confusion, etc. ensues.

Drugs that release DA

Amantadine, an antiviral agent, is useful in the treatment if PD and acts by altering the dopamine release. Side effects include dizziness, lethargy, sleep disturbance, etc.

Alternative medicine/Therapy

This means a replacement for mainstream medicine. Alternative medicine can have their own theories of disease and methods of diagnosis, as well as different treatments.

Acupuncture:

Acupuncture is part of traditional Chinese medicine and has been used for thousands of years. In Parkinson's, it may be helpful mostly for pain control. It is based on the premise that disease states are characterized by imbalances in flow of energy, and that insertion of needles along various points within energy channels can restore balance and health. Acupuncture appears to be safe, and many patients anecdotally describe benefit but there is not sufficient evidence to recommend it to patients with PD[14].

Alexander technique:

Alexander technique was invented by an actor, Frederick Alexander (1869–1955). He developed the method to treat himself when he developed problems with his voice. Later, he taught the system to others. Alexander technique trains you to think carefully about the way you use your muscles, recognizing and releasing muscle tension. There are only a few good scientific studies of Alexander technique. In one of these, it helped people suffering from back pain. In Parkinson's, it might help with co-ordination, balance, pain, fatigue and tremor [14].

<u>Aromatherapy:</u>

Aromatherapy uses oils made from a huge variety of flowers and plants. These 'essential oils' are usually massaged into your skin. They can also be inhaled or used in creams or in the bath. The theory is that essential oils have chemical properties that can ha ve all sorts of effects on both mind and body. People use aromatherapy for stress, pain, insomnia and depression and many other ailments. Some people see it more as a relaxing treat [14].

<u>Ayurveda:</u>

Ayurveda originates from India and is derived from ancient religious and philosophical writings called the Vedas. Ayurveda literally means 'the knowledge of life'. Ayurveda as *kampavata*, *kampa* meaning tremor and *vata* representing one of the dhosa, resembles Parkinson disease in its features. A common Ayurvedic treatment for *kampavata* comprises prominently of plant drugs Ashwagandha (*Withania somnifera* root) and Atmagupta (*Mucuna pruriens* seeds). *Mucuna pruriens* is a legume plant, known as 'velvet bean', which grows in tropical Asia and tropical Africa where it is native. It is used as food, but also in traditional medical practices [15].

Parkinson's is co-related with *kampavata* in Ayurveda. In old age *vata dosha* is more predominant in the body. This *vata* relocates in the brain and dries up the brain cells leading to tremors and instability. Parkinsons disease reflects massive vitiation of *vata* which occupies almost all channels of body. Weak digestive fire, disturbed digestion and presence of toxins are generally observed in the patient at gastrointestinal as well as cellular levels, which provide conducive environment for vitiation of *vata*. Digestive herbs, in conjunction with nerve tonics are administered to restore digestive function both in the gastro intestinal tract and at a cellular level. Specific diets and regimen are advised.

Mucuna pruriens has been used by Ayurvedic physicians in the past for the treatment of PD, it was felt that it should be reevaluated using modern methods of testing. There is a clear trend in favor of alternative medicine [16].

Herbal medicines

Herbal products for PD have been used worldwide in traditional medicine. Practitioners and patients employ HM as an adjuvant therapy of conventional treatment for the purpose of reducing dose of dopaminergic drugs, adverse event related to prolonged usage of dopaminergic agents and improving PD symptoms [17]. The herbs which show the significant effect in treating Parkinsonism are mentioned below:

- *Acanthopanax senticosus* Harms (family: Alariaceae)
- *Withania somnifera* (Family: Solanaceae)
- *Uncaria rhynchophylla* (family: Rubiaceae)
- *Nardostachys jatamansi* (Family: Valirenaceae)

- *Chrysanthemum morifolium* (Family: Asteraceae)
- *Cassiae semen* (family:Leguminosae)
- *Anemopaegma mirandu* (family:Bignoniaceae)
- *Hypericum perforatum* (Family:Hpericaceae)
- *Gastrodia elata* Blume (Family: Orchidaceae)
- *Centella asiatica* (Family: Umbelliferae)
- *Thuja orientalis* (Family: Cupressaceae)
- *Mucuna pruriens* (Family: leguminosae)
- *Ginkgo biloba* (Family: Ginkgoaceae)
- *Plumbago scandens* (Family: Plumbaginaceae)
- *Bacopa monniera* (Family: Scrophulariaceae)
- *Pueraria thomsonii* (Family:Fabaceae)

A huge heterogeneity in the herbal composition, drug formulation, dose, duration, combined therapy and control interventions was observed, which were the main obstacles to estimate the general effect size of herbal remedies [18].

Cymbopogon flexuosus

Cymbopogon flexuosus (Family: Poaceae), the plant used in the study, is available all over India, Sri Lanka, Burma and Thailand [19].The plant has been used in traditional system of medicine for the treatment of fever, stimulant and used as a flavouring agent, carminative and perfumery [20].

PLANT PROFILE

Cymbopogon flexuosus (Nees ex Steud.) Will. Watson

Family: Poaceae (Gramineae)

Common names: Cochin grass

Malabar grass

East Indian lemongrass

France Indian verbena

Vernacular names:

English: East Indian lemon grass

Malayalam: Chukkunaripullu / Inchipullu

Tamil: Karpoorapullu

Distribution:

India, Sri Lanka, Burma and Thailand

Chemical constituents:

The chemical composition of *C. flexuosus* oil has been reported. Some common constituents found in *Cymbopogon flexuosus* oil (CFO) such as geraniol (20%), geranyl acetate (12%), limonene (3.5%), ɑ-bisabolol (8.4%) [21].

The essential oil of *Cymbopogon flexuosus* is consisted mainly of citral (75-85%) which is mixture of geranial (citral a) and neral (citral b).

Other compounds present in prominent amounts reported in numerous previous studies are: Citronellal (0.37-8.04%), Citronellol (0.44-4.58%), Citronellyl acetate (1.2-3.6%), methyl eugenol (20.0%) and myrcene (0.1-14.2%) [19].

Fig.No.6: Parts of *Cymbopogon flexuosus* (lemon grass)

Parts Used:

Essential parts – Stalks and Leaves

Description:

It is a tall, fast-growing, lemon scented, perennial grass reaching a height of 1.5m. It has distinct, dark-green foliage and also produces seed [22]

Traditional uses:

Oil is used as stimulant, flavouring agent, as a carminative and also in perfumery. Citral is used in the synthesis of β-ionone from which vitamin A is prepared [20].

Reported activities:

Limonene and borneol present in the essential oil of *C. flexuosus* have anaesthetic properties [19]; antimicrobial [22, 23]; anticancer activity [24]; immunostimulatory and analgesic and anti- inflammatory [25] properties.

REVIEW OF LITERATURE

Anusha *et al.,* (2017) [25] investigated and reported the protective role of apigenin on rotenone induced rat model of Parkinson's disease: Suppression of neuroinflammation and oxidative stress mediated apoptosis. Apigenin (AGN), a non-mutagenic flavone found in fruits and vegetables, exhibits a variety of biological effects including anti-apoptotic, anti-inflammatory, and free radical scavenging activities. Unilateral stereotaxic infusion of ROT caused the loss of tyrosine hydroxylase (TH) immunoreactivity in striatum and substantia nigra. The mRNA expression of inflammatory markers and neurotrophic factors was quantified by reverse transcriptase polymerase chain reaction (RT-PCR). Administration of AGN significantly attenuated the upregulation of NF- κB gene expression in ROT induced group and prevented the neuroinflammation in substantia nigra pars compacta (SNpc).AGN exerts its neuroprotection in ROT model of PD and may act as an effective agent for treatment of PD.

Badawi *et al.,* (*2017*) [26] studied and reported the Sitagliptin and liraglutide reversed nigrostriatal degeneration of rodent brain in rotenone-induced Parkinson's disease. Their study investigated the possible relationship between pro-inflammatory cytokines and programmed nigral neuronal death in rotenone model of Parkinson's disease (PD). Sitagliptin and liraglutide efficacy to inhibit the inflammatory-apoptotic degenerative process were investigated. Sitagliptin (30 mg/kg/day, p.o) and liraglutide (50 lg/kg, s.c.) showed statistically significant ($p < 0.05$) effect on behavioral activity. Where, both doses improved the motor performance significantly in comparison with other doses in both cylindrical and catalepsy tests. Sitagliptin and liraglutide represent a promising strategy to mitigate the progression of PD by their anti-inflammatory, anti-apoptotic neurotrophic and neurogenic mechanistic activities.

Darbinyan *et al.,* (2017) [1] investigated and reported the Rotenone impairs hippocampal neuronal activity in a rat model of Parkinson's disease. Interactions between the dopaminergic systems and the hippocampus in synaptic plasticity and behavior are found. The Rotenone-induced animal model is commonly used in research studies involved in PD. Administration of rotenone causes alterations of electrical neuronal activity. Dose-dependent reduction of evoked neural activity and a reduction in firing strength were found in the hippocampus. Behaviorally, Rotenone rats showed a more consistent decrease in rearing across the 3 weeks, compared with animals in the control group. Thus, rotenone causes changes in hippocampal electrical activity and behavioral changes.

Adukwu *et al., (2016)* [21] performed and reported the antimicrobial activity, cytotoxicity and chemical analysis of lemongrass essential oil (*Cymbopogon flexuosus*) *and pure citral.* To determine the antimicrobial effects of lemongrass oil (*C. flexuosus*) and to determine cytotoxic effects of both test compounds on human dermal fibroblasts. Antimicrobial susceptibility screening was carried out using the disk diffusion method. Antimicrobial resistance was observed in four of five *Acinetobacter baumannii* strains with two strains confirmed as multi-drug-resistant (MDR).

Dhanalakshmi *et al.,* (2016) [27] examined and reported Vanillin Attenuated Behavioural Impairments, Neurochemical Defícts, Oxidative Stress and Apoptosis against Rotenone Induced Rat Model of Parkinson's Disease. Vanillin (4-hydroxy-3-methoxybenzaldehyde), a pleasant smelling organic aromatic compound, is widely used as a flavoring additive in food, beverage, cosmetic and drug industries. It is reported to cross the blood brain barrier and also displayed antioxidant and Neuroprotective activities. Rotenone treatment exhibited motor and non-motor impairments, neurochemical deficits, oxidative stress and apoptosis, whereas oral administration of vanillin attenuated the above-said indices. Akinesia, catalepsy and open field test are normally performed to assess the delay in initiating a movement, rigidity and locomotion and exploratory like behavior respectively in experimental rodents. Intraperitonial administration of rotenone showed a significant impairment in akinetic movement, cataleptic ability, locomotion activity as compared to control rats. In their study, Depletion of brain dopamine levels in rotenone treated rats resulted in behavioral anomalies as seen in PD. Enhancement of striatal dopamine and its metabolite levels by vanillin clearly indicate the efficiency of vanillin in protecting dopaminergic neurons.

Gupta *et al.,* (2016) [22] investigated and reported a study on antimicrobial activities of essential oils of different cultiviers of lemongrass (*Cymbopogon flexuosus*). Essential oils were isolated from one month old plants by hydro-distillation in mini Clevenger apparatus for 2 h. Antimicrobial activities were determined by agar well diffusion method. Lemongrass oils exhibited strong antimicrobial activity against all the microbes except E coli. The study revealed strong antimicrobial potential of the essential oil against pathogenic microbial strains which may be of high clinical importance in future.

Cortes *et al.*, (2015) [28] investigated and reported Protective efficacy of P7C3-S243 in the 6-hydroxydopamine model of Parkinson's disease. There are currently no therapeutic options for patients with Parkinson's disease that prevent or slow the death of dopaminergic neurons. We have recently identified the novel P7C3 class of neuroprotective molecules that blocks neuron cell death. After unilateral injection of 6-OHDA into the median forebrain bundle, rats were assessed for behavioral function in the open field, cylinder test, and amphetamine-induced circling test. Thereafter, their brains were subjected to neurochemical and immune histochemical analysis of dopaminergic neuron survival. Analysis was conducted as a function of treatment with P7C3 compounds, with administration initiated either before or after 6-OHDA exposure. When P7C3-S243 administration was initiated after 6-OHDA exposure, rats also showed protective efficacy in all measures, which included blocking dopaminergic neuron cell death in ipsilateral substantia nigra pars compacta, preservation of dopamine and its metabolites in ipsilateral striatum, and preservation of normal motor behavior.

Desai *et al.*, (2014) [29] investigated and reported the Modeling and optimization studies on extraction of lemongrass oil from *Cymbopogon flexuosus* (Steud.) Wats. Optimization studies for the extraction of essential oil from the leaves of lemongrass by hydro-distillation. The models based on partitioning thermodynamics and kinetic desorption were studied to understand the extraction behaviour. The process was found to be influenced by the initial desorption step, described by the two-site kinetic desorption model. The optimum conditions, using the Taguchi method, were obtained as: 1:14 (w/w) solid to water ratio, 25 mm size of the plant material, extraction time of 1.5 h and 500 W powers. Qualitative analysis of essential oil was performed and the maximum amount of citral (83.24%) was obtained under optimized condition.

Mahindra *et al.,* (2014) [30] investigated and discussed about Microwave Assisted Extraction of Lemongrass Oil. Lemongrass is an important member of the grass family grown mostly as a source of citral in its essential oil, which is widely for the production of artificial vitamin A. Essential oil of lemongrass was extracted by microwave-assisted hydro-distillation (MAHD) and the individual effects of process parameters; microwave power and irradiation time. Results showed that oil yield increases with increasing microwave power and irradiation time the maximum oil yield in MAHD was 1.72% for 90 minutes.

Samim *et al.,* (2014) [31] investigated and reported Neuroprotective Effect of *Ocimum sanctum Linn* on Rotenone Induced Parkinsonism in Rats. Experimental PD was induced by administration of rotenone, a neurotoxin which developed all the essential features of PD. PD is attributed to oxidative and inflammatory stress and hence drugs targeting these pathways hold promise as neuro-therapeutics. The behavioural alterations were evaluated by the open field test, pole test and rotarod test. Biochemical changes were assayed by estimating MDA, GSH and SOD. Histopathological study of the substantia nigra (SN) was also done. Treatment with lower and high dose of OS reversed the locomotor deficits and biochemical alterations due to rotenone which were supported by histopathological studies.

Sharma and Deshmukh, (2014) [32] examined and reported the Vinpocetine attenuates MPTP-induced motor deficit and biochemical abnormalities in wistar rats. Up-regulation in phosphodiesterase 1 (PDE1) expression and decreased levels of cyclic nucleotides (cAMP and cGMP) have been reported in patients and experimental animal models of Parkinson's disease (PD). Phosphodiesterase (PDE) inhibitors have been reported to be beneficial in cognitive and motor deficit states. The study is designed to investigate the effect of vinpocetine, a PDE1 inhibitor in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-Induced experimental PD-like symptoms in rats. Movement abnormalities were assessed by a battery of behavioral tests.

Ittiyavirah and Ruby, (2014) [33] examined the effect of hydro alcoholic root extract of *Plumbago zeylanical* (PZE)alone and it's combination with aqueous leaf extract *Camellia sinensis (AECS)*on rotenone induced Parkinsonism. The synergistic effect with AECS were assessed by the *ex vivo* antioxidant assays by measuring the various enzymes in the striatum region of brain such as Glutathione, Lipid peroxidation, Catalase and Superoxide dismutase. The various behavioral parameters analyzed were rearing, self-grooming, ambulation activity using open field apparatus and muscle rigidity using bar test. The administration of rotenone produced motor dysfunctions like catalepsy and muscle rigidity along with a reduction in locomotor activity.

Tyagi *et al.,* (*2014*) [34] investigated and reported the possible role of GABA-B receptor modulation in MPTP induced Parkinson's disease in rats. Accumulating evidence strongly suggests that gamma amino butyric acid (GABA) receptors play a crucial role in the pathogenesis of Parkinson's disease (PD). The study investigate the role of GABA-B receptor modulation in experimental models of MPTP-induced PD. Different behavioural tasks were performed and biochemical parameters were estimated, post treatment with baclofen significantly improved the motor abnormalities and attenuated the oxidative damage and neuro-inflammation in MPTP treated rats. CGP35348, GABA-B receptor antagonist, reversed the protective effect of baclofen GABA-B receptor play role in the neuroprotection.

Khurana and Asmita, (2013) [35] investigated and studied the ameliorative effect of *Sida cordifolia* in rotenone-induced oxidative stress model of Parkinson's disease. The study focused on the evaluation of aqueous extract, and its different fractions), against rotenone induced biochemical, neurochemical, histopathological and behavioral alterations in a rat model of Parkinson's disease (PD). *Sida cordifolia* is an important medicinal plant of Ayurvedic system of medicine, containing a number of poly-phenolic compounds. There is ancient Ayurvedic relevance for the use of this plant in the treatment of PD, the plant possesses significant *in vitro* and *ex vivo* anti-oxidant activity and encouraged its evaluation for the possible beneficial effect in neurodegenerative diseases based on its traditional use. As oxidative stress plays an important role in generation of rotenone-induced PD pathology, it is worth to explore the beneficial effect of this antioxidant plant in rotenone-induced oxidative stress model of PD.

Ain *et al.,*(2013) [36] investigated and reported an experimental design approach for the extraction of lemongrass (*Cymbopogon citratus*) oleoresin using pressurised liquid extraction (PLE). Extraction of lemongrass oleoresin was successfully optimised using Pressurised Liquid Extraction (PLE). Character impact compounds; neral, geranial and geraniol which constituted 72% oleoresin, were monitored during this optimisation study by using GCMSD. Based on maximum extraction of these compounds, the optimised operating conditions for PLE were a temperature of 167°C, a pressure of 1203 psi and a static time of 20.43 min. The quality of PLE extract was compared with conventional extraction methods, hydro-distillation and Soxhlet extraction. investigated and reported the Hydro-distillation of Essential Oil from *Cymbopogon flexuosus.* Hydro-distillation is a potentially useful method to extract essential oil from various plant materials and was used to separate essential oil from *Cymbopogon flexuosus* (lemongrass). The yield is dependent on various parameters like weight of raw material, volume of water, size of raw material and nature of raw material. The optimized conditions for the extraction were 50 g of raw material, 500 ml of water volume, plant size of 25 mm, and the yield obtained was 1.04 percent. The loss of about nine percent essential oil was observed during drying of leaves of lemongrass. Composition of the essential oil was analyzed by GC-MS and higher amount of oxygenated compounds, mainly citral, was found in each sample.

Swathi *et al.,* (*2013*) [37] investigated and reported the evaluation of rotenone induced Parkinson's disease on glutamate metabolism and protective strategies of *Bacopa monnieri.* The Neuroprotective effect of Rotenone (RT) induced Parkinson's disease (PD) with particular reference to glutamate metabolism in different regions of rat brain. The rats were divided into four groups of six in each, group 1 received Saline water, and group 2 received RT through i.p. route for 60 days to induce PD. The BM extract was given orally 20 days before induction of the PD to group 3 and group 4 received Levodopa (LD) orally, referred as drug control. The levels of glutamate content, Glutamate dehydrogenase (GDH), Glutamine synthetase (GS) and Glutaminase were measured. Glutamine content and activity levels of GDH, GS were significantly depleted and elevated glutaminase activity was found in the different brain regions of rat during RT induced PD when compared to controls rats.

Desai and Parikh, (2012) [38] studied and reported the Hydrotropic Extraction of Citral from Cymbopogon flexuosus (Steud.) Wats. A novel technique for the extraction of citral from the leaves of *Cymbopogon flexuosus* (Steud.) Wats. Using hydrotropic solutions (sodium salicylate and sodium cumene sulfonate) was investigated. The yield of citral was dependent on the concentration of hydrotrope, solid loading, temperature, and size of the plant material. Using the Taguchi method, the extraction was optimized, and both the hydrotropes gave the highest yield of citral at a concentration of 1.75 M, 5% solid loading, a temperature of 30 °C, and a size of 0.25 mm of the plant material. Using hydrotropic extraction, citral could be extracted under normal operating conditions, and the use of traditional organic solvents could be eliminated. As a simple and environmentally friendly technique, hydrotropic extraction could be utilized for the extraction of bioactive compounds from plants.

Riddle *et al.,* (2012) [39] explored and reported the Pramipexole and methamphetamine-induced reward-mediated behavior in a rodent model of Parkinson's disease and controls. Pramipexole (PPX) is a dopamine agonist that is FDA-approved for treatment of motor dysfunction in Parkinson's disease and restless leg syndrome. In a subpopulation of treated patients, PPX can lead to impulsive–compulsive disorders including behavioral addictions and dopamine dysregulation syndrome, a phenomenon that mirrors drug addiction. Methamphetamine (meth) and saline served as positive and negative controls, respectively. To model Parkinson's disease, the neurotoxin 6-OHDA was injected bilaterally into the dorsolateral striatum. The resulting lesions were verified functionally using a forelimb adjusting step and post mortem immunohistochemical staining of striatal tyrosine hydroxylase.

Adukwu *et al., (2011)* [40] investigated and reported the anti-biofilm activity of lemongrass *(Cymbopogon flexuosus)* and grape fruit *(Citrus paradisi)* essential oils against five strains of *Staphylococcus aureus*. Antimicrobial susceptibility screening was carried out using disk diffusion method.

Khuwaja *et al.,* (2011) [41] examined and reported the Neuroprotective effects of curcumin on 6-hydroxydopamine-induced Parkinsonism in rats: Behavioral, neurochemical and immunohistochemical studies. Curcumin, the active principle of turmeric used in Indian curry is known for its antitumor, antioxidant, antiarthritic, anti-ischemic and anti-inflammatory properties and might inhibit the accumulation of destructive beta-amyloid in the brains of Alzheimer's disease patients. A Parkinsonian model in rats was developed by giving 6-hydroxydopamine (10μg/2μl in 0.1% ascorbic acid–saline) in the right striatum. A significant protection of lipid peroxidation, glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase, tyrosine hydroxylase and D_2 receptor binding was observed in the striatum of lesioned group animals pretreated with 80mg/kg body weight of curcumin for 21 days as compared to lesion group animals. No significant alterations in behavior and biochemical parameters were observed in sham group animals. Their study indicates that curcumin, which is an important ingredient of diet in India and also used in various systems of indigenous medicine, is helpful in preventing Parkinsonism and has therapeutic potential in combating this devastating neurologic disorder.

Parikh and Desai, (2011) [42] investigated and reported the Hydro-distillation of Essential Oil from *Cymbopogon flexuosus.* Hydro-distillation is a potentially useful method to extract essential oil from various plant materials and was used to separate essential oil from *Cymbopogon flexuosus* (lemongrass). The yield is dependent on various parameters like weight of raw material, volume of water, size of raw material and nature of raw material. The optimized conditions for the extraction were 50 g of raw material, 500 ml of water volume, plant size of 25 mm, and the yield obtained was 1.04 percent. The loss of about nine percent essential oil was observed during drying of leaves of lemongrass. Composition of the essential oil was analyzed by GC-MS and higher amount of oxygenated compounds, mainly citral, was found in each sample.

Swarnkar *et al.,* (2011) [43] investigated and reported the Rotenone induced neurotoxicity in rat brain areas: A histopathological study. Rotenone a pesticide is known to induce neurotoxicity. Rotenone induced biochemical changes and cerebral damage in brain areas with neuromuscular coordination in rats. This study involves investigation of rotenone induced histopathological changes in the brain areas, striatum (STR) and substantia nigra (SN) using HE (hematoxylin and eosin) and CV (Cresyl Violet) staining after 1, 7, and 14 day of unilateral intranigral administration of rotenone (3, 6 and 12µg/5µl) in adult male SD rats. The study inferred rotenone causes neuronal damage which is evident by histopathological changes, impaired neuromuscular coordination and biochemical changes.

Chandrashekar and Prasanna, (2010) [24] investigated and reported Analgesic and Anti-inflammatory Activities of the Essential oil from *Cymbopogon flexuosus.* The anti-inflammatory activity was studied using acute or chronic treatments in rats. Analgesic effect of the essential oil was evaluated in acetic acid induced writhing and tail flick model. The essential oil exhibited significant anti-inflammatory activities in the acute carrageenan- induced rat paw edema and the chronic granuloma pouch models.

Cannon *et al.,* (2009) [44] performed and reported A highly reproducible rotenone model of Parkinson's disease. The systemic rotenone model of Parkinson's disease (PD) accurately replicates many aspects of the pathology of human PD and has provided insights into the pathogenesis of PD. The major limitation of the rotenone model has been its variability, both in terms of the percentage of animals that develop a clear-cut nigrostriatal lesion and the extent of that lesion. Male Lewis rats in three age groups (3, 7 or 12–14 months) were administered rotenone (2.75 or 3.0 mg/kg/day) in a specialized vehicle by daily intraperitoneal injection. All rotenone-treated animals developed bradykinesia, postural instability, and/or rigidity, which were reversed by apomorphine, consistent with a lesion of the nigrostriatal dopamine system. Animals were sacrificed when the PD phenotype became debilitating. Rotenone treatment caused a 45% loss of tyrosine hydroxylase-positive substantia nigra neurons and a commensurate loss of striatal dopamine.

Sharma *et al., (2009)* [45] investigated and reported anticancer activity of an essential oil from *cymbopogon flexuosus.* The essential oil from a lemongrass variety of *cymbopogon flexuosus* was studied for its *in vitro* cytotoxicity against twelve human cancer cell lines. The *in vivo* anticancer activity of the oil was also studied using both solid and ascetic Ehrlich and Sarcoma-180 tumor models in mice. In addition, the morphological changes in tumor cells were studied to ascertain the mechanism of cell death.

Woodley *et al.,* (2008) [46] investigated and reported the Enhanced function in the good forelimb of hemi-parkinson rats: Compensatory adaptation for contralateral postural instability? Postural instability is a major deficit in Parkinson's disease that is resistant to levodopa therapy and contributes to the risk of falling. One forelimb at a time, to normal rats as well as rats extensively depleted of dopamine by unilateral infusion of 6- hydroxydopamine (6-OHDA, given in the medial forebrain bundle) to produce a hemi-parkinsonian syndrome. The 6-OHDA rats showed severe postural instability in the impaired forelimb, but unexpectedly showed enhanced function in the non-impaired forelimb. The data suggest that the intact hemisphere may undergo rapid reorganization subsequent to unilateral dopamine depletion, which allows for compensatory function of the "intact" limb.

Ahmad *et al.,* (*2005)* [47] studied and reported Neuroprotective effects of *Withania somnifera* on 6-hydroxydopamine induced Parkinsonism in rats. The anti-parkinsonian effects of *Withania somnifera* extract, which has been reported to have potent anti-oxidant, anti-peroxidative and free radical quenching properties in various diseased conditions. 6-Hydroxydopamine (6-OHDA) is one of the most widely used rat models for Parkinson's disease. There is ample evidence in the literature that 6-OHDA elicits its toxic manifestations through oxidant stress. 6-OHDA has been reported to cause its dopaminergic toxicity by the process of lipid peroxidation and oxidant stress and there are many reports whereby antioxidants have been implicated in the protection against 6-OHDA toxicity.

Panov *et al.,* (2005) [47] examined and reported the Rotenone Model of Parkinson Disease multiple brain mitochondria dysfunctions after short term systemic rotenone intoxication. Chronic infusion of rotenone (Rot) to Lewis rats reproduces many features of Parkinson disease. Rot (3 mg/kg/day) was infused subcutaneously to male Lewis rats for 6 days using Alzet minipumps. Control rats received the vehicle only. Rotenone, when introduced *in vivo*, binds specifically to the mitochondrial Complex I (NADH:ubiquinone oxidoreductase) all over the brain.

Bashkatora *et al.,* (2004) [48] investigated and reported the chronic administration of rotenone increases levels of nitric oxide and lipid peroxidation products in rat brain. The complex I inhibitor rotenone is a neurotoxin that has been proposed to induce Parkinson-like degeneration. NO and TBARS were increased in the frontal cortex and in the striatum. Behaviorally, the rats exhibited akinesia and rigidity in the catalepsy test. These results show that chronic administration of rotenone over a long period is capable of increasing NO and TBARS in the cortex and striatum and mimics Parkinson's disease (PD)-like behavioral symptoms that are akinesia and rigidity in rats.

Brown *et al.,* (*1999)* [49] investigated and reported the Differences in Measures of Exploration and Fear in MHC-Congenic C57BL/6J and B6-H-2K Mice. Examine the strain and sex differences in MHC-congenic C57BL/6J and B6-H-2K mice with respect to exploration and fear motivated behaviors in the elevated plus maze, the elevated zero maze, and the open field. In the elevated plus maze and elevated zero maze, C57BL/6J mice spent more time in the open areas than B6-H-2K mice, suggesting that they were less fearful and more exploratory. No sex differences for exploration were found but male mice defecated more than females in the elevated plus maze. In the open field there were no significant strain or sex differences in measures of exploration or fear. There were no strain differences in the investigation of a novel object placed into the open field, but males investigated the novel object more than females.

Fredriksson and Archer, (1994) [50] scrutinized and reported Two experiments were performed to study the parametric effects of long-term administration of the neurotoxin, 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP), as a functional model of Parkinsonism in mice. DA depletions were severe at all five test intervals. These results offer functional and neurochemical evidence that MPTP treatment produces permanent damage to the nigrostriatal motor system in mice.

AIM AND OBJECTIVE

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects small regions in the brain that control movement, posture and balance. It is a complex disease that has many different symptoms, so that not everyone with the condition suffers from the same problems. There are up to 10 million people in the world (i.e., approximately 0.3% of the world population) and 1% of those above 60 years be affected with PD.

The pathology of PD involves the depletion of dopamine, degeneration of dopaminergic neurons in the nigro striatum, etc. Usually the inhibitory dopaminergic system and excitatory cholinergic system strike a balance in maintain the posture locomotion, motor reflexes, etc. In PD, the balance is affected due to the dopaminergic nerve degeneration and cholinergic nerves get upper hand leading to symptoms of PD.

Majority of population still have limited access or no access to modern medicines and rely on traditional ways of treatment. India being a country with rich floral diversity houses around 45,000 plant species. Use of herbal drugs for treating ailments is followed in Ayurveda, Yoga, Siddha, Unani, Homeopathy and Naturopathy forms of alternative medicines. Herbal medicines are now in great demand among the population in developing countries because they are inexpensive, better cultural acceptability, better compatibility with human body and minimal side effects.

Currently the PD is treated with the drugs those increase the dopaminergic activity like, precursors of dopamine (levodopa), drugs that inhibit dopamine metabolism (MAO inhibitors and COMT inhibitors), drugs that release dopamine (Amantadine), dopamine receptor agonist (Ergot derived: Bromocriptine, Non ergot: Pramipexole) and drugs those that suppress the cholinergic activity (Atropine and its substitutes). Our current medication have been shown to improve symptoms, by restoring more normal chemical balance in the brain between the dopaminergic and cholinergic nerves, thereby reducing the symptoms of PD. But, the altered pathological changes are not improved and also oxidative metabolism of some of the agents actually accelerates further damage, leading to irreversible and untreatable symptoms severely affecting the quality of life of the patient and to death.

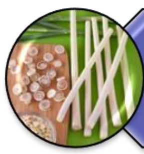
Alternative and traditional medicine is becoming an increasingly important therapeutic option in various morbidities including neurodegenerative disorders. In this line, WHO has recommended the evaluation of effectiveness of plants in condition where we lack a safer modern drug. Thus, a proper scientific evaluation and screening of plants by pharmacological and chemical investigations for the discovery of potential anti-parkinsonism agents is need of the hour with possible outcomes to prevent, delay, reduce and/or treat neurodegeneration.

Based on this background, the present research work on the validation of medicinal plant *Cymbopogon flexuosus* was carried out. The quintessential aim is to find out the effect of *Cymbopogon flexuosus* (lemongrass oil) against rotenone-induced Parkinsonism in rats. Objectives of the present study include the extraction of lemon grass oil from the plant *Cymbopogon flexuosus* leaves, qualitative evaluation of phytoconstituents present, testing for its toxicity, optimizing the rotenone-induced model for Parkinsonism in rats and studying the effect of the extracted oil against rotenone-induced Parkinsonism in rats.

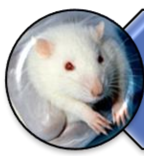
PLAN OF WORK

Review of literature

Collection and authentication of plant (*Cymbopogon flexuosus*)



Extraction of lemongrass oil from the leaves of *Cymbopogon flexuosus*



Acute toxicity study

In vivo study

Tabulation of results and Statistical analysis

MATERIALS AND METHODS

Cymbopogon flexuosus (Family: Poaceae), the plant used in the study, is available all over India, Sri Lanka, Burma and Thailand [18]. The plant has been used in traditional system of medicine for the treatment of fever, stimulant and used as a flavouring agent, carminative and perfumery [19]. The fresh leaves of this plant was used in this study. In this part of work the collection and authentication of the plant material, extraction, phytochemical screening and acute toxicity studies are described.

COLLECTION & AUTHENTICATION OF PLANT MATERIAL AND EXTRACTION & PHYTOCHEMICAL SCREENING OF OIL

Collection

Cymbopogon flexuosus (lemongrass) used in this work was collected in bulk from Ootacamund, Tamil Nadu, India during January 2017. A herbarium was prepared featuring the salient features of the plants and submitted for authentication. The plant was authenticated by **Dr. C. Murugan**, Scientist D & Head of Office, Botanical Survey of India, Tamil Nadu Agricultural University Campus, Coimbatore *viz.,* authentication letter bearing **No: 2948** dated **23.01.2017**

Drugs & Chemicals

Anhydrous sodium sulphate, n-hexane purchased from HiMedia, Mumbai. All the other chemicals and reagents used were commercially purchased and were of analytical grade.

Extraction of oil [36]

The lemongrass oil was extracted by hydro-distillation method. About 900g of fresh lemongrass was weighed and placed in a 500ml round bottom flask containing 250 ml of water and was subjected to hydro-distillation for 12 hours.

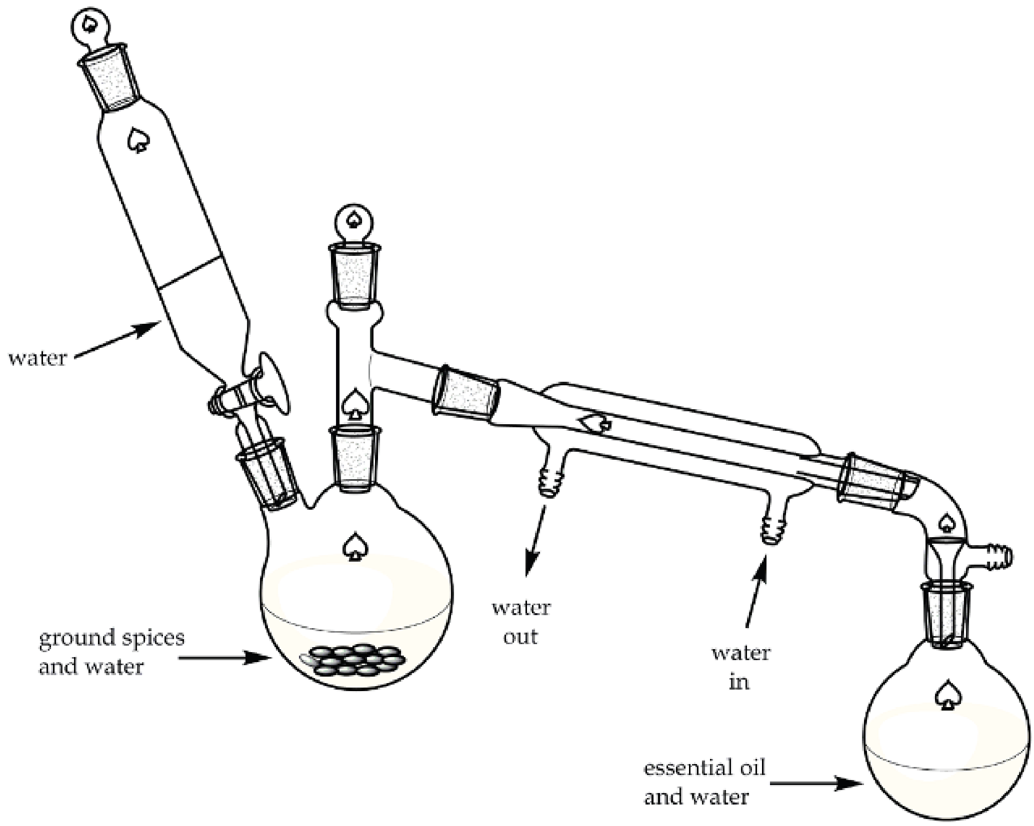


Fig.No.7: Diagramatic representation of distillation setup used for extraction of lemon grass oil from *Cymbopogon flexuosus* leaves

The distillate was saturated with sodium chloride and added with n-hexane and shaken for half an hour. Then, the n-hexane layer and hydro layer were separated by separating funnel. Anhydrous sodium sulphate was added to dehydrate the n- hexane layer and the dehydrated layer was further dried at 40°C, during which the oil separates out. The extracted lemon grass oil obtained from *Cymbopogon flexuosus* leaves (LOCFL) was stored in air-tight container and kept in room temperature in dark until further studies.

Phytochemical Screening of LOCFL

Following chemical tests were performed for the presence of phytochemical constituents in LOCFL as per the procedures explained by Trease and Evans [51].

A. Test for Carbohydrates

Molisch's test:

LOCFL was mixed with few drops of Molisch reagent (alpha naphthol) and concentrated sulphuric acid was added from sides of test tube. A purple coloured ring formation at junction indicates presence of carbohydrates.

Fehling's test:

Equal volume of Fehling's A and Fehling's B were mixed (1 ml each) and 2 ml of LOCFL was added followed by boiling for 5-10 minutes on water bath (Fehling's A solution composed of 0.5% of copper sulphate and Fehling's B solution composed of sodium potassium tartrate).The formation of reddish brown coloured precipitate (due to formation of cuprous oxide) indicates presence of reducing sugar.

Benedict's test:

It used for test reducing sugars and the Benedict's reagent composed mainly of copper sulphate and sodium hydroxide. To about 4ml of LOCFL, 1ml of Benedict's solution was added and heated almost to boiling. Green, yellow, orange, red or brown colour formation occurs in order of increasing concentration of simple sugar in the test solution (due to formation of cuprous oxide).

B. Test for proteins and amino acids

Biuret test:

To LOCFL in hot water, few drops of Biuret reagent (potassium hydroxide, copper sulphate and sodium potassium tartrate) was added, which turns blue reagent to violet. Usually in laboratory, it can also be done by adding few drops of 0.5% copper sulphate solution to the extract.

Millon's test:

Millon's test is usually positive for any compound containing a phenolic hydroxyl group. Millon reagent is a solution of mercuric and mercurous ions in nitric and nitrous acids. 1ml of LOCFL was taken in a test tube and few drops of Millon's reagent were added. The formation of white precipitate, which turns red after heating for 5 minutes on water bath indicates positive reaction (due to formation of a mercury salt of nitrated amino acid).

Ninhydrin test:

This test is mainly used to detect the presence of alpha-amino acids and proteins containing free amino groups. LOCFL when heated with Ninhydrin molecules, gives characterstic deep blue or pale yellow colour due to formation of comple between two ninhydrin molecules and the nitrogen of free amino acid.

C. Test for glycosides

Baljet test:

LOCFL when mixed with sodium picrate solution forms yellow to orange colour in presence of aglycones or glycosides.

Legal test:

To LOCFL equal volume of water and 0.5 ml of strong lead acetate solution was added, mixed and filtered. Filtrate was extracted with equal volume of chloroform and the chloroform extract was evaporated to dryness. The residue was dissolved in 2 ml of pyridine and sodium nitroprusside 2 ml was added followed by addition of sodium hydroxide solution to make alkaline. Formation of pink colour indicates the presence of glycosides or aglycone moiety.

D. Test for saponins

Foam test:

To 1ml LOCFL, 10-20 ml of water was added; shaken for few minutes, formation of frothing which persists for 60-120 seconds indicates the presence of saponins.

E. Test for steroids and triterpenoids

Libermann Burchard test:

LOCFL was evaporated to dryness and extracted with chloroform, add few drops of acetic anhydride followed by concentrated sulphuric acid from side wall of test tube to the chloroform extract. Formation of violet to blue coloured ring at the junction of two liquids, indicate the presence of steroid moiety.

Salkovaski test:

LOCFL was evaporated to dryness and extracted with chloroform, add concentrated hydrochloric acid from sidewall of test tube to the chloroform extract. Formation of yellow coloured ring at the junction of two liquids, which turns red after 2 minutes, indicates the presence of steroid moiety.

F. Test for flavonoids

Ammonia test:

Filter paper dipped in LOCFL was exposed to ammonia vapour. Formation of yellow spot on filter paper indicates the presence of this type of glycosides.

Concentrated sulphuric acid test:

LOCFL was treated with concentrated sulphuric acid to give a yellow colour.

Aluminium chloride test:

LOCFL was treated with 1% aluminium chloride. Formation of yellow colour indicates the presence of flavonoids.

G. Test for alkaloids and/or nitrogenous bases

Dragendorff's test:

LOCFL was treated with Dragendorff's reagent (potassium bismuth iodide). The formation of orange red colour or precipitate is an indication of the presence of alkaloids.

Mayer's test:

LOCFL was treated with few drops of Mayer's reagent (potassium mercuric iodide). The formation of a turbid or creamy-white precipitant is an indication of presence of alkaloids.

Hager's test:

LOCFL was treated with few drops of Hager's reagent (Saturated aqueous solution of picric acid). The formation of crystalline yellow precipitate is an indication of the presence of alkaloids.

Wagner's test:

LOCFL was treated with few drops of Wagner's reagent (dilute iodine solution). The formation of reddish brown precipitate is an indication of the presence of alkaloids.

Tannic acid test:

Few drops of tannic acid solution added to LOCFL. The formation of buff coloured precipitate is an indication of the presence of alkaloids.

H. Test for tannins and phenolics

LOCFL was treated with 5% ferric chloride to give a brown colour.

ACUTE TOXICITY AND *IN VIVO* STUDIES

EXPERIMENTAL ANIMALS

Healthy adult male Sprague Dawley rats weighing 220g±20% were procured from Kerala Veterinary and Animal Science University, Thrissur. The study protocol was approved by the Institutional Animal Ethical Committee (COPSRIPMS/IAEC/PG/P'COLOGY/03/2017-2018) and all procedures were performed in accordance with the recommendations for the proper care and use of laboratory animals.

Housing and feeding conditions

The animals were kept in cages under ambient temperature (22 ± 3°C) with 12 h light/dark cycle. They were fed with standard rat laboratory diet and drinking water *ad libitum*.

Drugs & Chemicals

Anhydrous sodium sulphate, bovine serum albumin, tri-chloro acetic acid, thiobarbituric acid and DTNB (Ellman's reagent) were purchased from HiMedia, Mumbai; glutathione, sodium pyrophosphate, rotenone was purchased from Sigma Aldrich, United states; Levodopa (Syndopa) were purchased from Sri Ramakrishna Hospital Pharmacy, Coimbatore. Alkaline phosphatase (ALP), Aspartate transaminase (AST), Alanine transaminase (ALT), Lactate dehydrogenase (LDH), obtained from Agappe Diagnostics, Cochin, India. All the other chemicals and reagents used were commercially purchased and were of analytical grade.

Instruments / Equipment

Semi auto-analyzer (Agappe Diagnostics Ltd, Mumbai), centrifuge (Remi Instruments Ltd., Kolkata) UV- Visible Spectrophotometer (JASCO V-630 spectrophotometer), pH meter, Digital balance (CAS-CBL), open field apparatus, rearing chamber (Ananya Enterprises, Coimbatore), elevated plus maze and wooden blocks were assembled by in-house carpenters. Sand-paper was purchased locally.

Acute Toxicity Study of LOCFL

Acute oral toxicity testing was carried out in accordance with the OECD guideline 420 Acute Oral Toxicity – Fixed Dose Procedure method [52].

Procedure

The acute toxicity study was done by two steps - Sighting study and Main study.

The purpose of the sighting study is to allow selection of the appropriate starting dose for the main study.

Sighting study was conducted using one animal and main study using five animals, in which one animal is taken from the sighting study. Healthy adult female (generally slightly more sensitive than male) Sprague Dawley rats weighing between 220±20%g body weight were procured and kept in cages under ambient temperature (22±3°C) with 12 h light/dark cycle. The animals were randomly selected, marked and kept in their cages for 5 days prior to dosing for acclimatization to laboratory conditions. An animal was fasted over-night but water provided *ad libitum* and received a single dose (2000mg/kg, body weight, *p.o.*) of lemongrass oil emulsion (prepared by adding LOCFL with water containing 0.5%w/v acacia gum as emulsifying agent). After the administration of the extract, food was withheld for further 3-4h.

Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24h (with special attention during the first 4 h) and daily thereafter for a period of 14 days. Once daily cage side observations included changes in skin and fur, eyes and mucous membrane (nasal), and also respiratory rate, circulatory (heart rate and blood pressure), autonomic(salivation, lacrimation, perspiration, piloerection, urinary incontinence and defecation) and central nervous system (drowsiness, gait, tremors and convulsions) changes.

If the animal dosed with 2000mg/kg b.w. during the sighting study survives and remains so without any toxic manifestations then the same dose is selected for the main study. The main study was done in 4 animals other than that taken for the sighting study (a total of 5 animals). The procedure of the main study is similar to that of the sighting study.

IN VIVO ACTIVITY

Selection of dose of the oil emulsion

LD_{50} was determined as per OECD guidelines for fixing the dose for biological evaluation. The LD_{50} of the LOCFL falls under category 5, with no death and no signs of acute toxicity at doses of 2000 mg/kg as per the Globally Harmonized System (GHS) of classifying toxic substances, can be considered to be 2000mg/kg b.w. Hence one-tenth of LD_{50}, *i.e.,* 200 mg/kg b.w. is fixed as the maximum dose for testing the biological activity. Two dose levels of 100 and 200 mg/kg b.w. are used in the biological evaluation of LOCFL to check any dose-dependent variation in biological response.

Experimental Design

Male Sprague Dawley rats weighing 220±20%g b.w. were divided into six groups, each consisting of six rats. The animals were numbered in each group using permanent marker in the base of their tails. The total treatment period was 21 days. Prior to the treatment the animals were trained for behavioural procedures using open field apparatus, rotarod, elevated plus maze and rearing chamber for three days. The various groups and treatments provided are described in the following table [1, 33]:

Table No.1: Group and Treatment

Group No	Group Name	Treatment
I	Vehicle Control	Coconut oil (1 ml/kg/day; *p.o.*)
II	Negative Control	Rotenone (2.5 mg/kg/day in coconut oil; *i.p.*)
III	Positive Control (Levodopa group)	Rotenone (2.5 mg/kg/day in coconut oil; *i.p.*) + Levodopa (10 mg/kg/day in 0.5%w/v carboxy methyl cellulose suspension; *p.o.*)
IV	Low Dose Test Group	Rotenone (2.5 mg/kg/day in coconut oil; *i.p.*) + LOCFL (100 mg/kg/day emulsion; *p.o.*)
V	High Dose Test Group	Rotenone (2.5 mg/kg/day in coconut oil; *i.p.*) + LOCFL (200 mg/kg/day emulsion; *p.o.*)
VI	LOCFL Alone Group	LOCFL (200 mg/kg/day emulsion; *p.o.*)

The treatment period is for 21 days. During the treatment period the body weight was checked every day prior to treatment and behavioural parameters were recorded every week. Twenty-four hours after the last administration the animals were bled under ether anaesthesia, euthansied by cervical dislocation and brains dissected out for further studies.

BEHAVIOURAL PARAMETERS

1. Open field test

Principle:

Parkinson's disease is a syndrome of varied etiology and its important features are bradykinesia, muscular rigidity, postural instability, loss of associated movements and tremors [53]. It is a movement disorder which cause decreased locomotor activity and it is measured by open field apparatus or by actophotometer. The open field is used for measuring anxiety and exploration as well as locomotion as it has large center arena.

Apparatus:

The open field apparatus constructed of clear plexiglass and measured 72×72cm with 36cm height, so rat could be visible through the walls. The floor of the chamber is divided into sixteen 18×18cm squares. A central square (18×18cm) was drawn in the middle of the open field.

Procedure:

Rats were carried to the test room in their home cages and were handled by the base of their tails at all times. Rats were placed into the center or one of the four corners of the open field and allowed to explore the apparatus for 5 minutes.After the 5 minute test, rat were returned to their home cages and the open field was cleaned with 70% ethyl alcohol and permitted to dry between tests. To assess the process of habituation to the novelty of the arena, rats were exposed to the apparatus for 5 minutes on 2 consecutive days [48].

Fig. No. 8: Picture of open field apparatus used in the study

Scores:

1. Line crossing

   Frequency with which the rat crossed one of the grid lines with all four paws.

2. Central square entries

   Frequency with which the rat crossed one of the red lines with all four paws into the central square.

3. Central square duration

   Duration of time the rat spent in the central square

4. Stretch attend posture

   Frequency with which, the rat demonstrated forward elongation of the head & shoulders followed by retraction to its original position.

2. Rotarod test

Principle

The Rotarod test is widely used to evaluate the motor coordination of rodents and is especially sensitive in detecting cerebellar dysfunction. The modified Rotarod test would be useful for evaluation of dopamine involvement in the acquisition of motor skill learning [54].

Instrument:

The apparatus consist of a horizontal wooden rod or metal rod coated with rubber with 3 cm diameter attached to a motor with the speed adjusted to 20 rotations per minute. The rod is 75 cm in length and is divided into six sections by plastic discs, thereby allowing the simultaneous testing of six rats. The rod is in a height of about 50 cm above the table top in order to discourage the animals from jumping off the roller [55].

Procedure

Turn on the Rotarod. Select an appropriate speed (20-25 rpm is ideal). Place the animal one by one on the rotating rod.

Fig.No.9: Picture of Rotarod apparatus used in the study

Note down the 'fall off time' when the rat falls from the rotating rod. A cut off time was 180 seconds at which the animal is taken off the rotating rod if it has not fallen by then.

3. Elevated plus maze

Principle

This degeneration of dopaminergic nigrostriatal system is largely responsible for the classical motor signs, including: resting tremor, muscle rigidity, and Bradykinesia. Furthermore, non-motor symptoms (NMS) are also becoming increasingly recognized as relevant symptoms in PD patients. NMS can include emotional and cognitive deficits, sleep disorders, and autonomic, gastrointestinal, and sensory dysfunction. Anxiety may also be comorbidity for PD patients, affecting up to 40% of individuals, sometimes in association with depression. The elevated plus maze was used to assess for anxious-like behavior [56].

Apparatus:

The plus-maze consists of two open arms, 50×10×40 cm and two closed arms, 50×10×40 cm, with an open roof, arranged so that the two open arms are opposite to each other. The maze is elevated to the height of 50cm.

Procedure

The rats are handled by the investigator on alternative days to reduce stress. Thirty minutes after i.p. administration of test/standard, the rat is placed in the center of the maze, facing one of the enclosed arms.

Fig.No.10: Picture of Elevated plus maze apparatus used in the study

The rats are housed in pairs for 10 days prior to testing in the apparatus. During the 5 minute test period the following measures are taken:

1. The number of entries into the open and enclosed arms

2. Time spent in the open arm and enclosed arm
3. The first preference of the animal to the open arm or closed arm

The procedure is conducted preferably in a sound attenuated room, with observations made from an adjacent room.

4. Catalepsy test

Principle

Neuroleptic or neurotoxins induced catalepsy in rats is used to evaluate the drugs for their antiparkinsonism effects. This exerts multiple events on dopeminergic signaling and produce DA related behavioural changes and catalepsy [57]. This activity used to assess extrapyramidal side effects such as, akinesia, rigidity and tremors, are called Parkinson's – like because Parkinson's disease the major clinical symptoms include difficulty to move and change posture (akinesia and rigidity) and tremors.

Neuroleptic-induced catalepsy has long been used as an animal model for screening drugs for Parkinsonism. It induces a behavioral state known as catalepsy in which the animals are unable to correct externally imposed postures [58].

Procedure

Two wooden blocks one being 3 cm high and the other 9 cm high.

Animals were treated with drug/standard/inducing agent and observed severity of catatonic response as follows:

Stage I: Rat moves normally when placed on the table, score – 0

Stage II: Rat moves only when touched or pushed, score – 0.5

Fig.No.11: Picture of Wooden blocks used in the study

3rd stage of catatonia **4th stage of catatonia**

Stage III: Rats placed on the table with front paws set alternately on a 3 cm high block fails to correct the posture in 10 seconds, score – 0.5 for each paw with a total of 1 for this stage

Stage IV: Rat fails to remove when the front paws are placed alternately on a 9 cm high block, score – 1 for each paw with a total score of 2 for this stage.

5. Rearing behavior

Principle

The rearing test is used to assess the exploratory behavior when they rear and contact the wall of the cylinder with their forepaws. The number of wall contacts made using one or both the forelimbs is recorded [45].

Apparatus:

The rearing chamber was made up of clear plexiglass acrylic sheets with the height of 30 cm and 20 cm in diameter. The chamber is height enough to prevent rats from jumping out, and wide enough to allow a small (1cm) gap between the base of the tail and the cylinder wall when the rat is on surface of the chamber with all forepaws.

Procedure

When placed in a clear cylinder, rats will engage in exploratory behavior, including rearing. During rearing behavior, the forelimbs will contact the wall of the rearing chamber. For this test, the rat was placed in a clear plexiglass chamber for 5 min.

To be classified as a rear, the animal had to raise forelimbs above shoulder level and make contact with the cylinder wall with either one or both forelimbs.

Fig.No.12: Picture of Rearing chamber used in the study

Removal of both forelimbs from the cylinder wall and contact with the table surface was required before another rear was scored.

This test has been successfully used previously to assess behavioral deficits in the rats receiving subcutaneous or intravenous rotenone [43].

6. Postural instability test

Principle

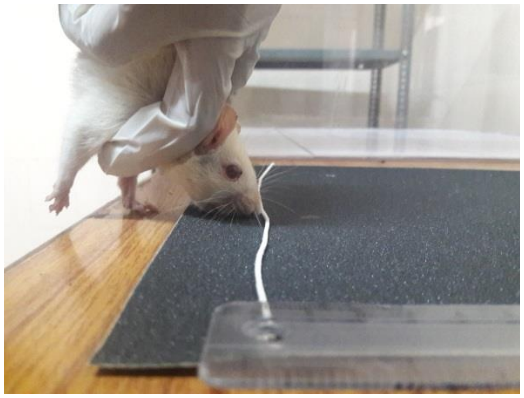
Postural instability is a hallmark feature of PD. Rotenone produces bilateral symptoms [43]. This is a new test that is a refinement of an earlier test variously referred to as the stepping test, adjusting-steps test, or bracing test which, in contrast to the present postural instability test, measured number of steps taken across a set distance of displacement also measured subsequent multiple steps, as well as sideways stepping.

Procedure

Rats were held almost vertically upside-down (in a “wheelbarrow”- like position) over a sandpaper-covered surface alongside a ruler, by a tester who was blind to the experimental condition of the rats.

This rough surface material induced stepping, rather than dragging or bracing, in response to imposed weight shifts. Viewed from above, the tip of the rat's nose was aligned with the zero line of the ruler, and one forelimb was gently restrained against the animal's torso by the experimenter while the animal was moved forward over the single planted forelimb until making a “catch-up” step to regain its center of gravity [45].

Fig.No.13: Postural Instability study by using Sand-paper



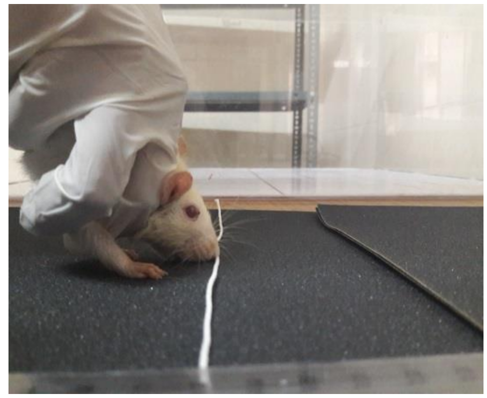


Figure 13 (a) **Figure 13 (b)**

Figure 1 – The angle at which the rat was held

Figure 2 – A rat made a catch-up step with the right limb after 9.5 cm of displacement

The new position of the nose tip indicated the displacement of the body needed to trigger a catch-up step in the unrestrained supporting forelimb.

We examined each forelimb independently while slowly shifting the center of gravity during weight support. We performed three trials on each forelimb on a given day of testing.

DISSECTION AND BLOOD COLLECTION

Twenty-four hours after the last administration the blood was collected by retro orbital puncture from the inner canthus of the eye under mild ether anaesthesia using capillary tubes in fresh pro-coagulation tubes and serum was separated by centrifugation and used for further studies.

Fig.No.14: Collection of blood by Retro orbital puncture

Serum parameters like,

1. Alkaline phosphatase (ALP)
2. Aspartate transaminase (AST)
3. Alanine transaminase (ALT)
4. Lactate dehydrogenase (LDH)

1. Alkaline phosphatase

Principle

Kinetic determination of ALP according to the following reaction

$$\text{Para-nitrophenyl phosphate} + H_2O \xrightarrow{ALP} \text{p-nitrophenol} + \text{Inorganic phosphate}$$

ALP is widely distributed throughout the body, but clinically important one for diagnostic reasons are in bone, liver, placenta and intestine.

Reagent preparation

Mix four volumes of reagent 1 (R1), which contains diethanolamine buffer (pH 10.2) 125 mmol/L, magnesium chloride 0.625 mmol/L with one volume of reagent 2 (R2) contains P-Nitro phenyl phosphate 50 mmol/L. This working reagent is stable for 30 days at 2-8°C.

Procedure

To 20 µl of sample, 1 ml of working reagent was added. Mix and incubate at 37°C for one minute. Measure the change in absorbance per minute during 3 minutes at 405 nm.

2. **Aspartate transaminase**

Principle

Kinetic determination of aspartate aminotransferase (AST) based upon the following reaction.

$$\text{L-Aspartate} + \text{alpha-ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L-Glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-Malate} + \text{NAD}^+$$

MDH: Malate dehydrogenase

Reagent preparation

Mix four volume of reagent 1 (R1) contains tris buffer (pH 7.8) 88 mmol/L, L-aspartate 260 mmol/L, LDH>1500 U/L, MDH>900 U/L with one volume of reagent 2 (R2) contains alpha-ketoglutarate 12 mmol/L, NADH-0.24 mmol/L. This working reagent is stable for 30 days at 2-8°C.

Procedure

To 100 µl of sample, 1 ml of working reagent was added. Mix and incubate at 37°C for one minute. Measure the change in absorbance per minute during 3 minutes at 340nm.

3. **Alanine transaminase**

Principle

Kinetic determination of Alanine transaminase (ALT) according to the following reaction.

$$\text{L-Alanine} + \text{alpha-ketoglutarate} \xrightarrow{\text{MDH}} \text{Pyruvate} + \text{L-Glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}^{+}$$

LDH- Lactate dehydrogenase

Reagent preparation

Mix four volume of reagent 1 (R1) contains tris buffer (pH 7.5) 110 mmol/L, L-alanine 600 mmol/L, LDH>1500 U/L, with one volume of reagent 2 (R2) contains alpha-ketoglutarate 16 mmol/L, NADH-0.24 mmol/L. This working reagent is stable for 30 days at 2-8°C.

Procedure

To 100 µl of sample, 1 ml of working reagent was added. Mix and incubate at 37°C for one minute. Measure the change in absorbance per minute during 3 minutes at 340nm.

4. **Lactate dehydrogenase**

Principle

Kinetic determination of lactate dehydrogenase according to the following reaction.

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightarrow{\text{LDH}} \text{L-Lactate} + \text{NAD}^{+}$$

Reagent preparation

Mix four volume of reagent 1 (R1) contains tris buffer (pH 7.4) 80 mmol/L, pyruvate 1.6 mmol/L, sodium chloride 200 mmol/L with one volume of reagent 2 (R2) contains NADH- 240 mmol/L. This working reagent is stable for 21 days at 2-8°C.

Procedure

To 10 µl of sample, 1 ml of working reagent was added. Mix and incubate at 37°C for one minute. Measure the change in absorbance per minute during 3 minutes at 340nm.

These parameters were determined using standard kits obtained from Agappe Diagnostics, Cochin, India using a Semi Auto-analyser (Mispa Viva, Mumbai, India).

Isolation of brain

Decapitate the animal with a pair of sharp, sturdy scissors or a single-edged razor blade. This is most easily done by first cutting through the skin and muscles around the circumference of the neck and then cutting the head off at the base of the skull, just behind the ears.

Cut through the skin on the top of the head, from the back of the head forward to between the eyes. Remove any muscle tissue that may still be attached to the base of the skull.

Locate the foramen magnum, a large opening at the back of the head where the spinal column enters the skull. Place one tip of the forceps into the foramen magnum, and crack and pull away the bone and tissue on either side of the opening. Hold the forceps horizontally and slightly angled downward.

Place the tips of the forceps in the eye sockets, and crack the piece of skull that lies between them.

Using the forceps, carefully begin to remove the skull. Start at the top of the foramen magnum and chip away the bone, up over the cerebellum and then forward toward the eyes. Continue removing bone (always holding the forceps horizontally) until the brain is exposed on three sides.

Once all of the bone has been removed from around the brain, hold the head upside down. At this point, it should be possible to carefully pry the brain away from the base of the skull with a flat metal spatula. The brain will still be attached to the skull by the optic and trigeminal nerves.

Sever these nerves with the edge of the spatula, and let the brain drop into a glass container, Label the cap with the rat's number or other identification [59].

Preparation of tissue homogenates

The brain was removed and washed immediately with ice-cold saline to remove blood. A 10% w/v brain homogenate was prepared in ice-cold potassium phosphate buffer (100mM, pH 7.4) followed by centrifugation at 5000g for 10 min. the resulting supernatant was used for the estimation of biochemical parameters.

A. Biochemical parameters

1. Estimation of total protein content
2. Estimation of malondialdehyde (MDA)

B. Determination of enzymatic antioxidants

3. Assay of catalase
4. Estimation of glutathione peroxidase (GPx)
5. Assay of superoxide dismutase (SOD)
6. Estimation of glutathione reductase (GSSH)

C. Determination of non-enzymatic antioxidants

7. Estimation of reduced glutathione (GSH)

A. Biochemical parameters

1. Estimation of total protein content

To 0.1ml of brain homogenate, 4.0 ml of alkaline copper solution was added and allowed to stand for 10 min [60]. Then, 0.4 ml of phenol reagent was added very rapidly and mixed quickly and incubated in room temperature for 30 min for colour development. Reading was taken against blank prepared with distilled water at 610 nm in UV-visible spectrophotometer. The protein content was calculated from standard curve prepared with bovine serum albumin and results were expressed as µg/mg brain tissue.

2. Estimation of malondialdehyde (MDA)

One ml of the homogenate was combined with 2 ml of TCA-TBA-HCl reagent (15% trichloro acetic acid (TCA) and 0.375% thiobarbituric acid (TBA) in 0.25 N HCl) and boiled for 15 min. precipitate was removed after cooling by centrifugation at 1000g for 10 min and absorbance of the sample was read at 535 nm against a blank without tissue homogenate. The levels of MDA were calculated using extinction coefficient calculation [61]. The values are expressed as nmoles/min/mg brain tissue.

B. Determination of enzymatic antioxidants

3. Assay of catalase

The reaction mixture contained 2.0 ml of homogenate and 1.0 ml of 30 mM hydrogen peroxide (in 50 mM phosphate buffer, pH 7.0). a system devoid of the substrate (hydrogen peroxide) served as a control. Reaction was started by the addition of the substrate and decrease in absorbance mentioned at 240 nm for 30 seconds at 25°C. the difference in absorbance per unit time was expressed as the activity and three parallel experiments were conducted. One unit is defined as the amount of enzyme required to decompose 1.0 M of hydrogen peroxide per minute at pH 7.0 and 25°C [62].

4. Estimation of glutathione peroxidase (GPx)

The reaction mixture consists of 0.2 ml of 0.4 M Tris buffer, 0.1 ml of 1.0 mM sodium azide, 0.1 ml of 0.042% hydrogen peroxide, 0.2 ml of 200 mM glutathione and 0.2 ml of brain tissue homogenate supernatant incubated at 37°C for 10 min. The reaction was arrested by the addition 0.1 ml of 10% TCA and the absorbance was taken at 340 nm [63]. Activity was expressed as nmoles/min/mg brain protein.

5. Assay of superoxide dismutase (SOD)

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.052 M, pH 8.3), 0.1 ml of 186 µM Phenazonium methosulphate (PMS), 0.3 ml of 300 µM nitro blue tetrazolium chloride (NBT), 0.2 ml of 780 µM NADH, 1.0 ml of homogenate and distilled water to a final volume of 3.0 ml. reaction was started by the addition of NADH and incubated at 30°C for 1 min. the reaction was stoped by the addition of 1.0 ml of glacial acetic acid and the mixture was stirred vigorously. 4.0 ml of n-butanol was added to the mixture and shaken well. The mixture was allowed to stand for 10 min, centrifuged, the butanol layer was taken out and absorbance was measured at 560 nm against a butanol blank [64].

A system devoid of enzyme served as the control and three parallel experiments were conducted.

6. Estimation of glutathione reductase (GSSH)

The enzyme activity was determined spectrophotometrically by the decrease in absorbance of NADPH at 340 nm. The reaction mixture contained 2.1 ml of 0.25 mM potassium phosphate buffer pH 7.6, 0.1 ml of 0.001 M NADPH, 0.2 ml of 0.0165 M oxidized glutathione and 0.1 ml of bovine serum albumin (10 mg/ml). The reaction was started by the addition of 0.02 ml of brain tissue homogenate with mixing and the decrease in absorbance at 340 nm was measured for 3 min against a blank [65]. Glutathione reductase activity was expressed as nmoles/min/mg brain protein at 30°C.

C. Determination of non-enzymatic antioxidants

7. Estimation of reduced glutathione (GSH)

Brain was homogenized in 10% w/v cold 20mM EDTA solution on ice. After deproteinization with 5% TCA, an aliquot of the supernatant was allowed to react with 150 µM DTNB. The product was detected and quantified spectrophotometrically at 416 nm [66]. Pure GSH was used as standard for establishing the calibration curve and three parallel experiments were conducted.

Histopathological Studies

Brain tissues isolated from rats were used for histopathological studies.

Preparation of the tissue for Histology

Fixation of solution: Picric acid (Saturated solution) – 75 ml; formaldehyde 40% - 25ml and glacial acetic acid – 5 ml [67]

Procedure: After sacrificing the rat by cervical dislocation, brain tissues were collected, washed in normal saline and fixed using the above mentioned fixative for 24 h and the tissues were sent for histopathological studies.

STATISTICAL ANALYSIS

Results were expressed as mean ± SEM of six animals in each group. The groups were compared using Student's Paired 't' test and One-way Analysis of Variance (ANOVA) with Tukey's test when and where appropriate. P values <0.05 were considered as significant.

RESULTS

Percentage yield of the LOCFL

The lemon grass oil was extracted from *Cymbopogon flexuosus* leaves using hydro-distillation process and the percentage yield of the LOCFL was found to be 1.10% w/w.

Phytochemical screening

The phytochemical screening of the LOCFL confirmed the presence of alkaloids, flavonoids, saponins, carbohydrates, amino acids and glycosides. The results were tabulated in Table 2.

Acute toxicity studies

LD_{50} was determined as per OECD guidelines for fixing the dose for biological evaluation. The LD_{50} of the LOCFL falls under category 5, with no death and no signs of acute toxicity at doses of 2000 mg/kg as per the Globally Harmonized System (GHS) of classifying toxic substances can be considered to be 2000mg/kg b.w. The observations made were tabulated in Table No. 3. Hence one-tenth of LD50, i.e., 200 mg/kg b.w. is fixed as the maximum dose for testing for biological activity. Two dose levels, i.e., 100 mg/kg and 200 mg/kg. were used in the biological evaluation of LOCFL to check if there is any dose dependent increase in the protection shown by the oil.

Body weight

The initial (0^{th} day) and final (22^{nd} day) body weights of rats used are summarized in Table No. 4. Initial values in control and experimental animals were not significantly different. The body weight of normal controls significantly increased by approximately 5.8% over their initial readings after 21 days of treatment, whereas there was a significant ($p<0.05$) decrease in the body weight of negative control rats (20.59%). Administration of the LOCFL emulsion, or standard (levodopa) in treatment group rats reversed this weight loss and there was no significant ($p>0.05$) difference between initial and final body weight in these animals.

BEHAVIOURAL PARAAMETERS

1. Open field test

The negative control rats showed significant decrease ($p < 0.01$) in number of line crosses and number of central square entries and time spent in Central Square when compared to vehicle control rats. It showed significant increase ($p < 0.01$) in duration of stretch attend posture. The treatment groups showed significant increase ($p < 0.001$) in line crosses, number of central square entries and time spent in central square when compared to negative control rats and there was a significant decrease ($p < 0.001$) in the duration of stretch attend posture when compared to negative control rats (Table No. 5)

2. Rotarod test

Negative control rats showed a significant decrease ($p < 0.001$) in fall off time when compared to vehicle control rats and the percentage decrease in time approximately 83.01 % when compared to vehicle control rats. Treatment rats with LOCFL and standard, resulted in significant increase ($p < 0.001$) in fall off time when compared to the negative control rats. There is no significant difference in percentage decrease in time in these animals. LOCFL alone rats showed no significant decrease or increase in the fall off time when compared to the vehicle control group. The values were comparable with that of standard (levodopa) summarized in Table No. 6.

3. Elevated plus maze

The negative control rats showed significant decrease ($p < 0.05$) in time spent and number of entries to the open arm when compared to the vehicle control rats. Treatment rats which showed significant increase ($p < 0.05$) in time spent and number of entries to the open arm when compared to the negative control rats. The LOCFL rats showed no significant increase or decrease in the open arm entries and time spent in that (Table No. 7).

4. Catalepsy test

Negative control rats showed significant decrease ($p < 0.01$) in catatonic response when compared to vehicle control rats. Treatment rats which were treated with LOCFL and standard resulted in significant increase ($p < 0.001$) in catatonic response when compared to negative control rats. LOCFL alone rats showed no significant decrease or increase in the catatonic response. The values were compared between the different groups are summarized in the Table No. 8.

5. Rearing behavior

In negative control rats, the number of rears decreased significantly ($p < 0.001$) when compared to the vehicle control rats. The treatment rats with LOCFL and standard, resulted in significant increase ($p < 0.001$) in the number rears when compared to the negative control rats. LOCFL alone rats showed no significant increase or decrease in number of rears when compared with the vehicle control rats. The results were summarized in Table No. 9.

6. Postural instability test

The negative control group showed significant decrease ($p < 0.01$) in size of the adjusting response when compared to vehicle control rats. Treatment rats which were treated with LOCFL and standard resulted in significant increase ($p < 0.01$) in size of the adjusting response. LOCFL alone rats showed no significant increase or decrease in size of the adjusting response when compared to vehicle control rats. The values were summarized in Table No. 10.

Serum parameters

The activities of serum marker enzymes like ALP, AST, ALT were significantly ($p < 0.01$) increased in negative control group when compared to vehicle control group. Treatment groups with LOCFL and levodopa showed significant reduction ($p < 0.01$) in the activities of ALP, AST and ALT when compared with negative control rats. The LDH activity were significantly increased ($p < 0.01$) when compared to the vehicle control rats. Rats treated with LOCFL and levodopa showed significant decrease ($p < 0.001$) in the activity (Table No. 11).

Tissue parameters

There was a significant ($p < 0.01$) decrease in amount of protein and an increase in the concentration of MDA in the brain tissues of negative control rats when compared with vehicle control rats. Reversal to near normal levels was seen with treated rats with LOCFL and levodopa (Table No. 12). The levels of enzymatic and non-enzymatic antioxidants in the brain tissues of various groups of experimental animals are presented in Table No. 13. The activities of enzymatic and non-enzymatic antioxidants in the negative control rats were significantly lower ($p < 0.05$) when compared with vehicle control rats. Rats fed with LOCFL and levodopa displayed significant ($p < 0.05$) increased activities as compared to negative control rats.

Table No. 2: Phytochemical screening of LOCFL

S. NO	CHEMICAL TEST		LOCFL
Carbohydrates			
1	A	Molisch's test	+
2	B	Fehling's test	+
3	C	Benedict's test	+
Proteins and Amino acid			
4	A	Biuret test	-
5	B	Millon's test	+
6	C	Ninhydrin test	+
Glycosides			
7	A	Baljet test	+
8	B	Legal test	+
Saponins			
9	A	Foam test	+
Steroids and Triterpenoids			
10	a	Libermann Burchard test	-
11	B	Salkowski test	-
Flavonoids			
12	A	Ammonia test	+
13	B	Concentrated sulphuric acid test	+
14	C	Aluminium chloride test	+
Alkaloids			
15	A	Dragendroff's reagent	+
16	B	Mayer's reagent	-
17	C	Hager's reagent	-
18	D	Wagner's reagent	-
19	E	Tannic acid test	-
Tannins and Phenols			
20	A	Ferric chloride test	-

LOCFL – Lemongrass oil obtained from *Cymbopogon flexuosus* leaves + denotes presence and – denotes absence of the phytoconstituents.

Table No.3: Acute toxicity studies of LOCFL

Observation	Score for Normal animal	Half an hour	Four hours	24 Hours	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Lethality		Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
Group Dispersion	0	0	0	0	0	0	0	0	0	0	0
Locomotor Activity	4	4	4	4	4	4	4	4	4	4	4
Restlessness	0	0	0	0	0	0	0	0	0	0	0
Lethargy	0	0	0	0	0	0	0	0	0	0	0
Writhing	0	0	0	0	0	0	0	0	0	0	0
Stereotyped behavior	0	0	0	0	0	0	0	0	0	0	0
Tremors	0	0	0	0	0	0	0	0	0	0	0
Twitches	0	0	0	0	0	0	0	0	0	0	0
Convulsions	0	0	0	0	0	0	0	0	0	0	0
Exophthalmos	0	0	0	0	0	0	0	0	0	0	0
Respiration	0	0	0	0	0	0	0	0	0	0	0
Alertness	4	4	4	4	4	4	4	4	4	4	4
Group startle response	4	4	4	4	4	4	4	4	4	4	4
NAD		Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

NAD – No abnormalities detected.

Table No.3 (Contd.): Acute toxicity studies of LOCFL

Observation	Score for normal animal	Time of individual observations									
		Half an hour	Four hours	24 Hours	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Loss if Righting reflex	0	0	0	0	0	0	0	0	0	0	0
Abnormal body carriage	0	0	0	0	0	0	0	0	0	0	0
Abnormal gait	0	0	0	0	0	0	0	0	0	0	0
Straub tail	0	0	0	0	0	0	0	0	0	0	0
Piloerection	0	0	0	0	0	0	0	0	0	0	0
Touch response	4	4	4	4	4	4	4	4	4	4	4
Fearfulness	4	4	4	4	4	4	4	4	4	4	4
Pinna reflex	4	4	4	4	4	4	4	4	4	4	4
Corneal reflex	4	4	4	4	4	4	4	4	4	4	4
Catalepsy	0	0	0	0	0	0	0	0	0	0	0
Passivity	0	0	0	0	0	0	0	0	0	0	0
Aggressiveness	0	0	0	0	0	0	0	0	0	0	0
Body tone	4	4	4	4	4	4	4	4	4	4	4
Grip strength	4	4	4	4	4	4	4	4	4	4	4
Paralysis	0	0	0	0	0	0	0	0	0	0	0
Cutaneous blood flow	4	4	4	4	4	4	4	4	4	4	4
Cyanosis	0	0	0	0	0	0	0	0	0	0	0
Salivation	0	0	0	0	0	0	0	0	0	0	0
Lacrimation	0	0	0	0	0	0	0	0	0	0	0
Ptosis	0	0	0	0	0	0	0	0	0	0	0
Pupil diameter	4	4	4	4	4	4	4	4	4	4	4
Pain response	4	4	4	4	4	4	4	4	4	4	4
Diarrhea	0	0	0	0	0	0	0	0	0	0	0
Vocalization	0	0	0	0	0	0	0	0	0	0	0
Increased urination	0	0	0	0	0	0	0	0	0	0	0
Grooming	4	4	4	4	4	4	4	4	4	4	4
NAD		Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil

NAD – No abnormalities detected.

Table No. 4: Effect of LOCFL on body weight in rotenone-induced Parkinson's disease

Group Name	Body weight (g)	
	Initial	Final (% change)
Vehicle Control	198.75 ± 7.58	211.00 ± 3.10 (↑5.8%)^{ns}
Negative Control	218.50 ± 0.64	173.50 ± 16.50(↓20.59%)*
Positive Control (Levodopa group)	200.66 ± 4.97	207.50 ± 5.85 (↑3.29%)^{ns}
Low Dose Test Group	195.00 ± 7.79	200.00 ± 8.24(↑2.5%)^{ns}
High Dose Test Group	183.00 ± 1.05	212.00 ± 5.07(↑13.67%)^{ns}
LOCFL Alone Group	207.66 ± 4.84	236.33 ± 7.53 (↑12.13%)^{ns}

Values are expressed as mean ± SEM (n=6).

Values in parentheses are the percent increase (↑) or decrease (↓) from their corresponding initial readings.

* denotes $p < 0.05$; ^{ns} denotes $p > 0.05$ when compared to initial readings; when compared to the initial readings (Student's Paired't'-test);

LOCFL – lemongrass oil of *Cymbopogon flexuosus* leaves.

Table No. 5: Effect of LOCFL on open field test in rotenone-induced Parkinson's disease rats

Groups	Day 0				Day 7				Day 14				Day 21			
	Line crosses	Central square		Stretch attend posture-Duration (sec)	Line crosses	Central square		Stretch attend posture-Duration (sec)	Line crosses	Central square		Stretch attend posture-Duration (sec)	Line crosses	Central square		Stretch attend posture-Duration (sec)
		Number of entries	Time spent (sec)			Number of entries	Time spent (sec)			Number of entries	Time spent (sec)			Number of entries	Time spent (sec)	
Vehicle Control	95.00 ± 9.46	2.00 ± 0.07	5.50 ± 0.50	28.25 ± 6.19	129.25 ± 6.88	16.02 ± 0.04	26.00 ± 2.00	20.00 ± 8.00	130.25 ± 5.77	16.25 ± 0.52	35.00 ± 1.78	40.00 ± 2.00	117.25 ± 15.41	15.78 ± 1.52	32.08 ± 1.86	22.50 ± 3.80
Negative Control	71.00 ± 3.53	3.00 ± 1.95	7.00 ± 1.95	36.20 ± 5.85	36.00 ± 7.16$^{\#\#\#}$	1.80 ± 0.80$^{\#\#}$	4.50 ± 1.80$^{\#\#\#}$	228.83 ± 24.62$^{\#\#\#}$	43.16 ± 4.62$^{\#\#\#}$	1.73 ± 0.77$^{\#\#\#}$	3.80 ± 0.83$^{\#\#\#}$	174.00 ± 24.00$^{\#\#}$	21.50 ± 4.22$^{\#\#\#}$	1.08 ± 0.18$^{\#\#}$	3.08 ± 0.06$^{\#\#\#}$	137.16 ± 20.32$^{\#\#}$
Positive Control (Levodopa group)	92.60 ± 3.05	4.00 ± 1.08	9.50 ± 2.84	31.00 ± 5.80	104.40 ± 11.33***	14.60 ± 1.07**	27.73 ± 0.83***	44.40 ± 7.35***	94.60 ± 6.24***	15.45 ± 0.83**	21.08 ± 0.08**	40.66 ± 4.48**	93.60 ± 6.34***	16.54 ± 1.83**	26.88 ± 1.06**	52.80 ± 3.39**
Low Dose Test Group	86.25 ± 4.73	3.40 ± 0.87	11.80 ± 3.05	36.80 ± 6.06	115.60 ± 7.91***	18.00 ± 1.58***	19.91 ± 0.09**	34.00 ± 6.06***	101.20 ± 5.26***	17.77 ± 0.73***	28.89 ± 1.08***	47.66 ± 12.38**	103.00 ± 6.33***	19.93 ± 1.91**	23.43 ± 1.08***	60.33 ± 12.44*
High Dose Test Group	91.80 ± 3.63	2.00 ± 0.54	4.80 ± 0.96	37.50 ± 6.98	144.33 ± 7.32***	21.60 ± 1.07***	33.03 ± 0.08***	47.33 ± 12.19***	131.16 ± 11.12***	21.01 ± 0.23***	43.08 ± 1.48***	22.66 ± 5.78**	117.16 ± 7.10***	23.12 ± 0.98***	46.06 ± 0.30***	58.75 ± 8.14*
LOCFL Alone Group	86.50 ± 7.85	4.00 ± 1.15	10.66 ± 3.71	32.33 ± 7.88	122.66 ± 7.96^{ns}	18.00 ± 0.50^{ns}	28.13 ± 0.33^{ns}	34.00 ± 19.00^{ns}	125.00 ± 4.72^{ns}	18.15 ± 1.26^{ns}	32.32 ± 0.38^{ns}	31.75 ± 4.09^{ns}	120.25 ± 7.64^{ns}	18.78 ± 1.34^{ns}	30.83 ± 1.68^{ns}	20.50 ± 2.50^{ns}

Table No. 6: Effect of LOCFL on Rotarod test in rotenone-induced Parkinson's disease rats

Treatment	Fall off time (sec)				
	Day 0	Day 1	Day 7	Day 14	Day 21
Vehicle Control	180 ± 0	180 ± 0	180 ± 0	180 ± 0	180 ± 0
Negative Control	180 ± 0	$25.50 \pm 2.21^{\#\#\#}$	$18.16 \pm 5.30^{\#\#\#}$	$19.16 \pm 3.71^{\#\#\#}$	$15.20 \pm 3.65^{\#\#\#}$
Positive Control (Levodopa group)	180 ± 0	$75.33 \pm 3.93^{***}$	$90.00 \pm 17.13^{**}$	$109.80 \pm 18.32^{***}$	$126.40 \pm 14.15^{***}$
Low Dose Test Group	180 ± 0	$84.00 \pm 4.05^{***}$	$136.00 \pm 11.12^{***}$	$128.00 \pm 9.75^{***}$	$133.00 \pm 7.21^{***}$
High Dose Test Group	180 ± 0	$99.00 \pm 2.22^{***}$	$128.33 \pm 8.90^{***}$	$150.00 \pm 7.41^{***}$	$144.33 \pm 8.67^{***}$
LOCFL Alone Group	180 ± 0	180 ± 0^{ns}	180 ± 0^{ns}	180 ± 0^{ns}	180 ± 0^{ns}

Values are expressed in mean ± SEM (n=6).
$^{\#\#\#}$ denotes $p < 0.001$ when compared to vehicle control group; **denotes $p < 0.01$ when compared to negative control group; ***denotes $p < 0.001$ when compared to negative control group; ^{ns} denotes $p > 0.05$ when compared to vehicle control group (One way ANOVA followed by Tukey test).

Table No. 7: Effect of LOCFL on elevated plus maze test in rotenone-induced Parkinson's disease rats

Treatment	Day 0						Day 7					
	First preference		Number of entries		Time spent (sec)		First preference		Number of entries		Time spent (sec)	
	Open arm	Closed arm	Open arm	Closed arm	Open arm	Closed arm	Open arm	Closed arm	Open arm	Closed arm	Open arm	Closed arm
Vehicle Control	+	-	2.66 ± 0.49	1.75 ± 0.47	162.28 ± 1.03	143.62 ± 2.82	+	-	14.30 ± 0.04	2.12 ± 0.57	232.75 ± 9.25	73.08 ± 1.26
Negative Control	-	+	2.80 ± 0.58	2.20 ± 0.37	153.82 ± 0.28	144.78 ± 2.93	-	+	3.01 ± 0.28$^{\#\#\#}$	12.63 ± 0.66$^{\#\#}$	67.33 ± 4.27$^{\#\#\#}$	243.38 ± 2.60$^{\#\#}$
Positive Control (Levodopa group)	+	-	2.00 ± 0.40	2.33 ±0.33	232.75 ± 2.95	78.62 ± 1.62	+	-	12.53 ± 0.28***	1.83 ± 0.44***	246.60 ± 3.75***	62.18 ± 4.62***
Low Dose Test Group	+	-	1.75 ± 0.25	2.00 ± 0.36	198.03 ± 2.81	103.73 ± 2.13	+	-	10.53 ± 0.05**	2.32 ± 0.32**	234.80 ± 3.55***	71.73 ± 3.48**
High Dose Test Group	+	-	2.50 ± 0.42	2.00 ± 0.36	173.33 ± 0.73	133.08 ± 1.37	+	-	11.10 ± 0.18**	2.78 ± 0.05**	226.40 ± 8.76**	67.08 ± 2.46***
LOCFL Alone Group	+	-	2.66 ± 0.49	2.00 ± 0.40	173.33 ± 1.82	132.73 ± 3.77	+	-	12.60 ± 0.63^{ns}	2.43 ± 0.04^{ns}	234.66 ± 5.60^{ns}	77.08 ± 2.16^{ns}

Treatment	Day 14						Day 21					
	First preference		Number of entries		Time spent (sec)		First preference		Number of entries		Time spent (sec)	
	Open arm	Closed arm	Open arm	Closed arm	Open arm	Closed arm	Open arm	Closed arm	Open arm	Closed arm	Open arm	Closed arm
Vehicle Control	+	-	12.16 ± 1.02	3.43 ± 0.04	253.08 ± 0.93	46.82 ± 1.83	+	-	11.63 ± 1.20	3.04 ± 0.08	243.00 ± 2.22	56.83 ± 1.82
Negative Control	-	+	2.53 ± 0.51$^{\#\#}$	12.86 ± 0.08$^{\#\#}$	68.76 ± 1.93$^{\#\#\#}$	238.64 ± 2.76$^{\#\#\#}$	-	+	2.04 ± 0.55$^{\#\#}$	13.57 ± 1.07$^{\#\#}$	43.82 ± 2.67$^{\#\#\#}$	256.62 ± 2.66$^{\#\#\#}$
Positive Control (Levodopa group)	+	-	12.51 ± 0.25**	2.48 ± 0.03**	248.43 ± 1.36**	62.02 ± 2.02***	+	-	12.36 ± 1.08**	4.32 ± 2.12**	258.62 ± 4.08***	46.83 ± 2.42***
Low Dose Test Group	+	-	11.43 ± 0.34**	3.82 ± 0.08**	272.83 ± 1.88***	34.44 ± 1.93***	+	-	11.62 ± 1.26**	3.46 ± 0.12**	268.08 ± 4.82	43.38 ± 1.84***
High Dose Test Group	+	-	14.84 ± 0.08**	2.08 ± 0.06***	264.45 ± 2.73***	46.48 ± 2.62***	+	-	13.32 ± 2.80**	2.06 ± 0.06***	256.82 ± 3.83***	62.73 ± 2.76**
LOCFL Alone Group	+	-	12.78 ± 0.12^{ns}	4.34 ± 0.04^{ns}	258.32 ± 2.86^{ns}	52.28 ± 2.28^{ns}	+	-	12.36 ± 1.57^{ns}	3.14 ± 0.06^{ns}	267.28 ± 1.08^{ns}	58.38 ± 2.83^{ns}

Values are expressed in mean ± SEM (n=6).

$^{\#\#}$ denotes $p < 0.01$ and $^{\#\#\#}$ denotes $p < 0.001$ when compared to vehicle control rats; ** denotes $p < 0.01$ and *** denotes $p < 0.001$ when compared to negative control rats.

^{ns} denotes $p > 0.05$ when compared to vehicle control group (One way ANOVA followed by Tukey test).

Table No.8: Effect of LOCFL on catatonia in rotenone-induced Parkinson's disease rats

Treatment	Catatonic response (Score)				
	Day 0	Day 1	Day 7	Day 14	Day 21
Vehicle Control	3.12 ± 0.12	3.00 ± 0.20	3.12 ± 0.12	3.12 ± 0.12	3.00 ± 0.35
Negative Control	2.37 ± 0.12	$1.25 \pm 0.34^{###}$	$1.50 \pm 0.34^{###}$	$1.08 \pm 0.20^{###}$	$1.16 \pm 0.33^{##}$
Positive Control (Levodopa group)	2.80 ± 0.12	$2.75 \pm 0.21^{**}$	$3.00 \pm 0.15^{***}$	$3.00 \pm 0.15^{***}$	$3.00 \pm 0.20^{**}$
Low Dose Test Group	2.90 ± 0.10	$2.58 \pm 0.23^{**}$	$3.10 \pm 0.18^{***}$	$2.80 \pm 0.25^{***}$	$3.20 \pm 0.12^{***}$
High Dose Test Group	2.90 ± 0.10	$2.66 \pm 0.16^{**}$	$2.91 \pm 0.08^{***}$	$3.08 \pm 0.08^{***}$	$3.00 \pm 0.25^{***}$
LOCFL Alone Group	2.83 ± 0.16	3.00 ± 0.20^{ns}	3.00 ± 0.20^{ns}	3.16 ± 0.16^{ns}	3.33 ± 0.16^{ns}

Values are expressed in mean ± SEM (n=6).

$^{###}$ denotes $p < 0.001$ when compared to vehicle control rats; ** denotes $p < 0.01$ and *** denotes $p < 0.001$ when compared to negative control rats.
^{ns} denotes $p > 0.05$ when compared to vehicle control group (One way ANOVA followed by Tukey test).

Table No. 9: Effect of LOCFL on Rearing Behavior in rotenone-induced Parkinson's disease rats

Treatment	Number of rears within 5 min				
	Day 0	Day 1	Day 7	Day 14	Day 21
Vehicle Control	14.75 ± 1.25	12.00 ± 0.81	10.25 ± 0.85	17.00 ± 0.70	16.25 ± 0.75
Negative Control	9.80 ± 0.37	9.50 ± 0.28	2.00± 0.57$^{###}$	1.66 ± 0.21$^{###}$	1.75 ± 0.47$^{###}$
Positive Control (Levodopa group)	14.00 ± 1.15	12.60 ± 1.50	12.40 ± 1.20***	13.40 ± 0.92***	14.20 ± 0.86***
Low Dose Test Group	13.00 ± 0.70	10.80 ± 0.86	12.00 ± 1.09***	10.20 ± 0.58***	10.40 ± 0.50***
High Dose Test Group	13.40 ± 1.24	11.20 ± 0.86	11.50 ± 0.76***	15.50 ± 1.38***	15.50 ± 0.88***
LOCFL Alone Group	16.75 ± 0.75	14.75 ± 0.75	14.25 ± 1.75^{ns}	12.33 ± 0.88^{ns}	16.00 ± 1.00^{ns}

Values are expressed in mean ± SEM (n=6).

$^{###}$ denotes $p < 0.001$ when compared to vehicle control rats; *** denotes $p < 0.001$ when compared to negative control rats.
^{ns} denotes $p > 0.05$ when compared to vehicle control group (One way ANOVA followed by Tukey test).

Table No. 10: Effect of LOCFL on Postural instability test in rotenone-induced Parkinson's disease rats

Treatment	Size of the adjusting response				
	Day 0	Day 1	Day 7	Day 14	Day 21
Vehicle Control	10.50 ± 0.64	10.00 ± 0.91	10.00 ± 1.08	10.75 ± 0.75	11.25 ± 0.75
Negative Control	13.16 ± 1.60	4.00 ± 0.44$^{\#\#\#}$	4.50 ± 0.64$^{\#\#}$	4.75 ± 0.47$^{\#\#\#}$	5.00 ± 1.00$^{\#\#}$
Positive Control (Levodopa group)	11.50 ± 0.76	8.50 ± 0.50***	11.40 ± 0.97***	10.60 ± 0.60***	9.80 ± 0.66*
Low Dose Test Group	11.66 ± 0.76	10.00 ± 0.81***	11.20 ± 0.96***	10.60 ± 1.07***	11.00 ± 0.70**
High Dose Test Group	15.66 ± 0.55	9.60 ± 0.67***	11.50 ± 0.76***	11.66 ± 0.66***	11.33 ± 0.91**
LOCFL Alone Group	12.25 ± 1.10	10.25 ± 0.62^{ns}	13.50 ± 0.64^{ns}	12.00 ± 1.15^{ns}	13.00 ± 1.15^{ns}

Values are expressed in mean ± SEM (n=6).

$^{\#\#}$ denotes $p < 0.01$ and $^{\#\#\#}$ denotes $p < 0.001$ when compared to vehicle control rats.
** denotes $p < 0.01$ and *** denotes $p < 0.001$ when compared to negative control rats.
^{ns} denotes $p > 0.05$ when compared to vehicle control group (One way ANOVA followed by Tukey test).

Table No. 11: Effect of LOCFL on serum marker enzymes and LDH in rotenone-induced Parkinson's disease rats.

Treatment	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	LDH (IU/L)
Vehicle Control	307.39 ± 71.42	19.53 ± 18.33	30.32 ± 7.07	41.09 ± 9.20
Negative Control	1625.11 ±122.65$^{\#\#\#}$	208.90 ± 35.38$^{\#}$	76.13 ± 8.47$^{\#}$	759.61 ± 83.18$^{\#\#\#}$
Positive Control (Levodopa group)	614.56 ± 214.43**	21.14 ± 31.09**	11.95 ± 5.39**	45.52 ± 12.82**
Low Dose Test Group	415.93 ± 132.68**	19.70 ± 4.02*	32.83 ± 5.45*	57.17 ± 9.77**
High Dose Test Group	407.35 ± 204.25**	1.17 ± 0.64*	7.43 ± 2.83***	32.74 ± 3.18***
LOCFL Alone Group	789.31 ± 106.62^{ns}	23.96 ± 9.63^{ns}	34.48 ± 4.71^{ns}	38.11 ± 6.45^{ns}

Values are expressed in mean ± SEM (n=6).

$^{\#}$ denotes $p < 0.05$ and $^{\#\#\#}$ denotes $p < 0.001$ when compared to vehicle control rats.
** denotes $p < 0.01$ and *** denotes $p < 0.001$ when compared to negative control rats.
^{ns} denotes $p > 0.05$ when compared to vehicle control group (One way ANOVA followed by Tukey's test).

Table No.12: Effect of LOCFL on protein and MDA in the brain tissues of rotenone-induced Parkinsonian rats

Treatment	Total protein (µg/mg wet tissue)	MDA (nmoles/min/mg protein)
Vehicle Control	1.35 ± 0.34	0.36 ± 0.08
Negative Control	0.56 ± 0.16$^{\#}$	0.90 ± 0.06$^{\#\#}$
Positive Control (Levodopa group)	1.16 ± 0.19*	0.35 ± 0.05***
Low Dose Test Group	1.20 ± 0.05*	0.45 ± 0.06**
High Dose Test Group	1.90 ± 0.24***	0.47 ± 0.06***
LOCFL Alone Group	1.25 ± 0.05^{ns}	0.76 ± 0.05^{ns}

Values are expressed in mean ± SEM (n=6).

$^{\#}$ denotes $p < 0.05$ and $^{\#\#}$ denotes $p < 0.01$ when compared to vehicle control rats.
* denotes $p < 0.05$; ** denotes $p < 0.01$ and *** denotes $p < 0.001$ when compared to negative control rats.
^{ns} denotes $p > 0.05$ when compared to vehicle control group (One way ANOVA followed by Tukey's test).

Table No. 13: Effect of LOCFL on enzymatic and non-enzymatic antioxidants in the brain tissue of rotenone-induced Parkinsonian rats

Groups	Enzymatic antioxidants				Non enzymatic antioxidants
	SOD (nmoles/min/mg protein)	CAT (µg/min/mg protein)	GPx (nmoles/min/mg protein)	GSSH (nmoles/min/ mg protein)	GSH (nmoles/min/mg protein)
Vehicle Control	15.85 ±2.31	29.86 ± 5.38	26.69 ± 3.30	15.58 ± 2.03	12.10 ± 0.50
Negative Control	6.56 ± 0.76	13.69 ± 1.52$^{\#\#}$	8.49 ± 0.56$^{\#\#}$	4.92 ± 0.85$^{\#}$	4.83 ± 0.94$^{\#\#}$
Positive Control (Levodopa group)	16.96 ± 2.92	30.25 ± 2.64***	21.63 ± 3.09*	15.09 ± 0.78*	12.94 ± 0.99**
Low Dose Test Group	15.03 ± 0.88	28.27 ± 1.70**	22.43 ± 2.91*	16.26 ± 1.29*	14.24 ± 1.31***
High Dose Test Group	21.45 ± 2.04	33.14 ±2.25***	42.60 ± 4.81***	23.16 ± 3.91***	16.17 ± 1.80***
LOCFL Alone Group	15.09 ± 0.62	27.93 ± 0.65^{ns}	30.16 ± 1.01^{ns}	14.65 ± 1.67^{ns}	12.78 ± 0.28^{ns}

Values are expressed in mean ± SEM (n=6).

$^{\#}$ denotes $p < 0.05$ and $^{\#\#}$ denotes $p < 0.01$ when compared to vehicle control rats.
* denotes $p < 0.05$; ** denotes $p < 0.01$ and *** denotes $p < 0.001$ when compared to negative control rats.
^{ns} denotes $p > 0.05$ when compared to vehicle control group (One way ANOVA followed by Tukey's test).

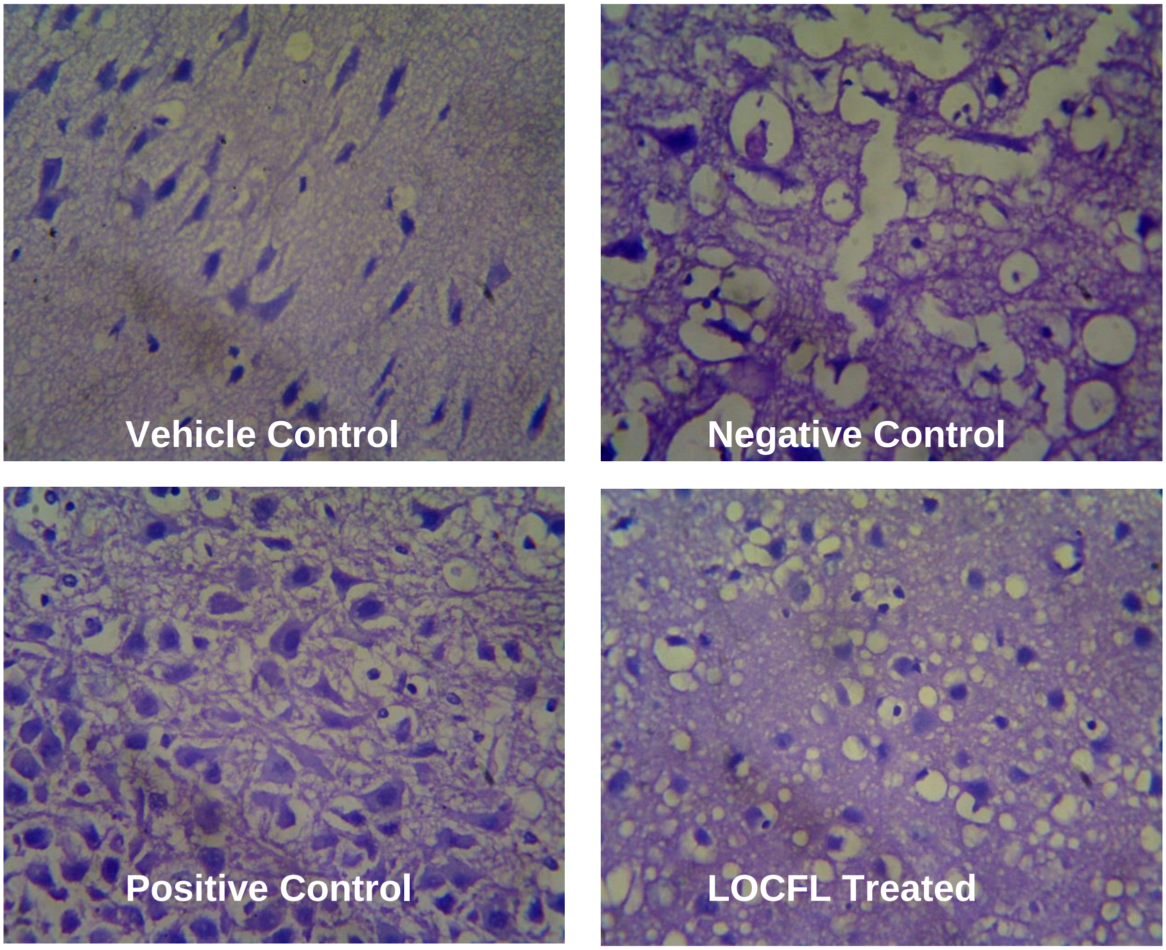


Fig. No. 15: Histopathology of brain tissue isolated from normal and rotenone-induced Parkinsonian rats

The **Vehicle Control** showed normal intact neurons with visible nuclei. The rotenone-induced Parkinsonian (**Negative Control**) rat brain tissue showed central vacuoles, decreased density of the cells, architecture completely altered featuring indisctint neuronal boundaries and invisible nuclei, also seen were haemorrhage and neuronal cell death. There is a significant reversal of the damage in levodopa-treated (Positive Control) and LOCFL-treated Parkinsonian rats.

DISCUSSION AND CONCLUSION

Parkinson's disease (PD) is the second most common neurodegenerative disease, with an estimated prevalence of about 0.3%, affecting 1–2% of people over 60 years of age. The pathological hallmark of PD is loss of dopaminergic neurons in the substantia nigra, and the presence of protein aggregates such as Lewy bodies involving synuclein alpha (SNCA) in the residual dopaminergic neurons [67]. The cause of chronic nigral cell death in PD and the underlying mechanisms remain elusive.

Plants, in the form of herbs, spices and foods, constitute an unlimited source of molecules available for improving human health. *Cymbopogon flexuosus* (lemon grass) belongs to a genus of plant species known for containing essential oil, which has a wide variety of chemical constituents used in perfumery, pharmaceutical industries and yield isolates which are used in high grade perfumery [23]. Essential oils and the phytoconstituents isolated from them are widely used to treat many human diseases [68]. Constituents of essential oils are widely known to have antioxidative property with several modes of actions as antioxidant, such as prevention of chain initiation, free radical scavengers, reducing agents, termination of peroxides, prevention of continued hydrogen abstraction as well as quenchers of singlet oxygen formation and binding of transition metal ion catalysts [69].

Animal models are only valuable as models for human diseases if they are actually reflect the diseased state in man, *i.e.,* the model should simulate the pathological, histological and biochemical changes of the disease and their resulting functional disturbances. Neurons are extremely dependent on mitochondria; they derive more than 90% of their ATP from mitochondrial respiration. The brain accounts for about 2% of body weight but receives about 20% of the cardiac output and consumes about 20% of the body's oxygen supply.

Rotenone, a mitochondrial NADH dehydrogenase inhibitor and selective inhibitor of complex I, act by markedly delayed opening of the mitochondrial permeabilty transition pore and increased the calcium capacity of mitochondria [70, 71, 72]. It induces neuronal injury through multiple pathophysiological mechanisms viz. complex I inhibition, oxidative damage, microglial activation, production of ROS, apoptosis and synuclein aggregation[73]. The resulting mitochondrial dysfunction may cause α-synuclein aggregation or neurodegeneration through oxidative stress or excitotoxicity[74]. Respiratory chain defect in Parkinson's disease (PD) provides not only a direct link with toxin models of Parkinsonism but also insight into the mechanisms involved in etiology and pathogenesis. Rotenone exposure accurately recapitulated the pathological, biochemical, and behavioral features of PD [70].

The vast majority of studies on health benefits of phytochemicals have focused on the fact that many of the active chemicals possess antioxidant activity. Neuroprotective effects of various phytochemicals, which possess fatty acids, phenols, alkaloids, flavonoids, saponins, terpenes etc., as phytoconstituents, are associated with reduced levels of oxidative stress [75].

Neuroprotective active phytochemical substances include. Lemon grass oil obtained from *Cymbopogon flexuosus* was found to possess most of the phytochemicals carbohydrates, proteins, amino acids, glycosides, saponins, flavonoids, etc. Hence it can be hypothesized that the plant/oil can be useful to be protective against neurotoxicity inflicted by rotenone.

The acute toxicity study was carried out as per the OECD guidelines No.420 [52] and was found that the oil is coming under the category 5 (considered to be safe for the use in animals and humans); since it was not producing any toxic reactions even tested at the highest dose, i.e., 2000 mg/kg body weight. The LD_{50} was fixed at 2000 mg/kg and two dose levels were tested, i.e., 100 mg/kg and 200 mg/kg. Two dose levels were used to ascertain any dose dependent increase in the protection shown by the oil.

The experimental design consisted of the use of rats under vehicle control (coconut oil alone); negative control (rotenone alone); positive control (rotenone and levodopa); two test groups (rotenone and two dose levels of lemon grass oil extracted from *Cymbopogon flexuosus*) and oil alone (lemon grass oil alone). The oil alone group was tested to learn any difference caused by treatment of oil alone without any neurotoxic substance.

Post treatment there was slight non-significant ($P>0.05$) increase in the body weight of the rats except for the rotenone-alone group, which had showed a significant ($p<0.05$) decrease. This may be attributed to the development of Parkinsonism in those rats. Possible determinants of weight loss in PD patients may be due to hyposmia, impaired hand–mouth coordination, difficulty chewing, dysphagia, intestinal hypomotility, depression, decreased reward processing of dopaminergic mesolimbic regions, nausea, and anorexia as the side effects of medication, and increased energy requirements due to muscular rigidity and involuntary movements [76].

Behavioural alterations and loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) are seen in toxin-induced Parkinsonism diseased rats as seen in idiopathic Parkinsonism patients. The same has been documented by many authors [72], [77], [78], [79], [80]. A variety of tests have been proposed for evaluation of motor deficits in rat models of Parkinsonism. They include, open field test, Rotarod, elevated plus maze, catalepsy, rearing behaviour, etc.

Open field and elevated plus maze tests are important test to evaluate the changes in locomotion, exploration and anxiety in animals. Changes in locomotion and anxiety are critical parameters in Parkinsonism patients. Anxiety disorders are usually under diagnosed and under treated in these patients [81]. Thus ability of lemon grass oil to reverse the rotenone-induced changes in locomotion and anxiety in rats can be evidenced.

Another widely used test is the “rotational behaviour” test usually using a Rotarod apparatus. It is based upon motor asymmetry induced by rotenone administration, leading to problems of sensitization, conditioning and priming [78]. Rotational behaviour is reproducible and easy to quantify.

Catalepsy is a state characterised by a patient keeping an uncomfortable, rigid and fixed posture despite external stimulus or resistance. There may also be decreased sensitivity to pain. It is a feature seen in catatonia. Catatonia is a major dysregulation syndrome that occurs in various medical conditions including Parkinsonism [82]. Drug-induced catalepsy can be used as a measure to evaluate effects on catatonia in Parkinsonism diseased rats.

The rearing behaviour or the cylinder test is one of the sensitive and reliable tests in detecting loss neuronal activity in the substantia nigra [80]. Postural instability is one of the many behavioral deficits observed in common animal models of PD. In the Parkinsonism diseased rat, there is a decreased reliance on the impaired forelimb for movements involving a response to weight shift. The animals preferentially initiate movement with the non-impaired forelimb, particularly for lateral movements during vertical exploration of surfaces [83].

In the present study, rotenone-induced Parkinson diseased rats showed significant changes in behavioural parameters which was reversed by presence of levodopa or lemon grass oil. Two doses of lemon grass oil also showed proportional increase in reversal of change in parameters.

An increase in serum parameters like serum glutamic-oxalacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT) and alkaline phosphatase (ALP) is seen in Parkinsonism diseased rats [84]. Elevation in serum lactate dehydrogenase (LDH) is seen in Parkinson's disorder [85].

Rotenone-induced Parkinsonism rats showed similar response and the same was reversed by treatment with levodopa and lemon grass oil.

Among the long list of neurological diseases linked to oxidative stress, not only will one find all sorts of acute disorders, but also, almost invariably, all of the prominent neurodegenerative disorders including Parkinson's disease (PD) [86]. Some of the oxidant-mediated alterations in cellular components are stable modifications and can thus be readily detected and quantified. The levels of enzymatic and non-enzymatic antioxidants in the brain tissue would be a good measure of oxidative status of the animal brain. In rotenone alone administered animals the oxidative stress was evident in the increase in levels of malondialdehyde, a thiobarbituric acid reactive substance, and a good indicator of oxidative stress. The levels of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, reduced glutathione are severely reduced in rotenone treated animals since the antioxidants are used up in the process of scavenging the free radicals. The levels are increased in levodopa and lemon grass oil treated animals.

The results of histopathological analysis also confirmed the extent of damage caused by rotenone in the brain and resultant reversal of the damage upon treatment with lemon grass oil. The findings correlate with results in the behavioural, serum and tissue parameters.

Conclusion:

Many phytochemicals have been reported to exert neuroprotective effects in various experimental models of neurological disorders. Although demand for phytotherapeutic agents is growing, there is need for their scientific validation before plant–derived substances gain wider acceptance and use. These type of investigations may provide a new source of beneficial neuropsychotropic drugs. They may also provide a phytochemical basis for some of the effects that these herbal preparations have on brain function and neuroprotection. Most of the current knowledge about CNS-active plants of cultural and traditional importance arose from ethnobotanical and ethnopharmaceutical uses, as for other natural active ingredients. Moreover, for a suitable neuroprotective agent, a very important property regards its ability to cross the blood-brain barrier (BBB), in order to reach the neurons in the nigrostriatum.

Finally, though the presence of receptors or transporters for phytochemicals in brain tissues remains to be ascertained and compounds with multiple targets appear as a potential and promising class of therapeutics for the treatment of Parkinsonism.

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