Evaluation of Anti-Parkinson Activity of Ayurvedic Formulation Saraswatha Ghrita in Rotenone Induced Parkinson's Mice Model

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DECLARATION

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EVALUATION CERTIFICATE

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Examination Centre: KMCH College of Pharmacy, Coimbatore-48

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CERTIFICATE

This is certify that the project title **Evaluation of Anti-Parkinson activity of Ayurvedic formulation Saraswatham Ghritham in MPTP induced mice model** has been approved by the IAEC Reg. No. 685/PO/Re/S/2002/CPCSEA.

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ABBREVIATIONS

ABBREVIATIONS	FULL FORM
ATP	Adenosine tri phosphate
BBB	Blood brain barrier
CMC	Carboxy methyl cellulose
CAT	Catalase
COMT	Catechol-O-Methyl Transferase
CPCSEA	Committee for the Purpose of Control and Supervision of Experimental Animals
DA	Dopamine
DAG	Diacylglycerol
DOPA	Dihydroxyphenylalanine
DOPAC	3,4-dihydroxy phenyl acetic acid
DMSO	Dimethyl sulfoxide
DTNP	5,5'-dithiobis-2-nitrobenzoic acid
G	Gram
GABA	Gamma Aminobutyric Acid
GSH	Reduced glutathione
5-HT	5- Hydroxytryptamine
IAEC	Institutional Animal Ethical Committee
i.p	Intraperitonial
L-dopa	Levodopa
LPO	Lipid peroxidation
LRRK 2	Leucine rich repeat kinase 2
MAO-A	Monoamine Oxidases Type-A

MAO-B	Monoamine Oxidases Type-B
MAOIs	Monoamine Oxidases Inhibitors
Mg/kg	Milligram/kilogram
MPDP+	1-methyl-4-phenyl-2,3-dihydropyridinium ion
MPP+	1-methyl-4-phenyl-pyridinium ion
MPTP	1-Methyl-4-Phenyl-1, 2, 3, 6-Tetra-Hydropyridine
ng/g	Nanogram per gram
nm	Nanometer
OECD	Organisation for Economic Corporation and Development
PBS	Phosphate buffer solution
p. o	Post Oral
PCD	Programmed cell death
PD	Parkinson ’s disease
REM	Rapid eye movement
ROS	Reactive oxygen species
rpm	Rotations Per Minute
SD	Standard deviation
sec	second
SEM	Standard Error Mean
SNpc	Substantia nigra par compacta
SG	Saraswatha Ghrita
SOD	Super oxide dismutase
TBA	Thio barbituric acid
µg/mg	Microgram

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1. INTRODUCTION

1. Introduction

Parkinson's disease (PD) is a second most neurodegenerative disorder caused by the progressive loss of mesencephalic dopaminergic neurons in the substantia nigra innervating the striatum. It was first described by neurologist James Parkinson in 1817 that he called ''Shaking Palsy'', or ''paralysis agitans''. The causes are unknown although risk factors in the genetic and toxic domain are being discovered. An important pathophysiological feature in PD is the loss of part of the dopaminergic neurons in the substantia nigra (SN) resulting in a specific dysorganisation of the complicated basal ganglia (BG) circuits. The relay functions at the level of the striatum are out of balance leading to disturbed subcorticocortical interactions.

Parkinson's disease (PD) is the second most common neurodegenerative disease, primarily affects people of ages over 55 years (approximately 1.5% to 2.0%), although young adults and even children can also be affected. Research on the pathogenesis of PD has rapidly advanced due to the development of animal models. Through the use of these models, the striatal dopamine deficiency could be associated with the motor symptoms of PD, and levodopa (dihydroxyphenylalanine or L-dopa) was first applied to compensate striatal dopamine losses. L-Dopa treatment still remains the standard of PD therapies. Unfortunately, long-time use of L-dopa results in dyskinesia (involuntary movements). Moreover, the specific etiology of PD is still unknown. Thus, the development of animal models is essential for better understanding pathogenesis and progression of PD and testing therapeutic agents for the treatment of PD patients. [1-5]

Parkinsonism is a clinical syndrome consisting of four cardinal features: bradykinesia (slowness and poverty of movement), muscular rigidity, resting tremor and an impairment of postural balance leading to disturbances of gait and falling. [6]

1.1 Etiologic classification

1.1. a Primary Parkinson's disease- Loss of pigmented neurons in the substantia nigra, chiefly in the medial and ventral segment allied with reactive gliosis.

Secondary Parkinson's disease- Caused by disorders like infection, trauma, neoplasm, toxins, atherosclerosis, drug intoxication etc. Drugs like neuroleptics, anti-hypertensives, anti-emetics etc are the common cause of drug induced Parkinsonism. 1-methyl-4-phenyl- 1, 2, 3, 6-Tetrahydropyridine (MPTP) produces a Parkinsonian syndrome due to the degeneration of locus ceruleus and substatia nigra, in users. [7, 8]

PD is a multifactorial disease, with both genetic and environmental factors playing a role. Age is the biggest risk factor for PD, with the median age of onset being 60 years of age[9] . The incidence of the disease rises with age to 93.1 (per 100,000 person-years) in age groups between 70 and 79 years. Additionally, there are cross-cultural variations, with higher prevalence reported in Europe, North America, and South America compared with African, Asian and Arabic countries [10,11].

Cigarette smoking

Cigarette smoking has been extensively studied with respect to PD, with The number of years smoking and the risk of PD, with the risk of developing PD being significantly reduced in heavy or long-term smokers compared with nonsmokers.The reasons underlying this associated reduced risk are not fully understood. Activation of nicotinic acetylcholine receptors on dopaminergic neurons by nicotine or selective agonists has been shown to be neuroprotective in experimental models of PD [12, 13]. Nevertheless, nicotine can also stimulate the release of dopamine, which is involved in the reward mechanisms; it is therefore difficult to confirm whether smoking prevents PD or whether PD helps prevent the habitual use of cigarettes. As a result of a reduction in dopamine in patients with PD, patients may be less prone to addictive behaviours, and thus less likely to smoke. This hypothesis is supported by the fact that patients with prodromal PD and PD were able to give up smoking much easier than controls, suggesting this association could be due to the decreased responsiveness to nicotine [14-18].

Caffeine

Caffeine is an adenosine A_{2A} receptor antagonist, which is believed to be protective in PD and has been shown to be neuroprotective in a mouse model of PD [19]. It has been previously reported that there is a 25% risk reduction in developing PD among coffee drinkers. Regular tea

drinkers also have been reported to have a lower risk of developing PD [20]. Additionally, in post-menopausal women, the effect of caffeine depended on whether the females were taking hormone replacement therapy including estrogens. As estrogen competitively inhibits caffeine metabolism, interactions between estrogen and caffeine may explain in part why PD risk is dependent on hormone replacement therapy in post-menopausal women [21].

Pesticides, herbicides, and heavy metals

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was first discovered to be associated with nigrostriatal degeneration when several people developed typical PD signs after injecting themselves with a drug contaminated with MPTP. MPTP is metabolized into the neurotoxin, MPP+ (1-methyl-4-phenylpyridinium), which is a mitochondrial complex-I inhibitor that selectively damages dopaminergic cells in the substantia nigra [22].The identification of MPTP as a cause of nigral degeneration led to the idea that PD could be caused by an environmental toxin. Since then, several studies have shown an association between pesticides and PD, with one case-control study showing an increased association with professional pesticide exposure in men and late-onset PD[23, 24].Paraquat (a herbicide which is structurally very similar to MPP+) and rotenone (a pesticide) are also selective complex-I inhibitors and induce dopaminergic depletion in animal models of PD. The relationship between exposure to these chemicals and the risk of developing PD has been investigated in other epidemiological studies. It has also led to the study of surrogate markers, including the association of farming, drinking well water, and living in rural areas with PD risk. Welding and heavy metal exposure (e.g., iron, copper, lead, aluminum, and zinc) have also been investigated, but the relationship between these and PD remains inconclusive.

Genetics

Although PD is generally an idiopathic disorder, there is a minority of cases (10–15%) that report a family history, and about 5% have Mendelian inheritance . Furthermore, an individual's risk of PD is partially the product of as-yet poorly defined polygenic risk factors [26]. The genes that have been found to potentially cause PD are assigned a "PARK" name in the order they were identified. To date, 23 PARK genes have been linked to PD. Mutations in the PARK genes demonstrate either autosomal dominant (e.g., *SCNA, LRRK2,* and *VPS32*) or autosomal recessive

inheritance (e.g., *PRKN, PINK1*, and *DJ-1*). The involvement of some of these genes has not been conclusively confirmed (PARK5, PARK11, PARK13, PARK18, PARK21, and PARK23), while others are considered risk factors (PARK3, PARK10, PARK12, PARK16, and PARK22) [27, 28].

The numerically most important genetic risk factors predisposing to PD are mutations in *GBA1*, a gene encoding β-glucocerebrosidase-a lysosomal enzyme responsible for the hydrolysis of glucocerebrosides[29]. *GBA1* mutations are known to cause Gaucher disease, which is the most common lysosomal storage disorder. Other genetic risk factors include the major histocompatibility complex, class II (HLA-DQB1) and gene encoding the protein tau, *MAPT* among others [30].

1.2 Epidimology of parkinson's disease:

Parkinson's disease ranks among the most common late life neurodegenerative diseases, affecting approximately 1.5% to 2.0% of the population older than age 60. The causes of Parkinson's disease (PD), the second most common neurodegenerative disorder, are still largely unknown. Current thinking is that major gene mutations cause only a small proportion of all cases and that in most cases; non-genetic factors play a part, probably in interaction with susceptibility genes. Numerous epidemiological studies have been done to identify such non-genetic risk factors, but most were small and methodologically limited [31].

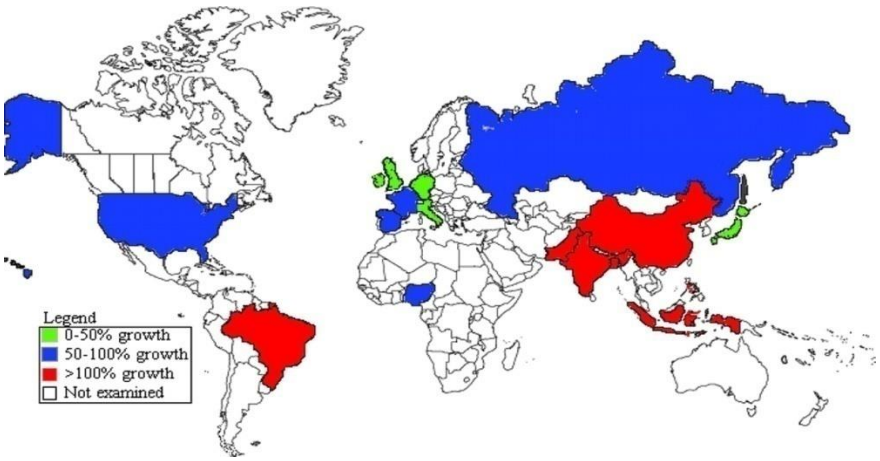


Figure-1: Epidimology of Parkinson's disease

1.1.5 SYMPTOMS OF PARKINSON'S DISEASE[32] :

TYPES OF SYMPTOMS	SYMPTOMS
MOTOR SYMPTOMS	• Brady kinesia • Rigidity • Tremor • Postural instability • Dysarthria • Dystonia
NON MOTOR SYMPTOMS Autonomic dysfunction	• Constipation • Orthostatic hypotension • Sexual dysfunction • Urinary retention • Sexual dysfunction • Sweating
Neuropsychiatric symptoms	• Anxiety • Cognitive impairment • Dementia • Depression • Panic disorder • Psychosis
Sensory symptoms	• Olfactory dysfunction • Paresthesias • Pain

Table 1: Various symptoms of PD

1.1.6 PATHOPHYSIOLOGY

Pathologically, PD is characterized by severe loss of substantia nigra (SN) dopaminergic neurons, visible in brain sections as depigmentation of the substantia nigra in the midbrain. It is estimated that approximately 60% to 70% of the SN dopamine cells are lost by the time a patient first presents for clinical evaluation, diagnosis, and treatment. [Figure-2]

1.1.6.1 Monoamine Oxidase (MAO-A and MAO-B) involvement in PD:

MAO exists as two isoforms with different substrate selectivities and different distributions in brain and between species. In man, dopamine is largely metabolised by MAO-B although it can also be a substrate for MAO-A. However, dopaminergic neurones in the striatum contain relatively little MAO-B but the A-isoform is present. Rather MAO-B is found extensively in glial cells and localised to the outer mitochondrial wall. Under normal physiological conditions, dopamine released into the synapse by impulse flow is rapidly 'inactivated' by the high affinity reuptake process that constitutes the dopamine transporter. In PD, the number of presynaptic terminals in the striatum is extensively depleted and now MAO-B in surrounding glial cells becomes a major focus for dopamine metabolism. This provides a targeted and disease specific mechanism through which dopamine degradation can be inhibited by the use of selective MAO-B inhibitors [33].

1.1.6.2 Mitochondria involvement in PD:

Mitochondria are involved in the regulation of apoptosis. Opening of the MTP and mitochondrial depolarisation seem to be part of the initiation of apoptosis. Consequently, cytochrome-c and the apoptosis inducing factors (AIFs) can exit mitochondria and activate proapoptotic proteins close to the outer mitochondrial membrane. Antiapoptotic proteins, such as Bcl-2, are also localised close to the outer mitochondrial membrane which makes mitochondria the location for proand antiapoptotic events. MPP+ opens the MTP and leads to cytochrome c release. Oxidative stress is caused by an imbalance between the generation of ROS and their enzymatic or non-enzymatic detoxification rate. Oxidative stress plays an important role in brain aging, neurodegenerative diseases and has long been associated with the death of dopaminergic neurons, due to production of toxic species through autoxidation and formation of neuromelanin. The iron and copper levels in SN are high and it contributes to autoxidation of dopamine and its metabolites,

leading to production of ROS. Oxidative damage of lipids, proteins and DNA as well as decreased level of an important antioxidant, reduced glutathione, have been detected PD patients autopsy brain. Oxidative damage induces SYN aggregation and impairs proteosomal ubiquitination and degradation of proteins, inducing LBs formation, one of PD's characteristic hallmarks. Mitochondrial activity can also be affected by environmental factors that possibly contribute to PD pathogenesis. The ability of the ROS generator, paraquat, to cause specific dopaminergic lesions strongly suggests involvement of oxidative stress in PD. Oxidative stress in PD is not restricted to the brain area alone: a variety of oxidative damage markers, in the serum and CSF of PD patients, suggest a systemic DNA/RNA oxidation. Oxidative stress in the central nervous system (CNS) comes not only from mitochondria-generated ROS, but also from microglia. Classic microglial activation is a source of intracellular and extracellular ROS, which can induce neuronal damage. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a membrane bound enzyme complex that catalyses the production of superoxide from oxygen, and is implicated as both the primary source of microglial-derived extracellular ROS and a mechanism of pro- inflammatory signalling in microglia. NADPH-oxidase is up regulated in the SN of PD patients [34] .

1.1.6.3 Role of Iron in Parkinsonism:

Abnormal levels of brain iron have been reported in Parkinsonism, which is characterized by degeneration of dopaminergic (DA) neurons. An animal model of Parkinsonism was used to clarify the contribution of the loss on nigrostriatal DAergic neurons to abnormal iron accumulation. In rats 6-OHDA induced unilateral DA depletion, brain iron deposition and its day-to-day stability can be studied in vivo using magnetic resonance imaging (MRI) scans and Perls'-DAB histochemical stain. The uptake and transport of iron by intrinsic cells of the striatum may vary, and this variability may have been exaggerated by the destruction of DAergic nigrostriatal neurons, which are known to modulate the activity of the intrinsic cells[35] .

1.1.6.4 Role of neuroinflammation in the pathogenesis of PD:

Glial cells, such as astrocytes or microglia, play important role in CNS homeostasis, mediating immune responses and reducing oxidative stress. When CNS regional homeostasis is disturbed, glial cells release cytokines to re- establish the balance and repair damaged cells. Such a

response is natural and beneficial for neurons; however repeated microglia and astrocyte activation evoke chronic inflammatory stress, leading to increased production of ROS and severe neuronal damage. Several cell, animal and human studies indicate involvement of neuroinflammation in the pathogenesis of PD [36].

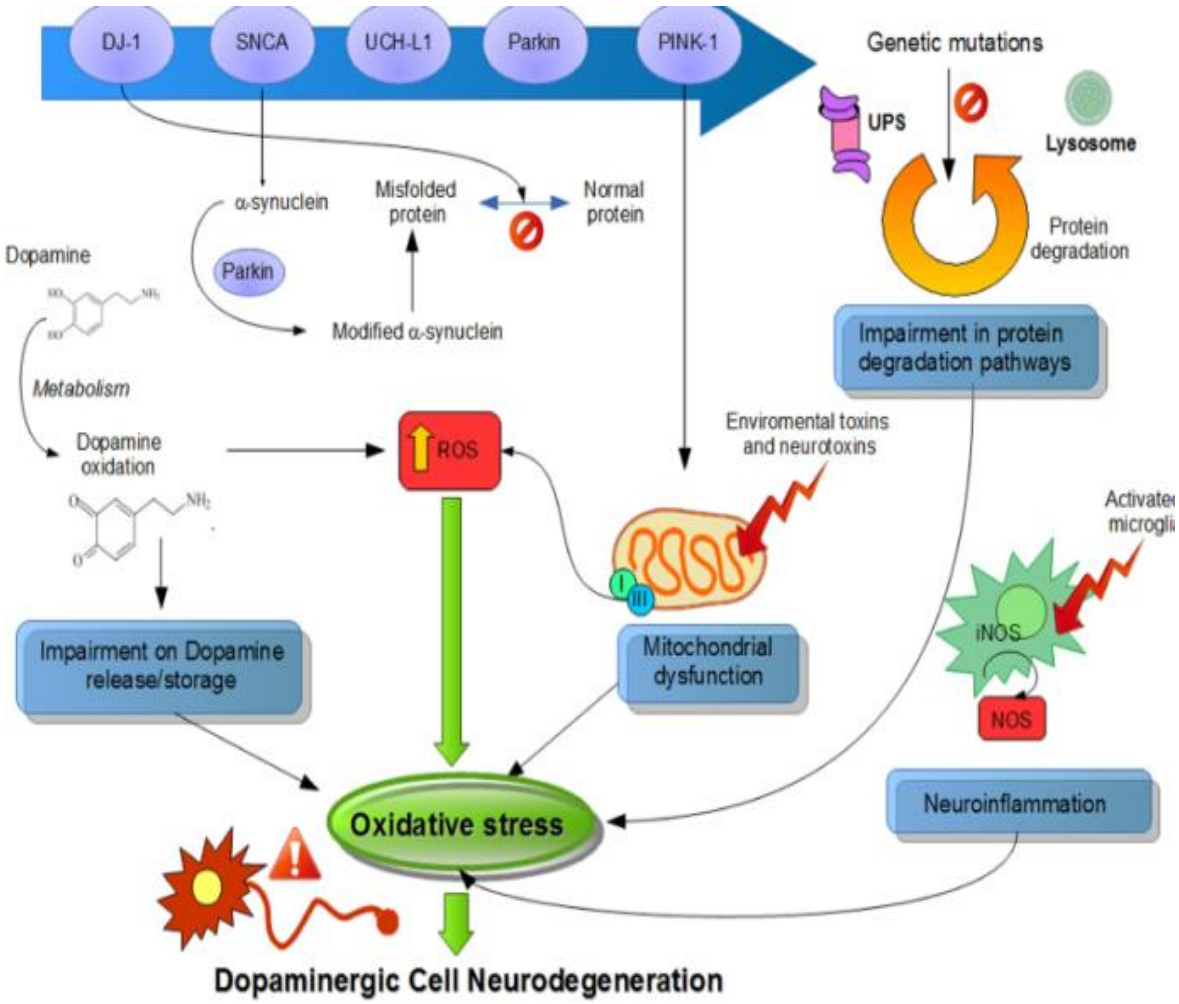


Figure-2: Molecular Mechanism of Parkinson's disease

1.1.7 DIFFERENTIAL DIAGOSIS OF PD [37] :-

S.NO	DIAGNOSIS	HISTORICAL FEATURES	SIGNS/SYMPTOMS	RADIOGRAPHIC FINDING
1.	Idiopathic Parkinson's disease	Difficulty with tasks, rigidity, tremor	Tremor, rigidity, bradykinesia, loss of balance, micrographia	No specific CT or MRI findings
2.	Drug induced parkinsonism	Previous use of antipsychotics, reserpine, metaclopramide	Tremor, rigidity, bradykinesia, often bilateral symptoms	Normal
3.	Vascular parkinsonism	Stepwise progression; CVA or TIA, co morbid cardiovascular disease	Fixed deficits from previous events	Lesions in white matter with or without basal ganglia
4.	Essential tremor	History in multiple family members, little evaluation	Tremor often bilateral, mild rigidity; no extra pyramidal symptoms; no response to L-dopa	SPECT shows normal dopaminergic system
5.	Progressive supranuclear palsy	Onset after 40 years of age; frequent falls	Vertical gaze paralysis, postural instability, resting tremor, dystonia, normal olfaction	MRI shows mesencephalon brain stem atrophy involving the superior colliculi

Table 2: Differential diagnosis of PD

1.1.8 PHARMACOLOGICAL TREATMENT

The current treatment method primarily targets symptoms by using anti-Parkinson drugs to replace DA. When drug therapy is not satisfactory, surgical treatments are recommended. Levodopa (L-dopa) to make and replenish the brain's supply of dopamine. L-dopa is often given along with carbidopa. Carbidopa delays the conversion of l-dopa in to dopamine until it reaches the brain. This prevents, or diminishes some of the side effects of l- dopa and reduces the amount of l- dopa needed [38].

1.1.8.1 CLASSIFICATION OF DRUGS:

A.DRUGS AFFECTING BRAIN DOPAMINERGIC SYSTEM:

1. **DOPAMINE PRECURSOR:** Levodopa
2. **PERIPHERAL DECARBOXYLASE INHIBITORS:** Carbidopa, Benserazide.
3. **DOPAMINERGIC AGONISTS:** Bromocriptine, Ropinirole, Pramipexole
4. **MAO-B INHIBITORS**: Entacapone, Tolcapone
5. **NMDA RECEPTOR ANTAGONIST(DOPAMINE FACILITATOR):** Amantadine

B.DRUGS AFFECTING BRAIN CHOLINERGIC SYSTEM:

1. **CENTRAL ANTI CHOLINERGICS**

   Trihexyphenidyl(Benzhexol), Procyclidine,Biperiden.

2. **ANTIHISTAMINICS:** Orphenadrine, Promethazine

- Bromocriptine, pergolide, pramipexole, and ropinirole. These drugs all mimic the role of dopamine in the brain
- Selegiline may delay the need of l- dopa therapy. When given with l-dopa , it seems to enhance and prolong the response of l -dopa.
- Anticholinergics may help to control tremor and rigidity. They appear to act by blocking the action of ach.

- Amantadine is an anti viral drug. It is effective at reducing many symptoms, but its efficacy wears off after several months. Effectiveness may return after a brief withdrawal [39].

1.1.8.3 EXPERIMENTAL THERAPIES [40]

1.1.8.3. a Cell Transplant Therapy

Transplant of fetal SN cells has been performed in several hundred patients to date in multiple centers throughout the world. While results have been encouraging in some individual patients, two double-blind placebo-controlled studies showed that consistent benefit was only seen in younger PD patients (age 60 or below), and side effects in some patients were significant. In particular, some patients developed off-medication dyskinesias even without any levodopa or other Dopaminergic medication.

1.1.8.3. b Gene Therapy

As of 2010, gene therapy has been tried in only a few PD patients. The only publicized trial is of delivery of the gene for glutamic acid decarboxylase (GAD) to the STN. GAD is a key enzyme in the production of the inhibitory neurotransmitter GABA. Gene therapy with GAD is meant to increase GABA production, reducing subthalamic nucleus (STN) activity in the manner of STN DBS.

1.1.8.3.c Physical and Occupational Therapy

Goals of physical therapy include maintaining or increasing activity levels, decreasing rigidity and bradykinesia, optimizing gait, and improving balance and motor coordination. PT program may include:

- Regular exercise.
- Stretching and strengthening
- Exaggerated or patterned movements, such as high stepping and weight shifting
- Mobility aids, orthotics
- Training in transfer techniques

1.1.9 VARIOUS IN-VIVO MODELS FOR PD:-

Animal models are valuable tools for studying the biology and genetics of human parkinson as well as for preclinical investigation of anti-parkinson therapeutics and parkinson preventive studies. Various animal models have been generated by genetic engineering, graft transplantation, and viral/physical/chemical induction. Studies from animal models of parkinson have been utilized for preclinical investigation of therapeutic efficacy and toxicity of chemicals and biologicals. Tremendous advances have been made in the generation of animal models of parkinson, which have become increasingly sophisticated by application of new technologies and integration of clinical information from patients. The goals are to faithfully recapitulate the human parkinson diseases in the animal models and apply them as preclinical tools, with the hope of successfully translating the basic knowledge into treatment and prevention of parkinson in humans.

The mouse has been the traditional animal model for basic and preclinical studies of parkinson, and other organisms including zebrafish play important and complimentary roles as models of parkinson research. Genetically engineered mouse models of parkinson have been generated by a variety of interventions such as chemical or physical mutagenesis, viral infection, insertion of transgenes, homologous recombination, and the recently developed gene edition. [41, 42]

1.1.9.1 MPTP induced PDs:

1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), is a potent neurotoxin & highly lipophilic. After systemic administration it rapidly crosses the blood-brain barrier, enters astrocytes and is metabolized to its active metabolite MPP+ by monoamine oxidase-B (MAO-B). MPP+ is able to inhibit complex 1 of the mitochondrial electron transport chain, resulting in the formation of ROS & leading to reduced ATP production.

Neuroprotection suggests preventing or slowing disease progression. Nevertheless, despite advances toward this goal, all current treatments are symptomatic; none halt or retard dopaminergic neuron degeneration. L-dopa treatment produces many distressing side effects, and its possible that metabolism of excess dopamine by the monoamine oxidase enzymes in the brain produces too much H2O2. An initial good response to symptomatic pharmacological treatment declines with time, and severe side effects develop and later on surgical interventions are to be

used. The progressive neurodegeneration in PD is not arrested by the currently used drug therapies. Hence, recent researches are focusing on finding therapies, preferentially herbal drugs [43].

1.1.9.2 Tremorine and Oxotremorine Antagonism:

The muscarinic agonist's tremorine and oxotremorine induce parkinsonism-like signs such as tremor, ataxia, spasticity, salivation, lacrimation and hypothermia. These signs are antagonized by anticholinergic drugs. The oxotremorine antagonism has been proven to be a reliable method for testing central anticholinergic activity. The overt isomorphism between the animal model and the symptoms of Parkinson's disease recommend this test for screening of anti-Parkinson drugs. However, the model measures only central anticholinergic activity [44].

1.1.9.3 Reserpine Antagonism:

Reserpine induces depletion of central catecholamine stores. The sedative effect can be observed in mice shortly after injection, followed by signs of eyelid ptosis, hypokinesia, rigidity, catatonia, and immobility. These phenomena can be antagonized by dopamine agonists. Locomotor activity and grooming scores of drug treated animals are compared with controls treated with reserpine and vehicle only by analysis of variance [45].

1.1.9.4 6-OHDA lesioned rats

6-OHDA is a neurotoxin, which, when directly injected into the medial forebrain bundle, striatum or SN, induces nigrostriatal DA neuronal degeneration. Although 6-OHDA leads to clear apoptosis of nigrostriatal dopaminergic cells, evidence indicates that the toxic effects of 6-OHDA are in part mediated through the activation of microglia. Direct administration of 6-OHDA into the SN of mice activates microglia and increases the number of activated microglia in the SN with the subsequent loss of dopaminergic neurons after 1 week. Furthermore, 6-OHDA-lesioned rats have been demonstrated to have increased levels of TNF-a in both SN and striatum.

The effects of striatal injection of 6-OHDA in rats was followed using PET scanning of presynaptic DA transporters following administration of transport site specific ligands. These authors observed progressive striatal neurodegeneration, whereas at the same time, an increase in activated microglia in the striatum and nigra occurred, which was initially focal but by 4 weeks had become widespread. This led the authors to conclude that neuroinflammation is a significant factor in the 6-OHDA neurodegenerative process [46].

1.1.9.5 ROTENONE induced PDs:

PD can be induced with mitochondrial complex I inhibitors such as the environmental toxins rotenone. Rotenone, a commonly used natural pesticide prepared from the roots of tropical plants, such as Derris elliptica, can freely cross cell and mitochondrial membranes. In vitro, rotenone has been shown to promote the accumulation and aggregation of alpha-synuclein and ubiquitin, cause oxidative damage, and endoplasmic reticulum stress, and lead to cell death. In vivo, chronic exposure of rats to rotenone induces PD-like symptoms, including dopaminergic neurodegeneration and the occurrence of cytoplasmic inclusions similar to Lewy bodies. Recent studies show that chronic exposure of Drosophila to rotenone recapitulates key features of Parkinsonism, including selective loss of dopaminergic neurons and locomotor deficits.[figure-3] Although there are also study showing contradictory results, most of the evidences are consistent and suggesting that rotenone exposure contributes to PD-like symptom and that rotenone-based Parkinson's disease models can be used to test potential compounds for PD intervention[47].

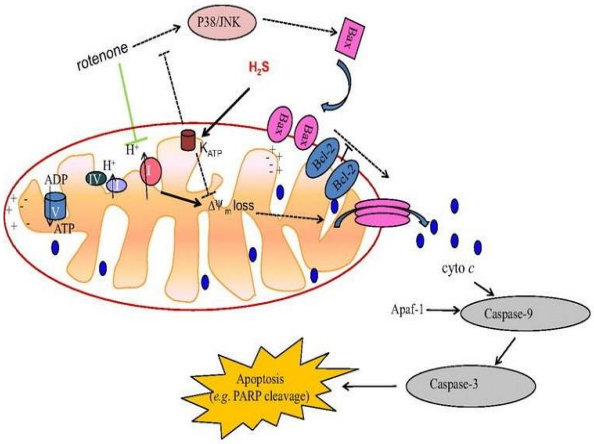


Figure 3:Molecular Mechanism of action of rotenone

1.1.10 PURPOSE OF THIS STUDY:

The existing conventional strategies that target PD are associated with numerous side effects and possess an economic burden. In recent years, there has been a growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants.

Ayurveda (Ayur = life, Veda = Knowledge) is concerned about the absolute wellness together with, psychological, physical, social wellness, economical as well as spiritual security. The prime goal of Ayurvedic medication is to facilitate people live long hence known as the science of longetivity), hale and hearty and balanced lives with no need for problematical surgery, chemical drugs or tormented through painful conditions. The basic principle of Ayurveda is the belief that distress and ailment results from disparity in three doshas (Vata, Pitta and Kapha) which are the three basic energy types of the body.

These doshas are the prime determinants of body type, energy levels, appetite, tendencies and moods and are considered and checked by the physicians to prescribe the essential medication. The major aspects of Ayurvedic restoration of balance includes tuning of the natural rhythms of our body and also synchronizing our body with nature and its cyclic patterns which helps to relieve stress and also restore thecircadian rhythm of our body.

Remarkably, in the context saraswatham ghritham as an ayurvedic product which has been efficacious in treating neurological issues has already been reported has found to the more exciting treatment. This products possess neuroprotective property [48], anti-oxidative [49, 50], anti-inflammatory [51, 52], nootropic , anti aging, anti depressant properties. It is commonly used in traditional medicine for memory enhancing and various other mental disorders from centuries and is known not to produce any toxic or adverse effect in human but probably there is no scientific evidence available on toxicity till date [53].

Based on this present study has been designed to evaluate the anti-parkinsonism effect saraswatham ghritham formulation, which can prevent further neuro inflammation along with neuronal protection, where the classical levodopa fails.

2. REVIEW OF LITERATURE

Several investigations have also been carried out on Antiparkinsons properties of plant-based extracts/phytochemicals. In what follows, the work carried out on Antiparkinsons medications in Ayureveda and pharmacological activities of different ingredients of Saraswatha gritham .The review is limited to 2019-2007.

Qi Zhang et al., (2019) reported the neurotoxicity of rotenone through intraperitoneal injection in mice and to investigate the global changes of phosphorylation proteomic profiles in rotenone-injured SH-SY5Y cells through a label-free proteomic analysis using a PTM Scan with LC MS/MS.The mice were intraperitoneally injected with different dosages of rotenone (1 mg/kg/d or 3 mg/kg/d) daily for 21 consecutive days. Rotenone caused a dose-dependent decrease in locomotor activities and a decrease in the number of Nissl-positive and tyrosine hydroxylase (TH)-immunoreactive neurons in the substantia nigra pars compacta (SNpc). This study indicated in rotenone-injured SH-SY5Y cells and provides molecular information for the neurotoxicity of rotenone [54].

Lakshmi Kakunuri *et al*., (2018) evaluated the antiparkinsonian activity of *Terminalia chebula (T. chebula)* fruit extracts by haloperidol-induced catatonia model in Sprague Dawley (SD) rats. In this study PD was induced by administering haloperidol (4 mg/kg p.o) daily for a week. All the treatment group animals received respective inducing, standard, and test treatment (100mg/kg.*p.o*) 30 min before the haloperidol administration. Based on the result they concluded that the Fruit extracts of *T. chebula* exhibited a significant antiparkinson's activity [55].

Ayodele Jacob Akinyemi *et al.*, (2018) investigated the effect of essential oils from Nigeria ginger and turmeric rhizomes on some cytokines in cadmium(cd) induced neurotoxicity. The study revealed that essential oil from ginger and turmeric rhizomes exerts anti-infammatory efect by preventing alterations of some cytokines/inflammatory biomarker levels, inhibits the acetylcholinesterase (AChE) and adenosine deaminase (ADA) in Cd treated rats. They concluded that essential oil from *ginger and turmeric rhizomes* exerts anti-infammatory properties in Cd induced neurotoxicity[56].

M Shelar *et al*.,(2017) reported that Standardisation of the SG was done by newly developed high performance thin layer chromatography (HPTLC) method .The simultaneous estimation of markers was carried out on silica gel precoated thin layer chromatography plates with 60F254 as the stationary phase and eluted using Toluene: Methanol: Triethylamine (9.2:0.5:0.3) as mobile phase. Optimum wavelength 282 nm was selected for detection and quantification. The method was validated as per ICH guidelines. The retention factor identified for standard such as BEB, PIP, 6-SHO and ASA. The developed HPTLC method was found to be simple, specific, accurate, precise and robust, thus can be used for routine analysis of SG for standardization with respect to selected active markers [57] .

Yuh-Chiang Shen *et al.*, (2017) determined the neuroprotective effect and mechanisms of action underlying the *Terminalia chebula* extracts and ellagic acid by using beta-amyloid induced cell toxicity in an undifferentiated pheochromocytoma (PC12) cell line. They were evaluated the cell toxicity and changes in intracellular reactive oxygen species (ROS) and calcium level to evaluate the neuroprotective effects of *T.chebula*. They were concluded *T. chebula* extracts e.g., ellagic acid is crucial to verify the neuroprotective efficacy [58].

Moses B. Ekong *et al.*, (2017) investigated the neuroprotective effects *Moringa Oleifera* (MO) leaf extract on aluminium-induced temporal cortical degeneration in rats. 24 male albino Wistar rats were grouped (n = 6) into control (1 ml/kg distilled water), l00 mg/kg aluminium chloride (AlCl3), 300 mg/kg MO, and 100 mg/kg AlCl3 and 300 mg/kg MO groups. There was an increased neuron specific enolase (NSE) and glial fibrillary acidic protein (GFAP) expressions in the AlCl3 group, while the MO group also showed increased NSE but decreased GFAP expression. They concluded that the MO protects against Al-induced neurotoxicity of the temporal cortex of rats[59] .

Meena J *et al.*, (2015) assessed the *Cyclea peltata* which has medicinal plant in the folk medicine is used in this study to analyse its antioxidant potential. Swiss albino mice were subjected to toxicity analysis on treatment with methanolic extract of the plant after inducing them with DAL cells. The antioxidant levels on the liver and kidney tissues revealed lower ranges in control group when compared to the treated groups. Based on The results they concluded that methanolic extract showed a reversed value towards the normal[60].

Radhey Shyam Tiwari *et al*., (2015) carried out the acute toxicity study of *saraswatham ghrita* carried out in wister mice(25±5) and observed for any adverse effects and mortality for 72 hrs after the oral administration of single dose of drug at several levels higher than the therapeutically equivalent dose. Their observations they concluded that the preparation as a whole was found to be safe in all routine lab investigation both in short and long term even on higher than the normal therapeutic dose [61].

Alyne Oliveira Correia et al., (2015)evaluated the neuroprotective activity of *Piper Nigram(pip)* in a model of PD. Male Wistar rats were grouped .All animals were subjected to behavioral studies, neurochemical, histological and immunohistochemical analyses. Based on the results they concluded *PIP* presented a neuroprotective action, probably a consequence of its antiinflammatory and antioxidant properties, making the drug a potential candidate for the treatment of neurodegenerative diseases as PD[62]**.**

Lucian Hritcu *et al*.,(2015) evaluated the possible anxiolytic, antidepressant and antioxidant properties of the methanolic extract of Piper nigrum fruits in beta-amyloid rat model of Alzheimer's disease. The antioxidant activity in the amygdala was assessed using superoxide dismutase, glutathione peroxidase and catalase specific activities, the total content of the reduced glutathione, protein carbonyl and malondialdehyde levels Based on the results results suggested that the methanolic extract ameliorates beta-amyloid-induced anxiety and depression by attenuation of the oxidative stress in the rat amygdala[63].

Anwesa Bag *et al*., (2013) assessed an anti-inflammatory, antioxidant, anti-lipid peroxidative and membrane-stabilizing effects of hydroalcoholic extract of Terminalia chebula fruits and also to establish a possible association between them. They were evaluated the carrageenin-induced inflammation in rats, Human erythrocyte hemolytic assay, Antioxidant potential of test fruit extract using TBARS and DPPH methods. Based on their study to found that the use of T. chebula fruits in the treatment of arthritic disorder, a potent anti-inflammatory agent[64]

Anu Elizabeth Joy *et al.*, (2012) assessed anticatalytic activity of MO of Neuroleptic induced catalepsy is an effective animal model for screening the antiparkinsonian activity of a chemical. The results indicate that administration of haloperidol (1mg/kg, i.p) significantly induced catalepsy in Swiss albino mice, which was significantly reversed by the ethanolic extract of Moringa oleifera(200mg/kg i.p). They finally confirmed that anticataleptic activity of Moringa oleifera can be due to its antioxidant potential or due to its effect on brain monoamines [65].

Arunachalam Muthuraman *et al*., (2011) investigated for protective effect of *Acorus calamus L. (AC)* in vincristine-induced painful neuropathy. Hydro-alcoholic extract of AC (HAE-AC, 100 and 200 mg/kg, p.o.) and pregabalin (10 mg/kg, p.o.) were administered for 14 consecutive days. Vincristine significantly induced neuropathic pain, mechanical hyperalgesia, allodynia , sciatic functional index and various biochemicals. Based on their study HAE-AC attenuated vincristine induced painful neuropathy, which may be attributed to anti-oxidative, anti-inflammatory, and calcium inhibitory actions [66].

David Raj Chellappan *et al.*,(2011) conducted Acute oral toxicity study as per OECD-423 guidelines and the extract was found to be devoid of any conspicuous acute toxicity in extract treated animals and no mortality upto 2 g/kg by oral route. Hydroalcoholic extract of *C. peltata* in a dose dependant manner (125 and 250 mg/kg. *p.o.*) showed significant gastric protection against the ethanol-induced gastric ulcer model in rats[67]

R.M.Santiago et al., (2010) reported the depressive like behaviour and neurotransmitter alterations in PD models induced by intranigral injection of MPTP, 6-OHDA and rotenone. The data indicate that MPTP, 6-OHDA and rotenone were able to produce anhedonia and behavioural despair. These altered behavioural responses were accompanied by reductions of striatal DA, homovanillic acid (HVA) and DOPAC restricted to the 6-OHDA group. Additionally, decreases on the hippocampal serotonin (5-HT) content were detected for the MPTP, 6-OHDA and rotenone groups[68].

Hyeri Kim *et al.,* (2008) evaluated the anti-inflammatory activity of *Acorus calamus leaf (ACL)* extract and to explore its mechanism of action on human keratinocyte HaCaT cells. HaCaT cells treated with polyinosinic: (polyI:C),(PGN) induced the inflammatory reactions. The anti-inflammatory activities of ACL were investigated using RT-PCR, ELISA assay, immunoblotting, and immunofluorescence staining. HaCaT cells induced the pro-inflammatory cytokines, IL-8, IL-6 expressions after treatment with polyI: C or PGN. Based on The results suggested that ACL inhibits the production of pro-inflammatory cytokines, effective in anti-inflammatory agent for the treatment of skin diseases[69] .

Fijesh P. Vijayan *et al.,* (2007) has evaluated the protective effect of a 70% methanolic leaf extract of *Cyclea peltata Lam* on cisplatin-induced renal toxicity. They measured the biochemical parameters in kidney tissues. The marked cisplatin-induced renal damage, characterized by a significant increased in biochemical enzymes level by the extract. They observed methanolic leaf extract could be used as a natural antioxidant against cisplatin-induced oxidative stress[70].

3. AIM AND OBJECTIVES

AIM

The aim of the present study is to evaluate the anti Parkinson activity of Ayurvedic formulation Saraswatham ghritham in Rotenone induced Parkinson mice.

OBJECTIVES:

- To standardize Ayurvedic Formulation Saraswatham Ghritham
- To evaluate anti Parkinson activity of saraswatham ghritham in Rotenone induced mice.
- To study the effect of treatment on Endogenous antioxidant enzymes
- To estimate effect of treatment on various Neurochemical parameters
- To evaluate effect of treatment on the histopathological changes in mice brain tissue

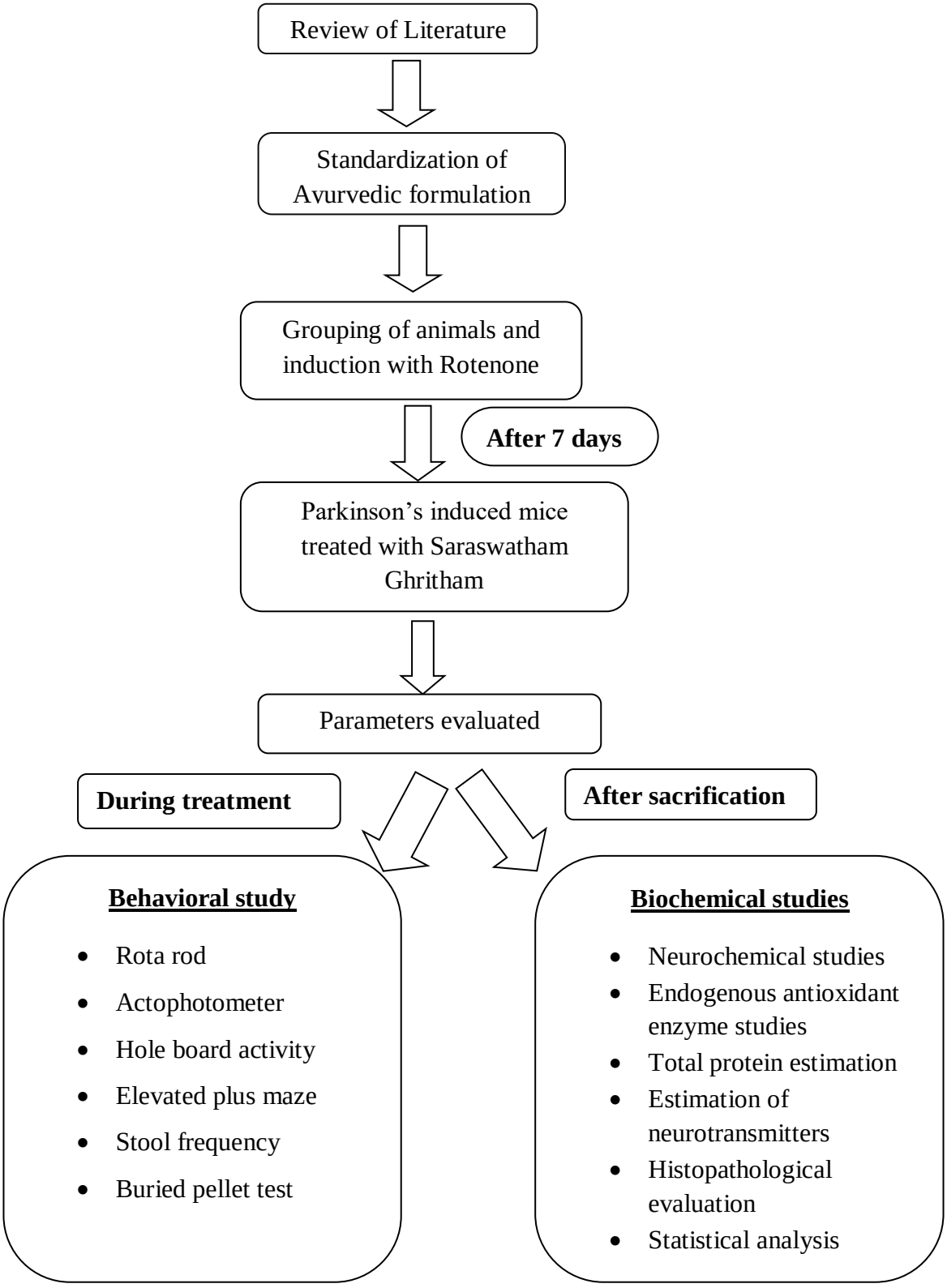
5. CONTENTS OF AYURVEDIC FORMULATION SARASWATHAM GHRITHAM

INGREDIENTS	QUANTITY IN EACH 10 GM	REPORTED ACTIVITY
Terminalia chebula (P.)	0.313 g	Antiparkinsonian activity Neuroprotective effect Anti-inflammatory, Antioxidant, anti-lipid peroxidative and membrane-stabilizing effects[31,32,33]
Zingiber officinale (Rz.)	0.313 g	Anti oxidant , anti Parkinson and neuroprotective effect[41,42,43]
Piper nigram (Fr.)	0.313 g	Anti inflammatory and antioxidative effect[44,45,46]
Cyclea peltata (Rt.)	0.313 g	Anti-oxidative, anti inflammatory and neuroprotective effect[36,37,38]
Acorus calamus (Rz.)	0.313 g	Anti-oxidative, Anti-inflammatory, and calcium inhibitory actions and Neuroprotective effects[34,35]
Moringa oleifera (Rt.Bk)	0.313 g	Antioxidant, anti catalytic and Neuroprotective effect[39,40]
Rock salt	0.310 g	Anti oxidant and anti inflammatory effect [49]
Goat's milk	10.9 ml	Rejuvenator and probiotics[47]
Ghee	10 g	Improve cognition[48]

Table 3: Contents of Ayurvedic Formulation Saraswatha Ghritham

Figure 4: Ayurvedic formulation saraswatham grutham and its label claim

4. PLAN OF WORK



Flow chart 1: schematic representation of project

6. MATERIALS AND METHODS

6.1 Procurement of Formulation

Ayurvedic formulation saraswatham ghritam (SG) was procured from Vaidyaratnam P.S. varier's ARYA VAIDYA SALA, kottakkal, kerala, india in month of October-2019.

6.2 Standardization of Ayurvedic Formulation

Physico- chemical studies like refractive index, specific gravity, acid value, saponification value, iodine value, determination of unsaponifiable matter, peroxide, viscosity, rancidity test and HPTLC were carried out as per the WHO guidelines, Ayurvedic Pharmacopoeia and Indian Pharmacopoeia [73,74,75] .

6.2.1.1Methodology:

The studies were done at KMCH College of Pharmacy, Coimbatore, tamilnadu,India as per standard procedure.

a. Refractive Index:

Placed a drop of water on the prism and adjusted the drive knob in such a way that the boundry line intersects the separatrix exactly at the centre. Noted the reading. Distilled water has a refractive index of 1.33217 at 28˚C. The difference between the reading and 1.3325 gives the error of the instrument. If the reading is less than 1.3320, the error is minus (-) then the correction is plus (+) if the reading is more, the error is plus (+) and the correction is minus (-). Refractive index of oil is determined using 1 drop of the sample. The correction if any should be applied to the measured reading to get the accurate refractive index. Refractive index of the test samples were measured at 28˚C.

b. Specific Gravity:

Cleaned a specific gravity bottle by shaking with acetone and then with ether. Dried the bottle and noted the weight. Cooled the sample solution to room temperature. Carefully filled the specific gravity bottle with the test liquid, inserted the stopper and removed the surplus liquid. Noted the weight. Repeated the procedure using distilled water in place of sample solution.

c. Acid Value:

Weighed 2-10 g of ghritha in a conical flask. Added 50 ml of acid free alcoholether mixture (25 +25ml) previously neutralised with the 0.1M potassium hydroxide solution and shaken well. Added One ml of Phenolphthalein solution and titrated against 0.1M Potassium hydroxide solution. End point is the appearance of pale pink colour. Repeated the experiment twice to get concordant values.

d. Saponification Value:

Weighed 2 g of the Amritaprasha ghritha into a 250 ml RB flask fitted with a reflux condenser. Added 25ml of 0.5M alcoholic potash. Refluxed on a water bath for 30 minutes. Cooled and added 1 ml of phenolphthalein solution and titrated immediately with 0.5 M Hydrochloric acid (a ml). Repeated the operation omitting the substance being examined (blank) (b ml). Repeated the experiment twice to get concordant values.

e. Iodine Value:

The sample was accurately weighed in a dry iodine flask. Dissolved with 10 ml of CCl4, 20 ml of iodine monochloride solution was added. Stopper was inserted, which was previously moistened with solution of potassium iodide and flask was kept in a dark place at a temperature of about 17 ºC for 30 min. 15 ml of potassium iodide and 100 ml of water was added and shaken well. This was titrated with 0.1N Sodium thiosulphate, starch was used as indicator. The number of ml of 0.1N sodium thiosulphate required (a) was noted. The experiment was repeated with the same quantities of reagents in the same manner omitting the substance. The number of ml of 0.1N sodium thiosulphate required (b) was noted. The experiment was repeated twice to get concordant values.

f. Determination of Unsaponifiable Matter:

Weighed 5 g of the Amritaprasha ghritha into the flask. Added 50 ml alcoholic KOH into the sample. Boiled gently but steadly under reflux condenser for one hour. The condensor was washed with 10ml of ethyl alcohol and the mixture was collected and transferred to a separating funnel. The transfer was completed by washing the sample with ethyl alcohol and cold water. Altogether, 50 ml of water was added to the separating funnel followed by an addition of 50 ml

petroleum ether. The stopper was inserted and shaken vigorously for 1 min and allowed it to settle until both the layers were clear. The lower layer containing the soap solution was transferred to another separating funnel and repeated the ether extraction six times more using 50 ml of petroleum ether for each extraction. All the extracts were collected in a separating funnel. The combined extracts were washed in the funnel 3 times with 25 ml of aqueous alcohol and shaked vigorously. And drawing off the alcohol-water layer after each washing. The ether layer was again washed repeatedly with 25 ml of water until the water no longer turns pink on addition of a few drops of Phenolphthalein indicator solution. The ether layer was transferred to a tarred flask containing few pieces of pumice stone and evaporated to dryness on a water bath. Placed the flask in an air oven at 85 ℃ for about 1 h to remove the last traces of ether. A few ml of acetone was added and evaporated to dryness on a water bath. Cooled in a desicator to remove last traces of moisture and then weighed.

g. Peroxide Value:

5 g of the Amritaprasha ghrita was weighed accurately into a conical flask, added 30 ml of mixture of 3 volumes of glacial acetic acid and 2 volumes of chloroform, added 0.5 ml of potassium iodide, allowed it to stand for 1 minute, add 30 ml of water titrate gradually with vigorous shaking with 0.1M sodium thiosulphate until the yellow color disappears. Add 0.5 ml of starch indicator continued the titration until blue color disappears.

Peroxide value= 10(a-b) / W

Where W= weight in g of the substance

h. Viscosity:

The given sample is filled in a U tube viscometer in accordance with the expected viscosity of the liquid so that the fluid level stands within 0.2 mm of the filling mark of the viscometer when the capillary is vertical and the specified temperature is attained by the test liquid. The liquid is sucked or blown to the specified height of the viscometer and the time taken for the sample to pass the two marks is measured.

Viscosity is measured using the formula:

$$\eta 1 = \frac{\rho 1 t 1 \text{ X } \eta 2}{\rho 2 t 2}$$

η1 – Viscosity of sample

η2 - Viscosity of water

t1 and t 2- time taken for the sample and water to pass the meniscus

ρ1 and ρ2 – Density of sample and water

X= Specific gravity of sample x 0.9961/specific gravity of water

η = Xx Time for samplex1.004/specific gravity of water x70sec

i. Rancidity Test:

1 ml of melted fat was mixed with 1ml of conc. HCl and 1 ml of 1% solution of phloroglucinol in diethyl ether and then mixed thoroughly with the fat acid mixture. A pink color indicates that the fat is slightly oxidized while a red color indicates that the fat is definitely oxidized.

j. Sample Preparation for HPTLC:

Sample obtained in the procedure for the determination of unsaponifiable matter is dissolved in 10 ml of chloroform this was followed for all the sample of Amritaprasha ghritha, and chloroform soluble portion was used for HPTLC. HPTLC: 4, 8 and 12 µl of the above sample of Amritaprasha ghrita was applied on a precoated silica gel F254 on aluminum plates to a band width of 8 mm using Linomat 5 TLC applicator. The plate was developed in toluene - ethyl acetate (9:1) and the developed plates were visualized under short UV, long UV, and after derivatisation in vanillin-sulphuric acid spray reagent it was visualized under white light and scanned under UV 254 nm, 366 nm and 620 nm. Rf , colour of the spots and densitometric scan were recorded.

6.3 Evaluation of Anti-Parkinson Study of Ayurvedic Formulation Saraswatha Ghrita

Species	Swiss albino mice
Age	3 months
Body weight	25-30g
Gender	Male
No of animals	46

Table 4: Selection of animal for MPTP induced Parkinson evaluation

Male Swiss albino mice 3 month of age, and 25-30 g body weight were offered by KMCH College of Pharmacy, Coimbatore. All the rats were kept at room temperature and allowed to acclimate in standard conditions less than 12 hr light/ 12 hr dark cycle in the animal house. Animals are fed with commercial pellet diet and water ad libitum freely throughout the study. The experimental procedure was approved by IAEC (Institution of Animal Ethical Committee).

6.3.1 EXPERIMENTAL DESIGN FOR ROTENONE INDUCED PARKINSON DISEASE

GROUP	NO OF ANIMALS	GROUP SPECIFICATIONS
GROUP I	8	Vehicle control
GROUP II	8	Standard Rotenone + levodopa 12mg/kg/selegiline10mg/kg *i.p*)
GROUP III	8	Only Rotenone 3mg /kg *i.p*
GROUP IV	8	Low dose (saraswatham ghritam 100 mg/kg oral)
GROUP V	8	Medium dose (saraswatham ghritam200 mg/kg)
GROUP VI	8	High dose (saraswatham ghritam 400 mg/kg)

Table 5: Experimental design for Rotenone induced Parkinson

6.3.2 Induction of Parkinson Disease [76]

6.3.2.1 Preparation and induction of ROTENONE solution

The Rotenone was purchased from sigma chemicals, Mumbai, India and was stored according to the manufacturer label (-20° C) to prevent its decomposition. The Rotenone solution was freshly prepared at 3 mg/kg. The Rotenone was dissolved in 2 % DMSO in normal saline and adjusts to pH 7.4 with potassium hydroxide. Rotenone injected *i.p* at the dose of 3 mg/kg body weight, 7 days. The solution should be used immediately after preparation. Rotenone solution is stable only for a period of 24 hours at 25˚C.

6.3.2.2 Preparation of Levodopa and Benzerazide

12mg/kg of levodopa suspension was freshly prepared by using 1% gum acasia and 10 mg/kg of selegilne was dissolved in distilled water. Levodopa and Selegiline was freshly prepared daily and given via oral to the standard group II for 21 days.

6.3.2.3 Preparation of sample

100 mg/kg, 200 mg/kg and 400 mg/kg of saraswatham ghritam were dissolved in natural ghee as a vehicle and it was daily prepared freshly and given via oral route to group IV, V, and VI respectively for 21 days.

6.4 Evaluation of behavioral parameters

6.4.1 MOTOR CO-ORDINATION TEST (ROTA ROD TEST)[77]

Principle

The rota rod performance test is carried out on a rotating rod that provides forced motor activity in animals. The animals were placed on a rotating rod which is placed horizontally, suspended above a cage floor, which is high enough to induce avoidance of fall. Animals naturally try to stay on the rotating rod avoid falling to the ground. The length of time (duration) the animal stay on the rod without falling, gives a measure of their coordination, balance, physical condition and motor-planning.

Procedure

Motor Co-ordination test was conducted using rota rod apparatus. Animal was placed individually on the rotating rod and trained for 3 min trail at 25 rpm on the day before the first day of testing. A cut off time of 180s was fixed and each animal performed 3 separate trials at 5 min interval. After each trial, 5 min rest period was given to alleviate stress and fatigue. Motor coordination can be tested by comparing the latency to fall on the very first trial between treatment groups. The time taken by animals to fall from the rotating rod was noted.

6.4.2 LOCOMOTOR ACTIVITY ON ACTOPHOTOMETER [78]

Principle

The locomotor activity (horizontal activity) can be easily measured using an actophotometer which operates on photoelectric cells which are connected in circuit with a counter. When the beam of light falling on the photocell is cut off by the animal, a count is recorded. An actophotometer could have either circular or square area in which the animal moves.

Procedure

The spontaneous locomotor activity of each animal was recorded individually, using Actophotometer. The apparatus was placed in a sound attenuated and ventilated room during the testing period. All the animals were placed individually in the activity cage for 3 min to habituate them before starting actual locomotor activity task for the next 3 min. the basal activity score was noted. The units of the activity counts were arbitrary and based on the beam breaks by movement of the animal. Counts/3 min is used as an index of locomotor activity.

6.4.3EXPLORATORY BEHAVIOR ON HOLE BOARD TEST [79]

Principle

When a animal is placed on the hole board apparatus, which is elevated to 25 cm from the base, shows anxiety as it is exposed to a new environment, thus showing characteristics head poking behaviour. Decrease in anxiety shows increased exploration of the holes. Whereas increased anxiety shows lower number of head poking.

Procedure

The hole board apparatus consist of a wooden board (40*40cm) placed 25 cm above the ground. It consists of 16 holes which is about 3 cm in diameter, spaced symmetrically in a diamond pattern. Animals were placed on the corner of the apparatus and were observed for the next 5 min for the number of head dipping. A head dipping is counted when the animal introduces its head into any hole of the box up to the level of the ears. The apparatus was thoroughly cleaned between each subject.

6.5 ESTIMATION OF BRAIN NEUROTRANSMITTER

6.5.1 Estimation of Serotonin, GABA and Dopamine [80, 81,82]

Preparation of tissue extracts

Reagents

- ✓ HCl – Butanol solution: (0.85 ml of 37% hydrochloric acid in one-litre n-butanol)
- ✓ Heptane
- ✓ 1 M HCl: (0.85 ml conc. HCl up to 100 ml H2O)

Procedure

At the end of experiment, rats were sacrificed and the whole brain was dissected out. 0.25 g of tissue was weighed and was homogenized in 5 mL HCl–butanol with motor driven Teflon coated homogenizer for about 1 min. The sample was then centrifuged for 10 min at 2000 rpm. An aliquot supernatant phase (1 mL) was removed and added to centrifuge tube containing heptane (2.5 mL) and 0.1 M HCl (0.31 mL). After 10 min of vigorous shaking, the tube was centrifuged under the same conditions as above in order to separate the two phases, and the overlaying organic phase was discarded. The aqueous phase was then taken either for 5-HT or NA and DA assay.

6.5.1.1 Estimation of dopamine

Reagents

- ✓ 0.4 M HCl: 0.34 ml conc. HCl up to 10 mL H2O
- ✓ Sodium acetate buffer (pH 6.9): 0.72 mL of 1 M acetic acid (6 µL of glacial acetic acid up to 1000 µL with distilled water) + 6.84 mL of 0.3 M sodium acetate (0.408 g of sodium acetate in 10 mL distilled water) and volume were made up to 25 mL with distilled water. pH was adjusted with sodium hydroxide solution.
- ✓ 5 M sodium hydroxide: 5 g of NaOH pellets dissolved in distilled water and volume was made up to 25 mL with distilled water.
- ✓ 0.1M Iodine solution (in Ethanol): 1 g of potassium iodide + 0.65 g of iodine dissolved in ethanol and volume was made up to 25 mL.
- ✓ Sodium thiosulphate solution: 0.625 g Na2SO3 in 2.5 mL H2O + 22.5 mL 5 M NaOH
- ✓ 10 M Acetic acid: 14.25 mL of glacial acetic acid dissolved in distilled water and made up to 25 mL.

Procedure

To 1 mL of aqueous phase, 0.25 mL 0.4 M HCl and 0.5 mL of Sodium acetate buffer (pH 6. 9) were added followed by 0.5 mL iodine solution (0.1 M in ethanol) for oxidation. The reaction was stopped after 2 min by the addition of 0.5 mL Na2SO3 solution. 0.5 mL Acetic acid was added after 1.5 min. The solution was then heated to 100°C for 6 min. When the sample reached room temperature, excitation and emission spectra were read from the spectrofluorimeter. The readings were taken at 330-375 nm for dopamine. Blanks for the assay were prepared by adding the reagents of the oxidation step in reversed order (sodium sulphite before iodine). Different concentration of dopamine and nor-adrenaline (1 mg/ml) was used as standard.

6.5.1.2Estimation of Serotonin

The serotonin content was estimated by the OPT method

Reagents

- ✓ O-phthaldialdehyde (OPT) reagent: (20 mg in 100 ml conc. HCl)

Procedure

To 1.4 mL aqueous extract, 1.75 mL of OPT reagent was added. The fluorophore was developed by heating to 100°C for 10 min. After the samples reached equilibrium with the ambient temperature, readings were taken at 360-470 nm in the spectrofluorimeter. Concentrated HCI without OPT was taken as blank. Serotonin (1 mg/mL) at different concentration was used as standard.

6.5.2 Estimation of brain GABA content

Preparation of tissue homogenate Animals were sacrificed by decapitation and the whole brain was rapidly removed. 0.5 g tissue was weighed and placed in 5 mL of ice-cold TCA (10% w/v). The tissue was then homogenized and centrifuged at 10,000 rpm for 10 min at 0°C. The supernatant was used for estimation of GABA content.

Reagents:

- ✓ Carbonate-bicarbonate buffer, 0.5 M (pH 9.95): 1.0501 g sodium bicarbonate and 1.3249 g sodium carbonate dissolved in distilled water and made up to 25 ml. pH adjusted to 9.95 if necessary.
- ✓ 0.14 M ninhydrin solution: 499 mg ninhydrin dissolved in 0.5 M carbonatebicarbonate buffer and made up to 20 ml.
- ✓ Copper tartarate reagent: 0.16% disodium carbonate, 0.03% copper sulphate and 0.0329% tartaric acid.

Procedure:

0.1 mL of tissue homogenate was placed in 0.2 mL of 0.14 M ninhydrin solution in 0.5 M carbonate-bicarbonate buffer (pH 9.95), and kept in a water bath at 60°C for 30 min. It was then cooled and treated with 5 mL of copper tartarate reagent. After 10 min fluorescence at 377/455 nm in a spectofluorimeter was recorded.

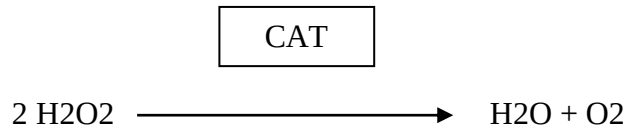
6.6 ESTIMATION OF ENDOGENOUS ANTI OXIDANT ENZYME LEVELS IN MICE BRAIN [83]

Procedure:

100 mg of the brain tissue was weighed and homogenate was prepared in 10ml tris hydrochloric acid buffer (0.5M; pH 7.4) at 4°C. The homogenate was centrifuged and the supernatant was used for the assay of cytoprotective enzymes namely catalase, superoxide dismutase, glutathione reductase and lipid peroxidation.

6.6.1 CATALASE: [CAT]

Principle:



In presence of CAT, H_2O_2 shows a continual decrease in absorbance when measured in UV range. The decomposition of H2O2 can be followed directly by the decrease in absorbance at 240nm (E240=0.00394±0.0002litres mmol-1mm-1). The difference in Absorbance (ΔA 240) per unit time is a measure of the CAT activity.

Reagents:

1. Preparation of Phosphate Buffer (PB):

- ✓ KHPO4 (Potassium dihydrogen phosphate): 1.703 g made upto 250 ml
- ✓ Na_2HPO_4 (Disodium hydrogen phosphate): 1.773 g made upto 250 ml

100mL of KH2PO4 solution and 150 ml of Na2HPO4 was mixed & pH was adjusted to7.

2. Preparation of PB- H_2O_2 solution: 50 ml of PB + 500 µl H_2O_2

Critical step:

The absorbance of PB- H_2O_2 solution was checked and it should be between 0.3-0.5. If the absorbance lies below this range, then H_2O_2 should be added to increase absorbance and if the absorbance lies above this range, PB was added to decrease absorbance. Time course between 0 & 60 second was selected. Auto zero was selected at 240 nm with PB.

Procedure:

- 3ml of H_2O_2 PB solution was added to 50µl of tissue homogenate.
- The above solution was kept in cuvette and absorbance was taken at 240nm.

6.6.2 SUPEROXIDE DISMUTASE [SOD]:

Principle:

The activity of SOD was determined by the method based upon the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome at alkaline pH. Inhibition of the chromogen formation by superoxide dismutase is linear with increase in enzyme concentration.

Reagents:

- ✓ Sodium carbonate buffer 0.1 M (pH 10.2): 1.05g of Na2CO3 in 100 mL of distilled water.
- ✓ Adrenaline (bitartarate) (final concentration- 250 µM)

Procedure:

- 1.85ml of sodium carbonate buffer was taken in the cuvette and 50µl of tissue homogenate was added followed by 100µl of Adr directly to the cuvette kept in the UV cuvette holder.
- Absorbance was read at 295 nm. The SOD activity (U/mg of protein) was calculated using the standard plot. (Photometric method).

6.6.3 GLUTATHIONE REDUCTASE [GSH]:

Principle:

GSH is a non-protein compound containing sulfhydryl group in its structure. Ellman's reagent (5, 5'-dithiobis-(2-nitrobenzoic acid) or DTNB is reduced by sulfhydryl compounds to an intensely yellow colour compound. The absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration.

Reagents:

- ✓ 5% TCA (Trichloro acetic acid): 5g in 100 ml of distilled water.
- ✓ PBS (0.2M) (pH=8.0): 0.218g NaH2PO4 + 2.641g of Na2HPO4 in 100ml distilled water
- ✓ DTNB (0.6mM): 20mg in 50ml of phosphate buffer

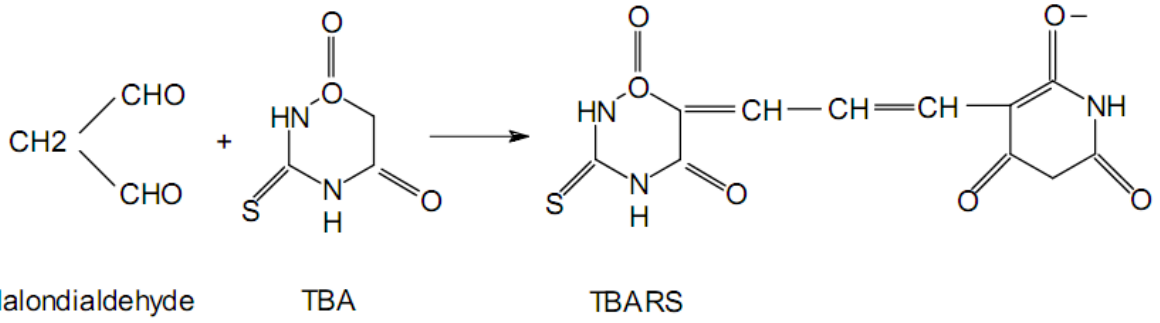
Procedure:

500 µl TCA solution was added to 500µl of tissue homogenate and then it was centrifuged. 500 µl of supernatant was Incubated with 3 ml of PBS and 500 µl of DTNB for 10min at room temperature. Absorbance was read at 412 nm.

6.6.4 LIPID PEROXIDATION:

Principle:

Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction. Malondialdehyde reacts with thiobarbituric acid to form red colour species (TBARS), which is measured at 535 nm.



Malondialdehyde TBA TBARS

Reagents:

- ✓ TBA-TCA-HCl reagent: [15%w/v TCA, 0.375%w/v TBA and 0.2ml of 0.25N HCl] this solution was mildly heated to assist the dissolution of TBA.

Procedure:

1ml of liver homogenate was combined with 2ml of TCA-TBA-HCl reagent and mixed thoroughly the solution was heated for 15min in a boiling water. bath. After cooling, the

flocculent precipitate was removed by centrifugation at 1000 rpm for 10min. The absorbance of the supernatant was measured at 532nm against a blank that contains all the reagents minus the liver homogenate. The malondialdehyde concentration of the sample can be calculated using an extinction coefficient of 1.56 x 105 M-1cm-1

6.7 Estimation of proteins

Requirements

- ✓ Alkaline copper reagent
- ✓ Solution A: 2% sodium carbonate in 0.1 N NaOH
- ✓ Solution B: 0.5% copper sulphate in 1% sodium potassium tartarate .50 ml of solution A was mixed with 1 ml of solution B just before use.
- ✓ Folin's phenol reagent (commercial reagent, 1:2 dilutions),
- ✓ Bovine serum albumin (BSA).

Principle

This method is a combination of both Folin-ciocalteau and biuret reaction which involves two steps

Step: 1 Protein binds with copper in alkaline medium and reduces it to Cu^{++}

Step: 2 The cu++ formed catalyzes the oxidation reaction of aromatic amino acid by reducing phosphomolybdotungstate to heteropolymolybdanum ,which leads to the formation of blue color and absorbance was measured at 640 nm.

Procedure:

To 0.1 ml of the homogenate, 0.9 ml of water, 4.5 ml of alkaline copper sulphate reagent were added and allowed to stand at the room temperature for 10 minutes. To this 0.5 ml of folin'reagent was added. After 20 minutes, the color developed was measured at 640 nm. The level of protein present was expressed as mg/g/ tissue or mg/dl.

6.8 Histopathological studies:

After behavioural and biochemical studies, the brains of different groups were perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and post fixed in the same fixative overnight at 48°C. The brains were embedded in paraffin and stained with Hematoxylin-Eosin. The hippocampus lesions were assessed microscopically at 40 magnifications

Fixation

Kept the tissue in fixative for 24-48 hours at room temperature

The fixation was useful in the following ways:

a) Serves to harden the tissues by coagulating the cell protein,

b) Prevents autolysis,

c) Preserves the structure of the tissue, and

d) Prevents shrinkage

Common Fixatives: 10% Formalin

Haematoxylin and eosin method of staining: Deparaffin the section by xylol 5 to 10 minutes and remove xylol by absolute alcohol. Then cleaned the section in tap water and stained with haematoxylin for 3-4 minutes and again cleaned under tap water. Allow the sections in tap water for few minutes and counter stained with 0.5% eosin until section appears light pink (15 to 30seconds), and then washed in tap water. Blotted and dehydrated in alcohol and cleared with xylol (15 to 30 seconds). Mounted on a Canada balsam or DPX Moutant and kept the slide dry and remove air bubbles.

6.9 Statistical analysis:

For in-vivo, the values are expressed as mean ± standard error of mean (SEM) of samples. All data were analyzed by one-way ANOVA followed by Dunnett's test using graph pad prism version 8.0software. The difference between the control and experimental groups were considered significant if, $p<0.05$.

7. RESULTS

7.1 STANDARDIZATION OF AYURVEDIC FORMULATION SARASWATHA GHRITA

Table 6: organoleptic characteristic of various sensory characters like colour, odour, taste etc,was carefully noted down in table.the SGstudied by organoleptic and morphological character like Rupa (colour), Rasa(taste), Gandha(odour), sparsha (touch), and so on.

1.	Colour	Muddy -Green
2.	Odour	Ghee like
3.	Taste	Low sweet
4.	Touch	Sticky
5.	Appearance	Viscous, semi solid

Table 7: Physiochemical parameter of SG:

S.NO	PARAMETER	RESUTS n=3 % w/w
1.	Specific gravity	0.9884
2.	Acid value	5.331
3.	Saponification value	112.63
4.	Iodine value	46.72
5.	Unsaponifiable matter(%)	1.003
6.	Peroxide value	0.18
7.	Rancidity	Fat is not oxidized

7.2 Screening of Anti Parkinson activity of SG by Rota Rod Test

7.2.a Rota Rod Test

The data obtained on the effect of treatment with various dose of SG on muscle grip strength is shown in Figures 5 and Table 8. The data reveal that induction with rotenone significantly decreases the muscle grip strength (13.500±0.342).Treatment with various doses of SG significantly increases muscle grip strength (34.833±0.654, 46.167±0.703, 11.000±0.258secs). Moreover, the highest dose of SG shows nearly equal significant effect as that of rotenone + levodopa/selegiline treated group (126.000±1.342secs) on 21 st day.

Table 8: Effect of various dose of SG (100 mg/kg ,200 mg/kg, 400 mg/kg) on muscle grip strength on rota rod. Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

GROUPS	Time spent on Rota rod (sec)		
	Day 7	Day 14	Day 21
CONTROL	178.167 ± 0.401***	178.167±0.401***	178.167±0.401***
ROTENONE+ LEVODOPA/SELEGILINE	38.667±0.211***	75.833±0.543***	126.000±1.342***
ROTENONE ONLY	14.667±0.715	13.833±0.401	13.500±0.342
LOW DOSE SG 100 mg/kg	17.667±0.211***	22.833±0.601**	34.833±0.654***
MEDIUM DOSE SG 200 mg/kg	21.667±0.422***	33.167±0.307***	46.167±0.703***
HIGH DOSE SG 400 mg/kg	31.000±0.477***	72.500±0.428***	111.000±0.258***

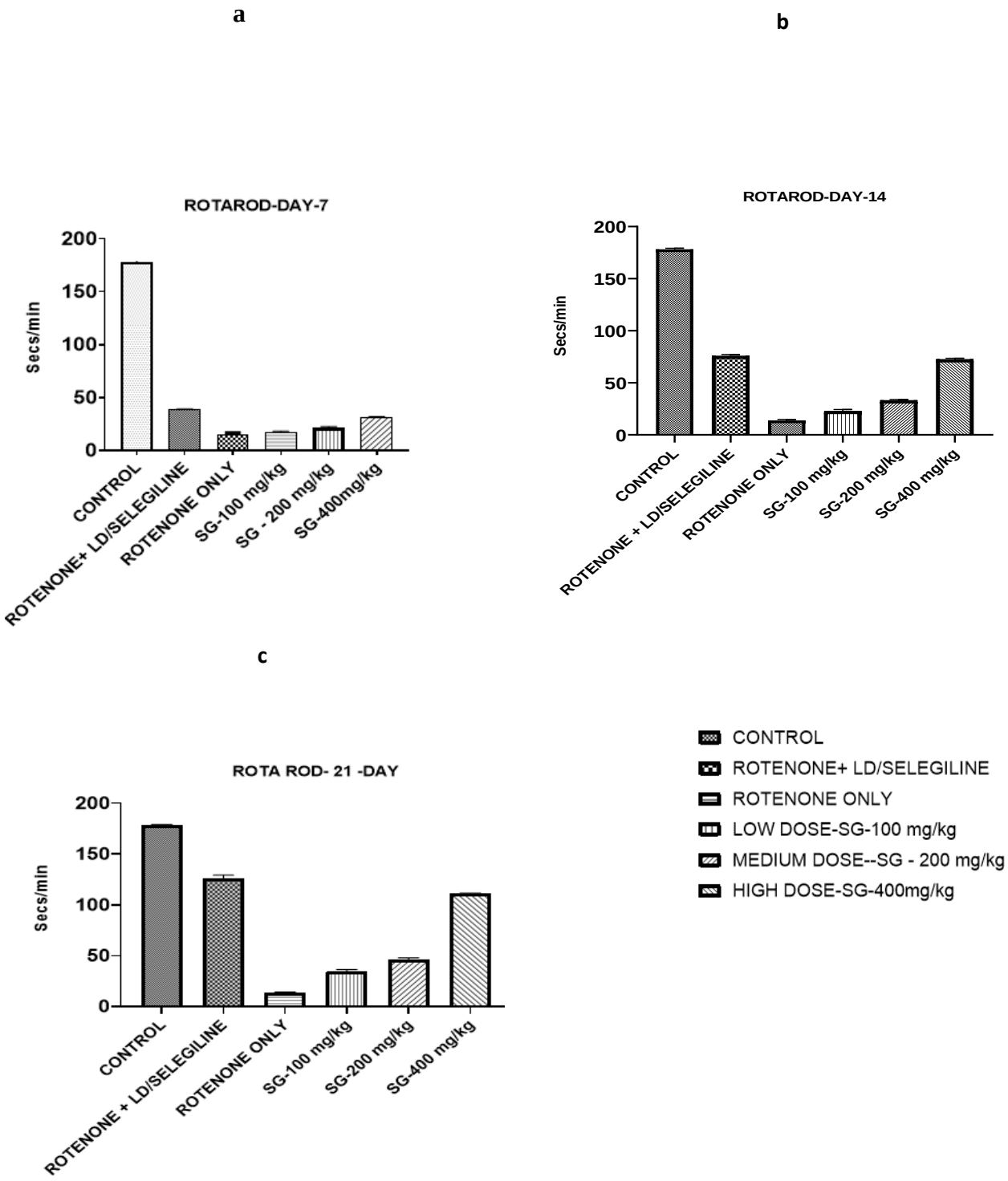


Figure 5: Effect of various dose of SG (100 mg/kg ,200 mg/kg and 400 mg/kg) on muscle grip strength on 7th 4 (a) , 14th4 (b) , 21 st 4 (c) day. Each value represents the mean ± SEM

(n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

7.2. b Screening of Anti Parkinson activity of SG by Hole Board Test

The data obtained on the effect of treatment with various dose of SG on muscle grip strength is shown in Figures 5 and Table 8. The data reveal that induction with rotenone significantly decreases the exploration behavior (5.333±0.211).Treatment with various doses of SG significantly increases exploration behavior (23.333±0.211, 37.000± 0.365 and 61.167±0.477). Moreover, the highest dose of SG shows nearly equal significant effect as that of rotenone + levodopa/selegiline treated group (69.500±0.764) on 21 st day.

Table 9: Effect of exploratory behavior of various dose of SG (100 mg/kg ,200 mg/kg, 400 mg/kg) on Hole board . Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01

GROUPS	Number of head pockings		
	Day 7	Day 14	Day 21
CONTROL	102.667±0.882***	104.333±2.275***	108.333±1.687***
ROTENONE+ LEVODOPA/SELEGILINE	39.167±0.307***	46.667±0.422***	69.500±0.764***
ROTENONE ONLY	5.333±0.211	5.500±0.224	5.333±0.211
LOW DOSE-SG (100 mg/kg)	11.500±0.500***	17.500±0.224***	23.333±0.211***
SG 200 mg/kg	20.667±0.333***	28.000±0.516***	37.000± 0.365***
SG 400 mg/kg	31.667±0.333***	40.500±0.224***	61.167±0.477***

and *** p<0.001 in comparison with Rotenone treated control group

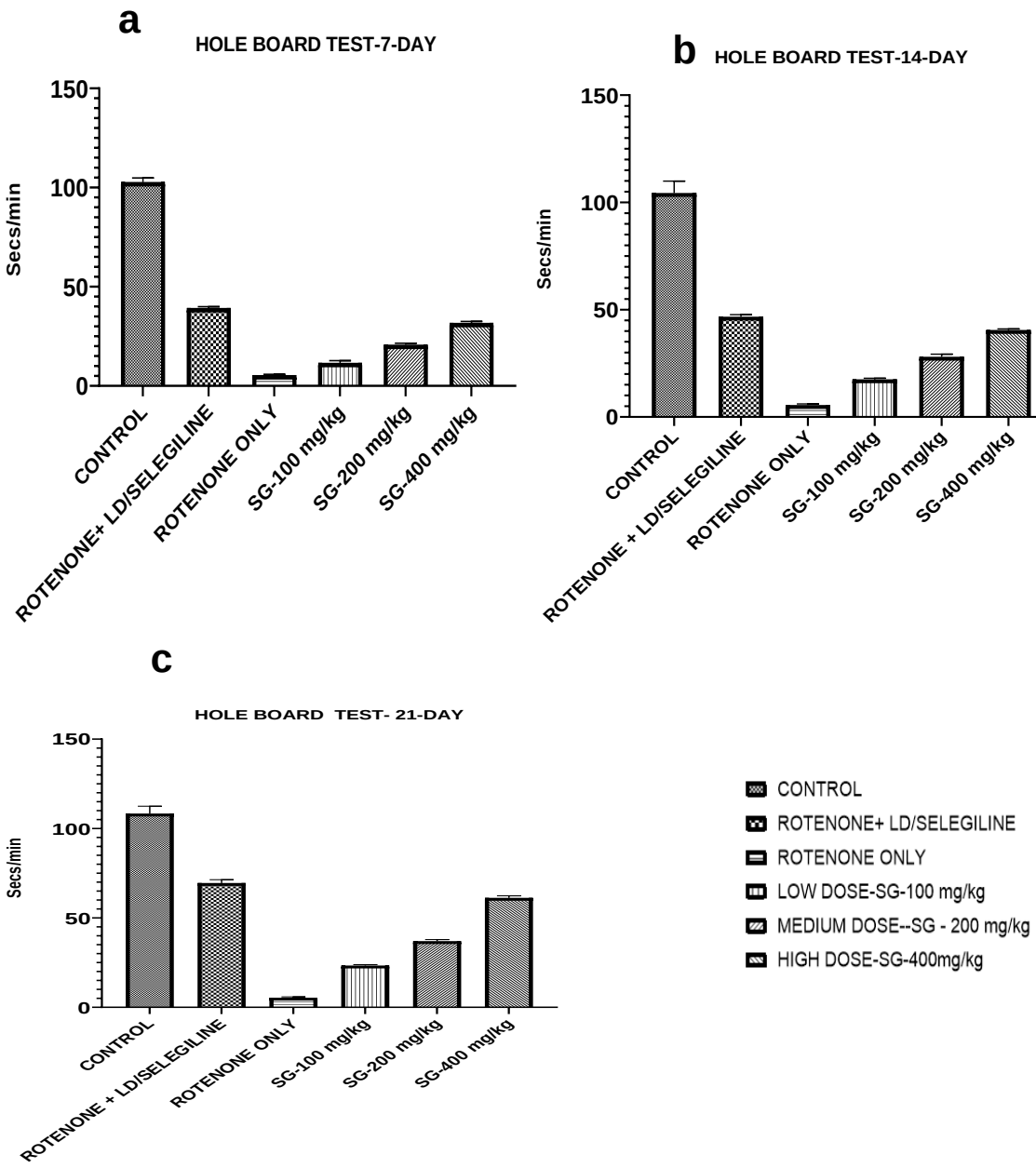


Figure 6: Effect of various dose of SG (100 mg/kg ,200 mg/kg and 400 mg/kg) on Hole Board test on 7th 4 (a) , 14th4 (b) , 21 st 4 (c) day. Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

7.2.c Screening of Anti Parkinson activity of SG by Burried Pellet Test

The data obtained on the effect of treatment with various dose of SG on olfactory assessment is shown in Figures 7 and Table 10. The data reveal that induction with rotenone significantly decreases the capacity of olfaction (25.167±0.167).Treatment with various doses of SG significantly increases the capacity of olfaction (11.333±0.211, 8.500±0.224, 5.500±0.224). Moreover, the highest dose of SG shows nearly equal significant effect as that of rotenone + levodopa/selegiline treated group (6.000±0.258) on 21 st day.**Table 10: Effect of olfactory assessment of SG on various dose (100 mg/kg, 200 mg/kg, 400 mg/kg). Each value represents the mean ± SEM (n=6). * p<0.1, **p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.**

GROUPS	Number of head pocking (mins/time)		
	Day 7	Day 14	Day 21
CONTROL	5.000±0.000***	5.000±0.000***	4.500±0.224***
ROTENONE+ LEVODOPA/SELEGILINE	15.000±0.000***	10.000±0.000***	6.000±0.258***
ROTENONE ONLY	25.000±0.000	25.5000±0.224	25.167±0.167
LOW DOSE-SG (100 mg/kg)	18.000±0.000***	16.333±0.21***	11.333±0.211***
MEDIUM DOSE -SG (200 mg/kg)	16.000±0.000***	12.333±0.333***	8.500±0.224***
HIGH DOSE-SG (400 mg/kg)	12.667±0.217***	10.000±0.000***	5.500±0.224***

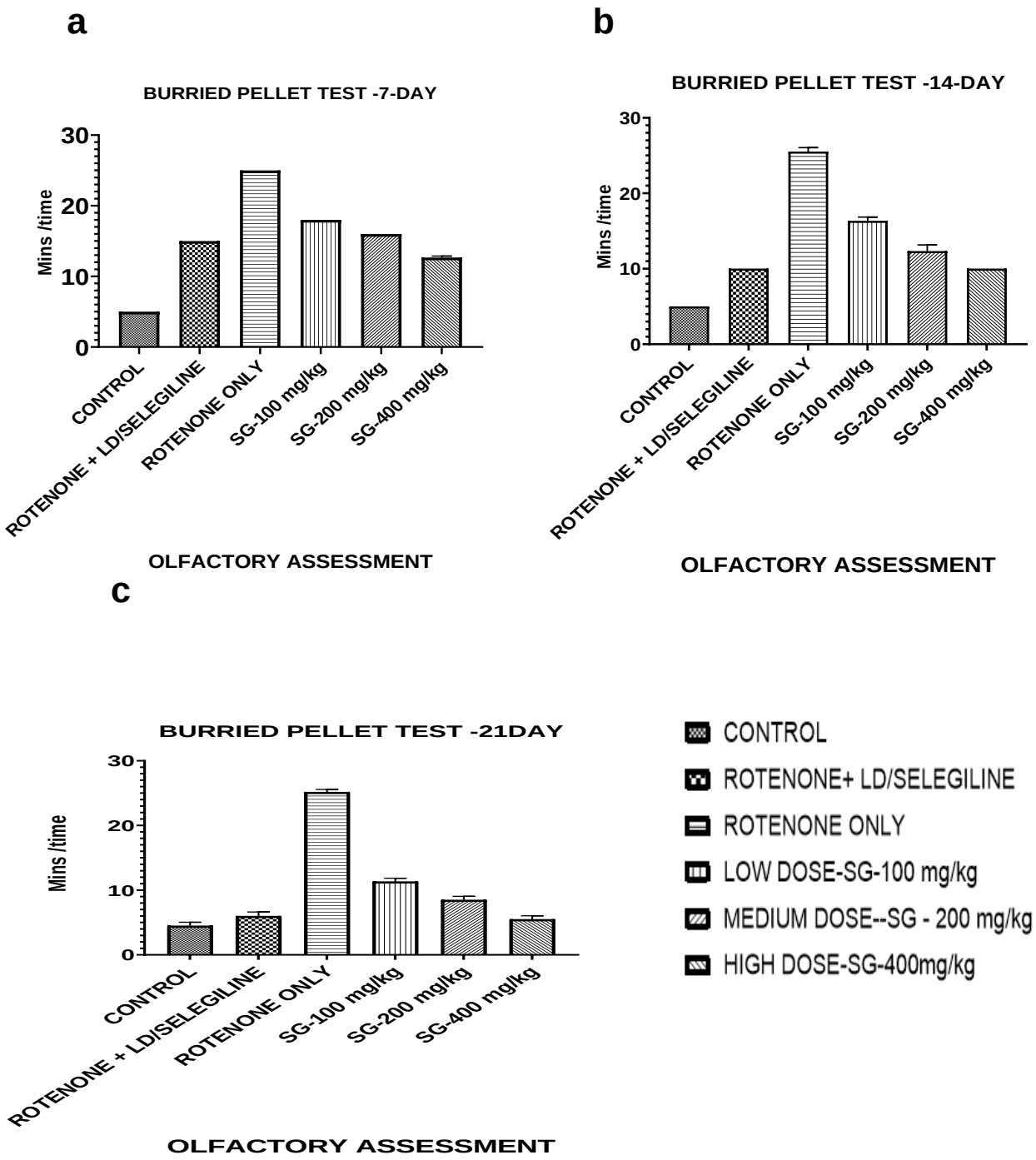


Figure 7: Effect of various dose of SG (100 mg/kg ,200 mg/kg and 400 mg/kg) on olfaction capacity on 7th 4 (a) , 14th4 (b) , 21 st 4 (c) day. Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

7.3 ESTIMATION OF NEUROTRANSMITTERS

7.3.a Effect of SG on brain dopamine level

The data obtained on the effect of treatment with various dose of SG on brain neurotransmitter dopamine is shown in Figures 8 and Table 11. The data reveal that induction with rotenone significantly decreases the dopamine level (88.872±0.234).Treatment with various doses of SG significantly increases (P<0.001) dopamine (low dose-94.578±0.197, medium dose-106.682±0.206 and high dose 128.380±0.341). Moreover, the highest dose of SG shows nearly equal significant effect as that of rotenone + levodopa/selegiline treated group (108.389±0.313).

7.3.b Effect of SG on brain serotonin level

The data obtained on the effect of treatment with various dose of saraswatham ghritham on brain neurotransmitter serotonin is shown in Figures 9 and Table 11. The data reveal that induction with rotenone significantly decreases the serotonin level (131.167±2.400).Treatment with various doses of SG significantly increases (P<0.001) Serotonin (low dose-167.333±1.085,mediumdose-179.833±0.910 and high dose- 194.000±0.730). Moreover, the highest dose of SG shows nearly equal significant effect as that of rotenone + levodopa/selegiline treated group (210.833±3.701).

7.3.c Effect of SG on brain GABA level

The data obtained on the effect of treatment with various dose of saraswatham ghritham on brain neurotransmitter GABA is shown in Figures 10 and Table 11. The data reveal that induction with rotenone significantly decreases the serotonin level (117.333±0.494).Treatment with various doses of SG significantly increases (P<0.001) GABA (low dose-167.333±1.085, medium dose- 179.833±0.910 and high dose-194.000±0.730). Moreover, the highest dose of SG shows nearly equal significant effect as that of rotenone + levodopa/selegiline treated group (156.833±0.307).

Table 11: Effect of of various dose of SG(100 mg/kg ,200 mg/kg, 400 mg/kg) on brain neurotransmitters like dopamine, serotonin,GABA Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

GROUPS	DOPAMINE (ng/gm tissue)	SEROTONINE (ng/gm tissue)	GABA (ng/gm tissue)
CONTROL	190.385±0.411***	269.083±2.087***	167.167±0.872***
ROTENONE+ LEVODOPA/SELEGILINE	108.389±0.313***	210.833±3.701***	156.833±0.307***
ROTENONE ONLY	88.872±0.234	131.167±2.400	117.333±0.494
LOW DOSE-SG (100 mg/kg)	94.578±0.197***	167.333±1.085***	125.500±0.342***
MEDIUM DOSE-SG (200 mg/kg)	106.682±0.206***	179.833±0.910***	135.500±0.428***
HIGH DOSE-SG (400 mg/kg)	128.380±0.341***	194.000±0.730***	157.167±0.749***

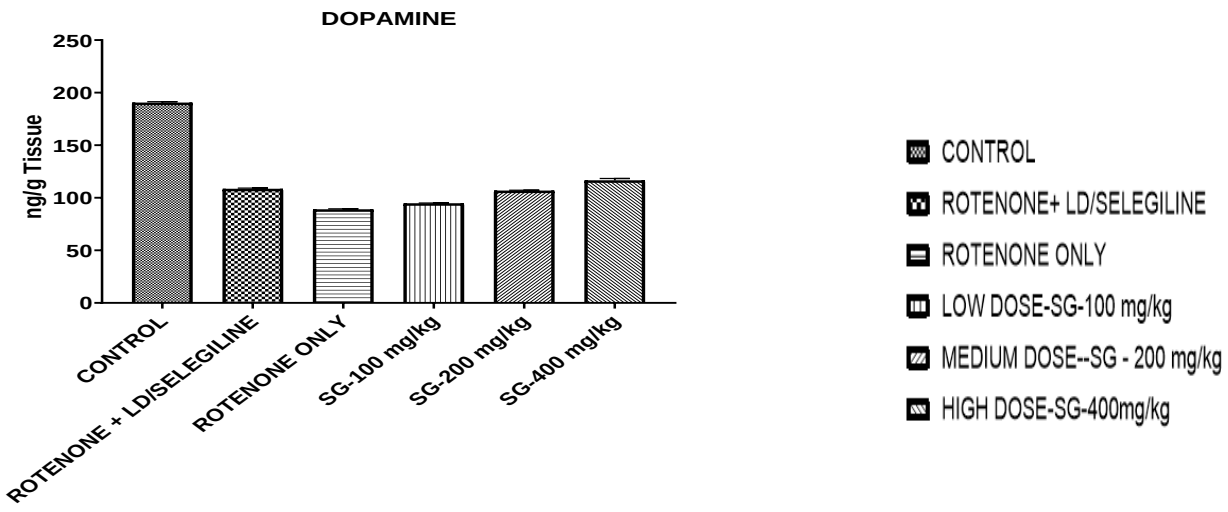


Figure8: Effect of of various dose of SG(100 mg/kg ,200 mg/kg, 400 mg/kg) on brain neurotransmitters like dopamine. Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

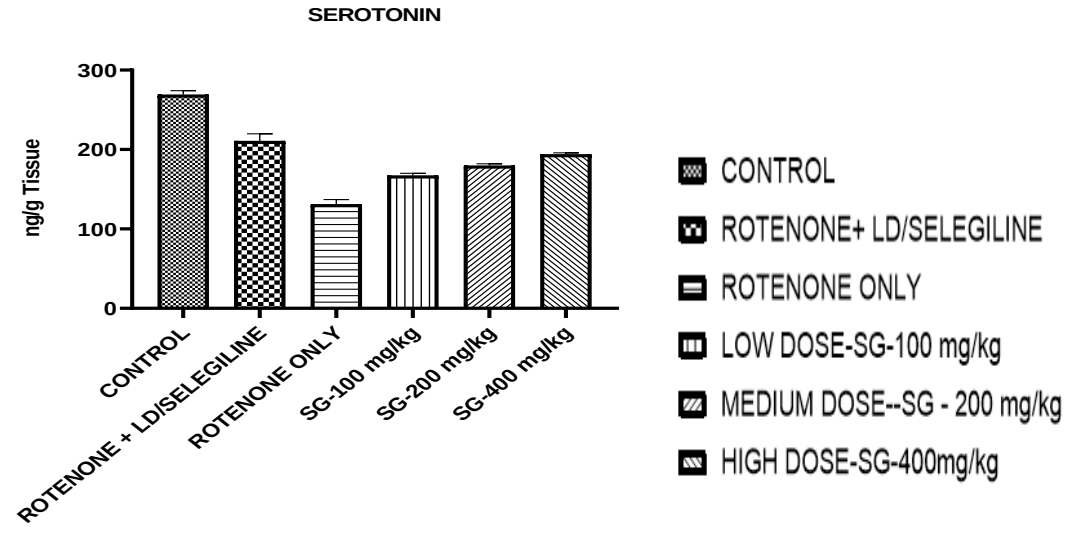


Figure 9: Effect of of various dose of SG (100 mg/kg ,200 mg/kg, 400 mg/kg) on brain neurotransmitters like serotonin. Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

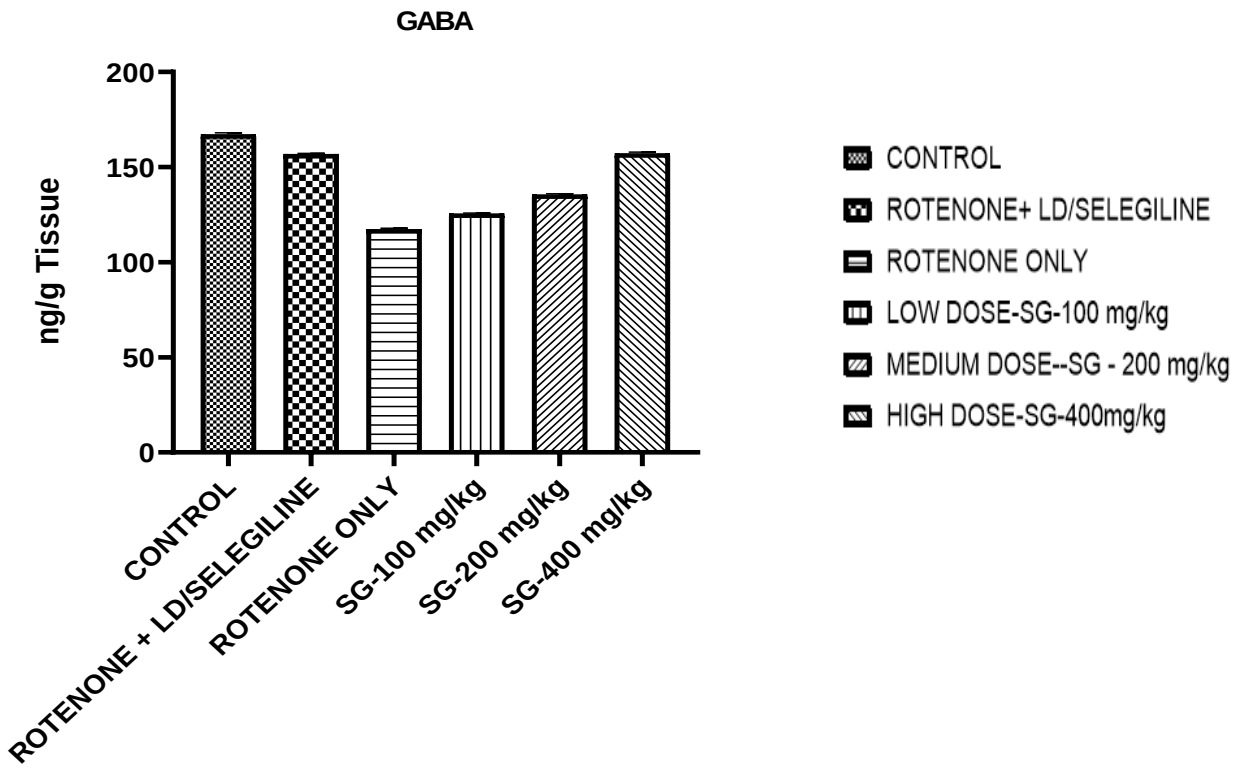


Figure 10: Effect of of various dose of SG (100 mg/kg ,200 mg/kg, 400 mg/kg) on brain neurotransmitters like GABA. Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

7.4 ESTIMATION OF TOTAL PROTEINS:-

7.4.a Effect of SG on brain protein level

The data obtained on the effect of treatment with various dose of saraswatham ghritham on brain neurotransmitter total proteins is shown in Figures 11 and Table 12. The data reveal that induction with rotenone significantly decreases the protein level (1.989±0.006).Treatment with various doses of SG significantly increases (P<0.001) the proteins (2.983±0.003, 3.667±0.070 and 4.079±0.052). Moreover, the highest dose of SG shows nearly equal significant effect as that of rotenone + levodopa/selegiline treated group (4.960±0.012).

Table 12: Effect of various dose of SG (100 mg/kg ,200 mg/kg, 400 mg/kg) on total proteins(mg/100mg of tissue). Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

GROUPS	LEVEL OF TOTAL PROTEIN (mg/100mg of tissue)
CONTROL	5.390±0.067***
ROTENONE+ LEVODOPA/SELEGILINE	4.960±0.012***
ROTENONE ONLY	1.989±0.006
LOW DOSE-SG (100 mg/kg)	2.983±0.003***
MEDIUM DOSE-SG (200 mg/kg)	3.667±0.070***
HIGH DOSE-SG (400 mg/kg)	4.079±0.052***

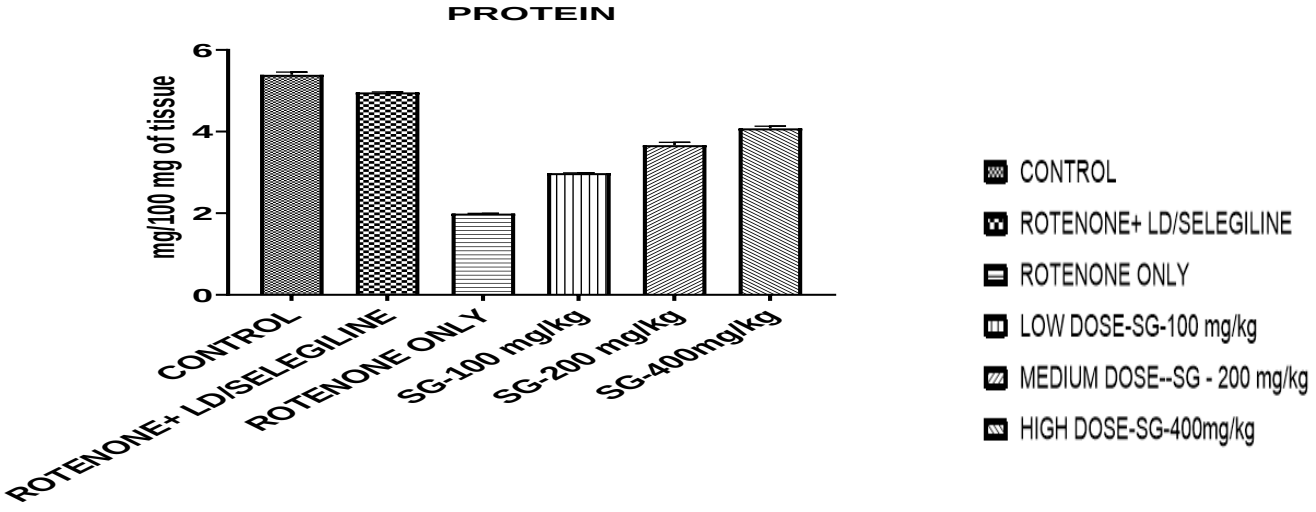


Figure 11: Effect of various dose of SG(100 mg/kg ,200 mg/kg, 400 mg/kg) on total proteins(mg/100mg of tissue). Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

7.5 Estimation of various anti oxidant enzyme levels

7.5.a Effect of SG on endogenous anti oxidant enzyme CAT level

The data obtained on the effect of treatment with various dose of saraswatham ghritham on brain endogenous anti oxidant catalase is shown in Figures 12 and Table 12. The data reveal that induction with rotenone significantly decreases the CAT level (5.553±0.064 U/mg of brain tissue).Treatment with various doses of SG significantly increases ($P<0.001$) the proteins (6.167±0.026, 7.090±0.042, 7.953±0.032 U/mg of brain tissue). Moreover, the highest dose of SG shows nearly equal significant effect as that of rotenone + levodopa/selegiline treated group (8.880±0.055 U/mg of brain tissue).

7.5.bEffect of SG on endogenous anti oxidant enzyme LPO level

The data obtained on the effect of treatment with various dose of saraswatham ghritham on brain endogenous anti oxidant LPO is shown in Figures 13 and Table 12. The data reveal that induction with rotenone significantly increases the LPO level (5.543±0.066 nmol of MDA/mg protein).Treatment with various doses of SG significantly increases ($P<0.001$) LPO (3.487±0.057, 3.134±0.013, 2.797±0.040 nmol of MDA/mg protein). Moreover, the highest dose of SG shows nearly equal significant effect as that of rotenone + levodopa/selegiline treated group (2.596±0.053 nmol of MDA/mg protein).

7.5.c Effect of SG on endogenous anti oxidant enzyme SOD level

The data obtained on the effect of treatment with various dose of saraswatham ghritham on brain endogenous anti oxidant SOD is shown in Figures 14 and Table 12.The data reveal that induction with rotenone significantly increases the SOD level (2.564±0.030 U/mg of brain tissue).Treatment with various doses of SG significantly increases ($P<0.001$) SOD (1.967±0.011, 1.553±0.064, 0.630±0.059 U/mg of brain tissue). Moreover, the highest dose of SG shows nearly equal significant effect as that of rotenone + levodopa/selegiline treated group (1.009±0.002 U/mg of brain tissue).

7.5.d Effect of SG on endogenous anti oxidant enzyme GSH level

The data obtained on the effect of treatment with various dose of saraswatham ghritham on brain endogenous anti oxidant GSH is shown in Figures 15 and Table 12. The data reveal that induction with rotenone significantly increases the GSH level (2.435±0.012 U/mg of brain tissue).Treatment with various doses of SG significantly increases (P<0.001) GSH (2.991±0.003, 3.053±0.046, 4.009±0.007U/mg of brain tissue). Moreover, the highest dose of SG shows nearly equal significant effect as that of rotenone + levodopa/selegiline treated group (4.075±0.037 U/mg of brain tissue).

Table 12: Effect of various dose of SG(100 mg/kg ,200 mg/kg, 400 mg/kg) on various endogenous antioxidant enzyme like CAT, LPO, SOD, GSH. Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group

Groups	CAT (U/mg of brain tissue)	LPO(nmol of MDA/mg protein)	SOD (U/mg of brain tissue)	GSH (µM/mg of brain tissue)
CONTROL	11.230±0.115***	1.725±0.127***	0.560±0.025***	5.494±0.075***
ROTENONE+ LEVODOPA/SELEGILINE	8.880±0.055***	2.596±0.053***	1.009±0.002***	4.075±0.037***
ROTENONE ONLY	5.553±0.064	5.543±0.066	2.564±0.030	2.435±0.012
LOW DOSE-SG (100 mg/kg)	6.167±0.026*	3.487±0.057***	1.967±0.011**	2.991±0.003*
MEDIUM DOSE-SG (200 mg/kg)	7.090±0.042**	3.134±0.013***	1.553±0.064**	3.053±0.046**
HIGH DOSE-SG (400 mg/kg)	7.953±0.032***	2.797±0.040***	0.630±0.059***	4.009±0.007***

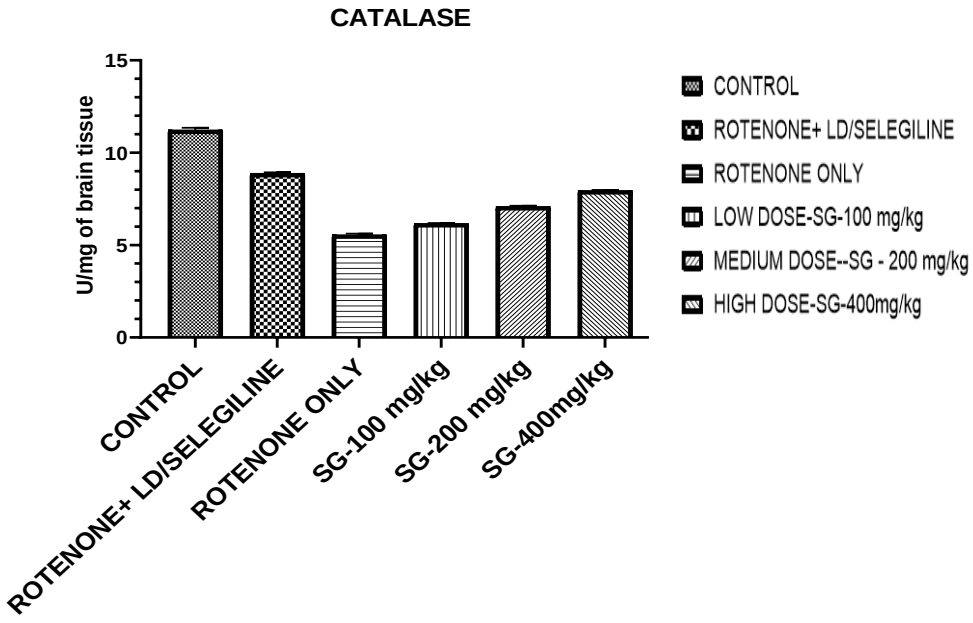


Figure 12: Effect of various dose of SG(100 mg/kg ,200 mg/kg, 400 mg/kg) on various endogenous antioxidant enzyme CAT. Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group

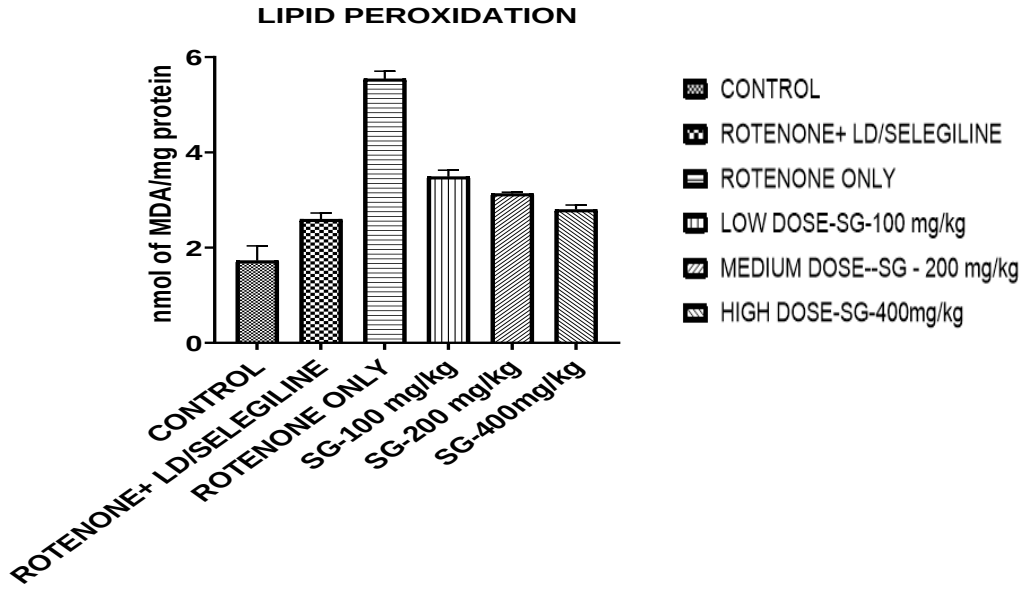


Figure 13: Effect of various dose of SG(100 mg/kg ,200 mg/kg, 400 mg/kg) on various endogenous antioxidant enzyme LPO. Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

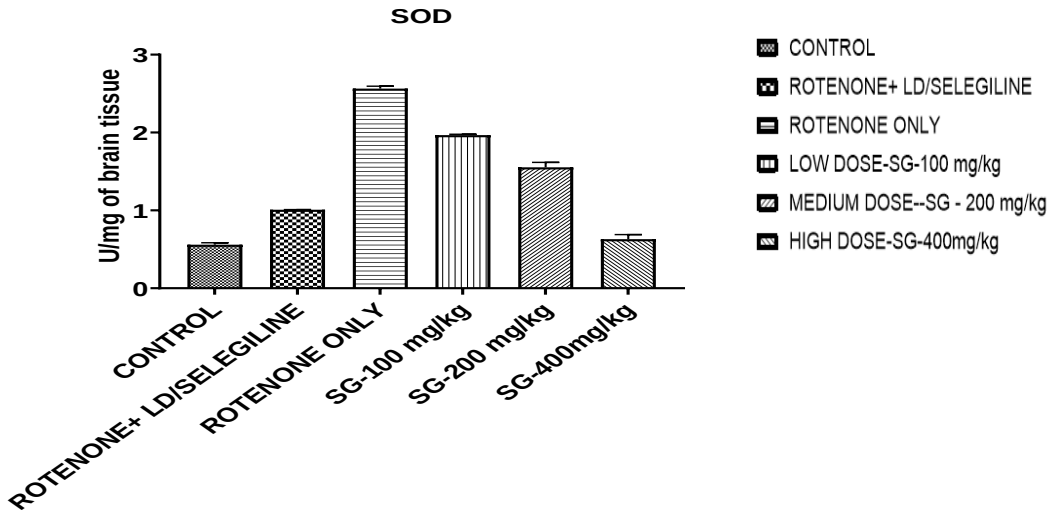


Figure 14: Effect of various dose of SG(100 mg/kg ,200 mg/kg, 400 mg/kg) on various endogenous antioxidant enzyme SOD.Each value represents the mean ± SEM (n=6). * p<0.1, ** p<0.01 and *** p<0.001 in comparison with Rotenone treated control group.

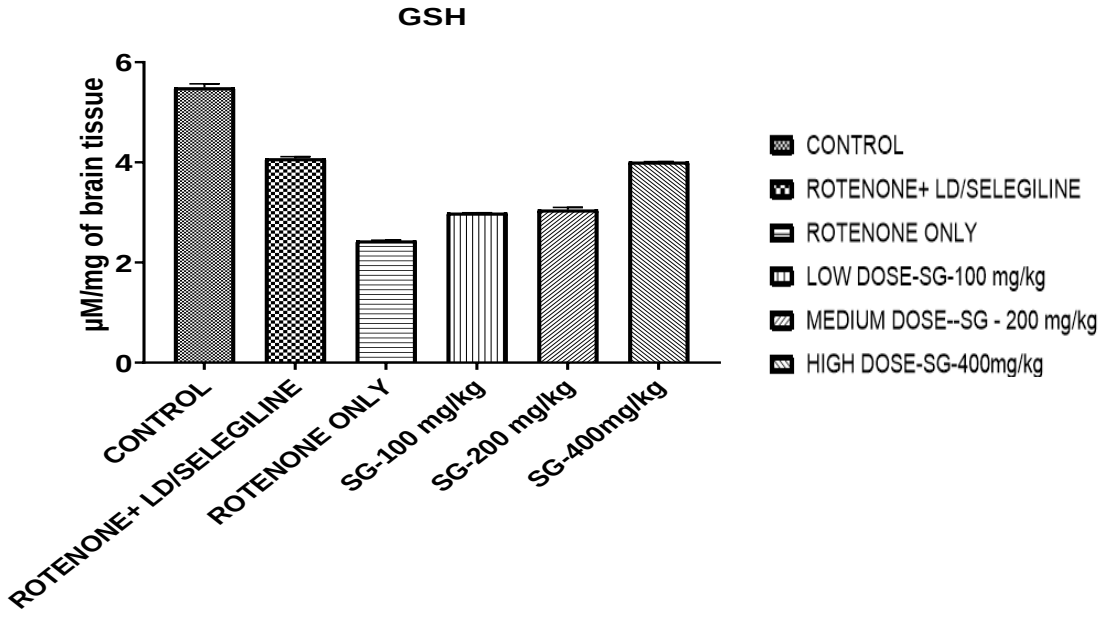


Figure 15: Effect of various dose of SG(100 mg/kg ,200 mg/kg, 400 mg/kg) on various endogenous antioxidant enzyme GSH .Each value represents the mean ± SEM (n=6). *

7.6 Effect of SG brain tissues of various groups of animals

The histopathology of mice from the control group(1) which shows normal astrocytes and glial cells.From the results the Mice brain cerebral cortex shows normal morphology.Brain tissue of standard group(2) which is treated with levodopa From the results Mice brain with cerebellum shows degeneration and mild gliosis. From the results the Rotenone induced(3) Mice brain cerebrum shows focal neuronal injury. Also show mononuclear inflammatory infiltration in the brain parenchyma with mild alter in the morphology.Brain tissue of test group which treated with lowest dose of SG(SG) (100 mg/kg-4). It shows normal astrocytes and glial cells. There is minimal reactive gliosis, with stromal oedema. The stroma showing minimal oedema and small foci of necrosis and inflammatory cell collections are seen.Brain tissue of test group with medium dose of SG (200 mg/kg-5). It also shows normal astrocytes and glial cell with small necrotic area than the lower dose treaded group.small areas of necrosis present in oedema,cerebellum also shows similar changes. It is the final group treated with highest dose of SG (400 mg/kg-6). It shows normal astrocytes and glial cells as normal tissue. Cerebellum also appear normal. One area shows choroid plexus which also appear normal

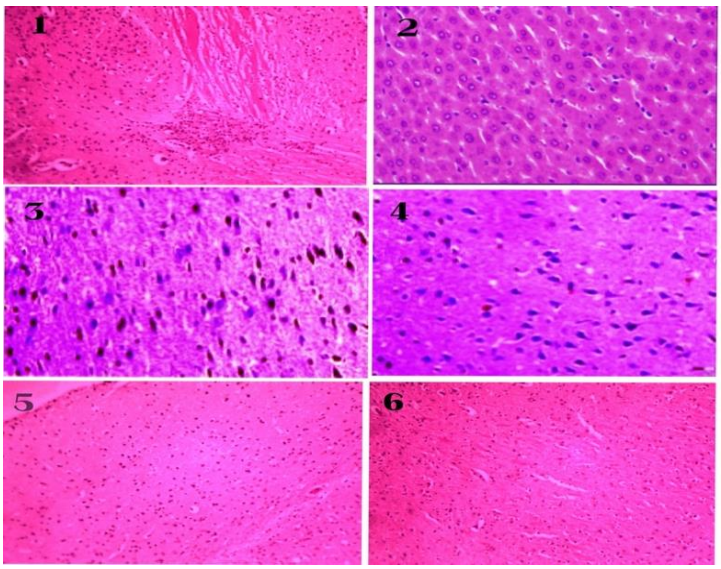


Figure 16: Histopathology of mice brain.

8. DISCUSSION

This study aims to evaluate the Anti-Parkinson activity of Ayurvedic formulation SG on rotenone induced Parkinson animal model by analyzing behavioral changes, estimation of catecholamine levels, various endogenous antioxidant enzymes levels and histopathological evaluation. PD is the second most neurodegenerative disease mainly occurred due to the degeneration of dopaminergic nigrostriatal pathway, which is cumulative effect of glutathione depletion, iron deposition, increased lipid peroxidation, oxidative DNA damage, mitochondrial dysfunction, and alterations in antioxidant enzymes activities. Although the etiology of PD is unknown, environmental factors, genetic predisposition, and genetic mutations have been shown to play an important role in the origin and development of the disease. It leads to develop the symptoms like the muscular incoordination, rigidity, tremor, bradykinesia, dyskinesia. Above symptoms of Parkinson disease are easily able to demonstrate on the Parkinson animal model.

The existing conventional strategies that are target the PD are associated with numerous side effects and economic burden also. In recent years there has been growing interest in alternative therapies, among that one of the leading choice is natural products. In Ayurveda medicinal plants and formulations are routinely used to cure neurodegenerative diseases mainly Parkinson's disease and its associated symptoms . In this context, Saraswatham ghrutham (SG), an Ayurvedic formulation which contains 7 major ingredients, *Terminalia chebula , Zingiber officinale*, *Acorus calamus, Piper longum, Piper nigram, Cyclea peltata and Moringa oleifera* which already reported for free radical scavenging activity, neuro protective and anti-inflammatory activity is one of the promising formulation for treating parkinsons disease mainly for reducing neuroinflammation has been explored in the present study.

On bases of initial observations, it is semi solid muddy green preparation made with decoction of 7 plant extractive juice in ghee, slightly sweetish, viscous, sticky and without sedimentation. Organoleptic evaluation of SG was performed, the results were satisfactory. The specific gravity of SG was 0.9884g/cm^3 showing the sample was not too dense. The saponification values was found to be 112% w/v, it gave an idea for molecular weight of an oil/fat. So this indicates SG contains lower molecular saturated fatty acids, which help in

better bioavailiablty. Higher the iodine value >55 w/v means the formulation is more susceptible to oxidation, free radical production, polymerization and rancidity. So iodine value intern reflects a products stability and shelf life. Here value is 46 so the product is stable. If acid values are high, then chances of photo oxidation and rancidity is more, in SG acid value is in permissible range 5.33. The rancidity test confirms there is no oxidation of fat, unrancid formulation. Fingerprint identity of SG by its unique R_f values was detected by HPTLC. So based on standardization tests the quality and identity of the product was confirmed for further animal tests.

After administration of rotenone in mice for a period of 7 days (3mg/kg ip) , the Parkinson's diseases was confirmed by various behavioral studies. Then based on protocol the various doses of SG were administrated. Rota rod test results were promising. As it is a standard apparatus for evaluation the muscle grip strength and muscle coordination. The loss of muscle grip is an indication of muscle weakness, which is classical symptom of Parkinson's disease. The differences in the fall off time from the rotating rod between the vehicle controls, rotenone treated control group and SG treated group taken as a index of muscle strength. Control group possess 100% of muscle grip strength. Rotenone treated group muscle grip strength is 8.3%, on 21 th day, this is a clear indication of muscle weakness with rotenone. Treatment with standard (levodopa and selegiline) and 100, 200, 400 mg/kg of SG, percentage of muscle grip strength is significantly increased compared with rotenone 81% ,21%,44% and 76%, resp.,

The hole board test is helpful for modeling anxiety in animals, in this test an anxiety like state may be reflected by an increase in head –dipping behaviors. After evaluation of the Hole board test readings the percentage of exploratory behaviour of Control group is 100%. In rotenone treated group there was a decreased percentage of exploration behavior 4.9%, which gives the picture of anxiety state induced by rotenone in mice. Treatment with standard (levodopa and selegiline) and 100, 200, 400 mg/kg of SG, percentage of exploration behavior is significantly increased compared with rotenone 63% and 21%,43%,59% resp.,

In early stages of Parkinson disease, the deficit in olfaction is a one of major issue. Buried pellet test is the classical screening test for evaluation of defect in malfunction of olfaction.

After evaluation of Buried pellet test readings the percentage of olfactory potential of Control group was 100%. In rotenone treated group there was a decreased percentage of olfactory potential 5%, which gives the picture of deficit in olfaction in rotenone induced mice. Treatment with standard (levodopa and selegiline) and 100, 200, 400 mg/kg of SG, percentage of olfactory potential was significantly increased compared with rotenone 53% and 23%, 46%, 88% resp., Results of actophotometer and elevated plus maze not significant in rotenone induced models. There was no variation after induction with rotenone.

Rotenone selectively damage the mitochondrial complex I, which cause changes in dopaminergic nigrostriatal system, resulting in changes in dopamine metabolites like DOPAC and homovanilic acid (HVA). So this causes the onset of motor symptoms, and there is a direct relationship between extent of dopamine loss and motor dysfunction. Many studies have revealed impaired behavioural responses within a short span of rotenone lessoned animals**.** Dopamine neurotransmitter was more affected in Parkinson's disease. Whereas other brain amines like norepinephrine, epinephrine and serotonin were much less affected than dopamine in Rotenone treated group.

Various dose SG treated group protective role of dopamine in the receptor binding density of mice is analyzed by spectroflourimetry dopamine levels when compared to the control group. Rotenone treated group dopamine level is 46 % on 21 th day, this is a clear indication of dopamine deficiency in rotenone treated group. Treatment with standard (levodopa and selegiline) and 100, 200, 400 mg/kg of SG, percentage significantly increased compared with rotenone 81% ,21%,44% and 76%, resp.,

The Rotenone treated group animal had 48.7% on 21 ST days. Rotenone are clearly decreased serotonin levels when compared to the control group.Treatment with standard drug(levodopa and selegiline) and 100, 200, 400 mg/kg of SG, percentage significantly increased compared with rotenone 78.37% and 62.18%, 66.00%,72.12% resp.,

The Rotenone treated group animal had 30% decreased Gamma –amino butyric acid (GABA) levels when compared to the control group on 21 ST day.Treatment with standard drug(levodopa and selegiline) and 100, 200, 400 mg/kg of SG, percentage significantly increased compared with rotenone 93% and 74.15%,80.75% and 91.12% resp.,

The neurotoxic effects of rotenone are mediated mainly through mitochondrial complex I (CI) of the electron transfer chain, on microtubules, and on proteosomes. CI of the mitochondrial respiratory chain is a critical initiator of the energy production process; deficits in this complex usually result in excessive generation of reactive oxygen species (ROS). It is a cardinal hallmark of PD. However, excess ROS production, as may occur with rotenone exposure, has the potential to cause cellular damage and may aid in generating further reactive species and initiating radical chain reactions. The motor deficits in Parkinsonian rat have been attenuated by antioxidant supplementation .One of the universally accepted etiologies of PD is the imbalance between free radical formation and the maintenance of the neuronal integrity through the endogenous antioxidant defense system resulting in oxidative stress. A surplus amount of free radical generation is thought to be the key module of neuronal damage in the brain. ROS threaten neuronal survival by their ability to propagate the initial attack on lipid rich membranes of the brain to cause lipid peroxidation.

Cell damage can be prevented by detoxification of free radicals, which eventually prevent the progress of LPO. In the present study we have observed an elevated level of LPO accompanied by a depleted GSH level in Rotenone induced PD mice brain. Our experimental finding reveals that treatment with various dose of SG reversing the elevated level of LPO and the depleted level of GSH which is concomitant with the previous observations where antioxidants were used as a remedy in experimental PD models. Oxidative stress to dopaminergic neurons of SNpc is believed to be one of the leading causes of neurodegeneration in PD. Oxidative stress promotes lipid peroxidation and alters the antioxidant defense system in the brain. The Rotenone treated group animal had 5.543±0.066 nmoles of MDA/mg protein increased LPO levels when compared to the control group when compared to the control group on 21 ST day.Treatment with standard drug(levodopa and selegiline) and 100, 200, 400 mg/kg of SG, percentage significantly

decreased compared with rotenone 2.596±0.053 and 3.487±0.057, 3.134±0.013, 2.797±0.040 nmoles of MDA/mg protein resp.,

GSH is a tripeptide in which thiol residue plays a major role on membrane protection. Its significantly depleted level may trigger the formation of lipid peroxidation and consequently disrupt the hemostat. A reduction in GSH content may also impair H_2O_2 and promote $^{*}OH$ formation. It has been further suggested that the decrease in GSH availability in the brain can promote mitochondrial damage by free radicals, and may result in selective inhibition of the complex-I activity. GSH plays a predominant role in removing excess of free radicals and hydroperoxidases and is a major defense system against oxidative stress in the brain. The Rotenone treated group animal had 2.435±0.012 µM/mg of brain tissue protein decreased the GSH levels when compared to the control group on 21 ST day.Treatment with standard drug (levodopa and selegiline) and 100, 200, 400 mg/kg of SG, percentage significantly decreased compared with rotenone 4.075±0.037 and 2.991±0.003, 3.053±0.046,4.009±0.007µM/mg of brain tissue resp.,

The catalase, which was found at a very low level of activity in the brain, detoxifies H2O2 to H2O. The loss of GSH and the formation of protein glutathione mixed disulfide (PrSSG) in the brain results in various membrane dysfunctions, such as inhibition on Na+K+-ATPase activity.The Rotenone treated group animal had 5.553±0.064 U/mg of brain tissue protein decrease the CAT levels when compared to the control group on 21 ST day.Treatment with standard drug (levodopa and selegiline) and 100, 200, 400 mg/kg of SG, percentage significantly increased compared with rotenone 8.880±0.055 and 6.167±0.026, 7.090±0.042, 7.953±0.032 U/mg of brain resp.,

SOD is upregulated in cells when O_2^- is produced in excessive levels. This observation suggests that SOD may play a role in the toxicity observed following acute treatment of rotenone, although ROS formation may not play a major role in rotenone -induced toxicity. The Rotenone treated group animal had 2.564±0.030U/mg of brain tissue protein decrease the SOD levels when compared to the control group on 21 ST day.Treatment with standard drug (levodopa and selegiline) and 100, 200, 400 mg/kg of SG, percentage significantly increased compared with rotenone 1.009±0.002 and 1.967±0.011,1.553±0.064, 0.630±0.059U/mg of brain tissue resp.,This study reports the therapeutic effect of an

ayurvedic formulation SG on acute rotenone induced behavioral and oxidative stress alterations in mice. Results of this study suggest SG is an effective compound for protection against rotenone induced motor impairment, ROS generation, cellular stress, and oxidative damage in the mouse brain. Histopathology results confirmed its effect on neuroinflamation. Necrosis, inflammatory cells and hemorrhage were clearly observed in rotenone induced brain tissue. But SG treated group brain tissues were perfectly normal. So this gives a clear confirmation that SG can be used for treatment of Parkinson disease.

9. CONCLUSION

From the present study, it can be considered that the Ayurvedic formulation Saraswatha Ghrita exhibited significant anti-parkinsonism activity in rotenone model in mouse. All the Parameters of formulation treated group animals have shown better results when compared with Rotenone-induced group and the standard L-dopa treated group.

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ABSTRACT

Parkinson's disease (PD) is a second most neurodegenerative disorder caused by the progressive loss of mesencephalic dopaminergic neurons in the substantia nigra innervating the striatum. The existing conventional strategies that target PD are associated with numerous side effects and possess an economic burden. In recent years, there has been a growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. The aim of the present study is to evaluate the anti Parkinson activity of Ayurvedic formulation Saraswatham ghritham (SG) in Rotenone induced Parkinson mice. For induction of parkinsons disese rotenone was injected i.p in mice at the dose of 3 mg/kg body weight, 7 days. After induction various doses of SG (100 mg/kg, 200 mg/kg and 400 mg/kg) were administrated (p.0) for 21 days. To find out its Antiparkinsons activity various behavioral studies, biochemical estimations, neurotransmitter evaluations and histopathalological studies have been performed. The results were promising like the behavioral studies the rota rode the high dose SG treated group (400mg/kg) the muscle grip strength(111.000±0.258 sec) was significantly increased compared to rotenone induced group (13.500±0.342). Treatment with SG results in regains the levels of endogenous antioxidant levels to normal values. Especially neuroprotective effect of SG was confirmed by histopathology. The inflammatory cells were absent , glial and astrocytes possess proper morphological features in SG treated group. So this study concludes the Antiparkinsons activity of saraswatha gritham.

Keywords: Saraswatha Ghrita , anti-parkinson and rotenone