Effect of Non-Invasive Neuro-Stimulation and Dietary Boosted Dopamine Levels in MPTP Induced Parkinson Mice Models

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Declaration

I certify that this research work titled "*Effect of Non-Invasive Neuro-Stimulation and Dietary Boosted Dopamine Levels in MPTP Induced Parkinson Mice Models"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

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I would like to dedicate this to my parents and grandparents for they are the ones who have always provided me with love and unconditional support.

Abstract

Parkinson's disease (PD) is one of the leading neurodegenerative disorders which involve degeneration of the dopaminergic neuron due to oxidative stress. The Centella asiatica whole plant extract (CAE) has been reported efficient as an anti-oxidant raising the glutathione levels in *Balb/c* mice model. Transcranial Direct Current Stimulation (tDCS) has also been approved effective for PD therapy in recent years. The present study was designed to investigate the effects of tDCS and CAE on the oxidative stress in mice model of PD induced by1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP).The animals were modulated by tDCS and orally treated with CAE. Behavioral alterations were observed after three weeks based on pole, grid and swim test. The mice were sacrificed for the measurement of the level of dopamine (DA) and reduced glutathione (GSH) in the brain and serum. The mice treated with MPTP had a reduced level of DA and decreased GSH activity, as well as behavior impairments.

Keywords: Parkinson's disease (PD), Centella asiatica Extract (CAE), Transcranial Direct Current Stimulation (tDCS),1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), Reduced glutathione level (GSH), Dopamine (DA).

Abbreviations

%	Percent
6-OHDA	6-Hydroxydopamine
ANOVA	Analysis of Variance
BDNF	Brain-derived Neurotrophic Factors
CAE	*Centella asiatica* Extract
CaMK	Calmodulin-dependent Kinases
$CHCl_3$	Chloroform or Trichloromethane
DA	Dopamine
DAT	Dopamine Transporter
DLPFC	Dorsolateral Prefrontal Cortex
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DTNB	5, 5'-Dithiobis (2-nitrobenzoic acid)
et al.	et alia
$FeCl_3$	Ferric Chloride
g	gram
GABA	gamma-Aminobutyric acid
GSH	Reduced Glutathione Level
H_2SO_4	Sulphuric Acid
HCl	Hydrochloric acid
IP	Intra- Paratonially
IRB	Internal Review Board
L-DOPA	L-3,4-dihydroxyphenylalanine
MAO-B	Monoamine Oxidase B
MAOBIs	Monoamine Oxidase type B Inhibitors
min	Minute
ml	milliliter
MPDP1	1-Methyl-4-Phenyl-2, 3-Dihydropyridinium
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NAD	Nicotinamide adenine dinucleotide
NaOH	Sodium Hydroxide
NH_3	Ammonia
NIH	National Institute of Health
nm	Nano-meters
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
OD	Optical Density
PARS	Protein Allosteric and Regulatory Sites
PBS	Phosphate-Buffered Saline
PD	Parkinson's Disease
pH	Power of Hydrogen
ROS	Reactive Oxygen Species
rTMS	Repeated-Transcranial Magnetic Stimulation
r-TNS	Transcranial random noise stimulation
SNpc	Substantia Nigra- pars compacta
Std.error	Standard Error
tACs	Transcranial Alternative Current Stimulation
TCA	Trichloroaceticacid
tDCs	Transcranial Direct Current Stimulation
TH	Tyrosine Hydroxylase
TMS	Transcranial Magnetic Stimulation
Tukey HSD Test	Tukey's Honest Significant Difference Test
UV	Ultra-violet
w/v	Weight/ Volume
α	alpha
β	beta
µg	micro gram
µl	microliter

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CHAPTER 1 : INTRODUCTION

Parkinson's disease is the second most common neurodegenerative disease in the world (Jain & Jain, 2018). It is caused by selective degeneration of dopaminergic neurons in the substantia nigra and reduced dopamine levels (Oertel & Fahn, 2003). Uncontrolled apoptosis takes part in underline mechanism of neuronal death that is responsible for many human neurodegenerative disorders including Parkinson's disease (Geng, 2007).

Parkinson's disease (PD) is characterized by bradykinesia (slowness of motion), rigidity (stiffness), resting tremor (uncontrolled shaking that can be restricted to a specific body segment like upper limbs or present in the entire body) (Wirdefeldt, Adami, Cole, Trichopoulos, & Mandel, 2011).

Parkinson's disease onset is mostly in 60s but with time it is emerging in younger individuals up to the age of 40s (Amin, Uddin, Rashid, & Sharmin, 2018).It is present worldwide and is estimated to effect 7-10 million people irrespective of the creed. In a worldwide epidemiological study of PD distribution was analyzed on the basis of age, geographic location, and gender. PD risk rises with age, as per geographical distribution a significant difference was seen in prevalence for individuals from ages 70 to 79 years with relatively more cases from North America, Europe and Australia compared with Asia. Males are relatively at a higher risk of PD than females (Pringsheim, Jette, Frolkis, & Steeves, 2014). Symptoms of PD are evident once 60% of the SNpc neurons have been compromised (Matsui & Takahashi, 2018). The cognitive abilities are compromised due to the effect of PD. The ability to concentrate on a task is undermined along with memory problems. Stress has an even worse effect on the onset and progression of PD by increasing the likelihood of further neuronal damage (Ou, Lin, Fang, & Liu, 2018).

Locomotor dysfunction is one of the primary clinical symptoms of PD that has been linked to the death of dopaminergic neurons (Xu et al., 2012). The axons are projected by the neurons towards the striatum and dopamine is utilized as their neurotransmitter. The reduced level of the dopamine is seen as the neurochemical imbalance in the onset of Parkinson's disease. The decreased expression of the pro-

apoptotic protein Bax results in neuronal apoptosis development (Fuxe, 1965; Vila et al., 2001).

The loss of dopaminergic neurons and reduced dopamine (DA) production results in unsteady body posture. The level of dopamine is reduced due to modulated tyrosine hydroxylase (TH), a precursor for dopamine. This enzyme is responsible for catalytic conversion of L-tyrosine to L-3, 4-dihydroxyphenylalanine (L-DOPA). PD is also characterized by increase in the level of peroxides and decrease in the level of antioxides (Lu et al., 2015). The bioenergetic and oxidative stresses in PD contribute to the neuronal cell death and decrease in the overall glutathione level (Cassarino et al., 1997).

The animal models are used to imitate the disease condition for experimentation. The *Balb/c* mouse model of PD produced by intra peritoneal administration of the neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) is widely used as the animal model (Bhatnagar, Goel, Roy, Shukla, & Khurana, 2017). MPTP is an inhibitor of the mitochondrial complex 1 that crosses the blood brain barrier and non-dopaminergic glial cells facilitate its conversion by using monoamine oxidase B into 1-methyl-4-phenylpyridinium (MPP+). MPP+ accumulates in substantia nigra causing complex I inhibition, leading to the cell death because of insufficient energy (Haleagrahara & Ponnusamy, 2010).

Dopamine replacement therapy is the main treatment for Parkinson's disease but it comes with side-effects like involuntary body movements, motor complications and likelihood of drug tolerance. TDCS is capable of modifying microglia activation in cerebral cortex of mice and rats. tDCs mode of action is multifactorial and elicits spontaneous neuronal activity rather than direct variation in resting membrane potential (Pikhovych et al., 2016).

Traditional herbal medication has been practiced for thousands of years and herbal plant extracts have biochemically been proved as a mixture of various antioxidant and neuroactive compounds (Shukla, Bhatnagar, & Khurana, 2012). Medicinal Molecules with anti-PD characteristics can turn tables in research development. The antioxidant activity of one such plant extract, *Centella Asitica* has shown promising results for preventing PD progression. Individual treatment with

madecassoside and asiaticoside, compounds isolated from *Centella asiatica*, have been reported to possess a neuroprotective role when tested in the rat model of Parkinsonism induced with MPTP (Xu, Qu, Zhang, Li, & Ma, 2013; Xu et al., 2012). Thus, in the present study, the MPTP mouse model was selected to evaluate the neuroprotective effects of combinatory therapy involving *Centella asiatica* extract (CAE) and trans-cranial direct current stimulation (tDCs) on an animal model of Parkinson's disease.

1.1- Thesis Overview

The rapidly increasing prevalence of Parkinson's disease is an alarming situation. The limitations of drug based treatment have opened doors for the research in more natural and non-invasive methods. It is hypothesized in this study that dopamine production can be individually increased by herbal extracts such as *Centella asiatica* and electrical current treatments such as transcranial Direct Current Stimulation (tDCs) thus they can together be more effective in reverting the effect of Parkinson's disease induced by MPTP in mice models. The objectives of this study are to induce Parkinson's disease through MPTP in *Balb/c* Mice models and test combinatory neuroprotective effect of tDCS and C.asiatica as theraputive agents on DA neurons of these disease models by conducting behavioral testing and biochemical analysis.

CHAPTER 2 : LITERATURE REVIEW

2.1- Parkinson's disease (PD)

Parkinson's disease (PD) is a neurological disease effecting movement of the body by constant degeneration based on unknown etiology. The dopaminergic neurons are subject to gradual loss in the substantia nigra, which leads to depletion of dopamine production, disruption; alteration in mitochondrial and proteasomal functions and up regulation of α-synuclein resulting in pathological and clinical abnormalities. The study of PD from a pathological perspective indicates that oxidative stress in the body and inflammation play vital roles in the disease. Oxidative stress is due to the increase in number of ROS and decreased level of glutathione. Microglial cells are responsible for generation of cytokines like TNF-α and IL-1β that are pro-inflammatory in nature thus aid as neuro-inflammatory molecules. In case of PD, Prostaglandins, platelet activating factor and cytokines together are involved in neurodegeneration (Farooqui & Farooqui, 2011; Oertel & Fahn, 2003).

The causes behind PD are unknown that account for approximately 90-95% of the cases however 5-10% are caused due to genetic factors such as inherited mutations. Abnormal dopamine metabolism generates hydrogen peroxide which leads to neurodegenerative progression. Monoamine oxidase is an important catalyst for deamination of neurotransmitters such as dopamine, serotonin and norepinephrine. The rapid degradation of these molecules helps regulate normal body functioning in terms of neurotransmission. However, in the case of PD loss of dopaminergic neurons leads to resting tremor, bradykinesia, gait disturbance and postural instability. The presence of Lewy bodies which are composed of α-synuclein, is the hallmark of PD. The other added features of PD include mitochondrial dysfunction, abundance of ROS, increased NO synthesis, inflammation and ubiquitin-proteasome system dysfunction (Matsui & Takahashi, 2018; Pringsheim et al., 2014; Wirdefeldt et al., 2011).

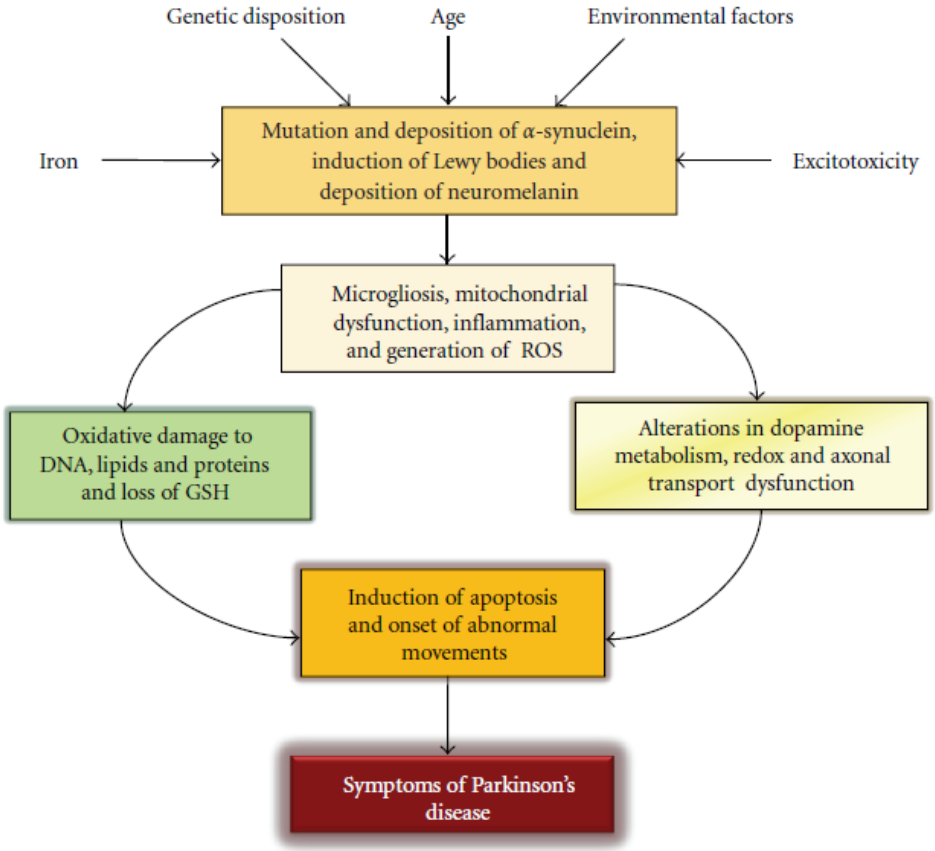

Figure 2.1: Factors and events associated with Parkinson's disease (Farooqui & Farooqui, 2011)

Parkinson's disease (PD) is the second in line to Alzheimer's as the most common neurodegenerative disease; it affects different regions in the brain including midbrain, brainstem, cerebral cortex, olfactory tubercle and peripheral nervous system (Pouclet et al., 2012). Motor impairments are thought to be the earliest and most striking physical disabilities that are together known as 'parkinsonism' (Wichmann & Dostrovsky, 2011).

Parkinson's disease (PD) traditionally has been termed as a movement disorder, characterized by stiffness, resting tremor and slowness of motion. It also comprises of the cognitive difficulties and other conditions that vary from patient to patient including change in taste and smell; difficulties in swallowing; nausea, vomiting, constipation; bladder dysfunction; sudden weight changes; dementia; hallucinations; anxiety; orthostatic hypotension; insomnia; leg swelling; excessive sweating; double vision and impulse control disorders (Marek et al., 2011).

Neurotoxin-based animal models have been produced using 6-hydroxydopamine (6-OHDA) or 1-methyl-1, 2, 3, 6-tetrahydropyridine (MPTP). The disease models have features of PD including an oxidative stress and increased death rate of DA neurons. The oxidative stress is due to the presence of reactive oxidative species (ROS) and per-oxynitrite. ROS are produced due to malfunctioned mitochondrial metabolism. There are certain limitations using the animal models but these studies are important to understand the neuro-toxic effects in humans (Blandini & Armentero, 2012; Santiago et al., 2010). 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) target those neurons that are particularly included in PD (Tillerson, Caudle, Reveron, & Miller, 2003).

In the past decade, a new technique has emerged in order to cater the effect of PD. The non-invasive brain stimulation termed as transcranial direct current stimulation (tDCs), has proved its competence to enhance neuroplasticity and improve learning in patients suffering with neurological disorders (Broeder et al., 2015). TDCs is used to induce weak electric currents through the scalp via two electrodes (anode and cathode) causing excitability in cortical tissue resulting in stimulation and inhibition respectively (Nonnekes et al., 2014). According to animal model studies, anodal tDCS helps stimulate dopamine release via the glutamate corticostriatal pathway in PD. Recently neuroprotective effect of tDCS in PD by reducing oxidative damage in dopaminergic neurons has also been reported (Benninger et al., 2010; Lu et al., 2015; Nonnekes et al., 2014).

2.2- 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)

MPTP metabolite MPP+ is the commonly used neurotoxin to produce the animal models of Parkinson's disease, as dopaminergic and cerebellar granule neurons are sensitive to MPP+ toxicity (Ayton et al., 2015; Smeyne & Jackson-Lewis, 2005). Caspase-3 plays important role in the apoptotic machinery (Shi, 2004) and Caspase-8, an activated upstream of caspase-3 plays a key apoptosis mediator in neurodegenerative disorders, including Parkinson's disease (Hartmann et al., 2001).

MPTP is a highly lipophilic compound. It quickly crosses the blood–brain barrier and enters the brain vicinity. The enzyme monoamine oxidase B (MAO-B) which is present in the non-dopaminergic cells metabolizes MPTP into 1-methyl-4-

phenyl-2, 3-dihydropyridinium (MPDP1) which is further converted into an active toxic compound, 1-methyl-4-phenylpyridinium (MPP1) by spontaneous oxidation. MPP1 escapes into the extracellular space and is readily available to be up taken by the dopaminergic neurons via high affinity plasma membrane dopamine transporter (DAT) (Brito-Armas et al., 2013). Transgenic mice like *Balb/c* or *C57bl/6* have increased DAT expression in their brain thus are more sensitive to MPTP (Leo & Gainetdinov, 2013).

Increase in concentration of MPP1 in the dopaminergic neuron causes impaired mitochondrial respiration by electron transport chain complex I inhibition. This leads to decrease in ATP formation (Meredith & Rademacher, 2011). MPP1 also causes an increased production of reactive oxidant species (ROS) which react with nitric oxide (NO) to produce potent oxidant like peroxy-nitrite. MPTP-toxicity is mediated by energy crisis and oxidative stress. Apoptotic machinery selective activation also leads to mitochondrial dysfunction (Venkateshappa et al., 2012).

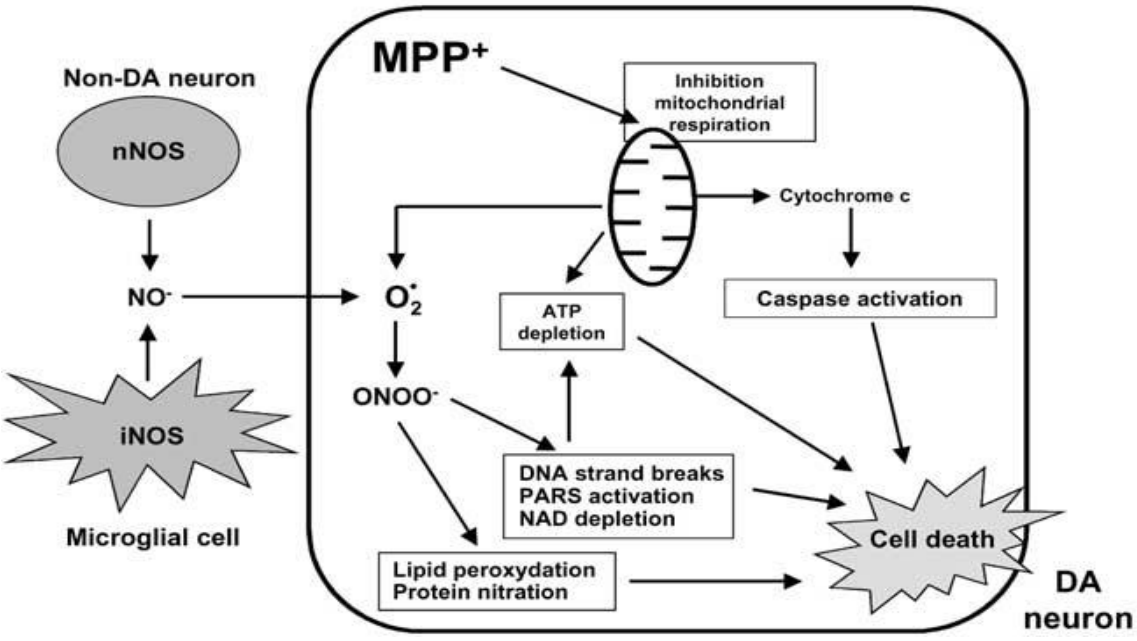

Figure 2.2: MPTP neurotoxicity in dopaminergic neurons (Przedborski & Vila, 2001)

MPP1 enzyme inhibitory action causes leakage of superoxides and deficiency of ATPs. Superoxides are confined within cells while NO- produced by non-dopaminergic neurons and microglial cells penetrate into neighboring neurons. The superoxides react with NO- to form per-oxynitrite, resulting in lipids, proteins, and DNA damage. Damaged DNA causes increased PARS activity and NAD depletion resulting in cell death. MPP1 also triggers caspase activation by cytochrome c release from mitochondria to the cytosol.

Two methods of MPTP administration have been widely used in mice models namely acute model and sub-acute model. The first consists of four 20 mg/kg intraperitoneal injections of MPTP in saline at 2 hours intervals within a single day causing extreme oxidative stress, resulting in excessive cell death (Lim et al., 2012). The second consists of 30 mg/kg/day intraperitoneal injection for 5 consecutive days by means of apoptosis leading to dopaminergic cell death (Anandhan, Essa, & Manivasagam, 2013; Dehay et al., 2010).

2.3- Transcranial Direct Current Stimulation (TDCS)

According to the non-invasive neuromodulation studies carried out in the healthy subjects, tDCS and transcranial magnetic stimulation (TMS) have shown improvement in neuroplasticity processes (Liuzzi et al., 2010). Repetitive TMS (rTMS) can be used to enhance motor function and temporary gain motor skill development in PD (Moisello et al., 2015; Zanjani, Zakzanis, Daskalakis, & Chen, 2015). Despite the fact that TMS has potential to modulate cortical excitability, it is more expensive and complicated to administer as compared to tDCS. TDCS is cost effective and safer technique keeping in view its therapeutic potential (Yokoi & Sumiyoshi, 2015). Transcranial alternating current stimulation (tACS) and transcranial random noise stimulation (tRNS) are also neuromodulatory techniques. Unlike TMS massive synchronized action potential is not discharged rather they change the threshold for discharge of stimulated neurons altering their cortical excitability as per the DCS principal mechanism of action. tACS targets the entrain brain by means of oscillations that have reverse polarities over the time period (Herrmann, Rach, Neuling, & Strüber, 2013; Woods et al., 2016).

The tDCS is target specific and is involved in modulation of spontaneous neuronal activity as it evokes weak constant electric current (Fritsch et al., 2010). In PD dopaminergic dysfunction of the striatofrontal network occurs which causes motor learning and behavioral deterioration (Gratwicke, Jahanshahi, & Foltynie, 2015). Studies have reported that performance modulations in working memory are attainable by tDCS application on parietal cortex region (Heimrath, Sandmann, Becke, Müller, & Zaehle, 2012). Increase in extracellular DA levels as a result of tDCS have also been reported in the animal studies however the animal studies are limited (Broeder et al., 2015). tDCS interaction with brain-derived neurotrophic factors (BDNF) promotes synaptic plasticity mainly due to antioxidative stress (Broeder et al., 2015; Fritsch et al., 2010). The neuroplasticity and motor learning due to tDCS is proposed to be modulated by intracellular signaling which causes increase in calcium influx into the targeted cortical neurons (Liuzzi et al., 2010). N- methyl-d-aspartate-receptor activation and GABAergic inhibition are responsible for adjusting the membrane potentials under the influence of tDCS (Flöel, 2014). Anodal polarization in the cerebral cortex and the firing pattern of neurons is linked with motor learning and behavioral interventions in PD are used to analyze this theory (De Xivry & Shadmehr, 2014). Anodal tDCS increases neuronal excitability, whereas cathodal tDCS causes inhibitory effect (Rossini et al., 2015). TDCS delivered to the dorsolateral prefrontal cortex (DLPFC) influences cognitive networks thus modulates cognitive function in healthy young and older subjects (Fertonani, Brambilla, Cotelli, & Miniussi, 2014; Harty et al., 2014; Miniussi, Harris, & Ruzzoli, 2013) although the exact relationship between the neuroplasticity, clinical motor and cognitive symptoms are not clear and further in-depth studies are needed (De Xivry & Shadmehr, 2014). The cortical excitability is altered in polarity specific way using anodal tDCS. The anodal stimulation depolarizes neuronal membrane which causes increase in the concentration of intracellular calcium ions in the post-synaptic neurons. This activates the protein kinases such as the calcium/calmodulin-dependent kinases (CaMK) which lead to increased transcription, translation and new glutamate receptor formation (Pang et al., 2011). In the long-term CaKM activates CREB (transcription factor), ultimately resulting in new protein formation (Rozisky, da Conceição Antunes, Brietzke, de Sousa, & Caumo, 2015).

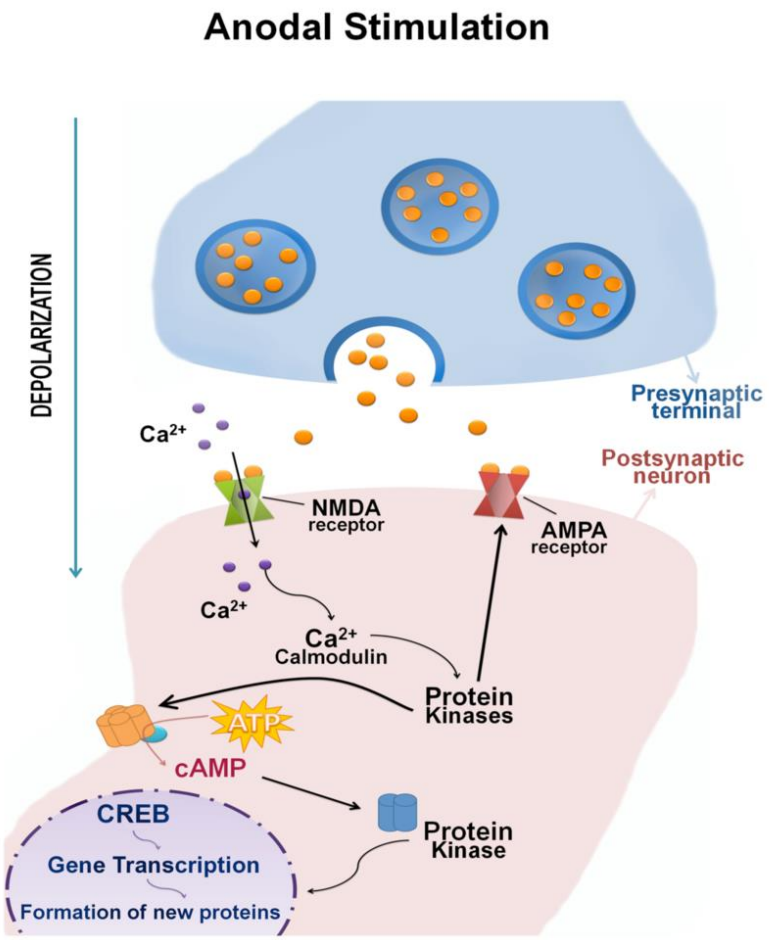

Figure 2.3: Mechanism of tDC anodal Stimulation (Podda et al., 2016)

In the case of cathodal stimulation hyperpolarization occurs and the serotonin and dopamine receptors are produced. This leads to inhibitory effect (Monte-Silva, Kuo, Liebetanz, Paulus, & Nitsche, 2010).

The reported tDC stimulations in humans and animals have current and electrode specifications for the experimentation. In one such study 1mA current is delivered by a pair of (saline-soaked) surface sponge electrodes (35 cm^2) with one of the electrodes placed over the representational area (involved in stimulation or inhibition) and the other electrode above the reference. The tDCS effects have lasted for approximately an hour after the stimulation in the healthy subjects (Paulus, 2011). Mice received daily tDCS of 0.2 mA for 10 minutes via an anodal electrode with a current density of 5.7 mA/cm^2 through a stimulation electrode. The anode electrode with an area of 3.5mm^2 was placed on the left frontal cortex via a plastic tube fixed on

the skull by dental ionomer cement. The cathode or reference electrode in the case of area 9 cm^2 was placed between the shoulders (Lu et al., 2015).

tDCS has to be administered in the patients of PD along with other pharmological compounds as part of the treatment thus the study of tDCS interacts with pharmacotherapy have been reviewed. Many of these studies carried out in healthy people have shown that drugs acting on neuronal transmission also influence the effects of tDCS (CAUMO et al., 2012). The effects vary from enhancement to inhibition dependent on the drugs used (Brunoni, Valim, & Fregni, 2011; Stagg & Nitsche, 2011). NMDA-glutamatergic receptors can alter plasticity induced by tDCS (Brunoni et al., 2012). Dextromethorphan (an NMDA-receptor antagonist) undermines the effect of both anodal and cathodal tDCS; lorazepam (GABA agonist) and D-cycloserine (partial NMDA agonist) selectively promote effects of anodal tDCS while carbamazepine (sodium channel blocker) and flunarizine (calcium channel blocker) suppress anodal tDCS effects. The studies in healthy subjects have turned tables by revealing that L-dopa causes anodal tDCS to facilitate inhibition and prolongs its effect even in case of cathodal tDCS (Kuo, Paulus, & Nitsche, 2014). The interaction studies are important for movement disorders like PD in order to plan future research or look for alternates (Ferrucci, Mameli, Ruggiero, & Priori, 2016).

2.4- *Centella asiatica* Extract (CAE)

Centella asiatica (L) Urban (Umbelliferae/Apiaceae family) is commonly known as Gotu Kola, Bharmi Buti, and Mandukparni and Hydrocotyl asiatica. It is a perennial creeping herb that has been part of the ancient Indian and Chinese medicine. It has been used in treatments for asthma, memory enhancement, strength promoting, wound healing, immune boosting, anti-anxiety, antiepilepsy and anti-stress (Shrivastava et al., 2013; Siddique et al., 2014). Centella asiatica has been clinically used in mentally retarded children and also in treatment of anxiety neurosis. This plant is also found to improve short-term memory and learning (Jansen, Brogan, Whitworth, & Okello, 2014). Centella asiatica has also shown a protective effect against lead acetate induced neurotoxicity (Meena, Pandey, Pandey, Arya, & Ahmed, 2012).

Popular treatments for PD are mainly symptomatic but in order to slow down the degenerative process it was necessary to identify such natural anti-apoptotic compounds that could be effective and reliable for long term use unlike the conventional pharmological medicines with long term side-effects. Dopamine Substitution is the most reasonable option currently available thus levodopa (L-dopa) has been used as the most effective medication for the past 40-50 years (Zesiewicz et al., 2010) initially produced by Birkmayer and Hornykiewicz in the 1960s (Riederer & Laux, 2011). There are certain limitations to it such that over stimulation of the dopamine receptors renders its normal performance. In the later stages of PD, L-dopa becomes ineffective against speech problems, gait issues, balance and swallowing problems (Coakeley, Martens, & Almeida, 2014).

CAE has anti-lipid per oxidative and free radical scavenging activities thus is an efficient and natural remedy for PD (Haleagrahara & Ponnusamy, 2010). MPTP-neurotoxicity can be countered by CAE by scavenging oxygen free radicals (Gohil, Patel, & Gajjar, 2010). The treatment has proven to be effective to attenuate MPTP-induced neurotoxicity in the rat model of Parkinsonism. Cerebellar granule neurons have been used to investigate the inhibitory and excitatory effect of CAE derivative echinacoside on caspase-3 and caspase-8 activation in the mouse model of MPTP-induced dopaminergic neuronal damage (Xu et al., 2013)

CHAPTER 3 : MATERIALS AND METHODS

3.1- Chemicals and Reagents

1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine hydrochloride (MPTP hydrochloride) and 5, 5'-Dithiobis (2-nitrobenzoic acid) DTNB were obtained from Med-Chem Express USA.

3.2- *C.asiatica* Extract

Fresh whole plant of C.*asiatica* was obtained from commercial market. The whole plant was cleaned, air-dried and powdered. The powder was soaked in double distilled water: ethanol (1:1) and placed in the shaking incubator at 25 ± 1° C for 2 days. The extracted solution obtained was then filtered through Whatmann No.1 filter paper, the yet fluidic extract was concentrated using the rotary evaporator at 78.2°C for 6 hours and remaining was air dried.

Figure 3.1:*C.asiatica* Extract

3.3- Phytochemical Testing

Presence of secondary metabolites or bioactive compounds was checked using phytochemical testing to verify the role of C.asiatica extract as an effective medicine for treatment of Parkinson's diseases.

3.3.1- Alkaloids

Hager's Test was used to detect the presence of Alkaloids. Few drops of Hager's reagent were dropped in the tube containing 2ml of CA extract. The formation of yellow precipitates indicated the presence of alkaloids.

3.3.2- Phenols

Few drops of 1% $FeCl_3$ were added to the tube containing 1ml CA extract. The colour shift to bluish black indicated the presence of phenols in the extract.

3.3.3- Anthraquinones

Borntrager's test was conducted to assess the presence of Anthraquinones. 3ml benzene and 5ml NH_3 were added to the tube containing 3ml of CA extract. Reddish violate colouration indicates the presence of Anthraquinones.

Figure 3.2: Alkaloid Test

Figure 3.3: Phenols Test

Figure 3.4: Anthraquinones Test

3.3.4- Flavonoids

1ml 10% lead acetate was added to 1ml extract for the detection of flavonoids. The yellow precipitations occurred indicating the flavonoids as positive.

3.3.5- Anthocyanins

2ml HCl and 1ml NH_3 were added to 2ml extract to detect the presence of anthocyanins by pinkish red to bluish violet colouration but the colour shift did not occur suggesting the absence of anthocyanins.

3.3.6- Tannins

Braymer's Test was conducted by adding 2ml water and few drops of $FeCl_3$ in 2ml extract. The transient greenish to black colour indicates the presence of tannins.

Figure 3.5: Flavonoids Test

Figure 3.6:Anthocyanins Test

Figure 3.7: Tannins Test

3.3.7- Leucoanthocyanins

5ml of Isoamyl alcohol was added to the tube containing 5ml of extract in order to detect the presence of Leucoanthocyanins. The organic layer turned into red colouration. This indicates the presence of this compound in the extract.

3.3.8- Phlobannins

Precipitate Test was used to identify phlobannins. 2ml 1% HCl was added to 2ml extract and boiled to form red precipitates.

3.3.9- Coumarins

3ml 10% NaOH was added to 2ml extract to form yellow precipitates thus indicating the presence of coumarins.

Figure 3.8: Leucoanthocyanins Test

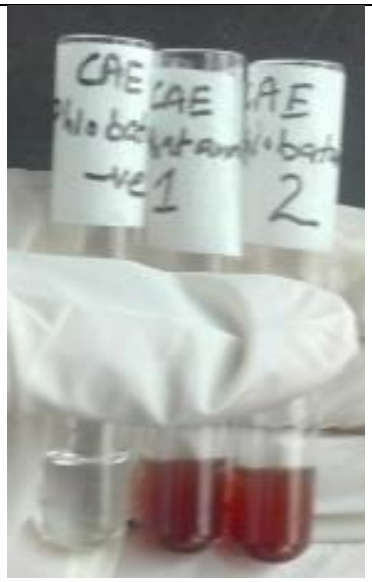

Figure 3.9: Phlobannins Test

Figure 3.10: Coumarins Test

3.3.10- Terpenoids

Ethanol, 2ml $CHCl_3$ and 3 drops of concentrated H_2SO_4 were added to 2ml of CA extract and heated over water bath for 2 minutes. The deep red colouration indicates the presence of terpenoids.

3.3.11- Diterpenes

2ml water and few drops of Copper acetate were added to the tube containing 2ml CA extract. The presence of diterpenes was indicated by the formation of emerald green colour.

3.3.12- Proteins

Also called as the Xanthoproteic Test, 1ml of concentrated H_2SO_4 was added to 1ml extract and boiled. The white precipitates turned yellow on boiling.

Figure 3.11: Terpenoids Test

Figure 3.12:Diterpenes Test

Figure 3.13: Proteins Test

3.3.12- Steroids

Salkowski's Test is conducted such that 2ml of $CHCl_3$ and 2ml concentrated H_2SO_4 are added to 2ml of extract. The reddish brown colouration formed at the interface indicates the presence of steroids.

3.3.13- Sterols

The second Salkowski's Test is conducted to identify sterols. Few drops of concentrated H_2SO_4 were added to the tube containing 1ml of the extract. The tube was shaken and left. The red colour appeared at the lower layer.

3.3.14- Saponins

Foam test was conducted such that 5ml water was added to 5ml of the extract and heated over water bath. The froth formation indicated the presence of saponins.

3.3.15- Resins

3ml acetone and 3ml HCl were added to 2ml extract and heated over water bath for about 30 minutes. Colour shift to red indicates the presence of resins however there was no colour change thus resins were absent.

Figure 3.14: Steroids Test

Figure 3.15:Sterols Test

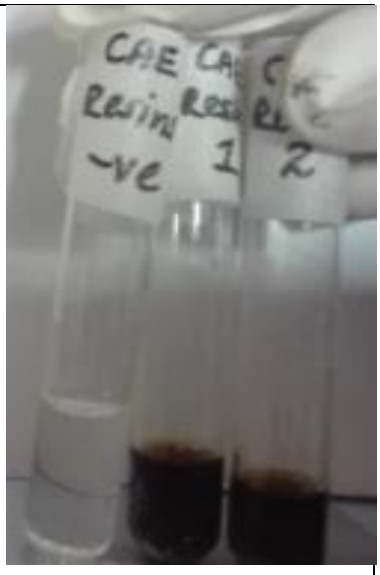

Figure 3.16: Resins Test

3.3.16- Glycoside

2ml $CHCl_3$ and 2ml concentrated H_2SO_4 were added to 2ml extract. The presence of glycoside was indicated by the reddish brown ring formed at the junction.

3.3.17- Cardiac Glycoside

2ml acetic acid, 1ml concentrated H_2SO_4 and few drops of $FeCl_3$ were added to 2ml extract. The violate colour below the brown colouration indicates cardiac glycoside presence.

3.3.18- Triterpenes

Few drops of concentrated H_2SO_4 were added to 1ml of CA extract. The tube was shaken and left to stand. The formation of faded yellow lower layer indicated triterpene's presence.

Figure 3.17: Glycoside Test

Figure 3.18: Cardiac Glycoside Test

Figure 3.19: Triterpenes Test

3.3.19- Amino Acids

The Ninhydrin Test was conducted such that few drops of Ninhydrin (Butanol) were added to 1ml of extract. The presence is indicated by violate colouration.

3.3.20- Carbohydrates

Fehling's test was conducted for the detection of carbohydrates. 1ml of Fehling's solution A and B were added to 2ml of the extract and heated. The carbohydrates are indicated by the colour shift from deep blue to red. The transition colours like yellow or orange suggest that there are mild sugars present in the extract as in the following case.

3.3.21- Deoxysugar

2ml acetic acid, few drops of $FeCl_3$ and 1ml of concentrated H_2SO_4 were added to 1ml extract. The formation of the brown ring identifies deoxysugars.

Figure 3.20: Carbohydrates Test

Figure 3.21: Deoxysugar Test

3.3.22- Animals

Balb/c mice were obtained from NIH. Mice were kept in cages at room temperature (25±2 ºC) and natural 12-12 hours light-dark cycle. Animals were given distilled water and fed with standard diet. Male mice (n=30) weighing 35-45 g and 4-6 months of age were used in experiments.

3.3.23- Ethics Statement

All experiments performed as per rulings of the Institute of Laboratory Animal Research, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011).

3.3.24- Study Design:

A 21 days long protocol was formulated to generate a Parkinson's disease mouse model by injecting MPTP and investigate the effect of transranial direct current (tDCs) and Centella asiatica extract (CEA) on the boosting of dopamine levels.

The animals were divided into 5 groups with 5 animals in each group. The control group received saline alone. MPTP alone group received 30mg/kg body weight of MPTP (HCL dissolved in 0.9% saline given intra- paratonially) for 5 days. The Centella asiatica + MPTP group received 300mg/kg CAE dissolved in 0.9% saline along with MPTP for 21days. The tDCs + MPTP group was given dose (time x current) of 2.0 (10mins x 0.2mA) via 0.2mA fixed current circuit with help of modified electrodes of 2.27 mm^2 anode on left frontal cortex and 9 cm^2 cathode on shoulder or tail along with MPTP for 7 days. The fifth group was given MPTP + CAE +tDCs was given CAE for 21 days and tDCs for 7 days following the above mentioned protocol. The behavioral tests were done on following the 21^{st} day. DTNB essay was used to detect the glutathione levels in the brain homogenate and blood respectively.

3.4- Animal Groups for Study:

Animals were randomly divided into five groups. Each group had a total of 5 animals of 4-6 months of age. Details of all the groups are as follows:

Table 3.1: Animal groups study design

Groups	No. of animals	IP injection	No. of days kept	tDCs/CAE
Control	5	None	21	None
MPTP only	5	30mg/Kg (3^{rd}-7^{th}day)	21	None
CAE+MPTP	5	30mg/Kg (3^{rd}-7^{th}day)	21	CAE 300mg/kg (1^{st}-7^{th} day)
tDCs+MPTP	5	30mg/Kg (3^{rd}-7^{th}day)	21	tDCs 0.2mA for 10mins (1^{st}-7^{th} day)
CAE+tDCs+ MPTP	5	30mg/Kg (3^{rd}-7^{th}day)	21	CAE 300mg/kg tDCs 0.2mA for 10mins (1^{st}-7^{th} day)

3.5- MPTP induction in Parkinson's disease Mice Model

Parkinson's disease was induced in mice model using MPTP hydrochloride peritoneal (IP) injections. Mice were weighed on alternative days and an IP dose of 30 mg/kg body weight was given for consecutive five days between 11 a.m-12 p.m. Two cages were used to separate the injected mice with the rest during the process of injecting MPTP.

Disturbance was avoided by careful handling to prevent the mice from becoming terrified or aggressive. The mouse was picked from its tail and placed on cage's lid. Securing the tail with one hand and gently forming a firm grip on the loose skin at the back of the mouse's shoulders with the other hand; to restrict the possible body and neck movement. The mouse was inverted such that the dorsal side was exposed. "Two man" procedure was practiced such that one person was responsible

for the orientation of the mouse and the other pricked the needle at a 30˚ angle into the abdomen in the right or left lower quadrant to avoid rupture of the abdominal organs (Figure 3.22). After IP injection was given the mouse was placed into the cage with other injected mice.

Figure 3.22: *Balb/c* Mouse receiving Intra-peritoneal Injection

CHAPTER 4 : RESULTS

4.1- Phytochemical Results

Table 4.1:Phytochemical analysis results of C.asiatica Extract

Phytochemical	**Presence (+/-)**
Alkaloids	+
Anthocyanins	-
Anthraquinones	+
Carbohydrates	+
Cardiac Glycoside	+
Coumarins	+
Deoxysugar	+
Diterpenes	+
Flavanoids	+
Glycoside	+
Leucoanthocyanins	+
Phenols	+
Phlobannins	+
Protein	+
Resins	-
Saponine	+
Steroids	+
Sterols	+
Terpenoids	+
Triterpenes	+

4.2- Behavioral Testing

Normality was tested by Shapiro-Wilk Test and Kolmogorov-Smirnov Test. Levine's test was used to determine variance in the data. Initially the mice were trained

to perform the task before the disease induction. However, the behavioral testing results were compiled on 21st day after the completion of the entire protocol.

4.2.1- Pole Test

The Pole test method was adapted from the protocol originally described by Ogawa et al. (1985). The mouse was placed on top of white tape marking on a vertical wooden pole 26 cm long (5 cm diameter) and the time taken for it to come to base of the pole was recorded. The experiment was conducted in triplicate and average was calculated. The base of the pole was placed in the home cage. When placed on the pole, animals oriented themselves in downward direction and descend the length of the pole back into their home cage.

Figure 4.1: Pole Test

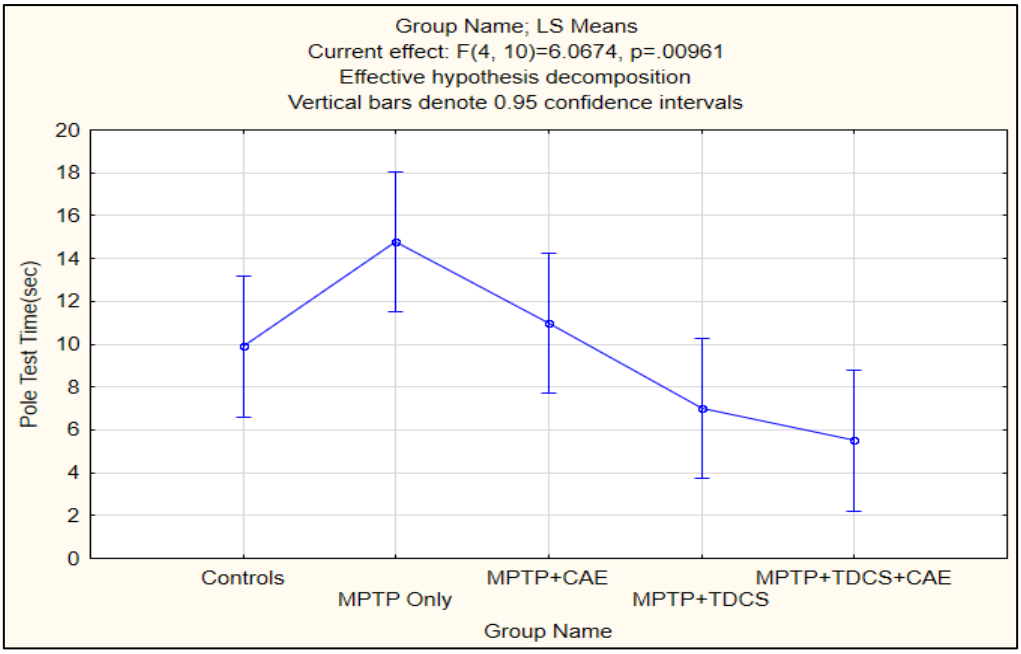

Figure 4.2 : Pole Test Results- One-way ANOVA was run to determine the time difference (sec) amongst groups (controls, MPTP only, MPTP+CAE, MPTP+TDCs and MPTP+ TDCs+ CEA) in completing the pole test.

The data was normally distributed, as assessed by Shapiro-Wilk's test (p> 0.05). The error bars represent mean and S.E.M. Statistically significant results were expressed by a p value of 0.00961.

Table 4.2: Pole Test Post-Hoc analysis- Tukey HSD Test was conducted to analyse the significant difference lies between MPTP only and MPTP+TDCs and MPTP only and MPTP+TDCs+CAE.

Tukey HSD test; variable Pole Test (Sheet1 in Behavioral)
Approximate Probabilities for Post Hoc Tests
Error: Between MS = 6.4823, df = 10.000

Cell No.	Group Name	{1} 9.8900	{2} 14.780	{3} 11.000	{4} 7.0000	{5} 5.5000
1	Controls		0.205933	0.981606	0.646735	0.286759
2	MPTP Only	0.205933		0.414910	0.024926	0.008397
3	MPTP+CAE	0.981606	0.414910		0.365000	0.134324
4	MPTP+TDCS	0.646735	0.024926	0.365000		0.946688
5	MPTP+TDCS+CAE	0.286759	0.008397	0.134324	0.946688	

4.2.1.1.Pole Test Test Post-Hoc Results

The group given tDCs and CAE and the group given tDCs alone has significant difference from the MPTP only group as per the results indicated by post hoc analysis.

It can be interpreted from the Pole test that the mice under the influence of MPTP only took the most time to cover the distance while the effect of tDCs on the mice resulted in better performance in both cases; with MPTP and with MPTP being congucated with CAE. The performance was even better than the controls which can be due to hyperactive state under stress influence.

4.2.2- Grid Test

An elevated metal square grid (41×41 cm2, with each grid cell 3.5×3.5 cm2; height: 41 cm) was used (Fig.4.3). The grid apparatus was located in a sound attenuated room with dim lighting. After each trial, 70% ethanol was used to clean the apparatus. A camera recorder was located below the apparatus with an angle of about 20–40 degrees. Behaviors on the grid were recorded and were analyzed later.

Figure 4.3: Grid-Walking Test

Grid-walking Test indicates motor deficits and the role of limb activity involved in precise movement and coordination (Chao et al., 2012). The slipping of paw through

the grid was marked as a negative point. Each wrong placement of foot was counted when the hind limb paw protrudes through the grid. Mice were monitored and recorded on videotape. Total number of steps was calculated and the number of wrong steps was also calculated. Percentage of the total correct steps to total number of steps was calculated. Mice were placed on one edge; forelimb and hind limb placement defects were analyzed for three trials and average was calculated.

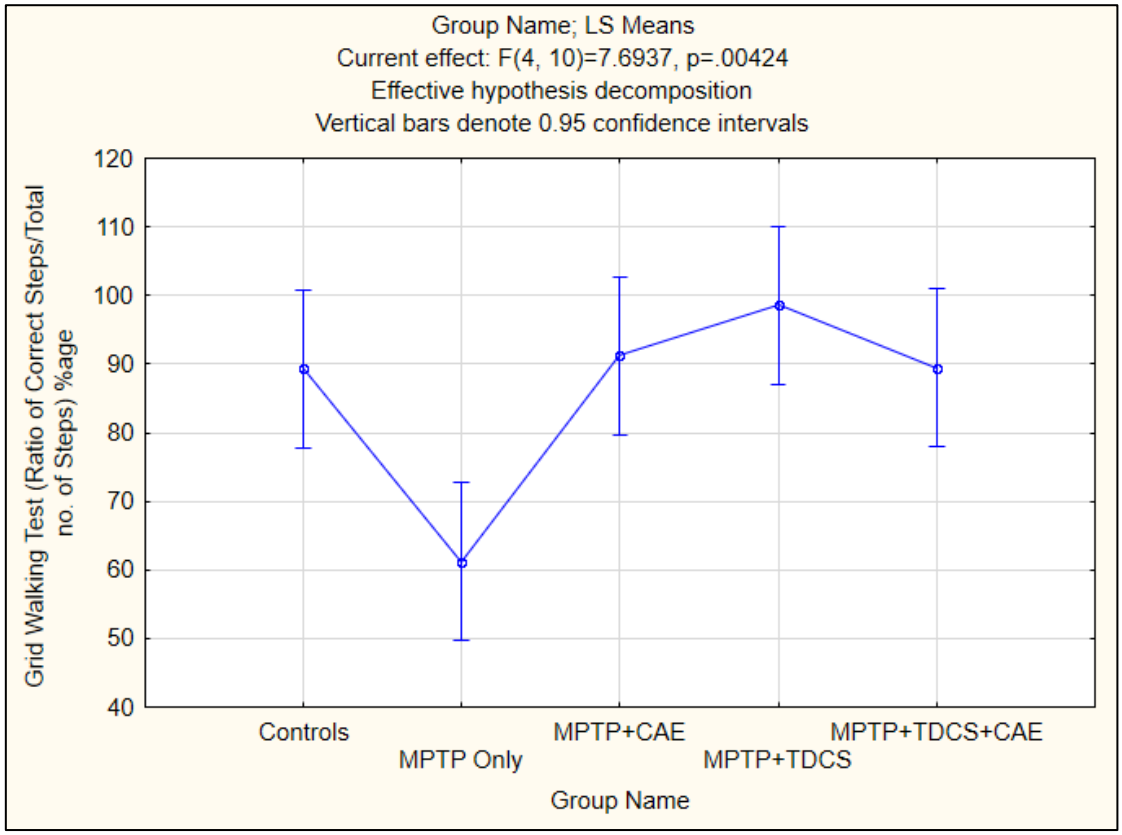

Figure 4.4: Grid Walking Test

Results- One-way ANOVA was run to determine the percentage of total correct steps to that of total number of steps amongst groups (controls, MPTP only, MPTP+CAE, MPTP+TDCs and MPTP+TDCs+CEA) in completing the grid walking test.

The data was normally distributed, as assessed by Shapiro-Wilk's test (p> 0.05). The error bars represent mean and S.E.M. Statistically significant results were expressed by a p value of 0.00424

4.2.2.1- Grid Walking Test Post-Hoc Results-

Tukey HSD Test was conducted to analyse the significant difference lies between MPTP only and all other groups. In case of the grid walking test best performance with least number of wrong stepping was done by the MPTP+TDCs group followed by the MPTP+TDCs+CAE group. The least number of correct stepping was done by the MPTP only group.

Table 4.3: Grid Walking Test Post-Hoc Results- Tukey HSD Test was conducted to analyse the significant difference lies between MPTP only and all other groups.

Cell No.	Tukey HSD test; variable Grid Walking Test (Sheet1 in Behavioral) Approximate Probabilities for Post Hoc Tests Error: Between MS = 79.980, df = 10.000 **Group Name**	{1} 89.347	{2} 61.237	{3} 91.187	{4} 98.537	{5} 89.460
1	Controls		0.021145	0.998988	0.720207	1.000000
2	MPTP Only	0.021145		0.014405	0.003365	0.020649
3	MPTP+CAE	0.998988	0.014405		0.846802	0.999220
4	MPTP+TDCS	0.720207	0.003365	0.846802		0.728664
5	MPTP+TDCS+CAE	1.000000	0.020649	0.999220	0.728664	

4.2.3- Swim Test

Swim-test was carried out on different days after MPTP treatment in water tubs (40 cm length×28 cm width×30 cm height). The depth of water was kept at 20cm and the temperature was maintained at 27±2 ◦C. The mice activity was recorded for a session of 2 minutes and 30 seconds from a certain angle (Figure 4.5).

Figure 4.5: Swim Test

The mice were trained in a practice session prior to final results recording on the 21st day. Mice were placed in clear tub filled with water up to 20cm with a temperature of 27 ± 2°C. After observing mice for 2 min 30 sec of swimming, they were removed from the water, immediately wiped dry and returned to their home cages.

4.2.3.1- Swim score scale for Classifying Swim Test

Swim-score scale used to classify the groups was such that 0 was allocated for hind part sinks with head floating; 1 for occasional swimming using hind limbs while floating on one side; 2 for occasional floating/swimming only and 3 for continuous swimming (Figure 31) (Donnan et al., 1987, Muralikrishnan and Mohanakumar, 1998). Categorical assessment was done for each subject in all groups.

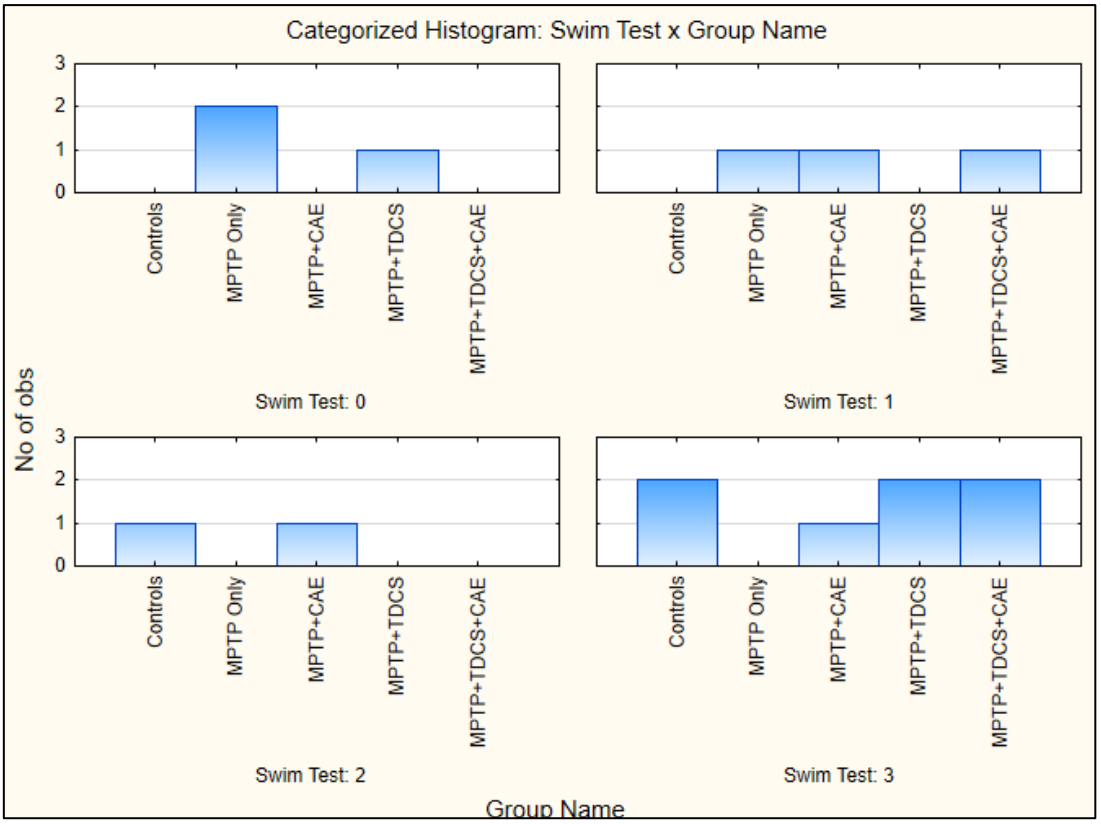

Figure 4.6: Swim Test categorical distribution of groups

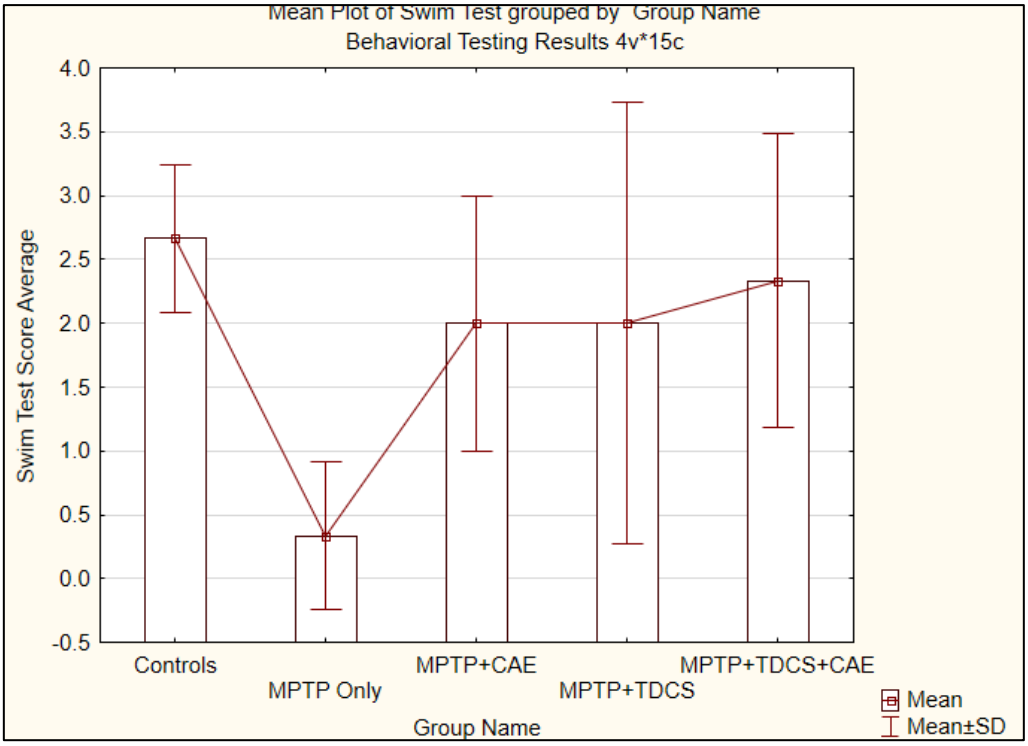

Figure 4.7: Swim Test -Average score of groups according to the categorical distribution with error bars representing the mean and standard deviation.

The control group showed the best performance approaching 3 which was the maximum according to the categorical distribution in case of the swim test while MPTP only group showed the worst performance approaching to 0 as per the categorical distribution. The combination of TDCs and CAE seems to have improved the behavior than that of TDCs or CAE alone.

4.3- Biochemical Testing

For the biochemical analysis, mice were sacrificed in the morning and the brains were carefully removed and immediately placed in -80℃ to avoid the disturbance in the enzyme, endogenous amine and anti-oxidant activity. Ice-cold PBS (pH 8.0; concentration: 15% w/v) was used to homogenize mice brains. Homogenized samples were centrifuged briefly to collect supernatant for biochemical estimation. Aliquot was taken from supernatants for assessment of Reduced Glutathione (GSH) to estimate dopamine production. Remaining supernatant was stored at -80℃.

4.3.1- Reduced Glutathione (GSH)

Reduced Glutathione (GSH) was determined by aliquoting 1 ml of supernatant from the mixture of 0.5 ml of tissue homogenate and 2 ml of TCA (Trichloroaceticacid). To this 1 ml aliquote, 0.5 ml of 0.0198% Ellman's reagent in 1% sodium citrate and 3 ml of phosphate buffer (pH 8.0) were added. The values of absorption (nm) are presented by One-Way ANOVA checked by photometric analysis at 412nm using a spectrophotometer.

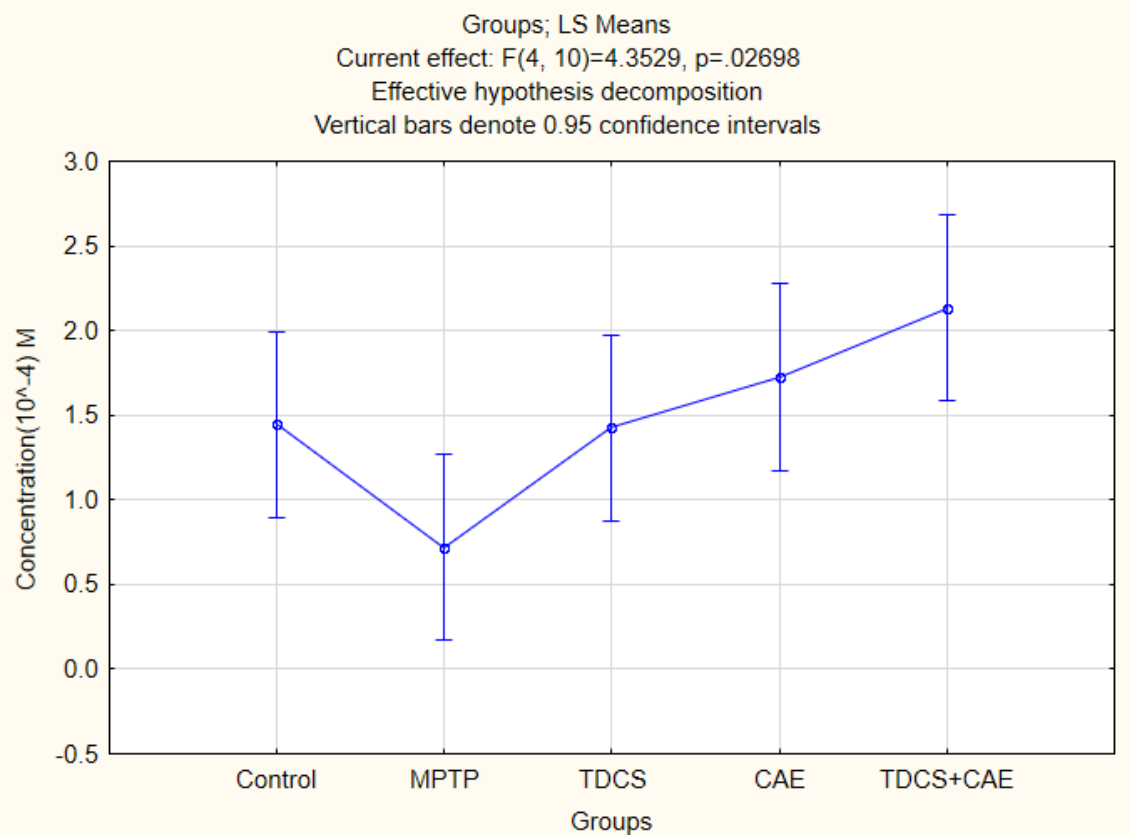

Figure 4.8: One-way ANOVA was run to determine the Concentrations in 10^-4 (M) values using the equation **concentration=absorption /constant*Dilution factor** on 412nm amongst groups (controls, MPTP only, MPTP+CAE, MPTP+TDCs and MPTP+TDCs+CEA).

The data was normally distributed, as assessed by Shapiro-Wilk's test (p> 0.05). The error bars represent mean and S.E.M. Statistically significant results were expressed by a p value of 0.02698

4.3.1.1 Reduced GSH Test Post-Hoc Results

There is significant difference between the MPTP only group and the tDCs and CAE group which suggests that they are most likely to show variation in comparison to rest of the groups.

Table 4.4: GSH Assay Post-Hoc Results-There is significant variation in the group with MPTP only and the group with MPTP+TDCs+CAE.

Cell No.	Tukey HSD test; variable Absorbtion (mmoles/g tissue) (Spreadshee Approximate Probabilities for Post Hoc Tests Error: Between MS = .00420, df = 10.000 **Group Name**	{1} .21836	{2} .10897	{3} .21531	{4} .26040	{5} .32260
1	Control		0.304177	0.999997	0.926516	0.344694
2	MPTP	0.304177		0.327768	0.097040	0.015903
3	TDCS	0.999997	0.327768		0.907868	0.320232
4	CAE	0.926516	0.097040	0.907868		0.764691
5	TDCS+CAE	0.344694	0.015903	0.320232	0.764691	

4.3.2- 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

DPPH assay was used to determine the free radical scavenging activity of C.*asiatica* extract. The DPPH molecule is a stable free radical but produces a deep violet coloration due to delocalization of the spare electron and absorption band in ethanol solution assessed by spectrophotometric analysis at about 517 nm. When the ethanolic solution of DPPH is mixed with that of ethanolic extract, donation of hydrogen atoms gives rise to its reduced form which is donated by the loss of the violet color.

An aliquot (0.5 mL) of ethanol solution containing CAE (0.97–250 µg/mL) was added to 1.5 mL of daily prepared ethanol DPPH solution (0.05 mM). The optical density (OD) change at 517 nm was measured 30 min later by a spectrophotometer. Absolute ethanol was used as blank. An ethanolic solution of DPPH was used as a negative control. Ascorbic acid was used as reference drug (0.97–250 µg/mL) in similar concentrations as that used for the extract. Tests were carried out in triplicate and ODs were recorded. The equation for calculation of percentage inhibition is as follows:

% Radical Inhibition =

{(Negative Control OD – CAE OD)/ Negative Control OD)} X 100

Table 4.5: DPPH free radical scavenging activity of Ascorbic acid. Extract. ODs, %inhibition, mean, standard deviation and standard errors are enlisted in the table.

Ascorbic Acid OD					
Conc. (µg/ml)	15	30	50	75	100
	0.08426	0.07422	0.04506	0.01934	0.00044
	0.08392	0.07435	0.04503	0.01934	0.00039
	0.08388	0.07417	0.04505	0.01929	0.00041
% inhibition	34.12041	41.97029	64.76935	84.87881	99.65598
	34.38624	41.86865	64.79281	84.87881	99.69507
	34.41751	42.00938	64.77717	84.9179	99.67944
Mean	34.30805	41.94944	64.77978	84.89184	99.67683
Std. Deviation	0.163257	0.072647	0.011943	0.022570	0.019676
Std. Error	0.094257	0.041943	0.006895	0.013031	0.011360

Table 4.6: DPPH free radical scavenging activity of *Centella asiatica* Extract. ODs, %inhibition, mean, standard deviation and standard errors are enlisted in the table.

Centella Asiatica Extract OD					
Conc. (µg/ml)	15	30	50	75	100
	0.10797	0.0922	0.08493	0.05813	0.04891
	0.107978	0.09260	0.08482	0.06125	0.047847
	0.10805	0.092299	0.08499	0.057966	0.048892
% inhibition	15.58249	27.91243	33.59656	54.55043	61.75919
	15.5763	27.59969	33.68256	52.11102	62.5901
	15.51994	27.8354	33.5498	54.6784	61.7735
Mean	15.54812	27.78251	33.60964	53.77995	62.04093
Std. Deviation	0.034466	0.162943	0.067342	1.446749	0.475650
Std. Error	0.019899	0.094075	0.038880	0.835281	0.274617

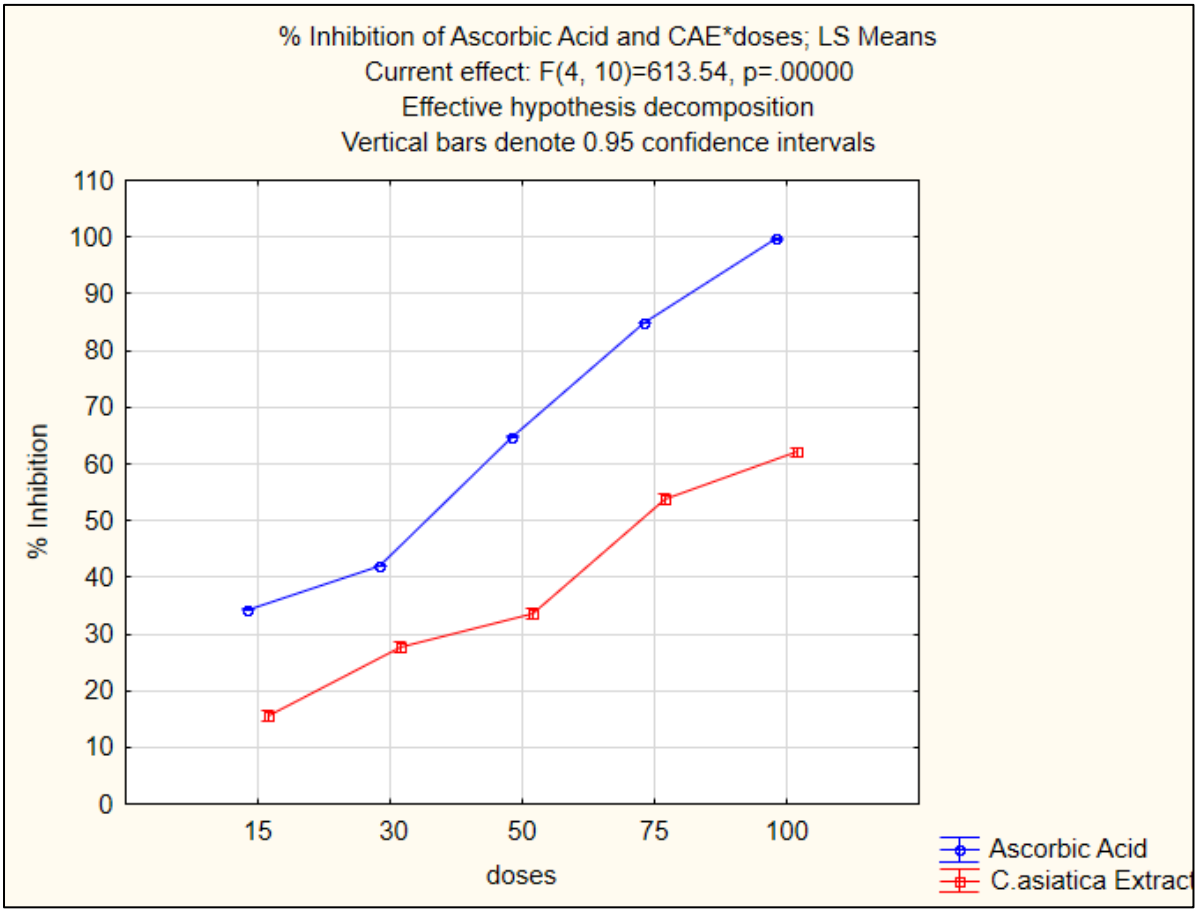

Figure 4.9: %inhibition of Ascorbic Acid and *C.asiatica* Extract-

Factorial ANOVA is used to determine the %inhibition of *C.asiatica* Extract and Ascorbic Acid with respect to different doses. The trend shows increase in %inhibition with increase in dose concentration

The blue linear pattern indicates the trend of %inhibition by ascorbic acid which is the standard for DPPH thus it has higher inhibitory values. The red linear pattern indicates the % inhibition of *C.asiatica* Extract. The values of inhibition are lesser than ascorbic acid however the pattern is similar.

Table 4.7: DPPH Assay Post-Hoc Analysis

Cell No.	Tukey HSD test; variable DV_1 (Spreadsheet5) Approximate Probabilities for Post Hoc Tests Error: Between; Within; Pooled MS = .23846, df = 19.984 doses	R1	{1} 34.308	{2} 15.560	{3} 41.949	{4} 27.783	{5} 64.780	{6} 33.610	{7} 84.892	{8} 53.780	{9} 99.677	**{10} 62.041**
1	A.A 15	A.A		0.000225	0.000179	0.000179	0.000179	0.755430	0.000179	0.000179	0.000179	0.000179
2	A.A 15	CAE	0.000225		0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
3	A.A 30	A.A	0.000179	0.000179		0.000225	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
4	A.A 30	CAE	0.000179	0.000179	0.000225		0.000179	0.000179	0.000179	0.000179	0.000179	0.000179
5	A.A 50	A.A	0.000179	0.000179	0.000179	0.000179		0.000225	0.000179	0.000179	0.000179	0.000210
6	A.A 50	CAE	0.755430	0.000179	0.000179	0.000179	0.000225		0.000179	0.000179	0.000179	0.000179
7	A.A 75	A.A	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179		0.000225	0.000179	0.000179
8	A.A 75	CAE	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000225		0.000179	0.000179
9	A.A 100	A.A	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179	0.000179		0.000225
10	A.A 100	CAE	0.000179	0.000179	0.000179	0.000179	0.000210	0.000179	0.000179	0.000179	0.000225	

4.3.2.1. DPPH Assay Post-Hoc Results

The trend of both Ascorbic Acid and C.asiatica Extract show increase in %inhibition with increase in dose concentration. The free radical scvanging activity of C. asiatica is less than that of Ascorbic Acid.There is significant variation in the dilutions of both ascorbic acid and *c.asiatica.*

The trend indicates that the extract has oxidative properties that increase % inhibition with concentration.

CHAPTER 5 : DISCUSSION

Parkinson's disease is a neurodegenerative disorder, characterized by resting tremor, postural instability and cognitive deficits (Wichmann & Dostrovsky, 2011). There have been advancements in the research related to PD in all fields like neuro-stimulatory, ethano-botany, pharmacotherapy and neurosurgery. The aim of all these strategies is to improve motor functioning and cognitive abilities thus making life of the patients better with minimal or no side effects. In recent years, various approaches with promising outcomes have surfaced. PD treatment include boosting dopamine by pharmacological compounds like MAOBIs, L-DOPA, amantadine, anticholinergic, β-blockers, dopamine agonists (Connolly & Lang, 2014). PD demands long-term treatment and despite the affectivity of the pharmacotherapy in short-term treatment, it eventually fails to deliver the same effect in the longer run due increased tolerance thus non-invasive treatments is a promising approach (Brunoni et al., 2012; CAUMO et al., 2012).

According to research, slowing its progression by improving the neuronal networking through neural-stimulation by tDCS, tACS, TMS, rTMS, tRNS (Woods et al., 2016) is under study. The interaction of tDCs and pharmacological drugs has proved to be elsewise (Kuo et al., 2014) thus a combination which is more natural with lesser side-effects is the need of the time. Practicing traditional medicine including use of extract prepared from ethno botanical important plants like *Centella asiatica* in combination with tDCs has been the focus of this study because of its neuroprotective effect which signifies its potential as a therapy for improvement in the treatment for PD.

The phytochemical analysis suggests that the presence of flavonoids, saponins, phenols, alkaloids, steroids, sterols and others in the CAE help to reduce the reactive oxygen species and provide a balanced oxidative environment in the mice models to up-regulate dopamine production (Haleagrahara & Ponnusamy, 2010). The DPPH assay also depicts the oxidative potential of *C.asiatica* Extract (Meena et al., 2012). The behavioral and biochemical analysis suggests that the mice induced with MPTP showed the most oxidative stress and the treatment with CAE alone and in

combination with tDCS showed improvement in the oxidative environment respectively.

The use of tDCs has cognitive, oxidative and motor improvements in healthy individuals and in PD animal models similar impact is also reported (Flöel, 2014; Kuo et al., 2014; Woods et al., 2016). The scope of this study includes role in reverting hypoxia and motor disabilities. The results of pole test show significant improvement in motion of the mice receiving it alone or in combination with CAE in comparison to mice receiving MPTP only. The grid test also showed similar results in terms of correct stepping due to improved motor function and cognitive alertness. The tDCs given to MPTP mice was better than the combination of tDCs and CAE in this case. Possible explanation to that is the neural networking improvement, reduced ROS and vigilance in the group due to regular sessions of tDCs. The swim test suggest that the normal group showed the best performance followed by the combinatory therapy group while the MPTP alone group showed the least mobility. The reduced GSH assay suggests that the least mitochondrial oxidative disruption is likely to occur in the presence of CAE and tDCs combination.

There are many future prospects that can be addressed in this field of research before its clinical practice can be carried out. The dopamine levels can be assessed by quantitative analysis to compare the groups, immunohistochemistry can be analyzed EEG recordings can be taken and most importantly the combination with commercial pharmacological drugs like L-DOPA can be studied to counter its adverse effects.

CHAPTER 6 : CONCLUSION

The combinatory therapy can be used to fight early signs of Parkinson's disease as suggested by the behavioral and biochemical analysis that there is significant difference in the cognitive abilities, performance of motor skills and encounter of the oxidative stress especially between the PD mice (diseased group) and the tDCs and CAE mice(treatment group). The results approve compatibility of the two modes thus long-term treatments can be carried out using the combination

CHAPTER 7 REFERENCES

Amin, K. R., Uddin, M. G., Rashid, M. M. O., & Sharmin, T. (2018). New insight in neuropharmacological activities of Dioscorea alata. *Discovery Phytomedicine, 5*(1), 1-6.

Anandhan, A., Essa, M. M., & Manivasagam, T. (2013). Therapeutic attenuation of neuroinflammation and apoptosis by black tea theaflavin in chronic MPTP/probenecid model of Parkinson's disease. *Neurotoxicity research, 23*(2), 166-173.

Ayton, S., Lei, P., Hare, D. J., Duce, J. A., George, J. L., Adlard, P. A., . . . Finkelstein, D. I. (2015). Parkinson's disease iron deposition caused by nitric oxide-induced loss of β-amyloid precursor protein. *Journal of Neuroscience, 35*(8), 3591-3597.

Benninger, D. H., Lomarev, M., Lopez, G., Wassermann, E. M., Li, X., Considine, E., & Hallett, M. (2010). Transcranial direct current stimulation for the treatment of Parkinson's disease. *Journal of Neurology, Neurosurgery & Psychiatry, 81*(10), 1105-1111.

Bhatnagar, M., Goel, I., Roy, T., Shukla, S. D., & Khurana, S. (2017). Complete Comparison Display (CCD) evaluation of ethanol extracts of Centella asiatica and Withania somnifera shows that they can non-synergistically ameliorate biochemical and behavioural damages in MPTP induced Parkinson's model of mice. *PloS one, 12*(5), e0177254.

Blandini, F., & Armentero, M. T. (2012). Animal models of Parkinson's disease. *The FEBS journal, 279*(7), 1156-1166.

Brito-Armas, J., Baekelandt, V., Castro-Hernandez, J., González-Hernández, T., Rodriguez, M., & Castro, R. (2013). Melatonin prevents dopaminergic cell loss induced by lentiviral vectors expressing A30P mutant alpha-synuclein. *Histol Histopathol, 28*(8), 999-1006.

Broeder, S., Nackaerts, E., Heremans, E., Vervoort, G., Meesen, R., Verheyden, G., & Nieuwboer, A. (2015). Transcranial direct current stimulation in Parkinson's disease: Neurophysiological mechanisms and behavioral effects. *Neuroscience & Biobehavioral Reviews, 57*, 105-117.

Brunoni, A. R., Nitsche, M. A., Bolognini, N., Bikson, M., Wagner, T., Merabet, L., . . . Pascual-Leone, A. (2012). Clinical research with transcranial direct current stimulation (tDCS): challenges and future directions. *Brain stimulation, 5*(3), 175-195.

Brunoni, A. R., Valim, C., & Fregni, F. (2011). Combination of noninvasive brain stimulation with pharmacotherapy. *Expert review of medical devices, 8*(1), 31-39.

Cassarino, D. S., Fall, C. P., Swerdlow, R. H., Smith, T. S., Halvorsen, E. M., Miller, S. W., . . . Bennett Jr, J. P. (1997). Elevated reactive oxygen species and antioxidant enzyme activities in animal and cellular models of Parkinson's disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1362*(1), 77-86.

CAUMO, W., Souza, I. C., Torres, I. L., Medeiros, L., Souza, A., Deitos, A., . . . Volz, M. S. (2012). Neurobiological effects of transcranial direct current stimulation: a review. *Frontiers in psychiatry, 3*, 110.

Coakeley, S., Martens, K. E., & Almeida, Q. J. (2014). Management of anxiety and motor symptoms in Parkinson's disease. *Expert review of neurotherapeutics, 14*(8), 937-946.

Connolly, B. S., & Lang, A. E. (2014). Pharmacological treatment of Parkinson disease: a review. *Jama, 311*(16), 1670-1683.

De Xivry, J.-j. O., & Shadmehr, R. (2014). Electrifying the motor engram: effects of tDCS on motor learning and control. *Experimental brain research, 232*(11), 3379-3395.

Dehay, B., Bové, J., Rodríguez-Muela, N., Perier, C., Recasens, A., Boya, P., & Vila, M. (2010). Pathogenic lysosomal depletion in Parkinson's disease. *Journal of Neuroscience, 30*(37), 12535-12544.

Farooqui, T., & Farooqui, A. A. (2011). Lipid-mediated oxidative stress and inflammation in the pathogenesis of Parkinson's disease. *Parkinson's disease, 2011*.

Ferrucci, R., Mameli, F., Ruggiero, F., & Priori, A. (2016). Transcranial direct current stimulation as treatment for Parkinson's disease and other movement disorders. *Basal Ganglia, 6*(1), 53-61.

Fertonani, A., Brambilla, M., Cotelli, M., & Miniussi, C. (2014). The timing of cognitive plasticity in physiological aging: a tDCS study of naming. *Frontiers in Aging Neuroscience, 6*, 131.

Flöel, A. (2014). tDCS-enhanced motor and cognitive function in neurological diseases. *Neuroimage, 85*, 934-947.

Fritsch, B., Reis, J., Martinowich, K., Schambra, H. M., Ji, Y., Cohen, L. G., & Lu, B. (2010). Direct current stimulation promotes BDNF-dependent synaptic plasticity: potential implications for motor learning. *Neuron, 66*(2), 198-204.

Fuxe, K. (1965). Evidence for the existence of monoamine neurons in the central nervous system. IV The distribution of monoamine terminals in the central nervous system. *Acta physioL scand, 247*, 39-85.

Geng, X., Tian, X., Tu, P., & Pu, X. (2007). Neuroprotective effects of echinacoside in the mouse MPTP model of Parkinson's disease. *European Journal of Pharmacology*, 564(561-563), 566-574.

Gohil, K. J., Patel, J. A., & Gajjar, A. K. (2010). Pharmacological review on Centella asiatica: a potential herbal cure-all. *Indian journal of pharmaceutical sciences, 72*(5), 546.

Gratwicke, J., Jahanshahi, M., & Foltynie, T. (2015). Parkinson's disease dementia: a neural networks perspective. *Brain, 138*(6), 1454-1476.

Haleagrahara, N., & Ponnusamy, K. (2010). Neuroprotective effect of Centella asiatica extract (CAE) on experimentally induced parkinsonism in aged Sprague-Dawley rats. *The Journal of toxicological sciences, 35*(1), 41-47.

Hartmann, A., Troadec, J.-D., Hunot, S., Kikly, K., Faucheux, B. A., Mouatt-Prigent, A., . . . Hirsch, E. C. (2001). Caspase-8 is an effector in apoptotic death of dopaminergic neurons in Parkinson's disease, but pathway inhibition results in neuronal necrosis. *Journal of Neuroscience, 21*(7), 2247-2255.

Harty, S., Robertson, I. H., Miniussi, C., Sheehy, O. C., Devine, C. A., McCreery, S., & O'Connell, R. G. (2014). Transcranial direct current stimulation over right dorsolateral prefrontal cortex enhances error awareness in older age. *Journal of Neuroscience, 34*(10), 3646-3652.

Heimrath, K., Sandmann, P., Becke, A., Müller, N. G., & Zaehle, T. (2012). Behavioral and electrophysiological effects of transcranial direct current

stimulation of the parietal cortex in a visuo-spatial working memory task. *Frontiers in psychiatry, 3*, 56.

Herrmann, C. S., Rach, S., Neuling, T., & Strüber, D. (2013). Transcranial alternating current stimulation: a review of the underlying mechanisms and modulation of cognitive processes. *Frontiers in human neuroscience, 7*, 279.

Jain, S., & Jain, A. (2018). Evaluations of additive effect of two Indigenous medicinal plants Cedrus deodara and Mucuna pruriens towards the treatment of Parkinsons disease. *Parkinsonism & Related Disorders, 46*, e87.

Jansen, R., Brogan, B., Whitworth, A., & Okello, E. (2014). Effects of five Ayurvedic herbs on locomotor behaviour in a Drosophila melanogaster Parkinson's disease model. *Phytotherapy research, 28*(12), 1789-1795.

Kuo, M.-F., Paulus, W., & Nitsche, M. A. (2014). Therapeutic effects of non-invasive brain stimulation with direct currents (tDCS) in neuropsychiatric diseases. *Neuroimage, 85*, 948-960.

Leo, D., & Gainetdinov, R. R. (2013). Transgenic mouse models for ADHD. *Cell and tissue research, 354*(1), 259-271.

Lim, L., Jackson-Lewis, V., Wong, L., Shui, G., Goh, A., Kesavapany, S., . . . Wenk, M. (2012). Lanosterol induces mitochondrial uncoupling and protects dopaminergic neurons from cell death in a model for Parkinson's disease. *Cell death and differentiation, 19*(3), 416.

Liuzzi, G., Freundlieb, N., Ridder, V., Hoppe, J., Heise, K., Zimerman, M., . . . Zwitserlood, P. (2010). The involvement of the left motor cortex in learning of a novel action word lexicon. *Current Biology, 20*(19), 1745-1751.

Lu, C., Wei, Y., Hu, R., Wang, Y., Li, K., & Li, X. (2015). Transcranial Direct Current Stimulation Ameliorates Behavioral Deficits and Reduces Oxidative Stress in 1-Methyl-4-Phenyl-1, 2, 3, 6-Tetrahydropyridine-Induced Mouse Model of P arkinson's Disease. *Neuromodulation: Technology at the Neural Interface, 18*(6), 442-447.

Marek, K., Jennings, D., Lasch, S., Siderowf, A., Tanner, C., Simuni, T., . . . Chowdhury, S. (2011). The parkinson progression marker initiative (PPMI). *Progress in neurobiology, 95*(4), 629-635.

Matsui, H., & Takahashi, R. (2018). Parkinson's disease pathogenesis from the viewpoint of small fish models. *Journal of Neural Transmission, 125*(1), 25-33.

Meena, H., Pandey, H. K., Pandey, P., Arya, M. C., & Ahmed, Z. (2012). Evaluation of antioxidant activity of two important memory enhancing medicinal plants Baccopa monnieri and Centella asiatica. *Indian journal of pharmacology, 44*(1), 114.

Meredith, G. E., & Rademacher, D. J. (2011). MPTP mouse models of Parkinson's disease: an update. *Journal of Parkinson's disease, 1*(1), 19-33.

Miniussi, C., Harris, J. A., & Ruzzoli, M. (2013). Modelling non-invasive brain stimulation in cognitive neuroscience. *Neuroscience & Biobehavioral Reviews, 37*(8), 1702-1712.

Moisello, C., Blanco, D., Fontanesi, C., Lin, J., Biagioni, M., Kumar, P., . . . Abbruzzese, G. (2015). TMS enhances retention of a motor skill in Parkinson's disease. *Brain stimulation, 8*(2), 224-230.

Monte-Silva, K., Kuo, M.-F., Liebetanz, D., Paulus, W., & Nitsche, M. A. (2010). Shaping the optimal repetition interval for cathodal transcranial direct current stimulation (tDCS). *Journal of neurophysiology, 103*(4), 1735-1740.

Nonnekes, J., Arrogi, A., Munneke, M. A., van Asseldonk, E. H., Nijhuis, L. B. O., Geurts, A. C., & Weerdesteyn, V. (2014). Subcortical structures in humans can be facilitated by transcranial direct current stimulation. *PloS one, 9*(9), e107731.

Oertel, W. H., & Fahn, S. (2003). Parkinsonism. In *Neurological Disorders (Second Edition)* (pp. 1021-1079): Elsevier.

Ou, Y.-K., Lin, C.-H., Fang, C.-W., & Liu, Y.-C. (2018). Using virtual environments to assess visual perception judgements in patients with Parkinson's disease. *Transportation Research Part F: Traffic Psychology and Behaviour, 56*, 322-332.

Pang, Z. P., Bacaj, T., Yang, X., Zhou, P., Xu, W., & Südhof, T. C. (2011). Doc2 supports spontaneous synaptic transmission by a Ca 2+-independent mechanism. *Neuron, 70*(2), 244-251.

Paulus, W. (2011). Transcranial electrical stimulation (tES–tDCS; tRNS, tACS) methods. *Neuropsychological rehabilitation, 21*(5), 602-617.

Pikhovych, A., Stolberg, N. P., Jessica Flitsch, L., Walter, H. L., Graf, R., Fink, G. R., . . . Rueger, M. A. (2016). Transcranial direct current stimulation modulates neurogenesis and microglia activation in the mouse brain. *Stem cells international, 2016*.

Podda, M. V., Cocco, S., Mastrodonato, A., Fusco, S., Leone, L., Barbati, S. A., . . . Grassi, C. (2016). Anodal transcranial direct current stimulation boosts synaptic plasticity and memory in mice via epigenetic regulation of Bdnf expression. *Scientific reports, 6*, 22180.

Pouclet, H., Lebouvier, T., Coron, E., des Varannes, S. B., Rouaud, T., Roy, M., . . . Derkinderen, P. (2012). A comparison between rectal and colonic biopsies to detect Lewy pathology in Parkinson's disease. *Neurobiology of disease, 45*(1), 305-309.

Pringsheim, T., Jette, N., Frolkis, A., & Steeves, T. D. (2014). The prevalence of Parkinson's disease: A systematic review and meta-analysis. *Movement disorders, 29*(13), 1583-1590.

Przedborski, S., & Vila, M. (2001). MPTP: a review of its mechanisms of neurotoxicity. *Clinical Neuroscience Research, 1*(6), 407-418.

Riederer, P., & Laux, G. (2011). MAO-inhibitors in Parkinson's Disease. *Experimental neurobiology, 20*(1), 1-17.

Rossini, P. M., Burke, D., Chen, R., Cohen, L., Daskalakis, Z., Di Iorio, R., . . . George, M. (2015). Non-invasive electrical and magnetic stimulation of the brain, spinal cord, roots and peripheral nerves: basic principles and procedures for routine clinical and research application. An updated report from an IFCN Committee. *Clinical Neurophysiology, 126*(6), 1071-1107.

Rozisky, J. R., da Conceição Antunes, L., Brietzke, A. P., de Sousa, A. C., & Caumo, W. (2015). TRANSCRANIAL DIRECT CURRENT STIMULATION AND NEUROPLASTICITY.

Santiago, R. M., Barbieiro, J., Lima, M. M., Dombrowski, P. A., Andreatini, R., & Vital, M. A. (2010). Depressive-like behaviors alterations induced by intranigral MPTP, 6-OHDA, LPS and rotenone models of Parkinson's disease are predominantly associated with serotonin and dopamine. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 34*(6), 1104-1114.

Shi, Y. (2004). Caspase activation: revisiting the induced proximity model. *Cell, 117*(7), 855-858.

Shrivastava, P., Vaibhav, K., Tabassum, R., Khan, A., Ishrat, T., Khan, M. M., . . . Islam, F. (2013). Anti-apoptotic and anti-inflammatory effect of Piperine on 6-OHDA induced Parkinson's rat model. *The Journal of nutritional biochemistry, 24*(4), 680-687.

Shukla, S. D., Bhatnagar, M., & Khurana, S. (2012). Critical evaluation of ayurvedic plants for stimulating intrinsic antioxidant response. *Frontiers in neuroscience, 6*, 112.

Siddique, Y. H., Naz, F., Jyoti, S., Fatima, A., Khanam, S., Ali, F., . . . Faisal, M. (2014). Effect of Centella asiatica leaf extract on the dietary supplementation in transgenic Drosophila model of Parkinson's disease. *Parkinson's disease, 2014*.

Smeyne, R. J., & Jackson-Lewis, V. (2005). The MPTP model of Parkinson's disease. *Molecular brain research, 134*(1), 57-66.

Stagg, C. J., & Nitsche, M. A. (2011). Physiological basis of transcranial direct current stimulation. *The Neuroscientist, 17*(1), 37-53.

Tillerson, J., Caudle, W., Reveron, M., & Miller, G. (2003). Exercise induces behavioral recovery and attenuates neurochemical deficits in rodent models of Parkinson's disease. *Neuroscience, 119*(3), 899-911.

Venkateshappa, C., Harish, G., Mythri, R. B., Mahadevan, A., Bharath, M. S., & Shankar, S. (2012). Increased oxidative damage and decreased antioxidant function in aging human substantia nigra compared to striatum: implications for Parkinson's disease. *Neurochemical research, 37*(2), 358-369.

Vila, M., Jackson-Lewis, V., Vukosavic, S., Djaldetti, R., Liberatore, G., Offen, D., . . . Przedborski, S. (2001). Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine mouse model of Parkinson's disease. *Proceedings of the National Academy of Sciences, 98*(5), 2837-2842.

Wichmann, T., & Dostrovsky, J. O. (2011). Pathological basal ganglia activity in movement disorders. *Neuroscience, 198*, 232-244.

Wirdefeldt, K., Adami, H.-O., Cole, P., Trichopoulos, D., & Mandel, J. (2011). Epidemiology and etiology of Parkinson's disease: a review of the evidence. *European journal of epidemiology, 26*(1), 1.

Woods, A. J., Antal, A., Bikson, M., Boggio, P. S., Brunoni, A. R., Celnik, P., . . . Kappenman, E. S. (2016). A technical guide to tDCS, and related non-invasive brain stimulation tools. *Clinical Neurophysiology, 127*(2), 1031-1048.

Xu, C.-L., Qu, R., Zhang, J., Li, L.-F., & Ma, S.-P. (2013). Neuroprotective effects of madecassoside in early stage of Parkinson's disease induced by MPTP in rats. *Fitoterapia, 90*, 112-118.

Xu, C.-L., Wang, Q.-Z., Sun, L.-M., Li, X.-M., Deng, J.-M., Li, L.-F., . . . Ma, S.-P. (2012). Asiaticoside: attenuation of neurotoxicity induced by MPTP in a rat model of Parkinsonism via maintaining redox balance and up-regulating the ratio of Bcl-2/Bax. *Pharmacology Biochemistry and Behavior, 100*(3), 413-418.

Yokoi, Y., & Sumiyoshi, T. (2015). Application of transcranial direct current stimulation to psychiatric disorders: trends and perspectives. *Neuropsychiatric Electrophysiology, 1*(1), 10.

Zanjani, A., Zakzanis, K. K., Daskalakis, Z. J., & Chen, R. (2015). Repetitive transcranial magnetic stimulation of the primary motor cortex in the treatment

of motor signs in Parkinson's disease: a quantitative review of the literature. *Movement disorders, 30*(6), 750-758.

Zesiewicz, T., Sullivan, K., Arnulf, I., Chaudhuri, K., Morgan, J. C., Gronseth, G., . . . Weiner, W. (2010). Practice parameter: treatment of nonmotor symptoms of Parkinson disease report of the quality standards subcommittee of the american academy of neurology. *Neurology, 74*(11), 924-931.