SAIKOSAPONIN B3 PROTECTS AGAINST MPTP-INDUCED PARKINSON'S DISEASE BY PREVENTING MITOCHONDRIAL OXIDATIVE DAMAGE

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SAIKOSAPONIN B3 PROTECTS AGAINST MPTP-INDUCED PARKINSON'S DISEASE BY PREVENTING MITOCHONDRIAL OXIDATIVE DAMAGE

A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science

In

Biomedical Sciences

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We hereby recommend that the dissertation prepared under our supervision by Neelam Shahzadi (NUST201463169MSMME62414F) titled: **Saikosaponin B3 Protects against MPTP-Induced Parkinson's Disease by Preventing Mitochondrial Oxidative Damage** to be accepted in partial fulfillment of the requirements for the award of MS degree with ___ Grade.

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I dedicate my thesis to my mother for her immense support, motivation & love.

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Neelam Shahzadi

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LIST OF ACRONYMS

%	Percent
°C	Centigrade
PD	Parkinson's Disease
DA	Dopaminergic Neurons
MPTP	1-methyl-4-phenyl-1,2,3,6-tertrahydropyridine
EEG	Electroencephalography
IP	Intraperitoneal
B	Beta
Hz	Hertz
min	minute
S	second
G	Gram

ABSTRACT:

Parkinson's disease is a neurodegenerative disorder that is linked with the loss of dopaminergic neurons of the substantia nigra pars compacta. In this disease, many projecting nerve fibers present in striatum are degenerated. Currently no efficient therapy is present to delay the neurodegenerative process, so modification of the neuro-protective therapy is critical clinical requirement. Saikosaponin B3 is a bioactive phytochemical of Bupleurum marginatum and B. falcatum. The structure, anti-cancer and anti-oxidative activities of Saikosaponin B3 have been discovered. This research is designed to evaluate the effect of anti-oxidative activity of Saikosaponin on Parkinson's disease as it is expected to attenuate the oxidative stress by increasing the activity of anti-oxidative enzymes. This study highlights the associated mechanism and interaction with pathways during neuro-protection therapy. MPTP-induced balb/c mice will be used as a model and the neuron response will be checked and evaluated after dosage of Saikosaponin B3 by EEG assesment. Later, the brain cells were isolated, purified and the activity was analyzed completely.

1. INTRODUCTION

1.1. Saikosaponin B3:

Saikosaponin B3 is a bioactive phytochemical of Bupleurum marginatum and B. falcatum. This plant is used in many traditional Chinese medicines for the treatment of cold, fever, pressure on chest and some related conditions (Hwang, 2013). Saikosaponin B3 is an important compound that was first isolated from Bupleurum chinese DC (Liang et al, 1997). Saikosaponin B3 has grabbed the attention as a useful chemical agent for treatment of several diseases. Some research has been done on its anti-oxidative activity as it has been reported to attenuate the oxidative stress by increasing the activity of anti-oxidative enzymes. It has been discovered recently so reported studies are not too much in number that describe the mechanism or activity of drug with pathways of body. In this research, • Therapeutic potential of Saikosaponin B3 will be assessed against Parkinson's disease by utilizing the anti-oxidative potential of the drug to lower the mitochondrial oxidative stress causing the neuro-degeneration.

1.2. Parkinson's disease:

Parkinson Disease is an acute degenerative disease of neurons. In this disease, dopaminergic neurons in the substantia nigra are lost that cause the symptoms of rigidity, tremors and bradykinesia. However, if the neural changes are dominant then it may cause completed non-motor conditions. There are many evidences that point out the contribution of abnormal mitochondrial activity and elevated oxidative stress in the Parkinson's disease (Schapira, 2008). There is a completed condition that takes place between the cellular machine and the mitochondria that impacts the cell life because an important role is played by mitochondria in the

oxidative phosphorylation and electron transport chain. Further mitochondria provide the free radicals in the cells that take part in the calcium homeostasis and also regulate the cell-death pathways. In these pathways a large number of genes take part that is also linked with Parkinson's disease. These genes have impact on the balanced conditions of mitochondrial fusion and fission; hence they have impact on the complicated structure of tubular network of mitochondria (Ashour, 2013). Further researches carried out on the association of mitochondrial function with PD have shown heterogeneity in pathophysiological factors and not in all the patients with PD mitochondrial dysfunction takes place. This element is very important in designing personalized therapies for the treatment of PD in the future. Understanding the mitochondrial interaction with the pathology of the PD is very helpful in developing the advanced therapies for disease. Henchcliffe and Beal (2008) have mentioned an evidence of the disturbed metabolism of mitochondria that came from the research involving in vitro cell cultures and the autopsy tissue samples from the individuals with Parkinson's disease. In this autopsy, an important part of the electron transport chain i.e. complex I is lowered at the frontal cortex that is also shown Parker et al (2008) and substantia nigra of the individuals with PD and also some researchers have shown decreased electron transfer rates and enhanced oxidative damage by the complex I in these patients (Keeney et al, 2006). This abnormality contributes in rendering the cells weak to Bax-induced cell death and also causes the loss of function of ells in the Parkinson's disease development. The disruption of electron transport chain is suggested to be systemic as there is reduced function of complex I observed in the platelets (Haas et al, 1995) and also the oxidative phosphorylation is disrupted in the skeletal muscles of the body (Pen et al, 1995). Some related studies have been done by occipital lobe (Rango et al, 2006) and temporoparietal region (Hu et al, 2000), by measuring the high energy phosphate level and high

cerebral lactate levels in the patients with PD. All these studies showed that there are abnormalities and dysfunction of mitochondria and also there is a change to the anaerobic mechanism in patients with Parkinson's disease. A study by Weisskopf et al (2007) showed that the elevated levels of the uric acid in serum that is actually a very active antioxidant actually lower the chances of Parkinson's disease.

There is an elevated oxidative stress by the disruption of the mitochondrial function. This mitochondrial dysfunction also leads to oxidative disruption of proteins, lipids (Dexter et al, 1989) and DNA (Zhang et al, 1999) and also a reduction in the concentration of reduced glutathione that is a critical antioxidant (Perry et al, 1986). These changes have been observed on the autopsy tissues taken from the brain of the patients suffering with Parkinson's disease. With the results it can be concluded that there is a strong connection between the oxidative stress and creation of the Lewy body elements that are actually important symptoms of Parkinson's disease. The damage caused by oxidative stress actually creates the ∝-synuclein and lead to the disruptions of the protein and associated functions in the brain (Jenner, 2003) the systemic influences of the disorders are also shown by Bogdanov et al (2008) who measured higher mean plasma 8-hydroxydeoxyguanosine levels in the individuals with the disease. Some more researches are being carried out that would be able to help in the recognition of the changes at the molecular level that are linked to the Parkinson's disease.

This research is designed to evaluate the effect of anti-oxidative activity of Saikosaponin on parkinson's disease as it is expected to attenuate the oxidative stress by increasing the activity of anti-oxidative enzymes. This study highlights the associated mechanism and interaction with pathways during neuro-protection therapy. MPTP-induced balb/c mice were used as a model and

the neuron response was checked and evaluated after dosage of Saikosaponin B3 by EEG recordings. Later, the brain cells were isolated, purified and the activity was analyzed completely

1.3. Research objectives:

Parkinson Disease has now become from a rarely reported disorder to one of the most common disease especially among the older people. There are a lot of studies that describe a link between the Parkinson's' disease and prevention of its symptoms with anti-oxidative drugs.

The main objective of the study is to assess the therapeutic potential of Saikosaponin B3 against Parkinson's disease by utilizing the anti-oxidative potential of the drug to lower the mitochondrial oxidative stress causing the neuro-degeneration. Dose and time dependent effect was checked to confirm an optimum potential dose.

The objectives of the study are reaped by testing the hypothesis that oxidative stress is an important mechanism in the development of Parkinson Disease and by identifying the underlying mechanism of Saikosaponin B3 in inhibiting the oxidative damage in brain cells. These objectives will help to discover and develop innovative antioxidant treatment strategies for preventing or attenuating the Parkinson Disease

1. LITERATURE REVIEW:

2.1 Parkinson Disease:

Parkinson disease was first described as a neurological disease in 1817 by James Parkinson. Parkinson is simply described with the symptoms like involuntary motions with decreased muscle activity in body parts and this happens even when there is a support, further, there is an affinity to bend the trunk, pacing up from walking to running and normal intellect is decreased (Parkinson, 2002).

Parkinson disease is the progressive neuro-degradation resulting because of the decrease of the dopamine containing cells of the brain part called, substantia nigra. At present no reliable test exists that can recognize and differentiate the Parkinson disease from other disorders that have similar physical symptoms. So according to Dauer and Przedborski (2003) the diagnosis is clinical on the account of examination and history. Most striking physical symptoms and disabilities caused due to Parkinson Disease (PD) are motor impairment that is collectively called Parkinsonism. Other symptpms that are shown by the people suffering from Parkinsonism are bradykinesia (slow movements), hypokinesia (inability to move), rest tremors and rigidity. Parkinsonism can result from drugs and sometimes from conditions like cerebral infarction, multiple system atrophy (MSA) and progressive sypranuclear palsy (PSP) (Daley, 2013).Most of the times causes of Parkinson's disease are idiopathic. However, there are some reasons apart from genetic reasons like Age related where failure of normal cellular processes causing vulnerability of Dopaminergic neurons and exposure to chemicals i.e., Pesticides, Herbicides, carbon monoxide, carbon disulfide, plant-derived toxins, Organic solvents, bacterial and viral infections.

Though PD is a motion disorder, there are other psychiatric problems associated with the disease as well like dementia, anxiety and depression. The condition may lead to pain and significant disability making the patient handicap with poor life quality. Caretakers and family may also be affected in an indirect manner. PD affects almost 100-180 people per 100,000 of the population. Every year about 4-200 people are affected from every 100,000. Disease prevalence is common in older age and also it is more prevalent in male population (National Collaborating Centre for Chronic Conditions, 2006).

2.2. MPTP-induced Parkinson's disease:

1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) that is used in the project to induce Parkinson's disease is actually a neurotoxin. MPTP is a precursor for MPP+ that leads to PD and creates almost all the symptoms permanently. This chemical is a lipid soluble neurotoxin that has the ability to cross the blood brain barrier and hence can enter the brain cells easily. When MPTP is administered, it leads to significant loss of SNpc neurons that causes loss of DA neurons. This is common in idiopathic PD as mentioned by Hare et al (2013) and Smeyne and Jackson-Lewis (2005).

MPTP reaches brain and is metabolized by the glial cells with the help of MAO-B enzyme. This creates MPDP according to Brooks et al (1989) that is a metabolite and is also metabolized to create MPP+, a pyridium species. This pyridium specie is then released from the glial cells and reaches the neurons with the help of dopamine transporter (DAT). Here as stated by Cui et al (2009) in electron transport chain of mitochondria, it interacts with complex I respiration. According to Gao et al, (2003) it further leads to harm by initiating the reactive microglia and generates free radicals (as shown in figure)

In administration of MPTP, a significant DA neuronal loss in the Substantia Nigra per compacta is clear after one week. Further, DA generation is also decreased in the terminal end inside the striatum. In this way, MPTP can lead to DA neuronal loss that shows the loss found in the Parkinson's' disease at last stage (Jackson-Lewis et al., 1995).

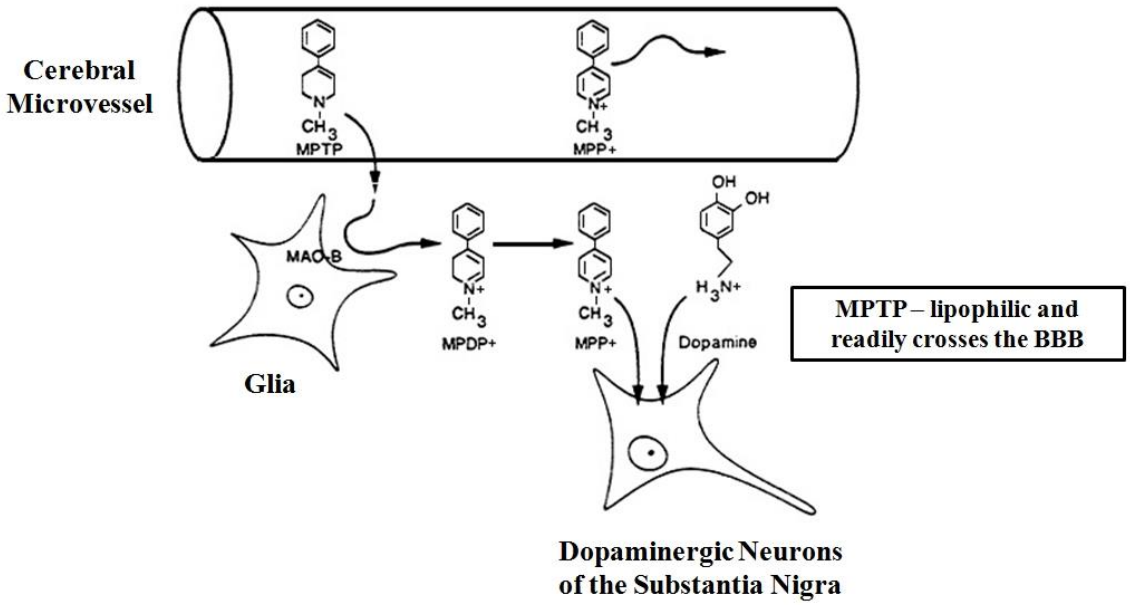

Figure 1: Schematic representation of mechanism of MPTP toxicity.

BBB- Blood brain barrier; MPTP- 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; MAO-Monoamine oxidase; MPDP+- 1-methyl-4-phenyl-2, 3-dihydropyridium; MPP+- 1-methyl-4-phenylpyridinium (Adapted from Ecobichon, D. J., & Klaassen, C. P. 2001).

2.3. Motor Deficits in PD

Parkinson Disease is a progressive disorder that has four major symptoms as mentioned earlier i.e. tremors, postural instability, bradykinesia and rigidity. Normally, these major symptoms appear only after major part i.e. 50-60% of the neurons is lost and also 80-85% dopamine from

Striatum has been diminished by then. Some emotional and cognitive impairment are also associated with the PD in addition to the motor impairments as stated by Rodriguez-Oroz et al., (2009).

2.4. Loss of dopaminergic neurons in PD

In Parkinson's disease, the DA neuronal loss takes place that leads to the loss of dopamine. This leads to short circuit inside the brain's control centre of movements leading to overstimulation of the target neurons. These are the reasons of the muscle tremor and other movement disabilities during Parkinson's' disease (Youdim and Riederer, 1997).

The generation of the symptoms may take years as degradation of dopaminergic SNc neurons in PD is a slow evolving process. These projections of SNc projections start to degrade before the degradation of striatum portions is started. This slow degradation may take years for non-motor signs to show up in relation to the motor signs and other conditions (Dickson, 2007).

Loss of dopamine chemical from basal ganglia leads to significant morphological changes and one of the most prominent changes is the reduction of sensitivity and thickness of the dendritic spines on MSNs that may affect the corticostriatal signal transfer (Villalba et al., 2006, Zaja-Milatovic et al., 2005).

The Dominergic receptors in the striatum subcellular portions may also be altered, hence, in comparison to the normal situation; more D1- receptors are bound to the plasma membrane. However, less receptor is bound to the cytoplasm in case of Parkinson disease (Guigoni et al. 2007).

2.5. Role of Mitochondria:

Mitochondria contribute much in the cell death by apoptosis as the membrane of mitochondria is made permeable by factors like cytochrome c and Bax etc that are released in the cytosol and cause the cell death (Green et al, 2004). Further when the mitochondrial membrane becomes

permeable many other pathways are triggered and all this happens at the time when there is great oxidative stress and also the electron transport chain is disrupted hence the activity of the membrane is damaged and pores appear in it. The pathways of apoptosis are hence triggered by the oxidative damage and disruption of electron transport chain in the cells. According to Beal, the threshold is set again for these activities as per required in reaction to the pro-apoptotic factors and Bax. There is also disruption in the metabolism of energy that is created in response to the mitochondrial damage and makes the cell prone to excitotoxicity. This whole condition results in the elevation of the creation of free radicals in the cells and also increases the injury of the cell organelles. The protein degradation mechanism controlled by ubiquitin–proteasomal system is also affected by the elevated oxidative stress and mitochondrial damage that leads to the many piles of damaged proteins accumulated in the cells (Beal, 1998).

There are many inhibitors of the complex I that create many causes of Parkinson's disease and lead to the disruption of the dopaminergic neurons. Some studies (Langston et al 1983) have shown that the PD is causes by the exposure to the 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP). This same chemical will be used in this research to induce the symptoms of disease in the subjects i.e. balb c mice. Many other chemicals and factors are there that lead to the loss of dopaminergic neurons ultimately causing the PD and in all these conditions there is generation of free radicals. According to McCord (2000) free radicals are very important in maintenance of normal cell activities like the oxygen radicals contribute in the biochemical tasks of the cell and in gene transcription and signal transduction etc. Nitric Oxide is also important in signaling and help in hemodynamics, thrombosis and angiogenesis etc (Zheng et al, 2000). Humans system constantly encounter the free radicals, some are taken in by the pollution and environment, some made by the body by cellular metabolism in the system. The

most common free radicals that are present in the human body are the superoxide, hydroxyl and nitric monoxide. Many chemical reactions take place in the body that led to the formation of the free radicals or the precursors of the free radicals (Gilgun-Sherki et al, 2001). Oxygen free radicals are also very common in the body. In human body there is a complete system of defense involving a complicated network of the antioxidants that work in response to the oxidative damage and stress.

2.6. Reactive Oxygen Species:

The reactive oxygen species (ROS) and oxygen free radicals (OFR) are produced by many normal cellular activities in the body. The human body works on aerobic metabolism that leads to the formation of these elements. Oxygen free radicals include hydroxyl radicals) and superoxide, while the ROS include peroxynitrile, hydrogen peroxide and hypochlorous acid etc (Halliwell, 1994). Along with the creation of these free radicals and reactive oxygen species the body also generates some antioxidants like citrulline, creatine, glutathione, zinc, vitamin A, C, E and selenium etc that are involved in regulation of these ROS and counteract their affects in the system thus a balance in maintained in the system with this mechanism. The system is regulated by some enzymes with anti-oxidation triggering activities and these enzymes help these antioxidants to perform their actions. Some of the most common anti-oxidant enzymes are glutathione peroxidase, superoxide dismutase and glutathione reductase etc. These enzymes are very important in smooth regulation of this free radical counter system (Bandyopadhyay et al, 1999). If somehow this system is disrupted and free radicals are produced in excess amount that are not counteracted then it will lead to the oxidative stress on the bimolecular level and ultimately cause damage in the system. This scenario creates many disorders like cancer, myocardial infarction, diabetes, cardiovascular diseases, aging and neurological degeneration in humans (Fang, 2002) some of the free radicals are very dangerous as NO is damaging to the cells

and it can block the functioning of some enzymes that are crucial for some of the very important cellular activities. If the oxygen is produced in excess that it can lead to the hypertension and may create spasmodic actions in the body (Huie, 1993). Reactive oxygen species are especially dominant in the brain tissues as the neurotransmitters that are present in the brain create the ROS as a result of metabolism. ROS cause oxidative stress in the brain. These chemicals affect the neurons where further mitosis doesn't take place and they are very sensitive to the free radicals so much damage is caused by ROS to the neurons (Gilgun-Sherki et al, 2001) According to Salganik (2001), the ROS impacts the cells causing oxidative damage and ultimately causing the apoptosis of the cells. There is need of antioxidant in the body to counteract the system of ROS generation. The anti-oxidant system consists of two major parts i.e. enzymatic system and non-enzymatic system. There are proteins involved in the anti-oxidant enzymatic system that help in the defense. These molecules are created by the body cells and help great in the regulation of whole mechanism. If this system is disturbed, there will be increased oxidative stress caused by ROS that will lead to the damage of cells and especially neurons causing the symptoms of PD. Oxidative stress cause further creation of free radicals and ultimately leads to the damage of the antioxidant defense system (Ceriello, 2000). The antioxidant elements cause antitumor, antibacterial, antiatherosclerotic, antiviral and anti inflammatory activities (Sala, 2002)

It has also been reported that taking natural antioxidants decrease the chances of cardiovascular diseases, cancer and diabetes (Yang et al, 2001) it has also been observed that antioxidants play an important role in inhibiting the generation of ROS and RNS (reactive Nitrogen species) and many other compounds that are not even free radicals. So if antioxidants are taken from outside then they can play preventive role in handling of the reactive oxygen species. In neurodegenerative diseases like PD the neurons from spinal cord and brain and lost that may

cause dementia or ataxia. Many studies have provided evidence of mitochondrial disruption and neuronal loss in the diseases like PD. The oxidative stress has a deep link to this neuron-degeneration (Emerit et al, 2004). The Parkinson disease that is an area of focus in this research has mentioned to be linked with the loss of the neurons in the brain. Neurotransmitter dopamine (DA) exists in these neurons and their nerve fibers extend to the striatum. The voluntary movements are controlled by these structures hence the damage to these neurons cause conditions like bradykinesia, tremors, muscular rigidity and postural imbalance. Most of the patients suffering from PD have idiopathic cases. Aging is an important trigger to the PD as well as the diet and chemicals that is taken in from the environment (Schapira et al, 2011).

PD is also caused by mutations in the associated genes however, in this research the cases that involve PD caused due to the oxidative stress and damage are considered as by the use of the Saikosaponin B3 the oxidative stress will be reduced as it has the potential to improve the activity of the enzymes that are ultimately involved in catalyzing the anti-oxidants (Uttara, 2009). However, it is interesting to notice that whether the disease is genetic or idiopathic the oxidative stress is common and prevalent in both cases and is involved in the mechanism of cellular damage in both conditions. In all the patients of PD the brain tissues autopsy shows the elevated levels of the oxidized DNA, proteins and lipids (Nakabeppu et al, 2007) and also the levels of the antioxidant enzymes are decreased hence there are chances in both the conditions that Saikosaponin B3 can increase the levels of the enzymes involve in counteracting the reactive oxygen species and ultimately treating the Parkinson's disease regardless of the type of the disease. It is already stated that oxidative stress is caused as a result of the imbalance in the formation of the ROS and the antioxidant activities of the cellular compounds that are naturally produce in the body to counter the negative effects of the ROS. Some of the enzymes like

monoamine oxidase and tyrosine hydroxylase are greatly involved in the production of the reactive oxygen species.

The brain is the most sensitive part of the body and the neurons that are involved in the neurotransmission are very much prone to the oxidative stress that is produced by these factors in the brain. Moreover, the neurons can also release compounds that catalyze the production of the oxidative stress. (Halliwell, 1992) A moderate level of stress even can start a series of reaction in the cellular environment that lead to the apoptosis and ROS are observed to be the most common and major source of the oxidative stress that lead to the damage of the DAergic neurons in the brain and it takes place during the metabolism of DA. Further, there is also mitochondrial damage and inflammation of the neurons that have been reported in the mentioned cases.

It has also been found out that DA neurons are also involved in creation of oxidative stress in the brain and cellular environment. The evidence of DA takes place and ultimately there is modification in the quinine that creates the oxidative stress and make the neuron cells prone to damage and loss of the neural functioning takes place that is also called dementia. Normally the DA neurons are stored in the vesicles however if it is in excess it can be found in the cytosol and it can be oxidized very easily and it will lead to the production of DA quinone. These quinone compounds have ability to modify the nuceophiles like sulfhydryls whose role is to help the cell in survival and prevent the cell death. When the quinone modify these elements and disrupt their function then it will ultimately promote the cell death as there will be no mechanism to help the cell in survival. This element also modifies the α-synuclein that makes the vesicle's membrane permeable and hence the DA neuronal cells are leaked from these pores to the outside environment in the cytosol (Lotharius et al, 2002) by this DA Parkin is also altered and leads to many cellular processes. Many researchers have observed the existence of Parkin in the brains

with the dementia problems (Lavoie et al, 2005). Hence the modification of the DA quinine is the cause of the inactivation of many enzymes that ultimately trigger other reactions causing the symptoms of PD in the brain. Hence if these elements are controlled by the proper regulation of the oxidative stress caused by the reactive oxygen species and mitochondrial dysfunction then it will be very helpful in the treatment of the PD disease.

2.7. The anti-oxidant activity of Saikosaponins:

The Saikosaponin B3 is found to be involved in triggering the antioxidant enzymes that ultimately help the antioxidants to overcome the effects of the free radicals and reactive oxygen species and hence it can ultimately help to stop the disruption of the mitochondria. The activity of the Saikosaponin B3 in controlling the negative impacts of the reactive oxygen species is observ4d in some researches in some other parts of the body like Zhang et al (2014) performed the research on the similar side and he found out that the reactive oxygen species are produced on increased level by heat stress that leads to the oxidative damage in the kidney. The Saikosaponin D that is extracted from the same Bupleurum falcatum plant from where the Saikosapnonin B3 is extracted helps in protecting the kidney from damage in such case. The oxidative damage to the kidney cells in the proximal tubular part of the kidney is prevented as this part was examined.

The mechanism is studied and it was found that Saikosaponin D lowers the oxidative damage by changing the activity of the anti-oxidant enzymes in the kidney cells. This mechanism can be used in the case of the PD as in the brain the oxidative stress is built due to the mitochondrial dysfunction similar to the case of kidney in the given research by Zhang et al (2014). Zhu et al (2009) also studied the activities of the Saikosaponins i.e. Saikosaponin D and A and found that

these compound s are actively involved in decreasing the oxidative damage as they increase the activity of the antioxidant enzymes.

2. MATERIAL AND METHODS:

3.1. Chemicals and Reagents

1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine hydrochloride (MPTP hydrochloride) was bought from Med Chem (Product catalogue# HY-15608).

3.2 Animals

Animal model was used in the project and BALB/c mice were bred and grown in standard animal house of Atta ur Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST). The age of mice was 4-6 weeks and all were female. Mice were housed in cages in natural light and dark circle of 12-12 hours. Animals were given distilled water and standard cat food consisting proteins, fat, fiber and moisture. Number of mice was 9, weighing 30-45 g.

Figure 2: Mice on day one

3.3. Ethics Statement

All the experiments performed at oncology lab were in compliance and accordance with the rules of Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Carbone, L. 2011). These standards are derived from the Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011. The protocol was followed after approval from the Internal Review Board (IRB), SMME, NUST.

3.4 Study Design:

A five day long experimental plan was formed to induce Parkinson disease in the animal model by administrating MPTP and later animals were treated with drug, Saikosaponin B3 for 10 days and the effects were later investigated with multiple tests. EEG analysis was performed for all the groups before and after treatment. Later the animals were decapitated for histological and biochemical studies. Experimental plan is given in Fig. 3

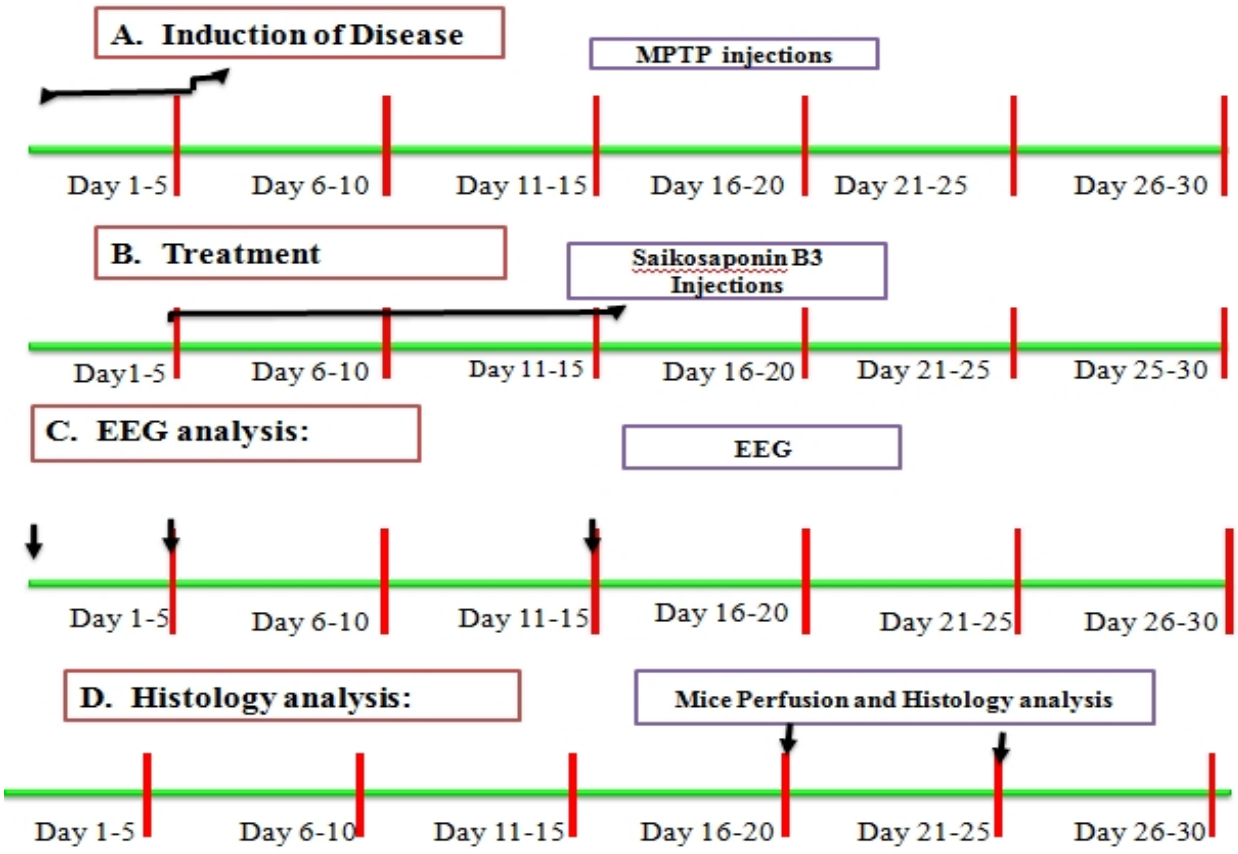

Figure 3: Experimental Plan for the study.

(A) Two groups of three Balb/c male mice each of 4-6 weeks of age was administered MPTP for 5 days (Intra-peritoneal injections) (B) For 10 days' same two groups of animals were treated with Saikosaponin B3 (tail vein injections) (C) On day 1, 5 and 15 EEG analysis of all the three group of animals was done including control, Parkinson disease group and treated group. (D) Mice were decapitated and perfused transcardially on day 20 and processing was done on brains for staining.

3.5. Animal Groups for Study:

Animals were divided into three groups based on their similar weights. There were 3 animals in each group of 4-6 weeks of age. Detailed group information is given below.

Table 1: Experimental design: Control group contained untreated and healthy Balb/c mice. Other two groups consisted of MPTP (diseased mice) and MPTP +Saikosaponin B3 (treated mice)

Sr No.	Groups	No. of animals	IP injection	No. of days
1.	Control	3	Vehicle	5
2.	PD mice	3	MPTP= 15mg/kg	5
3.	Saikosaponin B3 treated mice	3	Saikosaponin B3+MPTP= 15mg/kg	10

3.6. MPTP-induced mice model of Parkinson's disease:

Peritoneal injections of MPTP hydrochloride were given to develop mice model of disease. Two groups were given MPTP injections and BD Ultra-Fine II short needles (30 Gauge x 8 mm) were utilize to give IP injections. Mice were weighed everyday and an IP dose of 15 mg/kg body weight was injected to the mice daily between 11 am-12 pm for 5 days. The lid of the cage was removed with care to avoid any disturbance to mice. The animal to be injected was held smoothly by grasping the tail into the thumb and forefinger. The animal was lifted up from the cage on the cage lid holding the tail firmly. With the help of thumb and forefinger of second hand, loose skin was captured from over the shoulders and movement of animal's head was restrained. The mouse was lifted up by keeping a grip on the base of tail and scruff. The mouse

was then turned over and the body of mouse was held with support on the palm of hand. The body of the mouse was titled in a way that it exposes abdomen. Then the needle was inserted with the other hand into the abdomen at an angle of about 30˚. The penetration was minimal into the abdominal organs and MPTP was injected into the lower right or left quadrant of animal. The needle was later withdrawn carefully and mouse was released in to the cage.

3.7. Saikosaponin B3 Treatment:

One of the diseased groups was treated with Saikosaponin B3 by giving tail vein injections (15 mg/kg) for 10 days between 11 am-12 pm daily. BD Ultra-Fine II short needle (30 Gauge x 8 mm) was loaded with drug making sure that no air bubbles were present as bubbles can be fatal for animal if injected. Animal was picked up smoothly by capturing the tail into the thumb and forefinger. The animals were restrained either by using the cage and its lid as restrainer or held by the second person smoothly and firmly so that injections can be administered easily without movement of mice. The tail was wiped with alcohol and lateral tail vein was located. The syringe was held in dominant hand. The needle was injected in 2/3 way down the tail and the bevel up was nearly parallel to vein. The vessel was almost 2mm under the skin and the drug was injected freely without resistance. It was ensured that tail doesn't swell up otherwise the needle was removed and injected later on some other location. The needle was removed smoothly after injection and hemostasis (little pressure) was applied to the puncture site. Later the animal was placed in the cage.

3.8. EEG Methodology:

3.8.1. Placement of Electrodes:

Three cup electrodes were used, 1 cm in diameter each, filled with conductive gel and glued on the skin surface of mice's head with glass ionomer cement. Four imaginary quadrants of animal' head were selected and two electrodes were fixed on each side of mice's head (biopolar register) after trichotomy. An electrode was also glued with ionomer cement on the tail and it was taken as ground.

3.8.2. Method:

First of all the surgical instrument to be used were sterilized. Mouse was placed in the dissection tray and its head was shaved as well as some area near tail. The position for placing the electrodes was marked with the help of permanent marker. The electrodes were filled with conductive gel and were placed on the marks. Placement of positive and negative electrodes was made on head and the reference or Ground electrode was place on the tail of animal. After placing on the marks, the electrodes were fixed with the help of glass ionomer cement. After fixation, the electrodes were linked to the BioAmp wire of the Powerlab device. Mice were left in the Faraday's cage while recording EEG signal by using LabChart software. When the electrodes were fixed to position, they were linked to the amplifier and EEG was recorded while the mice were awake and freely moving. Recordings were made when the mice was at rest and also while it was moving. All the recordings were made at the same time of day i.e. 12:00 to 2:00 pm. EEG was recorded on healthy mice, later after development of Parkinson disease and then after treatment with the Saikosaponin B3

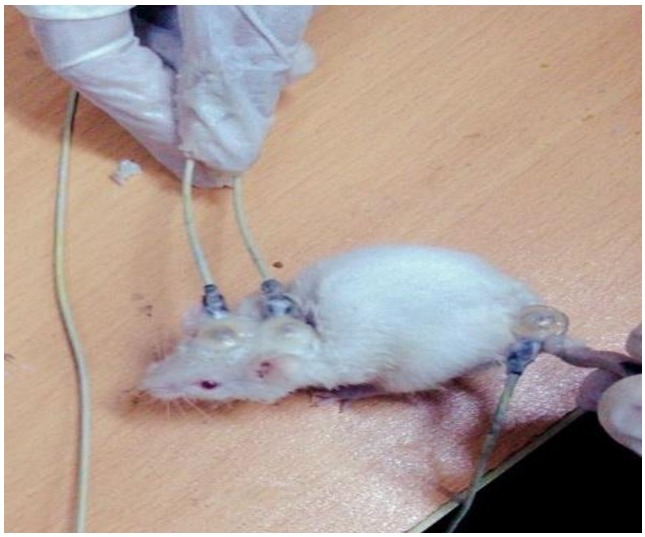

Figure 4: Electrodes placement for EEG-analysis.

3.8.3. Data analysis:

The EEG recorded were analyzed by using digital signal processing techniques implemented in Matlab® software (MathWorks, Inc., MA, USA). In order to measure the system accuracy by comparing with literature results, the digital signal collected was analyzed by using Fourier transform (FT). Finally, Mean FFT values in specifics band frequencies (beta) was calculated and expressed as mean±S.E.M, analyzed by ANOVA followed by Tukey test, considering level of $P<0.05$. The precision of one measurement was defined by comparing it with the mean of N measurement as described by NORTHROP (2005).

3.9. Histological Examination of Brain Regional Tissues

3.9.1. Tissue Perfusion/Fixation for Histological Assessment

Protocol of Gage et al (2012) was followed for heart perfusion of animal. Mice were weighed and deeply anesthetized by injecting anesthesia (zoletil +Rompun+ 1x PBS). Intra-peritoneal injections were administered (300µl/50g). Strernotomy was performed by making a midline incision that exposed the heart. It was ensured that mouse was still alive and heart was beating.

Normal saline (9% saline) was flowed in to the left ventricle with the help of a needle. The flow of normal saline was about 5ml/minute and the depth of inserted needle was nearly 5mm. the heart was held fixed in place with the help of forceps.

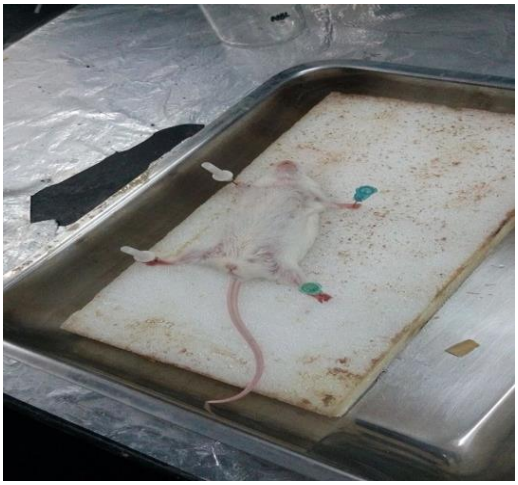

Figure 5: Mice is anesthetized deeply and fixed on tray before Strernotomy.

The blood was then allowed to flow out by making an incision in the right atrium. 90 ml of normal saline was injected and the procedure was repeated to flow 90 ml of 4% paraformaldehyde solution through the left ventricle.

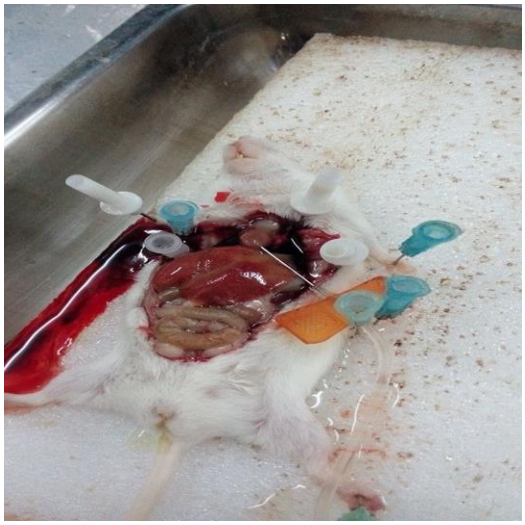

Figure 6: Needle is inserted and 4% paraformaldehyde solution is being run through the left ventricle and incision is made to the right atrium.

The organs of mice were observed to turn lighter in color that showed the perfusion was done right. Then the brain was excised and brain tissues were stored at 4˚C for 24 hours in 4% paraformaldehyde solution. Later the brain was transferred to 1x PBS and stored for 2 days before further processing with paraffin and embedding. Later, the brain tissues were stained with H & E staining after processing. Blocks and slides were prepared. Slides were visualized at 10X and 40 X resolutions by inverted microscope (Labomed, USA). The images were taken of the substantia nigra from brain by Pixel Pro™ image analysis software (Labomed, USA).

3.10. Protein Extraction:

Mice brains were placed at -80° C and then proteins were extracted with RIPA buffer. Half hemisphere of brain with substantia nigra was taken in a falcon tube and 3ml of RIPA buffer was added. The brain tissues were fully homogenized with repeated pipetting. The homogenized mixture was centrifuged at 15000 rpm for 10 min and the fluid was removed and cell debris was left at bottom. The taken fluid was again centrifuged for 5 min at 15000 rpm and supernatant was collected containing pure proteins and stored at -80° C for biochemical analysis.

3.11. Protein Quantification:

Cuvettes were washed with ethanol and samples were prepared. Bradford assay was performed with the help of UV spectrophotometer. Bovine Serum Albumin (BSA) was used as a standard protein and different concentrations were used to generate a standard curve. Total 1ml of mixture was used for every concentration. Samples were made in a dark room as BSA is sensitive to light. Samples were vortexed and incubated for 5 minutes at room temperature. A blank was also used containing water and Bradford reagent only and no protein. Bradford

reagent was used in each sample and absorbance was noted on 595 nm for protein. Same was repeated for Control, Disease and Treatment group proteins.

Table 2: Different concentrations of BSA/sample used and absorbance recorded at 595 nm

BSA Concentration/Sample	Water	Bradford Reagent	Total	Absorbance recorded
0ul	800ul	200ul	1000ul	0
20ul	780ul	200ul	1000ul	0.0441
40ul	760ul	200ul	1000ul	0.0974
60ul	740ul	200ul	1000ul	0.1384
80ul	720ul	200ul	1000ul	0.1898
100ul	700ul	200ul	1000ul	0.2549
Control (2ul)	798ul	200	1000ul	1.69
Diseased (2ul)	798ul	200	1000ul	1.86
Treatment (2u)	798ul	200	1000ul	1.92

A formula was generated with absorbance value of BSA and concentration of protein in the sample of three groups can be calculated from this formula. From the standard curve concentration of samples (control, diseased and treatment group) is found out to be 67.76, 74.56, and 76.96 in 2ul respectively. These values are generated from the formula $y = 0.025x - 0.0044$. The standard curve is shown in figure

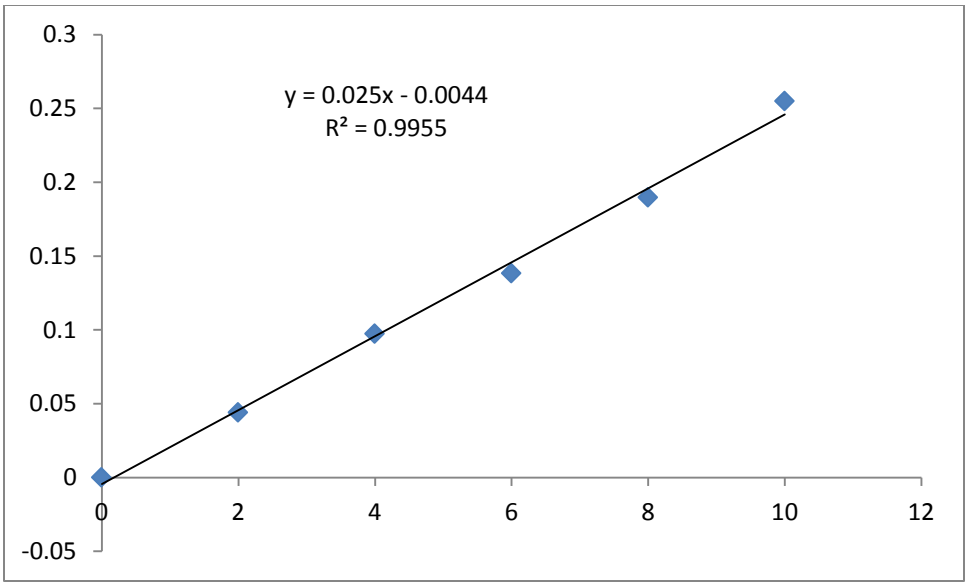

Figure 7: Standard Curve

3.12. Western Blot:

12% Separation gel solution was made and poured in to the assembly till the mark made on the stand. Iso-propanol was added in the remaining space for making the upper edge of the gel smooth. The gel is formed in 20-30 minutes. Now, iso-propanol was removed from the assembly and the empty portion above gel was dried with absorbent paper. 5%s stacking gel solution was prepared and mixed by vortexing for few seconds. This stacking solution was transferred very quickly to the assembly over the separation gel and comb was adjusted quickly. Gel is formed in 10-15 minutes.

Samples were prepared for loading according to the quantity determined by Bradford assay. I mixed the protein sample with 4X buffer and distilled water. Quantity of protein for every group's sample was taken according to the standard curve formula e.g. y = 0.025x - 0.0044

Table 3: Calculation of Samples for loading in gel

Sample	Absorbance	2ug	1ug	40ug Load	H2O	4X buffer	Total
Control	1.69	67.76	33.88	1.180638	13.81	5	20
Diseased	1.86	74.56	37.28	1.072961	13.93	5	20
Treatment	1.92	79.96	38.48	1.039501	13.97	5	20

Absorbance= Y values (1.69, 1.86, 1.92)

2ug (X value from y = 0.025x - 0.0044) = Qty of sample used in analysis by U spectrophotometer.

1ug= 2ug/2.

20ug Load= Desirable & variable load. 40/33.88= Loading Qty. divided by first value in 1ug column.

H2O= Highest value in 40ug column is subtracted from 15

4X buffer= 1/3 of total volume (15/3=5). Total= 40ug Load+H2O+4X buffer

Protein sample was mixed with 4X buffer and distilled water and centrifuged for a minute. The sample mixture is then placed in boiling water for 10 minutes to denature the proteins. Later the sample mixture was again centrifuged for a minute. Comb was removed from the gel and wells were washed with distilled water and dried with absorbent paper. 2ul running buffer is added in the wells and samples were loaded in the following manner.

4X (2ul)	Protein Marker (2ul)	Sample (20ug)	Sample (20ug)	Sample (20ug)		Sample (20ug)	Sample (20ug)	Sample (20ug)

1X running buffer was added in the running apparatus and gel was run for 2-2.5 hours at 100 volts. Right position of electrodes were taken care of while supplying power and running buffer was checked visually while it started moving upward after power is supplied. Gel was then transferred to the Nitrocellulose membrane after sample has been run.

The transfer assembly of gel is as follows:

Sponge	Filter paper	Gel	NC membrane	Filter paper	Sponge

Transfer cassette was closed and transfer buffer was added in the transfer apparatus. The transfer takes place at 90 volts for 25 minutes and then position of the cassettes were changed and run for 25 more minutes. When the transfer time was completed, NC paper was taken from the cassette and added to 1X ponceau stain solution. It made the bands visible.

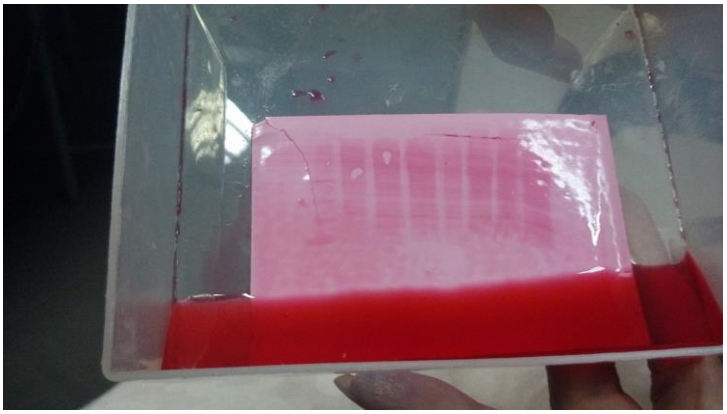

Figure 8: Visible bands of proteins transferred on NC membrane and stained with Ponceau stain solution

Later, NC paper was washed with distilled water and cut according to the molecular weight of the proteins. Range of the molecular weight was shown by Protein marker. NC strips were made

and placed in 5% non-fat milk at 4°C for 2 hours for blocking. After blocking, strips were placed in appropriate antibody for 16-48 hours at 4°C. I used Beta-Actin, Pro-caspase 3 and Catalase antibodies by Santa Cruz Biotechnology (SCBT), Inc. After incubation in primary antibody, washing was done with TBST for 5, 5, 10, 10, and 10 minutes changing TBST solution every time. Then, NC paper was incubated in respective secondary antibody for 2 hours at 4°C. Washing step was repeated again. Strips were then fixed in the X-ray cassette ECL-Kit solution (1:1) was poured on the strips. Finally the X-ray was fixed on them in the cassette and kept in dark room. 2 X-ray films were fixed first was taken for results after 2 hours and the other film was taken after 24 hours. X-ray was washed with developer, water and placed in buffer and bands were observed.

3. RESULTS

4.1. Effect of Transcranial direct current stimulation (tDCS) on beta oscillation in MPTP-induced Parkinson's diseases

The effect of Saikosaponin B3 on beta (β) waves in mice brain was investigated. The changes in β-oscillations were assessed by recording and measuring electroencephalography (EEG) of each animal. EEG of every mice was recorded with the help of cup electrodes and EEG was confirmed by further signal processing. Understanding the impact of Saikosaponin B3 on the EEG level is of great importance. Hence, in this research EEG was measured in the cortex region of mice brain. In both control and Saikosaponin B3 treated groups, normal β-activity was observed. However, increased β-activity was observed in MPTP group in comparison to the control group. In the drug treated group, β-activity was restored to some level in comparison to the control group. A prevalent frequency range from 13Hz to 35Hz, has been observed that are in accordance to the previously reported literature. Beta activity has been recorded for mice both in moving and resting position.

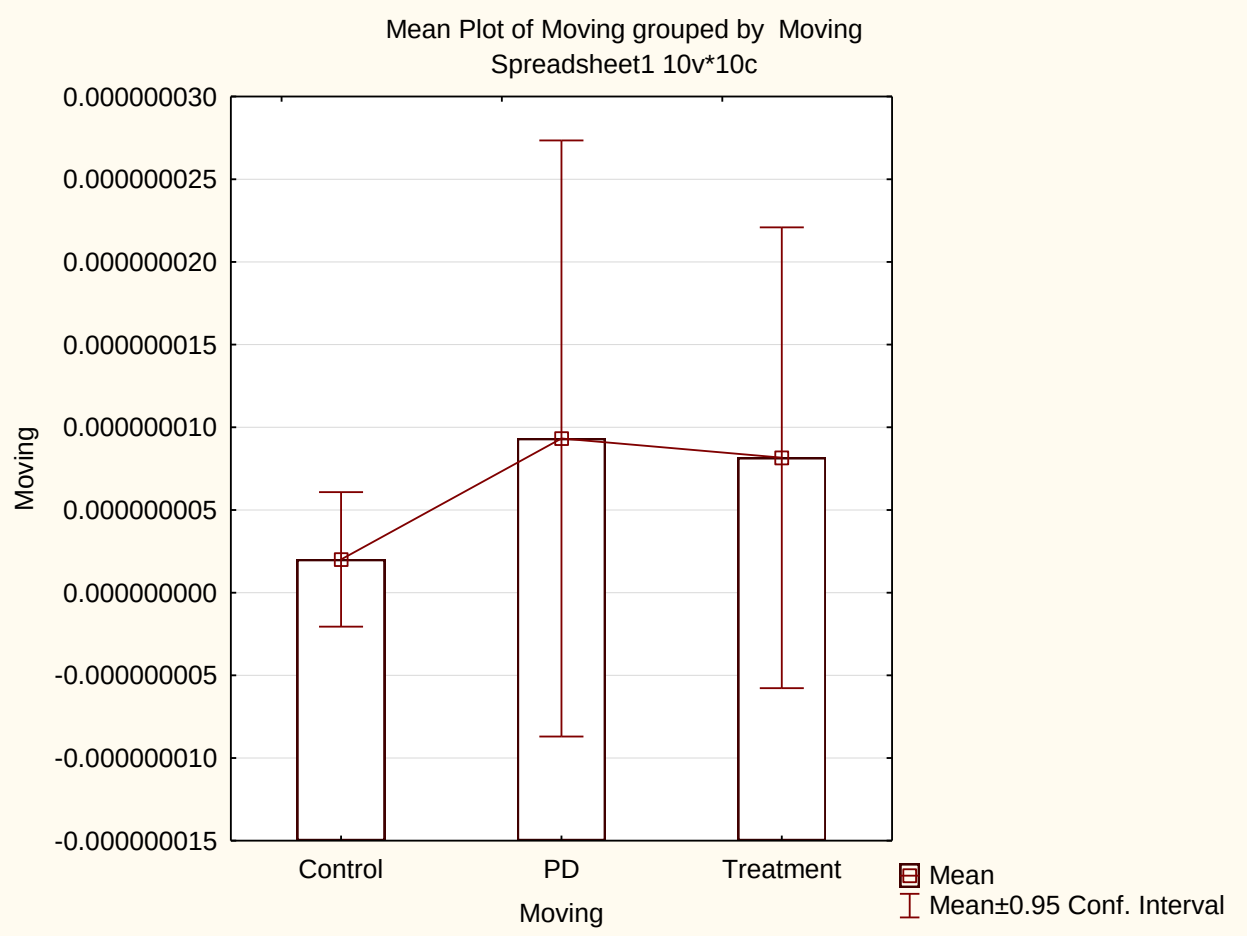

Figure 9: FFT- mean value analysis of change in β-activity after Saikosaponon B3 treatment (moving mice)

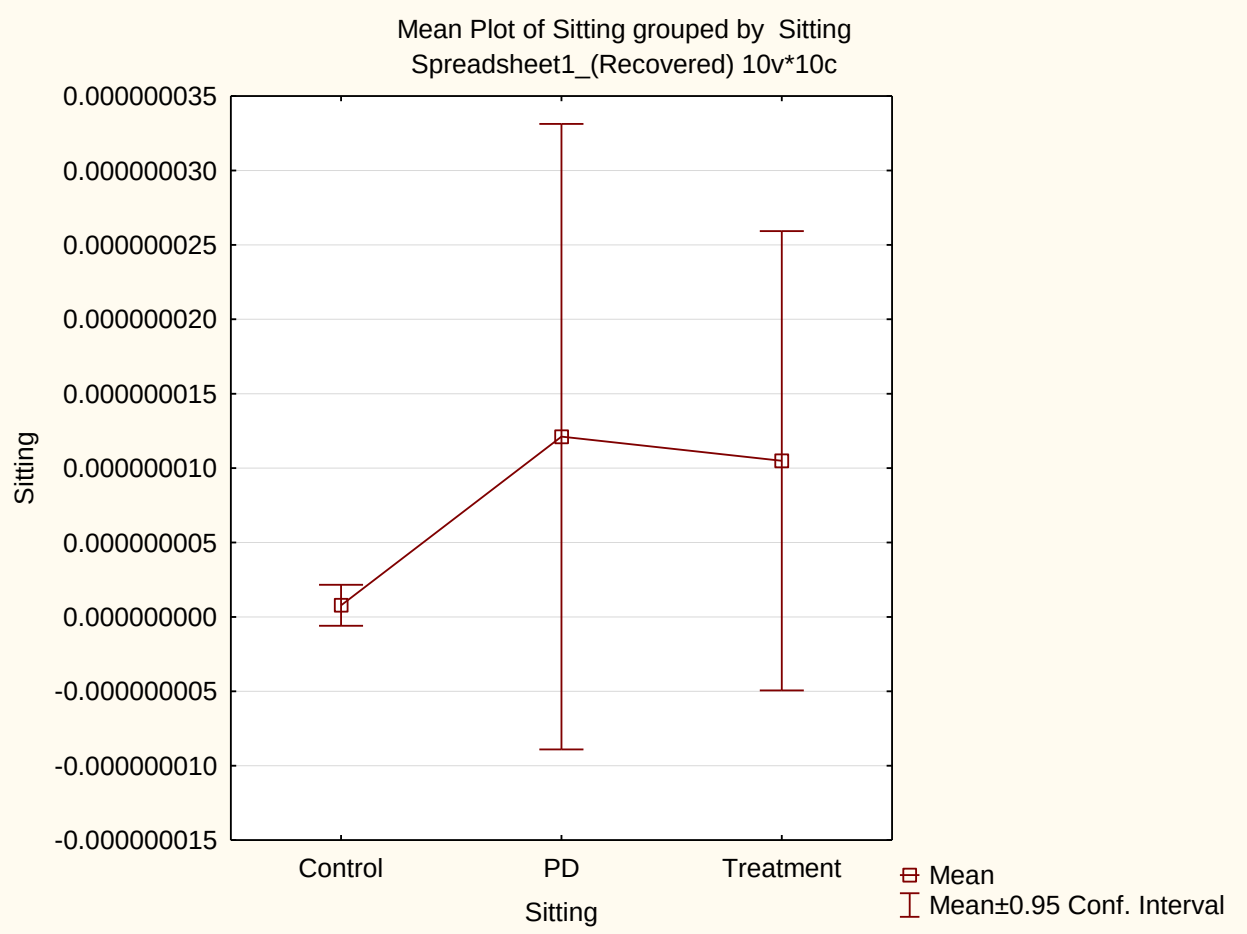

Figure 10: FFT- mean value analysis of change in β-activity after Saikosaponon B3 treatment (Sitting mice)

No significant difference was observed in control, MPTP and treatment group. However, significant rise in FFT- mean of β-activity can be observed in MPTP group in comparison to the control group. Significant reduction in β-activity can be observed in treatment group in comparison to MPTP group. Beta activity is overall more in mice while they are moving than at rest.

4.2. Effect of Saikosaponin B3 on histological features in MPTP-induced Parkinson's diseases

Histopathological assessment of substantia Nigra per compacta was done for all the three mice groups to check the morphological changes that took place in substantia nigra per compacta. The H & E staining showed a marked decrease in pigmentation and Nissl substances in disease (MPTP) group in comparison to the control. Saikosaponin B3 treated group showed an increase in number of pigmented neurons in

comparison to the diseased group. Number of cell bodies in the treated group is similar to the control group.

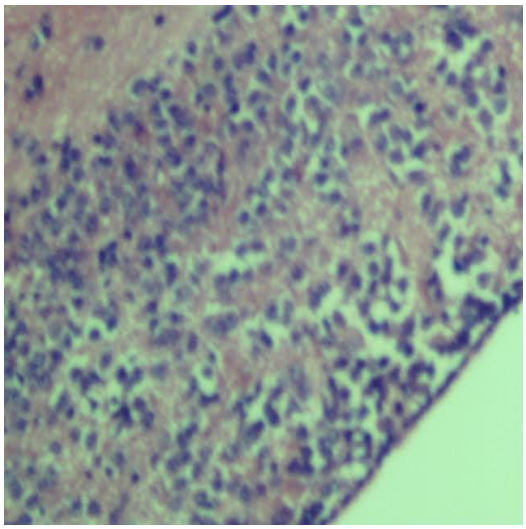

Figure 11

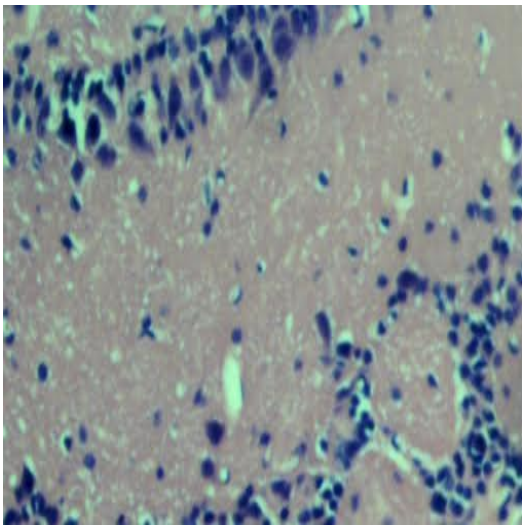

Figure 12

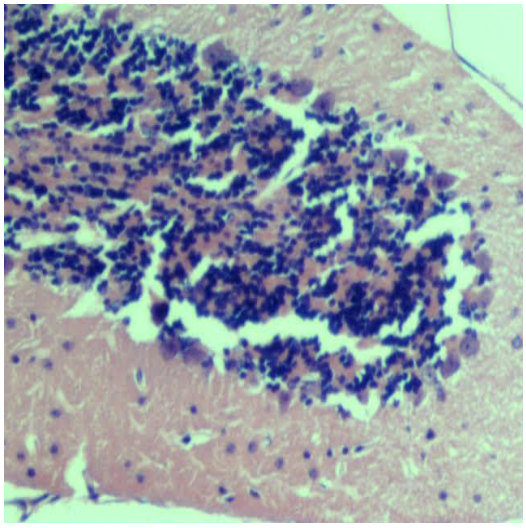

Figure 13

Figure 11: Histology of normal substantia nigra showing many pigmented neurons.

Figure 12: Histology of Parkinson's disease showing loss of pigmented neurons in substantia nigra.

Figure 13: Histology of treated substantia nigra showing many pigmented neurons as compared to diseased one.

4.3. Effect of Saikosaponin B3 on biochemical features in MPTP-induced Parkinson's diseases

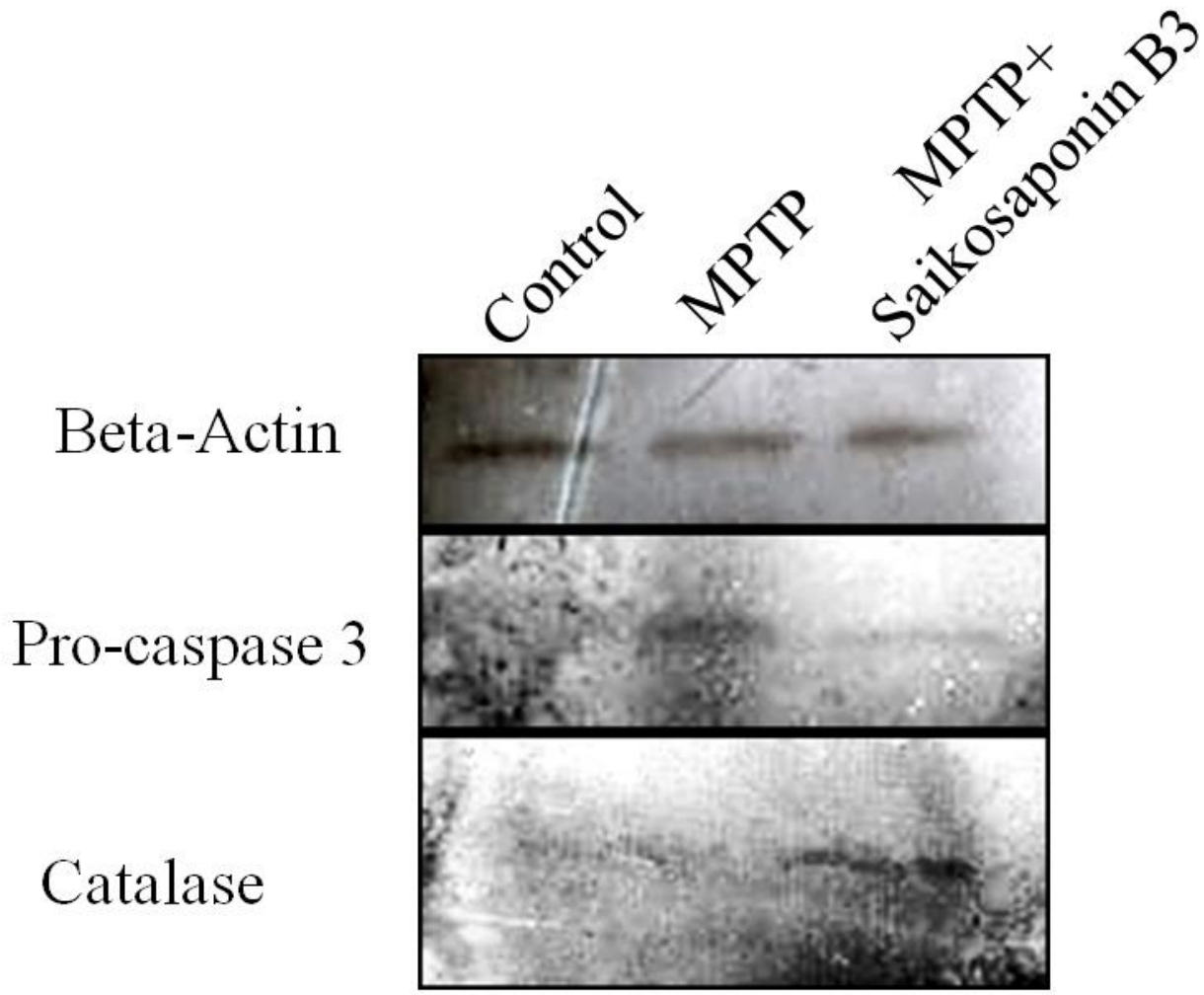

Figure 14: Results of western blot

Results show Beta-Actin present in all the three groups while increased Pro-caspase 3 in MPTP group as compared to the other two groups and increased Catalase in MPTP+Saikosaponin B3 group. (Treated group**)**

4. DISCUSSION

5. 1. Enhanced Beta activity in PD:

Enhanced Oscillations of beta (13–30 Hz) frequency band are prominent pathology in Parkinson's disease. These enhanced beta waves inside the cortico-basal ganglia-thalamic network are signatures of rigidity and bradykinesia in Parkinsonism (Arakelian et al., 2005). Cortico-basal ganglia-thalamic (CBT) networks are important modulators of the motor and cognitive function but when these neural circuits are compromised, disorders like Parkinson's disease take place. This motor deficit in Parkinson's disease is related to the enhanced beta oscillations inside the Cortico-basal ganglia-thalamic (CBT) network. No clear information is present about the types of cells in the CBT regions that are involved in these exaggerated oscillatory bands of beta frequency. So it is not thoroughly clear how the emergence of Beta oscillations takes place and how these are linked to the Parkinson's disease but according to Kondabolu et al (2016) striatal cholinergic interneuron are the mediators of increased beta oscillations in normal mice and create the motor deficits in Parkinson's disease. This is because the striatal cholinergic system is uninhibited due to the loss of dopamine (Kondabolu et al.) But according to Brittain et al (2014) a hypothesis can be developed that synchronization of this frequency band is an important element in computation across some neurons and when the synchrony increases, loss of information takes place and computational capacity is lost (Brittain, Sharott, & Brown) Ideally there should be ideal degree of network synchronization and if the levels are higher or lower than this optimal degree, it will lead to impaired behavioral performance as seen in Parkinson's disease. Although mechanism underlying this pathological

condition of beta oscillations is elusive, but as stated by McCarthy et al (2011) some mathematical models have been used to indicate that beta oscillations can be caused by inhibitory interactions of striatal medium spiny neurons. Here enhanced degree of cholinergic drive, pathology related to parkinsonian striatum, causes increased beta frequency oscillations in this model. The model by McCarthy et al gave the evidence of how the enhanced striatal network dynamic as is a reason for increased beta frequency waves in the Parkinsonism (McCarthy et al.)

Richardson (2013) discussed that neuronal oscillations from synchronized groups of neurons play an important role in determining pathological conditions in Parkinson's disease (Richardson) Little suggested that during cortical or subcortical sites are stimulated in the range of beta frequency that lead to slowing of movements thus creating the symptoms of Parkinson disease (Little & Brown) Levy et al (2002) suggested that depletion of dopamine let 15–30 Hz cortical beta oscillations reach the basal ganglia via cortical-subthalamic pathway that results in oscillatory synchronization and promote the symptoms of Parkinson's disease (Levy et al., 2002) According to Berardelli et al, (2001) the cortical deficit is prominent in the midline motor areas that causes abnormal pre-movement EEG activity and increased beta oscillations (Berardelli, Rothwell, Thompson, & Hallett, 2001) These oscillations of basal Ganglia can be reduced by dopaminrgic drugs, high frequency stimulation or neurosurgical lesioning so ultimately overcoming akinesia (Farmer, 2002) De Hemptinne et al also checked association β-band neuronal synchronization with abnormal activity in motor cortex (de Hemptinne et al.) Beta activity is enhanced in the Parkinson's disease in the animal models used in the experiment and a significant decrease in Beta activity is seen in the Saikosaponin B3 treated group

5.2. Histopathological changes after Parkinson induction and Treatement by Saikosaponin B3:

This study gives evidence that MPTP attenuates dopaminergic Neurons in Mice brain and Saikosaponin B3 reduces neuronal damage in Substantia Nigra Per compacta. This study enhances our knowledge regarding the neurobiochemical effect of Saikosaponin B3. Neurons in Parkinson's disease were well preserved after treatment by Saikosaponin B3 in comparison to those in MPTP group. The results are significant as they show that neuronal death after Parkinson Disease can be prevented by Saikosaponin B3. The mechanism involves the modulation of the activities of antioxidant enzymes. There can be some other phenomena involved and more animal studies must be performed to confirm this.

4.3. Increased Catalase and decreased Pro-Caspase 3 activity by Saikosaponin B3:

MPTP induced oxidative stress that increased the pro-caspase-3 activity. Pro-caspase-3 is a marker for apoptosis as mentioned by Labbé et al (2005). Moreover, oxidative stress also decreased the Catalase activity as per evidence by Escribano et al (2015). Catalase is an antioxidant enzyme so its activity is reduced in Parkinson disease

CONCLUSION

From our results, it is clear that Saikosaponin B3 prevent neurons damage in the cerebrovascular injured brain. These findings may be useful for studies about the therapeutic mechanism of anti oxidative drugs in case of PD.

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