A DISSERTATION ON

TO ASSESS THE EFFECT OF CURCUMA-OIL IN 6-HYDROXYDOPAMINE INDUCED EXPERIMENTAL MODEL OF PARKINSON'S DISEASE

SUBMITTED TO THE
DEPARTMENT OF BIOSCIENCES
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILLMENT
FOR THE
DEGREE OF MASTER OF SCIENCE
IN BIOTECHNOLOGY
BY

SHILPA KUMARI
M.Sc. Biotechnology (IV semester)
Department of Biotechnology Integral university, Lucknow

UNDER THE SUPERVISION OF
DR. SARIKA SINGH
(PRINCIPAL SCIENTIST)
DIVISION OF TOXICOLOGY
CENTRAL DRUG RESEACH INSTITUTE,LUCKNOW, UP

CSIR- Central Drug Research Institute

Certificate

Ref.: CSIR-CDRI/HRD&KRC/1595/2021-2022

Date: 27/05/2022

This certificate is issued to the candidate on successful completion of Skill Development Program for Post- Graduate Students

Name	:	Ms. Shilpa Kumari
Student	:	M.Sc. Biotechnology
Sponsor	:	Integral University, Lucknow
Topic	:	To assess the effect of curcuma-oil in 6-hydroxydopamine induced experimental model of Parkinson's disease
Duration	:	01/02/2022 to 02/06/2022

The candidate has fulfilled the prescribed requirements of the laboratory work, library consultation and completed the assigned task with sincerity and diligence.

Dr. Sarika Singh
Training Supervisor & Principal Scientist
Toxicology & Experimental Medicine

Vinay Tripathi
Training Coordinator & Chief Scientist
Head, HRD & Knowledge Resource Centre

Dr. D. Srinivasa Reddy
Director

CSIR-Central Drug Research Institute, Sector10, Jankipuram Extension, Lucknow 226031, U.P., INDIA; Website: www.cdri.res.in

INTEGRAL UNIVERSITY

Established Under U.P. Act No 09 of 2004 by State Legislation
Approved by University Grants Commission
Phone No.: +91 (0552) 2890812, 2890730,
3296117, 6451039, Fax No.: 0522-2890809

Kursi Road, Lucknow-226026, Uttar Pradesh (INDIA)

CERTIFICATE OF ORIGINAL WORK

This is to certify that the study conducted by **Ms. Shilpa Kumari** during the months Feb-June, 2022 reported in the present thesis was under my Co-supervision. The results reported by her are genuine and script of the thesis has been written by the candidate herself. The thesis entitled is "**To assess the effect of curcuma-oil on 6-hydroxydopamine induced experimental model of Parkinson's disease**" is therefore, being forwarded for the acceptance in partial fulfillment of the requirements for the award of the degree of M. Sc Biotechnology, Department of Biosciences, Integral University, Lucknow, (U.P).

Co-Supervisor

Dr. Mohammad Ashfaque.

Assistant Professor, Departments of Biosciences Integral University, Lucknow

E-mail: info@integraluniversity.ac.in **Web: www.integraluniv** INTEGRAL

INTEGRAL UNIVERSITY

Established Under U.P. Act No 09 of 2004 by State Legislation
Approved by University Grants Commission
Phone No.: +91 (0552) 2890812, 2890730,
3296117, 6451039, Fax No.: 0522-2890809

Kursi Road, Lucknow-226026, Uttar Pradesh (INDIA)

TO WHOM IT MAY CONCERN

This is to certify that **Ms. Shilpa Kumari**, a student of M. Sc. Biotechnology (II Year, IV semester), Integral University has completed her four months dissertation work entitled **"To assess the effect of curcuma-oil on 6-hydroxydopamine induced experimental model of Parkinson's disease"** successfully. She has completed this work from 1st of February to 2nd of June 2022 at CSIR-CDRI, under the guidance of Dr. **Sarika Singh.** The dissertation was a compulsory part of her M. Sc. degree.

I wish her good luck and future endeavors.

Dr. Snober S. Mir

Head,

Department of Biosciences,

Integral University, Lucknow

E-mail: info@integraluniversity.ac.in **Web: www.integraluniv** INTEGRAL

DECLERATION

I hereby declare that this project work titled "**To assess the effect of curcuma-oil on 6-hydroxydopamine induced experimental model of Parkinson's disease**" is a record of original work done by me under the supervision and guidance of **Dr Sarika Singh, Principal Scientist at CSIR-CDRI, Lucknow** and this project work has not formed on the basis for the award of any Degree/Diploma Association/Fellowship or similar title to any candidate of the university.

Signature with name

Place: Lucknow

Date:

ACKNOWLEDGEMENT

The work presented in this project would not have been possible without my close association withmany people. I take this opportunity to extend my sincere gratitude and appreciation to all those who made this project possible.

I gratefully acknowledge to **Dr D. Srinivasa Reddy, Director, CDRI, and Lucknow** to allow me to carry my research in the eminent institution of CSIR.

My sincere thanks to **Dr. Sharad Sharma, HOD Toxicology Division, CSIR-CDRI, Lucknow** forhis valuable guidance.

I am profoundly indebted to my supervisor **Dr. Sarika Singh, Principal Scientist, Department of Toxicology, CSIR-CDRI, Lucknow** for accepting me as a project trainee and her continuous support, guidance and suggestion to put in the best of my efforts in my research work.

It is a matter of immense pleasure and pride for me to present my report entitled "**To assess the effect of curcuma-oil on 6-hydroxydopamine induced experimental model of Parkinson's disease**" which is an outcome of my training in the Department of toxicology and experimental medicine, CDRI, Lucknow.

I express my grateful thanks to **Mrs. Anupama** for their support and cooperation for my work.

I am out of words in expressing my heartfelt thanks to **Mrs. Parul Gupta** and **Amrutha.K** for their enormous support, suggestions and valuable guidance throughout my tenure.

I am express my sincere gratitude towards my seniors for their constant guidance and enormous support throughout my project work **Mr. Abhishek Singh** and **Shubhangini Tiwari**.

I would like express my thanks to **Dr. Snober S. mir, Dean and Head Department of Bioscience and Biotechnology, Integral University** for her kind cooperation and support throughout my master's program.

I extended my warm thanks to my friends **Sargam, Vanshika ojha and Ankit madhesiya** for their cooperation during my work. I owe my special thanks and gratitude to my parents and family for their love, inspiration and constant support and encouragement that made me able to face all the hindrance in my path and to complete the project work. Above all, I am very thankful to "**Almighty**" for providing me strengthfor completion of my project work.

SHILPA KUMARI

CONTENTS

S.NO.	NAME OF CHAPTERS	PAGE NO.
1	INTRODUCTION	9-10
2	REVIEW LITERATURE	11-23
3	AIM AND OBJECTIVE	24
4	WORK PLAN	25
5	MATERIAL AND METHODS	26-40
6	RESULTS	41-47
7	DISCUSSIONS	48
8	CONCLUSION	49
9	REFERENCES	50-52
10	BIBILIOGRAPHY	53
11	APPENDICES	54-56

LIST OF FIGURES

S. NO	TITLE OF FIGURES
1	**Figure: 1** The brain has three main parts: the cerebrum, cerebellum and brainstem.
2	**Figure: 2** Structure of brain
3	**Figure:3** flow chart of nervous system
4	**Figure:4** Structure of neuron cell
5	**Figure:5** The intrinsic apoptotic pathway.
6	**Figure:6** The extrinsic apoptotic pathway.
7	**Figure:7** Chemical structure of dopamine
8	**Figure:8** Biosynthesis of Dopamine from L-tyrosine
9	**Figure:9** Structure of 6-OHDA.
10	**Figure:10** shown 6-OHDA and its mechanism of action.
11	**Figure:11** Western blotting pattern.
12	**Figure:12** Western blot images and graphs representing the quantification of band intensity of TH and caspase-3 expression.
13	**Figure:13** Pictorial representation of DNA damage in SN and STR regions of rat brain after 6-OHDA treatment.
14	**Figure:14** Bright field images of HE Staining after 3 days of 6-OHDA administration.
15	**Figure:15** Bright field images of CV Staining in SN and STR after 3 days of 6-OHDA administration.
16	**Figure:16** Graph representing the mean rotation/min of SD rats.
17	**Figure:17** Western blot images and graphs representing the quantification of band intensity of TH and caspase-3 expression.

ABBEREVEATIONS AND SYMBOLS

µg	MICROGRAM
µl	MICROLITRE
mM	MILIMOLAR
M	MOLAR
PI	PROPIDIUM IODIDE
APS	AMMONIUM PER SULFATE
BG	BASAL GANGLIA
CO	CURCUMA-OIL
BSA	BOVINE SERUM ALBUMIN
CV	CRESYL VIOLET STAINING
CNS	CENTRAL NERVOUS SYSTEM
CSF	CEREBROSPINAL FLUID
$CUSO_4.5H_2O$	COPPER SULFATE
DA	DOPAMINE
DPX	DEOXY PLASTICIZER XYLENE
DMSO	DIMETHYL SULFOXIDE
EDTA	ETHYLENE DIAMINE TETRA ACETIC ACID
HCL	HYDROCHLORIC ACID
HE	HEMATOXYLINE AND EOSIN STAINING
HRP	HORSELIN -RADISH PEROXIDASE
H_2O_2	HYDROGEN PEROXIDE
I.P.	INTRA-PERITONEAL
KCL	POTASSIUM CHLORIDE
KG	KILOGRAM
$MgCl_2$	MAGNESIUM CHLORIDE

NaCl	SODIUM CHLORIDE
Na2HPo4	SODIUM HYDROGEN PHOSPHATE
6-OHDA	6-HYDROXYDOPAMINE
OH^-	HYDROXYL ION
O.D.	OPTICAL DENSITY
PAGE	POLY ACRYL AMIDE GEL ELECTROPHORESIS
PFA	PARAFORMALDEHYDE
PBS	PHOSPHATE BUFFERED SALINE
PBS-T	PHOSPHATE BUFFERED SALINE TWEEN-20
PD	PARKINSON'S DISEASE
PNS	PERIPHERAL NERVOUS SYSTEM
PVDF	POLYVINYLIDENE DIFLUORIDE
SDS	SODIUM DODECYL SULPHATE
SD RAT	SPRAGUE DAWLEY RAT
SNPC	SUBSTANTIA NIGRA PARS COMPACTA
STR	STRIATUM
TH	TYROSINE HYDROXYLASE
TEMED	TETRAMETHYLETHYLENEDIAMINE
W/V	WEIGHT/VOLUME
LMP	LOW MELTING POINT

INTRODUCTION

Parkinson's disease (PD) is the second most common progressive neurodegenerative debilitating movement disorder, characterized by loss of dopaminergic neurons in the substantia nigra pars compacta. The characteristics symptoms of PD are bradykinesia, tremor and rigidity (Moustafa AA *et.al.*, 2016). Both environmental and genetically inherited factors have been implicated in the development of sporadic or familial PD, respectively. Reports that reveal involvements of several differentmechanisms in degeneration of dopaminergic neurons but still etiology remains enigmatic.

There are four characterized dopamine pathways in the brain. One of the four major dopamine pathways is the Nigrostriatal pathway, which is a bilateral dopaminergic pathway in the brain that connects the substantia nigra pars compacta (SNpc) in the midbrain with the dorsal striatum (i.e., the caudate nucleus and putamen) in the forebrain (Smith AD *et.al.*, 1990).The classic motor deficits of Parkinson's disease are caused by degeneration of dopaminergic neurons in the substantia nigra pars compacta, resulting in the loss of their long-distance axonal projections that modulate the striatum. In the current time the only treatment available is to minimize the symptoms of the disconnection as there is no such approach which can replace the Nigrostriatal pathway.

There are certain studies which show that the treatment of Parkinson's disease may improve by simultaneously replacing lost dopaminergic neurons in the substantia nigra and reconstructing theirlong-projecting axonal tracts to the striatum (Struzyna LA *et.al.*, 2018).

The present study involves the use of 6-Hydroxydopamine (6-OHDA) as a neurotoxin agent for inducing Parkinsonism in the Sprague Dawley Rats (Experimental model for the study). 6-OHDAis a neurotoxin used in scientific research to develop parkinsonism in rats to study degenerative mechanism. 6-OHDA is a highly oxidizable dopamine analogue that allows selective damage to catecholaminergic neurons such as, dopaminergic neurons of the substantia nigra pars compacta (SNpc) (Morelli M *et.al.*, 2012). It induces main cellular processes involved in PD, such as oxidative stress, neurodegeneration, neuroinflammation, and neuronal death by apoptosis (Hernandez-Baltazar D *et.al.*, 2017). 6-OHDA has a structural analogue of catecholamines, dopamine and noradrenaline, and exerts its toxic effects on catecholaminergic neurons. Apomorphine test carried out in experiments using animal models of Parkinson disease with unilateral lesions in the dopaminergic nigrostriatal system in which the

number and direction of animal rotations is quantified after apomorphine treatment (https://www.panlab.com/en/tests-solutions/apomorphine-rotation-test), it is one ofthe most widely used 6-OHDA-related experimental paradigms for PD is the evaluation of rotational behaviour in rats bearing a discrete degeneration of the dopaminergic nigrostriatal pathway (Ungerstedt U *et.al.,* 1970).

The ancient Indian system of medicine—Ayurveda—is concerned with the prevention, diagnosisand cure of diseases. Ayurveda describes a number of beneficial effects of the rhizomes and leavesof various species belonging to the Zingiberaceae family, especially those of *Curcuma longa* Linn.(Syn.*Curcuma domestica* Valeton). The rhizomes of the plants are popularly known as turmeric inEnglish or Haldi in Hindi. *C. longa* is one of the widely reputed medicinal plants, which are attributed with tonic, rejuvenating, anti-stress, anti-fatigue, anti-oxidant and apoptogenic properties (Priyanka Rathore *et.al.,* 2008). Modern interest in turmeric began in the 1970's when researchers found curcuminhas neuroprotective properties (Rao DS *et.al*., 1970). Chemical constituents of the curcuma oil used in the present study were patented by Ray Madhu, Pal R, Singh S, Khanna NM (2006) as an US patent (2006) (Ray M *et.al*., 2006).

All the toxicity experiments were checked in SD rats because rats and humans are almost similar species in such a way that both are mammals and give birth to young ones, both are warm blooded. More importantly, rats and humans are often suffered for the same disease; because of their sane basic physiology, similar organs and similar body plans. So here my works are mainly focused onSD rats because they are ideal for multipurpose models, safety and efficacy testing, aging, nutritionand a good surgical method.

REVIEW OF LITERATURE

THE BRAIN

The brain is an amazing three-pound organ that controls all functions of the body, interprets information from the outside world, and embodies the essence of the mind and soul. Intelligence,creativity, emotion, and memory are a few of the many things governed by the brain. Protected within the skull, the brain is composed of the cerebrum, cerebellum, and brainstem. The brain controls our thoughts, memory and speech, movement of the arms and legs, and the function of many organs within our body.

PARTS OF BRAIN

The brain is composed of the cerebrum, cerebellum, and brainstem.

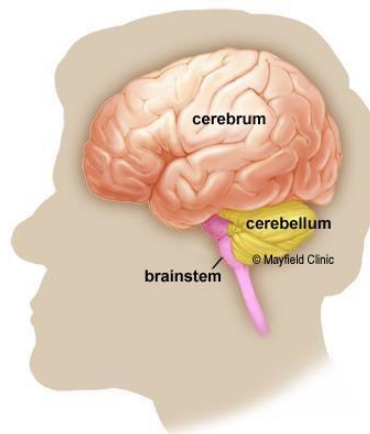


Figure: 1 The brain has three main parts: the cerebrum, cerebellum and brainstem.
(https://mayfieldclinic.com/pe-anatbrain.htm)

Cerebrum is the largest part of the brain and is composed of right and left hemispheres. It performshigher functions like interpreting touch, vision and hearing, as well as speech, reasoning, emotions,learning, and fine control of movement.

Cerebellum is located under the cerebrum. Its function is to coordinate muscle movements, maintain posture, and balance.

Brainstem acts as a relay centre connecting the cerebrum and cerebellum to the spinal cord. It performs many automatic functions such as breathing, heart rate, body temperature, wake and sleep cycles, digestion, sneezing, coughing, vomiting, and swallowing (Mayfield clinic, 2018).

The brain skull (cranium) helps to protect brain from injury. Brain is divided into several lobes:
The frontal lobes are responsible for problem solving, judgment and motor functions.
The parietal lobes manage sensation, hand writing and body position.
The temporal lobes are involved with memory and hearing.
The occipitals lobes contain brain's visual processing system (Mayfield clinic, 2018).

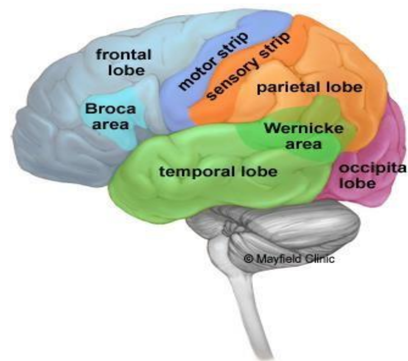


Figure: 2 Structure of brain (https://mayfieldclinic.com/pe-anatbrain.htm)

Substantia Nigra (SN)

The substantia nigra is a basal structure set within midbrain that plays a very important role in compensation and movement. Substantia nigra in Latin means "black substance" which appear darker than neighboring areas due to higher levels of neuromelanin in dopaminergic neurons. TheSN is a crucial player in brain performing eye movement, motor designing, reward seeking and learning. The co-dependence between the striate body and SN is seen during this way: once the SN is electrically stirred, no movement occurs; however, the symptoms of nigral degeneration dueto Parkinson's is an affecting example of the substantia nigra's effect on movement.

Striatum (STR)

The corpus striatum, additionally called striata nucleus is one amongst the nuclei within the neural structure basal ganglia of the prosencephalon. Striatum participates in motor and action planning, motivation and decision making etc. The corpus striatum could be an important part of the motor and reward systems.

The nervous system is split generally into 2 categories: the peripheral nervous system and also thecentral nervous system. The central nervous system is especially divided into 2 parts: the brain andspinal cord. The common adult human brain weighs 1.3-1.4 kg weight (approximately three pounds). The brain contains regarding one hundred billion nerve cells and trillions of support cellsreferred to as interstitial tissue.

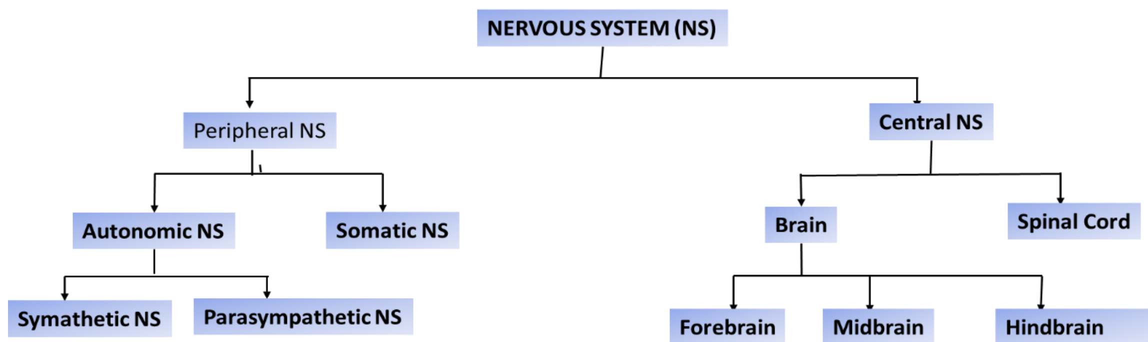


Figure:3 flow chart of nervous system

NEURON

Neurons are the information processing units of the brain which have a responsibility for sending, receiving, and transmitting electrochemical signals throughout the body. The neuron doctrine wasnamed and popularized by Heinrich Wilhelm Gottfried von Waldeyer-Hartz in 1891 (Stanley Finger, 2018). It is the basic computational unit of nervous system. Neurons, also known as nerve cells, are essentiallythe cells that make up the brain and the nervous system.

The central nervous system, which comprises the brain and spinal cord, and the peripheral nervous system, which consists of sensory and motor nerve cells all contain these information processing neurons.

Neurons are *excitable* cells. Depending on the information they receive, neurons generate electrical signals and propagate them along their processes. This capacity is due to the presence ofparticular proteins in their plasma membrane which allow the selective passage of ions: the ion

channels. Neurons are also *secretory* cells. Their secretory product is called a *neurotransmitter*. The release of a neurotransmitter occurs only in restricted regions, the synapses (Constance Hammond, 2015).

The function of a neuron is to transmit nerve impulses along the length of an individual neuron and across the synapse into the next neuron.

These are all major neuronal challenges and their mishandling eventually causes neurons to die. However, the underlying signaling mechanisms of how these factors induce the initiation of cell death remain elusive. In neurodegenerative diseases, apoptosis and necrosis are believed to be thetwo major death pathways for neurons (Hao Chi *et.al*., 2018).

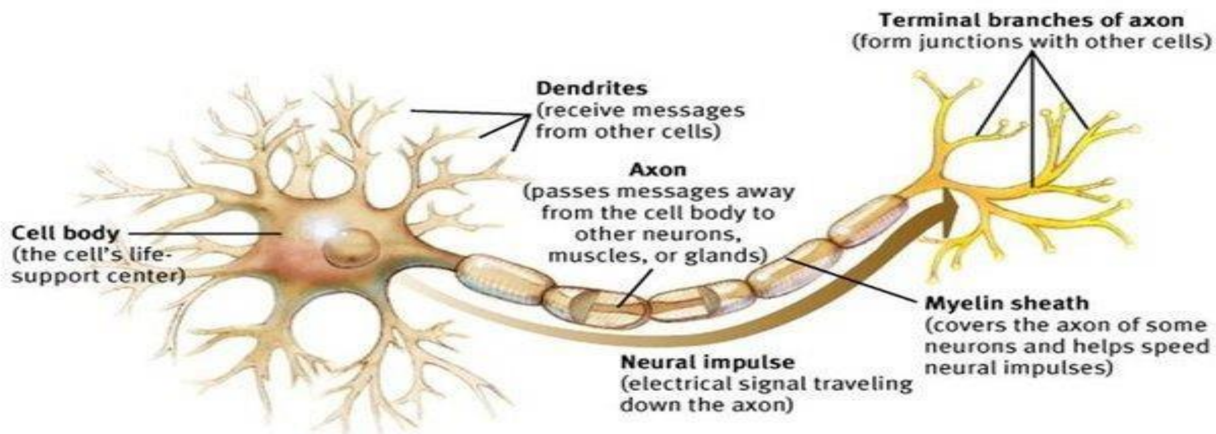


Figure:4 Structure of neuron cell

(Source: https://appsychology.com/book/biological/neuroscience/)

PARKINSON'S DISEASE

Parkinson's disease is a slow progressive neurodegenerative disease. It is also characterized as chronic disease. Parkinson's disease is also abbreviated as PD. In 1817, Dr. James Parkinson recognized it as a "shaking Palsy" (DeMaagd, G and Philip, A, 2015). PD is fourth most common neurological disease of the elders. Parkinson's disease caused due to loss of nerve cells in the part of the brain known as substantia nigra. These nerve cells are responsible for producing a chemical which acts as messenger called Dopamine. Dopamine helps to co-ordinate and control our body movements. The striatum is a part of the brain involved in regulating the coordinating muscle activity. The striatum consists of three parts: Globus pallidus, putamen and caudate nucleus. Insufficient levels

of dopamine from neuron of the substantia nigra synapsing on the neurons of the striatum isthought to be responsible for the Parkinsonism such as slow movement and abnormal walking About 1% of those >=65 yrs. Old and 0.4% of those >40 yrs. Old. The average age of onset isabout 57 yrs. As per Parkinson news today statistical data on PD, it is estimated that about seven to 10 million people are suffering from PD in the worldwide.

Familial statistic

Occurrence range of the disease is from 41 cases per 100,000 in fourth decades till now it is 1900 cases per 100,000, with average age of 80 or older than 80. According to the reports it is shown that 4% people with PD diagnosed before or at the age of 50. Men are 1.5 times more prone to have PD than women (parkinsonsnewstoday.com, 2022).

CAUSES

Currently, both the genetic changes and environmental factors are considered as cause of Parkinson's disease. Although, five percentages of PD cases result from genetic mutations in oneof key genes, while ninety-five percentages of PD cases are sporadic.

Genetics causes: Family studies suggests that autosomal dominant inheritance is linked with PD and more prevalent in males. (Thomas T *et.al.*, 2003), some pathological studies shows that substantial nigraldegeneration and presence of Lewy bodies. Chromosome number 4q21q23 is identified and assign as PARK1 locus and further studies reveals mutation on the exon 4 of gene encoding for the α- synuclein. It is missense type of mutation which results in substitution of alanine to threonine. Rapid progression of the disease is associated as clinical symptoms. Another locus such as PARK2 mutation, gene encoding for *Parkin* is responsible for causing Juvenile Parkinsonism. Also, ubiquitin hydrolase L1 (UCH-L1) is an enzyme which plays important role in hydrolyzing the ubiquitin proteins to produce ubiquitin monomers and remove unwanted proteins via ubiquitin- proteasome pathways. But due to mutation in UCH-L1, it is unable to perform its functions and leads to Parkinsonism. LRRK2 (leucine-rich repeat kinase 2) are responsible for autosomal dominant forms of PD, presumably by a gain-of-function mechanism. Loss-of-function mutationsin the genes encoding parkin, PINK1, and DJ-1 mediate autosomal recessive PD [17]. Many mutated dopaminergic genes are mainly associated with PD such Tyrosine hydrolase (11p15.5), Dopamine D2, D3 and D4 receptors (Thomas T *et.al.*, 2003).

Environmental causes: environmental factors are plays most role in causing PD over genetic factors. People living in the rural are more prone to PD due to they are in regular exposer of

herbicides and pesticides which contains some very harmful chemical that badly affects our brain.Reports reveals that, Dieldrin, an organochloride pesticide, is found in the brain of many PD patients while no trace in controls (Thomas T *et.al.*, 2003). and Dithiocarbamates, is also a pesticide found as environmental cause for PD. Dithiocarbamates elevates the level of MPTP toxicity and increases risk for PD. Rotenone, is widely used pesticide and a potential inhibitor of complex I and persuadesformation of Lewy bodies. Rotenone is found to be significant cause for PD. Few other toxins such as tetrahydroisoquinolines and β-carbolines are also involves in dopaminergic toxicity.

SYMPTOMS

Symptoms of PD are investigated as motor as well as non-motor. There are three key motor symptoms of PD such as bradykinesia, tremor and rigidity.

Table: 1 Motor symptoms of PD (Garcia Ruiz PJ *et.al.*, 2011)

Motor symptoms
Bradykinesia Slow movement Occurrence rate is 80% to 90% in patients
Tremor In 70% to 90% patients Initially distal, also involves hands, jaw, tongue, chin and leg
Rigidity Appears in 70% to 80% PD patients Protection from latent development in both flexor and extensor muscles with appendage loose. Frequently joined by "cogwheel" peculiarity

Disease progression is differing from person to person, in some people disease progression is quicker than other. As the disease progresses, the Shaking, or tremor, which affects the majority of patients may begin to interfere with daily activities. Other symptoms or non-motor symptoms includes dementia, psychosis sleep disorders, depression, autonomic dysfunction and other emotional changes; difficulty in swallowing, chewing, and speaking; urinary problems or constipation; skin issues (Khalil El Bayad *et.al.*, 2018).

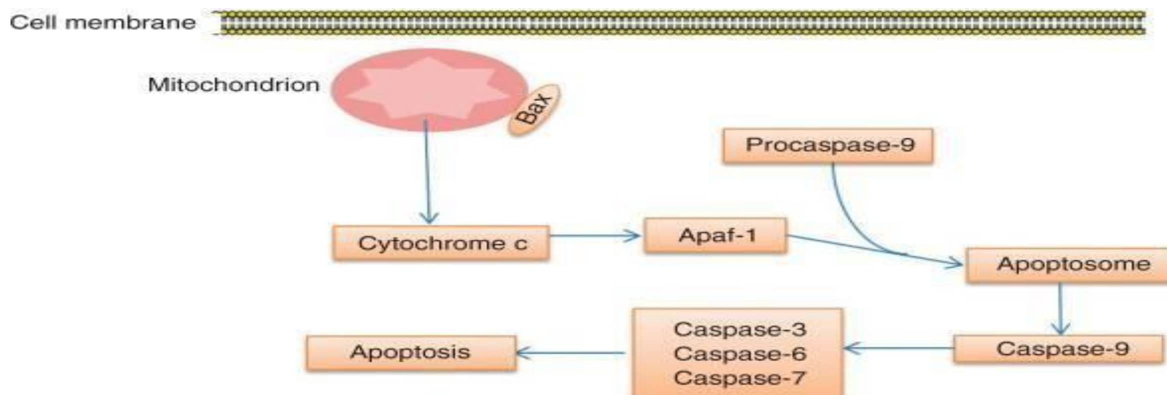
APOPTOSIS AND ITS ROLE IN PD

Apoptosis is the mechanism of programmed cell death. It is used during early development to remove unwanted cells and mediated by proteolytic enzymes called *caspases* which trigger cell death by cleaving specific proteins in the cytoplasm and nucleus. Studies shows that it is the mainmechanism of neuronal loss in Parkinson's disease patient by the identification of DNA fragmentation and apoptotic chromatin changes in dopaminergic neurons. The role of apoptosis inthe pathogenesis of Parkinson's disease was confirmed in post-mortem and in vitro studies that illustrated elevated activity of caspase-3 and increased expression of active caspase-3 in substantianigra pars compacta (Khalil El Bayad *et.al.*, 2018).

APOPTOTIC PATHWAYS

The apoptosis pathways were first described in genetic studies on the nematode, *Caenorhabditis elegans*. The apoptotic biochemical components are a group of molecules called the B-cell lymphoma (Bcl-2) family, apoptotic peptidase activating factor (Apaf-1) and caspases. Apoptosisis mediated by a number of initiator and executioner caspases and occurs via the intrinsic or extrinsic pathways. Activation of initiator caspase-9 mediates the intrinsic pathway which is also called the mitochondria-mediated pathway. Alternatively, activation of initiator caspase-8 mediates the extrinsic apoptotic pathway which is also called the cell death receptor-mediated pathway. Both initiator caspases converge onto a common pathway of executioner caspases involving caspase-3 and caspase-6 (Tompkins MM *el.al.*, 1997).

Figure:5 The intrinsic apoptotic pathway. proapoptotic proteins, like Bax, prompt the permeabilization of



the external mitochondrial membrane, prompting arrival of cytochrome c from the mitochondrial intermembranous space. Cytochrome c is then bound to Apaf-1, bringing aboutthe development of a multimeric Apaf-1/cytochrome c complex that initiates procaspase-9 framing the apoptosome. Thus, procaspase-9 is activated through proteolysis and therefore separated fromthis complex. Once enacted, caspase-9 activates killer caspases-3, - 6, and additionally - 7, whichintercede proteolytic occasions that in the long run led to apoptosis.

(Source: https://www.ncbi.nlm.nih.gov/books/NBK536724/)

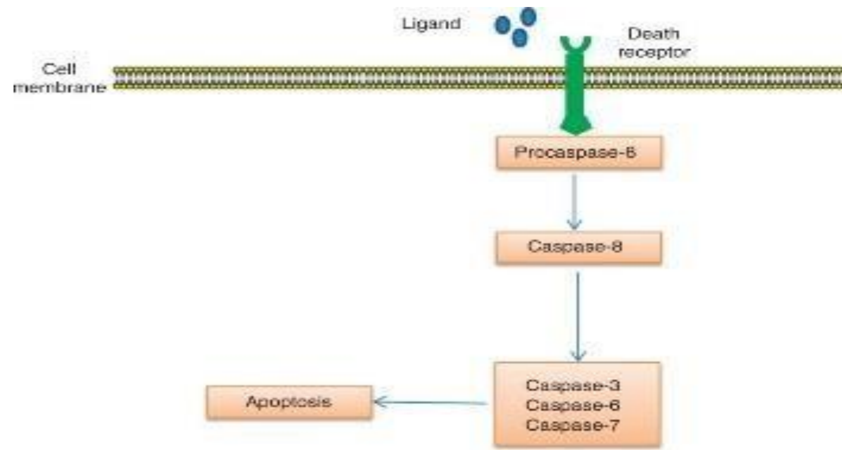


Figure:6 The extrinsic apoptotic pathway. Explicit death signal ligands tie to death receptors, bringing about receptor trimerization, and ensuing recruitment of explicit intracellular receptor- associated proteins, for example, procaspase-8. Procaspase-8 is then quickly cleaved into its activeform, which can actuate downstream killer caspases-3, 6, and additionally 7 that intercedeproteolytic occasions of cell proteins and structures leads to apoptosis. (https://www.ncbi.nlm.nih.gov/books/NBK536724/)

Dopamine and Parkinson's disease

A catecholamine neurotransmitter dopamine is a special type biological protein which is secreted by substantial nigra and other part of the brain region. It acts as messenger for neuron. It is found to be in elevated concentration in the striatum region and involved in extrapyramidal motor functions hence, regulation of motor activity. Researchers found that deficiency of dopaminergic neuron leads to appearance of Parkinson's symptoms. Initially, an amino acid precursor tyrosine which converts into L-dihydroxyphenylalanine (L-DOPA) then Tyrosine hydroxylase an enzymethat converts the L-DOPA into dopamine with the help of l-aromatic amino acid decarboxylase (AADC). L-DOPA is a precursor for dopamine synthesis hence, also a precursor for epinephrine (adrenaline) and norepinephrine (non-adrenaline).

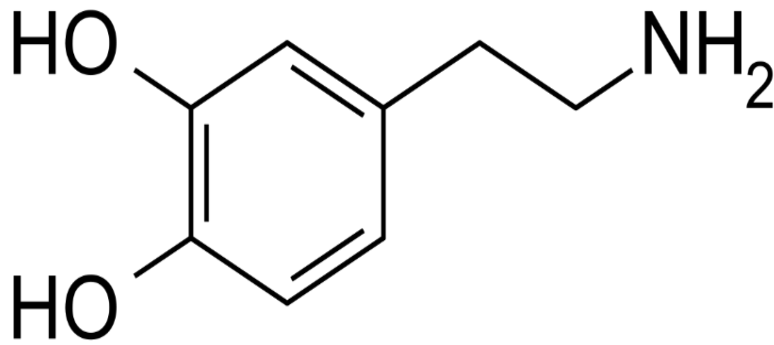


Figure:7 Chemical structure of dopamine (source: https://en.wikipedia.org/wiki/Dopaminergic)

Role of Tyrosine hydroxylase in PD

TH is a rate limiting enzyme. TH is highly homologous oligomeric protein. Its molecular weightis 60 KDa. Approximately, 50%-60% dopaminergic neuron death found in PD patients. Due to loss of dopaminergic neurons in the substantial nigra region of PD which leads to decrease in the level of dopamine. Loss of TH activity followed by a decrease in TH protein is considered to contribute towards DA deficiency and phenotypic articulation in PD (Nour S *et.al*., 2018). TH activity is controls via site-specific phosphorylation of three physiologically-directed sites in CNS such as Ser19, Ser31 and Ser40 (Tabrez S *et.al*., 2012). It has been accounted for that the phosphorylation of Ser19 doesn't straightforwardly impact TH action, however elevated phosphorylation of Ser40 can increase TH activity within specific limit and is related with uplifted DA turnover in neurodegenerative disorders (Haycock JW and Haycock DA, 1991).

Pathway for the biosynthesis of dopamine

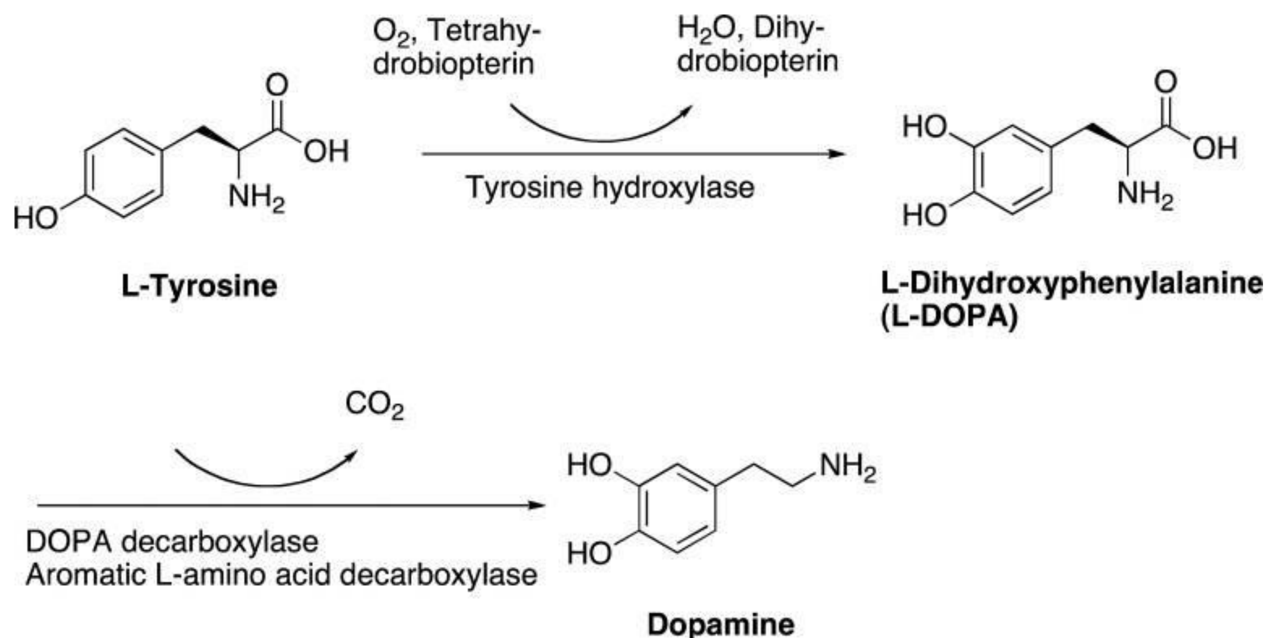


Figure:8 Biosynthesis of Dopamine from L-tyrosine (source: https://www.sciencedirect.com/science/article/pii/B9780124173026000015)

NEUROTOXICITY

It is a form of toxicity in which a biological, chemical, or physical agent generate a negative impacton the structure or function of the both central and peripheral nervous system or any. It happens when exposure to a substance, specifically, a neurotoxin/ a neurotoxicant that alters the normal activity of the nervous system in such a way as to cause permanent or reversible damage to nervoustissue (Salvatore MF *et.al.*, 2001). They may include symptoms like limb weakness or numbness, memory loss, vision, and/or intellect impairment, uncontrollable obsessive and/or compulsive behavior, delusions, headache, cognitive and behavioral problems and sexual dysfunction. Additionally, neurotoxicity has been found to be a major cause of neurodegenerative diseases suchas Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, etc.

In case of PD however, the degenerative process of neurons remains unclear. But there are recent studies shows some evidence suggest that the accumulation of unwanted and misfolded proteins due to involvement of oxidative stress plays a central role in pathogenesis in PD (Cunha-Oliveira T *et.al.*, 2008). A proper balance between the formation and degradation of cellular proteins is critical for neuronal survival,and alterations in degradation may lead to aberrant accumulation and aggregation of proteins, resulting in cellular toxicity and neurodegeneration, as seen in PD (Foley, P and Riederer, P *et.al.*, 2000).

6-OHDA- the 6-hydroxydopamine hydrobromide

Neurotoxin-based animal models such as the 6-hydroxydopamine hydrobromide (6-OHDA) which is a neurotoxic synthetic organic compound used to selectively destroy dopaminergic and noradrenergic neurons in the brain. Chemical formula is C8H12BrNO3. It's a light sensitive and highly oxidizable compound in nature. 6-hydroxydopamine hydrobromide (6-OHDA) is widely used neurotoxin to create PD model for *in-vivo* studies. 6-OHDA elevates the formation of 8- oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxodG), a biomarker of induced apoptosis and oxidative damaged DNA. 6-OHDA is structurally similar with catecholamines, dopamine and noradrenaline, and applies its toxic impacts on catecholaminergic neurons. The first successfully 6-OHDA induced model was created in 1968 by Ungerstedt in SN region of the brain.

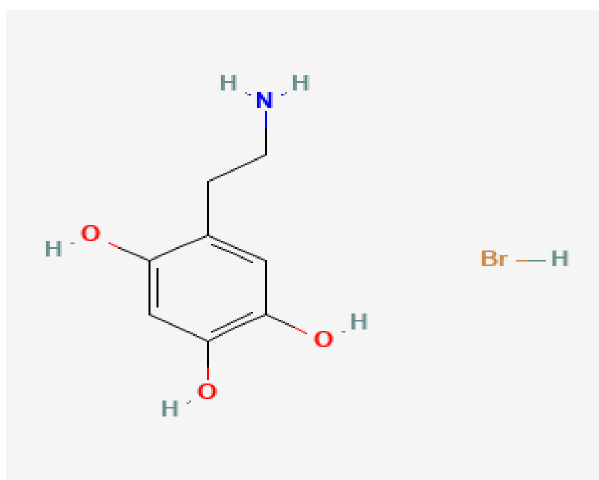


Figure:9 Structure of 6-OHDA (source: PubChem)

Mechanism of action of 6-OHDA

Till date there are three mechanisms of action are proposed for 6-OHDA neurotoxicity. 1) intra- or extracellular auto-oxidation of 6-OHDA, which leans toward the development of hydrogen peroxide and superoxide and hydroxyl radicals (Dawson *et.al.*, 2003), 2) Generation of hydrogen peroxide because of the activity of monoamine oxidase (Blum D *et.al.*, 2001), and 3) direct restraint of mitochondrial respiratory chain complex I (K. Chiba *et.al.*, 1984 and P. Jenner and A.H. Schapira, 1992).

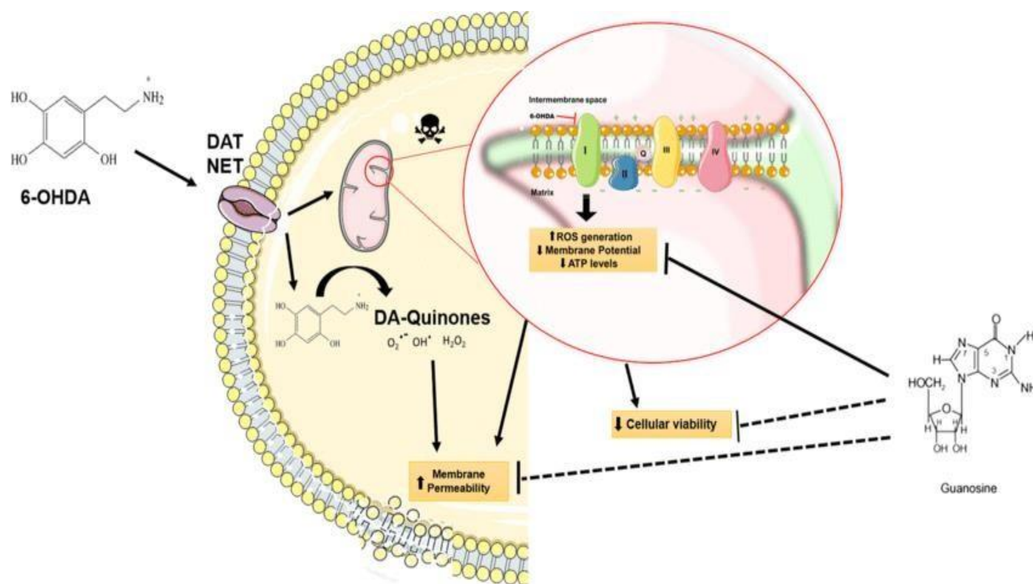


Figure:10 shown 6-OHDA and its mechanism of action (Source: https://www.researchgate.net/figure/Proposed-mechanism-of-neuroprotection-afforded-by-GUO-against-6-OHDA-toxicity-in-vitro-in_fig6_328881422).

Parkinson's disease treatment

Parkinson's disease is a chronic disease which required broad range of care, treatment, awareness, exercise and nutrition. There is no permanent cure for PD till date only the cure we have is the attenuating the neuronal death through medication and via surgery but this only provides reassurance from symptoms of PD. Few medications which are under development has three categories firstly, delivery of the drug via transdermal route, secondly, drug which are activated by non-dopaminergic process are being assessed, especially for their anti-dyskinetic agents and the third one is neuroprotective and neurotropic are under consideration.

L-DOPA/Carbidopa: Levodopa (L-dopa) is the metabolic precursor of the dopamine. It crosses BBB (blood brain barrier) where it is converted by endogenous dopa decarboxylase to dopamine. It is then stored in surviving nigrostriatal terminals. To decrease side effects, it is administered with a peripherals dopa decarboxylase inhibitor as Carbidopa which doesn't cross the blood.

Dopamine agonist: They are mainly used in the advanced stage of PD like Ergot derivatives (Bromocriptine) and non-ergot derivatives (Pramipexole).

MAO-B inhibitor: Inhibitor of this enzyme slows the breakdown of dopamine in the striatum e.g., Selegiline

Non pharmacologic therapy: Surgery

Gene delivery system for treatment for Parkinson's disease: it involves gene delivery via lentiviral vectors or liposomes.

Through, several symptomatic therapies are available for PD patients but still no therapy is available with hundred percent protection. Therefore, we need further improved therapeutics and remedy. In the present work, exploration of CDRI compound curcuma-oil was done to evaluate its role in 6OHDA mediated dopaminergic neuronal death.

AIM AND OBJECTIVE

The study was planned with the aim **"To assess the effects of curcuma-oil on 6-hydroxydopamine (6-OHDA) induced experimental model rats of Parkinson's disease."**

The proposed study has following objectives:

1. Validation of 6-OHDA induced behavioral response (stereotype behavior) and alterationin tyrosine hydroxylase (rate limiting enzyme of dopamine synthesis) and cleaved caspase-3 in experimental model of Parkinson's disease.
2. Histopathological assessments for neuronal morphology in 6-OHDA induced experimentalmodel of Parkinson's disease.
3. To assess the effect of Curcuma-oil on 6-OHDA induced Parkinson's disease specific pathological markers (stereotype behaviour, level of tyrosine hydroxylase and cleaved caspase-3) in both SN and STR regions of brain.

WORK PLAN

- Selection of animal/experimental model
- Design the model period
- Selection of toxin
- Selection of test compound
- Selection of route of administration
- Selection of parameters

MATERIALS AND METHODS

ANIMALS USED:

Sprague Dawley rats (SD Rats)

CHEMICALS USED:

Ammonium persulphate

Bisacrylamide

BSA standard solution

Chloral hydrate

Cresyl violet stain

Dibutyl phthalate Polystyrene Xylene (DPX)

Dimethyl sulfoxide (DMSO)

Disodium hydrogen phosphate (Na2HPO4)

EDTA

EGTA

Folin’s Reagent

Trichloroacetic acid

Hydrochloric acid

6-hydroxydopamine

Paraformaldehyde (PFA)

Phosphate buffered saline

Potassium chloride

Sodium bicarbonate

Sodium carbonate

Sodium chloride

Sodium dihydrogen phosphate

Sodium dodecyl sulphate

Sodium hydroxide

Sodium potassium tartarate

Sodium pyruvate

TEMED

Tris buffer

TritonX-100

Xylene

All the reagents were purchased from Sigma St. Louis, MO, USA and SRL, India

ANTIBODIES USED

TH (Tyrosine hydrolase)Caspase 3

Beta-actin

INSTRUMENTS USED

Homogenizer (Cole-ParmerLabGEN 7 Tissue Homogenizer, India)

Stereotaxic frame (Stoelting Stereotaxic Instrument, U.S.A)

Centrifuge (Sigma 3K30 Centrifuge)

ELISA Plate reader (BIOTEK Instruments, India)

Microtome (Leica RM 225, Leicamicrosystem, Germany)

Hamilton syringe (Hamilton,26 gauze, U.S.A)

Weighing Balance (Sartorius Quintix-3102 lab balance, German)

Fluorescent microscope (Nikon Eclipse E200, Japan)

Experimental Animals (Animal model (PD):

Adult male **Sprague–Dawley rats** (170–200 g) were procured from the National Laboratory Animal Centre of Central Drug Research Institute and experiments were performed according to internationally followed ethical standards and approved by the animal research ethics committee of CSIR – Central Drug Research Institute. Three animals per polyacrylic cage were housed withaccess to food and water ad libitum and were maintained in standard housing conditions. The HVAC (Heating, Validation and Air conditioning) system was used to control the temperature, validation and humidity.

The standard parameters were controlled at:

Temperature range of 22^{0}C to 25^{0}C

Relative humidity range of about 60-65%

Air changes of about 10-15 per hours

Light and dark cycle of 12 hours

All the animals were feed by the dry chow pellets produced by the animal house of CDRI. Food and free access to water was provided ad libitum during the entire study procedure. The animals were housed in polyacrylic cage (421x290x190mm) having rice husk as bedding material and

stainless-steel metal tops. Care was taken to avoid any distress to animals during the experimentalperiod. All the experiments and protocols were conducted in strict compliance according to the National CPCSEA guidelines on the "Proper Care and use of animals in laboratory research" and were approved by institutional animal ethics committee (IAEC) of Central Drug Research Institute, Lucknow.

NEUROTOXIN

6-OHDA was used as a neurotoxin at the dose of 12µg to induce the neurotoxicity by stereotaxic injection. To attains the final dose of 6-OHDA; 0.5 mg of 6-OHDA was dissolved in 250µl of 0.9% ascorbate saline solution (ascorbic acid as media). The solution of 6-OHDA was made freshdaily prior to surgery and can only be stored for a day at -20°C and covered with aluminum foil to protect it degradation from light because it is light sensitive compound.

STUDY DESIGN

Prior to the surgical process the rats were weighed and categorized in various groups according toweight. Experimental 6-OHDA model (induction of PD). For model validation animals were divided intwo groups:

Control

Treated (6-OHDA)

Table:2 Group of rats divided for model validation

S. No	Group name	Unilateral injection each at right striatum and SNpC region
1	Control	NO SURGERY
2	6-OHDA Treated	6-OHDA (6 µg in each SN and STR region)

Based on the study the rats were separated into three groups:

Control

6-OHDA

6-OHDA + Curcuma-oil

Preventive mode (3 days) and sacrifice at 10TH day 10. Total time period was 10 days (5+5 days).

Table:3 Group of the rats divided for study

S. No	Group name	Unilateral injection each at right striatum and SNpC region
1	Control	NO SURGERY
2	6-OHDA	6-OHDA (6 µg in each SN and STR region)
3	6-OHDA+Curcuma oil	6-OHDA (6 µg in each SN and STR region)

TEST COMPOUND

The ancient Indian system of medicine—Ayurveda—is concerned with the prevention, diagnosis and cure of diseases. Ayurveda describes a number of beneficial effects of the rhizomes and leaves of various species belonging to the Zingiberaceae family, especially those of *Curcuma longa* Linn. (Syn. *Curcuma domestica* Valeton). The rhizomes of the plants are popularly known as turmeric in English or Haldi in Hindi. *C. longa* is one of the widely reputed medicinal plants, which are attributed with tonic, rejuvenating, anti-stress, anti-fatigue, anti-oxidant and apoptogenic properties (Priyanka Rathore *et.al.,* 2008). Modern interest in turmeric began in the 1970's when researchers found curcumin has neuroprotective properties (Rao DS *et.al*., 1970). Chemical constituents of the curcuma oil used in the present study were patented by **Ray Madhu, Pal R, Singh S, Khanna NM (2006)** as an US patent (Ray M *et.al*., 2006). Curcuma oil (C.oil) has been obtained from the lipid soluble extract of rhizomes of *Curcuma species* of the Zingiberaceae family. The dried rhizomes were collected and identified botanically and samples have been preserved in the herbarium of the Botany department of Central Drug Research Institute, India, for future reference. The oil was analyzed by high resolution GC and GC/MS (Ray M *et.al*., 2006).

Oral dose of Curcuma oil (250mg/kg) was given to the respective group of rats before and afterwards on the 6-OHDA treatment. 30 minutes' prior on the day of 6-OHDA treatment for absorption of the drug and the same regime was followed on scarify of animals. These animals were then anesthetized according to their group and 6-OHDA (total 12µg; 6µg in each region) was unilaterally injected in substantia nigra (SN) and striatum (STR) regions of the rat brain through stereotaxic surgery. The coordinates for SN and STR were respectively from the bregma. Appropriate care was ensured post-surgery for early recovery. five days following 6-OHDA injection, intra-cardiac perfusion with 0.9% saline and decapitation was done after anesthetizing the rats to remove the brain. SN from the mid brain and STR from the fore brain regions were

isolated and the brain tissue was processed as per the requirements of respective biochemical estimations.

The animals will be sacrificed after 3 days respectively for each animal model and their brains willbe analysed, morphologically for neurotoxicity. The study will be performed in accordance with OECD test guidelines No. 433(1987).

SURGICAL PROCEDURE

REQUIREMENTS

- Chloral hydrate
- DMSO
- Betadine
- 6-OHDA dissolved in ascorbic acid media
- Scalpel blade
- Scissors, Forceps
- Needle
- Thread
- 1 ml syringe
- Bone wax
- 70% ethanol
- Petri plate
- Scale for measuring distance from bregma to specific regions
- Cotton
- Pen, marker for mark the rat number according to their body weight

Animal in various groups were anesthetized with intra-peritoneal (I.P) injection of chloral hydrateat dose of 300mg/kg. The chloral hydrate was dissolved in 0.9% normal saline and for each animalthe dose of anesthesia was calculated according to their body weight. The hair on the head of theanimal was removed by using a scissor and animal was placed on the stereotaxic frame for surgery.The cut was made on the skin of the head with the sterile scalpel to have a clear view of plus shaper(+) sign on the head called bregma skull point as (0,0) point.

The stereotaxic coordinates for SN and STR regions are as following

	For 200gm Rat	For 130-150gm Rat
STR	AP: 0.5-1mm	1mm
	L: 2.5-3mm	2mm
	D: 4.5-5.0mm	5mm
SN	AP: 5.8-6.04mm	5.8-6.0 mm
	L: 1.6-2mm	1.6mm
	D:7.5-8mm	8mm

The holes were drilled over the skull by sterile scalpel and 6-OHDA was infused bilaterally as per coordinates using 26 gauges Hamilton syringes. The syringes were kept straight while infusion and was further held for 5 minutes more for complete diffusion of toxin and to avoid backflow. After the injection the burr hole was sealed with bone wax and antiseptic powder sprayed afterwardand the skin was sutured. Microbicidal solution of Povidone-iodine was applied to avoid any infection. Proper postoperative care was provided until the animal recovered completely.

BEHAVIOURAL ASPECTS:

APOMORPHINE TEST: The apomorphine test has proved to be a popular test for screening the behavioral effects of a wide variety of lesions, drugs, and other experimental manipulations on thebrain of rodents.

This test is widely carried out in experiments using animal models of Parkinson disease with unilateral lesions in the dopaminergic nigrostriatal system in which the number and direction of animal rotations is quantified after apomorphine treatment (https://www.panlab.com/en/tests-solutions/apomorphine-rotation-test).

Protocol

Apomorphine-induced rotation test was performed to study the hypersensitivity of the lesioned striatum, assessed by injecting 1 mg/kg of apomorphine sc. (dissolved in a 0.9% saline solution) and tested over a 40 min session and rotation was checked.

BRAIN REMOVAL

REQUIREMENTS

- Scissors (one small and one large)

- Two 10ml syringes for perfusion
- Desiccators
- Anesthetic ether
- 0.9%saline-4.5gm in 500 ml distilled water
- Blade
- Forceps
- Cotton

STEPS

- Anesthetized rat's epidermal skin was cut from the abdominal to chest region.
- Flesh from the chest region was cut to expose out the heart.
- A tiny cut was made on the right side of the heart.
- 0.9% saline was injected into the left ventricle of the heart for perfusion.
- Then the rat's neck was cut to separate its head from the body.
- Then the epidermal layer and the flesh was removes from the top of its head.
- Three cuts were made (one straight and one each at slightly left and right side) at the spinalcord joining point.
- The skull layer was removed with the help of scissors and forceps.
- Then the bony part of the skull over the brain area and the membrane (meninges) was removed with the help of forceps.
- The brain was then carefully scooped out with the help of a scalpel and kept in 0.9% salinefor each brain tissue in different tubes and then immediately transferred to -20° Celsius for15 minutes before dissection or sectioning.
- Brains were taken out from -20°C s after 15 minutes and placed upside down (ventral sidefacing up) for dissection.
- The hindbrain part was cut away.
- For strata a cut was made at the forebrain anterior part to separate it into two parts (left andright).
- For SN a cut was made at the bottom of the midbrain (the part just above the hindbrain

- All the remaining parts were wrapped in an aluminum foil and discarded.

WETERN BLOT PROCEDURE

Sample preparation for western blot:

- Preparation of total lysate the rats were decapitated and brains were removed.
- Brain tissues (SN and STR) were collected separately and sonicated in a lysis buffer (Appendix-I)
- For sonication, Cole-Parmer 500- Watt Ultrasonic Processors was used at amplitude of20% for 1 minute with 5 seconds pulse ON and 5 seconds pulse OFF cycle.
- The homogenate was centrifuged at 10,000 x rpm for 20 minutes at 40C.
- Supernatant was collected and protein concentration was determined by Lowry's method.

PROTEIN ESTIMATION-by Lowry method

Table: 4 BSA standard

S. No.	BSA (µl)		Distilled water(µl)	2 N NaOH (µl)	Alkali (µl)	Distilled water (µl)		FCA (µl)	
1	A1	0	10	10	20	40	Kept at room temp. for 10 minutes	200	Incubate at 37ºC for 20 minutes
2	A2	1	9	10	20	40		200	
3	A3	2	8	10	20	40		200	
4	A4	3	7	10	20	40		200	
5	A5	4	6	10	20	40		200	
6	A6	5	5	10	20	40		200	
7	A7	6	4	10	20	40		200	
8	A8	7	3	10	20	40		200	
9	A9	8	2	10	20	40		200	
10	A10	9	1	10	20	40		200	
11	A11	10	0	10	20	40		200	

- All the reagents were added into each well of 96 well plates in duplicates and the absorbance were measured at 660nm in ELISA plate reader.
- A graph was plotted for absorbance verses protein concentration to get a standardcalibration

curve.

SDS – PAGE -PREPARATION OF GEL- RESOLVING GEL

COMPONENTS	12% (10 ml)	15% (10ml)
Water	3.3 ml	2.3 ml
Bisacrylamide	4.0 ml	5.0 ml
Tris-HCL (pH- 8.8)	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml
10% APS	0.1 ml	0.1 ml
TEMED	0.004 ml	0.004 ml

STACKING GEL

COMPONENTS	6% (5 ml)
Water	2.6 ml
Bisacrylamide	1 ml
Tris-HCL (pH – 6.8)	1.25 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml

- Arrange the assembly with glass and ceramic plates.
- Seal the plates at bottom with Agarose.
- Add 10 ml resolving gel (prepare freshly), add 1ml butanol, and leave it for 10 minutesto get it polymerized.
- Wash it with D/W.
- Add 5 ml stacking gel (prepare freshly), put comb in it and let it get polymerized for10 minutes. After 10 minutes, remove comb, mark the wells.
- Transfer it to electrophoresis chamber.
- Add electrode buffer to the chamber.

- Load samples (added with loading buffer and boiled for 5 minutes, beta-mercaptoethanol is added freshly to samples loading buffer).
- Run the current initially at 80 volts and after passing the stacking gel; increase the voltage to 100 volts.

Western Blotting:

- Blotting paper and foams are dipped in chilled transfer buffer for at least 15 minutes.
- PVDF membrane is activated in methanol for 10-15 minutes. And then kept in transfer buffer.
- Cassette: Positive (red) side of transfer cassette is placed down and a wet foam is placedover it – On wet foam two soaked filter papers are placed put the gel on PVDF membrane. Two wet filter papers- foam over it and then negative side of cassette (black). No bubbles should be trapped between gel and PVDF membrane. Tight the bolts.
- Transfer for 35 mins at 150mA and 12 volts in transfer buffer.
- Protein transferred to membrane confirmed by staining it with Ponceau stain.
- Stain with Ponceau for few minutes.
- Distain it with distilled water repeatedly until it become colorless.
- Block the non-specific sites by blocking agent for 2 hours to overnight depending onprotein and antibody
- Wash it with PBS-T (5 minutes, 3 wash).
- Incubate the membrane with primary antibody (anti caspase-3 antibody (1:500), anti β-actin (1:1000) and anti TH (1:1000) 4ºC overnight.
- Wash it with PBS-T (5 minutes, 3 wash).
- Incubate it with HRP conjugated secondary antibody (compatible with primary antibody, dilution to be standardized as per protein and antibody type) diluted in blocking solution containing 1% skimmed milk in PBS-T for 2 hours at room temperature.
- Wash it with PBS-T (5 minutes, 3 wash).
- Signals were developed by femto LUCENT plus-HRP chemiluminescent substrate (G-biosciences, USA) and detected under ChemiDoc HRS+ (Bio-Rad).
- Integrated density of bands was visible and normalized by β-actin using Image J software (NIH,

USA).

Figure:11 Western blotting pattern

COMET ASSAY:

Comet is also known as Single Cell Gel Electrophoresis (SCGE).

Principle: Comet assay was performed to investigate DNA damage in neurons. The comet assay(single-cell gel electrophoresis) is a simple method for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells. Cells embedded in agarose on a microscope slide are lysed withdetergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks.

Reagents

Stock solutions –

- Saline -0.9%
- 2.5M NaCl
- 500Mm Na_2EDTA (PH-8)
- Tris buffer (PH- 7.5)
- 3MnaOH

Working reagents –

- LMP Agarose (Low Melting Point) – 0.8%)
- Lysis buffer
- 0.5M EDTA

- 5M NaCl
- DMSO – 10%
- Triton –X (1%)
- Alkaline buffer (pH- 13)
- Tris buffer (PH – 7.5)
-

Procedure

- In vivo samples were chopped properly with the blade so as to form a homogenous samplemixed with PBS buffer.
- Chopped samples were then sieved and transferred into the Eppendorf tubes.
- Samples were mixed with 0.8% LMP agarose and spread on the frosted slides in a zig – zag manner evenly to form the thin layer of the gel on the slide (this is done in dark).
- The slides were kept at room temperature for 5 minutes.
- Slides were then transferred to the ice box and left for 10 minutes.
- After this the slides were incubated in Lysis buffer (500 ml) for 1 hour at 4°C.
- After one hour the slides were kept in the gel electrophoresis unit submerged in the alkalinebuffer (800 ml) and incubated as such for 20 min by setting the voltage (0.25-0.3 Amp).
- Current was switched on after 20 minutes for next 30 min.
- After 30 minutes of electrophoresis, slides were transferred into tris buffer for 20min at room temperature.
- Slides were kept in the humid chamber at 4°C.
- Slides were stained with 40µg/ml PI (Propidium iodide) and the cover slip was placed overit.
- Now, the images were taken by placing the slide on inverted Fluorescence microscope and grab the picture (around 50%).

HISTOPATHOLOGY

Fixing of Brain

- Fixation of the nervous tissue functions to preserve cells in their native state is regard to their morphology and localization of chemical constituents.
- Anaesthetized rats were trans-cardially perfused with saline and 4% paraformaldehyde (PFA). Brain was removed by decapitation and kept in 4% PFA for fixation.

Tissue processing

The brain was sectioned laterally and washed with tap water for 5-10 minutes to remove theexcess PFA from the tissue.

- The tissue was dehydrated by bathing them successively in graded series of mixtures of isopropanol followed by pure xylene.
- The tissue was then submerged in molten paraffin wax at 57 º Celsius for 2-3 hours, which removed the xylene, while infiltrating the tissue.
- This stage provides the hardness and support that the tissue requires for sectioning.

Wax Block preparation

- The main purpose of this step was to provide support to tissue that would facilitatesectioning using microtome.
- The moulds were used to fix the brain tissue forming a block.
- The block forming machine was maintained at 70°Celsius to melt the paraffin wax.
- The moulds were coated with glycerin for easy removal of the blocks without cracks.
- Molten wax was added to the mould placed on the hot platform.
- The brain was then removed from the final molten wax stage and placed carefully ontothe mould with warmed forceps.
- Molten wax was then poured onto the brain so that it was completely submerged.
- The entire mould was then placed on the cold platform to facilitate quicker solidification.

Sectioning

- Sectioning was done using a rotary microtome. This is an instrument, which consists of a sharp

metal blade held in a mixed position, and a chuck in which a block of wax with the tissue is held. The chucks move up and down by moving the rotor and fixed close to blade facilitating the sectioning of tissue.

- The wax block was trimmed with a blade to form a smooth surface. The microtome was set to cut the sections of 5 micro meter thickness.
- As sections were removed from the water bath and placed on an albumin coated dry glass slide. The slides were left over night on hot plate at 4° degree Celsius to enable the section to adhere tothe slide.

STANING

Cresyl Violet Stain

Cresyl violet is an organic compound. It is used in biology and medicine as a tool for histopathology stain. To assess neuronal shrinkage CV staining was done in brain sections of rat brain. Cresyl violet is a Nissl stain used for the endoplasmic reticulum. Therefore, Cresyl violetstains the cell's endoplasmic. The Nissl stain is used to study neurons under the light microscopeand is extremely useful since it distinguishes neurons and glia from one another.

Procedure

- The slides were kept in xylene (for deparaffinization) for 5 minutes.
- The slides were then transferred into 100% alcohol for 5 minutes.
- Then the slides were transferred into 95% alcohol for 5 minutes
- After 95% alcohol, the slides were transferred to 70% alcohol for 5 minutes.
- Then the slides were transferred to 50% alcohol for 5 minutes.
- After washing, the slides were kept in warm nissl stain (37-50 ^{o} Celsius) that can improve penetration and enhance even staining.
- Followed by staining, the slides were rinsed with distilled water.
- The slides were then deparaffinized by 95% ethanol (single dip).
- The slides were then kept in alcohol: xylene solution.
- From alcohol + xylene solution, the slides were transferred into xylene.
- Finally, the tissue was mounted with DPX.

HAEMATOXYLIN EOSIN STAINING:

This staining technique mainly involves the application of basic dye Hematoxylin, which colors the basophilic structures like nucleic acids with blue-purple hue, while alcohol based acidic eosin stains eosinophilic structures like cytoplasm as bright pink

Sequential changes were done by dipping the slides in the solution for definite time.

- Dip in xylene for 2-3 times for 10 mins.
- Dip the slides in 100% alcohol.
- Then in 90% alcohol for 5 min.
- Dip in 70% alcohol for 5 mins
- Dip into 50% alcohol for 5 mins
- Wash the slides under running water for 10 mins.
- Now, kept it in Hematoxylin for 30 secs
- Dip the slides in eosin for 2 mins.
- Then, given dips in two –two dips in acetone.
- Another dip in acetone.
- Then, dip the slides in xylene and given 2-3 dips.
- The slides were then visualizing under Bright field microscope and grabbing done.

RESULTS

Assessment of 6-OHDA induced Parkinson's disease model:

1. APOMORPHINE INDUCED ROTATION

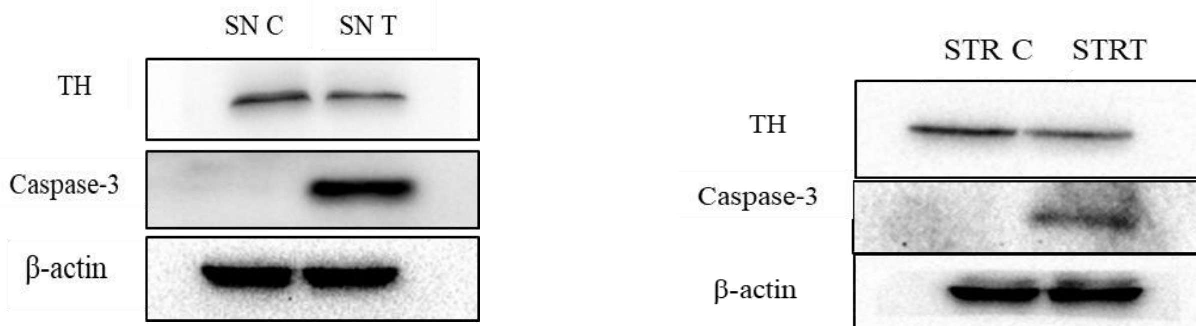
S.No.	Control	6-OHDA
1	No rotation	5(rotations/min)
2	No rotation	7(rotations/min)

Western Blot

Validation of 3-day PD model of 6-OHDA

Level of Parkinson's disease related Protein: In 6-OHDA treated group shows significantdecreased level of TH and increased level of cleaved caspase.

TH and Caspase-3 levels in 6-OHDA model



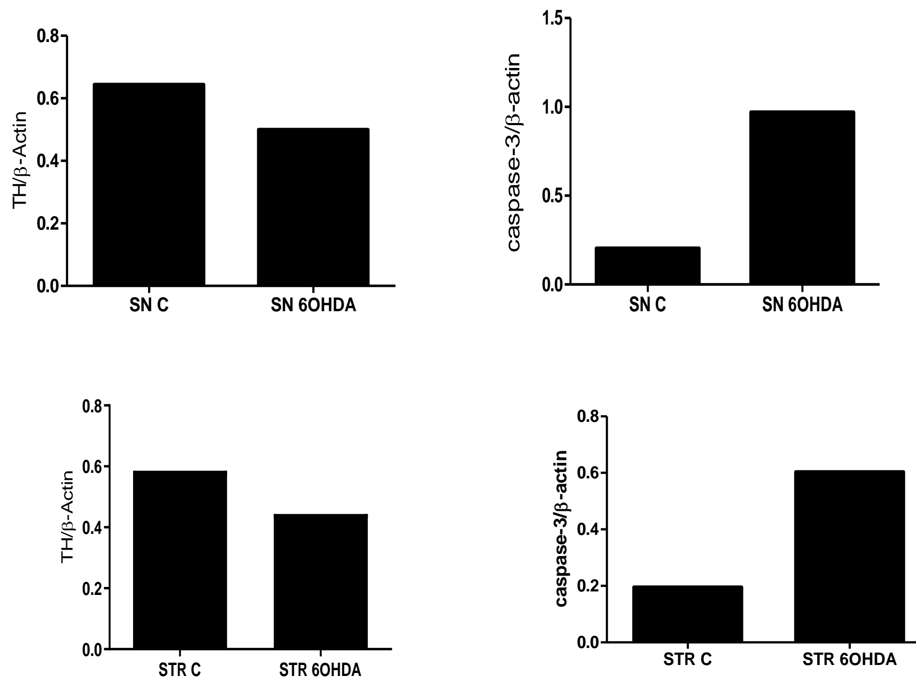


Figure:12 Western blot images and graphs representing the quantification of band intensity of TH and cleaved caspase-3 level. 6-OHDA treated groups in both SN and STR region showed decreased level of TH as comparedto control. While, increased level of caspase-3 levels in both the SN and STR regions of 6-OHDA treated group as compared to control.

Involvement of DNA damage (comet assay):

Single cell gel electrophoresis assay was performed to evaluate the DNA fragmentation in SN andSTR regions of brain after 6-OHDA treatment. DNA damage was observed in both SN and STR regions of 6-OHDA treated rats when compared to the control rat.

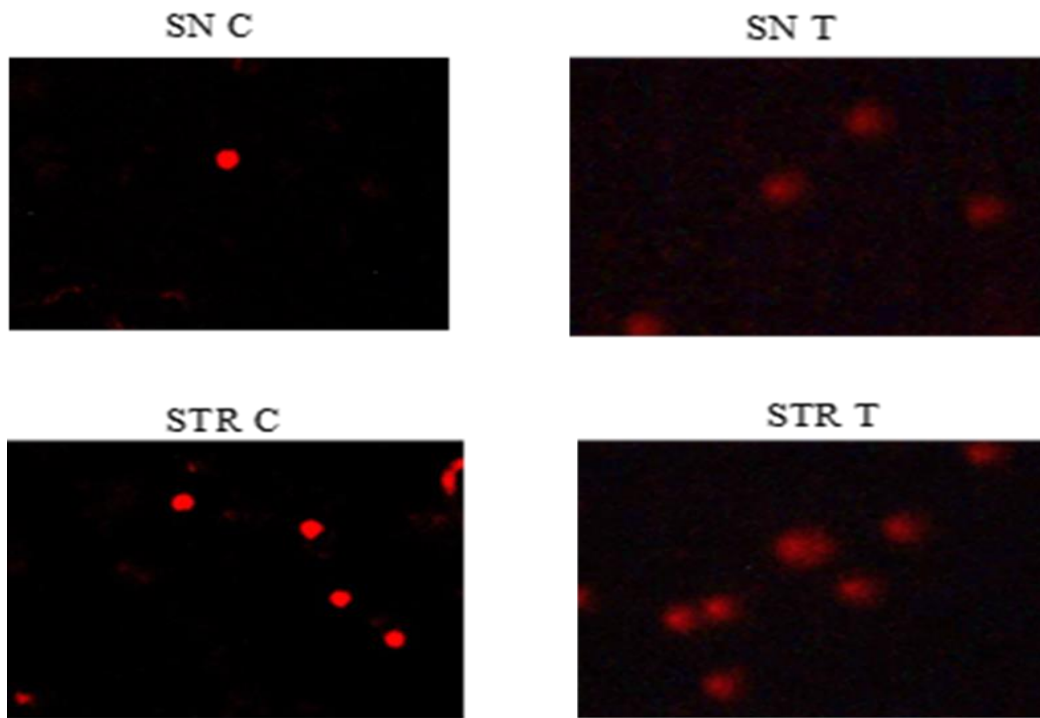


Figure:13 Pictorial representation of DNA damage in SN and STR regions of rat brain after 6-OHDA treatment.

HISTOPATHOLOGY:

HAEMATOXYLIN EOSIN STAINING OF SN AND STR REGION OF RAT BRAIN

To detect the degradation in nuclei and eosinophilic structure of cell. Hematoxylin stain nuclei blue and Eosin stain eosinophilic structure in various shades of red. Cell shrinkage was observed in 6-OHDA treated cell compared to normal cells.

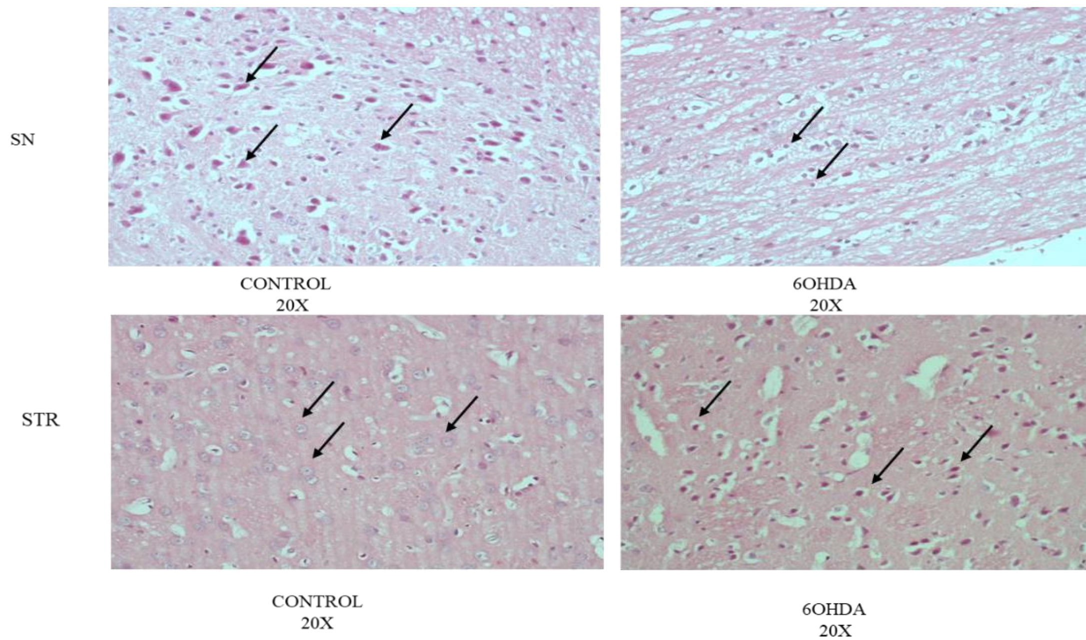


Figure:14 Bright field images of HE Staining after 3 days of 6-OHDA administration

Cresyl Violet Staining

To detect the morphological changes in neurons of brain tissue, CV staining was performed. Significant alteration in neuron cells morphology was found in 6-OHDA treated brain in both SNand STR region compared with control group.

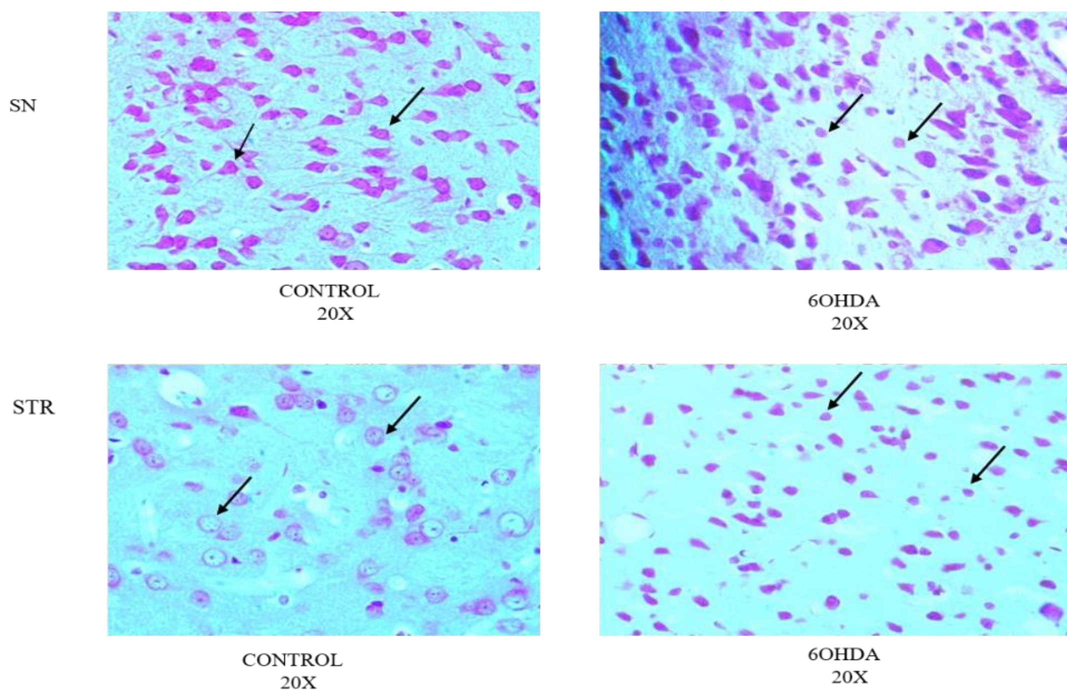


Figure:15 Bright field images of CV Staining in SN and STR after 3 days of 6-OHDAadministration.

The effect of Curcuma-oil on 6-OHDA induced neurotoxicity in SN and STR regions of brain.

APOMORPHINE INDUCED ROTATION

S. No	Control	6-OHDA	6-OHDA+Curcuma-oil
1	No rotation	5(rotations/min)	14(rotations/min)
2	No rotation	4(rotations/min)	No rotation
3	-	6(rotations/min)	2(rotations/min)
4	-	3(rotations/min)	No rotation

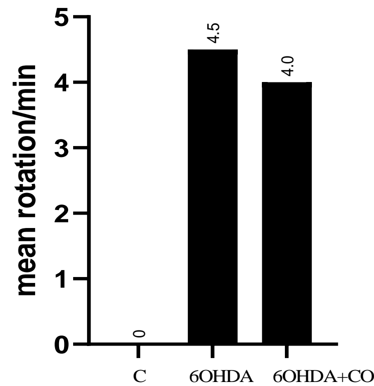
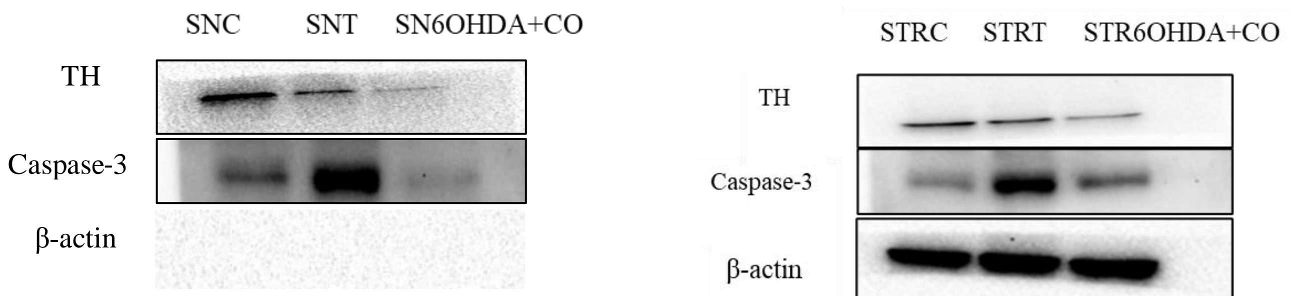


Figure:16 Table and graph representing the mean rotation/min of SD rats. 6OHDA treated rats shown significantly high number of rotation/min (4.5 rotation/min) as compared to 6OHDA+CO treated rat (4.0 rotation/min). No rotations shown by control rats when compare to 6OHDA and 6OHDA+CO treated groups.

Western blot

Western blot has been done to check the level of TH and caspase-3. TH is rate limiting enzyme inthe dopamine synthesis and Caspase-3 is an apoptotic factor that leads the death of the cell. Western blot shown the band intensity of TH and caspase-3 level in 6-OHDA treated group as wellas in 6-OHDA+CO treated group. 6OHDA group shown a depletion in TH level but increased level of cleaved caspase-3. Whereas, in case of curcuma-oil (CO) + 6-OHDA treated group, thereis a significant decrease in TH level along with an increased level of cleaved caspase -3.

TH and caspase-3 level



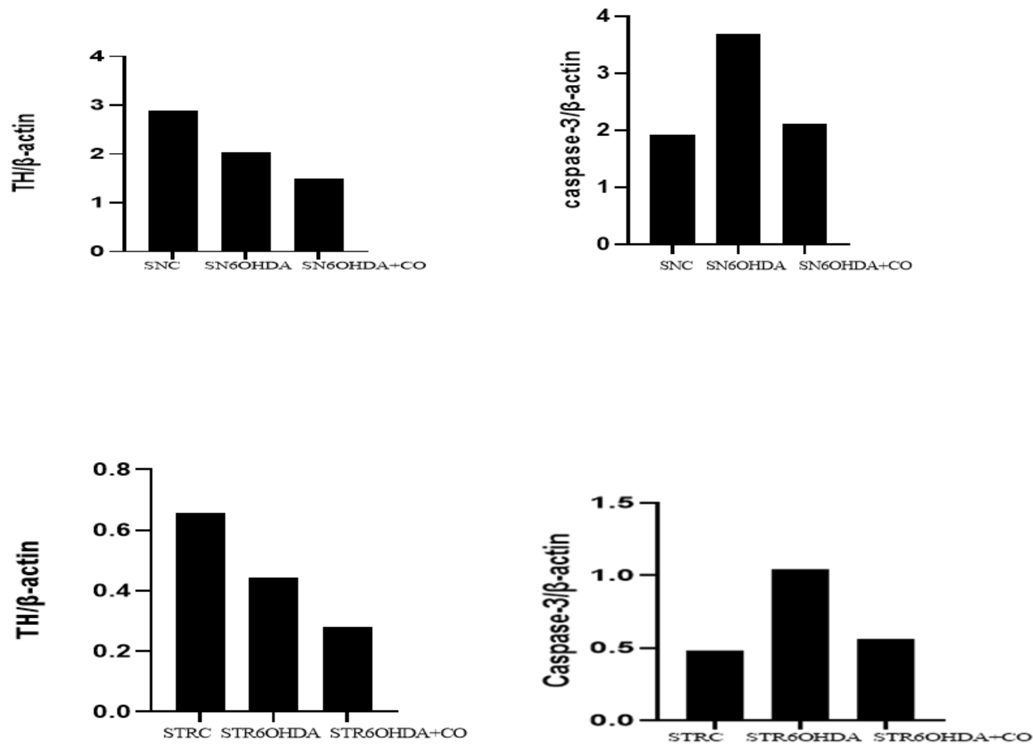


Figure:17 Western blot images and graphs representing the quantification of band intensity of THand caspase-3 level. 6-OHDA and 6OHDA+CO (curcuma –oil) treated groups in both SN and STR region shows significant depletion in TH level as compared to control. While, increased levelof caspase-3 in both 6-OHDA and 6OHDA+CO treated groups of SN and STR regions as compared to control.

DISCUSSION

PD pathogenesis involves dopamine depletion in substantial nigra and corpus striatum in post- mortem brains of PD patients correlated with an almost complete loss of dopaminergic neurons from the substantial nigra and degeneration of nerve terminals in the striatum.

The present study shown 6-OHDA administration in rat's brain induced a stereotype behaviour and depleted level of tyrosine hydroxylase along with augmented level of cleaved caspase-3 reflecting the disease specific pathological markers in experimental models. The validation of 6- OHDA administered experiment is done by using different parameters such as comet assay whichshown, DNA damage in SN and STR regions of rat brain after 6-OHDA treatment and morphological parameters were also observed with Cresyl violet staining and Hematoxylin & eosin staining in order to validate the 6-OHDA administered experiment, which shown the 6- OHDA induced shrunken neurons and distorted morphology of neuronal cells.

The effect of curcuma-oil on 6-hydroxydopamine induced experimental model of Parkinson's disease mainly in the SD rat brain regions specifically in STR and SN regions of the midbrain. The protein level mainly of TH and caspase-3 were estimated by western blotting. 6OHDA treatedgroup shown significant increase in Caspase-3 level, which was restored in 6-OHDA+curcuma- oil treated group, while TH depletion was observed in 6-OHDA treated group but no restoration of TH level seen in 6-OHDA+curcuma oil treated group. 250mg/kg dose of curcuma-oil shows only partial protection as revealed by caspase-3 level.

Preliminary findings indicated that curcuma-oil offered neuroprotective activity but needs further exploration to confirm the finding.

CONCLUSION

The preliminary findings of the present study shown that 6-OHDA administration in rat's brain induced a stereotype behavior and depleted level of tyrosine hydroxylase along with augmented level of cleaved caspase-3 reflecting the disease specific pathological markers in experimental models. Curcuma-oil treatment exhibited the protection against 6-OHDA induced neuronal apoptosis and reduced stereotype responses (numbers of rotations) however, no restoration of tyrosine hydroxylase enzyme was observed at the given dose regimen.

In conclusion, preliminary findings suggested that curcuma-oil has neuroprotective activity however, further detailed investigation is required.

REFERENCES

1. Moustafa AA, Chakravarthy S, Phillips JR, Gupta A, Keri S, Polner B, Frank MJ,Jahanshahi M. Motor symptoms in Parkinson's disease: A unified framework. Neuroscience & Biobehavioral Reviews. 2016 Sep 1; 68:727-40.

2. Smith AD, Bolam JP. The neural network of the basal ganglia as revealed by the study of synaptic connections of identified neurones. Trends in neurosciences. 1990 Jul1;13(7):259-65.

3. Struzyna LA, Browne KD, Brodnik ZD, Burrell JC, Harris JP, Chen HI, Wolf JA, Panzer KV, Lim J, Duda JE, España RA. Tissue engineered nigrostriatal pathway for treatment of Parkinson's disease. Journal of tissue engineering and regenerative medicine. 2018 Jul;12(7):1702-

4. Morelli M, Blandini F, Simola N, Hauser RA. Receptor Antagonism and Dyskinesia in Parkinson's Disease. Parkinson's Disease. 2012 Jan 1;2012.

5. Hernandez-Baltazar D, Zavala-Flores LM, Villanueva-Olivo A. El modelo de 6- hidroxidopamina y la fisiopatología parkinsoniana: Nuevos hallazgos en un viejo modelo. Neurología. 2017 Oct 1;32(8):533-9.

6. https://www.panlab.com/en/tests-solutions/apomorphine-rotation-test

7. Ungerstedt U, Arbuthnott GW. Quantitative recording of rotational behavior in rats after 6-hydroxy-dopamine lesions of the nigrostriatal dopamine system. Brain Res. 1970 Dec 18;24(3):485-93. doi: 10.1016/0006-8993(70)90187-3. PMID: 5494536.

8. Priyanka Rathore, Preeti Dohare, Saurabh Varma, Aparajita Ray, Uma Sharma, N. R. Jaganathanan & Madhur Ray. Curcuma Oil: Reduces Early Accumulation of Oxidative Product and is Anti-apoptogenic in Transient Focal Ischemia in Rat Brain. Neurochemical Research volume 33, pages1672–1682 (2008).

9. Rao DS, Sekhara NC, Satyanarayana, M. N. Satyanarayana, M. Srinivasan (1970). Effect of curcumin on serum and liver cholesterol levels in the rat. J Nutr 100:1307–1315.

10. Ray M, Pal R, Singh S, Khanna NM (2006). Herbal medicament for the treatment of neurocerebrovascular disorders. US patent number 6991814, grant date: 31-jan2006

11. https://mayfieldclinic.com/pe-anatbrain.htm

12. Stanley Finger. Origins of neuroscience: a history of explorations into brain function. Oxford University Press US. p. 48.2018 ISBN 978-0-19-514694-3. https://www.ncbi.nlm.nih.gov/books/NBK536724/.

13. Constance Hammond. Cellular and Molecular Neurophysiology (Fourth Edition), 2015. https://doi.org/10.1016/B978-0-12-397032-9.00001-7.

14. Hao Chi,1 Hui-Yun Chang,2,* and Tzu-Kang Sang. Neuronal Cell Death Mechanisms in Major Neurodegenerative Diseases. Int J Mol Sci. 2018 Oct; 19(10): 3082. Published online 2018 Oct 9. doi: 10.3390/ijms19103082.

15. DeMaagd, G., & Philip, A. (2015). Parkinson's Disease and Its Management: Part 1: Disease Entity, Risk Factors, Pathophysiology, Clinical Presentation, and Diagnosis. *P & T: a peer-reviewed journal for formulary management*, *40*(8), 504–532.

16. parkinsonsnewstoday.com, 2022.

17. Thomas T. Warner PhD, FRCP,Anthony H. V. Schapira MD, FRCP, FMedSci. Genetic and environmental factors in the cause of Parkinson's disease. 24 March 2003 https://doi.org/10.1002/ana.10487.

18. Garcia Ruiz PJ, Catalán MJ, Fernández Carril JM. Initial motor symptoms of Parkinson disease. Neurologist. 2011;17(suppl 1): S18–S20.

19. Khalil El Bayad, Ahmed Bouhouche, El Hachmia Ait Ben Haddou, Ali Benomar, Mohamed Yahyaoui, Abdelhamid Benazzouz and Wafa Regragui. April (2018). Non-Motor Symptoms of Parkinson's Disease and Their Impact on Quality of Life in a Cohort of Moroccan Patients. Front. Neurol., 04. https://doi.org/10.3389/fneur.2018.00170,

20. Tompkins MM, Basgall EJ, Zamrini E, Hill WD. Apoptotic-like changes in Lewy-body- associated disorders and normal aging in substantia nigral neurons. Am JPathol. 1997;150(1):119–31.

21. Nour S. Erekat. Stoker TB. Chapter 4 Apoptosis and its Role in Parkinson's Disease. Greenland JC editors. Brisbane (AU): Codon Publications; 2018 Dec 21.

22. Tabrez S, Jabir NR, Shakil S, Greig NH, Alam Q, Abuzenadah AM, Damanhouri GA, Kamal MA. A synopsis on the role of tyrosine hydroxylase in Parkinson's disease. CNS Neurol Disord Drug Targets. 2012 Jun 1;11(4):395-409. doi: 10.2174/187152712800792785. PMID: 22483313; PMCID: PMC4978221.

23. Haycock JW, Haycock DA. Tyrosine hydroxylase in rat brain dopaminergic nerve terminals: Multiple-site phosphorylation in vivo and in synaptosomes. J. Biol. Chem. 1991; 266:5650–5657.

24. Salvatore MF, Waymire JC, Haycock JW. Depolarization-stimulated catecholamine biosynthesis: involvement of protein kinases and tyrosine hydroxylase phosphorylation sites in situ. J. Neurochem. 2001;79(2):349–360.

25. Cunha-Oliveira T., Rego A.C., Oliveira C.R. 2008. "Cellular and molecular mechanisms involved in the neurotoxicity of opioid and psychostimulant drugs". Brain Research Reviews. 58 (1): 192–208.

26. Foley, P., Riederer, P., 2000. Influence of neurotoxins and oxidative stress on the onset and progression of Parkinson’s disease. J. Neurol. 247, 82–94.

27. Dawson, T.M., Dawson, V.L., 2003. Molecular pathways of neurodegeneration in Parkinson’s disease. Science 302, 819–822.

28. Blum D, Torch S, Lambeng N, Nissou M, Benabid AL, Sadoul R, Verna JM. Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution tothe apoptotic theory in Parkinson's disease. Prog Neurobiol. 2001 Oct;65(2):135-72. doi: 10.1016/s0301-0082(01)00003-x. PMID: 11403877.

29. K. Chiba, A. Trevor, N. Castagnoli Jr. Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase Biochem Biophys Res Commun, 120 (1984), pp. 574-578.

30. P. Jenner, A.H. Schapira. C.D. Marsden. New insights into the cause of Parkinson's disease. Neurology, 42 (1992), pp. 2241-2250.

BIBLIOGRAPHY

- https://mayfieldclinic.com/pe-anatbrain.htm
- https://appsychology.com/book/biological/neuroscience/
- https://www.ncbi.nlm.nih.gov/books/NBK536724/
- https://en.wikipedia.org/wiki/Dopaminergic
- https://www.sciencedirect.com/science/article/pii/B9780124173026000015
- PubChem
- https://www.researchgate.net/figure/Proposed-mechanism-of-neuroprotection-afforded- by-GUO-against-6-OHDA-toxicity-in-vitro-in_fig6_328881422
- Book from bookshelf of NCBI-Parkinson's Disease: Pathogenesis and Clinical Aspects

APPENDIX-I

LYSIS BUFFER

S. No	CHEMICALS	CONCENTRATION
1	HEPES	200 mM
2	Sucrose	250 mM
3	DTT	1 mM
4	KCl	10mM
5	EDTA	1mM
6	EGTA	1mM
7	MgCl2	1.5mM
8	Phenylmethanesulfonylfluoride fluoride(PMSF)	0.1mM
9	NP40	1%
10	protease inhibitor cocktail	1:100 ratio

APPENDIX-II

SDS PAGE-COMPONENTS OF RESOLVING GEL

S. No	COMPONENTS	12% (10 ml)	15% (10ml)
1	Water	3.3 ml	2.3 ml
2	Bisacrylamide	4.0 ml	5.0 ml
3	Tris-HCL (pH-8.8)	2.5 ml	2.5 ml
4	10% SDS	0.1 ml	0.1 ml
5	10% APS	0.1 ml	0.1 ml
6	TEMED	0.004 ml	0.004 ml

STACKING GEL COMPONENTS

S. No	COMPONENTS	6% (5 ml)
1	Water	2.6 ml
2	Bisacrylamide	1 ml
3	Tris-HCL (pH – 6.8)	1.25 ml
4	10% SDS	0.05 ml
5	10% APS	0.05 ml
6	TEMED	0.005 ml

APPENDIX-III

COMPONENT OF COMET ASSAY

STOCK SOLUTION COMPONENTS

S. No	COMPONENTS	CONCENTRATION/pH
1	Saline	0.9%
2	NaCl	2.5M
3	Na2EDTA	500Mm (PH-8)
4	Tris buffer	(PH- 7.5)
5	NaOH	3M

WORKING REAGENTS COMPONENTS-LYSIS BUFFER

S. No	COMPONENTS	CONCENTRATION/pH
1	EDTA	0.5M
2	NaCl	5M
3	DMSO	10%
4	Triton –X	(1%)
5	Alkaline buffer	(pH- 13)
6	Tris buffer	(PH – 7.5)

LMP Agarose (Low Melting Point) – 0.8%)

APPENDIX-IV

1X (1 Litre) PBS-T (Phosphate buffer saline-Tween-20)

S. No	CHEMICALS	CONCENTRATION (gram & ml)/pH
1	NaCl	8g
2	KCl	2g
3	Na2HPO4	4.54g
4	KH2PO4	2.04g
5	K2HPO4	2.610g
6	Tween-20	1ml

Adjust pH to 7.4.

APPENDIX-V

1X (1Litre) TRANSFER BUFFER

S. No	CHEMICALS	CONCENTRATION (gram &ml) pH
1	Tris	2.4g
2	Glycine	14.41g
3	SDS	0.2g
4	Methanol	200ml

APPENDIX-VI

1X (1Litre) RUNNING BUFFER

S. No	CHEMICALS	CONCENTRATION (gram & ml) pH
1	Tris	3g
2	Glycine	14.4g
3	SDS	1g