Dedicated to my beloved parents, Shri Binay Kumar Mishra (Papa) Late Shrawan Kumar Pandey (Father-in-law) and

Smt. Pushpa Mishra (Maa) Smt. Durga Pandey (Mother-in-law)

for their blessings, endless support and encouragement

### **DECLARATION BY THE CANDIDATE**

I hereby declare that the thesis **"To study the snake venom nerve growth factor-derived custom peptides for their application in preventing Parkinson's disease"** being submitted to Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur, Assam in partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology, has previously not formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition. Due to the unavailability of proper facilities at Tezpur University, the following experiments/sample analyses were carried out at other institutes:

- LC-MS/MS analysis of samples was performed at the Centre for Cellular and Molecular Platforms (C-CAMP) and Biokart, Bangalore, India. Transcriptomics and miRNA sequencing were performed at Biokart, Bangalore, India.
- 2. Confocal microscopic studies, Flow cytometry studies, and many other techniques were used at SAIC IASST, Guwahati 781035, India.
- 3. *In vitro* and *in vivo* experiments were performed at an animal experiment facility, IASST, Guwahati 781035, India.

Dupladhubolis

(Dev Madhubala)

Place: Tezpur

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Department of Molecular Biology and Biotechnology School of Sciences, Tezpur University



# **TEZPUR UNIVERSITY**

## **CERTIFICATE OF SUPERVISOR**

This is to certify that the thesis entitled "**To study the snake venom nerve growth factorderived custom peptides for their application in preventing Parkinson's disease**" submitted to the School of Sciences, Tezpur University in requirement of partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology is a record of research work carried out by Ms. Dev Madhubala under my supervision and guidance. All help received by her from various sources has been duly acknowledged. No part of this thesis has been submitted elsewhere for award of any other degree.

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Place: Tezpur Date:

Our Madhuleale

(Dev Madhubala)

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cells;  ${}^{\#}p \le 0.05$ , a significant difference between PT-treated cells with respect to mouse 2.5S-NGF and custom peptide pretreated PC-12 cells.  ${}^{\uparrow}p \le 0.05$ , a significant difference of mouse 2.5S-NGF treated cells with respect to the peptide (TNP and HNP) treated cells. (B) Treatment of cells with PT for 24 h followed by treatment with custom peptide for 4h. There is no significant difference in the cell viability in PT-treated cells with respect to mouse 2.5S-NGF, Vit C, and peptide post-treatment. (C) The cells were simultaneously treated with custom peptides and PT for 24 h followed by an assay of cell viability. There is no significant difference in the cell viability in PT-treated cells with respect to mouse 2.5S-NGF, Vit C, and peptide co-treatment. Values are mean  $\pm$  SD of triplicate determinations.

- Determination of the optimum time and doses of peptide for 4.11 101-102 neuroprotective activity against PT-induced toxicity. Time and dose-dependent neuroprotective activity was determined by MTT and LDH assay in PC-12 cells. (A) Pre-treatment of cells with custom peptides (100 ng/mL, ~ 71 nM) at a different time interval (0.5 h - 6 h) followed by PT treatment for 24 h at 37°C in a CO<sub>2</sub> incubator. \* $p \le 0.05$ , a significant difference between untreated (control) and PT-treated cells;  $^{\#}p \leq 0.05$ , a significant difference of PT-treated cells with respect to custom peptide (TNP and HNP) pretreated PC-12 cells for different time intervals (0.5 - 6 h). Significance of difference in the percent cell viability of custom peptide pre-treated cells incubated for 1 h with respect to 0.5-6 h, ^p < 0.05. (B) Pre-treatment of cells with different concentrations of custom peptides (12.5 ng/mL to 500 ng/mL) for 1h (optimum time) followed by PT treatment for 24 h at 37°C in a CO<sub>2</sub> incubator (C) LDH release assay to determine the protective effects of custom peptides on the LDH release of PT-induced PC-12 cell cytotoxicity when pre-treated with different concentrations of custom peptides (25 ng/mL to 500 ng/mL) for 1h followed by PT treatment for 24 h at 37°C in a CO<sub>2</sub> incubator. \* $p \le 0.05$ , a significant difference between untreated (control) and PT-treated cells;  $^{\#}p \leq 0.05$ , a significant difference of PT-treated cells with respect to custom peptide (TNP and HNP) pre-treated PC-12 cells for different time intervals (0.5-6 h). Significance of difference in the percent cell viability of custom peptide pre-treated cells at the dose of 100 ng/mL with respect to 12.5-500 ng/mL,  $p \leq 0.05$ . Values are mean  $\pm$  SD of triplicate determinations.
- 4.12 Determination of PT-induced intracellular ROS generation and its 103-104 reversal by pre-treatment with custom peptide (12.5 ng/mL to 500 ng/mL) and Vitamin C (positive control, 10000 ng/mL) for 1 h

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- Spectrofluorometric determination of intracellular ROS. \*p < 0.05, a 4.13 significant difference between untreated (control) and PT-treated cells; p < 0.05, a significant difference of PT-treated cells with respect to custom peptides (TNP and HNP) and Vit C pre-treated cells. Significance of difference in the fold change value between TNP and HNP at the dose of 12.5 to 100 ng/mL, p < 0.05. Values are mean  $\pm$  SD of triplicate determinations.
- 4.14 Reversal of PT-induced disruption of mitochondrial membrane potential (MMP) of PC-12 cells pre-treated with custom peptides (100 ng/mL, ~71 nM) for 1 h followed by the PT treatment for 24 h at 37°C in a CO<sub>2</sub> incubator. The PT-treated (10 mM) PC-12 cells pre-treated with or without custom peptide (~71 nM) were observed for the measurement of the ratio of red/green fluorescence intensity by JC-1 staining. (A) Confocal images of PC-12 cells stained with JC-1 dye to measure the MMP micrographed at the magnification of 40X. JC-1 red fluorescence represents normal MMP, whereas JC-1 green fluorescence indicates damaged MMP. The scale bar indicates the length as 20 µm. (B) Bar diagram representing the ratio of red/green fluorescence intensity quantified using Image J software. (C) The fluorescence signal intensity of JC-1 monomer and JC-1 aggregates was determined by flow cytometry analysis. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) is a mitochondrial uncoupling agent that depolarises the mitochondria taken as a positive control. (D) Bar graph representing quantitate analysis of the red and green fluorescence intensity detected by the flow

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- 4.15 Effects of the custom peptide (100 ng/mL) on inhibition of PT-induced apoptosis in PC-12 cells pre-treated with custom peptides (100 ng/mL, ~71 nM) for 1 h followed by the PT treatment for 24 h at 37°C in a CO<sub>2</sub> incubator. (A) The fluorescence intensity of Annexin V-FITC and Propidium iodide (PI) was determined by flow cytometry. (B) The bar graph represents a quantitative analysis of the percent cell death determined by flow cytometry analysis. \*p  $\leq$  0.05, a significant difference between untreated (control) and PT-treated cells; #p  $\leq$  0.05, a significant difference of PT-treated cells. Significance of difference in percent cell death between TNP and HNP, ^p  $\leq$  0.05. Values are mean  $\pm$  SD of triplicate determinations.
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- 5.3 (A) Confocal microscopic (40 X) studies of the *in vivo* binding of FITC-custom peptides (50  $\mu$ g/mL, 2 h) to CAM-1 mutant and compared with wild-type N2 strain *C. elegans*. The scale bar indicates the length as 100  $\mu$ m. (B) Bar graph representing fluorescence intensity between the CAM-1 mutant and N2 strains. \*p < 0.05, a significant difference between CAM-1 mutant and N2 strain of *C. elegans*. Values are mean  $\pm$  SD of triplicate determinations.
- Determination of the effect of the custom peptide on PT-induced 5.4 death of C. elegans. (A) worms were pre-incubated with mouse 2.5S-NGF (50 µg/mL)/quercetin (50 µg/mL, positive control) / vitamin C (100 µg/mL, positive control) and progressive concentration of custom peptides (12 µg/mL - 100 µg/mL) followed by the PT (10 mM) treatment. \*p < 0.05, a significant difference between untreated (control) and PT-treated cells;  $^{\#}p <$ 0.05, a significant difference between PT-treated cells and quercetin/ mouse 2.5S-NGF/ vitamin C and custom peptide pretreated C. elegans.  $p' \le 0.05$  Significance of difference in different concentrations for custom peptides. (B) worms were pre-incubated with quercetin (50 µg/mL, positive control) and custom peptides (50 µg/mL) for 2 h, 12 h, and 24 h followed by the PT (10 mM) treatment. \*p < 0.05, a significant difference between untreated (control) and PT-treated cells;  $^{\#}p < 0.05$ , a significant difference between PT-treated cells and quercetin (positive control) and custom peptide pre-treated C. elegans. p < 0.05, a significant difference between quercetin pre-treated C. elegans and the peptide (TNP and HNP) pre-treated C. elegans. (C) Worms were incubated with PT (10 mM) for 1 h and treated with custom peptides (12 µg/mL to 100 µg/mL). Freshly prepared custom peptides were added after 12 h of preincubation for 24 h pre-incubation condition. Worms were counted under a stereo zoom microscope for 30 s up to 24 h of treatments. Values are mean  $\pm$  SD of triplicate determinations. (D) Determination of the effect of the custom peptides on PTinduced death of cam-1 mutant compared with wild type N2 strain of C. elegans. Worms were pre-incubated with custom peptides (50 µg/mL) followed by the PT (10 mM) treatment. Percent neutralization was calculated against only paraquattreated worms.  $p^* < 0.05$ , a significant difference between wildtype and cam-1 mutant strain. (E) Restoration of chemosensory behavior in C. elegans pre-treated with custom peptides.

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- 5.6 Determination of dopaminergic neurodegeneration induced by PT in BZ555 C. elegans. (A) Confocal microscopic images (40 X) of DA neurons emerging GFP fluorescence signals in PTtreated BZ555 worms with or without pre-treated with custom peptides (50  $\mu$ g/mL). The scale bar indicates the length as 100 μm. (B) The bar diagram shows the GFP fluorescence intensity indicating the content of DA neurons in BZ555 worms, quantified using the Image J software. \*p < 0.05, a significant difference between untreated (control) and PT-treated worms; <sup>#</sup>p < 0.05, a significant difference between PT-treated and custom peptide-treated worms. Custom peptides inhibit the aggregation of  $\alpha$ -synuclein in transgenic NL5901 strains of C. elegans. (C) Confocal images of custom peptides (50 µg/mL)-treated NL5901 worms after 12 h of incubation. The scale bar indicates the length as  $100 \,\mu\text{m}$ . (D) The bar chart shows the fluorescence intensity representing the  $\alpha$ -synuclein protein accumulation in custom peptide-treated NL5901 worms for 12 h. \* (p < 0.05) a significant difference between control (CT) and custom peptides-treated C. elegans, # (p < 0.05) a significant difference between TNP and HNP treated C. elegans. Values are means  $\pm$  SD of triplicate determinations.
- 5.7 The qRT-PCR analysis shows the genes' expression in stress resistance, innate immunity, and apoptotic pathways in the PT-treated *C. elegans*, compared with the vitamin C (positive control)/custom peptide pre-treated *C. elegans*. The expression of mRNA was normalized using the housekeeping gene act-1. \* ( $p \le 0.05$ ) a significant difference between control (CT) and PT-

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#### **ABBREVIATIONS**

Abbreviation	Full form
AD	Alzheimer's Disease
ANOVA	One-Way Analysis of Variance
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BBB	Blood-Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
CD	Circular Dichroism
CID	Collision-Induced Dissociation
DAPI	4',6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
HD	Huntington's Disease
HNP	Heptadeca-Neuropeptide
L6	Rat Myoblast or Myogenic Cells
LDH	Lactate Dehydrogenase
MAPK	Mitogen-Activated Protein Kinase
MDA-MB-231	Human Breast Adenocarcinoma Cells
MCF-7	Michigan Cancer Foundation-7
EMEM	Eagle's Minimum Essential Medium
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
CCCP	Carbonyl Cyanide M-Chlorophenylhydrazone
CGC	Caenorhabditis Genetics Center
CTAB	Cetyltrimethylammonium Bromide
DBP	Disulfide Bridge Peptide
DCF	2',7'-Dichlorodihydrofluorescein
DTT	Dithiothreitol
DPPH	2,2-Diphenyl-1-Picrylhydrazyl

Abbreviation	Full Form
FPLC	Fast Protein Liquid Chromatography
HCL	Hydrochloride
H <sub>2</sub> DCFDA	2',7'-Dichlorofluorescein-Diacetate
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
LC <sub>50</sub>	Median Lethal Concentration
LC/ES-MS	Liquid Chromatography-Electronspray Tandem Mass
LC-MS/MS	Spectrometry
LPP	Liquid Chromatography-Tandem Mass Spectrometry
LD <sub>50</sub>	Median Lethal Dose
MALDI-TOF-	Matrix-Assisted Laser Desorption/Ionization -Time Of Flight -
	Mass Spectrometry
NCBI	National Center For Biotechnology Information
NGM	Nematode Growth Media
MMGBSA	Molecular Mechanics/Generalized Born Surface Area
MMP	Mitochondrial Membrane Potential
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NCBS	National Centre For Cell Science
NDs	Neurodegenerative Disorders
NGF	Nerve Growth Factor
P <sup>75</sup> NTR	P75 Neurotrophin Receptor
PC-12	Adrenal Pheochromocytoma Cell Line
PD	Parkinson's Disease
PDB	Protein Data Bank
PI	Propidium Iodide
PI3K	Phosphatidylinositol 3-Kinase Stimulation Of Protein Kinase B
	Signaling Pathways
PPI	Protein-Protein Interaction
PPP	Platelet-Poor Plasma
PRP	Platelet-Rich Plasma
PT	Paraquat
qRT-PCR	Quantitative Reverse Transcription-Polymerase Chain Reaction

Abbreviation	Full Form
RESPA	Reversible Reference System Propagator Algorithms
RMSD	Root Mean Square Deviation
ROS	Reactive Oxygen Species
RT	Rotenone
SD	Standard Deviation
SV-NGF	Snake Venom Nerve Growth Factor
TIP3P	Three-Points Water Model
TNP	Trideca-Neuropeptide
TrkA	Tropomyosin Receptor Kinase A Receptor
UHPLC-MS/MS	Ultra-Performance Liquid Chromatography-Tandem Mass
	Spectrometer
PBS	Phosphate buffered saline
PPP	Platelet poor plasma
PRP	Platelet rich plasma
Q-RT PCR	Quantitative reverse transcription polymerase chain reaction
Q-TOF	Quadrapole time of flight
RCSB	Research collaborator for structural bioinformatics
RNA	Ribonuclic acid
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase high-performance liquid chromatography
RP-UHPLC	Reversed-phase ultra-high-performance liquid chromatography
SGOT	Serum glutamic oxaloacetic transaminase
SGPT	Serum glutamic pyruvic transaminase
TBS	Tris-buffered saline
ТСА	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
WHO	World health organization
CNS	Central Nervous System
ALS	Amyotrophic Lateral Sclerosis
DALYs	Disability-Adjusted Life Years
Αβ	Amyloid-B

Abbreviation	Full Form
DAMP	Danger-Associated Molecular Pattern
IP3	Inositol 1,4,5-Triphosphate
PLCγ	Phospholipase Cγ
JNK	C-Jun N-Terminal Kinase
YFP	Yellow Fluorescence Protein
miRNA	MicroRNAs
NTFs	Neurotrophic Factors
6-OHDA	6-Hydroxydopamine
MPP+	1-Methyl-4-Phenylpyridinium Iodide