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Materials and Methods

Neurodegeneration is a process that occurs in both neuropathological circumstances and brain ageing (Kumar and Khanum, 2012). Neurological disorders are the leading cause of physical and cognitive disability across the globe, currently affecting approximately 15% of the worldwide population absolute patient numbers have considerably climbed over the past 30 years (Van Schependom and Dhaeseleer, 2023). They, as compared to metabolic or toxic disorders are identified by the slow loss of particularly vulnerable neuronal populations. The childhood neurodegenerative diseases have unclear pathophysiology and are sometimes categorized based on whether they affect the brain homogenously (diffuse encephalopathies) or preferentially affecting the cerebral cortex (poliodystrophies), the cerebral white matter (leukodystrophies), the basal ganglia (corencephalopathies) or the cerebellum, brainstem and spinal cord (spinocerebellar diseases) (Shang *et al.* 2022). Nanoparticles possess a wide array of applications in different fields such medicine, electronics, therapeutics and diagnostics (Bhumi and Savithramma, 2014) and they have more neuroprotective effect, which helps recover the neurodegenerative cells by inhibiting apoptotic processes (Ding *et al.,* 2020; Liu *et al.,* 2022). The secondary metabolites of plants including alkaloids, flavanoids and phenolic acids play a key role in improving regeneration and/or inhibiting neurodegeneration (Kamran *et al.,* 2020). Catechins are one of the most active compounds in the *Camellia sinensis* plant (green tea) (Khan and Mukhtar, 2013). B vitamins, lipids like linoleic and alpha-linolenic acids and trace minerals like magnesium, calcium, iron, zinc and selenium can be found in green tea leaves (Musial *et al.,* 2020). Free radicals, which are damaging chemicals, are neutralized by antioxidant substances. They help in preventing and combating disorders that might appear as a result of oxidative stress, which is a condition brought on by an accumulation of free radicals in the body (Stagos, 2019).

The study focuses on the synthesis of zinc oxide nanoparticles using *Camellia sinensis* (green tea) leaf extract capped with catechin which enhances bioactive potential and neuroprotective effect.

This chapter presents in brief the experimental procedures adopted for the study entitled '**Neuroprotective effect of synthesized zinc oxide nanoparticle-capped catechin'.**

The experimental design is divided into five phases.

Phase I: Evaluation of active compounds against neuroprotective target proteins using molecular docking studies

In silico assessment of molecular interaction between the drug for neuroprotective and proteins using Maestro Molecular Modeling Environment, (Maestro V: 11.8 Schrodinger_suite-2019) was carried out.

Phase II: Characterization of *Camellia sinensis* and identification of its bioactive compound

Preliminary phytochemical studies and antioxidant activity were done. Identification was done for an active compound using chromatographic and spectral studies such as Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC) and spectral studies involving UV-Vis spectrophotometer, Fourier Transform Infra-Red spectroscopy (FTIR), Nuclear Magnetic Resonance spectroscopy (NMR), Gas Chromatography-Mass Spectroscopy (GC-MS) and Liquid Chromatography Mass Spectrometry (LC-MS).

Phase III: Characterization of synthesized zinc oxide nanoparticles from *Camellia sinensis*

This was done and characterized using UV Visible Spectroscopy, Scanning Electron Microscope (SEM) with Energy-dispersive X-ray analysis (EDAX), X-ray Diffraction (XRD) analysis, Fourier Transform Infra-Red (FTIR) analysis and Zeta-Potential.

Phase IV: Synthesized zinc oxide nanoparticle-capped catechin their characterization and *in vitro* neuroprotective activity

Characterization of synthesized zinc oxide nanoparticle-capped catechin compound by UV Visible Spectroscopy, Scanning electron microscopy (SEM) with Energy Dispersive Spectroscopy (EDAX), X-ray Diffraction (XRD) analysis, Fourier Transform Infra-Red (FTIR) analysis and Zeta-Potential were carried out to evaluate the *in vitro* neuroprotective effect of synthesized zinc oxide nanoparticle-capped catechin.

Phase V: **Assessment *in vitro* neuroprotective activity of synthesized zinc oxide nanoparticle-capped catechin to neuro 2a cells**

Assessment of *in vitro* anti-oxidative role of synthesized zinc oxide nanoparticle-capped catechin cytotoxicity assay and assessment of *in vitro* Neuroprotective activity of CAT synthesized zinc oxide nanoparticle-capped catechin to neuro2a cells neuroblastoma cell lines as carried out.

The methodology is discussed under the following heads:

3.1. Phase I: Evaluation of active compounds against neuroprotective target proteins using molecular docking studies

3.1.1. Databases used for structural analysis

3.1.1.1 . PubChem

3.1.1.2 . Protein Data Bank (PDB)

3.1.2. Tools used for molecular docking analysis

3.1.2.1 . Maestro

3.1.2.2 . QikProp 3.0

3.1.2.3 . LigPrep 3.6

3.1.2.4 . Protein Preparation Wizard

3.1.2.5 . GLIDE (Grid based Ligand docking with Energetics)

3.2 Phase II: Characterization of *Camellia sinensis* and identification of its bioactive compound

3.2.1. Selection, Collection and Characterization of *Camellia sinensis* (green tea)

3.2.1.1. Selection, plant collection, identification and processing of *Camellia sinensis*

3.2.1.2. Preparation of extracts

3.2.2. Preliminary phytochemical screening of *Camellia sinensis*

3.2.2.1. Phytochemical screening

3.2.2.2. Determination of total phenol content

3.2.2.3. Determination of total flavonoid content

3.2.3. Antioxidant radical scavenging activity of *Camellia sinensis*

3.2.3.1. Determination of Diphenyl-Picryl Hydrazine (DPPH) radical scavenging activity

3.2.3.2. Determination of Ferric reducing antioxidant power radical scavenging activity

3.2.3.3. Determination of Total antioxidant capacity

3.2.4. Enzymatic antioxidants activity of *Camellia sinensis*

3.2.4.1. Estimation of catalase activity

3.2.4.2. Determination of peroxidase activity

3.2.4.3. Determination of polyphenol oxidase activity

3.2.4.4. Determination of superoxide dismutase activity

3.2.4.5. Determination of glutathione-S-transferase (GST) activity

3.2.5. Non-enzymatic antioxidant activity of *Camellia sinensis*

3.2.5.1. Determination of ascorbic acid

3.2.5.2. Determination of α-tocopherol

3.2.5.3. Determination of flavonoids

3.2.5.4. Determination of phenol

3.2.5.5. Determination of reduced glutathione

3.2.6. Identification of Active Constituents of *Camellia sinensis*

3.2.6.1. Isolation of active constituents by Column Chromatography

3.2.6.2. Separation of active constituents using Thin Layer Chromatography (TLC)

3.2.6.3. Determination of catechin content

3.2.6.4.Determination of active constituents of *Camellia sinensis* by High Pressure Thin Layer Chromatography (HPTLC)

3.2.6.5. UV-Visible absorption spectroscopy

3.2.6.6. Identification of active constituents using Fourier Transform Infra Red Spectroscopy (FT-IR)

3.2.6.7. Detection of active constituents using Nuclear Magnetic Resonance spectroscopy (NMR)

3.2.6.8. Identification of the active constituents using Gas Chromatography Mass Spectroscopy (GC-MS)

3.2.6.9. Identification of the active constituents by Liquid Chromatography Mass Spectrometry (LC-MS)

3.3. Phase III: Characterization of synthesized zinc oxide nanoparticles from *Camellia sinensis*

3.3.1. Synthesis of zinc oxide nanoparticles from *Camellia sinensis*

3.3.1.1. Preparation of plant extracts

3.3.1.2. Synthesis and optimization of zinc oxide nanoparticles from *Camellia sinensis*

3.3.2. Characterization of zinc oxide nanoparticles of *Camellia sinensis*

3.3.2.1. UV-Visible Spectroscopy of synthesized zinc oxide nanoparticles from *Camellia sinensis*

3.3.2.2. Study of surfaces topography of the zinc oxide nanoparticles using Scanning Electron Microscopy (SEM)

3.3.2.3. Elemental Mapping analysis of synthesized zinc oxide nanoparticles

3.3.2.4. Identification of the crystalline nanomaterial of the zinc oxide nanoparticles using X-ray powder diffraction (XRD)

3.3.2.5. Identification of the functional group of the zinc oxide nanoparticles using Fourier Transform Infra-Red (FTIR) Spectroscopy

3.3.2.6. Surface charge of the zinc oxide nanoparticles using Zeta potential

3.3.3. Antioxidant activity of synthesized zinc oxide nanoparticles

3.3.4. Assessment of neuroprotective effect of synthesized zinc oxide nanoparticles

3.3.4.1. Thin layer chromatography with bioassay detection for AChE inhibition

3.3.4.2. Acetyl cholinesterase inhibitory activity of synthesized zinc oxide nanoparticles

3.4 Phase IV: Synthesized zinc oxide nanoparticle-capped catechin their characterization and *in vitro* neuroprotective activity

3.4.1. Preparation of synthesized zinc oxide nanoparticle-capped catechin

3.4.2 Characterization of synthesized zinc oxide nanoparticle-capped catechin

3.4.2.1. UV-vis spectrocopy

3.4.2.2. Determination of surface topography of the synthesized zinc oxide nanoparticle -capped catechin using Scanning Electron Microscopy (SEM)

3.4.2.3. Identification of the crystalline material of the synthesized zinc oxide nanoparticle-capped catechin using X-ray powder diffraction (XRD)

3.4.2.4. Identification of the functional group of the synthesized zinc oxide nanoparticle-capped catechin using Fourier Transform Infra-Red (FTIR) Spectroscopy

3.4.2.5. Surface charge of the synthesized zinc oxide nanoparticle-capped catechin using zeta potential

3.4.3. Determination of antioxidant activity of synthesized zinc oxide nanoparticle-capped catechin

3.4.4. *In vitro* neuroprotective activity of synthesized zinc oxide nanoparticle-capped catechin

3.4.4.1. Determination of the Entrapment efficiency and Loading capacity

3.4.4.2. *In vitro* release study of synthesized zinc oxide nanoparticle-capped catechin

3.4.4.3. Qualitative evaluation of Acetylcholinestrase inhibition using thin layer chromatography

3.4.4.4. Assessment of Acetylcholinestrase Inhibition

3.5 Phase V: Assessment *in vitro* neuroprotective activity of synthesized zinc oxide nanoparticle-capped catechin to neuro 2a cells

3.5.1. Maintenance of cell lines

3.5.2. Treatment groups

3.5.3. Parameters analyzed

3.5.3.1. The cell viability MTT (dimethylthiazolyl -20, 50- diphenyl-2-H-tetrazolium bromide) assay

3.5.3.2. Assay of Lactate Dehydrogenase (LDH)

3.5.3.3. Determination of Cell Morphology changes

3.5.3.4. Determination of intracellular reactive oxygen species (ROS) level

3.5.3.5. Determination of Superoxide Dismutase (SOD)

3.5.3.6. Microplate assay for Acetylcholinestrase (AChE) activity

3.5.3.7. Cell cycle analyses by Flow cytometer

3.1. Phase I: Evaluation of active compounds against neuroprotective target proteins using molecular docking studies

Naturally occurring phytochemicals have various potential impacts in the biomedical area, particularly neuroprotective effects, as demonstrated by computational biology through molecular docking studies.

In silico molecular interaction studies were carried out using Maestro Molecular Modelling Environment (Schrodinger Module Version 9.4) the methodology of which is given below.

3.1.1. Databases used for structural analysis

3.1.1.1. PubChem

PubChem is a database of chemical compounds and their biological functions. The system is maintained by the National Center for Biotechnology Information, a component of the National Library of Medicine, which is part of the United States, National Institutes of Health. It can be accessed free of cost through a web user interface and millions of compound structures, descriptive datasets can be freely downloaded via File Transfer Protocol (FTP). Quercetin, kaempferol, C-methyl flavones, glibenclamide, acarbose and statin were retrieved from the PubChem database.

3.1.1.2. Protein Data Bank (PDB)

The Protein Data Bank (PDB) archive contains information about experimentally determined structures of proteins, nucleic acids and complex assemblies. The Research Collaboratory for Structural Bioinformatics PDB also provides a variety of tools and resources. It allows performing simple and advanced searches based on annotations relating to sequence, structure and function. The target proteins for neuroprotection were downloaded from PDB.

3.1.2. Tools used for Molecular docking analysis

3.1.2.1. Maestro

Maestro, an all-purpose molecular modeling environment is a single unified interface for all Schrodinger softwares which provide a powerful, fully integrated molecular visualization and analysis environment. Maestro is a powerful selection of analysis tool and an easy-to-use design, which makes it a versatile modeling environment for all researchers. It provides a clear insight into molecular properties as well as detailed intermolecular interactions.

3.1.2.2. QikProp3.0

Qikprop 3.0 is a quick, accurate, easy-to-use Absorption, Distribution, Metabolism and Elimination (ADME) prediction program designed by Professor William L. Jorgensen. QikProp predicts physically significant descriptors and pharmaceutically relevant properties of organic molecules, either individually or in batches. QikProp provides ranges

for comparing particular molecular properties with those of 95 per cent of known drugs. QikProp also flags 30 types of reactive functional groups that may cause false positives in high-throughput screening assays. The results from QikProp's rapid prediction of all ligands ADME of the necessary pharmacokinetic properties of viable drug compounds were saved (Friesner *et al.,* 2006; Friesner *et al.,* 2004).

3.1.2.3. LigPrep3.6

Computational methods have become an indispensable part of lead identification efforts. Nearly all methods require accurate 3D molecular models as a starting point. Efficient and accurate 2D to 3D conversion is therefore a key precursor to computational analyses. The preparation of ligand was done using LigPrep 3.6, a module on the Maestro window of Schrödinger which is of robust quality. Ligand preparation goes far beyond simple 2D to 3D structure conversions by including tautomeric, stereochemical and ionization variations, as well as energy minimization and flexible filters to generate fully customized ligand libraries that are optimized for computational analyses. It processes approximately one ligand per second, making it possible to convert the entire database at one time.

3.1.2.4. Protein preparation wizard

With Schrödinger suite of programs, the bulk of receptor preparation is carried out with the Protein preparation wizard. Schrödinger's protein preparation wizard is an easy-to-use tool for correcting common structural problems, namely, missing hydrogen atoms, ambiguous protonation states and flipped residues to form a reliable, all atom protein models in minutes instead of hours or days, while also ensuring the accuracy of all downstream modeling simulations. In this study, GLIDE Xtra Precision (XP) docking procedure was used. The docked complex structures were viewed with Glide Pose Viewer. The GLIDE XP mode uses more stringent scoring functions than Standard Precision (SP) mode. GLIDE XP was designed to remove false positives. The good ligand poses that were generated in GLIDE SP docking was used as input for GLIDE XP docking (Halgren *et al*., 2004).

3.1.2.5. GLIDE (Grid based Ligand Docking with Energetics)

GLIDE 5.9 (Schrodinger 9.4) searches for favourable interactions between one or more typically small ligand molecules and a typically larger receptor molecule usually a

protein. Each ligand must be a single molecule, while the receptor may include more than one molecule (e.g: a protein and a cofactor). GLIDE generates ligand poses that go through multiple hierarchical filters to assess the ligand-receptor interaction. GLIDE calculates a Gscore/Docking score based on chemistry. Score as follows:

Gscore=0.065*vdW+0.130*coul+Lipo+HB+metal+BuryP+RotB+sit
evdW = vander Waals energy

Coul = coulomb energy

Lipo = lipophilic term derived from hydrophobic grid
potential HB = hydrogen-bonding term

Metal = metal-binding term

Bury P = penalty for Buried polar groups

Rot B = penalty for freezing Rotatable
bonds Site = polar inter actions in the
active Site

3.2. Phase: II Characterization of *Camellia sinensis* and identification of its bioactive compound

Phytochemicals have numerous potential biological impacts, which are characterized by various analytical techniques such as UV-Vis spectrophotometry, FTIR and determination by different techniques like Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC), Nuclear Magnetic Resonance spectroscopy (NMR), Gas Chromatography-Mass Spectroscopy (GC-MS) and Liquid Chromatography Mass spectrometry (LC-MS).

3.2.1. Collection and Characterization of *Camellia sinensis* (green tea)

3.2.1.1. Selection and collection of Plant, Identification and Processing of *Camellia sinensis*

The leaves of *Camellia sinensis* (L.) Kuntze, also known as the green tea plant were collected from Aruvankadu in Nilgiris Distirct. The taxonomic identification of the plant was confirmed by the Botanical Survey of India, Coimbatore (Authentication No:

BSI/SRC/5/23/2019/Tech./185).The collected plant leaves were completely dried in the shade at room temperature. The plant material was then pulverized and used for further investigation (Plate 1).

Scientific classification

Kingdom	Plantae
Family	Theaceae
Order	Ericales
Genus	*Camellia*
Species	*Sinensis*

Plate 1. ***Camellia sinensis*** **(L.) Kuntze**

3.2.1.2. Preparation of extracts

The plant extracts were prepared using the solvents of increasing polarity, namely, hexane, acetone, ethyl acetate ethanol and methanol. 10 g of the air dried samples were taken and homogenized with 100 ml of the above mentioned solvents. The crude preparation was left overnight in the shaker at room temperature and then centrifuged at 4000 rpm for 20 minutes. The supernatant containing the plant extract was then transferred to a pre weighed beaker and the extract was concentrated by evaporating the solvent at 60˚C. The crude extract was weighed and dissolved in a known volume of dimethyl sulphoxide, to obtain a final concentration of 10 mg /5µl.

Isolation of flavonoids from the *Camellia sinensis* hydroethanolic (80:20) extract was done with 10 g of the powdered plant material and 100 ml of light petroleum ether (b.p 40˚-60˚C) in a Soxhlet apparatus for 18 hours to remove chlorophyll, non-flavonoid compounds and lipid dewaxing (Palanivel *et al.,* 2008).The treated material was dried and extracted with hydroethanolic extract using Soxhlet apparatus (Ansari *et al.,* 1976). This fraction is referred to as the flavonoid fraction of *Camellia sinensis*.

3.2.2. Preliminary phytochemical screening of *Camellia sinensis*

3.2.2.1. Phytochemical screening

The presence of alkaloids, phenols, flavonoids, tannins, glycosides, saponins, terpenoids and steroids in the hexane, acetone, ethyl acetate ethanol, methanol and hydroethanolic (80:20) extracts of *Camellia sinensis* was analysed. The procedure for phytochemical screening is explained in Appendix 1.

3.2.2.2. Determination of Total phenol content

The amount of total phenols was assayed by the Method of Aiyegoro *et al.,* (2010) as given in Appendix 2.

3.2.2.3. Determination of Total flavonoid content

Estimation of Total flavonoid was done by colorimetry using aluminium chloride (Chang *et al.,* 2002) as explained in Appendix 3.

3.2.3. Antioxidant radical scavenging activity of *Camellia sinensis*

3.2.3.1. Determination of Diphenyl-Picryl Hydrazine (DPPH) Radical Scavenging activity

Antioxidants react with DPPH and convert it to diphenyl-picryl hydrazine by donating OH group. The degree of discoloration from purple to yellow was measured at 519 nm according to the Method of Mensor *et al,* (2001) as described in Appendix 4.

3.2.3.2. Determination of Ferric reducing antioxidant power radical scavenging activity

Ferric reducing antioxidant power radical scavenging activity was estimated by the Method of Benzie and Strain (1996) as per the procedure given is Appendix 5.

3.2.3.3. Determination of Total antioxidant capacity

This was done by the Method of Lallianrawna *et al.,* (2013) as explained in Appendix 6.

3.2.4.Enzymatic Antioxidant activity of *Camellia sinensis*

3.2.4.1. Determination of Catalase activity

The catalase activity in the leaves of *Camellia sinensis* was assayed by the Method of Luck (1965). The procedure is explained in Appendix 7.

3.2.4.2. Determination of Peroxidase activity

Peroxidase activities of the samples were determined by the method proposed by Reddy *et al*. (1985). The protocol is detailed in Appendix 8.

3.2.4.3. Determination of Polyphenol oxidase activity

Determination of catechol oxidase in the *Camellia sinensis* leaves was done by the method followed by Esterbauer *et al.* (1977). The protocol is described in Appendix 9.

3.2.4.4. Determination of Superoxide dismutase activity

The activity of superoxide dismutase was determined by the Method of Misra and Fridovich, (1972). The procedure for the determination is presented in Appendix 10.

3.2.4.5. Determination of Glutathione-S-transferase activity

Glutathione-S-transferase activity using 2, 4 dichloronitrobenzene as substrate was assayed spectrophotometrically as described by Habig *et al*. (1974). Appendix 11 explains the method for this.

3.2.5. Non-enzymatic antioxidant activity of *Camellia sinensis*

Non-enzymic antioxidants such as ascorbic acid, α-tocopherol, flavonoids, polyphenol and reduced glutathione in the plant samples of *Camellia sinensis* were studied

3.2.5.1. Determination of Ascorbic acid

The determination of ascorbic acid in *Camellia sinensis* was studied by the procedure given in Appendix 12.

3.2.5.2. Determination of α-tocopherol

The activity of α-tocopherol in *Camellia sinensis* was studied as per the protocol given by Rosenberg (1942) the details of which are expressed in Appendix13.

3.2.4.3. Determination of Flavonoids

The flavonoid content of the plant samples was estimated by the procedure adopted by Cameron *et al.* (1943) as given in Appendix14.

3.2.4.4. Determination of Phenol

The total phenol content of the plant samples used in the study was estimated by the Method of Malick and Singh (1980). The detailed procedure is given in Appendix 15.

3.2.4.5. Determination of Reduced glutathione

The amounts of reduced glutathione in the samples were estimated by the Method of Boyne and Ellman (1972). The procedure for this is described in Appendix 16.

3.2.6. Identification of active constituents of *Camellia sinensis*

3.2.6.1. Isolation of active constituents by column chromatography

Separation of compounds from the hydroethanolic extract *Camellia sinensis* leaves, using column chromatography was performed by the method of Abdullahi *et al.* (2014) as given in Appendix 17.

3.2.6.2. Separation of active constituents using Thin Layer Chromatography (TLC)

Separation of flavanoids of the hydroethanolic extract of *Camellia sinensis*, using TLC was performed by the Method of Smith *et al*., (1997) as given in Appendix 18.

3.2.6.3. Determination of catechin content

Vanillin assay was carried out by the Method of Price *et al.,* (1978). The procedure for the determination of the activity of catechin is presented in Appendix19.

3.2.6.4. Determination of active constituents of *Camellia sinensis* by High Pressure Thin Layer Chromatography (HPTLC)

HPTLC is a valuable tool for the investigation of herbal products with respect to different aspects of their quality. The advantage of HPTLC over other techniques is that large numbers of samples can be simultaneously analyzed using small volumes of mobile phase unlike TLC, thus lowering analysis time and cost per analysis. The software used was win CATS 1.3.4 version. Separation of flavanoids of the hydroethanolic extract using HPTLC was performed by the method given in Khushboo *et al.,* (2009) as explained in Appendix 20.

3.2.6.5. UV-Visible absorption spectroscopy

A preliminary absorption spectral analysis was done by a survey scan of the hydroethanolic extract in a Shimadzu 1800 spectrophotometer. The instrument was set to scan mode and the absorption spectrum obtained in the range of 200 nm to 800 nm analyzed.

3.2.6.6. Identification of active constituents using Fourier Transform InfraRed Spectroscopy (FT-IR)

Functional group analysis of the bioactive constituents of hydroethanolic extract by FT-IR procedure was carried out using the Method of Nasir *et al.,* (2006) as seen in Appendix 21.

3.2.6.7. Detection of active constituents by Nuclear Magnetic Resonance (NMR) spectroscopy

Separation of the bioactive compounds of the hydroethanolic extract using NMR analysis was performed by the Method of Moore and Dalrymple, (1997) as described in Appendix 22.

3.2.6.8. Identification of active constituents of using Gas Chromatography-Mass Spectroscopy (GC-MS)

Separation of the bioactive compounds of hydroethanolic extract using GC-MS analysis was performed by the Method of Maciejewicz *et al.,* (2001) as expressed in Appendix 23.

3.2.6.9. Identification of active constituents by Liquid Chromatography-Mass spectrometry (LC-MS)

Separation of the bioactive compounds of the hydroethanolic extract of *Camellia sinensis* using LC-MS analysis was performed by the Method of Bouhafsoun *et al.,* (2018). The detailed procedure is given in Appendix 24.

3.3. Phase III: Characterization of synthesized zinc oxide nanoparticles from *Camellia sinensis*

Plant derived compounds have numerous potential biological impacts in the field of nanotechnology for both synthesis and encapsulation of nanoparticles using phytochemicals. The phytochemicals enrich the reducing and capping agents to synthesis the nanoparticles especially phenolic compounds in the aqueous extract of plants. These nanoparticles are preliminarily characterized by UV-Vis spectrophotometer and the crystalline structured nanoparticles confirmed by XRD, formation of nanoparticles characterized by SEM, identification of surface charge by zeta potential and determination of the functional group of nanoparticles by FTIR.

3.3.1. Synthesis of zinc oxide nanoparticles from *Camellia sinensis*

3.3.1.1. Preparation of plant extract

Aqueous extract: An aqueous extract of *Camellia sinensis* was prepared according to the method of Kumar (2012).10 g of thoroughly washed leaves were immersed in 100 ml of double distilled water at 60°C for 15 minutes. The extract was filtered using Whatman filter paper and stored at 4°C for further use.

3.3.1.2. Synthesis and optimization of zinc oxide nanoparticles from *Camellia sinensis*

Zinc oxide nanoparticles were synthesized using zinc acetate dehydrate (Zn $(CH_3COOH)_2$ 2H2O). The protocol described by Gnanasangeetha and Thambavani, (2014) is given in Appendix 25.

3.3.2. Characterization of zinc oxide nanoparticles of *Camellia sinensis*

Characterization of zinc oxide nanoparticles was done for the identification of the nanoparticles, by size, shape, surface area and dispersity. After completion of the nanoparticle synthesis process, the solution containing the nanoparticles were centrifuged to separate the nanoparticles. The process was repeated 2 to 3 times to remove unwanted debris and then dried in a hot air oven at 60˚C (Gnana Jobitha *et al.,* 2013).

3.3.2.1. UV-Visible Spectroscopy of zinc oxide nanoparticles from *Camellia sinensis*

The light nanoparticles exhibit unique and tunable optical properties on account of their surface plasmon resonance. A volume of 100 µl of synthesized nanoparticle sample was diluted with 900 µl solution for spectral analysis using an UV-Visible spectrophotometer (Shimadzu1800) in the 400 nm to 600 inm scanning range.

3.3.2.2. Study of surface topography of zinc oxide nanoparticles using Scanning Electron Microscopy (SEM)

The nanoparticle synthesized were characterized using high resolution Scanning Electron Microscopy (SEM). The sample was prepared by drop coating of the suspension of nanoparticles separately on a carbon-coated copper grid, by simply dropping a very small

amount of the sample on the excess solution and removed by blotting. The film on the SEM grid was then allowed to dry under a mercury lamp for 5 minutes. It was then subjected to SEM analysis.

3.3.2.3. Elemental Mapping analysis of synthesized zinc oxide nanoparticles

Elemental mapping leverages the compositional precision inherent in techniques such as Energy Dispersive Spectroscopy (EDS) microanalysis and combines it with high resolution imaging to present complex data in an accessible, visually striking format that assists AMA's (Advanced Micro Analytical) clients by communicating complex information in an efficient and compelling manner. Elemental mapping is based on compiling extremely specific elemental composition data across an area of a sample.

3.3.2.4. Identification of the crystalline nanomaterial of the zinc oxide nanoparticles using X-ray powder diffraction (XRD)

XRD is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell dimensions. The analyzed nanomaterial is finely ground, homogenized and the average bulk composition determined.

3.3.2.5. Identification of the functional group of the zinc oxide nanoparticles using Fourier Transform Infra-Red (FTIR) Spectroscopy

FTIR analysis is done to obtain the infrared spectra of absorption, emission and to ensure the formation of nanoparticles. An FTIR identifies to functional groups, formation and stabilization (capping material) of zinc nanoparticles. The advantage of using an FTIR is that it simultaneously collects spectral data in the 200 to 800 nm spectral ranges.

3.3.2.6. Surface charge of the zinc oxide nanoparticles using Zeta potential

Zeta potential analyzer is an analytical device for characterizing zeta potential as a property of interfacial layer in liquid particulates (dispersions, emulsions) and porous bodies. ISO Standard-13099 prepared that would describe the main principles underlying functioning of such devices. Zeta potential is not directly a measurable parameter. It should be calculated from other experimentally measurable properties of the system using appropriate theory.

3.3.3. Antioxidant activity of synthesized zinc oxide nanoparticles

Antioxidants activity was determined by the modified method of Rajeshkumar *et al.,* (2018) as detailed in Appendix 26.

3.3.4. Assessment of neuroprotective effect of synthesized zinc oxide nanoparticles

3.3.4.1. Thin layer chromatography with bioassay detection for AChE inhibition

The AchE inhibition activity of the samples was detected by using a Thin Layer Chromatography auto graphic assay (Machado *et al.,* 2015) as shown in Appendix 31.

3.3.4.2. Acetyl cholinesterase inhibitory activity of synthesized zinc oxide nanoparticles

The acetylcholinesterase inhibition activity was measured using the method described by Ingkaninan *et al.* (2003) as given in Appendix 27.

3.4. Phase IV: Synthesized zinc oxide nanoparticle-capped catechin their characterization and *in vitro* neuroprotective activity

Phytochemicals have properties such as anticancer, neuroprotective, antidiabetic, antifungal and antibacterial activities. The current study demonstrates the neuroproptective effects in *in vitro* based nano formulations. Zinc oxide nanoparticles were synthesised by using the *Camellia sinensis* leaf extract and encapsulating with catechin. This hybrid green synthesized zinc oxide nanoparticle-capped catechin has potentially active neuroprotective effect. The synthesized zinc oxide nanoparticle-capped with catechin was characterized by using different techniques such as UV-Vis spectrophotometer, XRD for identification of crystalline structured of nanoparticles, morphological identification of nanoparticles by SEM with the Energy Dispersive Spectroscopy (EDAX), identification of surface charge using Zeta potential and determination of the functional group of nanoparticles using FTIR. The antioxidant activity was evaluated and the neuroprotective effect of synthesized zinc oxide nanoparticle-capped catechin was analyzed.

3.4.1. Preparation of synthesized zinc oxide nanoparticle-capped catechin

Nanoparticle preparation was carried out by nanoprecipitation method (Arasoglu *et al.,* 2017) as seen in Appendix 28.

3.4.2. Characterization of synthesized zinc oxide nanoparticle-capped catechin

Nanoparticle identification was made possible through characterization, which included study of size, shape, surface area and dispersity. After completing the nanoparticle production, the solution was centrifuged to separate the nanoparticles and the process was repeated two or three times to remove undesirable debris. Then it was dried in hot air oven at 60˚C (Gnana Jobitha *et al.,* 2014).

3.4.2.1. UV-Visible spectroscopy

The UV–Vis spectrum of synthesized zinc oxide nanoparticle-capped catechin was measured for its maximum absorbance using a UV–Vis Double beam spectrophotometer (Shimadzu1800) in the wavelength range of 200–500 nm (Chandra *et al.,* 2019).

3.4.2.2. Determination of surface topography of the synthesized zinc oxide nanoparticle-capped catechin using Scanning Electron Microscopy (SEM)

The shape of the synthesized zinc oxide nanoparticle-capped catechin was analysed using SEM at 200 µm, 10 µm and 2 µm. The thin films of samples were prepared on copper grid coated with carbon by dropping 20–50µl of sample on the grid and later observing in the analyser. The extraneous materials were wiped by tissue paper and then allowed to dry under mercury lamp for 4-5 minutes (Manjul Gondwal and Pant, 2013).

3.4.2.3. Identification of the crystalline material of the synthesized zinc oxide nanoparticle-capped catechin using X-ray powder diffraction (XRD)

X-ray diffraction (XRD) is a versatile non-destructive analytical technique used to analyze physical properties such as phase composition, crystal structure and orientation of powder, solid and liquid samples. X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample.

3.4.2.4. Identification of the functional group of the synthesized zinc oxide nanoparticle-capped catechin using Fourier Transform Infra-Red (FTIR) Spectroscopy

The spectra of synthesized zinc oxide nanoparticle – capped catechin was analysed in the range of 400 to 4000 cm^{-1} using FTIR spectrophotometer (Shimadzu 2800). An FTIR measurement helps to identify the possible interactions between the zinc and bioactive molecules which may be responsible for the formation and stabilization (capping material) of zinc nanoparticles.

3.4.2.5. Surface charge of the synthesized zinc oxide nanoparticle-capped catechin using Zeta potential

Zeta potential analyzer is an analytical device for characterizing zeta potential as a property of interfacial layer in liquid particulates (dispersions, emulsions) and porous bodies. ISO Standard-13099, a multi-part document describes the main principles underlying functioning of such devices. Zeta potential is not a directly measurable parameter. It should be calculated from other experimentally measurable properties of the system using appropriate theory.

3.4.3. Determination of the antioxidant activity of synthesized zinc oxide nanoparticle-capped catechin

Antioxidant activity of the synthesized zinc oxide nanoparticle-capped catechin was determined by the modified method of Rajeshkumar *et al.* (2018) as given in the Appendix 26.

3.4.4 *In vitro n*europrotective activity of synthesized zinc oxide nanoparticle-capped catechin

3.4.4.1. Determination of the Entrapment efficiency and Loading capacity

The entrapment efficiency and loading capacity of synthesized zinc oxide nanoparticle- capped catechin were determined (Arasoglu *et al.,* 2017) with some modifications as shown in Appendix 29.

3.4.4.2. *In vitro* release study of synthesized zinc oxide nanoparticle-capped catechin

The *in vitro* release of catechin from the synthesized zinc oxide nanoparticle-capped catechin was conducted according to a previously published dissolution method (Derman and Akdeste 2015) as given in Appendix 30.

3.4.4.3. Qualitative evaluation of Acetylcholinestrase inhibition using thin layer chromatography

The AchE inhibition activity of the samples were detected by using a Thin Layer Chromatography autographic assay (Machado *et al.,* 2015) as shown in Appendix 31.

3.4.4.4. Assessment of Acetylcholinestrase Inhibition

The acetylcholinesterase inhibition activity of the synthesized zinc oxide nanoparticle-capped catechin was measured using the method described by Ingkaninan *et al.* (2003) as given in Appendix 28.

3.5. Phase V: Assessment *in vitro* neuroprotective activity of synthesized zinc oxide nanoparticle-capped catechin to neuro 2a cells

The *in vitro* antioxidant activity and assessment of neuroprotetive effects on neuro 2a cells for the green synthesized zinc oxide nanoparticle-capped catechin

3.5.1. Maintenance of cell lines

Neuro 2a (Neuroblastoma) cell lines were procured from the National Center for Cell Science Pune, India. The cells were grown in Minimal Essential Medium- Earle's Balanced Salt Solution (MEM-EBSS) media containing 10% Fetal bovine serum (FBS), 2 mM glutamine and 40 UmL-1 gentamicin at 37 ° C under 5% Carbon dioxide (CO_2). Synthesized zinc oxide nanoparticle-capped catechin and sterilised by incubation at 120˚C for 2 hours and suspended in culture medium. This stock solution was then serially diluted into doses ranging from 25 to 200 mg/ml. The samples were sonicated for a minimum of 30 minutes to produce a uniform suspension. Freshly sonicated synthesized zinc oxide nanoparticle-capped catechin suspensions were immediately applied to cells that had

attached for 24 hours in complete medium. The cells alone as the control group were not treated with synthesized zinc oxide nanoparticle-capped catechin.

3.5.2 Treatment Groups

The following treatment groups were set up for each parameter

1. Untreated negative control – cell line alone
2. Cells + commercial drug (rivatigmine)
3. Cells + synthesized zinc oxide nanoparticle-capped catechin

3.5.3. Parameters analyzed

3.5.3.1. Cell viability MTT (dimethyl thiazolyl-20,50-diphenyl-2-H-tetrazolium bromide) assay

The protocol for dose dependent inhibition of cell viability by MTT (dimethyl thiazolyl-20, 50-diphenyl-2-H-tetrazolium bromide) assay based on the presence of mitochondrial reductase enzyme in live cells, which converts the MTT into purple color formazan crystals, is presented in Appendix 32.

3.5.3.2. Assay of Lactate Dehydrogenase (LDH)

The activity of LDH in the medium was determined using a commercial LDH Kit according to the manufacturer's protocol. The optical absorption of the medium was then measured at 440 nm using UV-visible spectrophotometer as presented in Appendix 33.

3.5.3.3. Determination of Cell Morphology changes

The neuro 2a cells ($5x10^4$) were plated in a 6 well plate to study the effect of silver diamine fluoride on cell morphological changes. The cells were treated with and without Rivatigmine and synthesized zinc oxide nanoparticle-capped catechin (10-20 µg) for 24 hours. time point. After the treatment period, the cells were washed with PBS and observed under an Inverted phase contrast microscope (EVOS M7000)

3.5.3.4. Determination of intracellular reactive oxygen species (ROS) level

The intracellular ROS level in treated neuro 2a cells was analyzed by DCFDA (Dichlorodihydro Fluorescein Diacetate) staining. The detailed procedure is depicted in Appendix 34.

3.5.3.5. Determination of Superoxide Dismutase (SOD)

Superoxide Dismutase was measured with a SOD Assay kit (Sigma-Aldrich) according to the manufacturer's protocol manual given in Appendix 35.

3.5.3.6. Microplate assay for Acetylcholinestrase (AChE) activity

The acetylcholinesterase inhibition activity was measured using the method described by Marinelli *et al.,* 2017 (Appendix 36).

3.5.3.7. Cell cycle analyses by Flow cytometer

The cell distribution along the different phases of the cell cycle was examined in cells treated with10 µg/ml of Rivatigmine, synthesized zinc oxide nanoparticle-capped catechin and the control cells by evaluating the relative cellular DNA content with a flow cytometry technique BDFACS Canto^{TM}II (Becton and Dickinson Biosciences, Mountain View, CA, USA) and the data were analysed using BDFACS Canto clinical software. Histograms has been calculated by the percentage of occupancy of G0/G1, S and G2/M regions. The details of the procedure are explained by Valdiglesias *et al.,* (2011) in Appendix 37.

Based on the above experiments were carried out and the findings of the study discussed in the following chapter.