3.0 Materials and Methods

Previous sections detailed on the biopharmaceutical nature of *W. somnifera* and its metabolites with response to metal salts treatment. This section displays the materials and methods used to prove the biopharmaceutical activity of *W. somnifera* and its metal reduction ability.

3.1 Materials

3.1.1 Plant material

3.1.1a Media used

3.1.1b Initiation asnd maintenance of *in vitro* cultures

3.1.1c Field grown tissues of *W. somnifera*

3.1.2 Human cell cultures

3.1.3 Chemicals

3.2 Methods

3.2.1 Preparation of extract from plant parts

3.2.2 Preparation of standards

3.2.3 Preparation of metal salt solutions

3.2.4 Metals treatment on *in vitro* cultures

3.2.5 Synthesis of silver nanoparticles

3.2.6 Physical and Chemical characterization studies

3.2.6a UV visible spectrophotometry

3.2.6b Field emission scanning electron microscopy – Energy dispersive x-ray analysis

3.2.6c Transmission electron microscopy - Energy dispersive x-ray analysis

3.2.6d Chemical characterization by Fourier transform infrared spectroscopy

3.2.7 Biochemical and elemental profiling

3.2.7a HPTLC quantification of Withanolides

3.2.7b GC-MS analysis for metabolite profiling

3.2.7c Elemental analysis using ICP MS

3.2.8 Biopharmaceutical studies of *W. somnifera* extracts against *in vitro* Parkinson system

3.2.8a Cell viability assay

3.2.8b Analysis of ROS accumulation, Apoptosis and Mitochondrial membrane potential using Flow cytometry

3.2.8c Expression studies of mitochondrial gene targets in *in vitro* Parkinson’s system

3.2.8d Interaction studies of withanolides and rotenone against ETC complex I proteins

3.2.9 Statistical analysis

3.1 Materials

3.1.1a Media used

- Germination medium – Half strength Murashige Skoog (MS) basal media supplemented with 2% sucrose and 0.8% agar.
- Adventitious shoot induction medium – Full strength MS basal media supplemented with 3% sucrose and 0.8% agar.
- Adventitious shoot multiplication and maintenance medium – MS basal media supplemented with 1mg/L 6-benzylaminopurine (BAP), 3% sucrose and 0.8% agar
- Mass multiplication and treatment medium - MS basal media supplemented with 1mg/L BAP and 3% sucrose (without agar).
- The composition and preparation of the MS basal medium is given in **Appendix I**

3.1.1b Initiation and maintenance of *in vitro* cultures

The seeds of *W. somnifera* 'Jawahar 20' was obtained from Tamil Nadu Agricultural University (Coimbatore, India). The seeds were soaked in distilled water overnight and surface sterilized with sodium hypochlorite solution (2.3%) with Tween 20 (few drops) followed by sterilized double distilled water. The sterilized seeds were dried using filter paper and inoculated onto the germination medium. The cultures were maintained in darkness at 22±2ºC for germination. The germinated seedlings were allowed to grow under cool white fluorescent light at an intensity of 2000 lux and 16 hours photoperiod. After 15-20 days of germination, the seedlings were transferred to adventitious shoot induction medium. The healthy fresh shoots containing 2-3 nodes were excised from 30 days old cultures and sub cultured on to adventitious shoot multiplication and maintenance medium. Following that one-month-old shoots from adventitious shoot multiplication medium were transferred to mass multiplication and treatment medium for 15 days. Thus, the shoots were allowed to mature for 45 days in shoot multiplication medium prior to the treatment. The 45 days old shoots from mass multiplication and treatment medium were used for further studies.

3.1.1c Field grown tissues of *W. somnifera*

The dried shoots and roots from field grown plants of *W. somnifera* were obtained from Central Tobacco Research Institute (CTRI), Vedasandur, India.

3.1.2 Human cell cultures

The neuroblastoma SH-SY5Y cell lines were purchased from National Centre for Cell Science (NCCS), Pune, India and maintained in Gibco Dulbecco's Modified Eagle's Medium: Nutrient Mixture F12 (DMEM F12) media supplemented with 10% Fetal bovine serum (FBS) along with the 1% antibiotic-antimycotic solution and 1% L-Glutamine (200mM) in atmosphere of 5% CO_2, 18-20% O_2 at 37^0C maintained in the CO_2 incubator and sub cultured for every 2 days.

3.1.3 Chemicals

The nutrient salts used for plant tissue culture media preparation was purchased from HiMedia. For metal toxicity studies, the metal salts: silver nitrate and lead acetate purchased from Sigma Aldrich were used. The solvents used for the study were methanol (SRL), HPLC grade methanol (Rankem), toluene (Rankem), ethyl acetate (Rankem), formic acid (Qualigen) and silica gel plates 60 F^{254} for TLC (Merck). The standards withaferin A was purchased from Natural Remedies (Bangalore) and withanolide A and withanone from ChromaDex (USA). For animal cell cultures studies, DMEM F12 media (#AL189, Himedia), FBS (#RM10432, Himedia), 2',7'-dichlorofluorescein diacetate (H2DCFDA) (Life Technologies, Invitrogen), FITC Annexin V and Propidium Iodide (PI) (BD Biosciences) were used. Rotenone, a neurotoxic compound was purchased from TCI (Tokyo, Japan). For cell viability studies MTT (#4060, Himedia), DMSO (#PHR1309, Sigma) and PBS (#TL1006, Himedia) were used. For GCMS analysis, the derivatization chemicals: BSTFA (*N, O* bis(trimethylsilyl)trifluoroacetamide) and TMCS (trimethyl chlorosilane) (Sigma Aldrich) were used. The commercially available highest-grade chemicals and solvents were used for the present study.

3.2 Methods

3.2.1 Preparation of Extracts from plant parts

One gram of shoot powder was extracted using methanol following the protocol standardised by (Senthil et al. 2017). Briefly, the shoot powder was extracted four times with methanol at 1:50 sample to solvent ratio. The sample solvent mixture was sonicated for 20 mins and kept in shaker at room temperature for 2 hours and at 100 rpm. At the end of each extraction cycle, the extract was filtered using whatmann no.1 filter paper, pooled and concentrated using rotary vacuum evaporator at 100 rpm in a water bath at 45ºC. the resultant residue was dissolved in 10ml HPLC grade methanol and stored at -4ºC until further use.

3.2.2 Preparation of standards

The stock solutions of withaferin A, withanolide A and withanone was prepared using HPLC grade methanol in 1mg/ml concentration and stored at 4ºC. From the stock solutions, working solutions were prepared by diluting them to 0.5mg/ml final concentration.

3.2.3 Preparation of metal salt solutions

Silver nitrate stock solutions were prepared by dissolving $AgNO_3$ in sterilized double distilled water. For treatment, selected concentration of $AgNO_3$ for acute (1, 2, 4, 8 & 16mM) and chronic studies (0.25, 0.5, 1, 2, 4mM) was supplemented to the mass multiplication and treatment medium from stock solutions. Lead acetate stock solutions were prepared by dissolving the $PbAC_2$ in autoclaved double distilled water. For treatment, selected concentration of $PbAC_2$ for acute studies (0.3, 0.6, 1.2, 2.4 & 4.8Mm) and chronic studies (0.05, 0.1, 0.2, 0.4 & 0.8 mM) was added to mass multiplication and treatment medium from stock solutions (Table 3.1)

3.2.4 Metal treatment on *in vitro* culture

<u>Toxicity studies – acute & chronic</u>

The IVS cultures of *W. somnifera* was treated with two non-essential heavy metals namely silver nitrate and lead acetate for metal toxicity studies. The in vitro cultures were exposed to both metal salts at high and low concentrations for short and long duration of exposure to assess acute and

chronic toxicity to the metals (Table 3.1). A mature 45 days old IVS weighed approximately 5g from mass multiplication and treatment medium were subjected to metal toxicity studies.

Table 3.1. Concentration of metal salts and exposure conditions used on *in vitro* shoot cultures of *W. somnifera*

Experiment	Metal salt concentration (mM)		Duration of exposure to non-essential metal		
			T1 (12 hrs)	T2 (24 hrs)	T3 (48 hrs)
	Control (acute)	C (0)	CT1	CT2	CT3
Acute toxicity studies (H)	Silver nitrate (E1)	H1 (1)	E1H1T1	E1H1T2	E1H1T3
		H2 (2)	E1H2T1	E1H2T2	E1H2T3
		H3 (4)	E1H3T1	E1H3T2	E1H3T3
		H4 (8)	E1H4T1	E1H4T2	E1H4T3
		H5 (16))	E1H5T1	E1H5T2	E1H5T3
	Lead acetate (E2)	H1 (0.3)	E2H1T1	E2H1T2	E2H1T3
		H2 (0.6)	E2H2T1	E2H2T2	E2H2T3
		H3 (1.2)	E2H3T1	E2H3T2	E2H3T3
		H4 (2.4)	E2H4T1	E2H4T2	E2H4T3
		H5 (4.8)	E2H5T1	E2H5T2	E2H5T3
			T4 (4 days)	T5 (8 days)	T6 (12 days)
	Control (chronic)	C (0)	CT4	CT5	CT6
Chronic toxicity studies (D)	Silver nitrate (E1)	D1 (0.25)	E1D1T4	E1D1T5	E1D1T6
		D2 (0.5)	E1D2T4	E1D2T5	E1D2T6
		D3 (1)	E1D3T4	E1D3T5	E1D3T6
		D4 (2)	E1D4T4	E1D4T5	E1D4T6
		D5 (4)	E1D5T4	E1D5T5	E1D5T6
	Lead acetate (E2)	D1 0.05)	E2D1T4	E2D1T5	E2D1T6
		D2 (0.1)	E2D2T4	E2D2T5	E2D2T6
		D3 (0.2)	E2D3T4	E2D3T5	E2D3T6
		D4 (0.4)	E2D4T4	E2D4T5	E2D4T6
		D5 (0.8)	E2D5T4	E2D5T5	E2D5T6

Assessment of biomass accumulation

The metal salts treated IVS of *W. somnifera* were harvested and biomass was calculated after each treatment period.

3.2.5 Synthesis of silver nanoparticles

The shoot methanolic extract was prepared by the procedure given in section 3.2.1. For *ex vitro* synthesis of nanoparticles, field grown leaves of *W. somnifera* extract and silver nitrate solution was added in the ratio of 1:10. The reaction solution was kept in dark and cold (22±1ºC) condition for Ag NPs

synthesis. The reaction solution was kept in vortex shaker (SPINIX) for two to three times every day. The synthesis of Ag NPs was confirmed by the colour change of the reaction solution. Each experiment was repeated thrice.

UV visible spectrophotometry analysis was done for the reaction solution after 3 days (slight brown coloration occurred). The solutions were analyzed for its spectrum absorptions under ultra violet and visible spectrum regions, ranging from 200nm to 800nm. Once the stability of the synthesized nanoparticles was confirmed, the reaction solution was taken for further analysis. Following 10 days of experiment, reaction solution was vacuum dried by freeze dryer at -80˚C for 24 hours. 1 mL of sample was taken for freeze drying and the temperature of the vacuum column was maintained less than -80˚C throughout the process. The freeze-dried powdered sample from vacuum freeze dryer was used for physical characterization of nanoparticles.

3.2.6 Physical and Chemical Characterization studies

3.2.6a UV visible spectrophotometry

UV visible spectrophotometry analysis was done on experimental solutions containing silver nitrate and field shoot extracts of *W. somnifera* at different ratio and duration. The analysis was done at 200 to 1000nm range (UV-vis 1800 Shimadzu). HPLC methanol was used as a reference control.

3.2.6b Field emission electron microscopy & Energy dispersive x-ray analysis

Morphological study of *ex vitro* synthesized silver nanoparticles was done using Field emission scanning electron microscopy (FE SEM) analysis (MIRA 3 TESCAN with APEX EDAX). The synthesized silver nanoparticles in the powdered forms were gold sputter-coated to improve the conducting surface for nanoparticles. The surface morphology of synthesized Ag NPs and size and shape of the nanoparticles were analyzed using FESEM with EDX analysis.

3.2.6c Transmission electron microscopy & Energy dispersive x-ray analysis

For TEM analysis, the cryo- grounded cell lysate solution was prepared by standardized protocol Jain et al. (2014) with slight modifications. Briefly, 50mg of fresh young leaves taken from *in vitro* cultures of *W. somnifera* untreated, Ag and Pb treated shoots. The leaves were washed with HPLC grade water to remove any metal residues from the surface of the leaf. Then the leaves were placed in ice cold mortar and pestle with 2ml HPLC water for cryo-grounding. From the resultant shoot extract, 0.5ml of aliquot was taken for pulse-sonication on ice (95% amplitude for 10s followed by 15s rest with 5 pulses per cycle) with 3mm standard probe of the VCX500 ultrasonic processor with anti-noise cabinet (Sonics & Materials). The probe temperature was maintained at 20ºC. After sonication cycle, the leaf cell extract was centrifuged using table-top refrigerated centrifuge (Rota R-V/Fm, Plasto Crafts) at 4ºC for 30s at 10000 rpm. The sonication-centrifugation cycle was repeated three times to increase the leaf tissue and cell lysis. At the end of third cycle, the sonicated aliquot was pelleted by centrifugation and dissolved in 10µl of HPLC water and stored at -4ºC.

Optimum culture conditions treated IVS from metal toxicity analyses were selected based on the higher threshold concentration for a specific heavy metal (Ag/Pb) under aseptic condition. These shoots had the higher threshold for Ag or Pb and highest bioaccumulated Ag and Pb in their system. Thus, to analyse the nature of the bioaccumulated non-essential metal under chronic condition in the shoot cultures was done using TEM analysis. The 10µl of cell-lysed solution from sonication-centrifugation cycle was transferred onto the 400-mesh copper coated grids for ultra-structural analysis. Point and region analysis was performed at 120kV. EDAX spectrum was recorded for leaf metal composition and micrographs were captured at 40keV for 30-40s.

3.2.6d Chemical characterization by Fourier transform infrared spectroscopy

The chemical characterization of Ag NPs synthesized using field shoot extracts of *W. somnifera* done by FTIR analysis. 1µg of powdered

nanoparticles (sample) were dissolved in 10 µL of deionized water. Single drop of the sample was taken for FTIR analysis.

3.2.7 Biochemical and elemental profiling

3.2.7a HPTLC quantification of Withanolides

HPTLC profiling of withanolides was followed by the standardized protocol (Vinod et al. 2022). Briefly, for stationary phase, silica gel aluminium TLC plate and for mobile phase, the solvent system containing toluene, ethyl acetate and formic acid in 5:5:1 ratio respectively. The withanolides were applied as a standard in the concentration ranged from 0.5-2.0µg per band for quantification. The concentrated *W. somnifera* tissue samples were spotted along with standards as 6mm bands marked 8mm from the bottom. The samples were applied using CAMAG Linomat V sample applicator employed with a 100µl Hamilton syringe working under a stream of nitrogen (spraying rate: 150nLs-1). Followed by, the plate was kept in pre-saturated twin trough chamber for the development up to 80mm and visualized at 254 and 366nm. Densitometric scanning (TLC scanner III: CAMAG CATS 4) was carried out with Savitsky-Golay7 filter in the reflectance–absorbance mode at 223nm for withaferin A, 234nm for withanolide A and 231nm for withanone respectively at a scanning rate of 20mm s^{-1} and was analysed using visionCATS software. The Rf values and peak areas of the standards were compared to the resolved spots in the samples. The concentration of different withanolides in the metal treated samples was quantified using the linear regression curve obtained from the varying concentration of respective withanolides (standards: 0.5-2.0µg) which resolved at 0.54 (withaferin A), 0.72 (withanolide A) and 0.64 (withanone). Further, derivatization of the plates was done using the derivatization reagents namely 100% methanol (85ml), 100% glacial acetic acid (10ml), 97% sulphuric acid (5ml), and 98% Anisaldehyde (0.5ml) and kept in hot air oven at 110°C for about 10min for detection of spots.

3.2.7b GC-MS analysis of metabolite profiling

Briefly, plant extracts as described in section 3.2.1 were freeze-dried. For the derivatization of the dried plant extracts, 30 µl of methoxylamine hydrochloride in the concentration of 200µg/ml pyridine, 50 µL of (N, O-

bis(trimethylsilyl)trifluoroacetamide containing 1% trimethyl chlorosilane and 10µl of 2-chloronaphthalene in the concentration of 200µg/ml pyridine were added. Following that, the samples were kept in the incubator at 60ºC for 60 minutes and taken for GC-MS analysis (Shimadzu 2010 plus).

The sample injection was carried out with an ion source with an ionization energy of 70eV at 230ºC and helium used as the carrier gas with flow rate of 1ml/min was used for GC-MS analysis. The metabolites were separated using silica capillary column with dimensions of 30 m × 0.25 mm i.d. × 0.25 µm film thickness. Column flow was maintained as 1ml/min, column pressure as 61.3 kPa. The oven temperature was initially set as 70ºC with 1 min hold time and increased to 225ºC with 3min hold and finally 280ºC with 2 min hold. The mass spectra were observed between 35m/z and 650 m/z with scanning speed of 2500 scan/second. GC mass spectrum data interpretation was done using AMDIS (Automated Mass Spectral Deconvolution and Identification System) (ver. 2.71) and compound identification was done by NIST (the National Institute of Standards and Technology) mass spectral database. The relative amount of the metabolites in the plant extract were expressed as percentage (area) and then the datasets were subjected to principal component analysis (PCA) using XLSTAT.

3.2.7c Elemental analysis using ICPMS

Elemental analysis by ICPMS (Thermo fisher iCAP RQ ICPMS) done using the dried *W. somnifera* tissue powder. The essential metal, essential heavy metal and non-essential heavy metals for plants were quantified by the standard protocol given by the manufacturer (Thermo fished user guide). Briefly, 0.2g of powdered plant samples were digested using 6.0 ml of HNO_3 (67-70% concentration) in Anton Paar Multiwave GO microwave digestor for 30 minutes at 453K and made up to 50 ml in a volumetric flask. The solution without plant sample was used as blank.

3.2.8 Biopharmaceutical studies of *W. somnifera* extracts against Parkinson disease induced cells

3.2.8a Cell viability analysis

200µl of viable cell suspension at a density of 20000 cell per well were plated into 96-well plates and incubated for 24 hrs for cell confluence. After 70-80% confluence, the cells were pre-incubated with different concentration of standard drug (withaferin A) and plant extracts were added and incubated for 2 and 4 hrs at 37ºC in a 5% CO_2 incubator. After the incubation period, rotenone was added to the wells at the final concentration of 1µM and incubate for additional 24 hrs at 37ºC. The cells incubated with DMSO alone without rotenone is represented as negative control group. The cells incubated with rotenone without samples (standard drug and test samples) represented as positive control.

After 24hrs of rotenone incubation, the medium was removed and MTT was added to the wells at the concentration of 0.5mg/ml and incubated for 3 hrs under dark environment. After 3 hrs, the supernatant was removed from the wells and 100µl of DMSO was added for solubilizing the formazon crystals with stirring for 15 min on a microtiter plate gyratory shaker and the absorbance was read at 570 nm using an ELISA reader. The cell viability was then calculated using the formula:

$$\%\ viability = \frac{Absorbtion\ of\ treated\ cells}{absorption\ of\ untreated\ cells}\ X\ 100$$

The IC_{50} valuess were determined by linear regression equation: $Y = Mx + C$

Where, Y=50; M and C were derived from the graph.

3.2.8b Analysis of ROS accumulation, Apoptosis and Mitochondrial membrane potential using FACS study

The SH-SY5Y cells were cultures overnight in 96 well plates. The cells were pre-treated with plant extracts and standard drug then neurotoxicity was induced by exposing to 1µM rotenone for 24 hours. At the end of incubation period, the treated, control and vehicle control cells were subjected to quantification of ROS accumulation, apoptosis and mitochondrial membrane potential. The ROS scavenging activity of the *W. somnifera* shoot and root extracts towards rotenone-induced neuroblastoma cells were studied by

H2DCFDA (a fluorescent dye). For ROS accumulation study, the samples and rotenone treated cells after incubation period, were suspended in H2DCFDA solution and incubated for 30 min at 37ºC under dark condition. Followed by the cells were washed with PBS buffer and the emittance of fluorescence from the cells were measured at 488nm for excitation and 535nm for emission using flow cytometry (FACS) (Sekhar et al. 2015).

The apoptotic activity of *W. somnifera* extracts towards SH-SY5Y cells were determined by Annexin V FITC/Propidium iodide binding assay (Badmus et al., 2015). Briefly, the treated, control and vehicle control cells were washed with PBS and harvested by trypsin. Then the cells were added with 200µl of trypsin-EDTA solution for 3-4 mins at 37ºC. Subsequently, 5µl of FITC, 5µl of PI and 400µl of 1X binding buffer was added to the cells and analysed using FACS BD cell quest Pro version 6.0. The cells that were positive for Annexin V and negative for PI were identified as early apoptotic cells and positive for PI and negative for Annexin V were identified as late apoptotic cells (Fan et al. 2019).

For mitochondrial membrane potential measurement JC-1 (5,5,6,6-tetrachloro-1,1,3,3- tetraethylbenzimidazolylcarbocyanine iodide), a fluorescence probe was used and analysed by flow cytometer. Rotenone treated and W. somnifera tissue extracts pre-treated SH-SY5Y cells were incubated with JC-1 dye for 30 minutes and washed twice in PBS. MMP was measured by quantifying the relative fluorescence intensity by flow cytometer (BD FACSVerse) with excitation at 530nm and emissions at 585nm (Alpert et al. 2020).

3.2.8c Expression studies of mitochondrial gene targets in *in vitro* Parkinson's system

Isolation and quantification of total mRNA from SH-SY5Y cells

Total RNA was isolated from test samples treated, standard control treated, rotenone treated (positive control), untreated (negative control) SH-SY5Y cells. At the end of each treatment, the medium was removed from the wells into polystyrene tubes (12 x 75 mm). 500µl of PBS was used for washing the wells. Then the PBS was removed and the cells were incubated with 250µl

of trypsin-EDTA solution for 3-4 min at 37°C. After incubation period pour the removed medium back in to their respective wells and harvest the cells directly into polystyrene tubes (12 x 75 mm). Tubes were centrifuged for five minutes at 300 x g at 25°C and supernatant was removed. The cells were further washed with PBS two times and PBS was removed completely. Total RNA was isolated from all the treated samples by Qiagen RNeasy kit. The resulting RNA was treated with DNAse to degrade the genomic DNA contamination for RNA purification. The isolated RNA was quantified using UV-vis spectrophotometer using Qiaexpert. The quality of the RNA checked by determining the absorbance at 260 and 280 nm. The absorbance ratio at 260/280nm is 1.8-2 considered as good quality RNA. The isolated RNA was stored at -80ºC until further use.

Primer designing and validation

The primers for PINK1, DJ1 and GAPDH were designed using the software primer 3 and available reported literature (ref). GAPDH primer was constructed to use as a housekeeping gene to determine the homogenous gene expression, and quantity of cDNA in the neuroblastoma cells that used in the gene expression studies **(Table 3.2)**.

Table 3.2 Primer sequence for Real time quantitative PCR analysis

Primer name	Primer Sequence	Amplicon size
DJ-1 or PARK7	FWD: GACGGTCATCCCTGTAGATG (5’->3’)	174bp
	REV: GTAGAACCACCACATCATATGG (5’->3’)	
PINK1	FWD: GTACCAGTGCACCAGGAGAA (5’->3’)	210bp
	REV: GCTTGGGACCTCTCTTGGAT (5’->3’)	
GAPDH	FWD: GACAACAGCCTCAAGATCATC (5’->3’)	111bp
	REV: TGTGGTCATGAGTCCTTCCA (5’->3’)	

RT-qPCR conditions for expression profiling of mitochondrial genes (PINK1, DJ-1 & GAPDH)

The cDNA of the mitochondrial and a housekeeping gene namely PINK1, DJ-1 and GAPDH were amplified from isolated and purified RNA by reverse transcription (RT) process (5X IScript^{TM}, cDNA synthesis kit, Bio-Rad,

CA, USA). For cDNA synthesis, IScript cDNA synthesis kit, random hexamer and oligo dT primers were added in the concentration 5µl of reaction mix, 3µl of nuclease free water, 2 µg of purified RNA, and 2µl reverse transcriptase enzyme. The reaction mixer was incubated in PCR cycler at 25ºC for 5 min, 46ºC for 20 min and 95ºC for 1 min for priming, RT and RT inactivation respectively. The synthesized cDNA (first strand) was kept in -20°C until further use.

The relative quantification of the expression of mitochondrial genes was done in Qiagen Rotor Gene Q 5plex HRM using the SYBR Green Chemistry (Sensifast SYBR HiRoxkit, Bioline, USA). The RT qPCR reaction total volume was 25µl containing 1µl first strand cDNA, 12.5µl 2X SYBR Green Master Mix, 1µl forward primer in the concentration of 10µM, 1µl reverse primer in the concentration of 10µM, and 9.5µl of nuclease free water. Further, 40 cycles of RT qPCR were done in the optimized conditions like initial denaturation done at 95ºC for 5 min, denaturation at 95ºC for 10s, annealing at 60ºC for 20s and extension was done at 72ºC for 20s. The gene expression fold change was calculated using ΔΔCt method (ref) with housekeeping gene (GAPDH) as an internal control. The threshold cycle (Ct) value during the PCR reaction was calculated based upon the fluorescence data collection with 40 PCR cycles. For normalization of the Ct values, positive control (SH-SY5Y-untreated) and negative control (SH-SY5Y-Rotenone treated) were used. The RT qPCR reaction was done in triplicates.

3.2.8d Interaction studies on withanolides and rotenone against ETC complex I subunits

Rotenone, withaferin A, withanolide A and withanone interaction with selected subunits of electron transport chain complex I protein is reported in this study. The 3D structure of complex I protein (PDB ID: 5XTD) was retrieved from protein data bank (PDB) (https://www.rcsb.org/).The proteins were prepared by removing the water molecules and ligands associated with it using Pymol 2. The prepared proteins were subjected to rigid docking analysis individually against three withanolides and rotenone using Discovery Studio Visualizer 2022. The docking pose with the least binding energy among the

obtained ten binding poses was considered as the best docking confirmation. The binding pockets and the interacting residues were visualized using Discovery studio Biovia 2020. For multiple ligand simultaneous docking (MLSD), both withaferin A and rotenone dock parameter file (.dpf) was merged into one single file. For MLSD simulation studies, program loads both ligands in. PDBQT files. Following that, MLSD was carried using withaferin A and rotenone as ligands and 5XTD as a protein molecule.

3.2.9 Statistical analysis

All the experiments were done in triplicates (n=3) and the values were represented as Mean ± SE (standard error). Multivariate test with Duncan's multiples range test (DMRT) was done to analyse the statistical significance between the tested samples using SPSS (ver.16).