SPECIMEN FORMAT FOR THESES OF MONTH

Faculty	:	Biosciences
Department	:	Biochemistry, Biotechnology and Bioinformatics
Branch/ Area:	:	Plant Biotechnology and Neuroscience
Sub Subject Heading:	:	-
Candidate's Name	:	Kanimozhi Natarajan
Candidate's Address with email	:	11/25, K.T. Palayam (po), Oddanchatram (tk), Dindigul 624616
Title of the thesis	:	*In planta* Assimilation and Characterization of Metal Nanoparticles in *in vitro* shoots of *Withania somnifera* and its Therapeutic Evaluation using Rotenone-induced SH-SY5Y cells
(i) In Roman Script		-
(ii) In roman Script		-
Nomenclature of Degree:	:	Doctor of Philosophy
Month & Year of Enrolment:	:	July 2017
Month & Year of Registration:	:	July 2017
Month &Year of Submission:	:	August 2024
Month &Year of Award	:	January 2025
Name of Supervisor	:	Dr. Kalaiselvi Senthil
Designation of Supervisor	:	Professor
Centre/department/school in which research was conducted	:	Tissue culture laboratories, Department of Biochemistry, Biotechnology and Bioinformatics
University's Name & Address	:	Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore 641043

Abstract :

Withania somnifera is a predominant medicinal herb having economically valuable secondary metabolites and high reduction potential. Apart from being medicinal, *W. somnifera* also has hyper accumulation capability especially with heavy metals. Initially, metal bioaccumulation and metal reduction capability of field grown shoot tissues of *W. somnifera* was analysed using elemental analysis and extract based green synthesis of silver nanoparticles. The capability of *W. somnifera* to grow under the influence of heavy metal stress and accumulation of secondary metabolites is studied using *in vitro* shoot cultures. On confirmation of metal reduction capability, 45-days old *in vitro* shoot cultures were treated with different concentrations of silver nitrate and lead acetate salts at acute and chronic conditions. An Increase in biomass, primary and secondary metabolites (withanolides) was found to be accumulated in considerable amounts in metal salts treated *in vitro* shoots compared to *in vitro* control. Among the metal treated shoots, 1mM $AgNO_3$ treatment for 12 days period and 0.8mM PbAc treatment for 12 days period was selected as the optimum treatment conditions. The concentration of Ag in optimum AgNO3 treated shoot is 50.8ppm and Pb in optimum PbAc treated shoot is 405ppm. The presence of Ag and Pb nanoparticles in spherical and rod shape was confirmed using TEM and EDX analysis. In addition, the neuroprotective activity of metal treated IVS along with field grown tissues of *W. somnifera* was studied using Parkinson's disease cell model (SH-SY5Y cells). Compared to field grown tissues, $AgNO_3$ treated IVS exhibited increased neuroprotective activity against rotenone toxicity. Multiple ligand simultaneous docking revealed a binding of rotenone with withaferin A leaves complex I protein uninhibited. Thus, from the current study, we conclude that AgNO3 treated IVS along with increased withaferin A content has higher neuroprotective activity which may be used as a potential drug for toxins induced PD.

i) Major objectives:

1. To confirm the metal accumulation and reduction potential of field tissues of *W. somnifera*
2. To study the effect of heavy metal salts on *W. somnifera in vitro* shoot growth and withanolides production.
3. To quantify and analyse the nature of bioaccumulated metal ions in *in vitro* grown shoots.

4. To evaluate the neuroprotective potential of *W. somnifera* tissues in Parkinson's disease cell model.

ii) Hypothesis:

The *in vitro* shoots of *W. somnifera* exhibits tolerance to heavy metal stress and in planta formation of metal nanoparticles is possible which further increases its therapeutics.

iii) Methodology:

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 45^0 C. the resultant residue was dissolved in 10ml HPLC grade methanol and stored at -4^0 C until further use.

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 4^0 C. From the stock solutions, working solutions were prepared by diluting them to 0.5mg/ml final concentration.

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

Metal treatment on *in vitro* culture

Toxicity studies – acute & chronic

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.

Assessment of biomass accumulation

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

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 ± 1^{0} C) 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.

Physical and Chemical Characterization studies

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.

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.

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 20^0 C. After sonication cycle, the leaf cell extract was centrifuged using table-top refrigerated centrifuge (Rota R-V/Fm, Plasto Crafts) at 4^0 C 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 -4^0 C.

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.

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.

Biochemical and elemental profiling

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 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.

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 230^{0} C 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.

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.

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

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 37^0 C 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 37^0 C. 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.

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 37^0 C 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 37^0 C. 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).

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 -80^{0} C 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

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 25^{0} C for 5 min, 46^{0} C for 20 min and 95^{0} C 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 95^0 C for 5 min, denaturation at 95^0 C for 10s, annealing at 60^0 C for 20s and extension was done at 72^0 C 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.

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.

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).

iv) Findings:

The present study on "*In planta* assimilation and characterization of metal nanoparticles in *in vitro* shoots of *Withania somnifera* and its therapeutic evaluation using Rotenone induced SH-SY5Y cells" is formulated with the objective to study the heavy metal accumulation, reduction activity of *in vitro* shoot cultures of *W. somnifera* and evaluating the metal treated shoots for their therapeutic potential using *in vitro* cell line studies.

The study was carried out in three phases. In the first phase, the metal reduction capability of field grown shoots of *W. somnifera* was analysed using two metal salts: Silver nitrate ($AgNO_3$) and lead acetate (PbAc) solution. The green synthesis conditions were optimized and physical and chemical characterization was done to confirm the green synthesis of Ag NPs, and Pb NPs. In addition, metal bioaccumulation capability of field grown shoot and root of *W. somnifera* grown in non-contaminated soil was also studied. Once the reduction potential and bioaccumulation capability of field grown shoots of *W. somnifera* was determined, further studies were carried out using *in vitro* shoot. In the second phase, standardization of metal treatment using two abiotic stressors: AgNO3 and PbAc were studied for improved growth index and its influence on withanolides accumulation. The metal salts were treated under two different treatment conditions such as acute toxicity studied and chronic toxicity studies. Acute toxicity studies: high concentrations of metal salts were treated for shorter period which determines the threshold of *in vitro* shoot system for a specific metal treatment. Chronic toxicity studies: low concentrations of metal salts were treated for longer period which is to understand the shoots' ability to withstand metal treatment over a long duration. Chronic toxicity studies were usually conducted for field experiments because long duration and low concentration facilitates the plant to adapt to the stressors which may result in a different observation to that of acute studies. Acute and chronic metal toxicity studies on *in vitro* shoot resulted in increased growth index and Withanolide production. Healthy shoot cultures with increased GI and withanolides production was selected as the optimum culture conditions for each metal. This study also confers Ag and Pb bioaccumulation at optimum exposure condition. The ultra-structural studies were done to identify the nature of bioaccumulated metal in metal treated *in vitro* shoots of *W. somnifera*. In the third phase the therapeutic efficiency of AgNO3 treated *in vitro* shoots along with field tissue and WFA were studied using rotenone induced Parkinson's disease model in SH-SY5Y cell lines.

The metal bioaccumulation and reduction capability of field grown shoots (FGS) of *W. somnifera* is found to be increased compared to FGR. Initially, to identify the metal bioaccumulation capability of field grown tissues of *W. somnifera*, the field grown plants from non-contaminated soils were harvested, dried and acid digested tissues were taken for elemental analysis. The elemental analysis includes essential trace metals and essential heavy metals for plant growth and non-essential heavy metals for plant growth with totaling of 22 elements. Compared to FGR, FGS accumulated increased amount of both trace and heavy metals for plant growth which is normal considering that shoot is the major site for photosynthesis, metabolites production and accumulation. In addition, all trace and heavy metals from FGS and FGR found within permissible set for medicinal plant and edible plant. The presence of non-essential heavy metals is found to be increased in FGR for Cr, Co, As, and Hg content compared to FGS. On the other hand, increased amount of Cd, Ag, Pb, V and Li was found in FGS compared to FGR. Further, the selected non-essential heavy metals for plant growth Ag and Pb, both were found higher in FGS compared to FGR. Therefore, due to the increased metal bioaccumulation capability of FGS, metal reduction studies were conducted using FGS alone.

Green synthesis of metal nanoparticles was conducted using $AgNO_3$ & PbAc, two experimental conditions (Room temperature and 60ºC (Hot plant)) and FGS extracts. The green synthesis of Ag NPs was found to be positive for both experimental conditions. However, Ag NPs synthesized at 60ºC was unstable after 2 days whereas Ag NPs synthesized at room temperature was stable for more than 30 days which was confirmed using UV-vis spectrometry and taken for other physical and chemical characterization studies. Green synthesis of Pb NPs using both experimental conditions was failed. FESEM analysis on Ag NPs revelaed the cubic shape with size ranging from 20-80 nm. EDX analysis confirms that 67% percentage of Ag was present in the analysed samples. From FTIR spectrum the presence of phenols, terpenes and proteins as the surface molecules of Ag NPs which proves that plant phytoconstituents were responsible for the reduction and stabilization of Ag NPs.

In the second phase chronic metal toxicity study revealed that both metals did not exhibit any toxicity symptoms on IVS cultures. On the other hand, high concentration of metal treatment to shorter period exhibited toxicity symptoms in IVS. Among acute toxicity studies, E1H3T3 produced 1.8-fold increase in WFA content (1.097±0.01e mg/g DW) compared to CT3 (control shoots) (0.599±0.02a) but high concentration of silver treatment negatively correlated with GI at all concentration and durations. Further, E2H3T3 produced 1.6- and 1.97-fold increase in GI

(0.1772±0.01c) and WFA content (1.184±0.01c mg/g DW) respectively compared to CT3 (0.1196±0.02e; 0.599±0.02a mg/g DW) (control shoots). Whereas on chronic toxicity studies, E1D3T6 showed maximum GI (1.425±0.05c) and WFA content (2.568±0.08e mg/g DW) which was 4.5 and 3.4 fold greater than the CT6 (0.3143±0.002a; 0.764±0.02a mg/g DW) (control shoots). On the other hand, on Pb treatment, E2D4T6 showed maximum GI (2.211±0.04d) which was 7 fold greater than CT6 (0.3143±0.002a) whereas E2D5T6 showed maximum WFA (3.137±0.01f) which was 4.1 fold greater than CT6 (0.764±0.02a mg/g DW). Thus, among all acute and chronic metal toxicity studies, E1D3T6 and E2D5T6 which showed best elicitation activity was taken as optimum culture conditions for Silver and Lead, respectively.

Following that the HPTLC profiling of withanolides in E1D3T6, E2D5T6, control (CT6) shoots was performed in comparison to FGS and FGR against standard WFA, WTA and WN. Compared to control shoots (0.783 mg/g DW), all the other tested samples recoreded increased amount of WFA except FGR (0.608 mg/g DW). Maximum WFA content was observed in FGS (6.607 mg/g DW) following E2D5T6 (3.120 mg/g DW) and E1D3T6 (2.544 mg/g DW). There was no significant difference was observed in WTA content in all the analysed samples except that FGS (0.142 mg/g DW) had minor quantity of WTA compared to other tested samples. Compared to control (0.805 mg/g DW), WTA in E1D3T6 (0.600 mg/g DW) was decreased but in E2D5T6 (0.960 mg/g DW) increased. A maximum WN content was found in FGS (2.285 mg/g DW) following FGR (1.707 mg/g DW) and control (1.385 mg/g DW). Compared to control, WN content in both E1D3T6 (0.709 mg/g DW) and E2D5T6 (1.027 mg/g DW) was decreased significantly. Therefore, withanolides quantification on metal treated and control samples revealed that metal treatment positively correlated with WFA and WTA content and negatively with WN content compared to control, except for E1D3T6 where WTA level was decreased.

In addition, metabolic profiling of E1D3T6, E2D5T6 and CT6 was carried out in comparison to FGR and FGS using GC-MS. A total of 32 metabolites including 13 organic acids, 5 amino acids, 3 alcohols, 4 sugars and 7 other compounds was detected from GC-MS chromatogram. A significant difference in the levels of metabolites was found between E1D3T6, E2D5T6 and CT6 as well as FGS and FGR. Especially, isovanillic acid was present only in E1D3T6, E2D5T6 was not detected in FGS, FGR and CT6. In addition, aziridine and eicosane was found in all the tested samples except CT6. An increase in squalene content was observed in E1D3T6 and E2D5T6. The complexicity of this GC-MS data was further simplified with principal component analysis (PCA) in

which 32 metabolites detected were plotted against two factors F1 and F2 which contributed to 81.50% of the variation in the GC MS dataset. The biplot obtained showing the FGS with high metabolites to be positive for F2 and both E1D3T6 and E2D5T6 shoots with high metabolite content to be positive for both F1 and F2. The F1 accounting for 61.50% variation shows distinction between field samples (FGS & FGR) and metal treated samples (E1D3T6 & E2D5T6). The *in vitro* control shoots (IVS) and FGR accounted for low metabolite content with low F1 & F2 values.

The concentration of bioaccumulated metal in E1D3T6 and E2D5T6 was analysed using ICPMS. In elemental analysis, eight essential trace metals, four essential heavy metals and ten non-essential heavy metals were quantified in E1D3T6, E2D5T6 and control shoots. The Ag bioaccumulation in E1D3T6 was quantified as 50.8 ppm and Pb bioaccumulation in E2D5T6 was quantified as 405 ppm. Despite higher Pb concentration in their tissues, the shoots were in good shape and healthy. As expected, control was void of both Ag and Pb. Moreover, the percentage of Ag in E1D3T6 shoots was quantified as 2.54% and Pb in E2D5T6 was quantified as 15.14%.

The presence of increased heavy metal tolerance in *in vitro* shoot cultures of *W. somnifera* is observed. Resistance to the toxicity of heavy metal accumulation is observed with increase in macronutrients such as K, Ca and Fe in E1D3T6 and E2D5T6 compared to control. These increased macronutrients are reported to protect plants from biotic and abiotic stress. The concentration of K is increased to 26077.9 (E1D3T6) and 24779.14 mg kg-1 (E2D5T6) from 21109.36 mg kg-1 (control), Ca level is increased to 1833.64 (E1D3T6) and 1786.93 mg kg-1 (E2D5T6) from 1411.68 mg kg-1 (control), similarly, Fe level is increased to 85.101 (E1D3T6) and 82.1 mg kg-1 (E2D5T6) from 11.2 mg kg-1 (control) during heavy metals stress.

E2D5T6 shoot was analysed for the presence of possible nanostructures using TEM analysis. It was found that bioaccumulated Pb ions (405 mg kg-1) was in fact in the form of nanostructures in the shape of spherical and rod and the size was ranging from 25-40nm. Similar result was observed in E1D3T6 where synthesis and accumulation of Ag NPs was confirmed using electron microscopy and EDX analysis. In E1D3T6, Ag NPs were found in spherical and rod shaped and the size ranging from 20-90nm. Ag absorption, translocation and accumulation is much similar to Pb ion due to their similar ionic characteristics. The presence of increased secondary and primary metabolite content also increases the reduction and stabilization of Ag NPs at in planta level.

The reduction potential of *in vitro* shoots of *W. somnifera* towards Ag and Pb was confirmed. However, for further studies, E1D3T6 shoots i.e., 1mM AgNO3 shoots for 12 days period,

containing Ag NPs was taken to analyse its neuroprotective activity in rotenone induced SH-SY5Y cell line. E2D5T6 shoots was not used for therapeutic studies due to their heavy metal nature.

Rotenone is a pesticide/ isoflavone which capable of inhibiting mitochondrial electron transport chain complex I protein. Inhibition of complex I resulted in PD symptoms like decreased mitochondrial dysfunction, energy metabolism and increased dopaminergic neuronal loss in the substantia nigra. For neuroprotective studies, E1D3T6, control shoots, FGS, FGR and WFA was selected. Initially, MTT assay was done to analyse the cell viability of selected samples against rotenone induced SH-SY5Y cells. The result shows that FGS failed to revive the SH-SY5Y cells from rotenone induced toxicity. Further, E1D3T6, IVS, FGR and WFA with a IC50 value of 48.28 μg/ml, 43.64μg/ml and 49.57 μg/ml and 50.05 μg/ml exhibited increased cell reviving ability against rotenone induced toxicity. From these results, we concluded that FGS is highly toxic and CT6 which served as control for IVS cultures exhibited very low cell reviving capability. Hence, FGS along with CT6 were removed in further studies.

ROS generation, mitochondrial membrane potential and apoptosis in rotenone treated SH-SY5Y cells was analysed using flow cytometry assay. Increased ROS accumulation results in the damaged biomolecules which in turn acts as potential contributors to a range of diseases including neurodegenerative diseases. In control cells on treatment of rotenone, 55% ROS accumulation and aggregation was observed. However, pre-treatment with E1D3T6, FGR and WFA significantly reduced the ROS generation in SH-SY5Y cells after rotenone treatment to 16%, 36% and 36%, respectively.

Depolarization and hyperpolarization of mitochondrial membrane led to reduction in transmembrane potential which resulted in the activation of mitophagy and autophagy. In rotenone treated control SH-SY5Y cells, percentage of monomer level was increased to 69.74% due to mitochondria depolarization. On the other hand, during E1D3T6, FGR and WFA pre-treatment, the percentage of aggregates increased from 29.90% (control cells) to 86.81% (E1D3T6), 78.78% (FGR) and 71.11% (WFA) which suggests that mitochondrial transmembrane potential was reversed back to normal on treatment.

Apoptosis is a programmed cell death responsible for DA neuronal death in pathophysiology of Parkinson disease. Rotenone treatment causes mitochondrial damage which led to DA neuronal loss (apoptosis). Pre-treatment of E1D3T6, FGR and WFA showed higher percentage of

live cells 86.61, 83.17 and 84.89 respectively compared to 13.27% in rotenone treated control SH-SY5Y cells. In addition, Rotenone treated SH-SY5Y cells showed 49.26% early apoptotic cells (EAC) and 37.22% late apoptotic cells (LAC) which was reversed to 0.56, 4.09, 3.72% of LAC and 0.05, 11.37, 11.34% of EAC for E1D3T6, FGR and WFA pretreatment respectively.

For gene expression studies, two important mitochondrial genes such as PINK1 and DJ-1 were selected along with GAPDH as a housekeeping gene. PINK1 (PTEN-induced kinase 1), acts together with Parkin in the maintenance of mitochondrial quality control. Any disturbances reflect in increase of PINK1 gene expression as observed in rotenone treated SH-SY5Y cells. On pretreatment of E1D3T6, FR and WFA, the over expression of PINK1 gene was reverted to 5.7-, 3.5-, and 10.2-fold respectively from 18.5-fold in rotenone treated SH-SY5Y cells, restoring the normal function of neuronal cells.

Loss-of-function DJ-1 mutations are related to autosomal recessive early-onset Parkinsonism. Oxidative stress levels in mitochondria of SH-SY5Y cells upregulates DJ-1 expression. Compared to untreated cells, DJ-1 expression in rotenone alone treated cells is 7.5-fold up regulated. This effect was reversed when the SH-SY5Y cells were pretreated with E1D3T6, FR and WFA following rotenone treatment to 2.02, 5.58 and 3.7-fold respectively.

The mechanism of rotenone inhibiting ETC complex I chain is reported by many researchers. ETC complex I proteins has 44 protein subunits and the binding site for rotenone in ETC complex I proteins are NDUFS2, NDUFS7, ND1 and ND4. However, the mechanism of WFA and other important withanolides such as withanolide A and withanone with ETC complex I chain protein subunits is unexplored. Therefore, the present study was extended to analyse the interaction of three withanolides with selected complex I protein subunits using molecular docking analysis. We have selected important complex I protein subunits having rotenone binding site and NADH dehydrogenase enzyme active site. Total seven protein subunits were selected based on the literature survey such as NDUFV1, NDUFS1, NDUFV2, NDUFS2, NDUFS7, ND1 and ND4. Among the selected subunits, all three withanolides and rotenone was found to be bind with same active site having overlapping binding pocket amino acids. Among WFA and rotenone, overlapping binding pocket aminoacids that were found is Arg:111, Ser:205, Glu:204 and Met:197.

Multiple ligand simultaneous docking (MLSD) was performed for WFA and rotenone with selected protein subunits of complex I protein. The docking results shows that the binding occurred

between WFA and rotenone which inhibits the rotenone from binding with complex I protein. The two-ligands binding with each other instead of binding with receptor molecules indicates WFA forms a complex with rotenone preventing it from binding to receptor. Thus, leaving the complex I protein uninhibited. The absence of rotenone binding with the complex I protein results in the normal function of mitochondrial electron transport chain, normal ATP production and inhibition of DA neuronal loss. Therefore, WFA and WFA containing plant extracts might be used for the alleviating mitochondrial toxins induced parkinsonism in human.

Thus, the present study depicts the heavy metal bioaccumulation and reduction capability of *in vitro* shoots of *W. somnifera* resulting *in planta* synthesis and accumulation of Ag and Pb NPs. Further, silver treated *in vitro* shoots (E1D3T6) were found to be potent in reversing PD pathological symptoms compared to other test samples. Thus, it can be used for therapeutic purposes.

Examiners

Internal Examiner:

Dr. Dhiraj Bhatia,
Department of Biological science and Engineering,
Indian Institute of Technology,
Ghandhinagar, Gujarat, India.

External Examiner:

Dr. Christophe Hano
French National Institute for Agriculture, Food, and Environment (INRAE),
University of Orleans, France.