5.0 Summary and Conclusion

Plants have rich source of primary and secondary metabolites and all living organisms have been nutritionally depended on them directly or indirectly. Medicinal plants are capable of producing unique bioactive compounds that are specific to species or family of plants. The evolution of secondary metabolism in plants is an astonishing event which created thousands of bioactive compounds with unimaginable power to restore health in a human being. Though, researchers all around the world are trying to understand the vast application of secondary metabolites, we are still only a drop in the ocean. We still need to put enormous efforts to understand the dynamic plant kingdom and its phytocomponents. The present study adds a single step to this long walk ahead of us. Here, a medicinal and ayurvedic plant, *Withania somnifera*, is chosen to study its reduction and therapeutic potential under *in vitro* condition. As any other medicinal plant, *W. somnifera* has a unique secondary metabolite composition collectively called Withanolides which are steroidal lactones belonging to the group of triterpenoids. It is known to have anti-cancer, anti-diabetic, anti-inflammatory and neuro-protective activity. There are more than 40 withanolides reported till date and among them WFA, withanolide A and withanone are the most studied for its therapeutic properties. Field grown *W. somnifera* tissues are used in more than 200 traditional formulations. Further, *W. somnifera* has a hyperaccumulation capability that increases the percentage of heavy metal accumulation compared to non-accumulator plant species.

Taking into account the accumulator capacity of *W. somnifera* two heavy metals were selected (Pb and Ag) to see if *in vitro* cultures are capable of accumulating metals, if so, to what extent, the nature of accumulated metal and the therapeutic potential of these *in vitro* cultures. Pb was selected for its heavy metal and lower solubility and immobile in soil and Ag for its high reactivity, easily reducible and useful as a therapeutic agent.

Thus, 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: $AgNO_3$ 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 $AgNO_3$ treated *in vitro* shoots along with field tissue and WFA were studied using rotenone induced Pakinson'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 totalling 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. EDAX 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 componenet 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 EDAX 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 $AgNO_3$ 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. Though, Ag is considered as a heavy metal, it has increased pharmaceutical activity which can be useful in this study. However, due to its heavy metal properties and increased toxicity in a biological system, Pb was not selected for further studies.

Rotenone is used to induce Parkinson's disease symptoms in SH-SY5Y neuroblastoma cells. 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. ROS scavenging assay was done using H2DCFDA staining. 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. Mitochondirial membrane potential was anlaysed by JC-1 staining. 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). Apoptosis was analysed by Annexin V/ PI binding assay. 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 discturbance reflects 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 (positive control) 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 simulataneous 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.

Recommendations for further studies

- Apart from this, the impact of Ag and Pb on withanogenesis pathway shall be studied using multi-omics analysis.
- *W. somnifera* is an important ayurvedic medicinal plant with high metal reduction potential. Thus, its metal reduction potential towards other pharmacologically important metals shall be explored.
- Clinical trials of the *in vitro* developed shoot extracts are required to substantiate the findings of this study.