

## 4.0 Results and Discussion

Biogenically synthesized MNPs (Bio-MNPs), a class of MNPs is gaining considerable importance in the recent years. They are efficiently used for targeted drug delivery to a complex/compartmentalized organ system, as it exhibits lower toxicity, higher stability and bioavailability in a biological system. *Withania somnifera*, an Ayurvedic herb has pivotal applications in pharmacological field owing to its rich withanolides composition. Due to the medicinal and pharmacological properties and a high demand in the global medicinal plant market, cultivation of *W. somnifera* increased significantly in the recent years. Cultivation of *W. somnifera in vitro* under controlled conditions, reduces seasonal and geographical variation. By manipulating growth media with specific biotic/abiotic stressors, the production of unadulterated tissue powder and targeted withanolides has been reported. Additionally, *W. somnifera* and its important secondary metabolite withaferin A is known to protect neuronal dysfunction in human and animal models ([Saykally et al. 2017](#); [Dutta et al. 2019](#); [Paul et al. 2021](#)).

In the present study an attempt has been made to improve the secondary metabolite content by subjecting the *in vitro* shoot tissues to abiotic stress specifically heavy metal (lead: Pb) and a transition metal (silver: Ag) as an elicitor is reported here. The level to which *in vitro* shoots can withstand the metal stress was initially analysed by toxicity studies, followed by analysis of biomass, primary and secondary metabolite quantification. Additionally, bioaccumulation of these metal in *in vitro* shoot system and nature of bioaccumulated metal as metal nanoparticles (MNPs) is also investigated. To provide evidential support for therapeutic activity specifically neuro-protective activity of *in vitro* shoots, the PD cell model was used. The effect of Ag treated *in vitro* shoots against rotenone induced mitochondrial dysfunction in Parkinson's disease cell model (SH-SY5Y) is studied in comparison with field tissues of *W. somnifera*. In Parkinson disease, mitochondrial dysfunction is the major causative factor for neurodegeneration which is addressed here through gene expression studies using selected mitochondrial genes. The results of the study are presented and discussed in three phases as below.

#### **4.1 Metal reduction and accumulation capability of Field shoots (FS) of *W. somnifera***

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- 4.3.3 Mitochondrial membrane potential analysis of *W. somnifera* tissue extracts on rotenone induced SH-SY5Y cells
- 4.3.4 Anti-apoptotic effect of *W. somnifera* tissue extracts on rotenone induced SH-SY5Y cells
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#### **4.1 Metal reduction and accumulation capability of field shoots (FS) of *W. somnifera***

Green synthesis of nanoparticles using plant extracts has gained considerable interest in modern nanoscience due to their higher surface area, bio-stability, human and environmentally friendly nature. Generally, the plants with high reduction potential are used frequently for the green synthesis of metal nanoparticles (MNPs). Several metals have already been reduced by a specific plant extract resulting in various types of MNPs. These green MNPs or Bio-MNPs has major application in modern medicine as it exhibits very low toxicity in a biological system ([Singh et al. 2018](#)).

In the present study we report, the metal reduction potential of *W. somnifera* a medicinal plant indigenous to South East Asian countries especially in India. Owing to its withanolide content and other important specialized metabolite community, *W. somnifera* displays higher reduction potential towards various metal ions ([Shanmugapriya et al. 2022](#)). The field grown root (FGR) of *W. somnifera* is a well-known rejuvenator in ayurvedic formulations. However, field grown shoot (FGS) of *W. somnifera* has the higher secondary metabolite content and higher reduction potential than FR ([Alam et al. 2012](#); [Tebeka Simur 2018](#)). This may due to the presence of higher quantity of withaferin A in FS ([Vinod et al. 2022](#)). Withaferin A, one of the most studied metabolites has numerous pharmacological values including anti-cancer, anti-diabetic, anti-microbial, neuro-protective and cardio-protective activities. With this background information, FS was chosen for metal reduction/ nanoparticle synthesis study.

Apart from its high reduction potential, *W. somnifera* tissues are also known to have high metal bioaccumulation capability. To confirm this statement, FGS, FGR of *W. somnifera* tissues were subjected to elemental analysis along with *in vitro* shoot (IVS) and *in vitro* root (IVR) tissues. Further, to evaluate metal reduction capability of *W. somnifera* tissues, two metal salts: PbAc and AgNO<sub>3</sub> were selected. The extract from FGS were treated with both metal salts.

#### **4.1.1 Elemental analysis on Field tissues (FGS & FGR) of *W. somnifera***

The usage of field grown *W. somnifera* tissues in modern medicines, value added products, ayurveda and other traditional medicinal formulations are gaining attentions, which increases its demand in herbal market. Any field grown plants used for medicinal purposes must undergo elemental analysis before it hits the herbal market (Asgari Lajayer et al. 2017). In this study, we did an elemental analysis on field tissues (FGS and FGR) of *W. somnifera* which was obtained from a Central Tobacco Research Institute, India (a non-contaminated area). This work was done to identify the plants' ability to uptake and accumulate trace and heavy metals when it's grown in a non-contaminated soil.

#### **ICPMS**

The field tissues of *W. somnifera* was subjected to ICPMS analysis. A total of twenty-two heavy and trace metals were analysed in *in vitro* and field tissues of *W. somnifera*, and only 21 elements (except Sb) were detected. Field tissues were found to have all heavy and trace metals detected within the permissible limit set by FAO/WHO (except iron and copper) (WHO guidelines 2006). The concentration of all analysed metals was below the quantification limit (LOQ) value. Overall, the results showed that the FGS had the highest concentration of essential trace metals with K recording the highest followed by Ca, Mg, P, B and Cu in descending order (Table 4.1). Whereas in FGR, Ca recorded the highest concentration followed by K, Mg and P in descending order. Considering heavy metal composition in FGS the concentration of Pb, Ag, Ni, Cu, Zn and Ti were higher compared to FGR. However, increased concentration of Cd, Co, As, Cr and Hg was found in FGR than FGS. This may be due to the fact that metals are usually absorbed by root from soil (Li et al. 2023; Espinosa et al. 2023) and translocated to shoots by translocation factors in a plant dependant manner (Aziz et al. 2014; Espinosa et al. 2023). In the present study, Ag and Pb was found higher amount in FGS than FGR which confirms that FS has high translocating ability towards Ag and Pb compared to other heavy metals.

The study reported by [Kulhari et al \(2013\)](#) shows that *W. somnifera* had highest bio-accumulation ability when it comes to heavy metals. In our study, the plant samples were taken from non-contaminated soil shows that even at normal condition, *W. somnifera* holds recognizable amount of heavy and trace metals. However, the concentration of heavy metals in the analysed sample was within the permissible limit set by WHO/FDA (world health organization/food and drug administration). Further, FGS has the higher bioaccumulation capability compared to FGR of *W. somnifera* ([Kulhari et al. 2013](#)).

#### **The maximum permissible limit (MPL) set by various pharmacopeia**

According to the European Pharmacopeia (2007), the following MPL are available:  $1\mu\text{g.g}^{-1}$  Cd,  $2\mu\text{g.g}^{-1}$  Cr,  $0.1\mu\text{g.g}^{-1}$  Hg,  $5\mu\text{g.g}^{-1}$  Pb ([MARINESCU et al. 2020](#)). The Chinese Pharmacopeia recommends  $2\mu\text{g.g}^{-1}$  As,  $0.3\mu\text{g.g}^{-1}$  Cd,  $20\mu\text{g.g}^{-1}$  Cu,  $0.2\mu\text{g.g}^{-1}$  Hg ([Li et al. 2012](#)) and the WHO/FDA sets the following MPL:  $10\mu\text{g.g}^{-1}$  As,  $0.2\mu\text{g.g}^{-1}$  Cd,  $20\mu\text{g.g}^{-1}$  Cu,  $1\mu\text{g.g}^{-1}$  Hg,  $10\mu\text{g.g}^{-1}$  Pb,  $50\mu\text{g.g}^{-1}$  Zn ([WHO guidelines 2007](#)). Heavy metal concentration in field grown tissues of *W. somnifera* was found within permissible limit set by WHO/FDA. Regarding essential metals for plants, all the trace metals and essential heavy metals were found within normal value in all analysed samples.

Table 4.1 Essential and non-essential elemental composition and its reference values

| Elemental composition and its maximum permissible limit set by World Health Organization |                   |           |           |           |           |  |   |
|--|-------------------|-----------|-----------|-----------|-----------|--|---|
| S. no.   | Parameters        | FGR (ppm) | FGS (ppm) | IVR (ppm) | IVS (ppm) | Permissible Limit (Medicinal plants) (ppm) | Provisional tolerable weekly intake (Edible plants) |
| <i>Essential trace metals for plant growth</i>   |                   |           |           |           |           |  |   |
| 1  | 11B (Boron)       | 6.5711    | 31.449    | 10.589    | 21.2635   | NA   | NA  |
| 2  | 24Mg (Magnesium)  | 1433.08   | 7896      | 466.35    | 1282.32   | NA   | NA  |
| 3  | 31P (Phosphorus)  | 471.965   | 1941.1    | 2180.6    | 3679.81   | NA   | NA  |
| 4  | 39K (Potassium)   | 1451.96   | 27397     | 12780     | 21109.4   | NA   | NA  |
| 5  | 44Ca (Calcium)    | 3381.47   | 8689.2    | 1617.1    | 1411.68   | NA   | NA  |
| 6  | 55Mn (Manganese)  | 9.9144    | 41.164    | 220.68    | 195.555   | NA   | 126 mg  |
| 7  | 57Fe (Iron)       | 13.6292   | 10.502    | 6.259     | 11.203    | NA   | NA  |
| 8  | 95Mo (Molybdenum) | 0.3083    | 0.3311    | 7.9429    | 2.803     | NA   | 0.029 mg/kg   |
| <i>Essential heavy metals for plant growth</i>   |                   |           |           |           |           |  |   |
| 1  | 60Ni (Nickel)     | 0.8981    | 0.9871    | 0.0752    | 0.0032    | 1.5(WHO,2005)                              | 22 µg/ kg   |
| 2  | 63Cu (Copper)     | 3.9901    | 8.1969    | 1.2452    | 2.0273    | 20 (WHO/FDA)                               | 7 mg  |
| 3  | 66Zn (Zinc)       | 6.5135    | 15.586    | 62.25     | 42.2345   | 50 (WHO/FDA)                               | 100 mg  |
| 4  | 48Ti (Titanium)   | 1.143     | 3.667     | NA        | NA        | NA   | NA  |
| <i>Non-essential heavy metals for plant growth</i>                                       |                   |           |           |           |           |  |   |
| 1  | 52Cr (Chromium)   | 0.5084    | 0.2076    | 0.0494    | 0.0076    | 2 (EP)                                     | 0.021 mg/person                                     |
| 2  | 59Co (Cobalt)     | 0.2009    | 0.1635    | 0.1386    | 0.1452    | 5 (USP)                                    | 5 µg/kg   |
| 3  | 75As (Arsenic)    | 0.0418    | 0.0162    | 0.0106    | 0.0045    | 10(WHO/FDA)                                | 0.015 mg/kg   |
| 4  | 111Cd (Cadmium)   | 0.0213    | 0.0196    | 0.0075    | 0.0055    | 0.2(WHO/FDA)                               | 0.77-1.78 µg/kg                                     |
| 5  | 202Hg (Mercury)   | 0.3202    | 0.0002    | 0.0646    | 0.004     | 1 (WHO/FDA)                                | 1.6 µg/kg   |
| 6  | 107Ag (Silver)    | 0.002     | 0.015     | NA        | NA        | NA   | 5 µg/kg   |
| 7  | 208Pb (Lead)      | 0.1114    | 0.1132    | 0.085     | 0.0279    | 10 (WHO/FDA)                               | 3 mg/person   |
| 8  | 51V (Vanadium)    | 0.028     | 0.03      | NA        | NA        | 30 (US EPA)                                | 9 mg/kg   |
| 9  | 7 Li (Lithium)    | 0.024     | 0.026     | NA        | NA        | NA   | NA  |
| 10   | 121Sb (Antimony)  | NA        | NA        | NA        | NA        | NA   | NA  |

All essential trace metals were present at higher concentration in FGS compared to FGR, except Fe. The concentration of trace metals (B, Mg, P, K, Ca & Mn) in FGS is at least four-fold higher than FGR. Compared with *in vitro* tissues (shoot & root), field tissues (shoot & root) had similar elemental ratio for B and Fe. In the present study, higher concentration of trace elemental composition may be due to higher antioxidant potential of field grown shoots of *W. somnifera* compared to FGR. In the study on *Angelica keiskei* by [Ob et al \(2017\)](#), the antioxidant enzymes' biosynthesis and activations depend upon the concentration of elemental composition in plant cells. The concentration of Mn in FGS is 41.2 ppm and FGR is 9.91 ppm, but, an increased accumulation of Mn is observed in *in vitro* shoot (195.5 ppm) and root cultures (220 ppm). Similar trend was observed in IVS (42.2 ppm) and IVR (62.2 ppm) of *W. somnifera* for Zn concentration as well. Similar concentration of Mn (78.2 ppm) and Zn (39.5 ppm) was reported by [Rout et al \(2014\)](#) where *in vitro* plantlets were treated with different concentration of Fe for 7 and 14 days. The concentration of Mn in plants is depend upon the soil Mn concentration and bioavailability of Mn is dependent on the pH of the soil. It was also reported that excess Mn can inhibit the uptake of other essential macro-elements such as Ca, Mg, Fe and P ([Sieprawska et al. 2016](#)); similarly decrease in Ca, Mg, Fe and P level while increased accumulation of Mn was found in IVS of our present study compared to field samples (FGS & FGR).

B is an essential micronutrient for plant growth. The plant cell concentration of B ranged from 3-100 ppm. Concentration of B is present in shoot tissues of field grown and *in vitro* compared to root tissues which is due to accumulation of B in leaf tissues compared to root ([Reid and Fitzpatrick 2009](#)). In addition, B is known to facilitate the transport of P in plants which might explain the increased concentration of FS and IVS compared to FR and IVR (Table 4.1) ([Hänsch and Mendel 2009](#)).

In plants, a minimum K concentration of around 4000 ppm is maintained by plant cells. However, plant cells tend to accumulate more K ions in their vacuoles for nutritional requirements like protein synthesis, cuticle layer formation and maintenance of turgor potential. Also, K<sup>+</sup> is reported to be the

most abundant cation in plant cells which often exceeds the concentration of Ca (Mishra et al. 2014; Ragel et al. 2019). In our results, K was found to be highest among analysed metals in *W. somnifera* tissues and shoots recorded had the higher K content compared to root tissues of both field grown and *in vitro*. The accumulation of higher concentration of K in shoot tissues was reported in different genotypes of *Ipomoea batatas* field grown plants (Wang et al. 2015). P, is another important trace element found to be a main constitution in genetic materials (DNA & RNA), sugar phosphates, in cell membrane (phospholipid layer) and have an important role in plant growth and development (Karthika et al. 2018; Wieczorek et al. 2022). In *W. somnifera*, IVS (3679 ppm) and IVR (2180 ppm) had higher concentration of P than in field tissues (shoot and root). Similar concentration of P in *W. somnifera* shoot cultures were reported by Mishra et al. 2014. Mg is the second abundant trace element present in the plant cells in the concentration of ~600 ppm (Kleczkowski and Igamberdiev 2021). But in the present study, Mg was third abundant element next to K & Ca of all analysed samples. Our results shows that field tissues had higher concentration of Mg compared to *in vitro* tissues. However, IVR (466 ppm) had the minimum Mg content than normal required plant cell volume of Mg (600 ppm). It has been reported that high availability of cations (Ca, K, Mn) in the nutrient solution led to strong decrease in Mg content due to the competition of cations for uptake (Gransee and Führs 2013). In addition, Mg is the cation which is supplemented in low concentration compared to other cations (Ca, K) (Appendix 1). Hence, *in vitro* tissues accumulate less Mg compared to other cations (Table 4.1).

Mo is least abundant trace element (0.2 ppm) present in the plant tissues which is known to regulate biosynthesis and transportation of secondary metabolites in plants (Tejada-Jime'nez et al. 2013; Devkar et al. 2015). However, in the present study, an increased Mo concentration was found in both field grown and *in vitro* tissues of *W. somnifera*. Further, plant tissue level of Mo strictly depends on availability of soil Mo concentration and soil pH (Tejada-Jime'nez et al. 2013). Fe is an essential trace element for plant growth and its content ranges from 100 to 500 ppm (Karthika et al. 2018). Compared to normal Fe content in plant tissues, *W. somnifera* exhibited lower

Fe content in both Field tissues and *in vitro* tissues. Lowest concentration of Fe is observed in IVR (6.3 ppm) followed by FGS (10.5 ppm), IVS (11.2 ppm) and FGR (13.6 ppm). Fe serves as a major component in electron transport chain, cofactor for several enzymes, respiration and in protein synthesis, etc. (Mahawar et al. 2023). The report on Kulhari et al. 2013 shows that field grown *W. somnifera* had 17.44 & 19.13 ppm Fe in leaf and stem samples respectively (Kulhari et al. 2013) which is higher than our current results (FGS: 10.5 ppm). Ca is an essential macro element in plants, its concentration in shoots ranged from 2000 to 10,000 ppm of dry weight (Thor 2019). Ca was the second most abundant element after K in all analysed samples. Increased concentration of Ca was detected in FGS (8689 ppm) and FGR (3381 ppm) compared to IVS (1411 ppm) and IVR (1617 ppm). Ca is mainly required for plant growth, as structural elements, secondary messengers and abiotic stress detectors in plants (Karthika et al. 2018). A concentration of Ca less than 1000 ppm is considered as Ca deficiency in plants (Karthika et al. 2018). Both IVS and IVR had moderate amount of Ca in their tissue. Further, Ca was known to alleviate Ni toxicity in plants (Aziz et al. 2015). Increased concentration of Ni in FGS (0.9871 ppm) and FGR (0.8981 ppm) compared to IVS (0.0032 ppm) and IVR (0.0752 ppm) might be the reason behind increased concentration of Ca in field tissues compared to *in vitro* tissues of *W. somnifera*. Also, Ca is an important signalling molecule to regulate plant cell homeostasis under environmental stress via maintaining Ca transportation. Field shoots tend to encounter more environmental stressors compared to *in vitro* cultures (Feng et al. 2023). This may be another reason behind increased concentration of Ca in field tissues than in *in vitro* tissues.

Thus, the elemental analysis of field tissues (shoot and root) of *W. somnifera* confirmed the bioaccumulation of essential heavy metals such as Ni, Cu, Zn and Ti to be higher in field shoots rather than field root tissues. As for non-essential heavy metals only Ag, Pb and V (vanadium) is highly accumulated in field shoot tissues than field root of *W. somnifera*. Therefore, for further studies, only field shoots of *W. somnifera* was selected due to its high metal bioaccumulation ability and metal reduction capability. Further, Pb and Ag having high translocation factor was selected for further studies.

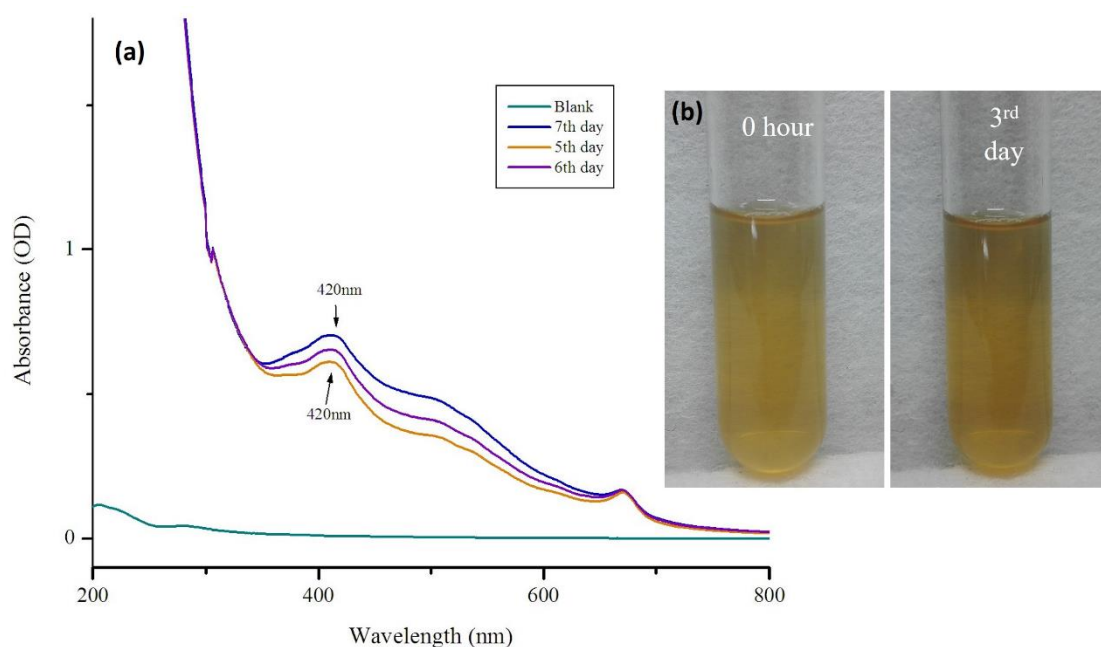
#### **4.1.2 Synthesis and characterization of metal nanoparticles**

Silver (Ag) is a highly reactive and a partial heavy metal which could easily be reduced to metallic silver (Ag<sup>0</sup>) when exposed to sunlight or UV light (Bhaduri et al. 2013). However, to analyse the metal reduction capability of FGS of *W. somnifera*, the experiments were done at dark environment without UV radiation of any type. Same experimental conditions were followed for lead nanoparticles synthesis.

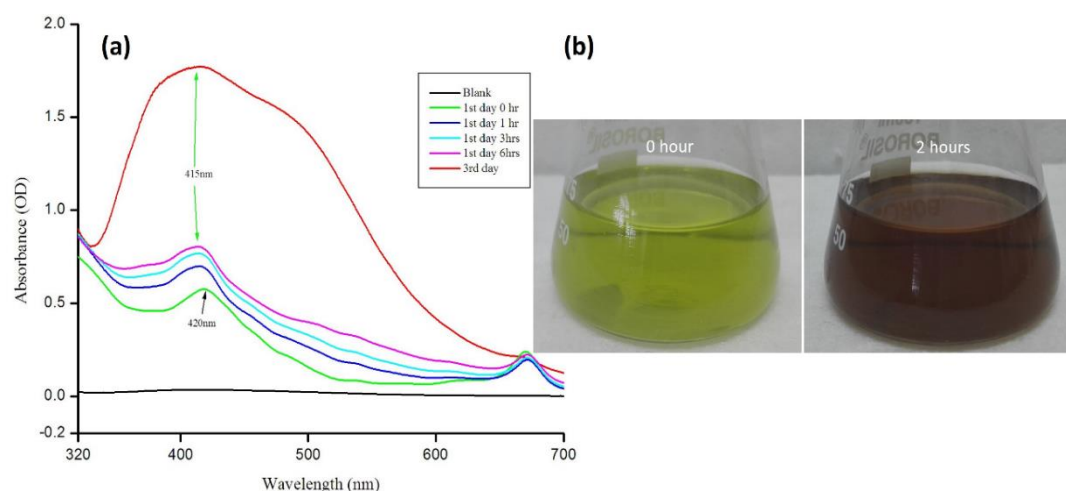
##### **4.1.2a Optimization of synthesizing conditions for silver nanoparticle (Ag NPs)**

For synthesizing Ag NPs, AgNO<sub>3</sub> was added with methanol extract of field grown shoot (FSE) of *W. somnifera* in the ratio of 1:10 and kept in a dark environment, using RT and HP method as described in section 3.2.4. In a RT method, colour change occurred at fifth day of the experiment. The solution colour changed from light brown to slightly dark brown in colour which may be due to the reduction of Ag ions in presence of *W. somnifera* FSE. The reduction of Ag ion in the experimental solution was analysed using UV-vis spectrophotometer. The characteristic surface plasmon resonance (SPR) band of Ag NPs was observed at 420nm in UV-vis spectrum (Figure 4.1), indicates that there is a high probability of reduction of Ag ion to Ag<sup>0</sup>. Though reports state that UV-vis spectra of Ag NPs show plasmon absorption bands at wavelengths ranging from 350nm to 690nm (Sivasankar et al. 2018) the ranges may vary depending upon the particle size and its SPR. Similar to our observation, SPR band at 420 nm for Ag NPs was reported by (Sivasankar et al. 2018) where Ag NPs was synthesized using exopolysaccharide of the fungi *Streptomyces violaceus* as a reducing and stabilizing agent. The UV-vis spectrum of Ag NPs and its varying wavelengths absorbance were discussed by (Bhaduri et al. 2013) and reported that the reduction of AgNO<sub>3</sub> in presence of sunlight as reducing agent and SDS (sodium dodecyl sulphate) as capping and stabilizing agent has given Ag NPs with a strong surface plasmon. Also, changing the concentration of SDS resulted in the nanoparticles of varying size (Bhaduri et al. 2013). In another study on *Jatropha curcas*, synthesis of Ag NPs and its size could be controlled by addition of varying concentration of AgNO<sub>3</sub> (Bar et al. 2009). Further, in the present study, the peaks' intensity increased

regularly until 10<sup>th</sup> day of the experiment and synthesized Ag NPs were stable even at 30<sup>th</sup> day of the experiment which shows that synthesized Ag NPs has a higher stability that may be due to the presence of natural capping agents in the plant extracts. The results were in correlation with a study on *J. curcas* seed extract-based synthesis of Ag NPs where stabilization of Ag NPs was found to be capping of protein through amide groups and cysteine residues in proteins (Bar et al. 2009). In a study on synthesis of Au NPs using *Panax ginseng* root revealed that high phytochemical content of a plant helped in the reduction, capping and stabilization of synthesized nanoparticles (Leonard et al. 2011).



**Figure 4.1 UV-vis absorption spectra of Ag NPs synthesized from *W. somnifera* shoot extract at room temperature**  
**a) Spectrum at various days (b) Colour change to brownish colour (at 3<sup>rd</sup> day) indicates the reduction of Ag ions.**



**Figure 4.2 UV–vis absorption spectra of Ag NPs synthesized from *W. somnifera* shoot extract at 60°C using hot plate.**

**a) Spectrum at various hours and day (b) colour change from green to dark brown (at 2 hours) indicates the reduction of Ag ions.**

In hot plate synthesizing method (HP method),  $\text{AgNO}_3$  and FSE of *W. somnifera* was added in 1:10 concentration and kept in hot plate for 1 hour at 60°C. A sudden colour change occurred from light green to dark brown colour within an hour of experiment which confirms the reduction of Ag ions. The experimental solution was analysed using UV-visible spectrophotometer before and after treatment. The UV spectrum (**Figure 4.2**) shows the presence of peak at 420nm; however, the peak shift towards lower spectrum (from 420 to 415 nm) area was observed after few hours of the experiment (**Figure 4.2**) and a broadened peak shows the instability of synthesized nanoparticles (nanoComposix 2012). Capriciousness of synthesized Ag NPs using hot plate method might be because of number of reasons such as temperature (Nurdin et al. 2016) which may impact capping and size of the nanoparticles (Hosseinpour-Mashkani and Sobhani-Nasab 2017), or may be hot plate accelerated Ag reduction in return it gives lower stability to the synthesized nanoparticles or both.

#### **4.1.2b Optimization of synthesizing conditions for lead nanoparticles**

In an attempt to synthesis Pb NPs using FSE of *W. somnifera*, the same procedure as silver nanoparticles was followed i.e., RT and HP method. No visible colour change was observed and reduction of Pb ions in the experimental solution could not be proved using UV spectrum. At this stage, the study was not continued, as no NPs formation could be detected, however, PbAc was used to treat *in vitro* shoots and analyse if *in planta* synthesis of Pb NPs is possible.

#### **4.1.2c Physical and chemical characterization**

Two metals used for the synthesis of nanoparticles via two different methods namely RT method and HP method. In the present study, Ag NPs synthesized from RT method had the higher stability than HP method; on the other hand, Pb was not reduced to its metallic form in both methods which was confirmed by UV-vis spectrophotometry. Therefore, Ag NPs synthesized from RT method (RT-Ag NPs) were further subjected to physical and chemical characterization. The synthesized RT-Ag NPs kept at cooling temperature ( $22\pm 2^{\circ}\text{C}$ ) for 2 days without any external disturbances. On the 3<sup>rd</sup> day the solution was centrifuged at 12,000rpm and  $-4^{\circ}\text{C}$  using Refrigerated centrifuge (Plastocrafts, Rota R-V/FM). The resultant pellet was washed with deionized water ( $\text{DIH}_2\text{O}$ ) and then concentrated using vacuum freeze dryer, temperature and vacuum was maintained at  $-130^{\circ}\text{C}$  and  $<10$  pa. The ensuing powder containing RT-Ag NPs was further taken for electron microscopy analysis.

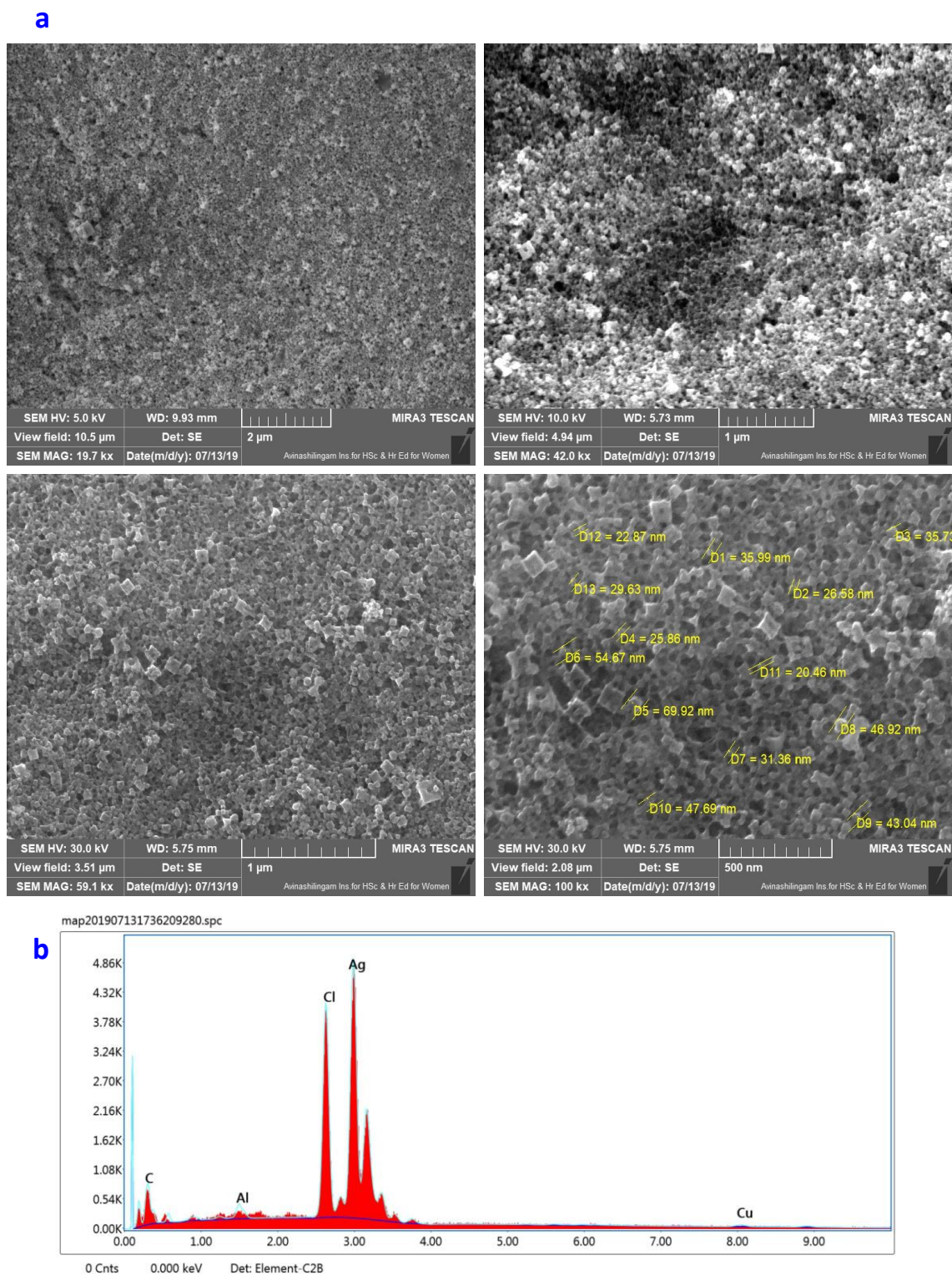
#### **Field-emission scanning electron microscopy with EDX analysis**

The RT-Ag NPs were analysed for its surface morphology using FESEM with EDX. The powder contained cubic shaped nanostructures with 49% Ag with additional elements such as 5% carbon, 4% aluminium, 41% chlorine and 1% copper (**Figure 4.3**). Other elements C, Cl, Al, & Cu may be from the plant extracts (FSE of *W. somnifera*). Similar study on synthesis of Ag NPs using *W. somnifera* tissues extracts has already been reported; however, in the previously reported study, the solvent used for tissue extracts was water rather than methanol. Further, *W. somnifera* water extract derived Ag NPs were mostly reported to be in a spherical form (Nagati et al. 2013; Marslin et al.

2018). On the other hand, in our study, *W. somnifera* tissues extracted with methanol resulted in Ag NPs with cubic shape and the size ranging from 20 – 70nm.

The synthesis of Ag NPs with cube shape was reported by many researchers. Most of the research focused on synthesizing cubic shaped Ag NPs was via chemical reduction method. For instance, Ethylene glycol as a solvent and a reducing agent, with poly(vinyl pyrrolidone) as a capping agent and a size controller, is reported to give Ag NPs with an average size of >10nm and in cubic shape (Skrabalak et al. 2007; Rycenga et al. 2008);(Fahad et al. 2019). In another study on *Eucalyptus macrocarpa* leaf extract derived Ag NPs resulted in cubic shaped nanoparticles range from 10-50nm in size (Poinern et al. 2013). Cubic shaped Ag NPs has enormous applications in medical field. These crystal silver nano cubes are useful structure for the production of gold nanocages that hold great potential for biomedical applications such as optical imaging contrast enhancement and photothermal treatment (Siekkinen et al. 2006);(Chen et al. 2010);(Li et al. 2022). Hence, the observance of cubic shaped Ag NPs obtained in the present study using *W. somnifera* FSE seems to be advantageous.

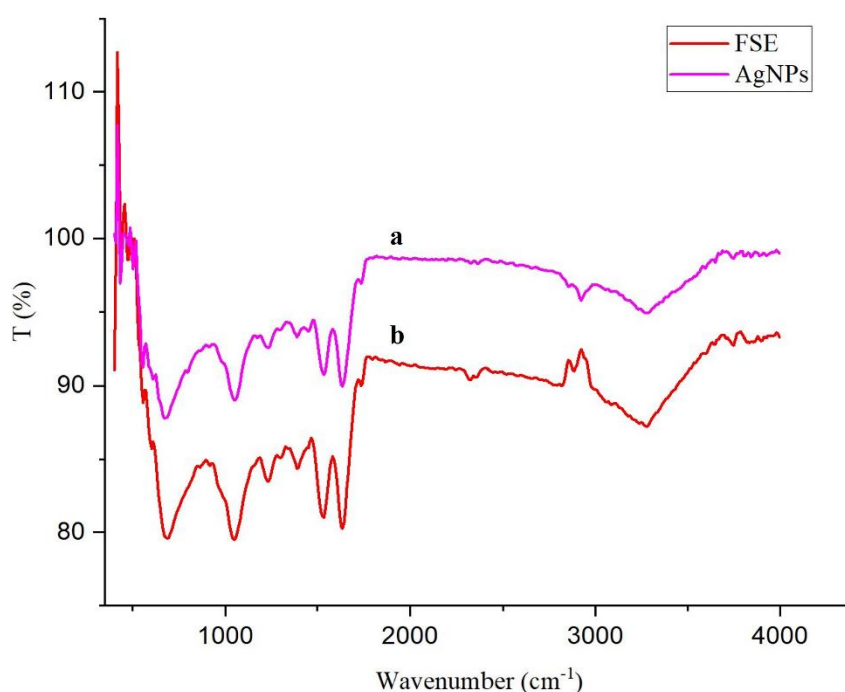
The presence of elements and atomic proportions of *W. somnifera* FSE-mediated Ag NPs was determined using energy-dispersive X-ray spectrometer. The analysis through FESEM-EDAX spectrum recorded a sharp peak between 2.9 keV and 3.4 keV **figure 4.3** confirmed the presence of an elemental silver signal of the silver nanoparticles (El-Khatib et al. 2020; Bhuyar et al. 2020). EDX analysis shows that the major peaks belong to Ag followed by Cl, Al and C.



**Figure 4.3 FESEM micrographs and EDAX spectrum synthesized Ag NPs. a) The crystalline nature of synthesized Ag NPs. b) EDAX spectrum displays the presence of Ag peak at 3keV**

## FTIR

Chemical composition on the surface of the synthesized Ag NPs was analysed by FTIR. The band at  $2924\text{cm}^{-1}$  indicates the presence of various amino acids, band at  $1735\text{cm}^{-1}$  corresponding to ketone and carbonyl groups, bands at  $1635\text{cm}^{-1}$  and  $1535\text{cm}^{-1}$  indicates N-H bending and N-O stretch of amino acids, bands at  $1234\text{cm}^{-1}$  and  $1049\text{cm}^{-1}$  are due to stretching vibration of C-O groups of esters and phenols, band at  $671\text{cm}^{-1}$  corresponds to C=C of alkene groups containing metabolites like terpene and terpenoid (Figure 4.4). The FTIR spectra bands corresponding to  $1635\text{cm}^{-1}$  and  $1535\text{cm}^{-1}$  indicates N-H bending and N-O stretch of amino acids demonstrating the presence of primary amines (Bhuyar et al. 2020). Likewise, another set of bands observed at  $1234\text{cm}^{-1}$  and  $1049\text{cm}^{-1}$  is assigned to stretching vibration of C-O groups of esters and phenols (Huang et al. 2007). The band at  $1735\text{cm}^{-1}$  attributed to ketone and carbonyl group of organic acids in the plant extracts (Mochochoko et al. 2013). All these measurements confirmed successful capping of Ag NPs with FSE of *W. somnifera*.



**Figure 4.4 FTIR spectrum of field shoot extracts (FSE) of *W. somnifera* and FSE derived silver nanoparticles.**

In conclusion, Elemental analysis on *W. somnifera* tissues revealed that field shoot has the higher bio-accumulation capability especially for Ag & Pb, compared to field root (**Table 4.1**). All-heavy metals are present within the permissible limit and its bioaccumulation efficiency at tissue level was confirmed. Among *W. somnifera* tissues, field grown shoot has the higher reduction potential and metal bio-accumulation capability towards Ag and Pb respectively, compared to field grown root. Unlike Lead (Pb), Silver was reduced in presence of FSE of *W. somnifera* at room temperature. Therefore, further studies mainly focused on the bioaccumulation efficiency and reduction ability of *in vitro* shoot cultures of *W. somnifera* under controlled condition for specific metals (Ag and Pb salts).

With this initial results, further study was constructed with following possible outcomes. Acute and chronic metal exposure conditions to optimise the culture conditions to determine higher biomass and withanolides yielding shoot cultures. The metals chosen for *in vitro* metal exposure studies are silver and lead. Pb was selected because of its heavy metal properties which could be used to determine hyperaccumulation potential of IVS of *W. somnifera* under controlled exposure conditions and also to see *in planta* formation of metal nanoparticles is possible. Ag was selected because of its heavy metal as well as its medicinal properties. A heavy metal and also a pharmacologically important heavy metal bioaccumulation was studied in order to determine its bioaccumulation and nature of bioaccumulation in IVS of *W. somnifera*.

## **4.2 *In planta* formation of metal nanoparticles, its characterization and its influence on secondary metabolite accumulation**

In the current study, two non-essential metal salts Lead acetate (PbAc) and Silver nitrate (AgNO<sub>3</sub>) were used as stressors to the *in vitro* shoot cultures of *W. somnifera*. *In vitro* shoots cultures of *W. somnifera* were treated with different concentration of metal salts at varying duration for optimizing cultures conditions to improve biomass and secondary metabolites. The studies were conducted using 45 days old shoot cultures. Shoots were grown and maintained in the adventitious shoot multiplication medium (solid) for 30 days and transferred to mass multiplication medium (suspension) for 15 days prior to the treatment. At 45<sup>th</sup> day, the shoots maintained in mass multiplication medium were treated with different concentrations of PbAc and AgNO<sub>3</sub> (Table 3.1). Primarily, the IVS were treated with metal salts starting on 45<sup>th</sup> day and 55<sup>th</sup> day for a long duration at lower concentration and short duration at higher concentration respectively such that harvesting time was synchronized for 57<sup>th</sup> day. After respective metal exposure, the shoots were immediately harvested and processed for further analysis. The exposure condition for both Ag and Pb treatments were selected based on maximum growth index and withaferin A for further studies.

### **4.2.1 Optimization of culture conditions for growth of *in vitro* shoots on exposure to abiotic stressors**

#### **4.2.1a Morphological changes observed in IVS on exposure to metal salts**

Silver nitrate (AgNO<sub>3</sub>) treatment to IVS of *W. somnifera* showed visible toxicity symptoms at higher concentration of Ag at both acute and chronic exposure periods. Acute exposure to Ag at higher concentration resulted in decreased shoot water content and whole shoot browning which leads to shoot senescence and death (Quah et al. 2015; Khan et al. 2019). However, in Ag prolonged exposure to higher concentration exhibits toxicity symptoms like callus browning and flaccidity of shoots which results in the lower biomass. The effect of Ag ions on plants causes senescence was also reported by ÖKTEM and KELEŞ (2018). At higher concentration (8mM & 16mM), Ag showed higher toxicity than Pb towards IVS of *W. somnifera*. Shoots were healthy at all Pb

exposure despite higher metal concentration and exposure period. Notably, Pb exposed shoots had large healthy leaves, higher biomass, increased branching on treatment with higher concentrations compared to control shoots. In contrast, as the concentration of Ag increases biomass of the shoot was also increased with healthy leaf and shoot. As it reaches a threshold of Ag for IVS of *W. somnifera*, healthy shoots start to flaccid, shoot senescence and cell/shoot death or rotting of the leaves was occurred.

Lead acetate (PbAc) exposure showed no harmful effects on the IVS at all concentration and treatment period studied. However, decrease in GI was observed at higher concentration (H4 & H5) to short duration (T1-T3) and lower concentration (D5) to higher duration (T6) yet shoots were in good health. In addition, though a in GI was observed, a significant increase in biomass was observed compared to control shoots ([Table 4.4](#)). Further, dark coloration of leaves and increased leaf surface ratio was observed on exposure to higher concentration of Pb for short duration when compared to exposure to long period of time at low concentrations where an increase in shoot regeneration was observed. This leaf coloration due to Pb exposure was reported by other researchers as well ([Dinu et al. 2020](#)). Except for change in leaf coloration on short exposure time to high concentrations of Pb, we did not find any other visible symptom of Pb even on a long-time exposure to low concentrations, probably because the plant has powerful metal detoxification and sequestration process which will be discussed in the next chapter ([section 4.2.2 & 4.2.3](#)). The darkened leaves were observed when basil plants were independently exposed to various heavy metals including Pb ([Dinu et al. 2020](#)).

In the present study, Pb showed lower toxicity towards IVS of *W. somnifera* and its GI. On the other hand, most of the studies showed that plants exposed to heavy metals resulted in adverse effects on germination and growth, inhibiting growth and development of plant tissues, yielding lower biomass following cell/tissue death in spite of certain plant species being identified to be tolerant to Pb even high concentrations ([Ali and Nas 2018](#); [Rehman et al. 2017](#)). For example, in the study on *Eclipta prostrata* exposed to different concentrations of PbNO<sub>3</sub> for 30 days showed significant increase in

the fresh weight (biomass) and higher Pb accumulation potential compared to other plant species studies (*Scoparia dulcis* and *Phyllanthus niruri*) (Chandrasekhar and Ray 2019). In addition, a study on Cd accumulation in two plant species namely *Solanum nigrum* and *Ricinus communis* discusses that total heavy metal accumulation was based on bioconcentration factor (BCT) as well as biomass production of the plant under heavy metal stress (Rehman et al. 2017).

#### **4.2.1b Standardization of metal salt concentration and treatment period for increase in GI and Withanolides content**

##### **4.2.1b(i) Acute toxicity studies**

This study was formulated considering the capability of hyperaccumulation and high reduction potential of *W. somnifera* integrating knowledge on live plant synthesis of nanoparticles reported elsewhere (Gardea-Torresdey et al. 2002; Parker et al. 2014). Therefore, in the present study, we report the absorption and reduction of silver by IVS of *W. somnifera* cultures under controlled condition, the conversion and accumulation of absorbed silver as nanoparticles.

Earlier studies have discussed on the elicitation of withanolides in cell suspension cultures of *W. somnifera* in presence of metals such as Cd, Cu, Ca, Al etc. (Baldi et al. 2008; Sivanandhan et al. 2014; Siddiqui et al. 2015; Mishra and Sangwan 2018), in whole plant cultures of *W. somnifera* using Fe, Zn, and Cu (Rout et al. 2013; Rout et al. 2015; Rout et al. 2019) and in adventitious root cultures of *W. somnifera* in presence of Al (Sivanandhan et al. 2012). *W. somnifera* is known to have a hyperaccumulation ability and shoot has the higher bioaccumulation potential compared to other parts of the plant (Khan et al. 2007). Thus, shoot cultures of *W. somnifera* (without root system) were treated with metal salts at different concentration and duration to understand its capacity to withstand high concentration of metal salts exposure for shorter duration (acute toxicity studies). Further, metal exposure to high concentration to short time period studies show the resistance and surviving ability of plants to a specific metal at a higher concentration.

### **Silver nitrate**

AgNO<sub>3</sub> a known pharmacologically important heavy metal was selected for metal toxicity studies against IVS of *W. somnifera*. Further, the effect on IVS of *W. somnifera* to Ag was analysed in terms of growth index, primary and specialized (withanolides) metabolites production. Silver nitrate treatment on shoot cultures of *Solanum tuberosum* have known to induce shoot organogenesis (Kaur et al. 2021). Numerous studies have shown that AgNO<sub>3</sub> is an efficient promoter for shoot organogenesis and micropropagation (Kaur et al. 2021; Turhan 2004; Sarropoulou et al. 2016). Apart from its elicitation effects, silver is also known for high reactivity and its application in medicinal field especially as anti-microbial agent.

### **Growth Index**
















Silver did not exhibit any severe toxicity symptoms or decrease in biomass of IVS of *W. somnifera* up to 24 hours exposure and 8mM AgNO<sub>3</sub> concentration (E1H4T2) (Plate 4.1). On increasing concentration of AgNO<sub>3</sub> (16mM) (E1H5) at all time periods mild toxicity symptoms like blackening and shrinkage of bottom/whole shoots were observed. Growth retardation was observed at higher concentration of AgNO<sub>3</sub> (8mM & 16mM; E1H4 & E1H5) at all treatment period. Even at 12 hrs treatment at higher concentration of AgNO<sub>3</sub> (8mM & 16mM) resulted in the growth retardation, bottom shoot blackening and mild drying or rotting of shoot cultures (E1H4T1 & E1H5T1) (Plate 4.1). The effect of Ag on IVS was studied here because most of the reported metal elicitation studies were performed using either root or cell suspension or callus cultures of *W. somnifera* (Sivanandhan et al. 2014; Thilip et al. 2019). AgNO<sub>3</sub> treatment to plants/plant tissue cultures was done with the expectation to increase shoot proliferation and micropropagation (Turhan 2004; Sarropoulou et al. 2015; Kaur et al. 2021). However, higher concentration of silver ions (Ag<sup>+</sup>) was shown to negatively affect the plant development due to its possible binding to the copper binding sites and it is also known to inhibit protein aquaporin resulting in the water imbalance in plant cells (Quah et al. 2015). Further, excess Ag<sup>+</sup> can lead to plant cell death as reported by Steinitz and Bilavendran (2011) similarly browning of cultures observed in the present study.

*Influence of duration on GI*

All shoot cultures were maintained in AgNO<sub>3</sub> solution along with mass multiplication medium for the specified period and concentration of AgNO<sub>3</sub> after which AgEIVS were immediately harvested. The result shows a significant increase in the GI at all duration (T1-T3) till H2. Afterward increase in the concentrations resulted in negative GI at all time period (T1-T3) of H5 and T2 & T3 of H4 which is presented as 0 in [table 4.2](#). Among IVS exposure to high concentrations of AgNO<sub>3</sub> to short time periods, T3 served as a rate limiting duration.

*Influence of Ag concentration on GI*

Elicitation mechanism in shoot cultures may enhance the production of secondary metabolite content in pharmacologically important medicinal plants. In addition, there are many parameters that can influence the biomass production during elicitor treatment ([Mahendran et al. 2018](#)). Elicitor concentration highly influences the biomass growth of shoot cultures. In high concentration of AgNO<sub>3</sub> treatment to short duration, IVS showed significant increase in GI from H1 to H2 after that significant reduction was observed. Among all shoots treated with high concentration of silver for short duration, H2 (2mM) exhibited increased GI compared to other AgNO<sub>3</sub> concentration (H1, H3-H5). Treatment of AgNO<sub>3</sub> to higher concentration (H4 & H5) resulted in the reduction in GI compared to control and other Ag treated shoot GI ([Table 4.2](#)). Similar study showing the increase in biomass in response to Ag concentration is reported only in a few studies and at higher silver concentration, and biomass of the plant was significantly reduced ([Bais et al. 2000](#); [Jiang et al. 2012](#)). However, most of the reports shows that Ag mainly decreases the biomass accumulation and growth index of the plant which were subjected to silver treatment ([Jiang et al. 2012](#)). On the other hand, when Ag ions were treated to *Arabidopsis thaliana* plants, no significant effect on biomass was observed at earlier concentrations (1.0 & 2.5 mg Ag<sup>+</sup>/L); further increase in Ag<sup>+</sup> concentration (5 mg Ag<sup>+</sup>/L) and duration (10 days) significantly decreased biomass ([Kaveh et al. 2013](#)).

| AgNO <sub>3</sub> acute treatment (E1) | 12 HOURS (T1)   | 24 HOURS (T2)  | 48 HOURS (T3)   |
|--|---|--|---|
| 1mM (H1)                               | <br>E1H1T1   | <br>E1H1T2   | <br>E1H1T3   |
| 2mM (H2)                               | <br>E1H2T1   | <br>E1H2T2   | <br>E1H2T3   |
| 4mM (H3)                               | <br>E1H3T1 | <br>E1H3T2 | <br>E1H3T3 |
| 8mM (H4)                               | <br>E1H4T1 | <br>E1H4T2 | <br>E1H4T3 |
| 16mM (H5)                              | <br>E1H5T1 | <br>E1H5T2 | <br>E1H5T3 |

E1H3T3 (2mM AgNO<sub>3</sub> for 48 hrs exposure) showing the maximum biomass among Ag acute treated shoots resulting in higher GI

Plate 4.1 Influence of AgNO<sub>3</sub> acute treatment (E1) on *in vitro* shoots of *W. somnifera*

### **AgNO<sub>3</sub> treatment on withaferin A accumulation**

In higher concentration of AgNO<sub>3</sub> treatment to short time, significantly increased WFA production was observed till E1H4 (8mM) for 12 hrs treatment (T1) (0.828 mg g<sup>-1</sup>). However, in T2 & T3 period, 4mM (E1H3) showed increased WFA rather than E1H4 which shows the adaptive nature of IVS of *W. somnifera* to Ag treatment to longer time period. Similar to our results, the secondary metabolite accumulation pattern changes depending upon the period of incubation with elicitors in *Ophiorrhiza mungos* cell suspension cultures (Deepthi and Satheeshkumar 2015; Mahendran et al. 2018). Further, higher WFA accumulation among acute treated shoots was found in E2H3T3 shoots (1.09 mg g<sup>-1</sup>). In high concentration exposure to short duration, a maximum of 1.10 mg g<sup>-1</sup> dry wt. of withaferin A was noted in E1H3 after 48 hrs (T3) of exposure and the least was in control (0.556 mg g<sup>-1</sup>) followed by (E1H1-E1H5) – T1 (1.2<sup>-1</sup>.6 mg g<sup>-1</sup>) exposed samples (Table 3.1).
















In IVS of *W. somnifera*, high concentration of AgNO<sub>3</sub> treatment to short time was found to be toxic at higher concentrations (H3, H4 & H5: 4, 8 & 16mM). Though, reduction in GI was observed at higher concentration to short period (H3 & H4) but increased WFA was observed at the same treatment namely, H3T1, H4T1, H3T2 and H3T3 (Table 4.3). This type of negative correlation between GI and secondary metabolite production in response to abiotic elicitors in plants was reported by Sá et al (2013) using *Mentha crispa* plantlets. The increased accumulation of WFA at each treatment period (T1-T3) was found to be almost 2-fold higher than their respective controls (Table 4.3). In 25µM AgNO<sub>3</sub> treatment to *P. abrotanoides* resulted in 2-fold increase in tanshinone content compared to control cultures (Zaker et al. 2015). In another study on *Gloriosa superba* cell suspension cultures treated with different concentration of AgNO<sub>3</sub> resulted in 4 to 5-fold increase in colchicine concentration compared to control (Mahendran et al. 2018). Therefore, high concentration of Ag exposure for short duration resulted in maximum of 1.9-fold WFA (1.09 mg g<sup>-1</sup>) accumulation at 4mM treatment to 48 hrs (E1H3T3) compared to control IVS (0.599 mg g<sup>-1</sup>).

### **Lead acetate**

All *in vitro* shoots treated with PbAc were harvested after a specific treatment period (**Table 3.1**), GI was calculated and subjected to HPTLC analysis for withaferin A quantification. Pb was known to increase secondary metabolites content in plants under specific concentration and treatment period ([Srivastava and Srivastava 2010](#); [Kisa et al. 2016](#)). Based on higher biomass (GI) and withaferin A production, optimum concentration and exposure period of Pb treatment to *W. somnifera* shoots were confirmed and taken up for further study.

### **Growth Index**

The inclusion of PbAc to the shoot suspension cultures of *W. somnifera* resulted in faster growth of shoots compared to control IVS. A high concentration of PbAc exposure to short period revealed that the shoots showed mild to severe toxicity symptoms. For instance, increased concentration of Pb treated IVS (2.4 & 4.8mM), H4 and H5 shoot leaves were turned dark green colour which associated with a lower growth index at T1 and T3 duration compared to control shoots. However, on 24 hrs (T2), PbAc treatment resulted in increased GI from H1 to H4 then GI was decreased at H5 (**Table 4.3**). Increased GI at H4 of T2 period indicates the adaptive mechanism of Pb treatment to IVS of *W. somnifera*. Further decrease in GI at H4 of T3 duration exhibits induction of Pb toxicity in IVS at increased Pb concentration and treatment period. Similar dose dependant increase in biomass till certain concentration after that reduction in biomass was observed in TiO<sub>2</sub> treated *H. perforatum* cultures ([Kruszka et al. 2022](#)). In PbAc toxicity study to higher concentrations for short duration (H4 & H5), shoots displayed heavy metal toxicity symptoms at higher treatment period (T3), which is in agreement with the decrease of plant biomass and dark green leaves reported in various other studies ([Islam et al. 2008](#); [Islam et al. 2011](#)).

| PbAc <sub>2</sub> acute treatment (E2) | 12 HOURS (T1)   | 24 HOURS (T2)  | 48 HOURS (T3)   |
|--|---|--|---|
| 0.3mM (H1)                             | <br>E2H1T1   | <br>E2H1T2   | <br>E2H1T3   |
| 0.6mM (H2)                             | <br>E2H2T1   | <br>E2H2T2   | <br>E2H2T3   |
| 1.2mM (H3)                             | <br>E2H3T1 | <br>E2H3T2 | <br>E2H3T3 |
| 2.4mM (H4)                             | <br>E2H4T1 | <br>E2H4T2 | <br>E2H4T3 |
| 4.8mM (H5)                             | <br>E2H5T1 | <br>E2H5T2 | <br>E2H5T3 |

E2H3T3 (2mM PbAc for 48 hrs exposure) showing the maximum biomass among Pb treated shoots resulting in the higher GI

Plate 4.2 Influence of PbAc treatment (E2) on *in vitro* shoots of *W. somnifera*

Table 4.2 Influence of AgNO<sub>3</sub> (E1) and PbAc (E2) acute treatment on Growth index of IVS of *W. somnifera*

| Acute treatment | T1             | T2            | T3                    |
|-----------------|----------------|---------------|-----------------------|
| Control         | 0.0160±0.004b  | 0.0603±0.04e  | 0.1196±0.02e          |
| E1H1            | 0.0121±0.002b  | 0.0431±0.005d | 0.0692±0.003d         |
| E1H2            | 0.0432±0.005d  | 0.0605±0.004e | <b>0.0930±0.005de</b> |
| E1H3            | 0.0274±0.001c  | 0.0026±0.001c | 0.0121±0.003c         |
| E1H4            | 0.0108±0.001b  | 0             | 0                     |
| E1H5            | 0              | 0             | 0                     |
| E2H1            | 0.0164±0.001ab | 0.0502±0.003a | 0.1131±0.02a          |
| E2H2            | 0.0168±0.002ab | 0.064±0.01ab  | 0.1524±0.01bc         |
| E2H3            | 0.0242±0.002b  | 0.0754±0.002b | <b>0.1772±0.01c</b>   |
| E2H4            | 0.0151±0.01ab  | 0.0957±0.004c | 0.1651±0.01c          |
| E2H5            | 0.0082±0.003a  | 0.0642±0.01ab | 0.1328±0.01ab         |

Values are presented as means ± SE of three replicates. Within each column, means followed by the same letter are not statistically different at  $p \leq 0.05$  according to DMRT. The mean difference is significant at the 0.05 level.

Table 4.3 Influence of AgNO<sub>3</sub> (E1) and PbAc (E2) acute treatment on withaferin A production in IVS of *W. somnifera*

| Acute treatment | Withaferin A (mg g <sup>-1</sup> ) |              |                    |
|-----------------|------------------------------------|--------------|--------------------|
|                 | T1                                 | T2           | T3                 |
| Control         | 0.556±0.01a                        | 0.583±0.02a  | 0.599±0.02a        |
| E1H1            | 0.620±0.02ab                       | 0.616±0.02a  | 0.737±0.01b        |
| E1H2            | 0.642±0.03ab                       | 0.925±0.01c  | 0.799±0.01cd       |
| E1H3            | 0.680±0.01b                        | 1.016±0.01d  | <b>1.097±0.01e</b> |
| E1H4            | 0.828±0.01c                        | 0.762±0.03b  | 0.826±0.01d        |
| E1H5            | 0.795±0.04c                        | 0.567±0.03a  | 0.766±0.01bc       |
| E2H1            | 0.783±0.02b                        | 0.832±0.03bc | 0.997±0.02b        |
| E2H2            | 0.910±0.01c                        | 0.915±0.04c  | 1.119±0.00c        |
| E2H3            | 0.982±0.01d                        | 1.021±0.01d  | <b>1.184±0.01c</b> |
| E2H4            | 1.022±0.01d                        | 1.030±0.04d  | 0.998±0.02b        |
| E2H5            | 0.891±0.02c                        | 0.800±0.01b  | 0.591±0.00a        |

Values are presented as means ± SE of three replicates. Within each column, means followed by the same letter are not statistically different at  $p \leq 0.05$  according to DMRT. The mean difference is significant at the 0.05 level.

#### *Influence of duration of PbAc treatment on GI*

The [table 4.3](#) shows significant increase in GI was observed at all PbAc treatment to short time period (T1-T3) compared to control and irrespective of the concentration. Compared to T1, T3 displays more than seven-fold increase in GI. Highest GI was observed at T3 period (0.1772) of H3 concentration. Similarly, abiotic elicitor treatment (Co) to *Salvia miltiorrhiza* cell cultures increased cell biomass at initial concentration and growth suppression was observed at high concentration of elicitor ([Zhao et al. 2010](#)). In addition, abiotic elicitor treatment on *Vitis vinifera* cell cultures, resulted in increased growth biomass at longer duration (24hrs) compared to shorter duration (4 hrs) ([Cai et al. 2013](#)). Regression analysis on high concentration of Pb treated IVS for short time shows that T1 is non-significant ( $P>0.09$ ) as compared to control. However, T2 ( $P>0.00$ ) seems to have a higher effect on GI compared to T3 ( $P>0.02$ ) but significant compared to control. Similarly, ANOVA results shows that the effect of T2 was higher ( $F=10.446$ ) than T3 ( $F=7.863$ ) on GI. Therefore, from statistical analysis, we confirmed that T2 was significantly more effective than T3 however, T3 was observed with higher GI than T2.

#### *Influence of Pb concentration on GI*

On high concentration of PbAc treatment for short time, H1 and H2 showed almost similar GI to control IVS. Increase in GI was observed from H1-H3 for T1 and H1-H4 for T2 and H1-H3 for T3 duration. Among different concentration studied, H3 of T3 showed maximum growth index (0.1772) compared to control (0.1196). Among different concentration of bio-Ag NPs effect on *F. indica* studied, 62.5 µg/ml resulted in higher biomass accumulation further increase in Ag NPs causes reduction in biomass ([Begum et al. 2020](#)). Further, at the highest PbAc concentration (H5), GI was approximately 50% of the control. Similar reduction in biomass at higher concentration of heavy metal treatment reported in *Spirodela polyrhiza* treated with different concentrations of Ag ([Jiang et al. 2012](#)).

#### **PbAc treatment on Withaferin A accumulation**

In a study on [Wink \(2016\)](#) reported that, though secondary metabolites are not important for plant growth and development, they have an important function in the plant defence mechanism. The evolution of the secondary

metabolic pathway in plants is a type of adaptation mechanism which was developed due to environmental stressors (Wink et al. 2016). Lethal (acute) concentrations of PbAc showed significant increase in the biomass and withaferin A content until a specific concentration and duration. Most of the studies reported that Pb treatment to plants resulted in the reduction of biomass and secondary metabolite production (Grejtovský et al. 2008; Sa et al. 2013; Rehman et al. 2017; Ni'am and Yuniati 2021).

All PbAc treated *in vitro* shoots were harvested after a specific treatment period (Table 3.1) and subjected to HPTLC analysis for withaferin A quantification. Pb was known to increase secondary metabolites content in plants under specific concentration and treatment period (Srivastava and Srivastava 2010; Kisa et al. 2016). Based on higher biomass (section 4.2.1a) and withaferin A production, optimum concentration and exposure period of Pb treatment to *W. somnifera* shoots were confirmed and taken up for further study.

### *Influence of PbAc concentration on withaferin A production*

*W. somnifera* IVS treated with higher concentration of Pb up to 4.8mM (1600ppm)(E2H5) was exposed for a shorter time period (12-48 hrs). The physiological observations proved that *W. somnifera* shoots exhibited high Pb tolerance and GI accumulation (Section 4.2.1a). Withaferin A accumulation found to be positively correlated with the PbAc concentration. In high concentration of PbAc treatment to brief exposure period resulted in the increase in withaferin A production up to 2.4mM of T1 (12 hrs), however, in later stages (2.4mM of 48 hrs and 4.8mM of 12, 24 & 48 hrs), it was significantly decreased compared to control IVS. Increased concentration of withaferin A in high concentration of Pb treated IVS was quantified at E2H2T3 shoots (1.2mM of 48 hrs) (1.184 mg g<sup>-1</sup>) (Table 4.3). Similar result was reported in *Ligusticum chuanxiong* showed a reduction in its bioactive metabolites such as ferulic acid and ligustilide under Pb and Cd exposure (Srivastava and Srivastava 2010; Zeng et al. 2020;). In addition, all IVS subjected to acute toxicity studies for different treatment period (T1-T3) had more than 1.5-fold increased WFA content compared to their respective controls (Table 4.3). Further, WFA accumulation was correlated with GI accumulation. Compared to control

shoots, a maximum WFA content was found at E2H3T3 and had 1.8-fold increased WFA accumulation.

#### **4.2.1b(ii) Chronic toxicity studies**

The metal detoxification mechanism in plants is species dependant and metal specific. Normally plants have differing abilities to metal treatment with few showing increased tolerance to heavy metal/metal contaminated medium, whereas most of them find it difficult to survive due to their lower metal tolerance and resistance ability (Mukherjee et al. 2016). Further *W. somnifera* were proved to tolerate heavy metals like Cd up to 45 days period at lower concentration ( $10^{-100}\mu\text{M}$ ) which shows a well-developed adaptive and Cd detoxification mechanism in *W. somnifera* (Mishra and Singh Sangwan 2019). Therefore, in the present study, *in vitro* shoot cultures of *W. somnifera* was treated with lower concentration of heavy metals such as Ag and Pb for longer treatment period.
















#### **Silver nitrate on GI**

Treatment of IVS to Ag for longer duration resulted in a significantly increase in GI from E1D1-D3 for T4-T6 (Table 4.4). The shoots were in good health until D3 (1mM) and T6 (12 days); however significant reduction in the growth index was observed beyond D3 at all the duration (T4-T6) i.e., D4 & D5. The fact that Ag increases growth of the plant at lower concentration and decreases growth index at higher concentration was also reported by Deepthi & Satheeskumar 2015 where biomass was increased till  $2.5\mu\text{M}$   $\text{AgNO}_3$  exposed for 10 days to cell suspension cultures of *Ophiorrhiza mungos* Linn after that biomass decreased. Out of different Ag treatment, the optimum concentration of  $\text{AgNO}_3$  was found to be 1mM (D3). 1mM of  $\text{AgNO}_3$  on the 12<sup>th</sup> day produced a significantly increased GI (1.425) and biomass (14.06 g). When compare to control shoots (0.314), a 3.16-fold increase in GI was observed with  $\text{AgNO}_3$  treatment (D3T6) for prolonged duration (Table 4.2). Therefore, current study depicts the influence of increased concentration of  $\text{AgNO}_3$  detrimental effect on IVS at higher concentration irrespective of the duration (Kruszka et al. 2022).

### *Influence of duration of Ag exposure on GI*

It has been reported by many researchers that elicitor treatments are very effective in plant growth and in the induction of specialized metabolite production. The age of the plant (Rout et al. 2013), the duration of exposure of elicitors (Xiao et al. 2010), concentration of elicitors (Hassan and Islam 2021) and type of elicitor (Park et al. 2016; Mahendran et al. 2018) plays an important role in increasing the effectiveness of these experiments.

The **table 4.4** shows, significant increase in GI was observed at all time period (T4-T6) irrespective of the concentration (D1-D5). During acute treatment, due to the higher concentration of Ag in the growth medium even at lower exposure period (T1-T3), the shoots GI was found to be decreased as the concentration and duration increases. In chronic treatment, shoots were in good health and improved GI was observed up to D3 of all duration. Thus, the treatment period was positively correlated with the GI and T6 showed highest GI (1.425) compared to T4 & T5. Lowest GI (0.142) was observed at T4 treatment period (Table 4.2). Hence, T6 served as a rate-limiting duration for increased production of GI under Ag treatment of IVS of *W. somnifera*. In AgNO<sub>3</sub> elicited *Ophiorrhiza mungos* Linn cell suspension had the highest biomass at 10<sup>th</sup> day of the experiment when compared to control cultures (Deepthi & Satheeskumar 2016); on the other hand, our results shows that T5 (8 days) had shown high biomass. However, the concentration used for the aforementioned study was 25µM AgNO<sub>3</sub> and cell suspension culture was used instead of matured IVS. Similarly, in a shake flask cultures of *S. miltiorrhiza* hairy roots biomass increased at earlier stages of AgNO<sub>3</sub> treatment (Xiao et al. 2010).

| AgNO <sub>3</sub> chronic treatment (E1) | 4 DAYS (T4)   | 8 DAYS (T5)  | 12 DAYS (T6)  |
|--|---|--|---|
| 0.25mM (D1)                              | <br>E1D1T4   | <br>E1D1T5   | <br>E1D1T6   |
| 0.5mM (D2)                               | <br>E1D2T4   | <br>E1D2T5   | <br>E1D2T6   |
| 1mM (D3)                                 | <br>E1D3T4 | <br>E1D3T5 | <br>E1D3T6 |
| 2mM (D4)                                 | <br>E1D4T4 | <br>E1D4T5 | <br>E1D4T6 |
| 4mM (D5)                                 | <br>E1D5T4 | <br>E1D5T5 | <br>E1D5T6 |

E1D3T6 (1mM AgNO<sub>3</sub> for 12 days exposure) showing the maximum biomass among Ag chronic exposed shoots resulting in higher GI

Plate 4.3 Influence of AgNO<sub>3</sub> chronic treatment on *in vitro* shoots of *W. somnifera*

#### *Influence of Ag concentration on GI*

Elicitation mechanism in shoot cultures may enhance the production of secondary metabolite content in pharmacologically important medicinal plants. In addition, there are many parameters that can influence the biomass production during elicitor treatment (Mahendran et al. 2018). Elicitor concentration highly influences the biomass growth of shoot cultures. Out of different chronic concentrations studied, a linear increase in GI was observed from D1-D3 was observed beyond which the GI was decreased significantly (D4 & D5). Compared to control (0.314), 4.54-fold increase in GI was observed at D3 (1mM) (1.425) which served as rate limiting concentration in AgNO<sub>3</sub> treated IVS.

In addition, our results corroborate the studies of Al-Khayri and Naik (2020) where the date palms suspension cultures treated with different elicitors [pectin, yeast extract, salicylic acid, cadmium chloride and silver nitrate] at lower concentrations showed a positive impact on biomass accumulation, and higher concentrations suppressed cell growth significantly (Al-Khayri and Naik, 2020). Similarly, Addition of AgNO<sub>3</sub> from 10 to 40µM significantly induces shoot multiplication which resulted in increased shoot number and length compared to control shoots (Bais et al. 2000). Instead, in IVS of *W. somnifera* 1000µM (1mM) AgNO<sub>3</sub> induced shoot multiplication and increased GI. It could be in relation with the bio-accumulation/ reduction potential of the selected plant species (Das et al. 2017; Kushwaha et al. 2018). Further, the effect of Ag NPs on *Fagonia indica* resulted in increased biomass accumulation in concentration and duration dependant manner. Optimum duration for Ag NPs treatment for higher biomass yield was found to be 125 µg/mL for 40days (Begum et al. 2020). Increased biomass and growth index of *W. somnifera* shoots even at higher concentration of AgNO<sub>3</sub> (1mM) might be due to the hyperaccumulation and high reduction potential of *W. somnifera* shoots (Pasricha et al. 2021).

#### **AgNO<sub>3</sub> treatment on Withaferin A accumulation**

In the present study, influence of Ag on IVS of *W. somnifera* under low concentration exposure to prolonged duration in terms of withaferin A production was investigated. There have been many reports that elicitor treatment is effective on plant growth and in the induction of secondary

metabolite. Ag is a well-known elicitor which was used to elicit numerous secondary metabolites in plants (Açıkgöz 2020). However, at higher concentration Ag elicits more toxic to plants than at lower concentration which was reported in numerous studies (Gonçalves et al. 2019).

*W. somnifera* IVS were treated with lower concentration of AgNO<sub>3</sub> up to 4mM for a prolonged duration in chronic toxicity studies. Similar to other reports, chronic exposure of Ag to plant cultures resulted in significant increase in secondary metabolite content up to specific concentration and a duration (Zhang and Wu 2003; Mahendran et al. 2018). On a prolonged Ag treatment to IVS resulted in decreases in WFA content which is in contrary with Pb chronic studies where prolonged Pb treatment increased WFA accumulation even at higher Pb concentration (2.4mM) (following section). This may be due to the lack of proper detoxification or sequestration mechanism for Ag ions in IVS of *W. somnifera* unlike for Pb ions.

On low AgNO<sub>3</sub> treatment to prolonged period, a significant increase in WFA was observed up to the concentration of D4 and D3 for T4, T5 and T6 respectively. Among T4 shoots, maximum WFA was found to be at D4 (1.98 mg g<sup>-1</sup>) which is 2.95-fold higher than T4 control shoots. Similarly, T5 shoots had increased WFA content at D4 (2.33 mg g<sup>-1</sup>) which is 3.2-fold higher than T5 control. On the other hand, among T6 shoots, D3 (2.57mg g<sup>-1</sup>) accumulated increased WFA which is 3.36-fold higher than T6 control (Table 4.5). The study reported by Mahendran et al (2018) showed that Ag treatment for 15 days resulted in increased colchicine and thiocolchicoside content compared to 30 days treatment. Increased WFA accumulation (2.57 mg g<sup>-1</sup>) was observed in E1D3T6 shoots i.e., 1mM AgNO<sub>3</sub> treated for 12 days period shoots which is coincided with the increased GI.

#### *Influence of AgNO<sub>3</sub> concentration on WFA production*

In prolonged Ag treatment to low concentration, a significant increase in WFA accumulation was observed until E1D5 (2mM) of T4 (1.20mg g<sup>-1</sup>) & E1D4 of T5 (2.33mg g<sup>-1</sup>) period beyond which the concentration decreased (Table 4.5). However, higher WFA accumulation was found in E1D3T6 (2.57 mg g<sup>-1</sup>) shoots compared to all acute, chronic treated and control shoots and at higher

doses (E1D4T6 & E1D5T6), withaferin A yield was declined; and minimum amount was found in control (0.669 mg g<sup>-1</sup> DW). Similarly, the report on *Agastache rugosa* cell culture treated with AgNO<sub>3</sub> increased the amount of rosmarinic acid in concentration dependant manner (Park et al. 2016). Thus, E1D3T6 served as a rate-limiting/ optimum growth condition for IVS of *W. somnifera*. Similarly, E1D3T6 shoots has given higher biomass among Ag treated shoots.

Thus, the synthesis of withaferin A was accumulated similarly with rapid increase up to 1mM (E1D3) for 12 days (T6) treatment of low concentration of AgNO<sub>3</sub> for prolonged duration and 2mM (E1H3) for 48 hours (T3) treatment of high concentration of AgNO<sub>3</sub> for short duration. A sharp rise in withaferin A was started in low concentration of Ag exposure for prolonged period and 3.36-fold increase was observed at E1D3T6 shoots. A positive correlation between the secondary metabolite content and elicitor concentration was showed up to certain concentration of AgNO<sub>3</sub> (1mM for chronic and 4mM for acute). For example, 1mM AgNO<sub>3</sub> triggered higher concentrations of withaferin A than 0.5mM AgNO<sub>3</sub>, but higher concentration of AgNO<sub>3</sub> (>2.0mM; D4 & D5) failed to enhance withaferin A content in prolonged exposure period (T6). This is in accordance with the root cultures of *Perovskia abrotanoides* treated with different concentration of AgNO<sub>3</sub> (0-50µM) resulted in 25µM AgNO<sub>3</sub> being more potent concentration for the increased production of tanshinone (Zaker et al. 2015). According to the characteristic of the shoot cultures in this study, 1mM AgNO<sub>3</sub> and 12<sup>th</sup> day was chosen because the accumulation of biomass and withaferin A were both increasing fast at this exposure. The concentration of withaferin A was dependant on the elicitor concentration and exposure time which was commonly reported by other researchers (Gonçalves et al. 2019; Açıköz 2020). In the study of *Salvia virgata* Jacq. shoot cultures, the treatment of Ag ions (2.5ppm) increased rosmarinic acid content at 5<sup>th</sup> day of treatment (Attaran Dowom et al. 2017), while in *Agastache rugosa* cell culture, higher concentration of rosmarinic acid was observed at 72 hours of Ag (30mg/ml) treatment (Park et al. 2016). Thus, aforementioned studies show that increases in secondary metabolites is elicitor concentration and treatment period specific.

Thus, E1D3T6 shoots i.e., 1mM AgNO<sub>3</sub> treated for 12 days period shoots were exhibited higher WFA accumulation (2.57 mg g<sup>-1</sup>) which is coincided with the increased GI. Hence E1D3T6 was concluded as optimum growth condition for IVS of *W. somnifera* and selected for further analysis.

### **Lead acetate**
















On prolonged exposure to low concentrations of Pb, a significant increase in biomass was observed at all concentration of Pb compared to control IVS (**Table 4.4**). However, higher GI was observed at D3T6 PbEIVS compared to other chronic PbEIVS. Generally, Pb exposure causes damage to the cell membrane and all the organelles to different extents depending on the type plant species and Pb concentration ([Usman et al. 2020](#)). Even though *W. somnifera* shows hyper-accumulator capability; it has a threshold for each metal/heavy metal. We determined the maximum threshold concentration and best exposure period of Pb for *W. somnifera* shoots under *in vitro* conditions as 0.8mM (E2D5) for 12 days exposure period (T6) and hence was selected for further analysis. In a study on low Pb treatment to prolonged period, results in the symptoms are correlated with previous reports in which changes in leaf colour and width in response to Pb stress was noted in *Avicennia marina* ([Yan et al. 2010](#)).

The evaluation of *W. somnifera* shoot growth parameters suggests that Pb has no adverse effects on the cultures at concentrations up to 0.8mM (E2D5). Thus, the 0.8mM Pb concentration was determined as a maximum threshold concentration for *W. somnifera* shoots cultures (E2D5). Among the five concentrations tested for chronic toxicity studies (D1-D5), GI increase positively correlated with all concentration at T4 & T5 period. However, maximum GI was observed at D4 (2.211) of T6 instead of D5 (2.160). Though increased biomass was observed at D5 treated shoots of T6, decrease in GI was observed at the same. Thus, biomass growth of *W. somnifera* shoots grown in suspension media supplemented with PbAc highly depends on the salt concentration and incubation time. This is in line with a study by [Deepthi and Satheeshkumar \(2016\)](#), where cell growth of *Ophiorrhiza mungos* in cell suspension media was highly dependent on the elicitor concentration, incubation time, and feeding time of the elicitors (yeast extract and silver).

Thus, the current results indicate that biomass (fresh weight) significantly correlates with long time exposure to lower Pb concentration. This result was in agreement with (Yan et al. 2010), where they observed that *Avicennia marina* plantlets exposed to Pb had a higher growth pattern at lower Pb concentration but a lower growth pattern at higher Pb concentration. Further, in D5T6 PbEIVS had higher biomass and lower GI but healthy greenish shoot development was observed with no visible toxicity symptoms. Therefore, for further studies D5T6 shoots were chosen instead of D4T6 (higher GI yielding) PbEIVS.

#### *Influence of duration of Pb exposure on GI*

In the current study, all Pb treatment periods (T4-T6) were positively correlated with increase in GI. Compared to T4 & T5, T6 duration had high GI at all chronic Pb concentration. Further, almost two-fold increase GI was observed between T4 and T6 duration (Table 4.3). Therefore, among all Pb treatment, T6 served as rate limiting duration which displays higher GI. However, in ANOVA results, T5 ( $F=582.15$ ) displays higher effect on GI compared to T6 ( $F=395.84$ ). This is in contrary with most of the reported results regarding Pb treatment on seedlings of the plant/ plant tissues. Several studies reported that Pb causes growth inhibition, decreases fresh biomass and growth index (Saifullah et al. 2016; Ni'am and Yuniati et al. 2021). However, only few studies reported on Pb induces growth in plants at lower concentrations (Deepthi and Satheeshkumar 2016). Further, other heavy metals like Cd treatment on *Solanum nigrum* causes increased biomass production and exhibits high tolerance against Cd *in vitro* (Ji et al. 2011). Therefore, high GI yielding shoots from T6 of chronic treatment was taken as optimum exposure period. Similar increase in plant biomass was observed in (Yan et al. 2010).

| PbAc <sub>2</sub> chronic treatment (E2) | 4 DAYS (T4)   | 8 DAYS (T5)  | 12 DAYS (T6)  |
|--|---|--|---|
| 0.05mM (D1)                              | <br>E2D1T4   | <br>E2D1T5   | <br>E2D1T6   |
| 0.1mM (D2)                               | <br>E2D2T4   | <br>E2D2T5   | <br>E2D2T6   |
| 0.2mM (D3)                               | <br>E2D3T4 | <br>E2D3T5 | <br>E2D3T6 |
| 0.4mM (D4)                               | <br>E2D4T4 | <br>E2D4T5 | <br>E2D4T6 |
| 0.8mM (D5)                               | <br>E2D5T4 | <br>E2D5T5 | <br>E2D5T6 |

E2D5T6 (2mM PbAc for 12 days exposure) showing the maximum biomass results in the higher GI

**Plate 4.4 Influence of PbAc chronic treatment on *in vitro* shoots of *W. somnifera***

#### *Influence of Pb concentration of GI*

Among Pb concentration studied, a linear increase in biomass was observed from D1 to D5 (0.05-0.8mM) except at higher duration T6 (12 days), a significant decrease in biomass was observed at D5. D4 for T6 (2.211) duration exhibits higher GI compared to control and other Pb treated IVSs. Almost seven-fold increase in biomass was observed at D4T6 as compared to control (0.314). Similarly, almost two-fold increase in GI was observed between T4 &T5 and T6.

Our results are corroborating with the study on abiotic metal elicitors effect on biomass accumulation in *F. indica* callus cultures is concentration dependant manner. Even higher concentration (125µg/ml- Ag NPs) of elicitor resulted in higher biomass (20g) compared to control (19.1) callus cultures (Begum et al. 2020).

#### **PbAc treatment on withaferin A accumulation**

All PbAc treated *in vitro* shoots were harvested after a specific treatment period (Table 3.1) and subjected to HPTLC analysis for withaferin A (WFA) quantification. Pb was known to increase secondary metabolites content in plants under specific concentration and treatment period (Srivastava and Srivastava 2010; Kisa et al. 2016).

The concentration of PbAc reaching up to 0.8mM (D5) for a prolonged duration (4-12 days) was selected for chronic toxicity studies. Contrary to other reported studies (Prasad et al. 2011; Kisa et al. 2016), prolonged Pb treatment had positive impact on *W. somnifera* shoots which displays a significant well-regulated detoxification and sequestration mechanism which includes increased withanolides production. On low Pb exposure to prolonged period, a significant increase in WFA content was observed in concentration and duration dependant manner. Both concentration and duration of exposure were positively correlated with WFA content and almost 3-fold higher than respective controls (Table 4.3).

Similar trend was observed in a study on *Zea mays* treated with different concentration of Pb (0-50 mg/kg) for 14 days period resulted in maximum phenolic content at 10 mg/kg treatment for 14 days (Kisa et al. 2016). Similarly,

*Catharanthus roseus* plantlets treated with 5mM Pb for 15 days period showed significant increase in alkaloid and serpentine accumulation in roots (Srivastava and Srivastava 2010).

*Influence of PbAc concentration on Withaferin A accumulation*

WFA accumulation found to be adversely associated with the concentration of PbAc (Table 4.4). At low Pb concentration to a prolonged treatment period increases the concentration of WFA up to 3.14 mg g<sup>-1</sup> (0.8mM). In addition, increased WFA content was observed in low Pb concentration exposure to long period even with a lower GI (E2D3T6), was in accordance with the study of Pb exposure on *Mentha crispa* (Sá et al. 2013). Further, maximum WFA concentration was found in 0.8mM treated PbEIVS (E2D5T6)(3.14 mg g<sup>-1</sup>) which is 2-fold lower than WFA of field shoot (6.60 mg g<sup>-1</sup>) and four-fold higher than control IVS (0.764 mg g<sup>-1</sup>) of *W. somnifera* (Table 4.5). This study demonstrates that after a prolong Pb treatment period (T6), compared with the control, Pb can stimulate the plant growth to a certain level (0.2mM- E2D3) which is independent to WFA (0.8 mM- E2D5) accumulation in PbEIVS. Our results are in accordance with the results reported by (Yan et al. 2010)(Deepthi and Satheeshkumar 2016)(Al-Khayri and Naik 2020).

Therefore, both high and low concentration of Pb treatment to short and long treatment period were found to have stimulation effects on the WFA production with low concentration of Pb treatment being more potent. Thus, the current study showed lower concentrations of Pb to a prolonged treatment period to be more effective in the production of significantly higher concentration of WFA compared to untreated and high concentration of Pb treated IVS at any particular time. From these results, it can be concluded that 0.8mM concentration of PbAc to 12 days duration (E2D5T6) were appropriate to induce elicitation activity in IVS of *W. somnifera*. Considering the type of elicitor (biotic/ abiotic), a specific concentration and treatment period (optimum growth condition) serve as a prerequisite for the elicitation of a specific secondary metabolite production. Beyond optimum condition resulted in decreased metabolite production which may be due to the feedback inhibition (Krzyzanowska et al. 2012).

**Table 4.4 Influence of AgNO<sub>3</sub> (E1) and PbAc<sub>2</sub> (E2) chronic treatment on GI of IVS of *W. somnifera***

| Chronic treatment | T4            | T5            | T6                 |
|-------------------|---------------|---------------|--------------------|
| Control           | 0.1607±0.003a | 0.23±0.003a   | 0.3143±0.002a      |
| E1D1              | 0.2061±0.005b | 0.2658±0.01a  | 1.009±0.05b        |
| E1D2              | 0.2683±0.01c  | 0.3920±0.01c  | 1.3037±0.07c       |
| E1D3              | 0.4465±0.02e  | 0.6088±0.06d  | <b>1.425±0.05c</b> |
| E1D4              | 0.3851±0.001d | 0.3648±0.01bc | 0.8876±0.05b       |
| E1D5              | 0.142±0.03a   | 0.2776±0.01ab | 0.3626±0.01a       |
| E2D1              | 0.7471±0.01b  | 0.9440±0.01b  | 1.5846±0.04b       |
| E2D2              | 0.7678±0.01b  | 1.4222±0.04c  | 1.8772±0.04c       |
| E2D3              | 0.8325±0.003c | 1.5255±0.03d  | 2.1807±0.05d       |
| E2D4              | 1.1554±0.01d  | 1.5557±0.02d  | <b>2.211±0.04d</b> |
| E2D5              | 1.2883±0.01e  | 1.6602±0.01e  | 2.1601±0.02d       |

Values are presented as means ± SE of three replicates. Within each column, means followed by the same letter are not statistically different at  $p \leq 0.05$  according to DMRT. The mean difference is significant at the 0.05 level

**Table 4.5 Influence of AgNO<sub>3</sub> (E1) and PbAc<sub>2</sub> (E2) chronic treatment on withaferin A production in IVS of *W. somnifera***

| Chronic treatment | Withaferin A (mg g <sup>-1</sup> ) |              |                    |
|-------------------|------------------------------------|--------------|--------------------|
|                   | T4                                 | T5           | T6                 |
| Control           | 0.669±0.01a                        | 0.722±0.01a  | 0.764±0.02a        |
| E1D1              | 1.263±0.02b                        | 1.429±0.06b  | 1.916±0.03bc       |
| E1D2              | 1.599±0.04c                        | 1.688±0.09c  | 2.297±0.12d        |
| E1D3              | 1.776±0.05d                        | 2.204±0.09de | <b>2.568±0.08e</b> |
| E1D4              | 1.982±0.03e                        | 2.328±0.09e  | 2.107±0.13cd       |
| E1D5              | 1.204±0.01b                        | 1.957±0.09d  | 1.731±0.04b        |
| E2D1              | 1.467±0.05b                        | 1.509±0.08b  | 1.858±0.05b        |
| E2D2              | 1.715±0.07c                        | 1.896±0.07c  | 2.105±0.07c        |
| E2D3              | 1.849±0.05c                        | 2.175±0.02d  | 2.404±0.10d        |
| E2D4              | 2.036±0.07d                        | 2.175±0.03e  | 2.713±0.06e        |
| E2D5              | 2.188±0.03d                        | 2.175±0.04e  | <b>3.137±0.01f</b> |

Values are presented as means ± SE of three replicates. Within each column, means followed by the same letter are not statistically different at  $p \leq 0.05$  according to DMRT. The mean difference is significant at the 0.05 level.

The current study reports that, more than 1.2mM PbAc concentration was found to be less tolerable to IVS of *W. somnifera*. Our results were similar to a study reported on Pb elicitation (1800mg/kg) of *Mentha crispa* L. resulted in the increased production of carvone (85-95%) compared to control plants (39.31%) (Sá et al. 2013). Similar trend was observed where lower concentration of Pb (25 mg/kg) elicited secondary metabolism in *Ocimum basilicum* L. resulted in increased production of methyl chavicol (Prasad et al. 2011). However, in our present study *W. somnifera* was found to be tolerable to Pb up to the concentration of 0.8mM (256 mg g<sup>-1</sup>) which is higher than other reported studies (Prasad et al. 2011; Kisa et al. 2016). This may be due to the hyperaccumulation capability of *W. somnifera* shoots.

Hence, Pb treatment to longer duration resulted in higher secondary metabolite content which was reported by many studies. Thus, current results indicates that lo concentration of Pb treatment resulted in the increased accumulation of WFA content (3.14 mg g<sup>-1</sup>) compared to high concentration of Pb treated (1.184 mg g<sup>-1</sup>) and control (0.765 mg g<sup>-1</sup>) IVS of *W. somnifera*.

Thus, 0.8mM (256 mg/kg) PbAc treated *in vitro* shoots of *W. somnifera* for 12 days treatment was concluded as optimum growth/elicitation condition for increased withanolides production.

#### **4.2.1c Comparative profiling of withanolides in *in vitro* and field grown tissues of *W. somnifera* (HPTLC)**

##### **Optimum AgNO<sub>3</sub> and PbAc treatment condition on withanolides accumulation**

###### *AgNO<sub>3</sub> treatment*

The study discusses the accumulation pattern of different withanolides in E1D3T6 shoots (optimum silver treatment), control IVS, field shoot and field root of *W. somnifera*. The accumulation of withanolides in Ag treated shoots was found to be higher for withaferin A and withanolide A and lower for withanone compared to control shoots. The production of secondary metabolites in plants is greatly influenced by type of elicitor used, feeding time of elicitor, duration of elicitation and type of plant species. In addition, the type of culture conditions used for the elicitation irrespective to type of elicitors used

may results in the identification of optimum growth condition (Zafari et al. 2016). Thus, identification of optimum growth conditions and enhancing specific secondary metabolite content for a specific plant is also dependant on type of plant culture (Açıkgöz et al. 2020) and type of elicitor specific (Park et al. 2016; Gonçalves et al. 2019). In the present study, optimum growth condition for Ag treatment in IVS of *W. somnifera* exhibiting higher GI and WFA was found to be 1mM AgNO<sub>3</sub> treatment for 12 days.

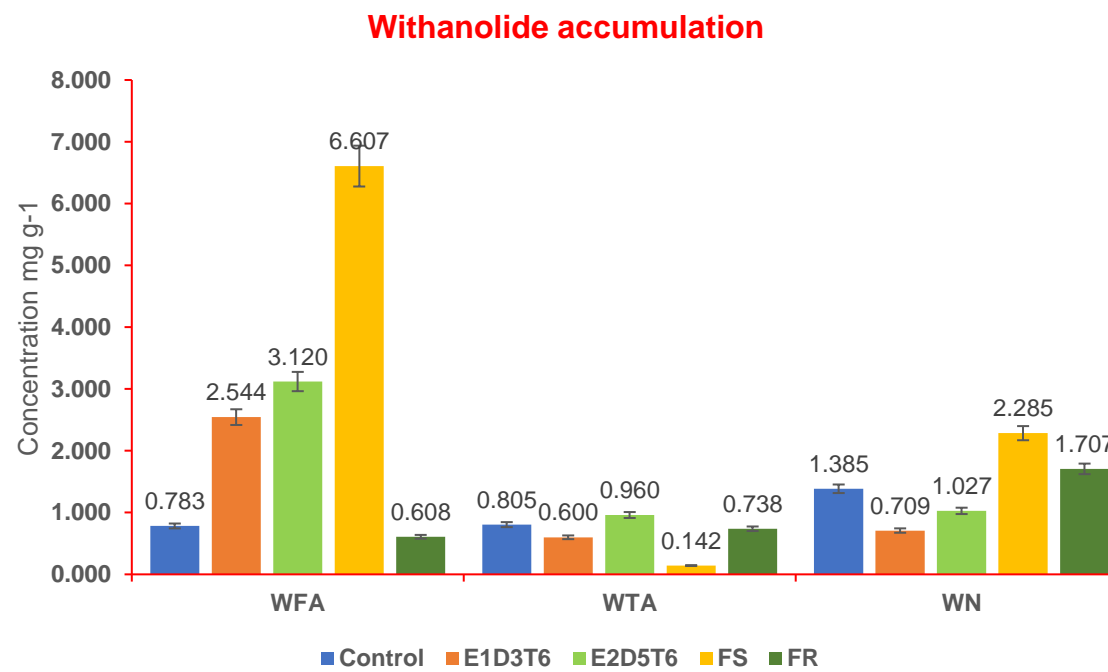
The exact mechanism of these abiotic elicitors action in promoting yield is not known yet but it probably up regulates genes encoding enzymes like sterol  $\Delta$ -7 reductase or enzymes of DOXP pathway which participate in withanogenesis (Agarwal et al. 2017). This was proved in hairy root culture of *S. miltiorrhiza* where the silver ions found to stimulate the production of tanshinones which can be dependent on both mevalonate and non-mevalonate pathways, due to induction of key enzymes (Ge and Wu 2005). Thus, the results obtained in the present experimentation indicate that abiotic elicitation could be a promising approach for triterpenoid (withanolides) enrichment. The process could also utilize *in vitro* shoots at large scale for production of pharmacologically important compounds i.e., withanolides.

#### *PbAc treatment*

Though various elicitors have proved to be effective to increase biomass and secondary metabolite accumulation in plants by many researchers, it is to be noted that the effect of elicitation on plants is also greatly influenced by the culture conditions (Kisa et al. 2016; Zafari et al. 2016). PbAc treatment to *W. somnifera* shoots encourages shoot growth and development up to 1.2 mM for 48 hrs (E2H3T3) in acute studies and 0.8mM for 12 days (E2D5T6) in chronic studies which also consistent with WFA accumulation. Further, chronic shoots exhibited more biomass and WFA accumulation compared to acute and control IVS. Thus, 0.8mM PbAc for 12 days treatment period established as an optimum exposure condition for IVS of *W. somnifera*. The above said culture conditions displayed higher growth index (2.211) and WFA (3.14 mg g<sup>-1</sup>) accumulation than other PbAc treated and control shoots (Figure 4.5). Similar trend was observed in *Prosopis farcta* seedlings treated with 0.4mM concentration of Pb for 4 days resulted in increased total phenolic content

(Zafari et al. 2016) which was similar to our results where 0.8mM resulted in increased WFA production in IVS of *W. somnifera*.

Therefore, E1D3T6 and E2D5T6 shoots were selected for further studies along with control IVS. The quantification of withaferin A (WFA), withanolide A (WTA) and withanone (WN) in the *in vitro* control, E1D3T6, E2D5T6 shoots in comparison with field shoot (FS) and field root (FR) were performed using HPTLC analysis against their respective standards. The standards resolved at R<sub>f</sub> (retention factor) 0.30 for withaferin A at 223nm, 0.46 for withanolide A at 234nm and 0.41 for withanone at 231nm. Further, PbAc treatment on IVS resulted in increased WFA (3.14 mg g<sup>-1</sup>) and WTA (0.96 mg g<sup>-1</sup>) accumulation but decreased WN (1.03 mg g<sup>-1</sup>) than control shoots (0.815; 0.805; 1.385 mg g<sup>-1</sup>) (**Figure 4.5**). Similarly, in AgNO<sub>3</sub> treated shoots (E1D3T6), increased concentration of WFA (2.54 mg g<sup>-1</sup>) and decreased WTA (0.600 mg g<sup>-1</sup>) and WN (0.709 mg g<sup>-1</sup>) than control was observed (**Figure 4.5**). Same trend was observed in study reported by Cai et al (2017) where MeJA (methyl jasmonic acid) was proved to be the best elicitor for furanocoumarin production and CaCl<sub>2</sub> and AgNO<sub>3</sub> found to had very little effect on furanocoumarin production in *Changium myrnioides* suspension cells. The result was coincided with our results where PbAc treatment decreases WN production *in vitro*. Many other studies detailed on the elicitor specific increase in secondary metabolites in plants (Kisa et al. 2016; Cai et al. 2017; Huang et al. 2021).



**Figure 4.5** Quantification of selected withanolides by HPTLC

#### **4.2.1d Comparative metabolic profiling of *in vitro* shoots of *W. somnifera* with field grown tissues (GC-MS)**

Every plant has a unique metabolite profile that could vary based on age of the plant, due to environmental stressors, type of tissues/plant species etc. Metabolic profiling of a plant dictates its biological activity, and its therapeutics. Here, metabolite profiling of CT6 (*in vitro* untreated control shoots), E1D3T6, E2D5T6 were done to identify the differential metabolite composition of *W. somnifera* under heavy metal stress using GC-MS analysis. In GC-MS, every metabolite has a unique ionic fragment, based on these fragments' m/z ratio, probability of metabolite can be identified using NIST library. In addition, peak area, area percentage and retention time of each metabolite were retrieved using AMDIS (Automated Mass Spectral Deconvolution and Identification System) software. The GC-MS chromatogram of all the analysed samples is given in [Appendix 2](#). A total of 32 metabolites comprising organic acids, sugars, alcohols, amino acids and other metabolites like squalene, neophytadiene were detected in all the analysed plant extracts ([Table 4.6](#)). The major role of neophytadiene in a biological system includes anti-inflammatory and anti-microbial activity ([Gonzalez-Rivera et al. 2023](#)). Similarly, the presence of neophytadiene in the methanolic extract of *Crataeva nurvala* was reported by [Moniruzzaman et al \(2018\)](#).

Squalene, an important precursor for the biosynthesis of triterpenoids including withanolides, sterols etc ([Grover et al. 2013](#)) was also found in all the analysed sample extracts. Aziridines are a reactive intermediate in the amino acid derivatives and its biological activity includes anti-microbial, anti-tumour activity. Though, aziridines are a synthetic compound, they also found in the various plant species. A study on *Pleurotus ostreatus* reported that higher amount (peak area: 56%) of aziridine was found in its n-hexane extracts ([Usman et al. 2023](#)). The presence of aziridine derivate was also reported in the methanolic leaf extract of *Azadirachta indica* ([Madhavan 2021](#)). Apart from this, the presence of phytol, phenol, eicosane, isovanillic acid and alanine was found in metal treated and field grown tissues of *W. somnifera* and absent in the *in vitro* control shoot extracts. Phytol is a type of terpene with anxiety relieving, anti-inflammatory activities and also precursors for vitamin E and K1

biosynthesis. Phenol is another important secondary metabolite in the plants having antiseptic, anti-inflammatory and antimicrobial activities and mainly present in the plants, microorganisms and marine organisms (Al-Owaisi et al. 2014). Eicosane is biological active lipid-based molecules involved in the innate immunity responses and found to present in certain plant parts including *Allium Chinese* (Rhetso et al. 2020). Isovanillic acid (a phenolic compound) is commonly found in crops, fruits and vegetables reported to have cytotoxicity, antimicrobial, antioxidant activities (Tavanappanavar et al. 2024; Matejczyk et al. 2024). The presence of specialized metabolites like phytol, eicosane, phenol, isovanillic acid in metal treated alone shows the effect of metal elicitation on specialized mechanisms of *W. somnifera*.

The concentration of organic acids including propanoic acid, pthalic acid, linoelaidic acid, stearic acid myristic acid and succinic acid is increased from CT6 to metal salts treated shoots extracts (E1D3T6 & E1D5T6). Similar increase in primary metabolites including organic acids, amino acids level was found in *Casuarina glauca* tissues under salt (NaCl) stress till specific concentration (200mM) (Jorge et al. 2017). This proves that the plants have a flexibility to alter its metabolic composition and reconfigure central metabolic pathways to withstand environmental stressors (Jorge et al. 2016) like heavy metal exposure in the present study.

Myo-inositol is an important signalling molecule in plants and a biologically active molecule reported to have improving metabolic syndrome like diabetes and polycystic ovary syndrome (DiNicolantonio and H O'Keefe 2022). An increased concentration of myo-inositol was found in metal treated tissue extracts of *W. somnifera* compared to CT6. Compared to *in vitro* shoots, field shoot extracts accumulated high myo-inositol level. Similarly, an increase in myo-inositol level was found in *Solanum pimpinellifolium* under salt stress (Razali et al. 2018). Thus, the metabolite profiling of CT6, E1D3T6 and E2D5T6 and FGS, FGR showed clear distinction in the metabolite composition and also accumulation of a few specialized metabolites was found only during metal treatment (E1D3T6 and E2D5T6).

Table 4.6 Relative percentage of the compounds detected in *in vitro* and field samples by GC-MS

| Class         | Compounds             | KEGG/ ChEBI ID | RT (min) | Fragmentation     | IVS          | E1D3T6 | E2D5T6 | FS     | FR     |
|---------------|-----------------------|----------------|----------|-------------------|--------------|--------|--------|--------|--------|
|               |                       |                |          | ion (m/z)         |              |        |        |        |        |
| Organic acids | Propanoic acid        | C00163         | 10.379   | 73, 147, 117      | 0.0340       | 0.0135 | 0.0151 | 0.0240 | 0.0010 |
|               | Phthalic acid         | C01606         | 22.001   | 149, 177, 127     | 0.0048       | 0.0058 | 0.0088 | 0.0020 | 0.0015 |
|               | Palmitic acid         | C00249         | 28.181   | 77, 57, 60        | 0.2240       | 0.1740 | 0.4256 | 0.0719 | 0.0825 |
|               | Linolenic acid        | C06427         | 30.399   | 79, 67, 217       | 0.0025       | 0.0195 | 0.0186 | 0.0853 | -      |
|               | Linoelaidic acid      | 5283446        | 30.300   | 67, 81, 95        | 0.0051       | 0.0426 | 0.0472 | 0.0467 | -      |
|               | Stearic acid          | C01530         | 31.211   | 73, 57, 60        | 0.0492       | 0.0506 | 0.0492 | 0.1210 | 0.2410 |
|               | 4-Hydroxybutyric acid | C00989         | 41.224   | 73, 147, 75       | 0.0003       | 0.0027 | 0.0031 | 0.0011 | 0.0002 |
|               | Butanoic acid         | C00246         | 20.914   | 174, 73, 147      | 0.0870       | 0.0041 | 0.0009 | 0.0013 | 0.0016 |
|               | Oxalic acid           | C00209         | 10.807   | 71, 72, 90        | 0.0006       | 0.0022 | 0.0047 | 0.0249 | 0.0019 |
|               | Myristic acid         | C06424         | 24.914   | 60, 127, 405      | 0.0444       | 0.0256 | 0.0267 | 0.1394 | 0.2121 |
|               | Benzoic acid          | C00180         | 10.895   | 43, 74, 73        | 0.0005       | 0.0026 | 0.0023 | 0.0022 | 0.0002 |
|               | Succinic acid         | C00042         | 11.841   | 55, 45, 101       | 0.0044       | 0.0074 | 0.0236 | 0.3650 | 0.0316 |
|               | Valeric acid          | C00803         | 22.488   | 60, 73            | -            | 0.0037 | 0.0018 | 0.0027 | 0.0006 |
|               | Alcohols              | Glycerol       | C00116   | 15.591            | 73, 147, 205 | 0.7901 | 0.0102 | 0.4260 | 0.0181 |
| Myo-inositol  |                       | C00137         | 30.786   | 73, 217, 305      | 0.3189       | 1.1280 | 3.8910 | 0.0528 | 0.0772 |
| Phytol        |                       | C01389         | 30.574   | 73, 71, 305       | -            | 0.0234 | 0.0276 | 0.0620 | 0.0830 |
| Sugars        | D-Glucose             | C00221         | 27.818   | 73, 147, 205, 160 | 0.4030       | 0.0662 | 2.7300 | 0.1400 | 0.1310 |
|               | D-Galactose           | C00984         | 28.485   | 73, 205, 319      | 0.4150       | 1.3400 | 2.2700 | 0.1170 | 0.7250 |
|               | Mannose               | C00936         | 28.173   | 73, 204, 147      | 1.1400       | 0.0287 | 0.0815 | 0.2440 | 0.0130 |
|               | D-Fructose            | C05003         | 26.267   | 73, 204, 437      | 0.0172       | 0.1390 | 0.1340 | 0.0920 | 0.0211 |
|               | Glycine               | C00037         | 16.225   | 174,73,86         | 0.0031       | 0.0051 | 0.0102 | 0.0047 | 0.0031 |

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*Results and Discussion*

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|        |                  |        |        |               |        |        |        |        |        |
|--------|------------------|--------|--------|---------------|--------|--------|--------|--------|--------|
|        | L-proline        | C00148 | 17.471 | 84, 56, 85    | 0.0150 | 0.0537 | 0.0901 | 0.0172 | 0.0011 |
| Amino  | Leucine          | C00123 | 26.448 | 117, 110, 274 | 0.0065 | 0.0146 | 0.0194 | 0.0081 | 0.0017 |
| acids  | L-tryptophan     | C00078 | 36.594 | 73, 147, 259  | 0.0004 | 0.0008 | 0.0015 | 0.0002 | 0.0003 |
|        | Alanine          | C00041 | 10.811 | 90, 58, 72    | -      | 0.0010 | 0.0049 | 0.0003 | -      |
| Others | Aziridine        | C11687 | 9.8525 | 98, 99, 100   | -      | 0.0076 | 0.0074 | 0.0004 | 0.0013 |
|        | Squalene         | C00751 | 39.704 | 69, 81, 95    | 0.0078 | 0.0793 | 0.0972 | 0.0588 | 0.0043 |
|        | Eicosane         | 43619  | 24.098 | 57, 71, 85    | -      | 0.0066 | 0.0136 | 0.0013 | 0.0018 |
|        | Neophytadiene    | 145817 | 25.868 | 68, 82, 95    | 0.0173 | 0.0379 | 0.0488 | 0.0258 | 0.0213 |
|        | Ribitol          | D00061 | 26.538 | 73, 103, 147  | 0.0084 | 0.0204 | 0.0184 | 0.0049 | -      |
|        | Phenol           | C00146 | 20.381 | 191, 57, 206  | -      | 0.0015 | 0.0018 | 0.0013 | 0.0005 |
|        | Isovanillic acid | 63798  | 21.319 | 168, 153, 97  | -      | 0.0005 | 0.0005 | -      | -      |

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### **Principal component analysis (PCA)**

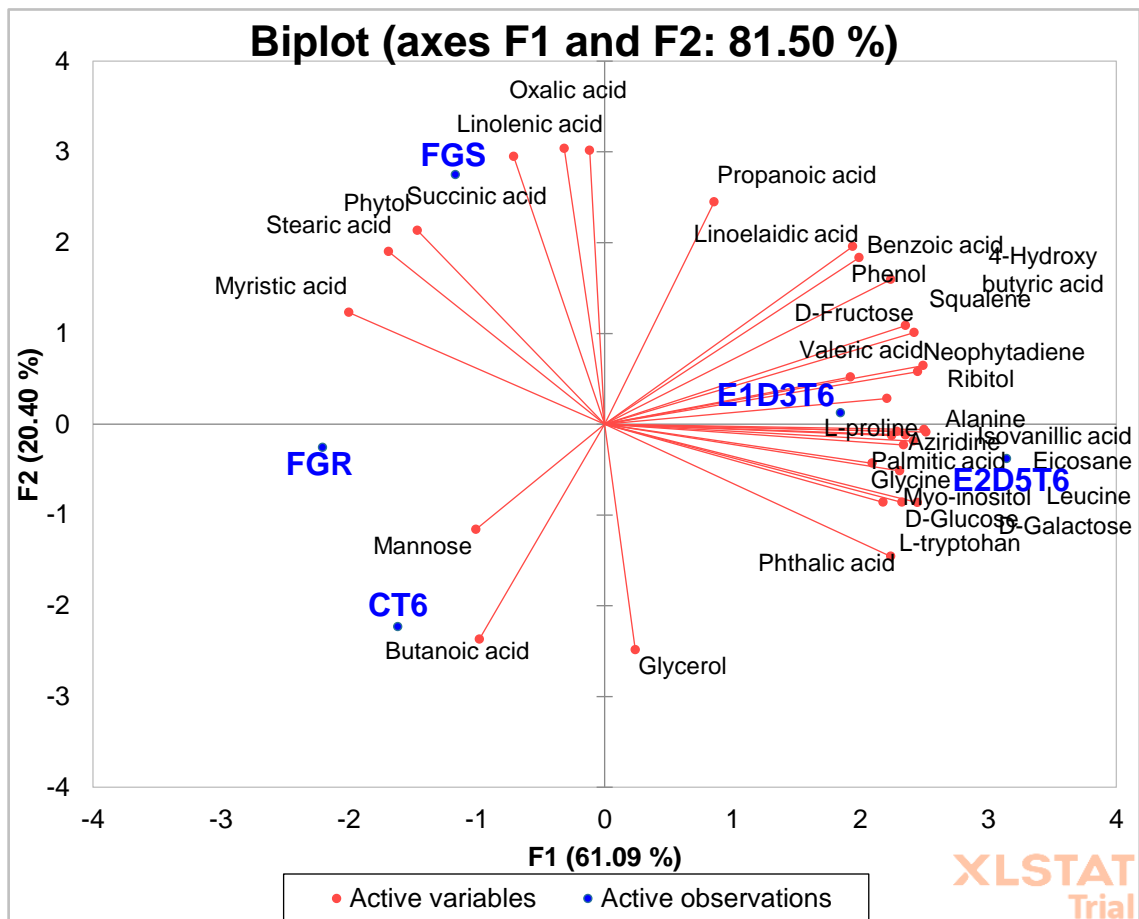
PCA was done to capture the most significant variation in the data via separating the datasets into principal components (factors/ eigen vectors). Here, large GC-MS datasets are analysed by PCA using XLSTAT and it was reduced into two major principal components comprising 81.50% of significant variance in the data (Figure). For PCA, 6 samples including *in vitro* control shoots (CT6), heavy metal treated shoots (E1D3T6 & E2D5T6) and field grown tissues (FGS & FGR) were selected. Among these sample extracts, 32 metabolites were identified and taken for PCA analysis which gave four principal components (factors). Each factor was represented by eigen value and percentage variance of total metabolite (Table 4.7). Factors with high % variance with higher eigen values tend to capture significant variance and comprises maximum contribution of metabolites (F1 & F2). The factors F1 (61.09%) and F2 (20.40%) together encompassed about 81.50% of variance in the dataset. Thus F1 & F2 selected for biplot construction with active variable (metabolites) and active observations (test sample extracts). In the biplot, F1 is represented by horizontal axis (X axis) and F2 is represented by vertical axis (Y axis). In figure 4.6, the metabolites are presented as red vectors and the length of vector indicates the quality of the metabolite in a particular factor dimension. The longer the vector better is its representation on that F dimension. Thus, the biplot shows that all the metabolites are well represented in the selected two dimensions. The biplot shows a clear distinction between the *in vitro* metal treated (E1D3T6 & E2D5T6) and CT6 and field grown samples (FGS & FGR) along the first dimension F1.

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 61.09% variation shows distinction between field samples (FGS & FGR) and metal treated samples (E1D3T6 & E2D5T6). The CT6 and FGR accounted for low metabolite content (Mannose & Butanoic acid) with low F1 & F2 values. Further, metal treated samples grouped in III (E1D3T6) and IV (E2D5T6) quadrant of the biplot with E2D5T6 with high metabolite content positive for F1 and E1D3T6 with high metabolite content positive for both F1 and F2 (Figure 4.6). The role of organic

acids in plants includes chelating agents, as carbon source under stress. Organic acids are intermediates of TCA cycle and during stress, the plant utilizes basic chemical components from these organic acids which help in growth and development of the host (Panchal et al. 2021).

**Table 4.7 Eigen values and percentage variability of the principal components**

| Principal Components (F) | Eigenvalue | Variability (%) | Cumulative % |
|--------------------------|------------|-----------------|--------------|
| F1                       | 19.550     | 61.094          | 61.094       |
| F2                       | 6.529      | 20.405          | 81.499       |
| F3                       | 3.640      | 11.375          | 92.874       |
| F4                       | 2.280      | 7.126           | 100.000      |



**Figure 4.6 PCA biplot showing distinction between *in vitro* control (CT6) and metal treated shoots and field samples of *W. somnifera***

Depending upon the plant species and type of stress conditions, the specific organic acid pathway is either up-regulated or down-regulated. Similarly various organic acids such as propanoic acid, valeric acid, benzoic acid, butyric acid is positive for both F1 & F2 showing increased accumulation of these organic acids in the metal treated samples (E1D3T6 & E2D5T6). Valeric acid helps plant to withstand abiotic stress reposes such as salt stress, and other environmental stressors. Under stress conditions, propanoic acid, valeric acid, benzoic acid and other organic acids are found to increase nutrient uptake, regulate plant metabolism and help the plants to cope with stress (Ellenita Gibe De Castro et al. 2020). Further, increased accumulation of organic acids such as isovanillic acid, palmitic acid, pththalic acid was found in E2D5T6 is positive for F1. The concentration of these organic acids helps the E2D5T6 shoots to cope with heavy metal, Pb induced stress. In correlation with our results, an increase in oxalic acid level was found in rice plants and an increase in citrate level was found in *A. thaliana* which results in the Pb tolerance (Osmolovskaya et al. 2018). Thus, heavy metals such as Ag and Pb stress on *in vitro* shoots of *W. somnifera* results in the metal specific increase in organic acids. Compared to CT6, an increased accumulation of organic acids was found in the E1D3T6 and E2D5T6. Similar metal stressor specific increase in organic acids level was reported by Dresler et al (2014).

#### **4.2.2 Bioaccumulation and metal reduction analysis on Pb exposed *in vitro* shoots (PbEIVS) of *W. somnifera***

##### **4.2.2a Bioaccumulation of Pb and other essential metals in PbEIVS of *W. somnifera* (ICP MS)**

The treatment of E2D5T6 shoots was found to be significantly performing well in terms of GI compared to other treatment. Thus, E2D5T6 shoots were analysed for bioaccumulated Pb level. Elemental composition of *W. somnifera* E2D5T6 and control (untreated) IVS (CT6) was analysed using inductively coupled plasma mass spectrometry (ICP MS). Including essential metals, non-essential metals, essential and non-essential heavy metals for plant growth, a total of 22 elements were quantified in *W. somnifera* shoot tissues. It is found that E2D5T6 shoots accumulated 405 mg kg<sup>-1</sup> Pb in its dry

weight of the tissue accounting to 15.14% of the elements analysed. As expected, control shoots are void of Pb.

For medicinal plants, the maximum permissible limit for heavy/trace metals is greater, than that permitted for edible plants. The European Pharmacopoeia states that the following MPL are readily available:  $1\ \mu\text{g g}^{-1}$  Cd,  $2\ \mu\text{g g}^{-1}$  Cr,  $0.1\ \mu\text{g g}^{-1}$  Hg, and  $5\ \mu\text{g g}^{-1}$  Pb (Marinescu 2020). Further, the Chinese Pharmacopoeia has established the following MPL:  $2\ \mu\text{g g}^{-1}$  As,  $0.3\ \mu\text{g g}^{-1}$  Cd,  $20\ \mu\text{g g}^{-1}$  Cu,  $0.2\ \mu\text{g g}^{-1}$  Hg (Li et al. 2012) and WHO/FDA recommends  $10\ \mu\text{g g}^{-1}$  As,  $0.2\ \mu\text{g g}^{-1}$  Cd,  $20\ \mu\text{g g}^{-1}$  Cu,  $1\ \mu\text{g g}^{-1}$  Hg,  $10\ \mu\text{g g}^{-1}$  Pb,  $50\ \mu\text{g g}^{-1}$  Zn (WHO guidelines 2007). The metal toxicity in plants is reported to inhibit plant growth and development, alteration in anti-oxidant enzyme production and photosynthesis and increased ROS production etc. On the other hand, some plants have tolerance against heavy metals using their intrinsic metal detoxification and sequestration mechanisms like vacuolar capture (Wang et al. 2021).

In our results, control IVS (CT6) contains all heavy and trace metals within permissible limit set by WHO/FDA. The shoots of selected treatment E2D5T6 contains all the metals within MPL except Pb. E2D5T6 bioaccumulated  $405.88\ \text{mg kg}^{-1}$  Pb in their leaf tissues within 12 days treatment period which is 40 times higher than MPL set for medicinal plants by WHO in different countries (WHO guidelines 2007) (more than 400-fold higher as compared to control). The same effect was obtained when the *Ocimum basilicum* L. was exposed to Pb results in the accumulation of  $407\ \text{mg kg}^{-1}$  Pb in their leaf tissues (Dinu et al. 2020). In another study on *W. somnifera* *in vitro* shoots exposed to  $500\ \mu\text{M}$  of Cd concentration resulted in the 2000-fold accumulation compared to control shoots within 30 days treatment period (Mishra et al. 2014). The above study proves that *W. somnifera* shoots have the hyperaccumulation ability regards to heavy metals such as Pb and Cd. Despite higher Pb concentration in their tissues, the shoots were in good shape and healthy (Plate 4.3 & 4.4). This may be attributed to the higher concentration of macronutrients K (24779 ppm), Ca (1786 ppm), & Fe (82 ppm) in E2D5T6 shoots that functioning as a protector during heavy metal stress

(Tripathi et al. 2014). Potassium (K) is found to be the most abundant element in the analysed Pb treated samples followed by Ca, P, etc. However, exposure to lethal concentration of Cd ( $150^{-1}000\mu\text{M}$ ) to *in vitro* regenerated *W. somnifera* shoots for 30 days exerted N, P and K deficiency resulted in the shoot growth retardation (Mishra et al. 2014); in contrast, our results exhibit, increase in the macronutrient levels (K & Ca) when exposed to higher concentration of Pb over a prolonged treatment period (Table 4.8).

**Table 4.8 Elemental composition of Pb treated *in vitro* *W. somnifera* shoots and MPL in medicinal plants**

| S. no.                        | Parameters        | CT6<br>(mg kg <sup>-1</sup> ) | E2D5T6<br>(mg kg <sup>-1</sup> ) | Permissible Limit<br>(mg kg <sup>-1</sup> ) |
|-------------------------------|-------------------|-------------------------------|----------------------------------|---|
| <b>Essential Metals</b>       |                   |                               |                                  |   |
| 1                             | 11B (Boron)       | 21.2635                       | 15.001                           | Nil   |
| 2                             | 24Mg (Magnesium)  | 1282.317                      | 769.07                           | Nil   |
| 3                             | 31P (Phosphorus)  | 3679.81                       | 1189.091                         | Nil   |
| 4                             | 39K (Potassium)   | 21109.36                      | 24779.14                         | Nil   |
| 5                             | 44Ca (Calcium)    | 1411.681                      | 1786.925                         | Nil   |
| 6                             | 55Mn (Manganese)  | 195.5545                      | 125.747                          | Nil   |
| 7                             | 57Fe (Iron)       | 11.203                        | 82.102                           | Nil   |
| 8                             | 95Mo (Molybdenum) | 2.803                         | 1.199                            | Nil   |
| <b>Essential Heavy Metals</b> |                   |                               |                                  |   |
| 1                             | 60Ni (Nickel)     | 0.0032                        | 0.079                            | 1.5 (WHO,2005)                              |
| 2                             | 63Cu (Copper)     | 2.0273                        | 0.501                            | 20 (WHO/FDA)                                |
| 3                             | 66Zn (Zinc)       | 42.2345                       | 21.274                           | 50 (WHO/FDA)                                |
| 4                             | 48Ti (Titanium)   | Nil                           | Nil                              | Nil   |
| <b>Heavy Metals</b>           |                   |                               |                                  |   |
| 1                             | 52Cr (Chromium)   | 0.0076                        | 0.0018                           | 2 (EP)                                      |
| 2                             | 59Co (Cobalt)     | 0.1452                        | 0.149                            | 5 (USP)                                     |
| 3                             | 75As (Arsenic)    | 0.0045                        | 0.003                            | 10 (WHO/FDA)                                |
| 4                             | 111Cd (Cadmium)   | 0.0055                        | 0.003                            | 0.2 (WHO/FDA)                               |
| 5                             | 202Hg (Mercury)   | 0.004                         | Nil                              | 1 (WHO/FDA)                                 |
| 6                             | 107Ag (Silver)    | Nil                           | Nil                              | Nil   |
| 7                             | 208Pb (Lead)      | 0.0279                        | 405.886                          | 10 (WHO/FDA)                                |
| 8                             | 51V (Vanadium)    | Nil                           | Nil                              | 30 (US EPA)                                 |
| 9                             | 7 Li (Lithium)    | Nil                           | Nil                              | 0.075                                       |
| 10                            | 121Sb (Antimony)  | Nil                           | Nil                              | Nil   |

EP – European Pharmacopeia; WHO/FDA - World Health Organization / Food and Drug Administration; US EPA - United States Environmental Protection Agency; USP – United States Pharmacopeia.

### **Importance of essential trace elements accumulation during heavy metal stress in plants**

Potassium (K), calcium (Ca) and iron (Fe) are three important essential trace elements for plant growth and development and also these metals are found to be increased during heavy metal stress in plants (Tripathi et al. 2014). K is an essential macronutrient for plant growth which protect plant from biotic and abiotic stresses (Wang et al. 2013; Karthika et al. 2018). During heavy metal stress, the role of K includes maintaining ion homeostasis and osmotic balance (Johnson et al. 2022), reduces ROS accumulation which results from heavy metal stress, enhanced production of secondary metabolites like flavonoids and phenols in plants and retains photosynthetic electron transport chain activity etc., (Dhiman et al. 2022). The concentration of potassium in control shoot (21109.36 mg kg<sup>-1</sup>) is increased after Pb treatment (24779.14 mg kg<sup>-1</sup>) (Table 4.8). Similarly, a study on Zahedifar et al. 2018 shows that significant role of K in basil plants where Cd distribution was negatively correlated with K content in the soil. Heavy metals Cd and Zn resistance was increased in sunflower when early addition of K than later applications during heavy metal stress (Elouear et al. 2016). Thus, K has an incomparable role in minimizing heavy metal stress in plants as reflected here.

Calcium (Ca) is another important element in alleviating plant growth inhibition during abiotic stress especially heavy metal stress (Aziz et al. 2015). Ca level in control shoot is 1411.68 mg kg<sup>-1</sup> which significantly increased after Pb treatment (1786.93 mg kg<sup>-1</sup>) (E2D5T6) (Table 4.8). It has been found that Ca is positively correlated with plant growth and biomass production under metal stress (Huang et al. 2017) which can provide plausible reasoning for increases in the biomass during Pb exposure in our study (section 4.2.1a). In addition, Ca also acts as a secondary messenger which involved in calcium-calmodulin pathway; thus, increase in Ca ion activates a protein calmodulin which in turn regulates heavy metal uptake, transport and metabolism in plants (Ghori et al. 2019).

Iron (Fe) is necessary for plant growth; however excessive Fe was toxic to plants. Fe is also found to alleviate Cd induced toxicity in poplar trees and

restore normal growth (Li et al. 2022). It was also found that the Fe improved plant growth, photosynthesis pigment and photosynthesis during heavy metal stress (ul Hassan et al. 2017) which correlated with the higher biomass and darkened leaves during Pb stress. Here, Fe content in control shoot was 11.2 mg kg<sup>-1</sup> which increased to 82.1 mg kg<sup>-1</sup> after Pb treatment. The induction in the Fe content during Pb stress might be because the shoots' effort to alleviate Pb toxicities like growth inhibition, decreased chlorophyll content etc. (Tripathi et al. 2018).

The concentration of Mg (769 ppm) and Mn (125) in the E2D5T6 shoots was decreased compared to CT6 (Mg: 1282 ppm; Mn: 195 ppm) (Table 4.8). Similar result was found in *Dittrichia* plants stress with Thallium (TI) leading to a decrease in Mg, K & Fe contents (Espinosa et al. 2023). In another study, Cd stress on pea plants resulted in the strong reduction in Mg, Mn & Ca (Rodríguez-Serrano et al. 2009). Thus, various studies revealed that K, Ca, & Fe alleviates heavy metal stress in plants (Tripathi et al. 2014; ul Hassan et al. 2017), in return the concentration of those metals were increased during the process. Further, IVS of *W. somnifera* exposed to Pb induced a major decrease in Mg, Mn & Zn concentration in IVS.

#### **4.2.2b *In planta* synthesis of Pb NPs in live PbEIVS of *W. somnifera* (TEM & EDAX)**

It has long been known that using plants to extract specific elements, particularly precious/ pharmacologically important metals from contaminated soils/aqueous waste could be an effective way to clean up the environment (Ghori et al. 2016). Pb was only recognized in the studies focusing on remediation and mining using plants (Hasnaoui et al. 2020). Still, there is no study describing the Pb bioaccumulation as nanomaterials i.e., *in planta* formation of lead nanoparticles (Pb NPs). The applications of metal nanoparticles (MNPs) in the nanoscience area are huge due to its remarkable properties they present; however, MNPs application in medicinal field is limited because of their chemical composition and increased reactivity (Sengul and Asmatulu 2020). The MNPs synthesized via green route using microorganisms, and plant tissue extracts (bio-MNPs) gained considerable interest in

bionanotechnology field due to their lower toxicity in a biological system (Kshtriya et al. 2021). In our study, E2D5T6 shoots were analysed for possible nanostructures using TEM analysis. We also found the presence of nanostructures in the analysed sample which was later confirmed as Pb NPs using TEM and EDAX studies. There are several reports on the applications of green synthesized Pb NPs which includes anti-oxidant, anti-inflammatory and anti-diabetic activities (Khan et al. 2023).

### **Absorption and reduction of Pb ions in PbEIVS**

After 12 days of Pb treatment, E2D5T6 shoots were harvested. This chronic exposure period facilitates the uptake of Pb via callus and translocation into shoots as Pb ions which was studied using ICP MS analysis (Table 4.5). Thus, E2D5T6 shoots were selected for ultra-structural analysis and elemental profiling.

When a plant is exposed to a heavy metal, they normally respond by either one of the following such as metal avoidance, metal tolerance and other alternatives by the production of stress related protein and specific hormone (Dalvi et al. 2013). Our study shows that the IVS of *W. somnifera* exhibits metal tolerance ability towards accumulated metal (Plate 4.3 & 4.4 and section 4.2.1a); in other words, intra-cellular metal detoxification mechanism of absorbed metals in plants were inactivated or converted to non-toxic byproducts within or outside the cells of a specific plant. It also proved from our current study that *W. somnifera* has a hyperaccumulation capability towards Lead (Pb) (heavy metal) rather an excluder (lower translocation factor >1) or indicator (metal stress causes toxic symptoms). Hyperaccumulator plants capable of translocating one or more heavy metals from soil and accumulates them in the above ground organs such as shoots/leaves, fruits rather retaining them in root tissues like excluders (Rascio and Navari-Izzo 2011; Asgari Lajayer et al. 2017). However, in a literature review by Asgari Lajayer et al. 2017, reported that a plant to be consider as a hyperaccumulator, it has to uptake and retain a specific concentration of metal (vary depending upon metal) in their greener regions. For instance, accumulation of more than or equal volume of 1000 mg/kg Pb is needed in the aerial parts of a plants to be

considered as Pb hyperaccumulator. The concentration of Pb in aerial part of PbEIVS of *W. somnifera* is found as 405 mg/kg. Further, PbEIVS found to hold 405 mg/kg (Section 4.3.1) in its leaf tissues which is much higher than non-hyperaccumulator species (<10 ppm). In addition, IVS showed no toxicity symptoms which may be due to its higher tolerance towards Pb. Thus, IVS of *W. somnifera* can be classified under a plant with a moderate hyperaccumulation capability.

Normally, metal uptake takes place in roots especially cell wall of root cells. Lead was reported to be one of the major pollutants that affects plants and environment (Kushwaha et al. 2018). There are many controversies regarding heavy metal especially, Pb uptake from the roots such as Pb normally precipitates on the root cell wall and free ions are transported by root cells via cationic exchangers on cell wall; on the other hand, there have been many reports on the crucial role of root plays in the alleviation of heavy metal toxicity by preventing their entry into the cell (Usman et al. 2020). Therefore, a clear picture on Pb absorption, translocation, sequestration and accumulation is needed to understand its specific route in plants which is discussed in following sections.

### **Absorption, translocation and sequestration of Pb ions in plants**

In this section, we are going to focus on the uptake of Pb by root cells. However, the current study emphasizes on the *in vitro* shoot cultures devoid of root system. Instead of roots, shoot cultures developed a callus system for immobilization and absorption of nutrients from culture media. Still, the uptake of Pb from soil to root cells and culture medium to callus cells both probably are the same. Therefore, the current section discusses the mechanism of uptake and translocation of heavy metals by root cells instead of callus cells. Pb uptake by the root occurs at radical cortex via binding to the -COOH group of acids or to the cell surface polysaccharides after that Pb passively penetrate the root cells then move through water (xylem cells) (Pourrut et al. 2013). On the other hand, mechanism of Pb uptake by root cells at a molecular level may vary depending upon the type of plant species. Most common and well-known mechanism is by calcium channels, a cation channel. Especially, the non-

selective cations channels such as voltage-insensitive cation channels and depolarization/hyperpolarization -activated calcium channels are reported to be the principal route for Pb ions entry point into root cells (Kushwaha et al. 2018). The inhibition of calcium or potassium ion channels via inhibitors confirms that the Pb enters through these channels into root cells (Pourrut et al. 2013). Aside from these finding, number of studies have reported other possible routes for Pb entry into plants (Wojas et al. 2007; Das et al., 2011) such as uptake and translocation of Pb using organic acids, etc.

Pb seems to be translocated to aerial part of a plant when they are in higher quantity in nutrient medium was reported by Asgari Lajayer et al. (2017). Once Pb is translocated to the apoplasts (root) it follows water flow and reaches endodermis followed by aerial part of the plants. Also, it appears that translocation of Pb using apoplastic pathway is blocked by the Casparian strips of endodermis. However, about 1% of the water flow (apoplast) to the aerial parts in the plant is bypasses the endodermis barrier in the roots which may be the predominant path for Pb translocation to aerial parts. On the other hand, in a plant with blocked apoplastic pathway, Pb translocation using water flow must travel with symplastic pathway which constitutes cell membranes and cytoplasm sequestration systems which prevents Pb ions from entering the cells. At this stage, Pb only penetrates the symplasts where cells are actively dividing i.e., apical meristem, or protoderm. The absence of secondary cell wall in young cells with thin plasma membrane make Pb to easily enter the cells; also, Pb was found to be confined to various cell compartments (vacuoles, vesicles, plasmotubules) within young symplasts (Jiang and Liu 2010; Wang et al. 2021). Still, only small amount of Pb was found to be present in symplast of a plant treated with Pb (Kupittke et al. 2007). Along with this, Pb complexed with organic acids may also could bypass the cell membrane and cytoplasmic barrier system using symplastic pathway. The exact path of Pb translocation in *W. somnifera* shoot system is yet another mystery that needs an investigation (Pourrut et al. 2013). Some studies also reported that lethal doses of Pb can penetrate most of the physical barriers including endodermis, cell membrane and enter the cytoplasm and other organelles of plant (Wang et al. 2021).

Among all the path Pb may take during translocation from medium-shoot via callus that mentioned earlier, there are few other studies reporting a synergistic effect between presence of plant growth regulator in the medium facilitated Pb translocation from root to shoot (Israr and Sahi 2008). There are many other studies also reported on the effect of plant growth regulator on heavy metal translocation and ameliorate heavy metal toxicity in plants (Xu et al. 2016; Mir et al. 2022).

#### **Detoxification mechanism in IVS of *W. somnifera***

In contrast with most reported studies, *in vitro* grown shoots of *W. somnifera* exerted high tolerance to Pb even at lethal concentration and prolonged treatment period. This shows that *W. somnifera* shoot system has well developed detoxification mechanism. Commonly detoxification occurs in plants under two stages passive and inducible mechanism. In passive mechanism, once Pb penetrates cell membrane of roots, its movement was restricted due to Pb binding with functional groups (-COOH, -OH, -SH) in cell walls especially pectin. As a result, plant can restrict movement of Pb to plasma membrane of the cell and also restricts cell toxicity (Krzyszowska et al. 2010). This may prove that Pb should be accumulated at Pb absorption site of plants in this case callus cells. On the other hand, we found that large quantity of Pb was translocated to aerial parts (shoot & leaves). This is due to the fact that callus cell walls contain minor quantity of pectin which was reported in strawberry callus cultures. Instead, cell walls of strawberry callus cell cultures had high extension content resulted in tightly cross-linked cell wall (Ric-Varas et al. 2020). The presence of these type of cell wall in callus cells of *W. somnifera* might be the fact that Pb was translocated to aerial parts of the cultures using apoplastic or symplastic pathways (previous section). The rapid deposition of extensin on callus cell walls was also observed during fungal elicitation of *Vitis vinifera* L. callus (Jackson et al. 2001). The extensive extensin deposition may stress related because induction of callus from shoot/leaf is also considered as stress to the plant cells (Ric-Varas et al. 2020).

### **In planta localization of nanostructures**

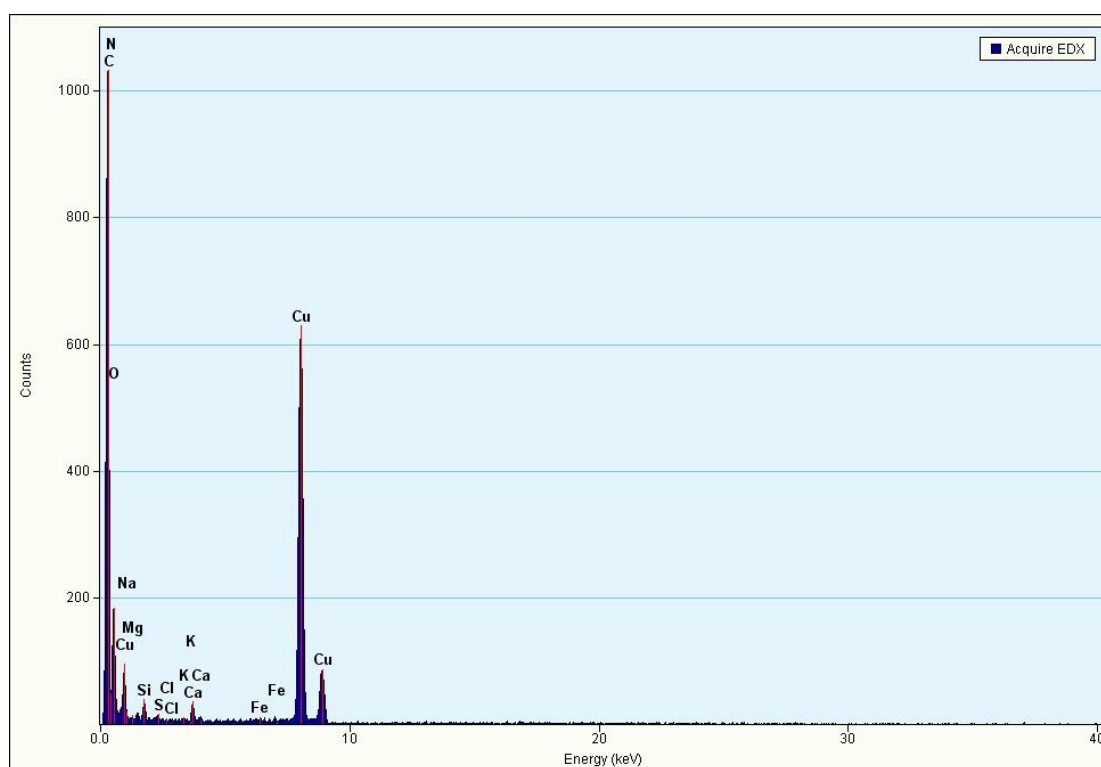
After bypassing above mentioned physical barriers, Pb was localized in the aerial part of the shoot cultures. However, the exact position of Pb inside plant cells were not studied here. Based on the previous reports, probable area for localization of accumulated Pb inside plant cells is discussed here. Plants exposed to Pb commonly accumulate Pb in their cell wall pectins' (Krzesłowska et al. 2010), intercellular spaces (Kupittke et al. 2007; Wang et al. 2021), plasma membranes, vacuolar compartmentalization (Wang et al. 2021), endoplasmic reticulum and cytoplasm (Lyubenova et al. 2012), vascular tissues of stems and leaves (Gupta et al. 2013).

### **In planta formation/synthesis of Pb NPs**

Localization of nanoparticles was assessed in the live leaf fractions of E2D5T6 *in vitro* shoot cultures of *W. somnifera*. Nanoparticles were in spherical and rod shaped and the size ranging from 25 to 40 nm (Figure 4.8). As expected, leaf fractions of *in vitro* control shoots (CT6) were void of any nanostructures (Appendix 3). The location of reduction of Pb ions that are absorbed and translocated to aerial parts such as shoots and leaves was reported in many studies. Those reports suggests that the absorbed metal gets reduced at the roots via root cell wall polysaccharides/organic acids or during translocation (root to shoot) in apoplasts/symplasts or at the greener regions (shoots and leaves) (Ric-Varas et al. 2020). The fact that nanoparticles can be formed and stored in plant tissues agrees with the reports of Marchiol et al (2014); Parker et al (2014) they suggested that live plants of *Medicago sativa*, *Brassica juncea* ad *Arabidopsis thaliana* has the ability to uptake and store metals namely Ag and PI (Palladium) as nanoparticles after treatment with the respective metal salts under *in vitro* condition. However, exact mechanism of live plant synthesis of metal nanoparticles is still largely unknown (Marchiol 2012). However, according to previous reports, the phytoconstituents (Karmous et al. 2022) present in *W. somnifera* shoots might have acted as both a reducing and stabilizing agent, which results in the formation of Pb NPs.

### Elemental composition

EDAX spectrum shows the position of various elemental peaks in control and E2D5T6 samples. More specifically, the features at the following energies- 2.4, 2.45, 2.7 keV - are present in E2D5T6 samples (Figure 4.9) alone compared to control (Figure 4.7). The energy peak at 2.4 & 2.45 keV in C5 samples confirms that Pb is in a reduced/metallic form. This is in line with Diba et al. (2021), where the Pb NPs were synthesized from two species of bacteria, *Bacillus* sp. A21 and *Oceano bacillus* sp. A22 and EDAX spectrum of the PbNPs shows that the Pb peaks were at 2.4 & 2.45 keV energy levels. In another study by Miri et al. (2018), the synthesized PbONPs peaks are present at ~2.45 keV. The biological applications and biocompatibility of synthesized PbNPs/PbONPs using green route was reported by various researchers (Miri



**Figure 4.7 EDAX spectra of control *in vitro* shoots**

at al. 2018; Açıkgöz 2020; Tailor and Lawal, 2021). According to the reports, Pb NPs/ PbONPs has higher antioxidant potential, antibiotics for the treatment of infectious diseases (Tailor and Lawal 2021), free radical scavenging activities (Açıkgöz 2020), anti-cancer and anti-diabetic activities (Miri at al. 2018; Açıkgöz 2020; Bratovcic 2020).

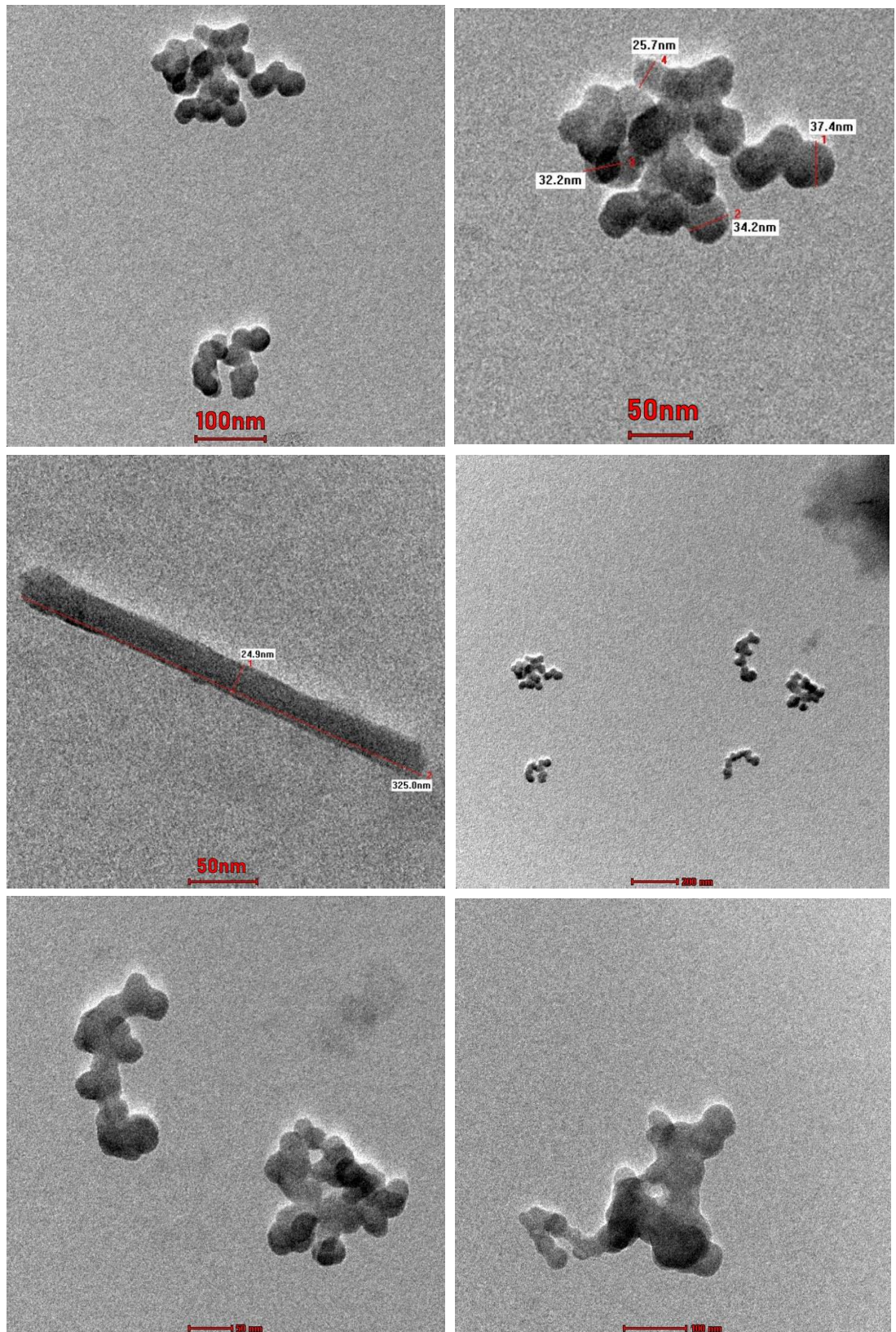
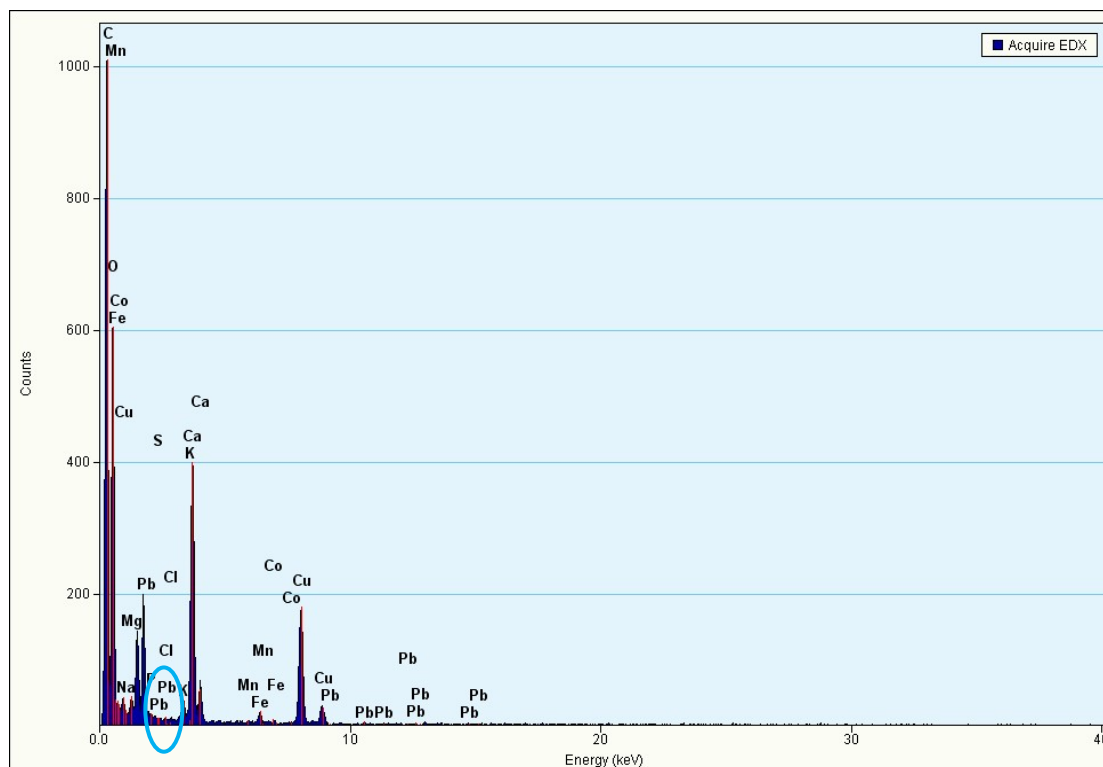


Figure 4.8 Electron micrograph of E2D5T6 leaf cells of IVS of *W. somnifera*



**Figure 4.9 EDAX spectra of E2D5T6 live tissue sample.**

The blue circle indicates that the presence of Pb at ~2.4 keV in metallic form i.e., Pb NPs

### **Nanoparticles synthesis by live plants**

There are many studies reported on metal uptake, nanoparticle synthesis and its accumulation inside the cell and cell wall of plants. The first report on nanoparticle synthesis and accumulation was published in 2002 by Gardea-Torresdey and colleagues. The study reports the ability of alfalfa plant to uptake zero valence gold from solid medium and store them as 4nm sized icosahedron shaped gold nanoparticles. The same group reported the synthesis of silver nanoparticles in the same plant in 2003 (Gardea-Torresdey et al. 2003). Following that, there are number of studies reporting the *in planta* synthesis of Ag, Au, PI (Marchiol et al. 2014) nanoparticles using plants such as *Medicago sativa*, *Brassica juncea*, *Festuca rubra* and *Arabidopsis thaliana* (Starnes et al. 2010; Taylor et al. 2014; Marchiol et al. 2014; Parker et al. 2014).

In the present study, *in planta* formation of Pb NPs in PbEIVS of *W. somnifera* is reported. The ability of *W. somnifera* to absorb, translocate and bioaccumulate Pb from nutrient medium to aerial parts of shoot cultures was identified via ICP MS analysis (Table 4.8). The reduction of Pb ions into Pb NPs

in live leaf tissue was investigated using TEM and EDX studies (Figure 4.8 & 4.9). Therefore, *W. somnifera* is an excellent system for phytoextraction, heavy metal reduction and for *in planta* synthesis of Pb NPs. Pb was also presented as the best elicitor for increased production of withanolides using IVS of *W. somnifera*. However, due to its heavy metal nature and its higher bioaccumulation in *in vitro* shoot cultures of *W. somnifera*, Pb was excluded from biopharmaceutical studies using human neuroblastoma cells (Section 4.3).

#### **4.2.3 Bioaccumulation, metal reduction analysis on Ag exposed *in vitro* shoots (AgEIVS) of *W. somnifera***

Silver is a highly reactive, transition and a heavy metal and highly reducible in nature. In the current study, the effect on AgNO<sub>3</sub> on IVS of *W. somnifera* under acute and chronic treatment period were studied. Optimum concentration of Ag treatment was selected based on high GI and withanolides production among all Ag treated shoots. The Ag bioaccumulation capability of *W. somnifera* shoots was analysed using ICP MS. Adsorption, transportation, sequestration, reduction and accumulation of Ag as ions or metalloid in the cells of Ag treated shoots were also investigated.

##### **4.2.3a Bioaccumulation of Ag and other essential metals in AgEIVS of *W. somnifera* (ICPMS)**

AgNO<sub>3</sub> 1mM treatment for 12 days period (E1D3T6) was concluded as the optimum concentration for increased GI and WFA production. Thus, E1D3T6 shoots were subjected to elemental analysis for Ag bioaccumulation. Elemental composition of E1D3T6 shoots and control IVS was analysed in comparison with field tissues (FS & FR) of *W. somnifera* using ICPMS analysis. Total twenty-two trace and heavy elements were quantified in *W. somnifera* shoots *in vitro* and field tissues. Among all the tested samples, E1D3T6 shoots found to bioaccumulated higher Ag content (50.8 mg kg<sup>-1</sup>). As expected, control shoots were void of Ag and field tissues had minor quantity of Ag in their tissues (>0.01 mg kg<sup>-1</sup>). Moreover, the percentage of Ag in E1D3T6 shoots was quantified as 2.54% (Table 4.9).

There are reports show that among field grown tissues, leaves and shoots had the highest bioaccumulation potential than others (Kulhari et al. 2013). As mentioned in the section 4.1.1, *W. somnifera* shoots has considerable metal bioaccumulation capability compared to root tissues. Therefore, in the present section, E1D3T6 shoots was compared control *in vitro* shoots for their elemental composition.

E1D3T6 found to accumulate lower Mg (727.5 ppm) compared to control *in vitro* shoots (1282 ppm). Further, 1.8-fold decrease in Mg content was observed in E1D3T6 shoots than in control. This may due to the presence of selective divalent metal ion transporters of the plant cell membrane and absorption of Ag divalent ions through Mg ion transporters resulted in the reduction of Mg content in E1D3T6 shoots (Tan et al. 2022).

In addition to Mg, Mn level was also decreased in E1D3T6 shoots and 1.5-fold reduction was observed compared to control. However, comparing to FS, both control and E1D3T6 shoots had higher Mn content. Supplementation of Mn in the nutritional medium may be the reason why increased concentration of Mn was found in *in vitro* than in field tissues. The concentration of Mn in the normal Indian soil was found to be approximately 215 ppm which is higher than WHO permissible limit (12 ppm). However, MPL for Mn in edible plants was reported to be 500 ppm by WHO (Anjali and Rani 2018) and considering that both treated and control shoots has Mn with WHO permissible limit. The concentration of P was 2-fold decreased in E1D3T6 shoots compared to control and almost similar concentration was found in both FS and E1D3T6 shoots.

**Table 4.9 Elemental composition of Ag treated *in vitro* shoots in comparison with field grown shoots of *W. somnifera***

| S. no.                        | Parameters      | Control (mg/kg) | E1D3T6 (mg/kg) | FS (mg/kg) | FR (mg/kg) | Permissible Limit (mg/kg) for medicinal plants |
|-------------------------------|-----------------|-----------------|----------------|------------|------------|--|
| <b>Essential Metals</b>       |                 |                 |                |            |            |  |
| 1                             | B (Boron)       | 21.26           | 15.088         | 31.45      | 6.5711     | NA   |
| 2                             | Mg (Magnesium)  | 1282.32         | 727.499        | 7895.97    | 1433.08    | NA   |
| 3                             | P (Phosphorus)  | 3679.81         | 1773.41        | 1941.089   | 471.965    | NA   |
| 4                             | K (Potassium)   | 21109.37        | 26077.91       | 27396.52   | 1451.96    | NA   |
| 5                             | Ca (Calcium)    | 1411.69         | 1833.64        | 8689.188   | 3381.47    | NA   |
| 6                             | Mn Manganese)   | 195.56          | 134.62         | 41.16      | 9.9144     | NA   |
| 7                             | Fe (Iron)       | 11.203          | 85.101         | 10.502     | 13.6292    | NA   |
| 8                             | Mo (Molybdenum) | 2.803           | 1.763          | 0.3311     | 0.3083     | NA   |
| <b>Essential Heavy Metals</b> |                 |                 |                |            |            |  |
| 1                             | Ni (Nickel)     | 0.0032          | 0.066          | 0.9871     | 0.8981     | 1.5 (WHO,2005)                                 |
| 2                             | Cu (Copper)     | 2.0273          | 0.385          | 8.1969     | 3.9901     | 20 (WHO/FDA)                                   |
| 3                             | Zn (Zinc)       | 42.2345         | 25.989         | 15.586     | 6.5135     | 50 (WHO/FDA)                                   |
| 4                             | Ti (Titanium)   | Nil             | Nil            | 3.667      | 1.143      | NA   |
| <b>Heavy Metals</b>           |                 |                 |                |            |            |  |
| 1                             | Cr (Chromium)   | 0.0076          | 0.0088         | 0.2076     | 0.5084     | 2 (WHO)  |
| 2                             | Co (Cobalt)     | 0.1452          | 0.141          | 0.1635     | 0.2009     | 5 (USP)  |
| 3                             | As (Arsenic)    | 0.0045          | 0.005          | 0.0162     | 0.0418     | 10 (WHO/FDA)                                   |
| 4                             | Cd (Cadmium)    | 0.0055          | 0.003          | 0.0196     | 0.0213     | 0.2 (WHO/FDA)                                  |
| 5                             | Hg (Mercury)    | 0.004           | Nil            | 0.0002     | 0.3202     | 1 (WHO/FDA)                                    |
| 6                             | Ag (Silver)     | Nil             | 50.801         | 0.015      | 0.002      | NA   |
| 7                             | Pb (Lead)       | 0.0279          | Nil            | 0.1132     | 0.1114     | 10 (WHO/FDA)                                   |
| 8                             | V (Vanadium)    | Nil             | Nil            | 0.03       | 0.028      | 30 (US EPA)                                    |
| 10                            | Li (Lithium)    | Nil             | Nil            | 0.026      | 0.024      | 0.075  |

Increased concentration of K (26077 mg/kg), Ca (1833 mg/kg) and Fe (85 mg/kg) was observed in E1D3T6 shoots compared to control (21109 mg/kg; 1411 mg/kg & 11 mg/kg). K is an essential trace element which has an important role in the alleviation of heavy metal stress in plants. In a study on *Cicer arietinum* treated with Cd resulted in the reduction of plant biomass which was retrieved by the addition of K and Ca (Ahmad et al. 2016). Similarly, in our study Ca and Fe both are increased during heavy metal stress (UI Hassan et al. 2017; Ghorri et al. 2019). They are known to protect plants/ plant cells from environmental stressors such as heavy metal stress (Karthika et al. 2018). Cadmium induced toxic stress on Indian mustard and *Lupinus albus* was reduced by Fe was reported by UI Hassan et al (2017).

The concentration Cr (0.008 mg/kg), Co (0.14 mg/kg) and As (0.005 mg/kg) was not influenced by Ag stress and both control and E1D3T6 shoots had same concentration (Table 4.9). The uptake of Ni was slightly increased during Ag stress (E1D3T6 shoots) compared to control from 0.0032 to 0.066 mg/kg. Ni is a known cofactor for several enzymes including redox enzymes superoxide dismutase and hydrogenases (Ghori et al. 2019). Thus, Ni content was increased which may due to induction of the aforementioned enzymes. The concentration of essential heavy metals such as Cu and Zn was reduced after Ag treatment. Both elements are naturally oxidized as two valence cations and due to its heavy metal properties, they probably replaced by Ag<sup>2+</sup> ions at cell membrane. In addition, Cu and Zn are important for plant growth and development but only required in small quantity (Varma et al. 2021). Due to metal detoxification mechanism in plants during abiotic stress, the plants tend to inhibits cationic transporters which caused decreased absorption and translocation of Cu and Zn compared to control IVS (Jalmi et al. 2018).

Therefore, treatment of Ag increased K, Ca & Fe and decreased Mg, P, Mn, Zn, Ni, Cu & Mo concentration in E1D3T6 shoots compared to control shoots. Further, there was no difference was noted in heavy metal concentration among E1D3T6 shoots and control shoots of *W. somnifera*. In E1D3T6 shoots bioaccumulated 50.8 ppm Ag content in their leaf tissues but no Ag was quantified in control shoots (Table 4.9). There are no MPL value was available in WHO for Ag in medicinal or edible plants. Thus, increased concentration of Ag present in IVS of *W. somnifera* may as well increases its pharmacological value to next level which is discussed in the next section. The nature of bioaccumulated Ag in *W. somnifera* leaf tissues was analysed and discussed in the following sections.

#### **4.3.3b *In planta* synthesis of Ag NPs in live AgEIVS of *W. somnifera* (TEM & EDAX)**

Ag is a highly reactive metal and it can be easily reduced using simple reducing agents like sugars. In this study, AgNO<sub>3</sub> was supplemented along with media components. Therefore, there is a high probability that added Ag reduced into Ag<sup>0</sup>, absorbed and translocated to leaf tissues or Ag ions reduced

*in planta* and accumulated in leaf tissues. Thus, the probability of formation of Ag NPs at *in planta* level was investigated using TEM & EDAX analysis in this section. There are several reports on the applications of green synthesized Ag NPs which includes anti-oxidant, anti-microbial, anti-inflammatory, anti-diabetic activities, used in electronic devices due to its good electrical conductivity (Yan and Chen 2019).

#### **Absorption and reduction of Ag ions/ Ag NPs in E1D3T6 shoots**

In the present study, *W. somnifera* treated with 1.0mM AgNO<sub>3</sub> for 12 days treatment period exhibits higher GI and Withaferin A accumulation compared to other AgNO<sub>3</sub> treated and control shoots. In addition, here, live leaf cell fractions of E1D3T6 shoots were prepared and analysed for possible nanostructures.

Silver easily oxidizes in liquid medium into ionic form (Ag<sup>2+</sup>) and can easily be reduced in presence of weak reducing agents (sugars and amino acids). The oxidation and reduction of silver was visual to naked eye when AgNO<sub>3</sub> solution was added to shoot mass multiplication nutrient medium. Colour change from transparent liquid medium to pure white colour followed by dark brown in colour after few hours of addition was observed. However, the absorption of Ag from nutrient medium as Ag<sup>2+</sup> (ionic form) or Ag<sup>0+</sup> (metallic form/nano form) is not confirmed. But absorption of Ag and bioaccumulation of Ag was confirmed by ICP MS analysis (Table 4.9). The results shows that 50.8 ppm Ag was bioaccumulated by E1D3T6 *W. somnifera* IVS which is 50 times higher than control shoots (0 ppm). This proves that *W. somnifera* has the ability to absorb, translocate and accumulate metal in their leaf cells. Even though absorption of Ag by E1D3T6 shoots was confirmed but site of reduction of Ag was unclear. Because there may be two possible routes that Ag enter into E1D3T6 shoots. 1) Initially Ag enters into callus tissues then it gets reduced inside plant cells using metabolic pathway components or 2) Ag may reduce in the nutrient medium by sugars (sucrose), hormone (BAP) or others (nicotinic acid, myo-inositol) etc., then passively enters the plant cells via passive diffusion.

The uptake and translocation of silver ions by plant roots was not reported much as far as we have studied. However, Ag NPs' uptake and translocation was reported frequently by many researchers. Thus, in the present study we focus on possible route for absorption, translocation and accumulation of Ag NPs instead of Ag ions by plants in tissues of a plant.

The uptake of Ag NPs by plant cells (root/callus) is mainly affected by particle size, its surface functional groups, type of plant species, treatment conditions and also type of plant tissue cultures studied. There are many studies reported on size dependant uptake and accumulation of nanoparticles in *Triticum aestivum* and *Arabidopsis thaliana* (Larue et al. 2012). In addition, the surface charge of the nanoparticles or elemental ion charge is highly dependent on the uptake of metal ions or metal nanoparticles in plant cells. Due to the presence of cell wall phospholipid bilayer, the cell surface is usually negative in charge which facilitates the uptake of positively charged metal ions/nanoparticles (Delsart 2016). Further, a study on *Oryza sativa* revealed that a preferential uptake and translocation of Au NPs via vascular system which shows that chemical properties of nanoparticles play a major role in the transportation within plants.

According to Shannon (1976), ionic radii of Ag<sup>+</sup> cation is 115 picometer or 0.12 nm in size. The porosity of young plant cell walls like interfibrillary and intermicellar spaces ranging in size from 3.5 nm to 14 nm (Delsart et al. 2016). Therefore, metal ions with the size of 0.1<sup>-1</sup>nm in range can easily enters young plant cell having immature walls rather than metal chelates which are preferably large in size (Delsart et al. 2016). Further studies have been confirmed that NPs with size below 100nm possess unique electrical, physico-chemical and biological properties so that they can readily pass through cell wall pores and reach plasma membrane (Wang et al. 2021). In our study, most of the NPs observed to be less than 100 nm in size (Figure 4.10). Thus, the mechanism of Ag NPs translocating through the cell wall is via cell wall pores. Subsequently silver ions may also enter the plant cell via cation exchangers or ionic transporters in the plasma membrane. Different type of ionic transporters or

exchangers used for heavy metal transport in plants are discussed in [section 4.2.2b](#).

### **Absorption, translocation and sequestration of Ag ions/Ag NPs in plants**

Based on existing literature, we found that AgNO<sub>3</sub> is a potential inhibitor of aquaporins in plants which in turn inhibits the uptake of mineral nutrients and metal ions. The presence of Ag ions in the medium significantly reduces the uptake of metal ions especially metal NPs by 92 % ([Hu et al. 2018](#)). This may be the reason why Ag (50.8 ppm) was bioaccumulated less by IVS of *W. somnifera* compared to Pb (405 ppm) ([Table 4.8](#)). In addition, translocation of Ag NPs in plants reported to be by both apoplastic and symplastic pathways. Further numerous studies have been reported on the translocation and penetration of Ag NPs into plant cells and accumulated inside vacuoles and other organelles ([Larue et al. 2012](#); [Mazumdar 2014](#)).

The uptake and translocation of Ag NPs is species specific and size dependant which was proved in tobacco seedlings where 3.5nm Au NPs are present in shoots and 18nm in size NPs are present in root outer surface ([Sabottwood et al. 2012](#)). However, in another study on *Arabidopsis thaliana* found to uptake SiO<sub>2</sub> NPs up to 200nm in size which proves that NP uptake is species dependant too ([Slomberg and Schoenfisch 2012](#)). In addition, like root cells, callus cells also secrete mucilage and exudates ([Barlow 2002](#)) which facilitate attachment, uptake and bioaccumulation of metal ions/ NPs in plant cells ([Azim et al. 2023](#)).

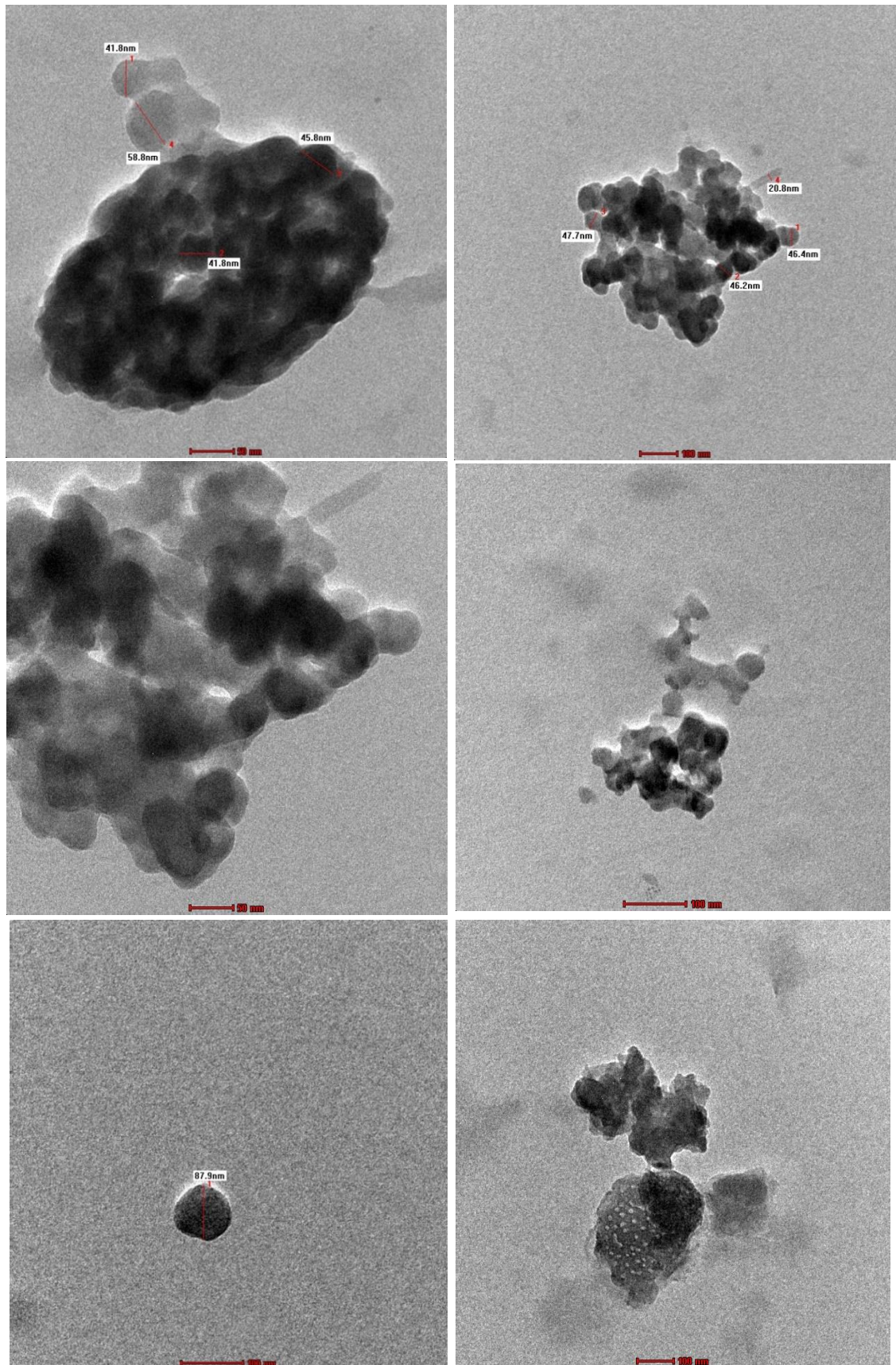


Figure 4.10 The electron micrograph of E1D3T6 leaf cells of IVS of *W. somnifera*



the plants as ionic form, then translocated in the plants and reduced to the elemental form (Gardea-Torresdey et al. 2003).

EDAX spectrum shows the elemental composition of E1D3T6 leaf tissues. The captured electron micrograph shows that most of the Ag was present at 2.5 to 3 keV confirms its metallic nature (Figure 4.10 & Figure 4.11). Point and regional analysis by TEM and EDAX was done simultaneously which confirms that the presence of nanostructures was in fact Ag NPs in figure 4.11 (Natarajan et al. 2024b). There are enormous reports on Ag NPs synthesis using plant extracts (Singh et al. 2018), microbial biomass/extracts (Bhuyar et al. 2020), individual components (phyto-constituents) (Raveendran et al. 2003; Ahmed and Baig 2014; Iram et al. 2014) or even using sun light as a reagent (Bhaduri et al. 2013; Tang et al. 2015) as a reducing agent. These studies shows that metallic silver was observed at ~3 keV in the EDAX spectrum (Bhaduri et al. 2013; Bhuyar et al. 2020). The presence of Ag peak at 3 keV (Figure 4.11) represent that Ag was in fact reduced by Phyto-constituents present in *W. somnifera* shoots which is probably withanolides or other secondary metabolites (Natarajan et al. 2024b).

Thus, the present study represents that the live shoot cultures of *W. somnifera* uptake metals in an ionic/metallic form via callus cells, transport them via apoplastic/ symplastic pathway (section 4.2.2a) and bioaccumulate them as Ag NPs in leaf tissues of *W. somnifera*. *W. somnifera* (Natarajan et al. 2022; Vinod et al. 2022) and Ag NPs (Siddiqi et al. 2018; Nakamura et al. 2019; Magdy et al. 2022) both have several pharmacological applications and the presence of both in the test sample may increase its pharmacological value to many folds. Therefore, E1D3T6 shoots were selected for neuro-protective study using Parkinson cell model in the following sections.

### **4.3 Evaluating the efficiency of *W. somnifera* extracts against Parkinson's disease using SH-SY5Y cells**

*Withania somnifera* and its important secondary metabolite withaferin A is known to protect neuronal dysfunction in human (Dutta et al. 2019) and animal models (Saykally et al. 2017). Its neuroprotective activity against Alzheimer's and Parkinson's disorder has been proved as early in the nineties (Schliebs et al. 1997; Mishra et al. 2000). Due to this capability, *W. somnifera* field tissues especially field root is being used in Ayurvedic formulations for neurological disorders (Paul et al. 2021). In this study, the effect of Ag treated *in vitro* shoots against rotenone induced mitochondrial dysfunction in Parkinson's disease cell model is studied in comparison with field tissues of *W. somnifera*. As *W. somnifera* plants are capable of accumulating heavy metals in metal contaminated field, tend to accumulate toxic heavy metals which makes them incompetent to be used for pharmaceutical applications. On the other hand, *in vitro* developed plants/tissues are biotic and abiotic contaminants free, usage of specific elicitor results in the increased production of withaferin A and selecting specific elicitors like AgNO<sub>3</sub> increases the plants bioactivity in humans. In this section, we are going to discuss on the effect of E1D3T6 shoots which was confirmed to store Ag NPs in their leaf tissues (Section 4.2.3b) on Rotenone induced Parkinson cell model. In Parkinson's disease, mitochondrial dysfunction is the major causative agent. Therefore, the effect of *in vitro* developed (E1D3T6) and traditionally used (FGR and WFA) *W. somnifera* tissue samples on mitochondrial gene expression and its dysfunction against PD cell model is reported here.

Rotenone is a toxic pesticide known to inhibit electron transport chain (ETC) complex I which in turn portrays symptoms of parkinsonism in both cell line and animal model (Luo et al. 2015). In addition, rotenone has the ability to reproduce hallmarks of PD such as motor dysfunctions, extranigral pathology (behavioural changes and autonomic dysfunction) and non-motor symptoms (hyposmia, gastroparesis, cognitive changes, etc) (Johnson and Bobrovskaya 2015; Carroll et al. 2021). The role of extracts of *in vitro* grown shoot tissues of *W. somnifera* on the restoration of mitochondrial function in rotenone induced

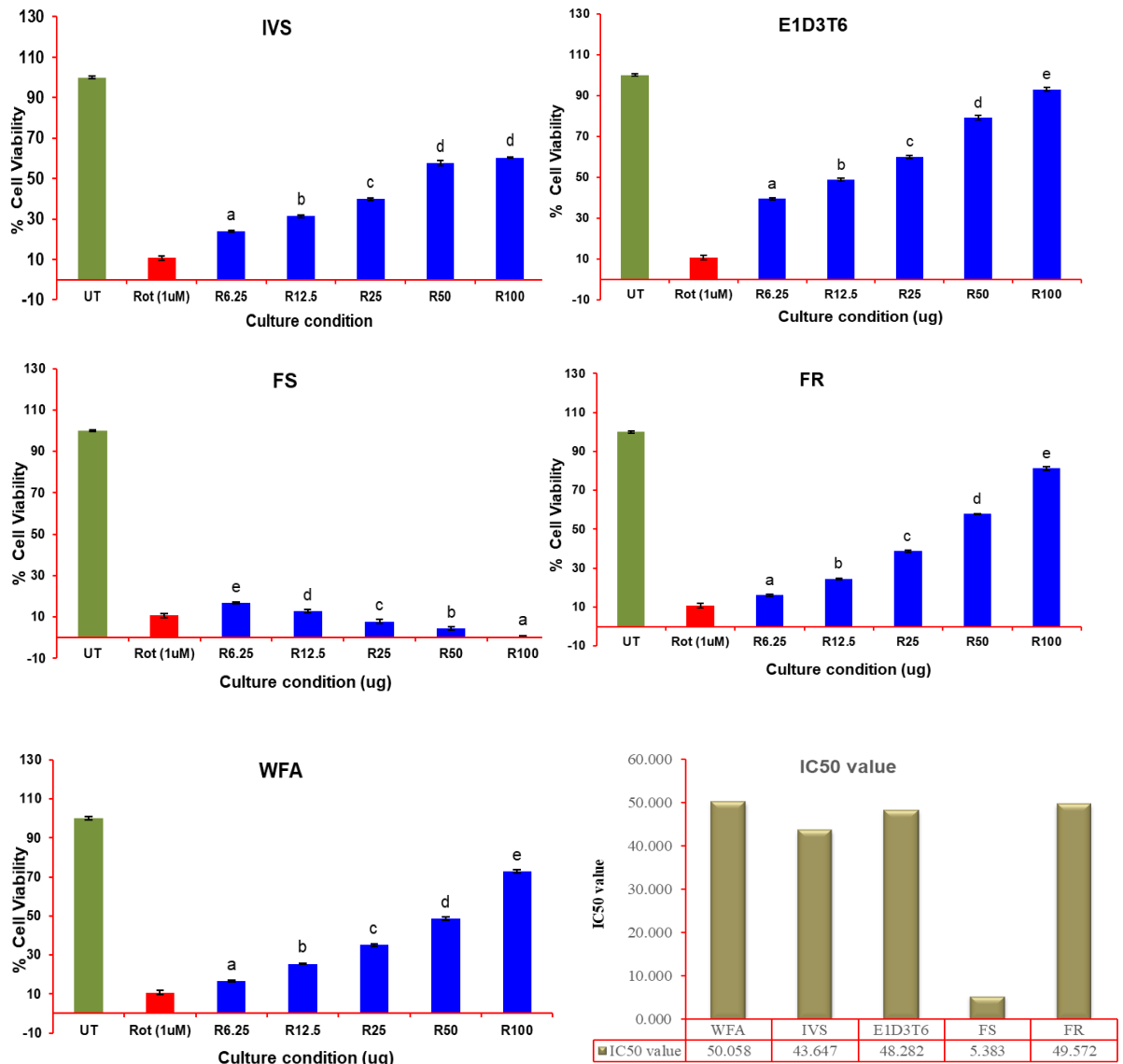
mitochondrial dysfunction in SH-SY5Y PD cell model, is reported for the first time. Hence in the present study rotenone is used to induce PD in SH-SY5Y neuroblastoma cells.

#### **4.5.1 Cytotoxicity assessment of *W. somnifera* tissue extracts on rotenone induced SH-SY5Y cells**

To understand the extend of toxicity of a foreign drug i.e., plant, microbial extracts or an organic compound against human cells by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is usually carried out. In MTT assay, MTT is reduced in presence of mitochondrial lactate dehydrogenase enzyme which results in the formation of insoluble formazan crystals. The purple-coloured crystals were dissolved in appropriate solvent, the resultant solution was read under 570nm using spectrophotometry. Only live cells can synthesize mitochondrial lactate dehydrogenase and form formazan crystals (Glancy et al. 2021); thus, from the intensity of peak and OD (optical density) value, the percentage of viable cells can be measured.

The optimal dose of Rotenone (mitochondrial toxin) to induce cytotoxicity and Parkinson disease pathogenesis in SH-SY5Y cells was selected as 1 $\mu$ M from reported studies (Zhang et al. 2017). All the plant extracts were tested in the concentration ranging from 6-100 $\mu$ g/ml and WFA in 6-100 $\mu$ M/ml. The viability of rotenone induced SH-SY5Y cells increased during *W. somnifera* tissue extracts treatment in a dose-dependent manner except FGS. All extracts and standard WFA exhibited reversal of rotenone induced SH-SY5Y cytotoxicity, except FGS (Figure 4.12). In addition, WFA, CT6 (IVS control), FGR and E1D3T6 shoots, all exhibited similar cell reviving capability with CT6 being slightly less effective against rotenone induced SH-SY5Y cells. An increase in SH-SY5Y cell viability along with increase in concentration of the plant extracts was observed during MTT assay. During E1D3T6 extract treatment, among all the concentration tested (6-100 $\mu$ g/ml), 100 $\mu$ g/ml extracts was found to increase cell viability to 86.26%. Similarly, FGR extracts (100 $\mu$ g/ml) and WFA (100 $\mu$ M/ml) treatment exhibited increased SH-SY5Y cell viability to 71.27% and 65.94% respectively. Though FS was found to be toxic,

all the other test samples (CT6 [IVS], E1D3T6, FGR and WFA) showed significant cell protective and increased cell viability potency.



\*All experiments were performed in triplicates. Values are means  $\pm$  standard errors of three replicates. The alphabets on the top of the error bars followed by the same letter are not statistically different at  $p \leq 0.05$  according to DMRT.

**Figure 4.12 SH-SY5Y Cell viability and IC50 of *W. somnifera* extracts – MTT assay**  
 IVS: *in vitro* control shoot (CT6); E1D3T6: AgNO<sub>3</sub> treated shoot; FS: field shoot (FGS); FR: field root (FGR) -extracts

In a study, vanillin was found to have a dose dependant increase in cell viability of rotenone injured SH-SY5Y cells and 100nM vanillin was reported to increase 84% cell viability after the injury (Dhanalakshmi et al. 2015). In yet another study by Saykally et al (2017), on *W. somnifera* root extracts at 20µg/ml concentration was found to prevent SH-SY5Y cell death from *in vitro* traumatic injury (Saykally et al. 2017). In addition, pretreatment of 0.5 mg/ml of KSM-66 (*W. somnifera* root powder) for 24 hours on 6-OHDA post-treatment of SH-SY5Y cells, 30% increase in cell viability was observed (Wongtrakul et al. 2021).

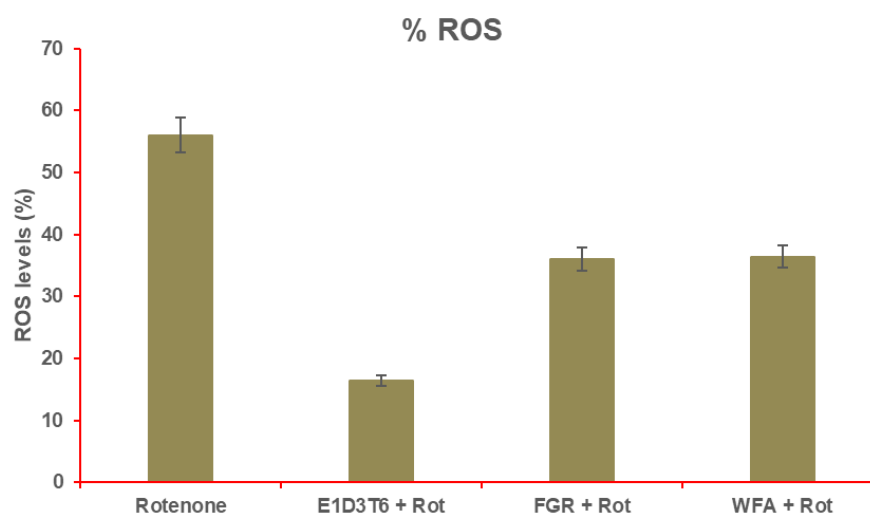
Hence, our result proves that *W. somnifera in vitro* and field tissue has neuroprotective activity against rotenone induced SH-SY5Y cells. Therefore, for further study, the concentration which showed maximum cell viability was selected, 100µg/ml for E1D3T6 and FGR and 100µM for WFA.

#### **4.3.2 ROS scavenging activity of *W. somnifera* tissue extracts on rotenone induced SH-SY5Y cells**

Reactive oxygen species (ROS) are produced intracellularly at various locations including mitochondrial electron transport chain complexes like complex I and complex III. Increased ROS accumulation results in the damaged biomolecules which in turn acts as potential contributors to a range of diseases including neurodegenerative diseases (Holmström and Finkel 2014). In addition, PD is characterized by high mitochondrial ROS generation which results in degeneration of dopaminergic neurons (DA neurons) causing cell death and impaired autophagy (El-Ghaiesh et al. 2020). As already discussed, in PD mitochondrial dysfunction is the major causative agent, thus, the accumulation of ROS will be higher due to damaged mitochondria in the DA neuronal cells (Johnson et al. 2015). Therefore, the effect of *W. somnifera in vitro* and field tissue extracts along with WFA in ameliorating mitochondrial function on rotenone induced SH-SY5Y cells/ PD cell model was studied.

Several reports are available on the enhancement of mitochondrial biogenesis using natural products (Baicalein), restored mitochondrial function in rotenone induced Parkinsonian rats and SH-SY5Y cells (Zhang et al. 2017). Also, rotenone is reported to be an inhibitor for mitochondrial ETC complex I

which resulted in the increased ROS accumulation, subsequently causing reduced glutathione and oxidative stress (Jhonson et al. 2015). Increase in generation of ROS in SH-SY5Y cells after treatment with rotenone for 4 hours was confirmed in the present study where 1 $\mu$ M rotenone injured cells were taken as a PD induced control (Figure 4.13). E1D3T6 shoots, FGR and WFA was selected as a test samples. 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 (Figure 4.13). The percentage of ROS generation significantly decreased in test sample and WFA treatment compared to control cells (Figure 4.14). Similar to our results, pretreatment with Danshensu to rotenone induced SH-SY5Y cells decreased ROS generation above 2-fold (Wang et al. 2021). In another study on MPP<sup>+</sup> induced ROS generation in SH-SY5Y PD model was suppressed by pretreatment with metformin as metformin pretreatment was found to recover mitochondrial membrane potential and suppresses ROS generation (Lu et al. 2016). In a review on PD by Lu et al (2016), reduction in ROS production contributes to the induction of AMPK-autophagy pathway and also inhibition of inflammasomes (IL-1 $\beta$ , TNF- $\alpha$  & NLRP3) which results in the attenuation of neuroinflammation in PD. The induction of AMPK-autophagy results in the clearance of dysfunctional mitochondria and suppression of ROS generation in DA neurons (Lu et al. 2016).



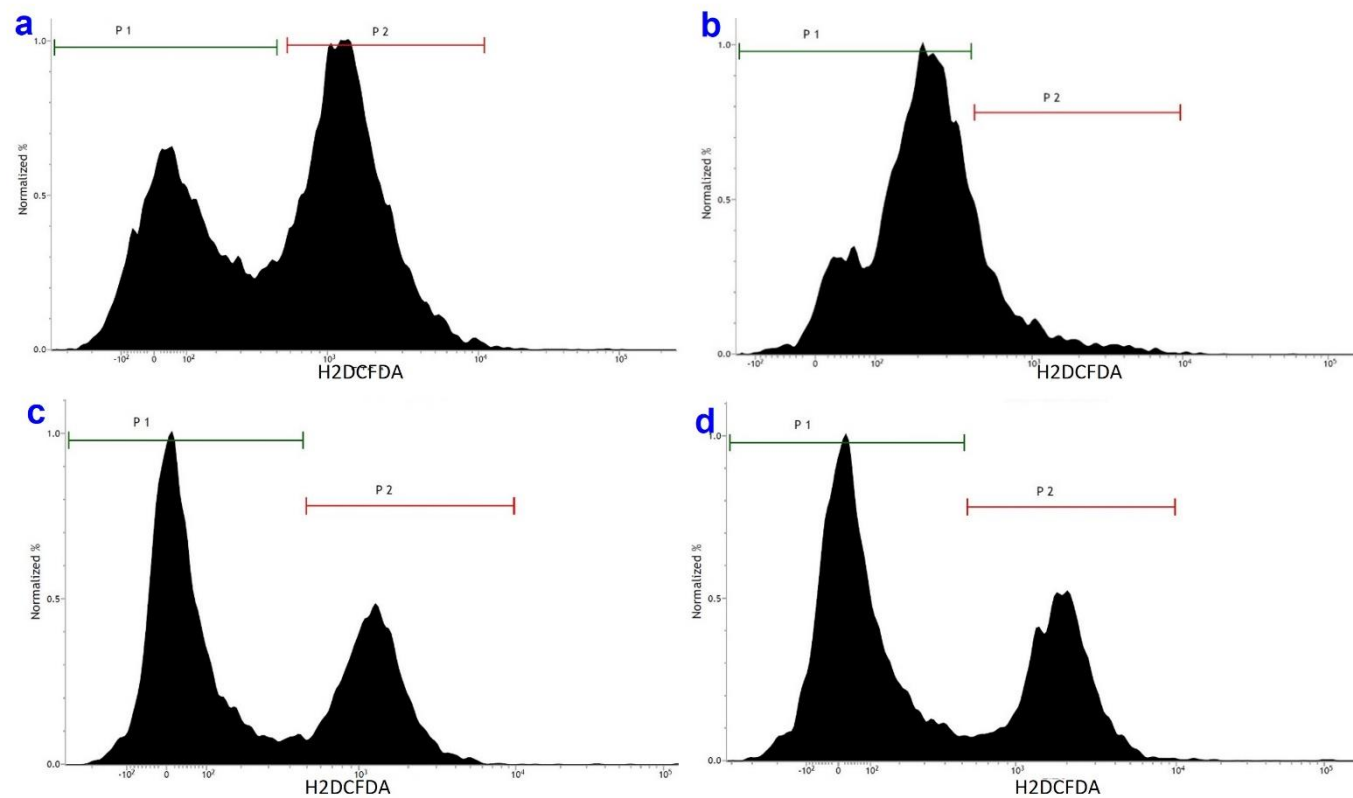
All experiments were performed in triplicates. The error bars represent the standard deviation in the data (n=3).

**Figure 4.13 Influence withaferin A and *W. somnifera* extracts on ROS levels (% DCF expression) in Rotenone induced SH-SY5Y cells**

In normal cells, continuous production of ROS is normal but they get removed by antioxidants like glutathione peroxidase, superoxide dismutase, etc. But when there is an imbalance between ROS generation and antioxidant activity, increased ROS generation results in oxidative stress which subsequently leads to mitochondrial dysfunction. The similar effect of increased oxidative stress and ROS accumulation caused by cisplatin treatment on MDA-MB231 cancer cells was reversed back to normal by the treatment of *W. somnifera* root extracts (Henley et al. 2017).

Pathological symptoms similar to PD in neuronal cell lines can be attained via pesticides like Rotenone or MPP<sup>+</sup> which inhibits complex I of ETC as deficiencies in ETC complex I are observed in familial PD pathology. The increment in ROS level causes the production of METC components leading to a reduction in ATP level and leakage of ROS into cytoplasm. ROS also negatively influences proteasomal and lysosomal activity. This leads to improper elimination of damaged or misfolded proteins ( $\alpha$ -synuclein) accumulated in DA neurons (Zucca et al. 2015). Accumulation of misfolded proteins and dysfunctional proteasomal systems in DA neurons appeared to be the main reagent for neurodegeneration in PD (Schapira et al. 2014).

Furthermore, increased ROS in cells causes peroxidation of membrane polyunsaturated fatty acids which leads to structural alterations of mitochondria that are found in PD pathophysiology (Zucca et al. 2015). Apart from these findings many other factors can induce ROS production in DA neurons such as PINK1 gene mutation (Wood-Kaczmar et al. 2008), DJ-1 and PGC1 $\alpha$  gene mutations (Dolgacheva et al. 2019; Anis et al. 2020), accumulation of wild type  $\alpha$ -synuclein in DA neuron (Martin et al. 2006), increased production of NADPH oxidases (NOX2), over-activated microglial cells (Xu et al. 2023), increased Fe content in mitochondria (Zucca et al. 2015) and the presence of Lewy-bodies in DA neurons (Chi et al. 2018). Apart from this, ROS is known to activate various signal transduction pathways and transcription factors including PI3K/Akt, MAP kinase, NF $\kappa$ B, Nrf2/Keap1, p53, and AMPK. Similarly, the attenuation of ROS accumulation in human microglial cells by *W. somnifera* extracts through inhibition of pro-inflammatory cytokines such as NF $\kappa$ B, TNF $\alpha$ , IL-1 $\beta$ , and IL-6 was reported by Gupta and Kaur (2019). Further, there are also reports on inhibition of iNOS expression and NO, RNS, and ROS production in Raw 264.7 cells by Withaferin A treatment (Oh et al. 2008). In addition, *W. somnifera* tissue extracts were found to be an excellent promotor for mitochondrial biogenesis which was found using C57BL/6J mice (Lee et al. 2020).



The H2DCFDA histograms obtained distinguishes the cells at P1 and P2 phases where P1 refers to negative expression region and P2 refers to the positive expression region. a) Rotenone (Rot) + SH-SY5Y cells; b) E1D3T6 + SH-SY5Y + Rot; c) FR + SH-SY5Y + Rot; d) WFA + SH-SY5Y + Rot.

**Figure 4.14 ROS accumulation in rotenone induced SH-SY5Y cells**

In a rotenone induced cell model of PD, rotenone was reported to act as an inhibitor for NADH-ubiquinone reductase of ETC complex I of mitochondria. Following the inhibition, restrained mitochondrial respiration occurs which generates increased ROS production and accumulation, abnormal mitochondrial morphology and reduced intracellular ATP (Xicoy et al. 2017). A study on rotenone induced mitochondrial dysfunction in *Drosophila melanogaster* was reversed when they were fed with *W. somnifera* extracts for 7 days. Inhibition of mitochondrial complex I-III and complex I -II was restored and ROS generation due to rotenone treatment was also normalized after co-exposure with *W. somnifera* extract (Manjunath and Muralidhara 2015). AMPK (AMP mediated protein kinase) is a cellular energy sensor that is activated on falling ATP level and restores energy homeostasis in cells (Peixoto et al. 2017). Further, rotenone inhibits the AMPK which further increases degeneration of dopaminergic neurons and cell death. Withaferin A was also found to induce AMPK activation in gentransgenic mice and also upregulated the level of AMPK phosphorylation for its activation (Guo et al. 2019). Therefore, in the present study we found that *W. somnifera* extracts especially E1D3T6 shoot extracts can be utilized to decrease ROS generation in neuronal cells which may pose life threatening effects in PD pathology.

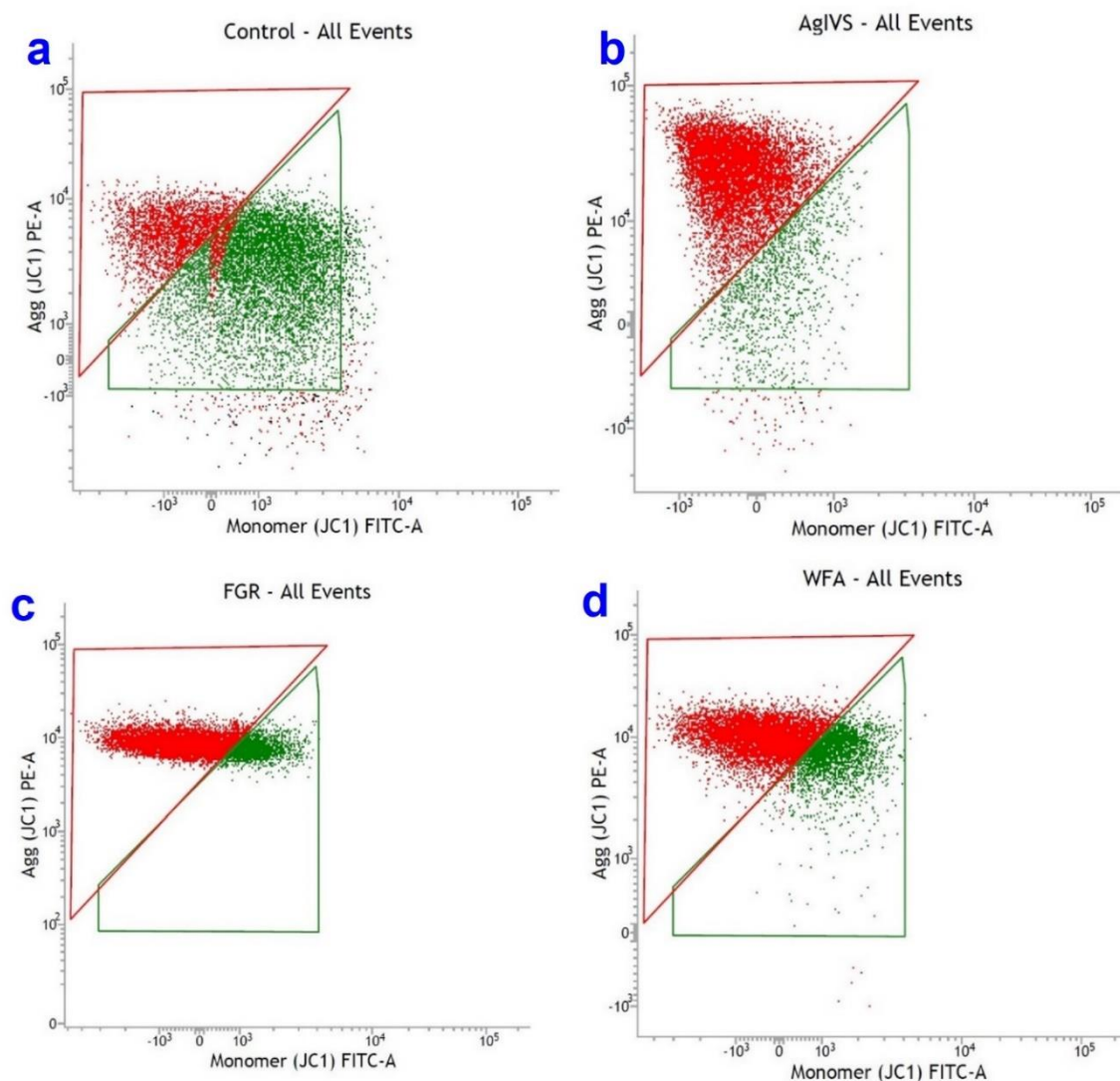
#### **4.3.3 Mitochondrial membrane potential analysis of *W. somnifera* tissue extracts on rotenone injured SH-SY5Y cells**

##### **Mitochondrial membrane potential in PD**

In addition to these factors, mitochondrial membrane potential (MMP) is reported to be one other important factor in PD. Depolarization and hyperpolarization of mitochondrial membrane led to reduction in transmembrane potential which resulted in the activation of mitophagy or autophagy. Damaged mitochondria due to toxins (pesticide like MPP+, rotenone, 6-OHDA), gene mutation or other environmental stressors causes reduction in MMP which does not allow PINK1 protein to enter mitochondria. Due to this fact, PINK1 gets accumulated on OMM which in turn activate parkin (phosphorylation) - a ubiquitination protein - and recruiting parkin to mitochondrial OMM for ubiquitination (Borsche et al. 2021). This type of mitophagy is regulated by PINK1/Parkin pathway components which includes

FBXO7 involved in mitophagy regulation. The mutations in FBXO7 gene are responsible for autosomal recessive form of early onset PD. Due to FBXO7 deficiency led to interruption in the mitophagy regulation which further leads to activation of intrinsic apoptotic pathway and loss of DA neurons (Nicoletti et al. 2021). Another important factor that causes reduction in MMP (depolarization of mitochondrial membrane) is intramitochondrial  $\text{Ca}^{2+}$  overload. Increased  $\text{Ca}^{2+}$  influx into mitochondria is caused by various factors including  $\alpha$ -synuclein aggregation and accumulation and PINK1 deficiency (Scorziello et al. 2020). For the maintaining calcium homeostasis in mitochondria, it was reported that overexpression of NCLX ( $\text{Na}^+/\text{Ca}^{2+}/\text{Li}^+$  exchanger) in DA neuronal cells resulted in the efflux of  $\text{Ca}^{2+}$  out of mitochondria which recovers MMP and mitochondrial dynamics and function.

In the present study, due to inhibition of ETC complex I during Rotenone treatment, MMP of neuronal cells were significantly reduced (depolarization of mitochondrial membrane) which resulted  $\text{Ca}^{2+}$  release into outer mitochondrial membrane space. In **Figure 4.15** monomers with red fluorescence represents depolarized mitochondrial membrane and aggregates with green fluorescence represents functional mitochondria with normal MMP. In Rot treated control SH-SY5Y cells, percentage of monomer level was increased to 69.74% due to mitochondria depolarization.

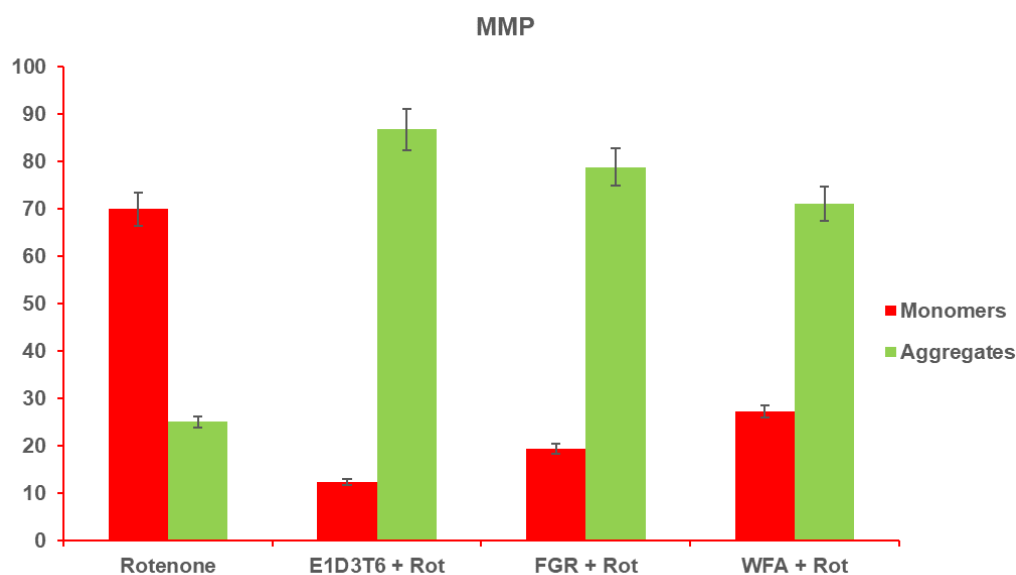


All experiments were performed in triplicates. a) Control (Rot + SH-SY5Y); b) T1 + SH-SY5Y + Rot; c) FR + SH-SY5Y + Rot; d) S1 + SH-SY5Y + Rot;

#### Figure 4.15 Mitochondrial membrane potential assessment using flow cytometry

On the other hand, during *W. somnifera* extracts and WFA pre-treatment, the percentage of aggregates was 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 (Figure 4.16). Among tested samples, E1D3T6 pre-treated rotenone induced cells exhibited increased level of aggregates (86.81%) compared to other samples (FGR and WFA) showing high neuroprotective activity. Similar to our results, in GSK621 (AMPK activator) pre-treated MPP<sup>+</sup> induced PC12

cells, the level of MMP was increased to normal (aggregates: ~80%) (Su et al. 2021).

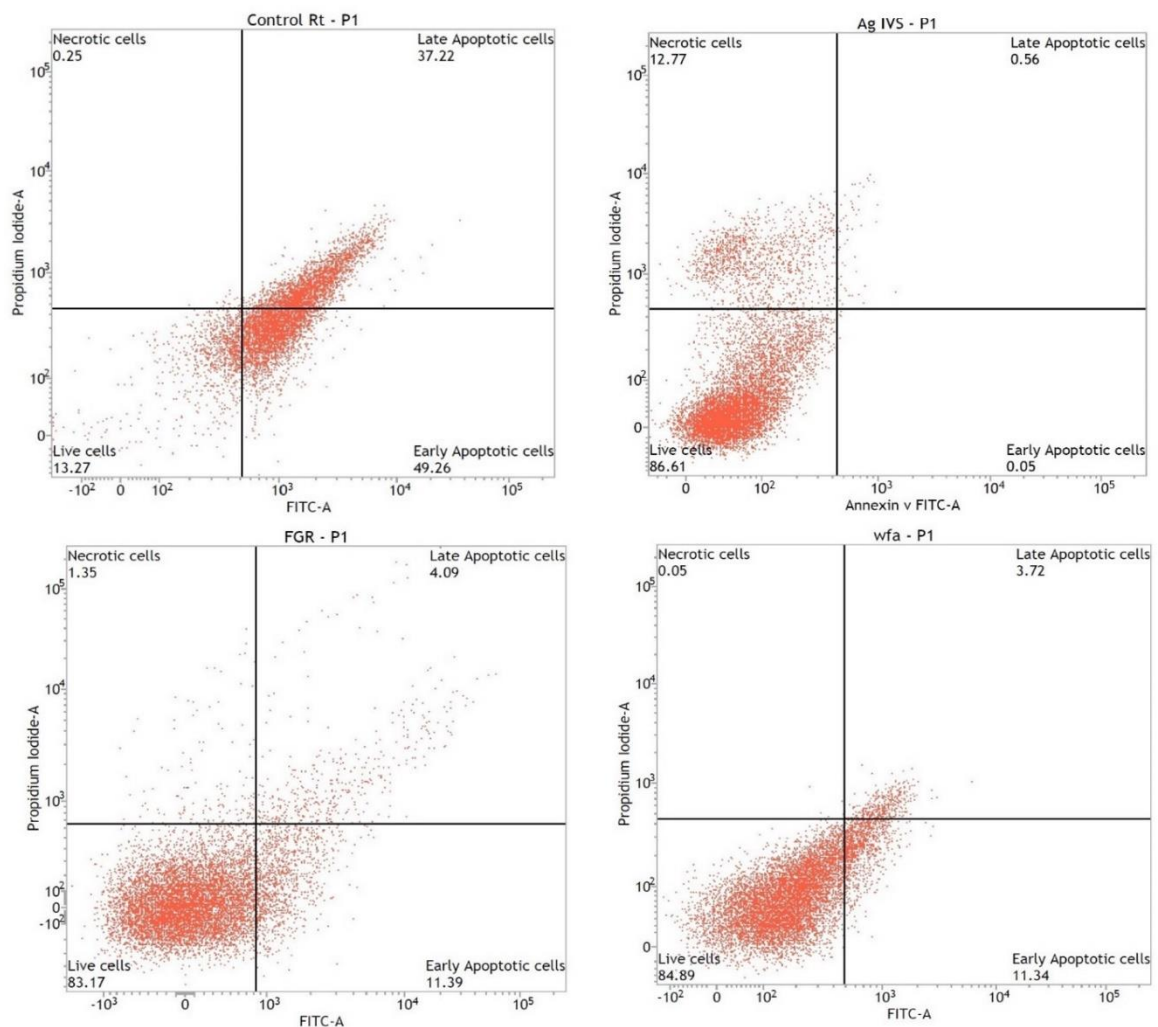


**Figure 4.16 Influence withaferin A and *W. somnifera* extracts on restoration of mitochondrial membrane potential in SH-SY5Y cells**

#### 4.3.4 Anti-apoptotic effect of *W. somnifera* tissue extracts on rotenone injured SH-SY5Y cells

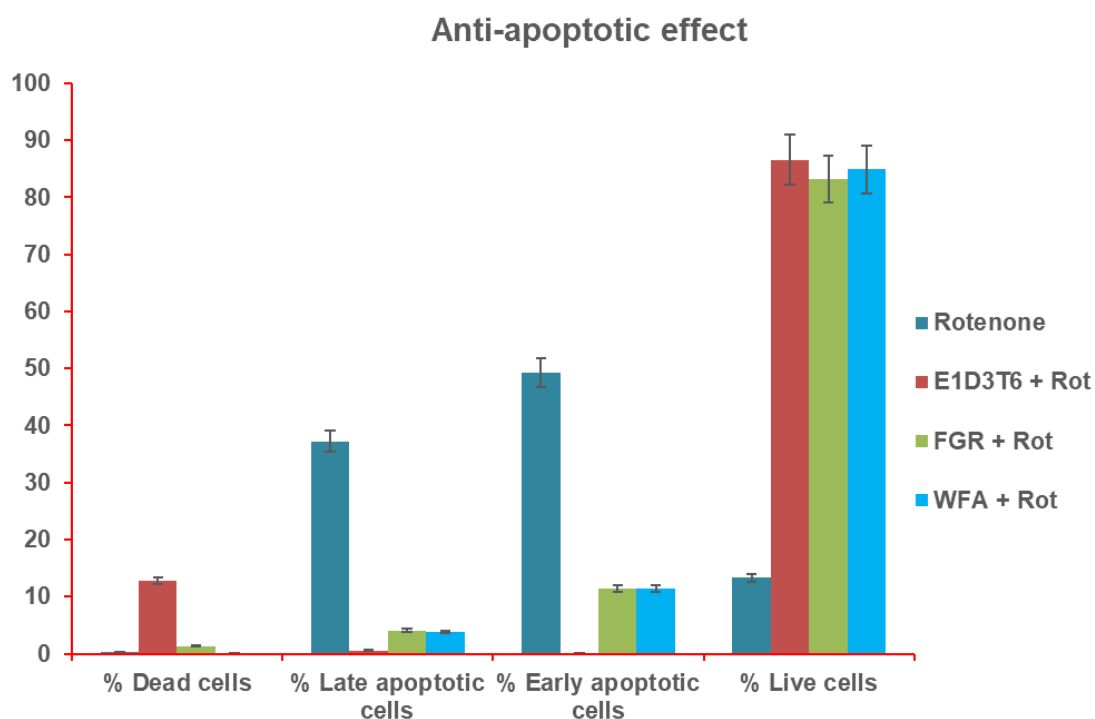
A flow cytometry analysis was performed to evaluate apoptotic activity in control and *W. somnifera* tissue extracts pre-treated rotenone induced SH-SY5Y cells. Apoptosis is a programmed cell death responsible for DA neuronal death in pathophysiology of PD (Enogieru et al. 2021). Apoptosis portrays distinct features like cell shrinking, membrane blebbing, and chromatin condensation. In PD, apoptosis is the main mechanisms for DA neuronal death. Due to its major role in PD, apoptosis pathway components become potential therapeutic targets in modern medicine (Erekat 2018). Generally, apoptosis occurs in cells through intrinsic apoptotic pathway (mitochondria mediated) and extrinsic apoptotic pathway (cell surface death receptor mediated). In PD, predominant apoptosis for neuronal degeneration is reported as intrinsic apoptotic pathway. Mitochondrial dysfunction and increased ROS production in the mitochondria led to the activation of caspase dependent/ independent apoptosis pathway in DA neurons in the substantia nigra pars compacta

(Venderova and Park 2012). Further, in PD pathology, increased caspase 3/7, caspase 9 and cleaved PARP activity was observed in SNpc (Enogieru et al. 2021). Thus, in DA neurons apoptosis was activated by intrinsic signals such as DNA damage, increased ROS and inhibition of ETC complex 1 causing activation of P53, and upregulation of pro-apoptotic factors including Bax, cytochrome c, Smac/DIABLO, HtrA2/Omi which led to the activation of intrinsic apoptotic pathway (Chi et al. 2018).



Quadranglar plots representing the Annexin V/PI expression in rotenone induced SH-SY5Y cells. a) Rotenone (Rot) + SH-SY5Y cells; b) E1D3T6 + SH-SY5Y + Rot; c) FR + SH-SY5Y + Rot; d) WFA + SH-SY5Y + Rot.

**Figure 4.17 Anti-apoptotic effect determined by flow cytometry**



**Figure 4.18 Influence of withaferin A and *W. somnifera* extracts on reversal of apoptosis in rotenone induced SH-SY5Y**

In the present study, the treatment of rotenone at the concentration of 1 $\mu$ M resulted in the inhibition of mitochondrial ETC complex I which causes mitochondrial dysfunction and neuronal loss due to increased ROS accumulation (Figure 4.17). Further, here we found that treatment of Rot resulted in the apoptosis of SH-SY5Y cells. The percentage of early and late apoptotic cells in Rot treatment were 47.26% and 37.22% and only 13.27% live cells were quantified (Figure 4.17). Thus, Rot treatment on SH-SY5Y cells causes significant number of cell loss which is due to the mitochondria dysfunction and energy deprivation due to inhibition of ETC in mitochondria. However, pre-treatment of E1D3T6, FGR and WFA to rotenone induced SH-SY5Y cells resulted in the reversal of apoptotic effect of rotenone on the neuronal cells. The anti-apoptotic effect of *W. somnifera* tissue extracts were already reported by Prakash et al (2014) on mice where anti-apoptotic protein Bcl-2 was increased significantly after 100 $\mu$ g/kg *W. somnifera* extract treatment. Similarly, in our laboratory we reported that the treatment of LPS on Raw 264.7 cells causes decrease in Bcl-2 levels which was reversed when the

cells were treated with methanol extracts of *W. somnifera* tissues (Vinod et al. 2022). Hence, the pre-treatment of E1D3T6, FGR and WFA showed higher percentage of live cells 86.61, 83.17 and 84.89 respectively, however, rotenone treated control cells showed only 13.27% live cells. In addition, rotenone treated SH-SY5Y cells showed 37.22% late apoptotic cells (LAC) and 49.26% early apoptotic cells (EAC) 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. During E1D3T6 treatment, the percentage of necrotic cells is higher 12.77 compared to FGR and WFA treatment (Figure 4.18). This may be that E1D3T6 may successfully reverse cells from EAC, but percentage of reversal from LAC may be lower. Similarly, in a study on MPP<sup>+</sup> treated SH-SY5Y cells, LAC level was increased to 44.81% and rutin pre-treatment decreased the apoptotic activity in a dose-dependent manner as 25  $\mu$ M (32.95%); 50  $\mu$ M (27.63%) and 100  $\mu$ M (22.38%) (Enogieru et al. 2021).

#### **4.3.5 Mitochondrial genes expression analysis on *W. somnifera* tissue extracts treated rotenone injured SH-SY5Y cells**

Mitochondrial dysfunction and elevated oxidative stress precede characteristic loss of dopamine-producing neurons from the midbrain in PD. DA neurons have high energy demand when mitochondrial function is affected during PD, which results in the loss of DA neuron in the SNpc. There are numerous mitochondrial genes which are found to be dysfunctional during PD among them DJ-1 and PINK1 are the two important genes that aids restore the mitochondrial function and increase DA neuron cell viability. DJ-1 regulates mitochondrial functions like oxidative stress, and it also removes the damaged mitochondria. Mutated DJ-1 resulted in abnormal mitophagy and it also changes mitochondrial dynamics via affecting Ca<sup>2+</sup> homeostasis. The role of PINK1 in normal neuronal cells is to recognize and bind to the outer membrane of damaged mitochondria and recruits' parkin, a E3 ubiquitin ligase, which led to the activation of mitophagy. Mutated PINK1 resulted in the presence of increased number of damaged mitochondria, decreased energy production which leads to DA neuronal death (Ammal Kaidery et al. 2019).

### Primer Validation

To study the expression of the above-said genes, real-time PCR was carried out. Primer validation was done to find the optimal concentration of forward and reverse primers. For the standardization of the optimum annealing temperature of the designed primers, gradient PCR was done using cDNA and temperature ranged between 50-60°C. The optimum temperature for specific annealing of each primer was fixed as 59°C. Primers were validated with SYBR reactions for amplification and melt curves. Amplification using all primer pairs (DJ-1/PARK7, PINK1, and GAPDH) gave the expected product size with no self-annealing or self-dimerization. In the melt curve plot, a single peak was detected for all primer pairs (Figure 4.19). PCR amplification efficiencies ranged from 90.30 to 97.19%.

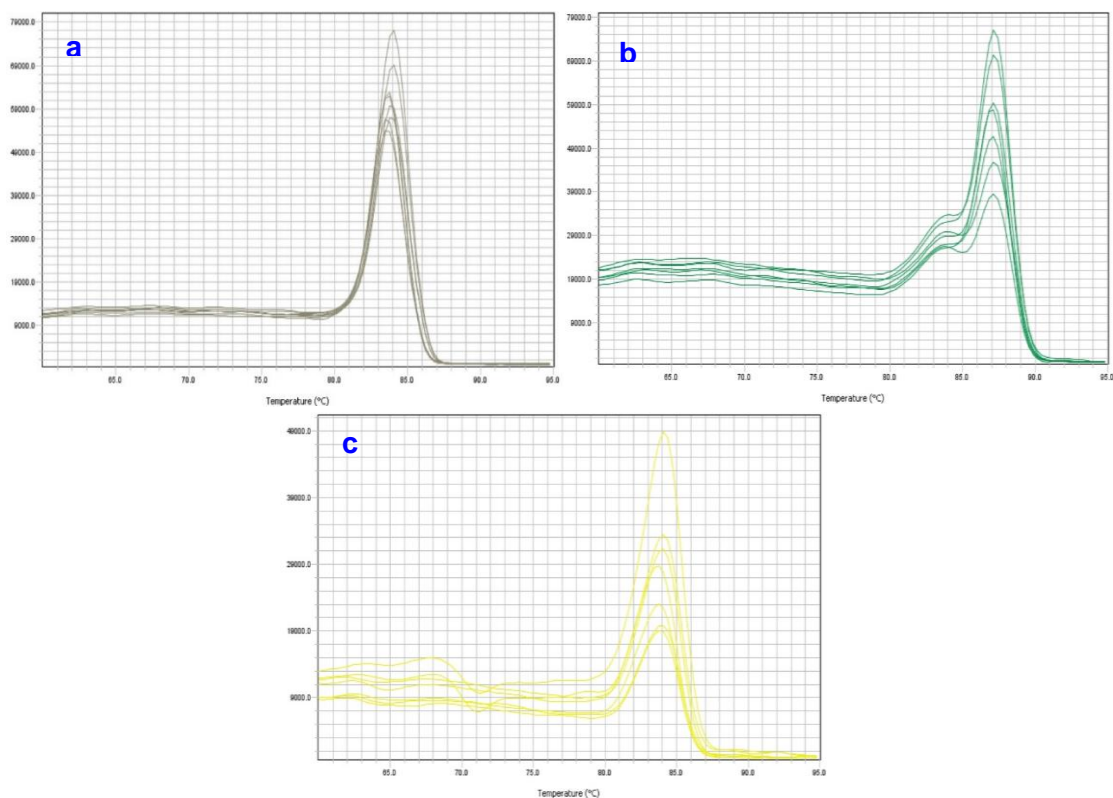


Figure 4.19 Melting curves of (a) DJ-1/PARK7, (b) PINK1 and (c) GAPDH primers

### **Gene expression study by Relative quantification using Real Time PCR**

Primer concentration 200nM with 80-95°C was selected and 50ng RNA equivalent cDNA was used per real time reaction. After total of 40 cycles, the fold changed calculated using  $\Delta\Delta C_t$  values. In addition, fold changes >1 of housekeeping gene (hGAPDH) is up regulation and <1 is down regulation.

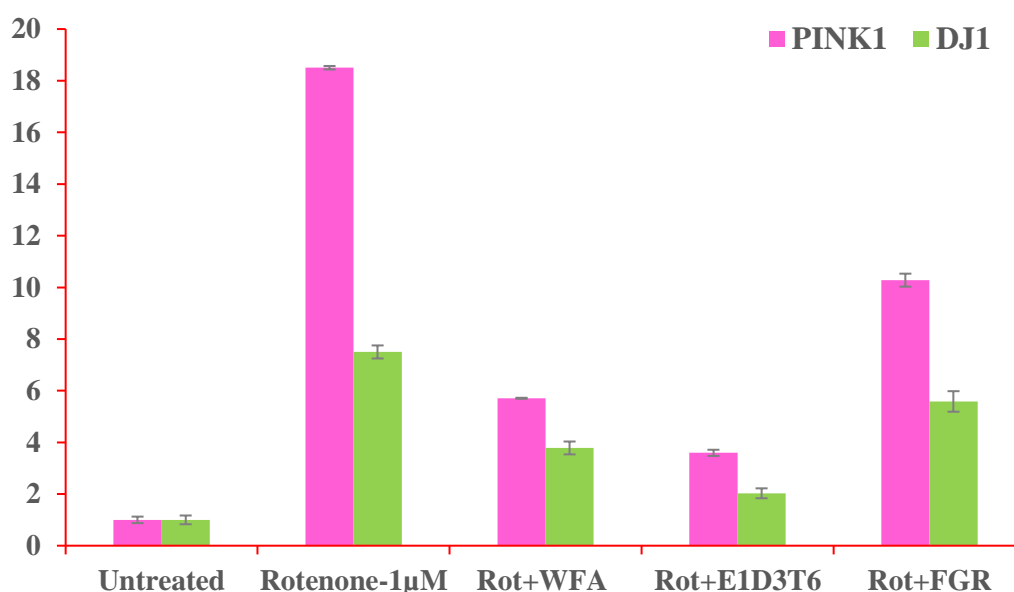
The mRNA expression of three PD related genes involved in mitochondrial function and one gene of housekeeping/ reference gene was measured in control SH-SY5Y cells, 1 $\mu$ M rotenone injured cells and *W. somnifera* tissues samples pretreated rotenone injured SH-SY5Y cells.

### **Role of PINK1 in mitochondrial function and PD**

PINK1 (PARK6) (PTEN-induced kinase -1) protein coding gene encodes a serine/threonine kinase having mitochondria membrane localization signal (OMS) at the N-terminal side. In normal cells, PINK1 acts together with Parkin in the maintenance of mitochondrial quality control. Mutation in either of these gene leads to oxidative stress mediated aggregation of  $\alpha$ -synuclein ([Sharma et al. 2019](#)) and mitochondrial dysfunction causing sporadic Parkinson's disease (PD).

In the present study, compared to untreated SH-SY5Y cell, the expression of PINK1 gene is significantly upregulated during rotenone treatment for 24 hours. Similar induction of PINK1/Parkin-mediated mitophagy and its related gene expression was reported in rotenone-treated silkworms ([Zhang et al. 2024](#)). In addition, about four-fold increase in the PINK1 expression was observed in CCCP (carbonyl cyanide m-chlorophenyl hydrazine) pre-treated SH-SY5Y cells ([Gómez-Sánchez et al. 2014](#)). The pretreatment of *W. somnifera* extracts such as silver treated *in vitro* shoots (E1D3T6), field grown root (FGR) and withaferin A (WFA) to rotenone induced cells was found to cause significant reduction in the PINK1 gene expression. On pretreatment of SH-SY5Y cells with E1D3T6, FGR and WFA, PINK1 expression was reverted back to 3.5-, 5.7-, and 10.2-fold from 18.5-fold in rotenone treated SH-SY5Y cells resulting in the restoration of normal function in DA neurons ([Figure 4.20](#)).

NFκB is a positive regulator of PINK1 gene, however, during stress conditions, NFκB upregulates PINK1 gene expression by binding to its promoter region (Duan et al. 2014; Soutar et al. 2018; Gonçalves and Morais 2021). NFκB was reported to be inhibited by withanolides, present in our test samples (E1D3T6, FGR and WFA) which may be a contributing factor in regulating the PINK1 expression (Heyninck et al. 2014; Sivamaruthi et al. 2023).



All experiments were performed in triplicates (n=3). Values are presented as mean± standard error

**Figure 4.20 Expression of mitochondrial genes PINK1 and DJ1 in Rotenone induced SH-SY5Y cells**

### **Role of DJ-1 in mitochondrial function and PD**

DJ-1 is an oxidative stress sensor and an antioxidant. In normal cells, the functions of DJ-1 include protease, glyoxalase, modulator of mitochondrial function, transcriptional regulator, increase neuronal survival from oxidative stress, molecular chaperone activity, co-activator for transcription factors such as P53, Nrf2 and NFκB. Inhibition or mutation in DJ-1 causes significant role in neurodegenerative diseases such as PD, Alzheimer's disease and Huntington's disease (Thomas et al. 2011; Sharma et al. 2019). Mutation in DJ-1 resulted in the aggregation of α-synuclein, increased oxidative stress in the

mitochondria led to increased mitochondrial fission, mitophagy and apoptosis which results in loss of DA neurons in SNpc. DJ-1 is normally present in cytoplasm during increased oxidative stress DJ-1 translocated to mitochondria and nucleus to protect neurons from cell death (Ma et al. 2014).

In the present study, treatment of Rot to SH-SY5Y cells resulted in the increased accumulation of ROS which resulted in the oxidative stress in DA neuronal cells. As we already know that the DJ-1 is an oxidative stress sensor, during increased oxidative stress which resulted in the mitochondrial dysfunction, DJ-1 is translocated to mitochondria for repair. Due to this fact DJ-1 expression in Rot treated SH-SY5Y cells (positive control) is significantly increased compared to untreated SH-SY5Y cells (negative control) (Figure 4.20). Similar results on the increased expression of DJ-1 in DA neuronal mitochondria were reported by Inden (2007) where chronic administration of rotenone to C57BL/6 mice (Inden et al. 2007). In addition, rotenone treatment on astrocytoma U251 cells resulted in the increased expression of DJ-1 and AKT phosphorylation due to increased accumulation of ROS and oxidative stress (Zheng et al. 2023). Due to inhibition of METC complex I by rotenone resulting in the accumulation of ROS in mitochondria which release of Ca<sup>2+</sup> ions from mitochondria causes decrease in MMP which further leads to oxidative stress in DA neuronal cells (Giorgi et al. 2012).

This loss of function of DJ-1 might have caused increased neurodegeneration in rotenone treated SH-SY5Y cells. However, pretreatment with E1D3T6 and FR extracts and WFA of SH-SY5Y cells following rotenone treatment causes restored DJ-1 expression to normalized levels (Table 4.10). 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, FGR and WFA following rotenone treatment to 2.02, 5.58 and 3.7-fold respectively. Withanolides found to interact with and activate number of transcription factors which involved in the human cell survival including NFkB, ERK1/2 AKT, Nrf, etc. and inhibits apoptosis regulating factors to ensure cell survival and proliferation (Neves et al. 2022). Activation of ERK signalling pathway in turn upregulates the DJ-1

gene expression upon stress stimuli (Neves et al. 2022) which results in the DJ-1 mediated neuro-protective activity during *W. somnifera* extracts pre-treatment.

This shows that oxidative stress formed due to rotenone treatment causing up regulation of DJ-1 in SH-SY5Y cells. But pretreatment of *W. somnifera* extracts and WFA standard decreased accumulation of ROS levels according to section 4.3.2 which resulted in a decrease in the oxidative stress levels. The above facts explains that the DJ-1 gene expression is reversed when oxidative stress level is decreased. Further, among the analysed test samples, SH-SY5Y cells pretreated with E1D3T6 extracts exhibited significant reversal than other tested samples (Figure 4.20).

**Table 4.10 Fold change of gene expression in SH-SY5Y cells**

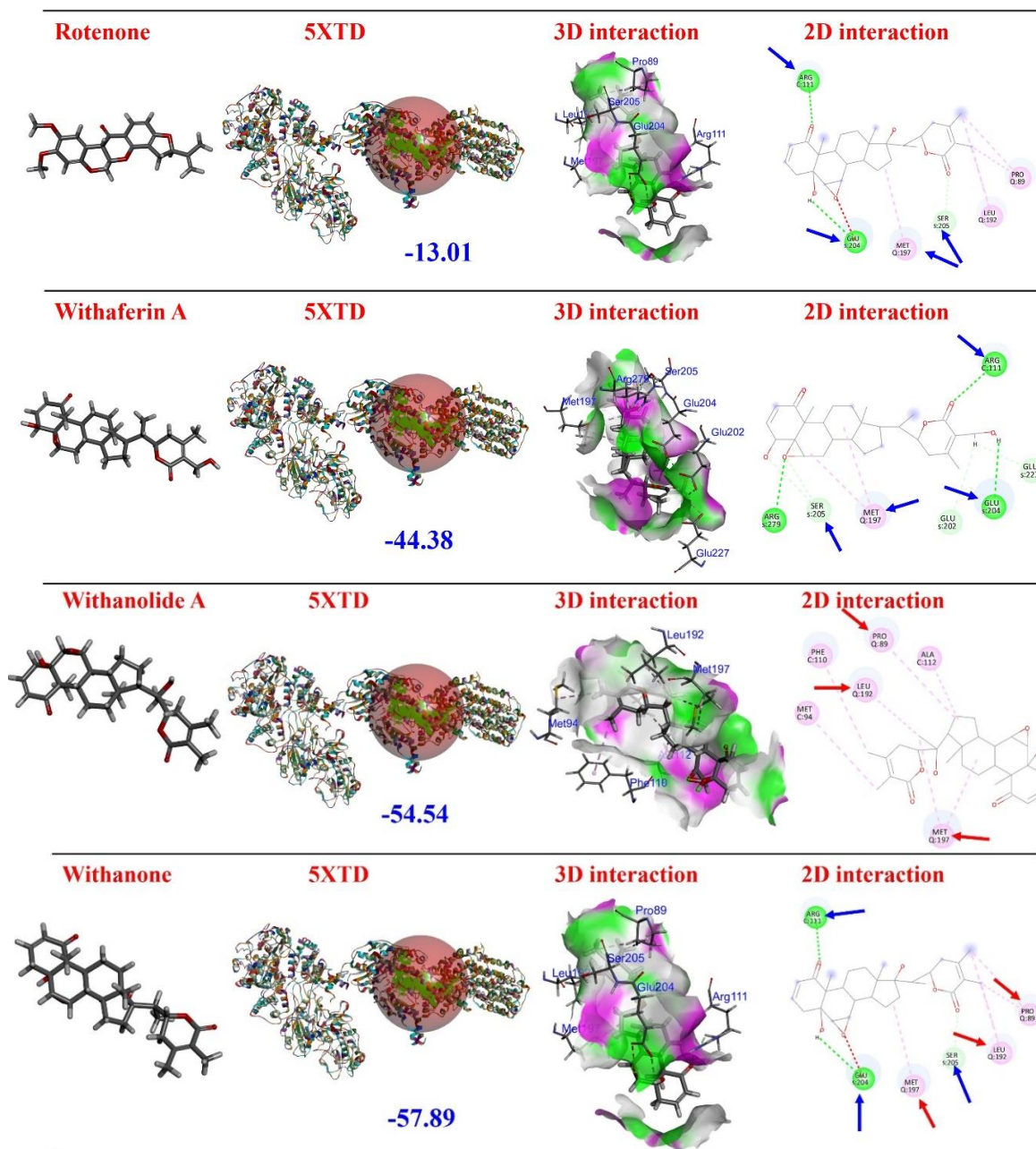
| Target gene         | PINK1      | DJ1       |
|---------------------|------------|-----------|
| Untreated           | 1±0.22     | 1±0.29    |
| Rotenone-1uM        | 18.50±0.12 | 7.50±0.44 |
| Rot+WFA1 -100uM     | 5.71±0.03  | 3.78±0.43 |
| Rot+E1D3T6-100ug/ml | 3.59±0.21  | 2.03±0.33 |
| Rot+FGR-100ug/ml    | 10.28±0.44 | 5.58±0.69 |

#### **4.3.6 Molecular docking analysis of Rotenone, withanolides with ETC complex I proteins**

The interaction among the proteins is essential to bring about any biological activity as any protein does not function alone (De Las Rivas and Fontanillo 2010). For the prediction of biological activity of a drug compound it is essential to identify its affinity towards the key proteins with maximum interaction with other proteins. We have selected ETC complex I protein for the molecular docking analysis because rotenone inhibits ETC complex I proteins and causes mitochondrial dysfunction in the PD. However, complex I protein is a very large protein having 44 protein subunits. Thus, we have selected seven specific protein subunits having active binding sites based on the already reported studies. Rotenone binding site in the complex I proteins is already

reported by [Pereira et al \(2023\)](#) as Q modules subunits such as NDUSF2, NDUFS7, ND1 and ND4. In addition to this, another three subunits of N module of complex I proteins such as NDUFV1, NDUFV2 was also selected for the present study ([Appendix 3](#)). Out of selected seven subunits, the PDB active site was located among NDUSF2, NDUFS7, ND1 and ND4 subunits. Therefore, binding of three withanolides, rotenone with selected seven subunits of complex I protein were analysed using molecular docking studies. The 3D structure of human complex I protein were obtained from RCSB PDB (5XTD). All the three withanolides along with rotenone was docked against complex I protein. Successfully, all the three withanolides and rotenone studied generated good docking binding energies against selected target complex I with minor changes in the binding pockets and its interacting residues ([Table 4.21](#)). The inhibition of complex I protein by rotenone results in the depleted ATP production in dopaminergic neuronal cells which further led to mitochondrial dysfunction ([Doke et al. 2021](#)).

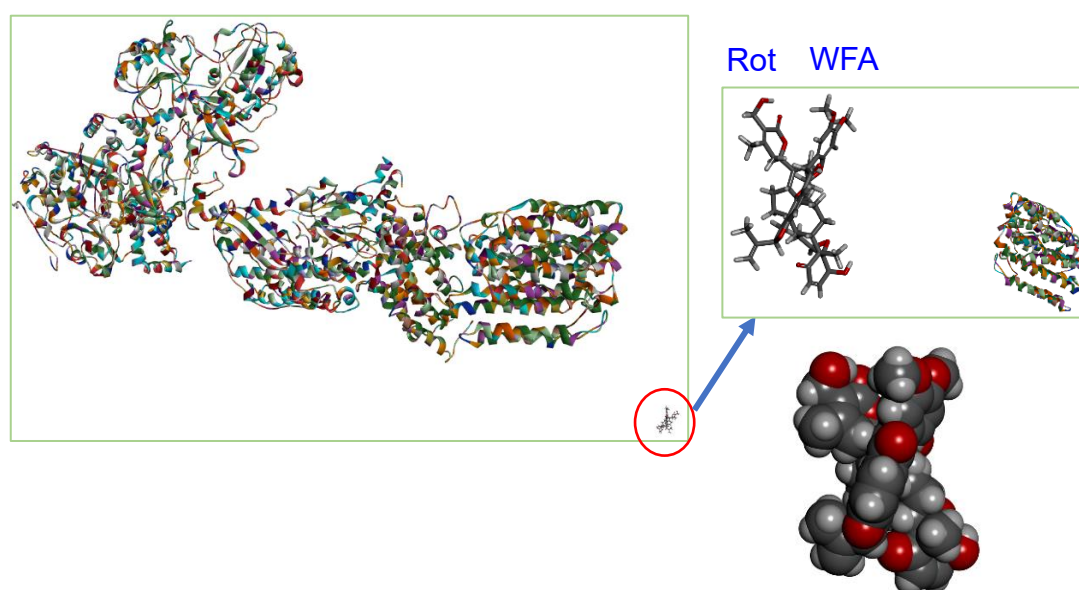
Docking against complex I protein showed interaction of similar amino acid residues with withaferin A, rotenone and withanone. The interaction residues were slightly different for withanolide A but overlapping amino acid residues was found between withanolide A and withanone. The binding energy of rotenone, withaferin A, withanolide A and withanone with complex I protein subunits is -13.01, -44.38, -54.54 and -57.89 kcal/mol respectively. All the selected ligands which interacted with two amino acids residues of complex I protein Met: 197. Rotenone and withaferin A binds with similar amino acids residues including Arg:111, Glu:204, Ser: 205 and Met:197.



Values are presented as **kcal/mol**

**Figure 4.21 Molecular docking of different withanolides with ETC complex I protein subunits**

The overlapping amino acid residues along with binding energies between withaferin A and rotenone indicate that withaferin A bind with rotenone binding pocket amino acids with highly negative binding energy (-44.38 kcal/mol). Among withanolides A and withanone, many overlapping amino acid residues were found including Pro:89, Leu:192, Met:197 with highly negative binding energy (-54.54 kcal/mol). However, due to the presence of alkyl bonding between these residues (Stefaniu et al. 2020; Umar et al. 2021) withanolide A was not selected for further studies. An overlapping residue between withanone and rotenone was also found including Arg:111, Glu:204, Ser:205 with highly negative binding energy (57.89 kcal/mol); however, the presence of unfavourable acceptor: acceptor bonding at Glu:204 was found (Ragi K et al. 2021). Thus, both withanolide A and withanone was not selected for further multiple-ligand simultaneous docking (MLSD) studies.



**Figure 4.22 Multiple ligand simultaneous docking of Withaferin A and Rotenone with ETC complex I protein**

MLSD is performed to analyse the interaction between substrate and inhibitor with the protein of interest at the same time. In MLSD, different conformations of multiple ligands can be docked with the receptor molecule simultaneously (Raghavendra et al. 2015). MLSD was done for withaferin A (substrate), rotenone (inhibitor) against complex I protein subunits. The docking results shows that the binding occurred between withaferin A and rotenone which inhibits the rotenone from binding with complex I protein. Similarly, strong ligand-ligand energetic coupling between two peptides Q and P of FVP peptides against SHP2 N-SH2 domain of phosphatase enzyme (Li and Li 2009). The binding of withaferin A and rotenone was found to be highly negative binding energy (rotenone: -89.584 & withaferin A: -46.471 kcal/mol) which is higher than its single ligand binding energies (-13.01 & -44.38 kcal/mol) with the protein molecule (Figure 4.22). The two-ligands binding with each other instead of binding with receptor molecules indicates WFA forms a complex with rotenone (Figure 4.22) preventing it from binding to receptor (Forli et al. 2016). Further, withaferin A and rotenone binding leaves the complex I protein uninhibited. The absence of inhibition of the complex I protein by rotenone binding results in the normal function of mitochondrial electron transport chain and normal ATP production. Therefore, withaferin A and withaferin A containing plant extracts might be used for the alleviating mitochondrial toxins induced parkinsonism in human.