2.0 Review of The Literature

In recent years, the demand for herbal products has increased owing to the worldwide shift in consumer choice towards phyto-therapeutics (Singh et al. 2016). According to the World Health Organization, it is estimated that 80% of the global population relies on herbal medicine to cure various health issues (Palhares et al. 2015). Among medicinal plants, *Withania somnifera* (L.) Dunal which belongs to the family Solanaceae has gained a lot of attention since ancient times due to its immense pharmaceutical potential (Kaur et al. 2021).

Medicinal properties of *W. somnifera* are imputed to the key active constituents known as withanolides and their glyco-conjugates present in different plant parts (Chatterjee et al. 2010). Elicitation with various biotic and abiotic elicitors has been widely applied to enhance the secondary metabolite production in shoot cultures as well as in plant cell cultures of different plant species. In *W. somnifera*, a number of biotic and abiotic elicitors have been identified which modulate major and minor withanolide biosynthesis in adventitious root, hairy root and multiple shoot cultures (Sivanandhan et al. 2012, 2014). In addition to enhancing the accumulation of specific product yield, the application of abiotic elicitor like metal salts in culture medium often stimulates its reduction and assimilation of metal ions as metal nanoparticles (MNPs). MNPs are receiving considerable interest from the scientific community due to the remarkable properties they present compared to the bulk of the same metal (Parker et al. 2014). The synthesis of MNPs using live plant species termed as *in planta* synthesis of MNPs. The application of these *in planta* MNPs include that they can be used in pharmaceuticals along with their host species if the host is a medicinal plant. In the present study, an ayurvedic medicinal herb *W. somnifera* shoots were used to synthesize MNPs at *in planta* level and its therapeutics application was confirmed in Parkinson's disease (PD) cell model.

The literature related to the present study entitled "*In planta* assimilation and characterization of metal nanoparticles in *in vitro* shoots of *Withania somnifera* and its therapeutic evaluation in Rotenone induced SH-SY5Y cells" is reviewed under the following headings in this chapter.

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2.1 *Withania somnifera* (L.) Dunal

Withania somnifera (L.) Dunal (Ashwagandha, Family: Solanaceae) is an important medicinal plant in the traditional Indian system of medicine for more than 3000 years. *W. somnifera* is widely distributed in Asia, Africa, Middle East and Mediterranean region. In India, it grows mostly in Punjab, Jammu and Himachal Pradesh (Baldi et al. 2008). About 2000 tons of *W. somnifera* roots are annually produced in India and the dried roots are sold at approximately $ 140 per quintal (Das et al. 2011). The root extract of *W. somnifera* is extensively used for the treatment of rheumatism, gynaecological disorders, bronchitis, arthritis, senile debility, tuberculosis and cardiac, skin and inflammatory diseases (Fatima et al. 2015; Sivanandhan et al. 2012). It also possesses a wide array of therapeutic properties, including anti-inflammatory, anti-tumour, anti-bacterial, anti-spasmodic, hypoglycaemic and hypolipidemic effects (Udayakumar et al. 2014). These therapeutic properties have been mainly attributed to its diverse array of secondary metabolites, including steroidal lactones (WFA, WTA), flavonoids and phenolics (dihydroxykaempferol, quercetin, quercetin-3-rutinoside, quinic acid, scopoletin and aesculentin), tropane alkaloids (tropine, pseudotropine, nicotine, withasomine, anaferine), withanone, ashwangandholide, withanolides dimer sulphide, 2,3 dihydrowithaferin A (viscosalactone B) and 27-hydroxywithanolide A (Chaurasiya et al. 2007; Chatterjee et al. 2010; Kapoor et al. 2022).

2.1.1 Morphology and taxonomic classification

W. somnifera is an evergreen, erect, small woody, tomentose shrub about 30-150 cm in height. Leaves are alternate, simple and petiolate, 4-10 cm long and 2-7cm broad. Flowers are monoceous or bisexual, yellow to green in colour, appearing in clusters of 4-25 flowers. Flowers are produced throughout the year with March –July as the peak flowering period. The fruits are small round berries, orange-red in colour, about 5-8mm in size and enveloped by a large brownish, papery and turgid calyx. Fruits contain many seeds. The seeds are kidney shaped, pale brown in colour, and about 2.5mm in size (Mirjalili et al., 2009). Roots are stout, fleshy, cylindrical and 1-2 cm thick, straight and unbranched. Roots bear fibrous secondary roots. Crown consists of 2-6 remains of stem bases which are thickened, nodes prominent on the side from

where petiole arises. It is short and uneven with characteristic odour, bitter taste and acrid (Khare, 2004).

Taxonomic classification

Kingdom: Plantae
Division: Angiospermae
Class: Dicotyledoneae
Order: Tubiflorae
Family: Solanaceae
Genus: Withania
Species: Somnifera

Vernacular names

Sanskrit: Asvagandha
English: Winter cherry
Hindi: Asgandh
Tamil: Amukira

(Gaurav et al., 2015)

2.1.2 Bioactive compounds

Primary metabolites such as carbohydrates, amino acids and lipids are used by higher plants to produce various secondary metabolites (Hatami and Ghorbanpour, 2016). Plant secondary metabolites are often referred to as compounds that have no directly essential role to the functioning of the plant; however, they do serve an important role in the interaction of plant with its environment for adaptation and defence (Akula and Ravishankar, 2011). Almost 100,000 of secondary metabolites have been identified with molecular weight less than 150 kDa and production of less than 1% of dry matter weight (Oksman-Caldentey and Inzé, 2004). Secondary metabolites also contribute to the specific odours, tastes, toxins and natural colours in plants, besides unique sources for food additives, flavours, and pharmaceutically active substances (Akula and Ravishankar, 2011). Based on their biosynthetic pathways plant secondary metabolites can be divided into the three most important groups including terpenes/terpenoids, phenolics and nitrogen compounds. Volatile terpenoids represented by mainly isoprene (C5), monoterpenes (C10) and sesquiterpenes (C15) constitute the largest group of plant secondary metabolites (Nagegowda 2010). Some of secondary metabolites containing nitrogen compounds are alkaloids, glycosides and phenolic compounds including phenolic acids, antocyanidin and lignin. Accumulation of secondary metabolites often occurs in plants subjected to different types of stresses, various elicitors and/or signal molecules (Hatami and Ghorbanpour, 2016) and

regulates by the several factors including genetic and evolution, growing conditions, physiological variations, climate, photoperiod, temperature, light, mineral elements, as well as heavy metals (Figueiredo et al., 2008; Street, 2012). Medicinal plant performance has been influenced by different environmental stresses (Figueiredo et al., 2008; Zheljazkov et al., 2011; Akula and Ravishankar, 2011; Asgari Lajayer et al., 2017), however, little information is available on heavy metals effects on quantity, quality and secondary metabolites biosynthesis in such plants. It has been reported that heavy metal stresses cause a significant increase of secondary metabolites in medicinal plants (Ali et al., 2006; Sinha et al., 2010).

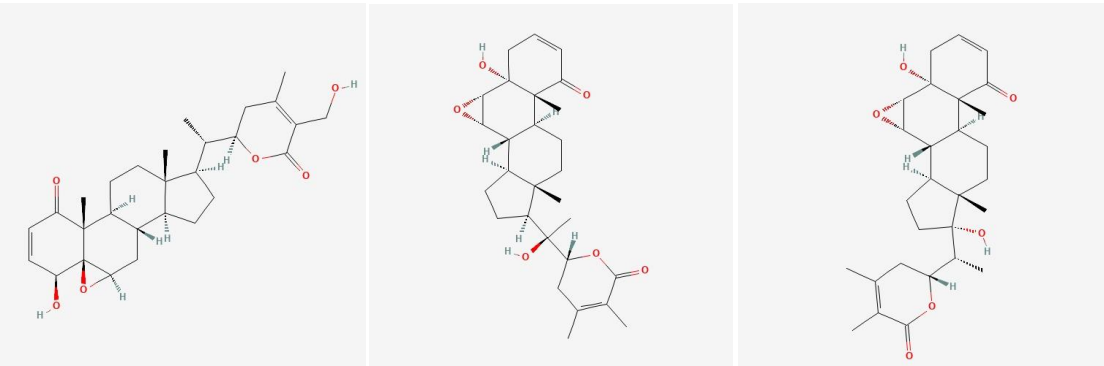
2.1.3 Withanolides

Withanolides, polyoxygenated C28 steroidal lactones with a chemical nomenclature of 22-hydroxy ergostane-26-oic acid 26, 22-δ-lactones, are the major chemical constituents of Withania species and are present in almost all parts of *W. somnifera*; however, the root is the product of commercial utilization and traditional formulation in practice. Many withanolides have been identified in the roots and leaves of *W. somnifera*. Withanolides are also present in 15 other genera of *Solanaceae*, plants of the families *Taccaceae* and *Leguminosae*, and in some marine organisms (Veleiro et al. 2005). More than 40 withanolides were reported from the aerial tissues, roots, and berries of *W. somnifera* (Mirjalili et al. 2009). Several therapeutic actions of *W. somnifera* were reported to have links to one or more withanolides. Withaferin A (WFA), withanone (WN), and withanolide A (WTA) are major withanolides in *W. somnifera* (Vinod et al. 2022). WFA inhibits cyclooxygenase-2 but not cyclooxygenase-1, which is desirable for a non-ulcerating, anti-inflammatory/chemotherapeutic drug. WFA was found to have immunosuppressive actions on B-lymphocyte proliferation (Jayaprakasam et al. 2003). WTA was reported as an important candidate for the therapeutic treatment of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, convulsions, and cognitive function impairment, as it can reconstruct neural networks (Kuboyama et al. 2005; Tohda et al. 2005). WTA has also attracted the interest of researchers due to its neuropharmacological properties that promote synaptic and outgrowth reconstruction at a very low

dose, i.e., 4.7 µg/kg (Kuboyama et al. 2005). The molecular mechanism studies for WFA and WTA demonstrate that the modulation of multiple targets, e.g., transcriptional factor, inflammatory cytokines, enzymes, growth factors, receptors, and other targets, thereby suggesting that these two compounds are potential candidates for cancer and neurological disorder treatments (Kumar et al. 2018).

Several active phytochemical compounds like alkaloids, flavonoids, saponins, phenols, carbohydrates, steroids, tannins, withanolides and sitoindosides have been found in various parts of this plant (Saleem et al. 2020). These compounds are held responsible for the ethanomedical and pharmacological efficacy of the plant (Nile et al. 2019). A total of 62 primary and secondary metabolites have been identified from leaves and 48 from roots, out of which 29 are common to both. It is also reported that the distribution of secondary metabolites varies significantly with respect to different tissues, developmental stages, and chemotypes (Chatterjee et al. 2010; Thirugnanasambantham et al. 2014).

Among the several phytochemical compounds reported in *W. somnifera*, the medicinal properties of this plant are attributed to a group of steroidal lactone compounds referred to as withanolides. The structures of about 40 withanolides have been elucidated so far all of which contain 22-hydroxy ergostane-26-oic acid 26, 22-lactone as the elementary backbone structure (Dar et al. 2015). The structures of the key withanolides of *W. somnifera* are depicted in **Plate 2.1.**



Withaferin A | Withanolide A | Withanone

Plate 2.1 Structure of key withanolides of *Withania somnifera*

2.2 Tissue culture and Abiotic stress

2.2.1 Tissue culture - callus, suspension, shoot/root culture

Plant tissue culture is a basic, fundamental science from the branch of plant biotechnology that helps in understanding the growth and development of plants at the cellular level. Tissue culture is defined as growing or culturing of desired cells, tissues, or organs on a designed sterile synthetic medium under controlled conditions of temperature, light, and humidity (Thorpe, 2007). Plant tissue culture techniques helped the commercial industry in the production of diversified metabolites, flavours, oils, colours, and pharmaceuticals from plants. This tissue culture technique, which existed in the early 1940s, helped in creating a billion-dollar market (Sahai and Knuth, 1985). Designing of the plant tissue culture medium was challenging in the initial stages, as it is very important to provide a favourable environment for a plant to grow, which simulates the nutrient availability from nature. An ideal plant tissue culture medium should have all the required minerals, nutrients, and vitamins. The source of the explant, type of media suitable to that particular explant, temperature, and many internal factors like solidifying agents, pH, and supplements are some important parameters to take into consideration to formulate a plant tissue culture medium. An addition of required plant growth regulators for the proper growth of the desired cell/tissue/organ cultures is an important milestone in the plant tissue culture technique (Sudheer et al. 2022).

In vitro propagation of *W. somnifera*

The poor viability and high dormancy exhibited by the *W. somnifera* seeds under field conditions has led to the search for alternative culture methods under optimised conditions (Khanna et al. 2013). Various nutrient medium, temperature and light conditions were then studied intensively and optimised *in vitro*. Soaking of seeds has reported to soften the seed coat thus increasing chances of germination (Vashistha et al. 2010). Further the increased rate of germination was observed under 16/8 hr photoperiod at 25 ± 2^{o}C and 3000lux light intensity (Khanna et al. 2013). Apart from seeds, all the plant materials like embryo, seedling, cotyledon, petiole, leaf, stem, shoot tip, root, nodes, and inter-nodes have shown the potency for callus and

adventitious shoot/root induction, differentiation, regeneration, flowering and fruit production (Singh et al. 2017).

The withanolides content and biomass in the plant were also found to be dependent on the growth stage with maximum accumulation at the exponential phase (7-28 days). The 28-day old plants in suspension were recorded with increased amount of withanolides (Sivanandhan et al. 2014). Senthil et al (2015) has reported a maximum WA accumulation (980 ± 0.97 µg/g DW) in 45 days old *in vitro* cultured leaves with a gradual decrease over extended period of culture. Similar synchronization with the age of cultures was found in WTA accumulation in root cultures. Among the various concentrations and combinations of plant growth regulators studied (BAP and Kinetin), MS medium supplemented with 4.44µM BAP showed 1.86-fold increase in WA content compared to control (Murugesan and Senthil et al. 2017). Though various workers described various methods for induction of callus tissue and withanolide production from callus tissue of *W. somnifera*, the callus failed to synthesis withanolides. Whereas multiple shoot cultures and transformed roots were capable to produce withanolides. Also, it is stated that the presence or absence of withanolides in any tissue type is dependent on the plant growth regulators used (Chakraborty et al. 2013).

2.2.2 Elicitation studies in *W. somnifera*

W. somnifera is being used in conventional systems of medicine and Ayurveda over thousands of years. Due to active constituents i.e. withanolides attributing to its pharmacological actions, *W. somnifera* achieving lot of attention in a variety of herbal preparations and formulations (Sangwan and Sangwan 2014; Sangwan et al. 2017). *W. somnifera* plants were reported to grow under different agro-climatic conditions across the country. Attempts are in progress to evaluate the yield and growth of plant under various stress conditions. The traditional cultivation methods of *W. somnifera* for withanolides drug preparation have been limited by a range of issues such as biotic and abiotic environmental factors, unpredictability of bioactive components, and lack of purity and standardized plant raw material for phytochemical analysis (Sivanandhan et al. 2016). Moreover, these methods are time consuming,

laborious, and not able to sustain the current *W. somnifera* global market requirement. The requirement of dried plant material for withanolide production has been estimated at about 12,120 ton, whereas the annual production is of about 5905 ton (Thilip et al. 2016). At the international level, there has been an ever-increasing demand for *W. somnifera* in larger quantities (Sivanandhan et al. 2012, 2016). Thakur et al (2019) reported that exogenous addition of elicitors was considered to be one of the most promising strategies for the increased production of secondary metabolites. The elicitor molecule in culture interacts with a plant membrane receptor which activates specific genes, resulting in the synthesis of secondary metabolites. Of various types of biotic and abiotic elicitors, methyl jasmonate, salicylic acid (SA), chitosan and fungal mycelial mat and Cd and Ag, have been proven as active elicitors for the production of secondary metabolites in plant cell/organ cultures respectively (Halder et al. 2019; Bhaskar et al. 2022).

Biotic elicitors

It is very much evident that biotic elicitors play a major role in elicitation of secondary metabolites. The production of WFA is found to be increased to a greater extent by introducing chitosan and Carrageenan elicitors to hairy root cultures of *W. somnifera* (Thilip et al. 2019). Further, Among the different organ cultures as well as elicitors tested, SA elicitation can be used for withanolides production (42–58-fold) in hairy root culture of *W. somnifera* (Sivanandhan et al. 2016). In another study using hairy root cultures of *W. somnifera*, chitosan proved to be the best elicitors for the increased production of withanolides. The results indicate chitosan elicitation would allow the optimized production of withanolides from leaf callus-derived adventitious roots (Sivanandhan et al. 2012). The elicitation strategy by the addition of cell extracts of *Verticilium dahaliae* as an elicitor resulted in 9.7-fold enhancement of WFA content in comparison to control cultures (2.65 mg/L) (Baldi et al. 2008).

Increased production of withanolides such as WTA (7.21 & 5.04 mg/g DW), withanolide B (4.23 & 2.59 mg/g DW), WFA (3.88 & 2.36 mg/g DW) and WN (6.72 & 4.32 mg/g DW) were achieved in the elicitation of cell suspension cultures of *W. somnifera* by *Gracilaria edulis* extract and L-glutamine with

picloram (Sivanandhan et al. 2013). In addition, biotic elicitation using seaweed extract powder of *Kappaphycus alverizii* on shoot cultures of *W. somnifera* yielded 18.56, 8.96, and 7.29 -fold increase in WFA, WT A and WN content compared to control shoots (Vinod et al. 2022). On the other hand, the studies of Sivanandhan and group that has reported an increase in SS (Squalene synthase), SE (Squalene epoxidase), HMGR (3-Hydroxy-3-methylglutaryl-coenzyme A reductase) and FPPS (Farnesyl pyrophosphate synthase) in hairy root cultures of *W. somnifera* on seaweed elicitation leading to 2.3-2.6 -fold increase in levels of withanolides (Sivanandhan et al. 2016). The expression of genes of MVA, MEP and withanolides biosynthetic pathways like HMGR, SS, SE, CAS, FPPS, DXR and DXS were upregulated by 12.5, 4.9, 2.18, 4.65, 2.34, 1.89 and 1.4 folds in the cell suspension cultures of *W. somnifera* treated with fungi (*A. alternata*, *F. solani*, *V. dahliae* and *P. indica*) (Ahlawat et al. 2017).

Abiotic elicitors

Plants possess natural ability to take up non-essential metals, including heavy metals such as Cd, Cu, and Ag. The uptake of metal triggers a set of complex changes in plant growth attributes as well as modulations at biochemical and physiological levels. Plants are able to develop certain mechanisms to tolerate metal stress by stimulating enzymatic antioxidants to overcome the oxidative injury caused by the metal stress, which is being reflected as a vital mechanism in plants (Rout et al. 2019). Metabolism of sugar, proline and phenylpropanoid/phenolics in coordination emerged as adaptive mechanism in *W. somnifera* under Cd stress which endorsed the tolerance and adaptive properties to *W. somnifera* under abiotic stress via different mode of actions. Carotenoids, proline, total phenolics and flavonoids contents were actively involved in ROS management and Cd detoxification mechanism (Mishra et al. 2018). Cd elicitation of *in vitro* multiple shoot cultures of *W. somnifera* showed that interplay of enzymatic as well as nonenzymatic responses constituted a system endeavor of tolerance of Cd accumulation and an efficient scavenging strategy of its stress implications. Sugar metabolism was also markedly modulated under Cd stress. Various other parameters including contents of photosynthetic pigments, phenolics, tocopherol, flavonoids, reduced glutathione, nonprotein thiol, ascorbate, and proline

displayed major inductive responses reflecting their protective role (Mishra et al. 2014). Similarly, copper treated *in vitro* grown plants of *W. somnifera* accumulated high amount of Cu and decrease in superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPX) activities seems to reflect its inability for eliminating the ROS resulting from Cu-induced oxidative stress.

On the other hand, total phenolic contents increased with increasing concentration of Cu compared to the control. Thus, *W. somnifera* have the ability to grow in Cu polluted areas by altering various physiological changes (Khatun et al. 2008). Similar study was performed by Rout where Cu treatment on *in vitro* plants of *W. somnifera* and its effect on synthesis of new proteins and isoforms of antioxidant enzymes such as GPX, SOD, and CAT was studied (Rout et al. 2013). In another study on shoot and callus cultures of *W. somnifera* exposed to KCl, NaCl, KNO_3, $NaNO_3$, and $CaCl_2$ resulted in increased antioxidants and enzymatic activities. Thus, these results suggest that the nonenzymatic and enzymatic antioxidant systems of both the tissues played a primary role in combating the imposed salt stress (Sabir et al. 2012). Zn and Fe is an essential metal for plant growth, Zn and Fe found to have an adverse effect on the morphological, biochemical, molecular, and elemental uptake in *in vitro* plantlets of *W. somnifera* when exposed to high concentrations. Also, overexpression of antioxidant enzymes (SOD, CAT & GPX) and their corresponding genes were resulted which indicates that *W. somnifera* has detoxification mechanisms to compact with Zn and Fe excess. Further, the proton-induced X-ray emission study confirmed an increasing order of uptake of and Fe Zn in plants by suppressing and expressing other elemental constituents which cause metal homeostasis (Rout et al. 2014, 2019). The effect of Cu and Zn on *in vitro* regeneration of *W. somnifera* cultures and change in photosynthetic pigments, and increased proline content (Fatima et al. 2011).

Apart from metal elicitation studies, ultraviolet B radiation stress on withanolides (WTA & WFA) and other secondary metabolite production were studied using field grown tissues of *W. somnifera*. In addition to this, the

enzymes of phenylpropanoid pathway such as phenylalanine ammonia lyase, cinnamyl alcohol dehydrogenase, 4-coumarate-CoA ligase, chalcone-flavanone isomerase, and di-hydroflavonol reductase were determined (Takshak and Agrawal 2014). Among the different wavelengths, red light was best for maximum biomass accumulation in callus cultures of *W. somnifera*. However, violet light condition was proven to be favouring the phenols and flavonoids synthesis in the callus cultures. Compared to other wavelengths, red light grown callus extract showed significantly higher content of chlorogenic acid, and WFA (Adil et al. 2019). Two months old *W. somnifera* plants were subjected to cold stress (4ºC) and analysed for withanolide A, WFA along with anti-oxidant enzymes such as SOD, GPX, CAT, GP and APX. It could be inferred from these observations that cold stress induces bioactive withanolides accumulation in *W. somnifera* as a mechanism for scavenging ROS (Mir et al. 2015). Low light stress significantly affected all the morphological parameters, and total withanolide content Highest withanolide accumulation was recorded under 75% shade condition; after exposure to stress for a period of 30 days (Jacob et al. 2014). Nanoparticles have elicitor activity for the enhancement of secondary metabolites biosynthesis in plants. *W. somnifera* was treated with four different NPs such as Zn-Ag NPs, Ni, and CdSe. Zn-Ag NPs showed elicitor activity for the enhancement of primary and secondary metabolites of *W. somnifera*; however, Ni showed suppressive activity by causing stress in the plants. Further, withanolides significantly decreased in CdSe treatment (Ramakrishna et al. 2011; Singh et al. 2019).

2.2.3 Metal stressors

All heavy metals are non-biodegradable, that is, they cannot be purged out naturally from the environment via any possible natural means, although some of them are reported to be immobile, that is, they can be taken up by plants root system via diffusion, endocytosis or through metal transporters. Nevertheless, some of these metals such as zinc, copper and nickel are essential micronutrients and are required in trace amounts as they act as cofactors for various enzymes. While other metals such as Cd, Pb, Hg and lead present in pesticides do not have any beneficial role and become toxic if their concentration exceeds a certain concentration (Alharbi et al. 2018). Plants

have various molecular and physiological mechanisms to overcome heavy metal stress which includes complex biochemical- and genomic-level processes. Plant uses various mechanisms to prevent exposure to heavy metals present in the soil by providing tolerance. Effects of heavy metals on production of bioactive compounds in some medicinal and aromatic plants are presented in **Table 1.**

Zinc stress

Zinc (Zn) is an essential element required by plant for its normal growth and development. Zinc is involved in carbohydrate, auxin and protein metabolism. It participates in enzymatic reactions as a cofactor and is involved in pollen formation and provides resistance against many pathogens. Deficiency in Zn can lead to severe yield loss in crops (Aziz et al. 2016). However, Zn is required in trace amounts only, and its excess can lead to Zn toxicity with dire consequences. Zn is a part of various enzyme-catalysed reactions in the cell, and its toxicity alters reactions requiring enzymes. Hence, in effect, it can cause retarded growth and senescence. Plants affected with Zn toxicity have visible symptoms of chlorosis, especially in younger leaves which are also spread to older leaves if the toxicity prolongs. Zn toxicity also induces accumulation of other heavy metals such as Cu and Mn in root and shoot (Nagajyoti et al. 2010; Ghori et al. 2019). Todeschini et al (2011) observed accumulation of Zn in cell walls of xylem and parenchyma cells. They also concluded that a significant increase in the number of calcium oxalate crystals was seen in leaves affected by Zn toxicity, deducing that Zn toxicity increases free calcium in the plants.

Cadmium stress

Plants faced with Cd toxicity usually have retarded growth, chlorotic leaves, brown root tips and decline. Cd accumulation inhibits Fe (III) reductase that leads to iron deficiency and in turn affects the photosynthesis. Photosynthesis is also affected due to reduced chlorophyll synthesis and inhibition of enzymes involved in CO_2 fixation. Cd also interferes with uptake of Ca, P, K, Mg and water and reduces the translocation and absorption of nitrate by inhibiting nitrate reductase. Water balance disturbance has also been

noticed which is caused by disturbed plasma membrane integrity during lipid peroxidation (Ghori et al. 2019). High levels of Cd also damage nucleolus leading to chromosomal fragmentation and aberration. Decomposition of mitochondria greatly hinders plant respiration. It is observed in radish that during Cd toxicity, Ca is displaced with Cd in calmodulin involved in cell signalling, causing inhibition of calmodulin-dependent phosphodiesterase activity (Rivetta et al. 1997). *Alternanthera bettzickiana* plants were treated with different levels of Cd and Pb to observe the responses of plants. It was observed that lower metal concentration results in increase in plant growth, biomass and photosynthetic pigment, whereas the higher metal concentration results in decrease in these aspects. Similar trend is obtained for the activities of superoxide dismutase, peroxidase, catalase and ascorbate peroxidase (Tauqeer et al. 2016; Ghori et al. 2019).

Copper stress

It has been found that Cu plays an important role in carbon assimilation and ATP synthesis in plants; furthermore, it has been established that it is an important constituent of plastocyanins and cytochrome oxidase, both of which are vital components of both photosynthetic and respiratory systems; nevertheless, Cu overload can cause oxidative stress in plants, which in turn causes an extensive damage to membranes, macromolecules and DNA (Yadav 2010). Plants under Cu stress show various different noticeable symptoms for, e.g., chlorosis and inhibited growth, in addition to ion leakage and retarded root growth (Bouazizi et al. 2010). Cu taken from the soil must be transported, distributed, and compartmentalized within different tissues and organelles for healthy plant growth and development. On the other hand, excessive Cu is characterized by a reduced plant biomass, leaf chlorosis, inhibited root growth, bronzing, and necrosis. Increased Cu leads to inhibition of stem and root development in *A. thaliana* with altered auxin homeostasis which caused by elevated NO production that inhibited PIN1 mediated auxin transport (Kolbert et al. 2012). Similar decreased root activities, physiological disorder and root cell membrane damage which led to the intracellular leakage of Cu^{2+} within root cells was observed in maize seedlings (Liu et al. 2014).

Table 2.1 The changes in Phytoconstituents of medicinal plants in response to heavy metal stress

S. No	Plant species	Heavy metal	Metal Concentration	Findings	Reference
1	*Artemisia annua* L.	Arsenic	0–4500 µg L−1	Enhanced artemisinin production and upregulation of artemisinin biosynthesis pathway genes	Rai et al. (2011)
2	*Mentha pulegium* L.	Copper and Zinc	0–25 mg kg−1 and 0–50 mg kg−1	Major essential oils components pinene, sabinene, 1,8-Cineol, and thymol were significantly increased	Asgari Lajayer et al. 2017
3	*Mentha crispa* L.	Lead	900, 1800, 3600, 7200, and 9000 mg kg−1	Improved production of carvone (major component of essential oils)	Sa et al. 2015
4	*Trigonella foenum-graecum* L.	Cadmium, Cobalt, Chromium, Nickel	0, 100, 300 and 500 µM; 0, 200, 400 and 800 µM; 0, 300 and 500 µM; 0, 200 and 400 µM	Stimulation of berberine synthesis in cell suspension cultures. Cd and Co stimulated biosynthesis of diosgenin, whereas, Cr and Ni inhibited the production of diosgenin	De and De 2011
5	*Helianthus annuus*	Copper	0, 0.125, 0.25, 0.5, 1 and 2 mg L1	The exudation of coumaric acid	Meier et al. 2012
6	*Silene paradoxa*	Copper and Nickel	5 µM	Over-production of phytoalexin and other phenolic compounds	Martellini et al. 2014
7	*Abutilon indicum*	Cadmium	0.5–4 mM CdCl2	Production of Stigmasterol	Rao et al. 2022
8	*Allium sativum*	Cadmium	0.05, 0.10, 0.15, and 0.20 mM CdCl2	Production of Alliin	Malik et al. 2020

9	*Anisodus acutangulus*	Silver	4.4583 mol L− 1 AgNO3	Increased production of Hyoscyamine, anisodine and scopolamine	Kai et al. 2012
10	*Bacopa monnieri*	Copper	45 mg L− 1 CuSO4	Production of Bacoside	Sharma et al. 2015
11	*Catharanthus roseus*	Silver	50 and 100 µM $AgNO_3$	production of Ajmalicine, vinblastine, vincristine, vindoline and catharanthine	Paeizi et al., 2018
12	*Corylus avellana*	Aluminium	50 and 100 µM $AlCl_3$	Production of taxol	Farrokhzad and Rezaei, 2020
13	*Ocimum basilicum*	Silver	5, 25, 50 and 100 µM $AgNO_3$	Production of Linalool Estragole	Açıkgoz, 2020
14	*Prosopsis farcta*	Lead	80, 160, 320, 400 and 480 µM Pb(C2H3CO2)2	Increased production of Ferulic acid, salicylic acid, daidzein, vitexin and phenolic acids. Enhanced activity of CAT, SOD, GPX and PAL.	Zafari et al. 2016
15	*Ulmus laevis*	Arsenic	0.06 mM (CH3)2As(O) OH and Na2HAsO4•7H2O	Production of Protocatechiuc, caffeic, p-coumaric and chlorogenic acids	Drzewiecka et al., 2018
16	*Zea mays*	Cadmium, Copper and Lead	10, 20, and 50 ppm CdCl2 and CuSO4, and Pb (NO3)2	Enhanced accumulation of Phenolic compounds such as chlorogenic acid and rutin	Kısa et al., 2016
17	*Oryza sativa*	Chromium	100 µM $K_2Cr_2O_7$	Accumulation of secondary metabolites Protocatechuic, p-hydroxybenzoic, vanillic, p-coumaric, caffeic and gallic acids	Dubey et al., 2018

18	*Withania somnifera*	Cadmium	5, 10, 20, 50, 100, 150, 200 and 300 µM $CdSO_4$	Increased accumulation of Phenols, flavonoids and lignins	Mishra and Sangwan, 2019
19	*Vitis vinifera*	Cd2+, Co2+, Ag+	5.0, 25, and 50 µM	Accumulation of Resveratrol	Cai et al. 2013
20	*Hypericum perforatum*	Chromium	0.01 and 0.1 mM	production of Protopseudohypericin, hypericin and pseudohypericin	Shakya et al. 2018

Mercury stress

Mercury acts as a toxic element for the plants and has no beneficiary effect at all (Ghori et al. 2019). Mercury toxicity causes visible symptoms of injuries depending on the area it affects. It may enter through water and bind to water channel proteins and obstruct the flow of water to plants. It can also affect the mitochondrial and chloroplast activity by interfering with electron transport chain and inducing oxidative stress along with membrane degradation and biomolecules oxidation (Nagajyoti et al. 2010). Hg stress on turf grass showed that, with the increase of the Hg stress intensity and the extension of time, the net photosynthetic rate, stomatal conductance and transpiration rate of leaf of the grass continued to decline, while the intercellular CO_2 concentration continued to rise (Guo 2015). Hg stress on wheat plants revealed that at >10μM concentration, Hg severely affect the plant growth, nutrient uptake and production of antioxidant enzymes (CAT and POX) (Sahu et al. 2012).

Lead (Pb) stress

Lead is counted among the major heavy metals that contaminate the soil resulting from smelting, mining and natural weather processes (Ashraf et al. 2016). Pb toxicity symptoms include chlorosis, stunted growth, inhibits photosynthetic apparatus and reduced root lengths. Once entered into the cell, Pb changes cell membrane permeability, hormonal changes, inhibition of various enzymes containing sulfhydryl group, reduction in water content, closes stomatal pores and disturbed mineral nutrition (Ghori et al. 2019). It was observed that the Pb accumulates largely in roots, followed by petiole and leaf tissues of water hyacinths. The activity of antioxidative enzymes also increases with increased lead stress; hence, it can be said that water hyacinths have efficient mechanisms to tolerate Pb toxicity (Malar et al. 2014). it was demonstrated in *Suaeda salsa*, Pb and Zn exposure induced osmotic stress as reveled by the increase in betaine shoot content in the presence of metals (Amari et al. 2017). White locust tree (*Robinia pseudoacacia*) grown in cadmium (Cd) and lead (Pb)-contaminated soils accumulated saponins, phenols, alkaloids and flavonoids in aboveground parts, remediating plant

health to some extent (Zaier et al. 2014). Pb stress retarded the plant growth and reduced chlorophyll contents in the leaves of *Daucus carota*.

A significant decrease was observed in photosynthetic attributes by Pb addition alone. However, exogenously applied GA_3 ameliorated the plant growth, phenolic compounds concentration and chlorophyll contents in the leaves of carrot cultivars under Pb stressed conditions (Ghani et al. 2021). Pb toxicity decreased chlorophyll and carotenoid contents and enhanced the expression of chlorophyllase in *Solanum Lycopersicon*. Further, addition of Jasmonic acid stimulated tolerance in tomato under Pb stress by increasing the levels of osmolytes, organic acids, polyamines, and metal ligation compounds, which showed their active involvement in tolerance against Pb toxicity (Bali et al. 2019). In mustard seedlings, combined application of 10− 7 M 24-EBL and 1 mM SA ameliorated the detrimental effects of Pb by reducing Pb uptake, increasing phenolic, polyphenol and flavonoid contents, and improving antioxidative capacities (Kumari et al. 2024). In maize, combined SA and sodium hydrogen sulfide mitigated Pb-triggered toxicity by improving reduced glutathione content and regulating amino acids metabolism, precursors for the biosynthesis of stress-triggered protein and defensive SMs against plant stress (Zanganeh et al. 2019).

Silver (Ag) stress

Metallic silver (Ag) has been used since ancient times for conserving water and food owing to the sustained release of silver ions (Ag^+) with antibacterial effects. In recent years, the antibacterial effect of Ag^+ and silver nanoparticles (Ag NPs) has been widely exploited in numerous consumer product and medical device applications. Paradoxically, the ever-increasing production of silver-containing consumer products and the release of Ag^+ and Ag NPs have raised great concerns due to their potential risks to humans and the environment (Yang et al. 2020). Ag is the second most toxic metal to aquatic organisms after mercury. Actually, Ag NPs can leach silver ions (Ag^+), which are persistent, bioaccumulative, and highly toxic to organisms. Therefore, the release of Ag NPs or Ag^+ ions into ecosystems raises great concerns about their safety and environmental toxicity. As plants are a vital part of ecosystem

and the primary trophic level in ecosystems, representing the base of the food chain, a good understanding of the impacts of Ag NPs on plants is of paramount importance for assessing their toxicity (Yan and Chen 2019). The released Ag^+ caused a fivefold increase in H_2O_2 production over controls plants of kiwifruit; moreover, the released Ag^+ damaged pollen membranes and inhibited germination to a greater extent which suggest that Ag^+ may exert its impacts mostly through chemical or physicochemical interactions with nucleic acids to induce DNA damage (Speranza et al. 2013).

The exposure of leaf disks to silver salt led to an increased accumulation of brachycerine. This response is probably the result of well-known induction of oxidative stress triggered by heavy metals (do Nascimento et al. 2013). $AgNO_3$ at higher concentrations strongly reduced root elongation and inhibited cell division compared to control plants and Ag strongly induced an increase of ROS formation compared to control (Cvjetko et al. 2018). Compared to Ag NPs, Ag^+ ions tend to cause more toxic. The differential modulation of genes involved in sulphur assimilation, GSH biosynthesis, GR, GST, and PCS shows the biological role of these pathways in dealing with Ag mediated stress response (Nair and Chung 2014). Similarly, Kaveh et al. (2013) reported that the number of genes upregulated was higher in Ag NPs exposed *A. thaliana* plants as compared to Ag^+ ions. Compared with Ag^+ ions, Ag NPs could alter the transcription of antioxidant and aquaporin genes, indicating that Ag NPs changed the balance between the oxidant and antioxidant systems, and also affected the homeostasis of water and other small molecules within the *A. thaliana* plant body (Qian et al. 2013). $AgNO_3$ treatment on rice plants resulted in significant reduction in growth and inhibited the levels of aflatoxins production (Ejaz et al. 2018). The production of camptothecin and cell biomass in $AgNO_3$ treated cell suspension cultures of *O. mungos* was increased significantly up to 25μM. Similarly, $AgNO_3$ elicitation has enhanced the production of isoflavonoids in cell cultures of *Genista tinctoria* L (Deepthi and Satheeshkumar 2016).

Nickel (Ni) stress

Nickel excess provokes several physiological and macroscopic responses including chlorosis, necrosis, compromised plant development and reduced root growth. These effects are commonly proposed to be general consequences of increased oxidative stress and competition with essential micro-elements, including iron (Seregin and Kozhevnikova 2006). Thus, it is now important to understand the fundamental mechanisms involved in nickel tolerance in plants. Previously, Lešková et al. (2017) systematically investigated the effects of metal excess in plants—including nickel, zinc and cadmium—on diverse physiological responses associated with iron deficiency, the kind of study that everybody wants to know the results of but no-one dares perform. In this issue, Lešková et al. (2020) have further investigated the response of Arabidopsis thaliana plantlets to high nickel at the transcriptomic, developmental and cellular levels. First, using A. thaliana gene expression microarrays, they identified 235 genes whose expression significantly changed >2-fold in response to 100 μM Ni exposure for 4 d. Fifty-six of these differentially expressed (DE) genes are known to respond to iron starvation, thus confirming that nickel excess triggers an iron deficiency response (Merlot 2020).

2.3 *In planta* synthesis of Nanoparticles

Nanotechnology

Nanotechnology has been defined as “the understanding and control of matter at dimensions of roughly 1-100 nm, where unique phenomena enable novel applications”. The concept of nanotechnology was put forward in 1959 by Richard Feynman in a lecture entitled “There’s Plenty of Room at the Bottom” at the annual meeting of the American Physical Society. About 15 years later, the term ‘nanotechnology’ was coined by Norio Tanigutchi while describing precision manufacturing at the scale of nanometres. The term ‘nanoparticle’ was coined from Greek work ‘nano’ that means ‘dwarf or small’ and when used as prefix it indicates size 10−9 one billionth of meter is equals to 1 nm (Jamkhande et al. 2019). The nanomaterial is defined as a material with any external dimension in the nanoscale or having an internal structure or surface structure in the nanoscale, approximately 1–100 nm size range. They

may be in the form of nanoparticles, nanofibers, nanotubes, nanocomposites and nanostructured materials.

Nanobiotechnology is the combination of nanoscience with biology that enables mankind to design and produce functionalized biological materials or devices taking advantage of elements or effects that occur at the nanometre scale (Srivastava et al. 2021). Nanomaterials are classified as carbon-based, metal-based, organic-based and composite-based nanoparticles. It has led to the increased production and applications of nanomaterials in a wide range of fields such as automotive, biomedical, cosmetics, defence, energy, electronics and pharmacology. The global market for nanotechnology products and applications was valued at $39.2 billion in 2016 and is expected to reach $90.5 billion in 2021 (Sengul and Asmatulu 2020).

Although the uses of nanoparticles have contributed significant advantages in many areas, the presence of nanoparticles raise toxicity in human and environment. In the recent years, a number of *in vitro* and *in vivo* studies have been performed to understand the toxicological impacts and possible hazards of different nanoparticle exposures to human and the environment. There is still a major gap in knowledge about the toxicity effects of nanoparticle exposures (Sengul and Asmatulu 2020). The accumulation and hazardous effect of MNPs towards human and environment raised a major concern which led to the increased production MNPs using biological materials such as plant extracts, microbial extracts, plant metabolites, etc and termed as green synthesis.

2.3.1 Green synthesis of metal nanoparticles

Within the realm of green chemistry, the synthesis of nanoparticles from various biological sources is a rapidly upcoming area that has caught the attention of researchers working in line with long term sustainability goals. Green synthesis using living forms is a bottom-up approach that is similar to the chemical reduction method with the difference in the use of a natural product instead of an expensive chemical reducing agent (Hussain et al. 2016). The chemical and physical methods of nanoparticle synthesis are gradually paving the way for ecofriendly biological methods due to cost and

environmental toxicity factors (Kumar and Yadva 2009). Use of living organisms (*in planta* synthesis) or their biomass could be an alternative to the conventional chemical and physical methods of nanoparticle synthesis (Mohanpuria et al. 2008). Apart from being inexpensive and ecofriendly, the biological process enables the recycling of expensive metal salts like gold and silver contained in water bodies. Biological synthesis of nanoparticles also allows easy separation of the nanoparticles from the reaction media, high stability due to binding of metabolites and fast reduction in a biological medium (Sintubin et al. 2011). Moreover, the coating of biological molecules on the surface of nanoparticles makes them biocompatible in comparison with the nanoparticles obtained by chemical methods (Srivastava et al. 2021).

There has been a recent uptrend in the demand of biologically synthesized nanoparticles in diverse areas ranging from biomedical sciences to catalytic applications, separation science and biosensors (Sharma et al. 2019). This is due to the fact that the nanoparticles synthesized using green synthesis methods are much more stable and safer as compared to those using classical methods. It is worthwhile to note that Ag has been the most investigated and explored metal for the biosynthesis of nanoparticles. This is due to the fact that Ag NPs have exclusive properties like good conductivity, chemical stability and catalytic activity that can be successfully utilized for industrial applications like in the preparation of biosensors, composite fibre, superconducting materials, cosmetics and electronics (Abbasi et al. 2014). Besides this, Ag NPs exhibit anti-inflammatory, anti-cancerous, anti-platelet and broad biocidal effect against a vast array of pathogenic microorganisms that facilitates its use in diverse areas of biomedical science (Srivastava et al. 2021).

Thus, live organism-based synthesis of NPs has considerable interest in the nanobiotechnological field. Among them, *in planta* synthesis of MNPs using live plant metabolites and accumulates within live plant cell organelles was reported only in few reports.

2.3.2 Metal nanoparticles bioaccumulation in plants

Metallic nanoparticles or metal nanoparticles, a new terminology has been originated in the field of nanoparticles in recent few years. The noble metal like gold, silver, and platinum having beneficial effects on health are utilized for the synthesis of NPs and designated as metallic nanoparticles (Bhattacharya and Mukherjee 2008). In addition, lead, titanium, copper, palladium, cadmium and other metals are used for the synthesis of NPs and their application includes energy and electronics sectors. Nowadays researchers are focusing on metal nanoparticles, nanostructures and nanomaterial synthesis because of their conspicuous properties that are useful for catalysis, composite like polymer preparations (Moura et al. 2017), disease diagnosis and treatment etc (Jamkhande et al. 2019).

Silver nanoparticles

Silver nanoparticles (Ag NPs) are larvicidal against filariasis, malaria vectors and other plasmodial pathogens (Srivastava et al. 2021). Among the various metallic nanoparticles (MNPs), Ag NPs have garnered prodigious interest during recent years due to their unique physicochemical and biological properties (Ahmed et al. 2016). It is estimated that nearly 500 tons of Ag NPs are produced every year and the global market of Ag NPs is expected to reach $ 2.45 billion by 2022 (Mohasseli et al. 2020). Ag NPs (1–100 nm) have broad applicability in the field of physics, chemistry, biology, medicine and material science (Islam et al. 2019). Ag NPs have high electrical and thermal conductivity, good chemical stability and pronounced optical, catalytic, magnetic and biological properties owing to their high surface-to-volume ratio (Hembram et al. 2018). Due to these distinct properties, Ag NPs are widely used in several different products, including textile coatings, food storage containers, air filters, deodorants, toothpaste (El-temsah and Joner 2012), bone cement, surgical instruments, surgical masks (Eby et al. 2009), wound dressings, tissue scaffolds, intermittent catheters, orthopaedic prostheses (Bhattacharya and Mukherjee 2008), topical creams, antiseptic sprays and other medical and pharmaceutical products (Kapoor et al. 2022).

Lead nanoparticles

Lead nanoparticles (Pb NPs) are a type of metallic NPs employed in diverse uses, including sensors, ceramics, glasses, pigments, batteries, and solar cells. The production of harmful chemicals and noxious contaminants is a major issue in the chemical synthesis of Pb-based NPs. Many research investigations on the eco-benign fabrication of Pb-based NPs employing microbial biomass and plant extracts without creating toxic waste have been performed to deal with these problems. Plants could be particularly useful for studying the biosynthesis of Pb-based NPs among green sources. The green synthesis of Pb-based NPs like Pb NPs, PbO NPs, and PbS NPs using diverse plant extracts and microbes in the absence of harmful capping agents has been discussed (Pagar et al. 2022).

Lead oxide (PbO) is known as an important industrial material, which has been widely utilized in batteries, gas sensors, pigments, ceramics, and glass industry (Miri et al. 2018). The colours that contain lead-based pigments seem to have interesting properties including rustproof, anti-bacterial, and anti-algae, which are extensively employed in shipbuilding, construction skeleton, and road construction. PbO is a semiconductor that has two crystalline forms, litharge (tetragonal crystalline structure) and massicot (orthorhombic crystalline structure) (Miri et al. 2018).

PbO NPs is extensively used in ceramics, pigments, glass, gas sensors, and battery manufacturing industries. PbO is prepared in various methods, such as chemical, physical and biological synthesis in different shapes and dimensions (Khan et al. 2023). Among different methods, green synthesis of PbO NPs has gained huge interest due to its simple and sustainable characteristics, which use non-toxic reaction media and solvents without affecting the environment (Szymanski and Dobrucka et al., 2023). The PbO NPs were used to obtain highly scatterable, deeply uncovered, and extremely large surfaces of small-size NPs (Noukelag et al., 2021). PbO NPs are widely utilized as efficient supports for organic reactions because of their high thermal and chemical strength, optical properties, minimal expense, and low toxicity, in

addition to their high photocatalytic movement and reproducibility (Chen and Mao, 2007).

Lead-containing compounds can cause different types of undesirable outcomes such as genetic toxicity, oxidative stress (Kordas et al. 2018), and neurological effects (Miri et al. 2018). As it is obvious, lead oxide has a great impact on the environment, it is necessary to find solutions for lead pollution. Nanotechnology and biosynthesis could be employed to produce metal oxide nanoparticles (Diallo et al. 2015). Biosynthesis of nano-sized lead oxide (PbO-NPs) could enhance its properties while reducing toxic substances during the synthetic procedure (Narayanan 2012).

2.3.3 Metal transportation in living plants

Once the metals are in the proper oxidative state or bound to the correct metallophore, they will be transported across the membrane by the different transition metal transporters. There are several families of them with distinct metal affinities and direction of transport. Transporters that introduce metals into the cytosol is listed here. ZIP transporters (Zinc resistance transporter, Iron-resistance transporter-like Proteins). These are a ubiquitous family of divalent metals transporters (mainly Fe2+, Zn2+, Ni2+, and Mn2+). Members from this family include the transporters responsible for iron and zinc uptake from soil (Lin et al. 2009). Ctr/COPT transporters (Copper transporter). They have only been found in eukaryotes, being known as COPT transporters in plants. In plants, COPT proteins have been suggested to play a role in copper uptake from soil and delivery to pollen (Sancenón et al. 2004). Nramp transporters (Natural Resistance-Associated Macrophage Protein). This family of transporters can be found in the three domains of life (Nevo and Nelson 2006). Some Nramp transporters have been proposed to be involved in iron and manganese uptake by the root epidermis (Cailliatte et al. 2010). YSL transporters (Yellow Stripe-like proteins). Yellow stripe is a phenotype identified in maize. In broad terms, YSL transporters are involved in metal uptake from soil in monocots and in long-distance metal distribution in both monocots and dicots (Conte and Walker 2011). MOT1 (Molybdate transporter type 1). In contrast to other transition metals, molybdenum is transported as the oxoanion

molybdate. Aside from the MOT1 family, an additional family, MOT2, has been identified also involved in molybdate uptake in *C. reinhardtii* (Tejada-Jiménez et al. 2011). MOT2 family members role in higher plants has not been determined yet.

Transporters that remove metals out of the cytosol is listed here. P1b-ATPases. They are a clade of the P-type superfamily of ATPases (which also includes the Na+/K+-ATPase, or the H+-ATPase. P1b-ATPases are involved in long-distance Cu+ and Zn2+ transport in plants, as well as metal transport into organelle (Andrés-Colás et al. 2006). CDF transporters (Cation Diffusion Facilitator). Members of this family are present in all organisms (Kolaj-Robin et al. 2015). Most plant CDFs, known as MTPs, have been associated to metal detoxification, although others could play a role in long-distance metal transport (Ricachenevsky et al. 2013). Ferroportins are only present in eukaryotes and they would be involved in iron/cobalt uploading of the xylem (Morrissey et al. 2009). VIT1/CCC1 families of transporters identified to transport Fe^{2+} and Mn^{2+} (Kim et al. 2006). Very little is known on their structure and transport mechanism, other than it is predicted to cross the membrane five times (González-Guerrero et al. 2016).

2.3.4 Detoxification mechanism

Vacuolar compartmentalization is central to heavy metal detoxification mechanism in plants. It depends on two vacuolar pumps (V-ATPase and V-PPase) and a set of tonoplast transporters, which are directly driven by proton motive force, and primary ATP-dependent pumps. While heavy metal non-hyperaccumulator plants largely sequester toxic heavy metal in root vacuoles, heavy metal hyperaccumulators usually sequester them in leaf cell vacuoles following efficient long-distance translocation. The distinct strategies evolved as a consequence of organ-specific differences particularly in vacuolar transporters and in addition to distinct features in long-distance transport.

The heavy metal induced vacuolar changes can proceed very rapidly. For example, Zn caused tubular vacuoles to turn spherical within 15 min in the hyphae of a *Paxillus involutus* isolate from a zinc-rich soil (Tuszynska et al. 2006). Following a longer exposure of 4 days to a subtoxic concentration of Zn,

approximately a threefold and 7-fold increase in vacuolar volume fraction occurred in *Oryza sativa* and *Triticum aestivum*, while a similar increase was absent in Secale cereale. The Zn-induced vacuolation was proposed to represent a compartmentalization mechanism aimed at averting the toxicity (Sharma et al. 2016). Recently, Fan et al (2011) demonstrated increased vacuolation in Cd (20 µM)-treated meristematic root cells of 3-day-old seedlings of *A. thaliana*. This study employed cellular morphometry for quantitative determination of number and size of vacuoles. Doubling the Cd concentration increased vacuolation in a larger proportion of exposed root cells than under control conditions (Fan et al. 2011). Cd-induced changes in vacuolar form were examined in the root tip cortical cells of A. thaliana transformed with GFP fused to a tonoplast protein. Within 24 h of Cd (100, 200 µM) treatment of 10-day-old seedlings, the complexity of vesicle pattern increased with a general expansion of vacuolar system. The magnitude of change essentially correlated with Cd concentrations.

Although vesicle transport is likely to be significantly in-volved in vacuolar compartmentalization of heavy metals, there are only a few studies addressing this issue. Using leaf cell protoplasts of Cd/Zn hyperaccumulator *Thlaspi caerulescens*, Leitenmaier & Kupper (2011) observed Cd-rich vesicle-like structures in the cytoplasm. Zn-rich vesicular structures have been shown in the root hairsof excessively Zn-treated Paulownia tomentosa (Azzarello et al. 2012). Another example of vesicle association is given by the iron transporter IRT2 whose protein fusion with fluorescent protein localizes to vesicles in root and does not rescue root iron uptake of irt1-1-deficient *A. thaliana* (Vert et al. 2009). Metal transporter proteins (MTPs) belong to the cation diffusion facilitator (CDF) family and function in heavy metal homeostasis, detoxification and hyperaccumulation in plants (Sharma et al. 2016).

2.3.5 Mechanism of metal reduction and *In planta* formation of nanoparticles

These living entities can serve as biological nanofactories for the production of nanomaterials due to their wide distribution along the ecological

boundaries, easy availability, safety in handling and presence of a broad range of metabolites (Jha et al. 2009). The size and shape of NPs are determined by the nature of biological entities, their concentrations and the type of organic reducing agents (Aromal et al. 2012). The dimensions of the nanoparticles are also strongly influenced by the type of growth medium parameters such as pH, temperature, salt concentration and exposure time (Srivastava et al. 2021).

Probable steps in the formation of MNPs under *in vivo* condition is detailed here. Initially, the cations formed after dissociation of metal salts are saturated to form hydroxyl complexes followed by crystallite growth of metal with oxygen leading to the formation of crystalline planes having different energy levels. This carries on till the activation of the capping agent that ultimately arrests the growth of high-energy atomic growth planes, the process ending with the formation of metal nanoparticles (MNPs). The newly formed nanoparticles are in a high surface energy state and tend to convert to their low-surface energy conformations by aggregation. The presence of higher con of reducing agents and stabilizing agents prevents the aggregation of nanoparticles and promotes the production of smaller NPs. Additionally, proteins can trap metal ions on their surface and convert them to their corresponding nuclei, which could further aggregate and, consequently, form NPs Considering the vast potentiality of microbes and plants as sources, the biological synthesis can serve as a green technique for the synthesis of nanoparticles as an alternative to conventional methods (Srivastava et al. 2021).

Another study on *A. thaliana* revealed that a potential involvement of Fe molecular machinery in mobilizing Au3+ into root system during synthesis of AuNPs at in planta level (Jain et al. 2014). Further the effect of *in planta* synthesis of AuNPs in *A. thaliana* at root transcriptome level was demonstrated the differential expression of the members of WRKY, MYB and BHLH gene families, which are involved in the Fe and other essential metals homeostasis. The proteome analysis revealed that Glutathione S-transferases were induced in the shoot and suggested its potential role in the biosynthesis AuNPs (Tiwari et al. 2016).

2.4 Characterization of green synthesized nanoparticles

2.4.1 Scanning electron microscopy

Scanning electron microscopy (SEM) is an important electron microscopy technique that is capable of achieving a detailed visual image of a particle with high-quality and spatial resolution. SEM is a multipurpose state-of-the-art instrument which is largely employed to observe the surface phenomena of the materials. The sample is exposed in SEM to the high-energy electron beam and gives information about topography, morphology, composition, chemistry, orientation of grains, crystallographic information, etc. of a material, and therefore SEM is a useful tool to be used for the characterization of materials. Morphology indicates the shape and size, while topography indicates the surface features of an object or “how it looks”, its texture, smoothness or roughness. Likewise, composition means elements and compounds that constitute the material, while crystallography means the arrangement of atoms in the materials (Akhtar et al. 2018).

2.4.2 Transmission electron microscopy

Transmission electron microscope TEM allows the direct visualization of nanostructures and is a vital tool for observing their morphology, size and structure. TEM has evolved over many years into a highly sophisticated instrument that has found widespread application across the scientific disciplines. Due to unparalleled ability to provide structural and chemical information over a range of length scales down to the level of atomic dimensions, TEM has developed into an indispensable tool for understanding the properties of nanostructured materials and in manipulating their behavior. The precise control of nanoparticles size, grain size, size distribution and homogeneity, lattice type, crystal structure, dispersion, chemical and physical property of phases such as number, morphology, and structure of the phases at the nano-level are characterized by TEM (Jafari Eskandari et al. 2020).

EDAX analysis

Energy dispersive X-ray analysis is performed in conjunction with SEM or TEM. It provides the elemental details of near surface elements of a sample and the overall positional mapping in it. Here a high energy electron beam

~ 10–20 keV is bombarded on a sample and X-rays emitted from the sample are collected by an energy dispersive spectrometer. The energy of the X-rays generated are characteristics of the atomic structure of the element from which it is emitted, and hence provides the elemental details of the sample. X-rays are generated in approximately 2 μm depth of the sample and, therefore, EDAX is generally a bulk characterization technique. The electron beam is scanned across the sample to verify the spatial uniformity and homogeneity (Gupta et al. 2020).

2.4.3 X-Ray diffraction and Fourier Transform Infra-Red spectroscopy

XRD is a non-destructive analytical tool to identify and characterize crystalline samples providing insight into crystal structure, chemical composition, unit cell information, and chemical bonding. X-rays have wavelengths an order smaller than the wavelength of light and can therefore provide the atomic level information of a sample. Typically, X-rays produced in a CRT are incident on a sample and the different lattice planes of the sample reflect these X-rays to produce constructive or destructive interference. For some of the directions, where constructive interference occurs, Bragg's law provides characteristic information about atomic structure of the sample. Bragg's law is given as: 2d sinθ ¼ nλ (9) where d is the lattice spacing, λ is the wavelength of X-rays, and θ is the diffraction angle, i.e., the angle where constructive interference of scattered X-rays occurs. Due to the random orientation of powdered material, all possible diffraction directions are attained by scanning through the sample. As each crystalline material has a unique crystal structure, its XRD pattern can be treated as its fingerprint for identification of its phase and composition (Gupta et al. 2020).

FTIR

The IR region of the EM spectrum is absorbed by a sample, transitions between its vibrational energy levels occur. Thus, IR spectroscopy reveals information about vibrational stretches of a molecule. Most IR spectrometers are built in the form of a Michelson interferometer. Interference of sample and reference beam is recorded at the detector. Final light intensity is plotted as a function of distance of one of the movable mirrors in the interferometer to

produce the Fourier transform. Thus, the final spectrograph consists of light intensity vs. wavenumber corresponding to the chemical bonding structure of the molecule under test. Each dip or peak in the final spectrum corresponds to specific bonds of the molecule relating to their various vibrational motions (Stuart 2004).

2.5 Therapeutic potential of withanolides with special reference to Neuroprotection

2.5.1 Adaptogenic activity of *W. somnifera*

W. somnifera has the most significant adaptogenic effects which mainly results from the complex steroidal withanolides found in the roots (White et al. 2016). Adaptogens are herbs that improve an individual's ability to cope with stress and adapt to change. The most recent definition of an adaptogen is "a class of metabolic regulators that enhances the body's ability to adapt to environmental factors and avoid the damage they could imply." A study was conducted on a group of horses given *W. somnifera* root extract. The animals were subjected to various stressors, such as heavy exercise, separation, and noise. After 21 days, a significant decrease in cortisol, epinephrine, glucose, triglycerides, creatinine, IL-6, alanine aminotransferase, and aspartate aminotransferase was observed in the treated group (Mikulska et al. 2023). This indicates the adaptogenic, antioxidant, and immune stimulating effects of *W. somnifera* (Natarajan et al. 2022). The adaptogenic effects of the standardised extract of *W. somnifera* root and *Panax ginseng* were also studied in rats subjected to chronic stress using the Foots hock method. Chronic stress induced the induction of hyperglycaemia, glucose intolerance, elevated plasma corticosterone levels, increased gastric ulcers, sexual dysfunction, cognitive deficits, immunosuppression, and mental depression. The entire range of the aforementioned disorders was significantly alleviated by the administration of *W. somnifera* extract and *Panax ginseng* prior to the stressor (Bhattacharya and Muruganandam 2003). Another study investigated the adaptogenic activity of a novel withanolide-free aqueous fraction from the roots of *W. somnifera* in rats and found that it exhibited significant anti-stress effects, including improved swimming endurance and reduced adrenal gland

weight, without causing any adverse effects (Singh et al. 2001; Mikulska et al. 2023).

2.5.2 Neuro-protective activity

W. somnifera has been shown to have beneficial role in many neurological conditions that include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, anxiety disorders, cerebral ischemia. *W. somnifera* supplementation has shown to attenuate symptoms and pathology in 6-hydroxydopamine-induced model of PD, by its antioxidant action as evident by the attenuation of lipid peroxide formation and improved thiol levels (such as GSH and GSSG) (Ahmad et al. 2005). In an experimental model of AD, *W. somnifera* root extract supplementation to the animals was shown to ameliorate behavioural deficits with enhanced amyloid beta clearance, thus protecting the neuronal cells from amyloid beta toxicity (Sehgal et al. 2012). *W. somnifera* supplementation attenuated lead-induced toxicity in glial cells by balancing the expression of glial fibrillary acidic protein, heat shock protein, mortalin and neural cell adhesion molecules. *W. somnifera* root extract was shown to markedly salvage the degenerating cells in the hippocampus of rats subjected to immobilization stress (John 2014). *W. somnifera* supplementation has shown to improve locomotor functions and movement patterns in PD, which is suggested to be due to induction of catecholamine neurotransmitters. *W. somnifera* supplementation has also been reported to improve neurotransmitter levels, antioxidant status and lower oxidative stress in animal model of Parkinson's disease (Sandhir and Sood 2017).

Furthermore, it rescued glial cells against lead induced toxicity by balancing up-regulation of GFAP and heat shock protein (HSP70), mortalin, and neural cell adhesion molecule (NCAM). Studies involving Swiss albino mice has revealed that *W. somnifera* was able to significantly decrease cognitive impairments by attenuating the oxidative stress induced by Bisphenol A (a major endocrine disruptor) induced toxicity (Birla et al. 2019). The leaf extract and its component, withanone prevented scopolamine-induced oxidative stress related down-regulation of neuronal and glial cell markers and DNA damage (Konar et al. 2011). *W. somnifera* has shown to reduce the lipid-

peroxidation and nitric oxide levels in cortex, hippocampus and striatum of rats exposed to AlCl3 toxicity (Elhadidy et al. 2018). Glyco-withanolides presented marked anti-oxidant activity in cortex and striatum of rat brain by activating superoxide dismutase, catalase and glutathione peroxidase activity in a dose-dependent manner. Additionally, extract of *W. somnifera* prevented streptozotocin-induced oxidative damage in mice (Parihar and Hemnani 2004). Withanone, one of the active constituents of *W. somnifera* has shown to protect the RA-differentiated neuron-like cells against NMDA-induced excitotoxicity by regulating accumulation of intracellular Ca2+, mitochondrial integrity, balance between pro- and anti-apoptotic proteins, caspase and cytochrome-C levels, in a Parp-1 dependent manner (Dar et al. 2017).

2.6 Parkinson's Diseases management

Parkinson's Disease (PD) is the second most common neurodegenerative disorder following Alzheimer's disease (Reeve et al. 2014). Although the pathogenesis of PD is not entirely understood, aging, genetic susceptibility, inflammation and apoptosis have been implicated (Chiang et al. 2017; Enogieru et al. 2018). There is also evidence suggesting that autophagy plays a critical role in the progression of PD (Nixon 2013). Therefore, a better understanding of the involvement of apoptosis and autophagy might help in the search for new and efficient treatment options for PD (Enogieru et al. 2021).

While the familial forms of PD, that have been described, involve mutations in a number of genes (Kieburtz and Wunderle, 2013), mitochondrial dysfunction, neuroinflammation and environmental factors are increasingly appreciated as key determinants of dopaminergic neuronal susceptibility in PD, and are a feature of both familial and sporadic forms of the disease (Ryan et al. 2015). In both cases, oxidative stress is thought to be the common underlying mechanism that leads to cellular dysfunction and, eventual cell death (Blesa et al. 2015). Other evidence for mitochondrial dysfunction related to oxidative stress and DA cell damage comes from findings that mutations in genes of proteins like α-syn, parkin, DJ-1, or PINK are linked to familial forms of PD. The convergence of all of these proteins on mitochondrial dynamics uncovers a common function in the mitochondrial stress response that might

provide a potential physiological basis for the pathology of PD (Blesa et al. 2015).

2.6.1 Parkinson's disease model

The commonly used immortalized cell line for CNS research is the human neuroblastoma cell line SH-SY5Y (de Medeiros et al. 2019). This cell line in its non-differentiated form resembles immature catecholaminergic neurons as it exhibits a correct neurite structure and expresses immature neuronal markers. Along with SH-SY5Y, there are some other non-neuronal cell lines also used in neurodegenerative disease research, such as the HeLa (Zhu et al. 2012), human embryonic kidney 293 (HEK293), and rat pheochromocytoma (PC-12) cell lines (Xicoy et al. 2017). Another human neuroblastoma cell line used in neurodegenerative disease research is SK-N-MC, which has a predominantly cholinergic phenotype. To transform a cell culture of neuron-like cells and non-neuron-like cells into a cellular model of AD or PD, cells must be exposed to specific neurotoxins to induce the pathogenesis of the disease as explained above. Exposure to the neurotoxin results in cell death, mimicking in vivo neurodegeneration, e.g., adding Aβ oligomers is required for modelling AD and adding 6-OHDA, MPTP, or rotenone is required for modelling PD.

Another strategy for developing cell models involves genetically modified, non-differentiated, immortalized cell lines. Transfection (Bailey et al. 2002) with wild-type or mutant variants of the genes for amyloid precursor protein, tau (Zhao et al. 2020) or α-syn (Xicoy et al. 2017) results in protein overexpression, which in turn leads to the formation of toxic aggregates. The accumulation of protein inclusions characteristic of AD and PD triggers a deleterious sequence of responses that mimic the characteristic cascade of neurodegeneration in vivo. Immortalized cell lines commonly used in such genetically modified models include Chinese hamster ovary, SH-SY5Y, LUHMES (Lund human mesencephalic), and HEK293 cells, the latter of which are particularly useful for studying tau pathology in AD (Houck et al. 2016), and the effects of α-syn mutations and other PD-associated genes (Cetin et al. 2022).

2.6.2 Parkinson's disease and *W. somnifera*

The herb *W. somnifera* may be alternatives because both have been connected with reducing oxidative stress and could therefore ameliorate age-related impairments. *W. somnifera* has shown to prevent neurotoxic and neurodegenerative conditions by modulating the antioxidant mechanisms (Dar et al. 2015). Comparative studies investigating the anti-oxidant potential of different natural product formulations containing *W. somnifera* have shown the highest activity compared to other formulations and standard ascorbic acid. It has demonstrated substantial (DPPH) free radical scavenging and hydrogen peroxide scavenging at 1000 g/mL and 100 g/mL concentrations respectively (Manwar et al. 2013). Besides being used as a neuroprotectant, the main impediment remains the blood brain barrier (BBB) penetrability of the active constituents of *W. somnifera*. There are no reports about the major withanolides crossing the BBB. However, a single report in prevalent literature has shown that withanamides (bioactive constituents from fruit of *W. somnifera*) traverse the BBB after intraperitoneal administration of extract (Vareed et al. 2014). In a study involving Wistar rats hydro-alcoholic root extract of *W. somnifera* was found to have no toxicity even at higher dose of 2000 mg/kg body weight. During the acute toxicity study, treatment with 2000 mg/kg of the extract for 14 days and with 500, 1000 and 2000 mg/kg for 28 days for sub-acute toxicity test, no discernible alterations were observed in the body weight, organ weight and haemato-biochemical parameters (Prabu et al. 2013). In another study, *W. somnifera* root extract was evaluated for prenatal developmental toxicity in wistar rats (Prabu and Panchapakesan 2015), no apparent changes were observed on mortality, structural abnormalities and changes in growth of mother as well as foetuses. Pharmacokinetic studies carried out for *W. somnifera* extract in different biological models have demonstrated that two major constituents WFA and WTA have been observed in plasma after 10–20 min after oral administration of the extract (Dar and Ahmad et al. 2020).

Prakash et al. (2014) further demonstrated anti-apoptotic and anti-inflammatory properties of *W. somnifera* could be significantly involved in beneficial effect in PD. Further, it was reported that supplementation with ethanolic root extract of *W. somnifera* (100 mg/kg bodyweight, i.p) for 9 weeks,

inhibited the enhanced expression of inducible nitric oxide synthase (iNOS) and astroglial activation marker glial fibrillary acidic protein in Maneb and paraquat induced model of PD. Manjunath and Muralithara (2015) have shown that neuromodulatory effect of *W. somnifera* against rotenone-induced PD model in *Drosophila melanogaster* is mediated via suppression of oxidative stress and its potential to attenuate mitochondrial dysfunctions. Supplementation of *W. somnifera* to leucine-rich-repeat-kinase-2 mutants (LRRK2) loss-of-function model of PD in drosphila resulted in increasing their lifespan, improved motor function and mitochondrial morphology (De Rose et al. 2016). Moreover, *W. somnifera* root extract has led to up-regulation of tyrosine hydroxylase and protected dopaminergic neurons. It has further replenished dopamine levels in the substantia nigra, and stabilized locomotor activity by attenuating inflammation, apoptosis and oxidative damage in Maneb-paraquat-induced mouse model of Parkinson's disease (Dhar and Ahmad et al. 2020).

W. somnifera is a natural product with promising pharmacological potential. It has extensive pre-clinical as well as clinical applications in Indian systems of Medicine. Different cellular and animal models have demonstrated that *W. somnifera* extracts and its active constituents mainly possess anti-oxidant properties and rescue neuronal cells against toxic insults and inflammation by modulating PI3K/Akt, MAPK signalling pathways, antiapoptotic cascades, thereby protecting the neurons from degenerative conditions such as in diseases like AD, PD and Huntington's disease. The claims for use of *W. somnifera* to ameliorate innumerable clinical conditions are overwhelmingly promising. However, robust clinical validation needs to be performed for its general medical use. In addition, different nanotechnological approaches may increases its clinical use.