Influence of Lead nitrate in *in vitro* Shoot Culture of *Withania somnifera*

Thesis submitted in Partial Fulfillment of the Degree of Master of Philosophy (M.Phil)

By

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CERTIFICATE

This is to certify that the dissertation entitled "**Influence of lead nitrate on withaferin production in *in vitro* shoot culture of *Withania somnifera***" submitted for the degree of **Master of Philosophy in Biochemistry** by **R.Kousalya** is the record of research work carried out by her during the period from 2018 -2019 under my guidance and supervision, and that this work has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other Title in this University or any other University or Institution of Higher Learning.

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Signature of the
Head of the Department

31/7/2019.

Signature of the
Supervisor

DECLARATION

I declare that the dissertation entitled **"Influence of Lead nitrate on Withaferin production in *in vitro* shoot culture of *Withania somnifera*"** submitted by me for the degree of Master of Philosophy (M.Phil.) is the record of work carried out by me during the period from 2018 to 2019 under the guidance of Dr. Kalaiselvi Senthil and has not formed the basis for the award of any Degree, Diploma, Associateship, Fellowship, Titles in this University or any other University or other similar Institution of Higher Learning.

Signature of the candidate

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R.Kousalya

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1.0 INTRODUCTION

Plants are one of the fascinating nature's gift which plays an important role in fulfilling the basic requirements of humans. They also produce a diverse range of bioactive molecules, making them a rich source for drug industry. The widespread use of herbal remedies and healthcare preparations, such as those described in ancient texts including Vedas, has been traced to the occurrence of natural flora with medicinal properties. Currently 80% of the world population depends on plant derived medicine as the first line of health care for human ailments because it has no side effect (Patel *et al.*, 2012).

Medicinal plants play a vital role for the development of new drugs. Herbal drug technology is used for converting botanical materials into medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge is important (Rasheed *et al*., 2012).

Among the plants known for medicinal value, those belonging to family Solanaceae are one of the most intriguing plant families and extensively utilized by humans. Among 98 reported genera, the genus *Withania somnifera* belonging to family Solanaceae is very important for their therapeutic potential. Among the worldwide list of twenty-six species, *Withania coagulans, Withania somnifera, Withania adunensis*, *Withania riebeckii* are examples of known important species of this genus which grows in different parts of the world (Atal and Kapoor, 1989; Sirkar, 1989).

In Ayurveda, *Withania somnifera* is known to promote physical and mental health and used to treat almost all the disorders that affect human health. *Withania somnifera* and *Withania coagulans* are the two most esteemed species of this genus having high medicinal significance. These species are natural sources of withanolides (steroidal lactones) which are used as ingredients in many formulations prescribed for a variety of diseases. Many pharmacological studies have been conducted to investigate the properties of *Withania somnifera* as a multi- purpose medicinal agent. Advances in biotechnology, especially *in vitro* culture techniques, molecular biology and metabolite profiling provides new insights for conservation and management of plant genetic resources and better harvesting of drugs from medicinal plants (Jain *et al*., 2012).

Secondary metabolites have ecological functions in that they protect plants against herbivorous, microbes or competing plants. Secondary metabolites usually occur in complex mixtures that also differ among plant organs (Powers, 2004).

Heavy metals are widespread in soil and aquatic ecosystems and to a relatively smaller proportion in atmosphere as particulate or vapors. Though several heavy metals are considered to be essential for plant growth, toxicity in plants is also observed depending on the plant species. Stress is an important factor in determining the composition of chemical compounds and therapeutic activity of medicinal plant, and heavy metals have also been used as stressors to induce accumulation of bioactive compounds. Studies have been reported that plants under the influence of stress caused by the action of heavy metals produce secondary metabolites, whose number increases with the concentration of the metal, but only to a certain point, beyond which a decrease in these metabolites can be observed (Jahangir *et al.,* 2008)

A high lead level in soil induces abnormal morphology in many plant species. Lead causes irregular radial thickening of roots, cell walls of the endodermis and lignifications of cortical parenchyma, it also induces proliferation effects on the repair process of vascular plants (Kaji *et al*., 2014). Lead administrated to potted sugar beet plants at rates of 100–200 ppm caused chlorosis and growth reduction. Low amounts of lead (0.005 ppm) caused significant reduction in growth of lettuce and carrot roots. Inhibitory effects of lead on growth and biomass production may possibly derive from effects on metabolic plant processes (Sharma and Dubey, 2010).

Plant tissue culture is a hugely successful viable technology for the multiplication of superior plants on large scale. Tissue culture in simple terms refers to the cultivation of the cells and tissues free from the mother plant in appropriate nutrient substrates under sterile conditions. The cells in culture divide multiply and produce compounds specific to the plants, and also differentiate. Since these plants are identical to mother plants, tissue culture has been effectively used for commercial production and delivery of plants by private enterprises (Murthy *et al.,* 2008). Adoption of elicitation methods have shown improved synthesis of secondary metabolites in adventitious root cultures. Development of large-scale culture methods using bioreactors has opened up feasibilities of production of secondary metabolites at the industrial levels (Murthy *et al.,* 2008).

Several studies on *in vitro* cultures of *Withania somnifera* and elicitation for secondary metabolite production using biotic and abiotic stressors have been reported, but no study has been performed until date to study the influence of heavy metals especially lead in culture media, on the growth, changes in secondary metabolites and absorption and accumulation of lead.

Hence the present study was formulated with the objective to study the uptake of lead nitrate by *in vitro* shoot cultures of *Withania somnifera* and its influence on withaferin A accumulation in leaf tissues.

2.0 Review of Literature

India has a rich culture of medicinal herbs and spices, which includes about more than 2000 species and has a vast geographical area with high potential abilities for Ayurveda, Unani, Siddha and traditional medicines. Plants are used medicinally in different countries and are a source of many potent and powerful drugs. But, only very few have been studied chemically and pharmacologically for their potential medicinal value. Herbal medicines are in great demand in both developed and developing countries as a source of primary health care owing to their biological and medicinal properties and also for their high safety margins and lesser costs. Traditional use of medicine is recognized as a way to learn about potential future medicines. Ayurveda has been a lively system of health care in India with an unbroken practice since 6000, years but its growth as an industry has commenced only a few years back. India's share in the global exports of herbal medicines is also low, at around 10 per cent only. Therefore, there is a need to transform Ayurveda into a dynamic, scientifically validated and evidence based industry which takes its roots from rich knowledge base of oral tradition and scripture (Jirge *et al*., 2014). Researchers have identified number of compounds used in mainstream medicine which were derived from "ethnomedical" plant sources (Temitope *et al.,* 2014).

Plant-produced secondary compounds have been incorporated into a wide range of commercial and industrial applications. Fortuitously, in many cases, rigorously controlled plant *in vitro* cultures can generate the same valuable natural products. Secondary products in plant cell culture can be generated on a continuous year-round basis; there are no seasonal constraints. Production is reliable, predictable, and independent of ambient weather (Karrupusamy, 2010).

The accumulation of secondary metabolites in plants is part of the defense response against pathogenic attack, which is triggered and activated by elicitors, the signal compounds of plant defense responses. Therefore, the treatment of plant cells with biotic and/or abiotic elicitors has been a useful strategy to enhance secondary metabolite production in cell cultures (Zhao *et al*., 2001). Hence biotechnological approaches, specifically plant tissue culture play a vital role in search for alternatives to produce desirable medicinal compounds from plants (Rao and Ravishankar, 2002).

The traditional use of 'Ashwagandha' was to increase energy, youthful vigour, endurance, strength, health, nurture the time elements of the body, increase vital fluids, muscle fat, blood, lymph, semen and cell production. It helps counteract chronic fatigue, weakness, dehydration, bone weakness, loose teeth, thirst, impotency, premature aging emaciation, debility and convalescence and muscle tension (Verma and Kumar, 2011). The plant root is rich in steroids and alkaloids and forms a constituent of several traditional Ayurvedic drug preparations. The major biochemical constituents of this plant are a class of secondary metabolites known as Withanolides. The biological activities of withanolides, especially of the dominant withanolide A and withaferin A, have been shown to have anti-cancerous activity (Szic *et al.*, 2014).

The literature relevant to the "**Influence of lead nitrate on Withaferin production in *in vitro* shoot culture of *Withania somnifera"*** is reviewed in this chapter under the following headings:

Withania somnifera: Ashwagandha

Secondary metabolites of *Withania somnifera*

In vitro culture Techniques

Influence of metal stress in *in vitro* cultured plants.

Analytical techniques used to study metal stress in *in vitro* cultured plants.

Chromatographic techniques

Spectral technique

XRD

Localization of accumulated compounds (FE-SEM)

Withania somnifera: Ashwagandha

W. somnifera (L) Dunal commonly known as "Ashwagandha", "Asgandh" and "Winter Cherry" belongs to family Solanaceae. It is widely distributed in warmer parts of the world. It mainly grows in Africa, the Mediterranean, and India. It is a small, woody, evergreen, erect, branching, tomentose shrub, 30-150 cm in height. Leaves are simple opposite, alternate, petiolate, elliptic ovate to broadly ovate, entire and estipulate.The tip of the leaf is acute, cuneate or oblique and glabrous upto 8 to 12 cm in length. Flower is shortly pedicillate and 4-6 mm in dia. Flowers are greenish or lurid yellow, small about 1 cm long; few flowers (usually about 5) born together in axillary, umbellate cymes (short axillary clusters). Fruits are globose berries, 6 mm in

diameter, orange red when mature, enclosed in the inflated and membranous persistent calyx. Seeds are smooth, discoid, 20-25 seed per fruit. Seeds are yellow, reniform and 2.5 mm in diameter. Roots are stout, fleshy, cylindrical and 1-2 cm thick, straight, unbranched, roots with fiber like secondary roots, outer surface buff to grey-yellow with longitudinal wrinkles; crown consists of 2-6 remains of stem base; stem bases variously thicked; nodes prominent only on the side from where petiole arises, short and uneven; odour characteristic; taste bitter and acrid (Khare, 2004).

Figure 2.1 *Withania somnifera* plant and its taxonomical classification

Kingdom	**:** Plantae
Sub-kingdm	**:** Trcheobionta
Super division	**:** Spermatophyta
Division	**:** Angiosperms
Class	**:** Dicotiledoneae
Order	**:** Tubiflorae
Family	**:** Solanaceae
Genus	**:** *Withania*
Species	**:** *somnifera*

(Singh *et al.,* 2011)

It is used in more than 100 therapeutic formulations in Ayurveda, Unani and Siddha. The roots of the plant are categorized as rasayanas, which are reputed to promote health and longevity by augmenting defence against disease, arresting the ageing process, revitalizing the body in debilitated conditions, increasing the capability of the individual to resist adverse environmental factors and by creating a sense of mental well being. The roots and leaves of Ashwagandha contain various alkaloids, viz., withanolides (Atta *et al.,* 1991) and withaferin (Devi *et al.,* 2000). The withanolides are steroidal compounds and bear resemblance, both in action and appearance to the active ginsenosides of Asian Ginseng. Studies show that the plant has been used as an antioxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent, antitumour, astringent and more recently to treat ulcers, bacterial infection, venom toxins and senile dementia. Clinical trials and animal research support the use of the plant for anxiety, cognitive and neurological disorders, inflammation, hyperlipidemia and Parkinson's disease (Gupta and Rana 2007).

Secondary metabolites of *Withania somnifera*

Withania has received much attention in recent years due to the presence of large number of alkaloids and steroidal lactones known as withanolides. Withanolides are naturally occurring polyhydroxy C28 steroidal lactones. In the basic structure of all withanolides a six- or five membered lactone or lactol ring is attached to an intact or rearranged ergostane skeleton (Maurya *et al.,* 2010). The term "withanolide" is a structural term that has been used for "withan" from the genus Withania, and "olide" is chemical term for a lactone. To this date, about 400 withanolides or closely related compounds have been discovered from 58 solanaceous species belonging to 22 genera (Eich, 2008).

Basically there are two major groups of withanolides as follows:

A- Withanolides with an unmodified skeleton

a) With a regular β -oriented side chain

b) With an unusual α -oriented side chain.

B- Withanolides with modified carbocyclic skeletons or side chains (Glotter 2003).

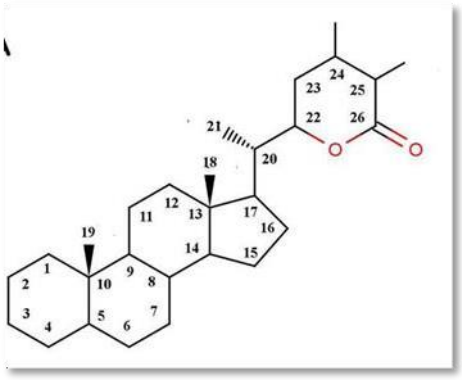
The pharmaceutically important compounds are withanolide A, withanolide B, withaferin A, and withanone (major constituents) and 12-deoxy withastramonolide, withanoside IV and withanoside V (minor constituents). Each withanolide is having a wide array of therapeutic values. Withanolide B, withaferin A and withanone also have remarkable activities in physiological and metabolic restoration, anti-arthritic, anti-aging, anti-cancer, cognitive function improved in geriatric states and recovery from neurodegenerative disorders (Mishra *et al*., 2000).

Withanolides generally contain a polyoxygenated ergostan skeleton. One of the characteristics is the ability to introduce oxygen functions in almost every position of the carboxylic skeleton and side chain of compounds of this type (Naz 2002). Withanolides are synthesized via the mevalonate pathway of terpenoids formation and arise from the initial cyclization of 3S-squalene-2, 3-epoxide (Kreis and Muller-Uri 2010).

The biological activities of withanolides, especially of the dominant withanolide A and withaferin A, have been studied extensively for its therapeutic properties. Withanolide A (5α, 20α-dihydroxy- 6α, 7α -epoxy-1-oxowitha- 2, 24- dienolide) and withaferin A (4β,27-dihydroxy-5β,6β-epoxy-1-oxowitha-2,24- dienolide) are the key

active withanolidal principles responsible for a diverse array of pharmacological activities. They have chemically similar back bone but differ in their side chain constituents (Sangwan *et al.,* 2007; Hemalatha *et al.,* 2006)

Figure 2.2.Chemical structure of Withanolide



Sabir *et al.,* (2008) reported the biotransformation potential of cell suspension cultures generated from *Withania somnifera* leaf, using withanolides, i.e. withanolide A, withaferin A, and withanone as precursor substrates. A noticeable conversion of withanolide A to withanone, and vice versa though at a lower level was observed. The type of reaction of this biotransformation appears be due to substitution of 20-OH group by 17-OH in withanolide A.

to

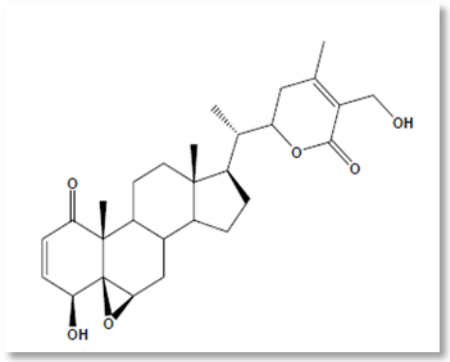


Figure 2.3.Chemical structure of withaferin-A

***In vitro* culture techniques**

The metabolic constituents, particularly the secondary metabolites differ with the variety of *W. somnifera* (L.), tissue type and sometimes with growth conditions (Abraham *et al.,* 1968*).* Also, depending upon the season, weather conditions and infection, the content of withanolides vary. Such variations often lead to inconsistent therapeutic and health promoting properties of various commercial *Withania* preparations (Dhar *et al.,* 2006).

The *in vitro* plant tissue culture technique provide an alternative to field grown plants harvested for the production of therapeutically valuable compounds (Jain *et al*., 2011). Production of secondary metabolites in tissue culture is usually higher when plant cells are organized into tissue/organs (Abozid *et al*., 2010). Gottlieb Haberlandt a German botanist is considered as the father of plant tissue culture, was the first to

separate and culture plant cells on Knop's salt solution in 1898 (Krikorian and Berquam, 1969).Plant tissue culture is a hugely successful viable technology for the multiplication of superior plants on large scale on a perpetual basis, production of novel and improved plants and for biosynthesis of products of industrial and medicinal value from natural plant resources. Tissue cultures having different morphology analyzed for withanolide production shows that, inherent biosynthetic capability of the donar plant was retained in cultures and they produced withanolides in *in vitro. In vitro* culture, is more advantage is over a single shoot formation for rapid clonal multiplication and its conservation (Valizadeh *et al*., 2009).

Micropropagation

Micropropagation is the process of vegetative growth and multiplication from plants tissues or seeds. It is carried out in aseptic and favorable conditions on growth media, using various plant tissue culture techniques (Zhou and Wu, 2006; Leifert *et al*., 1987; Bhojwani and Razdan, 1996).Tissue culture is based on concept of totipotency, the ability of plant cells and tissues to develop into whole new plant (Fowler 2000).In conventional cultivation, many plants do not germinate, flower and produce seed under certain climatic conditions or have long periods of growth and multiplication. Micropropagation techniques ensures a good regular supply of medicinal plants, using minimum space and time (Prakash and Staden,2007).The advantages of *In vitro* micropropagation of medicinal plant are given below;

- Higher rate of multiplication
- Enviromental can be controlled or altered to meet specific needs of the plant.
- Identification and production of clones with desired characteristics.
- Production of secondary metabolites.
- New and improved genetically engineered plant can be produced.
- Conservation of threatened plant species.
- Preservation of genetic material by cryopreservation

Callus induction and Multiplication

Callus is an undifferentiated mass of tissue which appears on explants within a few weeks of transfer into growth medium with suitable hormones. Callus formation occurs from reversed process of cell differentiation, known as dedifferentiation or

dedifferentiation. Different growth hormones are used to promote callus induction and development (Rout *et al*.,2000).

One major consequence of this dedifferentiation is that most plant cultures lose the ability to photosynthesis. The important consequences for the culture of callus tissue, as the metabolic profile will probably not match that of the donor plant. This necessitates the addition of other components such as vitamins and carbon as source to the culture medium, in addition to usual mineral nutrients. Callus culture is often performed in the dark (the lack of photosynthetic capability being no drawback) as light can encourage differentiation of the callus. During long term culture, the culture may lose the requirement for auxin or cytokinin (Valizadeh *et al*.,2009)

Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plants can subsequently be produced. It can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies (Gamborg, 2002).

Suspension culture

Suspension culture are formed *in vitro* when friable calli are grown on liquid media in suitable container and constantly agitated to provide suspension of free cells (Bhojwani and Razdan, 1996). Clonical flasks are used because of their large surface area which helps in maintaining liquid medium and continuous gas exchange (Vanisree *et al.,* 2004). Suspension culture are widely used in large scale production of secondary metabolites.

Suthar *et al.,* (2011) also reported *in vitro* cultured shoots of *B.serrata* showed maximum shoot length in MS liquid medium supplemented with 0.5 BAP with 0.05mg/L NAA, such shoots also produced highest number of leaves per clusters and increase in biomass was also observed.

The introduction of a liquid culture system for *in vitro* mass propagation facilitates the extensive reduction of plantlet production costs and this system would facilitate toward automation. Withanolide A (0.53 mg g-l DW), withanolide B (1.19 mg g-l DW), withaferin A (1.97 mg g-l DW) and withanone (1.71 mg g-l DW) were recorded in the multiple shoots grown in 20 ml MS liquid medium after 5 weeks of culture (Sivanandhan *et al.,* 2012).

Mir *et al.,* (2014) concluded that Withaferin A was produced in relatively high amounts in shoots cultured of half and full strength MS liquid media as compared to natural field grown plants. The establishment of *W.somnifera* multiple shoot cultures and the synthesis of withaferin A *in vitro* opens new avenues for bioreactors and large scale production of compounds.

Tissue culture plays a key role in phytoremediation research. It is proposed to have experimental convenience, shorter time span as compared with studies on whole plant systems. Tissue culture technique can be used in study of metal tolerance of a plant by exposing it in culture media containing known quantities of the specific heavy metal. Plants identified and screened by this method can then be tested for phytoremediation at polluted land, and also has the potential to study the effect of metal on whole plants. Several studies have been conducted to evaluate the effects of different heavy metal concentrations on living plants. Numerous *in vitro* experiments have focused on the effects of high concentrations of heavy metals on the regeneration of plants like accumulator, tolerant or sensitive to industrial pollution. Selection of plants under natural conditions of environmental pollution or *in vitro* may result in the selection of clones' accumulator to toxic metal ions Ashwini *et al.,* (2014).

Influence of metal stress on *in vitro* cultured plants

Plants exposure to the toxic levels of heavy metals causes the physiological and metabolic alterations (Villers *et al.,* 2011, Dubey, 2011). Heavy metals include cobalt, nickel, copper, zinc, selenium, silver, antimony, thallium, arsenic, cadmium, mercury, lead and thallium (Fergusson, 1996) Arsenic, cadmium, chromium, lead, and mercury are treated as most important elements because they are having high degree of toxicity and are ranked as elements, (Secchi *et al.*,2007). Each of these heavy metals has different sites of action within the plant, the most widespread evidence for the heavy metal toxicity is reduction of plant growth (Sharma and Dubey, 2007), and also causes chlorosis in leaf, necrosis, turgor loss, reduction in seed germination and a damaged photosynthetic apparatus, finally resulting in the plant death (Dolcorso *et al.*, 2008; Dolcorso *et al*., 2010).These effects are responsible for molecular, ultrastructural, and bio-chemical changes in the plant cells and tissues (Gamalero *et al.*, 2009).

Among these stresses, heavy metal toxicity has become a major attention because of the enhanced environmental pollution. Since metals are non-decomposable, they frequently lead to deleterious biological effects (Jaleel *et al*., 2009). High levels of the heavy metals also have direct effect on photosynthetic apparatus including thylakoids which decreases the rate of photosynthesis. It can also creates a barrier in the release of proteins, lipids, and elemental components if thylakoid membranes, resulting in the damage to light-harvesting complexes and photosystem II (PS II) (Hsu and Kao, 2004; Bakor *et al*., 2006)and causes reduction in Chl synthesis, which may be the cause of enzyme inhibition involved in the synthetic pathway (Boddi *et al* 1995; Shakya *et al*., 2008)

Some plant species have the ability to grow and develop in metal rich soils such as in the vicinity of mines or at industrial area (McIntyre *et al*., 2003). Few studies have also reported the negative impact of accumulation of heavy metals in food crops on human health (Nabulo *et al*., 2011; Uzu *et al*., 2011). To withstand heavy metal stress and metal toxicity, plants have evolved numerous defense mechanisms viz reduced heavy metal uptake, sequestration of metal into vacuoles, binding to phytochelatins, and activation of various antioxidants (Shahid *et al*., 2015). Different kinds of heavy metals uptake in various types of plants are listed below in Table.2.1.

Table.2.1. Effects of Various Heavy Metals on Plants

Heavy Metals	**Plants**	**References**
Arsenic (As)	Rice (*Oryza sativa*)	Marin and Pezeshki, 1993
	Tomato (*Lycopersicon esculentum*)	Barrachina *et al*., 1995
	Canola (*Brassica napus*)	Cox *et al*. 1996
	Chickpea (*Cicer arietinum*)	Srivastava *et al*., 2005.
	Maize (*Zea mays*)	Bakerand and Rosenqvist, 2004
Lead (Pb)	Rice (*Oryzae sativa*)	Kibria *et al*., 2010.
Cadmium (Cd)	Oil seed rape (*Brassica napus* L)	Li *et al*., 2009
	Garlic (*Allium sativum*), Maize (*Zea mays*),	Jiang *et al*., 2007
Chromium (Cr)	Tomato (*Lycopersicon esculentum*)	Shanker *et al*., 2003
	Onion (*Allium cepa*)	Nematshashi *et al*., 2000
Zinc (Zn)	Ryegrass (*Lolium perenne*)	Bonnet and Veisseire, 2000
Mercury (Hg)	Rice (*Oryzae sativum*)	Kibra, 2008
	Tomato (*Lycopersicon esculentum*)	Shaker *et al*.,2011

Cobalt (Co)	Radish (*Raphanus sativus*)	Jayakumar *et al*.2008
Nickel (Ni)	Winter rye grass (*Lolium perenne*)	Sheoran *et al*.,1990
	Wheat species (*Tritium species*)	Pandolifini *et al*., 1992
	Rice (*Oryzae sativa*)	Lin and Kao, 2005

Bieby *et al*., (2011) reported that metal uptake by plants depends on the bioavailability of the metal in the water phase and also on the metal retention time, and its interaction with other elements. Moreover, upon binding to the soil, the pH, redox potential, and organic matter content will all affect the tendency of the metal to exist in ionic and in available form. Plants may affect the soil composition due their ability to lower the pH and oxygenate the sediment, which affects the metal availability, increase in the heavy metals bioavailability by the addition of biodegradable physicochemical factors such as micronutrients and chelating agents.

Some heavy metals such as Co, Cu, Fe, Mn, Mo, Ni, and Zn are considered as beneficial for plant growth and development. Plants require them in a limited quantity, at high level these metal ions tends to create differential level of toxicity in plant that in turn leads to inhibited plant growth, halt enzymatic and metabolic pathways and also create damages to plant morphology and physiology that eventually reduce overall plant productivity (Arif *et al*., 2016).

Plants absorb essential and non-essential elements from the soil in response to concentration gradient and selective uptake of ions or by diffusion (Peralta-Videa *et al*., 2009). The absorption level of different element relies upon the different plant species. Root plays a significant role in the active uptake of metal ions. The mechanism is mainly started by the absorption of metal ions in the root tissue, the ions of Co, Cu, Fe, Mn, Mo, Ni, and Zn dissociates fromits complex forms at the root surface. The metals are heavily accumulated into the root apoplast (Krzesłowska, 2011). The adsorption of heavy metals on the root surface takes place in cationic form with negative cell wall due to the presence of cellulose, pectins, and glycoproteins that work as specific ion exchangers. The adsorption and translocation of metal ions occurs in xylem and phloem tissue through the root.

Das and Maiti (2007) observed that *J.curcas* leaves accumulated more Zn than Cu or Cd. Higher accumulation of Zn in leaves was similarly observed in red beet, field pumpkin, maize, barley, white cabbage (Sekara *et al*., 2005). Seedlings growth of *J.curcas* was not affected up to 500µm, but was greatly affected at 700µm.

Wu *et al.*, (2011) studied the metal (Al, Zn, Cu, Pb and Cd) accumulation of jatropha grown on metal contaminated soil as well as the effect of limestone addition. Root absorbs metal from the substrate and then transports it to leaves via stem. Thus the observed accumulation pattern was in the order of root>stem>leaves. The addition of limestone decreased the phytoavailability of soil metals.

Ghavri *et al.*, (2010) reported that stem tissues of plants grown in wasteland soil accumulated two fold; while leaves accumulated 2-4 fold higher Fe when compared to accumulation in garden soil. The accumulation pattern was similar in root, stem and leaves in soil containing 40% cow dung.

Islam *et al.*, (2008) reported that plant exposure to lead even at micro molar level it shows adverse effects on seed germination and growth germination is strongly inhibited by very low concentrations of $Pb^{2+,}$ it induced growth on seed germination has reported in various plants including *Oryza sativa, Zea mays* . The lead may speed up germination and induce several adverse effects on the length of radical in *E.argyi*.

In another experiment Kuboi T *et al*., (1986) observed Cd uptake in 34 plants species of 9 plant families and found that Cd content was low in Leguminosae, moderate in Cucurbitaceae and high in Cruciferae and Solanaceae.

Lead (Pb) is one of the heavy metals which have no known biological function. Numerous investigations had shown that plants can accumulate lead via root and shoot, and that the lead concentrations in plant tissues are significantly related to the lead levels in environment (Sawidis *et al*., 1995; Xiong 1997a). Excessive lead accumulated in plant tissue can be toxic to most plants, leading to decrease in seed germination, root elongation and biomass, inhibition of chlorophyll biosynthesis, as well as cell disturbance (Fargasova 1994; Xiong 1997b). In several studies, it has been reported that some plant species known as hyper accumulators, which are usually derived from heavy metal-contaminated areas, have the ability to accumulate unusually high content of heavy metals without dramatically being impacted in their growth and development (Baker and Brooks 1989; Xiong 1997 c).

Several evidence demonstrates that Pb toxicity does not appear when organic matter and other mineral nutrients are in abundant supply (Baumhardt and Welch, 1972), and that its toxicity occurs most commonly on waste heaps from mining operations where the organic matter and nutrient content of the soil are low

(Woolhouse, 1983). Wierzbicka (1989) reported that Pb toxicity in non-tolerant plants, demonstrating that Pb toxicity is associated with the disturbance of mitosis (Ahlberg *et al*., 1972; Ramel, 1973), toxicity of nucleoli (Liu *et al*., 1994), inhibition of root elongation (Lane and Martin, 1980), appearance of chlorosis (Johnson *et al*., 1977; Johnson and Proctor, 1977), inhibition of enzyme activities (Hamp *et al*., 1973) and reduction in photosynthesis (Bazzaz *et al*., 1974).Excess Pb causes a variety of toxicity symptoms in plants, such as reduced growth, chlorosis and darkening of the root system. Inhibition of root growth appears to result from Pb-induced inhibition of cell division of the root meristem (Eun *et al*., 2000). Lead inhibits photosynthesis, alters the mineral nutrition and water balance, modifies hormone levels and affects the structure and permeability.

The effect of different concentrations of lead in invitro shoot cultures of Datura inoxia was reported by Ashwini *et al*., (2014), this plant showed decrease in shoot proliferation and shoot length with increase in the concentration of lead in the medium upto 45mg/L increase in lead concentration to 50mg/L was found toxic to the plant.

Analytical techniques used to study metal stress in *in vitro* cultured plants

High Performance Thin Layer chromatography

High Performance Thin Layer Chromatography (HPTLC) is a powerful method equally suitable for qualitative and quantitative analytical tasks. Applications of HPTLC, such as identification and quantitation of constituents, impurities, active substances, process development and optimization, process monitoring, and cleaning validation have been demonstrated (Patel *et al*.,2012). Sharma and Nayak *et al* (2009) reported a simple, sensitive and accurate high performance thin layer chromatographic (HPTLC) method for the estimation of withaferin-A and withanolide-A in different plant parts such as, leaf, root, stem and fruit of two morphotypes of *Withania somnifera*. HPTLC of *W.somnifera* methanolic extract was performed on Si 60 F254 (20 cm × 20 cm) plates with toluene: ethylacetate: formic acid (5:5:1), as mobile phase. Quantitative evaluation of the plate was performed in the absorption-reflection mode at 530 nm. The method was validated for precision, repeatability, and accuracy. The average recovery of withaferin-A and withanolide A in two levels were 96.0 and 96.7%, showing the excellent reproducibility of the

method. Various analytical techniques like TLC, HPLC and HPTLC can identify the presence of compounds in plants and also quantify them. Densitometric HPTLC has been widely used for the phytochemical evaluation of the herbal drugs (Rakesh *et al*., 2009).

Preethi *et al*., (2014) quantified withanolide A and withaferin A present in 26 geographically different varieties of *Withania coagulans* root tissues using HPTLC, from that the author found that roots of *Withania coagulans*, AUF Wc 024 and AUF Wc 025 had maximum withanolide A accumulation of 1.17mg/g dry root powder. In another study, Pankajavalli *et al*., (2014) quantified both the dominant withanolides in field grown root and leaf tissues at different developmental stages and concluded that both the withanolides accumulate in optimum concentration at yellow berry stage life cycle of the plant.

HPTLC is being incorporated at a high rate in pharmacopeias and GMPs for botanical dietary supplements, and it is predicted that the use of HPTLC methods will also increase in worldwide pharmacopeias on synthetic drugs. An effort to encourage this was publication of a "stimulus article" on modern TLC in the Pharmaceutical Forum published by the U.S. Pharmacopeial Convention. To support further acceptance and application of HPTLC as real quantitative analytical technique, more stringent quality standards have to be applied by analysts. Methods used in pharmaceutical analysis must be sufficiently accurate, specific, sensitive and precise to conform the regulatory requirements. HPTLC procedures are highly diversified. Depending on the actual type of test; it can be used in a wide range of applications from simple qualitative identification to quantitative assay or purity test. There are a number of publications dealing with the development and validation of HPTLC methods. (Patel *et al*., 2012).

UV-Visible Spectroscopy

This is a spectroscopic technique that makes use of the ultraviolet and visible spectral regions. This involves absorption of Ultraviolet (UV) and visible radiations by particles accompanied by excitation of their electrons from lower to higher energy levels. Most molecules undergoing electronic excitation have to absorb light, though, some molecules require more energetic radiations (in the vacuum ultraviolet, <200

nm). A molecule has to contain either non-bonding orbital or valence bonds for it to absorb radiations in the UV-Vis regions (200 – 800nm).

When UV-Vis radiation passes through a material, part of it is absorbed by the material, causing the outer electrons to be moved from ground energy state to excited energy state. Normally, the electrons move from incomplete a molecular orbital. Detectors like photocell are then used to detect the radiations that have been transmitted. The amount of radiation absorbed is usually expressed as either transmittance or absorbance. A plot of the degree of absorption against the wavelength of incident radiation is then recorded on the read out meter (Dhivya *et al*., 2017).

To obtain a spectrum, the absorption of radiation by the sample is measured at various wavelengths. Each line in the spectrum represents the wavelength at which the energy of the incident radiation equals the energy required to effect a transition. Presence of an absorption band at a given wavelength indicates presence of a chromophore and its position depends on the chemical environment of the sample (Jain *et al*., 2016). Parameters like the pH, temperature, and concentration cause changes both in the intensity of the absorption maxima and wavelength.

Soomro MT (2008) reported that most of the phenolic compounds, such as flavonoids, anthroquinones, coumarins, anthocyanins, and other compounds containing conjugated double bond (s) with chromophore (s) in herbs have strong UV-Vis absorption. UV-VIS spectrophotometer in determination of heavy metals in medicinal samples is becoming popular in many laboratories because it provides an easy, economical, efficient, robust and rapid determination in low and high concentration at cheap cost.

XRD technique

X-ray diffraction techniques are analytical procedures which give data about the crystal structure, physical properties and synthetic composition of materials. It is a standout amongst the most useful characterization techniques in material and solid state science. X-rays are produced when high energy particles such as electrons are accelerated through a given voltage and then collide with a target. This result into electrons from the outer orbital immediately drops down to occupy the now vacant inner orbital. X-rays are released during these transitions in form of energies with

fixed values. The energies are material dependent, which implies that each material has its own characteristic X-rays patterns. This characteristic X-ray pattern can be utilized as a fingerprint‖ for identification of different materials. The powder patterns of most known solids are contained in the Powder Diffraction File (Treacy and Higgins, 2007).

The electron beam used in production of X-rays is provided by a heated tungsten filament. The beam is accelerated through a potential difference of several kV towards an anode. The X-ray tube is evacuated so as to prevent oxidation of the filament and the produced X-rays leave the X-ray tube via the windows.

Vijila *et al*., (2016) reported that when a monochromatic X-ray beam strikes a powdered sample that has crystals randomly arranged in every orientation, with some crystals oriented at Bragg angle, diffraction occurs. The diffracted beam is then detected by a movable detector or a photographic film. XRD analysis can be completed on different types of samples, however, the perfect sample is a crystalline powder squeezed into the specimen holder. The sample should also have a smooth surface and held at an angle of 45 degrees in the sample holder. Small volumes of solid sample taped on a microscope slide glass or a thin film deposited on a substrate may likewise be used. Better results are achieved with crystalline sample.

At the point when X-ray radiation with a wavelength λ is directed on a material, a diffraction peak is observed when the Bragg equation is fulfilled: $n\lambda = 2d \sin\theta$

Where, d is the lattice space of the material
θ the incidence angle
λ is the Cu Kα emission wavelength and is equal to 1.5418A˚.

A powder XRD pattern can also be used to determine the size of the particles using the Scherrer formula: $L = 0.9\lambda$

Where, L is the particle size
λ is the Cu Kα emission wavelength (1.5418A˚.)

This technique was utilized to determine crystallinity size of the lead treated sample . The analysis was conducted using a diffract meter operated at 45 KV within Cu Kσ radiation and a graphite monochromator that produces x-rays with a wavelength of 1.5418A°.

Field Emission Scanning electron microscopy (FE SEM)

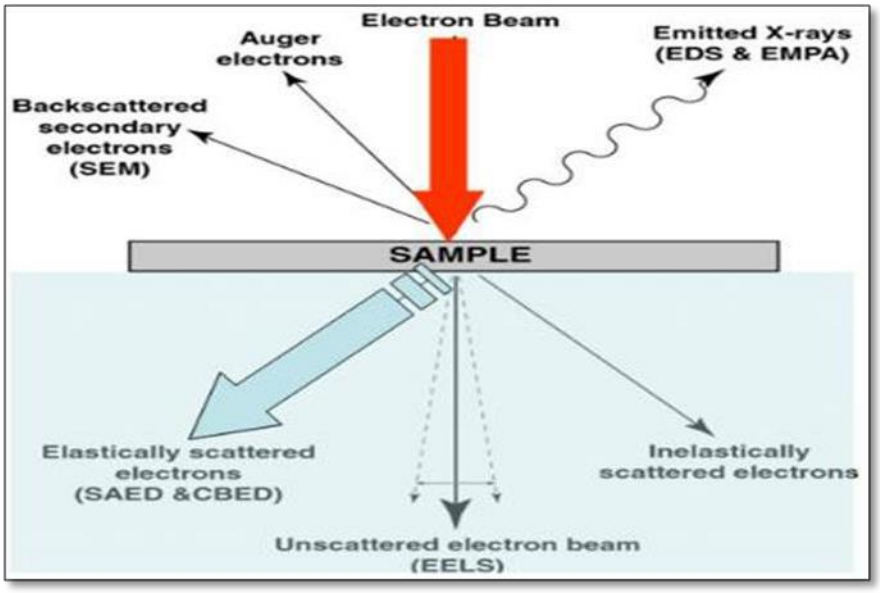
FE SEM is a microscopic technique which takes images of a specimen by scanning it using high energetic electron beam. The electrons interact with the specimen atoms producing signals containing data of specimen's surface composition, topography and electrical conductivity. In FE SEM, electrically non conducting specimens are covered with a thin conducting film such as of a metal or carbon (Duke, 2004).

FE SEM instrument is composed of two principle components; the electron column and the electronic console. The electronic console contains switches and control knobs for adjusting instrument‘s filament current, focus, magnification, accelerating voltage, contrast and brightness. Some of the modern electron microscopes uses electronic console in conjunction with the computer systems thus no need for bulky console for housing control knobs and switches. In such SEM instruments, all the primary controls are available through the mouse and keyboard of the computer system. The electron column consists of the electron beam that is produced under a vacuum and focused through a small diameter so as to scan across the specimen surface. The lower part of the column is known as the specimen chamber and houses the secondary electron detector. SEM samples are mounted onto the stage that is controlled by a goniometer (Sahi *et al*., 2002).

In scanning electron microscopy, two types of signals are used for the visual examination of the material surface; secondary and backscattered electrons. Secondary and backscattered electrons are continuously created from the specimen surface under electronic beam, though they result from two different types of interaction.

From the Figure 2.4**,** secondary electrons result from the inelastic collision followed by scattering of the incident electrons with the electrons of the specimen. These electrons are characterized by energies less than 50 eV and used to study the surface structure of the specimen with a resolution of approximately 10nm or better (Watt, 2005). Backscattered electrons result from the elastic collision and scattering interaction between 38 the incident electrons and nuclei or electrons of the specimen. They can be produced further from the specimen surface and reveals the topographical contrast as well as the atomic number contrast.

Figure 2.4.Schematic diagram showing different radiations produced during interaction of a material with an electron beam (Watt, 2005).



Scanning Electron Microscopy has been used to observe and differentiate the morphology of *in vitro* and in vivo grown *Passiflora edulis* and *var. edulis* and to observe the stomata and microcharacters in its leaves (Rubashiny Veeramohan *et al*., 2013). Similar study on stomatal structure of some cultured plant species has employed Scanning Electron Microscopy for its characterization (Budhindra Nath Hazarika *et al*., 2006).

Sahi *et al.*, (2002) have done their work on accumulation, speciation and cellular localization of copper in *Sesbania drummondii* by SEM-EDX. They found that the distribution of Cu within seedlings tissues predominantly accumulated in the corticular and vascular (xylem) regions of root tissues. In the stem, most of the Cu was found within the xylem tissue. However, the deposition of Cu within the leaf tissues was in the parenchyma.

In another experiment (Hu P J., *et al.* 2009) carried out to study the tolerance, accumulation and distribution of zinc and cadmium in hyperaccumulator plant *Potentilla griffithii*, the SEM-EDX pattern confirmed that highest Zn concentration was found in xylem parenchyma cells and epidermal cells, while for Cd, a gradient was observed with the highest Cd concentration in rhizodermal and cortex cells, followed by central cylinder.

3.0 MATERIALS AND METHODS

The various materials used and experimental techniques adopted for the present study entitled "**Influence of Lead nitrate on Withaferin production in *in vitro* shoot culture of *Withania somnifera***" are described below.

Materials

Plant material

Chemicals

Methods

Media preparation

Inoculation of the explants and culture and maintenance

Treatment with lead salts

Quantitative estimation of withaferin A

Extraction of secondary metabolites

Quantitative estimation of selected phytochemicals

Characterization of withaferin A using HPTLC

Spectral analysis

UV-Visible Spectrophotometer

X – Ray Diffraction (XRD)

Field Emission Scanning electron microscopy (FE SEM)

Statistical analysis

Materials

Plant material

Seeds of *Withania somnifera* Jawahar-20 variety were collected from the University of Agricultural Sciences, Bangalore. Surface sterilized seeds were germinated under *in vitro* condition and seedlings were maintained on half strength MS basal medium containing 2% sucrose with regular subculture. The shoots excised from 30 days old aseptic plantlets maintained *in vitro* were used as explants for further studies.

Chemicals

Chemicals used for this study were purchased from HiMedia unless otherwise mentioned. Double distilled water was used for the entire work. The solvents used are of LR grade Chloroform (Rankem), Ethyl acetate (Rankem), Methanol HPLC grade (Rankem), Formic acid (Qualigen), Pre-coated silica gel plates 60 F254 for TLC (Merck), Standard withaferin were purchased from Natural Remedies Bangalore, pyridine and methoxyamine were purschased from sigma (USA).BSTFA [N,O-bis (trimethisilyl) Trifluoroacetamide containing 1% TMCS (Trimethyl chlorosilane)] were obtained from alfa aesar (USA) and 2-chloronaphtahalene as internal standard for GC-MS were purschased from Tokyo Chemical Industry Co.,Ltd (Tokyo, Japan).

Methods

Media preparation

Full strength Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) were used for all the plant tissue culture experiments. The composition of stock solution is presented in **Appendix 1**. The macro, micronutrients, vitamins and myo-inositol were taken from the stock solutions according to the requirement. Sucrose (30 g/l) was added and mixed well. The pH of the media was adjusted to 5.6 - 5.8. Solidifying or gelling agent (agar, 0.8%) was added to the media and steamed to melt the agar. It was then dispensed in clean culture bottles (30 ml per bottle) and autoclaved at 15 lbs pressure at 121ºC for 20 minutes.

Inoculation of explants and culture maintanence

The working table of the laminar airflow chamber was first surface sterilized with 70% ethanol. Sterile petri dishes and tools (forceps, scalpels, sterile cotton, and sterile Whatmann no.1 filter paper) that were used for inoculation were kept in the laminar airflow chamber. The ultra violet light was switched on for 20 min. Hands were sterilized with 70 per cent alcohol prior to inoculation. The forceps and scalpels were dipped in 70% alcohol and flamed, cooled and used for inoculation. The shoot obtained from the germinated seedlings were used as the source of explants and is been inoculated on to the MS liquid basal medium, cultured at 25±2°C. A 16h photoperiod was maintained for all experiments.

Seeds of *W. somnifera* (L.) were germinated *in vitro* on half strength solid MS basal medium supplemented with 2% sucrose and maintained in dark at 25˚C. Shoots from *in vitro*-germinated seedlings were maintained on MS basal medium under standard culture conditions. Shoots were multiplied by established protocol in our laboratory; MS basal medium supplemented with 4.44 µM BAP with regular subcultures every 15 days. For large scale culture of shoots, multiplied shoot were transferred to liquid MS basal medium supplement with 4.44 µM BAP and 3% sucrose, in an airlift bioreactor (Biopia, Korea). The *in vitro* grown shoot samples were used for further studies.

Treatment with lead salts

The nodal sections of Withania were excised, trimmed at both the ends and inoculated in the culture bottles containing MS basal media supplemented with Benzylaminopurine (BAP). The explants were then cultured at 25ºC and observed regularly for contamination or for any other morphological changes. Each experiment had 3 replicates with three explants in each. A photoperiod of 16/8 hrs was maintained for all experiment. One month old *in vitro* shoots of *Withania somnifera* were transferred to MS basal media containing different concentration of lead Nitrate salts **Table 3.1**. Media without lead served as a control. The treatment groups along with the control were arranged in a randomized design and each contained three shoots. Then the shoots samples were harvested after 7 and 14 days respectively. The harvested shoot samples were used for further analysis.

Table.3.2.1.List of Lead Treatment

Treatments	Lead nitrate Concentrations (mM)	
	7 days	14 days
T0	0	0
T1	0.6mM	0.6 mM
T2	1.2mM	1.2 mM
T3	1.8mM	1.8 mM
T4	2.4 mM	2.4 mM
T5	3.0 mM	3.0 mM

Quantitative estimation of withaferin A

In vitro grown lead treated samples were harvested after 7 and 14 days. The wet weight and dry weight of each collected sample were noted. The dried samples were then subjected to the extraction and analysis of the secondary metabolites.

Extraction of secondary metabolites

The shoot and leaf tissues grown in solid media with different concentration of lead were shadow dried and powdered using a mortar and pestle. The secondary metabolites were extracted following procedure established earlier (Patel *et al.*, 2009, Parameshwari *et al.*, 2017) 1g of powder (1g) was extracted with 50ml methanol. A ratio of 1:50 sample to solvent was maintained throughout extraction. The extraction was carried out four times. Each time the extract was sonicated for 20 min and kept in shaker for 2 h at 100 rpm and filtered using whatmann No. 1 filter paper. All fractions were then pooled, filtered and evaporated to dryness using a rotary vacuum evaporator at 125 rpm in a water bath at 40°C. The residue was dissolved in 10 ml of HPTLC grade methanol and stored at -20°C until further analysis.

Preparation of Standard:

Standard solutions of Withaferin A (1.0mg/ml) were prepared using HPLC grade methanol and stored in refrigerator at 4ºC.From the stock solutions, working solutions were prepared by dilution with HPLC grade methanol.

Quantitative estimation of selected phytochemicals

The methanolic extract was used to estimate the phytochemicals following established procedures as given in appendix

S. No.	Phytochemical	Reference	Appendix
1	Flavanoids	Cameron *et al* ., (1943)	2
2	Phenols	Kumbhare *et al*., (2012)	3
3	Steroids	Wall *et al.*, (1952)	4

Characterization of Withaferin A using HPTLC

The High Performance Thin Layer Chromatography was performed on precoated silica gel aluminum plate (20 cmx10 cm) 60F254 (E.MERCK, GERMANY). The methanolic extract of *Withania somnifera* samples were loaded to the plates as 8mm bands, under a stream of nitrogen gas using the CAMAG (Switzerland) Linomat V semiautomatic sample applicator fitted with 100µl Hamilton syringe. The HPTLC plates were developed up to 80mm using the mobile phase Toluene:Ethyl Acetate:Formic acid in the ratio of (5:5:1) respectively in a Camag Twin trough glass chamber pre-saturated with the mobile phase solvents for 30 minutes at room temperature (25±2ºC).The developed plate was air dried and the image was captured under 254 and 366nm. The spots were visualized in HPTLC visualiser at 254nm, 366nm and also in white light. The plates were then scanned using Camag TLC scanner III at 223nm. Then the plates were derivatized in anisaldehyde sulphuric acid reagent and kept in hot-air oven at 110ºC for 10 minutes. The colour and the Rf values of the resolved spots were noted. Evaluation was done by peak area with linear regression. The amount of withaferin-A was computed from peak areas and were analysed by WINCATS software (Jirge *et al*, 2011).

Spectral analysis

UV-Visible Spectrophotometry

The extracts were subjected to spectral scan in a UV-visible spectrophotometer (UV-vis 1800 shimadzu) using HPLC methanol as reference. The characteristic peaks observed were recorded.

X- Ray Diffraction (XRD)

The X- ray diffraction (XRD) patterns of the samples were recorded on a PANalytical X'Pert PRO X-ray diffractometer using Cu Kα radiation (λ = 0.15406 Å). The crystalline size of samples was measured from the line broadening analyses using Debey- Schherer formula after accounting for instrumental broadening

$$D_{XRD} = 0.90 \lambda / \beta \cos \theta$$

Where, D is the crystal size

λ is the wavelength of the X-rays

β is the full width at half maximum of the diffraction peak

θ is the diffraction angle (Manimegalai *et al*.,2015)

Field Emission Scanning Electron Microscopy (FE-SEM)

Sample preparations

Samples of lead treated plant tissues were excised, cut into small portions (2 × 3 mm) and fixed for 2 h at 4°C in 0.1% (wt/vol) buffered sodium phosphate and 3% (wt/vol) glutaraldehyde at pH 7.2. Then they were post fixed with 1% osmium tetroxide (wt/vol) in the same buffer for 2 h, dehydrated in an ethanol series and embedded in Epon/Araldite epoxy resin (Electron Microscopy Sciences, Fort Washington, PA, USA). Serial ultrathin sections from each of the species were cut with a diamond knife, mounted on Cu grids, stained in uranyl acetate and lead citrate, and then observed under a Scanning electron microscopy (Marchiol *et al*., 2014)

Statistical Analysis

The significance level for quantitative estimations was carried out by ANOVA using Microsoft Excel 2007.

4.0 Result and Discussion

The present study entitled "**Influence of Lead Nitrate on Withaferin production in *in vitro* shoot culture of *Withania somnifera*"** is aimed to compare, withaferin A accumulation in *in vitro* shoots cultures in response to different concentrations of metal stress. The results obtained from the study are presented and discussed under the following sub headings:

Morphological changes and biomass accumulation in *in vitro* shoots cultured on media containing lead nitrate

Quantification of secondary metabolite in *in vitro* shoots cultured on media containing lead nitrate

Quantification of Withaferin A using HPTLC in stress treated plants

Analytical techniques used to study the accumulation pattern of leaf in *in vitro* cultured plants

UV-Visible Spectrophotometry

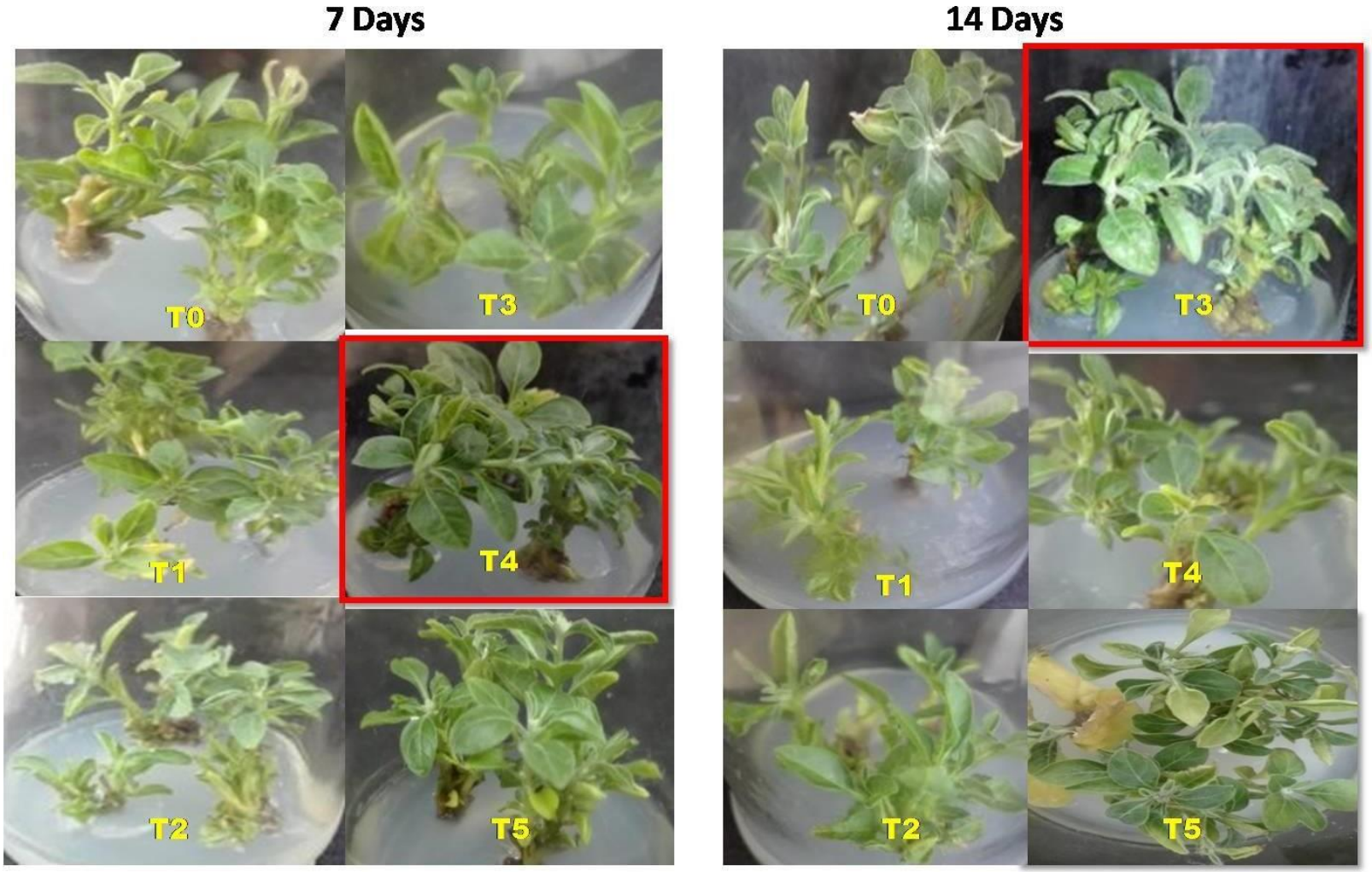
Scanning Electron Microscope (SEM)

X Ray Diffractions (XRD)

Morphological changes and biomass accumulation in *in vitro* shoot cultured on media containing lead nitrate

One month old shoot cultures of *Withania somnifera* were used for the present study. Approximately 2g of one month old shoots grown in MS media containing 4.4µM BAP were transferred to MS basal medium. This was done to ensure that the explants used for the present study do not have any residual influence of hormones and whatever response we observe will be solely due to the treatment of lead nitrate. These *in vitro* shoots grown on MS basal medium were now transferred to medium containing varying concentration of lead nitrate (T0-T5) (Table 4.1). Each treatment group consisted of three shoots and each experiment was repeated thrice. The shoots were observed regularly for morphological changes and harvested on 7^{th} day and 14^{th}day of treatment. The harvested shoots were weighed and then shade dried.

Figure 4.1.1 Morphological changes and biomass accumulation in *in vitro* shoot cultured on media containing lead nitrate for 7 and 14 days



The dried plants were then weighed and growth index was calculated using the formula:

$$GI = \frac{\text{Fresh weight of the harvested biomass - Fresh weight of the inoculum}}{\text{Fresh weight of the inoculum}}$$

The biomass accumulation of the shoots cultured in MS media containing varying concentration of lead nitrate is presented in Table 4.1. From Table 4.1 it is observed that all the treatment groups showed a significant difference compared to control except T1 which was on par with control even on prolonged growth period of 14 days.

Table 4.1.1 Biomass accumulation at 7 and 14 days of culture

Treatment	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**
7 days	1.25	1.09	1.68	1.9	1.95	1.03
14 days	1.44	1.31	1.04	1.33	1.14	1.09

ANOVA

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	0.19763	1	0.19763	1.82195	0.23497	6.60789
Columns	0.4239	5	0.08478	0.78158	0.60329	5.05033
Error	0.54237	5	0.10847			

The shoots cultured in MS media supplemented with low concentration of lead nitrate (T1) showed slow growth compared to control (T0). Among the treatment groups a gradual increase in growth rate was observed upto 2.4mM lead nitrate (T4) supplemented group after which it decreased (T5) on 7 days of culture. An increase in culture period to 14 days further reduced the growth rate in T2 and T3 and a maximum growth of 1.33g was observed in T3(1.8mM lead nitrate supplemented group).

Our observation is similar to observation of Wiszniewska *et al.*, (2015) for studies on Daphne species; where, an increase in fresh weight was recorded for cultures grown on media supplemented with lead nitrate upto a concentration of 1mM.

Although lead is a toxic heavy metal for plants, *Withania somnifera* showed significant growth in media containing lead upto a concentration of 1.8 mM. Further this stress has also increased the metabolite accumulation, thus increasing therapeutic potential of the plant. Similar response was observed in *in vitro* cultures of a solanaceae plant *Datura inoxia* which showed survival upto 45mg/l lead concentrations with a slight decrease in the number of shoots with increasing concentrations (Ashwini *et al*., 2014).

The ability of these plants to tolerate stress might be due their potential to selectively absorb the necessary nutrients and to maintain the appropriate nutrition of their photosynthetic system even from contaminated substrates (Zaier *et al*., 2010).

Phytochemical Analysis

Several specific reactions operating temporally and spatially are responsible for the production of secondary metabolites in medicinal plants. Changes of the environmental factors or placing a plant into tissue culture may also produce a new, and sometimes unexpected, secondary metabolic profile. The term quantitative analysis refers to the establishing and proving the identity of a substance. The active ingredients, after isolation, can be incorporated into the modern medicine system for the development of newer formulation for therapeutic ailments (Salwaan *et al*., 2012). The plant extracts were subjected to preliminary phytochemical screening for the detection of various plant constituents present.

Flavonoids

Flavonoids comprise the most common group of plant polyphenols and provide much of the flavor and color to fruits and vegetables. It has been recognized that flavonoids show antioxidant activity (Pourmorad *et al*., 2006). More than 5000 different flavonoids have been described. Interest in the possible health benefits of flavonoids has increased owing to their potent antioxidant and free-radical scavenging activities observed *in vitro*. The six major subclasses of flavonoids include the flavones (e.g., apigenin, luteolin), flavonols (e.g., quercetin, myricetin), flavanones (e.g., naringenin, hesperidin), catechins or flavanols (e.g., epicatechin, gallocatechin), anthocyanidins (e.g., cyanidin, pelargonidin), and isoflavones (e.g., genistein, daidzein). Most of the flavonoids present in plants are attached to sugars (glycosides), although occasionally they are found as aglycones (Ross *et al*., 2002).

Figure 4.2.1. Estimation of Flavonoids

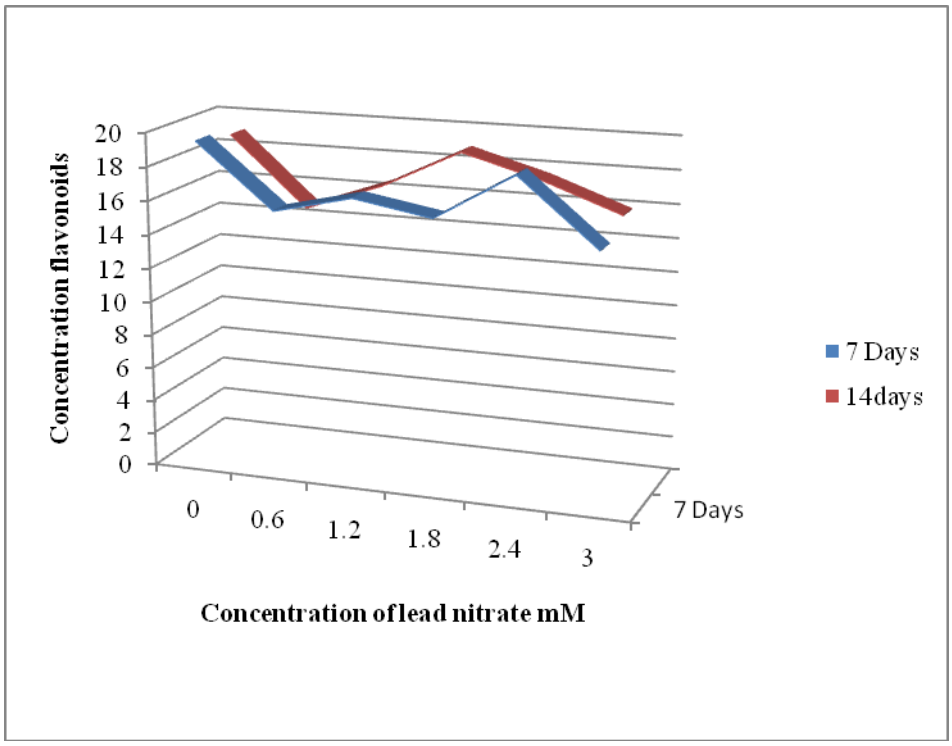


Table 4.2.1. Estimation of Flavonoids

Treatment	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**
7 days	19.43	15.64	16.74	15.94	18.73	14.85
14 days	18.99	14.86	16.53	18.84	17.53	15.84

ANOVA

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	960.3058	2	480.1529	217.827	5.69E-09	4.102821
Columns	14.45811	5	2.891622	1.311818	0.333397	3.325835
Error	22.04286	10	2.204286			

The concentration of flavonoids was estimated following method described by Cameron *et al*., 1943. Flavonoid content of shoots grown on media supplemented with varying concentrations of lead nitrate is presented in Table 4.2.1. The flavonoid content among all treatment groups was found to be significantly reduced compared to control (T0). Among treatment groups a gradual increase in flavonoid levels were

observed (T1- T4) which decreased with increase in concentration of lead to 3mM (T5). Similar results were observed in flavonoid content of shoots subjected to 14 days treatment.

Hafiz *et al.*, 2017 has reported a decrease in the phenol and flavonoid content with the increase in the concentration of Cd and Cu. Similar decrease in the phenolic and flavonoid content was reported in Hyacinthaceae plant in response to cadmium and aluminium treatment (Okem *et al.*, 2015).

Phytosterols

The major chemical constituents of the Withania genus, the withanolides, are a group of naturally occurring C28-steroidal lactone triterpenoids built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring (Mirjalili *et al.*, 2009).

Figure 4.2.2. Estimation of Phytosterols

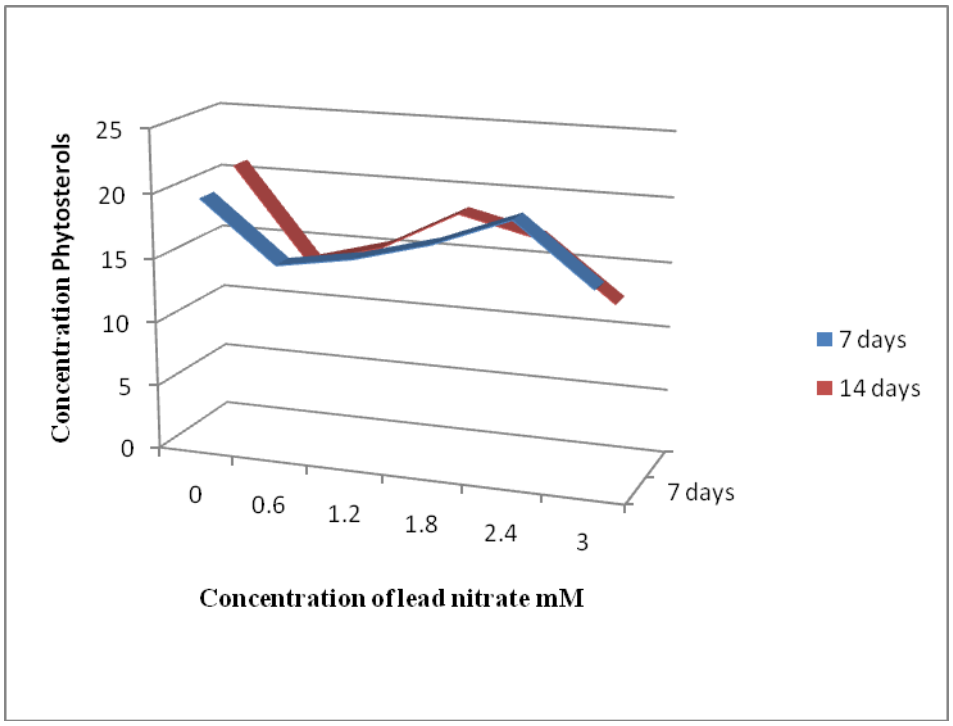


Table 4.2.2. Estimation of Phytosterols

Treatment	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**
7 days	19.54	14.84	15.82	17.43	19.75	15.13
14 days	20.93	13.72	15.34	18.45	17.05	12.52

ANOVA

Source of variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	927.0639	2	463.532	115.1423	1.25E-07	4.102821
Columns	38.2342	5	7.64684	1.899492	0.181437	3.325835
Error	40.2573	10	4.02573			

The concentration of phytosterol was estimated following method described by Wall *et al.,* (1952). The phytosterol content estimated in treated and control groups is presented in Table 4.2.2. Among control groups the concentration of phytosterols in 14 days old *in vitro* shoots of *Withania somnifera* recorded significantly higher levels than in 7 days old *in vitro* shoots. Among treatment groups a significant decrease in levels of phytosterol were observed with increase in concentration of lead nitrate when compared to control (T0). Among treated groups a gradual increase in phytosterol content was observed T1-T4 (14.84- 19.75mg/g) in 7 days and T1-T3 for 14 days (13.72-18.45 mg/g) after which the levels decreased. The toxicity of lead nitrate affects the phytosterol level at higher concentration and prolonged time period.

Phenol

Phenols are important secondary metabolite present in plants and animals. The concentration of phenols and other secondary metabolites in plants differ according to their environmental factors, geographical area, types of plant, part of the plant etc. The estimation of total phenol content in 11 medicinal plants from different geographical area was done by Johnson *et al.,* (2008) using Folin–Ciocalteu test and they identified *Prunus Africana* has the highest TPC 55.14mg/g of dry weight. Total phenolics content analysis on leafy vegetables and fruits was done by Chandra *et al.,* (2014). They found that basil (32.50mg/g) and chard (41.15mg/g) contains higher TPC than other leafy vegetables. The commonly used method to estimate total phenolic contents are Folin–Ciocalteu test with gallic acid as a standard.

. **Figure 4.2.3 Estimation of Phenols**

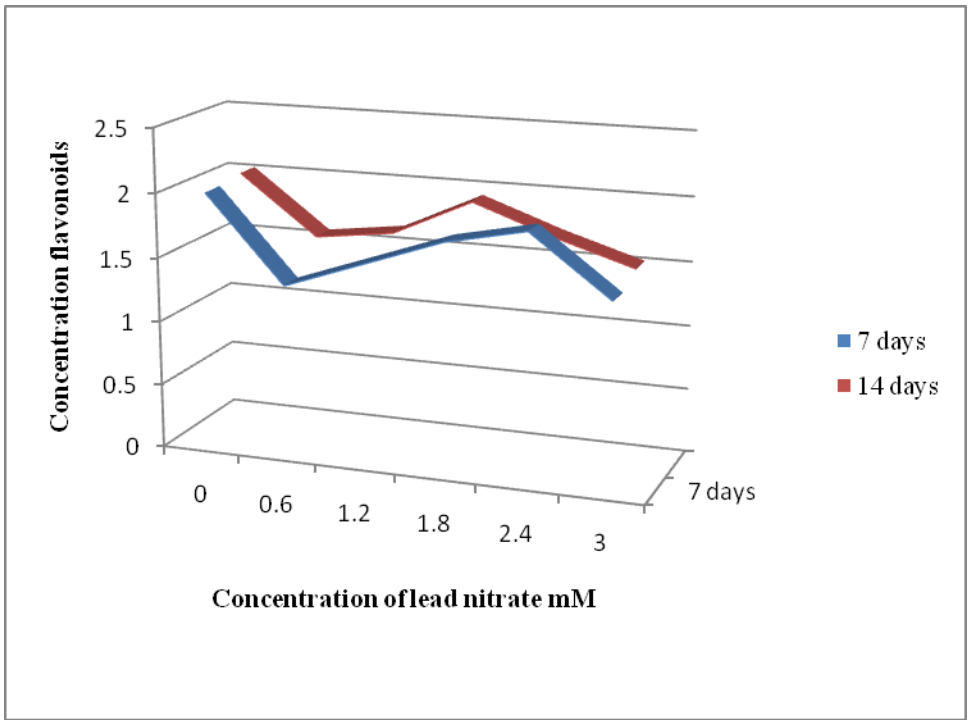


Table 4.2.3 Estimation of Phenols

Treatment	**T0**	**T1**	**T2**	**T3**	**T4**	**T5**
7 days	1.99	1.32	1.54	1.76	1.89	1.43
14 days	2.02	1.56	1.64	1.93	1.71	1.52

ANOVA

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	0.016875	1	0.016875	1.620259	0.259029	6.607891
Columns	0.508075	5	0.101615	9.756601	0.012916	5.050329
Error	0.052075	5	0.010415			

The phenol concentration was estimated following method described by Kumbhare *et al.*, (2012). The total phenol content estimated in treated and control group is presented in Table 4.2.3. Among treated groups a significant increase in phenol content was observed in T1-T3 (1.56-1.93 mg gallic acid equivalent/g of dry powder) after which the levels decreased with the maximum content of 1.93 mg gallic acid equivalent/g of dry powder (T4) in shoots grown for 14 days. When compared with control the levels of phenol decreased significantly, but among treatment groups,

there was a gradual increase from 1.32 - 1.89 mg gallic acid equivalent/g of dry powder (T1-T4) which decreased to 1.43 mg gallic acid equivalent/g of dry powder (T5) for7 days culture. A similar trend was observed for 14 days culture, with increase in levels only upto T3 (1.93mg/g) after which the phenol levels decrease significantly.

Suthar *et al.,* (2014) reported that treatment of seedlings with cadmium shows decrease from concentration 300 ppm ((1.793 mg g^{-1})) and again increased at 1000 ppm (2.185 mg g^{-1}) compared with control (1.856 mg g^{-1}).

In another experiment, the seedlings treated with Zinc,there was an initial decrease till 100ppm followed by step increase at 300ppm (1.793 mg g^{-1}) and followed by further decreased to (to 2.215 mg g^{-1})at 1000ppm,when compared with untreated seedlings (Saini *et al.,* 2018).

The studies of Chinmayee *et al*., 2014 on *Jatropha curcas* L. treated with lead and cadmium showed an increase in phenol content in leaves. The results are different from our observations. This may due to prolonged growth of *Jatropha curcas* L. in pot cultures with higher concentrations of lead (50-150mg/kg) of soil. To confirm our results, prolonged growth period should be carried out in future.

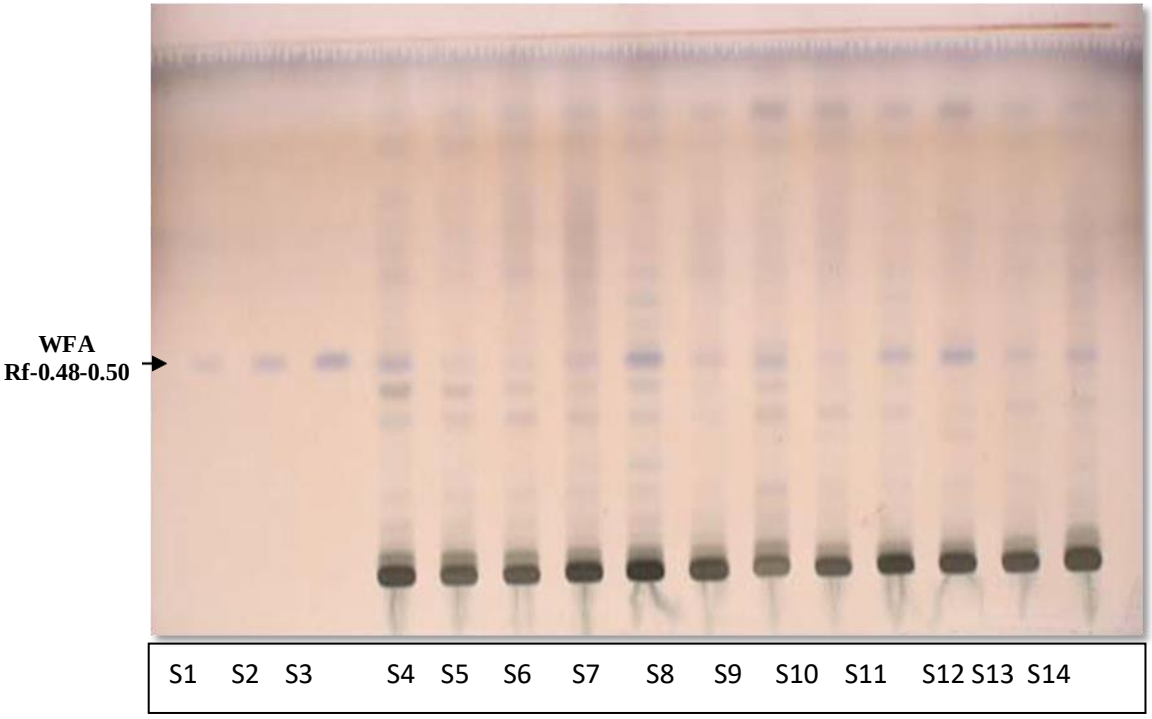
Quantification of major withaferin A

HPTLC has been used to provide excellent separation, qualitative and quantitative analysis of a wide range of compounds, such as herbal and botanical dietary supplements, neutraceutical, traditional western medicines, traditional Chinese medicines and Ayurvedic medicines and determination of radio labeled substances in chemical, biochemical, biological, pharmaceutical and medicinal samples.

The quantification of bioactive metabolites was performed using HPTLC. HPTLC often remains the method of choice because of its advantages of reliability in the quantification of analytes even at nano-gram levels and cost-effectiveness. Alam *et al.,* (2012) have concluded that the HPTLC method was found to be specific and accurate and can be used for qualitative estimation of crude extract of Withania and its polyherbal formulations. HPTLC method is especially suitable for the fingerprinting and high throughput analysis of botanical samples and herbal formulations. In the present study, a HPTLC analysis was performed for the quantification of significant metabolite withaferin A in the methanol extract of *W. somnifera in vitro* leaf tissues.

The major secondary metabolite of *W. somnifera*, namely withaferin A, was quantified by high performance thin layer chromatography (HPTLC; **Figure 4.2).** The methanolic extract of samples were applied to the plates as 8mm bands, under a stream of nitrogen gas, by means of a CAMAG (Switzerland) Linomat V semiautomatic sample applicator fitted with 100µl Hamilton Syringe. The loaded plates were developed in the chamber presaturated with the mobile phase (Toluene: Ethyl acetate: Formic acid, 5:5:1). The derivatized plates were viewed under 366nm and white light in an visvalizer and captured. The plates were then scanned at 223nm using a HPTLC scanner which is supported with WINCATS software. Calibration plots of peak area against concentration were linear in the range of 0.4 to 2.0 µg for withaferin A. Peak area versus concentration was subjected to least square linear regression analysis and the slope, intercept and correlation coefficient for the calibration over the concentration range was determined

Figure 4.3.1. HPTLC quantification of *W. somnifera* methanolic extracts of *in vitro* lead treated shoot samples



S1-S3 = Withaferin standards

S4 – S9 = Lead nitrate treated samples for 7 days

S10 – S15 = Lead nitrate treated samples for 14 days

Table 4.3.1. Peak area for the standard Withaferin A at 223nm

S.No.	Standard Withaferin A		Peak area
	Volume(µl)	Concentration (µg)	
1	2.5	0.25	416.40
2	5	0.5	830.54
3	10	1	1631.53

Figure 4.3.2. Chromatogram of standard and samples

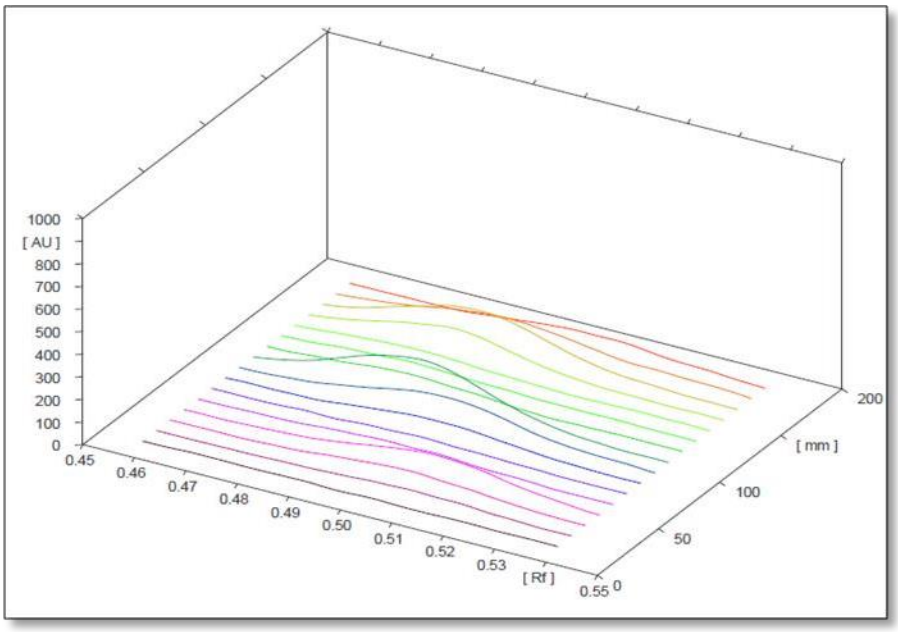


Figure 4.3.3. Linear regression graph for Withaferin A at 223nm

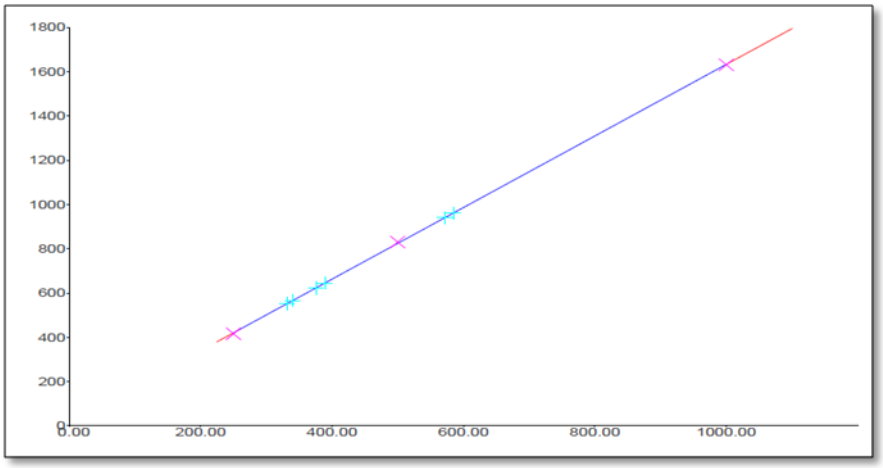


Table 4.3.2. Peak area and concentration of Withaferin A in *in vitro* shoots cultured on media supplemented with lead nitrate

Sample	Peak area		Concentration (mg/g)		Fold Increase	
	7 days	14 days	7 days	14 days	7 days	14 days
T0	565.59	623.66	0.255	0.281	-	-
T1	345.14	552.80	0.155	0.249	0.6	0.88
T2	940.38	1875.23	0.424	0.846	1.66	3.01
T3	2135.13	3038.66	0.964	**1.371**	3.78	**4.87**
T4	3577.06	1911.34	**1.615**	0.862	**6.33**	3.06
T5	645.51	962.04	0.291	0.434	1.14	1.54

From the results obtained for HPTLC analysis, extracts of *in vitro* lead nitrate treated shoot samples revealed the presence of more prominent spots. Significant differences were also observed in the accumulation patterns over 7days and 14 days treatment (Figure 4.2). Withaferin A in the samples was found to separate at Rf of 0.48 - 0.50 .The accumulation of withaferin A in the samples were calculated from the peak area obtained in comparison to the standard peak area.

The data obtained were subjected to two way ANOVA and the results are depicted in Table 4.3.3.

Table 4.3.3: Withaferin A concentration in *in vitro* Lead treated shoot samples

Treatment	T0	T1	T2	T3	T4	T5
7 days	0.255	0.155	0.424	0.964	1.615	0.291
14 days	0.281	0.249	0.846	1.371	0.862	0.434

ANOVA

Source of Variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Rows	0.00958	1	0.00958	0.10392	0.76022	6.60789
Columns	2.08706	5	0.41741	4.52946	0.06145	5.05033
Error	0.46078	5	0.09216			

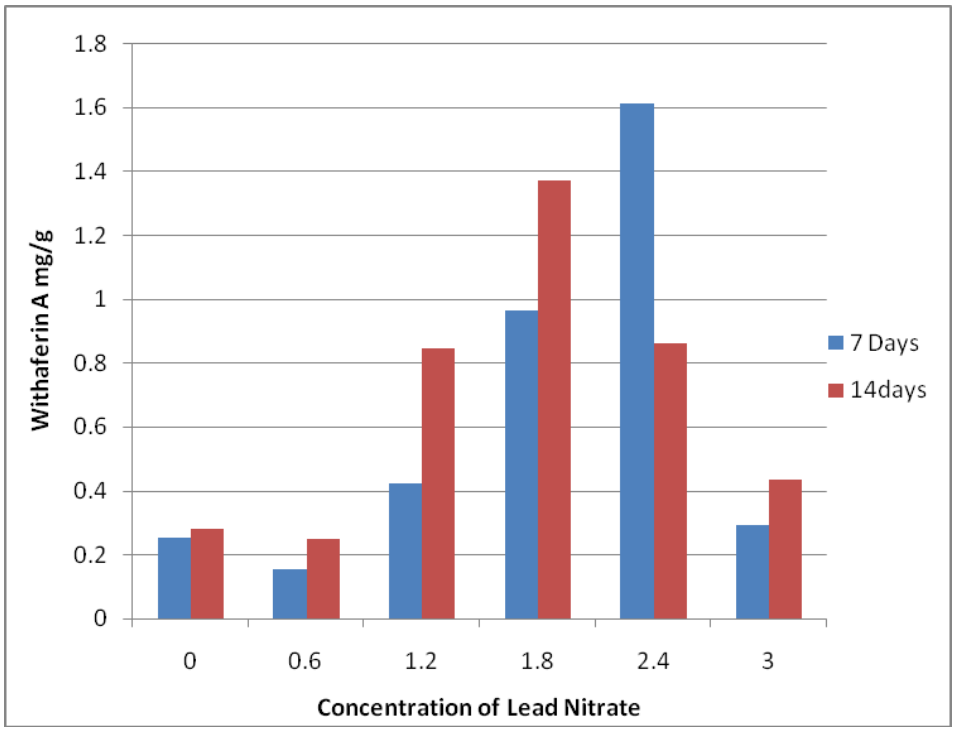
Similar to biomass, the lead concentrations were found to be proportional to the withaferin A accumulation upto a maximum of 1.615mg (2.4mM) in T4 for 7 days treatment and 1.37mg (1.8mM) in T3 for 14 days treatment after which a decrease in the accumulation was observed. The accumulation of Withaferin A was found to be

6.33 fold higher in T4 and 4.87 fold higher in T3 of 7 days and 14 days treated shoots respectively.

Nearly same increase in Withaferin A (approximately 7 fold) was observed using aluminium chloride as elicitor (10mg\L for 4 hours) in cultures of *Withania somnifera* (Sivanandhan *et al*., 2012). Similar results were obtained by Spollansky *et al*., (2000) in hairy root cultures of *Brugmanisa candida* on treatment with jasmonic acid and aluminium chloride. This shows the elicitation potential of the heavy metal lead towards increase in secondary metabolite content in plants.

Shajahan (2017) has reported that maximum Withaferin A (increase 4.03 fold) accumulation was observed in root culture of *Withania somnifera*, when treated with chitosan (100mg/l for 30 days) as an elicitor. Treatment of the shoots with 150µM of salicyclic acid for 4 hours showed 20 fold increase in Withaferin A (Sivanandhan *et al*., 2012). These studies suggest that, elicitation in any means, increases the secondary metabolite content in *in vitro* cultures, and can be exploited for commercial purpose.

Figure.4.3.4. Accumulation of withaferin A accumulation in *in vitro* tissues of *Withania somnifera* grown on media supplement with lead nitrate



Analytical techniques to study the accumulation of lead in *in vitro* cultured shoots

The lead nitrate treated *in vitro* grown shoots of *Withania somnifera* cultures were taken for analytical studies which includes UV-visible spectrophotometer, X Ray Diffractions (XRD) and Field Emission Scanning Electron Microscope (FE-SEM).

UV-Visible spectrophotometer

UV visible spectrophotometer was used to identify the accumulated lead as ions or particulates inside *Withania somnifera* plant. *W. somnifera* plant treated with lead nitrate showed significant changes in the metabolite and lead ions peak. The extracts of *in vitro* grown shoots of *Withania somnifera* were analysed by UV-visible spectrophotometer for the presence of lead metal. The standard lead nitrate showed peak at 295nm.The samples of treated groups also showed peaks at same wavelength indicating the uptake of lead in the leaf tissues. The absorbance of protein is also at the same wavelength which indicates the protein might combine with lead for its reduction. The peaks found at 665nm and 465nm found to be chlorophyll a and b, the peak identified at 410nm shows the presence of Beta carotene.

The lead nanoparticles was synthesized through green synthesis using *Zingiber officinale* and *Cuminum cyminum* extract gave the peak at around 239 nm and 335 nm (Delma *et al*.,2016 & Gandhi *et al*., 2018). Another study on *Cocos lucifera L* extract and lead nanoparticles synthesis followed by spectroscopic analysis showed peaks at 212nm. Even though the presence of peaks varied from 200 – 350 nm in green synthesized lead nanoparticles, the accumulation of lead particulates in live plants of *Withania somnifera* could vary in its UV absorption pattern (295nm). The samples were subjected to FE SEM analysis for the conforming the presence of lead.

Figure.4.4.1a. UV-Visible Spectrum analysis of 7 days Lead nitrate treated *in vitro* shoots of *Withania somnifera*.

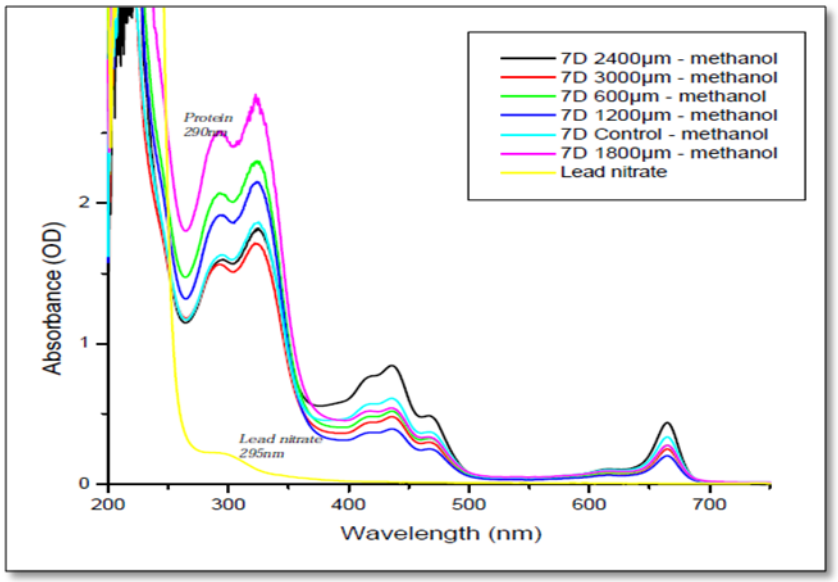
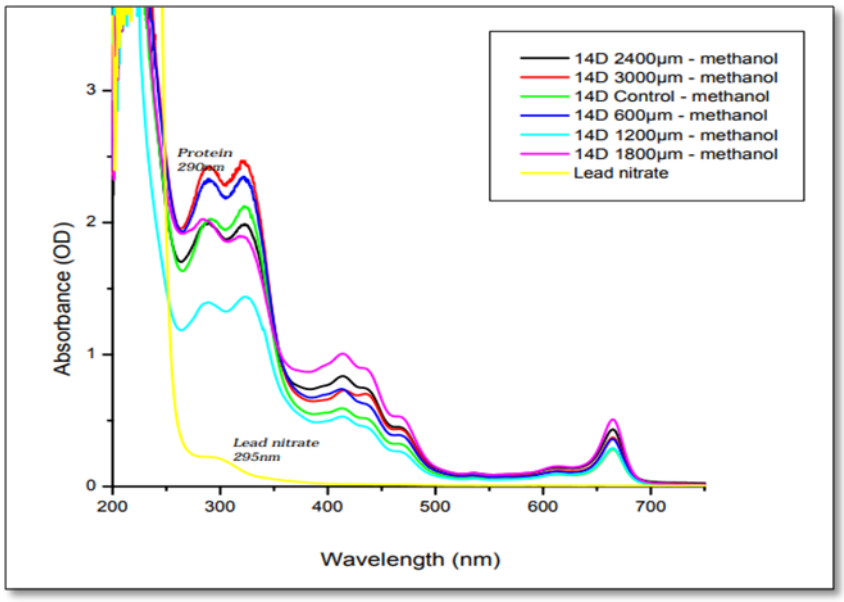


Figure.4.4.1b. UV-Visible Spectrum analysis of 14 days Lead nitrate treated *in vitro* shoots of *Withania somnifera.*



Field Emission Scanning Electron Microscopy (FESEM)

Field Emission Scanning Electron Microscopy (FESEM) images were measured and topographical analysis was performed based on the surface study. The FESEM studies provide the information on the morphology and size of the accumulated lead as metal or ions inside the plant tissues. One month old *Withania somnifera in vitro* plantlets were treated with different concentrations of lead nitrate containing agar medium. Leaf bits of *Withania somnifera* grown for 14 days on media

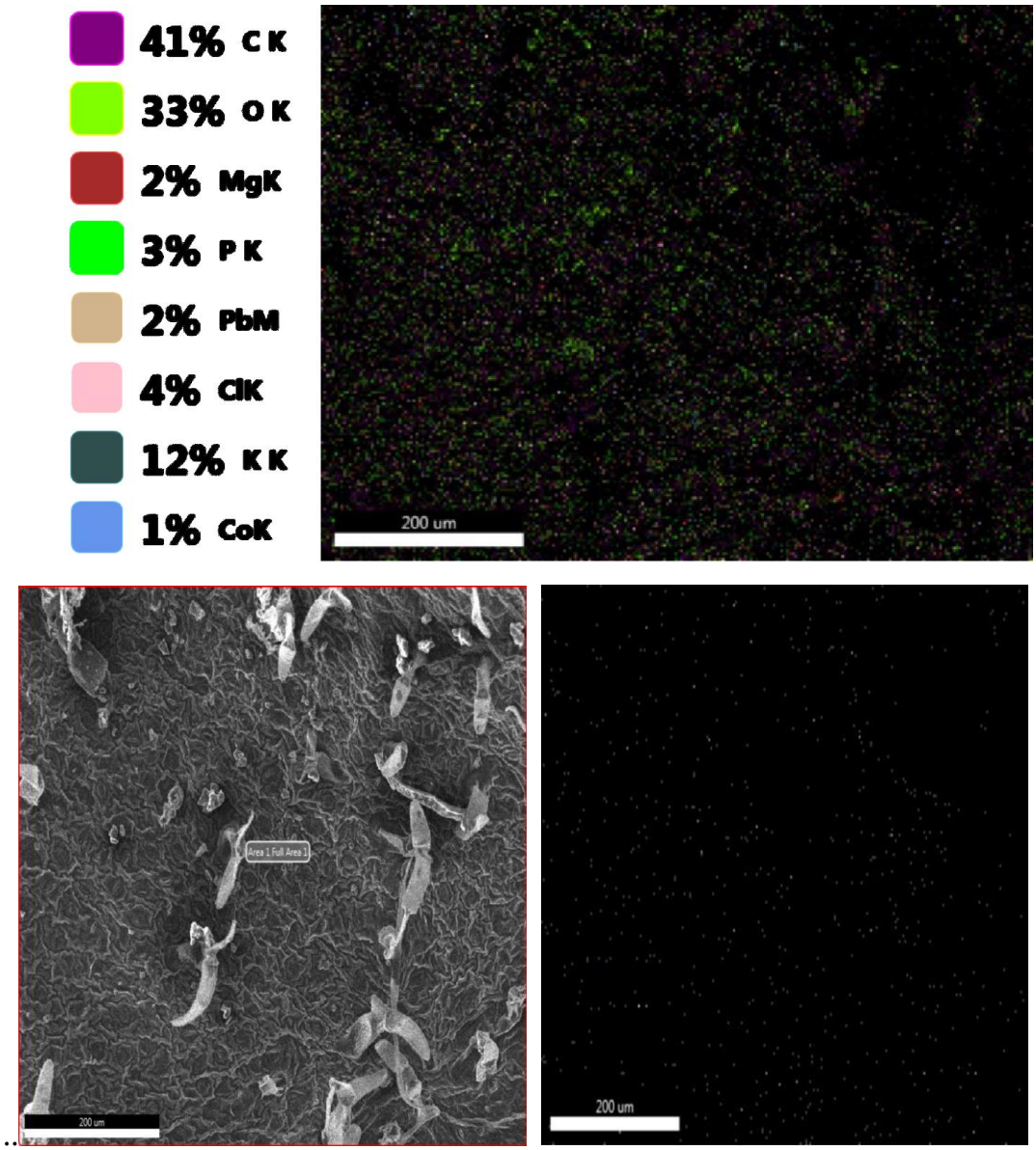
containing 3 mM lead nitrate were cut into 3.33mm piece and fixed by 3% glutaraldehyde at 4C for 2 hours and taken for FESEM analysis. The FESEM micrograph (Fig 4.12) shows the presence of several elements which are essential for the growth of the shoots cultured *in vitro* among which 2% lead was identified in the leaf of given sample which was further confirmed by EDAX spectrum. The EDAX spectrum also showed the presence of carbon 41%, oxygen 33%, magnesium 2%, potassium 12%, chlorine 4%, phosphorus 3% and cobalt 1% along with lead. This 2% of lead was found to be scattered all over the leaf tissues (Fig 4.12). The reduction of lead nitrate to lead may occur in the media or inside the plants during uptake. In conclusion, the lead in leaf tissues of *Withania somnifera* was from reduction, diffusion, transportation and accumulation of lead from the media. The atomic and weight percent value of lead observed as 0.07, 0.97 respectively.

A study conducted by Sahi *et al.,* 2002, where they transferred two weeks treated *Sesbania drummondii* plant grown on 1000 mg/L of $Pb(NO3)_2$ into nutrient medium (in absence of Pb) for a week in order to assess whether the absorbed Pb leaches back to the medium. The presence of Pb in stem and leaf in the same fashion under SEM confirmed the irreversible nature of uptake and translocation of lead in *Sesbania drummondii*.

Similar study on heavy metal toxicity in paddy plants was conducted by Alfaraas *et al.*, 2016. Treatment on paddy plants with different concentrations of lead and cadmium salts was done and analyzed for electron microscopic studies on root and leaf cross sections. They found that the accumulation of lead and cadmium in paddy plants were at concentration dependant manner and also at higher concentration of heavy metals cause the plants to physiological changes like chlorosis of leaf, reduction in root length and shoot height.

Cadmium stress on chickpea plants also proved that the toxicity of cadmium at 1000mg/l which caused opening of stomata, retarded root growth and distortion of tissue structure also observed (Mondal *et al*., 2013)

Figure 4.4.2a Elemental Mapping



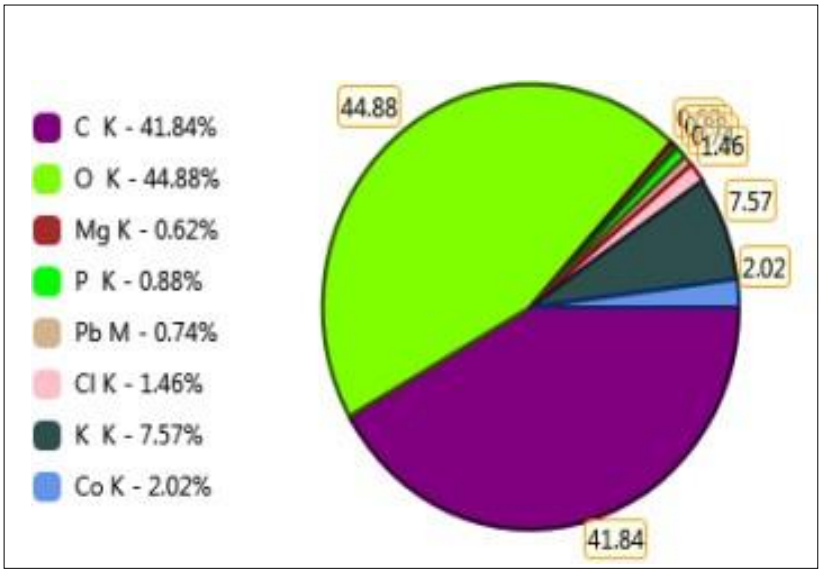
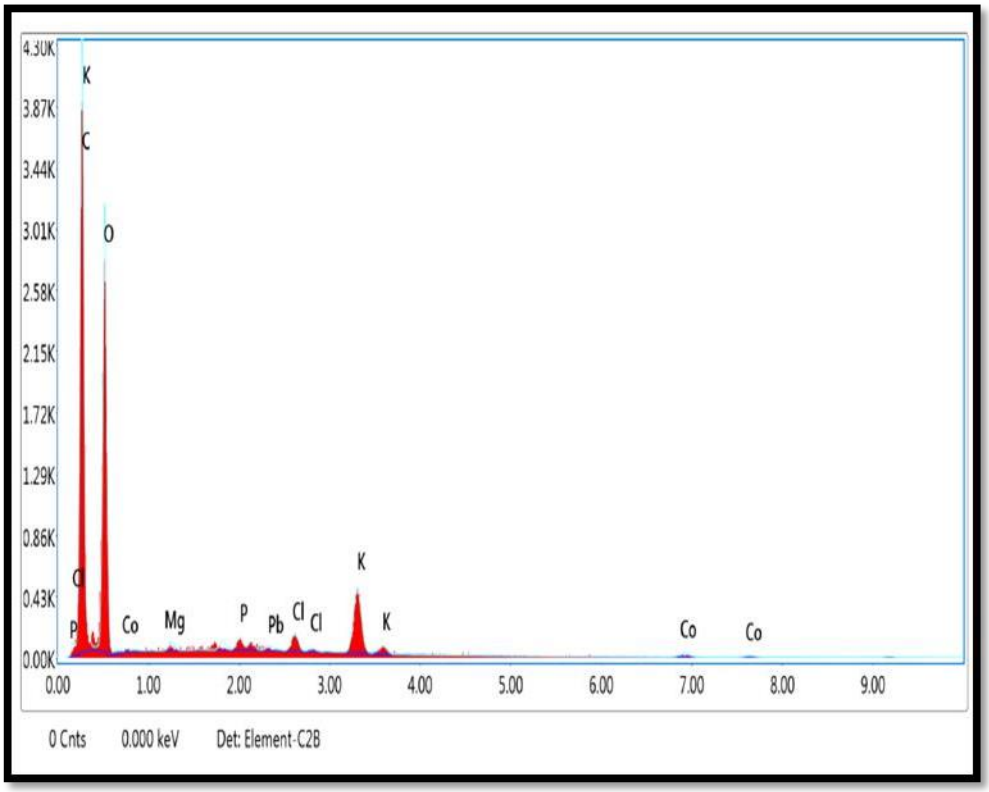


Figure 4.4.2b. EDAX spectrum of lead nitrate treated leaf tissues



XRD (X – Ray Diffraction)

The *Withania somnifera* 14 days 3.0mM lead nitrate treated *in vitro* shoots were taken for lead uptake and toxicity studies. The 3.0 mM 14 days shoots were shade dried and powdered with motor and pestle. The shoot powder was analyzed for crystalline nature of lead using XRD and the size was calculated.

The crystalline size and structure properties of synthesized lead were revealed by using X-ray diffractions. The XRD studies were carried out with Cu – κα radiation (κ – 0.154nm) and 2theta range from 20˚ to 80˚. The XRD image of lead nitrate treated *in vitro* leaves of *Withania somnifera* is shown in fig 4.8. The mean crystallite size (D) of the particles was determined from the XRD line broadening measurement using Scherrer equation

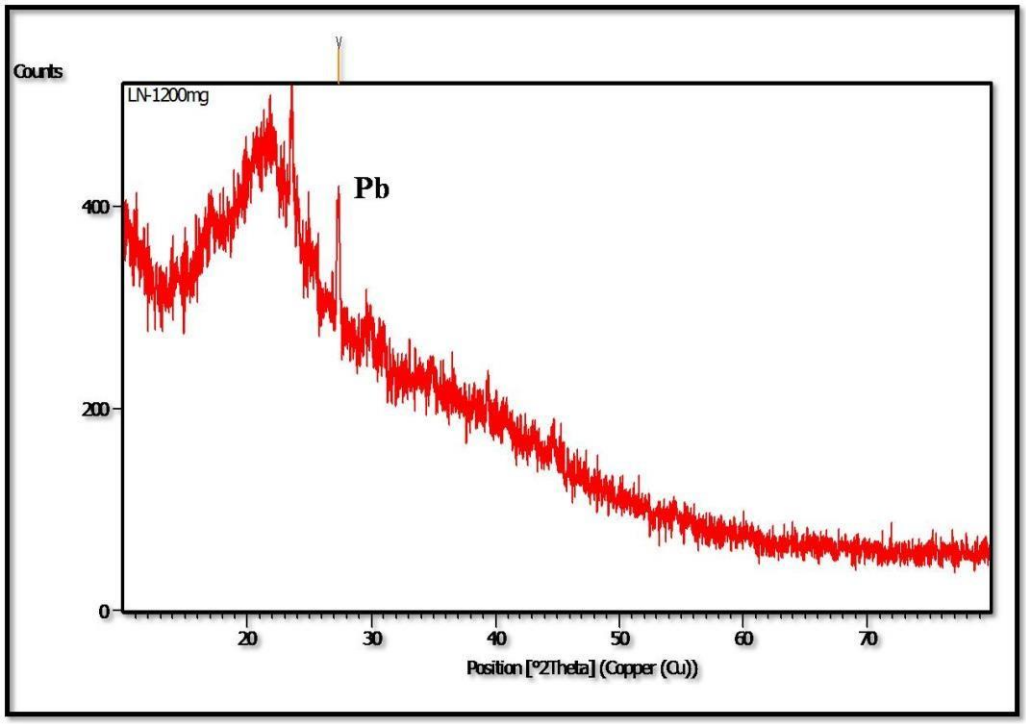
$$D = {}^{0.9\lambda}/_{\beta\ cos^{\epsilon}}$$

Where λ is the wave length (Cu – κα), β is the full width and half maximum (FWHM) of the lead nitrate 27.35 line is the diffraction angle. The calculated crystalline size of the powder is about 8.778 nm.

Similarly Delma *et al* (2016) has used XRD technique to study lead nanoparticles synthesized using *Zingiber officinale* extract and obtained diffraction peaks at 2θ values of 38.14 , 19.72 and 32.38 and the average particle size of lead nanoparticles was found to be 3nm according to Debye Scherrer equation.

2Ɵ values for lead nanoparticles synthesized by bacterial strains of *Bacillus toyonensis* were 26, 30, 43 and 51. The similar result was identified from our study that the lead from *in vitro* culture of *Withania somnifera* showed 2Ɵ values at 27.35 (Mathew and Krishnamurthy 2018).

Figure 4.4.3.1. XRD spectrum of in vitro shoots cultured on media containing lead nitrate (T5)



5.0 Summary and Conclusion

The result of the study "**Influence of Lead nitrate on Withaferin production in *in vitro* shoot cultures of *Withania somnifera***" is summarized as follows.

- *Withania somnifera* shoots were cultured in *in vitro* condition on MS media supplement with lead nitrate salt at different concentration. The maximum biomass and Withaferin A content was observed in 7 days when compared to control and 14 days treated shoots.
- Among the 7 days treated plants maximum biomass of 1.95±0.22g was observed in media supplemented with 2.4mM lead nitrate which is approximately 1.55 fold higher than that of untreated shoots.
- The maximum Withaferin A content was also observed in shoots grown in media containing 2.4mM lead nitrate for 7 days, which was approximately 6.33 fold higher than untreated shoots.
- The UV- visible spectrophotometric analysis of the standard lead nitrate solution showed peak at 295nm, the shoot samples also shows peak at the same wavelength indicating the presence of lead in the samples. The other peaks were found at 410 nm, 465nm and 665 nm indicates the presence of Beta carotene, Chlorophyll b and a respectively.
- The FE SEM analysis of the leaf tissue showed the appearance of lead particles scattered all over the surface of the tissues followed by the EDAX spectrum revealing the accumulation of 2% lead in shoots grown for the 14 days in media containing 3 mM lead nitrate .
- Further the X- Ray Diffraction studies revealed the crystalline nature of the lead particles accumulated in the shoots with the particle size of about 8.78nm.

Therefore, from the above results it can be concluded that lead though being a very toxic heavy metal, *Withania somnifera* has the capability to survive on it. Though toxicity affects its growth on the prolonged exposure it also acts as a good elicitor of the most important secondary metabolite withaferin A. Thus, *Withania somnifera* acts as a good phytoremedial plant which can be grown even in the contaminated soil. With further studies on the safety assessment of withaferin A isolated from the contaminated plants and its toxicity assessment, the plant can be grown even in industrial contaminated areas and its plant parts can be used as a source for withaferin A.

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Ingredients	**Composition (mg/ L)**	**Stock Solution (W/V) (g)**
MS Macro I (10 X)		**1000ml**
NH_4NO_3	1650	16.5

Composition of MS medium

KNO_3	1900	19
$MgSO_4.7H_2O$	370.6	3.7
KH2PO4	170	1.7
MS Macro II (10 X)		**1000 ml**
$CaCl_2.2H_2O$	439.8	4.398
Fe-Na EDTA (1000 X)		**100 ml**
Fe-Na EDTA	36.7	36.7
Micro Nutrients (1000 X)		**100 ml**
NaMoO4.7H2O	0.25	0.025
CuSO4.5H20	0.025	0.0025
CoCl2.2H2O	0.025	0.0025
$MnSO_4.4\ H_2O$	13.2	1.32
$ZnSO_4.4H_2O$	8.6	0.86
H3BO3	6.2	0.62
KI (1000X)	0.83	100ml
Myo-Inositol (100 X)		**100 ml**
Myoinositol	100	1
MS Vitamins (1000 X)		**100 ml**
Nicotinic Acid	0.5	0.05
Pyridoxine HCl	0.5	0.05
Thiamine HCl	0.1	0.01
Glycine	2	0.2

APPENDIX 2
ESTIMATION OF FLAVANOIDS
(Cameron *et al*., 1943)

PRINCIPLE

A Portion of plant was weighed and carried out in two steps, firstly MeOH: H_20 (9:1) and then MeOH: H_2O (1:1) solvent added to make liquid slurry and mixture left to 12hrs .Filtration to separate the extract from plant material was / carried out rapidly for using glass wool or cotton wool plug in neck of filter funnel two extracts were combined and evaporated 1/13 original volume or most of MeOH had been removed. Resultan aqueous extract was cleared if low polarity contaminants such as Fats, Terpenes, Chloroform and Xanthophylls' by extraction with hexane and or chloroform. This was repeated several times and extract combined. The solvent extracted aqueous layer containing bulk of Flavonoids was concentrated.

MATERIALS

• **Vanillin reagent** - 1%in 70%H2SO4

• **Catechin standard** -110 ug/ml

PROCEDURE

1. Aliquot of extract was pipetted into test tube and evaporated to dryness.
2. Then added 4ml of Vanillin reagent.
3. A standard was also treated in the same manner.
4. Then equal amount of distilled water was added.
5. Kept in boiling water bath for 15 minutes.
6. Took the readings at 360 nm.
7. Calculated the amount of flavonoids present in the sample.

CALCULATION

Express the amount in mg / g or 100 g sample.

APPENDIX-3

ESTIMATION OF PHENOL

(Kumbhare *et al.*, 2012)

MATERIALS

• **Standard:** 1ml of Gallic acid dissolved in 100ml of distilled water

• **Folin-Ciocalteau**

• **Sodium carbonate**

PROCEDURE

1. Pipetted out 0.5, 1, 1.5, 2, and 2.5 ml of Standards into a series of test tubes and 0.5ml

of the sample extract into another test tube.

2. 1.5ml of Folin-Ciocalteau reagent was added to all tubes.

3. Allowed tubes to stand in room temperature for 5 minutes.

4. Added 4.0ml of 20% sodium carbonate to each tubes.

5. Make up the volume to10 ml in all the test tubes.

6. All tubes were incubated at room temperature for 30 minutes.

7. Absorbance was measured at 765 nm.

CALCULATION

Express the amount in mg of gallic acid equivalent/ g of dry powder or 100 g sample.

APPENDIX –4

ESTIMATION OF PHYTOSTEROLS

(Wall *et al.*, 1952)

MATERIALS

• **Libermann Burchard Reagent :**(Acetic Anhydrate and Sulfuric acid) 0.5ml Sulfuric acid dissolved 10ml of a Acetic Anhydrates and kept in ice.

• **Standard:** 10mg Cholesterol dissolved in 10ml of Chloroform

PROCEDURE

1. Pipetted out 0.5, 1, 1.5, 2, and 2.5 ml of Standards into a series of test tubes and 0.2ml of the sample extract into another test tube.

2. Added 2ml of Libermann Burchard reagent to all the tubes.

3. Made up equal volume in all tubes with Chloroform.

4. Covered with carbon paper and Incubated at room temperature in dark for 30 minutes. A green colour was developed.

5. Read the absorbance at 640 nm.

CALCULATION

Express the amount in mg / g or 100 g sample.