Influence of Iron and Copper on *invitro* culture of *Withania somnifera*

R.Kousalya

(16PBC005)

Thesis submitted to

Avinashilingam Institute for Home Science and Higher

Education for Women, Coimbatore – 641043

In Partial Fulfilment of the Requirements for the Degree of

Master of Science in Biochemistry

April, 2018

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10/4/18

Signature of Head of the Department

10/4/2018.

Signature of the Supervisor with Designation

CEFTIFICATE

Certificate from the supervisor

I certify that dissertation entitled **“Influence of Iron and Copper on *invitro* culture of *Withania somnifera* ”** submitted for the degree of **Master of Science (M.Sc) in Biochemistry by R.Kousalya** is record of research work carried out by her during the period from 2015 to 2017 under my guidance and supervision and that this work has not formed the basis for the award of any degree, Diploma, Associate Ship, Fellowship other Titles in the university or any other university or institution of Higher Learing.

Signature of Head of the department **Signature of the Supervisor**

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R.Kousalya

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INTRODUCTION

INTRODUCTION

The term medicinal as applied to the plant indicates that it contains substances which modulate beneficially the physiology of sick mammals, and that it has been used by man for that purpose. Medicinally plants play a significant role in providing primary health care. They serve as therapeutic agents and from a source for important raw materials for the manufacture of traditional and moden medicine (Kumar and Janagam, 2011).

Withania somnifera is also known as Aswangandha or Indian ginseng belongs to the family solanaceae, it is commonly used as a domestic remedy for several diseases in India as well as other parts of the world. It is described as an herbal tonic and health food in the famous book of Vedas. Fruits, Leaves and Seeds have been traditionally used in the Ayurvedic system as aphrodisiacs, diuretics and for treating memory loss. It is chemically rich with its varied content of active compounds, such as withanolides, Sitoindosides and many useful alkaloids and used for centuries to treat a wide range of disease, constitutes a promising candidate as a multipurpose medicinal agent. Withanolide A, Withanoside IV and withanoside VI showed neuritic regeneration and synaptic reconstruction.

Secondary metabolite 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 plays a vital role in search for alternatives to produce a desirable medicinal compounds from plants (Rao and Ravishankar, 2002).

The term Wthanolide is 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 withanolide or closely related congers have been discovered in altogether 58 solanaceous species belonging to 22 genera.Withanolides have been discovered also in certain Tacca spp. Of the Taccaceae (taccalonolides) and Ajuga spp.,(Khan *et al*., 2003) as well as in some marine organisms.

Stress is an important factor in determining the chemical compound composition and therapeutic activity of medicinal plant. Many kinds of heavy metal were also used as elicitors to induce accumulations of bioactive compounds in Salvia miltiorrhiza, such

as Co2+, Ag+, Cd2+, Cu2+, Ce3+, La, Mn2+ and Zn2+ .The physiological and biochemical responses of medicinal plants to different heavy metals stresses and the detoxification / antioxidative pathways involved, all of which may lead to enhanced yield of secondary metabolites. Jahangir et al. (2008) 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.

Micronutrients in plants have been strengthened, and the effects of these micronutrients on Secondary metabolites production depend mainly on the concentration and type of element. Similar to macronutrients, inaccurate concentrations of micronutrients can cause crop damage related to toxicity because plants require micronutrients in small amounts. Iron (Fe) is an essential element and its absence reduces productivity in photosynthetic organisms(Jeong and Guerinot, 2009). Fe is a co-factor for proteins involved in cellular processes such as respiration, photosynthesis and cell differentiation (Broadley et al., 2012). It is required by AOEs because it catalyzes the reactions of electron transfer (Halliwell, 2006). Copper (Cu) is part of the structure of certain proteins, mainly those involved in photosynthesis (plastocyanins) and respiration (cytochrome oxidase) and in the electron transport chain (Pilon et al., 2006).

Among the available conventional chromatographic technique. HPTLC offers major advantages. HPTLC profile is quite helpful in setting up of standards for evaluating the purity and quality of Ayurvedic preparations. This will be helpful to overcome batch to batch variations in different Ayurvedic Churna / preparations (Meena *et al.,* 2010) (Garg *et al.,* 2013).

Gas Chromatography is normally used for direct analysis of compounds existing in traditional medicines and medicinal plants. In recent years GC-MS studies have been increasingly applied for the analysis of medicinal plants as this technique has proved to be a valuable method for the analysis of non polar components and volatile essential oil, fatty acids, lipids (Jie *et al.,* 1991) and alkaloids (Betz *et al.,* 1997).

Plant tissue culture can be a potential source for important secondary metabolites such as pharmaceuticals and food additives. Micropropagation techniques have been found to be useful in the propagation of a large number of threatened and endangered plants (Sarasan *et al*., 2006) the technology bears many advantages over conventional agricultural methods; production is independent of variatio n in crop quality or failure, yield of target compounds would be constant and geared to demand, there is no difficulty in applying good manufacturing practice to the early stages of production (Chattopadhyay *et al.,* 2007)

The present studt focuses to increase the secondary metabolites production by inducing metal ion stress to *Withania somnifera* was designed with the following objectives:

- To quantify the Withaferin A compounds using Withania somnifera a metal stressed plant by HPTLC method.
- Characterization of components present in Withania somnifera shoots samples using GC/MS.
- Identification of plants metabolites present in Withania somnifera metal stressed shoot samples using UV- Visible Spectrophotometer.

2.0. REVIEW OF LITERATURE

Medicinal plants as a functional group consist of the largest single group of plants with an approximate 30,000 species worldwide, covering over 5,000 genera and more than 1,000 families and sub-families. Indians also have one of the richest medicinal plants heritages. Around 8,000 species of plants are used by the people of India in primary health care for human, veterinary and agricultural applications .Medicinal plants are the essential source of nutrients and secondary metabolites (SMs) (Patra et al., 2013), frequently referred as bioactive compounds, such as Alkaloids, Phenolic compounds (PCs) and Terpenes (Jahangir et al., 2012).

Plant-produced secondary compounds have been incorporated into a wide range of commercial and industrial applications, and 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 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. It helps invigorate the body by rejuvenating the reproductive organs, just as a tree is invigorated by feeding the roots (Verma and Kumar, 2011).

Ayurveda has been a lively system of health care in India with an unbroken practice since 6000 years but 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).

In Ayurveda, *Withania somnifera* is considered a rasayana herb adverse because it promote health and longevity and increase capability of individuals to resist adverse environmental conditions .The plant root is rich in Steroids and Alkaloids and they have valuable constitute of traditional Ayurvedic drug preparations against many diseases

(Williamson, 2002). 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 studied extensively and, more recently, have been shown to have anti-cancerous activity (Szic *et al.,* 2014). Withaferin A has antibacterial, anti-tumor, anti-inflammatory and immune-modulatory properties(Ciddi, 2006).

The review of the works done on Ashwagandha is presented in this chapter under the following sub-headings:

2.1. *Withania somnifera*

2.2. Pharmacological properties of *Withania somnifera*

2.3. Chemical constituents present in *Withania somnifera*

2.4.Tissue culture of *Withania somnifera*

2.5. Effect of Elicitors

2.6. Gas Chromatography –Mass Spectrometry (GC-MS)

2.7. UV-Visible Spectrophometery (UV-VIS)

2.8. High Performance Thin layer Chromatography (HPTLC)

2.1.*Withania somnifera*

W. somnifera(L.) Dunal commonly known as “Ashwagandha”, “Asgandh” and “Winter Cherry” belongsto family Solanaceaeand widely distributed in warmer parts of the world. It is a small, woody shrub grows about two feet height. It mainly grows in Africa, the Mediterranean, and India. An erect, evergreen, tomentose shrub, 30-150 cm high, found throughout the drier parts of India. Roots are stout fleshy, whitish brown; leaves are simple ovate, glabrous; flowers are inconspicuous, greenish or lubrid-yellow, in axillary, umbellate cymes; berries small, globose, orange-red when mature, enclosed in the persistent calyx; seeds yellow, reniform. The roots are the main portions of the plant mainly used as therapeutically. The bright red fruit is harvested in the late fall and seeds are dried for planting. Whole plant, roots, leaves, stem, green berries, fruits, seeds, bark are used.

Figure 2.1 *Wthania somnifera* plant

Taxonomical Classification

Kingdom : Plantae

Subkingdm : Trcheobionta

Superdivision : Spermatophyta

Division : Angiosperms

Class : Dicotiledoneae

Order : Tubiflorae

Family: Solanaceae

Genus : *Withania*

Species : *somnifera*

(Singh *et al.,*2011).

Table 2.1BOTANICAL DESCRIPTION OF *W.somnifera*(Jain *et al.*, 2012)

	Description	***Withania somnifera*(L.) Dunal**
1.	Habit	Undershrub
2.	English Name	Winter Cherry, Indian Ginseng
3.	Vernacular Name	Ashwagandha
4.	Leaves	Alternate, broadly ovate, subacute,entire margins
5.	Inflorescence	Axillary, umbellate cymes
6.	Flowers	Monoecious
7.	Calyx	Accrescent, gamosepalous with5 sepals
8.	Corolla	Campanulate, greenish-yellowith 5 petals
9.	Androecium	Anthers 1.2 mm long, broadlyovate
10.	Gynoecium	Ovary ovoid/globose, glabrous
11.	Style	Filiform
12.	Stigma	Mushroom-shaped, 2-lamellate
13.	Fruit (Berry)	Globose, enclosed in thepersistent calyx, seeds yellow,reniform
14.	Seeds	Globose, enclosed in thepersistent calyx, seeds yellow,reniform
15.	Flowering	Throughout the year

It is one of the most valuable plants of the traditional Indian systems of medicines, is used in more than 100 formulations of ayurveda, unani and sidha and is therapeutically equivalent to ginseng (Seangwan *et al.*, 2009) biologically active chemical constituents present in *W.somnifera* are alkaloids (isopellertierine, anferine), steroidal lactones (withanolides, withaferins), saponins containing an additional acyl group (sitoindoside vii and viii), and withanoloides with a glucose at carbon 27 (sitonidoside XI and X).

The roots of *Withania somnifera*consist of primarily compounds known as withanolides, which has many medicinal properties. Withanolides are steroidal

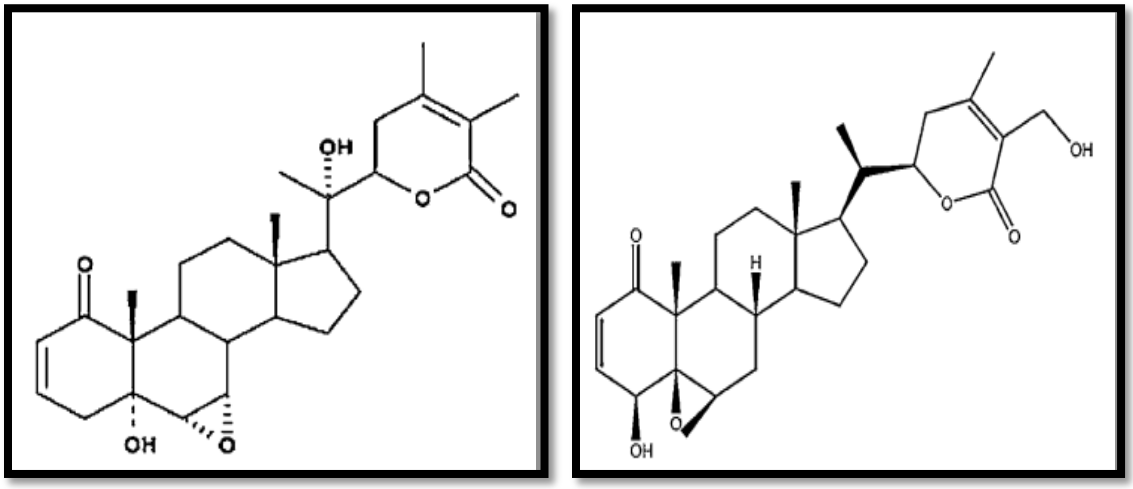
compounds and bear resemblance, both in action and appearance to the active ginsenosides of Asian Ginseng. Studies show that the plant has been used as an antioxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent, anti-tumour, astringent and more recently to treat ulcers, bacterial infection, venom toxins and senile dementia. The various withanolide steroidal lactones have been isolated from the leaves of *W. somnifera*(Glotter *et al.*, 2004)

The leaf of *W.somnifera* consists of steroidal lactones, which are called Withanolides. Much of *Ashwaganda's* pharmacological activity has been attributed to two main Withanolides, Withaferin A and Withanolide A. The Withanolides are a group of naturally occurring C28- steroidal lactones 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. The basic structure **(Figure 2.2.A)** is designated as the Withanolide skeleton.

Supe *et al*., (2011) reported that Withaferin A **(Figure 2.2.B)** is the most important of the Withanolides isolated from *Withania somnifera*to which the curative properties of the leaves are attributed. It has been receiving a good deal of attention because of its antibiotic and antitumour activities. Roots of *Withania somnifera*contains four steroidal lactones, called Withanolides, viz Withaferin A 5, 20a (R)-dihydroxy- 6a, 7a-epoxy-1 - oxo- (5a)-witha-2,24 - dienolide (m.p. 282-84 °C) and two minor Withanolides, of which one is probably 5a, 17a-dihydroxy-1-oxo-6a, 7a-epoxy-22R-witha-2, 24-dienolide (the so-called Withanone).

Figure 2.2.Structure of major withanolides

A.Withanolide A **B.Withaferin A**



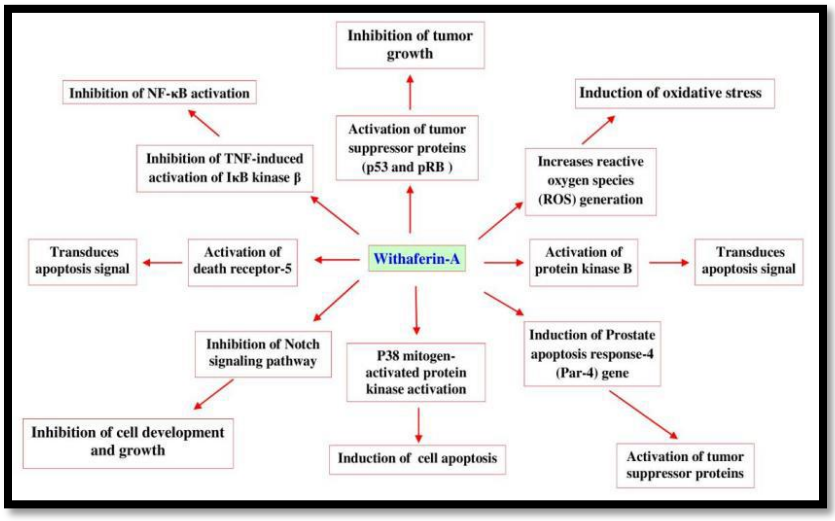
2.2.Pharmaceutical properties of *Withania* species

Withania somnifera, a commonly used herb in Ayurvedic medicine. Although the review articles on this plant are already published, those studies indicate Ashwagandha possesses antioxidant, anxiolytic, adaptogen, memory enhancing, anti-parkinsonian, antivenom, anti-inflammatory, anti-tumor properties. Various other effects like immune modulation, hypolipidemic, antibacterial; Antimicrobial Activity, Anticancer Activity, tolerance and dependence have also been studied. The results of studies conducted by Gupta and Rana (2011) are very encouraging and indicate this herb should be studied more extensively to confirm these results and reveal other potential therapeutic effects.

2.2.1a.Anticancer Activity

Ashwagandha is reported to have anticancer properties. Research on animal cellcultures has shown that the herb decreases the levels of the nuclear factor kappa B, suppresses the intercellular tumor necrosis factor, and potentiates apoptotic signalling in cancerous cell lines. One of the most exciting of the possible uses of Ashwagandha is its capacity to fight cancers by reducing tumor size. In one study, the herb was evaluated for its anti-tumor effect in urethane-induced lung tumors in adult male mice.Withanolides are highly oxygenated bioactive constituents which are responsible for ashwagandha's biological properties including antitumor activity (Mulabagalet al., 2011; Patel et al., 2013). *W.somnifera* enriched Withaferin-A induces apoptosis through mechanisms **(Figure. 2.3)** such as, inhibiting the activation of nuclear factor kappa-B (NF-κB) by preventing the TNF-induced activation of IκB kinase β via a thioalkylation sensitive redox mechanism (Oh and Kwon, 2009), activation of tumor suppressor proteins such as p53 and pRB (Wadhwa *et al*., 2013).

Figure.2.3. Withaferin-A induced cell apoptosis mechanisms(Kumar *et al.,* 2015)



2.2.1b.Anti-inflammatory Activity

Ashwagandha acts as an anti-inflammatory agent through the inhibition of complement, lymphocyte proliferation, and delayed-type hypersensitivity (Rasool & Varalakshmi, 2006). The extracts of *W. somnifera*show the anti-inflammatory effects in a variety of rheumatological conditions (Anbalagan *et al.,* 2001; Al-Hindawi *et al.,* 1999). The extract was found to decrease in the glycosaminoglycans content in the granuloma tissue by almost 100 percent and uncoupled with oxidative phosphorylation by significantly reducing the ADP/O ratio in mitochondria of granuloma tissue and increase in the Mg2+ dependent-ATPase enzyme activity and subsequent reduction in succinate dehydrogenase activity in the mitochondria of the granuloma tissue (Begum*et al.,* 2000).

2.2.1c.Antimicrobial activity

Antifungal and antibacterial properties have been demonstrated in the withanolides isolated from the ethanolic extract of the whole plant and leaves, respectively. The methanolic extract possessed maximum inhibitory activity against a spectrum of bacterial. It was also found that in crude extract of leaves of *W. somnifera*was tested against clinical pathogens *Staphylococcus aureus, Roteus mirabilis, Streptococcus mutans, Streptococcus sobrinus* and *Salmonella paratyphi* B, in 100 µl of extracts (100 mg/ml) which was able to inhibit the growth of all the pathogenic bacteria (Pandit *et al*., 2013).

2.2.1d.Antioxidant effects

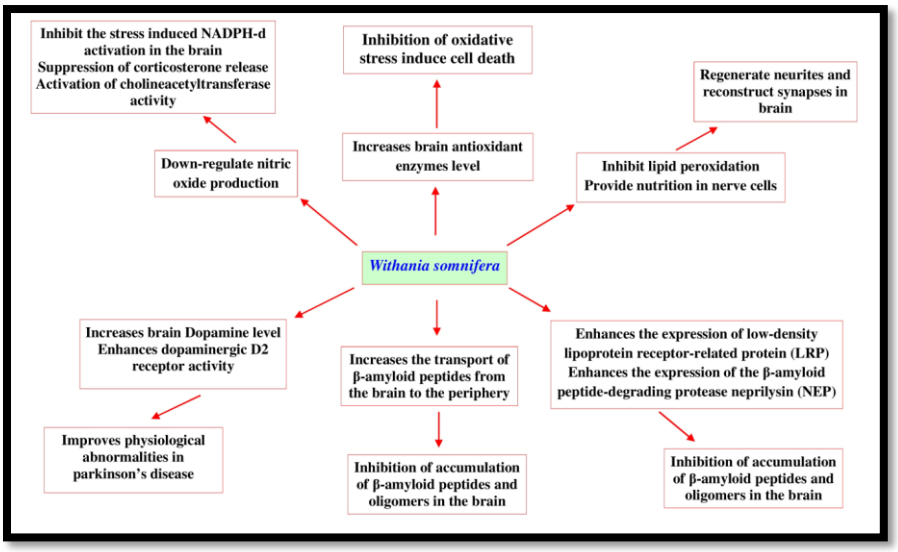
Free radical damage of nervous tissue may be responsible for neural loss in cerebral ischemia and may be involved in aging and neurodegenerative diseases, e.g., epilepsy, schizophrenia, Parkinson's, Alzheimer's and other diseases (Jesberger &Richardson 2005, Sehgal *et al.,* 2012). The active principles of *W. somnifera*, sitoindosidesVII-X and Withaferin A (glycol-Withanolides), are reported to increase levels of endogenous Superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GPX), and Ascorbic acid, with a concomitant decrease in lipid peroxidation (Dhuley, 1998; Bhattacharya, 2001; Jayaprakasam *et al.,* 2004; Bhatnagar, 2005; Mirjalali *et al.,* 2009).

2.2.1e.Neuroprotective activity

Neuroprotective activity of *W.somnifera* root extract could be because of presence of glycol-withanolides and their ability to inhibit lipid peroxidation. Phenolic compounds present in the root of *W. somnifera*play an important role on overall antioxidant activities of the plant (Bhatnagar et al.,2009). Different mechanisms of neuroprotective activity of

*W. somnifera*and its bioactive Withanolides are shown in the **(Fig.2.4).** *W.somnifera* root extract down-regulates nitric oxide production and induced NADPH-diaphorase activation in the brain via suppressing corticosterone release and activating the cholineacetyltransferase. Withanolide-A inhibits brain acetyl cholinesterase, which could be a therapeutic alternative for the treatment of neurodegenerative Alzheimer's disease (Grover et al., 2012).

Figure.2.4.Neuroprotective mechanisms of*Withania somnifera*.
(Vikas Kumar et al., 2015)



2.2.1f.Hepatoprotective Activity

Withaferin A at 10mg/kg dose showed significantly protective effect against CCl4-induced hepatotoxicity in rats. It was as effective as hydrocortisone dose for dose (Uddin *et al*., 2012).

2.2.1g. Anti-Aging

A significant improvement in hemoglobin, red blood cell count, hair melanin, and seated stature was observed. On treatment with the plant extract Serum cholesterol levels got declined and nail calcium was preserved. Erythrocyte sedimentation rate also decreased significantly on consumption of *W.somnifera* drugs **(**Verma and Kumar 2011).

2.2.1h.Anti-arthritic Activity

Ashwagandha powder has been found useful in acute rheumatoid arthritis and reduces the discomfort associated with arthritis (Bector *et al.,* 1968). This property has been attributed to the active principle *withaferin A.*

2.2.1i.Antistress activity

Bhattacharya (1987; 2000 & 2003) reported that Anti-stress activity associated with glycosides (sitoindosides VII and VIII) present in the plant of *W.somnifera*.. Ashwagandha is also used as a tonic for the treatment of spermatopathia and seminal depletion (Nadkarni, 1954) and the men who used the herb enjoyed higher vigour performance (Boone, 1998). The higher concentrations of inorganic elements like Fe, Mg, K and Ni in the roots of this plant play a significant role in the diuretic and aphrodisiac activity of the drug (Lohar *et al.,* 1992).

2.3.Chemical constitutents present in*Withania somnifera*

*Withania somnifera*is alsorich in iron. The roots of *Withania somnifera*consist primarily of compounds known as withanolides which are believed to account for its extraordinary medicinal properties. Apart from this they also contain chemical constitutent like withaniol, acylsteryl glucosides, starch, reducing sugar (Rani *et al* 2012,Sharma *et al.,* 2011).Madina *et al* (2007) stated that a novel 27β-hydroxy glucosyl transferase was purified from cytosolic fraction of *W. somnifera*leaves and studied for its biochemical and kinetic properties. The purified enzyme showed activity with UDP-glucose but not with UDP-galactose as sugar donor. It exhibited broad sterol specificity by glucosylating a variety of withanolides with β-OH group at C-17, C-21 and C-27 positions. It transferred glucose to the alkanol at C-25 position of the lactone ring, provided a -OH was present at C-17 in the sterol skeleton.

2.4.TISSUE CULTURE OF *Withania somnifera*

In conventional cultivation of many plants do not germinate, flower and produce seed under certain climatic conditions or have a long period of growth and multiplication. Micropropagation insures a good regular supply of medicinal plants, using minimum space and time (Prakash and Staden, 2007).Micropropagation is the process of vegetative growth and multiplication from tissues or seeds. It is carried out in aseptic and favourable conditions on growth media using various plant tissue culture techniques (Zhou and Wu, 2006). Many applications includes

1. Micropropagation using meristem and shoot culture to producelarge number of identical individuals.
2. Large scale growth of plant cells in liquid culture as a source of secondary products.

3. Removalof viruses by propagation from meristematic tissues.
4. Screening programmers of cells rather than plants for advantageous characters.

Suspension culture are formed *in vitro* when friable callus 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 (Fowler *et al*., 1993).

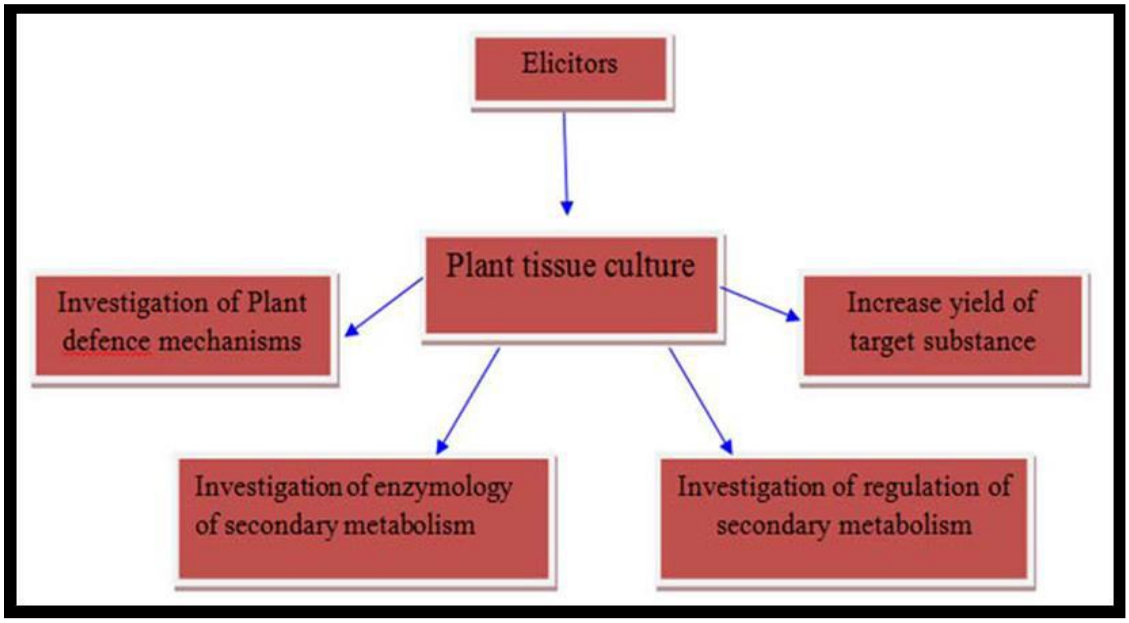
Suthar *et al.,* (2010) also reported *in vitro* cultured shoots of *B.serrata* showed maximum shoot length in MS liquid medium supplemented with 0.5 BAP with 0.05mg/L NAA, such shoots also produced highest number of leaves per clusters and increase in biomass was also observed.

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.

2.4.ELICITOR

"Elicitor is a scientifically described term for stress factors that directly or indirectly triggers the inducible defense changes in a plant system that results in an activation of array of protection mechanisms, including induction or expansion of biosynthesis of fine chemicals which do have a major role in the adaptation of plants to the stressful environment (*Goel et al*., 2011).In vitro culture, a wide variety of elicitors has been employed in order to modify cell metabolism. These modifications are designed to enhance the productivity of useful metabolites in the culturesof the plantcellsortissues. The cultivation period is particular, can be reduced by the application of elicitors, although maintaining high concentrations of product Rao, Ravishankar et al., (2002).

Figure 2.5 The employment of elicitor in various fields of plant tissue culture (Veersham, 2004)



2.4.2. Classification of Elicitors

Elicitors can be divided into two types on the basis of nature, biotic and abiotic. Biotic elicitors are the substances of biological origin, which includes polysaccharides originated from plant cell walls (chitin, pectin, cellulose, etc.) and micro-organisms. Abiotic elicitors consist of the substances that are of non-biological origin and are grouped into physical, chemical and hormonal factor(Poornananda *et al*., (2015).Further on the basis of plant elicitor interaction it may be classified into race specific and general elicitors [Staskawicz*et al*.,1999].

2.4.3.Abiotic Elicitors

Various types of plant hormones which act as Elicitors. The most common plant hormones like Salicyclic acid (SA) and Jasmonic acid (JA) are key signals for defense gene expression (Patel and Krishnamurthy, 2014). (Sivanandhan *et al*.,2012).

Salicyclic Acid with transgenic technology are highly enhanced the production of tanshinones in *S.miltiorrhiza* hairy roots. The higher production of withanolide A, withanone, and withaferin A was reported in the elicited–hairy roots of *W. somnifera*. It stimulated the production of alkaloids such as vincristine and vinblastine in periwinkle, the tropane alkaloid scopolamine in hairy root cultures of *Brugmansia candida*, and

pilocarpine in jaborandi leaves. Anthraquinone production was greatly increased in *Rubia cordifolia* after a SA treatment. SA also affects terpenoid secondary metabolism in plants. It induced accumulation of the triterpenoids ginsenosides in ginseng and glycyrrhizin in licorice (Siva *et al*., 2014, Naik and Al–Khayri, 2016).

Jasmonic Acid mainly constitute an important class of elicitors for many plant secondary metabolic pathways, which are typically manifested by the elicitation of secondary metabolite biosynthesis when plants face particular environmental stresses(PauwelsL *et al.,* 2009). Jasmonic Acid is an important signal molecule of plant in response to wound and pathogen attack (Wasternack C *et al.,* 2000). Jasmonic Acid can induce the production of a wide range of plant secondary metabolites such as rosmarinic acid, terpenoid indole alkaloid in various cell cultures(Krzyzanowska *et al.,*2012)

Silja *et al.,*2014).Jasmonic Acid elicited the production of Withanolide A, Withanone A, Withaferin A in the hairy root cultures of *W.somnifera*

2.4.4.BioticElicitors

Estrada *et al*., (2016) described that the application of biotic elicitors, that is, those of biological origin, in plant cell cultures constitutes an excellent system to enhance the production of secondary compounds with phytoalexinic properties, as well as to obtain more insight into the regulation of their biosynthetic pathways. Exogenous biotic elicitors include compounds released by microorganisms and other pathogens, or formed by the action of plant enzymes on microbial cell walls, e.g., microbial enzymes, fungal and bacterial lysates, yeast extracts, and polysaccharides from microorganism cell walls (e.g., chitin and glucanes).

2.6.GC-MS ANALYSIS

Gas Chromatography involves the principle of absorption and partition,it is important tools for separation of volatile components. It has gained widespread acceptance in numerous application areas, such as process control in chemical plants, quality control in the food industry, monitoring sample composition in the oil-industry, environmental and bio medical sciences. These are just a few examples in which gas chromatography has been applied. The combination of speed, sensitivity and a high resolving power in gas chromatography provides a very adequate technique for the

separation of complex samples. The coupling to spectrometric methods such as mass spectrometry (MS) for direct identification of unknown compounds is easy to establish.

Using GC-MS, the linear retention indices for all the compounds were determined by co-injection of the sample. The individual constituents were identified by their identical retention indices, referring to known compounds from literature (Adam, 1995) and also by comparing their mass spectra with either the known compounds or with Wiley mass spectral database (Rehana *et al*., 2016) or using NIST library. Medicinal plants are directly analysed their existing metabolic compounds by GC-MS technique. It mainly used for determination of thermochemical constants,for their purification of compounds and for their qualitative and quantitative analysis of mixtures.(sivakumar *et al*., 2011).

GC-MS also used for separation and analysis of multi component mixtures such as essential oils, hydrocarbons and solvents. Flame ionization detector and the Electron capture detector are high sensitivities gas chromatography can quantitatively determine materials present at very low concentrations. It follows, that the second most important application area is in pollution studies, forensic work and general trace analysis. Because of its simplicity, sensitivity, and effectiveness in separating components of mixtures, gas chromatography is one of the most important tools in chemistry. It is widely used for quantitative and qualitative analysis of mixtures, for the purification of compounds, and for the determination of such thermo chemical constants as heats of solution and vaporization, vapor pressure, and activity coefficients (Sermakkani *et al*., 2012).

2.5. High Performance Thin layer Chromatography (HPTLC)

HPTLC is used as reliable method for analysing several samples of divergent nature and composition at the same time (Sripathi *et al.*, 2011). High Performance Thin Layer Chromatography is one of the modern sophisticated techniques that can be used for wide diverse applications. It is a simple and powerful tool for high-resolution chromatography and trace quantitative analysis is made possible. It is most widely used for quick and easy determination of quality, authenticity and purity of the crude drugs and market formulations (Mamatha, 2011).

A Key feature of HPTLC includes,

- Simultaneous processing of sample and standard - better analytical precision and accuracy and less need for Internal Standard.
- Several analysts work simultaneously.

- Lower analysis time and less cost per analysis.
- Low maintenance cost.
- Simple sample preparation - handle samples of divergent nature.
- No prior treatment for solvents like filtration and degassing.
- Low mobile phase consumption per sample.
- No interference from previous analysis - fresh stationary and mobile phases for each analysis - no contamination.
- Visual detection possible - open system (Andola and Purohit, 2010).

Pankajavalli *et al*., (2014) quantified both Withanolide A and Withaferin A present in *in vitro* root cultures of *W. somnifera*and concluded that Withanolide A to be the dominant metabolite in *in vitro* root cultures.

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3.0. Materials and Methods

The various methods followed to conduct the present study entitled **"Influence of Iron and Copper on *in vitro* cultures of*Withania somnifera*"** are described below:

3.1. Materials

3.1.1. Plant material

3.1.1a. *In vitro* leaf cultures of *Withania somnifera*

3.1.2b. Chemicals

3.2. Methods

3.2.1. Culture and maintenance of *in vitro* tissues

3.2.2. Media preparation

3.2.3. Inoculation of the explants

3.2.4. Micronutrient manipulation in culture

3.2.5. Determination of withaferin A compound by GC-MS analysis

3.2.5a.Preperation of sample extract

3.2.5b.GC-MS analysis

3.2.6. Spectral analysis by UV-Visible Spectrophotometery

3.2.7. Quantitative estimation of WithaferinA compound

3.2.7a. Preparation of extracts

3.2.7b.preparation of standard

3.2.7b.Characterization of Withaferin A using HPTLC

3.1. Materials

3.1.1. Plant material

3.1.1a. *In vitro*shoot cultures of *Withania somnifera*

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

3.1.2b. 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 Chromodex,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).

3.2. Methods

3.2.1. Culture and maintenance of *in vitro* tissues

Seeds of *W. somnifera* (L.) were germinated on *in vitro*solid basal medium supplemented with 2% sucrose in the dark at 25˚C. Shoots from *in vitro*-germinated seedlings were maintained on MS basal medium under standard culture conditions. Shoots were multiplied on MS medium supplemented with regular subcultures once for every 15 days. Multiplied shoot branches were transferred to liquid MS basal medium with 1 mg/L BAP and 3% sucrose. For mass culture of adventitious shoots, the shoot branches from *in vitro*-induced adventitious shoots were transferred to liquid MS basal medium in a bubble column bioreactor (Biopia, Korea). The*in vitro* grown shoot samples were harvested after 8’16days of culture and secondary metabolites were analysed for the present study

3.2.2. Media preparation

Full strength of 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 5.8. 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.

3.2.3. Inoculation of the explants

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.

3.2.4.Micronutrient manipulation in culture medium

The various micronutrients like copper and ferrous EDTA used in MS basal medium were varied individually in the primary culture medium as well as in the secondary culture medium to optimize the level of individual micronutrient for best response towards induction of shoot buds from the nodal segments, biomass content in the regenerated plants. Different levels of micronutrients wereused in the liquid media were Cu(25µm-100 µm),Fe (25µm-100 µm) with a positive control and negative control. The positive control medium was the routine MS medium supplemented with standardized hormone levels where as the negative control medium was MS medium prepared without the respective micronutrient.The young and mature leaf samples were collected respectively after 16 days.The collected leaf samples were used for HPTLC analysis and GC-MS analysis.

Metals are influenced to alter the production of secondary metabolites by changing the aspects of secondary metabolism [Nasim *et al., 2010*]. Metals have become one of the main abiotic stress agents for living organisms because of their increasing use in the developing fields like industry, agrotechnics, high bioaccumulation and toxicity (Cai *et al.,* 2013*)*. Metals like Ni, Ag, Fe and Co have been shown to elicit the production of secondary metabolites in a number of plants. Many kinds of heavy metal were also used

as elicitors to induce accumulations of bioactive compounds in Salvia miltiorrhiza, such as Co2+, Ag+, Cd2+, Cu2+, Ce3+, La, Mn2+ and Zn2+ (Yan *et al.,* 2006*,* Shim *et al.,* 2014)

*3.*2.5 UV-Vis Spectroscopy

Ultraviolet – visible Spectroscopy (UV-1601 pc shimadzu spectrophotometry) or ultraviolet- visible spectrophotometer (UV-Vis) refers to absorption spectroscopy in UV-Visible spectral region.

3.2.6. Determination of withaferin A compound by GC-MS analysis

3.2.6a.Preperation of sample extract

Different concentration of dried sample (20mg),young and mature leaves of *W.somnifera* grown in suspension media, were extracted with 1 ml of 70% methanol to analyze metabolites by GC-MS. The sample extracts were sonicated for 30 minutes and centrifuged for 5 minutes at 2,000 rpm. Then the supernatant was filtered using a 0.45µm filter (PTFE SySartorius Stedim Biotech, Gottingen, Germany).After extraction of sample, 100µl of each sample was poured into GC vial for derivatization prior to GC-MS analysis.

The sample solutions in the GC vials were dried with nitrogen flow for about 5 minutes. To the dried vials added about 30µl of methoxylamine hydrochloride in pyridine, 50µl of BSTFA containing 1% of TMCS and 10µl of 2-chloronaphthalene respectively. After derivation,the samples were incubated at 60º C for 60 minutes and subjected to GC-MS analysis.

3.2.6b.GC-MS analysis

The GC-MS analysis was performed using 7890A Agilent GC system (Agilent Technologies,CA,USA)equipped with a 5975C MSD detector (Agilent Technologies and automatic sampler(7683 B series, Agilent Technologies).Electro impact ionization mode with ionization energy of 70 eV was used for GC-MS Detection. Analytes were separated on a fused silica capillary column of 5% phenyl methylpolysiloxane phase with dimension of 30m x0.25 mm i.d x0.25 µm film thickness. Helium was used as carrier gas at constant flow rate of 1.0 ml\minutes. The inlet temperature was mainted at 250ºC with an injection volume of 1.0 µl and a split ratio of 1:10 .The mass range was 50-700 Da and data were obtained in MSD with a full scan mode. The oven temperature was set upto to 70ºC and programmed to increase to 150ºC(at 5ºC\min) and then to 250ºC (at 3ºC\min; hold 2 minutes)and then to 320ºC(at 10ºC\min; hold 3 minutes).

3.2.7. Quantitative estimation of withaferin A compound

3.2.7a. Preparation of extracts

The leaf tissues grown in suspension media in different concentration were shadow dried and powdered using a mortar and pestle. Amount of powder (1g) was used for extraction. The samples were extracted with 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.

3.2.7b.Preparation of Standard:

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

3.2.7c. 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 HPTLC 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 tank. It was 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 visualizer under Densitometric scanning was performed with Camag TLC scanner III at 254nm, 366nm and also in white light. 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.

4.0 RESULT AND DISCUSSION

The traditional medicines are increasingly solicited through the traditional practitioners and herbalists in the treatment of infectious diseases. Medicinal plants play a vital role for the development of new drugs. The bioactive extract should be standardized on the basis of active compounds (Mathur and Agrawal, 2013).

The genus Withania (Family: Solanaceae) is a highly acclaimed genus of medicinal plants in the Indian Ayurvedic system of medicine because of its valuable pharmaceutical and nutraceutical properties. It is a small group of herbs distributed from the Canary Islands, the Mediterranean region and Northern Africa to the South-west of Asia (Hepper, 2013).

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). Standardization of herbal formulations is an essential factor in order to access the quality, purity, safety and efficacy of drugs based on the concentration of its active principles. Standardization of the drug is based on its composition and physico-chemical characteristics of the raw material used secondary metabolites in the influence of metal on *in-vitro* growth characteristics and the changes in secondary metabolites in *Withania somnifera.*

Owing to the importance of standardization of medicinal plants using various techniques, the present study was focused on understanding the results are discussed in this chapter under the following headings:

4.1 Biomass production in *in-vitro* shoot culture.

4.2 Spectral studies of *in-vitro* shoot extracts.

4.2.1 UV-Visible Spectrophotometry.

4.2.2 Gas Chromatography/Mass Spectrometry.

4.3 Extraction of secondary metabolites.

4.4 Quantification of Withaferin A by HPTLC

4.1 Biomass production in *in-vitro* shoot culture.

W. somnifera(L.) Dunal synthesizes a large array of pharmacologically active secondary metabolites known as withanolides. It has been extensively investigated in

terms of chemistry and bioactivity profiling. Shoots being the major source of desired phytochemicals, their indiscriminate harvest leads to the extinction of the at a fast pace.

Globally, there has been ever-increasing demand for health products containing *Withania somnifera* as a sole component in combination with other medicinal plant extracts or as a general health product. However, the quality of these products and the level of phytochemical constituents are highly affected by genotype and environmental conditions. For product consistency under these conditions, *in vitro* shoot suspension (IVSS) cultures could provide a replacement to field-grown plants for the production of therapeutically valuable compounds.

Cell and organ culture are reported to pocessess the potential to produce secondary metabolites in *in-vitro* plant as they have an active growth and metabolism in the culture within a shorter period. Therefore, the development of a fast-growing shoot system would offer a distinctive opportunity for producing raw material for drugs. The present is focused on selection of a right harvesting of plant grown in *in vitro* cultures to obtain maximum bioactive principles.

4.1.1. *In vitro* biomass production

Multiple uses of the plant species has necessitated its large scale collection as raw material to the pharmaceutical industry, leading to over exploitation and disappearance of the wild species. The conventional propagation of these valuable plants is difficult therefore *in vitro* propagation methods have been developed by many researchers (Kitanov and Pashankov, 2011) as a substitute to field grown plants.

The shoots of *W. somnifera*were established in suspension and analyzed for the increase in biomass. This was done by culturing them in bottles or flasks. In the present study, shoots (explants) of *W. somnifera*cultivar Jawahar 20 were cultured in Murashige and Skoog (MS) liquid medium supplemented with 30 g/L sucrose and 1 BAP for the establishment of shoot cultures. The *in vitro* shoot suspension (IVSS) were maintained and harvested at different combination of heavy metal stress harvesting at 16 days after culture. The fresh weight of the shoots was noted and growth index was calculated (Wu *et al*., 2008) and tabulated in **Table 4.1.**

The shoots were observed for biomass accumulation and *withaferin A* production. Biomass was calculated using the formula,

Figure 4.1 Image of *in vitro* tissues at different levels of Copper and Iron salt

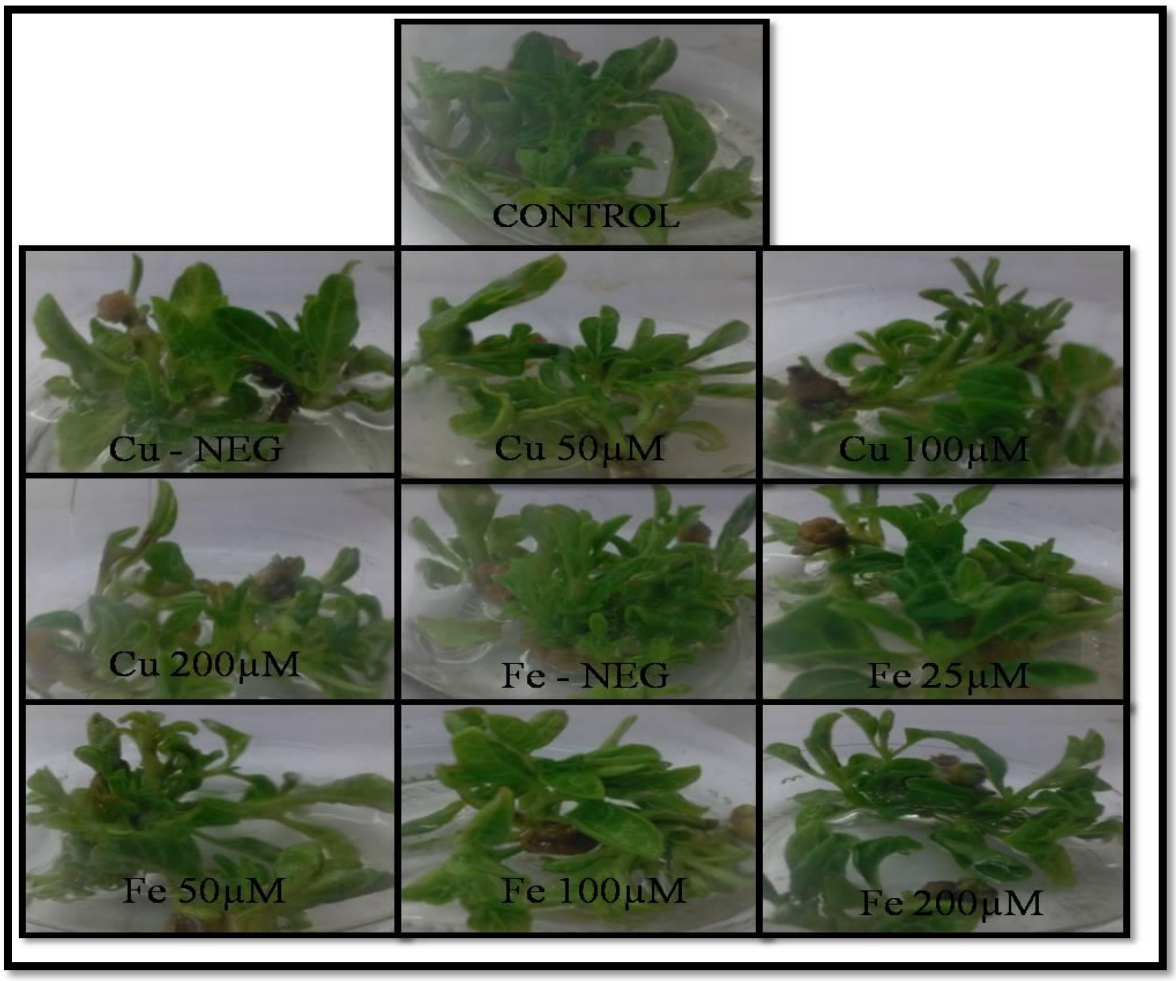


Table 4.1. Biomass of shoots in suspension culture

Samples	Biomass	Harvestedfresh weight (g/l)
C +ve	3.92	4.03±0.16
Cu -ve	2.94	3.39±0.63
Cu 50uM	3.51	3.76±0.36
Cu 100uM	3.11	3.64±0.75
Cu 200uM	2.05	2.91±1.22
Fe -ve	3.19	3.7±0.72
Fe 25mM	2.41	3.04±0.89
Fe 50mM	2.61	2.94±0.89
Fe 100mM	2.36	3.45±1.54
Fe 200mM	2.07	2.63±0.79

4.2.1: Ultraviolet - Visible Spectrophotometer Analysis:

The wavelength and amount of light that a compound absorbs depends on its molecular structure and the concentration of the compound used. The UV-vis spectra of different concentrations of Iron (Fe) and copper (Cu) treated *Withania somnifera* shoots were given in Figure 4.2 and 4.3.

In copper and iron stressed plant extracts, the peaks at 665nm and 465nm was showed an absorbance maxima within 2.0 and the absorbance maxima range is given in the Table 1, 2. Those peaks ware found to be Chlorophyll a. The peaks at 410nm and 440nm were Beta carotene.

The peaks for Chlorophyll b (approximately at 642nm, 453nm) was not found in UV-vis spectra from *W.somnifera* shoot samples. The reason may be, in plants the amount of chlorophyll b is lower than chlorophyll a in normal condition. And also the chlorophyll b is sensitive to salt stress (yasar akca*et al.,* 2012).

Figure: 4.2 UV-Visible Spectrum for Iron salts treated plants.

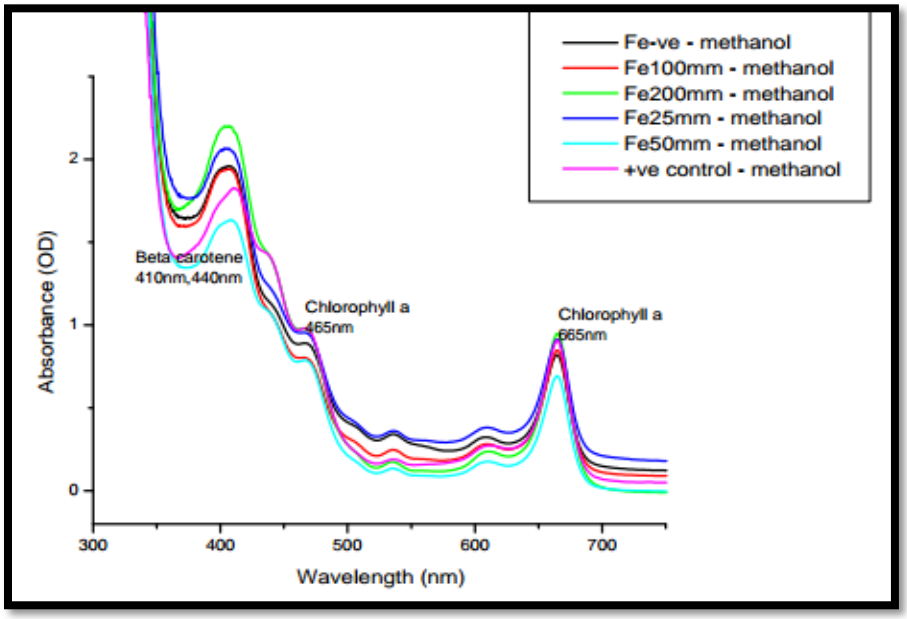
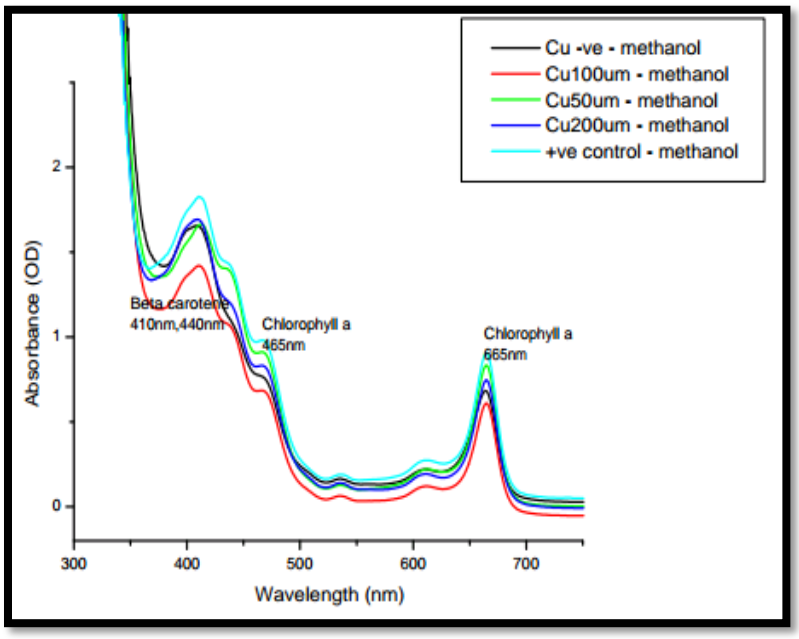


Figure 4.3 UV-Visible Spectrum for Copper salts treated plants.



The UV spectra obtained from shoot extracts of metal stressed *Withania somnifera* shown no difference in the peak area and the position of peaks were the same for all samples.Fourmajor peaks were identified at 410nm, 440nm, 465nm and 665nm. These peaks were identified as Beta carotene (at 410 and 440nm) and Chlorophyll a (at 465 and 665nm).

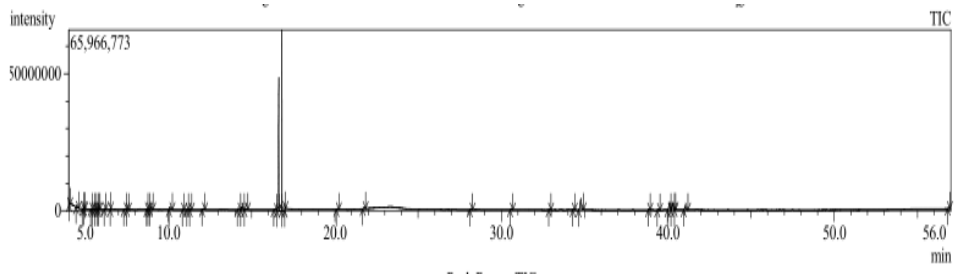
Hence, the amount and concentration of Flavonoids, Alkaloids, Pigments and other biochemical constituents of *Withania somnifera* was same during metal stressed condition. There was no new peak or components were identified, this indicates *Withania somnifera* metabolomics constituent level may tolerant to metal stress.

Gas chromatography / Mass Spectrometry:

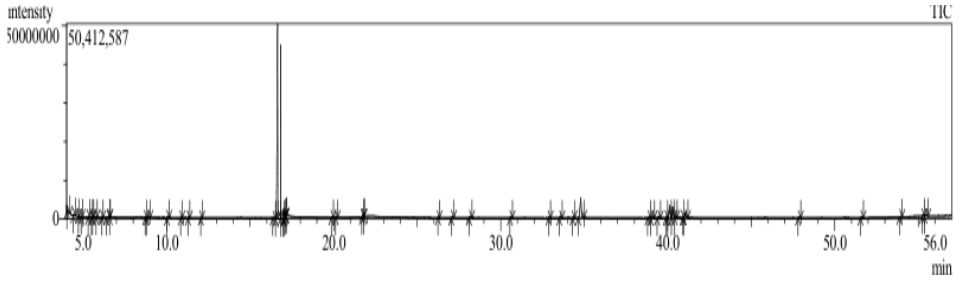
Gas Chromatography/Mass Spectrometry analysis separates chemical mixtures and identifies the components at molecular level. The methanolic extract of shoots of *Withania somnifera* stressed with metal salts (CuSO4 and FeSO4) was characterized qualitatively for the emission comparative screening test using GC-MS head space analysis. GC-MS spectra of the sample extract and their peaks and retention time (RT) in minutes are depicted in Figure. Table enumerates the compounds that were identified by head space GC/MS analysis in each of the 5 samples for copper (C+ ve, Cu –ve, Cu 50µM, Cu100µM, Cu 200µM) and 5 samples for Iron (C+ve, Fe –ve, Fe 50mM, Fe 100mM, Fe 200mM) under investigation. All the components of the sample extracts that had a matching retention time (RT) with 100% relative abundance.

Figure 4.4 Chromatogram for Iron salts stressed shoot extracts of *Withania somnifera*

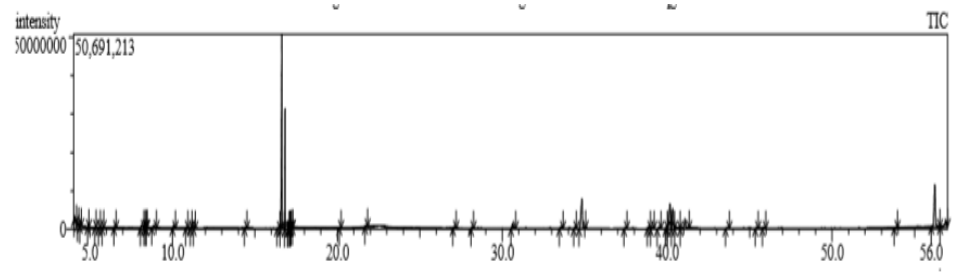
CONTROL



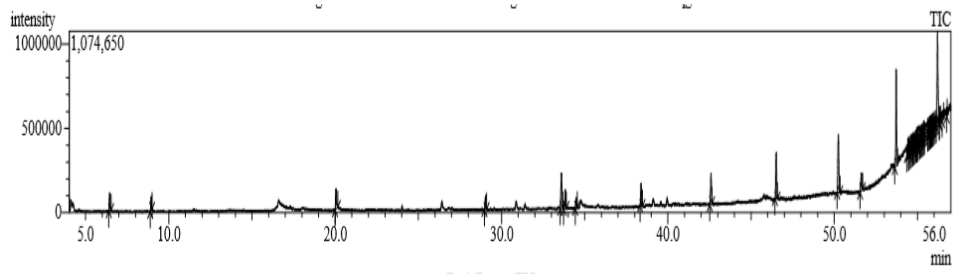
Cu - NEG



Cu 50µM



Cu 100 µM



Cu 200 µM

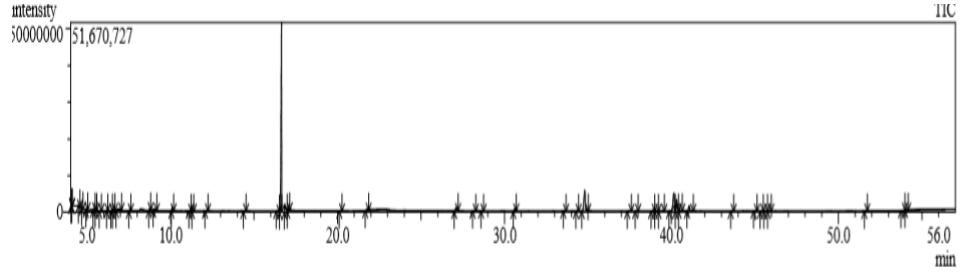


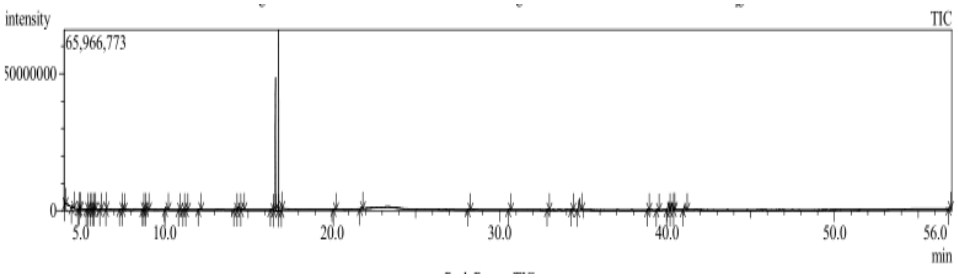
Table:4.2List of Compounds identified by GC MS in *in-vitro*cultures of *W. somnifera*

COMPOUND CLASSIFICATION	COMPOUND NAME	Various Metal ion concentration stress and compounds identified					
		C+ve	Cu -ve	Cu 50µM	Cu - 100µM	Cu - 200µM	RT
Sugar derivarives	Mannosamine			✓			8.44
	3,4-Anhydro-d-galactosan	✓				✓	11.153
Amide	Acetamide, N-ethyl-		✓	✓		✓	4.405
	Formamide, N,N-diethyl-		✓	✓		✓	5.314
Amino acid	DL-Proline, 5-oxo-, methyl ester	✓	✓	✓		✓	16.375
Nucleic acid	Thymine			✓			8.308
	Guanosine, 2'-O-methyl-				✓		56.485
Fatty acid	Dodecanoic acid	✓	✓	✓		✓	21.747
	Hexadecanoic acid, methyl ester	✓	✓	✓	✓	✓	33.593
	Tetradecanoic acid	✓	✓	✓		✓	28.182
	Palmitoleic acid					✓	43.632
	Octadecanoic acid		✓	✓		✓	41.057
	cis- 1,3-Eicosenoic acid			✓			43.628
	Oleic Acid					✓	37.448

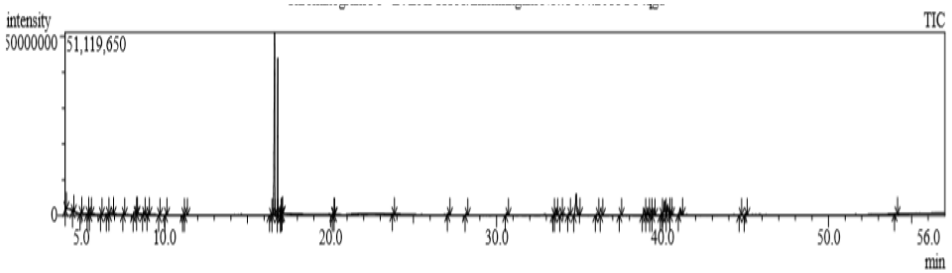
COMPOUND CLASSIFICATION	COMPOUND NAME	Various Metal ion concentration stress and compounds identified					
		C+ve	Cu -ve	Cu 50µM	Cu - 100µM	Cu - 200µM	RT
Diterpene	Phytol	✓	✓	✓		✓	39.497
Triterpene	Squalene			✓			53.846
Phenol	Phenol, 3,5-bis(1, 1-dimethylethyl)-					✓	20.163
	2,4-Di-tert-butylphenol		✓	✓			20.166
	Phenol, 2,3-dichloro-			✓			17.12
Organic compound	Imidazole, 2-bromo-4-methyl-5-nitro-				✓		55.286
Organic compound isomer of naphthalene	Azulene			✓		✓	11.326
Steroidal sapogenin	Pseduosarsasapogenin-5,20-dien			✓		✓	34.407
dibenzylbutyrolactol	Carissanol dimethyl ether			✓			56.226
Acid	Butanoic acid, 4-hydroxy-			✓		✓	4.894
Polyol compound	Glycerin					✓	6.919
Heterocyclic organic compound	Thiazolidine	✓					8.785

Figure 4.5 Chromatogram for Copper salts stressed shoot extracts of *Withania somnifera*

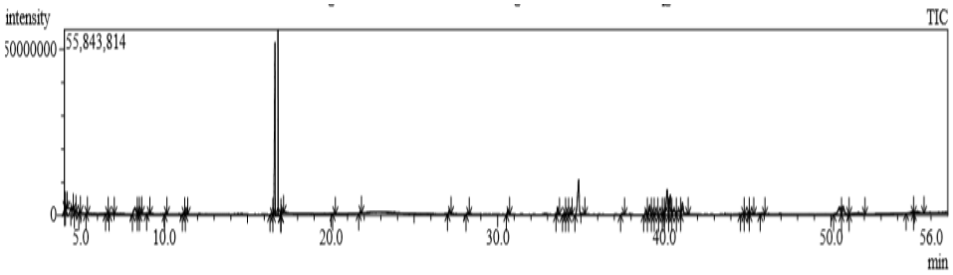
CONTROL



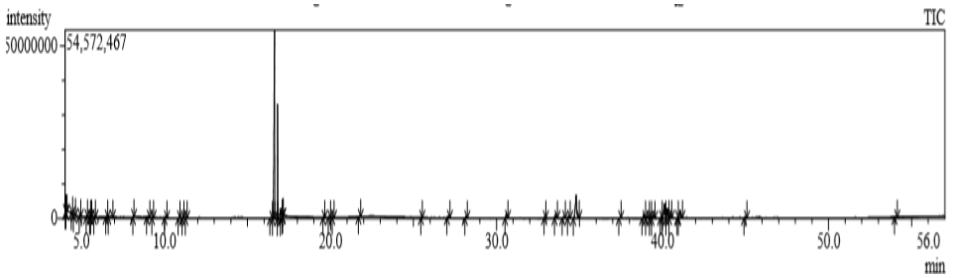
Fe – NEG



Fe 50mM



Fe 100mM



Fe 200 mM

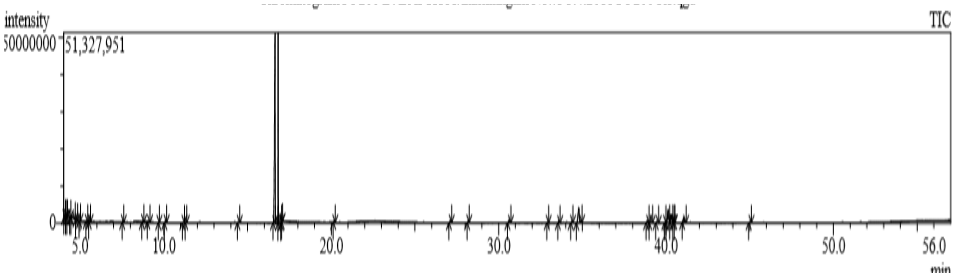


Table: 4.3 Compounds identified by GC MS in *in-vitro*cultures of *Withania somnifera*

COMPOUND CLASSIFICATION	COMPOUND NAME	Various Metal ion concentration stress and compounds identified					
		C+ ve	Fe- ve	Fe 50mM	Fe – 100mM	Fe - 200mM	RT
Sugar derivarives	3,4-Anhydro-d-galactosan	✓		✓		✓	11.153
Amide	Acetamide, N-ethyl-		✓	✓	✓	✓	4.405
	Formamide, N,N-diethyl-		✓	✓	✓	✓	5.314
Amino acid	DL-Proline, 5-oxo-, methyl ester	✓	✓	✓	✓		16.375
Nucleic acid	Thymine			✓			8.308
Fatty acid	Dodecanoic acid	✓		✓	✓		21.747
	Hexadecanoic acid, methyl ester	✓	✓	✓	✓	✓	33.593
	Tetradecanoic acid	✓	✓	✓	✓	✓	28.182
	Palmitoleic acid			✓	✓		43.632
	Octadecanoic acid		✓	✓	✓	✓	41.057
	Methyl stearate		✓	✓	✓	✓	39.947
	cis- 1, 3-Eicosenoic acid			✓			43.628
	Oleic Acid					✓	37.448
Diterpene	Phytol	✓	✓	✓	✓	✓	39.497
Phenol	Phenol, 3,5-bis(1, 1-dimethylethyl)-					✓	20.163
Phenol	Phenol, 2,3-dichloro-					✓	17.12
Organic compound, isomer of naphthalene	Azulene		✓				11.326

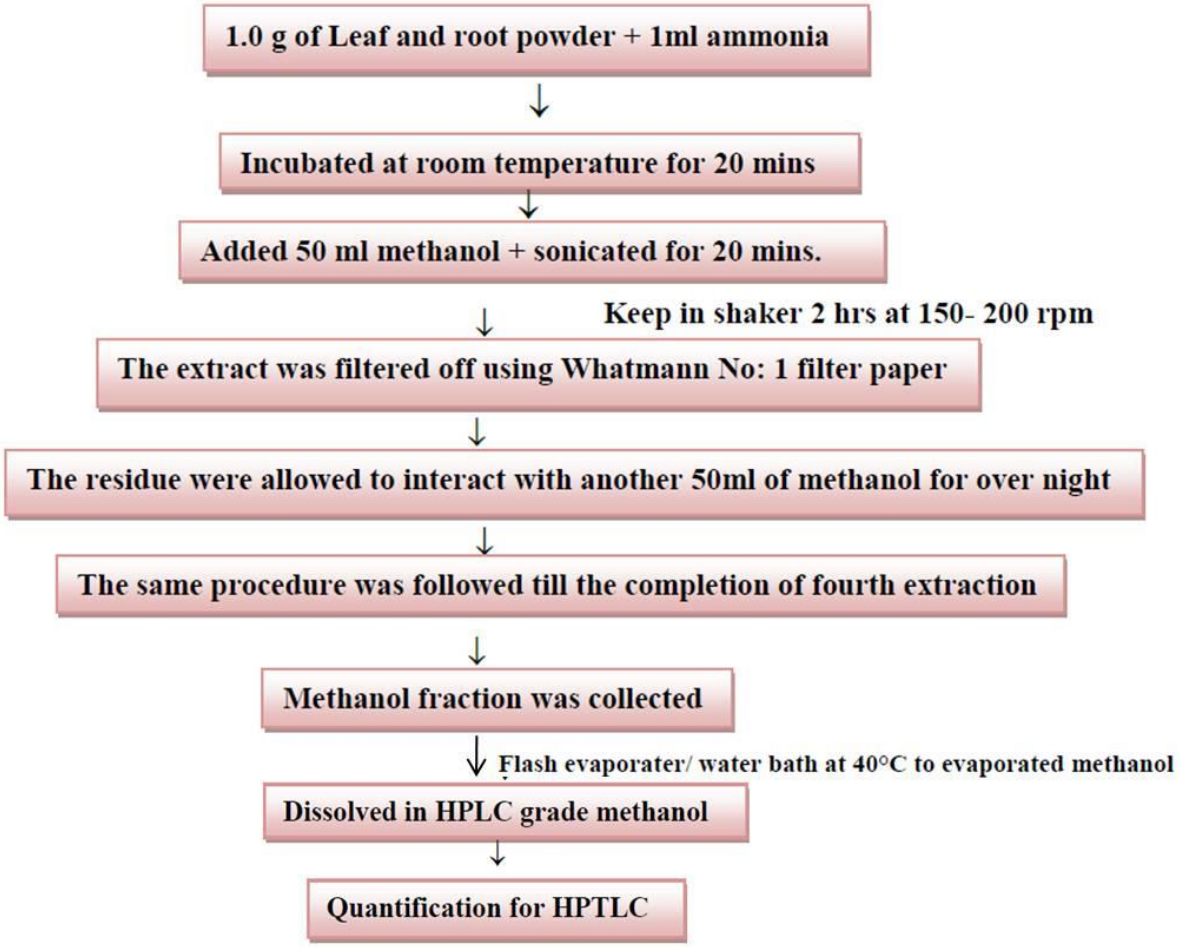
COMPOUND CLASSIFICATION	COMPOUND NAME	Various Metal ion concentration stress and compounds identified					
		C+ ve	Fe- ve	Fe 50mM	Fe – 100mM	Fe - 200mM	RT
Steroidal sapogenin	Pseduosarsasapogenin-5,20-dien			✓	✓		34.407
Dibenzylbutyrolactols	Carissanol dimethyl ether			✓			56.226
Acid	Butanoic acid, 4-hydroxy-		✓	✓			4.894
Polyol compound	Glycerin		✓	✓	✓		6.919
Heterocyclic organic compound	Thiazolidine	✓					8.785
Sesquiterpenoid alcohol	Guaiol		✓			✓	44.994
Phytosteroid	Digitoxigenin				✓		44.98

The GC-MS analysis has shown the presence of different phytochemical compounds in the methanolic extract of *Withania somnifera.* A total of 26 and 26 compounds were identified for Copper and Iron salts stressed plants. This analysis revealed that the methanol extract of *Withania somnifera*(shoots)contained mainly Fatty acids - Hexadecanoic acid, Octadecanoic acid, Dodecanoic acid and Tetradecanoic acid, Sugars – galactose derivatives and Aminoacids – Lysine, Valine and Proline.

The special metabolic components such as Di- and Tri-terpenes –phytol, Steroidal sapogenin and Squalene having anti-cancer, anti-oxidant, anti-tumor, chemo-preventive, pesticidal and sun-screen properties, Sesquiterpenoid- Guaiol having antibacterial and anti-tumor activity, Thiazolidine having anti-microbial activity, Naphthalene isomer – Azuelene, Phenols - 2,3-Dichloro phenol, 3,5-bis(1,1-dimethylethyl) phenol - Phenolics play important roles in plant development, particularly in lignin and pigment biosynthesis were also found.

4.3 Extraction of Secondary metabolites

In photochemical processing, extraction is an important step for the production of bioactive constituents from plant materials. The selection of a suitable extraction method is important for the standardization of herbal products, as it can be utilized to obtain the desire soluble constituents and for removing the materials which are not needed with the aid of solvents. Furthermore, the selection of suitable extraction process and optimization by various parameters are critical for up scaling the process. In the present study extraction was carried out using methanol as indicated in materials and method. Methanol was used as, it is a comparatively highly polar solvent and most of the compounds get extracted into it within a short period, and also the compounds are stable.



(Kalaiselvi S et al., 2017)

4.4 Quantification of Withaferin A by High Performance Thin Layer Liquid Chromatography

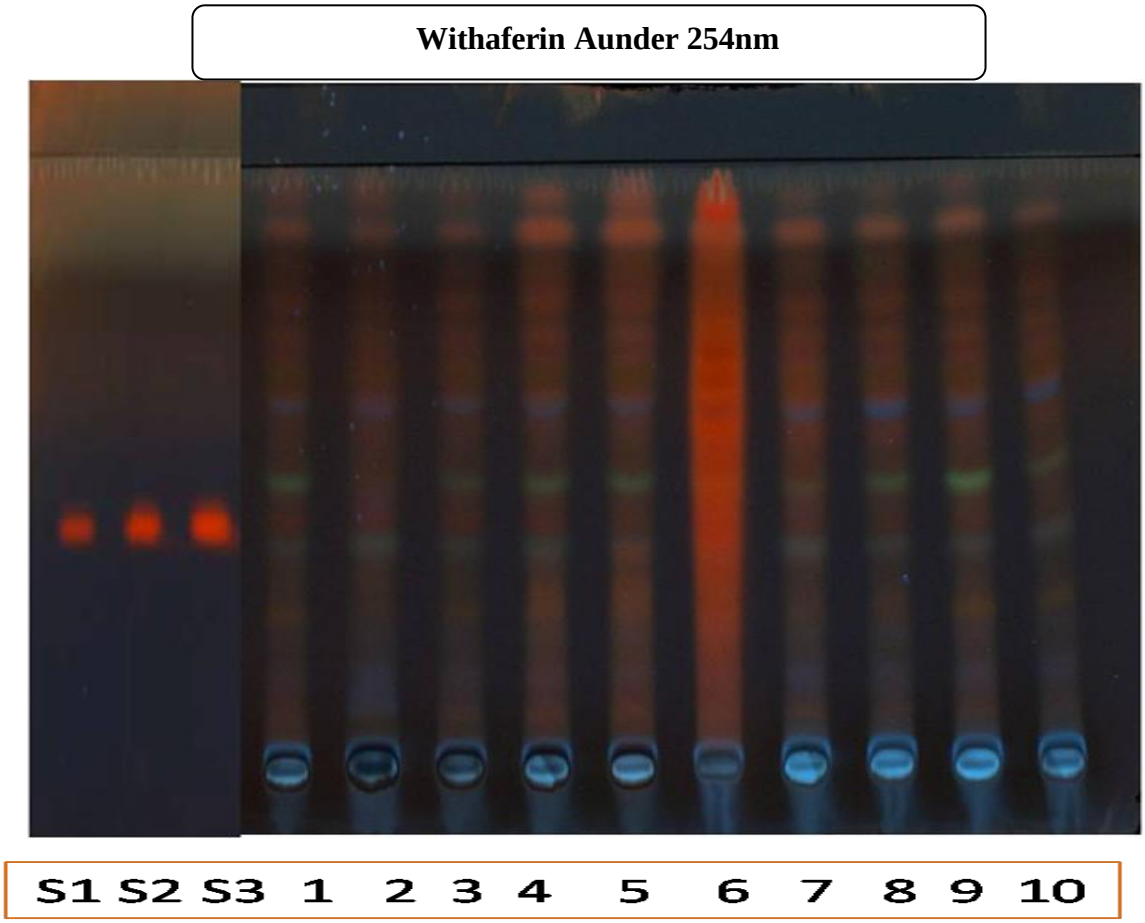
HPTLC has been used to provide excellent separation, qualitative and quantitative analysis of a wide range of compounds, such as herbal and botanical dietary supplements, neutraceutical, traditional western medicines, traditional Chinese medicines and Ayurvedic medicines and determination of radio labeled substances in chemical, biochemical, biological, pharmaceutical and medicinal samples. Various steps involved in research and development to bring any pharmaceutical substance or product to the market are supported by effective and efficient analysis and therefore effectual method development are of fundamental importance (Rashmin et al., 2012).

The result of HPTLC analysis was carried out in order to view secondary metabolite accumulation in the *in vitro* shoot extract of *Withania somnifera*. This was done on precoated silica gel aluminum plate $60F_{254}$. The Standard and different concentration of methanolic shoot extracts were applied to the plates as 8mm bands, under a stream of nitrogen, by means of CAMAG Linomat V semiautomatic sample applicator fitted with 100µl Hamilton HPTLC syringe. Linear ascending development to a distance of 6mm was carried out on twin trough chamber saturated the mobile phase, Toluene: Ethyl Acetate: Formic acid (5: 5: 1) as standardized earlier (Sharma et al.,2011).After run the plate were removed from the chamber and air dried and visualized at 254 and 366 nm. The plate was then subjected to scanning.It was performed with Camag TLC scanner III in the reflence absorbance mode Withaferin A at 223 nm according to the presence of secondary metabolite. Then, the plates were derivatized using concentrated sulfuric acid: methanol: glacial acetic acid: anisaldehyde in the ratio of 5: 85: 10: 5 and kept in hot air oven for 10 minutes at 110˚C, for the developed spots. Then the plate was visualized CAMAG TLC visualize and each compound was recorded at 366 and White R.

Withaferin A standard was visible as a clear spots with different RF values corresponding to each mobile phase used thus indicating the capillary of the solvents in the mobile phase to dissolve and carry the Withaferin A. The developed plates were then derivitized separately using anisaldehyde sulphuric acid. . The derivatized plates were viewed in a visualizer at 366nm was shown in Figure. Then plates were subjected to Densitometric scanning with CAMAG TLC scanner III using Savitsky – Golay 7 filter in the reflectance absorbance mode at 223nm at the speed of 200mm/s, the D2 and W

lamp was chosen to scan the plates and the slit dimensions were set at 4.00 X 0.30 mm, Micro. All the tracks were scanned and the peak was displayed. The Withaferin A peak was viewed as a separate spot with Rf values around 0.55-0.58 as obtained in the standardization of mobile phase. The peak analysis revealed the increase concentration of Withaferin A along the increase in the volume Fig 4.2

Figure 4.6. HPTLC fingerprint of various extracts under the influence of Mobile phase [Toluene:Ethyl acetate:Formic acid(5:5:1)]



S1-S3 = Standards

Lane 1 = W.somnifera in in vitro shoot

Lane 2 = W.somnifera in in vitro (without copper)

Lane 3 = W.somnifera in vitro shoot (Cu 50um)

Lane 4 = W.somnifera in vitro shoot (Cu 100um)

Lane 5 = W.somnifera in vitro shoot (Cu 200um)

Lane 6= W.somnifera in vitro shoot (without Iron)

Lane 7= W.somnifera in vitro shoot (Fe 25mM)

Lane 8= W.somnifera in vitro shoot (Fe 50mM)

Lane 9= W.somnifera in vitro shoot (Fe 100mM)

Lane 10= W.somnifera in vitro shoot (Fe 200mM)

Figure 4.73D View of Withaferin A at 223nm

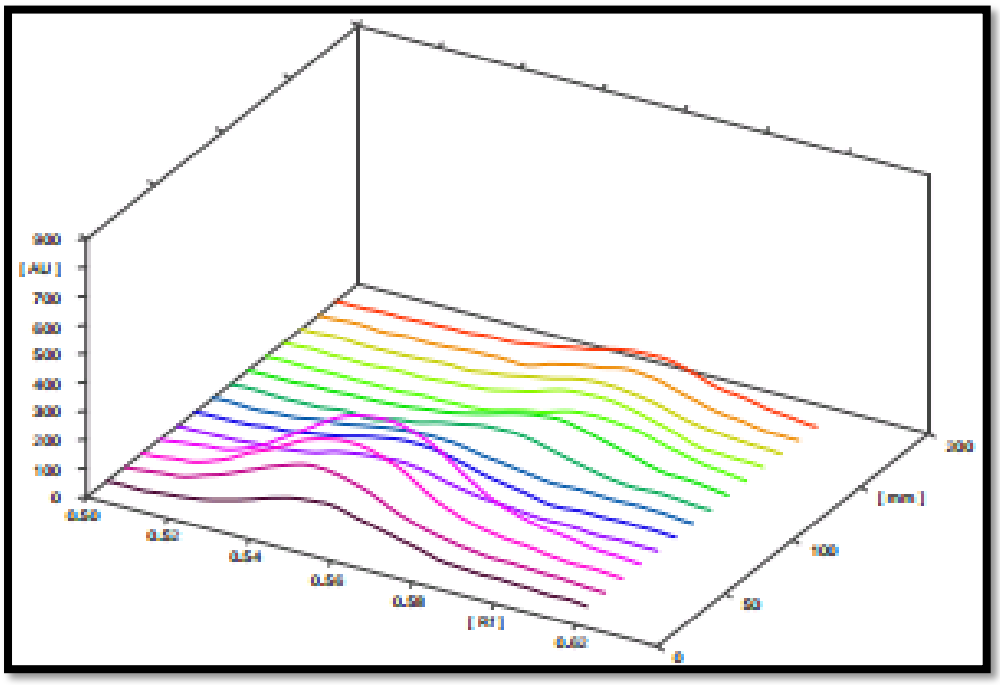


Table4.4 Peak area for the standard Withaferin A at 223nm

S.NO	STANDARDS		PEAK AREA
	Volume(µl)	Concentration (µg)	
1	5	5	3043.32
2	10	10	4618.17
3	15	15	6420.04

The amount of Withaferin A in each samples were quantified from the above peak areas in comparison with the peak area of the standards using the peak area as an evaluation mode and at multilevel calibration modes and a linear regression graph was obtained.

Figure.4.8 Linear regression graph for Withaferin A at 223nm

Regression r = 0.99981 Sdv = 0.79

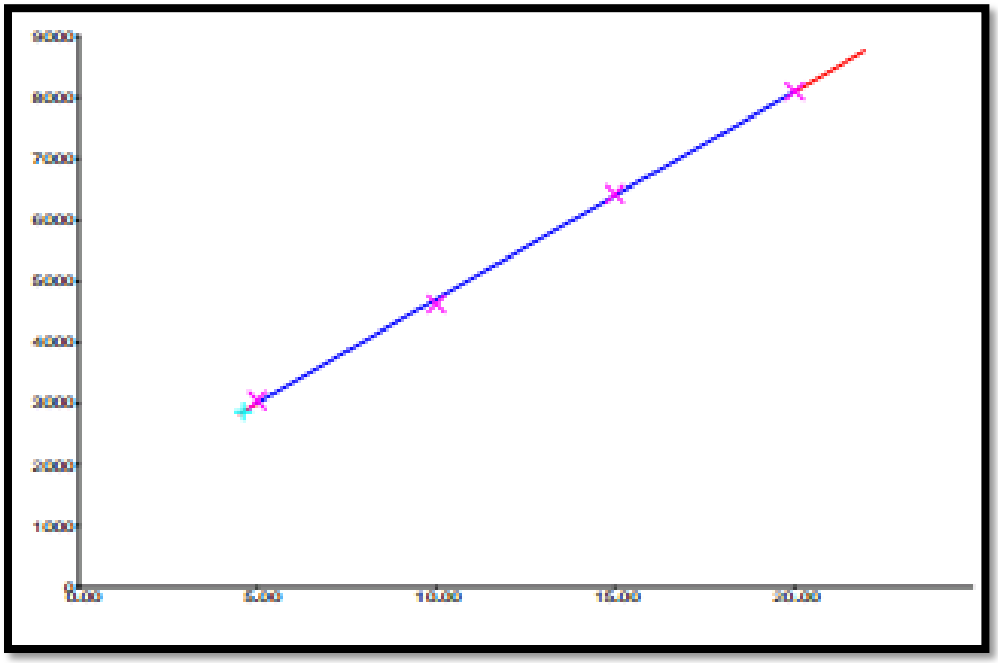


Table 4.5 Peak area and concentration of Withaferin A in shoot samples

S.NO	SAMPLE	Rf	PEAK AREA	Concentration per Gram tissue
1	CONTROL	0.62	255.94	2.8 mg
2	Cu - Neg	0.56	272.66	3.0 mg
3	Cu 50µM	0.57	516.29	5.6 mg
4	Cu 100µM	0.55	238.66	2.6 mg
5	Cu 200µM	0.50	172.53	1.8 mg
6	Fe - Neg	0.61	321	3.5 mg
7	Fe 25mM	0	0	-
8	Fe 50mM	0.54	147	1.6 mg
9	Fe 100mM	0.56	278.13	3.0 mg
10	Fe 200mM	0	0	-

The amount of Withaferin A in each samples is presented in table 4.5 respectively. The concentration of various secondary plant products are strongly dependent on the growing conditions (Kannan *et a l.,*2011) .The results revealed that higher amount of Withaferin A are present in Cu 50µM and Fe negative shoot extracts of *Withania somnifera* when compared to other samples.

Plant secondary metabolism is playing a crucial role in enhancing plants self protection ability and recording environmental information than primary metabolism (Martin *et al*., 2008). Extraction is the crucial first step in the analysis of medicinal plants because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. We have chosen methanol as extracting solvent since it was reported that remarkable observation of high yield of the extract can be obtained when methanol is used as an extracting solvent (Mohammedi and Atik, 2011). It was also reported that for the extraction of therapeutically desired active constituents, mostly methanol is used followed by ethanol, chloroform, and ethyl acetate. (Gupta *et al*.,2012). The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted. Different solvent systems are available to extract the bioactive compound from natural products.

The mobile phase used in the method gave good separation and resolution between peaks of Withaferin A standards and sample extracts. Preethi *et al*. (2014) have concluded that toluene : ethyl acetate: formic acid to be the best solvent system among the four solvent systems tested. Ray and Jha, (2001) showed that higher accumulation of Withaferin A was found to be maximum in shoots suspended in MS liquid medium supplement with BAP .

5.0 SUMMARY AND CONCLUSION

The results of the present study entitled **"Influence of zinc and manganese on *in-vitro* culture of *Withania somnifera*" are summarized as follows:**

- *Withania somnifera*shootswere cultured *in vitrounder the influence* two metal salts in four different concentrations.
- Secondary metabolites were extracted from all the samples using methanol as solvent.
- To establish the suspension culture, the shoots with apical node were subcultured into the suspension media supplemented with 4.44 µm BAP and allowed them to grow in orbital shaker.
- After one week of growth in suspension media the shoots were harvested fresh weight and dry weights were noted.
- The presence of Withaferin A in elicitor (metal salts) treated shoots were carried out using HPTLC.
- HPTLC analysis of samples showed good separation and resolution in toluene: methyl acetate: formic acid (5:5:1) as mobile phase and conc. H_2SO_4: Methanol:Glacial acetic acid: Anisaldehyde as spraying agent.
- The presence of Withaferin A compound was quantified by CAMAG Scanner at 223nm and the peaks were found at 0.55 Rf value, confirm the presence of Withaferin A most of the *in vitro* grown shoots.
- The results pertaining to GC-MS analysis led to the identification of a several metabolites present in the methanolic extract of *W. somnifera* tissues grown in *in-vitro* conditions. GC fraction of 70% methanolic extracts led to the identification of 26 metabolites in Copper salt stressed plant shoot tissues and 24 metabolites in Iron salt stressed plant shoot tissues of *Withania somnifera*.
- Specific compounds like phytol, squalene and digitoxigenin were also identified in *invitro* grown tissues, which are of commercial importance
- Ultraviolet–Visible Spectrophotometry was used for identification of presence of Phenols, Flavonoids, Pigments, Metals and etc., in plant extracts. The UV-Vis spectra were recorded (700-200 nm) for each plant extract. Two maxima

wavelengths specific to Beta carotene at 400-500nm and chlorophyll a at 465nm and 665nm were observed. The maxima wavelengths for Chlorophyll b have not been identified. There was no difference in composition of components among samples of *Withania somnifera* shoot extracts.

To conclude the present study, our results confirmed that *in-vitro*cultures of *Withania somnifera* has produced higher biomass and showed no morphological or physiological changes when cultured in media containing higher concentration of Copper and Iron. GC/MS analysis revealed the presence of Phytol, Squalene which are additional compounds with medicinal properties. Thus, *in-vitro* suspension cultures with varying media composition can be explored for synthesis of novel compounds.

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7.0 Appendix

Composition of MS medium

Ingredients	Composition (mg/ L)	Stock Solution (W/V) (g)
MS Macro I (10 X)		**1000ml**
NH_4NO_3	1650	16.5
KNO_3	1900	19
$MgSO_4.7H_2O$	370.6	3.7
KH2PO4	170	1.7
100 ml		
MS Macro II (10 X)		**1000 ml**
$CaCl_2.2H_2O$	439.8	4.398
100 ml		
Fe-Na EDTA (1000 X)		**100 ml**
Fe-Na EDTA	36.7	36.7
1 ml		
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
1 ml		
KI (1000X)	0.83	100ml
Myo-Inositol (100 X)		**100 ml**
Myoinositol	100	1
10 ml		
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
1 ml		