ANALYSIS OF SECONDARY METABOLITES IN THE *in vitro*, *in vivo* AND STORED ROOTS OF *Withania somnifera*

BY
SUPRIYA, S.
(07PBT21)

A thesis submitted to
Avinashilingam University for Women
Coimbatore – 641 043

*In Partial Fulfillment Of The Requirement For The Degree Of*
MASTER OF SCIENCE IN BIOTECHNOLOGY

APRIL 2009
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Certified as a bonafide research work

(Dr. R.Parvatham) \hspace{5cm} (Dr. R.Parvatham)
\textit{Signature of the} \hspace{5cm} \textit{Signature of the}
\textit{Head of the Department} \hspace{5cm} \textit{Guide}
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1.0 INTRODUCTION

“Peace and stillness are the great remedy for disease. When we can bring peace in our cells, we are cured.”

- The Mother

Indian medicinal plants are commonly used for the treatment of various ailments and are considered to be advantageous over the conventionally used drugs which are expensive and proven to have side effects (Arokiyaraj et al., 2007).

The exploitation of plants by man for the treatment of diseases has been in practice for a very long time. Herbal drugs constitute a major part in all the traditional systems of medicine (Mohanta et al., 2007).

Plants show enormous versatility in synthesizing complex materials which have no immediate obvious growth or metabolic functions (Akinmoladun et al., 2007). Thousands of secondary plant products have been identified and it is estimated that thousand more of these compounds still exist. Since secondary metabolites from natural resources have been elaborated within the living systems, they are often perceived as showing more “drug – likeness and biological friendliness than totally synthetic molecules” making them good candidates for further drug development (Kumaraswamy et al., 2008).

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products in the plant. The medicinal actions of plants unique to particular plant species or groups are consistent with the concept as the combination of secondary products in a particular plant is taxonomically distinct (Parekh et al., 2006).

*Withania somnifera* Dunal (WS), commonly known as Ashwagandha, has been used for centuries in Ayurvedic medicine to increase longevity and vitality. Western research supports its polypharmaceutical use, confirming antioxidant, anti-inflammatory, immune-modulating, and antistress properties in the whole plant extract and several separate constituents (Winters, 2006).
It is widely distributed from the Southern Mediterranean region to the Canary Islands and to South and East Africa. In India, the plant grows wild in North Western regions extending to mountaineous regions of Punjab, Himachal Pradesh and Jammu upto an altitude of 1500 m (Kumar et al., 2007).

The leaves and tuberous roots of *Withania somnifera* are medicinally important and are used in a number of preparations to treat diseases (Manickam et al., 2000).

The roots of the plant are categorised as rasayanas, which are reputed to promote health and longevity by augmenting defense against disease, arresting the ageing process, revitalising the body in debilitated conditions, increasing the capability of the individual to resist adverse environmental factors and by creating a sense of mental wellbeing (Gupta and Rana, 2007).

Phytochemically, the plant is unique in possessing the largest and structurally most diversified set of withanolides. The biologically active chemical constituents are alkaloids, steroidal lactones and withanolides (Mishra et al., 2000). Several modern molecular pharmacological studies have demonstrated linkage of these therapeutic actions to one or more withanolides present in the herb. The ethnopharmalogical properties of the plant include adaptogenic, anti-sedative and anti-convulsion activities, and the plant has been employed in the treatment of neurological disorders, geriatric debilities, arthritis and stress- and behaviour-related problems (Chaurasiya et al., 2007).

A regular development of high resolution analytical methods is necessary, for better phytochemical description, quality control of the drugs and also for the authentication of raw materials, characterisation and development of discrete chemotypes. Efficient techniques such as TLC, HPLC, GC-MS, spectroscopic studies like IR, UV, H- and C- NMR can be used to study the bioactive components in the crude as well as purified samples (Chaurasiya et al., 2007). These can then be subjected to analysis of their structure, based on which the drugs can be formulated for therapeutic use. TLC and HPLC are very much in use nowadays for the study of secondary compounds. TLC is an analytical technique which can be easily carried out in any laboratory. The technique requires only a standardized solvent system along with a developer for
the detection of spots. Although a simple method, the results obtained from TLC analysis are reliable, accurate and can be used for further analysis. Minute amount of samples can be analyzed by HPLC and the availability of instrumentation has made it a more common technique for analysis.

With this available information, a systematic approach was formulated to develop a finger print of *Withania somnifera* and it held the following objectives:

1. Extraction and analysis the phytochemical constituents

2. Compare the secondary metabolite content during development and storage

3. Develop a TLC and HPLC fingerprint for the roots of *Withania somnifera.*
2.0 REVIEW OF LITERATURE

Plants are potent biochemists and have been components of phytomedicine since times immemorial. Man is able to obtain from them a wondrous assortment of industrial chemicals. Plant based natural active constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc (Parekh et al., 2006). The medicinal values of these plants lie in their component phytochemicals, which produce definite physiological actions on the human body (Akinmoladun et al., 2007).

The systematic screening of plant species with the purpose of discovering new bioactive compounds is a routine activity in many laboratories (Parekh et al., 2006) and the present work titled “Analysis of secondary metabolites in the in vitro, in vivo and stored roots of Withania somnifera” is reviewed here under the following subheads.

2.1 Phytochemicals and their importance

2.2 Withania somnifera

2.3 Proven effects of Withania somnifera

2.4 Phytochemicals in Withania somnifera

2.5 Extraction and identification

2.6 Methods of detection

2.7 Chromatographic analysis
2.1 PHYTOCHEMICALS AND THEIR IMPORTANCE

Plant secondary metabolites have recently been referred to as phytochemicals. Phytochemicals are naturally occurring and biologically active plant compounds that have potential disease inhibiting capabilities. It is believed that phytochemicals may be effective in combating or preventing disease. The important ones of these phytochemicals are alkaloids, tannins, flavonoids and phenolic compounds (Akinmoladun et al., 2007).

ALKALOIDS

Alkaloids are principally of interest to humans because of their medicinal properties and many are widely used as drugs. They are basic plant substances and normally contain a nitrogen atom in their chemical structures as part of a heterocyclic ring. Their major occurrence is in the flowering plant and 40% of all plant families have at least one alkaloid bearing member. They can be present throughout the plant or alternatively restricted to certain tissues such as the root or bark (Harbourne et al., 1999). Alkaloids are a class of pharmacologically active compounds and several genes in the biosynthetic pathways for scopolamine, nicotine, and berberine have been cloned, making the metabolic engineering of these alkaloids possible (Sato et al., 2000).

PHENOLIC COMPOUNDS

Phenolic compounds are plant substances which possess in common an aromatic ring bearing one or more hydroxyl groups. They range from simple substances such as arbutin, eugenol, hydroquinone, khellin and myristicin to more complex structure of griseofulvin, podophyllotoxin, procyandin, tetrahydrocannabinol and usnic acid. The majority of phenolics are water soluble. They occur naturally combined with sugar in glycosidic form. They are located within the plant cell in the central vacuole. Several important types of plant polymeric material are phenolics, the lignans, melanins and tannins (Harbourne et al., 1999). Several phenolic compounds like tannins present in the cells of plants are inhibitors of many hydrolytic enzymes such as proteolytic macerating enzymes used by plant pathogens (Dash et al., 2008).
TANNINS

Tannins occur widely in vascular plants. Their occurrence in the angiosperms is particularly associated with woody tissues. Most tannins that have been purified and studied are biologically active (Harbourne et al., 1999). Tannins also complexes with proteins, divalent metals, cellulose, hemicellulose, pectin and other carbohydrates. High consumption of tannin is dangerous to health, being a phenolic secondary plant metabolite with one or more hydroxyl substitutes bonded to aromatic ring, it produces anthrocyanides, which is another toxic product on acid degradation (Monago and Akhidue, 2002).

QUINONES

Quinones are phenolic compounds that typically form strongly coloured pigments covering the entire spectrum. They play an important role in the respiration and photosynthetic electron transfer processes in plants. They act as electron carriers that function by converting between hydroquinones and quinines, thus acting as redox couples. They also act as phytochemical and non-phytochemical quenchers of energy in photosynthesis (Cseke et al., 2006).

TERPENOIDS

Terpene usually refers to a hydrocarbon molecule while terpenoid refers to a terpene that has been modified, by the addition of oxygen. They are the building blocks of other metabolites such as plant hormones, sterols, carotenoids, rubber, the phytol tail of chlorophyll, and turpentine. Terpenes are manufactured by plants in response to herbivory or stress factors, it has also been shown that flowers can emit terpenoids to attract pollinating insects; it has also been shown to attract beneficial mites, which feed on the herbivorous insects. Terpene emissions and subsequent attracting mechanisms have been shown to play an indirect role in plant defense mechanisms as well. Pharmaceutical and food industries have exploited them for their potentials.
and effectiveness as medicines and flavor enhancers. They have also shown antimicrobial activities (Zwenger and Basu, 2008).

SAPONINS

Saponins have the property of precipitating and coagulating red blood cells. Some of the characteristics of saponins include formation of foams in aqueous solutions, hemolytic activity, cholesterol binding properties and bitterness. These properties bestow high medicinal activities on the extracts. It has also been shown that saponins are active antifungal agents (Dash et al., 2008).

2.2 WITHANIA SOMNIFERA

Herbal medicine represents one of the most important fields of traditional medicine all over the world. To promote the proper use of herbal medicine and to determine their potential as sources for new drugs, it is essential to study medicinal plants, which have folklore reputation in a more intensified way (Parekh and Chanda, 2007).

Withania somnifera (L.) Dunal, commonly called Indian ginseng and a member of the family Solanaceae, is an important medicinal plant in the Ayurvedic and indigenous medicinal systems of India. The root extract is used for the treatment of tuberculosis, rheumatism, inflammatory conditions, cardiac diseases and as a general tonic (Murthy et al., 2008). It is known for its medicinal application since 2,500 years for the treatment of diseases and human ailments. It is also known to improve learning and memory retention in normal and deficient individuals and to provide non-specific host defense. The root of this plant contains various steroidal and other alkaloids generally known as “withania alkaloids” (Kumar et al., 2005).
TAXONOMICAL CLASSIFICATION

Kingdom : Plantae, Plants;
Subkingdom : Tracheobionta, Vascular plant
Super division : Spermatophyta, Seeds plant
Division : Angiosperma
Class : Dicotyledons
Order : Tubiflorae
Family : Solanaceae
Genus : Withania
Species : somnifera Dunal

The plant can be found growing in Africa, the Mediterranean, and India. An erect, evergreen, tomentose shrub, 30-150 cm high, found throughout the drier parts of India in waste places and on bunds. Roots are stout fleshy, whitish brown; leaves simple ovate, glabrous, those in the floral region smaller and opposite; flowers 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 used therapeutically. The bright red fruit is harvested in the late fall and seeds are dried for planting in the following spring (Gupta and Rana, 2007).

The roots of *W. somnifera* are extensively used in most of the Indian herbal pharmaceuticals and nutraceuticals (Misra *et al*., 2007). In Ayurveda, the roots are prescribed for gynaecological disorders, bronchitis, arthritis, rheumatism, inflammation, fevers, skin diseases (Kulkarni *et al*., 2000). They contain several alkaloids, withanolides, a few flavonoids and reducing sugars (Padmavathi *et al*, 2005).

The root is regarded as a tonic, aphrodisiac and is used in consumption, emaciation, debility, dyspepsia and rheumatism. A decoction of the root is also used for colds and chills. The plant is used in treating syphilis and a decoction of the root bark is administered in asthma, it is
used for all age groups, in both sexes and even during pregnancy without any side effects (Archana and Namasivayam, 1999).

The active pharmacological components of *W. somnifera* are steroidal lactones of the withanolide type. Several chemotypes exist that differ in their withanolide content. The principal withanolides in Indian *W. somnifera* are withaferin A and withanolide D. Both leaves and roots of the plant are used as the drug and steroidal lactones occur in both parts (Ray and Jha, 1999). Withanolide D has antitumour activity (Leyon and Kuttan, 2004).

2.3 PROVEN EFFECTS OF *Withania somnifera* EXTRACTS

ANTIBACTERIAL ACTIVITY

Both aqueous as well as alcoholic extracts of the plant (root as well as leaves) were found to possess strong antibacterial activity against a range of bacteria, as revealed by *in vitro* Agar Well Diffusion Method. The methanolic extract was further subfractionated using various solvents and the butanolic sub-fraction was found to possess maximum inhibitory activity against a spectrum of bacteria including *Salmonella typhimurium*. Oral administration of the aqueous extracts successfully obliterated *Salmonella* infection in Balb/C mice as revealed by increased survival rate as well as less bacterial load in various vital organs of the treated animals. Methanol and hexane extracts of both leaves and roots showed potent antibacterial activity (Gupta and Rana, 2007).

ANTI-INFLAMMATORY PROPERTIES

The effectiveness of Ashwagandha in a variety of rheumatologic conditions may be due in part to its anti-inflammatory properties. WS inhibited the granuloma formation in cotton-pellet implantation in rats and the effect was comparable to hydrocortisone sodium succinate treatment. Methanol extract of WS was given one hour before the implant and continued daily until the pellets were harvested on day 4 (Mishra *et al*., 2000). WS root extract reduced Freund’s complete adjuvant induced inflammation in rats; phenylbutazone was given as a positive control.
The glycoprotein found only in inflamed rat serum was decreased to undetectable levels in the WS group. Phenylbutazone, on the other hand, caused a considerable increase in the glycoprotein in both arthritic and healthy rats (Gupta and Rana, 2007).

ANTITUMOR MECHANISMS

Withania root extract diet showed 1.67 and 1.26-fold up-regulation of DTdiaphorase (DTD) and GST, respectively. Both are phase II liver enzymes that conjugate metabolites of cytochrome p450, which aids in liver detoxification of toxic phase I byproducts. Methanol extracts of WS root were able to down-regulate the expression of p34cdc2, a cell-cycle regulatory protein. This protein is expressed during cellular proliferation and downregulation arrests the cell cycle in the G2/M transition phase. The WS constituent most often identified as having the most potent antineoplastic activity (Winters, 2006).

Administration of WS ethanol extract and urethane reduced tumor incidence significantly. The histological appearance of the lungs of animals protected by WS was similar to those observed in the lungs of control animals. No pathological evidence of any neoplastic change was observed in the brain, stomach, kidneys, heart, spleen, or testes of any treated or control animals. In addition to providing protection from carcinogenic effects, WS treatment also reversed the adverse effects of urethane on total leukocyte count, lymphocyte count, body weight, and mortality (Mishra et al., 2000).

ANTIOXIDANT EFFECT

The brain and nervous system are relatively more susceptible to free radical damage than other tissues because they are rich in lipids and iron, both known to be important in generating reactive oxygen species. Free radical damage of nervous tissue may be involved in normal aging and neurodegenerative diseases, e.g., epilepsy, schizophrenia, Parkinson’s, Alzheimer’s, etc. An aqueous suspension of WS root extract was evaluated for its effect on stress-induced lipid
peroxidation (LPO) in mice and rabbits and it was found to prevent an increase in LPO (Gupta and Rana, 2007).

The active principles of WS, sitoindosides VII-X and withaferin A (glycowithanolides), have been tested for antioxidant activity using the major free-radical scavenging enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) levels in the rat brain frontal cortex and striatum. Decreased activity of these enzymes leads to accumulation of toxic oxidative free radicals and resulting degenerative effects. An increase in these enzymes would represent increased antioxidant activity and a protective effect on neuronal tissue (Mishra et al., 2000).

**ANTISTRESS EFFECT**

Antistress activity was studied in mice where an alcohol extract of WS root from defatted seeds of WS dissolved in normal saline and administered. The extracts approximately doubled the swimming time when compared to controls. Glycosides of WS (sitoindosides VII and VIII) exhibited significant antistress activity in forced swimming induced immobility in mice, restraint stress induced gastric ulcers in rats, restraint-induced auto-analgesia in rats, restraint stress effect on thermic response of morphine in rats, and morphine-induced toxicity in aggregated mice (Mishra et al., 2000).

The powdered root of *W. somnifera* and its aqueous suspension were administered in rats. The results indicate a significant increase in the plasma corticosterone level, phagocytic index and avidity index in rats subjected to cold swimming stress. In the rats pretreated with the drug, these parameters were near control values and an increase in the total swimming time was observed (Archana and Namasivayam, 1999).

**IMMUNOMODULATORY PROPERTIES**

The use of WS as a general tonic to increase energy and prevent disease may be partially related to its effect on the immune system. Glycowithanolides and a mixture of sitoindosides IX
and X isolated from WS were evaluated for their immunomodulatory and central nervous system effects (antistress, memory, and learning) in Swiss and Wistar strain albino rats. Produced significant antistress activity and augmented learning acquisition and memory retention in both young and old rats. Root extract of WS was tested for immunomodulatory effects in three myelosuppression models in mice: cyclophosphamide, azathioprin, or prednisolone. Significant increases (p<0.05) in hemoglobin concentration, red blood cell count, white blood cell count, platelet count and body weight were observed in WS-treated mice compared to untreated control mice also significant increase in hemolytic antibody responses toward human erythrocytes which indicated immunostimulatory activity (Mishra et al., 2000).

The aqueous suspension of Withania somnifera root powder was investigated for their in vivo and in vitro immunomodulatory properties. W. somnifera showed potent inhibitory activity towards the complement system, mitogen induced lymphocyte proliferation and delayed-type hypersensitivity reaction. Immunosuppressive effect of W. somnifera root powder has been reported, thus it could be a candidate for developing immunosuppressive drugs for the inflammatory diseases (Rasool and Varalakshmi, 2006).

ANTIPARKINSONIAN PROPERTIES

Parkinson's disease is a neurodegenerative disease characterized by the selective loss of dopamine (DA) neurons of the substantia nigra pars compacta. Administration of haloperidol or reserpine significantly induced catalepsy in mice. WS extract significantly inhibited these and provide hope for treatment of Parkinson's disease. Antiparkinsonian effects of WS extract have been reported due to potent antioxidant, antiperoxidative and free radical quenching properties in various diseased conditions. WS glycowithanolides (WSG) administered concomitantly with haloperidol for 28 days, inhibited the induction of the neuroleptic TD (Gupta and Rana, 2007).

2.4 PHYTOCHEMICALS IN Withania somnifera

Plants produce primary and secondary metabolites which encompass a wide array of functions. Primary metabolites include amino acids, simple sugars, nucleic acids, and lipids, are
compounds that are necessary for cellular processes. Secondary metabolites include compounds produced in response to stress, such as the case when acting as a deterrent against herbivores. Plants can manufacture many different types of secondary metabolites, which have been subsequently exploited by humans for their beneficial role in a diverse array of applications (Zwenger and Basu, 2008).

The phytochemical screening for the ethanolic extracts gave positive results for carbohydrates, alkaloids, glycosides, fixed oils and fats (Wagner et al., 1984).

The biologically active chemical constituents are alkaloids (ashwagandhine, cuscohygrine, anahygrine, tropine etc), steroidal compounds, including ergostane type steroidal lactones, withaferin A, withanolides A-y, withasomniferin-A, withasomidienone, withasomniferols A-C, withanone etc. Other constituents include saponins containing an additional acyl group (sitoindoside VII and VIII), and withanolides with a glucose at carbon 27 (sitoindoside IX and X). Apart from these contents, the plant also contains chemical constituents like withaniol, acylsteryl glucosides, starch, reducing sugar, hantreacotane, ducitol, a variety of amino acids including aspartic acid, proline, tyrosine, alanine, glycine, glutamic acid, cystine, tryptophan, and high amount of iron (Gupta and Rana, 2007). Leyon and Kuttan (2004) found that 70% methanolic extracts contained many other bioactive molecules such as starch, reducing sugars, glycosides, dulcitol and withaniol.

The aqueous-alcoholic soluble, chloroform-insoluble residue contains withanolide glycoside and sitoindoside components. These are potent bioactive constituents of ashwagandha. The commercially available extracts of ashwagandha obtained from old roots stock are either completely devoid of sitoindosides, or contain only traces of sitoindosides admixed with large amounts of toxic metabolites of withanolide aglycones, and polysaccharides (Ghosal and Shihbath 2004).

The 15 accessions of Withania somnifera were selected, which differed widely both qualitatively and quantitatively with respect to withanolides/glucowithanolides respectively. Structures of Withaferin-A was found to be present in the leaves as well as in the roots in all the
accessions of *W. somnifera* belonging to both RRL and CIMAP. But withanone was exceptionally absent in both leaves and roots of AGB 015 and AGB 025 but for RSS 030, RSS 027 and RSS 018 it is found to be present in roots while absent in leaves. The content of withanolide was found to be more in roots than in leaves (Dhar *et al*., 2006).

Withanolides have atoms with C-22 and C-26 bridged by δ-lactone functionality and an oxidized C-1 position. These compounds are specific for the Solanaceae family and in particular for the genus *Withania* (Ganzera *et al*., 2003).

Flavonoids and phenolic compounds namely 6, 8-dihydroxykaempferol, 3-O-rutinoside quercetin, 3-O-rutinoside 3-O-rutinoside-7-O-glucoside quinic acid, 4-O-caffeoylquinic acid and 4, 5-O-dicafeoylquinic acid from the aqueous ethanolic extract of *Withania somnifera* leaves (Kandil *et al*., 1994).

### 2.5 EXTRACTION AND IDENTIFICATION

"Ayurveda" roughly translated as the "knowledge of life" is a 5000+ year-old system of Indian home medicine using natural plant extracts. Ayurvedic medical system practices the use of dry powder or crude extract and assignment of bioactivities to a particular compound is not preferred. Interestingly, the mainstream pharmaceutical research is also on its way towards veering from mono-molecular or single target approach to combinations and multiple target strategies (Deocaris, 2008). Wild harvesting of medicinal plants can be problematic in terms of biodiversity loss, potential variation in medicinal plant quality and occasionally, improper plant identification with potential tragic consequences. The phytomedicine used today is based upon extraction and a further purification of active constituents (Briskin, 2000).

Various types of extraction can be applied to raw material resulting in significant changes in the quantities and proportions of active components affecting safety and benefits (Schilter *et

*Withania somnifera* plant parts were percolated four times with ethanol:water (1:1) at 25 ± 2°C. The aqueous ethanolic extracts were concentrated by evaporation at reduced pressure and temperature (50 ± 5 °C). The concentrate was extracted with chloroform and solubles dried under reduced pressure to yield total withanolide residue (Sharada et al., 2007).

Studies of Sangwan et al., (2004) showed that withanolides can be extracted using commercial products equivalent to 250 mg Ashwagandha using methanol as the solvent. The extracts were partitioned three times with 2.0 ml water and with equal volume of chloroform. The extracted withanolides were quantified by HPLC using standard Withaferin A.

Five new withanolides, namely withasomnilide, withasomniferanolide, somniferanolide, somniferawithanolide and somniwithanolide were isolated from silica gel column containing ethanolic extract of the stem bark after elution with petrol, chloroform and methanol in order of increment of polarity (Ali et al., 1997).

Methanolic extracts of the whole plant of *W. somnifera* were concentrated to a gum which was dissolved in methanol and defatted with petroleum ether. The defatted methanol extract was again evaporated and dissolved in water. The aqueous extract was extracted with chloroform, at different pH values. The fraction obtained at pH 7 was loaded on a Silica gel column and eluted first with chloroform and then with chloroform/methanol mixtures. The fraction obtained in chloroform-methanol (95:5) was again chromatographed by column chromatography, eluted with petroleum ether (40-60°) and petroleum ether/chloroform. The fraction collected on elution with chloroform-petroleum ether (80:20) was subjected to TLC using petroleum ether and chloroform. The fraction collected on elution with chloroform-petroleum ether (40:60) was purified by TLC to afford withasomidienone (Rahman et al., 1993).

A new chlorinated withanolide C was isolated from methanolic extracts of dried leaves of *Withania somnifera* after subjecting it to purification by silica gel column (Bessalle and Lavie,
1992). 5-dehydroxy withanolide R and withasomniferin-A was isolated after eluting the silica gel column containing methanolic extract of the aerial parts with hexane and hexane-chloroform mixtures (Rahman et al., 1991).

Withaferin A, 27-deoxy-14α-hydroxywithaferin A, 27-deoxywithaferin A, 27-deoxy-17α-hydroxywithaferin A, 17α-dihydroxy-1-oxo-6α,7α-epoxy-20R,22R-witha-2, 24-dienolide, withanolides N, O and withanolide P were isolated after eluting the silica gel column containing ethereal extracts of leaves of Withania somnifera with mixtures of chloroform and ethyl acetate. Similarly, Withanolide D, withanolide G, 27-hydroxy, 14α-hydroxy and 17α-hydroxywithanolide D were also isolated from leaves of Withania somnifera (Abraham et al., 1975).

2.6 METHODS OF DETECTION

Many techniques have been used to identify and quantify secondary metabolites. In the early 1990s, High-performance liquid chromatography (HPLC), with photodiode array detection was used to isolation and quantification. The coupling of HPLC and mass spectrometry methods, such as electrospray, thermospray, or fast-atom bombardment, have been widely used to provide molecular weight and characteristic fragment ions for structural elucidation, but these are very expensive techniques which are not readily available. Due to simplicity of methodology and low cost, techniques like paper chromatography and thin-layer chromatography have been applied routinely in many laboratories (Vendramini and Trugo, 2004).

In HPLC, UV is the most employed since it is by far the most common detector found in phytochemical laboratories. Fluorescence is one of the most sensitive detection methods for HPLC analyses. ELSD (Evaporative light scattering detector) is a mass detector which measures the scattered light generated by the non-volatile particles of analyte produced by the nebulization into droplets of the LC effluent. The signal intensity is related to the concentration of the solute in the effluent but not its chemical identity. ELSD is a universal, non-specific detector which can provide a stable baseline even with gradient elution. Furthermore, a number of volatile mobile-phase modifiers, such as NH₄OH, (C₂H₅)₃N, NH₄OAc, HCOOH, CH₃COOH, CF₃COOH, can be
used in order to obtain better selectivity. HPLC coupled with Diode Array Detector offers identification of polyphenols by scanning UV-Vis spectra of individual components, which spectral characteristics are unique, but not selective. At the same time HPLC-DAD determination methods of polyphenols differ in mobile phase solutions resulting in DAD scanned spectra deviation between different studies, aggravating the precise identification based on agreement to UV-Vis data from literature. HPLC–MS techniques have been successfully applied to the analysis of non-volatile molecules such as saponins. The major drawbacks of these interfaces were a poor reproducibility, which prevents their use for quantitative analyses, and a high fragmentation in the recorded spectra (Fuzzati, 2004). Mass spectrometry (MS) detection with molar weight determination of the individual components in the sample enables more precise identification of compounds eluted from the column (Mozetič and Trebše, 2004).

Gas chromatography (GC) is the most commonly adopted analytical technique for determination of residues in fruits and vegetables. But comparison of HPLC over Gas Chromatography, less volatile and larger samples can be used with HPLC. It was discovered that better separation of the components of the mixture occurs if the particles in the stationary phase are very small. However, it was also found that if very small particles were used in the column, then the liquid passed very slowly through the column. Therefore, a pump is used to force the liquid through the column. This is not necessary in GC but a shorter column can be used in HPLC because the separation is more efficient (Sundravadana et al., 2008).

2.7 CHROMATOGRAPHIC ANALYSIS

TLC is a very common technique for the fingerprint analysis of plant material and extract due to its easiness of use, low cost and versatility. TLC is still employed widely for the identification tests of plant derivatives. Asian and American ginseng can be discriminated for their ginsenosides composition by two-dimensional TLC using a mixture of chloroform, methanol and water (13:7:2) as the first developing solvent system (SS-I) and a mixture of water, n-butyl alcohol and ethyl acetate (5:4:1) as the second solvent developing system (SS-II) (Fuzzati, 2004).
Thin layer chromatography

Ethanol: water (1:1) extracts of the sample when further extracted with chloroform, was found to contain total withanolides. TLC analysis was conducted by spotting extracts of samples on pre-coated silica gel plates, developing the chromatograms with solvent system chloroform:methanol (96:4) for 2 h and visualizing the spots obtained by spraying of vanillin reagent [vanillin:boric acid:methanol:sulphuric acid in the ratio of 0.5 g:50 g: 500 cm3:10 cm3 (50 %)] and it revealed the presence of withanolides (Sharada et al., 2007).

The shade dried roots were ground and defatted with n-hexane and extracted with methanol, later subjected to silica gel column and eluted in n-hexane and ethylacetate with solvent gradient. The polarity was increased by sequentially adding ethyl acetate and methanol. The fractions collected were pooled based on their TLC pattern. Fractions yielded stearic acid, β-sitosterol, stigmasterol, withanolide B and withanolide A respectively. The fractions obtained were subjected to chromatography, crystallized and it yielded several previously reported important compounds such as witanolide A, D and withaferin A and also several new compounds (Misra et al., 2007).

Withanolides and glucowithanolides were found in the chloroform and n-butanol fractions of ethanol: water (1:3) extracts from dried roots and leaves of Withania somnifera. The withanolides were monitored by TLC with solvent system chloroform and methanol (19:1) and glucowithanolides by TLC with chloroform and methanol (4:1). The isolated pure withanolides/glucowithanolides were identified with the help of IR, NMR and mass spectroscopy data (Dhar et al., 2006).

Withaferin A and withanolide D were isolated and purified from fresh stalks and leaves of in vitro grown plant by TLC with benzene: ethyl acetate (1:4) as the solvent system and developed in methanol and chloroform (1: 14) (Lockley et al., 1976).

High performance liquid chromatography
HPLC is widely used technique because of its speed, sensitivity and adaptability to non-volatile and polar compounds. It is ideal for the analysis of saponins and sapogenins. Another advantage is its versatility due to the possibility of using different detection techniques such as ultraviolet (UV), evaporative light scattering (ELSD), fluorescence and mass spectrometry (MS) (Fuzzati, 2004).

A reliable and efficient HPLC method involving photodiode array detection (PAD) was developed for the analysis of the nine closely related withanolides of *W. somnifera*, viz. 27-hydroxy withanone, 17-hydroxy withaferin A, 17-hydroxy-27-deoxy withaferin A, withaferin A, withanolide D, 27-hydroxy withanolide B, withanolide A, withanone and 27-deoxywithaferin A. HPLC analysis was performed on a Waters (Milford, MA, USA) PAD (model 996) and separations were achieved using a Waters reverse phase column (150 × 3.9 mm i.d.; 4 µm) subjected to binary gradient elution. The two solvents used for the analysis consisted of water containing 0.1% acetic acid (solvent A) and methanol containing 0.1% acetic acid (solvent B). Gradient programming of the solvent system was carried out at 27°C and was: initially at 60% A, changed to 40% A at 30.0 min, maintained for the next 2.0 min, changed to 25% A at 45 min and then to 5% A at 54.0 min at a flow-rate of 0.6 ml/min and then at a flow rate of 1.0 ml/min the mobile phase was changed to 0% A at 55 min and this solvent composition was maintained until the run time reached 60 min. It resulted in well-resolved and symmetrical peaks for the nine withanolides (Chaurasiya *et al*., 2007).

TLC and HPLC analysis of undifferentiated and partially differentiated calli of *Withania somnifera* showing adventitious shoot bud primordia, multiple shoot cultures and regenerated plantlets revealed the *in vitro* synthesis of withanolides such as withanone, withaferin A, withanolide A, withanolide B, withanolide E (Sharada *et al*., 2007).

Ciddi, (2006) carried out HPLC by Phyton Inc, USA. The conditions of the analysis includes, gradient method with water with 0.1% TFA (tri-fluoro acetic acid) in Pump A, recovered acetonitrile (86%, 14% water) with a 0.1% TFA in pump B, with a column of Waters delta pack C-18, 150x4.6 mm, 4 µm with a guard column. The flow rate of mobile phase was
0.85 ml/min. The production of withaferin A for cultures transferred to production medium was confirmed.

The methanolic extract of the dried roots were defatted with hexane and the resulting residue was stirred and concentrated with ethyl acetate. This was subjected to silica gel column eluted with hexane and ethyl acetate mixtures. Successive column chromatographic and preparative HPLC purification of fraction III using a Phenomenex Luna column with water and methyl cyanide (1:1) as eluant, at a flow rate of 20 ml/min resulted in detection of ashwagandhanolide (Subbaraju et al., 2006).

High-performance liquid chromatography analysis demonstrated the presence of withanoloids in the hairy root extracts. The results of the study clearly indicate that there is enhancement of secondary metabolites in hairy roots, which is indicated through significant enhancement of the antioxidant activity, since these are the major constituents responsible for the activity. This is the first report on the presence of antioxidant principles in genetically modified roots of *W. somnifera*. The HPLC system used was Hewlett Packard’s (Palo Alto, CA) equipped with a quaternary pump fitted with a zorbax C18 (Hewlett Packard) analytical column (25 cm x 4.6 nm ID 5-μ particle size). The injection system (Rheodyne) used was 20 μL. Detection was done by an HP 1250 series variable wavelength detector at wavelength of 239 nm. The mobile phase consisted of water and methanol in the ratio of 8:2 with a flow rate of 0.8 mL/min. Total program time was 20 min. (Kumar et al., 2005).

Four withanolides namely withaferin-A, 12-deoxywithaframolide, withanolide A, and withanone in *Withania somnifera* were detected by HPLC in combination with Electrospray Ionization Mass Spectrometry (EIMS) (Khajuria *et al*., 2004).

The chloroform extracts of the commercial products and preparations of *Withania somnifera* were worked up and analysed by HPLC. Withaferin A. HPLC analysis was carried out on a reverse phase C-18 column (Waters) using methanol and water as mobile phase through a gradient elution of decreasing polarity under mild acidic conditions, with detection at 227 nm
using a photo-diode array detector. The analyses resulted in identification of withaferin A and six other structurally unidentified phytochemicals designated as WS 1-6 (Sangwan et al., 2004).
3.0 MATERIALS AND METHODS

This study is aimed at analyzing the secondary metabolites in the *in vitro, in vivo* and stored roots of *Withania somnifera*. The materials used for the analysis and the methodology followed is as follows.

3.1 Collection of sample

3.2 Preparation of samples for extraction

3.3 Sequential extraction of the secondary metabolite

3.4 Phytochemical analysis

3.5 Chromatographic analysis

3.5.1 Thin layer chromatography

3.5.2 High performance liquid chromatography

3.1 COLLECTION OF SAMPLE

<table>
<thead>
<tr>
<th>PLANT MATERIAL</th>
<th>SAMPLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried root</td>
<td>A</td>
</tr>
<tr>
<td>Stored root</td>
<td>B1, B2, B3 and B4 (2006-2009)</td>
</tr>
<tr>
<td><em>In vivo</em> root</td>
<td>C</td>
</tr>
<tr>
<td><em>In vitro</em> root</td>
<td>D</td>
</tr>
</tbody>
</table>
Sample A of *Withania somnifera* used for the phytochemical analysis and thin layer chromatography in the present study were obtained from the local market in Coimbatore. The samples for the study on sample B were obtained from the same place at different time periods (B1, B2, B3 and B4).

Dried mature seeds of *Withania somnifera* (Jawahar variety) were planted in the field maintained in the University campus. The seeds were sown in 10 pits of 25 seeds each. They were watered and their growth was monitored for a period of one month on daily basis. The roots of the one month old seedlings were used for the study on sample C.

Sample D were obtained from plants maintained in MS0 *in vitro*. They were grown for a period of one month and were used for analysis.

### 3.2 PREPARATION OF SAMPLES FOR EXTRACTION

The dried roots of *Withania somnifera* (samples A and B) obtained from the local market were finely powdered and sieved. For *in vivo* material (sample C), the plantlets were uprooted after one month, washed and the fresh roots were used for the study. The roots (sample D) grown *in vitro* were washed well with water to remove the agar from them. All the samples were air dried.

Five gram of sample A was used for the extraction for phytochemical analysis and 3.5g of sample B was used. A wet weight of 1g was taken for samples C and D.

### 3.3 SEQUENTIAL EXTRACTION OF THE SECONDARY METABOLITE

The sample A was extracted using solvents in the increasing and decreasing order of polarity. The order of the solvents is as follows: petroleum ether, chloroform, ethyl acetate and ethanol.
To 5g of the root powder, 20 ml of the solvent was added and incubated at 37°C for 8 hours in a shaker incubator. The mixture was then filtered through Whatmann No.1 filter paper and the filtrate was stored. To the residue added 20 ml of the next solvent. The procedure was repeated until the last solvent. The stored extracts were then used for the analysis.

Sample B was collected during the years 2006, 2007, 2008 and 2009. These were used to analyse the secondary metabolite content on storage. 3.5 g of the roots
were taken and extracted using 15 ml of the solvent. The petroleum ether extract was discarded, the following chloroform and ethyl acetate extracts were used for TLC. 1g of samples C and D were extracted in a same manner with 4ml of each solvent and analysed.

3.4 PHYTOCHEMICAL ANALYSIS

Sample A was tested to detect the presence of phytochemicals such as flavonoids, proteins, sugars, alkaloids, phenolic compounds, steroids, saponins, starch and quinone. The procedure followed is given in detail in Appendix 1.

3.5 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography was performed on Merck silica gel 60 F_{254} plate. A plate of height 5 cm and width 2.5 cm was taken and origin was marked at 0.5 cm from one end of the
plate. 20µl of the samples were spotted on the TLC plates and were placed in a chamber previously saturated with the solvent system chloroform: methanol in the ratio 9:1. After the run, the plates were air dried and sprayed using 10% sulphuric acid. The spots were developed by directly heating the air dried plate over a flame or on a hot plate. The results obtained were observed and recorded.

3.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC analysis was performed for the chloroform and ethyl acetate extracts of samples C and D. A Shimadzu HPLC Class VP series provided with two LC-6 AD pumps and variable wavelength programmable photo diode array detector SPD-M20 A. The column was maintained at a constant temperature of 27°C using the column oven CT0-10AS. The functioning of the system was controlled by system controller CBM-20A and Phenominex reverse phase C-18 (250 mm x 4.6 mm, 5 µm) was used. The HPLC system was equipped with class VP series version 6.14 software (Shimadzu).

The solvent used were of HPLC grade (Ranbaxy, India). All the solvents and samples were filtered through 0.22 µM pore size filter (Millipore Corporation, USA). Degassing of solvents was done by sonication for 40 minutes.

The modified method described by Chaurasiya et al., (2007) was followed for the analysis of withanolides. The two solvents used for the analysis consisted of solvent A – methanol with 0.1% TFA and solvent B – water with 0.1% TFA.

Gradient programming of the solvent system was carried out at 27°C and was: initially at 40% B for 3 minutes, changed to 0% B at 3.0 min, changed to 10% B at 6.0 min, and then to 20% B at 10.0 min, 25% B at the 12.0 min, at 15.0 min at 30% B and 100% B at the 20.0 min at a flow-rate of 1 ml/ min. The wavelength scan range of the Photodiode Array Detector was set to 190–350 nm and the chromatograms were recorded at 237 nm.
4.0 RESULTS AND DISCUSSION

The present study, “Analysis of secondary metabolites in the in vitro, in vivo and stored roots of Withania somnifera” is aimed at standardization of an extraction protocol for the isolation and analysis of secondary metabolites of *Withania somnifera* and comparing the content of withanolides on storage in the roots of *Withania somnifera*.

The study involved the collection of samples followed by their powdering and sequential extraction using solvents in the increasing and decreasing order of polarity and then the extracts were analysed using TLC and HPLC. The results are described and discussed under the following sub-heads:

4.1 Phytochemical analysis

4.2 Thin layer chromatography

4.3 High Performance Liquid Chromatography

4.1 PHYTOCHEMICAL ANALYSIS

The roots of *Withania somnifera* are widely being applied in the field of medicine and are well known for their antibacterial, antitumor, anti inflammatory and immunomodulatory. The root samples used in the study (samples A, B, C and D) were finely powdered and sieved and sequentially extracted using solvents of varying polarity, inorder to deduce specifically the solvent in which individual compounds of the roots are extracted. In this respect, each fraction collected was tested for the phytochemicals namely flavonoids, proteins, sugars, alkaloids, phenolics, saponins, starch, steroids, quinine and cellulose. The roots collected and stored in the year 2008 were chosen for the study to standardize the solvents for the sequential extraction and to anlayse which fraction contained the desired secondary metabolite. The results of this pilot study were further employed for the analysis of the *in vitro*, *in vivo* and stored roots.
Phytochemicals were analysed after sequential extraction with solvents in the order of increasing and decreasing polarity. The results are presented in Table 4.1.1.

### Table 4.1.1
Phytochemical analysis of *Withania somnifera* roots (2008) in the order of increasing polarity

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Proteins</td>
<td>–</td>
<td>✓</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sugars</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>✓</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>–</td>
<td>–</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Phenolics</td>
<td>✓</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Saponins</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Steroids</td>
<td>–</td>
<td>✓</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Quinone</td>
<td>–</td>
<td>✓</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cellulose</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

The extraction in the order of increasing polarity gave the following results as presented in Table 4.1.1. Flavonoids were present in all the four solvent extract and proteins were detected only in the chloroform extract. Sugars were present only in the ethanol extract. Alkaloids were found in the ethanol and ethyl acetate extract. Phenolics were present in the petroleum ether extract. Steroids were detected in chloroform and ethanol fraction and quinone was found to be present in chloroform extract. Cellulose was found to be present in all the extracts. Saponins and starch were not identified in any extracts. Table 4.1.1 depicts that number of positive results for various phytochemicals which are on the increasing polarity from petroleum ether to ethanol, petroleum ether is a less polar solvent and the number of components that are soluble in it are a few, whereas ethanol, is a more polar solvent and comparatively more components are present in that extract.
Previous studies on the phytochemicals in the various extracts have shown that petroleum ether extracts contain the phytochemicals such as phenolics, glycosides and tannins, chloroform extract possesses certain alkaloids, sterols and tannins and the ethanol extracts were found to contain alkaloids, sugars and sterols (Mallikharjuna et al., 2007 and Kumaraswamy et al., 2008).

Shade dried and powdered root, stem bark and leaves of Strychnos potatorum subjected to soxhlet extraction with petroleum ether, chloroform, 95% ethanol, and distilled water for 18h in the order of increasing polarity of solvents revealed the presence of alkaloids and sterols in all the extracts, flavonoids, phenols and saponins in the ethanol and aqueous extract, glycosides, lignins and tannins in the petroleum ether and chloroform extract (Mallikharjuna et al., 2007).

Sequential extraction of the bark of Betula utilis with solvents such as petroleum ether, chloroform, methanol, ethanol and water revealed the presence of carbohydrates and alkaloids in all the extracts (Kumaraswamy et al., 2008). However, in our study in the roots of Withania somnifera alkaloids were detected only in the ethyl acetate and ethanol extracts.

The ethanol extracts of Olax subscorpioidea showed the presence of tannins, alkaloids, glycosides, steroids and flavonoids (Ayandele and Adebiyi, 2007). In the present study the ethanol extract consisted of flavonoids, sugars, alkaloids, steroids and cellulose.

The results for the phytochemical analysis performed for the extracts in the order of decreasing polarity are tabulated in Table 4.1.2. Flavonoids were present in all extracts except that of petroleum ether. Proteins were found in the ethyl acetate and ethanol extracts. Sugars, alkaloids, steroids were present in the ethanol and ethyl acetate extracts. Phenolics were detected in the petroleum ether and chloroform extract. Quinone was found to be present only in the ethanol extract. Cellulose was found to be present in all the extracts. Saponins and starch were absent in all extracts. Table 4.1.2 indicates the presence of more compounds in the ethanol and ethyl acetate extracts and it gradually decreases towards the petroleum ether extract.
Table 4.1.2
Phytochemical analysis of *Withania somnifera* roots (2008) in the order of decreasing polarity

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Ethanol</th>
<th>Ethyl acetate</th>
<th>Chloroform</th>
<th>Petroleum ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>Proteins</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugars</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaloids</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenolics</td>
<td>–</td>
<td>–</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Saponins</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroids</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinone</td>
<td>✓</td>
<td>–</td>
<td></td>
<td>–</td>
</tr>
<tr>
<td>Cellulose</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Methanol extracts of *Ocimum gratissimum* were found to give positive results for alkaloids, tannins, steroids, terpenoids, flavonoids and cardiac glycosides (Akinmoladun *et al.*, 2007). The above table also indicates positive result for alkaloids, flavonoids and steroids in the ethanolic root extract, this may be since methanol and ethanol have polarity parameter 5.1 and 5.2 respectively.

The phytochemical screening of chemical constituents of the *Aquilaria agallocha* showed that the methanol soluble leaf extract contains high amount of carbohydrate and anthroquinone while protein, amino acid, alkaloid, tannin, glycoside and terpenoid occur in moderate amount. The methanol soluble bark extract contain amino acid, alkaloid, anthroquinone and terpenoid in high concentration whereas saponin, tannin, glycoside, fixed oil and fat were present in lesser amounts (Dash *et al.*, 2008).

Flavonoid were present in the petroleum ether extract in the order of increasing polarity but was not observed in the extract prepared in the order of decreasing polarity whereas phenolics and cellulose were present in petroleum ether extracts of both the orders. Chloroform extract shows similar result for flavonoids and cellulose in both the extracts. In the order of
increasing polarity presence of proteins, steroids and quinones was observed whereas in the order of decreasing polarity phenolics were present. In the increasing order of polarity ethyl acetate was found to possess flavonoids, alkaloids and cellulose, in addition to these it possessed proteins, sugars and steroids in the order of decreasing polarity. Flavonoids, sugars, alkaloids and steroids were present in the ethanol extract in both the methods of extraction, but the ethanol extract in the order of decreasing polarity was found to contain protein and quinone in addition to the above.

The ethanol extract possessed sugars predominantly which was clearly observed from charred spots on the TLC plates. Hence, for the further study on storage roots, *in vitro* and *in vivo* roots the chloroform and ethyl acetate extracts were used since most components were extracted in these solvents.

**4.2 THIN LAYER CHROMATOGRAPHY**

Thin layer chromatography enables the identification of secondary compounds, their fractionation and further study on the structure and therapeutic purposes can be performed. Thin layer chromatography was performed for all the extracts of sample A. From the TLC results it was observed that better results were obtained in chloroform and ethyl acetate fractions. Therefore, for the further analyses these solvent extracts were used for samples B, C and D since these extracts were found to produce better results for TLC in sample A.

20 µl of the sequentially extracted samples were spotted 0.5 cm above the lower end of the TLC plate. The samples were run up to the 0.5 cm mark made at the top of the plate and discrete spots appeared on developing the plates with sulphuric acid. In this method of detection the solutes are partially oxidized leaving behind a charred deposit of black carbon that is easy to distinguish. The solvent fractions in both the increasing and decreasing order of polarity resulted in discrete spots. A total of 18 spots were detected in the order of increasing polarity and 16 spots in the order of decreasing polarity.
TLC analysis of the various extracts of sample A

Table 4.2.1 depicts the results for the TLC of extracts in both the orders of polarity, petroleum ether extract had 2 spots with R\textsubscript{f} values 0.69 and 0.78; chloroform had 7 spots corresponding to R\textsubscript{f} values 0.2, 0.28, 0.4, 0.5, 0.59, 0.7, 0.74. Spots with R\textsubscript{f} values 0.19, 0.28, 0.59, 0.7, 0.74 were detected in the ethyl acetate extract, the ethanol extract resulted in similar spots except an additional spot at R\textsubscript{f} value of 0.28. Identical
<table>
<thead>
<tr>
<th>R&lt;sub&gt;f&lt;/sub&gt;</th>
<th>Increasing polarity</th>
<th>Decreasing polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Petroleum Ether</td>
<td>Chloroform</td>
</tr>
<tr>
<td>P'</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>0.78</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.74</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.7</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.69</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.59</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.5</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.4</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.28</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.2</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Total number of spots</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

P'-Polarity parameter

spots were observed in the chloroform, ethyl acetate and ethanol extracts at R<sub>f</sub> values 0.59, 0.7 and 0.74.

In the TLC analysis for the extracts in the decreasing order of polarity it was observed that ethanol and ethyl acetate had identical number of spots corresponding to the same R<sub>f</sub> values 0.2, 0.28, 0.59, 0.74 and 0.78. Chloroform extract showed 3 spots with R<sub>f</sub> values 0.59, 0.7, 0.78 and petroleum ether extract resulted only in one band at R<sub>f</sub> value 0.78.
The spot corresponding to the $R_f$ value 0.59 was found in the ethanol, ethyl acetate and chloroform extracts, and the spot at $R_f$ value 0.78 was observed in all the extracts.

The result for TLC analysis is represented in **Plate 4.1**. At $R_f$ 0.78 a spot was observed in the petroleum ether extract of both the increasing and decreasing orders. An additional spot at $R_f$ 0.69 was observed only in the petroleum ether extract of the increasing order of polarity. Chloroform extract of the increasing order resulted in 7 spots at $R_f$ values 0.2, 0.28, 0.4, 0.5, 0.59, 0.7 and 0.78, similar spots were observed between the chloroform extract of decreasing order at $R_f$ 0.2, 0.59 and 0.7 the absence of the other spots in the chloroform fraction of the decreasing order may be attributed to the solubility of the compounds in the previously used solvents namely ethanol and ethyl acetate. Spots were detected in the ethyl acetate extract of both the orders at $R_f$ values 0.2, 0.28, 0.59 and 0.74. Additional spot was observed at $R_f$ 0.7 in the order of increasing polarity and at 0.78 in the order of decreasing polarity. Ethanol extracts of both the orders had identical spots at $R_f$ values 0.2, 0.59 and 0.74, in the order of increasing polarity additional spot was observed at 0.7 and at 0.28 and 0.78 in the order of decreasing polarity.

The aprotic solvent soluble fraction of the extraction process, e.g. chloroform, contains mainly withanolide aglycones (Ghosal and Shibnath, 2004). The chloroform and ethyl acetate extracts of the order of increasing polarity showed the highest number of spots. These solvents were preferred for the next study on the analysis of variation in secondary metabolite content in the roots on storage.
In the study by Rahman et al., (1991) the methanolic extracts whole plant of *Withania somnifera* were evaporated, the gum obtained was dissolved in methanol and defatted with hexane. The fraction obtained at pH 7.0 was subjected to silica gel column chromatography and the fraction obtained was subjected to preparative TLC in methanol: chloroform (1:49) to afford S-dehydroxywithanolide-R and withasominiferin- A at $R_f$ 0.81 and 0.58, respectively.

Standard withaferin A and withanolide D were reported to have $R_f$ values of 0.34 and 0.51, respectively when analyzed on Merck silica gel TLC plates with the solvent system chloroform: ethyl acetate: methanol: benzene in the ratio 72:4:8:16 and sprayed with Liebermann–Burchard reagent (Ray and Jha, 1999).

The resolution of withanolide-A and withaferin-A has been achieved by using mobile phase consisting of toluene: ethyl acetate: formic acid (5:5:1). Spots corresponding to withaferin-A and withanolide-A were recorded at $R_f$ 0.33 and 0.53, respectively (Sharma et al., 2007).
TLC analysis of the various extracts of sample B

Table 4.2.2 represents the Rf values obtained for the storage roots from 2006-2009. The table shows identical spots at Rf values 0.20, 0.50, 0.59, 0.70 and 0.74 for all the extracts. Spot corresponding to Rf value 0.13 was detected in all the extracts except the ethyl acetate extract of the year 2009. A spot at Rf value 0.28 was exclusively identified in the chloroform extracts of the storage roots of 2006 and 2008. The samples C1, E1, C2, E2 and C4 only showed the presence of a spot at 0.40 which was absent in the other samples. A spot of the highest Rf value 0.78 was observed in the samples C1, E1 and C4 as depicted in Plate 4.2.

Hypericum perforatum plant material preparation includes dehydration or freezing and further storage, and these procedures can lead to variations in the secondary metabolite profile. The effect of drying, freezing and storage at -20 ºC on flavonoid and hypericin content in H. perforatum was evaluated. Leaves and stems of H. perforatum were dried at 25, 30, 50 and 70ºC, frozen in liquid nitrogen or frozen and then stored at -20ºC for 10, 20 and 30 days. Flavonoid and hypericin quantification was performed by High Performance Liquid Chromatography (HPLC). Levels of both
Plate 4.2

TLC analysis of the various extracts of sample B

|-------------|------------------|------------------|--------------------|------------------|------------------|--------------------|------------------|------------------|--------------------|------------------|------------------|--------------------|

Table 4.2.2

$R_f$ values for Thin Layer Chromatography of Sample B

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloroform (C1)</td>
<td>Ethyl acetate (E1)</td>
<td>Chloroform (C2)</td>
<td>Ethyl acetate (E2)</td>
</tr>
<tr>
<td>0.78</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.74</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.70</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.59</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.5</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.4</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.28</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.20</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>0.13</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>Total number of spots</td>
<td>9</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>
flavonoids and hypericin were affected by drying temperature. Drying at 25ºC resulted in lower levels of most of the metabolites analyzed while at 50ºC, levels of free and conjugated rutin, conjugated quercetin and quercitrin as well as hypericin content were maintained (Diniz et al., 2007).

Fresh ginseng is harvested during August through October, when growth and medicinal value of the ginseng roots are best. Therefore, it is necessary to store fresh roots for long time periods because of the imbalance of supply and demand. A problem which arises with red ginseng processed from roots stored for a long time is the occurrence of internal cavities which increase in severity with an extended storage period. The firmness of ginseng roots also declined during extended air storage. In controlled atmosphere storage the root firmness was maintained for five months. Starch content decreased with the extended storage periods. After one month of storage there was a big decrease in starch content. Content of major ginsenosides (Rgl, Re, Rd, Rc, Rb2, and Rb1) decreased during storage and the severity of internal cavity formation gradually increased during storage (Yun and Lee, 1998).

**TLC analysis of the various extracts of sample C and D**

Table 4.2.3 and Plate 4.3 represents the R_f values obtained for the TLC carried out in the chloroform and ethyl acetate extracts of the one month old sample C and D. Discrete spots were obtained. Sample C5 and C6 were found to possess spots at 0.28, 0.56, 0.68, 0.74 and 0.78. The ethyl acetate extracts of both samples had identical spots at R_f values 0.59, 0.7 and 0.78.
Table 4.2.3
R\textsubscript{f} values for Thin Layer Chromatography of Sample C and sample D

<table>
<thead>
<tr>
<th>R\textsubscript{f}</th>
<th><strong>Sample C</strong></th>
<th></th>
<th><strong>Sample D</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloroform (C5)</td>
<td>Ethyl acetate (E5)</td>
<td>Chloroform (C6)</td>
<td>Ethyl acetate (E6)</td>
</tr>
<tr>
<td>0.28</td>
<td></td>
<td></td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>0.56</td>
<td>✔</td>
<td></td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td>0.59</td>
<td></td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>0.68</td>
<td>✔</td>
<td></td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>0.7</td>
<td></td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>0.74</td>
<td>✔</td>
<td></td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>0.78</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td><strong>Total number of spots</strong></td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
Thin layer chromatography is a simple technique for the analysis of bioactive compounds. TLC was performed for the sequentially extracted root samples and discrete spots were observed. The $R_f$ value was calculated. The samples were further studied using HPLC.

### 4.3 HPLC ANALYSIS OF STORAGE ROOTS

HPLC analysis was performed for the stored roots of *Withania somnifera*. Roots collected during the years 2006, 2007, 2008 and 2009 were used for the study. The samples were evaporated and the residue was reconstituted in 500 µl of the respective solvent and filtered using 0.22 µm PTFE membrane. 20 µl of the filtered extract was injected for analysis. The secondary metabolite content in the stored roots was analysed.
The retention time and peak area for HPLC are tabulated in Table 4.3.1. Peaks were observed after the 8th minute of the 20 minute run. The number of peaks varied from 6 – 11 for each sample. The peaks are displayed in Plate 4.4. Similar peaks were observed between the samples.

<table>
<thead>
<tr>
<th>( R_f )</th>
<th>Increasing polarity</th>
<th>Decreasing polarity</th>
</tr>
</thead>
</table>

Accurately weighed quantities of the dried 50% alcoholic extracts were dissolved in a known volume of HPLC grade methanol:water (1:1). The solutions were filtered through a Millipore filter (0.45 µm) before injection into the HPLC system. The separation of withanolides was achieved on a RP-18 column using methanol:water (60:40) at a flow rate of 0.5 mL/min. The reasonable difference in the retention times of the marker compounds facilitated quantification of withanolides in samples. The retention times were 21.35 (WS-3), 25.24 (WS-12D), 28.40 (WS-1), and 31.01 min (WS-2), respectively (Khajuria et al., 2004).
Table 4.3.1
Retention time and peak area for HPLC analysis of storage roots

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Storage root (Year)</th>
<th>Retention Time (Mins)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2006, 8.23</td>
<td>5475349</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2007, 8.22</td>
<td>7884320</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2008, 8.26</td>
<td>190258699</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2009, 8.29</td>
<td>848962</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2006, 8.58</td>
<td>2909969</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2007, 8.53</td>
<td>1402220</td>
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<td>7</td>
<td>2008, 8.51</td>
<td>2590517</td>
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<td>8</td>
<td>2009, 8.57</td>
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<td>9</td>
<td>2006, 9.49</td>
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<td></td>
</tr>
<tr>
<td>12</td>
<td>2009, 9.52</td>
<td>3329720</td>
<td></td>
</tr>
</tbody>
</table>

Plate 4- HPLC Analysis of Stored roots
The methanol extracts of finely powdered fresh leaves and roots were further extracted with chloroform. The chloroform fractions were pooled and concentrated to a dry powder. A sample of the dry powder was dissolved in HPLC-grade methanol, filtered through a Millipore and subjected to HPLC analysis. The test set of withanolides consisted of 27-hydroxy withanone, 17-hydroxy withaferin A, 17-hydroxy-27-deoxy withaferin A, withaferin A, withanolide D, 27-hydroxy withanolide B, withanolide A, withanone and 27-deoxywithaferin A, a range that covers not only the major bioactive components (withaferin A, withanolide A, withanolide D), but also some closely related functional variants. Under these HPLC conditions, the mean retention times (Rt) for withanolides, were found to be 15.99, 18.69, 29.34, 32.09, 32.78, 35.39, 36.79, 8.65 and 49.99 minutes respectively (Chaursiya et al., 2007).

Dalavayi et al., (2006) conducted a study on the alcoholic extracts of Withania species. The HPLC chromatogram of standard withaferin-A at an optimum wavelength of 225 nm, showed a mean area of 6127383.6, at a mean retention time of 5.883 min. The HPLC chromatogram of leaf of *W. coagulans* corresponding to standard withaferin-A was showed at a retention time of 5.717 min, with an area of 2816990 at a wavelength of 225 nm. The HPLC chromatogram of root of *W. coagulans* corresponding to standard withaferin-A was showed at a retention time of 5.6 min, with an area of 936833 at a wavelength of 225 nm. The HPLC chromatogram of leaf of *W. somnifera* corresponding to standard withaferin-A was showed at a retention time of 5.771 min, with an area of 1384751 at a wavelength of 225 nm. The HPLC chromatogram of root of *W. somnifera* corresponding to standard withaferin-A was showed at a retention time of 5.451 min, with an area of 537152 at a wavelength of 225 nm. The variation in retention time of peak of withaferin-A in chromatograms of *W. somnifera* and *W. coagulans*, may be due to the presence of other chemical constituents.
5.0 SUMMARY AND CONCLUSION

The results of the study entitled “Analysis of secondary metabolites in the in
vitro, in vivo and stored roots of Withania somnifera” are summarized as follows.

The roots of Withania somnifera were sequentially extracted using solvents of
varying polarity. The extracts obtained were studied to analyse the presence of various
phytochemicals like phenolics, alkaloids, flavonoids, sugars, steroids, proteins,
saponins, quinones and cellulose. Phytochemical analysis was performed and the
presence of flavonoids, alkaloids, sugars, steroids, quinones and cellulose were detected.

Following the phytochemical analysis, thin layer chromatography was
performed for the root extracts. Discrete spots were observed on the plate after
developing with sulphuric acid. The chloroform and ethyl acetate extracts were found to
possess the highest number of spots and hence showed that most of the compounds are
extracted in these two solvents.

The initial phytochemical analysis and thin layer chromatography showed that
the chloroform and ethyl acetate extracts contain more secondary compounds than the
other extracts. Hence for the next phase of the study on storage roots the sequential
extract of the roots were prepared and the chloroform and ethyl acetate extracts were
used for the analysis.

Thin layer chromatography was performed for these extracts which revealed the
presence of discrete spots. Similar spots were observed in all the extracts at Rf values
0.20, 0.50, 0.59, 0.70 and 0.74 whereas certain spots were specific to one extract of a
particular root sample which showed that there exists slight variation in the secondary
compounds which may be due to storage.

The study on storage roots was followed by the analysis of one month old in
vitro and in vivo roots. The sequentially extracted roots were analysed using TLC. The
samples resulted in similar Rf which revealed the presence similar compounds in both
the samples.

To confirm the results obtained using TLC, HPLC was performed for the storage
roots. Separation of compounds was observed in the form of discrete peaks. Similarity
was observed for the samples of the year 2006 and 2008 and certain differences were also noted between the samples from the peaks obtained from HPLC analysis.

The method for sequential extraction was thus standardized, it was concluded that chloroform can be used as the solvent of choice for the extraction of most of the secondary metabolites from *Withania somnifera*. This method can be applied for the isolation and fractionation of withanolides and can also be further extended to analyse the structure of the previously reported compounds and also to determine the structure of novel compounds.


