Comparative Evaluation of Phytochemicals, Method Development and Karyotyping in *in vitro* and *in vivo* tissues of *Withania* species

Poorani, R
(12PBC010)

Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore – 641 043

In Partial Fulfillment of the Requirement for the Degree of

Master of Science in Biochemistry

March, 2014

CERTIFICATE

Comparative Evaluation of Phytochemicals, Method Development and Karyotyping in *in vitro* and *in vivo* tissues of *Withania* species

Poorani, R
(12PBC010)

Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women,
Coimbatore – 641 043

In Partial Fulfilment of the Requirement for the Degree of
Master of Science in Biochemistry

March, 2014

Signature of Head of the Department

Signature of Supervisor

ACKNOWLEDGEMENT

ACKNOWLEDGEMENT

"GOD IS INFINITE, BUT PEOPLE TRY TO COUNT THE LETTERS OF HIS NAME"

- Thomas Szasz

*I humbly placed my profound gratitude to **GOD ALMIGHTY** for everything he has done to me. This work would not have been possible unless He shower His abundant blessings.*

*I extend my sincere thanks to **Thiru T.S.K. Meenakshi Sundaram,** Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for all the good wishes rendered towards the successful completion of the study.*

*I placed on record my heartful thanks to Hon. Colonel. **Dr. Sheela Ramachandran,** Vice Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for the support and encouragement given during the time of study.*

*I expressed my sincere thanks to **Dr. Gowri Ramakrishnan MA.Dip Ed., (chennai), Ph.D (Mother theresa)** Registrar, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for extending all possible support for the successful completion of the study.*

*I wish to express my profound gratitude to Hon. Colonel. **Dr. Saroja Prabhakaran, MA.Dip Ed.,(chennai), Ph.D (Mother theresa)** Former Vice Chancellor, Director of The Hall of Residence, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her constant support and encouragement during the period of study.*

*I record my heartful of thanks to **Dr. A.Parvathi M.Sc., Dip.Ed., M.Phil.,Ph.D(Mathamatics)** Dean, Faculty of Science, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her moral support, motivation and encouragement given throughout the study period.*

*Any attempt at any level cannot be satisfactorily completed without the support and guidance of learned people. I would like to express my immense gratitude to my guide **Dr. Dr. Sivagami srinivasan Ph.D.,**Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for the opulent guidance rendered at every stage of the dissertation. She showed me different ways to approach a research problem and the need to be persistent to accomplish any goal. Without her dynamic guidance, valuable*

suggestions, untiring help, meticulous efforts and enduring support, this study would never have seen the light of the day.

I express my sincere thanks to ***Dr.K.Kalaiselvi Ph.D.,*** *for her constant encouragement throughout my studies and all my* ***Teachers*** *of the Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for their timely help and valuable suggestions rendered during the period of the study.*

I am indebted to all my ***Friends,*** *research scholars Mrs.****D.Pradeepa, Mrs.T.Pankajavalli, Ms. Preethi MP and K.Lavanya, D.Parameshwari,*** *and* ***Lab Assisants*** *for their selfless help, emotional support and constructive suggestions during various stages of my study.*

I often wonder, if one gets to see god in the mortal life they might be like parents who shower their best fortunes always on us. From the deepest depth of my heart to express my thanks, I bow my head to the feet's of my beloved ***Parents, M.Radhakrishnan, R.Dhanam*** *who's uncompromising life principles, love affection has been always unshared and showered upon me at all stages of life and giving me more than what I deserved in my life without her encouragement, co-operation and good wishes this task would have been impossible.*

I owe my deepest gratitude to ***R.Neelaveni, S.Dharani Priya,*** *and all my family members for their loving patience, unconditional support and encouragement to pursue my interests, even when beyond boundaries of language.*

A real friend is one who walks in when the rest of the world walks out*" All glories of this world are not worth a good friend. I deem it a great privilege to thank all* ***my friends*** *and* ***wellwishers*** *for their immense help in times of dire need and for their constant support.*

My thanks to everybody who has been a part of my life but I failed to mention.

Thank you all very much.

POORANI.R

CONTENTS

CONTENT

LIST OF TABLES

LIST OF FIGURE

LIST OF PLATE

LIST OF APPENDICES

INTRODUCTION

1.0 INTRODUCTION

Plants are one of the fascinating natures gift which plays an important role in fulfilling the basic requirements of humans. The contribution of plants to medicinal field have revolutionised this modern era. Currently 80% of the world population depends on plant derived medicine for the first line of primary health care for human ailments because it has no side effect (Patel *et al* 2012).

World Health Organization (WHO) has defined herbal medicines as finished labeled medicinal product that contain active ingredients, aerial or underground parts of the plant or other plant material or combinations (Barad *et al.*,2014). In Indian systems of medicine, a large number of drugs of either herbal or mineral origin have been advocated for various types of diseases and other different unwanted conditions in humans. Ayurvedic medicines are largely based upon herbal and herbo mineral preparations and have specific diagnostics and therapeutic principles.

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

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

The biological activities of withanolides, especially the dominant withaferin-A have been studied extensively and reported from the roots and leaves of both the species of *Withania*. Notable activities reported for this compound, include antiinflammatory, anticonvulsive, antitumor and antioxidant properties (Dalavayi *et al*.,2006).

The evolving of commercial importance of secondary metabolites has in recent years resulted in a great interest in secondary metabolism,particularly in the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology (Vanisree *et al.*,2004).

Withania species grow on a wide variety of drier areas in the sub-tropical and semi-temperate regions, enables the plant to favour different adaptogenic properties. Karyotype analysis was carried out to investigate the cytogenetical information about *Withania* species. It reveals morphologically distinct chromosome types.

With this available information, the present study was formulated with the following objectives:

1. To perform quantitative estimation of selected phytochemicals present indifferent *in vivo* and *in vitro* tissues of *Withania* species.
2. To develop a HPTLC method and quantify withanolide A and withaferin A *in vitro* and *in vivo* tissues of *Withania species* .
3. To perform histological elucidation of *in vivo* and *in vitro Withania coagulans* root powder.
4. To carry out karyotyping in fresh root tips of *Withania* species.

REVEW OF LITERATURE

2.0 REVIEW OF LITERATURE

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. It is necessary to develop methods for rapid, precise and accurate identification and estimation of active constituents or marker compounds as the qualitative and quantitative target to assess the authenticity and inherent quality. Through various analytical techniques like TLC, HPLC and HPTLC we can ascertain the presence of these compounds in plants and also quantify them. HPTLC offers many advantages over other chromatographic techniques such as unsurpassed flexibility (esp. stationary and mobile phase), choice of detection, user friendly, rapid and cost effective. Thus, HPTLC is most widely used at industrial level for routine analysis of herbal medicines (Jirge *et al*., 2011) .

The present work entitled "Comparative Evaluation of Phytochemicals, method Development and Karyotyping in *in vitro* and *in vivo* tissues of *Withania* species **"** is reviewed under the following sub heads:

2.1.Habitat

2.2.Pharmaceutical properties of *Withania* species

2.3.Compounds isolated from *Withania species*

2.4. High Performance Thin Layer chromatography

2.5..Secondary metabolites present in *Withania species*

2.6.Karyotyping

2.1.Habitat

2.1.1.Withania coagulans:

Withania coagulans Dunal is a rigid, gray-whitish small shrub, about 60-120 cm tall. The leaves are about 2.5-7.5 cm long and 1.5 cm broad, usually lanceolate oblong, sometimes ovate, obtuse, narrow at the base and very short stalked. The flowers are about 7-12 mm across, yellowish, and are dioecious and polygamous in nature. The flowers are found in axillary cymose clusters. The berries are about 7-12 mm in diameter, red, smooth and enclosed in leathery calyx. The seeds are dark brown, ear shaped, glabrous with sharp fruity smell.

2.1.1.1.Taxonomy:

Kingdom : Plantae

Division :Magnoliophyta

Class :Magnolipsida

Order :Solanales

Family :Solanaceae

Genus :*Withania*

Species :*W. coagulans*

(Gupta, 2012)

2.1.2.Withania somnifera:

Withania somnifera is a small, woody shrub in the Solanaceae family that grows about two feet in height. It 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.Parts used: Whole plant, roots, leaves, stem, green berries, fruits, seeds, bark are used (Pal *et al*.,2013).

2.1.2.1.Taxonomy:

Kingdom : Plantae

Division : Angiosperm

Class :Dicotyledoneae

Order :Tubiflorae

Family :Solanaceae

Genus :*Withania*

Species :*somnifera* Dunal

(Singh *et al.,2011)*

Table 2.1.3.BOTANICAL DESCRIPTION OF *W.coagulans* AND *W.somnifera* (Jain *et al.*, 2012)

s.no	Description	*Withania coagulans*(Stocks) Dunal	*Withania somnifera*(L.) Dunal
1.	Habit	Herb	Undershrub
2.	English Name	Vegetable Rennet, Indian Rennet	Winter Cherry, Indian Ginseng
3.	Vernacular Name	PanirBandh, Punir, PanirDodi	Ashwagandha
4.	Leaves	Alternate, elliptic lanceolate-coriaceous, obtuse, entire margins, glabrous, coated with minute stellate hairs on both the surfaces	Alternate, broadly ovate, sub-acute, entire margins
5.	Inflorescence	Axillary	Axillary, umbellate cymes
6.	Flowers	Dioecious	Monoecious
7.	Calyx	Campanulate, gamosepalous with 5 sepals clothed with fine stellate grey tomentum	Accrescent, gamosepalous with 5 sepals
8.	Corolla	Campanulate, greenish-yellow with 5 petals	Campanulate, greenish-yellow with 5 petals
9.	Androecium	Anthers long and filamentous in male flowers, smaller in female flowers	Anthers 1.2 mm long, broadly ovate
10.	Gynoecium	Ovary ovoid/globose, without style or stigma	Ovary ovoid/globose, glabrous
11.	Style	Glabrous	Filiform
12.	Stigma	Mushroom-shaped, 2-lamellate	Mushroom-shaped, 2-lamellate
13.	Fruit (Berry)	Globose, smooth, closely girt by the enlarged membranous persistent calyx	Globose, enclosed in the persistent calyx, seeds yellow, reniform
14.	Seeds	Globose, ear shaped, glabrous, enclosed in the persistent calyx yellow, reniform	Globose, enclosed in the persistent calyx, yellow, reniform
15.	Flowering	November-March*	Throughout the year

(Jain *et al.*, 2012)

2.2.Pharmaceutical properties of Withania species

In ancient system of medicine, many plants have been reported to cure various health problems and diseases. *Withania coagulans*(*W. coagulans*) Dual commonly known as 'Indian cheese maker' or 'vegetable rennet' belongs to family Solanaceae and is one of the important medicinal plants. *W. coagulans* Dunal is a gray-whitish small shrub distributed in east of the Mediterranean region extending to South Asia. It is a common medicinal plant in many parts of Pakistan and India. In Ayurvedic system of medicine, *W. coagulans* is widely used in diabetic cases. *W. coagulans* is also used to treat nervous exhaustion, disability, insomnia, wasting diseases, failure to thrive in children and impotence. The fruits of the plant are reported to be sedative, emetic, alterative and diuretic. Further, they are used for liver complaints, asthma and biliousness. The active compounds, in particular, withanolides isolated from the plant are considered to have antimicrobial, anti inflammatory, antitumor, hepatoprotective, anti-hyperglycemic, cardiovascular, immune suppressive, free radical scavenging and central nervous system depressant activities (Gupta *et al*.,2012).

Withania somnifera, a commonly used herb in Ayurvedic medicine. Although the review articles on this plant are already published, this review article is presented to compile all the updated information on its phytochemical and pharmacological activities, which were performed by widely different methods. Studies indicate ashwagandha possesses antioxidant, anxiolytic, adaptogen, memory enhancing, antiparkinsonian, antivenom, antiinflammatory, antitumor properties. Various other effects like immunomodulation, hypolipidemic, antibacterial, cardiovascular protection, sexual behaviour, tolerance and dependence have also been studied. These results are very encouraging and indicate this herb should be studied more extensively to confirm these results and reveal other potential therapeutic effects (Gupta and Rana,2007).

2.2.1. Antimicrobial, Antibacterial and Antifungal

Withaferin A exhibited a significant antibacterial activity against Gram-positive microorganisms at the concentrations 6–100 μg/ml. Antifungal activity of methanolic, petroleum ether and dichloromethane extract of aerial parts of *W.coagulans* against *Aspergillus niger*, *Candida albicans* and *Taeniarubrum* at doses of 12.5–50 μg/ml, 100, 150 and 200 μg/ml(Maurya *etal*.,2010).*Trichodermaviridis,Aspergillusflavus,Fusariumlaterifum,Aspergillusfumigatus,Trich ophytonmentogrophytes*and *Microsporumcanis (*Mughal *et al.*, 2011) has been shown. It was found to be more inhibitory to the filamentous fungi than to the yeast group of fungi.

The volatile oil obtained from fruits of *W. coagulans* had antibacterial activity against *Staphylococcus aureus* and *Vibrio cholerae* and was also found to have antihelmintic activity. 17β-Hydroxywithanolide K exhibited antifungal activity against human pathogens *Nigrosporaoryzae*, *Aspergillusniger*, *Curvularialunata*, *Stachybotrysatra*, *Allescheriaboydii*, *Drechslerarostrata*, *Microsporumcanis* and *Epidermophytonfloccosum* and plant pathogen *Pleurotusostreatus* (minimal inhibitory concentration 300 μg/ml) (Maurya *et al.,* 2010) **.**

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 bacteria. Oral administration of the aqueous fruit extracts successfully obliterated *Salmonella* infection in mice as revealed by increased survival rate, as well as less bacterial load in various vital organs of the treated animals The methanol, hexane and diethyl ether extracts from both leaves and roots of *W. somnifera* were evaluated for the antibacterial /synergistic activity by agar plate disc-diffusion assay against *Salmonella typhimurium* and *Escherichia coli (Jain et al.,2012).*

Antimicrobial property of *Withania somnifera*, in this study the crude extracts of *Withania somnifera* were successively extracted with polar to non polar solvents using soxhlet assembly. The extracts were then screened for their antimicrobial activity *in-vitro* against one gram positive bacteria (*Bacillus subtilis*), two gram negative bacteria (*Pseudomonas aeruginosa* and *Enterobactoraerogens*) and one fungus (*Aspergillus flavus*) by disc diffusion assay. Serial dilution method was used to determine minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration. The chloroform extract of calyx of highest activity against *B. Subtilis* (Singariya *et al., 2012).***.**The antibacterial activities of WSREt, WSFEt and WSLEt were determined using the agar well diffusion method following a published procedure with slight modifications . Briefly, for the evaluation of antimicrobial activities, a fresh 24 hr culture of bacteria was suspended in sterile distilled water to obtain a turbidity of 0.5 McFarland units. The final inoculum size was adjusted to 5×10^5 CFU/ml. Nutrient agar (nutrient broth+ 1.8% agar) was inoculated with the given microorganism by spreading the bacterial inoculum on the media. Wells (8 mm diameter) were punched in the agar and filled with 200 μl of the plant extracts (5 mg/ml). Negative control wells containing neat solvent (80% aqueousmethanol) or a standard antibiotic solution of tetracycline (100 μg/ml) (positive control) were run in parallel on the same plate. The plates were incubated at 37°C for 24 hr. Antibacterial activities were assessed by measuring the diameters of the zones of inhibition for the respective drugs. The relative antibacterial potency of a given preparation was calculated by comparing its zone of inhibition with that of the standard antibiotic tetracycline (Alam *et al.,2012*).

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 possessed maximum inhibitory activity against a spectrum of bacteria including *Salmonella typhimurium*. Moreover, in contrast to the synthetic antibiotic (viz. chloramphenicol), these extracts did not induce lysis on incubation with human erythrocytes, advocating their safety to the living cells. 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 (owais et al.,2005). In another study, the methanol, hexane and diethyl ether extracts from both leaves and roots of *Withania somnifera* were evaluated for the antibacterial/synergistic activity by agar plate disc-diffusion assay against *Salmonella typhimurium* and *Escherichia coli.* Different concentrations of Tibrim, a combination of rifampicin and isoniazid, were tested to find out the minimum inhibitory concentration (MIC), which came out to be 0.1 mg/ml for *S. typhimurium* and *E. coli*. From the six extracts tested, only methanol and hexane extracts of both leaves and roots showed potent antibacterial activity. A synergistic increase in the antibacterial effect of Tibrim was noticed when MIC of Tibrim was supplemented with these extracts (Gupta and Rana, 2007).

2.2.2.Antioxidant effect:

The importance of traditional medicinal plants is increasing now a days because of various advantages over the synthetic drugs. *Withania coagulans* Dunal was studied for its antioxidant activity. Various physicochemical parameters were studied such as ash value, total ash, acid insoluble ash, water soluble ash, sulphated ash alcohol soluble extractive value etc. Various qualitative phytochemical tests were done for the presence of alkaloids, glycosides, carbohydrates, flavonoids etc. It was observed that 50% ethanol extract of *Withania coagulans* contains carbohydrates, proteins, glycosides, steroids and sterols, anthraquinones and triterpenoids. The antioxidant activity of *Withania coagulans* Dunal was studied by DPPH and Nitric oxide method and it was observed that it has antioxidant activity. It showed more activity in DPPH method than Nitric oxide method. Preliminary Phytochemical study of 50% ethanolic extract of the root parts is found to contain carbohydrates, protein, some steroids, anthraquinone, flavonoids, tannin, phenolic compounds and triterpenoids are present. The antioxidant activity was determined and the plant extract showed low activity nitric oxide free radical inhibition method and moderate activity by DPPH method. The activity was compared with rutin and ascorbic acid (Singh *et al.,2012*)

The antioxidant capacities of the plant extracts were analyzed using free radical scavenging activity (DPPH), Reducing power, Competition of test fraction with DMSO, etc. The DPPH test is the oldest indirect method for determining the antioxidant activity based on the ability of free radical 2, 2–diphenyl-1- picrylhydrazyl to react with hydrogen donors react with phenol. The fractions isolated from *Withania somnifera* powder having ability to scavenge the free radical and useful to cure diabetes to some extent, all the fractions isolated from *Withania somnifera* Dunal. have antioxidant activity (Ahmed *et al.,2013).*

2.2.3.Anti Inflammatory activity:

The alcoholic extract of *Withania coagulans* showed significant anti-inflammatory effect in acute inflammation induced with egg albumin. Withanolide A from *Withania coagulans* showed significant anti-inflammatory effects in acute inflammation. The hydro alcoholic extract of *Withania coagulans* fruits showed significant anti-inflammatory activity in carragenin induced rat paw oedema model (*Gupta et al.,2013*).

Inflammation is a complex process occurring through a variety of mechanisms, leading to changes of local blood flow and the release of several mediators. Arachidonic acid serves as a substrate for cyclooxygenase which catalyzes the production of Prostaglandins, Thromboxane and Prostacyclin. Cyclooxygenase-1 (COX-1) and Cyclooxygenase-2 (COX-2) enzymes are responsible for the conversion of Arachidonic acid. Prostaglandins in turn, cause inflammatory responses in the body. The anti-inflammatory activity of withanolides was assessed in vitro using COX-1 and COX-2 inhibitory assays (Wube *et al*., 2008).

Research has explored the capacity of Ashwagandha to ease the symptoms of arthritis and other inflammatory conditions These studies have proven that the herb acts as an effective anti-inflammatory agent. Its naturally occurring steroidal content is much higher than that of hydrocortisone, a commonly-prescribed anti-inflammatory . The effectiveness of Ashwagandha in a variety of rheumatologic conditions may be due in part to its anti-inflammatory properties. Rats given powdered root of *Withania somnifera* orally one hour before being given injections of an inflammatory agent over a three day period showed that Ashwagandha produced anti-inflammatory responses comparable to that of hydrocortisone sodium succinate (Singh *et al.,2010*).

2.2.4.Immunomodulatory properties:

Withania coagulans Dun. (Stocks.)Vegetable Rennet. (Fam. Solanaceae.). This shrub has the property of coagulating milk, and has been used for preparing vegetable rennet ferment for making cheese. Six new withanolides, withacoagulins A-F (1-6, resp.), together with ten known withanolides, 7-16, were isolated from the aerial parts of *Withania coagulans*. Their structures were determined by spectroscopic techniques including 1D- and 2D-NMR (1H, 13C, HMQC, and HMBC) and MS experiments. These compounds, including the crude extracts of this herb, exhibited strong inhibitory activities on the T- and B-cell proliferation. From, that the results showed *Withania coagulans* has a immunosuppressive activity (Kumar *et al*.,2012).

Asgand showed a significant modulation of immune reactivity in animal models. Administration of Asgand was found to prevent myelo-suppression in mice treated with three immunosuppressive drugs viz. cyclophosphamide, azathioprin, and prednisolone. Treatment with Asgand was found to significantly increase Hb concentration, RBC count, platelet count, and body weight in mice. Administration of Asgand extract was found to significantly reduce leucopenia induced by cyclophosphamide (CTX) treatment. Administration of Asgand extract increased the number of β-esterase positive cells in the bone marrow of CTX treated animals, compared to the CTX alone treated group. Administration of Asgand extract was found to significantly reduce leucopenia induced by sub-lethal dose of gamma radiation. Withaferin A and Withanolide E exhibited specific immunosuppressive effect on human B and T lymphocytes and on mice thymocytes. Withanolide E had specific effect on T lymphocytes whereas Withaferin A affected both B and T lymphocytes (Pal *et al*.,2013)

2.2.5.Antitumor:

The genotoxic nature of any herbal drug is determined on the basis of presence of phytoconstituents. *W. coagulans* contains withanolides, which are reported for antitumor activity (Gupta, 2012)

Swiss albino mice fed a 2.5- and 5.0-percent 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. In this study, *Withania somnifera* did not up- or down-regulate phase I or p450 enzymes. This feature makes *Withania somnifera* compatible with other medications, since it is not likely to affect the half-life of pharmaceutical drugs. *Withania*

somnifera may also mitigate unregulated cell growth via the potent tumor suppressor gene p53, which regulates cell cycle proliferation. In research by Mathur *et al*,21 cells from Wistar rats exposed to UV β radiation demonstrated clusters of mutated p53 proteins, a precursor to carcinogenesis. Pretreatment of an extracted constituent of WS, 1-oxo-5β,6β-epoxy- with a-2-enolide, at 20 mg/kg body weight IP for five days prior to irradiation and 12 weeks following, resulted in no mutant p53 foci. These animals showed normal dermis and skin tissue, without evidence of necrosis or carcinogenesis, suggesting a possible role for *Withania somnifera* in conjucation with radiation (Padmavathi *et al*.,2005).

2.2.6.Hepatoprotective activity:

Protective effect of 3β-hydroxy-2,3-dihydro Withanolide F isolated from *W. coagulans* was tested against CCl_4-induced hepatotoxicity, and the compound was found to possess marked protective effect. A comparison of the protective properties showed that it is more active than hydrocortisone on a weight basis(Gupta, 2012).Withaferin A at 10mg/kg dose showed significantly protective effect against CCl_4-induced hepatotoxicity in rats. It was as effective as hydrocortisone dose (Singh *et al*.,2012).

P. Subramanian *et al.* investigated the influence of *W.somnifera* root powder on the levels of circulatory ammonia, urea,lipid peroxidation products such as TBARS (thiobarbituric acid and reactive substances), HP (hydroperoxides) and liver marker enzymes such as AST (aspartate transaminase), ALT (alanine transaminase) and ALP (alkaline phosphatase), for its hepatoprotective effect in ammonium chloride induced hyperammonemia. Ammonium chloride treated rats showed a significant increase in the levels of circulatory ammonia, urea, AST, ALT, ALP, TBARS and HP. These changes were significantly decreased in rats treated with *W.somnifera* root powder and ammonium chloride. Our results indicate that *W.somnifera* offers hepatoprotection by influencing the levels of lipid peroxidation products and liver markers in experimental hyperammonemia and this could be due to (i) the presence of alkaloids, withanolides and flavonoids, (ii) normalizing the levels of urea and urea related compounds, (iii) its free radical scavenging property and (iv) its antioxidant property. The exact underlying mechanism is still unclear and further research needed (Subramanian *et al.,2008*).

2.2.7.Hypolipidemic:

Administration of an aqueous extract of fruits of *W. coagulans* to high fat diet-induced hyperlipidemic rats for 7 weeks significantly reduced elevated serum cholesterol, triglycerides and lipoprotein levels. This extract also showed hypolipidemic activity in triton induced hypercholesterolemia (Jain *et al*.,2012)

The aqueous extract of fruits of *W. coagulans* (1 g/kg) showed 15% reduction in serum cholesterol level in Triton-induced hyperlipidaemic rats. In rats with a high fat diet-induced hyperlipidaemic, the aqueous extract at the same dose administered for seven weeks showed a significantly reduced body weight, elevated serum cholesterol, triglycerides and lipoprotein levels (Maurya et *al*., 2010)

The aqueous extract of *Withania coagulans*(1,000 mg/kg/day), was reported to have anti-atherosclerotic activity in normal and streptozotocin (STZ)-induced diabetes (Saxena., 2010**).**The extracted coagulin L from fruits of *W. coagulans* has antidyslipidemic effect on mice (Maurya *et al*., 2008). Hoda *et al* (2010) showed the aqueous and chloroform extracts of the fruits decreased triglyceride, total cholesterol, LDL and VLDL increased the HDL levels.

Withania somnifera root powder decreased total lipids, cholesterol and triglycerides in hyper cholesteremic animals. On the other hand, significantly increased plasma HDL-cholesterol levels, HMG-CoA reductase activity and bile acid content of liver. A similar trend also reported in bile acid, cholesterol and neutral sterol excretion in the hypercholesteremic animals with *Withania somnifera* administration. Further, a significant decrease in lipid-peroxidation occurred in *Withania somnifera* administered hypercholesteremic animals when compared to their normal counterparts. However, *Withania somnifera* root powder was also effective in normal subjects for decreasing lipid profiles (Visavadiya *et al.,2006*). In another study with aqueous extract of fruits of *Withania coagulans* to high fat diet induced hyperlipidemic rats for 7 weeks, significantly reduced elevated serum cholesterol, triglycerides and lipoprotein levels. This drug also showed hypolipidemicactivity in triton-induced hypercholesterolemia. The histopathological examination of liver tissues of treated hyperlipidemic rats showed comparatively lesser degenerative changes compared with hyperlipidemic controls. The hypolipidemic effect of *Withania coagulans* fruits reported to be comparable to that of an Ayurvedic product containing Commiphoramukkul ((Hemalatha et al., 2006). In another study, hypoglycemic, diuretic and hypocholesterolemic effects of roots of WS were assessed on human subjects. Six mild NIDDM subjects and six mild hypercholesterolemic subjects were treated with the powder of roots of WS

for 30 days. Suitable parameters were studied in the blood and urine samples of the subjects along with dietary pattern before and at the end of treatment period. Decrease in blood glucose was comparable to that of an oral hypoglycemic drug. Significant increase in urine sodium, urine volume, significant decrease in serum cholesterol, triglycerides, LDL (low density lipoproteins) and VLDL (very low density lipoproteins) cholesterol were observed indicating that root of WS is a potential source of hypoglycemic, diuretic and hypocholesterolemic agents (Gupta and Rana ,2007).

2.3.Compounds isolated from *Withania species:*

Withania has received much attention in recent years due to the presence of large number of alkaloids and steroidal lactones known as withanolides. Withanolides are naturally occurring polyhydroxy C_{28} steroidal lactones. In the basic structure of all withanolides a six- or five-membered lactone or lactol ring is attached to an intact or rearranged ergostane skeleton (Maurya *et al.,* 2010). The term "withanolide" is a structural term that has been used for "withan" from the genus Withania, and "olide" is chemical term for a lactone. To this date, about 400 witha-nolides or closely related congeners have been discovered in altogether 58 solanaceous species belonging to 22 genera (Eich, 2008).

Basically there are two major groups of withanolides as follows:

A- Withanolides with an unmodified skeleton

a) With a regular β -oriented side chain

b) With an unusual α -oriented side chain.

B- Withanolides with modified carbocyclic skeletons or side chains (Glotter 1991).

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

Withanolides possess antitumor, antibacterial, anti-inflammatory and immunosuppressive properties (Neogi *et a*l, 1988). This class of compounds does not occur in all members of the Solanaceae family. However, the occurrence of withanolides is not restricted to Solanaceae. They have also been reported from marine organisms (soft corals) and from members of plant families Taccaceae and Leguminoseae.

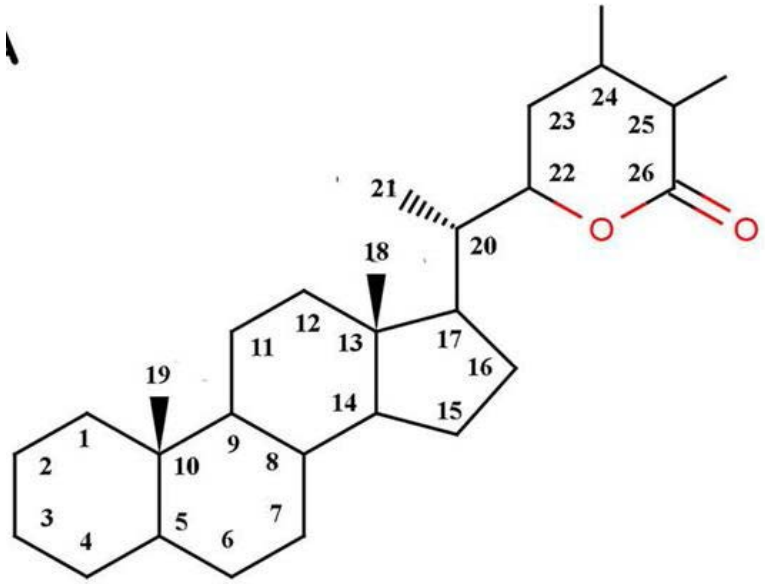


Fig 2.3.1 Chemical structure of Withanolide

Three withanolides, vizcoagulins P, Q and R, were isolated from *W.coagulans* (Rahman *et al.,* 1999). Coagulin-H isolated from W.coagulans has been identified to have immunosuppressive property (Mesaik *et al*., 2006).

One new withanolide, (17*S*, 20S, 22R)-14α, 15α, 17β, 20β-tetrahydroxy-1-oxowitha-2, 5, 24-trienolide) named coagulanolide along with four known withanolides have been isolated from *Withania coagulans* fruits and their structures were elucidated by spectroscopic techniques. (Maurya *et al.,* 2010). Different withanolides, withacoagin and coagulans reported from *W. coagulans*. Withaferin A (Steroidal lactones of withanolide series) had been isolated from fruits of *W. coagulans* (Khare, 2007).

Withanolides present in *withania spp.* is the main problem in analysis and isolation of these metabolites. The root extract of this species has recently been accepted as a dietary supplement in the United States. Harvesting roots is destructive for the plants and hence there is a growing interest in root culture as an alternative source for this important metabolite. Several properties of Withaferin A have been reported: antiangiogenesis through NF- κB inhibition;

cytoskeletal architecture alteration by covalently binding annexinII and apoptosis induction through the protein kinase C pathway in leishmanial cells . The primary molecular target of Withaferin A was shown to be the ß5 subunit of the proteosome (Yang *et al*., 2007). In view of these wide-ranging pharmacological activities, who have studied the production of withaferin A by plant tissue culture technique. Since many previous studies have been reported about withanolides production in tissue culture of the common species *W. somnifera* (Ciddi, 2006; Sharada *et al*., 2007; Padhyay *et al*., 2007; Sangwan *et al*., 2007), Withaferin A production was studied by the *in vitro* cultured roots of *W. coagulans*. These are the roots excised from *W. coagulans* sterile plantlets and continuously cultured on indole-3-butyric acid containing media. Production of secondary metabolites in tissue cultures is usually higher when plant cells are organized into tissues/organs. The expression of secondary metabolic pathways in organized cultures is not surprising because it mimics exactly what the plant does. Root cultures are typical examples that can be used for production of phytochemicals. Root cultures have been used as standard experimental system in studies of inorganic nutrition, nitrogen metabolism, plant growth regulation, and root development. However, the relatively slow growth remains the main disadvantage of this system (Abuzid *et al*.,2010)

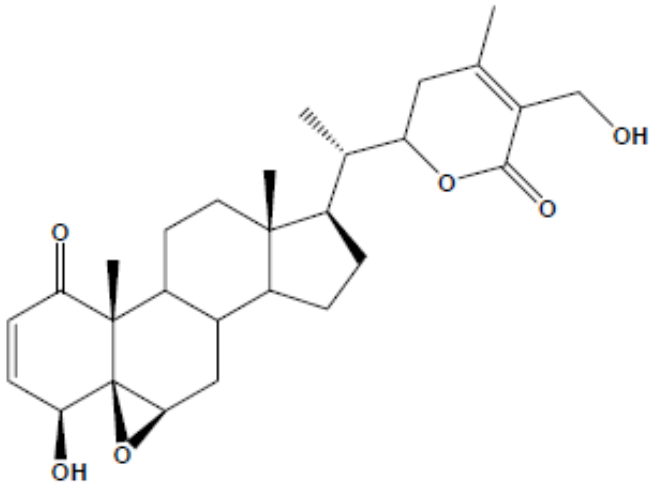


Fig 2.3.2 Chemical structure of withaferin-A

Withacoagin- a withanolide was reported to be isolated from dried root extracts of *withania coagulans* and structure elucidated as (20R, 22R)-5α, 20-dihydroxy- 1-oxowitha-2, 6, 24-trienolide by spectral analysis and chemical transformation and studied for their anti-tumor activity (Neogi *et al.*, 1988).

Six Withanolides, Withacoagulins A-F (1-6, resp.), together with ten known withanolides, 7-16, were isolated from the aerial parts of Withaniacoagulans. Their structures were determined by spectroscopic techniques including 1D- and 2D-NMR (1H, 13C, HMQC,

and HMBC) and MS experiments. These compounds, including the crude extracts of this herb, exhibited strong inhibitory activities on the T- and B-cell proliferation. (Huang *et al.,* 2009).

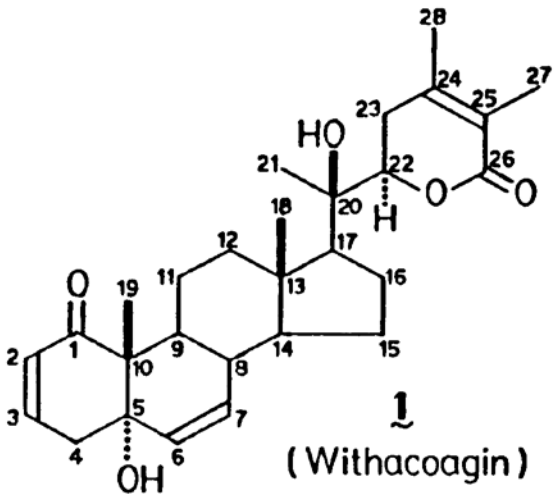


Fig 2.3.3

Phytochemical examination of the whole plant resulted in the isolation of 25 compounds, including 24 withanolides and one dimericlignan, bispicropodophyllinglucoside. In total nine compounds have been isolated from the fruits of *W. coagulans*, including ergosta-5, 25-diene-3β, 24ξ-diol and sitosterol-β-d-glucoside along with withanolides. Five withanolides have been isolated from the root of this plant.

2.4. High Performance Thin Layer chromatography

High Performance Thin Layer Chromatography (HPTLC) is a powerful method equally suitable for qualitative and quantitative analytical tasks. Applications of HPTLC, such as identification and quantitation of constituents, impurities, active substances, process development and optimization, process monitoring, and cleaning validation have been demonstrated (Patel *et al*.,2012)

Sharma *et al* and Nayak *et al* (2009) reported a simple, sensitive and accurate high performance thin layer chromatographic (HPTLC) method for the estimation of withaferin-A and withanolide-A in different plant parts such as, leaf, root, stem and fruit of two morphotypes of *Withania somnifera*. HPTLC of *W.somnifera* methanolic extract was performed on Si 60 F_{254} (20 cm × 20 cm) plates with toluene:ethylacetate:formic acid (5:5:1), as mobile phase. Quantitative evaluation of the plate was performed in the absorption-reflection mode at 530 nm. The method was validated for precision, repeatability, and accuracy. The average recovery of withaferin-A and withanolide-A in two levels were 96.0 and 96.7%, showing the excellent reproducibility of the method. Through various analytical techniques like TLC, HPLC and

HPTLC we can ascertain the presence of these compounds in plants and also quantify them. Densitometric HPTLC has been widely used for the phytochemical evaluation of the herbal drugs (Rakesh *et al*., 2009).

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

2.5.Secondary metabolites present in *Withania species:*

The chemistry of *Withania* species has been extensively studied and several groups of chemical constituents such as steroidal lactones, alkaloids, flavonoids, tannin etc. have been identified, extracted, and purified. At present, more than 12 alkaloids, 40 withanolides, and several sitoindosides (a Withanolide containing a glucose molecule at carbon 27) have been isolated and reported from aerial parts, roots and berries of *Withania* species (Mirjalili *et al.*, 2009).

2.5.1 Alkaloids

In the present study, the phytochemical and antioxidant properties of methanolic and aqueous extracts of fruits of *Withania coagulans*were compared and investigated to find out the number of different phytoconstituents present in the plant which makes it remarkable for its use by traditional practitioners. Phytochemical screening of both the extracts showed the presence of alkaloids (Mathur *et al.,2011*)

Roots of *W.somnifera* contain several alkaloids of medicinal value. These include 13 Dragendroff-positive compounds. The various alkaloids include withanine, somniferine, somnine, somniferinine, withananine, psuedo-withanine, tropine, psuedotropine, 3-α-gloyloxytropane, choline, cuscohygrine, isopelletierine, anaferine and anahydrine (Elsakka *et al.*, 1990).

2.5.2 Saponins

In the present study, the phytochemical and antioxidant properties of methanolic and aqueous extracts of fruits of *Withania coagulans* were compared and investigated to find out the number of different phytoconstituents present in the plant which makes it remarkable for its use by traditional practitioners. Phytochemical screening of both the extracts showed the presence of saponin. Further study showed high in vitro antioxidant activity in both of the extracts when compared to standard ascorbic acid but aqueous extracts showed higher antioxidant potential in comparison to that of methanolic extract (Mathur *et al.,2011*).

Saponins are a class of high molecular weight secondary metabolites containing glycosylated triterpenoids or steroidal alkaloids found in many plant species (Hostettmann and Marston, 1995; Morrissey and Osbourn, 1999). They are mainly characterized by an oligosaccharide chain, consisting of sugars attached at the C-3 position. Some saponins also have a glucose chain attached at C-26 or C-28 positions. Saponins are important components of a number of herbal medicines (Mishra and Simon, 2000; Chan *et al.,* 2002; Xu, 2001). Saponin present in *W.somnifera* includes sitoindoside VII and VIII that contain an additional acyl group and sitoindoside IX and X which are withanolides with a glucose at carbon 27 (Elsakka *et al*., 1990).

2.5.3 Flavonoids and Phenols

Withania coagulans and *Psidiumguajava*are more soluble in water. It gives more yield than other solvent. The Preliminary Chemical Examination shows the presence of Steroids, Saponin, ,phenol, Flavonoids, glucoside and Tannins in various extracts (Barad *et al*.,2014)

Kandil *et al.,* (1994) identified flavonoids and phenolic compounds namely 6, 8-dihydroxykaempferol, 3-0-rutinoside quercetin, 3-0-rutinoside 3-0-rutinoside-7-O-glucoside quinic acid, 4-0-caffeoylquinic acid and 4, 5-O-dicaffeoylquinic acid from the aqueous ethanolic extract of *W.somnifera* leaves.

2.5.4.Steroids:

The 50% ethanol extract root of *Withania coagulans* was subjected to preliminaryphytochemical studies. It has been observed that 50% ethanol extract of *Withania coagulans* contains carbohydrates, proteins,glycosides,steroids and sterols, anthraquinones andtriterpenoids (Singh *et al*.,2012).Phytochemical tests on Ashwagandha was done andsteroids, alkaloids, saponins were found to be present (Kushwaha *et al*.,2011).

2.6.Karyotyping:

Withania somnifera recently categorized as threatened plant of Rajasthan and known for its high medicinal value has been studied for existing genetic variations in four accessions collected from four different locations. From the present studies it is apparent that the gametic number of *Withania somnifera* is n = 24 and 2n = 48. From the combined information generated by present author and earlier workers, it is tentatively believed that *Withania somnifera* might be of allopolyploid origin with basic number of x = 12. In this case also precocious separation of rod bivalents but not structural hetrozygosity might be the reason for occurrence of univalents. From the detailed meiotic analysis, it is concluded that the species diversification is brought out by polyploidy in *W. somnifera*. Multidisciplinary approach for genome diagnostics with use of various marker systems might through more light on existing intra-specific genetic variation in *Withania somnifera* (Ram *et al*.,2012)

Root powder staining was carried out in order to find out any internal structures are present (Khandelwal, 2005).

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

The various materials used and experimental techniques adopted for the present study entitled " Comparative Evaluation of Phytochemicals , method development and Karyotyping in *in vitro* & *in vivo* tissues of *Withania* species"are described below.

3.1 MATERIALS:

3.1.1 Plant material

3.1.2 Chemicals & Equipments.

3.2 METHODS:

3.2.1 Extraction of secondary metabolites.

3.2.2 Quantitative evaluation of selected Phytochemicals in the tissues of *withania* species

3.3.3 HPTLC method development and quantification of withanolide and withaferin.

3.3.4 Histological Elucitation.

3.3.5 Karyotyping.

3.1 MATERIALS

3.1.1 Plant material

Dried root,stem,leaf samples of *Withania coagulans* from Varanasi ,imported Iran variety and JA134 variety of *Withania somnifera* were used.

S.NO	SPECIES	SAMPLE	*In vitro*	*In vivo*
1.	*Withania somnifera*	Root	30days	180 days
		Leaf	60 days	180 days
		Stem	60 days	180 days
2.	*Withania coagulans*	Root	60 days	Imported Iran variety
		Root	120 days	-
		Leaf	60 days	-
		Stem	60 days	-

3.1.2 Chemicals and Equipments

Himedia, Merck, Rankem chemicals were used for the entire study. HPTLC was performed on precoated Silica gel aluminum 60 F254 plates (E.MERCK, Germany) in a Semiautomatic CAMAG Linomat5 device. Colorimeter(ELICO) and spectrophotometer (Thermaspectronic,model Genesys 6) were used in the quantitative analysis to measure the absorbance of the products. Motic microscope (Motic,model B1-223 ASC) was used for karyotyping and histological elucitation.

3.2 METHODS:

3.2.1 Extraction of secondary metabolites from *Withania* species:

Fresh tissue samples were collected and kept in oven at 55^0c for overnight. The dried root, stem, leaf samples were ground well individually using pestle and mortar. 1g of tissues were extracted using methanol solvent. The extraction was carried out for four times using methanol solvent. (Patel *et al*., 2009).

Initially 1.0g of 120 days and 60 days old *Withania coagulans in vitro* root, 1g of 60 days old both *W.coagulans* and *W.somnifera in vitro* Stem and leaf, 1g of 30 days old *in vitro W.somnifera* root, *in vivo W.coagulans* root from Iran and 180 days old *W.somnifera in vivo* root.leaf,stem were weighed and treated with 1ml ammonia respectively for 20 mins .It was then sonicated for 20 min with 50 ml of the solvent and placed in a shaker for 2 hours at 104 rpm at 22^0C. At the end of 2hrs the extract was filtered using Whatmann no: 1 filter paper and the residue were again treated with 50 ml of the solvent. This step was repeated four times to obtain 200ml of the extract. These extracts were then concentrated by evaporation using a flash evaporator maintained at 45^0C and 150rpm. The solvent was evaporated and the residue was collected using HPLC graded methanol (Patel *et al*., 2009).

For quantitative analysis, the above protocol Patel *et al*., 2009 was followed for the extraction of *withania* samples.

3.2.2 Quantitative evaluation of selected Phytochemicals in the tissues of *Withania* species:

3.2.2.1 Estimation of flavonoids

To estimate the flavonoids present in 1g of *Withania coagulans and Withania somnifera* root, stem, leaf samples, and 0.5ml of extract was pipetted into test tube and the estimation was carried out by following the procedure of Hossain *et al*., 2011 (Appendix 1)

3.2.2.2 Estimation of saponins

The amount of saponins present in 1g of *Withania coagulans and Withania somnifera* root, stem, and leaf was calculated by following the estimation procedure by Bucco *et al*., 1977 (Appendix 2)

3.2.2.3 Estimation of alkaloid:

The amount of alkaloids present in 1g of *Withania coagulans and Withania somnifera* root, stem, and leaf was calculated by following the estimation procedure by Muthumani *et al.*, 2010 (Appendix 3)

3.2.2.4 Estimation of total steroids:

The amount of steroids present in 1g of *Withania coagulans and Withania somnifera* root, stem, and leaf was calculated by following the estimation procedure by Wall *et al*.,1952 (Appendix 4)

3.2.2.5 Estimation of total phenol:

The amount of total phenol present in 1g of *Withania coagulans and Withania somnifera* root, stem, and leaf was calculated by following the estimation procedure by Sadasivam & Manickam 1996 (Appendix 5)

3.2.3 HPTLC Method development and quantification of withanoloide and withaferin

The High Performance Thin Layer Chromatography analysis was carried out on 10x10cm precoated silica gel aluminum plate 60F254 (E.MERCK, Germany). The methanol extract of the samples were applied to the plates as 6mm bands, under a stream of nitrogen, by means of a CAMAG (Switzerland) Linomat V semiautomatic sample applicator fitted with a 100µl Hamilton HPTLC syringe. Linear ascending development to a distance of 8cm was carried out on 10x10cm twin trough chamber saturated for 30mins at room temperature(25ºC±2) with 20ml

of the mobile phase(three different mobile phases were used),the plates were dried in a current of air with the help of an air dryer. The banding patterns were visualized in 254nm, 366nm and white light and Densitometric scanning was performed with Camag TLC scanner III in the reflectance –absorbance mode at 540nm after spraying with either 5% Methanolic-Sulphuric acid or Anisaldehyde Sulphuric acid and operated by Win CATS software (1.3.0 Camag) (Jirge*et al*, 2011). The developed chromatograms were then compared on the basis of the intensity of spots obtained to finalise the best mobile phase for the development of chromatograms and the best derivitization agents to increase the intensity of the spots. These results were then applied to prepare a final HPTLC fingerprint containing *in vitro* leaf,stem,2nd ,3rd month old root and *in vivo* root (Iran)of *Withania coagulans* samples and also the *in vitro* and *in vivo* leaf, stem, root sample of and *Withania somnifera* were used. The final chromatogram was then observed to find the sample having maximum phytoconstituents by observing the spots obtained. The plates were then scanned and the peaks were analyzed to estimate the amount of withanolide and withaferin in each of the samples.

Different Mobile Phases Used

S.NO	MOBILE PHASES
1.	Toluene :Ethyl acetate:Formic acid(5:5:1)
2.	Chloroform:Methanol(9:1)
3.	Chloroform: Ethyl acetate: Methanol: Toluene(11.1:0.6:1.2:4.5)

3.2.4 Histological Elucitation:`

Finely ground powders of *coagulans* samples were used for staining the granules. Finely divided trace amount of sample was placed on a slide and placed a drop of staining solution specific for the compound to be stained. The sample was then smashed by placing a cover slip above the sample and applying pressure over it by rolling with a glass rod. The staining solution used depends on the compound to be stained. Starch granules were stained using iodine solution (Rinne *et al.,* 1994), alkaloids were stained using wagner's and dragendroff's reagent (Corsi *et al.,* 1998),saponins are stained using Para formaldehyde reagent, terpenoids are stained using 2,4 dinitro phenyl hydrazine(Mace *et al.*,1974) and p-dimethyl amino cinnamaldehyde (DMACA) was used for flavonoids (Feucht *et al.*,1993).

The stained material was observed under the motic microscope using which the granules were observed and the image was then captured using the motic software and saved.

3.2.5 Karyotyping:

RESULTS AND DISCUSSION

Root tips were excised from *W.coagulans* kept indoors in sunlight. The root tips collected were pretreated with 3ml of Isopropyl alcohol and 1ml of acetic acid for 2 hours. The root tips were hydrolysed in 50% HCl at room temperature for 2 seconds and stained with 1% aceto carmine.The material was then squashed and examined under motic microscope.Photographs were taken. A composite image was developed for most mitotic cells counts were made by printing digital images and assigning a number to each chromosome observed in the image. (Damayanti *et al*.,2010)

4.0 RESULTS 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, 2011).

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, 1991). Among the twenty-three known species of *Withania,* only two *W. somnifera* and *W. coagulans,* are economically significant and widely cultivated (Mirjalili *et al*.,2009).

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, physico-chemical characteristics and HPTLC profiling.

With the above knowledge, i.e importance of standardization of medicinal plants and the various techniques involved in it, the present study was focused in this aspect and the result were discussed in this chapter under the following headings:

4.1 Quantitative evaluation of selected Phytochemicals in the tissues of *Withania* species

4.2 HPTLC method development and quantification of Withanolide and Withaferin.

4.3 Histological Elucidation of *in vivo* and *in vitro W.coagulans* root powder.

4.4 Karyotyping.

4.1 Quantitative evaluation of selected Phytochemicals in the tissues of *Withania* species

The phytochemistry of *Withania* species has been studied extensively by several workers and several groups of chemical such as steroidal lactones, alkaloids, flavonoids, tannin etc. have been identified, extracted, characterized and isolated (Rahman *et al.,* 1993).

All plant parts synthesize some chemical themselves to perform their physiological activities. The medicinal value of these secondary metabolites is due to the presence of chemical substances that produce a definite physiological action on human body (Kubmarawa *et al*., 2008). The most important of these bioactive compounds are alkaloids, flavonoids, tannins and phenolic compounds (Duraipandiyan *et al.,* 2006).

Knowing this matter of concern, quantitative estimation of total flavanoid, saponin, alkaloids, total steroids, total phenol were analyzed on root, stem and leaf tissues of *in vitro* and *in vivo W.somnifera,*008 *in vivo* root variety of *W.cogulans* , *in vitro* root (60 and 120 days old),stem and leaf tissues of *W.coagulans* were evaluated.

4.1.1 Estimation of total flavanoids

The flavanoid content of the samples were estimated by $AlCl_3$ method and the results revealed that the flavanoid content in all samples ranged from 0.78- 28.05mg/g of tissue samples (table 4.1.1). *W.coagulans* root (008) had the least flavanoid content of about 0.78±0 mg/g dried root powder. The highest amount of flavanoid (28.05±0 mg/g) was found in *in vivo* leaf sample of *W. somnifera* and is obvious from Fig 4.1.1.

4.1.2 Estimation of saponin

The amount of saponin in the sample was estimated using Diosgenin standard as proposed by Bucco *et al* (1977),Uematsu *et al (*2000). The estimation of saponin showed that the samples varied in their saponin content from 3.3 to 26.28 mg/g of tissue samples , revealed from table 4.1.1.The least amount of saponin was found in *in vivo W.somnifera* root (3.34±0.15mg/g

of root sample).The highest saponin content (26.28±0.08 mg/g of root powder) was found in 60 days old *in vitro W.coagulans* root (Fig 4.1.2).

4.1.3 Estimation of alkaloids

The alkaloid content of the samples were estimated and the results revealed that the alkaloid content in all samples of *Withania* species ranged from 15.8-180.8 mg/g of dried tissue samples (table 4.1.1). *W.somnifera in vitro* root had the low amount of alkaloid content of about 15.88±0 mg/g dried root powder. The highest amount of alkaloid (180.84±0 mg/g of dried leaf tissue) was found in *in vitro* leaf sample of *W. somnifera (*Fig 4.1.3 *).*

4.1.4 Estimation of total steroids

The total steroid content of the samples were estimated by LB method and the results revealed that the total steroid content in all samples of *withania* species ranged from 4.13-28.08 mg/g of tissue samples (table 4.1.1). *W.coagulans* root (008) had the least flavanoid content of about 4.13±0.36 mg/g of dried root powder. The highest amount of steroid (28.08±0mg/g of dried stem tissue) was found in *in vitro* stem sample of *W. coagulans* (Fig 4.1.4).

4.1.5 Estimation of total phenol

The amount of total phenol in the sample was estimated using catechol standard as proposed by Sadasivam & Manickam (1996). The estimation of total phenol showed that the samples varied in their total phenol content from 0.8-2.75mg/g of tissue samples (table 4.1.1). The least amount of total phenol was found in *in vitro* 120 days old *Withania coagulans* root (0.88±0.04 mg/g of root dried powder). The highest total phenol content (2.75±0 mg/g of dried leaf tissue) was found in *in vivo* leaf sample of *Withania somnifera.*

The variation in amount of different phytochemicals in different samples may be due to the influence of the environmental condition they were grown on. There is a wide variation in the amount and type of chemical constituents in samples of different species, in samples that differ in method and time of collection (ICH Harmonised Tripartite Guidelines, 1996).

Table 4.1.1 QUANTITATIVE ANALYSIS OF PHYTOCHEMICALS

(Expressed in mg/g)

S.No	SAMPLE	TOTAL FLAVANOIDS	SAPONINS	ALKALOIDS	TOTAL STEROIDS	TOTAL PHENOLS
	In vitro					
1.	W.c root (120 Days)	2.92±0.16	**11.70±0.63**	16.99±0	9.49±0	0.89±0.04
2.	W.c root (60 days)	9.32±0	26.28±0.08	55.03±2.93	7.71±0.35	0.93±0
3.	W.c Leaf	16.55±0	17.46±0.07	116.47±0	26.29±0.35	2.19±0.04
4.	W.c Stem	6.37±0	18.47±0	40.40±0	28.08±0	2.28±0.04
5.	W.s Root	6.87±0	10.27±0.22	15.88±0	5.20±0.33	1.28±0
6.	W.s Leaf	19.84±0	19.58±0	180.84±0	20.93±0	1.71±0
7.	W.s Stem	8.67±0	10.20±0.07	46.26±0	5.56±0.35	1.97±0
	In vivo					
8.	W.c Root(008)	0.78±0	8.23±0	34.55±0	4.13±0.36	1.10±0
9.	W.s Root	3.74±0	3.347±0.15	46.26±0	8.42±0.36	2.75±0
10.	W.s Leaf	28.05±0	17.85±0	122.33±0	21.64±0	2.49±0
11.	W.s Stem	11.62±0	10.12±0.46	31.63±2.93	14.5±0	1.2±0

*****Data represents Mean± SE of duplicates, expressed in mg/g

*****W.c represents *Withania coagulans*

*****W.s represents *Withania somnifera*

Fig 4.1.1 Total flavanoids in *Withania* species

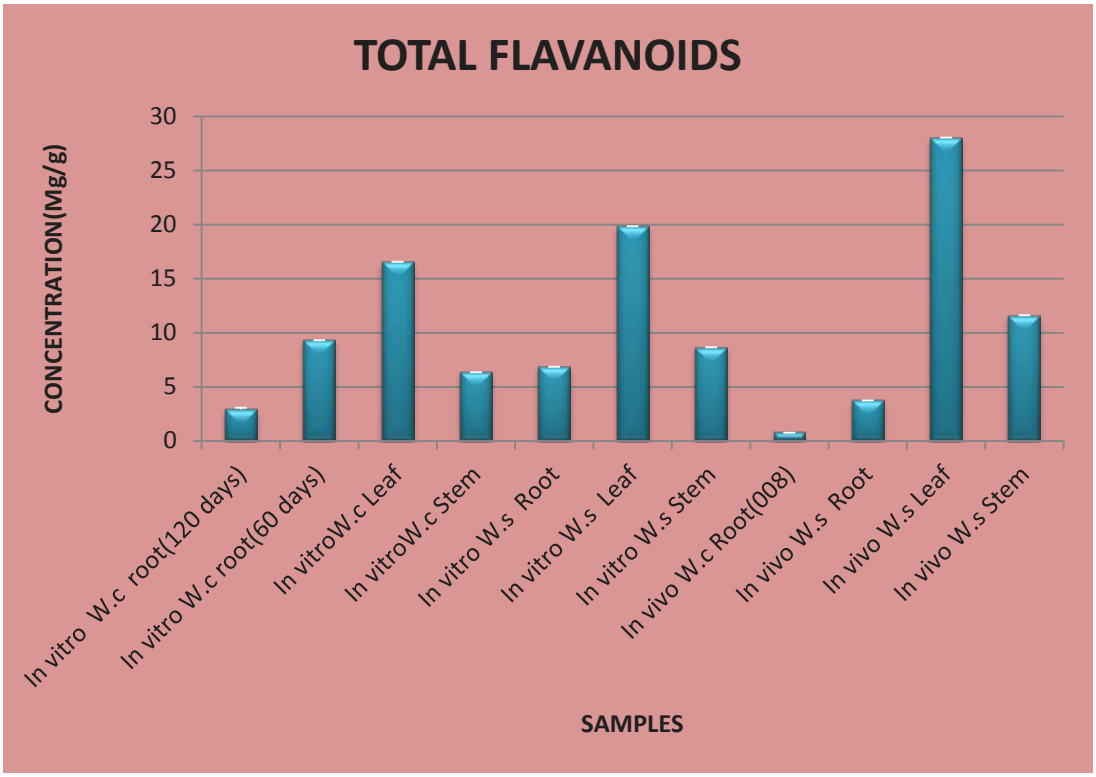


Fig 4.1.2 Total saponins in *Withania* species

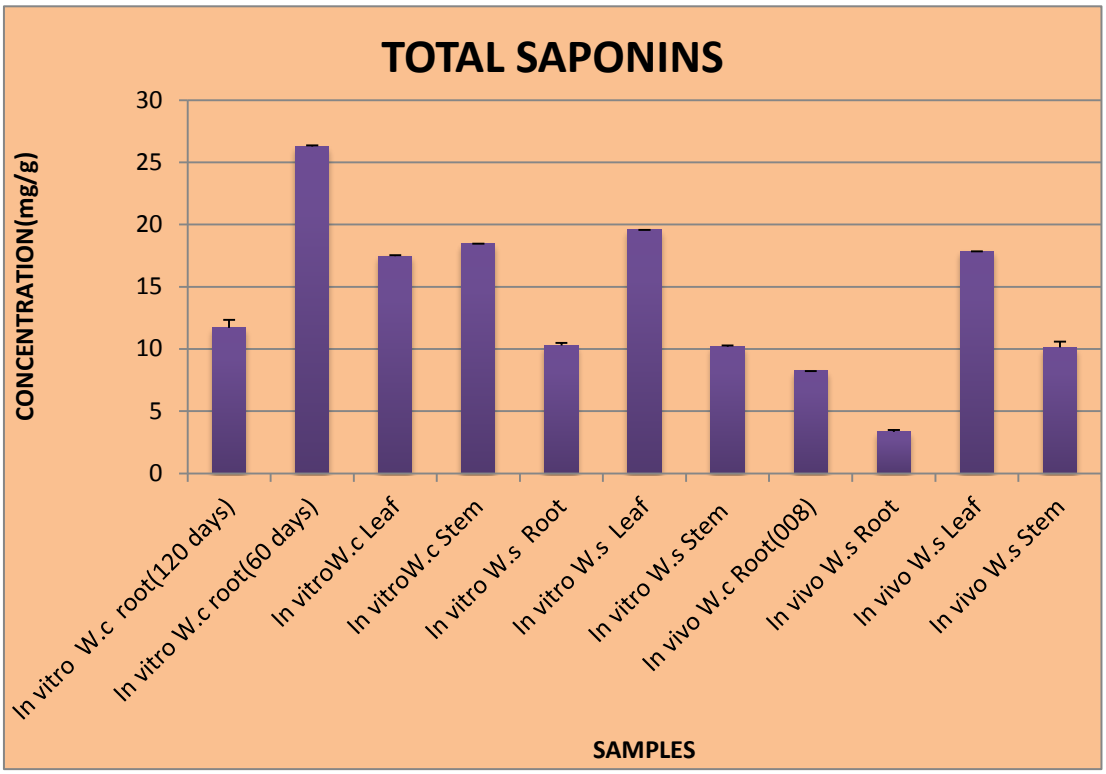


Fig 4.1.3 Total alkaloids in *Withania* species

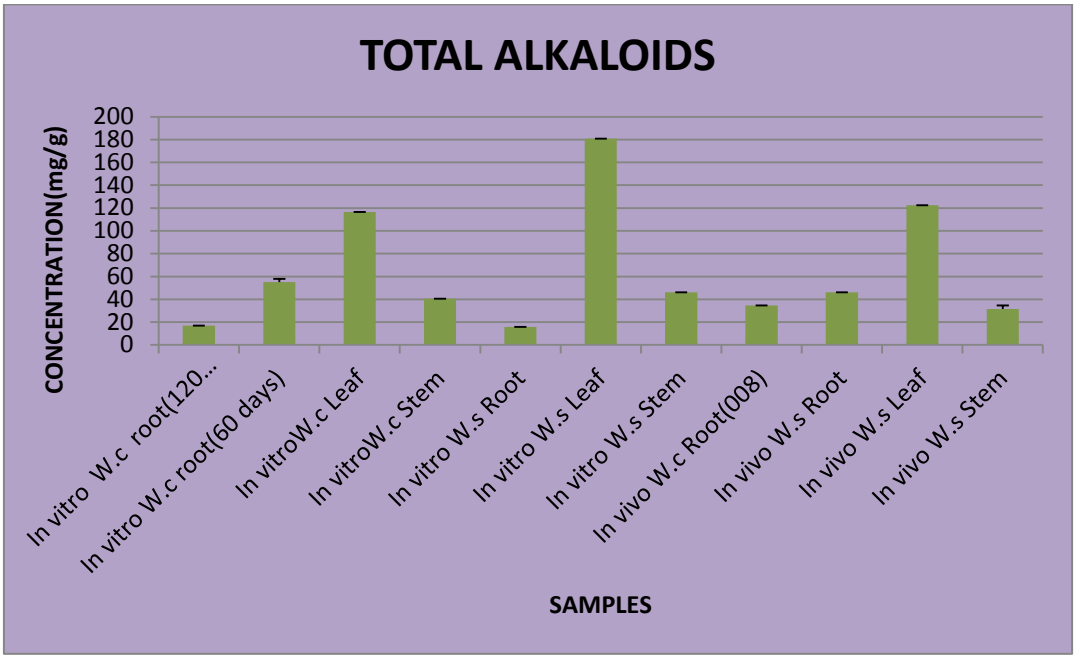


Fig 4.1.4 Total steroids in *Withania* species

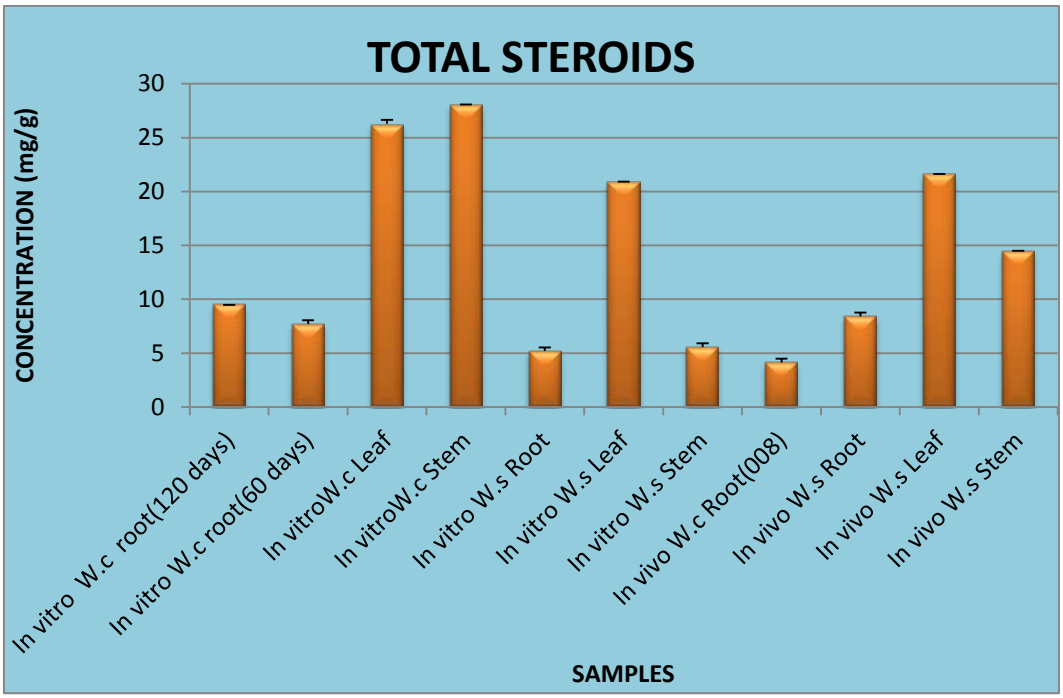
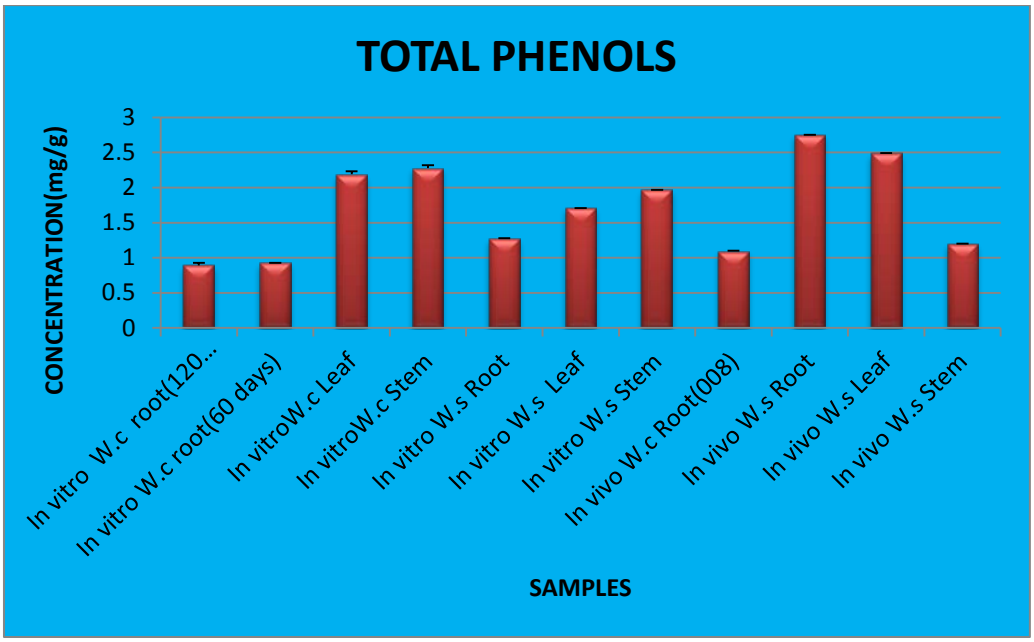


Fig 4.1.5 Total Phenols in *Withania* species



4.2 HPTLC method development and quantification of Withanolide A and Withaferin A:

HPTLC has been used to provide excellent separation, qualitative and quantitative analysis of a wide range of compounds, such as herbal and botanical dietary supplements, neutraceutical, traditional western medicines, traditional Chinese medicines and Ayurvedic medicines and determination of radiolabelled 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 methanolic extracts of *in vivo* and *in vitro* tissue samples of *W.somnifera* and *W. coagulans* were analysed by HPTLC using three different combinations of solvents in to get order to get a good separation and stable peak.Withanolide A and Withaferin A standard was visible as a clear spots with different Rf values corresponding to each mobile phase used thus indicating the capability of the solvents in the mobile phase to dissolve and carry the withanolide A and Withaferin A.The developed plates were then scanned using Camag TLC scanner III in the reflectance –absorbance mode at 530 and 230nm. The scan result showed Withanolide A and Withaferin A peak and the Rf value at which it was separated.The developed plates were then derivitized separately using anisaldehyde sulphuric acid. . The derivitized plates were viewed in a visualiser at 254nm and under white light.

4.2.1.Mobile phase-1 [Toluene:Ethyl acetate:Formic acid(5:5:1)]

Plate 4.2.1 HPTLC fingerprint of various extracts under the influence of Mobile phase-1 [Toluene:Ethyl acetate:Formic acid(5:5:1)]

Plate 4.2.1.a WithanolideA Under White light

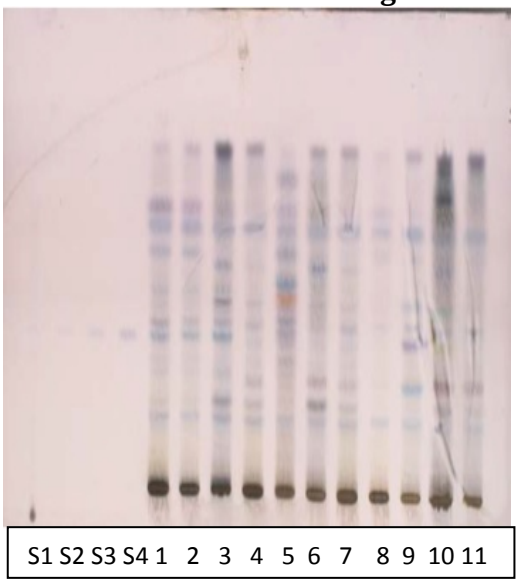
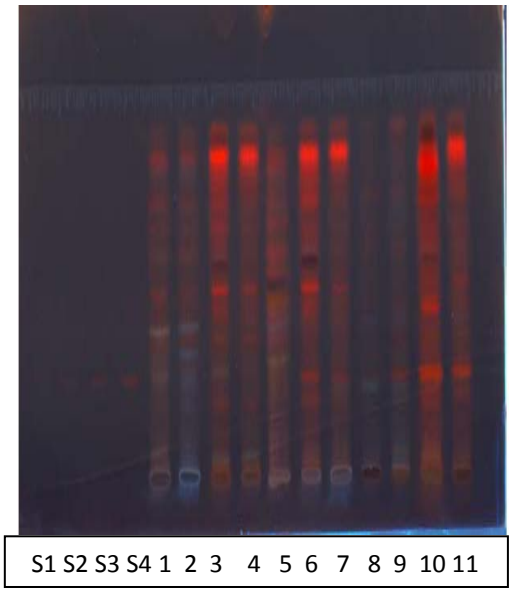


Plate 4.2.1.b WithanoloideA under 254nm

Plate 4.2.1.c WithaferinA Under White light

Plate 4.2.1.d WithaferinA under 254nm



Derivitization Agent: Anisaldehyde sulphuric acid

S1-S4 = Standards

Lane 1=*W.coagulans in vitro* root(60 days)

Lane 2= *W.coagulans in vitro* root(120 days)

Lane 3= *W.coagulans in vitro* leaf

Lane 4= *W.coagulans in vitro* stem

Lane 5= *W.somnifera in vitro* root

Lane 6= *W.somnifera in vitro* leaf

Lane 7= *W.somnifera in vitro* stem

Lane 8= *W.coagulans in vivo* root

Lane 9= *W.somnifera in vivo* root

Lane 10= *W.somnifera in vivo* leaf

Lane 11=*W.somnifera in vivo* stem

Table 4.2.1 Concentration of Withanolide A and Withaferin A in different samples under the influence of Toluene: Ethyl acetate: Formic acid (5:5:1) as mobile phase

S.NO	SAMPLE	Rf Value		PEAK AREA		CONCENTRATION (mg/g)	
		Withanolide A	Withaferin A	Withanolide A	Withaferin A	WithanolideA	Withaferin A
1.	*In vitro* W.c root (60 days)	0.43	0.30	1101.87	265.01	0.55	0.009
2.	*In vitro* W.c root (120 days)	0.43	0.28	1022.05	262.55	0.51	0.009
3.	*In vitro* W.c.Leaf	0.43	0.30	1497.43	435.55	0.75	0.032
4.	*In vitro* W.c.Stem	0.43	-	284.69	-	0.14	-
5.	*In vitro* W.s .Root	0.43	-	217.94	-	0.11	-
6.	*In vitro* W.s .Leaf	0.42	0.28	197.75	8667.44	0.099	1.12
7.	*In vitro* W.s.Stem	0.42	0.28	343.56	5299.31	0.17	0.68
8.	*In vivo* W.c Root(008)	-	0.28	-	1278.94	-	0.005
9.	In vivo W.s Root	0.43	0.28	2992.91	5434.19	1.55	0.69
10.	*In vivo* W.s .Leaf	0.44	0.28	499.14	17225	0.024	2.26
11.	*In vivo* W.s.Stem	-	0.29	-	10865.26	-	1.41

Fig 4.2.1 Linear regression graph of Withanoloide A

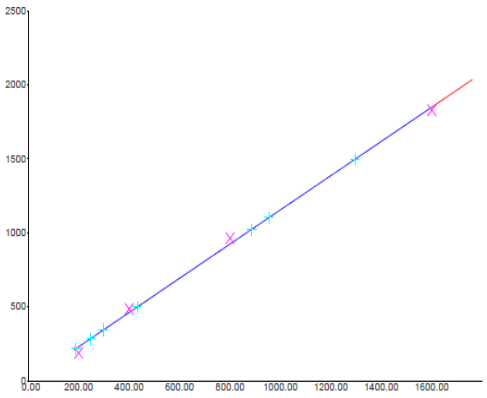


Fig 4.2.2 Linear regression graph of Withaferin A

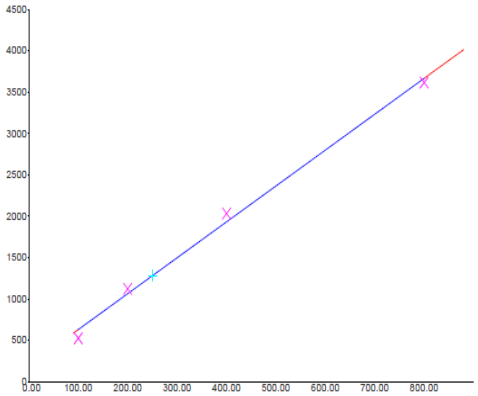
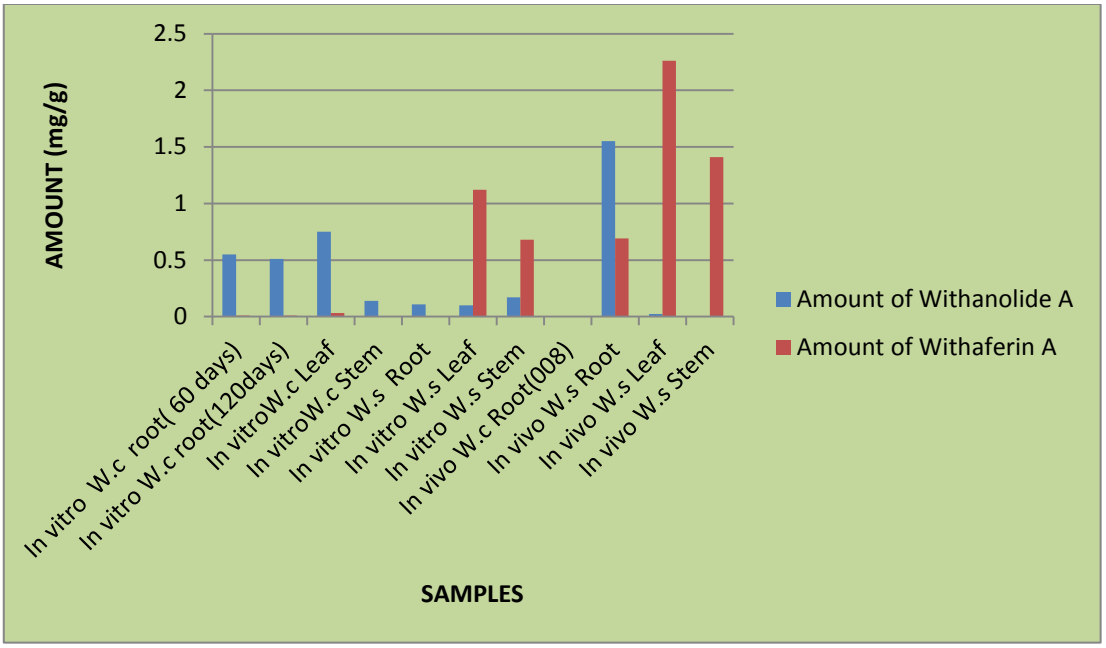


Fig 4.2.3 Concentration of Withanolide A and Withaferin A in different samples under the influence of Toluene: Ethyl acetate: Formic acid (5:5:1) as mobile phase



Toluene:Ethylacetate:Formic acid(5:5:2),gave good resolution for the separation of withanoloides at 530nm after derivitizing the plate with anisaldehyde sulphuric acid reagent (fig 4.2.3).The same result has already been reported by Sharma *et al*., (2007) and Patel *et al.*, (2009).This solvent system opted maximum compound separation in different tissue samples of *withania* species.Quantification of withanoloideA was not detected in *W.coagulans in vivo* root and *in vivo W.somnifera* stem .This might be due to the long term storage and influence of external factors .Concordantly,which was already reported by Vanisree *et al.*,(2003).

4.2.2.Mobile phase-2[Chloroform:Methanol(9:1)]:

Plate 4.2.2 HPTLC fingerprint of various extracts under the influence of Mobile phase [Chloroform:Methanol(9:1)]

Plate 4.2.2.a WithanolideA under White light

Plate 4.2.2.b WithanolideA under 254nm

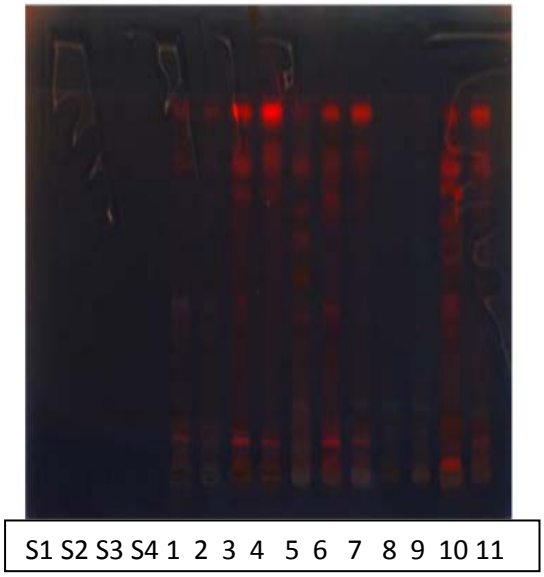


Plate 4.2.2.c WithaferinA under White light

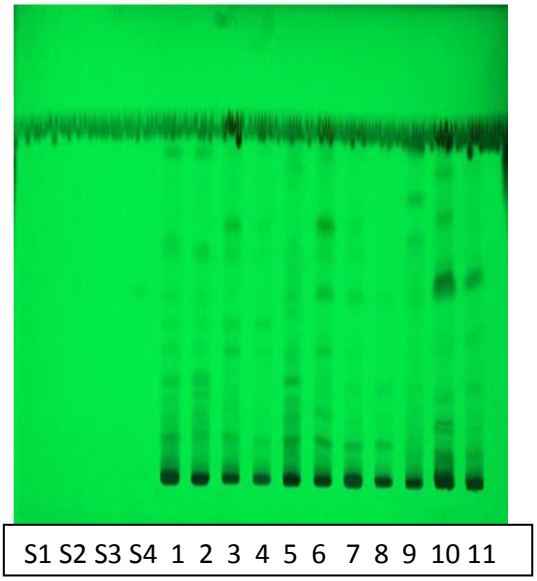
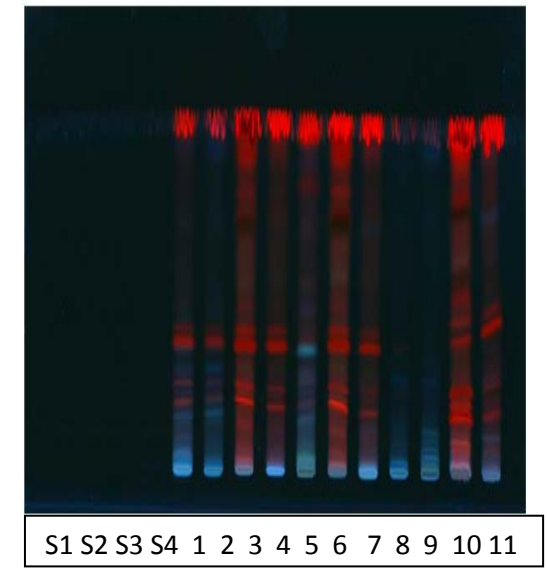


Plate 4.2.2.d WithaferinA under 254nm



Derivitization Agent: Anisaldehyde sulphuric acid

Lane 1=*W.coagulans in vitro* root(60 days)

Lane 2= *W.coagulans in vitro* root(120 days)

Lane 3= *W.coagulans in vitro* leaf

Lane 4= *W.coagulans in vitro* stem

Lane 5= *W.somnifera in vitro* root

Lane 6= *W.somnifera in vitro* leaf

Lane 7= *W.somnifera in vitro* stem

Lane 8= *W.coagulans in vivo* root

Lane 9= *W.somnifera in vivo* root

Lane 10= *W.somnifera in vivo* leaf

Lane 11= *W.somnifera in vivo* stem

Table 4.2.2 Concentration of Withanolide A and Withaferin A in different samples under the influence of Chloroform:Methanol (9:1) as mobile phase

S.NO	SAMPLE	Rf Value		PEAK AREA		CONCENTRATION (mg/g)	
		WithanolideA	Withaferin A	Withanolide A	Withaferin A	Withanolide A	Withaferin A
1.	*In vitro* W.c root (60 days)	0.37		314.04		0.0025	-
2.	*In vitro* W.c root (120 days)	-	0.58	-	197.43	-	0.013
3.	*In vitro* W.c.Leaf	0.36	0.60	643.28	622.29	0.033	0.023
4.	*In vitro* W.c.Stem	0.36	0.59	478.08	450.22	0.018	0.008
5.	*In vitro* W.s .Root	0.35	0.63	113.58	1438.95	0.016	0.092
6.	*In vitro* W.s .Leaf	0.35	0.60	316.63	13175.55	0.003	1.094
7.	*In vitro* W.s.Stem	0.34	0.59	164.36	7066.47	0.011	0.57
8.	*In vivo* W.c Root(008)	-	0.59	-	1990.42	-	0.14
9.	In vivo W.s Root	-	0.59	-	3456.41	-	0.26
10.	*In vivo* W.s .Leaf	-	0.61	-	9025.65	-	0.74
11.	*In vivo* W.s.Stem	-	0.64	-	2956.28	-	0.22

Fig 4.2.4 Linear regression graph of Withanoloide A

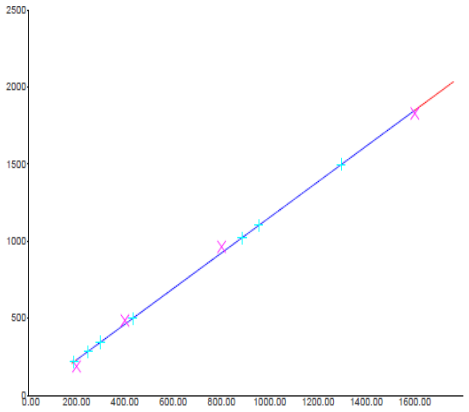


Fig 4.2.5 Linear regression graph of Withaferin A

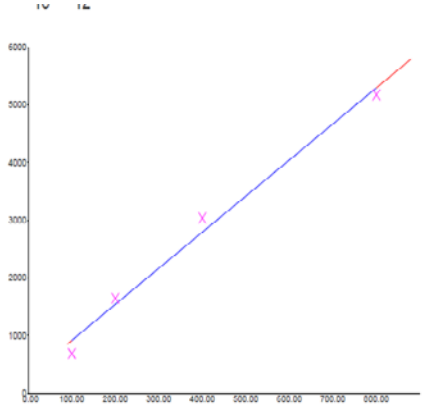
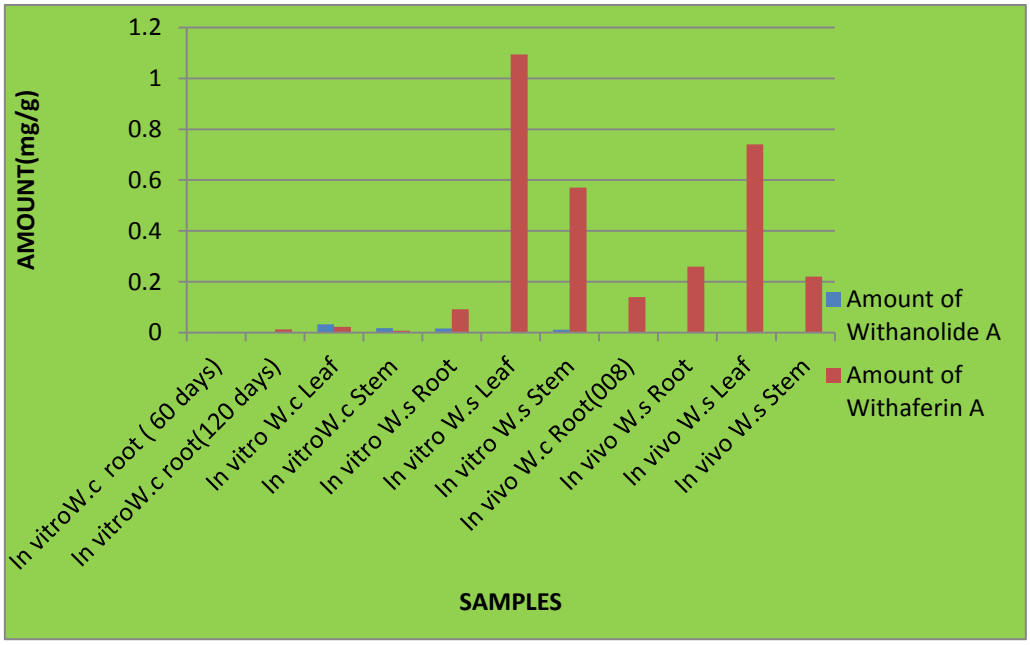


Fig 4.2.6 Concentration of Withanolide A and Withaferin A in different samples under the influence of Chloroform:Methanol:(9:1) as mobile phase



The two solvent system Chloroform:Methanol(9:1) gave a better resolution with maximum compound separation for the quantification of withaferin A at 230 nm before derivitizing the plate with anisaldehyde sulphuric acid reagent.The maximum quantification(1.094mg/g of dried tissue samples) was found in *W.s in vitro* leaf (fig4.2.6).WithaferinA was not detected in *W.coagulans* 60 days old root. It was already stated that withaferin quantification was found to be more in leaves than in root reported by Sharma *et al*., (2007).

4.2.3.Mobile phase-3 [Chloroform:Ethylacetate:Methanol:Toluene(11.1:0.6:1.2:4.5)]

Plate 4.2.3 HPTLC fingerprint of various extracts under the influence of Mobile phase 3

Chloroform:Ethyl acetate:Methanol:Toluene(11.1:0.6:1.2:4.5)

Plate 4.2.3.a WithanoloideA under White light

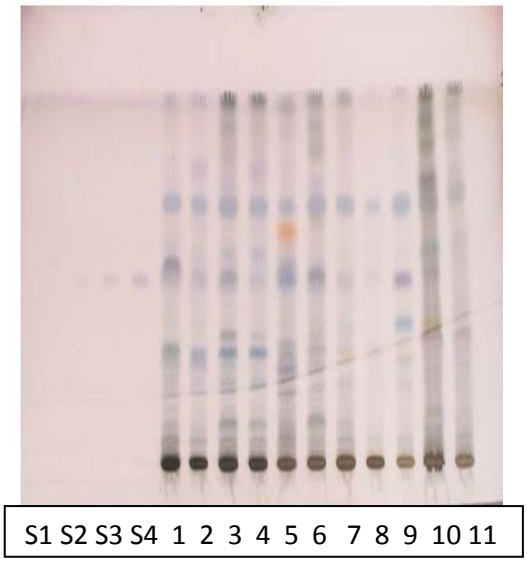


Plate 4.2.3.b WithanolideA under 254nm

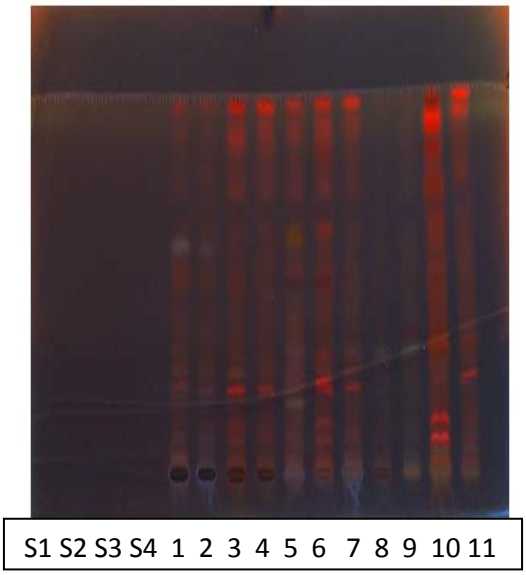


Plate 4.2.3.c Withaferin A under White light

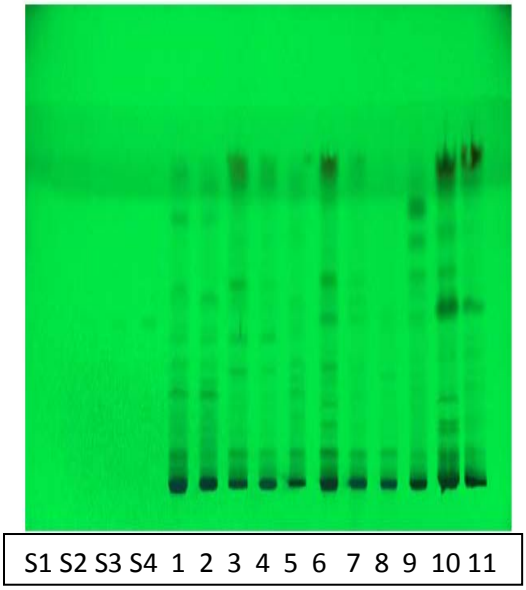
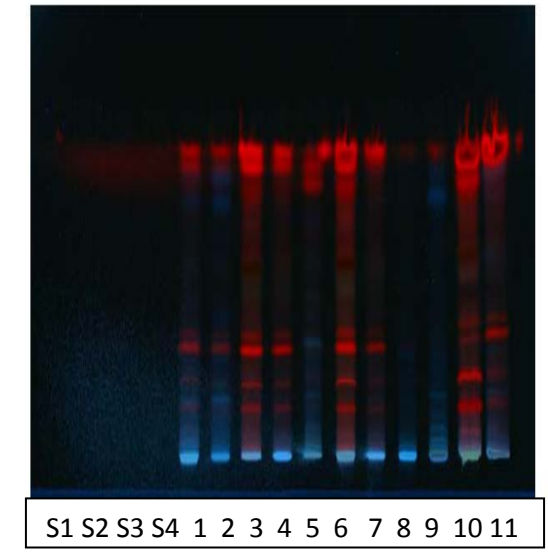


Plate 4.2.3.d Withaferin A under 254nm



Derivitization Agent: Anisaldehyde sulphuric acid

S1-S4 = Standards
Lane 1=*W.coagulans in vitro* root(60 days)
Lane 2= *W.coagulans in vitro* root(120 days)
Lane 3= *W.coagulans in vitro* leaf
Lane 4= *W.coagulans in vitro* stem
Lane 5= *W.somnifera in vitro* root
Lane 6= *W.somnifera in vitro* leaf
Lane 7= *W.somnifera in vitro* stem
Lane 8= *W.coagulans in vivo* root
Lane 9= *W.somnifera in vivo* root
Lane 10= *W.somnifera in vivo* leaf
Lane 11= *W.somnifera in vivo* ste

Table 4.2.3 Concentration of Withanolide A and Withaferin A in different samples under the influence of Chloroform:Ethylacetate:Methanol:Toluene(11.1:0.6:1.2:4.5) as mobile phase

S.NO	SAMPLE	Rf Value		PEAK AREA		CONCENTRATION (mg/g)	
		WithanolideA	Withaferin A	Withanolide A	Withaferin A	Withanolide A	Withaferin A
1.	*In vitro* W.c root (60 days)	-	0.51	-	163.62	-	0.11
2.	*In vitro* W.c root (120 days)	0.52	-	974.88	-	0.28	-
3.	*In vitro* W.c.Leaf	0.52	-	3850.32	-	1.15	-
4.	*In vitro* W.c.Stem	0.52	-	834.20	-	0.23	-
5.	*In vitro* W.s .Root	0.52	0.55	3430.87	771.81	1.025	0.019
6.	*In vitro* W.s .Leaf	0.52	0.53	3677.38	11781.13	1.10	1.62
7.	*In vitro* W.s.Stem	0.51	0.53	665.14	5011.68	0.18	0.61
8.	*In vivo* W.c Root(008)	0.52	0.54	280.47	1953.60	0.066	0.16
9.	In vivo W.s Root	0.51	0.53	1753.94	3591.50	0.51	0.39
10.	*In vivo* W.s .Leaf	0.52	0.53	403.07	6857.44	0.10	0.88
11.	*In vivo* W.s.Stem	-	0.55	-	4592.21	-	0.55

Fig 4.2.7 Linear regression graph of Withanoloide A

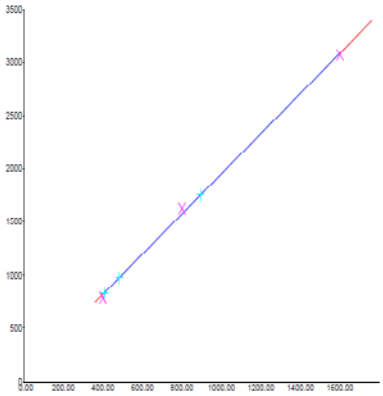


Fig 4.2.8 Linear regression graph of Withaferin A

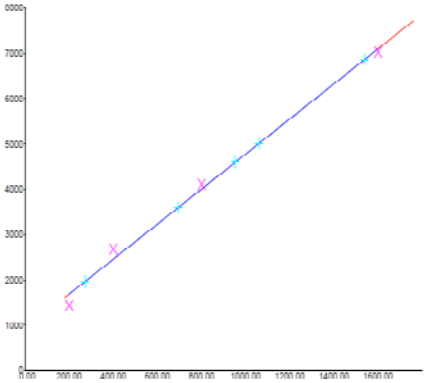
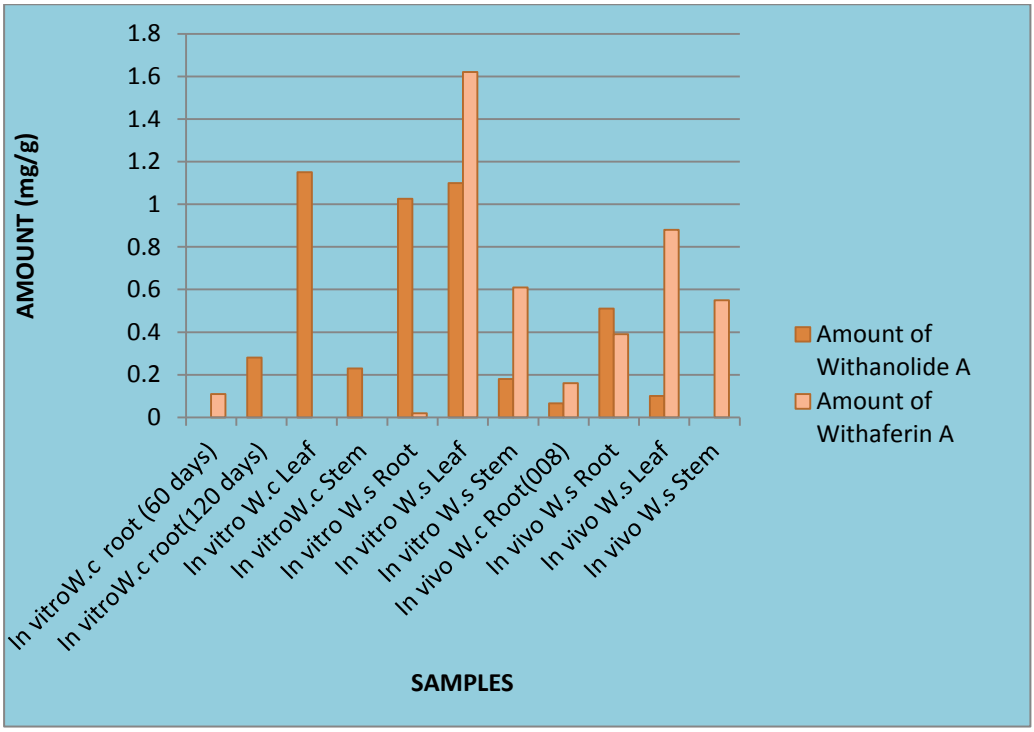


Fig 4.2.9 Concentration of Withanolide A and Withaferin A in different samples Chloroform:Ethyl acetate:Methanol:Toluene(11.1:0.6:1.2:4.5)



In Chloroform:Ethylacetate:Methanol:Toluene(11.1:0.6:1.2:4.5) mobile phase withanoloideA quantification was not detected in *in vivo W.coagulans* root and *in vivo W.somnifera* stem.Whereas,WithaferinA was not quantified in *in vitro* coagulans tissues, *W.coagulans* stem,leaf and 120 days old *in vitro* root tissue.This may be due to improper flow rate of mobile phase and the composition of mobile phase (Rashmin *et al.*,2012).

4.2.4.Comparative study of different mobile phases:

Among the three mobile phases used Toluene: Ethyl acetate: Formic acid (5:5:1) gave a better resolution of bands for Withanolide A and Chloroform:Methanol(9:1) for Withaferin A, Whereas in Chloroform:Ethylacetate:Methanol:Toluene(11.1:0.6:1.2:4.5) poor resolution of components was observed.

The accumulation of withanolide A and Withaferin A varies depending upon the mobile phase used and growing conditions.These results also revealed that the accumulation of Withanolide A is influenced by the geographical location in which it is grown. The concentrations of various secondary plant products are strongly depending on the growing conditions (Kannan et al., 2011) and also may be due to improper flow rate of mobile phase and poor polarity due to the composition of mobile phase (Rashmin *et al.*,2012).

4.3 Histological Elucidation:

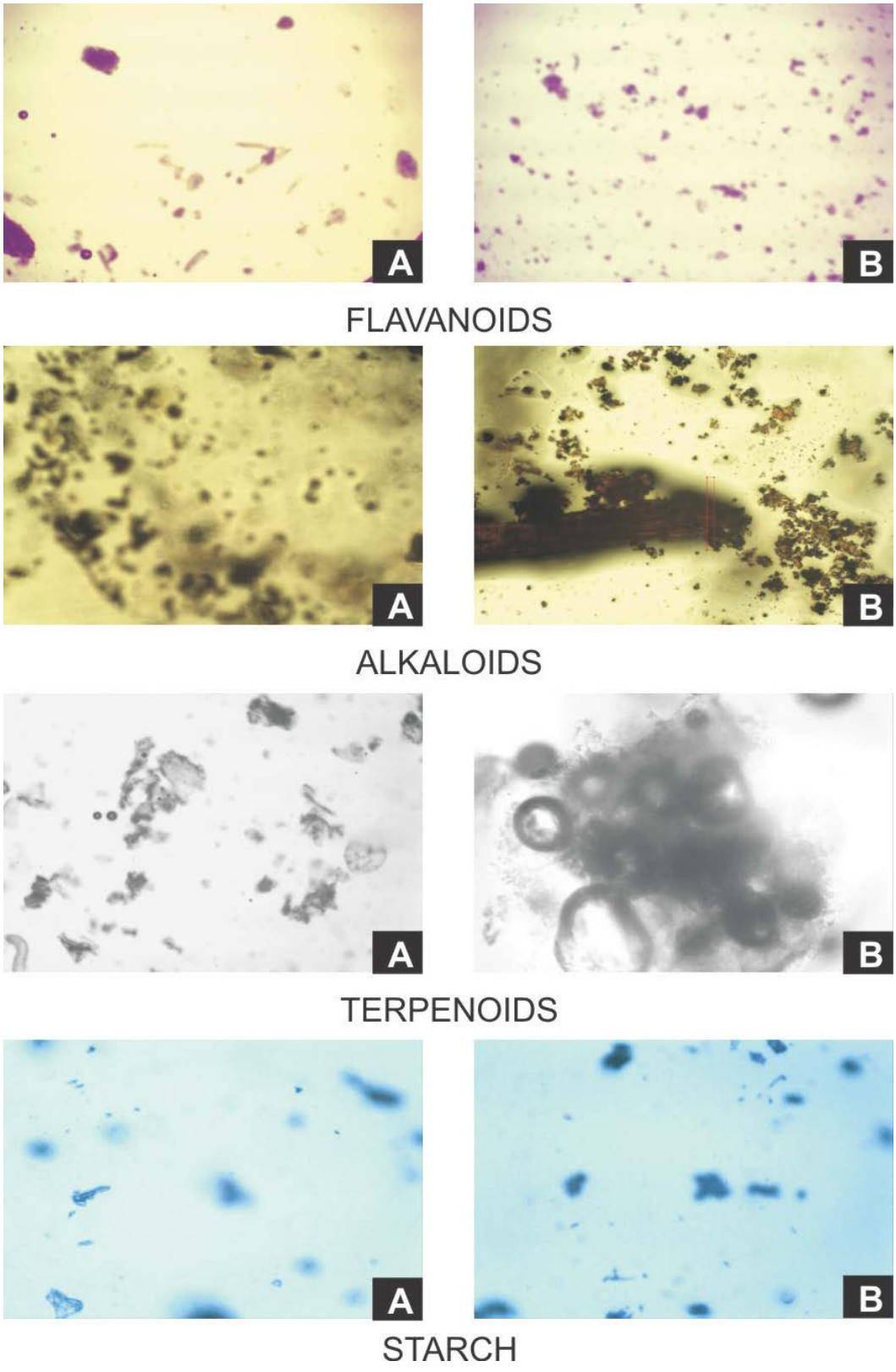
Histochemical tests were useful to localize *in situ* the main chemical classes of metabolites present in plant secretions. The use of *I. rubescens* in traditional Chinese medicine, the phytochemical studies and the proven pharmacological activities show the necessity of better knowledge of the secretory structures related to the production of secondary metabolites in this species. The secondary metabolites secreted by peltate trichomes were examined histochemically, which included terpenoids, flavonoids, carbohydrates, phenolics and alkaloids(Liu and Liu,2012). Histological studies was already carried out on *W.somnifera* root powder sample (Upton *et al*.,2000). So we focussed our study on *W.coagulans* in vivo and in vitro root powder sample.

With this known matter of concern, histochemical test of flavanoids, alkaloids, terpenoids and starch in both *in vitro* and *in vivo* root powder of *Withania coagulans* was carried out.

TARGET COMPOUND	REAGENT	COLOUR OBSERVED	*Withania coagulans* root powder	
			In vitro	*In vivo*
Flavanoids	Aluminium chloride	Violet	+ve	+ve
Alkaloids	Wagner's reagent	Black	+ve	+ve
Terpenoids	2,4-Dinitrophenylhydrazine	Black	+ve	+ve
Starch	Iodine solution	Blue	+ve	+ve

Upon Histological elucidation of *in vivo* and *in vitro* coagulans root powder,the localization of flavanoids,alkaloids,terpenoids and starch was found to be predominant in *in vitro* root than *in vivo* root(fig 4.3.1). This result was correlated with the phytocchemical quantification where the presence of flavanoids, alkaloids and terpenoids was found to be more in *in vitro* root of *W.coagulans* sample than *in vivo* condition(table 4.1.1).The accumulation of phytochemicals was found to be more in *in vitro* study than in *in vivo* condition. Our results are similar to the observation of Jain *et al.*,2012.

Fig 4.3.1 Histological Elucidation of *In vivo* and *In vitro W.coagulans* Root Powder



A- *In vivo W.coagulans* root powder

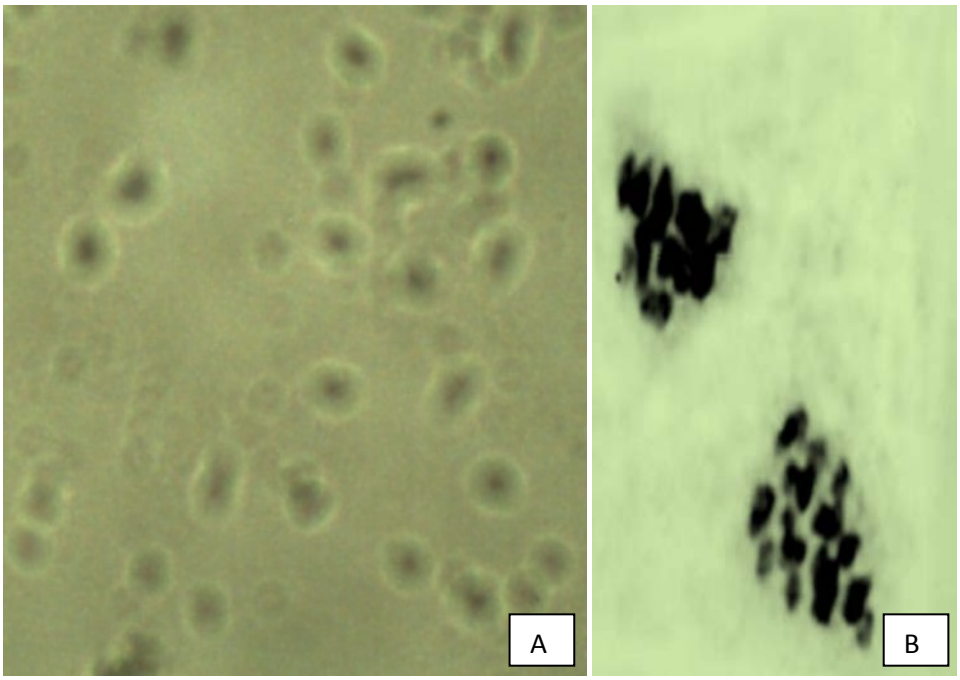
B- *In vitro W.coagulans* root powder

4.4 Karyotyping:

Karyotyping analysis was carried out in *Withania* species with sole objective to comprehend chromosome behaviour during mitosis.karyotypes of one species of each genus indicate that morphological diversification was accompanied by chromosomal changes,such as chromosome size,presence of satellite and karyotype formula(Ram *et al*.,2012).

The root tips of *Withania somnifera* collected were pretreated with 3ml of Isopropyl alcohol and 1ml of acetic acid for 2 hours. The root tips were hydrolysed in 50% HCl at room temperature for 2 seconds and stained with 1% aceto carmine.The material was then squashed and examined under motic microscope.photographs were taken. A composite image was developed for most mitotic cells counts were made by printing digital images and assigning a number to each chromosome observed in the image. The number of each chromosome observed in *Withania somnifera are* n=24,2n=48 (Fig 4.4.1).

Fig 4.4.1 karyotyping in *Withania somnifera* root tip

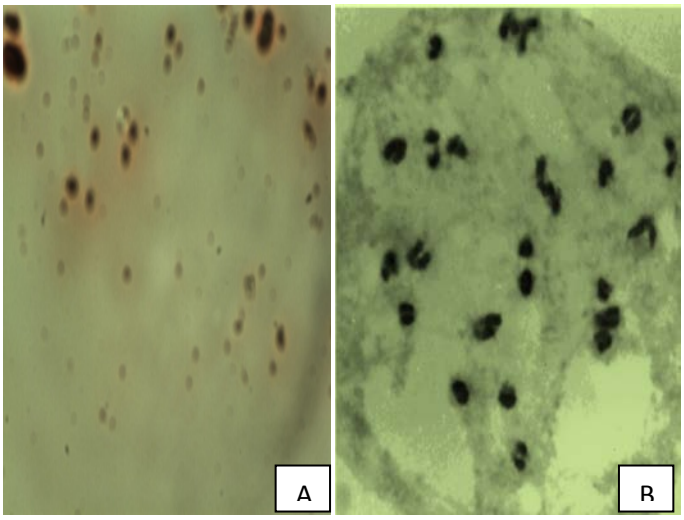


A-metaphase stage, B-Anaphase stage

The studies on the morphology of chromosomes indicate that mostly asymmetrical Karyotypes of cion hromosome have been observed. The derived basic chromosome number of the species is n=24 and the large number of species of *W.somnifera* studied have 2n=48 chromosomes and they may be considered as diploids.The reported chromosome numbers of *Withania somnifera* 2n=48 was reported by many others (Mir *et al.*,2010; Kumar *et al.*,2011;chatha *et al.*,1998;Baquar *et al.*,1965).

In case of *W.coagulans* karyotyping was carried out in *in vitro* fresh root tip sample. The basic number of chromosome was found to be n=24 and 2n=48(Fig 4.2.2).It was reported by (Baquar,1967). The result has yet to be confirmed.

Fig 4.2.2 Karyotyping in *Withania coagulans* root tip



A,B-Metaphase stage

SUMMARY AND CONCLUSION

5.0 SUMMARY AND CONCULSION

The results of the present study entitled **"Comparative evaluation of phytochemicals,method development and karyotyping in *in vitro* and *in vivo* tissues of *withania* species"** were summarized as follows.

Phytochemical analysis was studied on both *in vitro* and in *vivo tissue* samples of *withania* species. Methanolic extract of the dried tissue samples were used for the analysis of phytochemical components such as flavanoid, saponin, steroid, alkaloid and phenol. Phytochemical study revealed that the alkaloid (15.8-180.8 mg/g of tissue sample) and steroid content(4.13-28.08 mg/g of tissue sample) was found to be maximum in the *withania* species.

HPTLC quantification was carried on the tissue samples of *withania* species using different mobile phases viz., Toluene:Ethyl acetate:Formic acid(5:5:1), Chloroform:Methanol(9:1),Chloroform:Ethylacetate:Methanol:Toluene(11.1:0.6:1.2:4.5) and anisaldehyde sulphuric acid as the derivitizing agent.The study concluded that maximum compound separation with better resolution was found in Toluene:Ethylacetate:Formicacid(5:5:1)andChloroform:Methanol(9:1).Withanoloide quantification was found to be good in Toluene:Ethyl acetate:Formic acid(5:5:1)at 530nm after derivitizing the plate with anisaldehyde sulphuric acid reagent.Whereas, withaferin quantification was found to be maximum in Chloroform:Methanol(9:1) solvent at 223nm before derivitizing the plate with anisaldehyde sulphuric acid.

Histochemical analysis was carried out in both *in vitro* and *in vivo* root powder of *W.coagulans* species.Histological evaluation showed the localization of phytochemical accumulation, which was found to be more in *in vitro* samples than *in vivo* tissues.

Karyotyping was carried on fresh root tip of *in vitro W.somnifera* and *W.coagulans* species.In *W.somnifera* well defined mitotic stages was noted and the chromosome number was found to be 2n =48,diploid condition. In case of *W.coagulans* karyotyping was carried out in *in vitro* fresh root tip sample. The basic number of chromosome was found to be n=24 and 2n=48, the result has yet to be confirmed

BIBLIOGRAPHY

BIBLIOGRAPHY

- AbouZid SF, El-Bassuony AA, Nasib A, Khan S, Qureshi J and Choudhary MI(2010) Withaferin A Production by Root Cultures of *Withania Coagulans.* Int J App Resin Natural Products.3:23-27.

- Ahmed SA, Ansari AQ, Waheed MA and Juned AS(2013) Extraction and determination of antioxidant activity of *Withania somnifera* Dunal.EJEBAU.3:502-507.

- Alam N, Hossain M, Mottalib MA, Sulaiman SA, Gan SH and Khalil MI(2012) Methanolic extracts of *Withania somnifera* leaves, fruits and roots possess antioxidant properties and antibacterial activities.BMC.12:1-8.

- Bandopathyay M, Jha S and Tepfer D(2007) Changes in morphological phenotypes and withanolide composition of Ri-transformed roots of *withania somnifera*.plant cell Rep.26:599-609.

- Baquar SR, Akhtar S and Hussain A(1965) Meiotic Chromosome Numbers in Some Vascular Plants of Indus Delta I.*Botaniska Notiser*.118: 289-298.

- Baquar SR(1967) Cytomorphological studies in the family *Solanaceae* from west Pakistan.Genetica.38:388-397.

- Barad R,Soni P,Upadhyay S and Upadhyay U(2014) Effect of *Withania coagulans* and Psidium Guajava on diabetes mellitus,.International Journal of Pharmacotherapy.4(1):18-28.

- Bucco JC, Lambert F, and Sauvaire Y (1977) Spectrophotometric method for the determination of total steroidal sapogenin.Analyst. 102: 458–465.

- Chan RYK, Chen WF, Dong A, Guo D and Wong MS (2002) Estrogen-like activity of ginsenoside Rg1 derived from *Panax notoginseng*. *Journal of Clinical Endocrinology and Metabolism*. 87: 3691–3695.

- Chatha GS and Bir SS(1988) Cytological Evolution in Woody Taxa of Gamopetalae and Monochlamydae from South India.*Plant Sciences*. 9: 199-256.

- Ciddi V (2008) Withaferin A from the cultures of *Withania somnifera* .Indian Journal of Pharmaceutical Sciences.68:490-492.

- Corsi G and Biasci D(1998) Secretory Structures and Localization of Alkaloids in *Conium maculatum* L.(Apiaceae). *Annals of Botany.81:157-162.*

Dalavayi S, Kulkarni SM,Ulkarini , Itikala RL and Itikala S(2006) Determination of Withaferin-A in two *withania* species by RP-HPLC method.Indian Journal of Pharmaceutical Sciences.68(2):253-256.

Damayanti F, Lawn RJ and Bielig LM(2010) Genetic compatibility among domesticated and wild 1 accessions of the 2 tropical tuberous legume Vigna vexillata (L.) A. Rich. Crop & Pasture Science.61:1-25.

Duraipandiyan. V, Ayyanar. M and Ignacimuthu. S (2006) Antimicrobial Activity of Some Ethanomedicinal Plants Used by PaliyarTribe Tamil Nadu, India. *BMC Complementary and Alternative Medicine*. 635.

Eich E (2008) Solanaceae and Convolvulaceae Secondary Metabolites: Biosynthesis, Chemotaxonomy, Biological and Economic Significance: a Handbook Springer Verlag.Berlin Heidelberg.637.

Elsakka M, Grigorescu E, Stanescu U, Stanescu U and Dorneanu V (1990) New data referring to chemistry of *Withania somnifera* species. *Rev. Med. Chir. Soc. Med. Nat.Iasi,* 94(2): 385-387.

Glotter E (1991)Withanolides and related ergostane-type steroids. NAT PROD REP 8: 415–440.

Gupta GL and Rana AC(2007) Plant Review*Withania somnifera*(Ashwagandha): A Review.PhCogMag.1:129.

Gupta PC(2012). Withania coagulans Dunal an overview, International Journal of Pharmaceutical Sciences Review and Research.,1:32-39.

Gupta V and Keshari BB(2013) *Withania coagulan*s Dunal.(paneer Doda):A Review.IJAHM.3: 1330-1336.

Hemalatha S, Wahi AK, Singh PN and Chansuria JP (2006) Hypolipidemic Activity of Aqueous Extract of *Withania coagulans* Dunal in Albino Rats.*Phytother Res*.20:614. (B).

Hepper F.N, Hawkes JG, Lester RN, Nee M and Estrada E(1991) *Solanaceae III: taxonomy, chemistry, evolution* .Royal Botanic Gardens.211-227.

Hoda Q, Ahmad S, Akhtar M, Najmi AK, Pillai K and Ahmad SJ (2010)Antihyperglycaemic and antihyperlipidaemic effect of poly-constituents, in aqueous and chloroform extracts, of *Withania coagulans* Dunal. In experimental type 2 diabetes mellitus in rats. *Hum ExpToxicol.*29 (8): 653-658.

Hossain AM and Nagooru MR (2011) Biochemical Profiling and Total Flavonoids Contents of Leaves Crude Extract of Endemic Medicinal Plant *Corydylineterminalis*L. *Kunth.Pharmacognosy Journal*.3 (24): 25-30.

Hostettmann KA and Marston A (1995) Saponins, Cambridge Univ. Press, Cambridge, UK.

Huang CF, Ma L, Sun LJ, Ali M, Arfan M, Liu JW, and Hu LH(2009) ImmunosuppressiveWithanolides from *Withania coagulans*. Chemistry & Biodiversity.6: 1415-1426.

ICH Harmonised TripartiteGuidelines (1996) Validation of Analytical Procedures: Definition and Terminology/Methodology Q2A/Q2B, Geneva.

Jain R, Kachhwaha S and Kothari SL(2012) Phytochemistry, pharmacology, and biotechnology of *Withania somnifera* and *Withania coagulans*: A review.journal of Medicinal Plants Research.6: 5388-5399.

Jirge SS, Tatke PA and Gabhe SY(2011) Delvelopment and validation of a novel HPTLC method for simultaneous Estimation of Beta-Sitosterol-D-Glucoside and withaferin . Int J Pharm Pharm Sci.3:227-230.

Kandil FE, Sayed NHE, Douh AMA, Ishak MS and Mabry TJ (1994) Flavonol Glycosides and Phenolics from *Withania somnifera*. Phytochemistry.37: 1215-1216

Kannan N D and Kulandaivelu G (2011) Drought induced changes in physiological, biochemical and phytochemical properties of *Withania somnifera* Dun. Journal of Medicinal Plants Research Vol. 5(16):3929-3935.

Khandelwal KR (2005) Practical Pharmacognosy: Techniques and Experiments, India: Nirali Prakashan. 30-35, 146-147.

Khare CP (2007) Indian Medicinal Plants. Springer-Verlag. Berlin/Heidelberg

Kreis W, Muller-Uri F (2010). Biochemistry of sterols, cardiac glycosides, brassino steroids, phytoecdysteroids and steroidsaponins.In: Michael W (ed), Annual Plant Reviews., 40:304–363.

☐ Kubmarawa. D, Khan. ME, Punah.AM and Hassan(2008) Phytochemical Screening and Antimicrobial Activity of Extracts from *Parkiaclappertoniane*keay against Human Pathogenic Bacteria.*J. Med.Pl.Res*.2(12):352-355.

☐ Kumar A, Mir BA, Sehgal D, Dar TH, Koul S, Kaul MK, Raina SN and Qazi GN(2011) Utility of a Mul-tidisciplinary Approach for Genome Diagnostics of Cul-tivated and Wild Germplasm Resources of Medicinal *Withania somnifera,* and the Status of New Species, *W. ashwagandha,* in the Cultivated Taxon," *Plant Systemat-ics and Evolution.*291(3-4):141-151.

☐ Kumar UA, Manjunath A, Thaminzhmani T, Kiran YR and Brahmaiah Y(2012). A Review on Immunomodulatory Activity Plants.IJNDD.4:93-103.

☐ Kushwaha R and Karanjekar S(2011) Standardization of Ashwagandharishta formulation by TLC Method.International Journal of ChemTech Research.3:1033- 1036.

☐Liu MQ and Liu JF(2012). Structure and histochemistry of the glandular trichomes on the leaves of *Isodon rubescens* (Lamiaceae), African Journal of Biotechnology.,11:4069-4078.

☐Mace ME and Howell CR(1974) Histochemistry and identification of condensed tannin precursors in roots of cotton seedlings. *Canadian Journal of Botany.*52:2423-2426.

☐ Mathur D, Agrawal RC and Shrivastava V (2011) Phytochemical Screening and Determination of Antioxidant Potential of Fruits Extracts of *Withania coagulans,*Recent Research in Science and Technology., 3: 26-29.

☐ Mathur D and Agrawal RC(2011) *Withania coagulans*: a review on the morphological and pharmacological properties of the shrub.World Journal of Science and Technology. 1(10): 30-37.

☐ Maurya R, Akanksha and Jayendra (2010). Chemistry and pharmacology of *Withania coagulans*: an ayurvedic remedy, J Pharma Pharmacol.,62:153–160.

☐ Mesaik MA, HaqZu, Muradb S, Ismail Z, Abdullah NR, Gill HK, Rahman AR, Yousaf M, Siddiqui RA, Ahmade A and Choudhary MI (2006) Biological and molecular docking studies on coagulin-H, Human IL-2 novel natural inhibitor.MolImmunol.43:1855–1863.

☐ Mir BA, Loul S, Kumar A, Kaul MK, Soodan AS and Raina SN(2010) Intraspecific Variation in the Internal Transcribed Spacer (ITS) Regions of rDNA in *Withania somnifera* (Linn.) Dunal.*Indian Journal of Biotechnolog*.9:325-328.

☐Mirjalili MH, Moyano E, Bonfill M, Cusido RM and Palazón J(2009) Steroidal Lactones from *Withania somnifera*, an Ancient Plant for Novel Medicine.Molecules.14:2373-2387.

☐ Mishra LC and Simon BBS (2000) Dagenais, Scientific basis for the therapeutic use of *Withania somnifera* (ashwagandha): a review.,*Altern. Med. Rev*.5(4): 334–346.

☐ Morrissey JP and Osbourn AE (1999) Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiol. Mol. Biol. Rev.* 63: 708–724.

☐ Mughal T, Shahid S and Qureshi S (2011) Antifungal Studies of *Withania coagulans* and Tamarixaphylla.J App Pharm.3 : 289-294.

☐ Muthumani P, Meera R, Sweetlin and Devi P(2010) Phyto Chemical Investigation and Determination of Crude Alkaloidal Content(Solasodine) in S*olanum Leave D*unal(Dry and Fresh Berries). International Journal of Pharmaceutical & Biological Archives.,1(4):350-354.

☐ NayakP S, Upadhyaya S U and Upadhyaya A (2009) HPTLC method for analysis of withaferin-A in Ashwagandha *(Withania somnifera).* Journal of Planar Chromatography - Modern TLC 22(3): 197-200.

☐ Naz A(2002) Studies on the Chemical Constituents of *Withania coagulans* and Boswellia dalzielli. P.H.D thesis, international center for chemical sciences university of Karachi, Pakistan.

☐ Neogi, Partha, Kawai, Masao, Butsugan, Yasuo, Mori, Yuji, Suzuki and Makoto (1988) Withacoagin, a new withanolide from *Withania coagulans* roots. the chemical society of japan 61(12): 4479-4481 .

☐ Owais M, Sharad KS,Shehbaz A and Saleemuddin M(2005) Antibacterial efficacy of *Withaniasomnifera*(ashwagandha) an indigenous medicinal plant against experimental murine salmonellosis.*Phytomedicine.*12(3): 229-235 .

☐Padmavathi B, Rath PC, Rao AR and Singh RP(2005) Roots of *Withania somnifera* inhibit forestomach and skin carcinogenesis in mice. Evid Based Complement Alternat Med.2:99-105.

☐ Pal, KN, Junaid, N and Raman, B (2013) Pharmacological Profile of *Withania somnifera*(Ashwagandha).Journal of Botanical Sciences.2:6-14.

☐ Patel JB, Lahiri SK and Shah MB (2009) Development of a New Method for Identification and Estimation of *Withania somnifera* Root, and a Method for Quantitative Analysis of Withaferin A in Young and Old Roots.*Journal of Planar Chromatography*. Vol. 22 (4):283–286.

☐ Patel R, Mrunali P, Nitin D, Nidhi D and Bharat P(2012) HPTLC Method Development and Validation: Strategy to Minimize Methodological Failures. *Journal of Food and Drug Analysis.20:794-804.*

☐ Rahman A.U, Shabbir M, Yousaf M, Qureshi S,Dur-e-Shahwar, Naz A and Choudhary MI (1999) Three witha-nolides from *Withania coagulans.* Phytochemistry 52 (7): 1361-1364.

☐ Rahman AU, Abbas S, Shahwar DE, Jamal SA and Choudhary MI (1993) New Withanolides from *Withania* Spp. Journal of Natural Products.,56: 1000-1006.

☐ Rajendra CE, Shashidhara S, Hanumantharaju N, Nadaf MA and Mohan CG(2010). HPLC Estimation of Withaferin-A and Boswellic Acid in Formulated Gels.IJPBA.1:183-186.

☐ Rakesh SU,Patil PR, Salunkhe VR, Dhabale PN and Burade KB (2009) HPTLC Method for Quantitative Determination ofQuercetin in Hydroalcoholic Extract of Dried Flower of NymphaeastellataWild. International Journal of ChemTech Research .,1 (4): 931-936.

☐Ram H, Kumar A, Sharma SK, Ojha A and Rao SR(2012) Meiotic Studies in *Withania somnifera* (L.) Dunal.: A Threatened Medicinal Herb of Indian Thar Desert. *American Journal of Plant Sciences*.3:185-189.

☐ Rasheed NMA, Nagaiah K, Mehveen A, Rehana A, Waheed MA and Shareef MA(2012) Phytochemical evaluation and quantification of beta sitosterol in geographical variation of *Withania coagulans* Dunal by HPTLC analysis,Annals of phytomedicine.1(2):14-22.

☐ Rashmin P, Mrunali P, Nitin D, Nithi D and Bharat P(2012) HPTLC Method Development and Variation: Strategy to Minimize methodological Failures. Journal of Food and Drug Analysis.20:794-804.

☐ Rinne P, Tuominen H andJunttila O(1994).Seasonal changes in bud dormancy in relation to bud morphology,water and starch content,and abscisic acid concentration in adult trees of *Betula pubescens*, tree physiology.,14:549-561.

☐ Sadasivam S and Manickam A (1996) Biochemical methods.New age international.2:159-160.

☐ Sangwan RS, Chaurasiya ND, Lal P, Misra L, Uniyal GC, Tuli R and Sangwan NS (2007) Withanolide A Biogeneration in In vitro Shoot Cultures of Ashwagandha (*Withania somnifera* Dunal), a Main Medicinal Plant in Ayurveda. Chem.Pharm.Bull.55: 1371-1375.

☐ Saxena B (2010) Anti-hyperlipidemic activity of *Withania coagulans* in streptozotocin induced diabetes: A potent anti-atherosclerotic agent ,Drug Discoveries & Therapeutics4(5):334-340.

☐ Sharada M, Ahuja A, Suri KA, Vij SP, Khajuria RK, Verma V and Kumar A(2007)Withanolide production by *in vitro* cultures of *Withania somnifera* and its association with differentiation.Biologia Plantarum. 51:161-164.

☐ Sharma M, Kaur R and Puri S(2013) Quantification of withanolide A from *Withania somnifera* Dunal.in tropics of Himalaya using HPLC with DAD detector.IJBPR.4:702-705.

☐ Sharma V, Gupta AP, Bhandari P, Gupta RC and Singh B (2007) A Validated and densitrometric HPTLC Method for the Quantification of W ithaferin –A and Withanolide A in Different Part of Two Morphotypes of *Withania somnifera. Chromatographia* .66:801-804.

☐ Singariya P, Mourya KK and Kumar P(2012) AntimicrobialActivity of the Crude Extracts of *Withania somnifera* and *Cenchrus setigerus* In-vitro. *Pharmacognosy Journal*. 4 (27): 60-65.

☐ Singh A, Salwaan C, Mittal A and Singh P(2012) Investigation of the Pharmacognostical, Phytochemical and Antioxidant Studies of Plant *Withania coagulans* Dunal.Journal of Pharmacognosy and Phytochemistry.1:32-39.

☐Singh G, Sharma PK, Dudhe R and Singh S(2010) Biological activities of *Withania somnifera*.Annals of Biological Research.1:56-63.

☐ Singh N, Verma P, Pandey BR and Gilca M(2011)Role of *Withania somnifera* in Prevention and Treatment of Cancer:An Overview.International Journal of Pharmaceutical Sciences and Drug Research.3:274-279.

☐ Subramanian P, Harikrishnan B and Subash S(2008) Effect of *Withania Somnifera* Root Powder on the Levels of Circulatory Lipid Peroxidation and Liver Marker Enzymes in Chronic Hyperammonemia.E.J.chem.5:872-877.

☐ Upton R, Petrone C and Swish DBA(2000)Ashwagandha root *Withania somnifera* Analytical,Quality control,and Therapeutic monograph.American Herbal pharmacopoeia.1-25.

☐Vanisree M, Lee CY, Lo SF, Nalawade SM, Lin CY and Tsay HS(2004) Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures.Botanical Bulletin of Academia Sinica.45:1-22.

☐ Visavadiya NP and Narasimhacharya AV(2006) Hypocholesteremic and antioxidant effects of *Withaniasomnifera*(Dunal) in hypercholesteremic rats. *Phytomedicine*(In Press).

☐ Wall ME, Eddy CR, McClennan ML and Klump ME (1952) Detection and estimation of steroidal sapogenins in plant tissue. *Anul.Chem.* 24:1337-1341.

☐ Wube A A, Wenzig EM, Gibbons S, Asres K, Bauer R and Bucar F (2008) Constituents of the stem bark of Discopodium penninervium and their LTB4 and COX-1 and -2 inhibitory activities. Phytochemistry 69:982–987.

☐ Xu Y (2001) Perspectives on the 21st century development of functional foods: bridging Chinese medicated diet and functional foods. International Journal of Food Science and Technology, 36: 229–242.

☐ Yang H, Shi G and Dou QP (2007) The tumor proteasome is a primary target for the natural anticancer compound withaferin A isolated from ‘‘Indian Winter cherry’’. Mol. Pharmacol.71: 426–437.

APPENDICES

Appendices

APPENDIX –1

ESTIMATION OF TOTAL FLAVANOID $AlCl_3$ METHOD

Hossain *et al* (2011)

MATERIALS

- Standard 1mg/ml: 10 mg of quercetin dissolved in 10 ml of methanol to get 1mg/ml solution.
- 5% $NaNO_2$
- 10% $AlCl_3$
- 1mM NaOH

PROCEDURE:

1. Aliquots of 0.2, 0.4, 0.6, 0.8 & 1.0 ml of quercetin stock standard solution were taken in 6 different tubes.
2. 0.5 ml & 1.0 ml of aliquot from the plant extract was taken in separate tube.
3. To the standard and samples were mixed with 20ml of distilled water followed by 0.15ml of 5% $NaNO_2$ & incubated for 5 min 25 C
4. After that to all the tubes 0.15 ml of 10% $AlCl_3$ was added & allowed to stand for further 6 min.
5. Then, the reaction mixture was treated with 2.0ml of 1mM NaOH & immediately the reaction mixture was made to 5 ml with distilled H_2O.
6. The solution was mixed well again & the absorbance was measured against a reagent blank at 510nm.

CALCULATION

Express the amount in mg / g or 100 g sample

APPENDIX -2

ESTIMATION OF SAPONIN

Bucco *et al* (1977), Uematsu *et al* (2000)

MATERIALS

- **Standard:** 0.1 g Diosgenin dissolved in 10 ml of HPLC grade methanol.
- **Reagent A:** 0.5 ml Anisaldehyde in 99.5 ml Ethyl Acetate.
- **Reagent B:** 50ml con.H_2SO_4 in 50 ml Ethyl Acetate.

PROCEDURE

1. Pipetted out 0.2, 0.4, 0.6, 0.8 and 1.0 ml of Standard into a series of test tubes and 0.3ml of the sample extract into another test tube.
2. Make up the volume to 1ml in all the test tubes. A tube with 1 ml of ethyl acetate serves as the blank.
3. 0.5ml of Reagent A was added to all the tubes.
4. Equal amount of Reagent B was then added.
5. Kept in boiling water bath maintained at 60^0C for 20 minutes.
6. Cooled to room temperature and the absorbance were measured at 430 nm.

CALCULATION

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

APPENDIX –3

ESTIMATION OF ALKALOIDS

Muthumani *et al* (2010)

MATERIALS

- **Standard:** 10mg of pure caffeine and dissolve in 25ml of 20% acetic acid A.R., dilute as aliquot a further 10 times with 20% acetic acid. This solution contains 40mcg/ml
- **20% Acetic acid.**
- **Chloroform**
- **Anhydrous Na_2SO_4**

PROCEDURE

- Into four suitable separators were pipetted 0,1,2 and 3 of 40mcg/ml standard solution
- The volume of each was made up to 5ml with 20% acetic acid
- To each separator 5 ml of acetate buffer and 1ml of methyl orange were added.
- After shaking for 10 sec.
- 5 ml of chloroform was added.
- The separators were stopped and shaken for 3 min.
- After standing for a few minutes chloroform layers were withdrawn into dry test tubes, dried with small amount of anhydrous Na_2SO_4
- Absorbance read on a spectrophotometer at 420nm using 10mm cells.
- From the reading standard curves was constructed.

CALCULATION

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

APPENDIX –4

ESTIMATION OF TOTAL STEROIDS

Wall *et al (*1952)

MATERIALS

- **Libermann Burchard Reagent(acetic anhydrate & H_2SO_4):**0.5 ml of sulfuric acid dissolved in 10ml of acetic anhydrate and kept in ice
- **Standard:**10mg cholesterol dissolved in 10ml of chloroform.

PROCEDURE

- Pipetted out 0.5,1,1.5,2 and 2.5ml of standards into series of test tubes and 0.2 ml of the sample extract into another test tube.
- Added 2ml of Libermann Burchard reagent to all the tubes.
- Made up equal volume in all tubes with chloroform.
- Covered with carbon paper and incubated at room temperature in dark for 30 minutes.A green colour was developed.
- Read the absorbance at 640nm.

CALCULATION

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

APPENDIX –5

ESTIMATION OF TOTAL PHENOL

Sadasivam & Manickam (1996)

PRINCIPLE:

Estimation of phenol by folin – Phenol regent is based on the reaction between phenols and an oxidizing agent phosphomolybdate which results in the formation of a blue colour. The intensity of the colour developed was read colorimetrically at 650 nm.

REAGENTS:

- Folin – phenol reagent
- Sodium carbonate : 20%
- Catechol stock standard : 100 mg of catechol was dissolved in 100ml of distilled water.
- 80 % ethanol
- Working standard was prepared by diluting 10 ml of stock to 100 ml of distilled water

PROCEDURE:

- Different aliquots of the extract was pipetted out into test tubes.
- The volume in each tube was made up to 3ml with water and 0.5 ml folin ciocalteau reagent was added in to all the tubes.
- After 2 mins 2ml of sodium carbonate solution was added to all the tubes .
- Contents were mixed,thoroughly and tubes were placed in a boiling water bath for exactly one min.
- cooled and absorbance was measured at 650 nm against a reagent blank.
- The amount of total phenol present in the sample was calculated from the catechol standard.

CALCULATION

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