Quantification Of Major Withanolides And Antibacterial Activity in Over The Counter Health Supplements Containing *Withania Somnifera* Extracts

Anitha,K

(12PBT001)

Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women,

Coimbatore – 641 043

In Partial Fulfillment of the Requirement of the

Degree of Master of Science in Biotechnology

March, 2014

Quantification of Major Withanolides and Antibacterial Activity in Over The Counter Health Supplements Containing *Withania Somnifera* Extracts

Anitha, K

(12PBT001)

A Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women

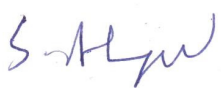
Coimbatore – 641 043

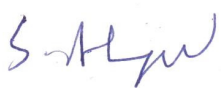
In Partial Fulfilment of the Requirement of the

Degree of Master of Science in Biotechnology

March, 2014

Signature of Head of the Department **Signature of Supervisor**





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 M.A., M.Phil., Ph..D.** 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 M.Sc., P.G.Dip., Ph.D.** 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(Matha matics)** 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. S. Annapurani Dip.Ed., M.Phil., Ph.D.,** professor and head 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 D.Parameshwari, K.Lavanya*** *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, D.Karuna moorthy, K.Sumathi*** *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 ***K.Arun prashath B.E., K.Selvaraj B.E., K.Aravind B.E.,*** *and all my family members for their loving patience, unconditional support and encouragement to pursue my interests, even when beyond boundaries of language.*

I express my special thanks to ***Shobana Nancy Charles, Irene Mariam Roy*** *for their help and suggestions in translating this work into reality.*

My thanks to everybody who has been a part of my life but I failed to mention. Thank you all very much.

\CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

1.0 INTRODUCTION

Nature is an infinite resource for drug development and created almost an inexhaustible array of molecular entities. It is not possible to obtain a precise figure for the total number of species existing on earth made an effort to express biodiversity in number, estimating the total number of species to be 10-100 million and plants existing range from 2,50,000 to 7,50,000. Only 5-10% of these species however have been acknowledged through scientific evaluation to have therapeutic value (Veeresham *et al.,* 2013)

Estimates indicate that approximately 30,000 to 75,000 species of medicinal plants are found on Earth. It is estimated that between 60 to 80 percent of people worldwide, have mainly confidence to traditional herbal medicines that they can provide basic needs to their health. Mobin believes that human life is directly or indirectly related to the plant. Medicinal plants are considered due to lack of easy access and low-cost and most of all side effects. Today, world-wide approach to the use of natural medications cause significant changes in all countries to identify and natural materials and plant and return to nature, so that in most of the world has knowledge of herbal medicine (phytotherapy) is located in research agenda for the scientific community (Zarezadeh *et al.,* 2007). The vast country of Iran with very different climates, have been very diverse flora in the world so that more plants that grow in this country or culture can be identified. Undoubtedly, the first step towards such plants with medicinal value is identified. The purpose of this survey (study) is gathering and identifying medicinal plant of Delafrooz Mountain in central Zagros (Yazdanpanah *et al*., 2013).

India is one of the 12 mega-diversity countries. It is estimated that around 70,000 plant species, approximately 7500 species have been recorded to have medicinal value. The 300 species are used by 7800 medicinal drug manufacturing units in India, which consume about 2000 tons of herbs annually. There are estimated to be more than 717,319 registered practitioners of ayurveda, siddha, unani and homeopathy in India and in recent years, the growing demand for herbal products has led to the extinction of many important plant herbs. Ashwagandha is also a rare and endangered plant. *W. somnifera* possess good immunomodulatory anti-inflammatory, anti-tumor, antioxidant, anticancer properties and many pharmacologically and

medicinally important chemicals; they protect the cells from oxidative damage and diseases. In present paper we have tried to unveil the therapeutic knowledge about Ashwagandha, which is used to exploit novel medicines. Considering its relevance, further research is required to explore the potential from this medicinal herb (Sharma *et al.,* 2011).

The important advantages claimed for therapeutic uses of medicinal plants in various ailments are their safety besides being economical, effective and their easy availability because of these advantages the medicinal plants have been widely used by the traditional medical practitioners in their day to day practice. According to a survey (1993) of World Health Organization (WHO), the practitioners of traditional system of medicine treat about 80% of patients in India, 85% in Burma and 90% in Bangladesh.

In traditional systems of medicine the Indian medicinal plants have been used in successful management of various disease conditions like bronchial asthma, chronic fever, cold, cough, malaria, dysentery, convulsions, diabetes, diarrhea, arthritis, emetic syndrome, skin diseases, insect bite etc. and in treatment of gastric, hepatic, cardiovascular & immunological disorders (Verma *et al.,* 2011).

W. somnifera(L.) Dunal commonly known as "Ashwagandha", "Asgandh" and "Winter Cherry" belongs to the family Solanaceae Juss., which has 1250 species, widely distributed in the warmer parts of the whole world. The genus *Withania* comprises 23 species including *W. somnifera* and *W. coagulans* (L.) Dunal, both are high in medicinal value and extensively used in Ayurvedic formulations as "Rasayana". Its roots and leaves are used in a number of preparations for their anti-inflammatory, anticonvulsive, antitumor, immunosuppressive and antioxidant properties besides for promoting vigor and stamina. Therapeutic value of its roots is considered comparable to that of *Panax ginseng* and it is often referred to as 'Indian ginseng". Pharmacological investigation suggests its safe and better utility than P. ginseng (Korean drug Ginseng) notably in view of "Ginseng abuse syndrome" of the latter. Moreover, Ashwagandha has a shorter life cycle; it takes only 8 months to reach maturity while Ginseng requires 7 years to develop fully. *W. somnifera* is increasingly becoming a popular adaptogenic herb and is available throughout the western world as a dietary supplement.

The medicinal properties of Ashwagandha are attributed to the group of compounds called Withanolides. A large number of Withanolides have been

identified in its roots and leaves. Withanolides are C–28 steroidal lactones. Withaferin A represents the first natural lactone of the Withanolide series isolated from *W. somnifera* shoots. Till date, about forty steroidal lactones structurally related to Withaferin A, have been reported.

W. somnifera roots are the major source of desired phytochemicals and their harvest necessitates the plant. The roots of 'wild' population are not preferred for drug production because of their rich carbohydrate and variable alkaloid content. The Withanolide profile and susceptibility of the plants to diseases and pests vary with the chemotype. According to an estimate, the annual requirement of the drug at about 9127.5 tons far exceeds the annual production of about 5905.1 tons under cultivation. Lack of post harvest storage technology for roots and adequate information on the genetic basis of yield contributing traits are other unfavorable factors. Long time gap between planting and harvesting, excessive exploitation of natural resources, non-availability of procedures for synthetic production of Withanolides, and ever increasing demand-supply ratio for the drug are reasons enough to apply modern bio-techniques in Ashwagandha. The herb has been identified by National Medicinal Plant Board of India as one of the thirty-two selected priority medicinal plants, which are in great demand in the domestic and international markets (Prajapati *et al.,* 2003) (Sharada *et al.,*).

Among the available conventional chromatographic techniques HPTLC offers major advantages. HPTLC method was validated on the basis of its selectivity, linearity, limit of detection (LOD) and limit of quantification (LOQ) according to ICH requirements (Gupta *et al.,* 2011). HPTLC profile is quite helpful in setting up of standards for evaluating the purity and quality of Ayurvedic preparations. This will be helpful to overcome batch to batch variations in different Ayurvedic churna/preparations (Meena *et al.,* 2010) (Garg *et al.,* 2013).

Antibiotics are one of our most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction. However, over the past few decades, these health benefits are under threat as many commonly used antibiotics have become less and less effective against certain illnesses not, only because many of them produce toxic reactions, but also due to emergence of drug-resistant bacteria. It is essential to investigate newer drugs with lesser resistance. Drugs derived from natural sources play a significant role in the prevention and treatment of human diseases. In many developing

countries, traditional medicine is one of the primary healthcare systems Herbs are widely exploited in the traditional medicine and their curative potentials are well documented (Dubey *et al.,* 2004) About 61% of new drugs developed between 1981 and 2002 were based on natural products and they have been very successful, especially in the areas of infectious disease and cancer. Recent trends, however, show that the discovery rate of active novel chemical entities is declining. Natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanisms of action. The effects of plant extracts on bacteria have been studied by a very large number of researchers in different parts of the world. Much work has been done on ethnomedicinal plants in India.

The present study focuses **"Quantification of major Withanolides and antibacterial activity in over the counter health supplements containing *Withania somnifera* extracts''** was designed with the following objectives:

1) To quantify the Withanolide A and Withaferin A in selected samples.
2) To evaluate the antibacterial activity of selected samples

2.0 REVIEW OF LITERATURE

Medicinal plants are the principal health care resources for the majority of people all over the world. The healing properties of herbal medicines have been recognized in many ancient cultures. The traditional medical systems such as Ayurveda, Siddha and Unani are part of a time-tested culture and honored by people still today. Despite the increasing use of medicinal plants, their future, seemingly, is being threatened by complacency concerning their conservation. Global demand for herbal medicines is accompanied by dwindling supply of medicinal plants due to over-harvesting, habitat loss and agricultural encroachment. As millions of rural households use plants for self-medication community involvement in monitoring use and status of medicinal plants can contribute to effective strategies for their sustainable use (Kumar *et al.,* 2011).

The work on **"Quantification of major Withanolides and antibacterial activity in over the counter health supplements containing *Withania somnifera* extracts"** are discussed under following headings.

2.1 *W. somnifera* – Ashwagandha

2.1.1 Distribution

2.1.2 Taxonomical classification

2.1.3 Botanical description

2.2 Traditional application of *W. somnifera*

2.3 Pharmacological importance of *W. somnifera*

2.4 Ayurvedic formulation containing Ashwagandha

2.5 Bioactive components in commercial products containing *W. somnifera*

2.6 High performance thin layer chromatography

2.7 Antibacterial activity of herbal supplements

2.1 *Withania somnifera* – Ashwagandha

Withania somnifera (W. somnifera), commonly known as ashwagandha, Indian ginseng, and winter cherry, has been an important herb in the Ayurvedic and indigenous medical systems for over 3000 years. Historically, the plant has been used as an aphrodisiac, liver tonic, anti-inflammatory agent, astringent, and more recently to treat bronchitis, asthma, ulcers, emaciation, insomnia, and senile

dementia. Clinical trials and animal research support the use of ashwaganda for anxiety, cognitive and neurological disorders, inflammation and Parkinson's disease (Bashir *et al*., 2013).The root of *W.somnifera* is a constituent of over 200 formulations in Ayruveda, Siddha and Unani medicines for the treatment of various physiological disorders (Shrivastava *et al*.,2013)

2.1.1 Distribution

The geographic distribution of *W. somnifera* extends widely from the Atlantic Ocean to the South - East Asia and from the Mediterranean region to South Africa and India. The cultivation of *W. somnifera* is spread over about 4000 hectares throughout India. Both wild and cultivated populations of this species exhibit enormous diversity in chemical constituents. Studies pertaining to the identification of morphological and physiological variations based on extremely diversified geographical distribution of *W. somnifera* have been conducted. An extreme degree of variability was also recorded in *W. somnifera* with respect to growth habit and morphological characteristics of plants in different parts of India and in other countries. As Indian natural population exhibits inheritable variations, its documentation and molecular confirmation can help to evolve promising high yielding cultivar. In addition, this will serve us genetic stock for future breeding programs (Udayakumar *et al.,* 2013).

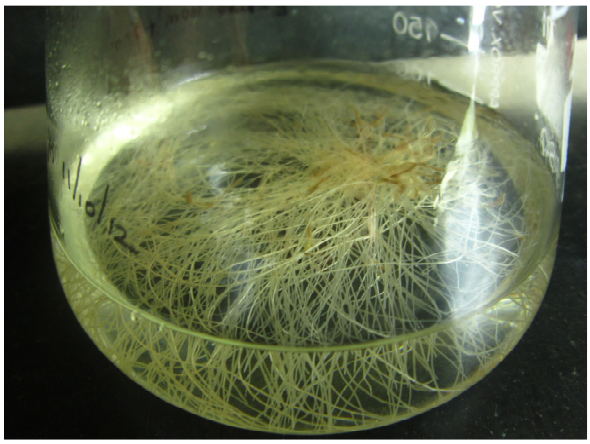
2.1.2 Taxonomical classification (Singh N *et al*., 2011)

Kingdom: Plantae
Division: Angiosperms
Class: Dicotiledoneae
Order: Tubiflorae
Family: Solanaceae
Genus: *Withania*
Species: *somnifera*

2.1 *Wthania somnifera* plant

***Withania somnifera in vivo* root**

***Withania somnifera in vitro* root**



2.2 Traditional application of *W. somnifera*

The plant *W. somnifera* is widely used in herbal formulations of Indian Ayurvedic medicine to attenuate a cerebral function deficit in the geriatric population, and to augment the faculty of learning and memory to provide a nonspecific host defense (Choudhury *et al*., 2014). *W. somnifera* is also used in Ayurvedic medicine, and it is one of the ingredients in many formulations to increase energy, improve overall health and longevity, and prevent disease. Indians used as both traditional and modern therapeutic agent cultivar are mostly preferred by drug manufacturers

Roots and leaves are used for medicinal purposes. The root contains several alkaloids including withanine, withananine, withananinine, pseudo-withanine, somnine, somniferine, somniferinine. The leaves of Indian chemotype contain 12 Withanolides, including Withanferin A. Steroidal lactones of withanolide series have been also isolated from it. Apart from its aphrodisiac activity it has been used for a number disease ailments like scrofula, rheumatism, anxiety neurosis, generalized weakness, inflammation and ulcers, due to its anti inflammatory, hypnotic, hepatoprotective, antibacterial, diuretic, antiarthritic, sedative narcotic and deobstruent properties without any known side effects. Its aphrodisiac properties have been also found effective in diabetics (Imtiyaz *et al*., 2013).The plant is also important for tropane alkaloids having anticancer, antiarthritic, anti-

rheumatic and anti-depression properties. The plant has gained popularity in Ayurvedic system of medicine due to its major medicinal properties like anti inflammatory, immune modulating, anti stress, vitality improver and repairment of the disorder related to nervous system (Kanungo *et al.,* 2013).

2.3 Pharmacological importance of *W. somnifera*

Numerous studies indicated that ashwagandha possesses antioxidant, antitumor, antistress, anti inflammatory, immunomodulatory, hematopoetic, anti ageing, anxiolytic, and antdepressive rejuvenating properties and also influences various neurotransmitter receptors in the central nervous system (Sharma *et al.,* 2011).It possesses immunomodulatory, anti inflammatory, antitumor, antioxidant, antistress, hemopoetic and rejuvenating properties (Mishra *et al.,*2000). Because of these properties,it is prescribed for many ailments such as sterility,rheumatism, memory loss and aging. Diseases like TB,chronic upper respiratory diseases and HIV been added to the list of Ashwagandha due to its strong immune stimulatory activity. It is also recognized as a blood tonic, especially in gynecological disorders including anemia and irregular menstruation Root is the economic important part but all the parts of the plant have shown remarkable importance in the field of pharmacology there are two cultivars of *W. somnifera* prevalent in Sri Lanka. They are local and Indian cultivar. Indian cultivar is mostly preferred by drug manufacturers because of its starchy nature. Root of local cultivar is fiberous and difficult to make powder for preparation of commercial drugs and home medicine as well. Because of this reason, local cultivar is identified as threatened plant. High demand of this plant resulted importation from other countries. Consequently, cultivation of the plant is immediately needed to make sure their availability to the pharmacological industry as well as to people who associated with traditional system of medicine (Shanmugaratnam *et al.,* 2013).

Ashwagandha is used for arthritis, anxiety, trouble sleeping (insomnia), tumors, tuberculosis, asthma, leukoderma (a skin condition marked by white patchiness), bronchitis, backache, menstrual problems, hiccups, and chronic liver disease (QamarUddin *et al.,* 2012). Ashwagandha is also used as an “adaptogen” to help the body cope with daily stress, and as a general tonic. Some people also use ashwagandha for improving thinking ability, decreasing pain and swelling (inflammation), and preventing the effects of aging. It is also used for fertility

problems in men and women and also to increase sexual desire. Many other pharmacological activities associated with aswagandha are antibiotic, aphrosidiac, astringent, anti inflammatory, diuretic, narcotic, sedative, and tonic. A decoction of *W. somnifera* roots and leaves is used as a nutrient and health restorative by pregnant women and the elderly. *W. somnifera* thickens and increases the nutritive value of the milk when given to nursing mothers. It has been reported that all of the major pharmacological activities of ashwagandha are due to their high content of polyphones and antioxidant activities. It has been reported that the antioxidant activities in a plant are dependent on some phyto constituents such as the phenolic compounds, the anthocyanin and ascorbic acids as well as many other important constituents (Kumari 2014).

2.4 Ayurvedic formulation containing Ashwagandha

Ayurveda is an ancient Indian medical system dating back to the Vedic period about 3000–1500 BC. It is considered to be one of the oldest of healthcare medical systems, and is based on sound scientific principles. The word Ayurveda (Sanskrit) is derived from the words *Ayur* and *Veda*. *Ayur* means life, while *Veda* means Science. Therefore, Ayurveda literally means the Science of Life. It is not just a medicinal system, but also a way of life. Ayurveda deals with the physical, as well as spiritual health (Mansi *et al.,* 2011).

W. somnifera is widely used in Ayurvedic medicine, and it is one of the ingredients in many formulations to increase energy, improve overall health and longevity, and prevent disease. Kushwaha *et al.,* 2012 study analyzed the efficacy of Ashwagandha root powder with water and with milk in treatment of hypertension. Roots of the plant are major source of active chemical substances and are traditionally used to cure ulcers, fever, cough, dyspnoea, consumption, dropsy, impotence, rheumatism, toxicosis and leucoderma. The pharmacological activities are mainly attributed towards the presence of different Withanolides mainly Withaferine A and Withanolide A. Ashwagandharishtha is the herbal formulation that contains roots of *W. somnifera* as a major crude drug along with 26 other herbs. It is mainly recommended for the treatment of impotency.

Ayurvedic formulations have numerous uses in Ayurveda. They affect or help to rectify the three doshas or humors in the body. Churna is a fine powder of well dried drug or drugs described in ancient literature. Quantitative estimation of

chemical markers of each ingredient in the poly herbal preparation required ideal separation technique. For herbal preparations (including polyherbal), there is an urgent need for scientific proof/validation with chemical standardization protocols/procedures, biological assays, animal models and clinical trials. HPTLC thus offers major advantages over other commonly available conventional chromatographic techniques. The proposed method was validated on the basis of its selectivity, linearity, limit of detection (LOD) and limit of quantification (LOQ) according to ICH requirements. HPTLC profile is quite helpful in setting up of standards for evaluating the purity and quality of Ayurvedic preparations. This will be helpful to overcome batch to batch variations in different Ayurvedic churna/preparations

Other uses of the formulation include syncope, epilepsy, cachexia, psychosis, emaciation, piles, digestive impairment, and neurological disorders. Quality of the crude drug used in manufacturing of such formulations depends on the amount of active ingredient presents. Literature survey indicated phytochemical variability in marketed preparations of *W. somnifera*. Therefore, standardization of these crude drugs has become necessary before their use for preparing formulations. Moreover, standardization of finished product is of equal importance to be considered in assuming the safety and efficacy product before use (Manwar *et al.,* 2012).

Ashwagandhadi lehya is a classical Ayurvedic polyherbal formulation included in Ayurvedic formulary of India. Lehya or Avaleh is one of the several groups of Ayurvedic formulations. It arises from Sanskrit root word Lih Aswadane, the form of medicine which can be tasted with help of tongue. It is a semi-solid preparation of drugs, prepared with the addition of jaggery, sugar or sugar candy and boiled with prescribed drug juice or decoction. The major ingredient of Ashwagandhadi lehya is Ashwagandha which is the most important medicinal plant mentioned in various Indian Systems of Medicine (Ayurveda, Siddha and Unani). It has been described in the *Nighantus* as tonic, alterative, pungent, astringent, hot and aphrodisiac and recommended for rheumatism, cough, dropsy, and senile debility. Besides *W. somnifera*, the other ingredients present are *Elettaria cardamonum*, *Myristica fragrans*, *Glycyrrhiza glabra*, *Hemidesmus indicus* and *Cuminum cyminum*. *Ashwagandhadi lehya* is therapeutically used for disorder of blood, Cachexia, Piles, Psychosis and is used as Rejuvenating agent and Aphrodisiac (Mansi *et al.,* 2011).

2.5 Bioactive components in *W. somnifera* commercial products

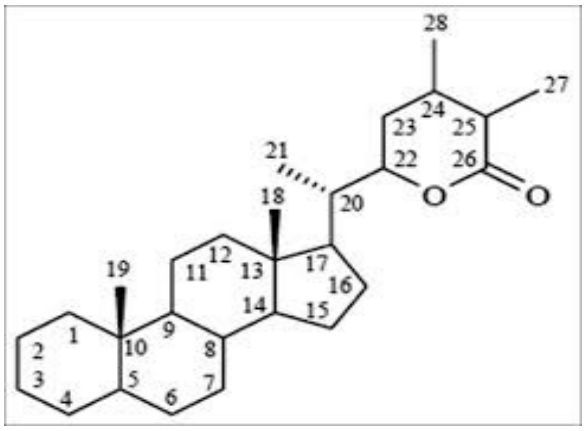
Over 35 chemical constituents are found in the roots of *W. somnifera*. The biologically active chemical constituents are alkaloids (isopellertierine, anferine), steroidal lactones (Withanolides, Withaferins), saponins containing an additional acyl group (sitoindoside VII and VIII), and sitoindoside is a Withanolide containing a glucose molecule at carbon 27. There are two main Withanolides, Withaferin-A and Withanolide D which are thought to be responsible for much of *Withania's* pharmacological activity. The main constituents found in roots of *W. somnifera* are Withanolides. Withanolides are steroidal and have resemblance, both in their action and appearance, to the active constituents of Asian ginseng known as ginsenosides (Kumari 2014).

Secondary metabolites are organic molecules that are not involved in the normal growth and development of an organism. While primary metabolites have a key role in survive of the species, playing an active function in the photosynthesis and respiration, absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of the organism's survivability, often playing an important role in plant defense. These compounds are an extremely diverse group of natural products synthesized by plants, fungi, bacteria, algae, and animals. Most of secondary metabolites, such as terpenes, phenolic compounds and alkaloids are classified based on their biosynthetic origin. Different classes of these compounds are often associated to a narrow set of species within a phylogenetic group and constitute the bioactive compound in several medicinal, aromatic, colorant, and spice plants and/or functional foods (Costa *et al*., 2007). 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 isolated. 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. The major chemical constituents of these plants, Withanolides, are mainly localized in leaves, and their concentration usually ranges from 0.001 to 0.5% dry weight (DW). The Withanolides are a group of naturally occurring C28- steroidal lactones built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring (Mirjalili *et al.,* 2009).

One of the most important Withanolides isolated from *Withania* extracts is the anticancer compound Withanferin A. The neuropharmacological properties of Withanolide A have also recently attracted interest, since it has been found to promote neurite outgrowth and synaptic reconstruction, and could thus be useful in treating neurological disorders such as Alzheimer's disease and Parkinson's disease. However, the levels of Withanolide A in *W. somnifera* plants are usually very low and, contrary to the other Withanolides, it occurs mainly in the roots. The Withanolide biosynthetic pathway is still unknown, but experiments with *W. somnifera* cultures have clarified some of its aspects. In general, undifferentiated calli and cell suspensions of *W. somnifera* do not produce Withanferin A Ray 1999, although shoot tips of this plant accumulated up to 0.16% of Withanferin A and 0.08% of withanolide D. Recently, a production of 0.2% of Withanolide A in in vitro regenerated shoots of *W. somnifera*, and 1% of Withanferin A have been reported. Hairy roots of this species are able to produce Withanolide D (Ray *et al.,* 1996), and sporadically, some root lines also produce Withanferin A. More recently, Murthy et al. described the production of Withanolide A, which has also been reported in in vitro regenerated roots (Mirjalili *et al.,* 2009).

Withanolides

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 isolated. 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. The major chemical constituents of these plants, Withanolides, are mainly localized in leaves, and their concentration usually ranges from 0.001 to 0.5% dry weight (DW). The withanolides are a group of naturally occurring C28- steroidal lactones built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring (Mirjalili *et al.,* 2009).



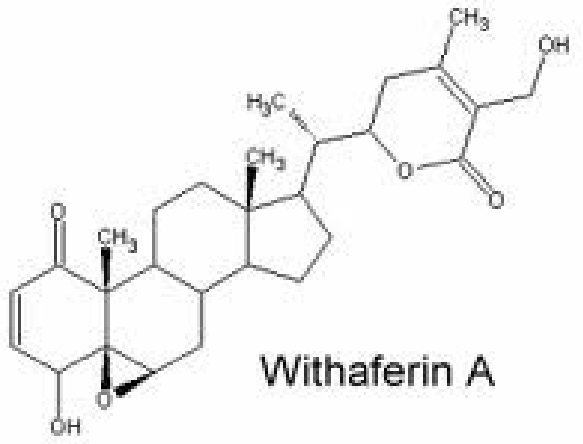
The basic structure of Withanolides.

The Withanolide skeleton may be defined as a 22-hydroxyergostan-26-oic acid-26, 22-lactone. There are many novel structural variants of Withanolides with modifications either of the carbocyclic skeleton or the side chain and these have often been described as modified Withanolides or ergostantype steroids related to Withanolides. These compounds are generally polyoxygenated and it is believed that plants elaborating them possess an enzyme system capable of oxidizing all carbon atoms in a steroid nucleus. The characteristic feature of Withanolides and ergosane-type steroids is one C8 or C9-side chain with a lactone or lactol ring but the lactone ring may be either six-membered or fivemembered and may be fused with the carbocyclic part of the molecule through a carbon-carbon bond or through an oxygen bridge. Appropriate oxygen substituents may lead to bond scission, formation of new bonds, aromatization of rings and many other kinds of rearrangements resulting in compounds with novel structures.

Withaferin A

Withanolides are a group of naturally occurring steroids based on ergostane nucleus and characterized by a lactone-containing side chain. Structural diversity of Withanolides present in *Withania* species 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 Withanferin A have been reported: antiangiogenesis through NF- κB inhibition cytoskeletal architecture alteration by covalently binding annexin II and apoptosis induction through the protein kinase C pathway in leishmanial cells. The primary molecular target of Withanferin A was shown to be the ß5 subunit of the proteosome. In view of these wide-ranging pharmacological activities, the production of Withanferin A by plant tissue culture technique is studied many previous studies have been reported about Withanolides production in tissue culture of the common species *W. somnifera.* Withanferin 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. (AbouZid *et al.,* 2010)



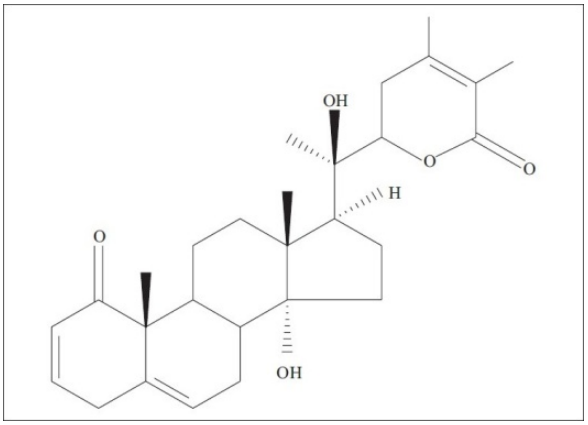
Withaferin A

Withanolide A

Withanolide A is an important secondary metabolite in *W. somnifera,* which is having a high medicinal value and possesses potent anti-tumor and antioxidant properties. In the study of Praveen N., *et al.,* (2010) Distribution of Withanolide A in various organs of *W. somnifera* was investigated by High Performance Liquid Chromatography (HPLC) method. The quantitative distribution of Withanolide A was

different in various organs tested, the accumulation was 386, 342, 272, 206, 102, 56, 35 and 23 /g g-1 DW in shoot tips, leaves, nodes, whole plant, internodes, roots, flowers and seeds respectively. The content of Withanolide A gradually decreased from aerial parts i.e., from young leaves to the root. In root, the root tip accumulated higher concentration when compared to middle and basal portion. The study provide the data base for the regulation and control of Withanolide A, moreover it provides the scientific evidence for the rational development and utilization of the *W. somnifera* resources.

Withanolide A has attracted interest due to its strong neuropharmacological properties of promoting outgrowth and synaptic reconstruction. Withanolide A is therefore an important candidate for the therapeutic treatment of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, convulsions, cognitive function impairment, as it is able to reconstruct neural networks (Praveen *et al.,* 2013).



2.6 High performance Thin Layer chromatography (HPTLC)

Chromatography is one of the fast emerging tools by which the quality control and fingerprint of herbs can be maintained. Using this technique, the identification of various chemical markers of the herbal drugs can be easily done and it also helps to identify the same herbs in combination. Application of TLC/HPTLC methodology in testing of phytoconstituents from individual herbal drugs and fingerprint

characteristic of the herbal plants are reviewed in this paper. Popularity of TLC/HPTLC analytical method for analysis of herbal drugs due to economic, rapid, simultaneously screening of large number of herbal samples and less time consuming methods. The different mobile phase, spraying reagent, property of herbal drugs and its phytoconstituents, TLC/HPTLC plates, trouble shooting of HPTLC, different developing solvents and chromatograms are pointed.

In view of this, high-performance thin layer chromatography (HPTLC) based methods could be considered as a good alternative as they are being explored as an important tool in routine drug analysis. A major advantage of HPTLC is its ability to analyze several samples simultaneously using a small quantity of mobile phase; this reduces the time and cost of analysis.13–15 In addition, it minimizes exposure risks and significantly reduces disposal problems of toxic organic effluents, thereby reducing possibilities of environment pollution. HPTLC also facilitates repeated detection of chromatogram with the same or different parameters. Furthermore, in the case of HPTLC, there are no restrictions on the choice of solvents and mobile phases; drug and lipophilic excipients can be dissolved in a suitable solvent that would evaporate during spotting on HPTLC plate leaving behind analyte as a thin band. Therefore, for such methods, extraction procedure is not required always and could be developed for analyzing drug without any interference from excipients. The present study described the development of environment friendly, simple, rapid, economic, and validated HPTLC method for routine estimation of PPD from bulk and pharmaceutical dosage forms such as tablets and MME formulations and solution developed in-house (Patel *et al.,* 2010).

Jirge *et al.,* developed and validated HPTLC method can be used to determine batch to batch variations and routine analysis by herbal manufacturers of Ashwagandha formulations.

2.7 Antibacterial activity in root culture samples herbal supplements

Antibiotics play a major role in clinical medicine in eradicating pathogens. Unfortunately, excessive/inappropriate use of antibiotics has contributed to the spread of important clinical and public health problems of antibiotics resistance. The present study aimed to examine a comparative analysis of the antibacterial efficacy of ethanol and methanol root extracts of *W. somnifera* with commercial antibiotics against the

bacterial strains. The ethanol and methanol root extracts of *W. somnifera* found to show equalent/potent antibacterial activity against the bacterial strains as compared to antibiotic discs. Methanol root extract of *W. somnifera* showed an effective antibacterial activity might be due to presence of numerous bioactive compounds. Thus, there is a tremendous need for future research to provide better evidence, to address the isolation and characterization of these bioactive components of methanol root extract of *W. somnifera* responsible for the antibacterial activity, offer an ecologically and economically safe drug compared to commercial antibiotics (Jeyanthi *et al.,* 2013).

Medicinal plants constitute an effective resource for both traditional and modern medicines, and herbal medicine has been shown to have genuine utility. In Nigeria, many plants are used in traditional medicine as antimicrobial agents but only few are documented. Plants based system of traditional medicine has continued to play an essential role in health care in many cultures. The increased use of plant derived products as alternatives to orthodox or synthetic drugs and increasing awareness of beneficial effects of natural products has resulted in increased interest in alternative therapies. Extracts from plants have been utilized for their antifungal, antiviral and antibacterial activities globally. Mastanaiah *et al.,* 2011 report the antibacterial activity of the chloroform, methanol and hexane of each sample was evaluated by using well diffusion method or couplet metho

3.0 MATERIALS AND METHODS

The materials used and methods followed for the study entitled **"Quantification of major Withanolides and antibacterial activity in over the counter health supplements containing *Withania somnifera* extracts"** are described in this chapter.

3.1. Collection of samples

3.2. Extraction of Withanolides from root culture samples and herbal supplements

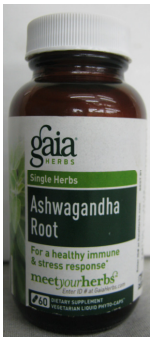
3.3. Assessment of Withanolides in root culture samples and herbal supplements using HPTLC

3.4. Assessment of antibacterial activity of root culture samples and herbal supplements

3.1 Collection of samples

Two months old *in vitro* root, mature *in vivo* root samples of *W. somnifera* were obtained from the Department of Biochemistry, Biotechnology and Bioinformatics in Avinashilingam university plant tissue culture laboratory. Herbal supplements containing *W. somnifera* as sole component or in combination with other herbal extracts. Gaia ashwagandha root,Himalaya ashwagandha were purchased from local medical shop. The other supplements of raja ashwagandha lehyam, ashwagandha chooranam were collected from tirupattur and finally ashwagandha paradise was purchased from US. The details of each supplement are given below:

Gaia ashwagandha root



Name of the supplement	Ashwagandha Root
Manufacturer	Gaia Herbs
Clinical Usage	For healthy immune and stress response
Dosage	One capsule two times daily
Mfd. Date	Not available
Exp. Date	December 2013
Cost	60Capsule/Rs.120
Amount of Withanolide	350mg

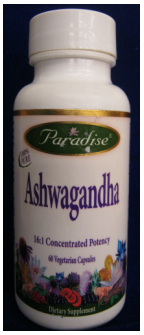
Himalaya ashwagandha

Name of the supplement	Ashvagandha
Manufacturer	Himalaya
Clinical Usage	Helps cope with life's daily stress
Dosage	one capsule twice daily
Mfd. Date	March 2013
Exp. Date	March 2016
Cost	60Capsule/Rs.100
Amount of Withanolide	250 mg

Ashwagandha chooranam

Name of the supplement	Ashwagandha Chooranam
Manufacturer	Raja sidhaa marundhagam
Clinical Usage	Not mentioned
Dosage	As direct by the physician
Mfd. Date	October 2011
Exp. Date	October 2016
Cost	100 g/Rs.55
Amount of Withanolide	Not mentioned

Ashwagandha paradise



Name of the supplement	Ashwagandha
Manufacturer	Paradise
Clinical Usage	Adaptogen
Dosage	One vegetarian capsule 1-3 times daily
Mfd. Date	Not available
Exp. Date	December 2013
Cost	£ 38/60 capsules
Amount of Withanolide	250mg

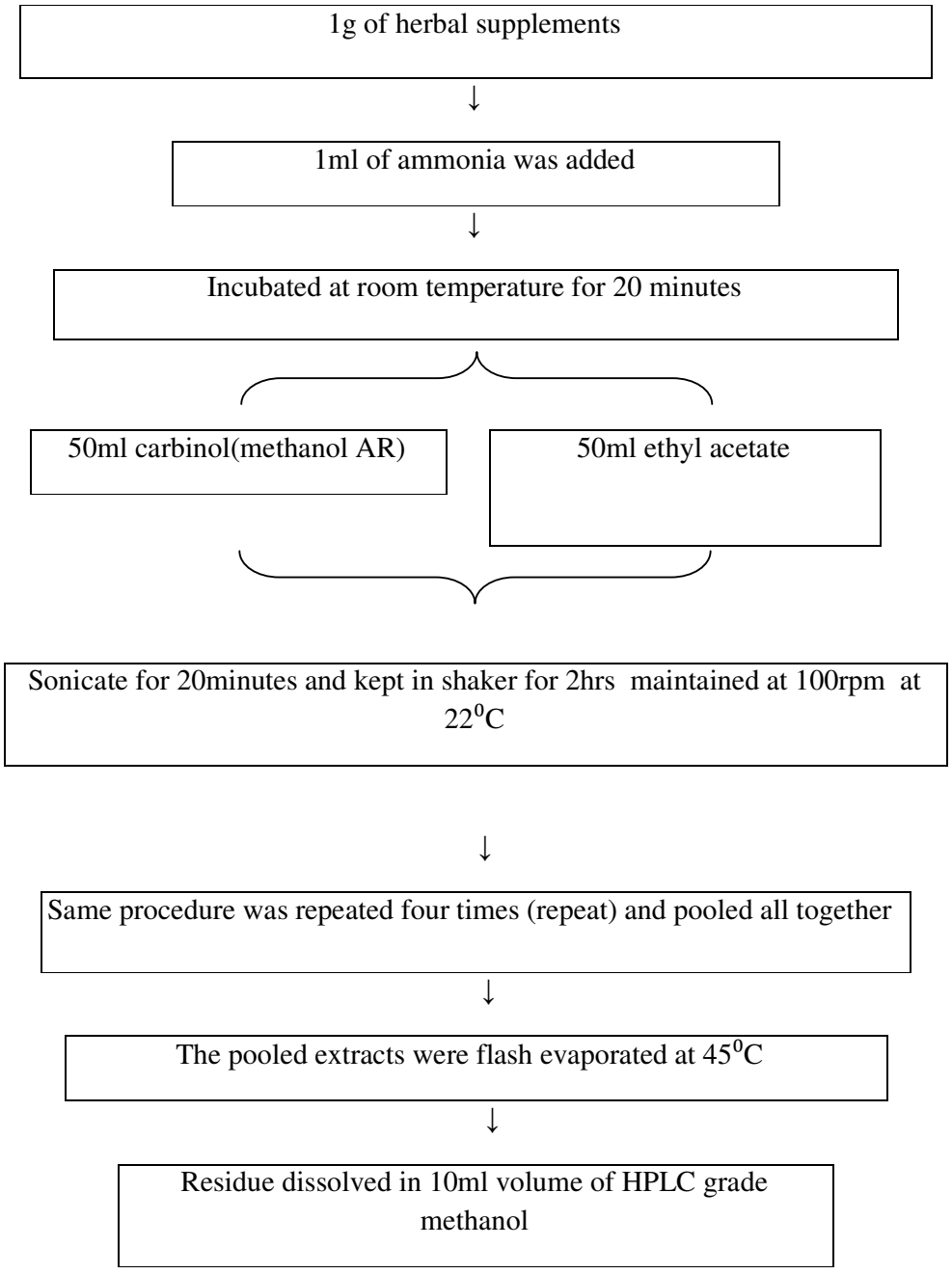
Raja ashwagandha lehyam

Name of the supplement	Raja Ashwagandhi Lehyam
Manufacturer	Raja sidhaa marundhagam
Clinical Usage	Enrich Power
Dosage	5mg twice daily with milk
Mfd. Date	January 2013
Exp. Date	January2018
Cost	125 g/Rs.50
Amount of Withanolide	Not mentioned

Fig.3.2

Extraction of Withanolides from root culture samples and herbal supplements (Patel *et al*., 2013)

Extraction was carried out using two solvents namely carbinol (methanol .AR) and ethyl acetate as given below:



3.3 Assessment of Withanolides using HPTLC

The extracts were analyzed for quantification of Withanolide using HPTLC.The sample was spotted in the form of bands of width 6 mm with a microlitre syringe (Camag) on precoated silica gel aluminium plate $60F_{254}$ (20 cm ×10 cm) with 250µm thickness. A constant application rate of 1.0 µl/s was employed and space between two bands was 5 mm. The slit dimension was kept at 4×0.3mm and 10 mm/s scanning speed were employed. The mobile phase consisted of Toluene (Qualigen), Ethyl acetate (Rankem) and formic acid (Qualigen) 5:5:1. Linear ascending development was carried out in 20 cm x 10 cm twin trough glass chamber presaturated with the mobile phase. The optimized chamber saturation time for mobile phase was 15 min at room temperature (25 ^{0}C ± 2) at relative humidity of 60% ± 5. The length of chromatogram run was 80.0mm. Subsequent to the scanning, TLC plates were dried in a current of air with the help of an air dryer. The plate was derivatized using Concentrated Sulphuric acid (Qualigen), Glacial acetic acid (Rankem), Anisaldehyde (Himedia), Carbinol AR (Himedia) and oven baked at 110^{0}C for a minute. Densitometric scanning was performed with TLC scanner III in the reflectance-absorbance mode at 530 nm for Withanolides and 223 nm for withaferin and operated by Win CATS software. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was carried out by comparing peak areas with linear regression (Jirge *et al.,* 2013).

3.4 Assessement of antibacterial activity of *in vitro*, *in vivo* root culture samples and herbal supplements

The extracts obtained above were screened for their antibacterial activity in comparison with standard antibiotics penicillin, streptomycin, canamycin, gentamycin, tetracycline *in vitro* by well diffusion method. The cup-plate agar diffusion method was employed to assess the antibacterial activity of the prepared extracts. The culture media was prepared by nutrient broth (1.3g/100ml) and agar agar (1.8g/100ml).20 ml of the inoculated nutrient agar were distributed into sterile petri dishes in each of these plates, 5 mm in diameter, were cut using a sterile gel sucker and the agar discs were removed. Using sterilized micropipettes 25µl of

different solvents with selected *W. somnifera* extracts was added into the well, allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37ºC for 18 hours. The respective solvents were used as controls. The diameters of the growth inhibition zones were measured after 24 hours of incubation and the mean values were tabulated (Velu 2012).

4.0 RESULTS AND DISCUSSION

Pharmaceutical importance of plants has led to the discovery and adoption of plant extracts which were commonly used in traditional medicine, as alternative source of remedy. A vast diversity of herbal ingredients, major proportion of which is derived from wild, provide the resource base to the herbal industry. *W. somnifera* (Ashwagandha) is a plant used in medicine from the time of Ayurveda, the ancient system of Indian medicine. The dried roots of the plant are used in the treatment of nervous and sexual disorders (Singh *et al.,* 2010).

The present study entitled **"Quantification of major Withanolide and antibacterial activity in over the counter health supplements containing *Withania somnifera* extracts"** is aimed to analyze the compounds present in herbal supplements containing Ashwagandha as the sole ingredients or as a major component. The results of the study are presented and discussed under the following headings:

4.1 Levels of Withanolides in the *in vitro*, *in vivo* root culture samples and herbal supplements in HPTLC analysis

4.2 Antibacterial activity of *in vitro*, *in vivo* root culture samples and herbal supplements

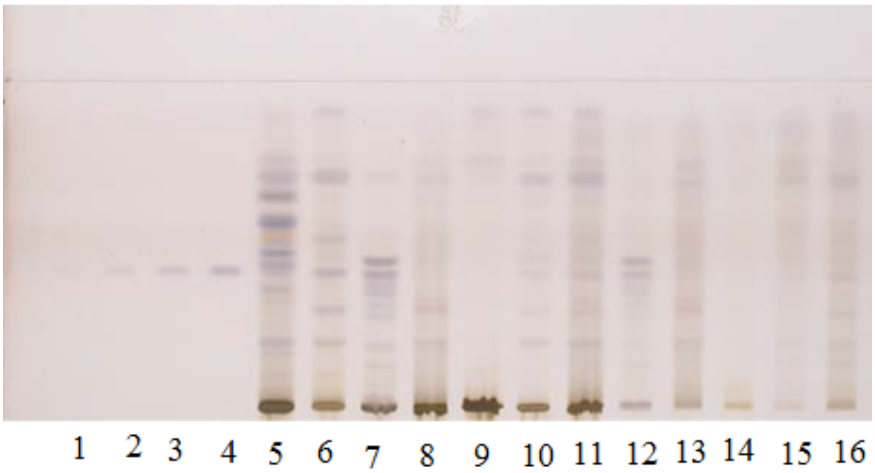
4.1 Levels of Withanolides in the *in vitro*, *in vivo* root culture samples and herbal supplements in HPTLC analysis

Methanol and ethyl acetate extracts of culture root samples and herbal supplements were subjected to HPTLC analysis. Uniform volume of 10µl *in vitro, in vivo* culture root samples and herbal supplements were taken for loading on the TLC silica plate mobile phase used toluene: ethyl acetate: formic acid (5:5:1) (Sharma *et al.,* 2007). The R_f value of the standard Withanolide A and Withaferin A were found to be 0.49 and 0.32 respectively

Plate 4.1

HPTLC profile of Withanolide A of root culture smples and herbal supplements

Derivatized plate at white R



Solvent system:

Toluene: ethyl acetate: formic acid (5:5:1).

Developing agent:

Con.H_2SO_4: glacial acetic acid: methanol: anisaldyhyde (5:10:85:0.5).

Table 4.1

Levels of Withanolide A in root culture samples and Herbal supplements in HPTLC analysis

S.No	Track	Sample Name	Concentration	Rf Value	Withanolide A in µg
Standard					
1	Lane 1	Standard 1	0.5	0.49	50.0
2	Lane 2	Standard 2	1	0.49	100.00
3	Lane 3	Standard 3	2	0.50	200.00
4	Lane 4	Standard 4	4	0.49	400.00
Methanol extracts					
5	Lane 5	root culture *(in vitro)*	10	0.29	50.381
6	Lane 6	root culture (*in vivo)*	10	0.30	150.94
7	Lane 7	Himalaya ashwagandha	10	0.31	10.678
8	Lane 8	Gaia ashwagandha root	10	0.29	0.408
9	Lane 9	Raja ashwagandha lehyam	10	-	-
10	Lane 10	Ashwagandha chooranam	10	0.30	8.541
11	Lane 11	Ashwagandha paradise	10	0.30	6.545
Ethyl acetate					
12	Lane 12	root culture *(in vitro)*	10	0.30	32.65
13	Lane 13	root culture *(in vivo)*	10	0.30	121.24
14	Lane 14	Himalaya ashwagandha	10	0.31	10.678
15	Lane 15	Gaia ashwagandha root	10	0.30	5.545
16	Lane 16	Raja ashwagandha lehyam	10	-	0
17	Lane 17	Ashwagandha chooranam	10	0.29	10.678
18	Lane 18	Ashwagandha paradise	10	0.31	9.892

*Values are mean of triplicates

Tables 4.1, plate 4.1 and figures 4.1 and 4.2 show the concentration of standard Withanolide A in methanolic extract of *in vitro, in vivo* root culture samples. *in vivo* root culture sample showed 150.94 µg/g of Withanolide A which was followed by 50.381 µg/g *in vitro* root culture sample; Himalaya Ashwagandha 10.678 µg/g; Ashwagandha chooranam 8.541 µg/g; Ashwagandha paradise 6.545 µg/g. Withanolide A content was found to be undetectable in Raja ashwagandha lehyam.

The ethyl acetate extracts showed the maximum content of Withanolide A in i*n vivo* root culture sample (121.24 µg/g) which was followed *in vitro* root culture sample (32.65 µg/g). Aswagandha chooranam and Himalaya ashwagandha showed same concenteration (10.678 µg/g) of Withanoliode A standard; Ashwagandha paradise showed 9.892 µg/g Withnolide A.

Levels of Withanolide A and Withaferin A present in culture root samples and herbal supplements are shown in Tables 4.1 and 4.2. Among the products, methanol and ethyl acetate extracts of *in vivo* root culture sample were found to contain high amount of Withanolide A (10.678µg/g). Methanol and ethyl acetate extracts of Ashwagandha paradise and the ethyl acetate extract of Himalaya ashwagandha contain highest amount of Withaferin A (51.462µg/g). Both the active principles were found to be absent in Raja ashwagandha lehyam.

The above results revealed in the methanol extract *in vivo* root culture showed the highest Withanolide A content when compared to ethyl acetate extract.

Plate 4.2

HPTLC profile of Withaferin A of root culture smples and herbal supplements

Derivatized plate at white R

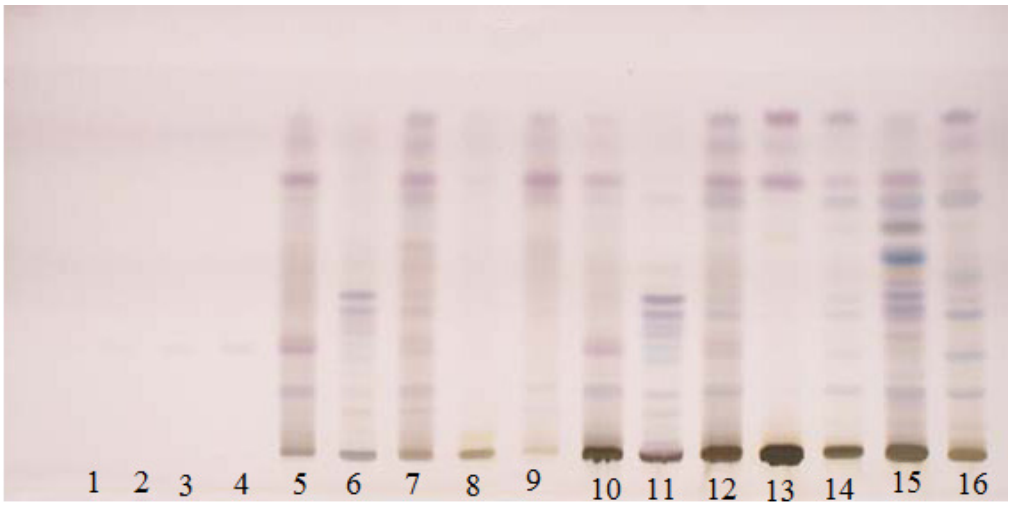


Table 4.2

Levels of Withanferin A in root culture samples and Herbal supplements in HPTLC analysis

S.No	Track	Sample Name	Concentration	Rf Value	Withaferin A in µg
Standard					
1	Lane 1	Standard 1	0.5	0.49	50.0
2	Lane 2	Standard 2	1	0.49	100.00
3	Lane 3	Standard 3	2	0.50	200.00
4	Lane 4	Standard 4	3	0.49	400.00
Methanol extracts					
5	Lane 5	Root culture*(in vitro)*	10	0.29	1.45
6	Lane 6	Root culture(*in vivo)*	10	0.30	43.54
7	Lane 7	Himalaya ashwagandha	10	0.31	10.678
8	Lane 8	Gaia ashwagandha root	10	0.29	10.678
9	Lane 9	Raja ashwagandha lehyam	10	-	0
10	Lane 10	Ashwagandha chooranam	10	0.30	9.591
11	Lane 11	Ashwagandha paradise	10	0.30	51.462
Ethyl acetate					
12	Lane 12	Root culture*(in vitro)*	10	0.30	0
13	Lane 13	Root culture*(in vivo)*	10	0.30	32.43
14	Lane 14	Himalaya ashwagandha	10	0.31	51.462
15	Lane 15	Gaia ashwagandha root	10	0.30	10.678
16	Lane 16	Raja ashwagandha lehyam	10	-	0
17	Lane 17	Ashwagandha chooranam	10	0.29	0
18	Lane 18	Ashwagandha paradise	10	0.31	51.462

*Values are mean of triplicates

Table 4.2, plate 4.2 and figure 4.1-4.2 show the levels of Withaferin A in root culture samples and herbal supplements in HPTLC analysis. Withaferin A, concentration was found to be higher in Ashwagandha paradise (51.462 µg/g) than that found in the i*n vivo* root culture sample (43.54 µg/g); Ashwagandha chooranam sample (9.591 µg/g); *in vitro* root culture sample (1.45 µg/g) of methanolic extracts. Himalaya ashwagandha and Gaia ashwagandha root showed same concenteration (10.678 µg/g) of Withaferin A in methanolic extract. among the extracts ethyl acetate extract of ashwagandha paradise was found to contain high content of withaferin A followed by Himalaya ashwagandha 51.462 µg/g. In same concentration when compared to other herbal supplements Gaia aswagandha root (10.678 µg/g).

Above the results the Ashwagandha paradise showed the same concentration of Withaferin A in both extracts (51.462 µg/g). Himalaya ashwagandha showed maximum concentration in both extracts(51.462 µg/g).

Fig 4.1

Levels of Withanolide A and Withaferin A in methanol extracts of culture root samples and herbal supplements

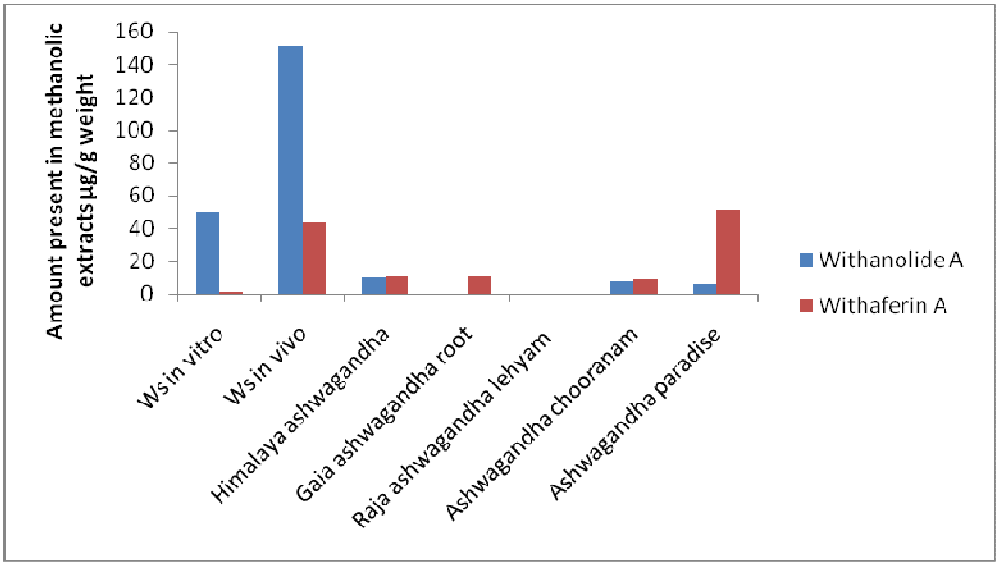
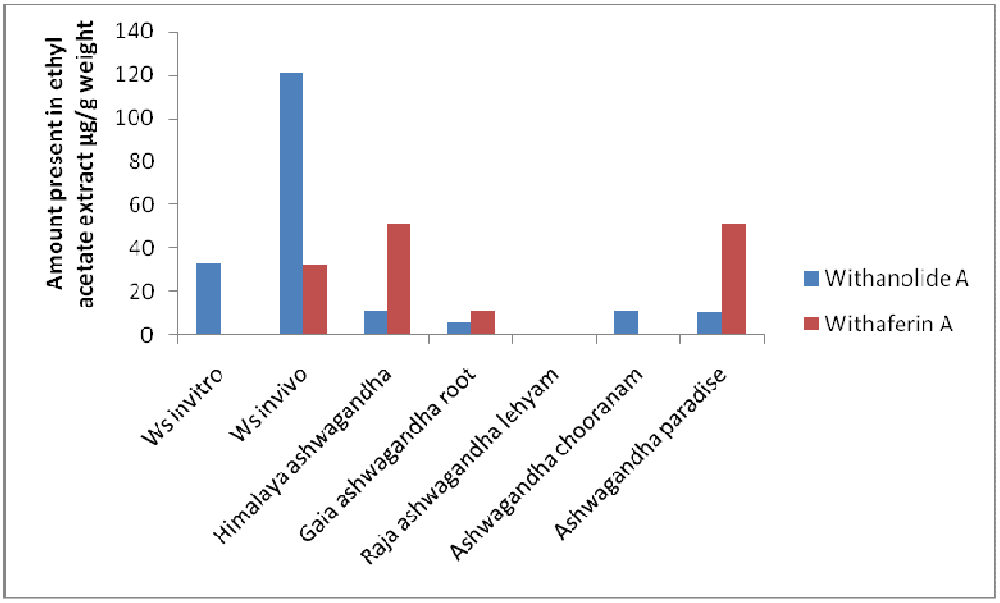


Fig 4.2

Levels of Withanolide A and Withaferin A in ethyl acetate extracts of culture root samples and herbal supplements



From the study it was observed that all the sample expect Raja ashwagandha lehyam showed the presence of Withanolide A and Withaferin A. Early studies of Patil *et al.,*2013 and Mirjalili *et al.,* 2009 also reported similar Withanolide content in *W. somnifera* root culture samples.

Linearity

The calibration graphs were constructed by plotting peak area against amount of drug (µg/µl). The stock solution (0.1µg/µl) of Withaferin A and Withanolide A was prepared in methanol. Different concentration of the stock solution for Withaferin A and Withanolide A (10µl) were loaded to a plate to provid 0.5, 1, 2, 4µg of Withaferin A and Withanolide A band separately. Amount of Withaferin A and Withanolide A in the sample (methanol extract and ethyl acetate extracts of *W. somnifera)* were computed from calibration curve (Fig 4.3 and 4.4).

Fig 4.3

Calibration curve for the standard Withanolide

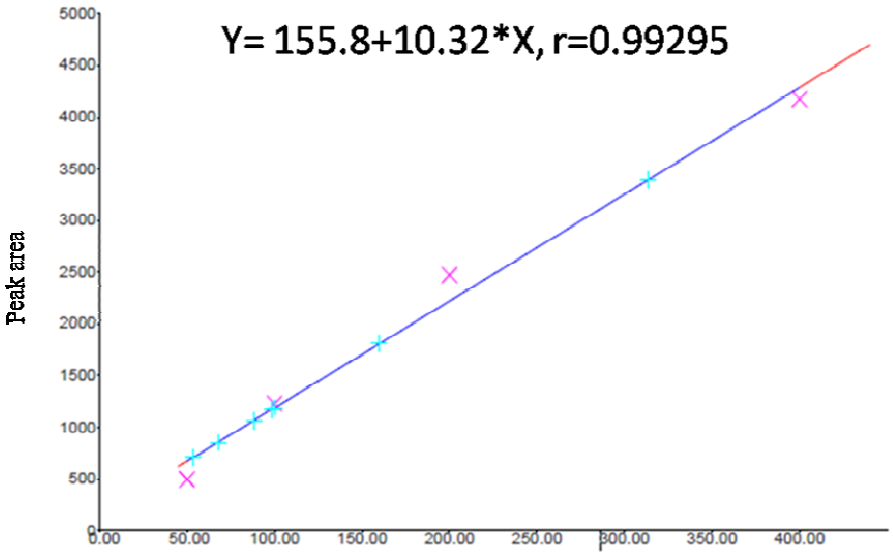
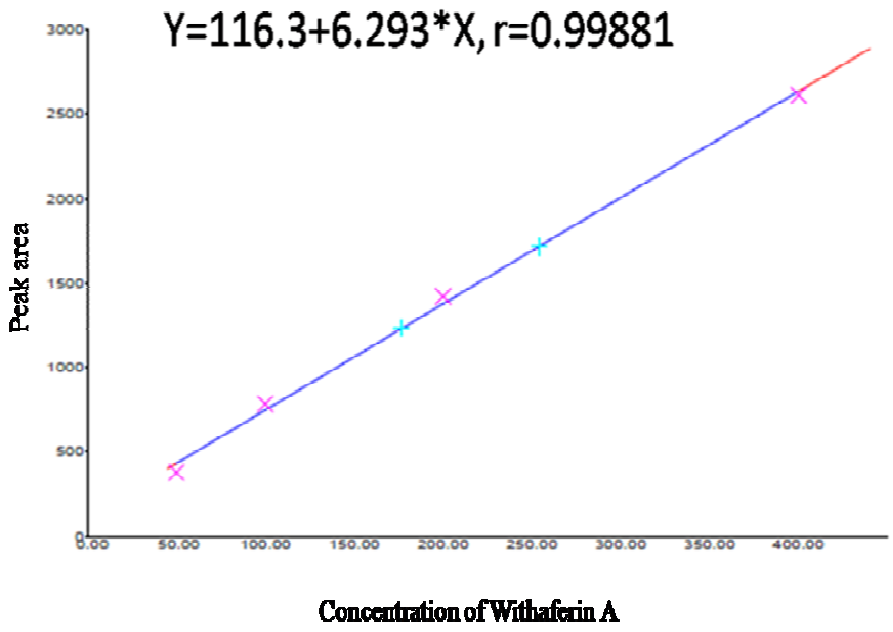


Fig 4.4

Calibration curve for the standard Withaferin A



Linear correlation between the peak area and applied concentration was found for the stand.the calibration plots of peak area against for concentration were linear in the range at 0.05 to 0.4µg in linear. In linear both Withanolide A and Withaferin A peak area versus concentration was subjected to least square linear regression analysis and the slope, intercept and correlation over the concentration range of Withanolide A(x). The linear equation for calibration plot was y=155.8+10.32x for Withanolide A and y=116.3+6.293x; the correlation coefficient(r) for Withanolide A and Withanolide A were r=0.99295 and r=0.99881 respectively. The calibration data and plot for Withanolide A are show in fig.4.3 and 4.4.

4.4 Antibacterial activity

Tables 4.3 and plates 4.5 to 4.9 show the antibacterial activity of methanol and ethyl acetate extracts of culture root samples containing *W. somnifera* and herbal supplements. In the present study, *in vitro*, *in vivo* root culture samples and five herbal supplements were selected and their methanol and ethyl acetate extracts were tested for the antibacterial activity against three bacterial cultures (*E.coli, B. subtilis, K. pneumoniae*). The antibacterial activity was assessed by the presence of zone of inhibition for the concentration of 1µg/ µl. In methanol and ethyl acetate extracts, *in vitro*, *in vivo* root culture sample of *W. somnifera* showed the zone of inhibition(3mm) and (5mm) respectively, in *K. pneumoniae* organism when compared to other test organisms of *B. subtilis* and *E.coli*. Ranges from 1mm to 10mm; 3mm to 6mm for *B. subtilis* culture, 1mm to 9mm; 2mm to 12mm for *E.coli*. 3mm to 8mm; 2mm to 13mm for *K. pneumoniae*. The results were shown in plate 4.5 to 4.8. Among the samples taken for the study ethyl acetate extracts of ashwagandha paradise showed maximum inhibitory effect against *B. subtilis* culture (10mm). Methanol extract of Gaia ashwagandha root showed maximum inhibitory action against *E.coli* culture (12mm). Methanol and ethyl acetate extracts of *in vitro* and *in vivo* root culture samples showed only minimum inhibition against all three bacterial cultures.

Jeyanthi *et al.,* 2013 reported that ethyl acetate and methanol root extracts of *W. somnifera* showed potent antibacterial activity against *B. subtilis, E.coli and K. pneumoniae* as compared to antibiotic discs. In addition Jamal *et al.,* 2013 also proved that the aqueous and ethanol extracts of *W. somnifera* showed for antibacterial

activities against *B. subtilis and E.coli.* Jain *et al.,* 2011 indicated that the methanolic and aqueous root extracts of *W. somnifera* might be exploited as natural drug for the treatment of several infectious diseases caused by *E.coli.* Singariya *et al.,* 2011 concluded water extract of leaves of *W. somnifera* showed highest antibacterial activity against *E.coli.*

Table 4.3

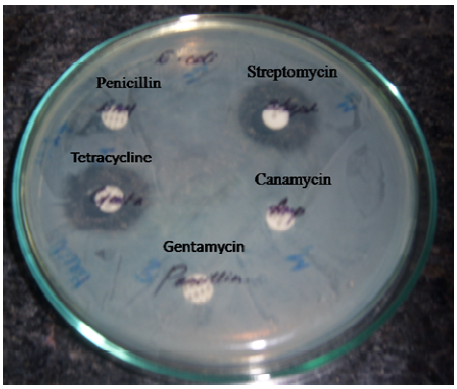
Antibacterial activity of methanol and ethyl acetate extracts of culture root samples and herbal supplements

Samples	Zone of inhibition in mm		
	B. subtilis	***E.coli***	***K. pneumoniae***
Standard(1µg/ µl)			
Penicillin	3	1	3
Streptomycin	2	5	3
Canamycin	4	6	1
Gentamycin	6	4	2
Tetracycline	5	5	7
Methanol extracts(1µg/µl)			
Root culture*(in vitro)*	0	2	3
Root culture*(in vivo)*	5	4	10
Himalaya ashwagandha	5	8	13
Gaia ashwagandha root	4	12	5
Raja ashwagandha Lehyam	3	2	6
Ashwagandha Chooranam	4	4	2
Ashwagandha Paradise	6	7	8
Ethylacetate extracts(1µg/µl)			
Root culture*(in vitro)*	0	2	5
Root culture *(in vivo)*	2	1	3
Himalaya ashwagandha	1	5	5
Gaia ashwagandha root	7	8	8
Raja ashwagandha Lehyam	6	2	6
Ashwagandha Chooranam	4	5	6
Ashwagandha Paradise	10	9	7

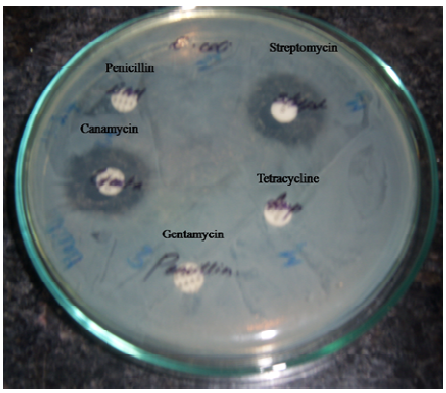
Plate 4.3

Antibacterial activity of standard antibiotics disc to *E.coli, B. subtilis* and *K. pneumoniae*

Antibiotic disc for *E.coli*



Antibiotic disc for *Bacillus*



Antibiotic disc for *Klebsiella*

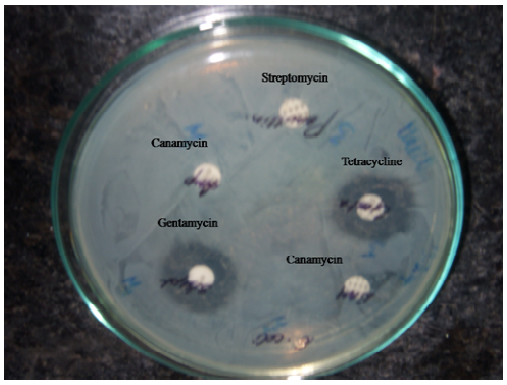


Plate 4.4

Methanol and ethyl acetate extracts in nutrient broth and agar agar (Controls)

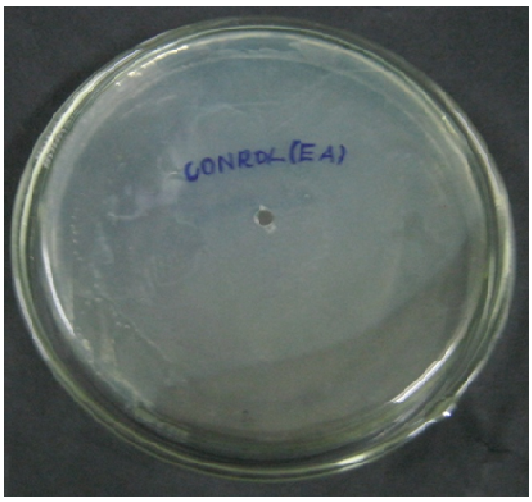
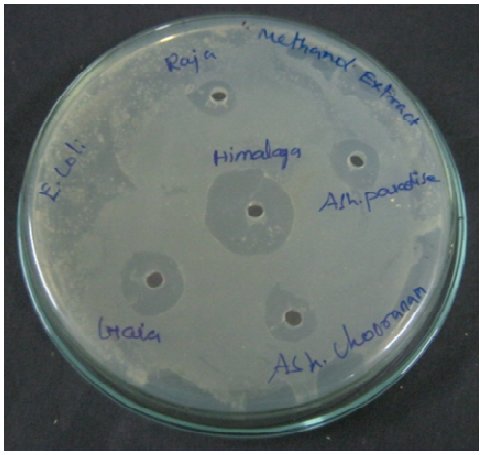
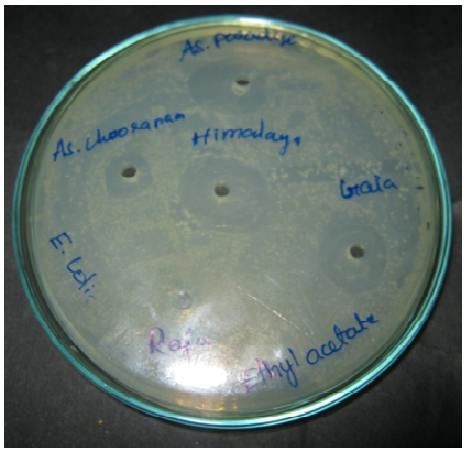


Plate 4.5

Antibacterial activity of methanol and ethyl acetate extracts of culture root samples and herbal supplements to *E.coli*





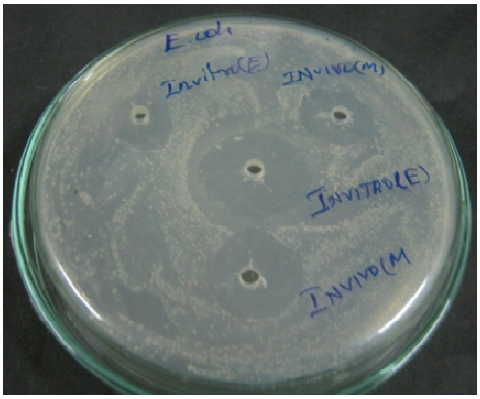
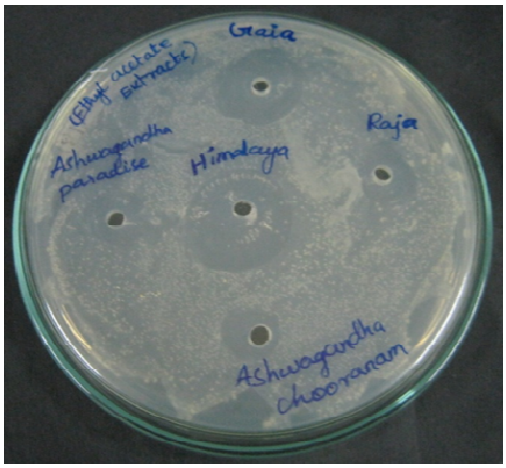
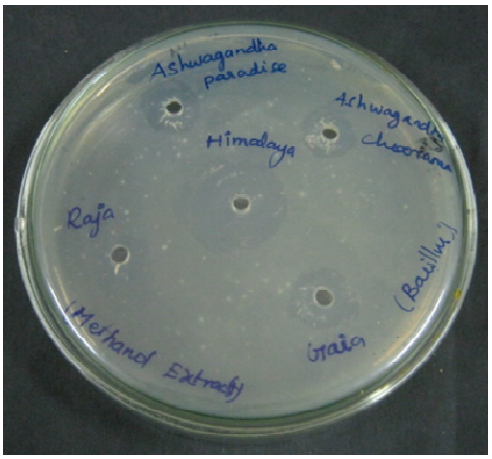


Plate 4.6

Antibacterial activity of methanol and ethyl acetate extracts of culture root samples and herbal supplements to *B. subtilis*





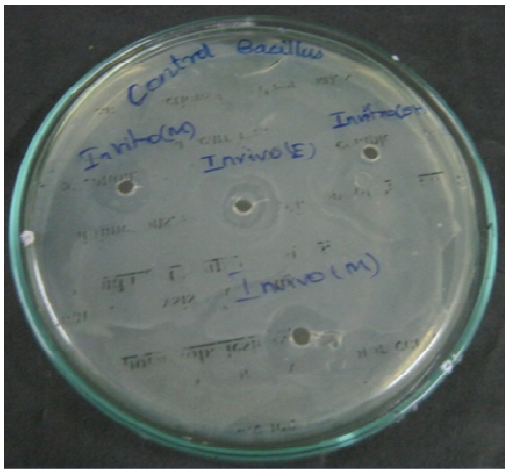
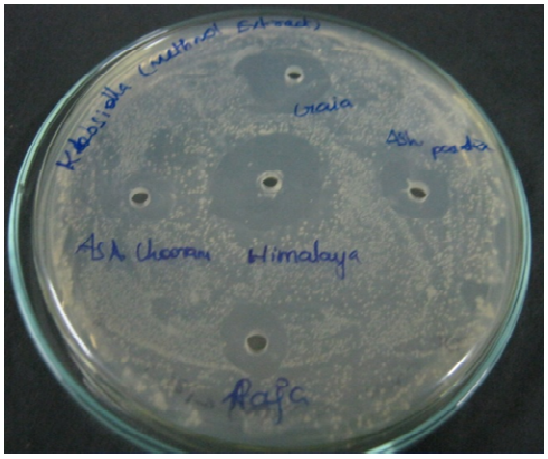
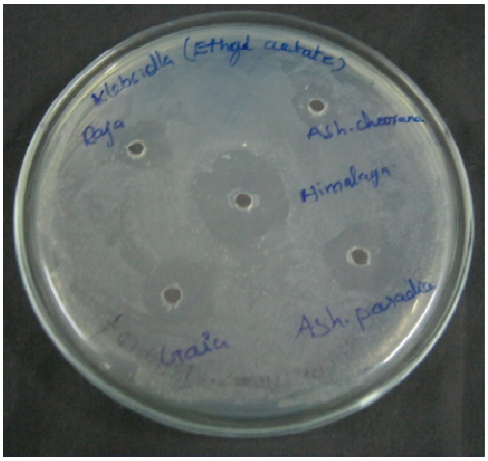
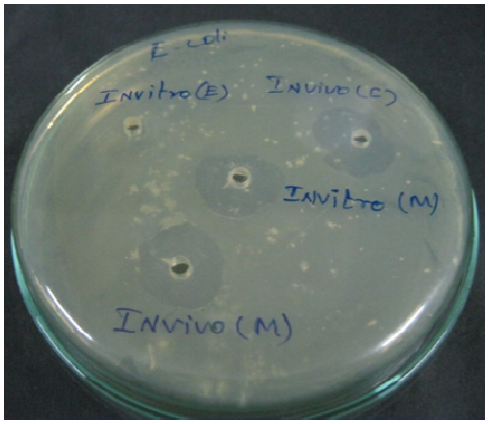


Plate 4.7

Antibacterial activity of methanol and ethyl acetate extracts of culture root samples and herbal supplements to *K. pneumoniae*







5.0. SUMMARY AND CONCLUSION

The results of the present study entitled **"Quantification of major Withanolides and antibacterial activity in over the counter health supplements containing *Withania somnifera* extracts"** are summarized as follows:

- *W. somnifera* in vitro and *in vivo* root samples were obtained from plant tissue culture laboratory. Five different herbal formulations were collected in different forms and from different locations
- Withanolides were extracted from all the samples using ethyl acetate and carbinol solvents.
- HPTLC analysis of products showed good separation and resolution in chloroform: ethyl acetate: methanol: toluene (7.4:0.4:0.8:3.0) as mobile phase and Conc.H_2SO_4: methanol: glacial acetic acid: anisaldehyde (5:85:10:0.5) as spraying reagent.
- All the samples contain Withaferin A and Withanolide A in different concentration. Among the samples methanol extract of Himalaya ashwagandha and ethyl acetate extract and of Himalaya ashwagandha and Ashwagandha paradise was found to contain high Withanolide A content (10.678µg/g) and ethyl acetate extract of Himalaya ashwagandha, Ashwagandha paradise and methanol extract of Ashwagandha paradise was found to contain high Withaferin A content (51.462µg/g) and. In contradictory, both the secondary metabolite contents were evaluated to be low in *in vivo* root of *W. somnifera.*
- Antibacterial activity was carried out in the methanol and ethyl acetate culture root sampls of *W. somnifera* for *in vitro* and *in vivo W. somnifera*. i*n vivo* methanol extract of *W. somnifera* roots showed maximum zone of inhibition (10mm) in test organism of *K. pneumoniae*.
- Among all herbal supplements, ethyl acetate extract of ashwagadha paradise and methanol extract of Himalaya ashwagandha showed

maximum zone of inhibition (10mm) and (13mm) in test organism of *B. subtilis and K. pneumoniae.*

To conclude, the present study could be used as a valuable analytical tool in the routine standardization of different herbal formulations to check the batch to batch variations. Standardization and development of reliable protocols for quality control of Ayurvedic formulations using modern techniques of analysis are extremely important so, the generated HPTLC fingerprints of the methanol and ethyl acetate extracts of in vivo and in vitro root culture samples. Also Withaferin A can be used as an appropriate bio marker for standardization of Himalaya ashwagandha and Gaia ashwagandha root products.

6.0 BIBLOGRAPHY

Abdullah E, Raus R. A and Jamal P (2012). Extraction and Evaluation of Antibacterial Activity from Selected Flowering Plants, ***American Medical Journal*****,** 3 (1), 27-32.

AbouZid S.F, Bassuony A, Nasib A, Khan S, Qureshi J and Choudhary M.I (2010). Withaferin A Production by Root Cultures of *Withania coagulans,* ***International Journal of Applied Research in Natural Products***, 3(1), 23-27.

Agostini-Costa T.S, Vieira R.F, Bizzo H.R, Silveira D and Gimenes M.A (2012). Secondary Metabolites, ***Chromatography and Its Applications*****,** 4(2), 132-164.

Amiri, M.S and Joharchi, M. R. (2013) Ethnobotanical investigation of traditional medicinal plants commercialized in the markets of Mashhad, Iran, ***Avicenna Journal of Phytomedicine*****,** 3(3), 254-271.

Bashir, H. S, Mohammed A.M, Magsoud A. S and Shaoub A. M (2013), Isolation of Three Flavonoids from *Withania Somnifera* Leaves (*Solanaceae*) and their Antimicrobial Activities, ***Journal of forest products & industries,*** 2(5), 39-45.

Borde V, Sonwane B, Sontakke V and Somwanshi B (2014). Isolation and purification of alkaloids from medicinal plants by HPLC, ***International Journal Current Microbiology Applied Science,*** 3(1), 414-423.

Choudhury MH, Chakraborty R, Chaudhuri UR (2014). Optimisation of Twin Screw Extrusion Process for Production of Snack from Aswagandha (*Withania sominifera*) Rice (*Oryza sativa*) and Chapra *(Fenneropenaeus indicus)* for Antioxidant Effect, ***British Journal of Applied Science & Technology*****,** 4(1), 1334-1352.

Dubey AK, Rajagopala S, Patel KS, Harisha CR and Shukla VJ (2011) Pharmacognostical and phyto-chemical standardization of ashtaangaavaleha: a polyherbal formulation. Pharmacie Globale**, International Journal of Comprehensive Pharmacy,** 11(5), 1-4.

Garg S, Mishra A and Gupta R (2013), Fingerprint Profile of Selected Ayurvedic Churnas/Preparations: An Overview, ***Alternative and Integrative Medicine,*** 2(6), 1-10.

Gupta V, Jain UK (2011) Status of piperine content in Ayurvedic formulation: method standardization by HPTLC. ***Research Journal of Pharmaceutical, Biological and Chemical Sciences,*** 3(2), 524-531.

Imtiyaz S, Alil SJ, Aslam M, Tariq M, Chaudhary SS (2013). *Withania somnifera*: a potent unani aphrodisiac drug, ***International Research Journal of Pharmaceutical and Applied Sciences*****,** 3(3), 59-63.

Jain P, Varshney R (2011). Antimicrobial activity of aqueous and methanolic extracts of *Withania somnifera* (Ashwagandha), ***Journal of Chemical and Pharmaceutical Research*****,** 3(5) 260-263.

Jamal Q, Munir S, Sherwan SK, Aleh M, Jabeen U, Malik SM, Hussain M (2013). Antibacterial activity of two medicinal plants: *Withania somnifera* and *Curcuma longa,* ***European Academic Research*****,** 6: 1335-1345.

Jeyanthi T, Subramanian P, Kumaravel P (2013). A Comparative Analysis of Antibacterial Activity of *Withania somnifera* Root Extract with Commercial Antibiotics, ***Asian Journal of Pharmaceutical Research*****,** 3(1) 98-102.

Jirge S.S, Tatke P.A and Gabhe S.Y (2011). Development and validation of a novel hptlc method for simultaneous Estimation of betasitosteroldglucoside and withaferin a, ***International Journal of Pharmacy and Pharmaceutical Sciences,*** 3(2), 227-230.

Kalisdha A. Indian Contribution to Medicinal Plants Research: A Scientometric Study, ***Journal of Advances in Library and Information Science*****,** 1(2), 65-70.

Kanungo S, Rout J.R and Sahoo S.L(2013). Evaluation of Antioxidant Enzyme Activities in *Withania somnifera* L. *in vitro* and *in vivo* Grown Explants, ***Iranian Journal of Biotechnology*****.** 11(4), 1-4.

Kasar R., Gogia A, Shah K, Vetriselvan and Anand C (2013). Critical Factor That Governs a Successful TLC/ HPTLC Analysis of Herbal Medicinal Products (HMPS), ***Research and Reviews: Journal of Pharmaceutical Analysis*****,** 2(4), 2347-2340.

Krishnamurthy S. R. and Sarala P (2010). Proximate Nutritive Values and Mineral Components of *Withania Somnifera* (Linn.) Dunal, ***Electronic Journal of Chemistry,*** 7(3), 985-996.

Kumari M (2014). Therapeutic Uses of Withania somnifera (Ashwagandha), ***International Journal of Green and Herbal Chemistry*****,** 3(4), 263-269.

Kushwaha S, Betsy A, Chawla P (2012). Effect of *Ashwagandha* (*Withania somnifera*) Root Powder Supplementation in Treatment of Hypertension, ***Ethno Medicine,*** 6(1), 111-115.

Mansi G, Bisht Deepa , Madan M.P, Sanjeev K.O, Sayyada K, Subha R and Ajay K.S (2011). Standardization of *Ashwagandhadi lehya*—An important *Ayurvedic* formulation of *Withania somnifera* ***Indian Journal of Traditional Knowledge,*** 10(4), 594-598.

Mastanaiah J, Prabhavathi N.B and Varaprasad (2011). Invitro Anti bacterial activity of Leaf Extracts of Lawsonia inermis, ***International Journal of PharmTech Research,*** 3(2), 1045-1049.

Meena AK, Rao MM, Panda P, Kiran and Yadav A (2010). Standardisation of Ayurvedic polyherbal formulation*,* ***Research journal of pharmaceutical biological and chemical sciences impact factor,*** 4(2), 11-14.

Meshram N, Kodopi N and Jimnani N (2009). antimicrobial activity of methanol extracts of medicinal plants against bacterial species, ***International Research Journal*****,** 1(3&4), 147-150.

Mirjalili M.H, Moyano E, Bonfill M, Cusido R.M and Palazón J (2009). Steroidal Lactones from *Withania somnifera*, an Ancient Plant for Novel Medicine, ***Molecules,*** 14(5), 2373-2393.

Negi MS, Singh A and Lakshmikumaran M(2000) Genetic variation and relationship among and within *Withania* species as revealed by AFLP markers, ***Genome.*** 4(3), 975-980.

Patel K, Singh R.B and Patel D.K (2013). Pharmacological and analytical aspects of withaferin A: A concise report of current scientific literature, ***Asian Pacific Journal of Reproduction,*** 2(3), 238-243.

Patel R.B, Patel M.R, Bhatt K.K and Patel B.G (2010). HPTLC method development and validation: Quantification of paliperidone in

formulations and in *vitro* release study, ***Analytical Methods*** 2(1), 525–531.

Pathak G.P, Khurana A, Sood D, Sharma N, Amir, and Vineet Sharma (2013). *In Vitro* Study of Antioxidant Activity of *Withania Somnifera* (Ashwagandha) Root, ***American Journal of PharmTech Research*****,** 3(5), 465-471.

Patil D, Gautam M, Sanjay M, Karupothula S, Gairola S, Jadhav S, Pawar S and Patwardhan B(2013). Determination of withaferin A and withanolide A in mice plasma using high-performance liquid chromatography-tandem mass spectrometry: Application to pharmacokinetics after oral administration of Withania somnifera aqueous extract, ***Journal of Pharmaceutical and Biomedical Analysis*****,** 80 (2), 203– 212.

Prajapati ND, Purohit SS, Sharma AK and Kumar T (2003) A handbook of medicinal plants – A complete source book. Agrobios, Jodhpur, India.

Praveen N, Murthy HN (2013). Withanolide A production from Withania somnifera hairy rootcultures with improved growth by altering the concentrations of macro elements and nitrogen source in the medium, ***International Journal of Pharma and Bio Sciences*****,** 35(3) 811–816.

Shanmugaratnam S, Mikunthan SG, Thurairatnam S (2013). Potential of *Withania somnifera* Dunal Cultivation as a Medicinal Crop in Jaffna District, ***American-Eurasian Journal of Agricultural and Environmental Science,*** 13(6) 357-361.

Sharada M, Aand A and Vij S.P (2012). Application of Biotechnology in Indian Ginseng (Ashwagandha): Progress and Prospects, Application of Biotechnology in Indian Ginseng (Ashwagandha), 3(6), 560-585.

Sharma V, Sharma S, Pracheta, Paliwal R (2011). Withania somnifera: A Rejuvenating Ayurvedic Medicinal Herb for the Treatment of various Human ailments, ***International Journal of PharmTech Research*****,** 3(4) 187-192.

Shrivastava AT, Pankaj K, Sahu (2013). Economics of Yield and Production of Alkaloid of *Withania somnifera* (L.) Dunal, ***American Journal of Plant Sciences*****,** 4(2), 2023-2030.

Singariya, Mourya KK, Kumar P (2011). Comparative microcidal activity of withania somnifera and cenchrus setigerus against the pathogenic micro-organisms, ***International Journal of Pharmacy and Pharmaceutical Sciences*****,** 3(2) 511-515.

Singh N, Verma P, Pandey BR, and Gilca M (2011). Role of *Withania somnifera* in Prevention and Treatment of Cancer:An Overview, organisms, ***International Journal of Pharmaceutical Sciences and Drug Research*****,** 3(2), 274-279.

Sundaresan, S. and Senthilkumar, B. (2013) A survey of traditional medicinal plants from the vellore district, tamilnadu, india, ***International journal of ayurvedic & herbal medicine*****,** 3(5), 1347-1355.

Udayakumar R, Kasthurirengan S, Mariashibu T.S, Sudhakar B, Ganapathi A, Kim E.J, Jang K.M, Choi C.W and Kim S.C (2013). Analysis of Genetic Variation among Populations of *Withania somnifera* (L.) in South India based on RAPD Markers, ***European Journal of Medicinal Plants,*** 3(2): 266-280.

Veeresham C and Chitti P (2013). Therapeutic Agents from Tissue Cultures of Medicinal Plants, ***Natural Products Chemistry & Research*****,** 1(1)18, 1-5.

Velu S, Baskaran C (2012). Phytochemical analysis and *in-vitro* antimicrobial activity of *Withania somnifera* (Ashwagandha*),* ***Journal of Natural Product,*** *Plant Resour*, 2(4), 711-716.

Verma S.K and Kumar A (2011). Therapeutic Uses Of *Withania somnifera* (Ashwagandha) With a Note on Withanolides and Its Pharmacological Actions, ***Asian Journal of Pharmaceutical and clinical research*****,** 4(1), 1-4.

Yazdanpanah E, Jahantab E and Javdani Z (2013). The introduction of medicinal plants of Delafrooz Mountain in the central Zagros (Kohgiluyeh region, Iran) for local jobs, ***International Journal of Agriculture and Crop Sciences*****,** 5(11), 1232-1234.

Zarezadeh A, Myrvakili S, Arabzadeh M (2007). Phenology and adaptation of plants grown in the collection of Yazd province. Quarterly, ***Journal - Medicinal and Aromatic Plants Research Research of Iran,*** 23 (2), 217-204.