STUDIES IN VALIDATION OF *WITHANIA SOMNIFERA* (L). DUNAL (ASHWAGANDHA) BY PHYSICAL, CHEMICAL AND BIOLOGICAL APPROACHES WITH RESPECT TO MAJOR BIOACTIVE WITHANOLIDES

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UNDER THE FACULTY OF SCIENCE

SUBMITTED BY

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CERTIFICATE

This to certify that the work incorporated in the thesis entitled **"Studies in Validation of *Withania somnifera* (L). Dunal (Ashwagandha) by Physical, Chemical and Biological Approaches with Respect to Major Bioactive Withanolides"** for the degree of '**Doctor of Philosophy'** in the subject of **Biotechnology** under the faculty of **Science** has been carried out by **Mr. Santosh Tatyaba Devkar** in the Department of Herbal Biotechnology Laboratory (IRSHA) and Center for Innovation in Nutrition Health Disease Laboratory (CINHD-IRSHA) at Bharati Vidyapeeth Deemed University, Medical College Campus, Pune during the period from 2012 to 2015 under the guidance of Dr. Suresh D. Jagtap and Prof. Mahabaleshwar V. Hegde.

Place: Pune

Date: / /

Dr. A. C. Mishra
(Director, IRSHA)

CERTIFICATION OF GUIDE

This is to certify that the work incorporated in the thesis entitled **"Studies in Validation of *Withania somnifera* (L). Dunal (Ashwagandha) by Physical, Chemical and Biological Approaches with Respect to Major Bioactive Withanolides"** Submitted by **Mr. Santosh Tatyaba Devkar** for the degree of '**Doctor of Philosophy**' in the subject of **Biotechnology** under the faculty of **Science** has been carried out in the Department of Herbal Biotechnology Laboratory (IRSHA) and Center for Innovation in Nutrition Health Disease Laboratory (CINHD-IRSHA) at Bharati Vidyapeeth Deemed University, Medical College Campus, Pune during the period from 2012 to 2015 under my guidance.

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled **"Studies in Validation of *Withania somnifera* (L). Dunal (Ashwagandha) by Physical, Chemical and Biological Approaches with Respect to Major Bioactive Withanolides"** submitted by me to the Bharati Vidyapeeth Deemed University, Pune for the degree of '**Doctor of Philosophy'** (Ph.D.) in the subject of **Biotechnology** under the faculty of **Science** is original piece of work carried out by me under the supervision of **Dr. Suresh D. Jagtap** and **Prof. Mahabaleshwar V. Hegde**. I further declare that it has not been submitted to this or any other university or Institution for the award of any degree or Diploma.

I also confirm that all the material which I have borrowed from other sources and incorporated in this thesis is duly acknowledged. If any material is not duly acknowledged and found incorporated in this thesis, it is entirely my responsibility. I am fully aware of the implications of any such act which might have been committed by me advertently or inadvertently.

Place: Pune

Date: / /

Mr. Santosh Tatyaba Devkar
Research Student

This work is dedicated to

God,

My Beloved Guides

&

My Family…

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Santosh Tatyaba Devkar

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LIST OF ABBREVIATIONS

Abbreviation	Full Form
%	Percent
1,2 DWM	1,2 deoxywithastramonolide
^{13}C-NMR	Carbon nuclear Magnetic Resonance
1H-NMR	Proton nuclear magnetic resonance
ADP	Adenosine Diphosphate
AOT	Acute Oral Toxicity
API	Active Pharmaceutical Ingredients
API	Ayurvedic Pharmacopoeia of India
apo(a)	Apolipoprotein(a)
AST	Aspartate Transaminase
ASU	Ayurveda, Siddha and Unani
BA	Bioavailability
BHC	British Herbal Compendium
BHP	British Herbal Pharmacopoeia

Caco-2	Colorectal Adenocarcinoma
CAT	Catalase
CCl_4	Carbon Tetrachloride
CETP	Chlolesteryl Ester Transfer Protein
CHP	Chinese Herbal Pharmacopoeia
cm	Centimetre
COX	Cyclooxygenase
CPCSEA	Committee for the Purpose of Control and Supervision on Experimental Animals
CTX	Cyclophosphamide
CV	Cyclic Voltammeter
CVD	Cardiovascular Disease
CVS	Cardiovascular System
DMSO	Deuterated Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
Dox	Doxorubicin
DPPH	2,2-Diphenyl-1-picryl-hydrazyl

DTNB	5, 5´-dithiobis (2-nitro benzoic acid)
ECG	Electrocardiogram
EDTA	Ethylene Diamine Tetraacetate
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
GC/MS	Gas Chromatography/Mass Spectroscopy
gm	Gram
GMP	Good Manufacturing Practices
GPX	Glutathione peroxidise
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
ha	Hectare
HDL	High Density Lipoprotein
HPLC	High Pressure Liquid Chromatography
HPTLC	High Pressure Thin Layer Chromatography
hr	Hour

HRT	Hormone Replacement Therapy
i.p.	Intra-peritoneal
I/R	Ischemia- and Reperfusion
IAEC	Institutional Animal Ethics Committee
IBD	Inflammatory Bowel Disease
IDL	Intermediate Density Lipoprotein
IHM	Indian Herbal Medicines
IL- 1	Interleukin-1
IR	Infrared
ISO	Isoprenaline
JSHM	Japanese Standards for Herbal Medicine
kg	Kilogram
LCAT	Lecithin Cholesteryl Acyl Transferase
LDH	Lactate Dehydrogenase
LDL	Low Density Lipoprotein
LPL	Lipoprotein Lipase

LPO	Lipid Peroxidation
LPS	Lipopolysaccharides
MDA	Malondialdehyde
MDCK	Madin-Darby Canine Kidney Epithelial Cells
Mg	Milligram
mg/dl	Milligram/decilitre
mg/kg	Milligram /kilogram
MI	Myocardial Infarction
min	Minute(s)
mL	Millilitre
MS	Mass Spectrometry
NADH	Nicotinamide Adenine Dinucleotide Reduced form
NADP	Nicotinamide Adenine Di-nucleotide Phosphate
NaOH	Sodium Hydroxide
NBT	Nitroblue Tetrazolium
ng	Nanogram

nm	Nanometre
NMR	Nuclear Magnetic Resonance
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
°C	Degree centigrade
OECD	Organisation for Economic Co-operation and Development
OTA	Ochratoxin A
p.o	Per OS
p-407	Poloxamer 407
PCR	Polymerase Chain Reaction
PG	Panax ginseng
PGN	Peptidoglycans
PO	Pharmacopoeia Committee
q	Quintal
QA	Quality Assurance
QC	Quality Control
RBC	Red Blood Cell
RH	Related Humidity

ROS	Reactive Oxygen Species
SED	Erythrocyte Sedimentation
SOD	Superoxide Dismutase
t	Ton
TBA	Toxic Baile Acids
TC	Total Cholesterol
TCA	Trichloracetic Acid
TLC	Thin layer Chromatography
TNBS	Trinitro Benzyl Sulfonic Acid
TNF-α	Tumor Necrotic Factor- α
TTC	Triphenyltetrazolium Chloride
US-FDA	United States Food and Drug Administration
USHP	United States Herbal Pharmacopoeia
UV	Ultraviolet
VLDL	Very Low Density Lipoproteins
WF A	Withaferine A
WHO	World Health Organization

WN A	Withanolide A
WN B	Withanolide B
WNN	Withanone
WS	*Withania somnifera*
WS IV	Withanoside IV
WS V	Withanoside V

Chapter 1

Introduction, Review of Literature and Objectives

1.1. Introduction

Ayurveda – wisdom of life, the Indian traditional medicine is a science developed mainly by the experience and wisdom of our ancestors. As against this, modern medicine (allopathy) is a recently developed, experiment-based science of well-defined chemicals with known mechanisms of action, and possible side-effects and toxicity. As the former is experience-based and the latter is experiment-based, the former is regarded as arbitrary and the latter as more exacting science and hence the latter has wider acceptance. It is obvious therefore that there is a need to put Ayurveda on a firmer scientific footing, to reap its full benefits and also integrating with modern medicine.

According to some sources up to 80% of people in India use some form of traditional medicine: a category which includes Ayurveda. Ayurveda is the Indian traditional medical science, practiced for over 5000 years (Aneesh et al., 2009). Ayurvedic medicine is a system of Indian traditional medicine, which native to the Indian subcontinent, and is a form of alternative medicine. The oldest known Ayurvedic texts are the Susruth, Samhita and the Charaka. These classical sanskrit texts are among the foundational and formally compiled works of Ayurveda. There are two ways to approach Ayurvedic principles and terminology; one may either focus on the historical foundation (as evidenced in the oldest Ayurvedic texts, going back to the early centuries of the Common Era) or, alternatively, a description may take an ethnographic approach and focus on the forms of traditional medicine prevalent across India today. Ayurveda stresses mainly the use of plant-based medicines for treatments. Plant-based medicines are derived from roots, leaves, fruits, barks and seeds of medicinal plants. Some animal products may also be used: for example, milk, bones, and gallstones. In addition, fats are also used both for oral administration and external use in treatments (Patwardhan et al.,

2005). Minerals, including sulphur, arsenic, mercury, lead, copper and gold regarded as toxic in modern medicine are often also included. This practice of adding minerals to herbal medicine is known as Rasa Shastra. Although Ayervedic medicines have specific healing power, some formulations also provide the vital life-support as antioxidants (Aqil et al., 2006).

Herbal medicinal products are complex mixtures, which originate from natural sources. Naturally there is a perception that Ayurvedic formulations are safer than synthetic chemicals. Hence, great efforts are necessary to guarantee with assured therapeutic potential. By carefully selecting the plant material and a standardized manufacturing process, the pattern and concentration of constituents should be kept as constant as possible, as this is a pre-requisite for reliability and reproducible therapeutic results.

Although, Indian Herbal Medicines (IHM) has a great therapeutic potential they are not well received in world market as Traditional Chinese Medicines (TCM). IHM have a great potential to become a leader in Global Market. The major hurdle for the IHM to become a leader in Global Market is the lack of quality control. Indian Herbal products do not normally specify the concentration(s) of active principles and stability of the product. Scientific authentication for IHM is lacking and quality control, standardization; scientific methods of production and evaluation are missing (Cai et al., 2004; Patwardhan et al., 2005; Aneesh et al., 2009).

1.1.1. Ayurveda: Herbal Medicine

Ayurveda emphasizes the relationship between man and plants throughout the development of human culture. Due to the toxicity and side-effects of allopathic medicines the use of herbal medicine has led to a sudden increase in the number of herbal drug manufactures. Herbal medicines as the major remedy in traditional

system have been used since antiquity. The herbal medicine/ Ayurvedic practices is popular even today because of its health benefits in many parts of the world and has made a great contribution towards maintaining human health (Patwardhan et al., 2005; Patwardhan and Mashelkar, 2009).

In olden times, herbal doctors/ Vaidyas used to treat patients on individual basis and prepare personalized medication according to the requirement of the particular patient. But the scenario has changed now; herbal medicines are being manufactured on large scale in Pharmaceutical Industrial Units, where manufacturers come across many problems such as availability of good quality raw material, authentication of raw material, availability of standards, proper standardization, formulation methodology and quality control parameters (Patra et al., 2010).

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 (WHO, 2008). In almost all the traditional systems of medicine, the quality control aspect has been considered from its inspection by Rishis, Vaidyas and Hakims. However, in modern scientific approach there is a need to change in approach by standardization of scientific methods and quality control in terms of modern methodologies. Thus, today quality assurance is the thrust area for the evaluation of traditionally used medicinal plants and herbal formulations.

In the western world, as the people are becoming aware of side-effect of the synthetic drugs, there is an increasing interest in the herbal product remedies. Natural products from plant, animal and minerals have been the basis of the treatment of human disease(s). Accordingly, by today's estimate about 80% of people in developing countries rely on traditional medicines for their primary health care. Alternate medicine is the need of the day (Dubey et al., 2004).

Herbal medicines are currently in demand and their popularity is increasing day-by-day. In the healthcare sector WHO recommends and encourages the use of traditional herbal remedies because huge amount of raw material is easily available. They are comparatively safe because of their low toxicities. Till today most of the villagers relied on herbal remedies as this is a perception by the common man that it will spare him of the side-effects of the allopathic drugs and will cure magically. However, plants are very complex in their composition and their therapeutic activity depends on their chemical constituents, which may vary depending on geographical location, age and harvesting processes. Also improper authentication of herbs, adulterations by microorganism, pesticide residue etc. has made standardization of herbal drugs a matter of primary importance and urgency.

At present no official standards are available for the herbal preparations. Manufactures who are doing some testing of their formulation have fixed their own parameter of quality control most of which are arbitrary in nature. At present it is very difficult to identify the presence of all the ingredients as claimed in any formulation. Hence the first important task is to evolve such parameter by which the presence of all the ingredients can be identified. Various chromatographic and spectrophotometric methods can be used for the evaluation of physicochemical properties and for identifying the presence of different ingredients. Wherever possible, these methods can be applied for quantitative estimation of bioactive group of compounds like alkaloids, flavonoids, polyphenolic compounds or estimation of a particular compound.

In polyherbal Ayurvedic preparations it will be very difficult if we want to estimate each and every ingredient in term of its chemical constituent. But if few major constituents having particular therapeutic action indicated in the label can be

pinpointed then these constituents should be estimated quantitatively along with the other parameters through which presence of all ingredients can be confirmed. Combined, well-coordinated efforts from scientific workers of different disciplines are required for validation and standardization of herbal medicines.

1.1.1.1. Advantages of Herbal Medicine

1. Herbal medicine have long history of use and better patient tolerance as well as acceptance.
2. Medicinal plants constitute renewable source, which is our only hope for sustainable supplies of cheaper medicines for the growing world population
3. Availability of medicinal plants is not a problem especially in developing countries like India having rich agro-climatic, cultural and ethnic biodiversity.
4. The cultivation and processing of medicinal herbs and herbal products is environmental friendly.
5. Prolonged and apparently uneventful use of herbal medicines may offer testimony of their safety and efficacy.
6. Throughout the world, herbal medicine has provided many of the most potent medicines to the vast arsenal of drugs available to modern medical science, both in crude form and as a pure chemical upon which modern medicines are structured.

1.1.1.2. Limitations of Herbal Medicines

Like any other branch of science and technology, present scenario of herbal medicine has some limitations summarized below:

1.1.1.2.1. Ineffective in Acute Medical Care

Herbal medicines are not very effective to treat any acute illness. As most of the medicines are designed to work at molecular level of physiology, the drug takes its time to deliver the results. However, there are a few herbal medicines which work instantly in acute

conditions like diarrhea. On the other hand, modern system of medicine is better equipped for management of acute conditions.

1.1.1.2.2. Inadequate Standardization and Lack of Quality Specifications

This is the most often criticized aspect of herbal medicines. One important fact is that a herbal preparation is administered for its holistic value. Each herbal ingredient in the herbal preparation has an array of chemical constituents with complex molecular formulae; each herbal preparation is a source of polypharmacy within itself. As a result, standardization of herbal preparation or its ingredients becomes a highly complex issue. Standardization of herbal drugs by known marker compounds may not provide a complete answer. Despite this major limitation, pharmaceutical industry strives hard to have in-house specifications based on the quantification of marker compounds. Therefore, a consensus is being arrived at to incorporate the qualitative finger-printing together with other physicochemical parameters of quality protocols for herbal medicines in an ongoing process and this shortcoming could possibly be overcome due course (Aneesh et al., 2009).

1.1.1.2.3. Lack of Scientific Data

There is a lack of exhaustive literature on herbal medicines and evidence based scientific data in support of the medicinal activity and data on their safety and efficacy. Hence there is a need to incorporate certain quality control parameters, pharmacological evaluations on modern lines. WHO guidelines clearly direct that it is not necessary to carry out detailed toxicological evaluation of herbs and herbal preparation originating from traditional system of medicine (Aneesh et al., 2009).

1.2. Validation and Standardization of Herbal Drugs

1.2.1. Validation of Herbal Drugs

Validation is a process of establishing documentary evidence demonstrating that a procedure, process, or activity carried out in production or testing that maintains the desired level of compliance at all stages. In Pharma Industry it is very important, apart from final testing and compliance of product with standard that the process adapted to produce itself must assure that process will consistently always produce the expected results. Here the desired results are established in terms of specifications of the final product. Qualification of systems and equipment is therefore a part of process of validation. It is a requirement of food and drug, and pharmaceutical regulating agencies like FDA's good manufacturing guidelines are practiced.

1.2.2. Need of Validation

Reasons for validation is, US FDA or any other food and drugs regulatory agencies around globe not only ask for a product that meets its specification but they do ask for a process, procedures, intermediate stages of inspections, testing adapted during manufacturing are designed such that when they are adapted they produce consistently similar, reproducible, desired results which meets the quality standard of product being manufactured. Such procedures are developed through the process of validation. This is to maintain and assume higher degree of quality of food and drug products. Validation is “Establishing documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes”. A properly designed system will provide a high degree of assurance that every step, process, and change has been properly evaluated before its implementation. Testing a sample of a final batch is not considered sufficient evidence that every product within a batch meets the required

specifications (Patwardhan and Mashelkar, 2009; Choudhary and Sekhon, 2011).

1.2.3. Standardization of Herbal Drugs

Standardization is the process of developing and implementing technical standards. Standardization can help to maximize compatibility, inter-operability, safety, repeatability, or quality. Standardization is the process by which one or more active ingredients of herb are identified, that can be verified for all batches of the herb produced by a single manufacturer to contain the same amount of active principles. The purpose of quality control is to ensure that each dosage unit of the drug product delivers the same amount of active ingredients as far as possible, free of impurities (Mosihuzzaman and Choudhary, 2008; Shinde et al., 2009). As herbal medicinal products are complex mixtures which originate from biological sources, great efforts are necessary to guarantee a constant and adequate quality. By carefully selecting the plant material and a standardized manufacturing process the pattern and concentration of constituents of herbal medicinal products that should be kept as constant as possible as this is a pre-requisite for assuring reliable and reproducible therapeutic results.

1.2.4. Need of Standardizations

In recent years there is a spurt in the interest in Ayurvedic forms of medication. Globally, there is a shift towards the use of medicines of herbal origin. As the shortcomings and the dangers of modern medicine have started getting more apparent, majority of Ayurvedic formulation prepared from herbs are being increasingly preferred.

It is the cardinal responsibility of the regulatory authorities to ensure that the consumers get the medications which guarantee: purity, safety, potency and efficacy. This duty is discharged by the regulatory authorities by rigidly following various standards of quality control prescribed for raw materials and finished products in

pharmacopoeias controlling manufacturing through statutory imposed "Good Manufacturing Practices" (GMP). All these procedures logically would apply to all types of medications whether included in modern system of medicine or in one of the traditional system such as Ayurvedic system of medicine. Unfortunately, the Ayurvedic pharmacopoeias and the formulations have been exempted from the standard that applies to modern medicine. Modern medicines are continuously undergoing changes with respect to improvements in the standard of purity, safety and efficacy, and are being strictly regulated. On the other hand maintaining the quality of Ayurvedic medication becomes the sole responsibility of the manufacturer who escapes statutory strict regulations and consumer is not assured of its efficacy (Mosihuzzaman and Choudhary, 2008).

Herbal products have been enjoying renaissance among the customers throughout the world. However, one of the impediments in the acceptance of the Ayurvedic formulation is the lack of standard quality control profile. The quality of herbal medicine i.e. the profile of the constituents in the final product has implication in efficacy and safety. Due to complex nature and inherent variability of the constituents of plant source, it is difficult to establish quality control parameter and modern analytical technique are expected to help in circumventing this problem.

The quality control (QC) of crude drugs and herbal formulations is of paramount importance in justifying their acceptability in modern system of medicine. But one of the major problems faced by the herbal drug industry is non-availability of rigid quality control profile for herbal materials and their formulations. Quality control of synthetic drugs offers no problems with very well defined parameters of analysis. In contrast, herbal products represent a number of unique problems when quality control aspects are considered. These

relate to the nature of the herbal ingredients present therein which are complex mixtures of different secondary metabolites that can vary considerably depending on environmental and generic factors. Furthermore, the constituents responsible for the claimed therapeutic effects are frequently unknown or only partly explained. These complex aspects of herbal drugs are further complicated by the use of combination of herbal ingredients being used in traditional practice. It is not uncommon to have as many as five different herbal ingredients in one product. Thus batch to batch variation starts from the collection of raw material itself in the absence of any reference standard for identification. These variations multiply during storage and further processing (Shinde et al., 2009).

The task of laying down standards for quality control of herbal crude products and their formulations involves biological evaluation for a particular disease area, chemical profiling of the material and laying down specifications for the finished product. Therefore, in the case of the herbal medication and products, the word "Standardization" should encompass entire field of study from cultivation of medicinal plant to its clinical application.

Plant material and herbal remedies derived from them represent substantial portion of global market and in this respect internationally recognized guidelines for their quality assessment and quality control are necessary. WHO has emphasized the need to ensure quality control of medicinal plant products by using modern technique and by applying suitable parameters and standards. In order to overcome certain inevitable shortcoming of the Pharmacopoeial monograph other quality control measures must be explored. Quality control has wide connotation and covers many aspects of drug manufacture, distribution and is not restricted to final product analysis either regulatory or otherwise. While engaging in this task, it must be realized that some of the quality control

practices that work excellently with modern drug, these may not be appropriate with Ayurveda, Siddha and Unani (ASU) drug (WHO, 2008).

1.2.5. Current Regulations for Standardization of Crude Drugs

In India a great deal of knowledge exists amongst ordinary people about the traditional use of herbal medicine. It is difficult to quantify the market size of the traditional Indian medicines, since most practitioners formulate and dispense their own recipes. The present annual turnover of product manufactured by large companies is estimated at approximately US $300 million compared to a turnover of approximately US $2.5 billion for modern drugs. According to the study on the attitude, modern medicine practitioners are relatively unfamiliar with Ayurvedic product even though some are practiced. They are willing to try an Ayurvedic product if its efficiency is scientifically proven and would try it for aliments such as cough, cold, diarrhea, stomach problem, reproductive diseases, liver and skin diseases.

Patent proprietary Ayurvedic medicines are sold over the counter in pharmacies. These products appear to represent a major share of branded traditional medicine in India. Nevertheless systems like Ayurveda still need to gain an empirical support of modern medical sciences to make them credible and acceptable for all. An innovative research effort to define the advantage of traditional system of medicine with respect to their safety and efficacy could result in a better utilization of these complementary systems of medicine. Internationally several pharmacopoeias have provided monographs stating parameter and standard of many herbs and some products made out of these herbs. These include:

- Pharmacopoeia Committee (PC)
- Chinese Herbal Pharmacopoeia (CHP)
- United States Herbal Pharmacopoeia (USHP)

- British Herbal Pharmacopoeia (BHP)
- British Herbal Compendium (BHC)
- Japanese Standards for Herbal Medicine (JSHM)
- Ayurvedic Pharmacopoeia of India (API)

These have laid down parameters for herbs and herbal products to maintain their quality in their respective nations. Government of India has brought out Ayurvedic Pharmacopoeia India, which recommends basic quality parameters for eighty common Ayurvedic herbal drugs.

1.2.6. Quality Control of Crude Herbal Drugs

According to WHO it is the process involving the physicochemical evaluation of crude drug, covering the aspects such as selection and handling of crude material, safety, efficacy and stability assessment of finished product, documentation of safety and risk based on experience, provision of product information to consumer and product promotion.

- *Qualitative Chemical Evaluation:* It covers identification and characterization of crude drug with respect to phytochemical constituent.
- *Chromatographic Examination:* Includes identification of crude drug based on use of major chemical constituent as marker.
- *Qualitative Chemical Evaluation:* A criterion to estimate amount the major class of constituents.
- *Extractive Values:* These are indicative of the approximate measure of chemical constituents of crude drug.
- *Toxicological Studies:* Pesticide residue, potentially toxic elements, and microbial count provide an approach to minimize their effect in final product.
- *Macro and Microscopic Examination*: For Identification of right variety and search of adulterants

- *Foreign Organic Matter*: Removal of matter other than source plant to get the drug in pure form
- *Ash Values:* Total ash, sulfated ash, water soluble ash and acid insoluble ash are the criterion to judge the identity and purity of crude drug
- *Moisture Content:* To check moisture content helps prevent degradation of product.
- *Crude Fiber:* To determine excessive woody material: criterion for judging purity.

1.2.6.1. Physical Evaluation (Physical Approach)

It includes appearance, colour, odour, pH, clarity (solutions) and freedom from visible particulate contamination, size range of particulate contamination (large volume parenterals), particle size distribution (suspensions), micelle size distribution (micellar solutions), resuspendability (suspensions), viscosity, moisture content (powders for reconstitution), phase separation (emulsions).Physical approach also includes cyclic voltammeter analysis (Hoyle and Santos, 2009; Psotova et al., 2011).

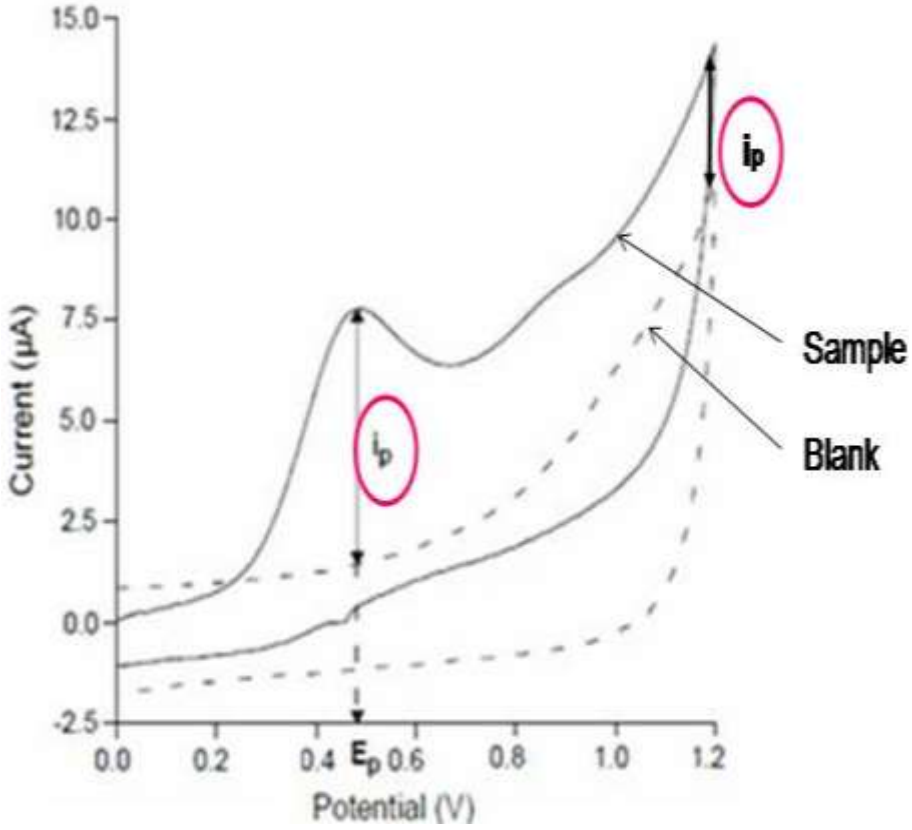
Each monograph contains detailed botanical, macroscopic and microscopic descriptions of the physical characteristics of each plant that can be used to ensure both identity and purity. Each description is accompanied by detailed illustrations and photographic images which provide visual documentation of accurately identified material.

1.2.6.1.1. Cyclic Voltammeter

In cyclic voltammeter the electrons are withdrawn from the electrode (for electrochemical reductions) or donated to the electrode (for oxidations), and a current flows in the external electrical circuit. A voltammogram is a plot of the current as a function of the applied potential. The shape of a voltammogram depends on the type of indicator electrode and the potential ramp that are used. Physical characteristics i.e. voltammogram corresponds to the concentration of antioxidant potential of tested sample. The potential at the maximum of anodic wave reflects the reducing ability of antioxidants present in the tested sample (**Fig. 1.1).**

Fig. 1.1: Cyclic Voltammogram.

The anodic peak current (ip) corresponds to the concentration of antioxidants. ip is difference between tested sample and blank. The potential at the maximum of anodic wave reflects the reducing ability of antioxidants present in the tested sample.



1.2.6.2. Chemical Evaluation (Chemical Approach)

A chemical method for evaluation covers the isolation, purification and identification. Chemical analysis of the drug is carried out to assess the potency of vegetable and animal source material in terms of their active principles. The chemical tests include color reaction test; these tests help to determine the identity of the drug substance and possible adulteration (Shinde et al., 2009).

A number of analytical techniques are used for qualitative and quantitative chemical evaluation such as: Colorimeter, Spectrophotometer, High Performance Thin Layer Chromatography (HPTLC), High Pressure Liquid Chromatography (HPLC) etc.

1.2.6.2.1. Colorimeter

A colorimeter is a device used to measure a colored substance or from which a colored compound can be desired. It is a device that measures the absorbance at particular wavelengths in visible range by a given solution. This device is most commonly used to determine the concentration of a known solute in a given solution by the application of the Beer-Lambert law, which states that the concentration of a solute is proportional to the absorbance.

1.2.6.2.2. Spectrophotometer

Spectrophotometry is the quantitative measurement of the transmission or reflection properties of a material as a function of wavelength. Spectrophotometry uses photometers that can measure a light beam's intensity. However they can also be designed to measure the diffusivity on any of the listed light ranges that usually cover around 200 nm - 2500 nm using different controls and calibrations. Within these ranges of wavelength, calibrations are needed on the machine using standards that vary in type depending on the wavelength of the photometric determination.

1.2.6.2.3. High Performance Thin Layer Chromatography (HPTLC)

Chromatography is a collective term for a set of laboratory techniques for the separation of mixtures. Thin layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures. The mixture is dissolved in a solvent called the mobile phase which carries it through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different rates causing them to separate. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus achieving the separation. The HPTLC is an enhanced form of TLC. A number of enhancements can be made to the basic method of TLC to automate the different steps, to increase the resolution achieved and to allow more accurate quantitative measurements.

1.2.6.2.4. High Pressure Liquid Chromatography (HPLC) and Mass Spectroscopy (MS)

It is a technique used to separate, identify, and to quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts differently with the adsorbent material, causing differential flow rates for the different components and leading to the separation of the components as they flow out the column.

HPLC methods are historically divided into two different subclasses based on stationary phases and the corresponding required polarity of the mobile phase. Use of octadecylsilyl (C18) and related organic modified particles as stationary phase with pure or pH adjusted water organic mixtures such as water-acetonitrile and water-methanol are used in techniques termed reversed phase liquid

chromatography (RPLC). Use of materials such as silica gel as stationary phase with neat or mixed organic mixtures are used in techniques termed normal phase liquid chromatography (NPLC). RPLC is most often used as the means to introduce samples into the Mass Spectroscopy (MS), in Liquid Chromatography Mass Spectroscopy (LCMS) instrumentation. LCMS is an analytical chemistry technique that combines the physical separation capabilities of HPLC with the mass analysis capabilities of MS. LCMS is a powerful technique that has very high sensitivity and selectivity and so is useful in many applications. Its application is oriented towards the separation, general detection and potential identification of chemicals of particular masses in the presence of other chemicals (i.e., in complex mixtures), e.g., natural products from natural products extracts, and pure substances from mixtures of chemical intermediates.

1.2.6.3. Biological Evaluation (Biological Approach)

Full and accurate characterization of plant material requires a combination of physical and chemical tests. Microscopic analyses of plants are invaluable for assuring the identity of the material and as an initial screening test for impurities, while biological analysis is important for assuring the chemical activity of the drug with the help of biological assessments i.e. *in vivo* and *in vitro* assay.

1.1.6.3.1. In vivo

In vivo studies are those in which the effects of various biological entities are tested on whole living organisms, usually animals including humans, and plants as opposed to a partial or dead organism, or those done *in vitro*, i.e., in a laboratory environment using test tubes, petri dishes etc. Examples of investigations *in vivo* include: the pathogenesis of disease by comparing the effects of bacterial infection with the effects of purified bacterial toxins; the development of antibiotics, antiviral drugs, and new drugs generally

as well as new surgical procedures. Consequently, animal testing and clinical trials are major elements of *in vivo* research. *In vivo* testing is often preferred over the *in vitro* test system because it is better suited for observing the overall effects of an experiment on a living system.

1.1.6.3.2. In vitro

In vitro studies are performed with cells or biological molecules studied outside their normal biological environments, for example proteins are examined in solution, or cells in artificial culture medium. Colloquially called "Test Tube Experiments", these studies in biology and its sub-disciplines are traditionally done in test-tubes, flasks, petri dishes etc., but which now involve the full range of techniques used in molecular biology and larger commercial applications.

1.2.7. Stability and Shelf-life

The purpose of shelf-life is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, and enables recommended storage conditions, re-test periods. There are several types of stability studies such as Accelerated Testing, Intermediate Testing and Stress Testing. Accelerated are studies designed to increase the rate of chemical degradation or physical change by means of exaggerated storage conditions. Intermediate studies carried out at 30ºC/60%RH are, intended for extrapolation to long term storage at 25ºC. Stress testing Active Pharmaceutical Ingredients (API): studies elucidate intrinsic stab it's of API, during development. Normally, more stressful than 'accelerated' testing and finished product: Studies of effect of 'severe' conditions, e.g. freeze/thaw cycling for suspensions and emulsions, low humidity for aqueous liquids in moisture-permeable containers.

1.3. Antioxidant, Inflammation and Bio-availability

1.3.1. Antioxidant

In general antioxidant is any substance that when present at low concentrations significantly delays or prevents oxidation of cell constituents such as proteins, lipids, carbohydrates and DNA. An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death of the cell. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions. These antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenols (Volko et al., 2007).

In nature there is a wide variety of naturally occurring antioxidants (natural antioxidant) which vary in their composition, physical and chemical properties, mechanisms and site of action. Some of the main categories can be described below:

- *Plants antioxidants:* Fatty acids, Phenols (Lignan), and Secondary metabolites such as; alkaloids, terpenoids, glycosides etc.
- *Vitamins and minerals antioxidants:* Vitamin A, C and E are essential nutrients also function as antioxidants and prevent peroxidative damage in the biological system. Minerals like selenium, manganese, zinc etc. are well known antioxidants as they are part of antioxidant enzymes.
- *Enzymes antioxidants:* Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidise (GPx), etc. are well known enzymes present in cells which act as antioxidants by transforming reactive oxygen species and reactive nitrogen species into stable compounds.

- *Low molecular weight antioxidants compounds:* bilirubin and some polyphenols come under this category.
- *Higher molecular weight antioxidants compounds:* These include some bio-molecules present in the system.

1.3.2. Inflammation

Inflammation is a non-specific, localized immune reaction of the organism which tries to localize the pathogenic agent. Many consider the syndrome a self-defense mechanism. It consists of vascular, metabolic, cellular changes triggered by the entering of pathogen agent in healthy tissues of the body. The inflammatory reaction takes place at the microcirculation level and it is comprised of following changes:

- Tissue damage
- Cellular – vascular - cellular response
- Metabolic changes
- Tissue repair

1.3.2.1. Anti-inflammatory Action

Anti-inflammatory property refers to the characteristics of a substance that reduces inflammation. Anti-inflammatory drugs make-up about half of analgesics, remedying pain by reducing inflammation as opposed to opioids, which affect the central nervous system.

Non-steroidal anti-inflammatory drugs (NSAIDs), alleviate pain by counteracting the cyclooxygenase (COX) enzyme. On its own COX synthesizes prostaglandins causing inflammation. The NSAIDs prevent the prostaglandins from being synthesized, reducing or eliminating the pain (Vane and Botting, 1998).

Some common examples of NSAIDs are: aspirin, ibuprofen, and naproxen. The newer COX-specific inhibitors are not classified together with the traditional NSAIDs even though they presumably share the same mode of action. On the other hand, there are

analgesics that are commonly used along with anti-inflammatory drugs but they have no anti-inflammatory effects. An example is paracetamol also called acetaminophen in the U.S. (and sold under the brand name of Tylenol). As opposed to NSAIDs which reduce pain and inflammation by inhibiting COX, paracetamol has been shown in 2006 to block the re-uptake of endocannabinoids which only reduces pain, possibly explains why it has minimal effect on inflammation. Long-term use of NSAIDs can cause gastric erosions, which can lead to stomach ulcers and in extreme cases can cause severe haemorrhage resulting in death (Vane and Botting, 1998).

1.3.3. Bioavailability

In pharmacology, bioavailability (BA) is a subcategory of absorption and is the fraction of an administered dose of unchanged drug that reaches the systemic circulation; one of the essential principal pharmacokinetic properties of a drug.

When a medication is administered intravenously, its bioavailability is 100%. However, when a medication is administered by other routes (such as oral), its bioavailability generally decreases due to incomplete absorption that may vary from patient to patient. Bioavailability needs to be considered when calculating dosages of drugs for non-intravenous routes of administration.

In view of the above stated problem of herbal medication, the present investigation was planned to develop simple methods for standardization and validation of herbal medicine. Ashwagandha is a very popular herb used in several herbal formulations and it considered as the best suited herb for our investigations. The present study illustrates a short view of medicinal properties of Ashwagandha, the herb of our choice.

1.4. Review of Literature

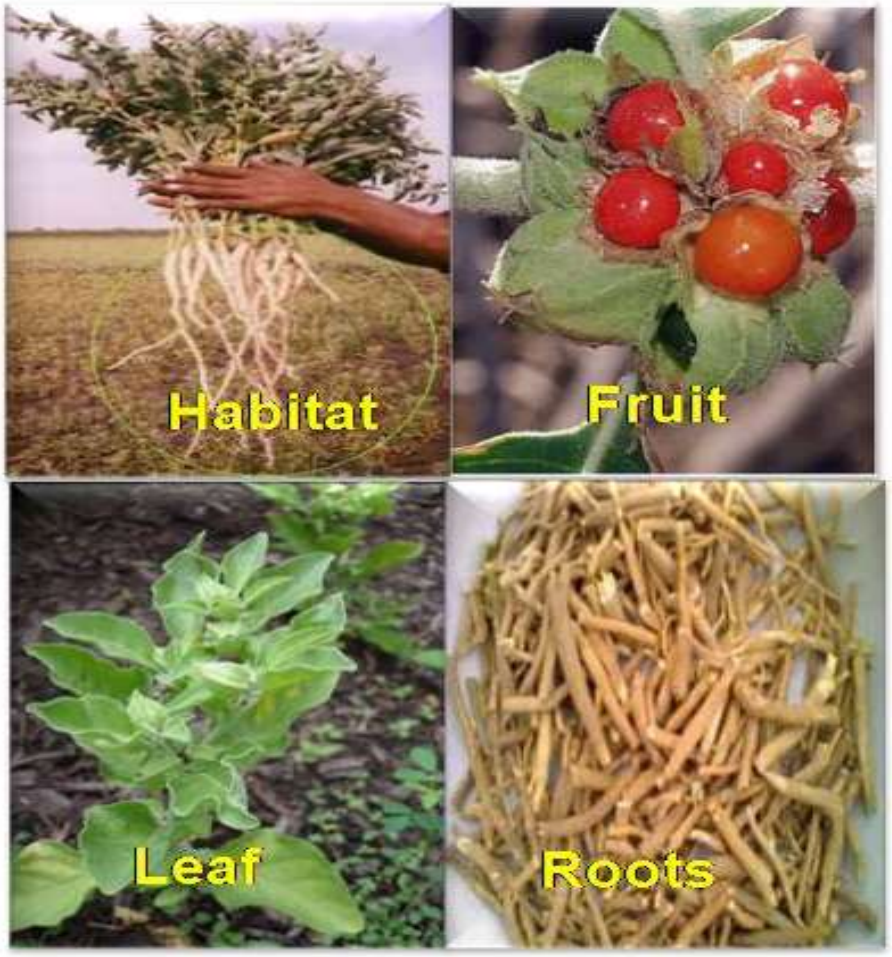
1.4.1. *Withania somnifera:* Description

Withania somnifera (L.) Dunal, (Solanaceae) commonly known as Ashwagandha, Asgandh, Winter Cherry and Indian ginseng belongs to the family Solanaceae, which has 1250 species. Ashwagandha grows widely throughout the drier and subtropical parts of India and is well represented in Maharashtra, Gujarat, Rajasthan, Madhya Pradesh, Uttar Pradesh, Punjab plains extending to the mountainous regions of Himachal Pradesh and Jammu and Kashmir where it ascends up to an elevation of 1800 m above sea level (Nigam and Kandalkar, 1995). The species dwells in a variety of phytogeographic regions differing from each other in climate and edaphic characters (Singh and Kumar, 1998). It has also been reported to grow in Pakistan, Afghanistan Palestine, Egypt, Jordan, Morocco, Spain, Canary Island, East Africa, Congo, Madagascar and South Africa and occupies areas which differ in their soil, rainfall, temperature and altitudinal profiles (Nigam and Kandalkar, 1995).

1.4.1.1. Physiology

Ashwagandha is a small, woody shrub that grows to about the height of 30-150 cm (**Fig. 1.2**). The aerial part, especially the stem, leaves and calyx are sparsely covered with fine hairy tomentum. Leaves are simple, petiolate, entire, shiny smooth, ovate and glabrous, 5-10 cm long and 2.5 – 7 cm wide (**Fig. 1.2**). Flowers are inconspicuous, greenish or yellow, in axillary umbellate cymes, bisexual; fruit berry in a persistent calyx; seeds small, flat, yellow, reniform, and very. The plant required 8-9 months for complete growth (Atal et al., 1975; Singh and Kumar, 1998; Kothari et al., 2003) (**Fig. 1.2)**. The bright red fruit is harvested in the late fall and seeds are dried for planting in the following spring. The berries have been shown to have an emetic effect. The roots are the mainly used therapeutically (**Fig. 1.2**).

Fig. 1.2: Habitat, Leaf, Fruits and Roots of Ashwagandha.



1.4.1.2. Soil Conditions for Germination

Ashwagandha grows well in sandy loam or light red soil having pH 7.5 to 8.0 with good drainage. Black soils or such heavy soils are suitable for cultivation. The seeds are first planted in small areas or in a nursery in lines to allow them to germinate. The seeds are sown about 2 cm deep into the soil at a distance of about 10 cm. The amount of seeds germinated should be based upon a final transplanted density of about 1 kg of seeds per hectare. The line method of sowing is preferred at this germination stage as it promotes the development of a healthy root system compared to being sown using the broad casting method (Singh and Kumar, 1998; Kothari et al., 2003). The germination is best carried out just before the arrival of the monsoon and a few light showers during this stage produce the optimal seedlings. Once the young plants are about 30 days old, they are ready for transplanting into the main farming fields. In these fields they are planted at a distance of approximately 60 cm into well ploughed and aerated soil. While the 30 day old seedlings are a guideline, in practice the transplanting corresponds to the actual arrival of the first monsoon rains (Obidoska et al., 2004; Kambizi et al., 2006; Shetty and Nareshchandra, 2012).

1.4.1.3. Water Management

Ashwagandha is grown as late rainy season (kharif) crop. The semi-tropical areas receiving 500 to 750 mm rainfall are suitable for its cultivation as rain fed crop (Singh and Kumar, 1998).

1.4.1.4. Temperature

The crop requires relatively dry climate during its growing period. It can tolerate a temperature range of 20°C to 38°C and even low temperature as low as 10°C. The plant grows from sea level to an altitude of 1500 m above sea level (Singh and Kumar, 1998; Kothari et al., 2003).

1.4.1.5. Grading of roots

Maturity of the crop is judged by drying out of leaves and yellow-red berries. Roots can be graded in the following 3-4 grades as per their length and thickness. A grade roots: root pieces upto 7cm long and diameter 1.0 - 1.5 cm, solid, bright and pure white. B grade root: root piece upto 5 cm long and diameter 1 cm, bright and white. C grade root: root pieces upto 3-4 cm in length, diameter less than 1 cm, solid, side branches. Lower Grade: small root pieces, semi-solid, very thick, yellowish, and chopped. The superior grade roots are stout and long and fetches premium price. Ashwagandha gives 3 to 5 q of dry roots and 50 to 75 kg of seeds/ha in well managed fields. The dry root yield goes up to 6.5 to 7.0 q /ha under scientific crop management. There are instances where farmers have achieved root yields as high as 1 t/ha. Commercially, roots of 6 to 15 mm diameter and 7 to 10 cm length are preferred. Alkaloid percentage in roots ranges from 0.13 to 0.31% (Kothari et al., 2003).

1.4.1.6. Profits from Ashwagandha Cultivation

As per 2001 figures and using an INR-USD exchange rate of 40, the net profits observed on an average for Ashwagandha cultivation are US $600 per hectare. Cultivation in Western countries would likely yield much higher profits in view of the recent increase in the popularity of Ashwagandha in these regions. The roots of Ashwagandha were mainly used for medicinal purposes (Singh and Kumar, 1998; Kothari et al., 2003). Throughout the world Ashwagandha is being used in the form of decoction, infusion, ointment, powder and syrup. In view of its varied therapeutic potentials, it has also been the subject of considerable modern scientific attention. Ashwagandha roots are a parts of over 200 formulations in Ayurveda, Siddha and Unani medicine which are used in the treatment of various physiological disorders (Mirjalili et al., 2009; Shetty and Nareshchandra, 2012). Formulations of

Ashwagandha are available in the world market; some of the products are shown the **Fig. 1.3**.

Fig. 1.3: Ashwagandha formulations.

1.4.2. Pharmacological Properties of Ashwagandha

Ashwagandha is considered as one of the most important plant of Indian Ayurvedic medicinal system for over 3000 years (Chatterjee et al., 1995; Bone and Morgen, 1996; Mishra et al., 2000; Mirjalili et al., 2008). Ashwagandha appears in WHO monographs on Selected Medicinal Plants and an American Herbal Pharmacopoeia monograph is also forthcoming (Mirjalili et al., 2009). **Fig. 1.4** gives a clear cut idea of usefulness of Ashwagandha in various disorders. The detailed pharmacological properties are shown in the **Fig. 1.4**.

Fig. 1.4: Pharmacological properties of Ashwagandha.

1.4.2.1. Anti-oxidant Effects of Ashwagandha

Since traditionally Ayurvedic use of Ashwagandha has been used for management of many diseases associated with free radical oxidative damage, it has been considered likely the effects may be due to a certain degree of antioxidant activity. Brain lipids contain large proportion of omega 3 fatty acids which because of their unsaturation index are prone to oxidation and can generate free radicals under stressful conditions. These free radicals can attack and damage almost any vital molecules and structures in our body, irreversibly and permanently causing disease and death. The brain uses nearly 20% of the total oxygen supply (Ames et al., 1993). Free radical damage of nervous tissue may contribute to neuronal loss in cerebral ischemia and may be involved in normal aging and neurodegenerative diseases, e.g., epilepsy, schizophrenia, Parkinson's, Alzheimer's, and other diseases (Jesberger et al., 1991; Scarfiotti et al., 1997). There has been some evidence to suggest that free radicals and some reactive nitrogen species trigger and increase cell death mechanisms within the body such as apoptosis and in extreme cases necrosis (Halliwelland Gutteridge al., 2007).

Panda et al. (1997) studied free radical scavenging activity of root powder of Ashwagandha in mice and it is observed that root powder possesses free radical scavenging activity, which may be responsible for its pharmacological effects. Bhattacharya et al. (1997) studied the antioxidant effects of Ashwagandha in the brain. The active principles of Ashwagandha, sitoindosides VII-X and withaferine A (glycowithanolides) have been tested for antioxidant activity using the major free-radical scavenging enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidise (GPX) levels in the rat brain frontal cortex and striatum. It was noted that the administration of active glycowithanolides of Ashwagandha (10 or 20 mg/kg, i.p for 21 days) increases levels of all the enzymes. This implies that Ashwagandha does have an antioxidant effect in

the brain which may be responsible for its diverse pharmacological properties.

In another study, an aqueous suspension of Ashwagandha roots extract was evaluated for its effect on stress-induced lipid peroxidation (LPO) in mice and rabbits (Dhuley et al., 1998). It was noted that the blood levels of LPO increased by administration of 0.2 mg/ kg of lipopolysaccharides (LPS) from *Klebsiella pneumoniae* and 100 mg/kg of peptidoglycans (PGN) from *Staphylococcus aureus*. Simultaneous oral administration of Ashwagandha extract (100 mg/kg) prevented the increase in LPO.

1.4.2.2. Anti-inflammatory Action of Ashwagandha

The effectiveness of Ashwagandha in a variety of rheumatologic conditions may be due in part to its anti-inflammatory properties, which have been studied by several authors. In a study by Anbalagan et al. (1981) powdered root of Ashwagandha (1 g/kg suspended in 2% gum acacia, 50 mg/ml) was given orally one hour before the induction of inflammation by injection of Freund's complete adjuvant to rats and continued daily for three days. It was found that Ashwagandha caused dose-dependent suppression of α2-macroglobulin (an indicator for anti-inflammatory drugs) in the serum of rats inflamed by sub-plantar injection of carrageenan suspension. The doses of Ashwagandha root powder were 500, 1000, 1200, 1500mg/kg given as suspension orally 3-4 hours prior to induction of inflammation. Dose of 1000 mg/kg showed maximum anti-inflammatory effect (Anbalagan et al., 1984).

In a study by Begum et al. (1987) was shown that Ashwagandha decreased the glycosaminoglycans content in the granuloma tissue by 92%, compared with 43.6% by hydrocortisone (15 mg/kg) treatment and no effect by phenylbutazone treatment (100 mg/kg). Ashwagandha also uncoupled the oxidative phosphorylation by significantly reducing the ADP/O ratio in mitochondria of granuloma tissue. It increased the Mg^{2+} dependent-

ATPase activity and also reduced the succinate dehydrogenase activity in the mitochondria of the granuloma tissue; no such effect was produced by the reference drugs. Begum et al. (1988) examined the effect of Ashwagandha (root powder, 1000 mg/kg, orally daily for 15 days) on paw swelling and bony degenerative changes in Freund's adjuvant- induced arthritis in rats and found that Ashwagandha caused significant reduction in both paw swelling and degenerative changes as observed by radiological examination. The reductions were better than those produced by the reference drug, hydrocortisone (15 mg/kg). Hindawi et al. (1992) found that methanol extract (10 mg/kg) of Ashwagandha inhibited the granuloma formation in cotton-pellet implantation in rats and the effect was comparable to hydrocortisone sodium succinate (5 mg/kg) treatment.

In a double-blind, placebo-controlled crossover studies, 42 patients with osteoarthritis were randomized to receive a formula containing Ashwagandha or placebo for three months. Patients were evaluated for one month and during the pretreatment all previous drugs were withdrawn. Pain and disability scores were evaluated weekly while erythrocyte sedimentation rate (ESR) and radiological studies were conducted monthly during both the pretreatment and treatment phases. In these study the Ashwagandha formulation significantly reduced the severity of pain ($p<0.001$) and disability ($p<0.05$) scores (Kulkarni et al., 1991).

Somasundaram et al. (1983a) have conducted studies on the mechanism of action for the anti-inflammatory properties of Ashwagandha. 3.5% formalin injected in the rats hind leg footpad showed a decrease in absorption of ^{14}C-glucose in drug untreated rat's jejunum. Glucose absorption was maintained at the normal level by both Ashwagandha and the cyclooxygenase inhibitor oxyphenbutazone. Similar results were obtained in parallel experiments using ^{14}C-leucine absorption from the jejunum.

(Somasundaram et al., 1983b) These studies suggest cyclooxygenase inhibition may be involved in the mechanism of action of Ashwagandha. In a study by Pawar et al. (2011) it was shown that rectal gel application of Ashwagandha root extract provides anti-inflammatory and muco-restorative activity in TNBS-induced Inflammatory Bowel Disease (IBD).

1.4.2.3. Anti-stress Effect of Ashwagandha

Singh et al. (1982) studied the anti-stress effect of Ashwagandha. An alcohol extract of seeds of Ashwagandha dissolved in normal saline was given (100 mg/ kg i.p; single dose) to mice and the swimming performance was observed in water at 28ºC-30ºC. It was observed that Ashwagandha extracts approximately doubled the swimming time when compared to controls.

Ashwagandha prevented both weight increase of the adrenals and reduction in ascorbic acid content of the adrenals which was normally caused by swimming. Based on this observation Bhattacharya et al. (1987) suggested that Ashwagandha induced a state of nonspecific increased resistance during stress. Glycosides of Ashwagandha (sitoindosides VII and VIII, 50 to 100 mg/kg) exhibited significant anti-stress activity in forced swimming induced immobility in mice, restraint stress induced gastric ulcers in rats and morphine-induced toxicity in aggregated mice (Bhattacharya et al., 1987).

The alcohol extract of Ashwagandha (100 mg/kg) reduced the increased levels of blood urea nitrogen, blood lactic acid and adrenal hypertrophy in stress induced rat, but did not affect changes in thymus weight and hyperglycemia (Dadkar et al., 1987). Ashwagandha root powder (100 mg/kg orally as an aqueous suspension daily for seven days) given before the swimming test in water at 10ºC increased total swimming time, indicating better stress tolerance in rats and also reversed the cold swimming-

induced increases in plasma corticosterone, phagocytic index, and avidity index to control levels (Archana et al., 1999). Grandhi et al. (1994) conducted a comparative study for anti-stress activity. Finely powdered roots of Ashwagandha and *Panax ginseng* (PG), suspended in 2% acacia (100 mg/kg in 1 ml oral) were given to mice daily for seven days; the swimming test was given on day 8. It is noted that significant increased anti-stress activity, as measured by the swimming endurance test, with both compounds but was better in the Ashwagandha group than in the PG group. If these results could be reproduced in humans, it would support the use of Ashwagandha in nervous exhaustion due to stress and in cachexia to increase body weight.

1.4.2.4. Anti-tumor Properties of Ashwagandha

The antitumor and radio sensitizing effects of Ashwagandha have been studied extensively. One study evaluated the anti-tumor effect in urethane-induced lung adenomas in adult male albino mice (Singh et al., 1986). Simultaneous daily oral administration of Ashwagandha ethanol extract (200 mg/kg) and urethane (125 mg/kg) for seven months reduced tumor incidence. The histological appearance of the lungs of animals protected by Ashwagandha was similar to those observed in the lungs of control animals. No pathological evidence of any neoplastic change was observed in the brain, stomach, kidneys, heart, spleen, or testes of any treated or control animals. Ashwagandha treatment also reversed the adverse effects of urethane on total leukocyte count, lymphocyte count, body weight, and mortality. The growth inhibitory effect of Ashwagandha was also observed in Sarcoma 180 (S-180), a transplantable mouse tumor (Singh et al., 1986).

Ethanol extract of root of Ashwagandha daily 400 mg/kg dose for 15 days produced complete regression of tumor after the initial growth. A 55% regression was obtained at 1000 mg/kg; however, it was a lethal dose in some cases. Ashwagandha was also found to act

as a radio- and heat sensitizer in mouse S-180 and in Ehrlich ascites carcinoma (Devi et al., 1992; Devi and Sharada, 1995; Devi, 1996a). Antitumor and radio sensitizing effects of withaferine were also seen in mouse Ehrlich ascites carcinoma *in vivo* (Sharad et al., 1996). These studies are suggestive of antitumor activity as well as enhancement of the effects of radiation by Ashwagandha.

1.4.2.5. Androgenic Activity of Ashwagandha

Ambiye et al. (2013) studied on spermatogenic activity of Ashwagandha root extract in oligospermic patients and reported that there was 167% increase in sperm count, 53% increase in semen volume and 57% increase in spermmotility. The significant improvement and regulation were observed in serum hormone levels with Ashwagandha treatment. Kalani et al. (2012) reported that the Ashwagandha increases circulating cortisol levels and improves insulin sensitivity. Gupta et al. (2013) reported that the Ashwagandha not only reboots enzymatic activity of metabolic pathways and energy metabolism but also invigorates the harmonic balance of seminal plasma metabolites and reproductive hormones in infertile men.

Ilayperuma1 et al. (2002) reported that Ashwagandha root extract induced a marked impairment in libido, sexual performance, sexual vigour, and penile erectile dysfunction. Ahmad et al. (2009) reported that the Ashwagandha inhibited lipid peroxidation and protein carbonyl content and improved sperm count and motility. He also reported that the Ashwagandha significantly increased serum Testosterone (T) and Luteinizing hormone (LH) and reduced the levels of Follicle-stimulating hormone (FSH) and Prolactin (PRL). Shukla et al. (2011) investigated the effect of Ashwagandha on apoptosis and intracellular reactive oxygen species (ROS) concentration of spermatozoa and the metal ions copper, zinc, iron and gold in seminal plasma from infertile men. It was noted that the Ashwagandha improves semen quality by combating oxidative stress

and cell death and improving essential metal concentrations. Abdel-Magied et al. (2001) reported that notable increase in testicular weight of animals treated with Ashwagandha extract. Mahdi et al. (2011) reported that the decrease in stress improved the level of anti-oxidants and improved overall semen quality in a significant number of individuals.

1.4.2.6. Estrogenic Activity of Ashwagandha

Nagareddy and Lakshmana, (2006) studied the effect of Ashwagandharoot extract on osteoporosis. The ethanolic root extract contains oestrogen-like withanolides for anti-osteoporotic activity. The author observed significant increase in serum ALP levels and excretion of urinary Ca and P in withanolide treated group. Khazal et al. (2013) studied the effect of Ashwagandha root extract on Estrogen Receptor-Positive Mammary Carcinomas. The authors found that in treated group the rate of cell division, in the mammary tumours was significantly reduced.

1.4.2.7. Effects of Ashwagandha on the Alzheimer's Disease

Thoda et al. (2000) reported that methanolic extract of Ashwagandha roots significantly promoted formation of neurites in human neroblastoma SK- N- SH cells with the effect being dose- and time dependent. The levels of mRNA of denritic markers MAP2 and PSD-95 were found to increase markedly however, that of axonal marker Tau was not.

Kuboyama et al. (2006) found that withanoside IV induced neurotic outgrowth in cultured rat cortical neurons. In Aβ (25-35) injected mice oral administration of withanoside IV at a dose of 10 μmole/day significantly improved memory deficits and prevented loss of axons, dendrites, and synapses; the aglycone of withanoside IV, sominone was identified as the main metabolite responsible for observed beneficial effects. Sominone 1 μmole by itself was able to induce the axonal and dendritic regeneration and synaptic

regeneration in culture rat neurons damaged by 10 μmole Aβ (25-35). Kuboyama et al. (2005) discuss the mechanism of action of Ashwagandha extracts on *in vitro* and *in vivo* models of neuro degenerative diseases such as Alzheimer disease and sipnal cord injury.

Nakayama et al. (2007) examine the effect of withanoside IV in mice with spinal cord injury (SCI) it was found that in SCI the myelin levels in axons, white matter, gray matter and CNS is decrease. Treatment with withanoside IV (10 μmole/kg body) resulted in increase axonal density with increase myelin levels in peripheral nervous system (PNS); the loss of CNS myelin was not affected. The authors suggest that oral administration of withanolide IV may ameliorate locomotors function by facilitating both axonal regrowth and increase in PNS myelin levels. Konar et al. (2011) reported that administration of scopolamine resulted in down regulation of the expression of BDNF and GFAP in dose and time dependent manner. Treatment with alcoholic extract of Ashwagandha leaf markedly attenuated these effects. Similarly effects was noted in IMR32 neuronal and C6 glioma cells the authors concluding that scopolamine besides the blocking cholinergic receptors, may induce memory loss by causing oxidative stress; leaf extract of Ashwagandha and withanone may serve as potential preventive and therapeutic agents.

Choudhary et al. (2005) reported isolation of withanolide derivatives form Ashwagandha these compounds were found to be potent inhibitors of Acetylcholinesterase (AChE) and Butryl choline esterase (BCHE) also these withanolides were found to posses calcium antagonistic ability and were safe in human nutrophil viable assay. The authors proposed that these compounds have potential for treatment of AD and associated problems. Zhao et al. (2002) reported isolation of five new withanolide derivatives which at concentration of 1 μM promoted neurite outgrowth neuroblastoma

SH-SY5Y cell line. Rao et al. (2013) found that methanol:chloroform (3:1) extract Ashwagandha reversed β amyloide induced toxicity in human nuraonal cells its implication in HIV-Associated nerocognitve disorders.

Babu et al. (2007) reported that Ashwagandha root powder and ethanolic extract of Ashwagandha protected from collagen glycation and cross linking in Tail tendons obtained from rats incubated with 50 mM glucose. The activity of ethanolic extract was comparable to met forming a known atiglycin agent the author suggested that Ashwagandha could have therapeutic role in the prevention of glycation induced pathogenesis in diabetes mellitus and aging. Based on computational model Grover et al. (2012a) suggested that withanolide A may be useful as AChE inhibitor and may thus provide substantial neuroprotective ability. Sehgal et al. (2012) observed that in the brains of middle egged and old APP/PSI AD transgenic mice. Ashwagandha reversed AD pathology by enhancing low density lipoprotein receptor related protein liver (LRP) LRP but not a β degrading protease neprilysin (NEP) in liver.

1.4.2.8. Effects of Ashwagandha on the Cardioprotection and Cardiopulmonary System

Mohanty et al. (2004a, 2004b) reported strong cardioprotective effect of Ashwagandha in the experimental model of isoprenaline-induced myonecrosis in rats. Augmentation of endogenous antioxidants, maintenance of the myocardial antioxidant status and significant restoration of most of the altered hemodynamic parameters may contribute to its cardioprotective effect. The author also concluded that Ashwagandha significantly reduced myocardial injury and emphasizes the beneficial action of Ashwagandha as a cardioprotective agent (Mohanty et al, (2004b).

Sandhu et al. (2010) showed that Ashwagandha increased velocity, power and maximum oxygen consumption (VO_2 max)

whereas *Terminalia arjuna* (Arjuna) increased VO_2 max and lowered resting systolic blood pressure. When given in combination, the improvement was seen in all parameters except balance and diastolic blood pressure. Thus, Ashwagandha may be useful for generalized weakness and to improve speed and lower limb muscular strength and neuromuscular coordination.

Ashwagandha may be useful as a general tonic due in part to its beneficial effects on the cardiopulmonary system. The effect of total alkaloids from the roots of Ashwagandha known as Ashwagandholine (AG) was studied on the cardiovascular and respiratory systems in dogs and frogs. The study found that the hypotensive effect was mainly due to autonomic ganglion blocking action and that a depressant action on the higher cerebral centers also contributed to the hypotension. These studies were found to be consistent with the use of Ashwagandha as a tranquilizing agent (Malhotra et al., 1961).

1.4.2.9. Effects of Ashwagandha on the Endocrine System

Panda et al. (1998) studied on efficacy of Ashwagandha in regulating thyroid function and based on the observations author suggested that Ashwagandha provides protection from free radical damage in the mouse liver. In another study Ashwagandha root extract were given to mice (1.4 g/kg, daily for 20 days) and it was noted that the treatment significantly increased the serum levels of 3,3',5-triiodothyronine (T_3) and tetraiodothyronine (T_4), while the hepatic concentrations of glucose 6-phosphatase activity and hepatic iodothyronine 5'-monodeiodinase activity did not change significantly. Ashwagandha significantly reduced hepatic LPO and increased the activity of SOD and catalase. The results suggest that Ashwagandha stimulates thyroidal activity and also promotes hepatic antioxidant activity (Panda et al., 1998).

A combination formula of Ashwagandha, *Tinospora cordifolia* (Guduchi), *Ocimum sanctum* (Tulasi,), *Picrorhiza kurroa* (Kutki), and *shilajit* was found to cause a dose-related decrease in streptozotocin-induced hyperglycemia (Ghosal et al., 1989).

1.4.2.10. Hemopoetic Effect of Ashwagandha

Administration of Ashwagandha extract was found to significantly reduce leukopenia induced by cyclophosphamide (CTX) treatment in Swiss albino mice (Davis and Kuttan, 1998). Total white blood cell count was in normal range in CTX-plus-Ashwagandha group. In the CTX-plus-Ashwagandha mice, the cellularity of the bone marrow was significantly increased compared to the CTX-alone treated group. Similarly, the number of alpha-esterase positive cells in the bone marrow of the CTX-plus-Ashwagandha mice increased compared to the CTX alone mice. The major activity of Ashwagandha may be the stimulation of stem cell proliferation. These studies indicated that Ashwagandha reduced CTX-induced toxicity and may prove useful in cancer chemotherapy (Davis and Kuttan, 1998).

1.4.2.11. Hepatoprotective Effects of Ashwagandha

Elberry et al. (2010) investigated antihepatotoxic Ashwagandha extracts against carbon tetrachloride (CCl_4)-induced hepatic damage in rats. The antihepatotoxic activity was assessed by measuring aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), reduced glutathione (GSH), tissue content and malondialdehyde (MDA) as well as histopathological examination. It was observed that significant antihepatotoxic effect by reducing significantly the levels of AST, ALT and LDH. However, significantly increased the GPx, GR and GST activity with increased GSH tissue contents and decreased production of MDA levels. Furthermore, alleviated histopathological changes were noted rats liver treated with CCl_4.

1.4.2.12. Hypoglycemic and Diuretic Effect of Ashwagandha

Andallu and Radhika, (2000) reported significant increase in urinary Na, urine volume, significant decrease in serum cholesterol, triglycerides, LDL and VLDL levels in human patients. Thus, it indicates that Ashwagandha is a potential source of hypoglycemic, anti-diuretic and hypocholesterolemic agent.

1.4.2.13. Immunomodulatory Activities of Ashwagandha

The use of Ashwagandha as a general tonic to increase energy balance and prevent disease may be partially related to its effect on the immune system. Ghosal et al. (1989) evaluated the immunomodulatory and central nervous system effects (antistress, memory, and learning) of glycowithanolides and a mixture of sitoindosides IX and X isolated from Ashwagandha in Swiss mice and Wistar rats. The author observed that both extracts produced statistically significant mobilization and activation of peritoneal macrophages, phagocytosis, and increased activity of the lysosomal enzymes; it also produced significant anti-stress activity in mice and rats and augmented learning acquisition and memory retention in both young and old rats (Ghosal et al., 1989).

Ziauddin et al. (1996) investigated the immunomodulatory effects of Ashwagandha in three myelosuppression models in mice: cyclophosphamide, azathioprin, or prednisolone. It was observed that significant increases in hemoglobin, red blood cell count, white blood cell count, platelet count, and body weight were observed in Ashwagandha treated mice. The authors also reported that the significant increases in hemolytic antibody responses toward human erythrocytes which indicated immunostimulatory activity in Ashwagandha treated mice (Ziauddin et al., 1996). The effect of Ashwagandha was also studied on the functions of macrophages obtained from mice treated with the carcinogen ochratoxin A (OTA). It was observed that OTA treatment of mice for 17 weeks significantly decreased the chemotactic activity of the macrophages.

Interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) production was also markedly decreased (Dhuley et al., 1997).

Senthilnathan et al. (2006) reported that the carcinogen as well as the paclitaxel effects on the immune system, and the toxic side effects on the immune system are more reversible and more controllable by Ashwagandha. These results collectively suggest the immunomodulatory activity of Ashwagandha root extract.

1.4.2.14. Effects of Ashwagandha on Nervous System

Total alkaloid extract (ashwagandholine, AG) of Ashwagandha roots has been studied for its effects on the central nervous system. AG exhibited a taming effect and a mild depressant (tranquilizer) effect on the central nervous system in monkeys, cats, dogs, rats and mice. AG had no analgesic activity in rats but increased Metrazol toxicity in rats and mice, amphetamine toxicity in mice, and produced hypothermia in mice. It also potentiated barbiturate, ethanol, and urethane induced hypnosis in mice (Malhotra et al., 1965a).

Effects of sitoindosides VII-X and withaferine isolated from aqueous methanol extract of roots of cultivated varieties of Ashwagandha were studied on brain cholinergic, glutamatergic and GABAergic receptors in male Wistar rats (Schliebs et al., 1997). The compounds slightly enhanced Acetylcholinesterase (AChE) activity in the lateral septum and globus pallidus, and decreased AChE activity in the vertical diagonal band. These changes were accompanied by enhanced M1-muscarinic-cholinergic receptor-binding in lateral and medial septum as well as in frontal cortices, whereas the M2-muscarinic receptor-binding sites were increased in a number of cortical regions including cingulate, frontal, piriform, parietal, and retrospinal cortex. The data suggest the compounds preferentially affect events in the cortical and basal forebrain cholinergic-signal transduction cascade. The drug-induced increase in cortical

muscarinic acetylcholine receptor capacity might partly explain the cognition-enhancing and memory-improving effects of Ashwagandha extracts in animals and in humans (Schliebs et al., 1997). Ashwagandholine, total alkaloids extracted from extract of Ashwagandha roots caused relaxant and antispasmodic effects against various agents that produce smooth muscle contractions in intestinal, uterine, tracheal, and vascular muscles (Malhotra et al., 1965b). The pattern of smooth muscle activity was similar to that of papaverine, but several folds weaker, which indicated a direct musculotropic action. These results are consistent with the use of Ashwagandha to produce relaxation.

Ashwagandha may be useful in various central nervous system (CNS) disorders, particularly its indication in epilepsy, stress and neurodegenerative diseases such as Parkinson's and Alzheimer's disorders, Tardive Dyskinesia, Cerebral Ischemia, and even in the management of drug addiction (Kulkarni and Dhir, 2008a; Ahmad et al., 2005; RajSankar et al., 2007; Kumar et al., 2009; RajSankar et al., 2009a, 2009b; Prakash et al., 2013).

1.4.2.15. Rejuvenating Effect of Ashwagandha

The growth-promoting effect of Ashwagandha was studied for 60 days in a double-blind study of 60 healthy children, age 8-12 years, who were divided in five groups. It is noted that administration of Ashwagandha slightly increase in hemoglobin, packed cell volume, mean corpuscular volume, serum iron, body weight, and hand grip, and significant increases in mean corpuscular hemoglobin and total proteins ($p<0.01$) at the end of 60 days when compared to the initial level and the placebo group. The study demonstrated that Ashwagandha may be useful as a growth promoter in growing children (Singh et al., 1982).

In another clinical trial, Ashwagandha purified powder was given 3 g/day for one year to 101 normal healthy male volunteers,

age 50-59 years (Boneand Morgen, 1996). All subjects showed significantly increased hemoglobin and RBC count, and improvement in hair melanin and seated stature. They also showed decreased ESR rate, and 71.4% of the subjects reported improvement in sexual performance. Thus, these studies indicate that Ashwagandha may useful in younger as well as older populations as a general health tonic.

1.4.3. General Toxicity Studies

An important consideration when investigating the medicinal properties of an unknown compound is diligent evaluation of its potential for harmful effects, usually evaluated through toxicity studies. Malhotra et al. (1965a, 1965b) was used Ashwagandholine to determine acute toxicity. It is noted that the acute LD_{50} was 465 mg/kg (332-651 mg/kg) in rats and 432 mg/kg (299-626 mg/kg) in mice.

In an antistress-effect study, an alcoholic extract of defatted seeds of Ashwagandha dissolved in normal saline was used to study LD_{50} in albino mice (Singh et al., 1982). The acute LD_{50} was 1750 mg (p.o). In another anti-stress study, aqueous-methanol extracts of the root from one-year-old cultivated WS (SG-1) and equimolar combinations of sitoindosides VII and VIII and withaferine-A (SG-2) were studied for acute toxicity (Grandhi et al., 1994). The acute LD_{50} of SG- 1 and SG-2 by intra-peritoneal administration in mice was 1076 mg/kg and 1564 mg/kg, respectively.

Sharma et al. 1986 carried out long-term study, Ashwagandha roots were boiled in water and administered to rats in their daily drinking water for eight months while monitoring body weight, general toxicity, well being, and number of pregnancies, litter size, and progeny weight. The estimated dose given was 100 mg/kg/day. In the second part of the study, the estimated dose was 200 mg/kg/day given for four weeks as above while monitoring body

temperature, body weight, cortisol value in heparinized plasma, and ascorbic acid content of the adrenals. The liver, spleen, lungs, kidneys, thymus, adrenals, and stomach were examined histopathologically and were all found to be normal (Sharma et al., 1986). The Ashwagandha group was devoid of any toxic effects after eight months of daily dosing in this study.

Ashwagandha promote physical and mental health, rejuvenate the body in debilitated conditions and increase longevity. Having a wide range of activity, it is used to treat almost all disorders that affect the human health. Thus, Ashwagandha is known as an **Indian Ginseng**.

1.4.4. Phytochemicals of Ashwagandha

Withania species has been extensively studied and several groups of chemical constituents have been reported. Several bioactive alkaloids and sterollactone based Phytochemicals, e.g. ashwagandhine, cuscohygrine, isopelletierine, anaferine, anhygrine, tropine, sitoindosides, saponins, withanolides, withanamides, and glycowithanolides have been isolated from different parts of this plant (Kapoor et al., 2001; Jamal et al., 1991; Choudary et al., 1996; Bandyopadhyay et al., 2007; Mirjalili et al., 2009; Matsuda et al., 2001; Mishra et al., 2005, 2008; Rahman et al., 1993; Shabbir et al., 1999; Naz and Choudhary, 2003). Chatterjee et al. (2010) reported that the total of 62 major and minor primary and secondary metabolites from leaves and 48 from roots were identified. 29 of these were common to the two tissues. These included fatty acids, organic acids, amino acids, sugars and sterol based compounds. 11 bioactive sterol–lactone molecules were also identified. The structures of some of the phytochemicals identified in Ashwagandha were shown in **Fig. 1.5.** Chatterjee et al. (2010) also suggested that withaferine A and withanone were the major metabolites present in the leaf as shown by NMR and withaferine A and withanolide A are major metabolites in the root.

Several researchers have proved that the medicinal values are mainly attributed to withanolides present in the roots of (Ali and Shuaib, 1997). Thus, among all secondary metabolites, withanolides are the most important Phytochemicals present in roots.

1.4.4.1. Withanolides in Ashwagandha

The withanolides are a group of naturally occurring C28- steroidal lactones built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring. The basic structure is designated as the withanolide skeleton (**Fig. 1.6)** (Glotter et al., 1971; Tursunova et al., 1977; Christen et al., 1986; Glotter, 1991; Alfonso et al., 1993;

Alfonso and Kapetanidis, 1994). Among all the withanolides withanoside IV, V, withaferine A, withanolide A and withanolide B are the major bioactive compound (Sangwan et al., 2008; Chatterjee et al., 2010).

Withaferine A (4β, 27-dihydroxy-1-oxo-5β, 6β-epoxywitha-2-24-dienolide, **Fig. 1.5**; **1.7**) was the first member of this group of compounds to be isolated from the well-known South-Asian medicinal plant Ashwagandha **(Table 1.1)**. The structural novelty and interesting biological activities elicited by this compound led to a thorough chemical investigation of the plant and numerous compounds with similar structural features were isolated (Leet et al., 1982; Tursunova et al., 1977; Glotter et al., 1991) (**Fig. 1.7; Table 1.1**). Some other important withanolides present in the roots of Ashwagandha shown in the **Fig. 1.7**.

Fig. 1.5: Some of the phytochemicals identified in Ashwagandha

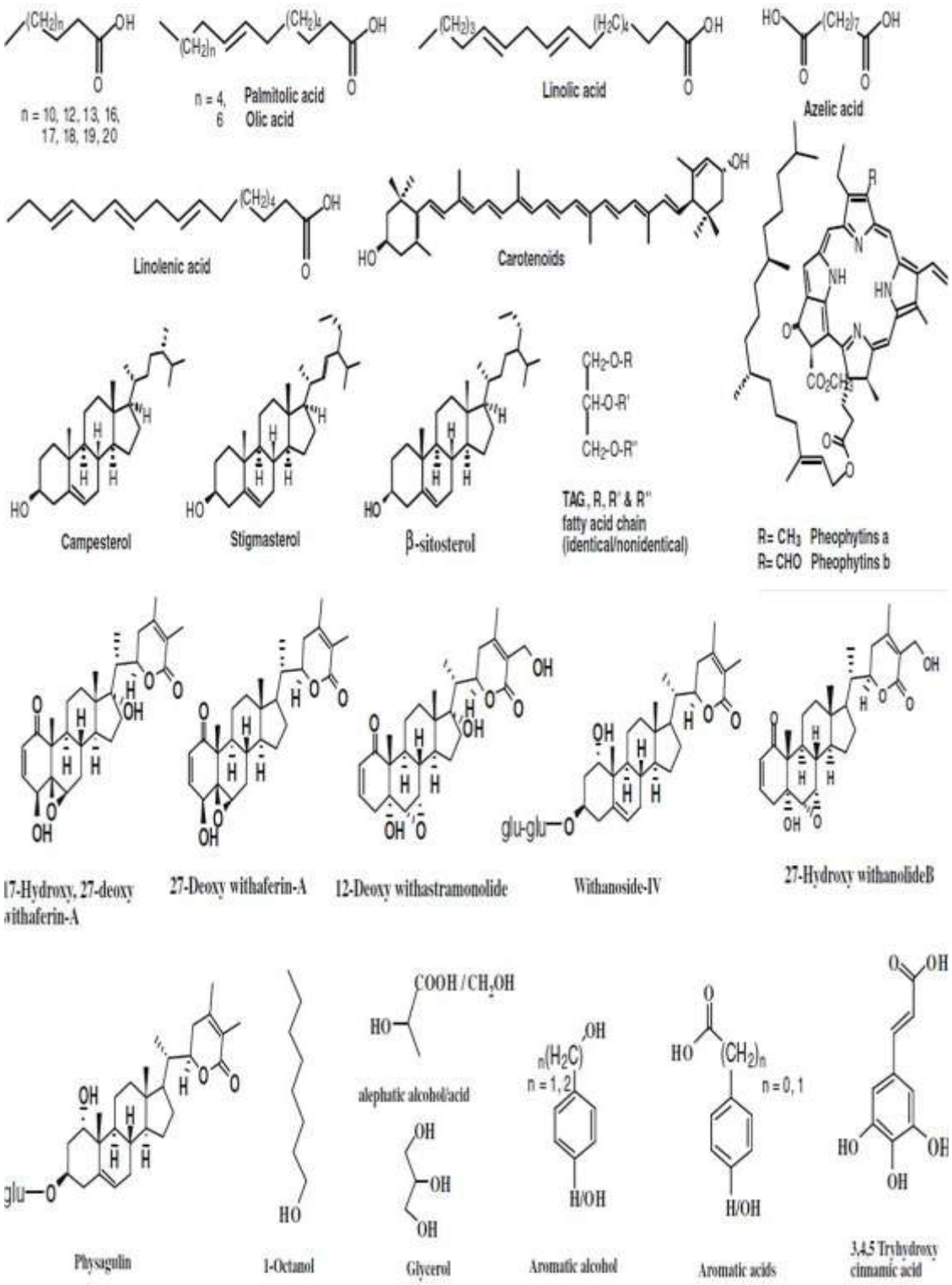


Fig. 1.6: Basic structure of withanolides

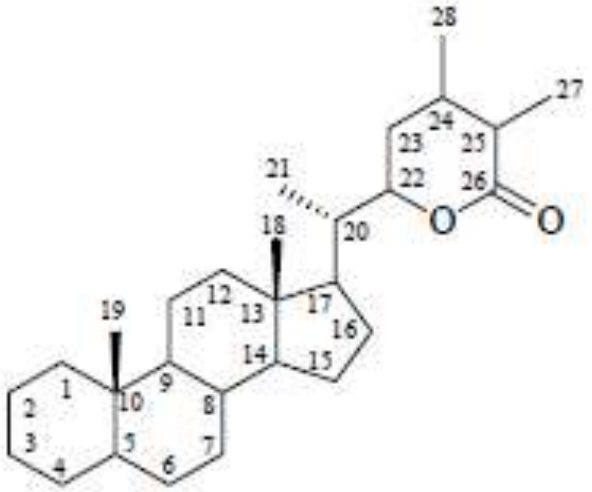


Fig. 1.7: Structure of major bioactive withanolides present in the root of Ashwagandha.

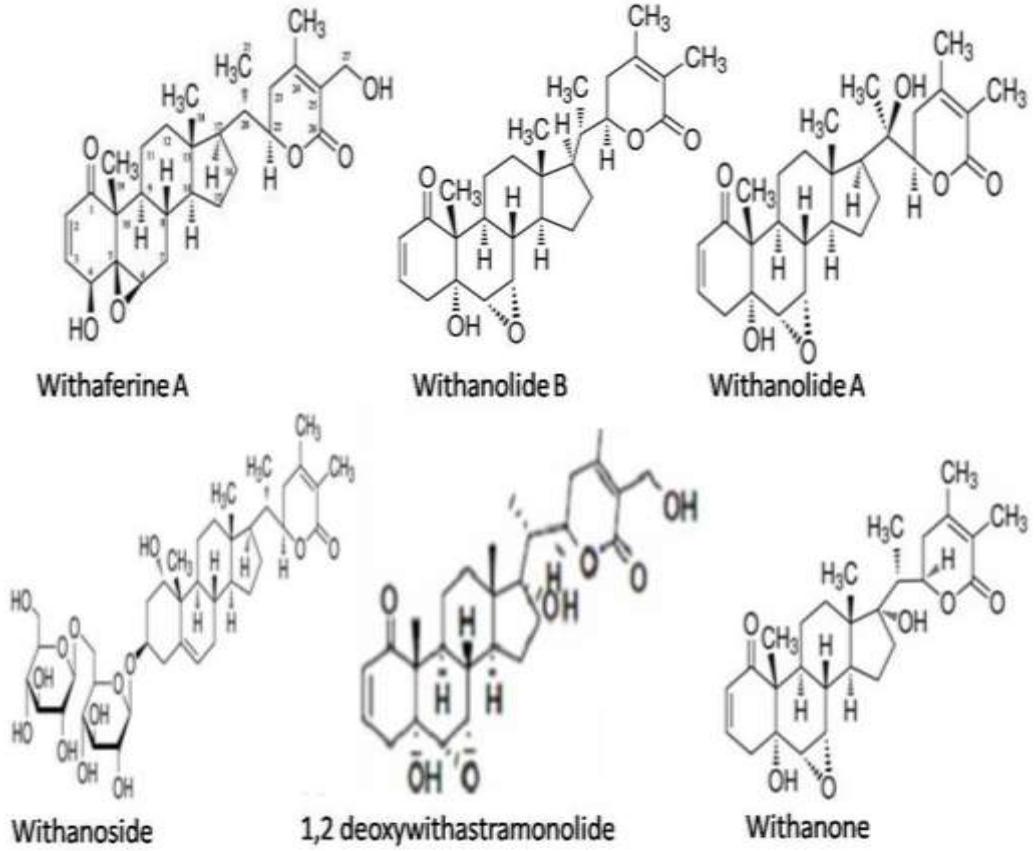


Table 1.1: Withanolides in Ashwagandha.

Withanolides	Reference
Withaferine A	Glotter et al., 1971, 1991; Gupta et al., 1996
Withanolides F, E, and 4β-hydroxy-withanolide E	Lavie et al., 1972
Withanolide Q and R,	Kirson et al., 1975
Withanolides S and T	Sharma et al., 1981
Jaborosalactone A, withanolide Y	Bessalle et al., 1987
Sitoindoside IX, X and withasomidienone	Ghosal et al., 1989; Rahman et al., 1993; Shabbir et al., 1999;
Withanolide A, withanosides IV and VI	Glotter et al., 1991; Zhao et al., 2002
Withanoside VIII, withanoside IX, withanoside XI, withanolide A, withanoside IV, withanoside VI and coagulin	Zhao et al., 2002
Physagulin D (1→6)-β-d-glucopyranosyl-(1-4)- β -d-glucopyranoside, 27-O- β - d-glucopyranosylphysagulin D, 27-O- β -d-glucopyranosylviscosalactone B, 4, 16-dihydroxy-5 β, 6 β -epoxyphysagulin D, 4-(1-hydroxy-2,2-dimethylcyclopropanone)-2, 3-dihydrowithaferin A, withaferin A, 2, 3-dihydrowithaferin A, viscosalactone B, 27-desoxy-24, 25-dihydrowithaferin A, sitoindoside IX, physagulin D, and withanoside IV	Jayaprakasam et al., 2003
Withanone, 27-hydroxy withanolide A, iso-withanone and 6α,7β-epoxy-1 β,3 β, 5α-trihydroxy-witha-24-enolide	Lal et al., 2006
Withanolide Z	Pramanick et al., 2008
Withaferin A and witharistatin	Benjumea et al., 2009
Withanolidesulfoxide	Vanisree et al., 2009
Physagulin D and withastraronolides	Ahuja et al., 2009

1.4.4.2. Other Components in Ashwagandha

Examination of Ashwagandha roots has resulted in the isolation of a new dimeric thiowithanolide, named Ashwagandhanolide (**Fig. 1.8**) (Ahuja et al., 2009). A purification of the methanol extract of Ashwagandha fruits yielded withanamides, A-I (**Fig. 1.9**). The structure of these compounds was determined by using serotonin, glucose and long-chain hydroxyl fatty acid moieties (Jayaprakasam et al., 2004). In quantitative analysis of Indian chemotypes of Ashwagandha by TLC densitometry Gupta et al. (1996) detected alkaloids in all the above mentioned plant parts, with the highest content found in leaves (Gupta et al., 1996). Extraction with 45% alcohol yields the highest percentage of alkaloids. The isolation of nicotine, somniferine, somniferinine, withanine, withananine, pseudowithanine, tropine, pseudotropine, 3α-tigloyloxytropane, choline, cuscohygrine, dl-isopelletierine and new alkaloids anaferine and anhygrine has been described (Kapoor et al., 2001; Gupta and Rana, 2007). The reported total alkaloid content in the roots of Indian Ashwagandha varies between 0.13 and 0.31%, though much higher yields (up to 4.3%) have been recorded in plants of other regions/countries. In addition to the alkaloids, the roots are reported to contain starch, reducing sugars, hentriacontane, glycosides, dulcitol, withanicil, an acid and a neutral compound. The leaves are reported to contain five unidentified alkaloids (yield 0.09%), chlorogenic acid, calystegines (nitrogen-containing polyhydroxylated heterocyclic compounds) withanone, condensed tannin and flavonoids. The berries have amino acids. Four types of peroxidases have been purified and characterized from Ashwagandha roots (Kapoor et al., 2001; Johri et al., 2005)

Fig. 1.8: Ashwagandhanolide, a new compound isolated from Ashwagandha.

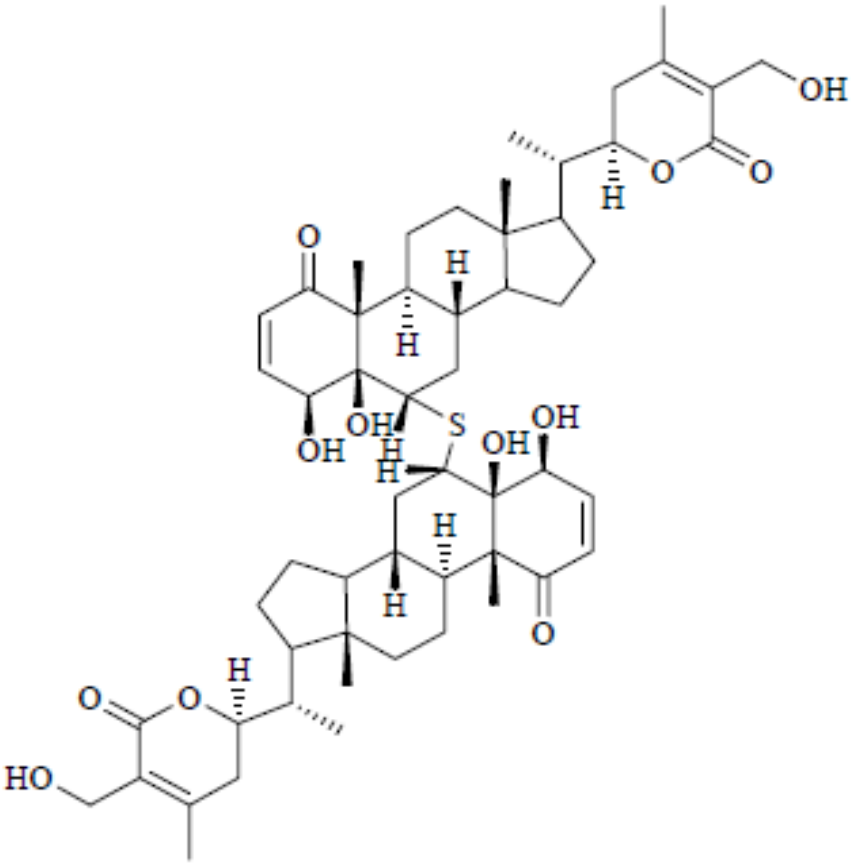
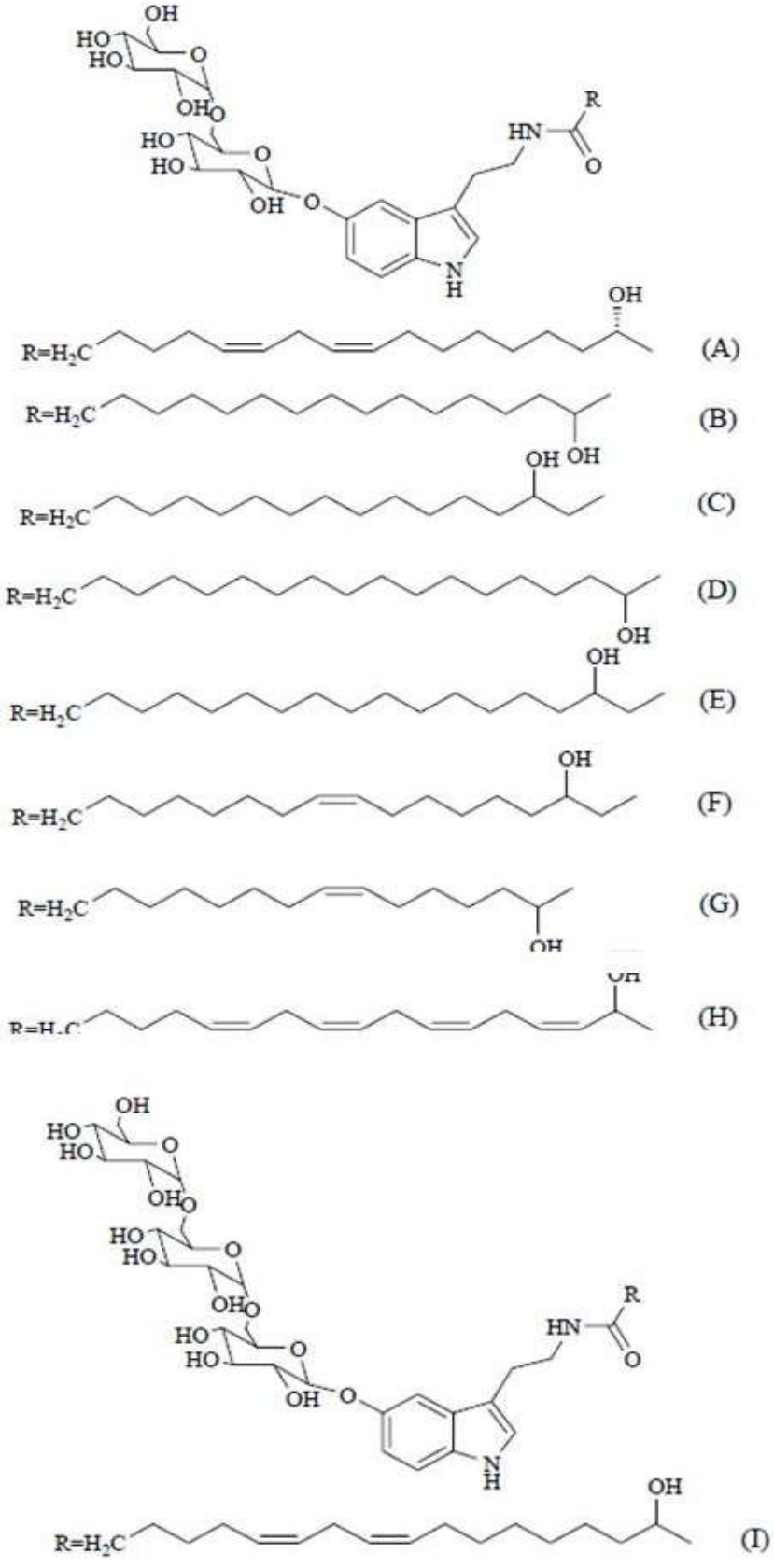


Fig. 1.9: Different withanamides (A-I) isolated from Ashwagandha fruits.



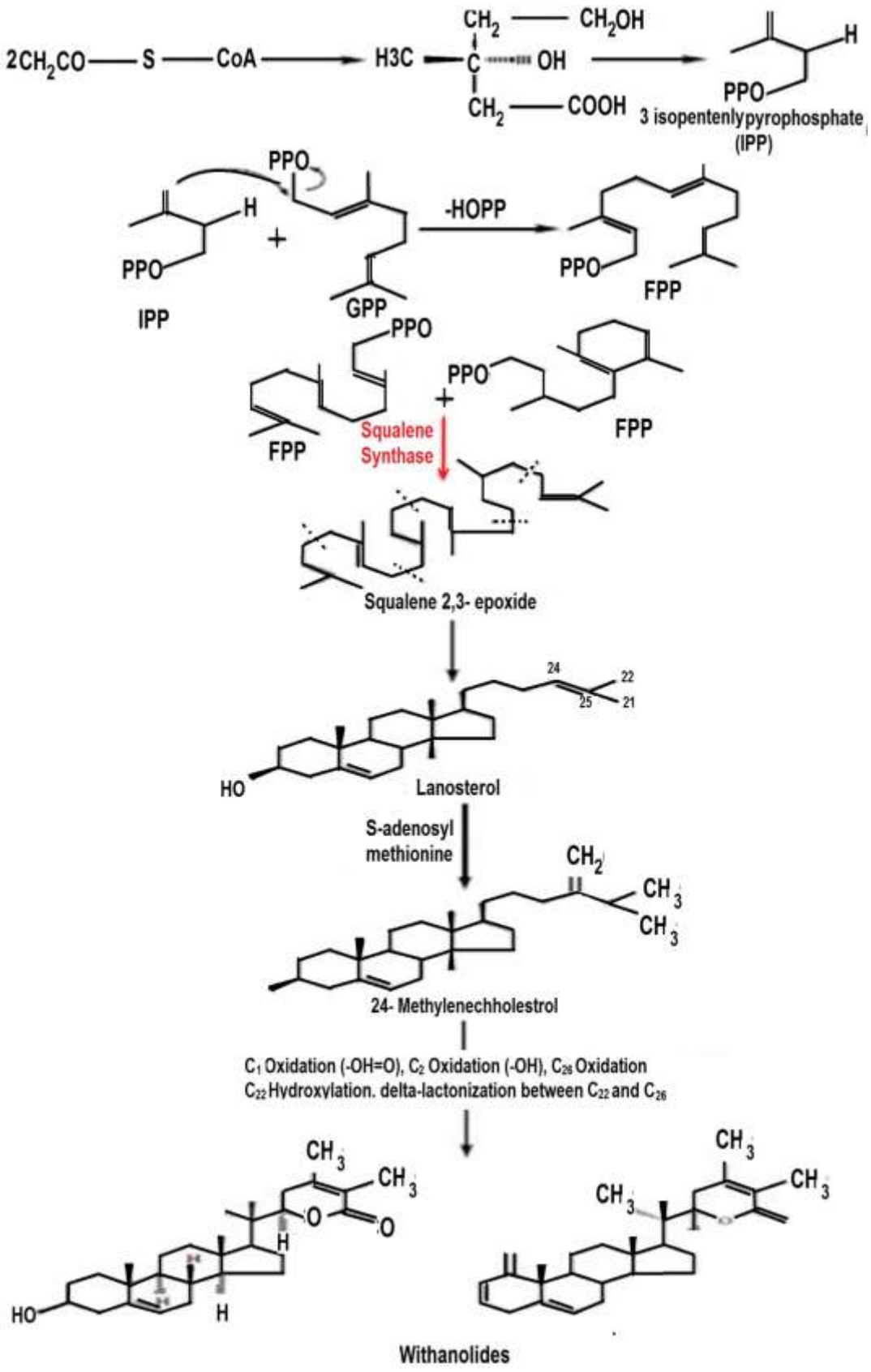
1.4.5. Biosynthesis Pathway of Withanolides

The biosynthetic pathways of withanolides and other chemical constituents of Ashwagandha are not fully known, but the possible biosynthetic pathway was described by several researchers (Kirsonet al., 1977; Nittala et al., 1981; Ray and Gupta, 1994). **Fig. 1.10** shows an overview of the most important steps in the withanolide biosynthetic pathway. It has been reported that, except for a very few exceptions, the plants that synthesize the 20-H withanolides are unable to produce the 20-OH counterparts and vice versa (Kirson et al., 1977).

It is assumed that withanolides probably take similar biosynthetic route as cholesterol in mammals. The first step in the biosynthesis of cholesterol is the activation of acetate by its conversion to acetyl Co-enzyme A (acetylCoA). Two units of acetylCoA are combined and metabolized to mevalonic acid. Only the R- form of mevalonic acid is used by the living system to produce terpenes, while the S- form is metabolically inert. The (R)-mevalonic acid is converted into isopentenyl pyrophosphate (IPP) through the loss of 1 carbon atom. The molecule of 3-isopentenyl pyrophosphate (IPP) can condense in a head-to-tail manner with its isomer, 3,3-dimethyl allyl pyrophosphate (DMAPP), to give geranyl pyrophosphate (GPP). A condensation reaction of transgeranyl pyrophosphate with another molecule of IPP yields farnesyl pyrophosphate (FPP). The enzyme squalene synthase catalyses the condensation of two molecules of farnesyl pyrophosphate in a head-to-head manner in the presence of NADPH to produce squalene. Oxidation of squalene by atmospheric oxygen is catalyzed by NADPH-linked oxide to afford squalene 2,3-epoxide. The latter undergoes ring closure to form lanosterol which is then converted into a variety of different steroidal triterpenoidal skeletons. The bioconversion of lanosterol to 24-methylenecholesterol is still not fully understood. The sequence of reactions and intermediates may

also differ slightly among organisms. 24-Methylenecholestrol may be a biosynthetic precursor of steroidal lactones. It has been proposed that the hydroxylation in C22 and δ-lactonization between C22 and C26 of 24-methylenecholestrol yields withanolides (**Fig. 1.10**). It has also been suggested that the α, β-unsaturated ketone in ring A of common withanolides may be produced through the sequence (Manitto et al., 1981; Velde et al., 1981).

Fig. 1.10: Important steps in the biosynthesis of withanolides



1.4.6. Pharmacological Activities of Individual Withanolides

Withanolides are a group of naturally occurring oxygenated ergostane type steroids i.e. steroidal lactone, having lactone in side chain and 2-en-1-one system in the ring. Ashwagandha has several pharmacological activities which are mainly attributed to withanolides. Withanolides have shown a wide range of pharmacological activities including anti-inflammatory, anti-arthritic, angiogenesis inhibitor, anti-cholinesterase, antioxidant, anti-bacterial, immunomodulatory, and above all, anti-tumor. **Fig. 1.11** represents the shematic diagram of pharamacological activity of individual withanolides for more clarification.

1.4.6.1. Withaferine A

As earlier described in section 1.4.4.1 withaferine A (WF A) is the first member of the withanolide group (**Fig. 1.7**). Its Molecular formula is $C_{28}H_{38}O_6$ and molecular weight is 470.6. WF A is white crystalline powder which is soluble in chloroform, ethyl acetate and alcohol. WF A is insoluble in petroleum ether and water. The maximum UV absorption i.e. λ_{max} of WF is 227nm. WF A is very well known and it has several medicinal properties. **Fig. 1.7** shows the shematic diagram of pharamacological activity of individual withanolides.

Srinivasan et al. 2007 reported that the WF A inhibits survival of both androgen-responsive and androgen-refractory prostate cancer cells by a Par-4–dependent mechanism. As Par-4 up-regulation induces apoptosis in most tumour cells. Stan et al. 2008a studied on WF A treatment causes G2 and mitotic arrest in human breast cancer cells and he reported that G2-M phase cell cycle arrest may be an important mechanism in anti-proliferative effect of WF A against human breast cancer cells. Stan et al. 2008b reported that the WF A treatment decreased viability of MCF-7 (estrogen-responsive) and MDA-MB-231 (estrogen-independent) human breast cancer cells in a concentration-dependent manner.

The tumors from WF A-treated mice exhibited reduced cell proliferation and increased apoptosis compared with tumors from control mice. These results point towards an important role of FOXO3a in regulation of WA-mediated apoptosis in human breast cancer cells Stan et al 2008b. Ryeong Hahm et al. (2011) shows that the WF A suppresses Estrogen Receptor-α Expression in Human Breast Cancer Cells.

Vaishnavi et al. 2012 demonstrate that WF A has a strong binding to the target cells (cancer cells i.e. mortalin, p53, p21 and Nrf2); it showed high cytotoxicity towards cancer cells and was safe for normal cells. Khedgikar et al. (2013) stated that *in vitro* proteasome inhibition by WF A simultaneously promoted osteoblastogenesis by stabilizing RunX2 and suppressed osteoclast differentiation, by inhibiting osteoclastogenesis. It is reported that the WFA is a proteasomal inhibitor promotes healing after injury and exerts anabolic effect on osteoporotic bone (Khedgikar et al., 2013). WF A also has Anti-tumor, apoptotic, anti-angiogenesis, radiosensitizing and anti-inflammatory activities (Devi and Sharada, 1995; Mohan et al., 2004, 2007; Sabina et al., 2007; Sen et al., 2007; Yang et al., 2007; Oh et al., 2008).

1.4.6.2. Withanolide A

Molecular formula of withanolide A (WN A) is $C_{28}H_{38}O_6$ and molecular weight is 470.6. WN A is white crystalline powder which is soluble in chloroform, ethyl acetate and alcohol. WN A is insoluble in water. The maximum UV absorption i.e. λ_{max} is 227 nm. WN A is also very well known withanolide of Ashwagandha (**Fig. 1.7).**

Tohda et al. (2000, 2004) reported that the WN A improves Aβ (25-35) induced memory impairment, neuronal atrophy and synaptic loss in the cerebral cortex and the hippocampus and subsequent treatment with withanolide A induced significant reconstruction of

pre-synapses and post-synapses, in addition to regeneration of both axons and dendrites in the neurons.

Kobuyama et al. (2005) reported that neurite outgrowth, which supports the potential neuritogenic role of WN A. Zhao et al. 2002 reported that WS IV showed significant neurite outgrowth activity at a concentration of 1 mM on a human neuroblastoma SH-SY5Y cell line. Several researchers have been reported that WN A was very effective as a neurological, immunological and anti-stress agent **Fig. 1.7**(Kobuyama et al., 2002; Malik et al., 2007, 2013; Kour et al., 2009).

1.4.6.3. Withanoside (IV-V)

Withanolides have glucose moiety at C_3 position (**Fig. 1.5; 1.7)** called as glyco-withanolides, thus they are polar and also have higher molecular weight. Molecular formula of withanoside IV (WS IV) and withanoside V (WS V) are $C_{40}H_{62}O_{15}$ and $C_{40}H_{62}O_{14}$ respectively. Molecular weight of WS IV is 782.92 and WS V is 766.92. Both are white crystalline powders which are soluble in chloroform, ethyl acetate and alcohol; sparingly soluble in water and insoluble in petroleum ether. The maximum UV absorption for both withanolides are i.e. λ_{max} is 227 nm. WS IV plays an important neuro-regenerative role. Thus in spinal cord injury WS IV and V improve hind limb function and increase the myelin layer in peripheral nervous system**.**

Kuboyama et al. (2006) found that Withanoside IV, V, VI induced neuritic outgrowth in cultured rat cortical neurons. In Aβ (25-35) injected mice oral administration of withanoside at a dose 10 μmole/day significantly improved memory deficits and prevented loss of axons, dendrites, and synapses; the aglycone of withanoside, sominone, was identified as the main metabolite responsible for observed beneficial effects. Sominone 1 μmole by itself was able to induce the axonal and dendrite regernation and synaptic

regeneration in culture rat neurons damaged by 10 μmole Aβ (25-35).

Nakayama et al. (2007) examined the effect of WS IV in mice with spinal cord injury (SCI) it was found that in SCI the myelin levels in axons, white matter, gray matter and CNS is decrease. Treatment with WS IV (10 μmole/kg body) resulted in increased axonal density with increase myelin levels in peripheral nervous system (PNS); the loss of CNS myelin was not affected. The authors suggest that oral administration of WS IV may ameliorate locomotors function by facilitating both axonal re-growth and increase in PNS myelin levels. Zhao et al. 2002 reported that WS IV showed significant neurite outgrowth activity at a concentration of 1 mM on a human neuroblastoma SH-SY5Y cell line.

1.4.6.4. Withanolide B

Molecular formula of withanolide B (WN B) is $C_{28}H_{38}O_5$ and molecular weight is 454.60. WN B is white crystalline powder which is soluble in methanol, chloroform, ethyl acetate and alcohol. WN B is insoluble in water. The maximum UV absorption i.e. λ_{max} is 224 nm (**Fig. 1.7).**

Ichikawa et al. 2006 investigated the effect of the WN B on NF-κB and NF-κB-regulated gene expression activated by various carcinogens. It is observed that withanolides suppressed NF-κB activation induced by a variety of inflammatory and carcinogenic agents, including tumor necrosis factor (TNF), interleukin-1β, doxorubicin, and cigarette smoke condensate.

1.4.6.5. 1, 2-Deoxy Withastramonolide (27-Hydroxy Withanolide)

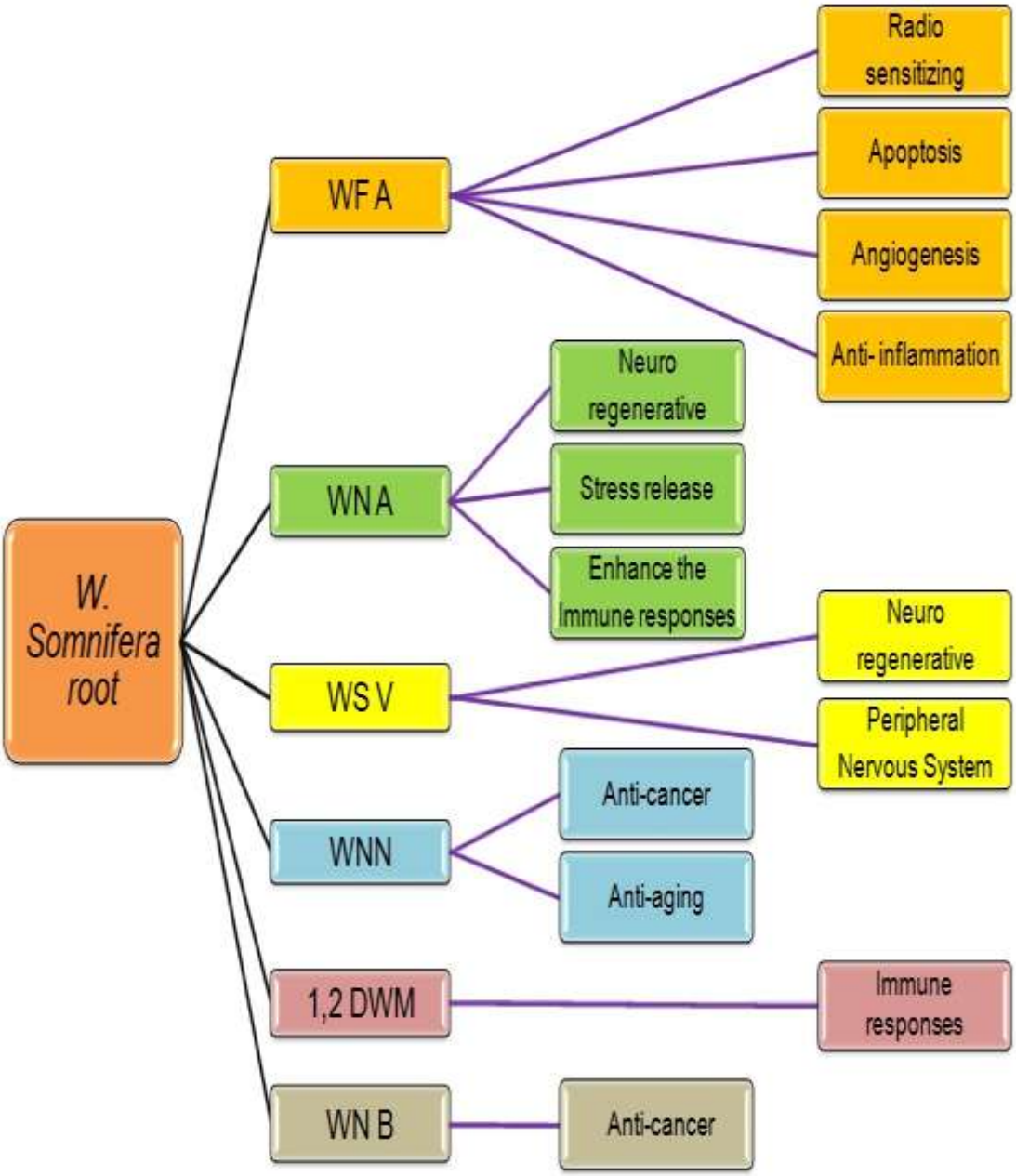
Molecular formula of 1, 2 deoxywithastramonolide (1, 2 DWM) is $C_{28}H_{38}O_6$ and molecular weight is 470.61. 1, 2 DWM is white crystalline powder which is soluble in chloroform, ethyl acetate and alcohol. 1,2 DWM is insoluble in hexane and water. The maximum UV absorption i.e. λ_{max} is 235 nm (**Fig. 1.7).** Zhao et al. 2002 reported that WN B showed significant neurite outgrowth activity at a concentration of 1 mM on a human neuroblastoma SH-SY5Y cell line.

1.4.6.6. Withanone

Molecular formula of withanone (WNN) is $C_{28}H_{38}O_6$ and molecular weight is 470.60. WNN is white crystalline powder which is soluble in chloroform; while sparingly soluble in methanol, ethyl acetate and alcohol. WNN is insoluble in water. The maximum UV absorption i.e. λ_{max} is 229 nm (**Fig. 1.7).**

Konar et al. (2011) studied on protective role of withanone on scopolamine-induced changes in brain cells. Author suggested that besides cholinergic blockade, scopolamine-induced memory loss may be associated with oxidative stress. WNN may serve as potential preventive and therapeutic agents for neurodegenerative disorders. Withanone has been tested on normal human fibroblasts and it was noted that it have both anticancer and anti-aging activity (Widodo et al., 2009). Priyandoko et al. 2011 reported that withanone protects cells from MAA-induced toxicity by suppressing the ROS levels, DNA and mitochondrial damage, and induction of cell defense signaling pathways. Vaishnavi et al, 2012 demonstrate that WNN has a weak binding to the targets (cancer cells i.e. mortalin, p53, p21 and Nrf2); it showed milder cytotoxicity towards cancer cells and was safe for normal cells.

Fig. 1.11: Shematic diagram of pharamacological activity of individual withanolides.



1.4.7. A Biotechnological Approach to Withanolide Production

Ashwagandha shows several pharmacological properties which are mainly attributed to secondary metabolites i.e. withanolides present in the roots. Thus, several researchers have carried out many biotechnological studies of *Withania* species to enhance the production of withanolides. Large-scale plant cell cultures may be cost-effective and also allow the production of higher amounts of withanolides in a relatively short period of time. Roja et al. (1991) studied on tissue cultures of an Indian chemotype of Ashwagandha from axillary meristems using MS agar medium supplemented with 2,4-D, IAA, NAA, BA, coconut milk or kinetin, either alone or in combination (Roja et al., 1991). He reported that callus cultures failed to synthesize withanolides, but multiple shoot cultures synthesized significant amounts of withanolides. Ray et al. (2001) studied on multiple shoot cultures of Ashwagandha from single shoot tip explants and their potential for the production of WF A and withanolide D. The author observed that supplementation of MS solid agar medium with 1.0 mg BA/L and 4% sucrose enhanced accumulation of both WF A (0.16%) and withanolide D (0.08 %). MS liquid medium containing 1.0 mg BA/L and 10% coconut milk favored a maximum increase in biomass (27 fold), induced micro shoots (37.6) as well as accumulation of WF A (0.14%).

Direct rooting from leaf explants of Ashwagandha has been achieved on half-strength MS medium supplemented with 15 g/l sucrose, and different concentrations of growth regulators (Wadegaonkar et al., 2006). The concentration of alkaloids increased compared to field grown roots. WN A biogeneration in shoot cultures of Ashwagandha has been reported (Sangwan et al., 2007). The production of WN A in the cultures varied considerably according to the hormone composition of the culture media as well as the genotype used as the explants source.

The hairy root system based on *Agrobacterium rhizogenes* (A. rhizogenes) inoculation has become popular as a method of producing secondary metabolites synthesized in plant roots (Toivonen et al., 1993; Palazon et al., 1997). White et al. (1980) carried out hairy root transformation of Ashwagandha by three different strains of *A. rhizogenes* (A4, LBA 9402 and LBA 9360) and analyzed the specificity and frequency of their withanolide production with special reference to WF A. The best response in terms of transformation ability and growth of the hairy roots was obtained with strain A4, followed by LBA 9402; LBA 9360 failed to induce a transformation event. The production of WF A was studied in the A4-induced hairy root lines at different growth phases (4, 10 and 24 weeks) using HPLC and maximum levels were observed in the media and hairy roots of 10-week-old cultures.

Transformation of Ashwagandha with wild type nopaline and octopine strains of *A. tumefaciens* has been reported (Ray et al., 2001). It is observed that withanolide synthesis in shooty teratomas was much higher (0.07–0.1% WF A and 0.085–0.025% withanolide D) than in non-transformed shoot cultures. Bandyopadhyay et al, (2007) have reported the presence of TR-DNA in all the transformed callus lines of Ashwagandha obtained after infection with *A. rhizogenes* A4, thus confirming the effects of aux genes on root line phenotypes. The accumulation of WF A was maximum (0.44% dry weight) in the transformed hairy root lines. All the rooty callus lines accumulated both WF A and withanolide D. Some of the callus lines produced both WF A (0.15–0.21% dry weight) and withanolide D (0.08–0.11% dry weight), and they grew faster than the transformed root lines (Bandyopadhyay et al., 2007).

Ray et al. (1996) have been studied on hairy root culture and he reported root cultures synthesized several withanolides, from which withanolide D was isolated and identified. The productivity of withanolide D in transformed roots (0.181 mg/L) was higher than in

untransformed root cultures (daily production of 0.181 and 0.026 mg/L, respectively).

1.4.8. Effects of Environmental Parameters on Content of Active Components

It is known that the medicinal properties of Ashwagandha are attributed to withanolides and Ashwagandha roots were used in several medicinal formulations. Thus, variations in withanolide content leads to inconsistent product formulations resulting in unreliable therapeutic and health promoting effects (Patwardhan et al., 2009).

Therefore the concentration of withanolides in roots is a very crucial factor during Ayurvedic preparations. The several researchers have shown that the secondary metabolite differs with location, cultivation practices, soil nutrients, tissue type and varieties of Ashwagandha (Kumar et al., 2007; Kubsad et al., 2009; Murthy andNagella, 2012; Nasira et al., 2012). Thus, the selection of appropriate plant material for preparing the Ayurvedic formulation is important.

1.4.9. Status of Indian Herbal Medicine in International Market

Herbal medicine has been used in India for thousands of years and is increasingly been used worldwide during the last few decades as evidenced by rapidly growing global and national markets of herbal drugs. The global pharmaceutical market was worth US $550 billion in 2004 and is expected to exceed US $900 billion by the year 2009. According to WHO estimates, the present demand for medicinal plants is US $14 billion a year and by the year 2050 it would be US $5 trillion. Due to high prices and harmful side-effects of synthetic drugs, people rely more on herbal drugs and this trend is growing, not only in developing countries but in developed countries too.

Traditional Chinese Medicine (TCM) uses over 5000 plant species, while India uses about 7000 for export of herbal

formulations in world market (**Fig. 1.12**). India has 2.4% of world's area with 8% of global biodiversity. In India, around 25,000 effective plant-based formulations are used in traditional and folk medicine (Aneesh et al., 2009; WHO 2008). The Ayurvedic Pharmacopoeia of India gives monographs for 258 different Ayurvedic drugs. Presently, Indian systems of medicine use more than 1100 medicinal plants of which most are collected from the wild. The Indian Herbal Medicine (IHM) has a great potential to become a leader in Global market. Ashwagandha, Shatawari, Harar and Ashoka occupy about 20% of IHM market which could mean that Ashwagandha is one of the most important medicinal plant in Ayurveda (**Fig. 1.13**).

In the international market TCM is well accepted as compared to IHM since the latter lack in quality control/quality assurance and do not provide assurance of batch to batch consistency, safety levels, defined dosage, contaminants, adulterants. Also the products do not normally specify, what are the active principles and their stability; no simple and reliable methods/techniques are used for the validation of the Herbal product (Gibert 1998; Jiang et al., 2000; Cheng 2000; Aneesh et al., 2009). In view of this here we studied in validation of *Withania somnifera* (L).Dunal (Ashwagandha) by physical, chemical and biological approaches with respect to major bioactive withanolides.

- **Physical Approach:** Cyclic voltammeter analysis
- **Chemical Approach:** Chromatographic and other chemical analysis
- **Biological Approach:** *in vivo* and *in vitro* analysis

Fig. 1.12: Growing export rate of Traditional Chinese Medicine (TCM) in the World market as compare to Indian Herbal Medicine (IHM).

Global market for herbal medicine is about US $ 62 billion. TCM is leading with US $19 billion; while IHM is on third rank with US $1 billion. IHM used 7000 and TCM used 5000 species of medicinal plants for export. It cloud mean that IHM has great potential but it is not well established as compare to TCM in the world market. TCM increased their exports by 120 million US $ in the year 2007; while IHM able to increased by 16 million US $ only (Aneesh et al., 2009).

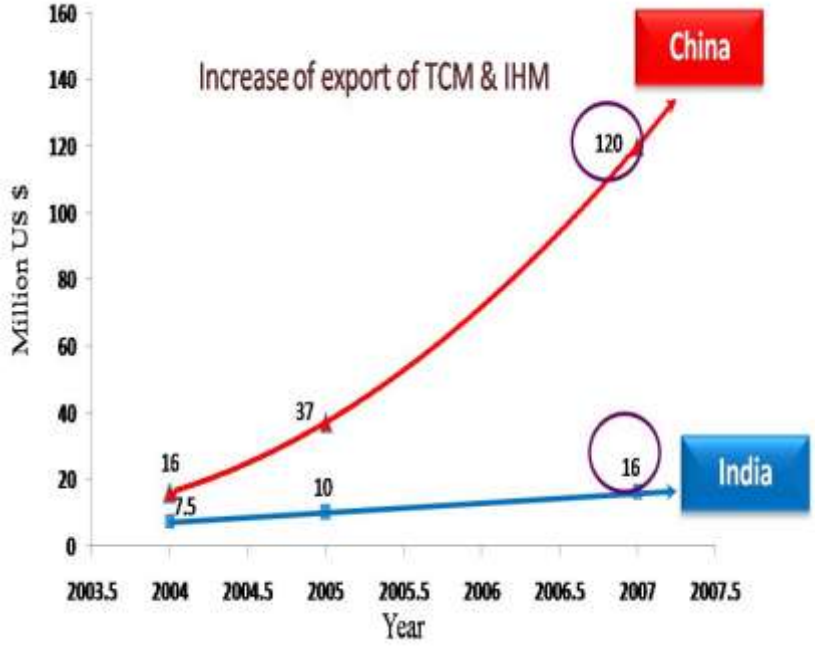
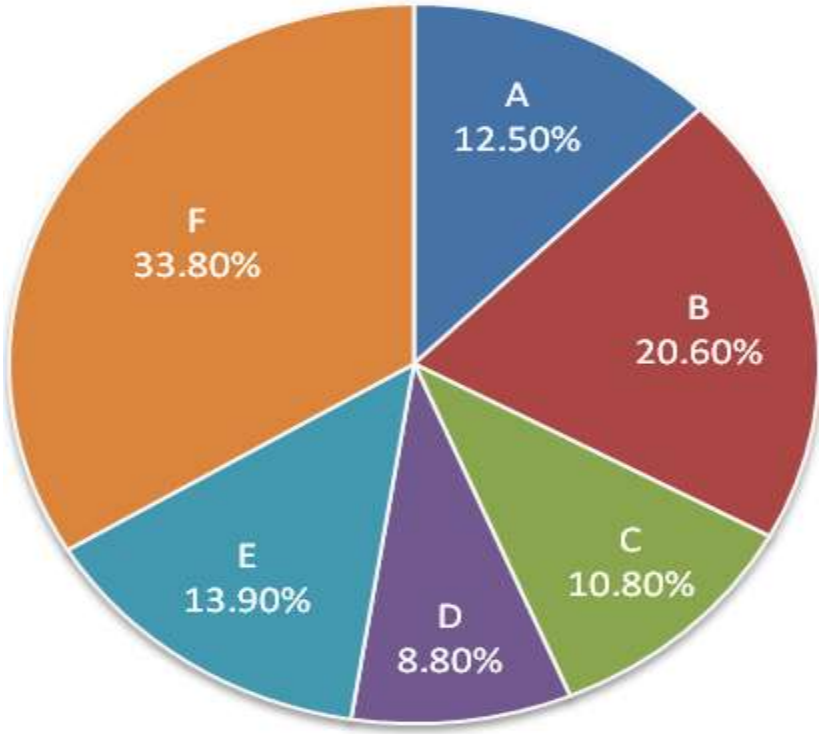


Fig. 1.13: Status of Ashwagandha in IHM (Aneesh et al., 2009).
A. *Emblica officinalis Gaertn* (Amla); **B.** *Asparagus racemouse* Willd (Shatawar), *Withanta somnifera* Dunal (Aswagandha), Terminalia *chebula* (Harar), *Saraca asoca* (Ashoka); **C.** *Aegle marmelos* (Bel), *Cassia angustifolia Vahl* (Sonapaatri/Sana), *Adhatoda vasica* (Adusa/Arusa); **D.** Piper longum (Pippali), *Bacopa monnieri* (Brahmi), *Sida cordifolta* (Kanghi), *Ocimum sanctum* (Tulsi); **E.** *Bambusa bambos Druce*. (Vansalochan), *Boerhaavia diffusa* (Punarnava), *Azadirachta indica A. Juss*. (Neem), *Solanum nigrum* (Mokoya), *Woodfordia fruticosa Kurz* (Dhataki), *Andrographis paniculata* (Kalmegh), *Syzygiumaromaticum* (Ling/lavang), *Tinospora cordfolia* (Giloe, Guduchi); **F.** Others



1.5. Aims and Objectives

1.5.1. Scope of Work

Herbal medicinal products are complex mixtures, which originate from biological sources, great efforts are necessary to guarantee a constant and adequate quality. By carefully selecting the plant material and a standardized manufacturing process, the pattern and concentration of constituents should be kept as constant as possible, as this is a prerequisite for reproducible therapeutic results. The therapeutic properties of several medicinal plants have been reported in Ayurveda. However, information on standardization, validation, or stability is wanting. Therefore studies were undertaken to correlates the biological activity of therapeutic significance (e.g. antioxidant and anti-inflammatory activities) with chemical constituents and physical characteristics (e.g. voltammogram) for standardizing and validating the herbal products. In the present thesis these parameters were evaluated using Ashwagandha as a representative.

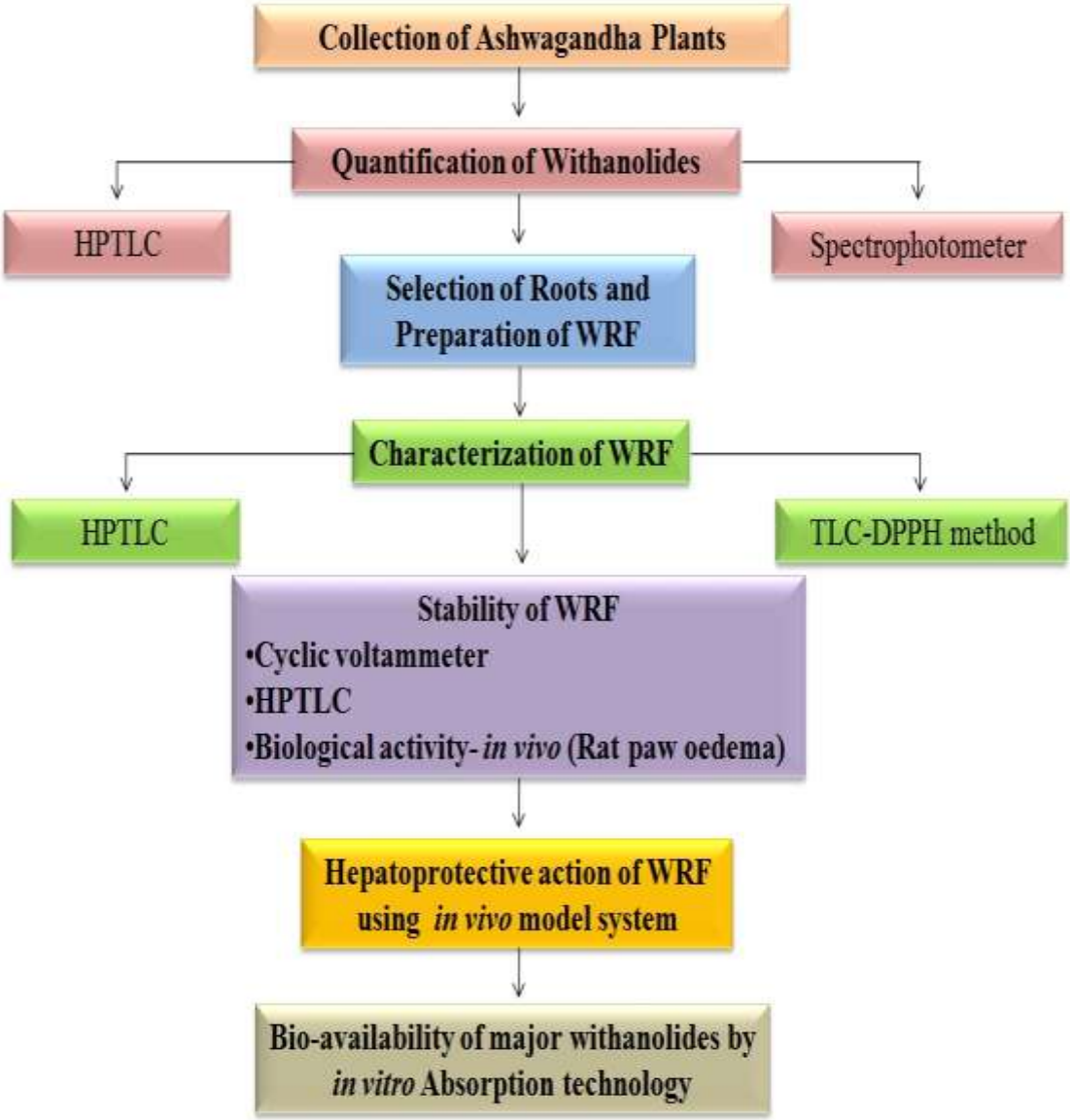
1.5.2. Aims and Objectives

- To study the effect of macro and micronutrients on the accumulation of withanolides content
- To select the plant material for preparation of Withanolide rich fraction (WRF)
- To quantify Withanolides in WRF by HPTLC and spectrophotometeric methods
- To evaluate the Antioxidant potential of individual components by TLC-DPPH method
- To evaluate the stability of individual component of WRF
- To evaluate the anti-inflammatory action using *in vivo* model system
- To evaluate the bio-availability of major withanolides using MDCK cell culture system

1.5.3. Plan of Work for Attaining the Objectives

- ✓ Collection of nitrogen, phosphorus, potassium and calcium deficient as well as control plants from the field (NAIP-ICAR Component 3 Project)
- ✓ Extracts of roots to be used to analyze nutrient content, withanolides content and free radical scavenging activity
- ✓ Selection of plant material for preparation of Withanolide Rich Fraction
- ✓ Optimization of withanolide rich fraction (WRF) according to the method of Chaurasiya et al. (2008) with some modifications from selected plant materials
- ✓ Estimation of antioxidant potential of individual components present in WRF by TLC-DPPH method
- ✓ Estimation of stability, shelf-life and biological activity of WRF components
- ✓ Estimation of Hepato-protective effects of WRF in Acetaminophen intoxicated rats
- ✓ Evaluation of bioavailability of major withanolides by *in vitro* absorption model

Fig.1.14. Schematic Representation for Plan of Work



Chapter 2

Influence of Macronutrient Deficiencies on Molybdenum Content in Ashwagandha Roots and its Correlation with Withanolide Content

2.1. Introduction

For complete growth of medicinal plants, it requires 17 elements: carbon, hydrogen, oxygen, nitrogen, phosphorus, potassium, calcium, magnesium, sulphur, iron, manganese, zinc, copper, boron, molybdenum, chlorine and nickel (HamLin, 2006). If nutrients are not available during growth phase of the plants, deficiency symptoms will occur and quality of product will deteriorate. Essential elements used by plants in relatively large amounts for growth are termed as macronutrients. The major macronutrients are nitrogen (N), phosphorous (P), potassium (K) and Calcium (Ca). Molybdenum (Mo) is a micronutrient that governs biosynthesis and transport of secondary metabolites (HamLin, 2006). All these macronutrients (N, P, K, and Ca) deficiencies may influence micronutrient uptake including Mo (a micronutrient). Micronutrients are important for promoting plant growth and influence biosynthesis of secondary metabolites (Brady and Weil, 1999).

Secondary metabolites differ with location, cultivation practices, soil nutrients, tissue type and varieties of Ashwagandha (Kumar et al., 2007; Kubsad et al., 2009; Nagella and Murthy, 2012). It is known that the medicinal properties of Ashwagandha are attributed to withanolides. Ashwagandha roots are used in several medicinal formulations in the form of decoctions, infusions, ointments, powder and syrups. It may be expected that variations in macro- and micronutrients can influence the growth of plants and also biosynthesis/ transport of withanolides (Suryapujari et al., 2010). Such, variations leads to inconsistent product formulations resulting in unreliable therapeutic and health promoting effects (Patwardhan et al., 2009). Hence the objective of the present study was to examine how the contents of withanolides are influenced under macronutrient deficiency conditions.

2.2. Material and Methods

2.2.1. Chemicals

Ferric chloride hexahydrate, orthophosphoric acid, sulphuric acid, methanol, chloroform, nitric acid, hydrochloric acid, 2, 2-diphenyl-1-picryl-hydrazyl, dichloromethane, toluene, acetone, diethyl ether, cholesterol were procured from local supplier.

The standard withanoside V (WS V), withaferine A (WF A), 1, 2 deoxywithastramonolide (1, 2 DWM), withanone (WNN), withanolide A (WN A) and withanolide B (WN B) were purchased from Natural Remedies Pvt. Ltd. Bangalore India. Pre-coated silica gel plates [0.2mm thickness, $60F_{254}$ (20 cm X 20 cm)] were obtained from Merck Ltd. Mumbai, India.

2.2.2. Plant Materials

Ashwagandha var. JS-134 was cultivated under Indian Council of Agricultural Research- National Agriculture Innovation Project III (ICAR-NAIP III) in Vidarbha region of Maharashtra, India (**Fig 2.1**). Based on the macronutrient deficiency symptoms nitrogen deficient (-N), phosphorus deficient (-P), potassium deficient (-K), calcium deficient (-Ca), as well as healthy (control) Ashwagandha fresh plants were identified and collected in the month of November and December 2011 from different areas of Vidarbha (Suryapujari et al., 2010). In each group 15 plant sample were used for investigation.

Fig. 2.1: Cultivation of Ashwagandha var. JS-134 under National Agriculture Innovation Project - Sustainable Rural Livelihood Security in Backward Districts of Maharashtra (NAIP-III).

2.2.3. Determination of Nitrogen, Phosphorus, Potassium and Calcium

Estimate of nitrogen (N), phosphorus (P), potassium (K) and calcium (Ca) were carried out as per the method described by Suryapujari et al. (2010).

2.2.4. Determination Molybdenum (Mo)

1 g root samples were subjected to acid digestion with 5 mL of concentrated HNO_3. To the digested sample 2 mL concentrated HCl was added and the samples were diluted to 25 mL with deionized water.

Stock solution 1 g Mo was dissolved in minimum amount of deionized water and $HN0_3$, and 8 mL HCl was added and was diluted with deionized water to 1L. Working Mo standards 25, 50 and 100 ppm were prepared by diluting the stock solution. Determination of Mo content was carried out by Atomic Absorbance Spectroscopy (AAS) (Hanlon, 1998).

The amount of Mo was estimated by formula:

$$\text{Mo (ppm)} = \frac{\text{(absorbance of sample x dilution factor)}}{\text{Sample weight}}$$

2.2.5. Preparation of Root Extracts for Determination of Withanolides Content

As per the method described by Chaurasiya et al. (2008) the fresh root (5.0 g) were finely powdered in liquid nitrogen and extracted overnight in 20 mL of methanol–water (25:75, v/v) at room temperature (25°C) on a shaker and filtered. The filtrate was further extracted with n-hexane (3 × 60 mL). The n-hexane fraction was discarded and the methanol–water fraction was further extracted with 3 times 60 mL chloroform. The chloroform fractions were pooled and concentrated to a dry powder. The residue was weighted and redissolved in chloroform to give 20 mg/mL samples.

2.2.6. Preparation of Standard Withanolide Solutions

Stock solutions (1 mg/mL) were prepared in chloroform. 4–24 µL of working standards (50 ng/mL made in chloroform) were applied to HPTLC plate to obtain six point calibration curves. The chloroform extracts of the roots were subjected to HPTLC analysis for the quantification of the individual withanolides.

2.2.7. Preparation of Colour Reagent for Total Withanolide Determination

8 mL of stock solution (21.5 g ferric chloride hexahydrate dissolved in 100 mL orthophosphoric acid) was diluted to 100 mL with conc. sulphuric acid.

2.2.8. Determination of Total Withanolides (TW) Content

A modified spectrometric method was used to determine total withanolides content in roots. For this purpose 1 mL chloroform extract was used for the development of color by adding 2 mL of glacial acetic acid and 21 mL of color reagent (Mishra, 1994). After keeping for 5 min in an ice bath, the optical density was recorded in a spectrophotometer at 540 nm. The concentration of withanolides was calculated using cholesterol as standard.

2.2.9. Quantification of Major Withanolides (MW)

Quantification was carried out by HPTLC chromatography using dichloromethane: toluene: methanol: acetone: diethyl ether (7.5:7.5:3:1:1 v/v) as the mobile phase. The chromatogram was run for 80 mm. Quantitative evaluation of the plate was performed in the absorption reflection mode at 235 nm, tungsten (W) and deuterium (D2) lamp, using slit width: 4.00 x 0.30 mm, data resolution 100 µm/step, scanning speed: 20 mm/s, and base line correction was used (Sharma et al., 2007)

2.2.10. Estimation of Free Radical Scavenging Activity by 2, 2-diphenyl-1-picryl-hydrazil (DPPH) Method

Free radical scavenging activity of Ashwagandha extract was measured by 2, 2-diphenyl-1-picryl-hydrazil (DPPH) method with some modification (Shimada et al., 1992).

1 mL of 0.1 mM solution of DPPH in methanol was added to 0.5 mL of 100 µg extract of all groups. After 30 min absorbance was measured at 517 nm. The percentage of DPPH inhibition was calculated by using equation:

$$\text{Inhibition (\%)} = [(A_0-A_1)/ A_0] \times 100$$

Where,

A_0= Absorbance of the blank

A_1=Absorbance of 100 µg of chloroform root extract

2.2.11. Statistical analysis

The results are expressed as mean of ± standard deviations. The data were analyzed by standard computer program: Excel (2003) and statistical analysis was carried out by one-way ANOVA test.

2.3. Results

2.3.1. Symptoms of Nitrogen Deficiency

Leaves get yellow and detached earlier. Yellowing starts from tip of leaf to lower portion. Before shedding, young leaves curved and flower and fruit formation delays by 17 days. Nitrogen deficiency leads to reduced shoot and root growth. Plants had dull appearance and excess deficiency led to complete defoliation and death of plant prematurely (**Fig. 2.2**). Average nitrogen content was reduced 49.8% in deficient sample when compared with healthy sample (**Table 2.1**).

2.3.2. Symptoms of Phosphorous Deficiency

Leaves became yellow and turned into brown color from centre to other part of leaf. Defoliation of old leaves was noticed earlier than new leaves. Leaves became curved and misshapen. Leaves started

coiling inside due to phosphorous deficiency (**Fig. 2.2**). Phosphorous content was reduced by 88.0% in deficient sample when compared with healthy sample (**Table 2.1**).

2.3.3. Symptoms of Potassium Deficiency

Brown spots were observed on potassium deficient leaves. Burning of leaves at tip was observed and plants started drying from shoot to root. Leaves showed brown and yellow patches (**Fig. 2.2**). Potassium content was reduced by 88.7% in deficient samples when compared with healthy samples (**Table 2.1**).

2.3.4. Symptoms of Calcium Deficiency

Deficiency of calcium showed rotting of tip of leaf, roots and shoots. Seeds were malformed and cracking of capsules was observed. In case of excess deficiency, complete drying of plant was observed and seeds remained immature (**Fig.2.2**). Calcium content was reduced by 38% in deficient samples when compared with healthy sample (**Table 2.1**).

Fig. 2.2: Macronutrient deficiency symptoms in Ashwagandha plant; healthy (control), nitrogen deficient (-N), phosphorus deficient (-P), potassium deficient (-K) and calcium deficient (-Ca) plant.

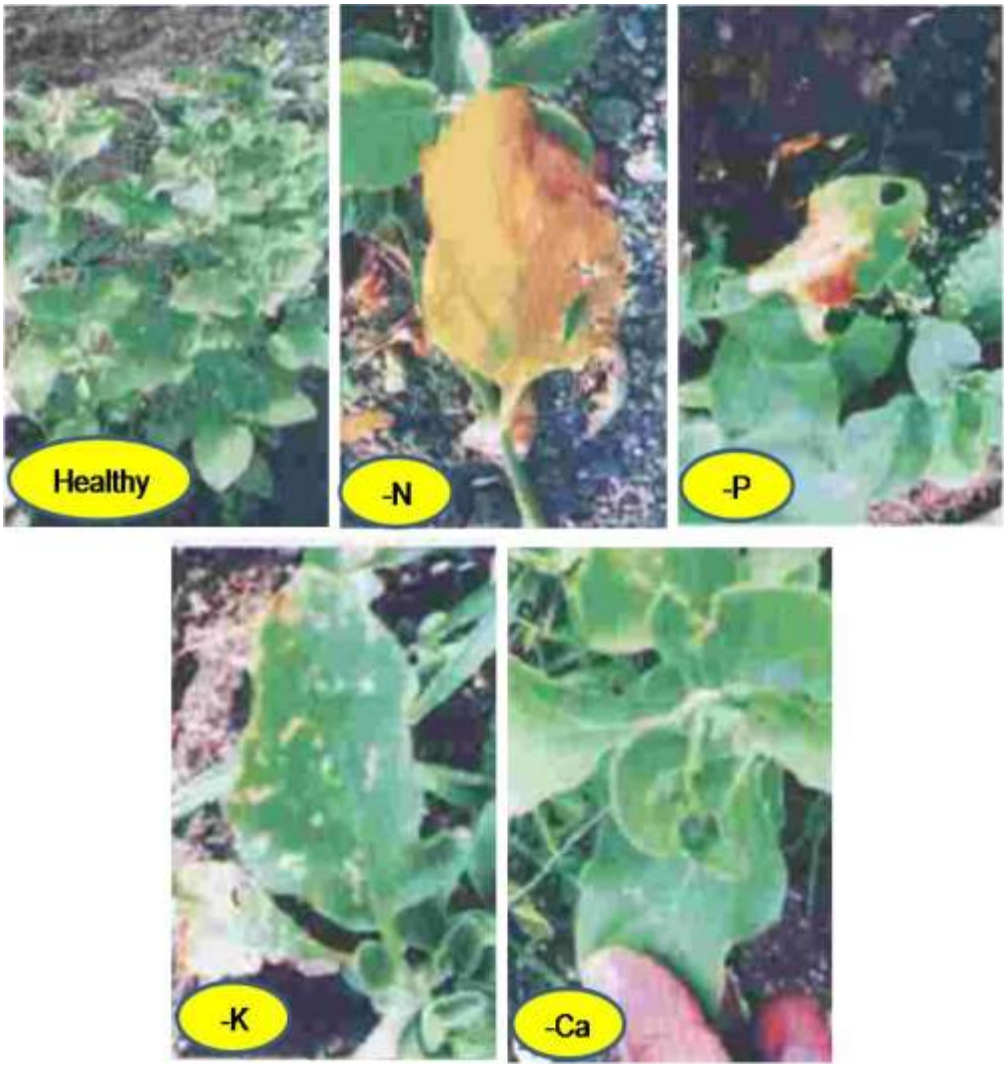


Table 2.1: Concentration of nitrogen, phosphorus, potassium and calcium in the roots of deficient plant as compare to healthy plants. Each value is mean of fifteen independent samples.

	Concentration of N		**Concentration of P**		**Concentration of K**		**Concentration of Ca**	
	Control	**-N**	**Control**	**-P**	**Control**	**-K**	**Control**	**-Ca**
% Content	1.44	0.94	1.10	0.22	1.02	0.15	1.30	0.92
% Reduction	49.8		88.0		88.74		38.0	

healthy (control), nitrogen deficient (-N), phosphorus deficient (-P), potassium deficient (-K) and calcium deficient (-Ca)

2.3.5. Major Withanolide (MW) by HPTLC

A clear-cut separation of major withanolides was achieved by HPTLC shown in **Fig. 2.3A. Fig. 2.3B** shows concentration-dependant HPTLC pattern of standard withanolides as well as the withanolides from the root extract of the experimental samples. On the basis of *Rf* values of 0.7, 0.58, 0.61, 0.68, and 0.79 for WS V, WF A, 1, 2 DWM, WN A and WN B, respectively, individual withanolides were identified. The concentrations of major withanolides are given in the **Table 2.2.**

It can be noted that deficiency of phosphorus, potassium and calcium had only marginal effect on the content of all withanolides. However, deficiency of nitrogen had significant deleterious effect. Thus content of WS V was totally abolished while there was an overall 35-36% reduction in the MW (**Table 2.2).**

2.3.6. Total withanolides (TW) Content by Spectrophotometric Analysis

TW content were reduced by 10.38%, 11.81%, 15.03% and 37.21% in Ca, K, P and N deficiency plants as compare to healthy plant (**Table 2.2**).

2.3.7. Molybdenum Content

It can be noted that nitrogen deficient plant had the lowest Mo content (7.02 ± 2.1 ppm) and potassium deficient plants had highest Mo content (33.5 ± 3.3 ppm) comparable to that of control (33.9 ± 1.6 ppm). Phosphorus deficient (13.1 ± 1.6 ppm) and Calcium deficient (17.1 ± 0.9 ppm) plants had intermediate values **(Table 2.2**).

Deficiency of Ca and P lowered the Mo content by 50 and 60% respectively while deficiency of N caused a significant 80% reduction. It is of interest to note here that despite 50 and 60% reduction in Mo content in Ca and P deficiency there was no significant decrease in the individual and TW contents **(Table 2.2**). However, when concentration of Mo was 7 ppm in nitrogen deficient

plants, the levels were affected significantly as pointed out above. Hence it maybe suggested that Mo levels of up to 13 ppm or so may be an absolute requirement for maintaining normal withanolide concentration; levels below this lead to adverse effect (**Table 2.2**).

With a view to elaborating the dependence of withanolide contents on Mo, the concentration of individual withanolides was plotted against Mo concentration from different 15 independent observations for control and nitrogen deficient groups. These plots are shown in **Fig. 2.4**. It is clear that for the control group a good correlation between withanolide content and Mo concentration was obtained; regression (R^2) values ranged from 0.76 to 0.99 (**Fig. 2.4A**). Even for nitrogen deficient group R^2 values ranged from 0.63 to 0.99 implying good correlationship (**Fig. 2.4B).**

2.3.8. Free Radical Scavenging Activity

Free radical scavenging activity of potassium deficient plant was highest (32.9 ± 1.7), whereas that for nitrogen deficient plants was lowest (24.7 ± 2.2) as compared to control plant (35.3 ± 1.1). Calcium deficient (31.7 ± 1.5) and Phosphorus deficient (30.5 ± 2.1) plants had intermediate values **Table 2.2.** The decrease in antioxidant activity seems to correlate with the reduction in TW and MW content in various deficiency conditions.

Fig. 2.3: (**A**) HPTLC chromatogram at 235 nm of major withanolides of Ashwagandha. (**B**) Image of HPTLC plate of Ashwagandha at 235 nm. 1-6 tracks for standards mixture; 9, 16 (Control); 8, 15 (-Ca); 7, 14 (-K); 11, 13 (-P), and 10, 12 (-N).

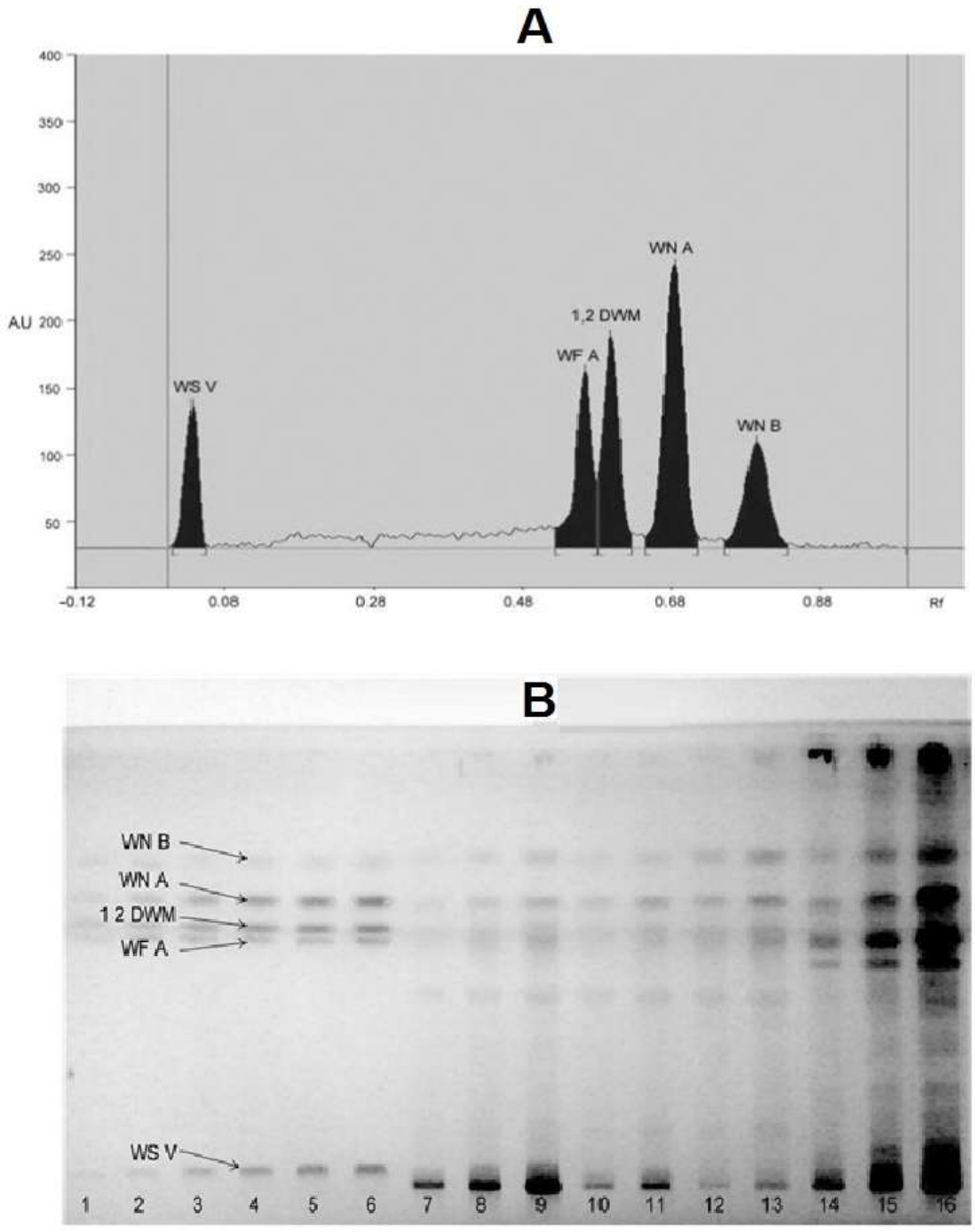
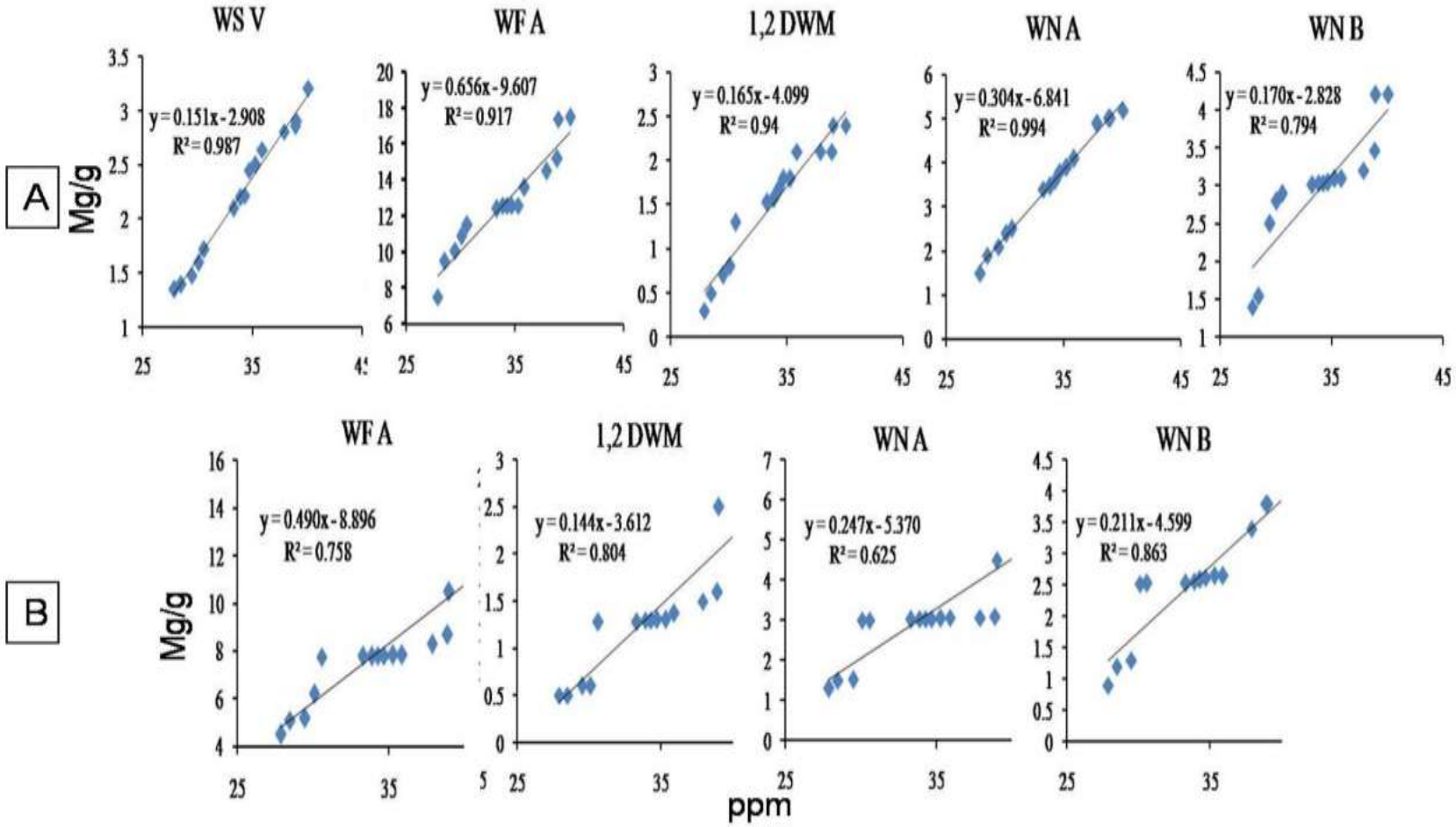


Table 2.2: Effect of macronutrient deficiency on withanoside V (WS V), withaferine A (WF A), 1, 2 deoxywithastramonolide (1, 2 DWM), withanolide A (WN A), withanolide B (WN B), major withanolides (MW), total withanolides (TW), molybdenum (Mo) content and antioxidant activity of healthy (control), calcium deficient (-Ca), potassium deficient (-K), phosphorus deficient (-P) and nitrogen deficient (-N) Ashwagandha plant. Each value is mean ± standard deviation (SD) of fifteen independent samples.

Sample	**Control**	**-Ca**	**-K**	**-P**	**-N**
WS V (mg/g)	1.97 ± 0.6	1.54 ± 0.7	1.14 ± 0.8	1.54 ± 0.8	0.0 ± 0.0
WF A (mg/g)	12.7 ± 2.7	10.25 ± 3.0	12.57 ± 3.4	9.25 ± 2.2	7.79 ± 2.2
1,2 DWA (mg/g)	1.52 ± 0.6	0.52 ± 0.3	0.76 ± 0.2	0.46 ± 0.8	1.31 ± 0.6
WN A (mg/g)	3.52 ± 1.2	4.72 ± 1.7	3.68 ± 1.7	4.31 ± 0.8	3.05 ± 1.2
WN B (mg/g)	2.97 ± 0.7	3.41 ± 0.7	2.57 ± 1.1	4.13 ± 1.4	2.59 ± 0.9
MW (mg/g)	22.9 ± 2.2	20.4 ± 3.8	20.7 ± 4.9	19.6 ± 3.1	14.7 ± 3.4
TW (mg/g)	55.9 ± 1.7	50.1 ± 1.1	49.3 ± 1.7	47.5 ± 4.7	35.1 ± 2.7
Mo (ppm)	33.9 ± 1.6	17.1 ± 0.9	33.5 ± 3.3	13.1 ± 1.6	7.02 ± 2.1
Reduction in MW (%)	--	11.08	9.68	14.17	35.75
Reduction in TW (%)	--	10.38	11.81	15.03	37.21
Free radical scavenging activity at 100µg (%)	35.3 ± 1.1	31.7 ± 1.5	32.9 ± 1.7	30.5 ± 2.1	24.7 ± 2.2

Fig.2.4: Graph of regression (R^2) analysis of WS V, WF A, 1, 2 DWM, WN A and WN B with Mo content in the roots of control (A) and in –N plants (B).Each value is mean of fifteen independent samples



2.4. Discussion

N, P, K and Ca are essential macronutrients for normal growth of the plants. It is reported that in healthy plants the average concentration of N, P, K and Ca were 15000 ppm, 10000 ppm, 10000 ppm and 5000 ppm respectively; while average concentration of Mo concentration was 50 ppm (Brady and Weil, 1999). The results of the present studies indicate that the macronutrient deficiency was observed in collected plant materials and deficiency symptoms can be characterized physical appearance in field. 45 kg N/ha and 26 kg P/ha have been reported to be optimum for normal growth of Ashwagandha (Nasira et al., 2012). Secondary metabolites differ with location, cultivation practices, soil nutrients, tissue type and varieties of Ashwagandha. Similar results were noted in the present studies. Root length and root diameter are important selection criteria for economic yield (Das et al., 2011). The medicinal properties of Ashwagandha are attributed to the withanolides content in the roots which are used in several medicinal formulations (Ali and Shuaib, 1997). Thus, critical concentration of macronutrients (N, P, K and Ca) and also possibly of Mo are important for the proper growth as well as withanolides content for commercial and therapeutic applications.

It is clear from the data presented (**Table 2.2)** that deficiency of Mo had dramatic effect on the withanolide contents; deficiency of other macronutrients had a marginal effect. The regression analysis studies showed that there was a positive correlation between withanolide contents and Mo content in the roots in control as well as N deficiency conditions **(Fig. 2.4).** Molybdenum functions as cofactor of many important plant enzymes involved in redox processes: nitrate reductase, xanthine dehydrogenase, aIdehyde oxidase, and probably sulfite oxidase (Zimmer and Mendel 1999). There is no known transporter for Mo. It is believed that Mo is transported by sulphate (SO_4^{2-}) transporter. This assumption is

based on the fact that Mo has structural similarity to SO_4^{2-}. Transporter of SO_4^{2-} is an energy-dependent process (Ferrari andRenosto, 1972). In other words it is a process of active transport. Therefore one would presume that Mo transport is also an energy-dependent active transport process. This would mean that the plants will accrue Mo by an energy-dependent process; concentration-dependent passive transport is not involved. In other words the plants will acquire the requisite quantity of Mo irrespective of its content in the soil. In this context it is of interest to note that the concentration of Mo in soil shows wide range of variation of 0.5 to 5 ppm; a tenfold variation (Sharma and Chatterjee, 1997).

It has been shown that uptake of Mo is interlinked with N metabolism; especially role of nitrate reductase has been emphasized (Axler et al., 1980; HamLin, 2006). Results of present studies are consistent with the above observation. The results would imply that only a severe deficiency of N would impair the withanolides content of the roots together with Mo content. The content of withanolides in roots is the most important criterion for therapeutic use. Hence it may be suggested that to the soils deficient in N manures rich in N e.g. urea, be added.

Thus it is important to maintain normal levels of these macronutrients N, P, K and Ca and especially of N for not only getting the good yield of the roots but also for getting good amounts of withanolide in the roots. It is also evident from the present work that N metabolism dependent Mo uptake influences the WF A accumulation, which is a major pharmaceutically important withanolide.

2.5. Conclusions

Regression analysis of the data emphasizes the importance of Mo in withanolide synthesis which is crucial for pharmacological properties. Micronutrient in the soil, where the herb is grown does influence the quality and quantity of the herbal product. Thus, these results bring out the importance of quality control of the herbal product at the level of plantation of the herb. For further studies plants with adequate quantities of macronutrients in the soil (control Group) were selected to prepare concentrated withanolide fraction **which is described in the next chapter (Chapter 3).**

Chapter 3

Characterization of WRF by using HPTLC and Thin Layer Chromatography-2, 2-Diphenyl-1-picrylhdrazyl (TLC-DPPH) Method

3.1. Introduction

In previous chapter (Chapter 2) it has been shown that the levels of withanolides in roots vary with nutrients in the soil and can affect the quality of herbal product. The study emphasized the importance of quality controls of the herbal product at the level of plantation of the herb. It is known that the pharmacological properties of Ashwagandha are mainly attributed to its withanolides. The multicomponent extracts shows better medicinal efficiency than purified components, i.e., the strength of herbal medicine increased with the mixture of secondary metabolites instead of a single component (Mishra et al., 2000; Patwardhan et al., 2009). Keeping this in mind a withanolide rich fraction was prepared from the roots of Ashwagandha. With a view to characterization of fraction the antioxidant potential and free radical scavenging activity of individual components contained in a complex mixture were evaluated.

Characterization of fraction was carried out by using thin layer chromatography-2, 2-diphenyl-1-picrylhdrazyl (TLC-DPPH) assay. Higher resolution with more number of separated compounds on TLC plate is desirable for TLC-DPPH assay, therefore it is important to get higher resolution with more bands on a single TLC plate. With this view in mind a new method was developed for simultaneous separation of major withanolides. Several workers have attempted quantitative measurement of free radical scavenging potential of individual components in complex mixture for plant extracts by TLC-DPPH method (Ciesla et al., 2011a, 2011b; 2012; Olech et al., 2012; Pozharitskaya et al., 2008). However, analysis of Ashwagandha root fraction by TLC-DPPH method has not been attempted so far. The present study reports for the first time improved TLC-DPPH method for screening biologically active compounds in Ashwagandha root fraction.

3.2. Materials and Methods

3.2.1. Chemicals

As described in section 2.2.1 (Chapter 2).

3.2.2. Plant Materials

Selected Healthy fresh roots of Ashwagandha (as detailed in chapter 2 of the thesis) were used to prepare Withanolide Rich Fraction (WRF).

3.2.3. Preparation of Root Extracts for Determination of Withanolide Content

3.2.3.1. Extraction by Chaurasiya et al. (2008)

As described in section 2.2.5. (Chapter 2)

3.2.3.2. Isolation of WRF by Chaurasiya et al. (2008) With Some Modification

The roots were dried in shade and pulverized to coarse powder, which was then subjected to Soxhlet extraction using methanol–water (25:75, v/v) as the solvent. The extract was concentrated under vacuum and then dried in vacuum desiccator. Part of the dry extract (0.5 g) was hydrolyzed for 1 h in hot water bath with 20 mL of 50% (v/v) methanol containing 1.2 M HCl. After hydrolysis, the solution was filtered through Whatman filter paper No. 1. To the hydrolyzed sample 20 mL of freshly prepared chloroform: methanol (3:1, v/v) mixture was added. The mixture was shaken vigorously and allowed to stand for 15 min to separate out the chloroform phase. The chloroform layer was carefully removed and evaporated to dryness under vacuum. The residue was weighted and redissolved in chloroform to obtain stock hydrolysate containing the withanolide rich fraction (20 mg/mL).

3.2.4. Preparation of Standard Solutions

Stock withanolide solutions were prepared in chloroform. Aliquots 4, 8, 12, 16, 20 and 24 µL of working standards (50 µg/mL) applied to the HPTLC plates for preparing six point calibration curves. The extracts were also subjected to HPTLC for separation and quantification of withanolides.

3.2.5. Quantification of Withanolides by High Performance Thin Layer Chromatography (HPTLC)

The samples and standards were applied to the HPTLC plate as 6 mm wide bands with constant application rate of 150 nL/s, using an automatic TLC sampler (LINOMAT V) under a flow of N_2. The linear ascending development was carried out in a CAMAG twin trough chamber (10 x 10 cm). The HPTLC chamber was pre-saturated with mobile phase: dichloromethane: toluene: methanol: acetone: diethyl ether (6.5:7:4:1.5:1 v/v) for 20 min, and for the linear ascending development of the chromatogram was allowed to run up to 90 mm. Quantitative evaluation of the plate was performed in the absorption reflection mode at 235 nm, with slit width set at 4.00 X 0.30 mm, data resolution 100 µm/step, scanning speed: 20 mm/s, and base line correction was applied.

The experiments were performed in triplicate and limit of detection (LOD), limit of quantification (LOQ), accuracy and precision were calculated based on these results. The software winCATS and ImageJ provides the values of area under the curve in AU for HPTLC as well as TLC-DPPH plates (details given below).

3.2.6. DPPH Test

After separation and quantification of compounds, the TLC plate was immersed for 5 seconds in freshly prepared 0.2% (w/v) DPPH solution prepared in hexane: acetone (9.5:0.5 v/v) and excess DPPH was allowed to drip, after which the plate was kept in the dark for 30 min and then scanned using a flat-bed scanner class MF4320d

(CANON IMAGE), for screening free radical scavenging activity of the compounds. Concentration-dependent calibration curves for DPPH positive reaction for individual withanolides were also performed in triplicate.

3.2.7. Image Processing by Means of the ImageJ Program

The results of TLC–DPPH test were documented by flat-bed scanning, saved in the form of jpg documents and further processed by means of an open source and free program ImageJ, developed at the National Institute of Health in the USA (National Institute of Health, 2012). In case of DPPH staining, the results change with time. Therefore it is of crucial importance to precisely define the time that elapses between immersion and documentation. The results were documented every 5 min and after comparison it was decided to process images taken 30 min after staining. Subsequently the images chosen were processed by means of the ImageJ program, with the use of a modified procedure described by Olechet et al. (2012). First, colour images were turned into 8-bit type images (Image/Type/8-bit). Subsequently the images obtained were denoised by applying the following steps: Process/Filters/Median/Radius – 5 pixels. Then the baseline drift was removed (Process/FFT/Band pass Filter/Filter large structures down to – 40 pixels; filter small structures up to – 0 pixels).The images processed in the aforementioned way were then inverted (Edit/Invert). In order to change the video scan images into chromatograms, resembling those obtained in high-performance liquid chromatography (HPLC), a rectangular selection tool was used to outline the tracks. The line profile plots were obtained in the same way as described in the original procedure (Olech et al., 2012). The areas under the individual peaks were measured and compared with the corresponding area obtained for withanolides and components of WRF with a recognized free radical scavenging potential.

3.2.8. Limits of Detection and Quantification

In order to estimate the limit of detection (LOD) and limit of quantification (LOQ) of both HPTLC and TLC-DPPH assay, chloroform (blank) was spotted six times using the same method as explained by Waksmundzka and Wawrzynowicz, (2002); signal: noise ratio was determined as 3:1 and 10:1 for LOD and LOQ, respectively.

3.2.9. Validation of the Method

3.2.9.1. Linearity

Working standard solutions of WS V, WF A, 1, 2 DWM, WNN, WN A and WN B in chloroform as mentioned above (4, 8, 12, 16, 20 and 24 µL) were applied to TLC plate using an automatic TLC sampler (Linomat V) for preparing six points calibration curves. The regression equation and correlation coefficient were calculated from the calibration curves. Linearity was observed in the range 200-1200 ng for all the standards.

3.2.9.2. Selectivity

Each withanolide was separated with the baseline return.

3.2.9.3. Accuracy

To the known concentration of pre-analyzed sample, 200 ng of WS V, WF A, 1, 2 DWM, WNN, WN A and WN B were added and the recovery examined. The experiments were conducted in triplicate to examine recovery and accuracy of the method.

3.2.9.4. Precision

In order to determine precision, 400 ng standard mixture was applied in triplicate to different tracks and analyzed to determine variation arising from the method and Standard deviation (SD) was calculated followed by calculation of relative standard deviation in percentage (% RSD) by using the formula;

% RSD = 100SD/ x

Where,

'x' is the average value of standards applied on HPTLC plate.

3.2.10. Statistical analysis

Results are expressed as mean ± standard deviations. The data were analyzed by standard computer program: Excel (2003) and statistical analysis was carried out by one-way ANOVA test.

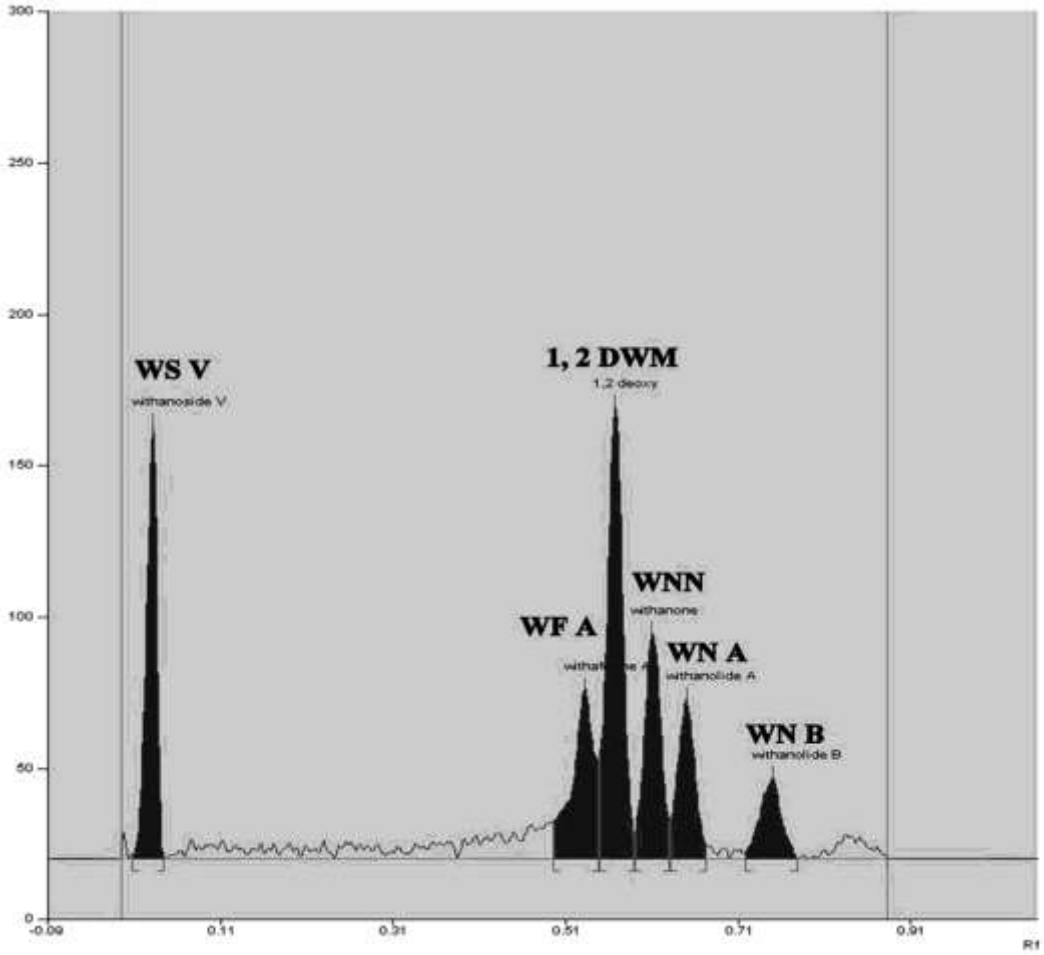
3.3. Results

3.3.1. Yields of Major Withanolides in Extracts

Various compositions of mobile phases were tested to get clear-cut resolution of WS V, WF A, 1, 2 DWM, WNN, WN A and WN B, with symmetrical and reproducible peaks **(Fig. 3.1).** Best separation was achieved by using mobile phase consisting of dichloromethane: toluene: methanol: acetone: diethyl ether (6.5:7:4:1.5:1 v/v). The method described here gives clear-cut separation and easy identification and quantification of all six major withanolides. The symmetrical, well-formed peaks obtained at 235 nm after separation for the standards mixture enables easy identification and quantitative analysis (**Fig. 3.1**).

Peaks corresponding to WS V, WF A, 1, 2 DWM, WNN, WN A and WN B, were recorded at *Rf* 0.07, 0.54, 0.61, 0.63, 0.68 and 0.79, respectively. The calibration curves were linear in the range from 200-1200 ng for all the above-mentioned withanolides. In linearity study, standard deviation (SD) for all standards was less than 3% and regression (r) value was close to 1. According to Chaurasiya et al. (2008) extraction method the yield of WS V-1.9 mg/g, WF A- 12.7 mg/g, 1, 2 DWM-1.52 mg/g, WNN- ND, WN A-3.52 mg/g and WN B-2.97 mg/g were observed.

Fig. 3.1: HPTLC chromatogram at 235 nm of standard withanolides of Ashwagandha.



Whereas in the yield of WS V-0.86 mg/g, WF A- 12.9 mg/g, 1, 2 DWM-1.92 mg/g, WNN- 1.52 mg/g, WN A-5.24 mg/g and WN B-4.52 mg/g were observed in modified Chaurasiya et al. (2008) extract. Thus, by the proposed modified method we obtained quantitatively higher yields of WNN, WN A and WN B. **This fraction is designated as Withanolides Rich Fraction (WRF)**. The concentration of WF A is much higher than other withanolides; 1, 2 DWM and WNN are present in very low amount in both root extracts (**Table 3.1**).

3.3.2. Antioxidant Potential of Components of WRF by TLC-DPPH Method

TLC-DPPH method has been developed for the quantification of withanolides and simultaneously estimates antioxidant potential of each constituents present in a complex mixture. A TLC–DPPH rapid test was used to assess the free radical scavenging activity of constituents in WRF for the first time.

The LOD and LOQ of withanolides on TLC and TLC-DPPH, average recovery and relative standard deviation, i.e., % RSD for HPTLC are summarized in **Table 3.2**. The recovery is more than 97% and %RSD was less than 2% for all studied markers. *Rf* values of withanolides and unknown compounds are summarized in **Table 3.3**. The WRF contains six bioactive withanolides and eleven unknown compounds (**Table 3.3**; **Fig. 3.2A**). Concentration of WF A is much higher than of other withanolides which is followed by WN A and WN B. WS V, 1, 2 DWM and WNN are present in low amount **Table 3.3. Fig. 3.2B** shows the photo camera image of HPTLC-DPPH plate. The densitometer traces of antioxidant peaks visualized after DPPH treatment (**Fig. 3.2C)**. In withanolides, WS V and WN B show highest antioxidant potential. In unknown compounds peak no. 17 at R*f* 0.92 has highest antioxidant potential and peak no. 16 at *Rf* 0.87 has recorded lowest antioxidant potential. It is also found that

some of the unidentified components had significant antioxidant potential (**Table 3.3**).

Table 3.1: The regression coefficient equation from calibration curves as well as concentrations of major withanolides in old extract and in new extract. Values are given as averages of three independent experiments

Compound	*Rf*	Regression via area	r	% sdv (SD)	Concentration (mg/g)	
					Chaurasiya et al; 2008	Modified Chaurasiya et al; 2008
WS V	0.07	Y = 29.17 + 1.226*X	0.98	2.5	1.9	0.86
WF A	0.54	Y = 90.31 + 1.261*X	0.99	1.5	12.7	12.9
1,2 DWM	0.61	Y = 92.40 + 1.912*X	0.99	2.3	1.52	1.92
WNN	0.63	Y = 52.40 + 1.209*X	0.98	2.4	---	1.52
WN A	0.68	Y = 139.0 + 3.693*X	0.99	1.9	3.52	5.24
WN B	0.79	Y = 70.84 + 2.646*X	0.99	1.7	2.97	4.52

Table 3.2: LOD, LOQ, accuracy, and precision of the method. Values are given as averages of three independent experiments.

Compound	**LOD (ng)**		**LOQ (ng)**		**Accuracy of HPTLC analysis**		**System precision (%) RSD for 400 ng(HPTLC)**
	HPTLC	TLC-DPPH	HPTLC	TLC-DPPH	% Average recovery (HPTLC)	Recovery amount per 200 ng of standard (HPTLC)	
WE V	60	80	250	300	97	196	1.8
WF A	120	120	350	350	98	196	1.16
1,2 DWM	80	160	300	400	99	199	1.14
WNN	80	150	300	400	98	197	1.75
WN A	60	130	250	350	98	196	1.75
WN B	80	120	300	350	99	198	1.6

Fig. 2: (A) HPTLC chromatogram of constituents of Ashwagandha root extract at 235 nm. The ordinate represents AU units and abscissa represents *Rf* values; (B) Photocopy of HPTLC plate after treatment with DPPH solution; (C) Densitometer tracess of antioxidant peaks visualized after DPPH treatment. The peaks were visualized using ImageJ program software as detailed in the text. The ordinate represents arbitrary units and abscissa represents *Rf* values.

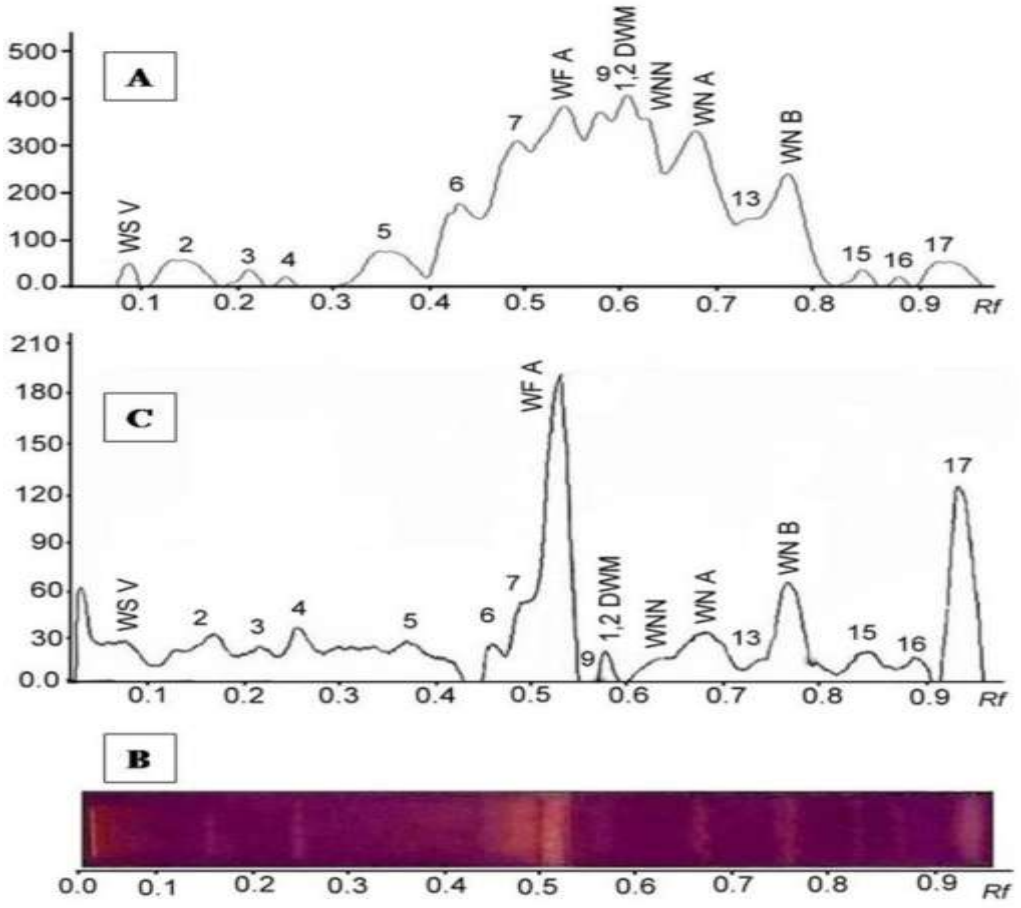


Table 3.3: Concentration and free radical scavenging potential of constituents of Ashwagandha root extract. Values are given as averages of three independent experiments.

Peak No.	*Rf*	Compound/ Components	Area by HPTLC (AU)	TLC-DPPH free radical scavenging area	Withanolide content by HPTLC (mg/g)	TLC-DPPH free radical scavenging area/Withanolide content by HPTLC	TLC-DPPH free radical scavenging area/ Area by HPTLC
1	0.07	WE V	1008	301.24	0.86	350.3	0.29
2	0.14	unknown 1	2620	468.97	-	-	0.17
3	0.21	unknown 2	806	404.09	-	-	0.50
4	0.25	unknown 3	201	626.21	-	-	2.12
5	0.37	unknown 4	2318	513.62	-	-	0.22
6	0.45	unknown 5	2112	221.6	-	-	0.10
7	0.48	unknown 6	4221	778.26	-	-	0.18
8	0.55	WF A	6971	3769.5	12.9	292.2	0.54
9	0.57	unknown 7	2694	0	-	-	0
10	0.61	1,2 DWM	3593	91.07	1.92	47.4	0.025
11	0.63	WNN	3233	90.05	1.52	59.24	0.02
12	0.66	WN A	5620	935.69	5.24	178.6	0.16
13	0.73	unknown 8	995	0	-	-	0
14	0.77	WN B	4727	1353.74	4.52	299.5	0.28
15	0.85	unknown 9	812	254.97	-	-	0.31
16	0.87	unknown 10	215	111.02	-	-	0.51
17	0.92	unknown 11	2348	2392.79	-	-	1.01

3.4. Discussion

Medicinal properties of Ashwagandha roots are primarily attributed to withanolides present in the roots (Ali and Shuaib, 1997). With the improved procedure for extraction of withanolides from Ashwagandha roots, in the present studies it was possible to get significantly higher concentration of withanolides as compared to earlier procedure (Chaurasiya et al., 2008).

Higher resolution with more number of separated compounds on TLC plate is desirable for TLC-DPPH assay, attempt were made to get higher resolution (more bands) on a single TLC plate. Although many HPTLC methods are available for the separation, they were able to separate three withanolides in the extract whereas with the present new method described here it was possible to separate six withanolides simultaneously (**Table 3.4; Fig. 3.1**). It was also possible to resolve WS V, WF A, 1, 2 DWM, WNN, WN A and WN B by following the modified method. Earlier workers have used methanol for preparing TLC-DPPH solution (Ciesla et al., 2011a, 2011b, 2012; Olech et al., 2012; Pozharitskaya et al., 2008; Waksmundzka and Wawrzynowicz, 2002). However, it was noted that methanol was not suitable solvent for TLC-DPPH assay of withanolides because the spots diffuse. Therefore, attempts have made to try combinations of different solvents for preparing DPPH solution. Hexane: acetone (9.5:0.5 mL v/v) system gave clear free radical scavenging bands. The compounds which possessed antioxidant activity showed free radical scavenging bands on TLC plate after incubation in DPPH solution (Ciesla et al., 2011a, 2011b). The DPPH itself has purple color and antioxidant active components bleach the purple color and thus appear as white bands (**Fig. 3.2B**). The densitometer eliminates the background and gives peaks for corresponding antioxidant areas (**Fig. 3.2C**).

Table 3.4: HPTLC methods used for the quantification of withanolides

Sr. No	Mobile Phase	Compound	Separation	Reference
1	toluene: ethyl acetate: formic acid (5:5:1 v/v)	Only WF A and WN A	2	Sharma et al. 2007
2	chloroform: ethyl acetate: methanol: benzene (74:4:8:24 v/v)	Only WF A, WN A and WNN	3	Sangwan et al. 2007
3	dichloromethane: methanol: acetone: diethyl ether (15:1:1:1 v/v)	Only WF A, 1,2 DWM and WN A	3	Shrivastav et al. 2008
4	toluene: dichloromethane: methanol: acetone: diethyl ether (6.5:7:4:1.5:1 v/v)	WS V, WF A, 1,2 DWM, WNN, WNA and WN B	6	Proposed method

ImageJ, an open source image processing program is used in quantitative measurements of free radical scavenging potential of individual compound in the fraction. Area under the peak is directly proportional to antioxidant potential of individual compound (**Fig. 3.2C**). Intensity of peak is estimated by ImageJ software and values are summarized in **Table 3.3**. Several researchers reported that WF A, WN A, WS V, WNN and 1, 2 DWM are biologically active, among all these WF A and WN A are potent and in present investigation the similar results were observed (Sabina et al., 2008; Mohan et al., 2007; Malik et al., 2008; Kour et al., 2009; Kobuyama et al., 2005; Sen et al., 2007; Yang et al., 2007; Nakayama et al., 2007). WF A has highest antioxidant activity followed by WN B, WN A, WS V, 1,2 DWM and WNN respectively, while in unknown components peak no 17 i.e. unknown 11 at *Rf* 0.92 has highest antioxidant potential (2392.79) and peak no 16 i.e. unknown 10 at *Rf* 0.87 has recorded as a lowest antioxidant potential (111.02) as shown in **Table 3.3**. Except for peak 9 and 13 remaining unknown compounds have antioxidant activity and values are summarized in **Table 3.3**. Interestingly after considering the ratio of TLC-DPPH free radical scavenging area / Withanolide content, the antioxidant potential of individual withanolides becomes clear. Surprisingly, WS V and WN B shows highest antioxidant potential as compared to WF A and WN A; while 1, 2 DWM and WNN shows lowest antioxidant potential (**Table 3.3)**. As for the unknown components, the attempts were made to evaluate their antioxidant potential in terms of the ratio of TLC-DPPH free radical scavenging area/ Area by HPTLC. These values are also summarized in **Table 3.3**. Surprisingly unknown 2 and 10 components had comparable activity to WF A. WE V and WN B had practically similar activity which is comparable to unknown 9. Unknown 3 had highest activity followed by unknown 11. For other unknown compounds the ratio ranged from 0.1 to 0.22. From the present study it was observed that WS V has strongest activity in known withanolides, and peak 17 among the several unknown compounds.

The most surprising feature was high antioxidant potential of specified unknown peaks. In this context it may be mentioned that the efficacy of Ayurvedic medicine lies in synergetic action. It is possible that the high antioxidant potential of the unknown components may potentiate the efficacy of Ashwagandha for management of various therapeutic activities by acting as enhancers (Hegde et al., 2008).

3.5. Conclusions

TLC-DPPH method provides a selective tool for screening plant extracts for the presence of biologically active compounds. This technique has several advantages, i.e. simplicity, flexibility, high throughput or direct access to the compounds separated. This method may be useful in quality control of Ayurvedic products containing Ashwagandha roots. An indepth analysis of the unknown peaks with high antioxidant activity can open door for efficient management/ application of Ashwagandha treatment.

It is also expected to be useful in validating both raw material and finished products, and also be useful in shelf-life determination.

Chapter 4

Studies on the Stability, Shelf-life and Biological Activity of WRF and its Components

4.1. Introduction

Stability testing is an important aspect of drug development as it affects quality, safety, and therapeutic efficacy of the product. Stability testing is carried out at distinct phases including pre-formulation, formulation development, product development, and post-marketing. For long-term therapeutic efficacy it is important to confirm the stability of product as this is often not prepared daily but the preparation is used repeatedly for several days or weeks. In such case biological activity and stability assume important (Goppel and Gerhard, 2004; Plubrukarn et al., 2006; Patil et al., 2010).

Stability testing especially of herbal drugs, is challenging because of the inherent physicochemical complexities. Further, herbal drugs are now considered as active substances in their entirety and there may be challenges in the selection of the assay or of the parameters and protocols that profile their stability characteristics (Patil et al., 2010; Patra et al., 2010). A chemical is considered to be unstable compound when it is degraded by non-enzymatic processes. Degradation may be caused by several mechanisms, the most common being hydrolysis, oxidation, or light-catalyzed degradation. Compounds that are highly unstable may not be suitable as drug candidates since it may be difficult to maintain a therapeutically effective formulation (Mosihuzzaman and Choudhary, 2008). The regulatory agencies such as the United States Food and Drug Administration (USFDA) and the European Medicinal Evaluation Agency advocate (EMEA) that the stability of herbal drugs generally should not be based entirely on the assay of the active constituents or characteristic markers (EMEA 2009).

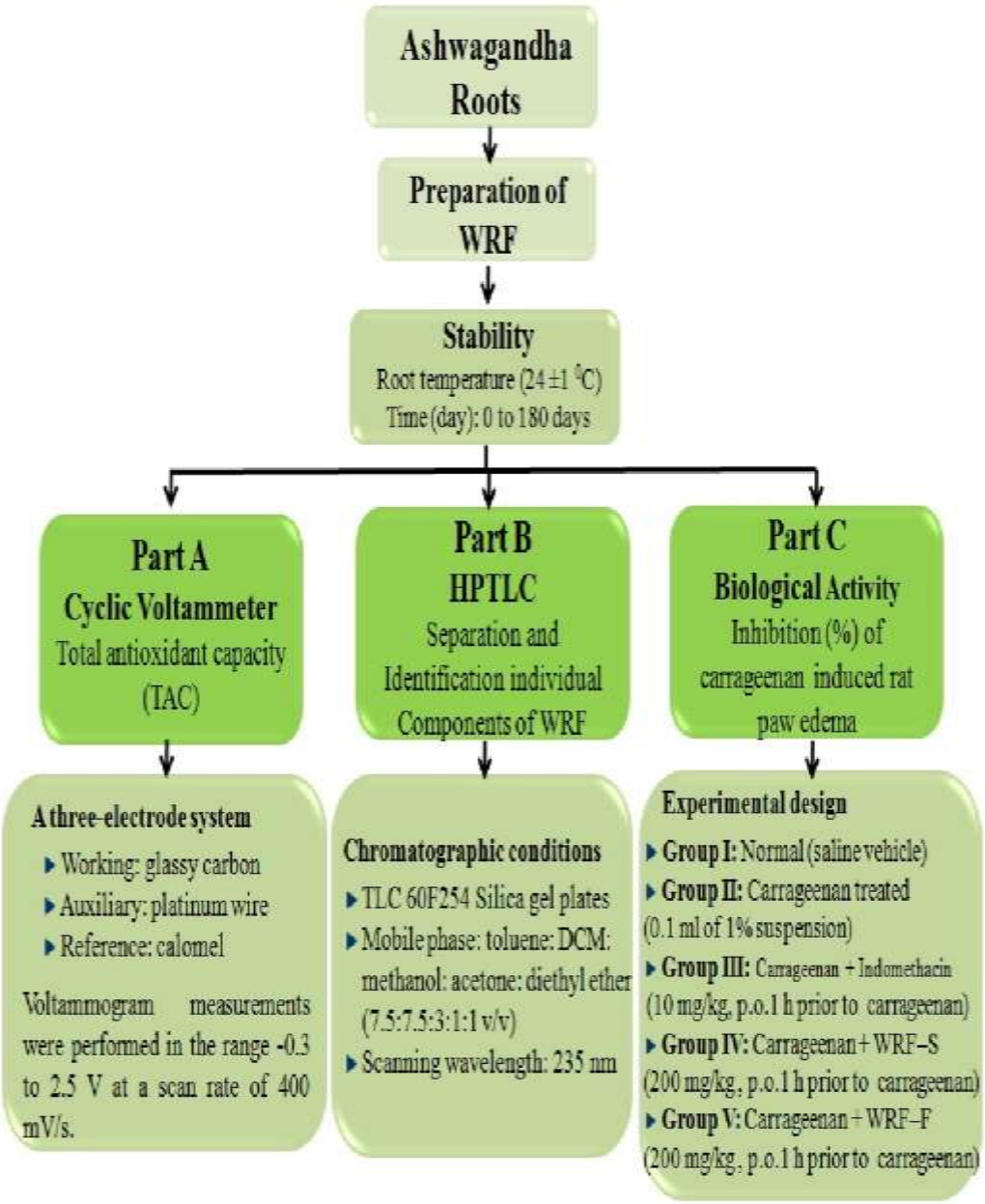
Electrochemical properties of foods and biological samples can be used for the evaluation of their reducing/antioxidant capacity. The anodic peak current (ip) characterizes the reducing power of antioxidants and provides information on the type and ability of antioxidants to donate electrons. The anodic current corresponds to

the concentration of antioxidants (**Fig. 1.1** of Chapter 1). Cyclic voltammeter is a simple, rapid and economical technique. It can be used to estimate total antioxidant capacity of foods/biological samples (Momen-Beitollahi et al., 2010; Hoyle and Santos, 2010; Psotová et al., 2001).

Studies on the chemical stability of withaferine A and withanolide A have been reported; however, study of physicochemical stability and its correlation with biological activity at a pre-formulation stage have been rarely attempted (Kopleman et al., 2001; Sahoo et al., 2009; Patil et al., 2010). Therefore studies were undertaken to correlate the biological activity of therapeutic significance (anti-inflammatory activity) with chemical constituents and physical characteristics (e.g. chromatogram and voltammogram) for estimation of shelf-life of WRF. The plan of work is shown in **Fig. 4.1.**

This study may help in determining the shelf-life of individual components of WRF. The total antioxidant capacity and biological activity profile at different time intervals, as well as the shelf-life of WRF of Ashwagandha and its compounds is described in this chapter.

Fig. 4.1: Schematic diagram for stability study of WRF.



4.2. Materials and Methods

4.2.1. Chemicals

Carrageenan and Indomethacin were purchased form S.D. Fine Chemicals, Mumbai, India. The source of standard withanolides and other chemicals have already described in section 2.2.2 of Chapter 2.

4.2.2. Plant material

As described in section 3.2.2 of Chapter 3.

4.2.3. Preparation of Sample for Stability Study

WRF was prepared as per method described in section 3.2.3.2 of Chapter 3. For stability studies WRF was weighted and redissolved in methanol: water (8:2 v/v) to obtain stock WRF suspension (10 mg/mL). WRF samples were stored for six months in the glass stoppered bottle under room temperature conditions (24 ± 1°C). From day 0 to day 180 of preparation test samples were withdrawn at different time intervals and were examined for total antioxidant capacity, stability and biological activity.

4.2.4. Estimation of Total Antioxidant Capacity (TAC) of WRF by Cyclic Voltammeter

The total antioxidant capacity of WRF by using PGSTAT 101, Autolab (Metrohm,Switzerland). It is a three-electrode system; working electrode is a glassy carbon (Autolab GC, 8 mm in diameter), auxiliary electrode is platinum wire and reference electrode was calomel. All cyclic voltammogram measurements were performed in the range (–0.3) – (2.5) V at a scan rate of 400 mV/s as per described by method Hoyle and Santos, (2010) with some modification. The samples were analyzed triplicate. The ip values were used for calculation of $t_{1/2}$ and t_{99}. The $t_{1/2}$ and t_{99} were calculated by using the first order kinetics equation.

4.2.5. Estimation of Shelf-life and Stability of Individual Component of WRF by HPTLC

The samples and standards were applied to the HPTLC plate as 6 mm wide bands with constant application rate of 150 nL/s, using an automatic TLC sampler (LINOMAT V) under a flow of N_2. The detailed procedure for chromatography described in Section 3.2.5 of Chapter 3.

WS V, WF A, 1, 2 DWM, WN A and WN B were used as external reference standards. The fingerprint was validated on the basis of intermediate precision and repeatability of relative peak areas (RPA) and retardation factor (*Rf*) of selected peaks in the chromatograms. The similarity of month wise fingerprints was compared. The samples were analyzed triplicate. The area under the curve (AUC) was used for calculation of $t_{1/2}$ and t_{99}. The $t_{1/2}$ and t_{99} were calculated by using the first order kinetics equation.

4.2.6 First Order Kinetics Equation

$$\text{Decay constant (k)} = \frac{2.303}{t} \text{ X log } \frac{[a]}{[a-x]}$$

$$\text{Half life } (t_{1/2}) = \frac{2.303}{k} \text{X log 2}$$

$$\text{Shelf life } (t_{99}) = \frac{2.303}{k} \text{X log 100}$$

Where,

t = time in days

[a]= ip value/ concentration on day 0

[x]= ip value/ concentration of fraction decayed at time t

[a-x]= ip value/ concentration of fraction at time t

$t_{1/2}$= time required for 50% decay

t_{99}= time required for 100% decay

4.2.7. Estimation of Biological activity by Rat Paw Edema Model

4.2.7.1. Experimental Protocol Approval

The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Bharti Vidyapeeth, Pune and the experiments were performed in accordance with the guidelines of Committee (CPCSEA/103/12).

4.2.7.2. Animals

The old adult male albino rats of Wistar strain (8-10 week; 180-220 g) were obtained from the National Institute of Biosciences, Pune. The animals were maintained at 24 ± 1^0C with relative humidity of 45-55% and 12:12 hr dark/light cycle. The animals had free access to standard pellet chow (Pranav Agro Industries Ltd., Sangli, India) and water throughout the experimental period.

4.2.7.3. Acute Oral Toxicity Tests for WRF

Acute oral toxicity test was performed in male Swiss albino mice for the WRF according to the OECD guidelines using AOT 425 software. Acute oral toxicity of WRF was studied up to 2000 mg/kg dose.

4.2.7.4. Experimental Design

The animals were randomly divided in 5 groups of 6 rats each as given below:

Group I: Normal: Sterile saline solution (0.9% NaCl, 0.1 mL) was injected into the left paw as the control reference.

Group II: Carrageenan treated: Injecting inflammatory agent carrageenan 0.1 mL of 1% suspension into the right hind paw planter surface.

Group III: Indomethacin treated: The animals were administered a single dose of 10 mg/kg of Indomethacin in distilled water p.o. 1 hr prior to injection of Carrageenan.

Group IV: WRF–S (200 mg/kg): The animals were administered a single dose of 200 mg/kg of 1% aqueous solution of WRF-S

in distilled water p.o. 1 hr prior to injection of Indomethacin**.**

Group V: WRF–F (200 mg/kg): The animals were administered a single dose of 200 mg/kg of 1% aqueous solution of WRF-F in distilled water p.o. 1 hr prior to injection of Indomethacin**.**

Where,

WRF-S: Stored WRF for six month

WRF-F: Fresh WRF

At the end of the experimental period, the foot volumes of the animals were determined by Plestimographic method (Winter et al., 1979). The foot volume measurements were taken before the injections and then at hourly intervals i.e. 1 hr, 2 hr, 3 hr, 4 hr, 5hr, 6 hr and at 24 hr after the injection of the inflammatory stimulus.

Measurements of the volume difference between the right and the left paws in comparison with both the negative control group; treated with saline solution, and the positive control; treated with Indomethacin (Moraes et al., 2010).

4.2.8 Statistical analysis

The results were expressed as mean ± standard deviations. The data were analyzed by standard computer program: Excel (2003) and statistical analysis was carried out by one-way ANOVA test. p value, (<0.05) are considered as significant.

4.3 Results

4.3.1. Total Antioxidant Capacity of WRF by Cyclic Voltammeter

Various compositions of solvents were tested to get clear-cut voltammogram of WRF and it was achieved by using methanol: water (8:2 v/v). For cyclic voltammogram measurements different ranges of voltage were tested; and it was found that the range (–0.3) to (2.5) V at a scan rate of 400 mV/s showed best results.

It was noted that the anodic peak current (ip) values are directly proportional to its antioxidant potential (**Fig. 1.1**). Values of the ip of WRF at different time intervals are given in **Table 4.1**. **Fig. 4.2** represents the voltammogram of 0, 30, 90, 120, 150, 180 day old WRF. It is clear that the height of voltammogram gets reduced with time. A plot of ip versus time showed an exponential pattern for reduction of antioxidant potential of WRF on storage (**Fig. 4.3A**). Plot of log (ip) values versus time were plotted again to check whether this reduction pattern followed 1st order kinetics and it was observed that the plot resolved in two components; namely component I and component II **(Fig. 4.3B)**.

The stability was evaluated in terms of the half-life ($t_{1/2}$) and shelf-life (t_{99}). The fast moving (degrading) component I had $t_{1/2}$ of 31.3 days and t_{99} of 208 days, while slow moving (degrading) component II had $t_{1/2}$ of 478 days and t_{99} of 3174 days (**Table 4.2; Fig. 4.3B**).

Table 4.1: Anodic peak current (ip) of WRF at different time intervals. Values are given as averages of three independent experiments.

Day	ip (A) X 10^{-5}
0	1.67
3	1.53
6	1.46
9	1.35
12	1.25
15	1.20
18	1.15
21	1.08
24	1.03
27	1.01
30	0.99
37	0.98
46	0.98
53	0.97
60	0.95
67	0.95
74	0.94
81	0.94
88	0.92
95	0.91
102	0.89
109	0.88
116	0.87
123	0.86
130	0.84
137	0.83
144	0.82
151	0.82
158	0.81
165	0.79
172	0.78
180	0.76

Fig. 4.2: Effect of storage at room temperature on antioxidant potential of WRF. Voltammogram of WRF evaluated monthly up to 6 month under room temperature. The difference between blank and test sample is anodic peak potential (ip) which is directly proportional to antioxidant potential. The ip values are given in the **Table 4.1.**

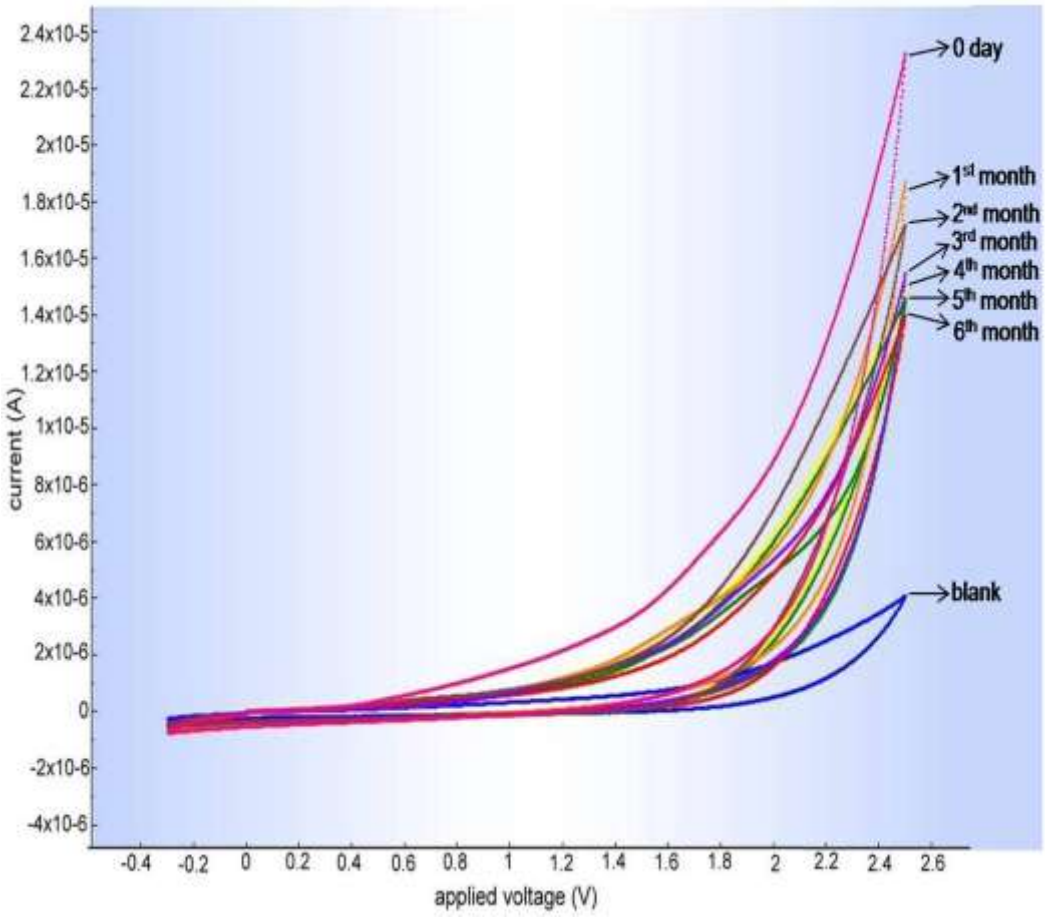


Fig. 4.3A: Plot of anodic peak current of WRF versus time. The ordinate represents anodic peak current in ip (A) units and the abscissa represents time in days. Each point represents average of three independent experiments

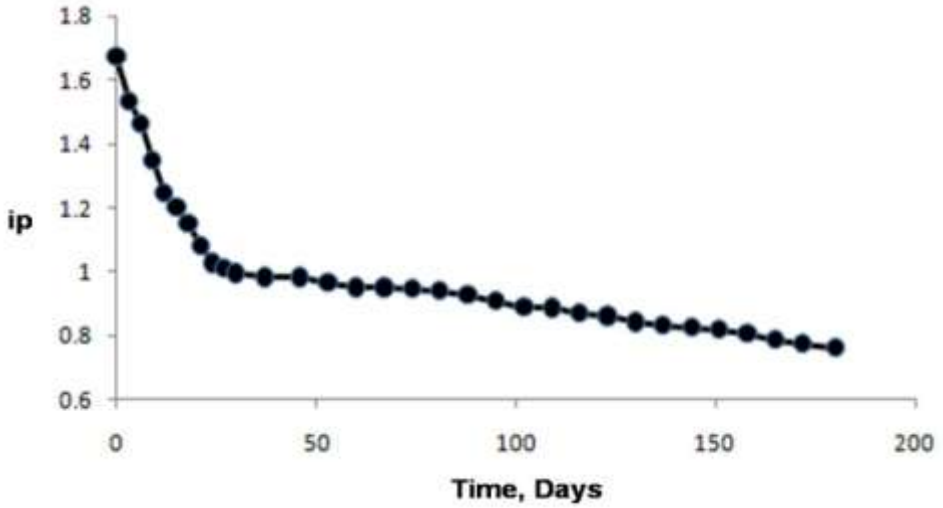


Fig. 4.3B: Decay of anodic peak current of WRF with time. The ordinate represents Log of anodic peak current in ip (A) units and the abscissa represents time in days. Each point represents mean of three independent experiments

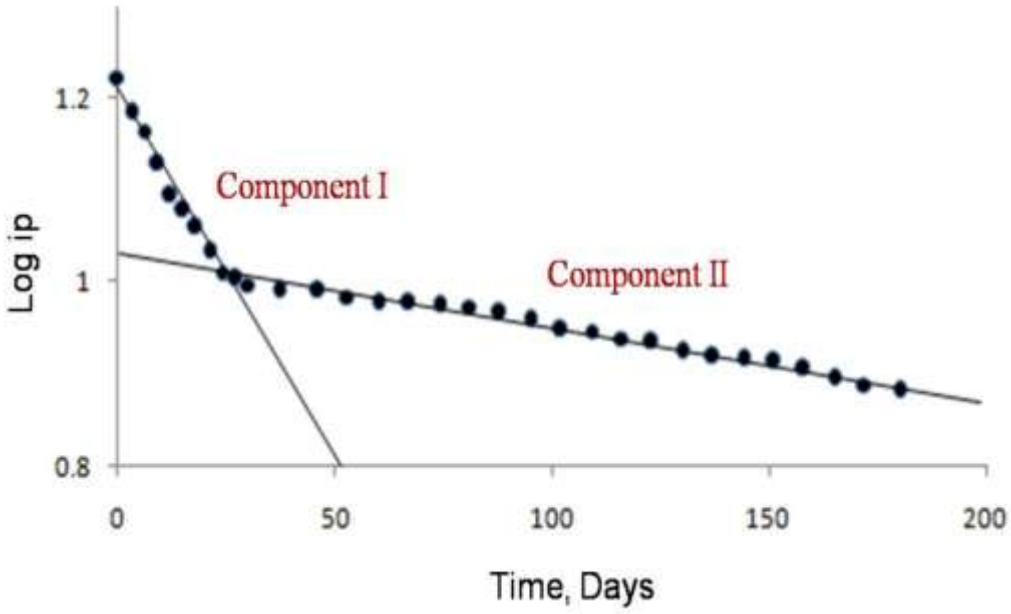


Table 4.2: $t_{1/2}$ and t_{99} values of WRF based on ip.

Samples	Component I		Component II	
	$t_{1/2}$	t_{99}	$t_{1/2}$	t_{99}
WRF	31.3 ± 1.3	207.8 ± 8.8	477.5 ± 21.9	3174 ± 145.6

4.3.2. Stability of Individual Components of WRF by HPTLC

Identification of major withanolides was on the basis of *Rf*. The WRF contains five bioactive withanolides and nine unknown compounds **(Fig. 4.4).** The stability was evaluate on the basis of concentration i.e. Area Under the Curve (AUC) of components at different time intervals (**Table 4.3).** Area of WS V, WF A, 1,2 DWM, WN A and WN B at 0 day were 1907, 3623, 4393, 3557 and 1366 AUC, respectively; while after on 180th day the area were 725, 1952, 2644, 2536 and 645 AUC, respectively (**Table 4.3)**. In WRF there are 9 unidentified components and the concentration at 0 day ranged from 175.3 to 3316.1 AUC, while on 180th day it raged form 120.3 to 2101.9 AUC (**Table 4.4)**.

In unidentified peaks the concentration of unidentified peak 7 was highest, followed by peak 8, 2 and 6, while peaks 1, 3, 4 and 7 were present in low amounts (**Table 4.4; Fig. 4.4**). Concentrations of unidentified components were reduced very fast in first 21 days and then the reduction occurred at slow rate. Among all unidentified peaks, peaks 3 and 9 degraded very fast followed by peak 4, 3 and 9 which were completely degraded by 21 days; peak 4 took 109 days for complete degradation (**Table 4.4; Fig. 4.4**).

The plot of log (AUC) versus time is shown in **Fig. 4.5 and 4.6**. The plots resolved in two components for the major withanolides, while for the unidentified components, the plots resolved in either one or two components (**Fig. 4.5, 4.6**). Estimation of $t_{1/2}$ and t_{99} of individual components was carried out by applying the first order kinetic equation. Component I of WS V was fast decaying with $t_{1/2}$ of 39.9 ± 1.2 days and t_{99} of 265.2 ± 8.0 days, while component II was slow decaying with $t_{1/2}$ of 279.2 ± 8.2 and t_{99} of 1855.4 ± 54.2 days. For WN A component I was the fast decaying with $t_{1/2}$ of 104.1 ± 3.2 days and t_{99} of 691.6 ± 21.2 days, while component II was slow decaying with $t_{1/2}$ of 597.9 ± 26.2 and t_{99} of 3974 ± 173.8 which is the highest value and WS V was lowest as compared to other major

withanolides. WF A, WN B and except t_{99} value of 1,2 DWM had intermediate values (**Table 4.5; Fig. 4.5**).

The $t_{1/2}$ and t_{99} values of unidentified peaks indicated that the peak 3, 4 and 9 are very unstable at room temperature, while peak 2 and 7 are most stable as compared to other components (**Table 4.6; Fig. 4.6)**. It is also noted that the concentrations of major withanolides and unidentified components decreased on storage.

Fig. 4.4: Effect of storage at room temperature on WRF components. Month wise chromatograms are shown in overlay plots. Also refer to **Tables 4.3** and **4.4** for results.

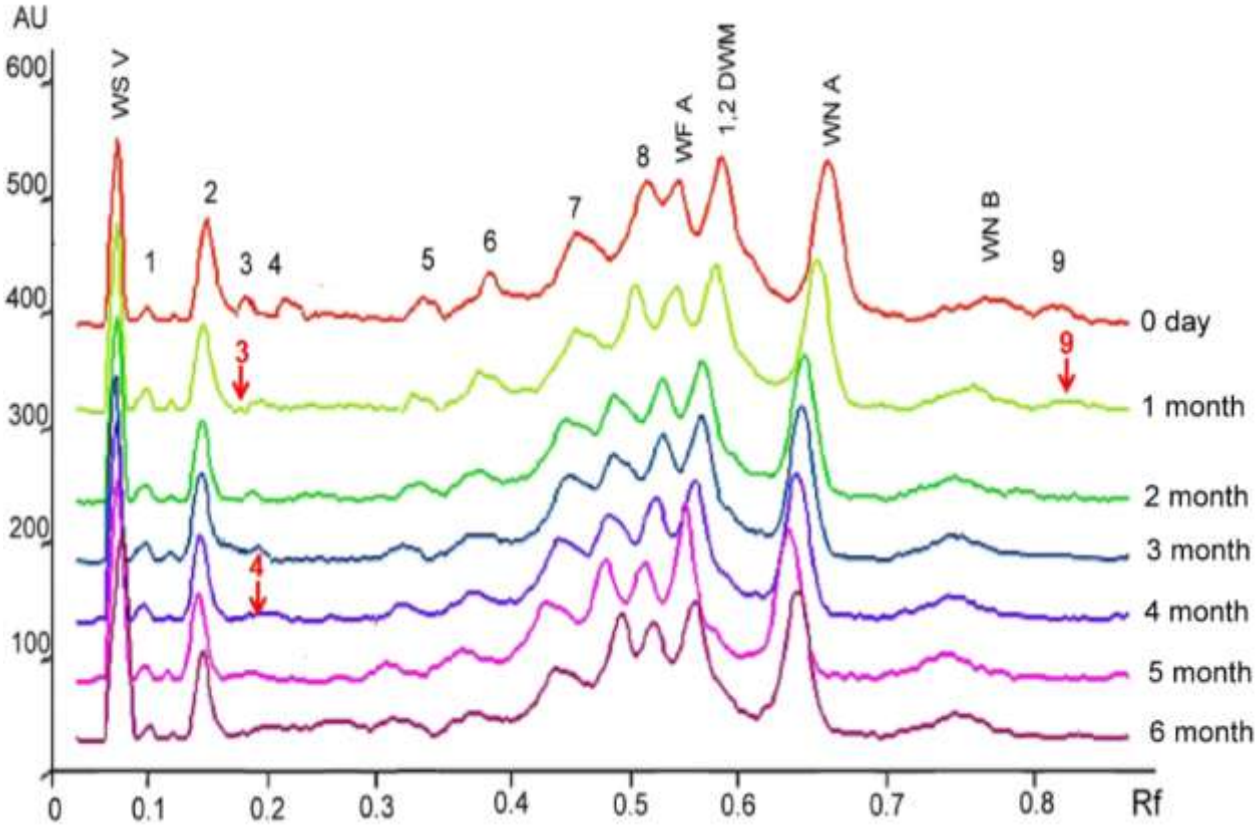


Table 4.3: Concentration (Area, AU) of major withanolides of WRF by HPTLC. Values are given as averages of three independent experiments.

Day	Major withanolides of WRF (Area AU)				
	WS V *Rf* 0.05	**WF A** *Rf* 0.55	**1,2 DWM** *Rf* 0.58	**WN A** *Rf* 0.65	**WN B** *Rf* 0.74
0	1907	3622.6	4393.2	3557.1	1365.7
3	1811.4	3441.4	4273.6	3474.9	1297.4
6	1700.3	3200.5	4101.6	3399.1	1206.6
9	1656.1	3141.5	4025.4	3331.0	1150.3
12	1512.6	3028.7	3932.4	3297.3	1100.9
15	1501.2	2812.7	3802.5	3251.1	1073.3
18	1450.5	2720.7	3712.4	3174.5	998.2
21	1352.5	2607.1	3573.3	3104.6	956.2
24	1216.4	2571.6	3487.4	3061.3	922.2
27	1119.5	2569.4	3350.9	2994.5	901.6
30	1115.0	2550.4	3302.9	2942.7	890.8
37	1075.4	2536.1	3200.3	2889.5	880.5
46	1028.5	2495.5	3102.7	2870.6	869.5
53	995.3	2454.1	3099.0	2846.5	855.3
60	982.4	2402.4	3086.1	2806.3	831.3
67	956.7	2369.6	3069.4	2796.7	824.2
74	940.4	2358.5	3047.8	2776.4	809.1
81	903.7	2299.5	3000.0	2751.3	801.3
88	875.6	2250.4	2985.8	2744.5	793.2
95	856.4	2233.6	2956.3	2731.9	785.2
102	845.6	2222.5	2925.5	2721.6	776.4
109	824.8	2202.5	2901.3	2700.6	765.3
116	804.0	2199.3	2894.2	2696.3	755.4
123	801.4	2158.4	2850.4	2689.3	748.2
130	780.2	2154.6	2834.9	2675.3	735.9
137	774.2	2144.6	2809.1	2650.4	723.4
144	761.2	2129.2	2805.9	2627.2	697.5
151	754.7	2087.3	2775.7	2593.1	689.9
158	740.2	2071.7	2748.0	2573.9	672.4
165	734.2	2023.9	2699.6	2565.2	662.5
172	730.2	1997.5	2674.2	2545.6	655.7
180	724.7	1951.5	2643.6	2535.7	645.2

Table 4.4: Concentrations of unknown components of WRF by HPTLC. Values are given as averages of three independent experiments.

Day	Unknown Component (Unidentified peaks) of WRF (Area AU)								
	1 *Rf* 0.08	**2** *Rf* 0.11	**3** *Rf* 0.15	**4** *Rf* 0.21	**5** *Rf* 0.34	**6** *Rf* 0.39	**7** *Rf* 0.43	**8** *Rf* 0.52	**9** *Rf* 0.80
0	175.9	1187.2	175.3	281.2	580.4	1037.6	3316.1	3199.0	387.6
3	175.3	1146.9	166.5	271	509.4	985.5	3198.5	2907.1	301.6
6	172.4	1098.2	154.9	265.3	475.9	902.5	3100.5	2756.2	234.8
9	171.4	1025.6	146.5	259.3	408.2	864.3	2890.4	2648.2	190.6
12	170.2	988.5	123.2	251.2	371.4	809.3	2710.3	2590.1	125.4
15	170.1	941	110.8	245.3	352.1	775.9	2682.6	2545.0	--
18	169.8	913.5	95.4	241.3	335.4	729.5	2561.0	2442.8	--
21	169.2	875.4	--	238.3	332.4	714.7	2552.1	2434.5	--
24	168.2	850.6	--	234.3	315.8	696.9	2516.9	2364.7	--
27	166.7	840.3	--	232.3	313.1	681.4	2473.5	2343.1	--
30	166.5	820.4	--	232.3	310.9	676.6	2472.1	2307.9	--
37	165.2	813.5	--	225.2	306.5	658.7	2407.2	2305.5	--
46	164.9	796.1	--	214.8	300.5	640.4	2394.7	2245.1	--
53	164.5	780.2	--	209.4	295.5	635.7	2392.3	2203.7	--
60	164.1	772.2	--	190.4	290.3	629.4	2375.7	2179.5	--
67	157.7	770.3	--	183.3	283.6	615.4	2364.9	2150.7	--
74	152.7	769.3	--	173.2	280.9	601.2	2351.9	2103.2	--
81	150.7	769.2	--	160.8	275.5	595.6	2341.9	2055.5	--
88	148.7	765.2	--	155.8	269.9	594.2	2336.5	2001.8	--
95	145.3	763.5	--	146.5	268.8	591.4	2329.8	1994.3	--
102	143.2	760.4	--	142.5	264.6	590.3	2309.1	1987.5	--
109	140.6	758.4	--	133.5	259.3	586.4	2293.9	1980.2	--
116	138.5	758.2	--	--	255.2	586.3	2260.3	1970.2	--
123	134.1	755.3	--	--	251.3	580.3	2258.4	1967.4	--
130	132.1	753.6	--	--	249.5	580.3	2244.7	1962.0	--
137	131.3	753.3	--	--	245.3	579.4	2199.5	1956.6	--
144	128.6	751.3	--	--	245.2	574.2	2189.2	1950.9	--
151	126.8	747.1	--	--	238.7	572.2	2187.0	1928.6	--
158	124.4	744.2	--	--	237.5	570.1	2185.0	1901.2	--
165	123.2	735.8	--	--	234.5	565.4	2178.3	1892.4	--
172	122.6	730.2	--	--	230.6	560.3	2129.2	1889.3	--
180	120.3	725.4	--	--	226.8	545.8	2101.9	1872.3	--

Fig. 4.5: Decay of Area (AU) with time of major withanolides of WRF. The ordinate represents Log of Area (AU) units and abscissa represents time in days. Each point represents a mean of three independent experiments.

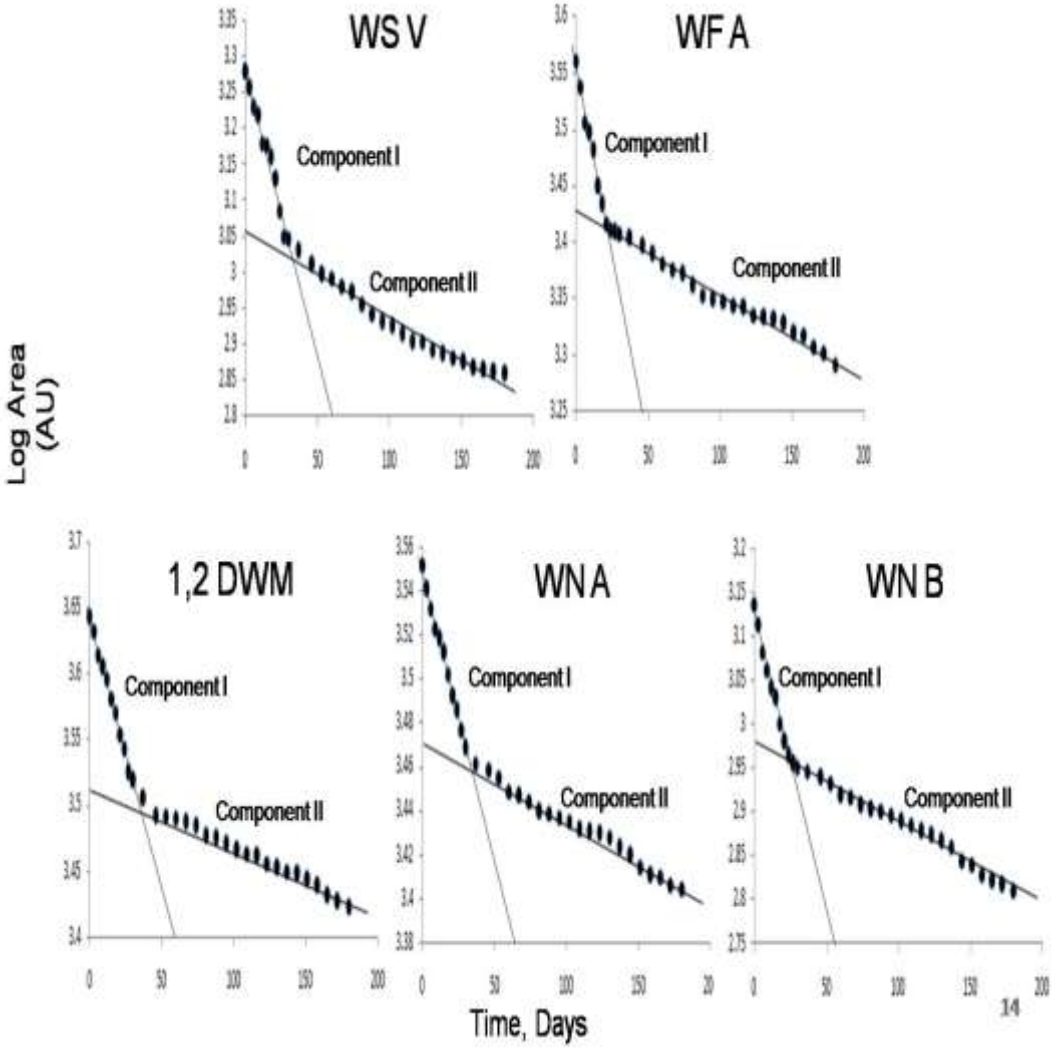


Fig. 4.6: Decay of Area (AU) with time of unknown components of WRF. The ordinate represents Log of Area (AU) units and abscissa represents time in days. Each point represents a mean of three independent experiments.

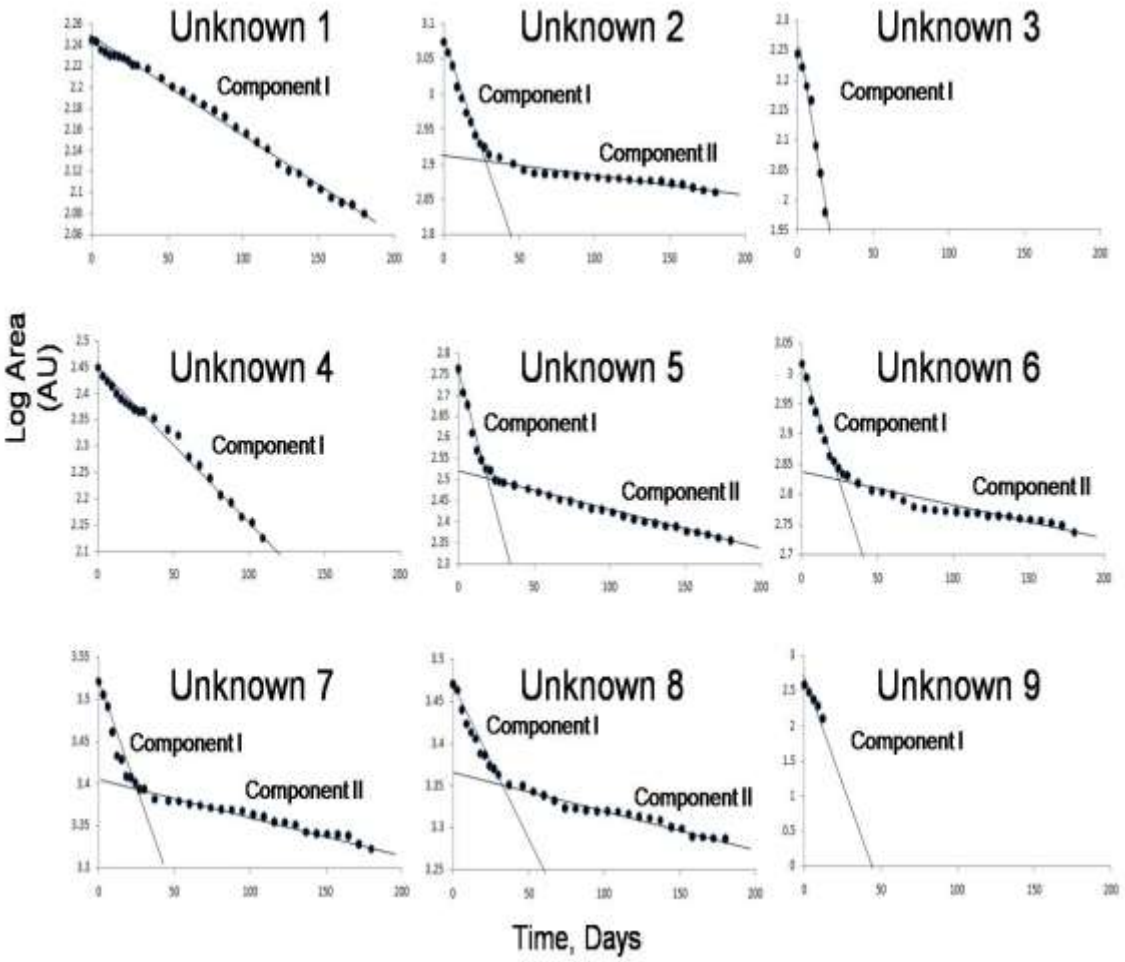


Table 4.5: $t_{1/2}$ and t_{99} values of major withanolides of WRF by HPTLC.

Major withanolides of WRF	Component I		Component II	
	$t_{1/2}$	t_{99}	$t_{1/2}$	t_{99}
WS V	39.9 ± 1.2	265.2 ± 8.0	279.2 ± 8.2	1855.4 ± 54.2
WF A	45.5 ± 2.3	302.6 ± 15.2	363.1 ± 9.4	2414 ± 62.4
1,2 DWM	73.8 ± 2.1	508.2 ± 14.0	668.4 ± 39.9	8884 ± 265
WN A	104.1 ± 3.2	691.6 ± 21.2	597.9 ± 26.2	3974 ± 173.8
WN B	42.47 ± 2.0	282.2 ± 13.4	341.7 ± 10.0	2272 ± 66.2

Table 4.6: $t_{1/2}$ and t_{99} values unidentified components of WRF by HPTLC.

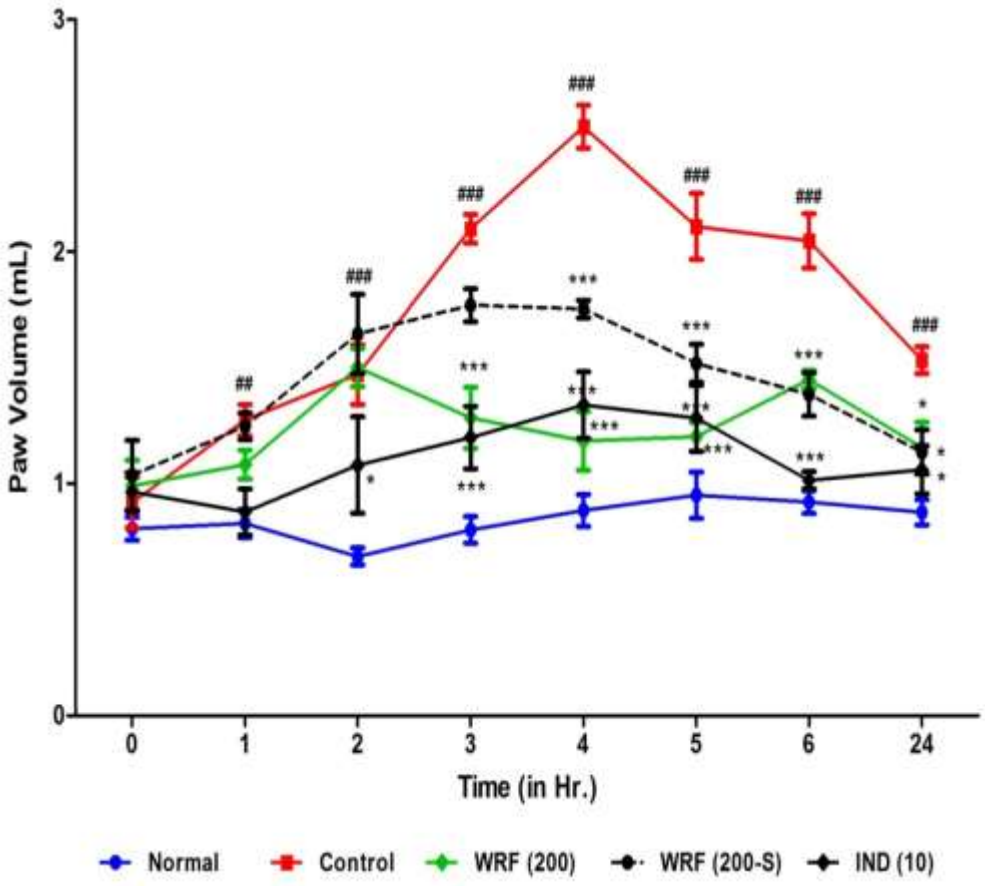
Unknown components of WRF	**Component I**		**Component II**	
	$t_{1/2}$	**t_{99}**	**$t_{1/2}$**	**t_{99}**
Unknown 1	350.8 ± 14.7	2332 ± 97.6	---	----
Unknown 2	49.5 ± 1.8	329.0 ± 12.2	669.6 ± 43.7	4450.4 ± 290.4
Unknown 3	29.27 ± 3.3	194.5 ± 22.2	---	---
Unknown 4	95.75 ± 3.8	636.2 ± 25.4	---	---
Unknown 5	24.9 ± 2.2	165.4 ± 14.8	613.8 ± 8.2	2040.2 ± 27.0
Unknown 6	38.3 ± 1.8	254.4 ± 12.0	754.8 ± 48.6	2508.6 ± 161.4
Unknown 7	55.37 ± 2.9	368.0 ± 19.4	1272.0 ± 50.0	4226.8 ± 162.8
Unknown 8	47.64 ± 5.0	316.6 ± 33.4	533.8 ± 15.4	1773.8 ± 51.2
Unknown 9	8.3 ± 0.3	52.4 ± 2.2	---	---

3.3. Biological Activity of WRF by Carrageenan induced Rat Paw Edema Model

Acute toxicity studies showed no toxic signs and symptoms such as restlessness, respiratory distress, diarrhea, convulsions and coma; WRF was safe up to 2000 mg/kg.

Biological activity measured as the percent inhibition of carrageenan-induced rat paw edema. At 3rd, 4th, 5th, and 6th hr the inflammation was significantly high in carrageenan treated group as compared to normal group. At 4th hr the inflammation was highest however, WRF-F and WRF-S (200 mg/kg) treatment resulted in significant inhibition of the inflammatory process. Inflammatory process was significantly inhibited ($p < 0.01$) by WRF-S (50%) and WRF-F (80%) at the 4th hour as compared to normal group (**Fig. 4.7**). Indomethacin, the positive control used in this test, inhibited the edema by 85.2% at a dose of 10 mg/kg, when compared to the untreated control animals ($P < 0.01$).

Fig. 4.7: Inflammation studies; Paw volume in mL versus time in hours of the experimental groups. WRF (200) represents Fresh WRF, while WRF (200-S) represents Six Month Stored WRF.



Data are expressed as mean ± SEM (n = 6) and analyzed by one-way ANOVA followed by Dunnett's test for each parameter separately. *P < 0.05, **P < 0.01 and ***P < 0.001 as compared to negative control group and #P < 0.05, ##P < 0.01, ###P < 0.001 as compared to normal group.

4.4. Discussion

In Ayurveda, Indian Herbal Medicine (IHM), the hidden therapeutic power in several medicinal plants has been reported. However, no proper information exists as regards the standardization of the formulation, validation, or stability of these herbal formulations (Mosihuzzaman and Choudhary, 2008; Choudhary and Sekhon, 2011; Sagar et al., 2005). These formulations don't have general acceptance. Detailed stability profiling of extracts at preformulation stages may help the formulator to identify inherent weaknesses of the extract at an early stage and enable him to choose few but rational stability-indicating parameters for further stages of development. Plants/ Herbs may have inconsistent growth and bioactive content (Patra et al., 2010). Investigations of stability of herbal drugs are rarely reported because of the additional cost during the manufacture. Therefore it is required to develop reliable and cost effective methods for stability estimation (Choudhary and Sekhon, 2011). The therapeutic properties of several medicinal plants have been reported in Ayurveda. However, information on standardization, validation, or stability is wanting. In the present investigation these parameters were evaluated in WRF by using simple, reliable and cost-effective techniques.

Drug stability means the ability of the pharmaceutical dosage form to maintain the physical, chemical and therapeutic properties during the time of storage and usage by the patient (Choudhary and Sekhon, 2011). The results suggest that the ip values of WRF decreased from 1.67 X 10^{-5} to 0.76 X 10^{-5} on day 0 to 180 day, implying 54% reduction in antioxidant potential during storage. It was reported that generally pharmacological activity of drugs was affected on long storage at room temperature and similar results were observed in the present investigation (Plubrukarn et al., 2006; Patil et al., 2010; Shinde et al., 2009). Antioxidant potential and content of withanolides were reduced very fast in first 21 days and then the reduction occurred slowly (**Fig. 4.2**).

It is reported that in a complex mixture some components are fast degrading/ moving while some of them are slow degrading/ moving i.e. component I and II, respectively (Dave et al., 2002). It is evident that the system resolved the total antioxidant activity in two components. This is due to the presence of stable and unstable components in the complex mixture (Patil et al., 2010). Component I, the fast decayed with $t_{1/2}$ of about one month and t_{99} of 7 months, while component II was a slow degrading with $t_{1/2}$ of 16 months and t_{99} of 106 months i.e. about 9 years.

Misico and Forzani, (2003) reported the application of cyclic voltammeter in measurement of hydrolysis decay time constants of monoarylimino derivatives of β-lapachone. In the present studies cyclic voltammeter has been used for the first time to measure the stability of complex mixture i.e. WRF which gave a gross picture of shelf life of the mixture.

Stability of WRF was determined using HPLTC method. Among all the major withanolides, 1,2 DWM and WN A were found to be most stable, while WS V was less stable as compared to other withanolides. Patil et al. (2010) notated that WN A and WF A are stable components and which is consistent with results of the present studies. The plot of log (AU) versus time in days resolved in two components for the major withanolides, while for unknown (unidentified) components, the plots resolved in either one or two components. It is possible that residual component I can have influenced the $t_{1/2}$ and t_{99} of component II. It could mean that the values of component II somewhat higher than shown table (**Table 4.2 and 4.5**). Thus, in order to verify whether spill over from component I is influencing the values of $t_{1/2}$ and t_{99} of component II of withanolides the stability of standard WS V has been carried out by cyclic voltammeter. **Fig. 4.8** shows the decay of anodic peak current of WS V with time. A single straight line observed, which could mean that there is influence of some co-migrating components

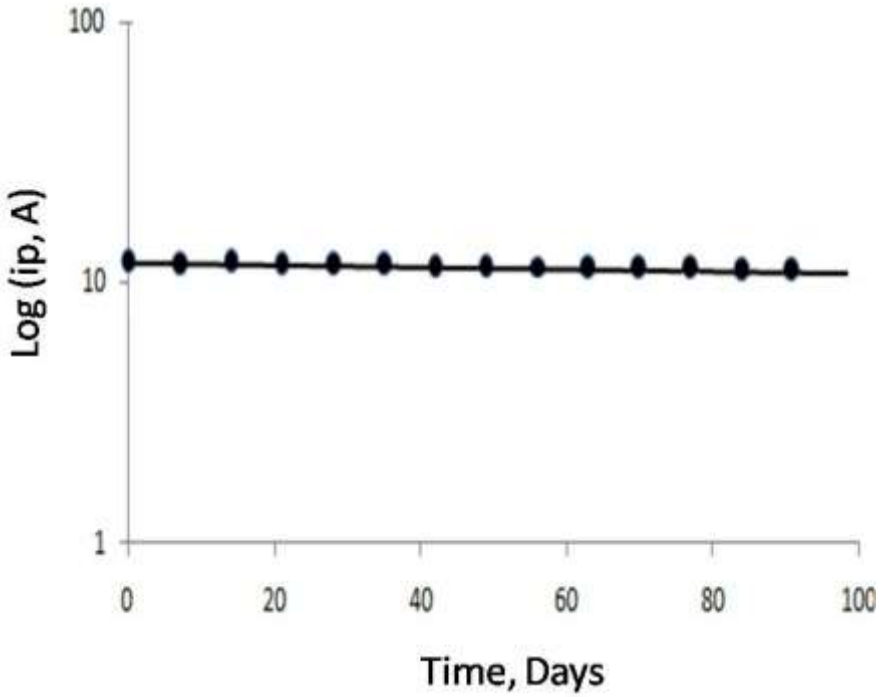
present in extract. The $t_{1/2}$ and t_{99} of standard WS V were 702.5 ± 53.8 days and 4670.0 ± 357.6 days, respectively. Thus, major withanolides in the WRF are stable for considerable longer period which may be attributed to the protective action of co-migrating unknown components.

WRF-S inhibited inflammatory process (paw volume) by 50%; while WRF-F was more effective and inhibited inflammatory process by 80% on the 4th hour. Based on the results it may be infer that the WRF has anti-inflammatory activity and on storage this activity gets reduced. It is possible that the fast decaying components may have anti-inflammatory activity which on storage gets decreased. The therapeutic potential and its correlation with withanolide content have already been pointed at in 2.3.7 of Chapter 2. It was noted that the decrease in antioxidant activity seems to correlate with the reduction in withanolide content and similar results were observed in this investigations. Therefore antioxidant activity of withanolides/- unidentified components may be potentiating the immunomodulatory activity of WRF.

4.5. Conclusions

The overall stability of WRF is investigated at room temperature for 6 months by using simple, reliable and cost effective techniques. This technique may beneficial to check the stability of the active principle and the potency of the herbal formulation and state the shelf-life of the herbal product.

Fig. 4.8: Decay of anodic peak current of WS V with time. The ordinate represents Log of anodic peak current in ip (A) units and the abscissa represents time in days. Each point used represents mean of three independent experiments.



Chapter 5

Hepatoprotective Effect of WRF in Acetaminophen Intoxicated Rat

5.1. Introduction

Use of acetaminophen (APAP) for therapeutic purpose was approved by the U.S. Food and Drug Administration in 1955 (Ahmad et al., 2012). APAP, commonly known as paracetamol is a commonly used analgesic and antipyretic drug which is generally regarded as safe and efficacious compared to aspirin especially for paediatric treatment (Anderson et al., 1999). However, overdose of APAP causes severe hepatotoxicity and necrosis in both humans and experimental animals (Katyare and Satav, 1991; Larson et al., 2005). The action of APAP as an analgesic drug is either through inhibition of prostaglandin synthesis or through active metabolite *p*-aminophenol. *p*-Aminophenol is conjugated to arachidonic acid by amide linkage to form the active archidonic acid metabolite: AM404; AM404 exerts therapeutic effect through cannabinoid receptors (Ottani et al., 2006). APAP is primarily metabolized in the liver by glucuronidation and sulfation. However, a small proportion undergoes cytochrome P450 (CYP_{450})-mediated bioactivation to from highly toxic metabolite N-acetyl-p-benzoquinoimine (NAPQI), which is rapidly quenched by glutathione (GSH) (James et al., 2003). After an overdose of APAP, elevated levels of NAPQI are generated which cause the depletion of glutathione (GSH). Subsequent to covalent adduct formation with of NAPQI, oxidative stress, lipid peroxidation and inhibition of plasma membrane Ca^{2+}-ATPase activity which disrupts Ca^{2+} homeostasis, together lead to necrosis (Moor et al., 1985). Although the precise biochemical mechanism of cell necrosis is not fully understood, it is generally believed that there is simultaneous involvement of GSH depletion, lipid peroxidation and oxidative stress (Randle et al., 2008). Overdose of APAP in humans is fairly common and is often associated with liver damage and compromised antioxidant metabolism in the kidneys (Ghosh and Sil, 2007).

Overdose APAP of can cause acute liver failure in humans as well as in experimental animals (Larson et al., 2005; Katyare and Satav, 1991). Silymarin -a known hepatoprotective agent - is a flavonoid obtained from the plant *Silybum marianum* also known as milk thistle. Silymarin comprises of three isomers: silybinin, silydianin and silychristin, of which silybinin is quantitatively the most important (Kshirsagar et al., 2009). Silybinin has been shown to inhibit the function of Kupffer cells which are well recognized for their fibrogenic mediator activity (Jeong et al., 2005). In addition, silymarin stabilizes the lipid structures in the hepatocellular membranes; this may apply in general to all cell membranes (Jeong et al., 2005).

The liver is a vital organ which regulates several important metabolic functions and is also responsible for maintaining homeostasis of the body. A number of chemical agents and drugs which are used routinely produce cellular as well as metabolic liver damage. The traditional system of medicine has a major role in the treatment of liver ailments (Mohan, 2012). The antioxidant and hepato-protective activity of Ashwagandha is known but detailed information on anti-inflammatory markers is not well known (Elberry et al., 2010). Thus, it offers an opportunity to work in detailed on mechanism of anti-inflammation of WRF. Therefore in this work the cell death pathway via anti-inflammatory pathway were focused.

5.2. Materials and Methods

5.2.1. Chemicals

1,1',3,3'-Tetraethoxypropane, crystalline beef liver catalase, reduced glutathione, 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), Folin-phenol reagent and silymarin were purchased from S.D. Fine Chemicals, Mumbai, India.

Sulphanilamide, naphthalamine diamine, and other analytical grade reagents and solvents were obtained from Loba Chemi Pvt. Ltd, Mumbai, India. RT-PCR was performed according to instructions provided by manufacturer (Biotools B & M Labs, Spain).

5.2.2. Plant Material

As described in section 3.2.2 of Chapter 3.

5.2.3. Preparation of WRF

Isolation and characterization of WRF was described in section 3.2.3.2 of Chapter 3.

5.2.4. Animals

Adult male albino rats of Wistar strain (8-10 week old) were used. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy, Bharti Vidyapeeth, Pune and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA/103/12).

5.2.5. Acute Oral Toxicity Tests for WRF

Acute oral toxicity test was performed for the WRF in male swiss albino mice according to the OECD guidelines using AOT 425 software.

5.2.6. Experimental design

The animals were randomly divided in 6 groups of 6 rats each as given below:

Group I: **Normal**: The animals were administered a single daily dose of 10 mg/kg of 1% aqueous solution of gum acacia p.o. for 14 days.

Group II: **APAP Treated:** The animals were administered a single daily dose of 10 mg/kg of 1% aqueous solution of gum acacia p.o. for 14 days 2 h prior to oral administration of APAP suspension (750 mg/kg) for 14 days.

Group III: **Silymarin Treated:** The animals were administered a single daily dose of 25 mg/kg of 1% aqueous solution of Silymarin in distilled water p.o. 2 h prior to oral administration of APAP suspension (750 mg/kg) for 14 days.

Group IV: **WRF (50 mg/kg):** The animals were administered a single daily dose of 50 mg/kg of 1% aqueous solution of WRF in distilled water p.o. 2 h prior to oral administration of APAP suspension (750 mg/kg) for 14 days.

Group V: **WRF (100 mg/kg):** The animals were administered a single daily dose of 100 mg/kg of 1% aqueous solution of WRF in distilled water p.o. 2 h prior to oral administration of APAP suspension (750 mg/kg) for 14 days.

Group VI: **WRF (200 mg/kg):** The animals were administered a single daily dose of 200 mg/kg of 1% aqueous solution of WRF in distilled water p.o. 2 h prior to oral administration of APAP suspension (750 mg/kg) for 14 days.

The selection of the dose regimen of APAP was according to Ahmad et al. (2012).

At the end of the experimental period, i.e. on the 15th day the animals were anesthetized using anesthetic ether and blood was withdrawn by retroorbital puncture and collected in tubes containing EDTA. After collecting the blood the animals were killed by cervical dislocation, the liver was excised and divided in two portions which were stored at -80°C. These portions were then used for biochemical estimations, RT-PCR studies and histopathological examination.

5.2.7. Serum Analysis

The blood samples were subjected to centrifugation at 7000 rpm using Eppendorf cryocentrifuge model No. 5810, at 4^0C to obtain clear serum samples. The levels of albumin, direct bilirubin, total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), triglyceride (TG), cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were measured by using commercially available kits according to the procedures provided by the manufacturer (Accurex Biomedical Pvt. Ltd., Mumbai, India).

5.2.8. Estimation of Oxidative Stress

20% (w/v) liver homogenates were prepared in 0.1 M tris-HCl buffer pH 7.4 and used for determination of malondialdehyde (MDA), nitric oxide (NO), total protein, reduced glutathione (GSH) and superoxide dismutase activity (SOD) according to methods reported previously (Kandhare et al., 2013).

5.2.9. Estimation of Total Antioxidant Capacity (TAC)

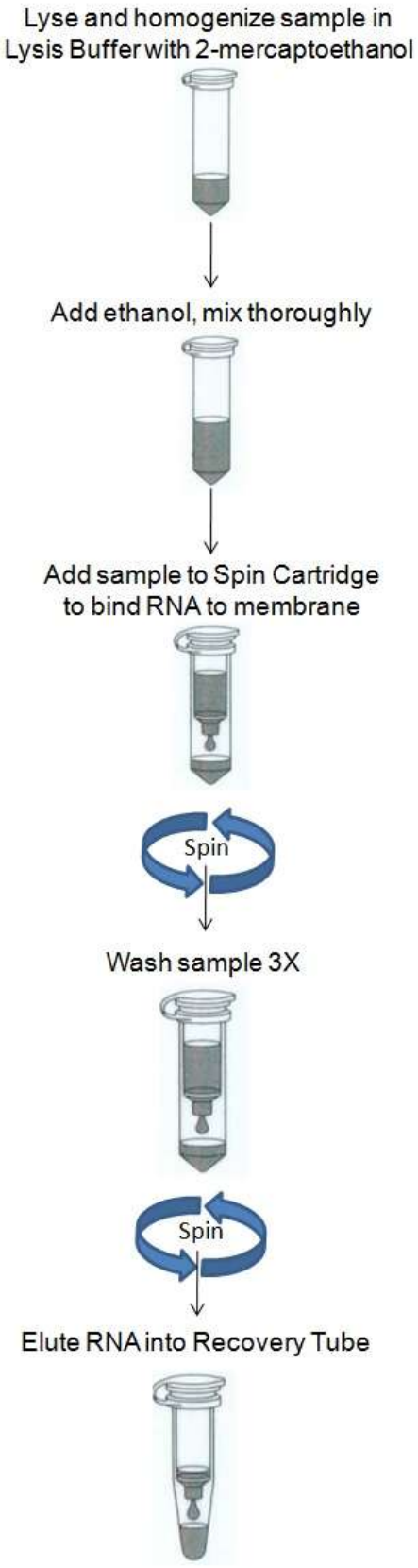
Liver homogenates (20% w/v) were prepared using phosphate buffered saline (pH 7.4) and the total antioxidant capacity was determined by using Autolab, PGSTA 101 cyclic voltammeter (Metrohm, Switzerland). A three-electrode system was used for the study. The working electrode: glassy carbon (Autolab GC) 8 mm in diameter was polished before each measurement; platinum wire served as an auxiliary electrode and saturated calomel electrode as the reference electrode. The cell contained 2 ml of phosphate buffered liver homogenate. All cyclic voltammogram measurements were performed in the range (–0.2) – (1.3) V at a scan rate of 400 mV/s. Each sample was analyzed in triplicate (Momen-Beitollahi et al., 2010).

5.2.10. RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

5.2.10.1. RNA Extraction

The RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed as per standard protocol provided by Biotools B & M Labs, Spain. Briefly, the liver tissue (30-40 mg) was disrupted in liquid nitrogen using mortar and pestle. To this 350 µl lysis buffer (provided in the kit) and 3.5 µl β-mercaptoethanol were added followed by vigorous vortexing. The lysate was loaded onto a filtering column in collection tube, and centrifuged for 1 min at 11,000 x g. The clarified lysate was mixed with 70% ethanol (350 µl) and loaded onto the RNA binding column in collection tube followed by centrifugation for 30 sec at 11,000 x g. Desalting buffer DBR (350 µl) and rDNase reaction mixture (95 µl) were added and incubated at room temperature for 15min. Following successive washings with wash buffer, RNA was re-suspended in RNAse-free water (60 µl) and centrifuged. The elute containing pure RNA was stored at -80°C until used for analysis (**Fig. 5.1**).

Fig 5.1: Schimatic digram of RNA isolation.



5.2.10.2. RT-PCR Analysis

Single-stranded cDNA was synthesized from total cellular RNA using reverse transcriptase polymerase chain reaction. Briefly, total RNA (2µl) was treated with 100 mM magnesium sulfate solution (10 µl), the primers (5 µl) and PCR astringent (12 µl). The volume was made up to 50 µl with nuclease free water. The primer sequences for TNF-α, Il-1β, iNOs and COX-II were synthesized by Amnion Biosciences Pvt. Ltd., India (**Table 5.1**). Amplification of β-actin served as a control for sample loading and integrity. Polymerase chain reaction products were detected by electrophoresis on a 1 % agarose gel containing ethidium bromide. Size of amplicons was confirmed by using 100-bp ladder (Amnion Biosciences Pvt. Ltd., India) as a standard size marker. The amplicons were visualized and images were captured using gel documentation system (Alpha Innotech Inc. USA). Expression of the genes was assessed semi-quantitatively by generating densitometry data for band intensities in different sets of experiments and analyzing the gel images using software Image J program (Version 1.33, USA). The band intensities were compared with constitutively expressed β actin which served as a control for sample loading and integrity. The intensity of product mRNAs was standardized against that of the β-actin mRNA from each sample and results were expressed as product (primer) mRNA/ β-actin mRNA ratio.

Table 5.1: Primer sequences for TNF-α, IL-1β, iNOs, COX-II and β-actin

Gene	Primer sequences (5'–3')	Length (bp)
TNF-α	5'-CAGCCTTGTCCCTTGAAGAGAACC-3' 5'-TACTGAACTTCGGGGTGATTGGTCC-3'	295
IL-1β	5'-TGATGTTCCCATTAGACAGC-3' 5'-GAGGTGCTGATGTACCAGTT-3'	378
iNOs	5'-ACCTTCCGGGCAGCCTGTGA-3' 5'-CAAGGAGGGTGGTGCGGCTG-3'	238
COX-II	5'-ACACTCTATCACTGGCATCC-3' 5'-AAGGGACACCCTTTCACAT-3'	548
β-actin	5'-CCTCTATGCCAACACAGTGC-3' 5'-CCTGCTTGCTGATCCACATC-3'	764

5.2.11. Histological Examination

The liver tissue was kept in 10% formalin for 24 h at room temperature and dehydrated by passing through grades of ethyl alcohol (70, 90 and 100%). Following this, the samples were placed in xylene for 1h with the solvent being replaced 3 times. The tissue was then fixed in paraffin wax, and sections of 3-5 µm thickness were obtained using a microtone. The samples were stained with hematoxylin and eosin. The specimens were mounted on slides uing Distrene Pthalate Xylene (DPX) as mounting medium. Sections were examined under a light microscope for inspection of the histopathological features and infiltration of cells. The changes in histological features were graded as Grade 0 (not present or very slight) l; Grade 1 (mild); Grade 2 (moderate); and Grade 3 (severe).

5.2.12. Statistical Analysis

The data were analyzed by one way analysis of variance test (ANOVA) using Graph Pad Prism 5.0 software (Graph Pad, San Diego, USA). Dunnett's test was applied for posthoc analysis. The results are given as mean ± SEM. Values of $P < 0.05$ were considered to be statistically significant.

5.3. Results

5.3.1. Characterization of WRF

This is already described in Chapter 3.

5.3.2. Acute Toxicity Studies

Acute toxicity studies showed no toxic signs and symptoms such as restlessness, respiratory distress, diarrhea, convulsions and coma; WRF was safe up to 2000 mg/kg.

5.3.3. Effects of WRF on Serum Parameters

APAP treatment resulted in significant decrease in serum albumin level with significant increase in the direct bilirubin, total bilirubin, ALP, AST, ALT and LDH activities. Treatment with WRF (50, 100 and 200 mg/kg) did not show any significant change in the serum albumin level. However, direct bilirubin and total bilirubin levels decreased significantly in the WRF (200 mg/kg) treated group as compared to APAP-treated rats. There was significant and dose-dependent decrease in the ALP, AST, ALT and LDH in the WRF (100 and 200 mg/kg) treated rats as compared to the APAP-treated group. Treatment with silymarin significantly lowered the elevated levels of direct bilirubin, total bilirubin, ALP, AST, ALT and LDH as compared to the APAP-treated rats (**Table 5.2**).

5.3.4. Effects of WRF on Plasma Lipid Parameters

In APAP-treated rats TG, cholesterol and LDL levels were significantly elevated whereas HDL level decreased significantly as compared to normal rats. WRF treatment (100 and 200 mg/kg) significantly restored the altered levels of TG, cholesterol, LDL and HDL in a dose-dependent manner. Silymarin treated rats also showed significant reduction in elevated levels of TG, cholesterol, LDL and restored the altered levels of HDL (**Table 5.3**).

5.3.5. Effect of WRF on Antioxidant Parameters

The MDA and NO levels increased significantly in the APAP-treated rats; the elevated level of MDA and NO decreased significantly in a

dose-dependent manner following WRF treatment. Silymarin treated rats also showed decreased level of MDA and NO (**Table 5.4**).

The anodic peak potential (ip) characterizes are shown (**Fig. 1.1**). ip corresponds to the concentration of antioxidants in terms of total antioxidant capacity (TAC). The potential at the maximum of anodic wave reflects the reducing ability of antioxidants present in the liver tissue. As can be noted there was significant decrease in the TAC in APAP-treated rats as compared to normal controls. The decreased levels of TAC showed dose-dependent increase by WRF treatment (**Fig. 5.2, Table 5.4**).

There was significant decrease in the GSH and SOD levels in the APAP-treated rats as compared to normal controls. The decreased levels of GSH and SOD showed dose-dependent increase following WRF (100 and 200 mg/kg) treatment. Silymarin treated rats also showed elevated GSH and SOD levels (**Table 5.4**).

Table 5.2: Effect of treatment with WRF on APAP-induced alteration of serum levels of albumin, direct bilirubin, total bilirubin, ALP, AST, ALT and LDH in rats

Animals	Albumin (mg %)	Direct bilirubin (mg %)	Total bilirubin (mg %)	ALP (IU/l)	AST (IU/l)	ALT (IU/l)	LDH (mg %)
Normal	3.0 ± 0.4	0.1 ± 0.04	0.1 ± 0.02	39.2 ± 3.0	125.8 ± 7.8	21.8 ± 1.8	203.5 ± 48.62
APAP-treated	1.6 ± 0.1$^{\#\#}$	0.5 ± 0.08$^{\#\#\#}$	0.2 ± 0.02$^{\#\#\#}$	217.9 ± 15.8$^{\#\#\#}$	293.5 ± 29.1$^{\#\#\#}$	142.0 ± 10.2$^{\#\#\#}$	2989 ± 362.2$^{\#\#\#}$
Silymarin (25)+ APAP	1.6 ± 0.2	0.3 ± 0.02*	0.1 ± 0.02**	126.2 ± 8.9***	146.4 ± 19.8***	33.4 ± 7.7***	2470 ± 192.1
WRF (50)+ APAP	1.5 ± 0.2	0.4 ± 0.04	0.2 ± 0.03	199.9 ± 23.4	249.3 ± 11.3	127.2 ± 21.6	2626 ± 383.7
WRF (100)+ APAP	1.7 ± 0.2	0.3 ± 0.06	0.2 ± 0.03	159.1 ± 18.4*	200.6 ± 26.8*	75.1 ± 7.9**	1588 ± 305.6**
WRF (200)+ APAP	2.4 ± 0.0	0.2 ± 0.01***	0.1 ± 0.03*	129.2 ± 8.1**	132.3 ± 26.3***	50.0 ± 7.7***	1155 ± 295.9***

WRF: Withanolide rich fraction, ALP: Alkaline phosphatase, AST: Aspartate transaminase, ALT: Alanine transaminase and LDH: Lactate dehydrogenase. Data are expressed as mean ± SEM (n = 6) and analyzed by one-way ANOVA followed by Dunnett's test for each parameter separately. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to control group and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared to normal group.

Table 5.3: Effect of treatment with WRF on APAP-induced alteration in serum TG, cholesterol, LDL and HDL in rats

Treatment	TG (mg %)	Cholesterol (mg %)	LDL (mg %)	HDL (mg %)
Normal	63.1 ± 4.0	14.3 ± 1.3	1.1 ± 0.3	65.3 ± 4.7
APAP-treated	159.5 ± 4.4$^{\#\#\#}$	48.8 ± 5.4$^{\#\#\#}$	5.6 ± 0.7$^{\#\#\#}$	21.5 ± 2.8$^{\#\#\#}$
Silymarin (25)+ APAP	106.5 ± 7.1***	27.2 ± 2.9***	1.6 ± 0.6***	40.6 ± 5.4*
WRF (50)+ APAP	141.8 ± 9.3	45.5 ± 2.1	5.6 ± 0.7	28.8 ± 5.7
WRF (100)+ APAP	116.2 ± 11.8**	33.7 ± 2.7**	3.4 ± 0.5	43.7 ± 6.0*
WRF (200)+ APAP	91.46 ± 6.0***	19.5 ± 1.5***	2.6 ± 0.4**	61.1 ± 2.2***

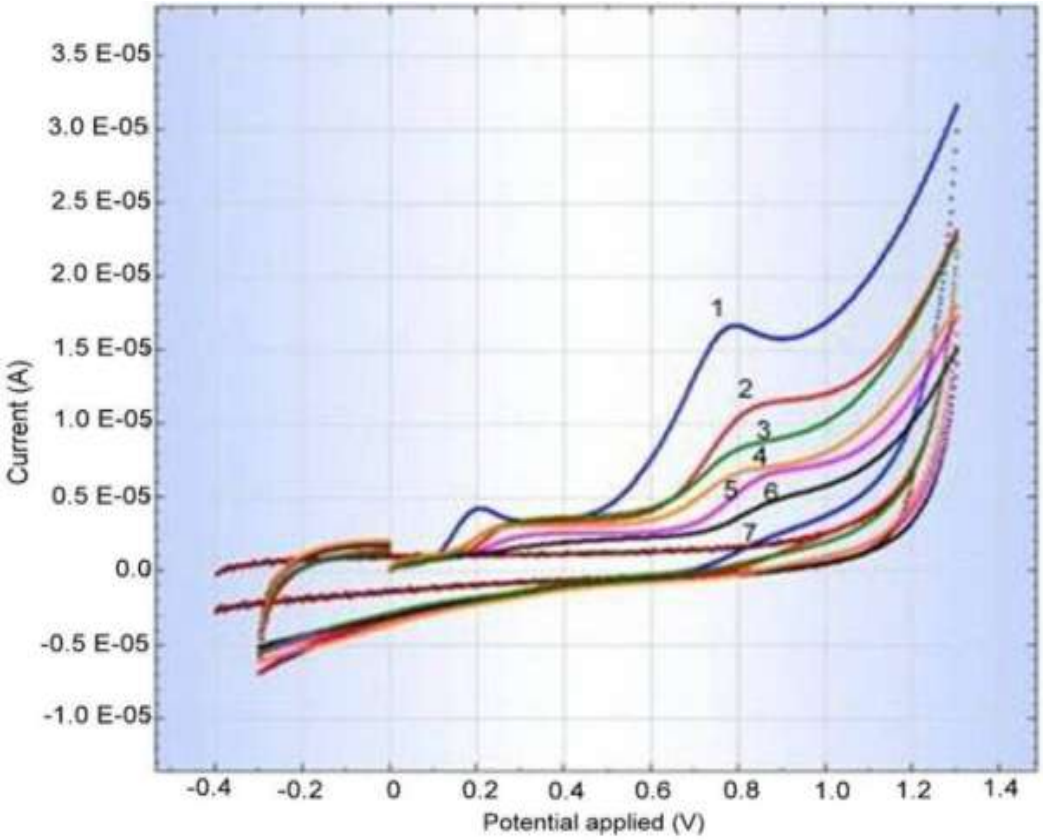
TG: Triglyceride, LDL: Low-density lipoprotein and HDL: High-density lipoprotein. Data are expressed as mean ± SEM (n = 6) and analyzed by one-way ANOVA followed by Dunnett's test for each parameter separately. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to control group and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared to normal group.

Table 5.4: Effect of treatment with WRF on APAP-induced alteration on antioxidant parameters in rat liver.

Treatment	MDA (nM/mg protein)	of NO (µg/mL)	GSH (µg/mg of protein)	Total antioxidant capacity is given as ip (A) X 10^6	SOD (U/mg of protein)
Normal	0.4 ± 0.1	128.4 ± 9.2	0.42 ± 0.03	12. ± 0.1	1.2 ± 0.07
APAP-treated	1.7± 0.2$^{\#\#}$	304.9 ± 16.1$^{\#\#\#}$	0.10 ± 0.01$^{\#\#\#}$	3.27 ± 0.5$^{\#\#\#}$	0.2 ± 0.04$^{\#\#\#}$
Silymarin (25)+ APAP	0.8± 0.2*	207.9 ± 7.1**	0.37 ± 0.06***	6.62 ± 0.5***	0.9 ± 0.06***
WRF (50)+ APAP	1.5 ± 0.1	282.5 ± 18.3	0.17 ± 0.01	4.12 ± 0.5	0.3 ± 0.05
WRF (100)+ APAP	1.1± 0.2*	228.6 ± 19.8*	0.29 ± 0.04*	5.52 ± 0.4	0.6 ± 0.07**
WRF (200)+ APAP	0.63± 0.2**	204 ± 31.4**	0.32 ± 0.05**	6.18 ± 0.4**	1.0 ± 0.04***

MDA: Malondialdehyde, NO: Nitric Oxide, GSH: Glutathione Peroxidase and SOD: Superoxide dismutase. Data are expressed as mean ± SEM (n = 6) and analyzed by one-way ANOVA followed by Dunnett's test for each parameter separately. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to control group and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ as compared to normal group.

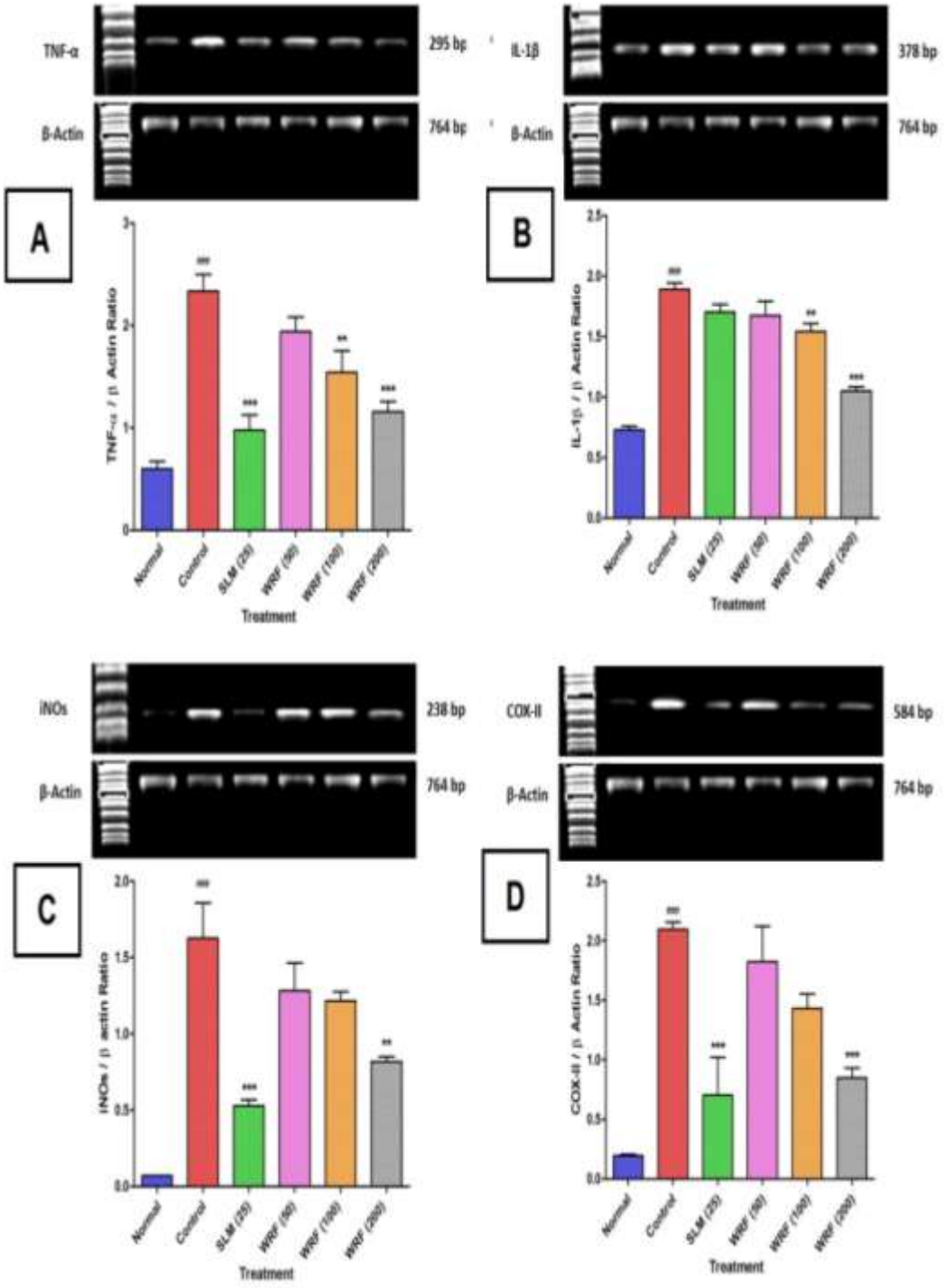
Fig. 5.2: Superimposed cyclic voltammograms of rat liver phosphate buffer saline; (1) normal, (2) Silymarin, (3) WRF 200, (4) WRF 100, (5) WRF 50, (6) APAP-treated and (7) blank (phosphate buffer alone).



5.3.6. Effect of WRF on TNF-α, IL-1β, iNOS and COX-II mRNA Expression

There was significant up-regulation in the TNF-α, IL-1β, iNOS and COX-II mRNA expression in APAP-treated rats as compared to normal controls. WRF (100 and 200 mg/kg) treatment significantly down-regulated TNF-α and IL-1β mRNA expression in a dose-dependent manner. Up-regulated iNOS and COX-II mRNA expression was significantly reduced only after treatment with 200 mg WRF. Silymarin treatment showed significant down-regulation in TNF-α, iNOS and COX-II mRNA expression but failed to produce any significant alteration in IL-1β mRNA expression (**Fig. 5.3**).

Fig. 5.3: Effect of WRF treatment on APAP-induced alteration in reverse transcriptase analysis of mRNA expression of TNF-α (A), IL-1β (B), iNOs (C) and COX-II (D).



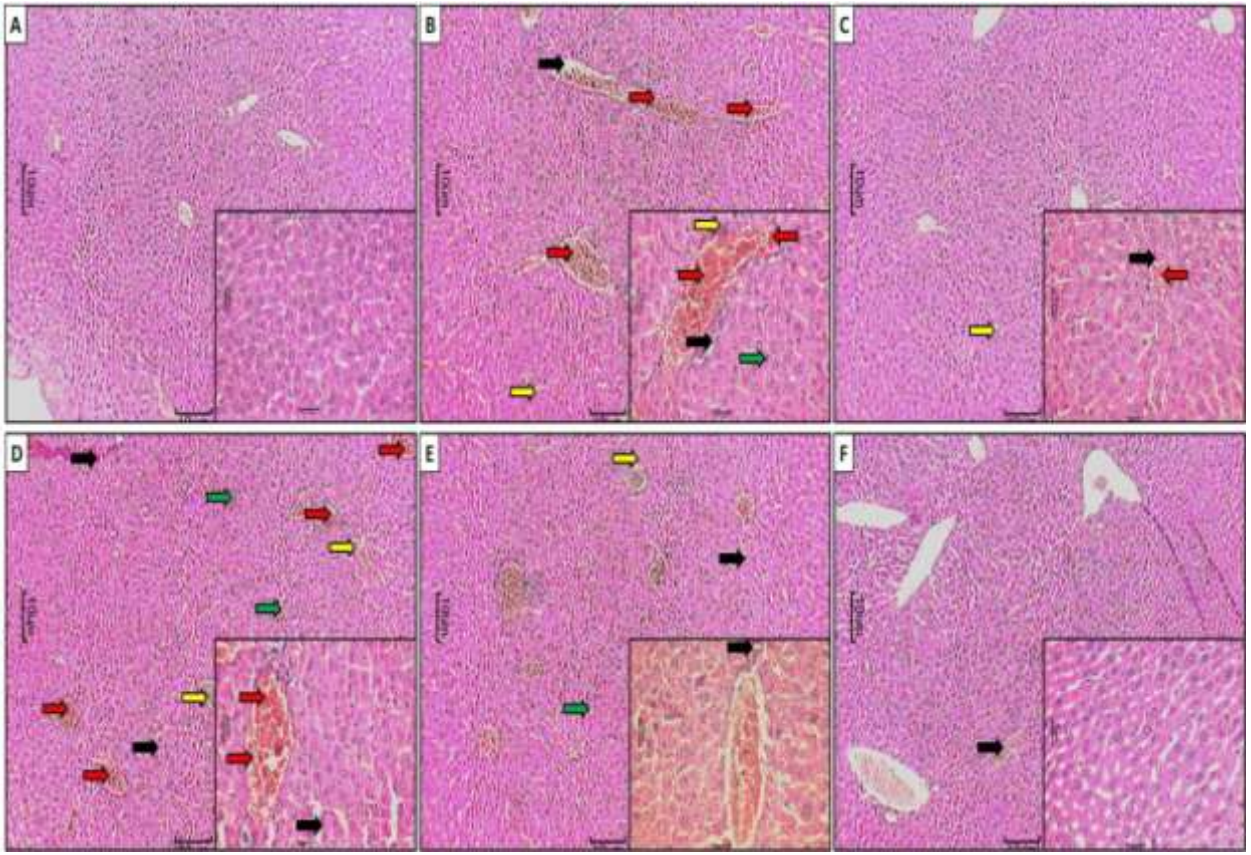
Data are expressed as mean ± S.E.M. and analyze by one-way ANOVA followed by Dunnett's test. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 as compared to control group and #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 as compared to normal group.

5.3.7. Histopathological Observations

Presence of the normal central vein with portal triads without any evidence of necrosis (grade 0) and inflammatory infiltration (grade 0) reflected the normal architecture of liver tissue (**Fig. 5.4A**). There was no evidence of vesicular or fatty changes (grade 0). However, presence of mild degree of congestion (grade 1) was noted. Histopathological studies of the liver tissue from APAP-treated rats showed presence of centrilobular necrosis (grade 3) with inflammatory infiltration (grade 3) around the centrilobular veins with periportal degeneration. It showed the focal hyaline degeneration of hepatocytes along with macrovesicular fatty changes (grade 4) (**Fig. 5.4.B**). Liver from silymarin treated rats showed mild necrosis (grade 2) and scant number of inflammatory cells mainly macrophages (grade 1) were seen around centrilobular veins. There was no evidence of periportal degeneration. However; it showed vacuolization (grade 1) with macrovesicular fatty changes (grade 1) (**Fig. 5.4.C**). Liver from WRF (50 and 100 mg/kg) treated rats showed necrosis (grade 3 and grade 2, respectively) and congestion around central vein (grade 2). Inflammatory cells mainly macrophages and plasma cells (grade 3) were seen along with macrovesicular fatty changes (grade 3 and grade 1, respectively). It also showed focal hepatic hyaline degeneration (**Fig. 5.4.D and 5.4.E**). Histology of liver tissue from WRF (200 mg/kg) treated rats showed normal architecture. Hepatocytes were unremarkable; central veins and portal triads were normal. However, it showed mild necrosis (grade 1) without any infiltration of inflammatory cells. There was no evidence of macrovesicular fatty changes or congestion (**Fig. 5.4.F**; **Table 5.5**).

Fig. 5.4: Effect of WRF treatment on APAP-induced pathological alteration in rat liver**.**

Photomicrograph of sections of liver of normal (A), APAP-treated (B), Silymarin (25 mg/kg) treated (C), WRF (50 mg/kg) treated (D), WRF (100 mg/kg) treated (E) and WRF (200 mg/kg) treated (E) rats. H&E staining at 40 X and 100 X (inset).



Inflammatory infiltration (red arrow), congestion (yellow arrow), pykonsis (green arrow) and necrosis (black arrow).

Table 5.5: Effect of WRF on APAP-induced histology alterations in rat liver

Treatment	Necrosis	Congestion	Vesicular fat	Inflammatory cells	Pykonsis	Vaculization	Plasma cells
Normal	-	+	-	-	-	+	-
APAP-treated	+++	+++	++++	+++	++	+++	++++
Silymarin (25)+ APAP	++	++	+	+	-	+	+
WRF(50)+ APAP	+++	++	+++	+++	++	+++	+++
WRF(100)+ APAP	++	++	+	-	+	+	-
WRF(200) + APAP	+	-	-	-	-	-	-

Note:

-, No abnormality detected,

+, Damage/ active changes up to less than 25%,

++, Damage/ active changes up to less than 50%,

+++, Damage/ active changes up to less than 75%,

++++, Damage/ active changes up to more than 75%.

5.4. Discussion

Overdose of APAP in human is fairly common and is often associated with hepatic damage in humans as well as animals (Larson et al., 2005; Katyare and Satav, 1991). Ashwagandha shows several pharmacological activities; hpatoprotective activity is one of them (Malik et al., 2013). Hence detailed investigations were undertaken to study the hepatoprotective properties of WRF employing APAP-induced hepatotoxicity in rat model.

In WRF concentration of WF A is much higher than that of other withanolides which is followed by WN A while WN B. WS V, 1, 2 DWM and WNN are present in low concentration (**Table 3.3 in Chapter 3**). WF A has highest antioxidant activity followed by WN B, WN A, WS V, 1,2 DWM and WNN, respectively (**Table 3.3 in Chapter 3**).

The estimation of enzymes in the serum is a useful quantitative marker of the extent and type of hepatocellular damage. The increase in the serum AST and ALT levels has been attributed to the damaged structural integrity of the liver (Ahmad et al., 2012). In the present study significant elevation in the marker enzymes viz. AST, ALT, ALP and LDH, and levels of total bilirubin and direct bilirubin in serum were noted following APAP administration. The increased levels of these serum biomarkers in APAP-intoxicated rats indicate a deterioration of the hepatic structure/ function due to liver membrane damage (Jaeschke et al., 2003).

It has been reported that aqueous extract of roots of Ashwagandha (500 mg/kg) lowered the increased activities of liver markers enzymes (Malik et al., 2013). It has been suggested that the prevention of the leakage of intracellular enzymes in serum of APAP-intoxicated rats might be due to the membrane stabilizing activity of WRF of Ashwagandha (Ganguly et al., 2009).

Reduced serum albumin is a hallmark of liver diseases and Increased levels of serum bilirubin indicate impaired capacity of the liver to store bilirubin (Jaeschke et al., 2003; Ganguly et al., 2009). In the present studies it was observed that WRF at higher concentration could offer some protection in this regard.

It has been well documented that significant alterations occurred in lipoprotein and cholesterol metabolism after APAP intoxication (Kaushal et al., 1999; Malik et al., 2013). With increased availability of free fatty acids, the triglycerides levels were significantly increased in APAP-induced toxicity which leads to decreased hepatic release of lipoprotein and increased esterification of free fatty acids (Malik et al., 2013). Histological examination of the liver tissue from APAP-treated rats showed macro-vesicular fatty changes (grade 4) (**Fig. 5.4.B**) this also supports the above findings. Treatment with WRF significantly reduced cholesterol and triglyceride levels which correlated with histopathological findings in which liver tissue from WRF treated rats did not show any evidence of macro-vesicular fatty changes (**Fig. 5.4.F**).

Insult to the cell caused significant leakage of the LDH from cell cytoplasm into the serum (Shiva Kumar et al., 2014). Results of the present studies are consistent with this finding (**Table 5.2**). In the diagnosis of hepatic damage serum LDH plays an important role (Shiva Kumar et al., 2014). WRF significantly prevented the increase in the level of serum LDH implying hepatic protection.

It has been reported that the highly reactive intermediate of APAP metabolism NAPQI has great affinity for GSH which causes depletion of cellular GSH leading to damage to the liver cells due increased reactive oxygen species (ROS) (Mitchell et al., 1973). SOD plays an important role in eliminating the superoxide radicals. GSH is a non-enzymatic biological antioxidant which plays an important role in quenching of free radical species such as hydrogen peroxide, superoxide and alkoxy radicals (Visnagri et al., 2013). Decreased

levels of GSH and SOD correlates with the toxic effects of ROS produced by APAP which is consistent with previously reported observations. Treatment with WRF caused elevation of GSH and SOD level indicating protection from APAP-induced liver damage (Olaleye and Rocha, 2008).

Membrane lipid-peroxidation (in terms of MDA) reflects the damage to the cellular membranes structure via destruction of the double bonds in the unsaturated fatty acids and is considered to be a reliable marker of oxidative stress (Kandhare et al., 2013). This is evident from the reduction of antioxidant status of APAP intoxicated rats (Malik et al., 2013). In the present investigation increase in the MDA level was noted in the APAP-treated rats. Administration of WRF significantly lowered the elevated level of MDA which corroborates the results of earlier reports for Ashwagandha root powder and aqueous extracts suggesting its protective role in APAP mediated liver injury (Malik et al., 2013).

Elevated oxidative stress caused release of pro-inflammatory mediators such as nitric oxide (NO) via inducible nitric oxide synthase in APAP intoxicated liver which results in cellular dysfunction (Ahmad et al., 2012). Results of the present investigations are consistent with the finding of previous reports where administration of APAP significantly increased NO level in liver (Ahmad et al., 2012). An effort has been made to improve hemodynamic properties of the liver by inhibiting the hepatic microvascular constriction by administration of compounds with NO scavenging or nitric oxide synthase (NOS) inhibitors (Malik et al., 2013). Treatment with WRF significantly reduced the elevated level of NO suggesting its protective role in APAP-induced hepatotoxicity (**Table 5.4**).

Cyclic voltammeter (CV) can be of use to determine the total antioxidant capacity (TAC) in plasma or in other biological fluids,

tissue homogenates and skin (Momen-Beitollahi et al., 2010). For first time CV was used to measure the TAC of liver in APAP-treated and WRF treated rats. The decrease in antioxidant levels in APAP-intoxicated rats as compared to treated rats was observed; the CV analysis also showed that APAP caused a significant decrease in liver TAC. The elevated level of oxidative stress in APAP-treated rats correlated well with the results of CV where TAC of liver tissue decreased significantly. These results also correlate with other antioxidant parameters (**Table 5.4**).

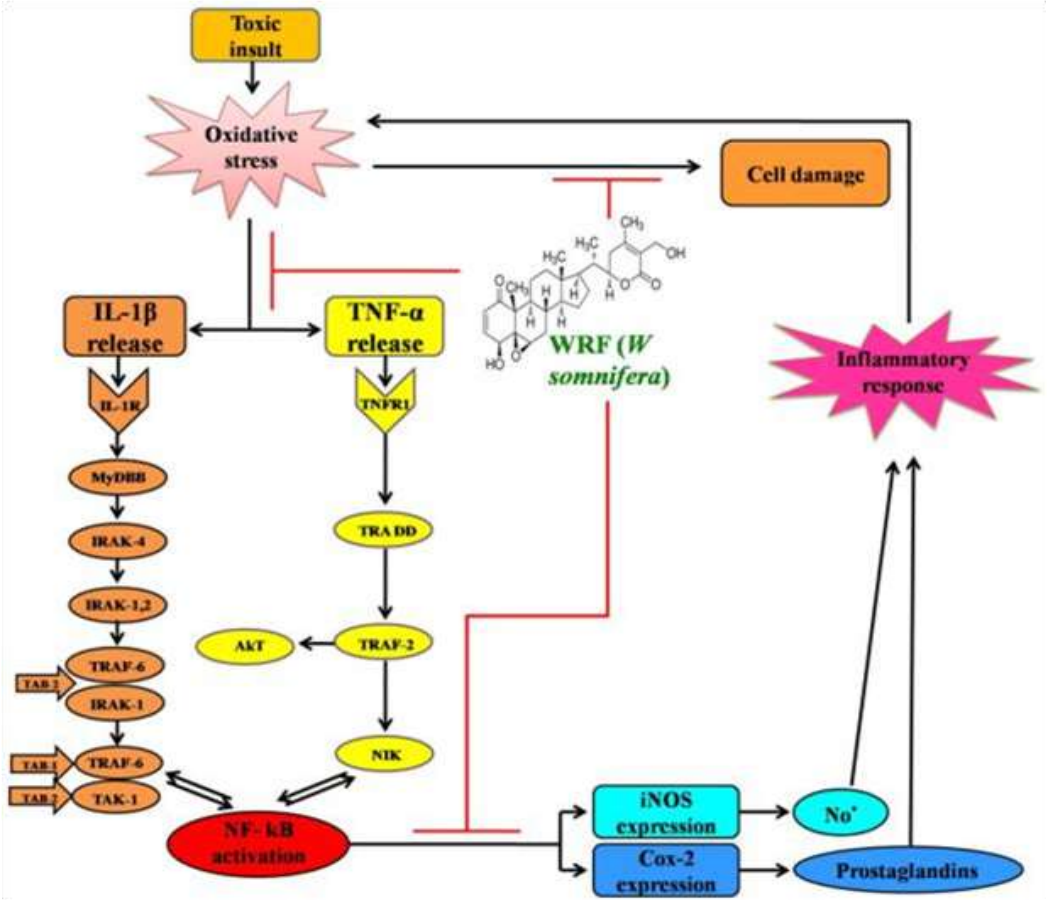
It has been reported that NF-κB (nuclear transcription factor-κB) plays decisive role in regulation of various gene expressions such as IL-1β, TNF-α, and COX-2. These genes play critical role in tumorigenesis, various autoimmune diseases and inflammation (Visnagri et al., 2013). *In vitro* studies carried out in murine J774.2 monocytes and macrophages showed that APAP caused significant activation of NF-κB, caused release of pro-inflammatory mediators TNF-α and IL-1β and thus elevated oxidative stress (Al-Belooshi et al., 2010). It has been reported that APAP-induced hepatotoxicity is associated with elevated levels of pro-inflammatory cytokines such as TNF-α and IL-1β (Tilg et al., 1992). Cytokines are associated with mediation of various inflammatory conditions. In an array of diseases, TNF-α is pro-inflammatory cytokine which in turn triggers a cascade of interleukins like IL-1 β, IL-6 and IL-8. IL-1 β which is synthesized as 31 kDa precursor peptides and converted into mature cytokine of 17 kDa which triggers inflammatory response resulting in tissue necrosis (Ahmad et al., 2012). Results of the present investigations are in accordance with the findings of previous investigators (Ahmad et al., 2012). Treatment with WRF significantly lowered the elevated oxidative stress and thus in turn down-regulated expression of pro-inflammatory cytokines (TNF-α and IL-1β). The results of the present studies are in agreement with findings of previous investigators where administration of alcoholic

extract of Ashwagandha showed inhibition of the cytokines activity in mice by down-regulation of NF-κB (Grover et al., 2010).

NF-kB has been reported to regulate the gene expression and subsequent mRNA translation of iNOS. Various studies have demonstrated that APAP intoxication leads to up-regulation of iNOS protein and nitric oxide production in hepatocytes (Ahmad et al., 2012). In the present investigation we noted up-regulation of NF-kB, iNOS and nitric oxide production was noted in the APAP treated group. WRF administration inhibited iNOS expression and nitric oxide production thereby alleviating hepatic inflammation and damage. Inflammatory insult by APAP caused overproduction of prostaglandins controlled by release of rate-limiting enzyme cyclooxygenase-2 (COX-II) (Ahmad et al., 2012). Data of the present studies show overexpression of COX-II synthesis, which is up-regulated by inflammatory stimuli or lipid hyperoxidation induced synthesis of PG, which causes liver injury. Treatment with WRF significantly down-regulated COX-II expression and thus reduced the inflammation, confirming the anti-inflammatory activity of WRF. Earlier reports state that leaf extract Ashwagandha and withaferine A inhibited the expression of inflammation markers TNF- α, NF-kB, COX-II and IKKβ (Kaileh et al., 2007; Grover et al., 2010, 2012b). We observed similar results in our present studies. Based on the results of the present studies it may be suggested that WRF down-regulates NF-kB regulated gene products such as COX-II and iNOS induced by TNF-α and IL-1β activation (**Fig. 5.3**). **Fig. 5.5** shows the proposed mechanism of hepatoprotective activity of WRF.

Fig. 5.5: Scheme showing the proposed mechanisms of hepatoprotective activity of WRF

Toxic exposure could induce oxidative stress in the liver due to its metabolization into highly reactive free radicals. Oxidative stress triggers the IL-1β and TNF-α release from Kupffer cells and injured hepatocytes, which further activates NFkB, allowing its nuclear translocation. Consequently, NFkB stimulates the expression of iNOS and COX-2 at the level of transcription, translation, and the enzyme level. The final products of iNOS and COX-2, NO• and prostaglandins, respectively, contributes to nitrosative stress and, on the other hand, initiate the cascade of inflammatory response in injured liver. Inflammation, in turn, is associated with the release of highly reactive oxygen and nitrogen species from inflammatory cells, further exacerbating oxidative and nitrosative stress. WRF prevents oxidative damage, as indicated by the decrease in lipid peroxidation, and improves the antioxidant status. Furthermore, WRF suppresses the inflammatory response by down regulating the proinflammatory cascade initiated by TNF-α and IL-1β, and attenuates nitrosative stress by the iNOS inhibition.



5.5. Conclusions

From present studies it may be concluded that the WRF exercises its hepatoprotective effect in APAP-treated rats through its antioxidant potential. The results also suggest that cyclic voltammeter may be an alternative rapid method for determining loss of antioxidant as a measure of toxicity. The findings of the present studies also suggest that the anti-inflammatory activities of WRF of Ashwagandha are possibly mediated through suppression of the TNF-α and IL-1β.

Chapter 6

Evaluation of the Bioavailability of Major Withanolides of Ashwagandha

6.1. Introduction

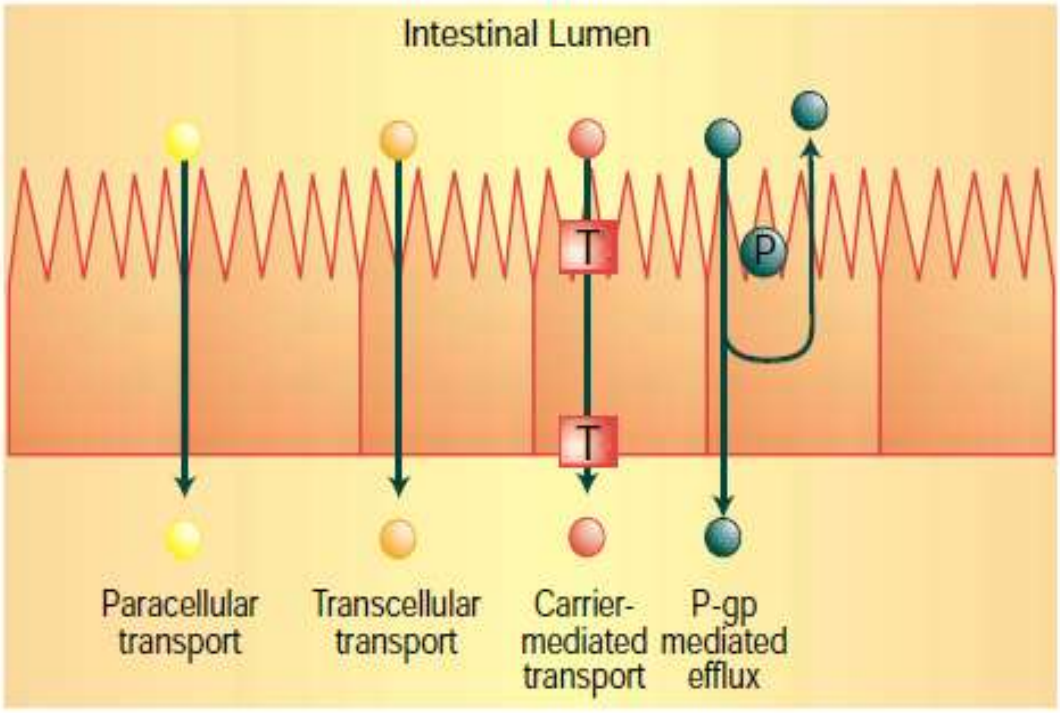
Therapeutic efficacy and stability of WRF was evaluated and reported in the previous chapters of this thesis. It was noted that the content of withanolides in roots are very important for the therapeutic activity of Ashwagandha. Many researchers have reported that individual withanolides have pharmacological activity. It means that individual withanolides can be used as therapeutic drug. The estimation of bioavailability of compounds is the most important initial step in drug designing and drug development. Therefore, it becomes essential to evaluate the bioavailability of individual withanolides. *In vitro* cell culture systems may therefore be a good option for rapid bioavailability testing of withanolides.

The U.S. Food and Drug Administration (FDA) define bioavailability as "the rate and extent to which the active drug ingredient is absorbed from a drug product and becomes available at the site of drug action". In practice it is rare that drug concentrations can be determined at the site of action (Dalton and Yates, 2007). Hence, usually bioavailability refers to the absorption of a drug from an *in vitro* absorption model gastrointestinal tract following oral administration of a dosage form. The dosage form may be any type of product, including a solution, suspension, tablet, capsule or powder. Bioavailability can also refer to other types of dosage form, such as intramuscular injections, ointments and other topical preparations, transdermal patches, and implants, which also require an absorption step prior to reaching the systemic circulation (Sachan et al., 2009; Makanikar and Parekh, 2011). Although many compounds show a promising *in vitro* therapeutic potential, they cannot be used *in vivo* due to bioavailability problems. The intestinal absorption of a compound may occur via passive diffusion (paracellulary or transcellularly), or via carrier-mediated active transport (eg. D-glucose, dipeptides). The intestinal absorption of a compound may be hindered by P-glycoprotein (P-gp), an ATP-

dependent multidrug for the permeation of compounds across the MDCK cell monolayer (**Fig. 6.1**). The membrane transport properties of novel compounds can thereby be assessed using these differentiated cell monolayers (Engman et al., 2001).

The intestinal epithelium plays an important role in drug absorption and transportation. Thus it becomes important to determine the bioavailability of a drug in the intestine. *In vitro* cell culture may therefore be useful model system to quickly assess the bioavailability of the given drug (Pang et al., 1996; Owens et al., 1976). A Sino-Veda MDCK *in vitro* cell culture system shows characteristics similar to *in vivo* intestinal epithelium (Tam et al., 2000). In view of this for the evaluation of bioavailability profiles of major withanolides, Sino-Veda MDCK cell culture system was used.

Fig. 6.1: Mode of absorption of compounds in intestinal lumen.



6.2. Materials and Methods

6.2.1. Standard Withanolides

The authentic withanolides described in section 2.2.1 of Chapter 2 were used for bioavailability study. The purity of the standards was established by HPLC analysis **(Fig. 6.2)**.

The bioavailability studies were facilitated through active collaboration with **Sino-Veda (Canada)**. They had developed a patented method, standardized with Ginseng for *in vitro* bioavailability studies. Therefore in order to carry out bioavailability of withanolides, standard withanolides were sent to Sino-Veda laboratory (**Edmonton, Canada**) and the results are presented below.

6.2.1. HPLC-MS Analysis

For evaluating the permeability of major withanolides, standard authentic individual withanolides were diluted with Hank's buffered saline and tested for permeability by the Sino-Veda's proprietary cell culture system (**Fig. 6.3)**. For determining the concentration of withanolides, high performance liquid chromatography (HPLC) coupled to diode array absorbance detection (DAD) and positive mode electrospray ionization mass spectroscopy (ESI) was employed. A Phenomenex Luna 3µ C18 (2) 100A 15 cm X 4.60 mm column equipped with a guard column (security guard C18) and column heater set at 40°C was used. For HPLC-MS analysis mobile phase A was 5 mM ammonium acetate (pH 3.0 made with formic acid) in 18 mega Ω water, and mobile phase B was HPLC grade acetonitrile. Based on withanolides behavior in cell culture system the mobile phase gradient program was modified to obtain better resolution. Flow rate was 0.7 mg/mL and solvent gradient program for WF A, WN A, WN B, WNN and 1,2 DWM was 0-15 min A: B (60: 40%), 15-20 min A: B (15: 85%) and 20- 25 min A: B (60: 40%). For 0-15 min A: B (70: 30%), 15-20 min A: B (15: 85%) and 20- 25 min A: B (70: 30%). Mobile phase program for WS V was 0-15 min A: B (70: 30%),

Fig. 6.2: The major withanolides of Ashwagandha and their HPLC elution profile.

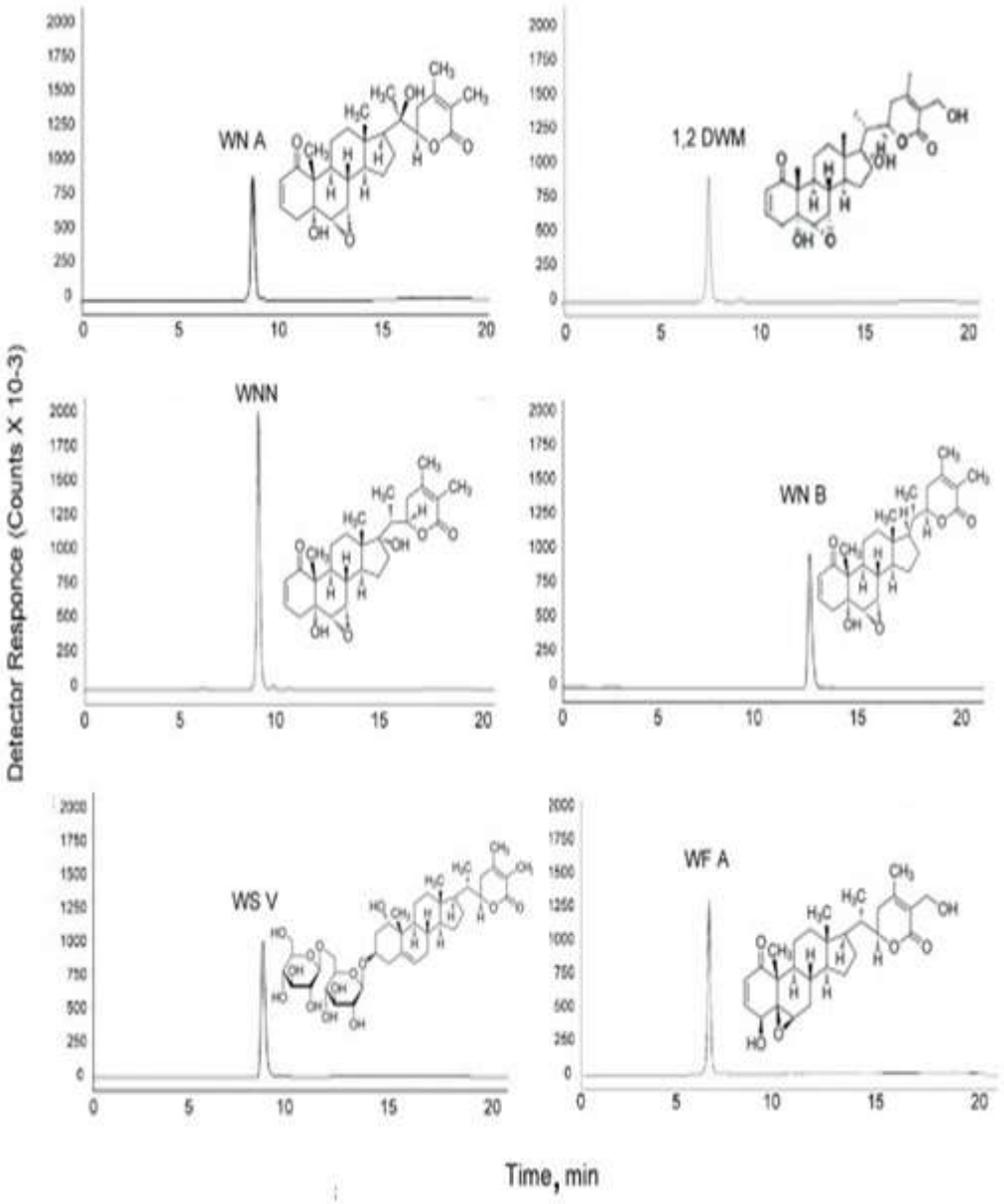
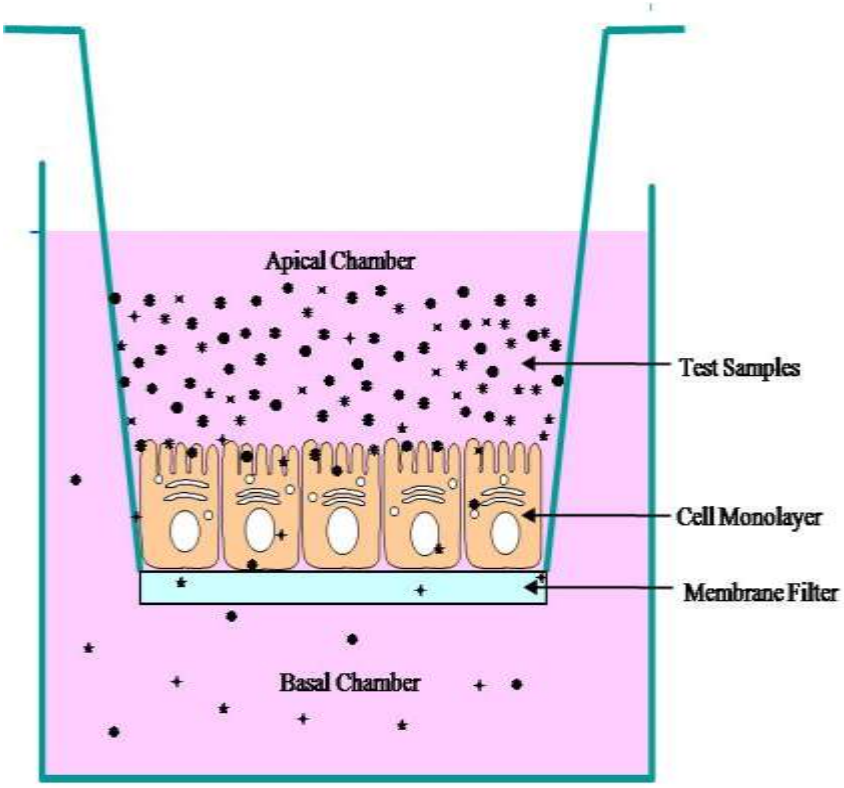


Fig. 6.3: The cell culture chamber.

Samples to be tested for permeability are incubated under control conditions and allowed to pass through the cell monolayer. Samples collected from apical and basal chambers are analyzed by HPLC-MS for ascertaining permeability of different withanolides.



15-20 min A: B (75: 25%) and 20- 25 min A: B (70: 30%). DAD detector conditions specific signals were collected at 205 nm (bandwidth 16), 210 nm (bandwidth 8), 254 nm (bandwidth 16), 270 nm (bandwidth 16) and 280 nm (bandwidth 16). All spectra were scanned from 190 nm to 400 nm with 2 nm step.

Electrospray mass spectrometer conditions were; Positive mode, gas temp 350°C, drying gas 13 L/min, neb pressure 60 psig, vaporizer 350°C and capillary voltage 3000 V. Scan conditions were low mass 150, high mass 600, fragmentary 70, gain 1, threshold 150, step size 0.20. Selected ion mode signals monitored at various rages. Electrospray mass spectrometer selected ion mode signals monitored at 471.3, 488.4 (WN A), 455.4, 472.4 477.4 (WN B), 471.2, 453.2, 493.2 (WNN), 471.3, 493.2 (WF A, 1, 2 DWM) and 407.4, 425.4, 443.4, 767.4, 784.6, 789.4 (WS V) except WS V was the high mass is 850.

Madin Darby Canine Kidney cells (MDCK) were used in the proprietary Sino-Veda cell culture system. The MDCK cells show absorption characteristics similar to human intestinal epithelium and suited for rapid permeability screening (Irvine et al., 1999; Hugger et al., 2002). Cells were cultured for 3 days and monolayers with Trans Epthelial Electric Resistance (TEER) between 80 to 120 ohm.cm^2 were used in the study. Stock solutions of standards were prepared in methanol and then further diluted with Hank's buffered saline supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to desired concentrations. Lucifer Yellow was added to the test solution as an indicator for monitoring the integrity of membrane monolayer. Incubation was carried out on a shaker (50 – 70 rpm) at 37^0 C for one hr. Samples were collected from apical donor side before incubation, and form basal receiver side after incubation (**Fig. 6.3)**. All withanolides were tested at 2 µg/mL to ascertain that they did not disrupt membrane integrity and the chemical concentrations at the receiver side were

above their quantifiable limits. Apical chamber sample was diluted 1: 20 and the injection volume was 20 µL for LCMS analysis. For basal chamber samples no dilution was necessary and 40 µL was injected.

6.3. Results

The bioavailability of standard withanolides was assessed with the help of standardized proprietary cell cultured system mentioned above (**Fig. 6.3**). Under these HPLC conditions the mean retention times (*Rt*) for withanolides WF A, 1, 2 DWM, WN A, WS V, WNN and WN B, respectively, were 6.8, 7.75, 9.01, 9.2, 9.31 and 13.1 min. (**Table 6.1). Fig. 6.4 and 6.5** shows representative mass spectrometric selected ion mode chromatograms for the individual withanolides which were collected from apical and basal permeability chambers. A single peak was observed in all chromatograms except for WNN and WNB which showed an extra peak possibly representing a degraded product. WNN and WNB and the unidentified degraded /derivatized products were highly permeable. By contrast, WS V seems to be partially permeable. A standard curve was generated and amounts quantified in the experiments were well within the range of standard curve. Permeability was measured in terms of efflux pump (cm/sec) and was calculated as follows:

$$P_{eff} = \frac{\text{Receiver Volume}}{\text{Membrane Area X Donor Concentration}} X \frac{\text{Receiver Concentration}}{\text{Incubation Time}}$$

P_{eff} values of WN A, WNN, 1, 2 DWM, WN B, WS V and WF A were 4.05 X 10^{-05}, 2.06 X 10^{-05}, 1.97 X 10^{-05}, 1.80 X 10^{-05}, 3.03 X 10^{-06} and 3.30 X 10^{-07} respectively (**Table 6.1)**.

Table 6.1: P_{eff} values and permeability of Ashwagandha standard.

Chemicals	Rt (min)	P_{eff} (sec/cm)	Permeability
		Mean	
WN A	9.01	4.05×10^{-05}	High
WNN	9.31	2.06×10^{-05}	High
1, 2 DWM	7.75	1.97×10^{-05}	High
WN B	13.1	1.80×10^{-05}	High
WS V	9.2	3.03×10^{-06}	Low
WF A	6.8	3.30×10^{-07}	Impermeable

Fig. 6.4: Apical and Basal distribution pattern for highly permeable withanolides. Degradation product is denoted by D.

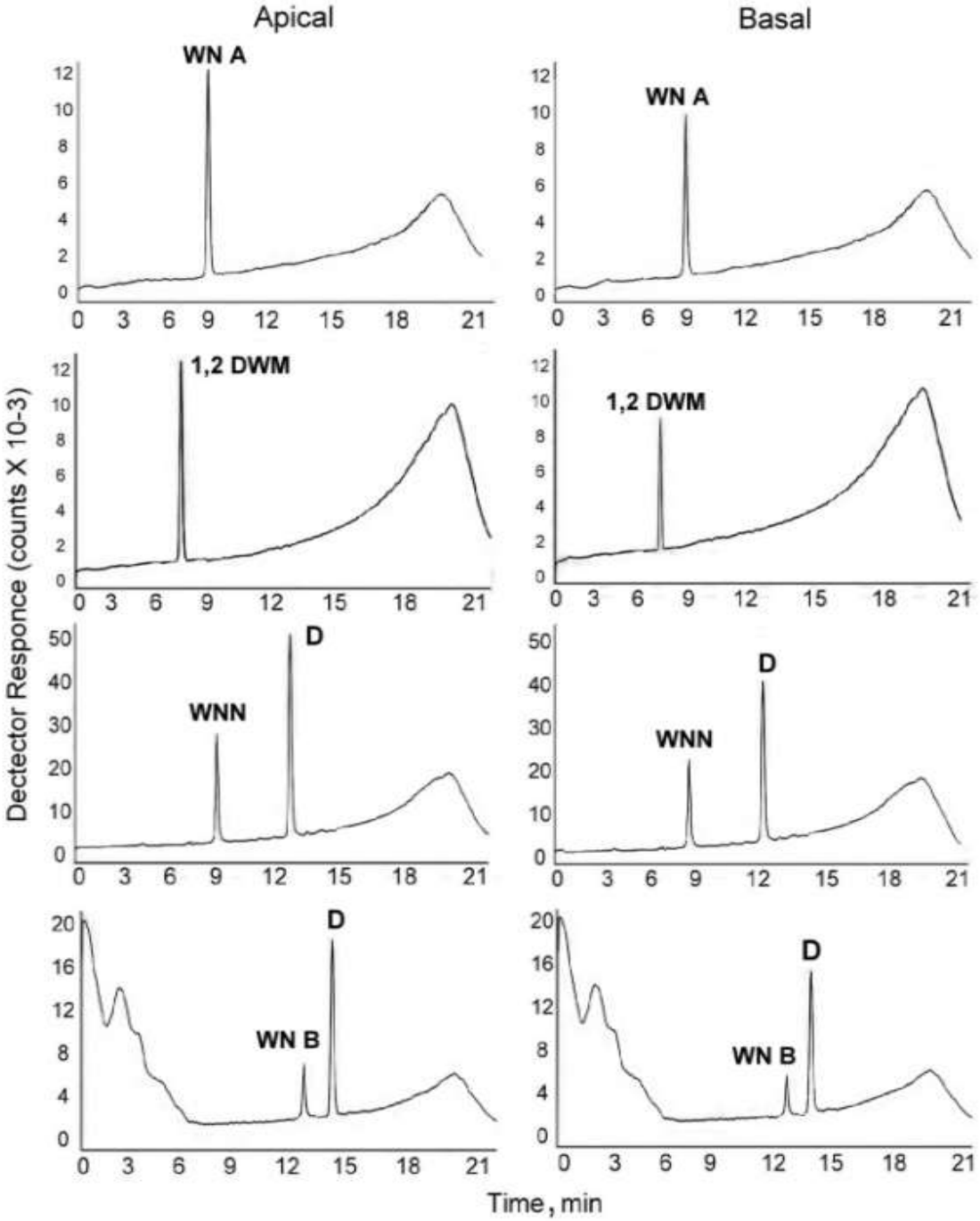
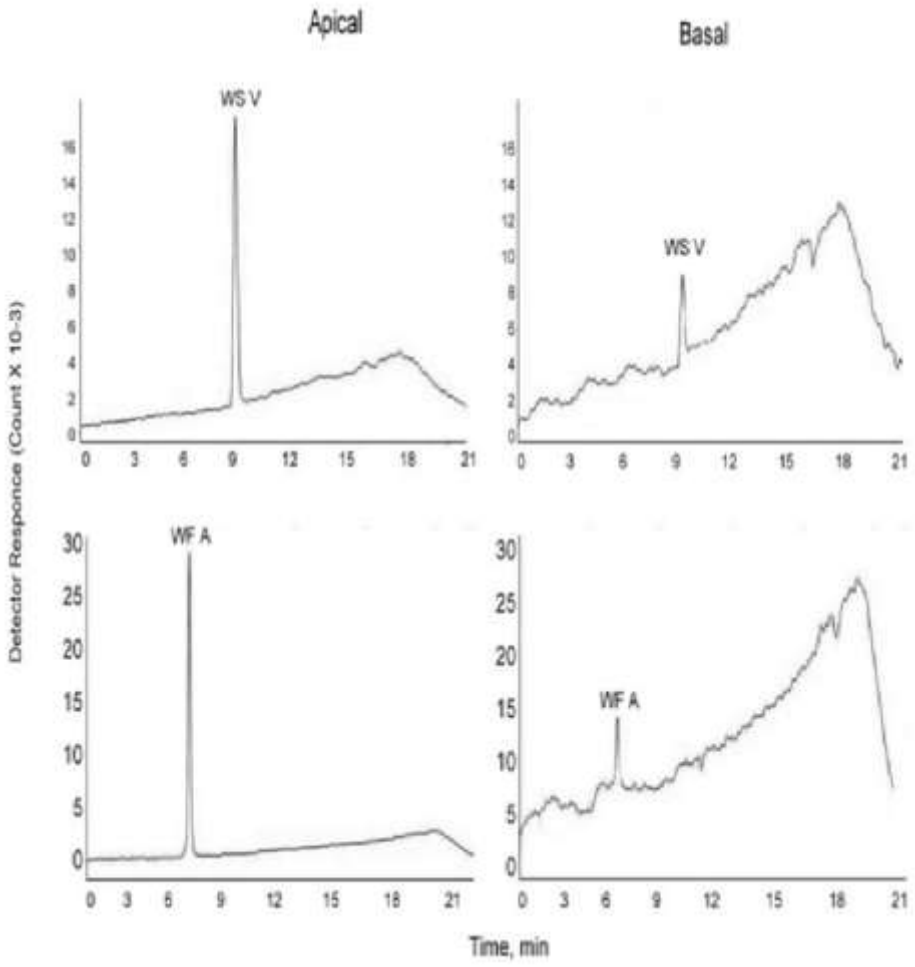


Fig. 6.5: Apical and Basal distribution pattern for low and impermeable withanolides.



6.4. Discussion

Biological activities of some individual withanolides of Ashwagandha have been evaluated by *in vitro* assays and it was noted that withanolides have therapeutic activity (Yang et al., 2007; Sen et al., 2007; Mohan et al., 2007; Devi and Sharada, 1995; Sabina et al., 2008; Kobuyama et al., 2005; Malik et al., 2007; Kour et al., 2009; Nakayama and Tohada, 2007). The detailed pharmacological activities of major withanolides have been described in section 1.4.6 of Chapter 1.

In vivo bioavailability study of WN A and WF A has been carried out by Patil et al. 2013. However, *in vitro* assay for evaluation of bioavailability of withanolides have not been carried out. *In vitro* cell culture systems may therefore be a useful model to study the absorption behavior of withanolides (Engman et al., 2001; Pang et al., 1996). In view of this the bioavailability of withanolides was carried out by using proprietary Sino-Veda (Canada) MDCK cell culture system (Lin et al., 2004; Tam et al., 2000).

It has been reported that among all withanolides WN A is most stable and bioavailable under *in vivo* condition (Patil et al., 2010, 2013); the present investigation has shown similar results. Several researchers studied biological activity under *in vitro* culture for WN A (Kobuyama et al., 2005; Malik et al., 2007; Kour et al., 2009), it indicates that WN A is permeable under *in vitro* conditions, and this is in accordance with the present observation. Cells grown under the proprietary conditions have a narrow range of paracellular permeability (1 X 10^{-06} to 1 X 10^{-05} cm/sec). In present study the P_{eff} value of WN A was 4.05 X 10^{-05} which indicates high permeability. The permeability observed in these cells correlates directly to the permeability in human system (Sun et al., 2002). In the present investigation it was noted that WNN, 1, 2 DWM, and WN B are highly permeable (**Fig. 6.4)**. **Fig. 6.4** also shows that significant metabolic and or degradation product appeared in WNN and WN B.

Apparently, of the various withanolides tested in the present investigation only WNN and WNB seem to be modified and the rest seems to be stable. Putative modified products of WNN and WNB are unidentified and need further investigations. In present investigation P_{eff} value of WS V was 3.03 X 10^{-06} observed, signifying their low permeability as compared to other above mentioned withanolides. WS V have glucose moiety at C_3 position (**Fig. 6.2)**, thus they are polar and also have higher molecular weight. It is known that hydrophobic interior of the cell membrane- the lipid bilayer- serves as a barrier to the passage of polar and high molecular weight molecules (Alberts et al., 2002). The gut has glucosidases which could hydrolyze the glucose moiety, thus facilitating the absorption of such compounds. In view of this it may be suggested that further studies on these lines are required.

Several researchers have reported that WF A is a highly biologically active compound (Yang et al., 2007; Sen et al., 2007; Mohan et al., 2007; Devi and Sharada, 1995; Sabina et al., 2008). After oral administration of aqueous extract of Ashwagandha, the WF A was more bioavailable compared to WN A (Patil et al., 2013). Surprisingly and paradoxically it was observed that the P_{eff} value of WF A was the lowest (3.30 X 10^{-07}) implying that it may be impermeable in the *in vitro* model i.e. in the MDCK cells or that it may be metabolized as it passes through the cell layer and we are unable to measure the metabolite. However, as mentioned above, after oral administration of aqueous extract of Ashwagandha to mice there was significant absorption resulting in high concentration of WF A in the plasma (Patil et al., 2013). It seems that the process of WF A absorption is more complex and that MDCK cells *in vitro* model possibly does not provide the exact *in vivo* environment. However, this possibility needs to be verified further by direct experiments using different cell culture system such as Human Epithelial Colorectal Adenocarcinoma (Caco-2) cell

monolayer (Irvine et al., 1999). Although both CaCo2 cells and MDCK cells are predictors for passive properties and lack of active transporters, the discrepancy seen in the present studies could likely be attributed to the lack of active transporters or species difference.

6.5. Conclusions

Based on the Sino-Veda absorption model the absorption characteristics of the tested withanolides, it may be concluded that WN A, WNN, 1, 2 DWM and WN B were highly permeable; whereas WS V showed low permeability. Surprisingly WF A, the highly biologically active withanolide was found to be either impermeable or WF A metabolized on passing through the cell layer. It is likely that WFA *in vivo* absorption is more complex and needs further in depth investigations. The study demonstrates the usefulness of the technology for rapid testing of bioavailability of drugs.

Chapter 7

Summary and Conclusions

7.1. Summary

7.1.1. Preamble: Integration of Ayurveda with modern medicine

Ayurveda is a treasure of wisdom gained by Indian Rishis and Vaidyas about the therapeutic power of several herbal formulations for curing several human ailments. It is time tested experiences of over 5000 years. Unlike the modern medicine, Ayurveda lacks proper standardization and validation; seems arbitrary, and therefore does not find universal acceptance. On the other hand, modern medicines are tailor-made synthetic proprietary drugs, designed with fair knowledge of the disease at molecular level; seems precise, well targeted to the disease in particular and accepted all over the world being experimentally validated standardized therapy. The modern drugs go through rigorous, stringent regulatory norms, before being introduced for human use. All the same, these modern drugs being synthetic are more likely to have side effects than herbal medicines, which are natural. In order to reap the full benefits of ancient wisdom and bring them in line with modern medicine, it is necessary to put these herbal formulations also through validation, standardization, and stability criteria to make it more acceptable in the world market. Therefore this study was undertaken to pave a path for validation standardization and stability of herbal formulations.

7.1.2. Ashwagandha: A chosen herb for validation standardization and stability studies

Ashwagandha root is one of the most popular herbal parts used in many Ayurvedic formulations. Therefore, root of Ashwagandha was selected as the best suited herbal part to develop methods of validation standardization and stability studies.

It is generally considered that plants and plant parts are inherently richer in antioxidants and Ayurveda wisely exploits the specific secondary antioxidant metabolites for a given particular

disease in specific organs. Ashwagandha roots have a class of steroidal lactones called withanolides that have documented medicinal properties useful in the treatment of several diseases.

For Ayurvedic medicine to be effective and to be accepted in the world market, it has to be consistent in quality with an assurance of curative potential over a period of time that it stands on the shelf before it reaches the consumers.

7.1.3. Pertinent issues to address the problem

There are several pertinent issues that need to be tackled to address this problem. Some of the important issues are;

- Firstly, the best plant variety, with proven medicinal property has to be selected.
- Secondly, the plant has to be grown under ideal condition, nutritional support and proper agronomical inputs, so as to get maximum medicinal value.
- Thirdly, the method of formulation by all the manufacturers should be standard, well defined, uniform, to get the assured curative potential in the final product.
- Fourthly, the active principles, if known, be quantified and stated on the label.
- Fifthly, the antioxidant (anti-inflammatory) value be physically, chemically measured and stated.
- Sixthly, when the herbal formulation is being orally administered, it is necessary to ascertain how much active principle components of the herbal formulation is being absorbed and assimilated in human system to get the desired curative effects on the disease of the patient.
- Seventhly, the actual medicinal value to be checked in vivo, in animal model before being introduced into the market.
- Eighthly, the shelf-life of formulation should be noted on the label of final product.

It is in view of the above, In the present studies physical, chemical and biological approach were used to validate, standardize and conduct shelf-life stability studies by taking Ashwagandha as an example. Highlights and conclusions based on the above theoretical surmise of validation, standardization and stability was given below:

7.1.3.1. Quality control in the farmer's field

- It is observed that macronutrients N, P, Ca and K deficiencies in the soil do affect the withanolide contents in the roots.
- It was also observed that Mo contents in roots vary under different soil macronutrients deficiency conditions.
- Further, It conclude that Mo in roots correlate with withanolide contents in roots.
- Mo is a micronutrient to be regulated in the soil for high quality, withanolide rich root products.

7.1.3.2. Efficient extraction of roots

- An efficient method of getting withanolide rich fraction (WRF) has been developed.

7.1.3.3. Fractionation and quantification of withanolides by HPTLC

- Solvent system has been developed for fractionation and quantization of withanolides in WRF.
- WRF has higher content of withaferine A followed withanolide A and withanolide B

7.1.3.4. Simple quick antioxidant assay of withanolides

- The fractionated withanolides on HPTLC plates can individually assessed for their antioxidant activities and thus assist in quantitative and qualitative standardizations of Ashwagandha formulations.

7.1.3.5. Quicker physical technique (Cyclic Voltammeter) for quality assessment at different stages in the production of formulation and for shelf life

- The technique identified two components in WRF, one fast decaying and the other slow decaying.
- Among the major withanolides 1, 2 deoxywithastramonolide is most stable followed by withanolide A.

7.1.3.6. Biological assessment of Anti-inflammatory and hepatoprotective activities

- Assessment of anti-inflammatory activity provides a tool for biological assessment.
- As it has been found anti-inflammatory activity has a shorter life, it may be attributed to slow decaying components.
- Hepatoprotective activity may be attributed to the antioxidant potential of WRF.
- Anti-inflammatory of WRF possibly mediates through the suppression of TNFα and IL-1β.

7.1.3.7. Bioavailability

- Sino-Veda – Canada MDCK cell culture is a suitable model for quickly in vitro assessing bioavailability of herbal formulations.
- It was observed that non polar aglycone withanolides has higher penetrability to pass through biological membrane.
- Polar glycosylated withanoside V showed low permeability.
- However, withaferine A, regarded as most potent withanolide, surprisingly showed no permeability.
- This observation needs to be confirmed with more appropriate cell line $CACO_2$.

7.1.4. Conclusions

Ayurveda, a treasure of therapeutic knowledge of medicinal herbs, can be made popular, on par with modern medicine, only by putting these formulations through strict rigors, of standardization and validation.

The standardizations have to begin at the framers field, continue for method of extraction / fractionation / concentration, of active principles, upto formulation. Validation involves the determination of potency by quick antioxidant assay, anti-inflammatory or by specific biological assays. Efficacy must also be ensured through the bioavailability and shelf-life of the active principles in the product.

In conclusion, it may be stated that although validation, standardization, stability information of Ayurvedic formulations are very complex. However, the problem is not insurmountable, with the proper holistic approach; one can achieve this as demonstrated in this study, with Ashwagandha root as an example.

7.2. Accomplishments

Taking Ashwagandha as example,

- The importance of quality control at the farmer's field has been demonstrated,
- The precise extraction for the concentration of active principles increases efficacy,
- Antioxidant assay by cyclic voltammeter gives a quick handle for evaluation of the potency,
- Anti-inflammatory and hepatoprotective activities have been employed to demonstrate therapeutic value,
- A new approach for the investigation of bioavailability *in vitro* absorption technology has been employed,
- Although the method has been employed with Ashwagandha as an example, the same can also be applied to most of the Ayurvedic products

7.3. Limitations of the Work

- Although, Mo deficiency has been shown to affect withanolide concentration in roots, other nutrients may also be involved in determining the concentrations of withanolides in Ashwagandha roots.
- HPTLC used in the study, is quick and cheap, convenient, however resolution power of HPTLC is limited, and therefore HPLC although costly, may have been a better choice.
- The cyclic voltammteric method for validation has a limitation that the active principles, insoluble in water may be difficult to be evaluated by this method
- Bioavailability studies could have been carried out by using Caco-2 cells rather than MDCK cells.

7.4. Future Prospects

Life is a play of redox. It is the ability of living system to neutralise oxidative stress that keeps the life going. Therefore antioxidant in food supplementations and herbal medicines are supportive to life by protection form diseases. Therefore it is our considered opinion that therapeutic power in herbal formulations may reside, particularly in its antioxidant activity.

A simple inexpensive, reliable method of assessing antioxidant power in any herbal formulations can be achieved by cyclic voltammeter. A cyclic voltammeter can quickly validate the most of Ayurvedic formulations and keep track on its stability, efficacy during formulation process and storage.

The application of such a simple technique as first fairly reliable measure of efficacy can be a valuable method of validation of Indian Traditional Herbal Medicine. This method can be further explored for general application, of all herbal formulations

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List of Publications

❖ List of Publications

1. **Devkar ST**, Badhe YS, Jagtap SD, Hegde MV. (2012) "Quantification of major bioactive withanolides in Withania somnifera (Ashwagandha) roots by HPTLC for rapid validation of Ayurvedic products" JPC-Modern TLC 25(4): 290-294.

2. **Devkar ST,** Jagtap SD, Katyare SS, Hegde MV. (2014) "Estimation of Antioxidant Potential of Individual Components Present in Complex Mixture of *Withania sominfera* (Ashwagandha) Root Fraction by Thin-Layer Chromatography–2, 2-Diphenyl-1-Picrylhdrazyl Method" JPC-Modern TLC 27(3): 157–161

3. **Devkar ST,** Jagtap SD, Suryapoojari S, Katyare SS, Hegde MV. (2014) "Effect of macronutrient deficiency on withanolides content in the roots of *W. somnifera* and its correlationship with molybdenum content" Pharmaceutical Biology 53(4): 518-523.

4. **Devkar ST**, Sloley BD, Jagtap SD, Lin J, Tam YK., Katyare SS, Hegde MV. "Evaluation of the bioavailability of major withanolides of *Withania somnifera* using an *in vitro* absorption model". (Communicated)

5. **Devkar ST**, Kandhare AD, Zanwar AA, Jagtap SD, Katyare SS, Bodhankar SL, Hegde MV. "Hepatoprotective effects of Withanolide Rich Fraction (WRF) of *Withania somnifera* (L.) in Acetaminophen intoxicated rats". (Communicated)

6. **Devkar ST,** Jagtap SD, Katyare SS, Hegde MV. "Studies on the stability, shelf-life and biological activity of Withanolide Rich Fraction (WRF) and its components of Ashwagandha". (Communication).

❖ Conference

Devkar ST, Jagtap SD, Katyare SS, Hegde MV. (2015) "Studies on the stability and therapeutic potential of Withanolide Rich Fraction (WRF) from Ashwagandha roots" 2nd International Congress of Society for Ethnopharmacology: 77-78.

❖ Awards and Certificates

Certificate 1: TLC/ HPTLC Training Certificate by Anchrom (June, 2012).

Certificate 2: Certificate for attending seminar on Research and Methodology: Sangamner College, Sponsored by UGC Pune (October, 2012).

Certificate 3: Dr. P. D. Sethi Best Research Paper Award-2012.

Certificate 4: Certificate for Oral Presentation at 2nd International Congress of Society for Ethnopharmacology (SFE-2015).

Certificate 5: Certificate for Indian Pharmacological Society Sponserd, Wrkshop on Pharmacology (IPS-2015).

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TLC/ HPTLC Training Certificate by Anchrom (June, 2012)

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Place : Pune

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