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.,
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 Ayurvedic 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 ailments 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 Voltammetry

In cyclic voltammetry 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 30°C/60%RH are, intended for extrapolation to long term storage at 25°C. 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. Ashwagandhha 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 7 cm 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 in 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, 1500 mg/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.
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 28°C-30°C. 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 10°C 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 signal 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 withanolone 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 (CCL₄)-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 CCL₄.
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 LD₅₀ 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 LD₅₀ in albino mice (Singh et al., 1982). The acute LD₅₀ 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 LD₅₀ 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 withanolone 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.

<table>
<thead>
<tr>
<th>Withanolides</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Withaferine A</td>
<td>Glotter et al., 1971, 1991; Gupta et al., 1996</td>
</tr>
<tr>
<td>Withanolides F, E, and 4β-hydroxy-withanolide E</td>
<td>Lavie et al., 1972</td>
</tr>
<tr>
<td>Withanolide Q and R,</td>
<td>Kirson et al., 1975</td>
</tr>
<tr>
<td>Withanolides S and T</td>
<td>Sharma et al., 1981</td>
</tr>
<tr>
<td>Jaborosalactone A, withanolide Y</td>
<td>Bessalle et al., 1987</td>
</tr>
<tr>
<td>Sitoindoside IX, X and withasomidienone</td>
<td>Ghosal et al., 1989; Rahman et al., 1993; Shabbir et al., 1999;</td>
</tr>
<tr>
<td>Withanolide A, withanosides IV and VI</td>
<td>Glotter et al., 1991; Zhao et al., 2002</td>
</tr>
<tr>
<td>Withanoside VIII, withanoside IX, withanoside XI, withanolide A, withanoside</td>
<td>Zhao et al., 2002</td>
</tr>
<tr>
<td>IV, withanoside VI with coagulin</td>
<td></td>
</tr>
<tr>
<td>Physagulin D (1→6)-β-d-glucopyranosyl-(1-4)-β-d-glucopyranoside, 27-O-β-d</td>
<td>Jayapramasam et al., 2003</td>
</tr>
<tr>
<td>glucopyranosylphysagulin D, 27-O-β-d-glucopyranosylviscosalactone B, 4, 16-</td>
<td></td>
</tr>
<tr>
<td>dihydroxy-5 β, 6 β -epoxyphysagulin D, 4-(1-hydroxy-2,2-dimethylcyclopropanone)</td>
<td></td>
</tr>
<tr>
<td>2, 3-dihydrowithaferin A, withaferin A, 2, 3-dihydrowithaferin A, viscosalactone</td>
<td></td>
</tr>
<tr>
<td>B, 27-desoxy-24, 25-dihydrowithaferin A, sitoindoside IX, physagulin D, and</td>
<td></td>
</tr>
<tr>
<td>withanoside IV</td>
<td></td>
</tr>
<tr>
<td>Withanone, 27-hydroxy withanolide A, iso-withanone and 6α,7β-epoxy-1 β,3 β,</td>
<td>Lal et al., 2006</td>
</tr>
<tr>
<td>5α-trihydroxy-witha-24-enolide</td>
<td></td>
</tr>
<tr>
<td>Withanolide Z</td>
<td>Pramanick et al., 2008</td>
</tr>
<tr>
<td>Withaferin A and witharistatin</td>
<td>Benjumea et al., 2009</td>
</tr>
<tr>
<td>Withanolidesulfoxide</td>
<td>Vanisree et al., 2009</td>
</tr>
<tr>
<td>Physagulin D and withastraronolides</td>
<td>Ahuja et al., 2009</td>
</tr>
</tbody>
</table>
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) withanine, 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-methylenecolesterol 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 schematic diagram of pharmacological 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. \( \lambda_{\text{max}} \) of WF is 227nm. WF A is very well known and it has several medicinal properties. Fig. 1.7 shows the schematic diagram of pharmacological 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. \( \lambda_{\text{max}} \) 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 C₃ 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 C₄₀H₆₂O₁₅ and C₄₀H₆₂O₁₄ 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. λₘₐₓ 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 regeneration 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. \( \lambda_{\text{max}} \) 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 Withaframolide (27-Hydroxy Withanolide)

Molecular formula of 1, 2 deoxywithastramonolide (1, 2 DWM) is C\textsubscript{28}H\textsubscript{38}O\textsubscript{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. \( \lambda_{\text{max}} \) 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\textsubscript{28}H\textsubscript{38}O\textsubscript{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. \( \lambda_{\text{max}} \) 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:** Schematic diagram of pharmacological 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 and Nagella, 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 racemose* 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 cordifolia* (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), *Syzygium aromaticum* (Ling/lavang), *Tinospora cordifolia* (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 spectrophotometric 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

- **Collection of Ashwagandha Plants**
- **Quantification of Withanolides**
  - HPTLC
  - Spectrophotometer
- **Selection of Roots and Preparation of WRF**
- **Characterization of WRF**
  - HPTLC
  - TLC-DPPH method
- **Stability of WRF**
  - Cyclic voltammetry
  - HPTLC
  - Biological activity *in vivo* (Rat paw oedema)
- **Hepatoprotective action of WRF using *in vivo* model system**
- **Bio-availability of major withanolides by *in vitro* Absorption technology**