CHAPTER 1

INTRODUCTION
Since the dawn of history, man has been in search of ways to find cure and relief from mental and physical illness. The history of medical botany dates back to the origin of civilization. In the Indian subcontinent Ayurveda along with Unani and Siddha medicinal systems are quite popular. Ayurvedic approach is dominant among them. Ayurveda is thought to be the oldest system of healing in the world, pre-dating even Chinese medicine. Today it is actively promoted as an alternative to conventional western medicine.

The name Ayurveda is derived from two Indian words: *ayur*, meaning *life* and *veda*, meaning *knowledge* or *science*. Ayurveda is more than a system of healing. It is a way of life encompassing science, religion and philosophy that enhances well being, increases longevity and ultimately brings self-realisation.

Ayurveda evolved over 5,000 years ago in the far reaches of the Himalayas from the deep wisdom of spiritually enlightened prophets or *Rishis*. Their wisdom was transmitted orally from teacher to disciple, and eventually set down in Sanskrit literature known as the *vedas*. These writings, dating approximately 1500 BC, distilled the prevailing historical, religious, philosophical and medical knowledge and formed the basis of Indian culture. The most important of these texts are the *Rigveda* and the *Atharvaveda*. *Rigveda*, one of the oldest repositories of human knowledge mentions the use of 67 plants for therapeutic use and *Yajurveda* enlists 81 plants whereas *Atharvaveda* describes 290 plants of medicinal values. In about 800 BC, the first Ayurvedic medical school was founded by Punarvasu Atreya. He and his students recorded medical knowledge in treatises that in turn influenced *Charak*, a scholar who lived and taught around 700 BC. His writings, the *Charak Samhita*, describe 1,500 plants, identifying 341 plants as valuable medicines. This major reference text is still consulted by Ayurvedic practitioners. The second major work was the *Susruta Samhita*, written a century later, which forms the basis of modern surgery and is still in use and mentions 395 medicinal plants.

THE VALUE OF HERBAL MEDICINE

The importance of Ayurveda is proved particularly by its timelessness, as it has existed as an unbroken tradition for thousands of years. Following the rise of the Mogul
Empire in the 16th century the dominance of Islamic medicine, Unani Tibb, led to the partial repression of Ayurveda in India. In the 19th century, the British dismissed it as nothing more than native superstition and in 1833 they closed all Ayurvedic schools and banned the practice. Great centers of Indian learning thus fell apart, and Ayurvedic knowledge retreated into villages and temples. At the turn of the century, however, some Indian physician and enlightened Englishmen begun to re-evaluate Ayurveda. and by the time India became independent in 1947 it had regained its reputation as a valid medicinal system. Today, Ayurveda flourishes side by side with Unani Tibb and the western conventional medicine and is actively encouraged by the Indian government as a cost-effective alternative to the western drugs.

Ayurveda is not only a medical science that deals solely with treatment of disease, but it offers practical guidelines, which are applicable to every facet of daily existence. It seeks to reconcile health and lifestyle with the universal aspects of existence, and thus enhances the well being, longevity and harmony of all those who practice it. For these reasons, Ayurveda is known as a science of lasting benefits to anyone seeking an alternative to modern medical practices.

The period of 16th and 17th century witnessed a spurt in the development of medicinal botany and most of the world famous medicinal drug came into light during this period, the period is called “The Age of Herbal Medicines”.

**CHANGE IN ATTITUDES TO HERBAL MEDICINE**

The most noticeable change towards herbal medicine in the developed countries of this century has been because of the interest shown by the ordinary people. From being regarded as “old fashioned” and distrusted, herbs such as ginseng and guarana which are now hailed as wonder drugs. The change in attitude began in the 1960s, when the ‘hippie’ movement advocated a nature living, initiating “alternative” medicine and therapies. The growth of the conservation movement and the founding of companies using only natural products in an environmentally friendly way were also major factors. As a result, increasingly wide ranges of herbs are now available as fresh, dried, as ingredients of cosmetics, perfumes, and over-the-counter medicines. Jojoba oil (from *Simmondsia chinensis*) was unheard of 20 years ago; it was promoted as a substitute for
sperm whale oil in industrial high performance lubricants as part of the campaign to save whales, and went on to become a revolutionary new emollient in skin and hair-care products.

Interest in herbal medicine throughout the world is increasing. In the West, people often cite the risk of side effects from powerful modern drugs as a reason for turning to gentler plant medicines. In the developing world, a lack of hard currency to pay for imported pharmaceuticals is encouraging a reappraisal of traditional folk remedies. This trend towards natural medicine has been helped by our growing concern with environmental issues, such as the destruction of rainforests and the loss of rare species of plants and animals. Although the therapeutic effects of many herbs have not been scientifically proven, research is continually being done to learn more about the way in which these plants work, and to identify the active ingredients that give them their healing properties. Scientists hope that such research may help uncover new active plant ingredients that can form the basis of drug to fight cancer or Aids; these drugs will join the many thousands of other widely used synthetic remedies derived originally from medicinal herbs.

**THE BENEFITS OF HERBAL MEDICINE**

Besides the advances and advantages of conventional medicine, or biomedicine as it is also known, it is clear that herbal medicine has much to offer. We tend to forget that in all but the last fifty years or so, humans have relied almost entirely on plants to treat all types of illnesses, from minor problems such as coughs and colds to life threatening diseases such as tuberculosis and malaria. Today, herbal remedies are coming back into prominence because the efficacy of conventional medicines such as antibiotics, which once had near-universal effectiveness against serious infections, is on the wane. Over the years, infectious organisms have developed resistance to synthesized drugs. The herb "ging hao" and its active constituent, artemisin, for example, are now being used to treat malaria in areas of the world where the protozoa causing the infection no longer respond to the conventional treatment.

Herbal medicine is often complementary to conventional treatment because it provides safe, well-tolerated remedies for chronic illness. It is experiencing a dramatic
renaissance in the western countries, particularly because no effective conventional treatment exists for many chronic illnesses like asthma, arthritis, liver disorder and irritable bowel syndrome. In addition to this, concern over the side effects of biomedicine is encouraging people to look for more gentle forms of treatment. It is estimated that 10-20% of hospital patients in the West is hospitalized because of the side effects of the allopathic treatment.

IMPORTANT OF WHOLE PLANT

Although herbal medicine is ultimately about the action of whole plant, it is important to understand the action of individual constituents of a plant. The whole herb is worth more than the sum of its parts and scientific research is increasingly showing that the active constituents of many herbs, for example there in ginkgo (Ginkgo biloba), interact in a complex way to produce the therapeutic effect of the remedy as a whole. Plants contain hundreds, if not thousands, of different chemical constituents that interact in complex ways. Frequently we simply do not know in detail how a particular herb works – even though its medicinal benefits are well established. The pharmaceutical approach in understanding how the whole herb works is like working on a jigsaw puzzle where only some of the pieces have been provided. Furthermore, although it is very useful to know that a plant contains certain active constituents, such information can be misleading on its own. For example, tea (Camellia sinensis) and coffee (Coffea arabica) contain approximately the same level of caffeine. Tea however contains greater quantity of tannins (which give tea its sour taste). These constituents reduce the amount of nutrients and drugs that are absorbed from the intestines into the blood stream, and consequently less caffeine is absorbed. As a result, and true to most people’s experience, tea is less stimulating than coffee.

This example reveals a couple of fundamental truths about herbal medicine. Firstly, the experience of the herbal practitioner and of the patient often provides the most reliable guide to the medicinal effect of individual herbs. Secondly, the value of medicinal herb cannot be reduced simply to a list of active constituents.
FADE OF TRADITIONAL MEDICINE

The introduction of abstract medicine in the form of base chemicals and pharmaceuticals during the 18th and 19th centuries has demonstrated methods for bringing quick relief from sufferings and this earned them instant admiration and popularity. This system of allopathy made rapid advances during the 19th and 20th centuries as a result of the advances made in biological, chemical and pharmaceutical sciences. New discoveries of sulfa drugs, synthetic, antibiotics and other chemotherapeutic agents in quick succession swept all other medicinal systems off their feet. Universal adoption of modern medicine based on sound experimental data, toxicity studies and human clinical trials in turn obliterated the use of most of the traditional systems of medicine and the practice of traditional medicine eventually receded more or less to the country side.

The plant kingdom has long served as the main resource base for the traditional medicines. This began to change in the 1930's with the advent of synthetic chemistry and was cemented in the 1950's with the introduction of laboratory-bred 'wonder drugs' such as the antibacterial sulfonamides or sulfa drugs. Predictably, the American pharmaceutical industry quickly lost interest in natural products as source of potential new medicines.

Synthetic drugs like sulfonamides and diazepam (a sedative) have led some chemists to have the illusion that synthetic chemistry is the sole future of new drug discovery.

RE-ESTABLISHMENT OF TRADITIONAL MEDICINE

Now days, there is renewed interest in traditional medicine. During the past decade, there has been an ever-increasing demand especially from the developed countries for more and more plant drugs containing medicinally useful alkaloids, polyphenolics, steroids, glycoside and terpenoid derivatives. The revival of interest in natural drugs especially derived from plants, started mainly because of the widespread belief that 'green' medicine is healthier than synthetic products. This has led to the rapid spurt of demand for health products like herbal teas, ginseng etc. during the 1980's. Similar
tendency has been shown towards a general increasing preference in the utilization of natural flavours, dyes, preservatives etc. rather than the less expensive synthetics.

THE RESOURCE BASE OF TRADITIONAL MEDICINE

The resource base of all traditional medicine is mainly the plant, animal products and also minerals. The major classical systems of medicine like Ayurveda, Sidha and Unani together use about 1200 plant species for medicinal purpose. But the tribals of India are using over 7500 plant species for treating a variety of ailments. The medicinal plants used in the classical systems are drawn from different regions of the world whereas the medicinal plants used by tribal community or village folk practitioners are location specific. Figure 1.1 Shows Indian medicinal plants used in different systems.
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PLANT-DRUG RESEARCH AND IMPLICATIONS

Plant-drug research has been associated with a number of problems; some of them are listed below:

1. In India, medicinal plants used by traditional practitioners bear different names in different geographical areas. The names are also different in different languages. In some cases different plants are known by the same name which add to the confusion and has resulted in controversial identity of many plants. This brings out the importance of botanical authentication of medicinal plants in the field of herbal drug research.

2. There is inconsistency in plant drugs and the major causes include ontogenetic, ecotypic, genotypic and chemotypic variations and also variations due to harvesting period, method of drying and storage conditions. These variations include geographical and age-wise variation.

3. In herbal, Ayurvedic or Unani drugs, the practitioners invariably use polyherbal formulations (containing more than one medicinal plant), whereas, for the modern scientist it is easier to investigate single plant drug.

4. For scientific study, appropriate experimental models are not available for validating claims of some very important and useful drugs like “Rasayana”, therefore commonly accepted in Ayurveda and in modern terminology are known as vitalizers, rejuvenators, adaptogens, immunomodulators etc.

5. Major national laboratories resorted to more broad based screening of plants by including, apart from plants which are well known and time tested by traditional system of medicine, those which are not mentioned by these system. Some undertook studies largely on individual initiatives. In spite of all these efforts, however, the ultimate goal of producing inexpensive, safe and efficacious drugs for most diseases encountered in our country remains to be achieved.

6. The lack of scientifically planned clinical trials on traditionally used medicinal plants is the major bottleneck. Recently, Indian Council of Medicinal Research has adopted new strategy of disease oriented approach of carrying out clinical trials on medicinal plants selecting certain “refractory diseases” i.e. those for which modern medicine has not been able to offer a satisfactory or a lasting remedy.
The novelty and the popular trend on the part of the phytochemists and pharmacologists to continue efforts to find out an "active principle" responsible for the pharmacological action and therapeutic efficacy of a plant, in many instances, has led to frustrating results. Phytochemists and pharmacologists engaged in medicinal plant research may have to change their approach to holistic plant material or with semi pure principles or even crude extracts.

Except for some research institutes, few facilities exists in India for carrying out pre-clinical evaluation including toxicity tests on medicinal plants.

Clinical evaluation of plant drugs is an area which is handicapped due to lack of facilities and proper orientation of scientists of different disciplines.³

RESEARCH NEEDS IN TRADITIONAL MEDICINE

The intrinsic value of traditional medicine in imparting health care to people, particularly to the rural mass of the developing countries is now well recognized. The common notion that all remedies of natural origin are harmless and carry no risk to the consumer is not fully true. Many plants, animal products and minerals used in traditional remedies are highly toxic. There are methods prescribed in certain traditional systems like Ayurveda, Sidha and Unani for neutralizing the toxic effects of the toxic products. In such cases proof of safety should take precedence over establishing efficacy. It is also desirable to scientifically evaluate the traditional remedies to ensure safety, efficacy, stability, standards and dosage formulations. Efforts should be made to determine the composition of the therapeutic principle. Correct labeling of the constituents of herbal remedies is critical for safety evaluation and quality control. Scientific validation and fixing standards for sample particularly single plant based remedies are comparatively easy. But such evaluations may pose problems with compound formulations containing several ingredients like those from the organized systems like Ayurveda, Sidha, and Unani.

The therapeutic effect of the formulations with multiple ingredients is possibly due to compound effect or synergetic effect of a number of compounds. It is also possible that the active compounds when isolated in pure form although active may be very toxic. But, in the natural form, it is in association with other compounds either derived from
the same plant or other materials in the formulation, the toxic effect is either minimized or eliminated.

The evaluation of traditional medicine requires an integrated approach that combines the concept and theoretical foundations, particularly of the classical traditions like Ayurveda, Sidha or Unani with the modern scientific knowledge, technology and tools. It has been estimated that 250,000 to 750,000 species of flowering plants exist on the earth and it is very difficult to determine accurately how many of these species have been used in traditional medicine. A reasonable estimate would be about 10%. However, perhaps only about 1% of these is acknowledged through scientific studies to have a real therapeutic value when used in extract form by humans. As world population is increasing day by day, global estimates indicate that over 3/4th of the world population cannot afford the product of the western pharmaceutical industry and have to rely upon the use of traditional medicines, which are mainly derived from plants. This fact is well compiled by W. H. O. in an inventory of medicinal plants. To reduce the financial burden on developing countries which spends 40–50% of their total health budget on drugs, W.H.O. encourages, recommends and promotes the inclusion of herbal drugs in national health care programs because such drugs are easily available, at a price within the reach of common man, are time tested and thus considered to be much safer than the modern synthetic drugs. The interest in herbal drugs in the western European countries is evident from the fact that two volumes of British Herbal Pharmacopoeia have been published and $33 million worth of literature on herbal product was sold in the USA in 1990. The “green wave” in the utilization of medicinal plants resulted in higher consumption.

STANDARDIZATION OF HERBAL MEDICINE

The therapeutic use of medicinal plants has gained considerable momentum in the world during the past decade. The overuse of synthetic drugs with impurities results in higher incidence of adverse drug reactions in more advanced communities has motivated mankind to go back to nature for safer remedies. However, it should be ensured that commercial formulations based on medicinal plants are safe, effective and of standard quality.
Today, over the world, there is a great deal of interest in Ayurvedic system of medicine and thus the demand for various commonly used medicinal plants in the production of Ayurvedic medicine is ever increasing. Due to varied geographical locations where these plants grow, they are known by different vernacular names. This leads to a great deal of adulteration or substitution encountered in the commercial markets. An important factor, which can contribute to the consistent quality of herbal products, is to have adequate control on the quality of the herbs. Therefore reproducible standards of each plants are necessary for effective quality control.

WHO has also issued “Guidelines for Quality Control Methods for Medicinal Plant Materials” in 1992 with a clear objective to provide general test methods for current botanical evaluation and identification of medicinal plants widely used in the traditional and home remedies. In addition to the test methods, some suggestions regarding general limits for contamination are also included.

What is missing from the attitude to health is the sense that we are responsible for our own well being. Many minor ailments are often of our own making, brought about by poor diet, lack of exercise or “burning the candle at both ends”. Modern drugs may effect rapid apparent cures, but they cannot solve the problems we persistently bring on ourselves.

Standardization of herbal medicine can be divided into four phases.

**PHASE I**

It consists of various steps such as survey, botanical authentication, efficacy testing, safety testing and chemical testing.

- **Survey**
  The survey can be done in two ways, Ethnobotanical and/or Literature. In this survey the main emphasis is given on parts of the plant used or uses of plants, method of preparation of herbal remedies, usefulness against which disease and type of the patient, other varieties or sub-species of herb used and lastly pharmacology and phytochemical work.
• **Authentication**

An appropriate body botanically authenticates the plant species. If contaminants or adulterants are likely to be used, these are also authenticated.

• **Efficacy Testing**

Efficacy is tested on animal model(s), keeping the form and route of medicine similar to that used in traditional systems.

Many of the herbs, which are used medicinally in Europe, have a traditional reputation for their uses, but there is little scientific or medical documentation in respect of their active constituents, pharmacological actions or clinical efficacy. Examples of these groups include avens, boneset, burdock, clivers, damiana, etc. For other herbs, documented phytochemical or animal data may support traditional uses, but evidence of human efficacy is limited.

• **Safety Testing**

An acute toxicity and sub acute toxicity test of the species of interest is performed. The safety of all medicinal products is of the utmost importance. All applications for new medicines undergo extensive evaluation of their risk-to-benefit ratio and, once granted, products are closely monitored for the occurrence of adverse effects. The safety of herbal remedies is of particular importance in that the most of these products are self-prescribed and are used to treat minor and often chronic conditions.

The extensive traditional use of plants as medicines has enabled these medicines with acute and obvious signs of toxicity to be well recognised and their use avoided. However, the presumption that traditional use of a plant for perhaps many hundreds of years established its safety is not necessarily true.

• **Chemical Testing**

In this chemical testing general methods of test quality like extractive values in different solutions, proximate analysis etc., of raw material (powder) are performed.

Also chromatographic fingerprint pattern, to authentic species from raw material is developed.

In order to obtain marketing authorization, manufacturers of herbal remedies requires to demonstrate that their product meet acceptable standards of quality, safety and efficacy$^5$. 
QUALITY

Compared with conventional preparation, herbal products present a number of unique problems when quality aspects are considered. These arise because of the nature of the herbal ingredients, which are complex mixture of constituents, and it is well documented that levels of plant constituents can vary considerably depending on environmental and genetic factors. Furthermore, the constituent responsible for the claimed therapeutic effects are frequently unknown or only partly explained and this precludes the level of control which can routinely be achieved with synthetic drug substances in conventional pharmaceuticals. The position is further complicated by the traditional practice of using combinations of herbal ingredients, and it is not uncommon to have as many as 5 herbal ingredients in one product.

Herbal Ingredients

Control of starting material is essential in order to ensure reproducible quality of herbal remedy. Following are to be considered in the control of starting materials.

- *Authentication and Reproducibility of Herbal Ingredients*
  Herbal ingredients must be accurately identified by macroscopical and microscopical composition with authentic material or accurate descriptions of authentic herbs. Even when correctly authenticated, it is important to note that different batches of the same herbal ingredients may differ in quality due to a number of factors.

- *Inter/Intra – Species Variation*
  There is considerable inter and intra species variation in constituents, which is genetically controlled and may be related to country of origin.

- *Environmental Factors*
  These factors are climate, altitude and growing conditions, which affect the quality of herbal ingredient.

- *Time of Harvesting*
  The optimum of harvesting should be specified, as it is known that the level of constituents in a plant can vary during the growing cycle or even during the course of a day.
• **Plant Part Used**
Active constituents usually vary between plant parts and it is not uncommon for an herbal ingredient to be adulterated with parts of the plant not normally utilized.

• **Post Harvesting Factors**
Storage conditions and processing treatment can greatly affect the quality of an herbal ingredient. Inappropriate storage after harvesting can result in microbial contamination, and process such as drying may result in a loss of thermolabile active constituents.

• **Adulteration/Substitution**
Instances of herbal remedies adulterated with other plant material and even with conventional medicines have been documented. Reports of herbal products devoid of known active constituent have reinforced the need for adequate quality control of herbal remedies.

• **Identity Tests**
In order to try and ensure the quality of herbal remedies, it is essential therefore not only to establish the botanical identity of herbal ingredient but also to ensure batch to batch reproducibility. Identification tests include simple chemical tests, e.g. colour or precipitation and chromatographic tests. Thin-layer chromatography is commonly used for identification purpose but for herbal ingredients containing volatile oils a gas-liquid chromatographic test may be used. The aim of such tests is to confirm the presence of active principle(s); it is frequently the case that the nature of the active principle has not been established. In such a cases chemical and chromatographic tests help to produce batch to batch comparability and the chromatograms may be used as a “fingerprint” for the herbal ingredient.

• **Assay**
For those herbal ingredients with known active principles, an assay should be established in order to set the criteria for minimum accepted percentage of active substance(s).

• **Ash Value**
Incineration of an herbal ingredient produces ash, which constitutes inorganic matter. Treatment of the ash with HCl results in acid-insoluble ash, which consists mainly of silica and may be used to act as a measure of soil present. Limits may be set for ash and acid insoluble ash of herbal ingredients.
• **Foreign Organic Matter**
It is not possible to collect herbal ingredient without small amount of related parts of plant or other plants. Standards should be set in order to limit the percentage of such an unwanted plant contaminant.

• **Pesticides**
Herbal ingredients, particularly those grown as cultivated crops, may be contaminated by DDT or other chlorinated hydrocarbons, organophosphates, carbamates or polychlorinated biphenyls. Limit tests are necessary for acceptable level of pesticides contamination of herbal ingredients.

**PHASE II**

Steps involved in this phase are pharmacology, chemistry, cultivation and study of variations of medicinal plants.

• **Pharmacology**
In this step the activity of medicinal plants are narrowed down to a particular extract or to a specific phytochemical.

• **Chemistry**
The isolation and structure elucidation of active extract of phytochemicals are done in this step and thus generating standards for medicinal plants. This standard is used to develop a specific method to determine quality of medicine.

• **Cultivation**
The cultivation of plant species is attempted in order to get authentic material in required quantities.

• **Study of variation**
Using the analytical method developed for standard, seasonal variations, geographical variations and variations due to age of plants are studied. Stability testing of raw material and medicine in original form is performed.
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PHASE III

The formulation is made in modern manner like capsules, tablets, syrups, etc. Again this newly developed formulation is used to evaluate the safety, efficacy and stability of the medicine.

PHASE IV

Newly developed formulation is used for clinical trials, in the presence of practitioners of both modern and traditional therapies.

Clinical Efficacy

Newly developed formulations are measured up to the standards by employing following criteria:

1. Content
2. Percent strength
3. Purity
4. Safety
5. Clinical efficacy
6. Bioavailability

Now a days, it is understood that percent chemical strength is not only the correct amount of the labelled drugs but also release drug upon administration to the patient. Thus clinical effectiveness and bioavailability are added to the criteria for effective drug product development. A drug therefore, should not only be safe but beneficial as well and its therapeutic claims must be based upon sound clinical evidence.

Bioavailability

The most important property of the dosage form is its ability to deliver the active ingredients to its site of action in sufficient amount to elicit the desired pharmacological response. This property of the dosage form is referred to as its physiological availability or bioavailability. More about bioavailability is discussed in chapter – 4.
As discussed earlier, in case of standardisation of herbal medicine, efficacy testing, safety testing and chemical testing are the important parameters. These parameters are evaluated by taking the help of analytical techniques and thus analytical chemistry plays an important role in standardisation of herbal medicine. Also in clinical studies, the amount of active ingredient present in biological fluid is monitored with the help of modern analytical techniques.

Standardisation of herbal medicine is a **multidisciplinary approach**. The boundaries set by the classical division of research into chemistry; botany, zoology etc. need to be traversed if we have to do justice in evaluating these medicines.

The role of analytical chemist here is that of an ‘all rounder’. Along with his natural area of chemical analysis he has to dwell into the areas of pharmacology and toxicology.

The development of modern analytical techniques e.g. high performance thin layer chromatography (HPTLC) with facility to perform densitometric scanning in situ (on plate) has put a very powerful tool in the hands of chemist to do his bit in standardisation of plant based medicine. Analytical chemistry continues to expand, as developing technology and changing society impose new demands on it. In order to meet their demands, a host of new analytical techniques and instruments have been developed.

**SELECTION OF ANALYTICAL METHOD**

Once the purpose of analysis is established, the following important factors are considered in choosing an analytical method.

**Limit of detection**

Limit of detection is important especially in the case of trace analysis, when one has to decide whether a contaminant is present below or above a maximum acceptable concentration. Ideally, the limit of detection of the method selected should be at least one tenth of the concentration to be measured. In some cases, the limit of quantification should be considered, if it is necessary to detect the presence of an analyte and also to determine the amount present with a reasonable degree of certainty.
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Accuracy
A high degree of accuracy is not often important for trace analysis where the concentration of the contaminant is well below the permitted level. When the permitted allowed concentration of contaminant or permitted additive is close to the maximum allowed, accuracy becomes more important.

Precision
It is expressed as degree of agreement among individual test results when procedure/method is applied to a homogeneous sample – usually expressed as standard deviation (SD) / relative standard deviation (RSD).

Speed
If a large number of samples are to be analysed, a method, which is rapid, is to be preferred so that the data can be acquired quickly at minimum efforts and cost.

Sample size
The amount of sample available is normally not a limiting factor. However in case of clinical chemistry, no patient is willing to donate large volumes of blood for analysis. Similarly, in forensic work, the sample material may be limited in size. Improved detection levels can sometimes be achieved by taking a large weight of sample, but there are limits to this approach.

Cost
Analytical chemists have to be concerned with the cost of analysis. Analysis requiring techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy will be more expensive than classical techniques because of high cost of equipment and high grade of staff required for interpreting the data and also high cost of maintenance of the equipment.

Safety
The need for special facilities for work involving neutron activation analysis and radio chemical measurement and other safety factors may also influence our choice of method. For example one may wish to avoid the use of methods, which requires toxic solvents such as benzene, chlorinated hydrocarbons or reagents like potassium cyanide if alternative procedures are available.
Specificity

The degree of discrimination between the analyte and the other substance present or extracted from the matrix must be carefully considered. Attention will be paid to the clean up procedures to be used. This discriminating power of the detection system testing using likely interfering compounds may be necessary.

Robustness

Robustness of the selected method must be kept in mind before selecting a method of analysis. The selected method must give reproducible results in a different laboratory under different environment conditions. For example the results of the analysis must not vary with changes in the brand of the reagents used, slight changes in pH, time of heating etc. The method must also be reproducible with slight changes in temperature, humidity etc.

In conclusion the choice of the method will depend on several factors. The selected method must be adequate for the decision one has to take when the results are obtained. For that, one must have a clear picture in his mind as to why the analysis is being carried out and what one has to achieve. Frequently more than one technique can be used to detect the same analyte.

CLASSIFICATION OF ANALYTICAL METHODS

Analytical methods can be broadly classified as wet-chemical methods which includes gravimetric and volumetric analysis and instrumental analysis which includes optical methods (e.g. emission and absorption spectroscopy etc.), separation methods (e.g. chromatography), and electroanalytical methods (e.g. potentiometry, voltammetry etc.). Figure –1.2 shows classification of different analytical methods.
Chromatographic technique is a powerful separation technique and it is used for identification and quantification of components in a complex matrix of sample. The present research work employs a high performance thin layer chromatographic (HPTLC) technique for determination and quantification of piperine, an active alkaloid present in black pepper and high performance liquid chromatographic (HPLC) technique for determination and quantification of trovafloxacin, an antibacterial drug from human plasma.
ADVANTAGES OF HPTLC TECHNIQUE IN STANDARDIZATION OF HERBAL MEDICINE

- As sample clean up taken place on the pre-adsorbent layers, minimal sample preparation is required. Prime steps involved in sample purification (extraction, separation of phases, evaporation and reconstitution of analyte) are eliminated resulting in saving time and avoidance of loss of analyte due to these steps. Thus herbal extract can be directly applied to the pre-adsorbent layers.

- Since the herbal extract is the complex matrix containing various components like alkaloids, fatty acids, proteins, glucose etc. they can be directly applied and separated on a plate. But in case of HPLC, samples require purification and clean up before being introduced into the column. Strongly adsorbed impurities and unwanted components if present in sample can damage the HPLC column.

- Accuracy of the method is higher than HPLC.

- There is extreme flexibility for various steps [stationary phase, mobile phase, developing technique, detection (pre-and post chromatographic derivatization)].

- Choice of solvents for dissolving sample is not very critical as it is to be removed completely before developing the chromatogram.

- Strongly adsorbed impurities or presence of solid particles do not interfere, as plates are not re-usable.

- In-situ derivatization possible and routinely employed.

- Solvents need no prior treatment like filtration and degassing and solvents of analytical grade are suitable.

- Mobile phase consumption per sample is extremely low, thus reducing the acquisition and disposal cost.

- Multiple development after each scanning possible to separate certain slow moving solutes.

- Sorbent requires no regeneration as TLC/HPTLC plates are disposable but in case of HPLC, column requires regeneration after each chromatographic run, which is a time consuming process.
ADVANTAGES OF HPLC TECHNIQUE IN CLINICAL STUDIES

In clinical studies, HPLC technique is mainly used for determination of active component (moiety) from biological fluids. Often in body, active component converts to its metabolite/s and quantification of that metabolite/s is necessary. HPLC technique is highly suitable in such cases and has several advantages over other separation techniques in determination of the drug from biological fluids.

- Most important analytical parameter in this technique is the resolving power (specificity), which is relatively high. The resolving power helps in separating the active component from plasma interference.
- Accuracy of the recovery experiment is better than that achieved with HPTLC.
- As compared to HPTLC, precision of HPLC is high.
- In measurements by HPLC technique, capacity factors ($k'$) are reproducible.
- Resolution is better in HPLC than in HPTLC.
- Substances sensitive to light and oxygen give least problems, as it is a closed system.
- HPLC columns are reusable.

STATISTICS

Statistics refers to quantitative information or to methods of dealing with quantitative information. The quantitative information or data are analysed by specific method called statistical methods.

The various functions of statistics are as follows
- It represents facts in a definite form
- It simplifies mass of figures.
- It facilitates comparison
- It helps in formulating and testing hypothesis
- It helps in prediction
- It helps in formulation of suitable policies
Statistical science is a large field, with application in the pharmaceutical sciences. Statistical methods are an integral part of development, evaluation, and marketing of drug products. It finds significant applications in pharmacological screening of new drugs, toxicological tests, biochemistry, drug metabolism studies, clinical testing including analysis of pharmacokinetic data, pharmaceutical dosage forms, research and development, quality control, consumer testing, market research and marketing. When any chemical analysis is performed, it is influenced by several uncertainties. Therefore it is practically impossible to perform a chemical analysis in a way where results are totally free from errors or uncertainties.

When an experiment or study is performed, the results obtained are in the form of numerical values or measurements. The results obtained from experiments are meaningless unless some conclusion can be drawn on the basis of such experiments: statistics allow us to make certain decisions based on these results.

It is important to understand the precise definitions of the various terms to avoid any ambiguity. The various statistical operations that are used in analytical chemistry are summarised.

**Error**

The term error is defined as the departure of the measured value from its true value. Thus, greater the difference between the two values, greater will be the magnitude of the error involved.

**Accuracy and Precision**

Accuracy is defined as the closeness of the observed value to the true or accepted value, whereas precision is defined as the closeness of the measurement as compared to other measurements which are carried out in a similar manner. Thus it can be said that accuracy is a measure of the reliability of any measurement and precision is a measure of reproducibility of a measurement.

In case of figure (a), the results obtained are highly accurate and precise. In case of figure (b), results obtained are less accurate but highly precise. Figure (c) shows that the results obtained are highly accurate but less precise and in case of figure (d) results obtained are less accurate and less precise.
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a. high accuracy high precision  
b. low accuracy high precision

c. high accuracy low precision  
d. low accuracy low precision

Accuracy is expressed in terms of error namely absolute error and relative error. 
Precision being reproducibility of results are expressed in terms of mean, median, 
standard deviation and coefficient of variance.

**Central tendency**
A large proportion of observations in a group of observations on some variables has a 
tendency to cluster around some central value. This is known as central tendency. The 
most commonly used measures of central tendency are:

**Mean**
It is calculated by adding all the values in the data and then dividing by the total 
number of measurements.

\[ X = \frac{\sum x}{n} \]

**Median**
Median is one of the measures of central tendency. It divides the ordered sequences of 
data into two equal groups, half of the values will be less than the median and half will 
have values greater than the median. Thus if the middle observation of the data that is 
arranged in increasing or decreasing order is the median. If the number of observations
‘n’ is odd there will be one and only one middle value or median i.e. \( \frac{1}{2} (n+1)^{th} \) observation from either end of the ordered sequence. If ‘n’ is even there is strictly no middle observation but the median is defined by convention as the mean of two middle observations, i.e. the \( \left( \frac{n}{2} \right)^{th} \) and \( \left( \frac{n+1}{2} \right)^{th} \) observation from either end in the ordered sequence.

**Deviation**

The difference between the observed value and arithmetic mean of the set of observations in known as deviation

\[ d_i = X_i - X \]

**Mean Deviation**

Mean deviation is defined as the arithmetic mean of all deviations irrespective of all the sign of the deviation

\[ \bar{d} = \frac{d_1 + d_2 + d_3 + \ldots + d_n}{n} \]

**Standard Deviation**

The standard deviation of an infinite set of statistical data is theoretically the mean of the squares of the difference between the individual value \( x_i \) and the mean of the infinite observation \( \mu \).

Scientific experiments often have a small number of observations. Standard deviation is calculated using \( (n-1) \) because it represents better estimate of the standard deviation of the population from which the sample is taken.

\[ S = \sqrt{\frac{(x_i - \mu)^2}{n-1}} \]

For large number of observations \( (n-1) \approx n \).

Therefore,
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Variance
The variance of a set of data is a square of the standard deviation. Variance measures the extent of which the data vary amongst themselves.
Variance = (standard deviation)$^2$.

Coefficient of Variance
The relative measures of dispersion is expressed by the term coefficient of variance for a set of measurements having 'X' as the mean and 'S' as the standard deviation. The coefficient of variance can be computed as

Coefficient of variance = $\frac{S \times 100}{X}$

Confidence Interval
The statistical interval set about the mean 'X', so that, for a given number of replicate measurements and for a given probability level the population mean 'µ' lies within the specified range. These statistical limits are known as confidence limits and the interval so generated is called as a 'confidence interval'. The confidence interval is calculated as follows:

Confidence interval = $X \pm \frac{S.D.}{\sqrt{N}} \times \alpha$

Where, X is the observed mean, S. D. is standard deviation for the experiment containing N number of observations. 'z' is the student 't' value obtained from the student 't' table for the given level of confidence and for a given degree of freedom. Confidence intervals are usually calculated at 95 % and 99 % level of confidence. The smaller the confidence interval at 95 % and 99% levels of confidence, the more accurate are the results.
**Regression Analysis**

The statistical tool with the help of which we are in a position to estimate or predict the unknown values of one variable from known values of another variable is called as regression. In regression analysis the average relationship between the two variables is revealed and thus it is possible to make a prediction of the dependent variable. The variable whose value is influenced is called as the dependent variable ‘Y’; the variable which exerts the influence, is called as the independent variable ‘X’. In case of linear regression, it will be seen that a unit change in the value of the independent variable (X) will produce a constant and absolute change in the dependent variable (Y). When the two variables have a linear relationship the regression line can be used to determine the value of the dependent variable. The correlation coefficient obtained during the analysis of a regression line plays an important role in deciding the relationship between the independent and the dependent variable.

**The Least-Squares Method for Deriving Calibration Plots**

Most analytical methods are based on a calibration curve in which a measured quantity y is plotted as a function of the known concentration x of a series of standards. (Figure 5 shows a typical calibration curve) A plot of resulting data approximates a straight line. However, not all the data fall exactly on the line because of the random errors in the measuring process. Thus, we must try to derive “best” straight line from the points. A statistical technique called the method of least squares provides the means for objectively obtaining an equation for such a line and also for specifying the uncertainties associated with its subsequent use.

In applying the method of least squares, one has to assume that there is a linear relationship between the peak area (y) and the analyte concentration (x) as by the equation

\[ y = mx + b \]

Where b is the intercept (the value of y when x is zero) and m is the slope of the line. One has to also assume that any deviation of individual points from the straight-line
results from errors in the measurement. That is, the prepared standards carefully enough so that the random errors in the preparation process are negligible with respect to those in the measurement process. Usually it is possible to satisfy this assumption.

The vertical deviation of each point from the straight line is called a residual value. The line generated by the least-squares method is the one that minimises the sum of the squares of the residuals.

**STATISTICAL ANALYSIS OF PLASMA DRUG LEVEL**

The plasma drug level data should be analysed statistically in order to establish whether any difference between the plasma concentration time curves of each drug products under the test are simply due to biological and experimental variations or due to their actual bioavailability differences.

**IN VITRO - IN VIVO CORRELATION**

In vivo - in vitro correlation refers to the establishment of a relationship between a biological parameter or parameter derived from biological property produced by a dosage form and physico-chemical property or characteristic of the same dosage form. The biological properties used are the plasma concentration form or AUC (area under curve) obtained following drug administration of the dosage form. The physico-chemical properties are characterised by dosage forms in vitro dissolution behaviour viz., percentage drug release under a given set of conditions.

The simplest way to demonstrate a correlation is to plot the fraction absorbed in-vivo versus the fraction released in vitro. This relationship is often linear with a slope of 1. The intercept may or may not be zero depending upon whether there is a lag time before the system being to release drug in-vivo, or the absorption rate is not instantaneous resulting in the presence of so finite quantity of dissolved but unabsorbed drug. In either case, it is a point-to-point or levels A correlation when the relationship is linear with slope of 1.
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IN-VITRO CORRELATION BY NELSON AND WAGNER

- Using the dissolution profile graph the time required for 50% dissolution should be estimated as shown in figure 1.3

Figure 1.3
Dissolution profile

\[ x^* = \% \text{ dissolved before getting absorbed} \]
Using the Nelson-Wagner model the fraction absorbed should be estimated as shown in table 1.1

**Table 1.1**

**In vitro data - percentage fraction absorbed (Nelson - Wagner model)**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Mean plasma concentration $C_t$</th>
<th>$\int_0^t C_t , dt$</th>
<th>$K \int_0^t C_t , dt$</th>
<th>$C_t + K \int_0^t C_t , dt$</th>
<th>Percentage fraction absorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.5</td>
<td>2.40</td>
<td>0.60</td>
<td>0.05</td>
<td>2.45</td>
<td>41.5</td>
</tr>
</tbody>
</table>

A graph showing the percentage fraction absorbed against time should be presented as shown in figure 1.4

**Figure 1.4**

**Percentage fraction absorbed versus time curve**
• Using this graph, the time for 50% of absorption is to be estimated

• The intensity factor is to be calculated as

\[
\text{Intensity factor} = \frac{\text{Time for 50\% absorption}}{\text{Time for 50\% dissolution}}
\]

• Transform \( T \) (in vivo time point) to the corresponding in vitro time point applying to the equation

\[
T = \frac{\text{In vivo}}{\text{Intensity factor}}
\]

• The percentage absorption at \( T_{\text{in vivo}} \) and percentage dissolution at \( T_{\text{in vivo}} \) should be plotted on Y and X axis respectively as shown in figure 1.5

Figure 1.5

**Percentage fraction absorbed versus percentage dissolved**

A slope = 1 of this line indicates a good in vitro-in vivo correlation. The value of the positive intercept of X axis gives the percentage drug dissolved before getting absorbed or percentage drug dissolved during lag time (figure 1.4).

Lag time can be estimated as the time corresponding to this intercept on dissolution profile (figure 1.3).
SELECTION OF PLANT

Black pepper (Piper nigrum linn.), a native of India, is the oldest and widely used spice of the world. In India black pepper is cultivated mainly in the states of Kerala, Tamilnadu, Karnataka and to a small extent in Assam and Maharashtra. Two crops are harvested in a year, one from August to September and the other from March to April; hence it is easily available throughout the year. High annual yield of commercial pepper makes it available to common man at affordable prices.

Fresh green pepper finds its use in pickles especially in Kerala. It finds a major use in the meat industry of America for curing and preservation of meat. It is widely used as a flavouring agent in many cuisines of the world. In modern medicine, black pepper is rarely prescribed whereas in the Indian medicinal systems it holds an important place. In combination with long pepper and ginger it has been used for many centuries and this combination is known as ‘Trikatu’ as three acrids. Black pepper appear in many ayurvedic preparations like Madhumalini Vasant, Laghumalini Vasant, Suvarnamalini Vasant, Gasex tablets, Amritharishtha, Trikatu etc. Black pepper fruits are used as diuretic, carminative, stimulent, anticholiner, antiasthmatic, in malarial fever, in paraplegia and arthritic diseases. Extracts and essential oil of the fruit shows biological activities like antibacterial, antifungal, taenicial and larvicidal.

Published work reveals that the main alkaloid, piperine, present in black pepper enhances bioavailability of drugs when it is co-administered making it a potentizing agent.

The results of several experiments performed to assess the potantizing property of piperine have revealed that the increase in bioavailability of a drug under investigation was not uniform and in some cases no increase in plasma drug concentration was observed.

To conduct bioavailability study in healthy human volunteers, the following facilities are required.
• A well equipped clinical facility that can house at least twelve volunteers.
• Full time doctors, pharmacologists, and assistants
• A laboratory equipped with sophisticated instruments for blood plasma processing, storage and to carry out its analysis

Owing to importance of black pepper in Indian medicinal system and its effectiveness as a potantizing agent was one of the reason for the selection of plant.
Piper nigrum Linn.: A review

Family: Piperaceae
English – Black Pepper

Habitat:
This perennial climber shrub is cultivated in the hot and damp parts of India.

Parts used:
Dried fruit

Pharmacological Action:
Black pepper is acrid, pungent hot, carminative, also used as antiperiodic. Externally, it is rubefacient and stimulant to the skin and also a resolvent. The extracts and essential oils of black pepper are reported to have antibacterial and antifungal activity. The fruits exhibited taenicideal activity.

Medicinal properties and uses:
The paste of black pepper is a rubefacient and stimulant, it is used for treatment of boils, relaxed sore throat, piles, paralytic affection, rheumatic pain, headache, protapsed rectum, toothache, debility, diarrhea, cholera, disorders of the urinary systems, cough, gonorrhea, and malarial fever. Pepper is mainly employed as an aromatic stimulant in cholera. Black pepper was used by Malay women as an abortifacient. It is thelminitic and appetizer, and it also increases the digestive capacity. Piperine from black pepper is responsible for its CNS depressant activity.

Pharmacognostical characteristics:
A stout, glabrous, woody creeper, much swollen at the nodes. Leaves broadly ovate. Flowers unisexual and bisexual, in slender, drooping spikes, berries in racemes, rather fleshy, one sided, yellow, turning red when ripe. The fruit are distinguished by the absence of peripheral sclereids of pericarp. Dark brown mesocarp zone, asymmetrical thickened wall of the endocarp, shining seed coat layer and yellow pigment cell in the kernel.

Chemical constituents:
Black pepper consists of an alkaloid piperine (5 to 9 %), piperidine (5%), a balsamic volatile essential oil (1 to 2 %) and fats (7%); mesocarp contains chavicine, a balsamic volatile oil, starch, lignin, gum, fat, proteids, etc. chavicine is a soluble pungent concrete
Plate 1.1
Black pepper leaves

Plate 1.2
Black pepper fruits
resin.

*Chemical and Analytical Work on P. nigrum.*

Black pepper is a spice, which is used, in daily life as a food. Because of the increasing demand of black pepper, the possibility of adulteration increases, therefore becomes necessary to check the quality of the spice. Different physical and chemical methods are being used to check quality of the spice.

In early years, various attempts were made to identify adulterants in black pepper\textsuperscript{15-28}. In 1909 G. Graff\textsuperscript{29} showed that the most reliable determination next to the ash value, was the crude fibre. He also showed his inability to distinguish a grounded product made entirely from whole black pepper of medium grade from a mixture of especially good whole pepper with pepper shells. He tried to set grades of pepper on the basis of crude fibre content.

Since black pepper is bitter in taste, some attempts were made in early days, to evaluate relation between chemical constituents and its taste. H. Staudinger\textsuperscript{10} and his colleges reported that the taste of pepper was not only because of piperine, the main alkaloid, but it was also because of different components present in pepper.

In 1900 to 1950, the main work done on pepper was for identification, isolation and characterisation of new compounds like volatile oil, chavicine, piperidine, piperritine etc\textsuperscript{31-39}.

With introduction of a different instrumental method in chemical analysis, there is a significant change in research area. These instruments can be used for quantitative as well as qualitative analysis.

Francesco Duro\textsuperscript{40} used a volumetric method (Idometric titration) to determine the piperine in Piper nigrum fruits. Horac D. Graham\textsuperscript{41} showed that the piperine can be estimated by colorimetrically using Labat reagent. In his work he measured the colour at a wavelength 660 nm. In his another work he treated piperine with nitric acid and then with alkali which gives red colour which is stabilized by thiourea. This colour was determined at 490 nm\textsuperscript{48}.

Some more attempts were made to determine piperine by the method of colourimetry or spectrophotometry\textsuperscript{42-47}.

In 1947, O. Hanc and F. Santevy\textsuperscript{49} used a polarographic method based on the reducibility of piperine on a Hg-drop electrode, to determine the piperine content.
After the introduction of *Gas Chromatographic* method in the field of instrumental analysis, several attempts were made to determine the volatile compounds from black pepper.

**W. G. Jennings** and **R. E. Wrolstad**\(^5^0\) use made of gas chromatography for separation of black pepper oil into 23 volatile compounds including \(\alpha\)-pinene, \(\beta\)-pinene and \(\beta\)-caryophyllene etc. **Richard H. M. and W. G. Jennings**\(^5^1,5^2\) reported several hydrocarbons and oxygenated terpenes in pepper oil. Several compounds have been determined by gas chromatography\(^5^3-5^6\).

HPLC technique were often used to determine the several compounds in black pepper and now a days, it is a very popular and familiar separation technique used for quantification of piperine. There are several HPLC methods used for determination of piperine from black pepper\(^5^7,6^6\).

**Experimental Biology:**

Wide ranges of pharmacological activities are shown by black pepper\(^6^7\). It is proved experimentally that pepper is insecticide\(^6^8,6^9\), carcinogenic\(^7^0,7^1\), hepatoprotective\(^7^2\), antioxidant potential of piperine\(^7^3\) etc.

Experimental evidences showed that the use of black pepper or its active principle, piperine, when co-administered with other drug enhances the bioavailability of number of drugs. This might be the reason why Ayurvedic materia medica mentions this herb as one of the essential ingredient of number of prescriptions and formulations used for a wide range of disorders ranging from disorders of the urinary systems, cough, gonorrhea, to malarial fever.

In 1929, **Bose**\(^7^4\) while describing the anti-asthmatic property of vasaka leaves (Adatoda Vesica) affirmed that addition of long pepper (which contains piperine as an active ingredient) to vasaka increased its efficacy as anti-asthmatic agent.

Experimental evidences show that the use of black pepper or piperine enhances the bioavailability of a number of drugs like vasicine, spartain, sulphadiazine and tetracycline\(^7^5,7^6\). In studies carried out on animals as well as human volunteers it was observed that piperine, the active principle from the pepper\(^7^7,7^8\) was the major compound responsible for enhancement in bioavailability. Enhancement of blood levels of the drug such as rifampicin\(^7^9\), phenytoin\(^8^0\), pentabarbitalone\(^8^1\), theophylline and
propranolol\textsuperscript{82} was recorded when these drugs were co-administered with piperine in human volunteers in the range of 1.0 to 30.0 mg as total dose.

**Pharmacology and Pharmacokinetics of Piperine**

Piperine shows CNS Stimulant activity in rat\textsuperscript{83} and it was also demonstrated that piperine possessed CNS depressant properties in laboratory animals and produced hypnotic effect\textsuperscript{84}. Piperine exhibited analgesic activity against tail-clip pressure: antipyretic activity against typhoid vaccination and writhing syndrome in mice and anti-inflammatory activity against carrageenin-induced oedema in rats\textsuperscript{85}. Piperine interacted with Serotonergic system of rat brain and it was proved that this action of piperine could be responsible for its reported anti-epileptic activity\textsuperscript{86}. Piperine was found to deplete substance in the rat spinal cord\textsuperscript{87}. The neuromuscular transmission was affected by piperine; the activation of sensory terminals as well as direct action on sensory receptors has been reported in rat\textsuperscript{88}. The anti-fungal activity of piperine has also been documented\textsuperscript{89} and it is suggested that it could be due to the effect of piperine on the bioenergetic functions of the cell like its inhibitory effect on mitochondrial electron transport in the rat liver\textsuperscript{90}.

The pharmacokinetic profile of piperine has revealed 97% absorption irrespective of mode of dosing whereas 3% gets excreted in the faeces; but piperine has not been detected in urine. Maximum concentration of piperine was found in the stomach and small intestine by 6 hr. after oral administration and by 24 hr. only traces of piperine remained in serum, kidney and spleen. The increased excretion of uronic acids, conjugated sulphates and phenols as a consequence of scission of the methylenedioxy groups of piperine, glucuronidation and sulfation appeared to be the major steps in its disposition\textsuperscript{91}.
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