Chapter 2
Natural Products: Drug Discovery and Development

Inder Pal Singh, Furkan Ahmad, Debanjan Chatterjee, Ruchi Bajpai, and Neha Sengar

2.1 Drug Discovery and Development

The classical drug discovery and development is one of the most challenging efforts that directly benefit the mankind by providing new medicines. The process is long time consuming and cost intensive despite the recent technological advances. It is estimated that 1 out of 10,000 molecules synthesized or isolated is finally approved for human use. The cost of developing a new drug is close to 1.5–2 billion US dollars and takes up to 15 years from discovery stage to drug approval. The major cost is for clinical trials as compared with the preclinical drug discovery process, making a careful strategization of initial drug discovery process all the more important. Therefore it is more important to identify the possible dropouts at an early stage. The three major reasons for compounds failing to be successful drugs are toxicity, lack of efficacy, and lack of bioavailability [1]. Currently, the medicinal chemists have the advantage of guiding principles that have developed over the decades of experiences in order to design and optimize lead compounds. The first guidelines introduced as Lipinski's rule of five states that orally active drug should obey these five criterion: molecular weight less than 500 Da, calculated partition coefficient clog*P* less than 5, hydrogen bond donors less than 5, hydrogen bond acceptors less than 10, and number of rotatable bonds more than 10. Several other parameters were later added to the five proposed by Lipinski. These drug design guidelines were later followed by several others such as quantitative estimate of drug-likeness (QED). These computation models provide target molecules based on desirable ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties and these models are fairly able to predict the above properties. However, some of the most

I. P. Singh (✉) · F. Ahmad · D. Chatterjee · R. Bajpai · N. Sengar
Department of Natural Products, National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar, Punjab, India
e-mail: ipsingh@niper.ac.in

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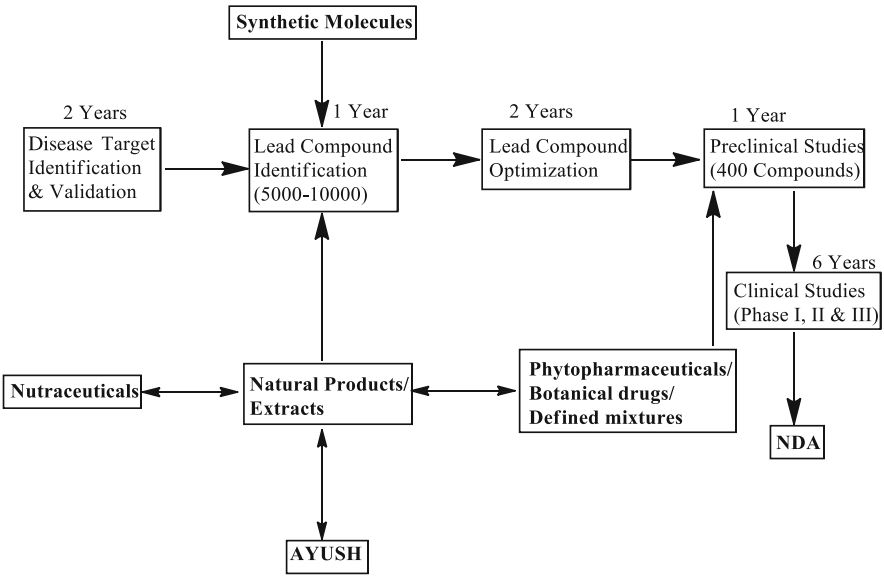


Fig. 2.1 Drug discovery and development process stages with approximate time lines [3–5]

important natural product drugs such as taxol, amphotericin B, vancomycin, and several others do not follow Lipinski's rule of five [2].

The process of drug discovery and development proceeds through several stages as shown in Fig. 2.1. Discovery begins with target identification, choosing a specific target involved in the biochemical pathway by understanding the disease condition at genetic, protein, and cellular level. This is followed by validating the target by confirming that it is actually involved in the disease and can interact with the drug. Several thousand molecules (synthetic, natural, semisynthetic, natural-mimics) are evaluated for interaction with the drug target and one or more lead molecules are selected. The promising compounds are evaluated for early safety studies. ADMET properties of each promising compound are studied. The potential drug molecules should be absorbed in the bloodstream, distributed to the site of action, metabolized, excreted from the body, and should be nontoxic. Lead optimization involves modifications in molecular structure to obtain more effective and safe molecules. A number of structural motifs can impart desirable properties to lead molecules in order to improve ADMET properties. The leads thus obtained are subjected to preclinical studies to determine if the drug is safe for human testing through a battery of in vitro and in vivo assays. Large quantities of drug are required for the development process. Therefore the scale-up of the drug is required at this stage to start with the clinical trials. Investigational New Drug (IND) application is filed before the trial can begin. The application includes the results of preclinical work, chemical structure, its manufacturing details, and any side effects. Phase I clinical trial is performed on small group of healthy volunteers in order to see the safety of drug in humans and study pharmacokinetics and pharmacodynamics. Phase II trials are conducted on a

small group of patients to evaluate efficacy and to see possible short-term side effects. Phase III trials are conducted on larger number of patients (1000–5000) to generate statistically significant data on safety and efficacy of the drug. This is the most expensive part of drug discovery and development process. New Drug Application is submitted after the completion of three phases of clinical trials. Phase IV trials after the approval of drug as larger number of patients use the drug involve monitoring for any adverse reactions and long-term safety of the drug [3].

2.2 Natural Products for Human Health

Historically, plants have been used since millennia for the treatment of many diseases and illnesses. Natural products (NPs) have long been a traditional source of drug molecules and modern drug discovery programs have relied extensively on natural sources in the past. Natural product (NP) chemistry has produced a huge diversity of secondary metabolites from a vast array of natural resources including plants, microorganisms, and animals both from terrestrial and marine origin. Several of these NPs are successful drugs or are drug candidates. The use of NPs as traditional medicines across several ancient civilizations is well documented in history.

The proteins, fats, nucleic acids, and carbohydrates known as primary metabolites are essential for living organisms and are the products of primary metabolism. In contrast, secondary metabolites are not essential for growth and development of an organism and are biosynthesized through several fundamental processes such as photosynthesis, glycolysis, and Krebs cycle. The most important building blocks involved in biosynthesis of secondary metabolites are acetyl coenzyme A, shikimic acid, and mevalonic acid resulting in an infinite diversity through several biosynthetic pathways, and it is the secondary metabolites that have played a major role in drug discovery efforts in the past [3].

Despite the enormous contribution of NPs in drug discovery, the last century being the most productive source of leads for drugs, however, there was a diminished interest in NPs in the pharmaceutical industry in the late 1980s and 1990s. This was in spite of the fact that there were major technological advancements in chemistry and biology. The combinatorial chemistry and high-throughput screening failed to show the anticipated impact or success. The pharma industry still faces challenges for the discovery and development of new drugs and requires major changes and new paradigms. It requires concerted efforts from biologists, synthetic and medicinal chemists, structure biologists, biotechnologists, computational experts, and clinicians to meet the challenges for new drug discovery and development. Discovery and development of natural products as modern drugs is a hugely challenging task. Besides being successful drugs, natural products also form the basis of several traditional medicine systems such as Ayurveda, Sidha, Unani, Homeopathy, Traditional Chinese medicines (TCM), Kampo, Tibetan medicine, etc. [6]. A new drug class regulated in India is phytopharmaceuticals which contain

partially purified extracts from traditionally used medicines with defined minimum four constituents. NPs also play a great role in beneficial effects of nutraceuticals. Aspects of natural products in modern drug discovery and the development of Ayurveda, Siddha, and Unani (ASU) drugs is covered in this chapter.

2.3 Natural Products in Modern Drug Discovery and Development

Drug discovery and development of natural products is a challenging task, which may be based on folklore, traditional or alternative medicine, and is entirely different from developing herbal remedies or dietary supplements or nutraceuticals. The process of finding and discovering a new and effective drug from Mother Nature involves isolating and purifying a single active molecule, characterizing the molecule followed by the entire process as shown in Fig. 2.1. Many drugs that are available in the market were discovered from natural sources; the approved drugs are either NPs or botanical drugs (defined mixtures) or are natural product derivatives. David Newman and Gordon Cragg, in a series of reviews over the last 20 years, have analyzed the role that NPs have played as sources of new drugs. In cancer alone, about 175 small molecules were approved during the time period from 1940s to 2014 and out of these 85 (49%) were either natural products or their derivatives. The other area where NPs have played a prominent role is the infectious diseases. During the period 1981–2014, a total of 174 approved drugs for cancer included 17 NPs, one botanical drug (defined mixture), and 38 NP derivatives. Besides these, 33 were biological macromolecules. NPs have also made enormous contribution in antibacterial area: 140 approved drugs included 11 NPs and 71 NP derivatives. NPs drugs were also approved as immunosuppressant (5), immunostimulant (3), hypocholesterolemic (4), antiparasitic (2), and one each for several other disease conditions. Four botanical drugs (defined mixtures) were also approved during this time period. This analysis by Newman and Cragg clearly provides an optimistic scenario for the important role of natural products in modern drug discovery [7].

Natural resources play a major role as starting materials for drug discovery. It has been estimated that nearly 75,000 species of higher plants exist and only 10% have been used in traditional medicine. Only 1–5% of these have been studied scientifically. Scaffold diversity is one of the most important features of a compound library that determines its success in screening hits when identifying bioactive molecules. Structural diversity in natural products is enormous underlining their importance in drug discovery and development. NPs often provide selective and specific biological activities based on mechanisms of action, examples are the HMG-CoA reductase inhibition by lovastatin and tubulin-assembly by paclitaxel. Pharmacologically active compounds from plants and microbes represent an important pipeline for a new investigational drug [8].

2.3.1 Historical Perspectives

Natural products have been used since ancient times for the treatment and prevention of many diseases. The earliest natural products were described on clay tablets in cuneiform from Mesopotamia era (2600 B.C.), oils from *Cupressus sempervirens* (Cypress) and Commiphora species (myrrh); these are still in use to treat coughs, colds, and inflammation. The Ebers Papyrus (2900 B.C.) is an Egyptian medical text compilation, which has 700 plant-based drugs ranging from gargles to ointments. The first and most famous well-known example of a natural product as a drug is morphine that was isolated from *Papaver somniferum* L. (opium poppy). Morphine was commercialized by Merck in 1826. Heroin (diacetylmorphine) was prepared by boiling crude morphine (isolated from the plant *P. somniferum*) with acetic anhydride. Codeine is also easily obtainable by simple methylation of morphine. The first NP-based semisynthetic pure drug was Aspirin (anti-inflammatory agent). It was derived from the natural product salicin isolated from the bark of the willow tree *Salix alba* L. and introduced by Bayer in 1899. Another example is penicillin from *Penicillium notatum* discovered by Fleming in 1929. Digitoxin is cardiotonic glycoside isolated from *Digitalis purpurea* L. (foxglove), which is frequently used in congestive heart failure. Paclitaxel (Taxol), the most widely used drug for breast cancer, was isolated from the bark of *Taxus brevifolia* (Pacific Yew). Pilocarpine, an alkaloid containing a histidine ring, was isolated from *Pilocarpus jaborandi* (Rutaceae). Pilocarpine has been shown to be effective in chronic glaucoma. Pilocarpine is also used to treat dry mouth (xerostomia) resulting from radiation therapy in cancer patients. Quinine and artemisinin are well-known anti-malarial drugs [9].

2.3.2 Sources of Natural Products

Natural products (NPs) with therapeutic potential can be obtained from various natural resources that include plants, animals, marine organisms, bacteria, yeasts, molds, fungi, etc. Therefore, it becomes very important to do a thorough literature search so that proper natural resource is selected. Most of the discoveries in the past from natural products have come out of serendipity. The selection can be based on phenotypic screening or target-based screening. There has been an explosion of data in biomedical sciences in the last 3–4 decades, and the main focus of drug discovery has been on drug targets using rational drug design strategies. Drug discovery in the past was mainly driven by serendipity with limited knowledge on molecular mechanisms of disease. The important factors to consider in choosing a starting point (plant/NP) are the history, folklore, medicinal use, availability, literature on pharmacology, toxicology, etc. Huge number of internet resources, retrieval databases, books, and primary literature are available to aid the process of selection of right resource for the intended purpose.

2.3.2.1 Plants

Plants produce a variety of different classes of compounds/secondary metabolites with diverse biological properties. In the last seven decades, several plant-derived compounds have been approved as antineoplastic agents; these include vinblastine, vincristine, vinorelbine, etoposide, teniposide, paclitaxel, docetaxel, topotecan, and irinotecan. Plants have provided useful drugs for many other disease conditions. NPs isolated and identified from both higher and lower plants will undoubtedly continue to provide useful drugs (Fig. 2.2).

2.3.2.2 Animals

The skin of poisonous Ecuadorian frog is a source of Epibatidine which is ten times more potent than morphine and has led to a new class of analgesics. Teprotide from the extract of Brazilian viper led to the development of enalopril and captopril, which are effective angiotensin-converting enzyme (ACE) inhibitors and have proved to be anti-hypertensive drugs (Fig. 2.3).

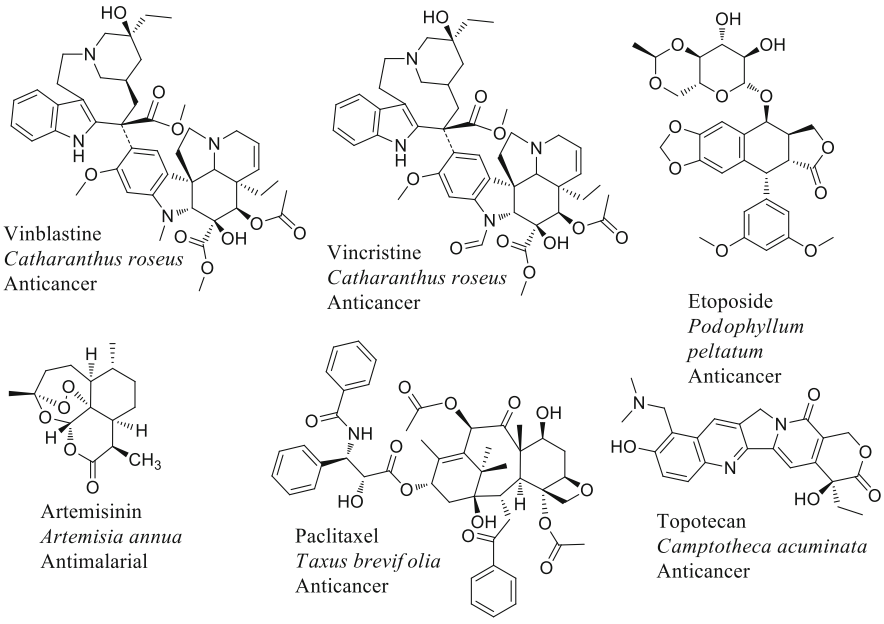


Fig. 2.2 Drugs from plants

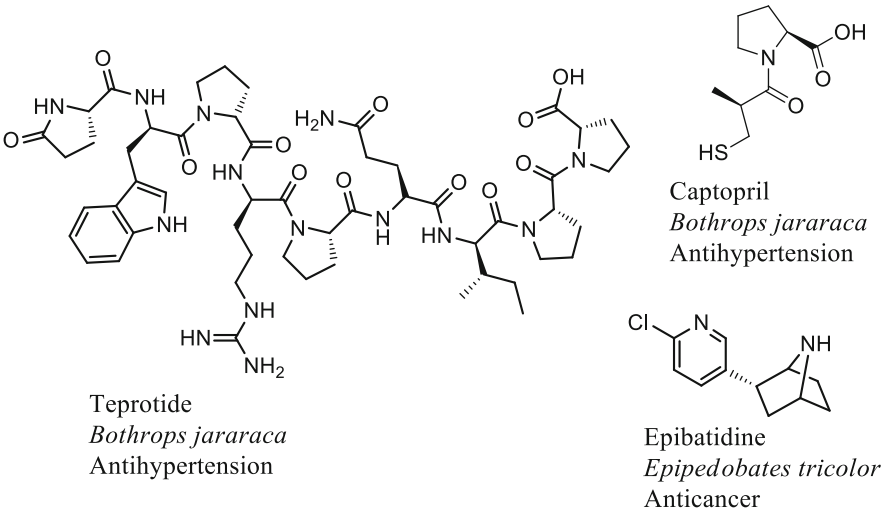


Fig. 2.3 Drugs from animals

2.3.2.3 Marine

Marine environment has proven to be a very rich source of bioactive secondary metabolites with unusual skeletons not encountered in terrestrial environment. More than 70% of earth surface is covered by oceans which have biodiversity even greater than rainforests and provide greater opportunity for discovering novel drug molecules. The first marine natural product to become a successful drug was cytarabine (Ara-C, cytosine arabinoside) isolated from *Cryptotheca crypta*, which is currently used for treatment of leukemia and lymphoma. Plitidepsin, a depsipeptide, was isolated from the Mediterranean Tunicate *Aplidium albicans*. It is effective against various types of cancers. Ecteinascidin 743 (terbactedin, yondelis) was isolated from the ascidian *Ecteinascidia turbinata* and is used in soft tissue sarcomas and ovarian cancer. Discovery of Halichondrin B obtained from sponge *Halichondria okadai* as anticancer agent led to the approval of eribulin for metastatic breast cancer in 2010 (Fig. 2.4).

2.3.2.4 Microbial

Microorganisms have already been proven as an excellent source of novel NPs primarily with antibiotic potential as well as several other therapeutic areas. The first antibiotic penicillin was discovered by Flemming from *Penicillium chrysogenum (*formerly *notatum)*. Mitomycin is an antimitotic agent and rapamycin is an immunosuppressive agent. Chloramphenicol isolated from *Streptomyces venezuelae* is used in typhoid. Doxorubicin isolated from the fungus *Streptomyces*

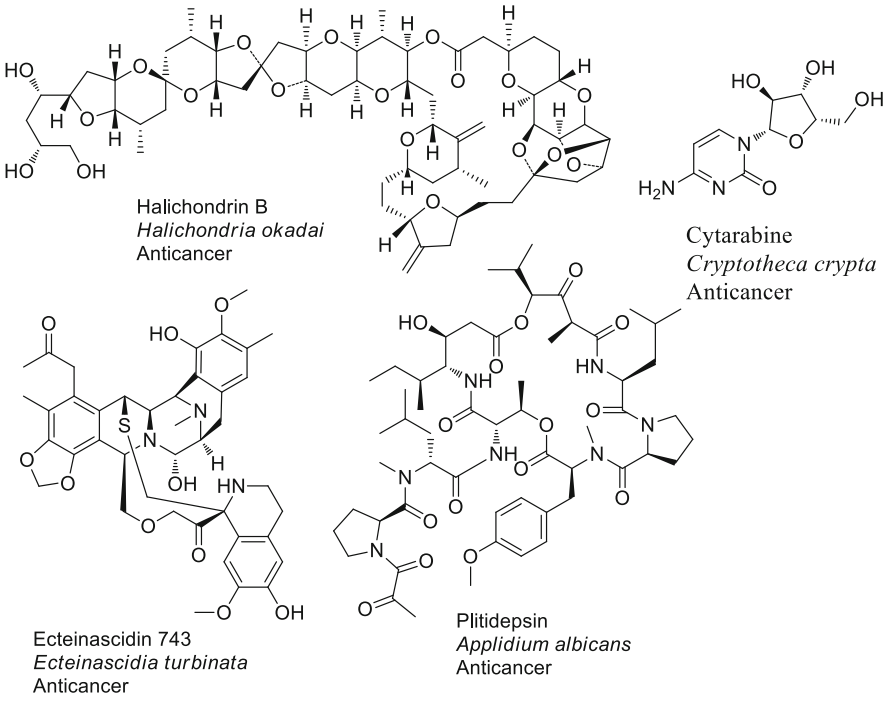


Fig. 2.4 Drugs from marine organisms

peucetius is used in the treatment of leukemia as well as Hodgkin and non-Hodgkin lymphomas [9] (Fig. 2.5).

2.3.3 Extraction

After careful selection of the natural resource for the discovery of known, new or novel drug molecules, the raw material has to be extracted with a suitable method/solvent system so that the target class of molecules is extracted. Extraction is a process of removal of one or more component from liquid, semisolid, or solid raw material that may be a plant part, or microbial broth, or animal tissue, etc. It is the first step in the analysis of plant material and isolation and characterization of secondary metabolites. This is an intensive and time-consuming exercise, and together with the fact that amounts of active ingredients in natural raw materials are fairly low, the extraction and isolation of NPs is usually considered a bottleneck in the application of NPs in drug development. Solvent extraction is the most widely used method; the solvent penetrates into the solid matrix solubilizing the solute that diffuses out of the matrix. Any factor that would increase the solubility or diffusivity of the solute will facilitate the extraction. Therefore the properties of the extraction

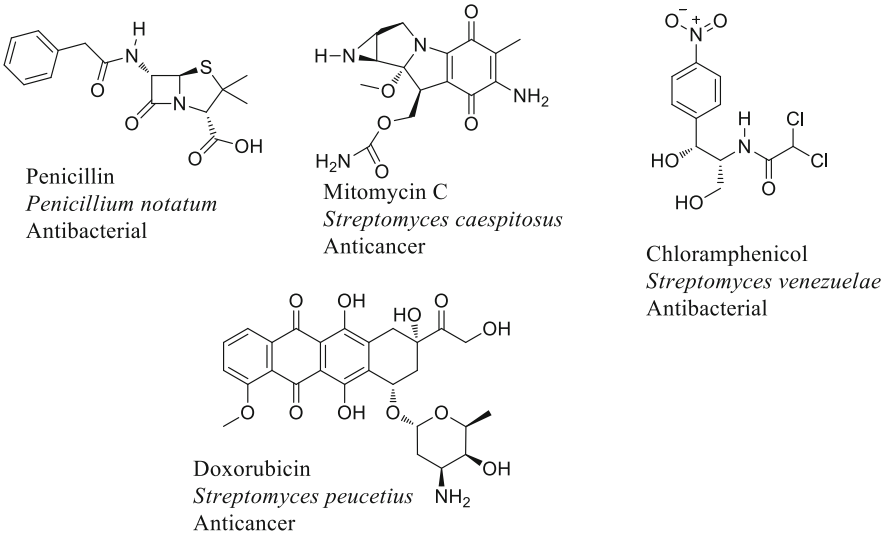


Fig. 2.5 Drugs from microbial sources

solvent, the particle size of the raw material, solvent-to-solid ratio, the extraction temperature, and the extraction duration considerably affect the extraction efficiency. The selection of solvent is crucial for solvent extraction; solvents with a polarity value close to the polarity of solute to be extracted are likely to give better extraction efficiency [10].

Several extraction methods have been employed for obtaining crude extracts from raw materials. **Maceration** is a very simple extraction method in which plant material is soaked in a suitable solvent with stoppered container kept at room temperature for a period of a minimum of 3 days with intermittent agitation followed by filtration. The disadvantage of this process is long extraction time, lesser efficiency, and huge solvent consumption. The major advantage is its suitability for the extraction of thermolabile components. **Percolator** is a conical vessel with an open top and adjustable bottom and has a closure to allow the passage of the fluid at a definite rate. The plant material is moistened with a sufficient amount of solvent prior to placing in the percolator. The material is placed in a percolator vessel so as to allow passage of fluid and complete contact with the plant material. The percolator must be filled with liquid and bottom outlet is opened for definite flow rate. The wet mass is pressed to extract the maximum residual fluid retained. Finally, an extract is obtained by filtration. This process is less time consuming and gives more complete extraction. In hot continuous extraction (**Soxhlet extraction**), the finely divided crude drug is placed in porous bag or thimble, the extracting solvent in the flask is heated and its vapors condensed into the condenser. The condensed extractant drops down into the thimble containing the crude drug. The liquid contents containing the extract siphon into the flask when the level of liquid in the chamber rises up to the top of the siphon tube. This process is continuous and carried out until a drop of solvent

from the siphon tube does not leave a residue when evaporated. In Soxhlet extraction, larger amount of drug can be extracted with a small quantity of solvent. The major disadvantage is the raised temperature which may degrade thermolabile compounds. **Countercurrent extraction** (CCE) is a liquid–liquid extraction process in which two immiscible liquids move in the opposite direction in continuous contact with each other. In this process, crude material is crushed and prepared in the form of fine slurry. The material to be extracted (fine slurry) moves in one direction within a cylindrical extractor where it comes in contact with extraction solvent. Finally, concentrated extract comes out at one end of extraction assembly while the marc (residue left after extracting the desired constituents) comes out from the other end [11]. The **microwave-assisted extraction** (MAE) uses microwave as a source of energy to heat solvent in contact with the sample. MAE is very fast and uses very small amount of solvents and gives improved yield. In this process, microwave radiation interacts with dipoles of polarizable material (solvent and sample) making it a selective method for polar compounds. **Accelerated solvent extraction** (ASE) employs high temperature and pressure for extracting various constituents from the crude drug. The crude drug is packed with sand in an extraction cell forming layers of sand and sample. The automated extraction technology is able to control temperature and pressure and is a very fast technique usually complete within an hour. ASE is very efficient technique, despite using high temperature and pressure; it still can be used for thermally unstable compounds as the extraction time is very short. **Enzyme assisted extraction (EAE)** is a very safe, green, and novel approach for the extraction of bioactive compounds. The pressed material is treated with an enzyme such as cellulase, pectinase, and α-amylase. The use of this hydrolytic enzyme gives better yield due to the action of enzymes on the cell wall and membrane inside the cell that facilitates the release of the natural products [12]. **Ultrasound extraction (sonication)** uses ultrasound ranging from 20 to 2000 kHz, the ultrasound waves increase the surface contact between solvent and sample, the ultrasound energy breaks the cell wall that facilitates the release of compounds. It is a more effective extraction method for thermolabile compounds as moderate temperature is used and is very fast technique. The use of ultrasound more than 20 kHz may affect active compounds through the formation of free radicals [11]. **Super-critical fluid extraction (SCFE)** uses supercritical fluid as an extracting solvent. Carbon dioxide is the most commonly employed supercritical fluid (SCF). A supercritical fluid is more likely a gas but with solvation characteristics of a liquid. Supercritical carbon dioxide is good for extraction of non-polar solutes; for more polar compounds, small amount of methanol or ethanol is added as an auxiliary solvent. Major advantage of SCFE is that the gas is easily recycled, chemically inert, non-flammable, a natural substance, easily available, and easily removed from the product [10]. **Decoction** is the process in which a crude drug is boiled in water for certain time period, then it is cooled and the mixture is strained or filtered. This method is suitable for extracting water-soluble and heat-stable compounds. Generally roots, leaves, and flowers are boiled in water for about 15 min, while branches and other hard parts can require up to an hour [11]. **Aqueous alcoholic fermentation** is commonly employed in traditional medicine preparation such as Ashavas and

Arishtas in Ayurveda. The drug either in powder form or decoction is kept with jaggery and flowers of *Woodfordia fruticosa* at defined temperature for a certain period of time. Fermentation generates alcohol in situ, which serves as a preservative [11].

Once the material is extracted, two different strategies may be adopted for further research. In the olden times, where the main focus was on delineating the chemistry of different plants, the approach adopted was straight forward isolation and identification of compounds followed by biological evaluation. Most of the NP research before 1970s followed the above approach. The result was isolation and characterization of a huge number of natural products from different plant sources, which may or may not have shown or evaluated for any activity. For the isolation of a specific class of compounds, the individual fraction may be assayed through physicochemical data such as analyzing each fraction by nuclear magnetic resonance (NMR) or mass spectrometry. The selection of plants for chemical investigations was indeed based on their ethnopharmacological information, traditional uses, or folkloric reputations. The other approach is the bioassay-guided (mainly in vitro) isolation of NPs as lead compounds. The crude extracts are subjected for preliminary screening against various biological or pharmacological assays in order to find the active extracts for a given assay relevant to the disease condition. A bioassay is an analytical procedure for qualitatively or quantitatively measuring the concentration or potency of target molecules/extracts by evaluating effect on living cells or tissues. A variety of preliminary bioassays to detect properties such as antifungal, anticancer, anti-HIV, antibacterial, and antidiabetic are available for various disease conditions and can be used to prioritize the extracts in terms of activity. These bioassays should be simple, fast, and sensitive since the amounts of active constituents in extracts may be very small. Bioassays could involve the use of in vivo systems (whole animal experiments), or in vitro systems (e.g., cultured cells). In vivo studies are more relevant to clinical conditions and can also provide toxicity data simultaneously. However, in vitro bioassays are microplate-based and require a small amount of extract, fraction, or compound for the assessment of activity [3].

2.3.4 Isolation and Purification

Each crude extract from a natural source usually contains a huge number of secondary and primary metabolites in a percentage usually ranging broadly between 1.0 and 0.0001 percent of the total biomass. The activity shown by the crude extract may be due to a single compound or to a mixture of compounds. Therefore, it becomes important to isolate and purify the NPs from the crude extract by chromatographic techniques. It is relatively easier to purify a compound which may be 1% of the biomass, but extremely difficult to purify a compound which is 0.0001%. Isolation is a process in which separation of a single pure compound occurs from many related molecules in the mixture. The design of isolation protocol is very important for successful accomplishment of this objective. First, it should be

determined whether the objective of isolation is an unknown compound responsible for a particular biological activity, a known compound, a class of NPs within an organism, or structurally related compounds such as isomers or all the secondary metabolites produced by organisms. The isolation strategy will differ depending on what is to be isolated. For example, if an unknown compound responsible for a particular biological activity is to be isolated, bioassay-guided isolation should be tried where bioactivity at each stage of isolation is determined and only active fractions are taken forward for further isolation steps. For a known compound, it would be best to follow the literature methods used for isolation of given compound. For diastereoisomers, separation on normal phase or reverse phase chromatography can be tried, whereas for enantiomers, chiral chromatography is the answer. Ionic compounds are best separated using reverse phase chromatography, ion-pair chromatography, or ion-exchange chromatography. Biomolecules are separated using size exclusion chromatography. The general features of molecules that are helpful for the isolation process are its solubility, acid-base properties, charge, stability, and molecular size. For an unknown molecule, qualitative analytical assays such as TLC or the above properties would suggest the most suitable methods for isolation. Hydrophobicity or hydrophilicity of extract and compounds present in the extract is determined by trying to dissolve compound in a range of polarity solvents such as water, methanol, acetonitrile, and ethyl acetate. Acid-base properties are determined by dissolving the compound/extract in different pH solvents [10].

Another important question is the purpose of isolation, whether it is to obtain sufficient amount for chemical characterization or to generate a sufficient amount for biological evaluation. A pure compound can be characterized even with a few mg of the sample, with high-resolution NMR, it is possible to run all 1D and 2D NMR experiments with as small as 1–2 mg of the sample. In vitro experiments can also be conducted with few mgs of the sample. However, animal experiments require higher amounts of sample usually in gram quantities. Isolation strategies would vary depending on the above requirements. Further, 90–95% purity is good enough for chemical characterization, whereas more than 99% purity is required for biological evaluation. There are examples where even 0.1–0.2% impurity in a molecule has led to wrong assignment of biological activity to a major compound.

2.3.4.1 Chromatographic Techniques for Isolation

A variety of chromatographic techniques are available to a separation scientist for the isolation and purification of NPs. It is seldom possible to purify a NP using a single technique; rather it is generally a mix of techniques used one after the other to achieve final purification of a compound. A crude extract is generally first divided into different polarity fractions by liquid–liquid extraction using solvents in a wide polarity range, starting from the nonpolar hexane to chloroform to ethyl acetate to butanol. This would generally provide five fractions, the hexane fraction containing nonpolar compounds such as terpenoids, ethyl acetate fraction containing medium polar compounds such a flavonoids, and butanol fraction containing alkaloids,

glycosides, or other polar compounds. These fractions are then subjected to different chromatographic techniques to isolate a pure natural product. A brief introduction to various chromatographic techniques is given below, for details on chromatography the reader is referred to several excellent books on chromatography.

2.3.4.2 Thin-Layer Chromatography (TLC)

It is a preferred method for qualitatively analyzing natural mixtures before proceeding with other chromatographic techniques. A crude extract dissolved in a suitable solvent is spotted on TLC plate using microcapillary and allowed to run using different strength mobile phases. Various compounds migrate to different distances on TLC plate depending on their adsorption on the stationary phase. Less polar compounds move a greater distance on silica based and show high R_f value, whereas more polar compounds move a lesser distance and show low R_f value. The developed plate is visualized under UV or by derivatizing with suitable reagents, which produce distinct colors to indicate the presence of certain classes of natural products. For example, spraying with ninhydrin reagent shows amino acids as brightly colored spots on TLC plate. The adsorption of solute on silica stationary phase may involve one or several mechanisms including dipole-dipole interaction, hydrogen bond interaction, π-complex formation, or steric factors. The preliminary TLC experiments indicate suitable stationary and mobile phases for further preparative chromatographic procedures.

2.3.4.3 Preparative Thin-Layer Chromatography (PTLC)

It is a thin-layer chromatography technique aimed at isolating compounds in smaller amounts (10–50 mg) for structure elucidation. Silica, alumina, cellulose, C_8, and C_{18} reverse-phase precoated plates are available; however, silica is most commonly used stationary phase for preparative separations. Stationary phases with particle size distribution range (5–40 μm) are used in layer thickness 0.5–2 mm plates. The sample dissolved in the solvent at a concentration of 10–20 mg/mL is applied. The band of desired compound is scrapped off and extracted with a suitable solvent. The purity of isolated compound can be assessed by analytical TLC. PTLC is simple and cost effective compared with instrumental techniques; however, separation efficiency is poorer compared with HPLC or other instrumental techniques. PTLC is preferred if ΔR_f is high between the two compounds to be separated [10].

2.3.4.4 Open Column Chromatography (CC)/Vacuum Liquid Chromatography (VLC)/Flash Chromatography (FC)

Once the TLC analysis has given indications of a suitable stationary phase and mobile phase, the crude extract is subjected to further fractionation using CC/FC/

VLC. A suitable stationary phase and mobile phase is selected based on literature, polarity of the compounds to be separated, or the results from analytical TLC showing good resolution of the compounds to be separated. A column is packed with stationary phase to sample ratio ranging from 1:30 to 1:300 based on the complexity of the mixture. The sample is loaded either dissolved in a solvent, preferably the mobile phase, or as a solid adsorbed on the stationary phase support. The column is developed and fractions are collected with mobile phase of increasing strength. The eluted fractions are monitored by TLC. Compared with CC which is very time consuming, FC and VLC are very fast techniques and can be easily performed with available equipment/glassware. **Flash Chromatography (FC)** is an air-pressure-driven column chromatography optimized for rapid separation. Smaller silica gel particles (40–63 μ) and small positive pressure (10–15 psi) are used to run the column. A column of 5 inch bed is prepared with the stationary phase and a flow rate of 2 + 0.1 inches/min is maintained in order to achieve good resolution, which is achievable in reasonable period of time if the recommended conditions are followed. A mobile phase that shows an R_f value of about 0.35 on analytical TLC for the compound of interest is selected for isocratic elution. Under these conditions, chromatography is usually complete in 15–30 min and requires very small amount of solvents. On the other hand, **Vacuum liquid chromatography (VLC)** is vacuum-driven chromatography (20–70 mmHg). It was originally proposed to reproduce the results of analytical TLC on a preparative scale using column. A Buchner funnel is used as a column and the stationary phase, usually TLC grade silica or alumina, is filled up to a height of about 5 cm, the same as height of a TLC plate. Gradient elution of column is mostly preferred but isocratic can also be used, 10–15 mL fractions are collected at each polarity stage for a sample load up to 1 g. On normal phase silica gel, petroleum ether/hexane with increasing amounts of more polar solvents such as methylene chloride, diethyl ether, ethyl acetate, or acetone followed by increasing percentage of methanol are used as mobile phase for gradient elution. Usually 20–25 fraction will elute all the compounds from the column. The column is dried after each fraction is collected in a manner similar to repetitive runs of a TLC plate. A packed column can be reused for similar separation by washing the column with methanol. The advantage is reduced time for separation, reduced solvent consumption, simple and less expensive, and universally available apparatus. All these chromatographies can be performed using normal phase silica gel, reverse phase silica gel, or bonded phases.

The fractions so obtained are then subjected to final purification using instrumental techniques such as MPLC, HPLC, and countercurrent chromatography.

2.3.4.5 Low-Pressure and Medium-Pressure Liquid Chromatography (LPLC and MPLC)

LPLC columns use approximately 40–60 micron particle size enabling high flow rates and pressures up to 10 bars. The pressure in MPLC columns can go up to 40 bars. These low pressures also allow the use of refillable glass columns. The

packing material is filled into columns manually and held in place with porous glass frits. Prepacked columns are also available for use. These systems can be used to separate samples from milligrams to gram scale.

2.3.4.6 Countercurrent Chromatography (CCC)

It is a form of support-free liquid–liquid chromatography in which two immiscible liquids prepared by mixing two or more solvents serve as the stationary and mobile phase, an instrument keeps one phase stationary and the other is pumped through it as mobile phase. The principle of separation is the partitioning of solute between two immiscible liquid phases. The relative proportion of solute in each phase is determined by the partition coefficient. In practice, the phase which contains more of the solute is kept stationary. The major advantage of this chromatography is that no solid support is used in the column and there is no irreversible adsorption of solute, no tailing, no loss of material and minimal solvent consumption. Two variants, droplet countercurrent (DCCC) and centrifugal partition chromatography (CPC) rely on gravitational field and centrifugal force for the retention of liquid stationary phase, respectively. In DCCC, droplets of mobile phase pass through an immiscible stationary liquid phase for continuous partitioning of solute between two phases. These droplets can move either in ascending or descending mode, depending on whether the stationary phase has higher or lower density than the mobile phase. A typical DCCC instrument contains 200–600 interconnected glass columns of approximately 2–3 mm diameter. The choice of two liquid phases is crucial for separation, binary solutions are impractical, ternary or quaternary mixture forming two phases are generally used in CCC. CPC uses centrifugal force to speed up separation and achieves higher flow rate than DCCC. Choice of mobile phase and stationary phase can be guided by TLC.

2.3.4.7 High-Performance (or High Pressure) Liquid Chromatography (HPLC)

HPLC can be used for final separation of partially purified fractions to obtain pure natural products. HPLC can be done in analytical, semi-preparative, or preparative mode. Samples from microgram range to gram to kilogram range can be processed on HPLC. High resolution achieved in HPLC is due to very fine particle size of the stationary phases used in HPLC columns. Most commonly, a particle size of about 5 micron is used in HPLC columns. The correct operating conditions (mobile phase and stationary phase) can be selected on the basis of initial TLC runs using normal phase silica gel or reverse phase silica TLC plates. The analytical HPLC conditions can be optimized by changing certain parameters such as retention factor (k'), selectivity (α), or plate number (N). An increment in any of these parameters will generally lead to a better separation or increased resolution between the compounds. These parameters generally depend on strength and type of mobile phase, type of

stationary phase, length of column, particle size of stationary phase, temperature, etc. The selection of suitable stationary phase is very important to achieve the desired results; huge variety stationary phases are available in HPLC columns. However, most of the applications on HPLC are done using nonpolar reverse phase C18 columns and polar mobile phases (methanol/acetonitrile/water). Analytical HPLC is normally used for obtaining information (qualitative and quantitative) on sample mixtures, the amounts injected are generally very small, usually 5–10 micrograms, and cannot be used to isolate pure NPs. In order to obtain sufficient amounts of pure NPs, semi-preparative or preparative HPLC is used. The particle size in preparative or semi-preparative HPLC is smaller than that used for MPLC, generally 5–30 μm; mostly about 5–10 μm; in semi-preparative HPLC. The analytical column is usually 0.46 cm ID while semi-preparative columns are about 1 cm internal diameter [12].

2.3.5 Structure Elucidation of NPs

Once a pure NP is isolated, it is characterized for physicochemical properties such as color, nature of compound, melting point, and optical rotation. The structure elucidation of a NP is a challenge as the natural compounds exhibit tremendous structural diversity. The advancements in spectral techniques have made structure elucidation simpler and faster. The chemical structure is elucidated by interplay of different spectral techniques such as ultraviolet spectroscopy (UV), infrared spectroscopy (IR), mass spectrometry (MS), nuclear magnetic resonance (NMR), optical rotatory dispersion (ORD), and circular dichroism (CD). In spectral techniques, the sample is subjected to excitation by photons from different regions of electromagnetic spectrum. The first step in structure elucidation is determining the molecular weight and molecular formula of the compound using mass spectrometry or elemental analysis.

2.3.5.1 UV-Visible Spectroscopy

UV spectroscopy is highly sensitive with detectability up to 10^{-9} M and provides information on UV absorbing chromophores present in the molecule. The absorption of radiation in the UV/visible region by a molecule results in electron transitions from lower energy levels to higher energy levels; all organic molecules absorb UV/visible light in a wavelength up to 800 nm. The only electronic transitions possible in alkanes are from low-energy σ orbital to high-energy σ^* antibonding orbital, which require very high energy and occur at very short wavelength. Consequently no useful structural information about saturated organic molecules can be extracted. However, low energy transition in molecules containing multiple unsaturations fall in the UV region of 200–400 nm and can be detected normally by the UV spectrometer and therefore provide structural information. For example, UV spectroscopy can easily distinguish among conjugated diene, triene, tetraene, α,β-unsaturated carbonyl groups, etc. Empirical rules to correlate UV absorption

with structural moieties were given by Woodward and Fieser, which can fairly predict the UV absorption for simple organic molecules [13].

2.3.5.2 Fourier-Transform Infrared Spectroscopy (FTIR)

Absorption of electromagnetic radiation in the infrared region effects changes in vibrational and rotational energy levels. Fourier-transform infrared spectroscopy is a powerful tool for the identification of functional groups present in the natural compounds. Specific functional groups show IR absorption bands in defined region of the spectrum; for example, a carbonyl group appears in a range from 1650 to 1850 cm^{-1} depending on other features in the vicinity of carbonyl group. The carbonyl group in different functionalities appears in the region:

Amide	Acid	Ketone	Aldehyde	Ester	Anhydride band I	Acid chloride	Anhydride band II
1650	1700	1715	1725	1735	1760	1800	1810 cm^{-1}

Apart from functional group identity, the other important structural information obtained from IR includes the presence and nature of H-bonding (intermolecular or intramolecular), ring size (for example, cyclohexanone, cyclopentanone, cyclobutanone), tautomerism (keto-enol), conformational isomerism, etc. Any factors that cause increase in bond strength would lead to IR absorption at higher wavenumber and vice versa (Fig. 2.6).

2.3.5.3 Mass Spectrometry

Mass spectrometry is a powerful analytical tool for the identification of unknown compounds, quantification of known compounds, and to elucidate the structures of molecules. It gives information about the molecular mass, molecular formula, and fragmentation pattern. The mass spectrometry analyzes ions in gas phase produced

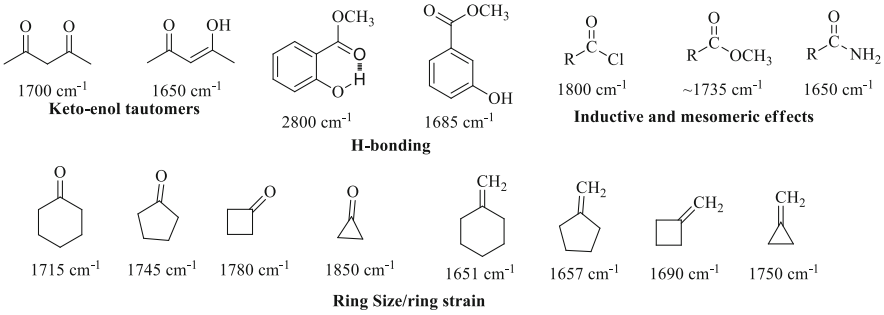


Fig. 2.6 Factors affecting absorption frequency in IR

by ionizing sample molecules by one of the several available ionizing techniques and measuring their mass-to-charge ratio (*m/z*) and relative abundance. Most commonly used ionization techniques are electron impact mass spectrometry (EIMS), chemical ionization mass spectrometry (CIMS), electrospray ionization mass spectrometry (ESIMS), and fast atom bombardment mass spectrometry (FABMS). In EI, the sample molecules in gas phase are bombarded by high-energy electrons (usually 70 eV) and are converted into high-energy positively charged ions (molecular ion or parent ion) by abstracting one electron to generate a radical cation. The molecular ions can fragment further into smaller ions (fragment ions or daughter ions). Mass spectrometry is highly sensitive and only picomolar concentration of the sample is required. MS has found wide applications in the screening of drug candidate, drug degradation analysis, and characterization of chemical compounds, drug metabolism, pharmacokinetics study and bioavailability studies. It is also frequently applied in dereplication process in natural products chemistry.

2.3.5.4 Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance (NMR) concerns magnetic properties of atomic nuclei, ^{1}H and ^{13}C or other magnetically active nuclei such as ^{19}F and ^{31}P or any other nuclei whose spin quantum number is greater than zero. The hydrogen nucleus possesses both the electric charge and mechanical spin and behaves like a tiny bar magnet and generates its own magnetic field. When this spinning nucleus with spin quantum number $I = 1/2$ is placed in a strong magnetic field B_0, it will orient itself in two directions (energy states), one aligned in the direction of the applied magnetic field (+1/2, lower energy) and the other aligned against the magnetic field ($-1/2$, higher energy), the energy difference between two states ($\Delta E = h\nu_0$) being proportional to the external applied field strength. The spinning nucleus will also precess around the axis of external applied field just like a spinning top does under the earth's gravitational field, at a precessional frequency (ν_0) that is proportional to the strength of external applied field. The irradiation of precessing nuclei with a radiofrequency matching ΔE will result in absorption of energy by nuclei in the lower energy state to jump to higher energy state. This absorption of energy is measured and recorded in the form of NMR spectrum. All the protons in a molecule do not experience the same applied magnetic field due to their different chemical environment and therefore absorb at slightly different radiofrequency, thus showing separate signals at different chemical shift values (δ) for different types of protons present in the molecule. The above is an oversimplification of the theory of NMR.

NMR provides information about the number, type, and electronic environment around the nucleus being studied. Advanced NMR experiments also give information of connectivities between the adjacent nuclei within the molecule as well as information on stereochemistry of the molecule. The number of signals in the ^{1}H NMR indicate the number of types of protons, the relative intensity of the signals gives the relative number of protons in that signal, splitting of a signal (a signal is split into $n + 1$ lines, $n =$ no. of protons on adjacent carbons) indicates the number of

neighboring hydrogens, the chemical shift indicates the chemical environment of the proton, the coupling constants suggest the stereochemistry of the molecule. The ^{1}H NMR spectrum of ethyl acetate shows three signals in ratio of 3:3:2 indicating there are three different types of protons. The signal at δ 1.3 has a relative intensity of 3, is split into three lines suggesting this signal is for three protons adjacent to a methylene group. The second signal for three protons appears as a singlet at δ 2.1 suggesting there are no protons on adjacent carbons. A signal at δ 4.2 for two protons appears as a quartet suggesting a methylene group adjacent to a methyl group. The ^{13}C NMR on the other hand does not show splitting and relative ratio of numbers. It provides information on number of carbon atoms and their chemical environment. More structural information is provided by advanced NMR experiments. Common one-dimensional (1D) NMR experiments include ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, ^{31}P NMR, and DEPT; two-dimensional (2D) techniques include ^{1}H-^{1}H COSY,^{1}H-^{1}H DQF-COSY,^{1} ^{1}H-^{1}H NOESY,^{1}H-^{1}H ROESY, TOCSY, ^{1}H-^{13}C HMBC,^{1}H-^{13}C HMQC,^{1}H-^{13}C HSQC, HSQC-TOCSY, etc. [14].

2.3.5.5 Hyphenated Techniques

It is a coupling of two different techniques with the help of an interface. It is an online combination of a chromatographic separation with a sensitive spectroscopic detector. GC-MS is a technique coupling of gas chromatography and mass spectrometry. Compounds that are volatile, small, and stable at high temperature can be analyzed by GC-MS. These two techniques are highly compatible with each other as sample is in the vapor phase in both the techniques. GC-MS has high resolving power and sensitivity and accomplishes rapid analysis of sample with good accuracy and precision. However, only volatile samples or those samples which can be volatilized after derivatization can be analyzed by GC-MS. In comparison, LC-MS is a combination of liquid chromatography (HPLC) and mass spectrometry. It is the most widely used method suitable for analysis of nonvolatile, thermally labile, and charged molecules. LC-NMR is least sensitive, however provides useful information towards the structure elucidation of compounds. LC-NMR is used for the analysis of complex mixtures of all types, but particularly for analysis of natural products and drug metabolites [10].

2.3.6 Preclinical Studies and Clinical Trials

Preclinical studies: These involve in vitro and in vivo studies to determine if the drug is safe for human testing. Wide range dosages for the study drug are given to the animal or to an in vitro substrate to obtain preliminary results such as efficacy, toxicity, and pharmacokinetic information and to assist decision-making to go-ahead for further testing of the drug. The mechanism of action, efficacy, and safety is determined during preclinical studies.

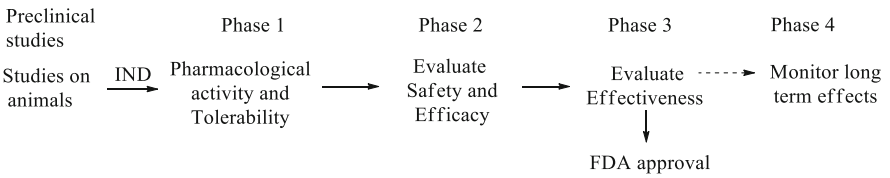


Fig. 2.7 Phases of clinical trials

Clinical trials: Clinical trials are research investigations in which people volunteer to test new treatments or tests to prevent, detect, treat, or manage various diseases or medical conditions. World Health Organization (WHO) defines a clinical trial as "*any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes*." Clinical trials are essential for the development of new interventions to determine their safety and efficacy (Fig. 2.7).

2.3.6.1 Phase I

This is the first stage of testing on small group (20–100) healthy volunteers. The main objective is to establish the safety of the drug in humans. The trials are designed to assess the safety (pharmacovigilance), tolerability, pharmacokinetics, and pharmacodynamics of a drug; the absorption, metabolism, and elimination of the drug from the body. Any possible side effects are also investigated. These trials are conducted in an inpatient clinic, where the subject can be under the supervision of full staff. The subject is usually monitored until several half-lives of the drug have passed. Phase I trials also include dosing studies so that the appropriate dose for therapeutic use can be identified. Phase I trials mostly include healthy volunteers; however, there may be some exceptions such as patients who have an end-stage disease and lack other treatment options. This exception most often occurs in cancer and HIV drug trials.

2.3.6.2 Phase II

Once the initial safety of the study drug has been confirmed in Phase I trials, Phase II trials are performed on larger groups (100–500) of patients to evaluate the effectiveness of the drug and to examine the short-term side effects or adverse reaction. Phase II studies are divided into two phases such as Phase IIA and Phase IIB. Phase IIA is specifically designed to assess dosing requirements (how much drug should be given to the subject) and Phase IIB is specifically designed to study efficacy (how the drug works at the prescribed dose).

2.3.6.3 Phase III

Phase III studies are performed on large and diverse patient groups (1000–5000) and are designed to assess and generate significant data on the safety, effectiveness, and overall benefit-risk of the drug. The Phase III trials are the most expensive and time consuming. If the phase III clinical trials are satisfactory, then necessary documents are prepared for regulatory approval, which include description of the methods and results of human and animal studies. These documents also include all manufacturing procedures, details of formulation, and shelf life studies.

2.3.6.4 Phase IV

This phase of the clinical trial is also known as Post Marketing Surveillance. It involves the safety surveillance (pharmacovigilance) and ongoing technical support of a drug. The safety surveillance is designed to detect any rare or long-term adverse effects on a much larger patient population and longer time period than was possible during the Phase I-III clinical trials. Adverse effects reported in Phase IV trials may result in a restricted use or withdrawal of drug [15].

2.3.7 Discovery and Development of Eribulin

2.3.7.1 Extraction and Isolation of Halichondrin B

Halichondrin B, a macrocyclic lactone, was isolated from marine sponge *Halichondria okadai* collected from south of Tokyo in the mediolittoral zone of the Pacific Ocean. Approximately 600 kg specimens were collected and stored in a freezer; the frozen specimens were crushed in a blender with MeOH, kept for a period of 3 days, and filtered. The brown color filtrate was concentrated under reduced pressure at low temperature. The remaining aqueous solution was extracted with *n*-butanol saturated with water. The combined organic layers were concentrated under reduced pressure, the crude extract was further dissolved in 70% aqueous MeOH and washed with *n*-hexane. The 70% aqueous MeOH layer was concentrated under reduced pressure to give an oily material which was charged on TSK G3000S polystyrene gel column; the bioactive fractions (B-16 melanoma cells) were eluted with 50% ethanol and then 60% ethanol. Each fraction was further separated by repeated chromatography on LiChroprep RP-C8 column and YMC Pack A2l2 (C-8) column to yield eight active compounds (12.5 mg halichondrin B from 600 kg sponges) [16]. Halichondrin B was also isolated from other sponges with very less yield, 0.4 mg, 1.8×10^{-8}% from *Axinella* sp., 8 mg, 3.5×10^{-9}% from *Phakellia carter* [17, 18], and 2 mg, 4×10^{-5}% from *Lissodendoryx* sp. [19]. Further isolation was done for the most potent compound Halichondrin B from *Lissodendoryx* sp., which yielded 310 mg from 1 ton biomass [20] (Fig. 2.8).

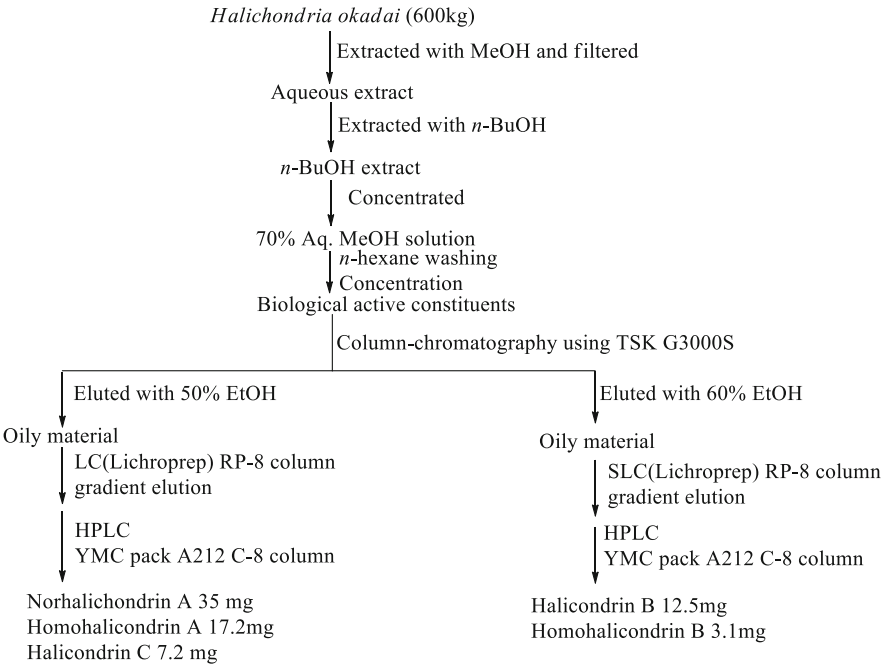


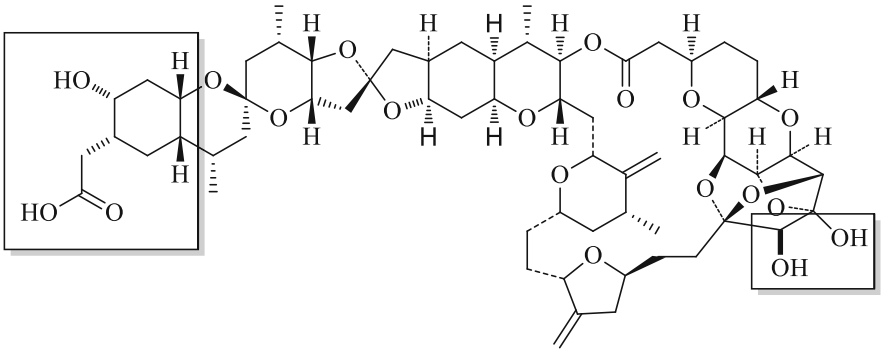
Fig. 2.8 Isolation of halichondrins

2.3.7.2 Characterization of Halichondrin B

The structure of norhalichondrin A (MW 1126) was established by single crystal X-ray analysis after preparing its ester with *p*-bromophenacyl bromide. The absolute configuration was determined by applying exciton chirality method. The structure of halichondrin B was determined by comparison of its spectral data with that of norhalichondrin A. The molecular weight (1110) of halichondrin B was obtained by FDMS *m/z*, 1133 $[M + Na]^+$ for the molecular formula $C_{60}H_{86}O_{19}Na$ suggesting one oxygen less in halichondrin B. Acetylation with acetic anhydride and pyridine gave a triacetate suggesting the presence of three hydroxyl groups. The structure was established by a comparison of COSY spectra of two compounds and assigning the partial structures from C1 to C14. Further the chemical shifts and coupling constants of each proton from C15 to C44 were assigned which were consistent with norhalichondrin A. A complete analysis of proton, carbon, ^1H-^1H COSY, and ^1H-^{13}C COSY led to the complete structure of halichondrin B [21] (Fig. 2.9).

2.3.7.3 Biological Activity of Halichondrin B

Halichondrin B showed in vitro cytotoxicity IC_{50} of 0.093 (ng/mL) against B-l6 melanoma cells. In vivo antitumor activity of halichondrin B was evaluated for B-16



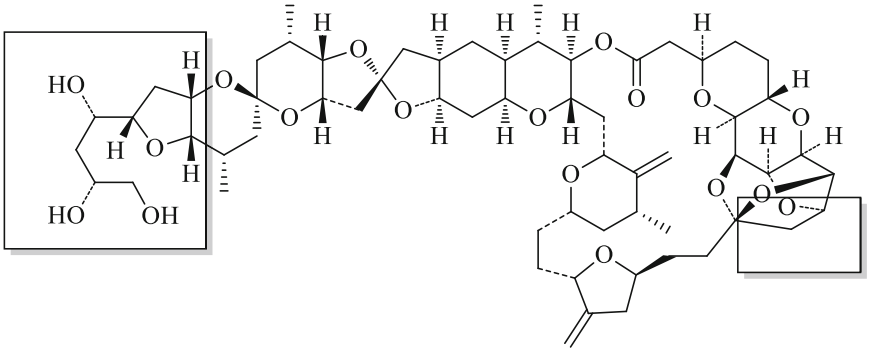


Fig. 2.9 Structures of halichondrins

melanoma, P-388 leukemia, and L-1210 leukemia in mice model. The mean survival time increased by almost 200% at a dose of 2.5–20 μg/kg in B-16 melanoma assay. Antitumor activity against P-388 leukemia and L-1210 leukemia also increased the mean survival rate of animals. Halichondrins inhibit microtubule dynamics by inducing nonproductive tubulin aggregates, leading to suppression of spindle microtubule. This mechanism differs from that of other tubulin-interactive drugs such as vinblastine or paclitaxel [22].

2.3.7.4 Optimization/Medicinal Chemistry

Halichondrin B was isolated in very small amount from sponges and showed very good activity at very low doses; it was necessary to obtain large amounts of this natural compound for further preclinical and clinical studies. The total synthesis of this big molecule with large number of chiral centers was a huge challenge. Kishi and coworkers accomplished the total syntheses of halichondrin B and norhalichondrin B [23]. However, the synthesis of halichondrin B was not cost

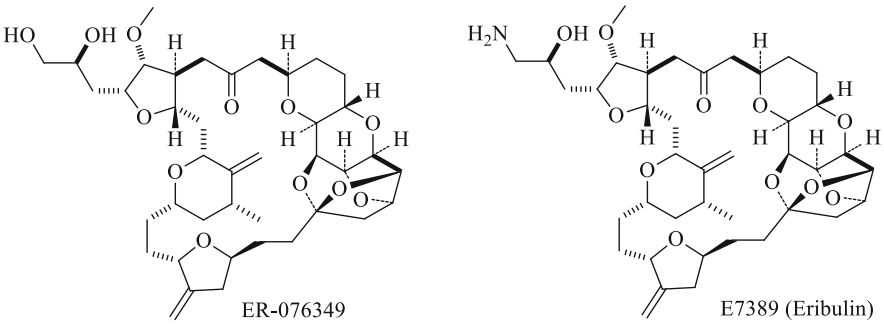


Fig. 2.10 Semisynthetic derivatives of halichondrin B

effective. An intermediate compound, a macrolactone diol, obtained during the total synthesis of halichondrin B showed IC_{50} 4.6 nM against human colon cancer cell lines that was comparable to parent compound. This promising result encouraged further studies to perform the structure activity relationship for this active intermediate. Two leads ER-076349 and E7389 were obtained after several modifications in the macrolactone diol (Fig. 2.10).

2.3.7.5 Preclinical Studies on Eribulin

Eribulin showed better antiproliferative activity than ER-076349, vincristine, and paclitaxel against MDA-MB-435 (IC_{50} 0.09 nm). It also showed high activity; at nanomolar or subnanomolar concentration against colon cancer COLO 205 and DLD-1, prostate cancer DU 145 and LNCaP, melansoma LOX, leukemia HL-60, lymphoma U-937 cells, this activity was better than vinblastine or paclitaxel [22]. Activity shown by eribulin in NCI 60 cell line screen was similar to halichondrin B. Eribulin also showed synergistic effects with several other conventional drugs in SK-BR-3 cell line. Eribulin showed tumor regression and increased life span in breast, lung, ovary, colon, melanoma, pancreatic, and fibrosarcoma in human tumor xenograft investigations in mice. Eribulin did not show any significant adverse effects in mice at maximum tolerated dose. Eribulin inhibits cancer cell growth through mitotic block at G_2-M phase disrupting the mitotic spindle formation [24].

2.3.7.6 Clinical Trials on Eribulin

Phase I

Several studies were conducted at different locations for the Phase I trials. The first study was done on 40 patients with advanced solid tumors. Eribulin was administered at a starting dose 0.125 mg/m^2/week in patients, dose escalation was done up to

2.0 mg/m^2/week. In the second study on 32 patients (1-h i.v. infusion, 1.4 mg/m^2) on days 1, 8, and 15 of a 28-day cycle suggested that 1.4 mg/m^2 dose level could not be administered in week 3 dose due to adverse effects. Therefore maximum tolerated dose was suggested to be 1.0 mg/m^2. The third study was done on 21 patients at doses ranging from 0.25 to 4 mg/m^2 with 1 h infusion on day 1. The MTD in this schedule was established as 2.0 mg/m^2. A dose of 1.4 mg/m^2 on days 1 and 8 every 3 weeks was recommended for phase II studies. The other studies in phase I were conducted on patients with renal dysfunction and advanced urothelial cancer, and combination with gemcitabine in patients with advanced solid tumors. The most common adverse events were neutropenia, alopecia, nausea, and fatigue among others; however, it was found to be fairly safe.

Phase II Trial

Phase II trial of eribulin was conducted in metastatic breast cancer patients (pretreated with either anthracycline or taxane) at 23 sites in the USA with bolus 1.4 mg/m^2/week on days 1 and 8 of a 21 day cycle. Eribulin achieved an overall response rate (ORR) of 11.5% and median overall survival was 275 days. Another phase II study sponsored by Eisai showed median overall survival of 10.4 months. Another study in Japan on MBC patients showed median overall survival of 331 days. Phase II trials were also conducted for non-small cell lung cancer, prostate cancer, ovarian cancer, sarcoma, pancreatic cancer, urothelial tract cancer (UC), and squamous cell carcinoma of the head and neck [24].

Phase III Trials

Total 762 patients were recruited and divided into eribulin treated groups (508) and other treatment of physician's choice (TPC) groups (254) with median age of 55.2 years. The median overall survival was 13.1 months for eribulin and 10.6 months for TPC. Median progression-free survival was (PFS) was 3.7 months for eribulin and 2.3 months for TPC. ORR was 12% (0.4% complete response, 11.5% partial response) for eribulin and 5% (0 CR; 5% PR) for TPC. About 10% of patients showed serious adverse effect—for eribulin arm 12% and for TPC arm 7%. Eribulin has been approved to treat the patients with metastatic breast cancer by the United State Food and Drug Administration (USFDA) in 2010. The discovery and development of eribulin is reviewed in detail by Swami et al. [24]. Complete discovery and development process of eribulin spanned 25 years, from isolation of Halichondrin B in 1985 to approval by USFDA in 2010 for treatment of metastatic breast cancer [24].

2.3.8 Discovery and Development Process of Taxol

2.3.8.1 Extraction and Isolation

Taxol was isolated for the first time from the bark of *Taxus brevifolia* Nutt. in 1966. The bark of *Taxus brevifolia* was extracted with alcohol and the concentrated extract was then portioned with water and chloroform. The isolation was performed by using bioassay-guided fractionation with 9KB cell and various leukemia systems. The chloroform fraction was first separated on florisil, followed by Sephadex LH-20 and silica gel. Final purification was done by crystallization from aqueous methanol and yielded 0.02% of taxol [25]. Other isolation methods have also been reported. In vivo bioguided isolation was performed with solid tumor in Walker-256 intramolecular rat carcinoma (5WM). The bark of *Taxus brevifolia* (12 kg) was extracted with 95% ethanol and the concentrated extract was partitioned with water and chloroform. Active chloroform fraction was applied to Craig Countercurrent Distribution (CCD) with a series of repetitions. The yield of pure taxol was 0.004% (0.5 g). This process was tedious and time consuming. Taxol has also been reported from different parts of other *Taxus* species in range of 0.001–0.016%. *Taxus brevifolia* is very slow growing plant and it takes about 200 years to reach maturity. Therefore, sufficient amount of taxol from *Taxus brevifolia* was practically not possible. It has been reported that baccatin III and 10 deacetylbaccatin III can be used for semisynthesis of taxol and its derivatives. Baccatin III and deacetylbaccatin III were isolated from *T. baccata*, *T. wallichina*, and *T. brevifolia* in considerable amount [26]. Elaborate studies were conducted in 1960s to find suitable species or varieties and parts of those trees which could generate maximal amount of taxol.

2.3.8.2 Structure Elucidation of Taxol

Taxol showed M^+ at *m/z* 853 corresponded to the composition $C_{47}H_{51}N0_{14}$. Hydroxy, ester, keto, and amide functionalities were established by IR spectroscopy. Characteristic moieties were also established through ^1H NMR. Wall and coworkers established the structure of taxol in 1971 by single crystal X-ray analysis after preparing its derivative *p*-bromobenzoate ester. Complete assignment of all proton and carbon signals using 1D and 2D NMR was reported in 1992 [27].

2.3.8.3 Optimization/Medicinal Chemistry

Semisynthesis of Taxol from Baccatin III and Deacetylbaccatin III

Adequate supplies of taxol were required for preclinical and clinical studies. Semisynthesis of taxol from readily available congeners ultimately provided sufficient supplies of taxol for commercialization. 10-Deacetylbaccatin (10-DAB) is

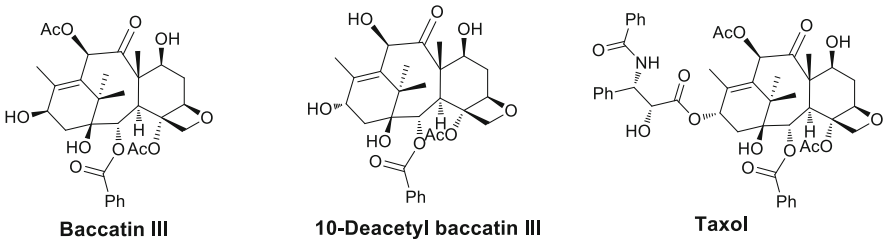


Fig. 2.11 Structures of taxol, Baccatin III, and 10-DAB

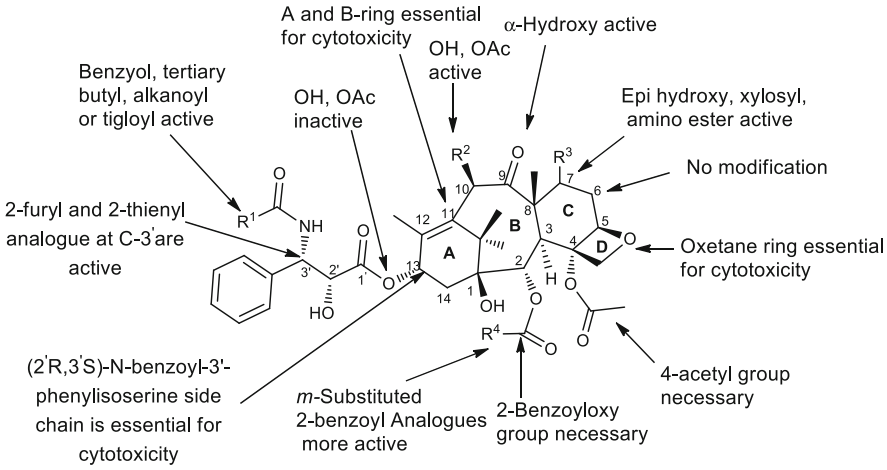
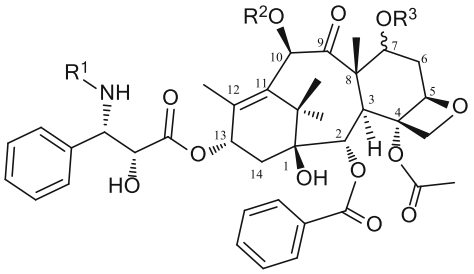


Fig. 2.12 Structure activity relationships in taxol

relatively more abundant taxane in the needles of English Yew *Taxus baccata* and its isolation is simpler than isolation of taxol. Therefore, a major breakthrough in the development of taxol came after semisynthesis of taxol from 10-deactylbaccatin III and baccatin III (Fig. 2.11) [28].

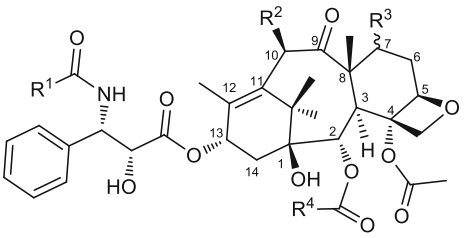
A huge number of analogs were synthesized for evaluation of anticancer activity along with several other naturally occurring taxanes. These included analogs/derivatives of 10-deacetyl taxol, 7-*epi*-taxol, 7-xylosyltaxol, modifications at C-7 and C-10 hydroxyl group, modifications at C-2, 4, 6, 9, and 19, and modifications in the C-13 side chain. A brief glimpse of medicinal chemistry of taxol is shown below and the structure activity relationships are summarized in Fig. 2.12 [28].



	Compound	Substitution			Biological activity				
		R^1	R^2	R^3	KB[a]	P388[b]	J774.2[c]	ID_{50}[d]/ ID_{50} (taxol)	ED_{50}[e]/ ED_{50} (taxol)
1	Taxol	Bz	Ac	β-OH	0.001	+	S	1.0	1.0
2	7-Xylosyltaxol	Bz	Ac	β-Xylosyl					0.4
3	Cephalomannine	Tigloyl	Ac	β-OH	0.004	+	M/S	1.5	
4	10-Deacetyltaxol	Bz	H	β-OH	0.003	+	M	1.3	
5	10-Deacetylcephalomannine	Tigloyl	H	β-OH	0.03	+	M	5.0	
6	7-Xylosylcephalomannine	Tigloyl	Ac	β-Xylosyl					0.5
7	10-Deacetyl-7-xylosyltaxol	Bz	H	β-Xylosyl					0.6
8	10-Deacetyl-7-epi-taxol	Bz	H	α-OH	0.03				
9	7-epi-taxol	Bz	Ac	α-OH	3×10^{-5}			3.0	
10	10-Deacetyl-7-epi-cephalomannine	Tigloyl	H	α-OH	0.05				

[a]ED_{50} in μg/mL for growth inhibition of KB cells
[b]Activity against P388 cells; + = Increase in life span >25%
[c]Growth inhibition of macrophages, i.e., J774.2 cells; *S* Strong, *M* Medium
[d]Microtubule disassembly
[e]Microtubule assembly activity

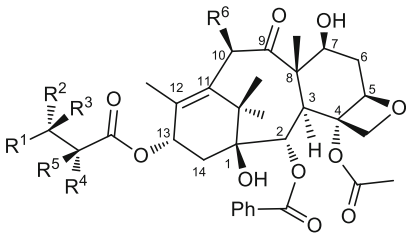
Modification at Diterpene Nucleus of Taxol and Taxotere Analogs



	Compound	Substitution				Biological activity
		R^1	R^2	R^3	R^4	ED_{50}[a]/ED_{50} (taxol)
1	Taxotere	OBu^t	OH	OH	Ph	0.5[b]
2	10-Deacetyltaxol	Ph	OH	OH	Ph	1.3[b]
3	10-Acetyltaxotere	OBu^t	OAC	OH	Ph	0.5[b]
4	10-Deacetoxytaxol	Ph	H	OH	Ph	0.5[c]
5	2-(3-Cyanobenzoyl)-2-debenzoyltaxol	Ph	OAc	OH	*m*-CNPh	0.2[d]
6	2-cyclohexylcarbonyl-2-debenzoyltaxotere	OBu^t	OAc	OH	Cyclohexyl	1.0[e]

[a]Microtubule assembly activity
[b]Microtubule disassembly
[c]Microtubule assembly
[d]P388 Murine leukemia cells
[e]$ED_{50}/ED_{50(Taxotere)}$ against P388 leukemia cells

Modification at C-13 Side Chain of Taxol



	Substitution						Biological activity				
	R^1	R^2	R^3	R^4	R^5	R^6	KB[a]	P388[b]	J774.2[c]	ID_{50}[d]/ $ID_{50(taxo)}$	ED_{50}[e]/ $ED_{50(taxo)}$
1	Ph	BzNH	H	OH	H	Ac	1.0	1.0	1.0	1.0	1.0
2	Ph	H	OH	H	OH	H				3.0	
3	Ph	H	H	H	OH	H				3.5	
4	Ph	H	H	H	OH	Ac			38.0		
5	Ph	BocNH	H	H	H	H				4.1	
6	Ph	H	H	OH	H	Ac					13.2
7	Ph	H	H	OH	H	H				4.5	
8	Ph	H	NH_2	H	OH	H				30.0	
9	Ph	NH_2	H	OH	H	Ac				44.0	
10	H	BzNH	H	H	OH	Ac			>500		>100

[a]ED_{50} in μg/mL for growth inhibition of KB cells
[b]Activity against P388 cells
[c]Growth inhibition of macrophages, i.e., J774.2 cells
[d]Microtubule disassembly
[e]Microtubule assembly activity [33]

2.3.8.4 Preclinical Studies on Taxol

In the initial studies, taxol was evaluated in vitro as well as in vivo. Taxol showed cytotoxicity to KB cells with ED_{50} 6.7×10^{-5} μg/mL. In vivo activity was performed against murine L1210, P388, and P1534 leukemias, the Walker 256 carcinosarcoma, sarcoma 180, and Lewis lung tumor. A number of in vitro studies against a variety of cell lines including human leukemias, melanomas, and carcinomas lung, CNS, breast, kidney, ovary, and colon showed taxol to be active with $IC_{50} < 2.5$ nM [28]. Taxol exhibited significant cytotoxic activity on human prostatic cancer cells at a concentration of 10 nM [29]. Taxol was evaluated against the human tumors transplanted into athymic mice (breast, endometrium, ovary, brain, lung tumor) at dose of 12.5 mg/kg subcutaneous injection every day for five consecutive days out of seven over a period of 3 weeks. Among all evaluated xenograft tumors, taxol was found most active against breast tumor, four out of five tumors disappeared in mice [30]. In mice, LD_{10} of taxol was found at 70 mg/m^2/d in 5-day schedule, whereas in dogs no toxicity was observed at dose of 22.5 mg/m^2 with the single dose and 7.5 mg/m^2/day in 5-day schedule [31]. It was shown by Horwitz and coworkers that taxol binds reversibly to microtubules and this binding site was different from the binding sites of GTP, colchicines, podophyllotoxin, and vinblastine. Taxol promotes microtubule assembly and stabilizes microtubules, which are stable even after treatment with calcium or low temperatures. Taxol induces formation of abnormal spindle asters during mitosis. Taxol has also been shown to prevent transition of cell from G0 phase to S phase.

2.3.8.5 Clinical Trials of Taxol

Phase I

Phase I clinical trial of taxol was initiated in 1983 under Division of Cancer Treatment (DCT). In one trial, a total of 20 patients (7 male and 13 female) with median age of 52 years (range 32–69 years) with metastatic cancer of colon, sarcoma, melanoma, lung, head and neck, ovary, and uterus were selected. Taxol was administered intravenously daily for 5 days at 3-week intervals and with starting dose 5 mg/m^2/day daily, and with highest dose 40 mg/m^2/day for 5 days [36]. A number of phase I trials were conducted by different research groups. The recommended dose for phase II trials was 150–250 mg/m^2/day. Adverse effects like leukopenia, neutropenia, hypersensitivity, neuropathy, and mucositis were noted [31–33].

Phase II

The phase II clinical trials of taxol were conducted at a number of institutes. In one trial, 47 patients were selected for treatment of advanced ovarian cancer of taxol. The patients were treated with varying dose 110–250 mg/m^2/day 22 days for 24-h infusion which showed 30% response rate [34]. Another study was conducted on 34 patients with metastatic ovarian cancer. Taxol was administered with dose ranging from 180 to 250 mg/m^2 for 24-h continuous infusion with 20% total response [35]. In another clinical study a group of 25 metastatic breast cancer patients were given 250 mg/m^2 of drug by 24-h infusion at 21 day intervals. This treatment showed total response rate of 56% (12% complete and 44% partial) [36]. Other research groups have reported the response rate of taxol 22–62 for treatment of metastatic breast cancer and 20–48 for ovary cancer. Phase II clinical trial was also evaluated for non-small cell lung, small cell lung, melanoma, renal, prostate, colon, cervix, gastric, pancreas, bladder, and head and neck lymphoma cancers. It has been observed that response rate also depends on the type of prior therapy [28].

Phase III

In a study on 391 patients, the response was obtained from 382 patients. Taxol was administered in dose ranging from 175 or 135 mg/m^2 for 24 or 3 h infusion. Response rate was found slightly higher at dose of 175 mg/m^2 (20%) than at 135 mg/m^2 (15%). Taxol administered as 24 h infusion showed high neutropenia. A longer time to progression was reported at a dose of 175 mg/m^2 [37]. In another trial on 471 patients with metastatic breast cancer patients, taxol was intravenously administered at a dose of 175 or 135 mg/m^2 every 3 weeks as a 3-h infusion. The higher dose 175 mg/m^2 showed overall response 29%, complete response 5%, median time to disease progression 4.2 months, and median survival time of 11.7 months [38]. Taxol was approved by United State Food and Drug Administration (USFDA) for the treatment of refractory ovarian cancer and refractory or anthracycline-resistant breast cancer in 1992 and 1994, respectively. Due to its unique mode of action, the extensive research has been done to develop more dugs, resulting in approval of docetaxel (Taxotere), cabazitaxel, and larotaxel by USFDA.

2.4 AYUSH (Indian Systems of Medicine)

Traditional systems of medicine have been used throughout the world since prehistoric periods. The World Health Organization (WHO) defines traditional systems of medicine as an aggregate of the knowledge, skills, and practices of different cultures

used to maintain health and cure diseases. Awareness of plants and their healing properties have been found closely linked to human life and its social and cultural well-being. About 80% of the population of developing and underdeveloped countries relies on the traditional systems of medicine for the treatment of ailments. Several types of traditional systems of medicine are practiced worldwide such as the Indian Systems of Medicine (Ayurveda, Siddha, Unani), Traditional Chinese Medicine, Traditional Japanese Medicine, Traditional Korean Medicine, Traditional Aboriginal Medicine, Traditional African Medicine, and Russian Herbal Medicine [6, 39]. It is estimated that Ayurveda uses 1200 species of plants while Siddha and Unani include 900 and 700 species of plants, respectively, in their pharmaceutical preparations [40]. In 1995, the Government of India established the Department of Indian Systems of Medicine and Homoeopathy (ISM&H) under the Ministry of Health and Family Welfare for the growth and development of Ayurveda and other popular systems of Indian Medicines. In November 2003, Department of Indian Systems of Medicine and Homoeopathy was renamed as the Department of Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homeopathy (AYUSH). Government of India upgraded the Department of AYUSH to a full Ministry of AYUSH in 2014. This ministry takes decisions for policy formulation and implementation of schemes and programs. The Ministry of Health and Family Welfare (AYUSH) has also recognized Sowa-Rigpa traditional system of medicine after the Indian Medicine Central Council Amendment Bill 2010 [41, 42].

2.4.1 Ayurveda

Ayurveda is the oldest and holistic system of Indian medicine that is popularly practiced in India and several other countries including Pakistan, Nepal, Bangladesh, and Sri-Lanka [42]. Ayurveda is a Sanskrit word (Ayur means life and Veda means the science or knowledge), so it is the science of life. It is believed that Ayurveda originated as a divine science of healing, transferred from the Hindu deity Lord Brahma to Dakshas and then further to Ashwini twins and to Lord Indra. It is assumed that medicinal information is recorded in the oldest scriptures Rig Veda and Atharva Veda [43].

The knowledge and experiences of practitioners and scholars have been documented in many texts since ancient times and have been translated into different languages including Greek (300 BC), Persian and Arabic (700 AD), Chinese (300 AD), and Tibetan [44]. The Ayurvedic literature originated from Indian Vedic times during 500–1500 BC, two main texts Charaka Samhita and the Sushruta Samhita (1000 BC) are the foundation of Ayurveda and these two were influential scriptures on traditional medicine during this era. The Charaka Samhita contains

knowledge about foods, lifestyle, and drugs which give longevity, good health, and help disease prevention [43]. Sushruta Samhita is the oldest textbook of surgery and is divided into five parts, Rogaharas (physicians), Shaylyaharas (surgeons), Vishaharas (poison healers), Krityaharas (demon doctors), and Bhisagatharvans (magic doctors) [45]. Sushruta Samhita contains 184 chapters with description of 1120 illnesses, 700 medicinal plants, 64 preparations from mineral sources, and 57 preparations based on animal sources [46]. Charaka Samhita and Sushruta Samhita were later updated in the form of Astanga Sangraha and Astanga Hrdaya. Madhava Nidana (diagnosis of disease), Bhava Prakasa (information related to plant and diet), and Sarngadhara Samhita (formulation and dosage form) [47] also contributed towards Ayurveda. The eight major divisions of Ayurveda are Kayacikitsa (internal medicine), Salyatantra (surgery), Salakya (diseases of supra-clavicular origin), Kaumarabhrtya (pediatrics, obstetrics, and gynecology), Bhutavidya (psychiatry), Agadatantra (toxicology), Rasayanatantra (rejuvenation and geriatrics), and Vajikarana (aphrodisiology and eugenics) [45, 47]. The following concepts are basic principles of Ayurveda.

2.4.1.1 The Five Elements

According to Ayurveda, everything in the universe is composed of energy and this energy exists in five different states of density, giving rise to five factors or elements, namely Vayu (air), Jala (water), Aakash (space or ether), Prithvi (earth), and Teja (fire). Every person is made of these five basic elements called as Pancha Mahabhoota and helps understand physiology and pathology of the body. **Vayu (air)** exists in the gaseous state which is light, dry, clear, and dispersing. Air affects respiration, excretion, expansion, contraction, and voluntary and involuntary movements of body. **Jala (water)** is liquid, has no shape, and it holds all things together. It is present in body in the form of blood, stools, urine, saliva, and mucus. Water also controls taste buds and taste perception. **Aakash (space or ether)** has no physical shape and is associated with ears and throat. **Prithvi (Earth)** earth is a stable and solid matter. The characteristic feature is solidity, stability, and rigidity and is associated with nose and smell. **Teja (Fire)** is an energy and form without any matter. Fire has ability to convert a solid matter to liquid to gas and vice versa and is associated with eyes.

2.4.1.2 The Three Humors (Doshas)

The five elements are responsible individually or collectively to form the three basic humors of the human body which have different composition. The three humors are called as "Tridoshas," namely Vata dosha (wind/air), Pitta dosha (bile), and Kapha dosha (phlegm). The blood was also considered as a fourth dosha in some old

schools of thought, but over periods the concept of three dosha has prevailed. These doshas play an important role in regulating the physiological and pathological functions of the human body [43]. **Vata dosha** is composed of air and ether which has great force, its action focuses on all movements in the body, it controls flow of blood, breathing, elimination of wastes. It is considered the most important of the three doshas. **Pitta dosha** contains fire and water. Pitta dosha controls metabolism and certain hormones linked to appetite. **Kapha dosha** is composed of water and earth and is concerned with stability in mind and body. It controls muscle growth, body strength, and immune system. It is believed that the body is composed of seven elements which is called "Saptadhatu," named as Chyle (rasa dhatu), blood (raktadhatu), fat (medhadhatu), flesh (mamsadhatu), bone (asthidhatu), marrow (majjadhatu), and semen or female reproductive tissue (shukradhatu) [42].

2.4.1.3 The Gunas (Quality)

According to Ayurveda, human beings possess three gunas, viz. sattva, rajas, and tamas. Sattva is the quality of purity and clarity of perception which is responsible for goodness and happiness. Rajas is the quality of all movements, and enjoyment, pleasure and pain, effort and restlessness. Tamas is the quality representing darkness, heaviness, inertia, and materialistic attitudes. These gunas are responsible for psychological constitution [42].

Ayurveda known as the Ashtanga-Ayurveda has eight distinct branches, *kayabalagrahaurdhwangashalyadaunshtrajaravrushan,* i.e., (i) general medicine, (ii) surgery, (iii) ear, nose, throat, eye, and mouth disease, (iv) psychiatry, (v) midwifery and pediatrics, (vi) toxicology, (vii) rejuvenation and tonics, and (viii) aphrodisiacs. Ayurvedic system of medicine promotes overall health rather than just treating the diseases.

2.4.2 Yoga and Naturopathy

Yoga originated in India and it has been associated with spiritual, self-care, and health practices and started thousands of years ago [48]. The term Yoga is derived from Sanskrit root "yuj" meaning "to join," "to balance," or "to unite." Yoga provides the ability to control the mind by Yoga Sutra. Yoga is a general practice for the growth of humans to divine heights which includes techniques useful for therapeutic applications in making life healthier. Several streams of yoga are reported but major four streams or schools are popular. **Karma Yoga** (the path of work) teaches to do service without any desire of fruit. **Gyana Yoga** (the path of knowledge) teaches humans to be rational, intellectual, and to acquire spiritual knowledge studying the scriptures and practices of meditation. **Bhakti Yoga** (worship or the control of emotions) is worship and surrender to the divinity. The bhakti path provides control over emotional instabilities. **Raja Yoga** (the path or Yoga for

mind culture) is famous "Ashtanga Yoga" that provides all-round development of human beings. These are Yama, Niyama, Asana, Pranayama, Pratyahara, Dharana, Dhyana, and Samadhi [49].

2.4.2.1 Asana

It is a process to stay comfortable by adopting easy physical posture. Asana provides healthy body controlling power over mind. The Asanas are started gradually and then the postures are maintained for a longer time providing deep relaxation. By doing asana, the energy is channeled making the mind active and releasing the stress. The three stages in Asanas are Sthira, Cira, and Sukha. Sthira is making asana stable that requires much effort, full concentration, and will power in the beginning. The second stage is Cira that gives relaxation and third one is Sukha stage is the bliss. It starts from the body level moving towards muscular level to the breathing level to the emotional level, thereby managing the balance and calmness of mind. By doing so, one achieves Samatva, the ideal state of body and mind. Asana are classified as cultural, relaxational, and meditative postures. Relaxation postures are Savasana, the Makarasana (the crocodile postures), Sithila Dhanurasana and Sithila Taḍasana. The Padmasana, Siddhasana, the Vajrasana, and the Sukhasana are called the meditative postures. All the other postures are called cultural postures [50, 51].

2.4.3 Naturopathy

Naturopathy is a drugless alternative system of healing that was practiced in eighteenth and nineteenth centuries. The theory of naturopathy based on the sound philosophy, principles, techniques, and science. Naturopathy was first practiced by the Hippocratic School of Medicine in 400 BC [52]. Naturopathy was introduced to North America in 1895 and this medicinal system was at its peak in 1920–1930. In India, Naturopathy has been practiced since ancient times in the form of fasting, dugdha kalapa, and taking bath in holy rivers. Translations of Louis Kuhne's famous book "The New Science of Healing" in Telugu by Shri Venkata Chelapati Sharma in 1894 and into Hindi and Urdu in 1904 by Shri Shroti Kishan Swaroop led to the revival of naturopathy in India. Naturopathy recognizes existence of vital curative force within the body. It is believed that naturopathy has no side effect. Naturopathy advocates that the cause of all diseases in body is accumulation of morbid matter in body and the treatment is the elimination of morbid matter from the body. Acute diseases are considered self-healing efforts of the body. The human body itself has the healing power and prevents itself from diseases. It treats all aspects including physical, mental, social, and spiritual at the same time. Naturopathy treats food as the only medicine. Various components of naturopathy include diet therapy, fasting therapy, mud therapy, hydrotherapy, massotherapy, acupressure, acupuncture, chromotherapy, air therapy, and magnet therapy. As per diet therapy, all foods

must be taken in their natural form. The diets are classified into eliminative diet, soothing diet, and constructive diet. The diet should consist of 20% acidic and 80% alkaline foods. Fasting is considered as a therapy in various conditions. Fasting leads to increased insulin sensitivity resulting in reduced plasma glucose and improved glucose tolerance and reduced levels of oxidative stress. It also leads to enhanced immune function. Use of different forms of water such as solid, liquid, and vapor at different temperatures such as hot and cold baths, saunas, and wraps for treatment of disorders constitutes hydrotherapy. Massage with different lubricants such as mustard oil, olive oil, coconut oil, rose petals, and neem leaf powder constitutes massotherapy. Massage involves acting on the body applying pressure, tension, motion, vibration, etc. to target tissues such as muscles, tendons, ligaments, skin, and joints. The seven fundamental modes of massage are touch, effleurage, friction, petrissage, tapotement, vibration, and joint movement. Massage can affect vasodilation of arteries, stimulation of peristalsis, changing muscle tone, and stimulate heart and produce several other physiological effects. Medical research has shown that massage is highly beneficial in pain, anxiety, and depression. Acupressure is ancient healing that uses fingers or blunt objects to press key points "acu points" to stimulate self-curative ability of the body. Acu points release muscular tension and promote circulation of blood to help healing. Acupressure has been documented for treatment of over 3000 conditions. It is highly effective in headaches, eyestrain, neck pain, backache, arthritis, anxiety, and insomnia. Acupuncture uses pricking with fine needles into specific points on body to relieve pain. Acupuncture has been practiced in China since ancient times and the text documenting acupuncture dates back to 305–204 B.C. It has been shown to be effective in depression, headaches, nausea, vomiting, arthritis, sciatica, asthma, insomnia, etc. In magnet therapy, magnets are used on the body for the treatment. It helps in balancing the energy and improves circulation to the applied area [52–54].

2.4.4 Siddha

Siddha is one of the oldest systems of medicine that originated in South India (Tamil Nadu). All the literatures are available in the Tamil language. The Siddha medicinal system is popularly practiced even today for treatment of several diseases. It is largely therapeutic in nature. The principles and doctrine of Siddha is similar to Ayurveda. Siddha system believed that the human body is the replica of the universe and so are the food and drugs. This system also accepts the five-element theory and the tridosha theory as in Ayurveda. In Siddha medicinal system, the diseases are identified through pulse, urine, and different anatomical features such as the tongue, voice, complexion, eyes, touch (to find dry, warm, cold, sweating condition), and stools [45]. Metals and minerals are extensively used in this system. There are water-soluble inorganic alkalies and salts, water-insoluble mineral drugs that emit vapors when put in fire or when heated both natural and artificial. Metals like gold, silver, copper, tin, lead, and iron are regularly used after thorough processing through

incineration. Even mercury and its different salts like mercuric chloride and red oxide of mercury are used in this system. Sulfur also finds usage for therapeutic applications in Sidha. Sidha system is used for the treatment of psoriasis, STD, urinary tract infections, gastric diseases, liver diseases, arthritis, and allergic disorders [55].

2.4.5 Unani

Unani system of medicine was started by Great philosopher and physician Hippocrates in Greece. Unani system of medicine was introduced and popularized in India by various Arab and Persian practitioners in the eleventh century. The famous books were written as Kitab-al-shifa (Book of Healing) and the Canon of Medicine. This system was known as UnaniTibb (Unani being the Arabic word for "Greek" and Tibb an Arabic word for "medicine") in 980 CE (WHO 2010). Unani system of Medicine was intensively propagated in nineteenth century by great Hakim Ajmal Khan. The Unani medicine is practiced in India, Pakistan, Bangladesh, Sri-Lanka, Nepal, China, Iraq, Iran, Malaysia, Indonesia, Central Asian and Middle Eastern countries, African and European countries. The Unani system of Medicine is sometimes called Unani-Tibbor Hikmat. According to Unani medicine system, the cause of disease is a natural process and symptoms are the responses to disease. The human body works on the basis of self-preservation mechanism. The fundamental theory of the Unani system is "humoral theory" of Hippocrates. According to this theory, four humors, namely Dam (blood), Balgham (phlegm), Safra (yellow bile), and Sauda (black bile), which maintain the body balance are present in the human body. Blood (Dam) is hot and moist, phlegm (Balgham) is cold and moist, yellow bile (Safra) is hot and dry, and black bile (Sauda) is cold and dry [56]. The human body is made up of seven components, which are Arkan (element), Mizaj (temperament), Akhlat (humors), Aaza (organs), Arwah (sprit), Qowa (faculties), and Afaal (functions). Unani system believes that every human body has its own temperament (mizaj) and this is expressed as sanguine, phlegmatic, choleric, and melancholic. Human body contains four elements, fire, water, earth, and air, and each of these has its own temperament. Disease diagnosis and treatment prescription is based on these components [57].

2.4.6 Homeopathy

Homeopathy is another form of alternative medicine introduced by German physician Samuel Hahnemann in 1796. The term homeopathy is derived from Greek words, homeo meaning "similar" and pathos meaning "suffering." Homeopathy is a relatively newer system of medicine compared with the other systems mentioned above. The Organon of Rationale Medicine written by Samuel Hahnemann is the

Bible of homeopathy. Homeopathic system of medicine is commonly based on the use of herbs. Homeopathic medicines cure the patients with very low concentrations and are supposed to cause effects similar to normal concentrations. Homeopathy system is based on the principle of Similia Similibus Curantur (like cured by like). Homeopathy believes that the vital force regulates the self-healing capabilities of good health, which equilibrate the mind and body. The weakening of the body causes illness. Samuel Hahnemann developed a method "potentization," a process in which a substance is diluted with alcohol or water and is shaken vigorously during preparation. According to this method, homeopathic agents are diluted until there is not a single molecule left in the final product. It is believed that higher potencies (i.e., more diluted remedies) are more effective than lower potencies [58, 59]. Homeopathic medicines originate from plants, animals, and minerals. Mahendra Lal Sircar, medical doctor who publically converted his practice into homeopathy in 1867, was the first Indian homeopathic physician [60, 61].

2.4.7 Sowa-Rigpa

Sowa-Rigpa is Tibetan system of medicine. It is believed that this is one of the oldest and well-documented medicinal systems. Sowa-Rigpa is a Tibetan word (Sowa meaning to nourish, heal, correct and Rigpa meaning science or knowledge, perception or erudition). Sowa-Rigpa is practiced in Tibet, India, Mongolia, Bhutan, China, and Nepal. Sowa-Rigpa is widely practiced in Himalayan states of India like Sikkim, Arunachal Pradesh, and regions of Darjeeling, Lahaul and Spiti, and Ladakh. The theory and practice is similar to Ayurveda. Sowa-Rigpa is also popularly known as Amchi system. Sowa-Rigpa is based on the principle of *Jung-wa-lan,* which means five elements (Panch-mahabhuta in Ayurveda), and Ngepa-Sum (Tridohsa in Ayurveda). It is believed that all activities in universe are composed of sa (earth), Chu (water), Mai (Fire), rlung (air), and Nam mkha (space), which in Sanskrit mean Dharti, Jal, Agni, Vayu, and Akash, respectively. The physiology, pathology, pharmacology, and materia-medica of Sowa-Rigpa was developed on the basis these theories. Sowa-Rigpa system of medicine was recognized in India in 2011 after the Indian Medicine Central Council Amendment Bill 2010 [40, 62].

2.4.8 Development of AYUSH Drugs

General guidelines for drug development of Ayurvedic formulations were recently published by Central Council for Research in Ayurvedic Sciences in 2018 in the form of three series, (1) drug development of Ayurvedic formulations, (2) safety toxicity evaluation of Ayurvedic formulations, and (3) clinical evaluation of Ayurvedic interventions. According to Rule 3a of Drugs and Cosmetics Act, 1940 "Ayurvedic, Siddha or Unani drug" includes all medicines for internal or external

use for the diagnosis, treatment, mitigation, or prevention of disease or disorder in human beings or animals and manufactured exclusively in accordance with formulae described in authoritative books of Ayurveda, Siddha, and Unani Tibb systems of medicine. Ayurvedic drugs are majorly classified as classical drugs (raw/crude drugs, extracts, compound formulations, herbo mineral formulations), various dosage forms (asava and arista, arka, avaleha/leha/paka, kvatha, chuma, guggulu, chuma, ghrita/taila, lavanaksara, lepa, vati and gutika/pills, netrabindu and anjana, parpati, pisti, mandura, rasayoga, lauha, dhupa, bhasma), and patented and proprietary drugs (syrup, ointment, capsule, granules, confectioneries, dusting powder, tablet, suppositories) [63]. The raw drugs are generally procured from wild/cultivated sources. Different plant parts are used in preparation of drugs and these may include roots, fruits, bulbs, seeds, flowers, etc. Forty different parts have been listed in the general guidelines. The chemical composition and the content of active constituents vary between different parts. Good agriculture and collection practices guidelines published by WHO are an important step in ensuring the quality of herbal medicines. The protocol for development of AYUSH drugs is compiled in these guidelines and is divided into eight phases as listed in Table 2.1.

2.4.8.1 Prevalence Survey

Literature search is the key point for the development of drugs and vast literature on traditional systems of medicine in practice is available in the public domain. It is reported that globally about 35,000 plants are used for medicinal purposes [64]. The herbs/plants contain biologically active compounds that impart medicinal values to these plants. For the purpose, the medicinal plants can be divided into major categories, (1) herbs which occur in databases that provide detail and coherent description of the history and theory of use from Ayurvedic, Unani, Siddha, and Homeopathy medicine and (2) herbs used as folk medicine but lack literature information on their history and theory of use. The number of medicinal plants listed in Ayurveda (1200 species), Siddha (900 species), and Unani (700 species) [40] and thousands of formulations documented in the Ayurvedic Formulary of India, National Formulary of Unani Medicine, and Siddha Formulary of India can tremendously increase the success rate for developing medicinal formulations with sound scientific evidence.

2.4.8.2 Collection of Plant Materials

Good Agricultural Practices (GAP) of medicinal plants is defined as a cultivation program designed to ensure optimal yield in terms of both quality and quantity of any crop intended for health purposes. The supply and demand of plant materials has increased worldwide due to increased popularity of herbal medicines. Several reports have demonstrated adverse effects of many herbals that might be due to use of wrong plant species, adulteration with undeclared other medicines and/or potent

Table 2.1 General research guidelines and methodologies for drug development for Ayurveda, Siddha, and Unani medicine

Preparatory phase		
Phases	Activity	Considerations
1	Prevalence survey and formulation of drug/combination for specific targeted indication/activity	Appropriate basis of literary survey, previous clinical data of ingredients/any other data of folklore claims, classical evidences
Drug developments phases		
2	Collection of raw drugs	Current good agricultural practices, good field collection practices and Ayurvedic textual methods
3	Botanical identification/pharmacogonostic /chemical studies of ingredients	Based on available guidelines and classical methodology
4	Formulation of SOPs and standardization, stability studies, quality assurance	Considering the classical methods and currents available physical/chemical, biological parameters for standardization
5	Preclinical safety studies (acute/subacute/chronic studies as per the clinical use of the drug)	With appropriate animal ethical clearances as per available guidelines
6	Animal studies for biological activity/ efficacy	Specific/mechanism of action activity for clinical correlation
7	Design of study and formulation of clinical protocols (bulk preparation of quality assured drug for clinical trial, packing labelling, etc. as per need at appropriates time)	As per current guidelines and adopting classical methodology
8	Execution of clinical trial	Approval of IEC/IRB and CTRI Registration Trial conduct Trial monitoring Trial coordination Data analysis Publication

substances, contamination with toxic and/or hazardous substances, overdosage, inappropriate use by healthcare providers or consumers, and interaction with other medicines. Intrinsic (genetic) and extrinsic (environment, collection methods, cultivation, harvest, post-harvest processing, transport, and storage practices) factors are responsible for the safety and quality of raw materials of medicinal plant and final products. The environmental contaminations in medicinal plants are heavy metals, pesticides, herbicides, mycotoxins, and pathogenic microorganisms, all related to plant growth in polluted soils, cultivation requiring the use of pesticides, harvest, and storage of the raw materials in unclean warehouses. World Health Organization (WHO) has published guidelines for Good Agriculture and Collection Practices (GACPs) to ensure the affordable and sustainable supply with good quality of medicinal plant materials. WHO also addressed the impact of cultivation and collection of medicinal plants on the environment and ecological processes and the

welfare of local communities [63, 65]. According to GACPs, proper documentation is required to minimize the contaminations of plant material.

2.4.8.3 Botanical Identification/Pharmacogonostic/Chemical Studies of Ingredients

Starting plant materials are the foundation of the any finished products, therefore determination of authenticity of plant material is essential to maintain the consistency of efficacy and safety of any finished products. Following parameters are recommended to ensure the quality of raw material.

Passport Data of Plant Material (Place and Date of Collection)

The cultivation and harvest/collection time, the place of cultivation and collection, the name of the part collected, and the chemicals or other substances used during cultivation or storage such as fertilizers, pesticides, herbicides, and fumigants should be documented [65, 66].

Foreign Matter

The raw material should be free from any foreign matter such as molds or insects, and other animal contamination including animal excreta. Plant material should be free from any poisonous, dangerous, or harmful foreign matter. Take about 100 g plant material and spread in a thin layer in an appropriate dish or tray. Examine the sample in daylight with eye or magnifying glass to separate the foreign matter. Weigh the foreign matter and calculate the excluded foreign matter in percent with reference to drug sample.

Macroscopic and Microscopic Characters

The identity and the degree of purity of plant materials can be observed through their characteristic macroscopic and microscopic features and should match the Pharmacopoeial standard as a reference. The macroscopic identity of plant materials is based on shape, size, color, surface characteristics, texture, fracture characteristics, and appearance of the cut surface. Microscopic examination is performed with chemical reagent for identification of plant materials. Microscopic characteristics features include the presence or absence of hairs (trichomes), canals, oil glands, particular cell types, pollen or seed morphology, and vascular traces.

Loss on Drying (Moisture Content)

It is a method to determine water and volatile matter in the raw material. The sample (10 g) is prepared by chopping, grounding, or shredding of the unground or unpowdered material to a thickness of not more than 3 mm. Care should be taken to avoid moisture loss during sample preparation. A suitable amount of sample is placed in evaporating dish for drying at 105 °C for 5 h and weighed. This is repeated after 1 h interval till the difference between two successive weighing corresponds to not more than 0.25%.

pH Value

Determination of pH value gives information about acidity or alkalinity of a solution. The pH value can be determined with a pH meter.

Ash Values

It is a method to measure the total amount of material remaining after ignition. Two types of ash are determined, physiological ash (derived from the plant tissue itself) and non-physiological ash (the residue of the extraneous matter, e.g., sand and soil). Ground sample is accurately weighed in a tared platinum or silica dish and heated at a temperature not exceeding 450 °C till free from the carbon, coal and reweighed. The obtained total ash is boiled with dilute HCl and ignited to determine the remaining acid-insoluble ash. Water-soluble ash is the difference between the total ash and the residue after treatment of the total ash with water.

Extractive Values

The extractive values indicate the amount of chemical constituents extracted with solvents from a given amount of sample. The air-dried and coarsely powdered sample (5 g) is macerated with 100 mL of water in a closed flask for 24 h, with frequent shaking during first 6 h and kept for 18 h. The percentage of water-soluble extractive value is calculated and compared with reference. Alcohol soluble extractive value is calculated after maceration with alcohol.

Volatile Oil (If Oil-Bearing Plants)

Volatile oils possess specific characteristics like odor, oil-like appearance, and ability to volatilize at room temperature. The volatile oil in sample is extracted by distilling with a mixture of water and glycerin using clevenger apparatus.

Test for Heavy/Toxic Metals

Analytical techniques like atomic absorption spectrophotometry or inductively coupled plasma mass spectrometry techniques are used for the determination of heavy metal elements and some nonmetal elements in the atomic state. The permissible limits of lead (10 ppm), arsenic (3 ppm), cadmium (0.3 ppm), and mercury (1 ppm) are given in API (API IX).

Pesticide Residue

A pesticide is any chemical or mixture of chemicals which are used for preventing, destroying, or controlling any pest, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport, or marketing of plant drugs. The plant material and products should be free from pesticide chemicals. Mostly chlorinated hydrocarbons and related pesticides like aldrin, chlordane, DDT, dieldrin, HCH, and a few organophosphorus pesticides like carbophenothion have a long residual action. Therefore these pesticides should be tested in the medicinal plant materials [73]. Several methods are employed for extraction, purification, and identification of pesticides.

Microbial Contamination

Microbial tests should be applied to raw plant materials for Enterobacteria and also for fungal count as per Ayurvedic Pharmacopoeia of India. Besides, microbial tests for specific pathogens like *Escherichia coli*, *Salmonella* spp., *Staphylococcus aureus*, and *Pseudomonas aeruginosa* should also be performed.

Aflatoxins

Aflatoxins are hazardous to health even in very small amounts. Aflatoxins (B1, B2, G1, and G2) are highly dangerous and precaution should be taken during aflatoxin test. Qualitative and quantitative analysis of aflatoxins can also be performed by analytical techniques like TLC and HPLC [67].

Chemical Standardization

Quality control and authentication of raw materials are the main concern for the acceptability of herbal medicines. Several assays are reported for determination of different classes of bioactive compounds such as total tannins, sugar contents, total phenolics, and total flavonoids. Many of the biological activities shown by plant drugs are ascribed to the phenolics contents, which are assayed

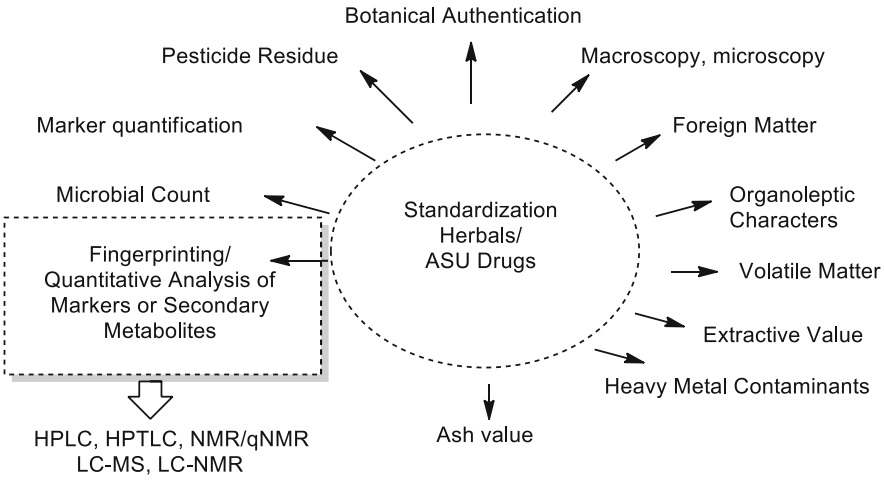


Fig. 2.13 Standardization of ASU drugs

spectrophotometrically using Folin Ciocalteu reagent. Total sugars, total reducing sugars, and total nonreducing sugars are estimated by Nelson-Somogyi photometric method.

In plants, the secondary metabolites are generally present inside the cells. Therefore, grinding of the raw material and breaking tissue are required to enhance the extractive yields. Standardization of plant extracts is very important as quality of plants material plays significant role in ensuring quality of the finished products. Traditional drugs can be single herbs or polyherbal. The identification and quantitation of one or two markers or medicinally active constituents in extracts are used for the quality and authenticity of herbal medicines. The extracts of single herb or polyherbal drugs may contain hundreds of compounds belonging to different chemical classes and some of these may be present in very low concentration, but may be highly active. Further, different compounds may be acting on different therapeutic targets leading to the overall observed benefits of these drugs. Therefore, standardization with respect to one or two markers may not provide true whole picture for quality control in terms of therapeutic benefits of extracts. This may also not account for the synergistic effects within these drugs. On other hand, employing a single analytical technique may also be inadequate for the effective analysis of complex herbal medicine, chromatographic and spectral fingerprints using TLC, HPTLC, HPLC, LC-MS, and NMR may be generated. Therefore, standardization of extract with identification and quantification of maximum chemical constituents is required to assure the reproducibility and repeatability of biological and clinical efficacy. Standardization parameters for quality control are summarized in Fig. 2.13.

Complete metabolite profiling of *Eugenia jambolana* fruit pulp has been reported. Sixty-eight chemically diverse metabolites were identified in the fruit pulp using qNMR, HPLC, LC-MS, GC-MS, and MALDI-TOF. The identified compounds included anthocyanins, anthocyanidins, amino acids, sugars, phenolics, and volatile

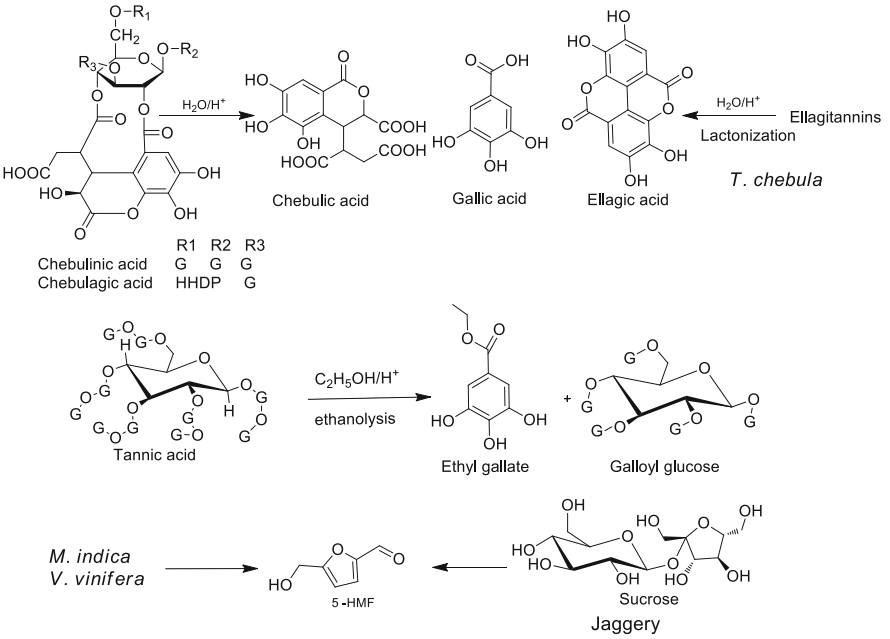


Fig. 2.14 Chemical changes during fermentation in Abhayarishta

compounds. Twenty-five metabolites were identified in the *n*-butanol and aqueous-methanolic extracts by qNMR. The main advantage of using qNMR is that reference standards of natural products being quantified are not required. Any commercially available compound of known purity can be used as an internal standard provided its NMR signals do not overlap with the NMR signal of the analyte being quantified. Further qNMR does not require the separation of analytes. HPLC and HPTLC require authentic reference standards for the preparation of calibration curves [68].

Arishtas are important Ayurvedic formulations that are prepared by anaerobic fermentation of decoctions of plant materials with jaggery. Fermentation generates alcohol which helps preserve these formulations. It has been shown that fermentation alters the chemical composition of the finished products. The amounts of chemical constituents in fermented product differed from that observed in the raw materials, which was explained in terms of chemical transformations occurring during fermentation (Fig. 2.14). Abhayarishta contains *Terminalia chebula* (pericarp), *Vitis vinifera* (fruits), *Embelia ribes* (fruits), and *Madhuca indica* (flowers). It was shown that chebulinic acid and chebulagic acid, two major constituents and marker compounds of *Terminalia chebula*, completely disappeared in the formulation. There was an increase in amounts of chebulic acid, ellagic acid, and gallic acid. This suggested that gallotannins and ellagitannins hydrolyzed during fermentation and were converted into monomeric constituents. Similarly, ethyl gallate, which is not present in any of these plants, appeared in the finished product. This was considered to form through ethanolysis of tannic acid and galloyl glucose. This

study underlines the importance of standardization of raw materials as well as the finished products to ensure quality control [69].

2.4.8.4 ASU Formulations

ASU drugs can be formulated into a variety of dosage forms depending on the nature of chemical constituents and the intended uses. Pre-formulation studies are essential for developing a suitable herbal formulation (Fig. 2.15).

2.4.8.5 Stability Studies/Shelf Life

Stability depends on several factors like type of compounds present in the drug, packing material used, storage, etc. Stability studies of drugs give information about shelf life and storage conditions. This is important for providing evidence for the quality of a drug substance over a period of time under the influence of variety of environmental factors such as temperature, humidity, and light. Stability test of drugs can be studied by storing the sample at standard storage and accelerated storage conditions and test them at defined time intervals. Alternately, drugs can be evaluated by selecting samples manufactured over a period of 5 years and crossing 6 months. The stability test should be performed with suitable parameters like the physical, chemical, biological, and microbiological attributes. Stability for chemical parameters may include color reaction, pH value, weight variation, disintegration, bulk density, extractive values, and estimation of biomarker or marker compounds by suitable analytical methods. If possible, a suitable bioassay can be used. The date of expiry should be displayed on the label. The guidelines for stability studies are prescribed in the Ayurvedic Pharmacopoeia of India, Part-I,

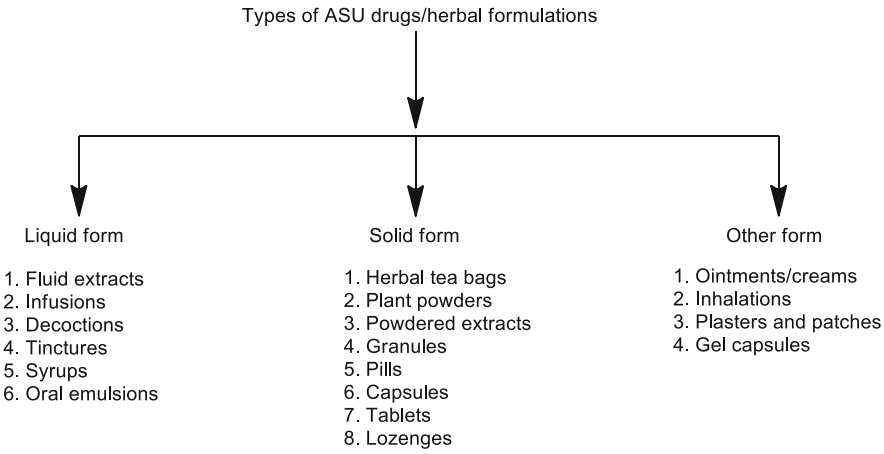


Fig. 2.15 Types of herbal formulations

Volume-VIII. In general, the shelf life of AYUSH drugs varies from 1 year (arka) to 10 years (asava and arista) for different dosage forms.

2.4.8.6 Preclinical Safety/Toxicity and Efficacy

The importance of safety and toxicity of food and drugs is known since ancient times and there are evidences that preclinical testing Vishaana/Virudhanna/Pareeksha on animals was well known in Ayurveda. Sushruta mentions testing of food /drugs on birds and animals to establish safety. The toxicity of some plants, metals, and minerals was well known to ancient Ayurveda practitioners as processing (shodhana, marana) of these drugs to make them safe is well documented in Ayurveda. It is important to do comprehensive literature search on plant ingredient of drug preparations before starting the preclinical safety/efficacy studies. The literature search should then be extended to gather information on closely related plant species for chemotaxonomic correlation. Generally, preclinical studies are performed to characterize toxicity with respect to target organs, dose dependence, and relationship to exposure. These data help for clinical experiment in the human trial for establishing safe starting dose and identify potential adverse effects. Ministry of AYUSH issued guidelines for safety requirements for different categories of ASU drugs. Recently, CCRAS has given detailed protocols for conducting safety/toxicity of ASU drugs; these include single-dose toxicity studies (Acute Toxicity), repeated-dose oral toxicity study, reproduction and developmental toxicity studies, and special toxicity tests. Several parameters are listed for toxicity studies in CCRAS manual for preclinical studies for different category of drugs (CCRAS Guidelines Series II) [70].

For any ASU drugs given in Section 3(a) of Drugs and Cosmetics Act 1940, which are manufactured and prescribed as per ASU texts, there is no requirement of any preclinical safety and efficacy data. Even if there are any changes in the dosage form, but the drugs ingredients are as per texts, still there is no requirement of preclinical safety and efficacy data. If these drugs are to be used for a new indication, preclinical efficacy data is required. For patented and proprietary drugs defined under Section 3(h) of the act, containing crude drugs/aqueous extracts or hydroalcoholic extracts as per text, not preclinical data are required. However, for patented and proprietary drugs containing other than aqueous/hydroalcoholic extract of any other solvent extract, detailed preclinical safety and efficacy data is required. These include, for oral preparations, acute and long-term toxicity in rats, reproductive and developmental toxicity, genotoxicity, and carcinogenicity. Dermal toxicity, photo toxicity, and allergenicity tests are required for topical formulations. Similar detailed toxicity and efficacy studies are required for patented or proprietary drugs defined under Section 3(h) containing any of the ingredients of Schedule E(1). Complete details required for licensing are given in CCRAS guidelines Series II. The efficacy study is required for proof and documentation of indicated claims [70].

2.4.8.7 Clinical Study

Ayurvedic texts have mentioned two-way approach of experimentation and drug trials, feasibility of test interventions, observational design and system validation are mentioned in Sushruta Samhita. Clinical studies for ASU drugs and patent and proprietary medicines are divided into Phase I, Phase II, Phase III, and Phase IV. Phase I determines the maximum tolerated dose (both single and multiple dose) and early measurement of drug activity. It also determines the nature of expected adverse reactions. Phase II explores the efficacy of drug for the indications and to determine any short-term side effects. The study should be able to determine the dose, therapeutic regimen, and target population (mild versus severe disease) for Phase III trials. The primary objective of Phase III trials is the confirmation of therapeutic benefits. This study should be designed to confirm the preliminary evidences on safety and efficacy in intended indication. It may explore dose–response relationships, usage in wider population in different stages of the disease, safety and efficacy in combination with other drugs. Phase IV trials are post-marketing surveillance and include additional drug–drug interactions, dose response or safety studies, any mortality/morbidity studies, epidemiological studies, etc. Trials for classical Ayurvedic drugs prepared as per Ayurvedic description with same textual indications can start directly from phase III/IV trial, whereas for drugs with new indications, trials can start directly from Phase II. Clinical trials should start from Phase I for patented and proprietary medicines containing Schedule E-1 ingredients (poisonous substances). These clinical studies are mainly of two types, observational study and experimental study. Observational study explores cause and effect relationships where the investigator observes the participants by asking questions, taking measurements, or studying clinical records. In experimental study, participants receive medical products/drugs as per the approved protocols and a comparison of placebo to a standard drug is done. Trial design is very important for any successful clinical trial. Complete details and requirements to conduct clinical trials are provided in CCRAS guidelines Series III. These guidelines also give detailed requirements for issuing of license for ASU drugs [71].

Several new drugs have been developed by the research councils (CCRAS, CCRH, CCRUM, and CCRS) and several others are being developed. AYUSH 82 as an antidiabetic and AYUSH SG as an anti-rheumatoid drug have been developed by CCRAS. AYUSH PJ-7 for Dengue fever, AYUSH Manas for mental retardation, and several other drugs for various conditions are currently being developed.

2.4.8.8 Development of AYUSH 82 for Diabetes

Ayush 82 consists of four plants: Amer Bija (*Mangifera indica* Linn seed), Jambu Bija (*Eugenia jambolana* seed), Karvellaka Bija (*Momordica charantia* Linn seed),

and Gudmar (*Gymnema sylvestre* leaf). This polyherbal ASU formulation was developed based on the reported antidiabetic potential of these four plants.

Mangifera indica belongs to the family Anacardiaceae. It is an important medicinal plant of the Ayurvedic and indigenous medicinal systems. The fruit, bark, leaves, and seeds possess antidiabetic, antioxidant, antiviral, anti-inflammatory properties. A number of studies have proved the effect of *M. indica* for antidiabetic activity. Various classes of bioactive compounds, terpenoids, flavonoids, alkaloids, coumarins, terpenoidal saponins, polyphenolics, tannins, etc. have been reported from different parts of the *M. indica* [72]. The ethanol and aqueous extracts of leaves and stem bark of *M. indica* exhibited significant hypoglycemic effect in type 2 diabetes rat model at dose of 250 mg/kg body weight [73]. The ethanolic extract showed decreased blood glucose level and also restored the levels of glycated hemoglobin in streptozotocin-induced diabetic rats at dose of 300 mg/kg b.w./day for 21 days [74]. The methanol and aqueous extracts of seed kernel showed significant reduction in blood glucose level in diabetic rats. Purified mangiferin from *Mangifera indica* leaves showed significant antidiabetic, antihyperlipidemic, and antiatherogenic activity at a dose of 10 and 20 mg/kg b.w. [75]. The ethanolic extract of mango fruit peel showed significant reduction in blood glucose level in streptozotocin-induced diabetic rats [76].

Momordica charantia L., commonly known as bitter melon or bitter gourd, belongs to family Cucurbitaceae. It is traditionally used for the management of diabetes since ancient times [77]. Studies have been reported for antidiabetic effect of *M. charantia* [78]. The methanol extract of *M. charantia* fruit orally administered to alloxan monohydrate-induced diabetic rats for 30 days showed a significant decrease in triglyceride, low-density lipoprotein and a significant increase in high-density lipoprotein level. A significant effect was also observed for oral glucose tolerance [79]. *M. charantia* fruit juice showed the increment of β cell in the pancreas of streptozotocin (STZ)-induced diabetic rats as compared to untreated diabetics rats [80]. Several phenolic acids, flavonoids, phytosterols, and terpenoids have been reported [81].

Gymnema sylvestre belongs to the family Asclepiadaceae and is popularly known as gurmar. It is mentioned in Ayurveda for the treatment of several ailments, especially diabetes management. First experimental pharmacological study was reported in 1930 [82]. It possesses a variety of biological activities including antidiabetic, antihyperlipidemic, antiobesity, antioxidant, immunomodulatory, anti-inflammatory, anticancer, and wound healing [83]. The water-soluble extract of *Gymnema sylvestre* leaves was administered (400 mg/day) to 27 patients with insulin-dependent diabetes mellitus on insulin therapy. The water-soluble extract enhanced endogenous insulin, possibly by regeneration/revitalization of the residual *beta* cells in insulin-dependent diabetes mellitus [84]. Daily oral administration of methanolic extract of *Gymnema sylvestre* leaves (100, 200 and 400 mg/kg b.w.) and glibenclamide (5 mg/kg) in normal and streptozotocin-induced diabetic rats for 28 days showed significant reduction in blood glucose level with no histopathological change [85]. Gymnemic acid has been reported as an active compound with antidiabetic properties and inhibited glucose absorption [86].

Eugenia jambolana Lam. (Syn. *Syzygium cumini* Skeels or *Syzygium jambolana* or *Eugenia cuminii* Druce.) belongs to the family Myrtaceae. It is commonly known as Indian blackberry, jamun, java plum, Portuguese plum, black plum, Indian blackberry, jambu, jambul, and jambool. In traditional system of Indian medicine the fruit pulp and seed extracts are being used for the treatment of several diseases including diabetes. Jamun has been used for the treatment of diabetes alone or in combination with other antidiabetic plants in Europe [87]. A number of pharmacological activities like antioxidant, hepatoprotective, anticancer, anti-inflammatory antibacterial, antifungal, and gastroprotective activities have been reported [88]. Metabolite profiling of jamun fruit and pulp has shown the presence of phenolic acids, anthocyanins, flavonoids, carotenes, and phytosteroids [68, 89]. The anti-hyperglycemic effect of aqueous and alcoholic extracts was evaluated in diabetic animals. The different doses 32 mg/kg and 120 mg/kg of alloxan monohydrate for mild (plasma sugar >180 mg/dL, duration 21 days) and moderate (plasma sugar >280 mg/dL, duration 120 days) diabetes, respectively, and 150 mg/kg of streptozotocin for sever diabetes (plasma sugar >400 mg/dL, duration 60 days) were administered. The reduction of glucose level in moderate and severe diabetic rats was 55.62 and 17.72%, respectively, [90]. The hypoglycemic activity of ethanolic extract of seeds of *E. jambolana* was evaluated in alloxan-induced diabetic rabbits. The induced diabetic rabbits were treated with ethanolic extract with a dose of 100 mg/kg for (1 day), (7 days), and (15 days) for sub-diabetic, mild diabetic, and sever diabetic rabbits, respectively. The glucose level significantly reduced in sub-diabetic and mild diabetic rabbits [91].

Acute Toxicity

Administration of Ayush 82 orally in Swiss Albino (I. B) mice revealed no pre-terminal deaths, toxic signs, or abnormal behavior in the animals at 10 times of intended therapeutic dose.

Subacute Toxicity

Subacute toxicity studies of Ayush 82 was performed in Wistar rats which showed no significant effect in the blood biochemistry, hematology, and weight of the vital organs in comparison to the control suggestive of its safety.

Clinical Efficacy

Total 886 patients was selected in which 497 patients completed cases and 389 patient dropouts at Council's peripheral Central Research Institutes. The Ayush 82 was administered thrice daily. The results showed significant reduction in fasting and post prandial blood sugar level along with clinical improvement. No

adverse effects were reported in the patients during the treatment period. The recommended dose is 15 g/day, which is divided into three doses along with 500 mg Shuddha Shilajita twice daily [92].

2.5 Summary

Nature is the richest source of medicines for the prevention and treatment of various diseases. Most of the blockbuster drugs available in the market are derived from natural sources. The process of natural product-derived drug discovery involves taxonomical identification of targeted plant followed by extraction, fractionation, isolation, and characterization. However, lesser yield of hit molecules from natural sources drives the focus mainly to semisynthetic derivatives of lead molecules derived from nature. The other major challenges faced by natural-product-based drug discovery and indigenous systems of medicines include poor bioavailability of most natural compounds, time-consuming process for discovery of novel skeletons, lack of strict regulations for the standardization of traditional formulations, etc. Despite the supremacy of synthetic drugs, there is a re-emergence of plant-based drugs in recent years. Molecules from traditional sources have great ethnopharmacological value. Natural-product-based drug discovery will continue to be a promising area in the field of research and development.

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