Chapter 1

Chapter-1: Introduction

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Chapter -1

Introduction

1.1. Folklore medicinal plants as pharmacotherapeutics:

The last couple of decades have seen a resurgence of interest in the use of herbal products. The interest may have been revived by factors such as inadequate treatments for chronic conditions and the need to overcome the short comings of the established conventional healthcare systems. The popularity is also activated by the advantage of safety and cost control, which is generally perceived in phytotherapy. This interest in the herbals, as a form of health provider, has given momentum to a rapid and enormous amount of research work on the components of the plants, their pharmacological properties, and method to isolate them and bring out medicinal or health supplements and improve them.

1.2. Isolation of bioactive compounds

From the time when more than 80% of the world’s population was found to use medicinal plants for the purpose of primary health care, it is not astonishing to discover that in several countries have recognized and established a systematic traditional system of medicine.
Fig. 1.1: Natural Products and its related studies

Recent research in herbal drug investigation area is mainly concentrated in terms of regularization of plant material extracts. Hence the quality can be measured by the mixtures of specially obtained with an assistance of suitable methods incorporated for evaluating the biological activity. The changes has been occurred for quite a lot of different reasons, comprising the readiness of predictable in vitro bioassay, even though it is very difficult to measure sometimes because of their interfering substances and these would be the valuable conditions in evaluating the worth of a particular plant material.

Different sources of obtaining a molecule are varied by its chemical nature and also with their biological activity. Certain molecules reveal specific biological activities that can make them crucial tools in pharmaceutical research and distinctive originals for the development of drugs. For discovering new bioactive molecules, different types of bioassays are employed.
Mostly the bioassay is used for the detection of bioactive compounds in the extract preferably for the quantification of isolated moiety. Furthermore, these assays may be used to guide the compound fractionation technique that leads to the isolation of bioactive substances in pure form\(^2\).

This approach calls for thorough and specific understanding of the desired physiological / pharmacological activity. The technique involved in extracting the drug using three to five solvents of increasing polarity, which are known to exhaust all kinds of phytoconstituents present in them. These different fractions and sub-fractions are then tested for the desired biological activity. At each step the active fraction is identified and the inactive fraction is not given any importance for further purification. The process of activity guided fractionation is continued till a single bioactive compound is isolated\(^3\).

### 1.3. High–Throughput Screening

While performing *in-vivo* biological evaluation it was necessary to collect large number of plant materials for primary screening modality, then only it is possible to achieve a considerable amount of the extract for specific study designed, accordingly the availability of the extracts can be acquired for possible testing. Very selective *in-vitro* assays have been developed gradually and drastically reduced the requirement of materials very minimal quantity.
Understanding of the basic biological process which is related to a number of major disease states that is linked with the better availability of enzymes, cell systems, receptors and genetic switches have been allowed the automated bioassay systems development. Which will be helpful in screening the tens of thousands samples within a short period of time, also these biological screening methods are economical, faster, result oriented and productive. So these can be called as High throughput Screening⁴,⁵.

High-throughput screening is a concept, which involves the use of miniaturized assays to screen several thousand of molecules against new biochemical and/or other genomic targets within a relatively short time. A large number of compounds can be tested in an automated manner, for activity as inhibitors or activators of specific biological assays. The HTS assays can be classified broadly into two distinctive categories are as Single-Target Specific Bioassays and Multi-Targeted Functional Bioassays.

1.4. The Bioassays:

Any pharmacological method, which is rapid, reproducible, economical and sample hungry (has a high sample throughput), can be used as a monitor for fractionating natural products. The major limitation is that most of the bioactive compounds are present in very small quantities naturally. On the contrary, amount of sample that can be easily generated using preparative chromatographic techniques is generally in milligram quantities whereas for pharmacological
evaluation often gram quantities of samples are required. Therefore bioassays that need very small quantities of sample (in milligrams) are most suitable for bioactivity guided fractionation. The number of such bioassays is very limited because of which often general non-specific bioassays like isolated guinea pig ileum test, Brine shrimp lethality test etc., have to be employed.

![Scheme for Bioactivity-Guided Fractionation and Structural Elucidation of isolated pure compound](image)

**Fig.1.2:** Scheme for Bioactivity-Guided Fractionation and Structural Elucidation of isolated pure compound

Four major roles of bioassays can be renowned, i.e. prescreens, monitors, screens and secondary testing.
For determining a large number of samples and to check their desired type are either biologically active or not can be done by a bioassay. These assays also used for selecting substances for secondary testing determined in a screen. A bioassay is applied to guided fractionation of a crude plant substances towards isolation of the pure bioactive compounds can be determined in a monitor. It must, therefore be fast and cheap, have high capacity and be readily available to the phytochemists. In the secondary testing, in the multiple models and test conditions the lead compounds are evaluated to select candidates for drug development towards clinical trials. Secondary testing is characterized by an expensive, low capacity and slow bioassays.7.

The technique for the detection of natural plant products biological activity and mixtures can best be separated into two groups for biological screening purposes are as follows. Specialized and General screening bioassays; Depending on the aims of the screening program, either a general screening or a broad screening bioassays has to be performed, which can pick up many different effects. A specific assay, which is aimed at finding some effect next to a specific disease; the most useful broad screening bioassay is probably if one is randomly screening selected organisms for any kind of biological or pharmacological activity. The drawback of using a general test for monitoring and screening is that one does not know, until the active compound has been isolated, if the work was worth doing.8.
Since, in most phytochemical laboratories engaged in the bioactivity guided isolation of active molecules from natural product mixtures no complex bioassays can be used, for the rapid screening of extracts and fractions the efforts have been made to introduce inexpensive, single ‘front-line’ or ‘bench-top’ bioassays. Care must be taken in the interpretation and predictive ability of these screening bioassays, but in general, they interesting preliminary information on the pharmacological potential of the plant extracts under study\textsuperscript{9}.

\textbf{1.5. Carbohydrate Metabolism}

Alpha amylase involves in the breakdown of starch (polysaccharides) and the enzyme bounded in epithelium of the small intestine helps in cleavage of other short chain polysaccharide by alpha glucosidase enzyme. The carbohydrate becomes enzymatically decomposed (hydrolysis) in to simple sugars in small intestine. As the simple sugars (monosaccharides) are absorbed via specific and non-specific passage\textsuperscript{10}.

\textbf{1.5.1. Enzymatic Cleavage of Starch:}

Depending on its origin, starch is a mixture of amylopectin and amylose of variable composition. Amylase has an optimal enzymatic activity at pH 7 and is inactivated in the acid environment of the stomach, so that breakdown of starch commences in the duodenum by the pancreatic amylase. Both pancreatic and salivary amylase can cleave only 1,4 linkages of starch. Thus, the main products of starch
hydrolysis are maltose and maltotriose from amylose, and from amylopectin, in addition, the so-called alpha-limit dextrins.

Only very little free glucose results from the primary breakdown of starch since alpha amylase represents endoenzyme that is capable of hydrolyzing only in the middle of glucose chains, but exhibits no activity as regards to the cleavage of end molecules. Both the unavailable and dietary fibre is then subjected within the colon to bacterial breakdown and fermentation.

1.5.2. Final Carbohydrate Digestion by brush border disaccharidases

The monosaccharides necessary for the absorption are provided from starch degradation products and from dietary disaccharides by specific enzymes of the membrane of small intestine contain brush border. These enzymes are synthesized in maturing enterocytes and embedded in the apical membrane, where they form an integral part of the membrane in close structural and functional proximity to the glucose-carrier protein. Among the alpha glucosidases of the brush border membrane, a distinction is made between the three enzymes (maltases, glucoamylases and sucrases) in man. Their specificities for the various breakdown products of starch overlap. They affect the release of glucose from the products of predigestion of starch in a mutually comprehensive manner.
Role that the alpha amylases & alpha glucosidases have in the intraluminal is generation of glucose. A delay in the absorption of carbohydrates may be achieved, by dietary fibres, alpha amylase inhibitors or alpha glucosidase inhibitors. Simple representation of the concept of α- amylase and α-glucosidase inhibition, the bold arrows indicate the points at which acarbose delays the production of monosaccharides and as a consequence their intestinal absorption

1.5.3. Alpha Amylase Inhibitors

1,4- D-glucanglucanohydrolase a proteinaceous digestive enzyme which is commonly found in cereals and legumes available as primary phyto metabolites in plants. The influence of alpha amylase enzymes helps in inhibition of carbohydrate reuptake in diabetic and obese patients.\textsuperscript{11}

The alpha amylase inhibitor may also inhibit pancreatic alpha amylases at a pH of 6-7, owing to the residual activity developed at a neutral pH.\textsuperscript{12} Specific inhibitors of animal alpha amylases were found in plants, particularly wheat and beans, 40 years ago. It was not until 1973, however, that and alpha amylase inhibitor from wheat was reported to decrease postprandial plasma glucose rise in response to raw, but not to cooked wheat starch.\textsuperscript{13} Subsequently, the amylase inhibitor (phaseolamin) present in white and red kidney beans (Phaseolus vulgaris) was successfully isolated.\textsuperscript{14}
1.5.4. Alpha Glucosidase Inhibitors

The use of alpha glucosidase inhibitors provides the clinical with a pharmacological means of modulating postprandial (after meals) blood glucose levels by delaying carbohydrate digestion and absorption. Improved regulation of blood glucose concentrations will help avoid the longer-term complications of diabetes.

The inhibitors of alpha glucosidase enzyme have cyclohexene and 4,6-dideoxyaminomando-D-glucose unit (called Acarvosine). Although the cyclohexene and amino sugar portion of Acarvosine are essential for the inhibition of alpha glucosidase activity, the specifying of this action appears to be determined by the number of glucose molecules linearly α-1, 4-coupled to the core\textsuperscript{15}.

Acarbose was the first available alpha glucosidase inhibitor. Alpha-glucosidase inhibitors have proven useful for improving the glycaemic control when utilised as an adjuvant to the standard therapy involving oral antidiabetic drugs, dietary restrictions, and subcutaneous insulin\textsuperscript{16}.

1.5.5. The Need for Isolation of Bio-Actives

The major reasons for isolation of bioactive compounds from medicinal plants are: a) Search for novel molecules that may form the basis for new drugs; b) Standardization of herbal products where the crude drugs, their extracts or the combined formulations are used as such in therapy.
More than 85% of higher plants are not surveyed adequately for potentially valuable biological activity and the plant kingdom was received adequate attention as a source of potent and effective medicinal substances. Here, it has been estimated that over half of the world’s 25 top selling pharmaceuticals for 1991 owed its origin to a natural source substance. Another statistic shows that 120 plant derived chemical compounds are used as drugs currently. Many of these compounds are extracted and purified directly from medicinal plants. These may form the basis of new lead compounds for further exploitation\textsuperscript{17-19}.

The main Indian traditional systems of medicine namely Ayurveda, Siddhha and Unani are primarily plant based systems. The demand for herbal products throughout the world is growing exponentially. Standardization of herbal products is essential for several reasons. The western concept standardization of a plant drug means clear knowledge of the active compound(s), it’s quantification, and it’s pharmacology including the mode of action at molecular level, complete toxicity profile and also detailed information on the pharmaco-kinetic and bio-availability aspects. Such information is almost absent for herbal drugs\textsuperscript{20}. “Lack of standardization of polyherbal drug formulations is a serious problem in validating efficacy and maintaining quality during the manufacture of herbal drugs”. Thus another reason, why activity guided fractionation needs
to be taken up seriously, is standardization of phytomedicines / herbal products\textsuperscript{21}.

In the past some of the major natural product screening programmes have not been very successful because of the lack of multi-disciplinary approach. The chemists have only been interested in the chemical composition of the plants and isolating new compounds without any attention to their biological activities. The pharmacology’s on the other hand have never bothered to look at the chemical aspects and thus only the crude drugs as such or at the best their extracts have been pharmacologically evaluated. Very little effort has been put in trying to attribute the activity through activity-guided isolation or by testing each of the isolated compounds for biological activity. The latter works out to be very expensive as the number of compounds in a crude drug can be many.

1.6. Bioactivity Guided Fractionation

Bioactivity guided fractionation/separation which is thus very important for developing new drugs using leads from natural products\textsuperscript{22}. Any compound, which is unique to a plant and is present in convenient detectable quantities, can be used as a maker for standardization of the plant\textsuperscript{23}.

However while the detection and quantification of a marker can ensure genuineness of the crude drugs it does not guarantee the bioactivity or efficacy of the crude drug. For both biomarker based
standardization and bioactive-based standardization, the process of activity-guided fractionation is a pre-requisite. If the bioassay used in general and non-specific then the isolated compounds can be labeled as the ‘bio-markers’ for that crude drug. But if the bioassay used in specific, for a particular bioactivity then the compound isolated can be called as the ‘bio-active molecule’ for that crude drug, for that particular bio-activity\textsuperscript{24}.

Activity guided fractionation is a multidisciplinary approach involving pharmacological evaluation of a given mixture (crude drug or a formulation), followed by separation by means of extraction using solvents of increasing polarity. Each of these successive extracts is then tested for biological activity and the extracts, which are not active, are discarded and the most active extracts are further separated into fractions.

Each of these fractions is again tested for activity and the active fractions again separated to obtain sub-fractions. This process is repeated till the pure active compound(s) are obtained \textsuperscript{25}. For the purpose of fractionation, normally preparative chromatographic techniques are used like column chromatography, flash chromatography, vacuum liquid chromatography, CCTLC (Centrifugal Circular Thin Layer Chromatography), preparative TLC and sometimes semi-preparative and preparative HPLC. These techniques have been reviewed in detail with special emphasis to natural products.
After the active compound(s) are identified, apart from quantification of actives in the crude drug its extract and final formulations. Several other aspects of standardization also become possible like detection of photochemical variations due to seasonal, ecological chronological and geographical reasons; bio-availability studies, pharmaco-kinetic and stability studies etc\textsuperscript{26}.

1.6.1. Minimal amount of sample:

Once the chemist reaches up to the fraction level, he can generate only milligram quantities of samples. Most of the \textit{in-vivo} pharmacological methods require at least gram quantities of sample in order to evaluate the desired biological activity. Thus bioassay, which requires only milligram quantities of samples, can be utilized for directing the fractionation.
Fig. 1.3. Bioactivity Guided Fractionation

1. Crude plant Material
   - Test for activity
2. Successive extracts
   - Test for activity
   - Fraction of active extracts only
     - Discard inactive extracts
3. Further separation to obtain sub fractions
   - Test for activity
     - Discard inactive sub-fractions
4. Final separation to obtain pure compounds
   - Test for activity
     - Discard inactive
5. Isolation and identification of the bioactivities
6. Structure elucidation and characterization
   - Qualification of these bioactive(s) in the crude herb, its extract and the finished formulation
7. Detailed pharmacological profile dose response curves
   - Decision on the NLT % of the bioactives for obtaining the desired extent of bioactivity
Fig. 1.4: BROAD CLASSIFICATION OF BIOASSAYS FOR PHYTO PHARMACOLOGICAL SCREENING

- BIOASSAYS
  - SPECIFIC ASSAYS
  - NON-SPECIFIC ASSAYS (BROAD SCREENING ASSAYS)
    - USING LOWER ORGANISMS
      - BACTERIA
      - FUNGI
      - INSECTS
      - HELMINTHS
      - NEMATODES
      - MOLLUSCUS
      - PROTOZOA
      - VIRUS
    - USING SUB CELLULAR SYSTEMS
      - ENZYMES
      - RECEPTORS
      - ORGANELLES
    - USING CELLULAR SYSTEMS
    - USING ORGANS OF VERTEBRATES
    - USING WHOLE ANIMALS
    - MISCELLANEOUS