

**APPROACHES TOWARDS THE PRECLINICAL TESTING  
AND STANDARDIZATION OF MEDICINAL PLANTS  
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## **INTRODUCTION**

Before the introduction of modern medicines, disease treatment was entirely managed by herbal remedies. It is estimated that about 80% of the world population residing in the vast rural areas of the developing and under developed countries still rely mainly on medicinal plants. Medicinal plants are the only affordable and accessible source of primary health care for them, especially in the absence of access to modern medical facilities. Studies reveal that there are more traditional medicine providers than the allopathic providers especially in the rural areas (WHO 2002).

The use of traditional medicine has increased in developed countries also, mainly due to the failure of modern medicine to provide effective treatment for chronic diseases and emergence of multi-drug resistant bacteria and parasites. The adverse effects of chemical drugs, questioning of the approaches and assumptions of allopathic medicine, their increasing costs and greater public access to information on traditional medicine has also led to an increase in interest in alternative treatments (WHO 2002). Plant extracts have become a source of hope as a wide group of medicinal plant preparations are available that have been used over the centuries almost exclusively on the basis of empirical evidence. Hence, it has become necessary to revisit the importance of these herbal medicines.

Increasing interest by multinational pharmaceutical companies and domestic manufacturers of herbal-based medicines is contributing to a significant economic growth of the global medicinal plants sector. However, a large proportion of medicinal plant research is focused on nutraceuticals, chronic and metabolic disorders (diabetes, cardiovascular, etc.) and other diseases like HIV/AIDS, malaria, etc. **Whereas, the common diseases of resource poor communities such as diarrhoeal diseases and acute respiratory tract infections (ARI) are often not addressed.** Moreover, unlike the rural communities who use fresh/dried plant material or their crude extracts, the industry lays importance on isolation of active principles or standardized fractions since crude extracts are not patentable. However, it is often seen that a crude extract is more active compared to the isolated active fractions e.g. *Cirriformia tentaculata* loses its activity upon fractionation with hexane (Kicklighter *et al.* 2003).

It is generally believed that standardization of the plant material is not required when used by the rural communities for their primary health care. But, regardless of whether the medicinal plant is to be used by local communities or by industry, a systematic approach is required for a plant identified from traditional medicine, as is done in modern medicine. It is necessary to focus on all aspects of medicinal plant research: from cultivation, ethno-pharmacology, utilization, isolation and identification of active constituents to efficacy evaluation, pharmacology, safety,

standardization, formulation and clinical evaluation. Animal toxicity studies are required to establish the potential adverse effects.

Artuso (1997) has outlined the entire process which includes formulating an appropriate strategy and he estimates that the entire process would take more than 10–20 years. This approach is very demanding since there is an estimated 250,000 species of higher plants present on this earth (Ayensu and DeFilipps, 1978). However, this scenario would change with use of the high throughput advanced screening methods that are available today. **Another approach than can prove to be a highly productive and cost effective in development of safe, effective and acceptable therapeutic agents is reverse pharmacology which is based on the documented therapeutic effects of plants in ancient texts** (Vaidya, 2006).

This paper will discuss the approaches that need to be considered while studying medicinal plants. It focuses on aspects of the medicinal plant research: from collection of plant material, to efficacy and safety evaluation through preclinical studies and phytochemical standardization.

## **SOURCE OF PLANT MATERIAL**

The prominent mode of obtaining medicinal plants is wild harvesting and most of the industrial requirement is still met through wild collection (Lange 1998). Though many medicinal plants are commonly available in the wild and can be freely harvested, uncontrolled collection and sale of large quantities of plant material from the forest can lead to destruction of many forest plants especially the endemic species that have a restricted geographical distribution. For example, medicinal plants like *Curcuma caesia*, *Rauwolfia serpentina* were reported to occur abundantly (IUCN 1994) in central India. However, due to their growing economic importance and rampant harvesting, these plants have now been categorized as critically endangered (Prasad and Patnaik 1998). The present deteriorating condition of medicinal plants in forests needs immediate attention not only for conservation but also for propagation. Countries can protect their biodiversity in medicinal plants by working with industry towards monitoring and maintaining controlled non-destructive harvesting with habitat management.

Cultivation of medicinal plants would seem as a commercially attractive option to companies because they have greater control over supply of the plant material and it is easier to control post-harvest treatment. Moreover, cultivation can reduce the dependence on collection of plants from wild and thus have the potential to save wild populations and conserve their genetic diversity.

Cultivation in kitchen gardens can lead to easy availability of medicinal plants and can be an effective means of self reliance in supply of these plants for rural communities. Communities can cultivate plants with multiple uses such as those with both medicinal and commercial value (e.g. guava) or those, which are indicated against multiple disease conditions (e.g. Nirgudi for worms, cough, aches and pains; Adulsa for wet and dry cough; guava for viral/bacterial diarrhoea, swollen gums and kidney infections) (Satyavati 1987).

The feasibility of cultivating medicinal plants would, however, depend on a

number of factors such as the ability of the species to thrive under mono culturing. The economic viability will depend on the demand and market prices. Moreover, cultivation of medicinal plants requires intensive care and management and the conditions and duration required can vary depending on the quality of the medicinal plant material required. Risks of contamination from pollution by hazardous chemicals should be avoided. Moreover, introduction of non-indigenous plant species into cultivation can lead to detrimental consequences on the ecological balance of the region (Sharma *et al.* 2005).

A point that needs specific consideration is that cultivated plants are sometimes considered qualitatively inferior to the wild collections. The medicinal properties in plants are due to the combinations of secondary products. Different plants would have different combinations of these secondary products that would often be taxonomically distinct in individual plants resulting in unique medicinal properties (Wink 1999). Secondary metabolites that are generally produced for defense against predators, pathogens or competitors or for protection/adaptation to environmental stress related to changes in soil conditions, temperature, water status, light levels, UV exposure, and mineral nutrients in their natural habitats; and are responsible for most of the biological activities. Therefore, **the secondary metabolites may not be expressed in optimum quantities when cultivated under optimum conditions to obtain better vegetative yields**. For example, the wild ginseng roots are 5-10 times more valuable than cultivated roots because the cultivated roots lack the characteristic shape of wild roots (Robbins 1998). These beliefs were also reflected in the conclusion reached through research on *Arnica montana* by the herbal company Weleda (Ellenberger 1998). Analysis of the biochemical properties of the cultivated plants showed differences when compared with wild plants that grow in poor meadows with acidic soils in mountainous areas of Europe. The rhizomes of the cultivated stocks had lost much of *Arnica's* characteristics, reducing its commercial potential.

## SELECTION OF PLANTS

As per WHO guidelines (WHO 2003), the plant selected for collection should be taxonomically same as recommended by the national pharmacopoeia or other related documents. If a new plant is being selected for collection then it should be properly identified and documented. The botanical identity, scientific name including genus, species, subspecies or variety and family of the plant should be recorded. If available, the local name should also be verified. Complete taxonomical identification is an important factor during selection as taxonomy of the plant species can play an important role in their biological activity. This was observed in our study with two varieties of *Zingiber officinale* wherein one variety showed immune enhancing properties while the other did not (unpublished data). Information regarding environmental conditions, such as topography, geology, soil, climate and vegetation at the collection site, should be obtained. Information such as the geographical distribution of the plant, its abundance, whether it is threatened or endangered, shrub/fast growing tree etc should also be obtained. It is of immense importance that a voucher specimen be deposited in a national or regional herbarium for authentication and further consultation by other researchers.

Several reviews have described approaches that can be used for selecting plants of

potential therapeutic interest (Verpoorte 2000, Phillipson and Anderson 1989, Kinghorn 1994, Vlietinck and Vanden Berghe 1991, Farnsworth 1996, Farnsworth and Bingel 1977). In general, the search for the medicinal plants can follow three main routes: random, ethno (including ethnobotanical, ethnomedical and ethnopharmacological) and ecological search (Fabricant and Farnsworth 2001). Random search is extremely laborious and the success rate could be very low (Basso *et al.* 2005). Nevertheless, important drugs such as taxol, derivatives of camptothecin and homoharringtonine have been discovered by the National Cancer Institute (NCI) in collaborations with the United States Department of Agriculture (USDA) using this method (Cragg *et al.* 1999).

The ethnobotanical, ethnomedical or ethnopharmacological approach uses information obtained from ethnobotanical survey such as the geographical distribution of the plant, its abundance, whether it is threatened or endangered, shrub/fast growing tree, easily cultivable, easily identifiable (with minimum varieties) etc. Information such as the season of collection, parts that are used and whether those parts are seasonal/replenishable and if there is any reported toxicity, are also required. The information can be obtained from traditional medical practitioners and other people such as village elders and local women who are traditional users of medicinal plants. Undertaking of an ethnobotanical survey should be by a team of local botanists, traditional healers and medical practitioners. While the traditional healers would identify medicinal plants for treatment of different diseases, the botanist can carry out appropriate taxonomical and botanical characterization of these medicinal plants, whereas the medical practitioners would help in proper identification of the disease conditions (e.g. differentiate between muscle pain and pain due to arthritis) and help in understanding whether a treatment is curative or is alleviating the symptoms only or whether it is a placebo effect.

Frequency of quote is an important indicator of the usage of the plants by the community. However, information obtained from the community may not always be reliable. It is possible that people may quote a particular plant more frequently since it is easily available, easily recognizable or resembles a certain disease feature e.g. seeds of *Bixa orellana*, which have a bright red arillus, are used in herbal mixtures used for treating bloody diarrhoea (Kufer *et al.* 2005). People may also quote plants about which they have gained information from personal communication or books. In addition, publications on medicinal plants are often compilations from other texts and seldom from personal experience, making evaluation difficult.

Ethnomedical information is available from ancient texts of different systems of medicines such as Ayurveda, Unani, Kampo, and traditional Chinese medicine. However, while using the ancient texts, one must consider the fact that the plants may have evolved over a period of time resulting in changes in their phytochemical composition and hence their medicinal properties and therefore validation is required.

Nevertheless, the success rates of the ethno-based approaches are substantially higher than those of random screening since the continued use of crude preparations are, in fact, comparable to small scale clinical trials. Tests carried out at the NCI for antineoplastic activity using this approach yielded positive activity

in the order of 2 to 5 times higher than random screening (Lewis and Elvin-Lewis 1995).

Plants with medicinal properties can also be selected using an ecological approach. The absence of predation in areas infested with herbivores, for example, can indicate the presence of toxic compounds. Selection can also be based on an approach called zoopharmacognosy, a variation to the ecological approach, which proposes the selection of plant species regularly ingested by animals, mostly primates for reducing pain, microbial or worm infestations (Berry *et al.* 1995).

It has to be specifically understood that there are certain differences in approaches when selecting plants for an industrial or a rural application. The rural community requires medicinal plants for their primary health care and hence focuses more on selection of plants for treatment of common diseases such as diarrhoea, malaria, pneumonia, wound infections, etc. On the other hand, pharmaceutical industry requires medicinal plants for formulation of herbal drugs for commercial gain and hence focuses more on urban problems such as metabolic disorders, chronic diseases, and multi-drug resistance among infectious pathogens. Whether for rural community or for industrial application the selection of plant should be based on its therapeutic efficacy in terms of its effect on the causative agent or on the host. From the rural perspective, since the understanding of disease in terms of causative agents is not possible in the community, it is important that the plant formulation should address the common causative agents resulting in a given symptom e.g. diarrhoea which is caused by various infectious agents including bacteria, viruses and protozoa. The plants selected for utilization by rural communities, should be able to control the respective diseases or else at least act as a stop gap until further medical aid becomes available. Moreover, these plants should be easily available so that the users of these medications can become self reliant.

## **COLLECTION OF MEDICINAL PLANTS**

Good collection practices are necessary for the long term survival of wild populations and their habitats. WHO guidelines (WHO 2003) can be followed while collecting medicinal plant materials.

**Medicinal plant materials should be collected in the proper season** so as to ensure the best possible quality of both the starting material as well as the finished product. Seasonal variations can affect the chemical composition of the plants and thus its biological activity. This was demonstrated in one of our studies where the decoctions of *Psidium guajava* leaves collected in two different seasons showed variable antibacterial activity against six bacterial strains, the November collection being more active than the March collection (unpublished data). In most cases, maximum accumulation of chemical constituents occurs at the time of flowering which then declines at the beginning of the fruiting stage (Mendonca-Filho 2006). The time of harvest should also depend on the plant part to be used since it is well known that depending on the plant species the level of biologically active constituents can vary in different parts at different stages of the plant growth and development. For example, Kursar *et al.* (1999) found that younger leaves of tropical rainforest plants contained secondary metabolites that were either present in very little quantities or totally absent in matured leaves. The

extracts from these younger leaves showed better biological activity when tested for anticancer activity or activity against *Bacillus subtilis* and *Artemia salina* (brine shrimp). It also applies to other components in the plant material such as the toxic components. Climatic conditions, e.g. light, rainfall, and temperature (including daytime and nighttime temperature differences) also influence the physical, chemical and biological qualities of medicinal plants. The water and temperature stress related increase in the content of active constituents such as the total phenolic compounds was shown by Nacif de Abreu and Mazzafera (2005) in *Hypericum brasiliense*. Hence the best time of collection should be determined according to the levels of the biologically active constituents rather than the vegetative yield.

Information such as the correct plant parts that are used (roots, leaves, fruits, etc.) and whether these parts are seasonal or replenishable should be obtained. The collection levels and the collection practices should also be known before initiating collection. It is necessary that the collection practices employed should be non-destructive. For example, while collecting roots, the main root should not be cut or dug up or while collecting bark, the tree should not be girdled or completely stripped of its bark. Parts that are not required or are decomposed and any foreign matter such as soil or toxic weeds should be removed during collection.

Collection of medicinal plants should not be done from places that are prone to or close to sources of contamination such as areas where high levels of pesticides or other possible contaminants are used or found e.g. roadsides, drainages, mine tailings, garbage dumps and industrial facilities which may produce toxic chemicals or active pastures that may lead to microbial contamination. Quality control ensures that the plant material is not contaminated with microbes, pesticides, heavy metals or other toxic agents (Mendonça-Filho 2006) and that the final product is of consistent high standard.

Rapid and safe transportation of the collected plant materials should be arranged in advance. Handling of the plant material such as cleaning, drying and storage, should be carried out by trained personnel.

## **PROCESSING OF PLANT MATERIALS AND THEIR PREPARATION**

Preliminary processing of the plant material that can be done include elimination of undesirable materials and contaminants, washing to remove soil, sorting and cutting. It would be advisable to dry the plant materials prior to transportation if the processing facilities are located away from the collection sites. Cross contamination of the different collected plants or plant parts should be avoided during transportation. The plant materials should be protected from conditions that may cause deterioration such as rain, moistures, etc during or after transportation till the processing begins. The plant material that needs to be used fresh should be delivered as quickly as possible to the processing facility to prevent microbial fermentation or thermal degradation.

Specific processing methods are often required, to reduce drying time, to detoxify the inherent toxic constituents, to reduce side effects or to enhance therapeutic effects. For example, the methods and temperatures used for drying may have a considerable impact on the quality of the resulting medicinal plant materials.

Shade drying is the preferred method for drying plant material since it can maintain or minimize loss of color of leaves and flowers; and the lower temperatures can prevent the loss of volatile substances in the plant materials (Ibanez *et al.* 2003, Bartram 1995). However, plants can be dried in a number of other ways: in drying ovens/rooms and solar dryers, by indirect fire, baking, lyophilization, microwave, or infrared devices. Pre-selection, peeling the skins of roots and rhizomes, boiling in water, steaming, soaking, pickling, distillation, fumigation, roasting, natural fermentation, treatment with lime and chopping are some of the common processing practices. All processed medicinal plant materials should be protected from contamination and decomposition as well as from insects, rodents, birds and other pests, and from livestock and domestic animals.

Medicinal plant preparations can be prepared in several ways that usually vary based upon the plant being used, and sometimes, the condition for which it is being used. These preparations can be in the form of infusions, decoctions, tinctures, macerations, fresh juices, etc. Some other methods include hot baths, powdered plants, steam inhalation and even aromatherapy. **Adherence to the method of preparation as mentioned in the ancient texts or by traditional practitioner is necessary** depending on the form of preparation or the plant used as they may hold important information for obtaining an effective herbal preparation. A juice of a plant may be recommended instead of decoction/powder if the active ingredients are volatile or thermo labile e.g. fresh leaf juice of *Adhatoda vasica* is used for reducing blood glucose level of diabetic patients (Ahmad *et al.* 2007). Sometimes, it is possible that due to the difficulty in preparation of the extracts and the time required, whole fresh material (e.g. leaves) or dried powder is used instead of the required extract for treatment. This may lead to potential toxicity which would otherwise not be observed due to the elimination of the toxic constituent during extraction. In this context, an example that can be cited from our study is the extraction of negligible amounts of the toxic component karanjin from the leaves of *Pongamia pinnata* in the aqueous decoction (Brijesh *et al.* 2006).

The medicinal property of plants is closely related to the different classes of phytoconstituents (such as essential oils, alkaloids, acids, steroids, tannins, saponins, etc.) present in the plant, each of which would have a preferred effective method of extraction, facilitating maximum yield in the preparation. For example, preparing a decoction might extract a group of anti-inflammatory plant steroids to treat arthritis and yet when the same plant is prepared in alcohol different antibacterial alkaloids are extracted instead. (<http://www.rain-tree.com/prepmethod.htm>)

## **STORAGE**

Storage can also influence the physical appearance and chemical quality of plant materials and hence it is necessary to maintain appropriate storage conditions so as to increase their shelf life. It is customary to store the plant material in dried form since preparations like decoctions/infusions can only be stored for a few days. Dried plant materials can be stored in whole, crushed or powdered forms in storage conditions that include use of cloth bags, clear glass bottles and plastic. Plant materials that are used fresh should be stored under refrigeration, in jars or sandboxes, or using enzymatic or other appropriate conservation methods.

However, they should be used as quickly as possible to avoid microbial contamination. Shelf life of plant material is usually ignored due to the general belief that the plant materials do not have an expiry date, however, dried plant materials usually retain their activity for about six months only. It is observed that the powdered plant material degrades faster than the whole or crushed plant material (unpublished data). Different types of plastics can be used which prevent absorption of moisture and oxidation of the plant material by preventing the exchange of gasses to increase the shelf life of the plant material.

## **BIOLOGICAL STUDIES**

Biological screening is necessary to provide a scientific basis for validating the traditional utilization of medicinal plants. A great number of screening programs are ongoing worldwide for new plant based bioactive molecules. Several researchers have worked on medicinal plants with activity against different ailments. Preclinical pharmacological studies and randomized clinical trials form an important part of the biological screening of medicinal plants. Preclinical studies usually serve to verify the data on mechanisms of action reported in animals or humans. However, a pharmacological effect observed *in vitro* or in animal models, for both safety and efficacy needs to be reconfirmed by clinical studies and the information obtained from the preclinical studies can form the basis for further clinical trials. (Lipsky and Sharp 2001, Bleicher *et al.* 2003, Dove 2003, Kenakin 2003, Knowles and Gromo 2003, Verkman 2004).

## **CLINICAL STUDIES**

Clinical studies are necessary to confirm the pharmacological effects of medicinal plants before they can be integrated into conventional medical practice. Well-established, randomized controlled clinical studies facilitate the acceptance of herbal medicines in different regions and in people with different cultural traditions. This would be especially true in case of some unrelated effects of therapy contributing to efficacy that may be difficult to measure pre-clinically. These studies should be carried out on the basis of information obtained from official national compendia and relevant literature or traditional medical practitioners. The general principles for the clinical studies that apply to conventional drugs should be followed when testing a new herbal preparation, a new indication for an existing formulation, or a significantly different dosage form or route of administration (WHO 2000). Well recorded case reports can contribute towards useful information at such times and put forward new hypothesis and stimulate further study (Morris 1989). However, double blind clinical trials may not be required when an extensive and detailed database of case studies is available. Such a database is especially important when a particular treatment is individualized.

The methods and guidelines used for clinical validation of modern medicines must be applied to herbal products even though the latter has a holistic approach to treatment. However, conventional concepts of clinical research design may be difficult to apply when using clinical research to evaluate various systems and practices of traditional medicine (WHO 2000). This could be due to the fact that herbal remedies are individualized (each person has certain predispositions to disease and susceptible to factors like environment, genetics, dietary and lifestyle)

therapies and hence depend on the proficiency (including the skills and experience) of medical practitioners.

Clinical studies, in some cases, must be adapted to deal with the specifics of herbal medicines. Single-case studies, as per the theories and concepts of traditional medicine, for the evaluation of efficacy and randomization can allow for the individualization of treatments. Methods such as randomization and use of placebo may not always be possible. Patients previously treated with plant preparation having a characteristic organoleptic property cannot be randomized into control groups or a placebo may not be possible when the plant preparation has a strong smell or taste as is the case of certain essential oils.

The number of patients required for undertaking clinical trial of medicinal plants is large not only since the study design needs to be adequate and statistically appropriate but also to cater to the control, confounders and placebo groups to provide sufficient evidence for judging efficacy of the plant under study. The increase in patient number also increases the time commitment and the expenses involved.

Therefore only a limited number of plants can be subjected to clinical trials. Hence, it is essential to undertake appropriate preclinical testing to short list plants for clinical evaluation.

## **PRECLINICAL STUDIES**

Preclinical testing helps in collection of important efficacy and safety data before clinical trials can be carried out. The preclinical evaluation and authentication of medicinal plants involves documentation and testing of their pharmacological efficacy *in vitro* (cells) and *in vivo* systems and studies of toxicology, specificity, biopharmaceutical properties and drug interactions.

The preclinical studies help in determining the therapeutic effect of the plant in question and also elucidate the efficacy and/or the mechanism of action including cell interactions, cell-environment interactions, intracellular activity, and genetic studies. Plants with novel and/or multiple mechanism(s) of action can also be identified. The advantage of these studies is that one can easily study and compare the efficacy of different plants in a cost effective manner and design rational drug combinations. This requires proper designing of screening assays that have significant impacts on the outcome of the overall drug discovery process. The selected assay should be able to mimic the *in vivo* dynamics as far as possible with high sensitivity and specificity.

The basis for designing a screening assay is the identification of valid target. An estimated 30-40% of experimental drugs fail due to an inappropriate target (Butcher 2003) and hence it is important to develop new screening assays with newer and more appropriate targets. It is crucial to establish the role of the target in question in the cause or symptoms of a disease (Williams 2003). Pharmacological manipulation of the target should consistently lead to desired phenotypic changes. The desired changes must also be reproducible in at least one relevant animal model (Drews 2003). Emphasis has to be placed on assessment of

assay quality and validation of the parameters being used.

Assay formats employed in screening can be either cell-based or biochemical. Though the logistics of cell-based assays are more challenging than with biochemical assays due to requirement of significant investments in cell culture infrastructure (Moore and Rees 2001), the current trend in drug discovery is clearly shifting towards cell-based assays. Cell-based screening has multiple advantages. It can provide biologically more relevant information on the nature of the activity (Moore and Rees 2001, Johnston and Johnston 2002). In addition, information regarding cellular membrane permeability and cytotoxicity can also be obtained.

Approaches that are commonly used for studying the pharmacological effects of medicinal plants are: use of single bioassay for screening multiple plants and use of multiple bioassays for studying single plant. The latter approach has been used widely for metabolic diseases. Unfortunately, when screening plants for infectious diseases the assay system is often limited to testing for antimicrobial activity. However, this approach is not always appropriate. Plants can exhibit their efficacy against infectious diseases by mechanisms other than antimicrobial activity. When screening plants for immuno-enhancing properties, often synthetic antigens and immunological assays are used which do not have any biological relevance to disease(s) in question.

The importance of using relevant and where necessary multiple bioassays for screening medicinal plants for infectious diseases is highlighted in our studies. Decoctions of two plants viz. *Cyperus rotundus* (unpublished data) and *P. pinnata* (Brijesh *et al.* 2006) were screened for their antidiarrhoeal activity. The different bioassays used were: antibacterial, anti-giardial and anti-rotaviral assay; adherence to and invasion of bacterial pathogens to epithelial cells; ganglioside monosialic acid-enzyme linked immunosorbent assay for *E. coli* heat labile toxin (LT) and cholera toxin (CT); and suckling mouse assay for *E. coli* heat stable toxin (ST). These assays in addition to the antimicrobial action screened the plants for colonization (adherence and invasion) and enterotoxins – the two most important features of diarrhoeal pathogenicity and thus define the possible mechanism(s) of action of *C. rotundus* and *P. pinnata* in infectious diarrhoea. It was observed that though both plants did not have marked antimicrobial action, they were effective antidiarrhoeal agents with different mechanism(s) of action. CT and LT were affected though there was no effect on ST. *P. pinnata* inhibited bacterial adherence to epithelial cells whereas *C. rotundus* inhibited both bacterial adherence to and invasion of epithelial cells. These results showed that the antidiarrhoeal activity of the plant could be due to its action on various parameters other than just the antimicrobial activity. Different plants can show activity in different assays determining their usefulness in different forms of diarrhoea. The study highlighted the necessity of looking at different parameters and not just concentrating on singular assays like antimicrobial activity for determining the biological efficacy of plants.

## Limitations of preclinical studies

1. Suitable pharmacological models have not yet been developed for many common diseases with unknown, or multi-factorial origins (Hamburger and Hostettmann 1991).
2. Some compounds which show good activity *in vitro* may be metabolized *in vivo* into inactive metabolites. Alternatively, extracts may only show *in vivo* activity due to the metabolism of inactive compounds into active forms (Farnsworth 1993).
3. The pharmacological investigation of drug interactions in multi-compound preparations is difficult due to the presence of constituents from several plants where some plants may show less specific activity and some plants may have been added to reduce the toxicity of the more therapeutically effective plants (Taylor *et al.* 2001).
4. Some of the most common side effects are difficult to recognize in animal models e.g. nausea, nervousness, lethargy, heartburn, headache, depression, stiffness, etc.
5. Extrapolation of *in vitro* dose to *in vivo* animal models and humans is difficult.

## Toxicity Studies

Toxicological evaluation of medicinal plants has often been neglected since prolonged and apparently uneventful use usually is considered as a testimony of its safety. However, a history of traditional usage is not always a reliable guarantee of safety since it is difficult for traditional practitioners to detect or monitor delayed effects (e.g. mutagenicity), rare adverse effects, and adverse effects arising from long-term use (Ernst 1998) such as for food supplements and nutraceuticals e.g. *Glycyrrhiza glabra*, which is used for conditions like bronchitis and peptic ulcers causes not only hypertension, weight gain and hypokalaemia but also low levels of aldosterone and anti-diuretic hormone on excessive or prolonged usage (Newall *et al.* 1996). The use of herbal preparations may also lead to hypersensitivity reactions ranging from transient dermatitis to anaphylactic shock (Ernst 1998). Many widely used medicinal plants have been implicated as possible causes of long-term disease manifestations such as liver and kidney diseases. The widespread use of *Scenecio*, *Crotalaria* and *Cynoglossum* has been implicated in the occurrence of liver lesions and tumours, lung and kidney diseases in certain areas of Ethiopia (Addae-Mensah, 1992).

The absence of any such documentation, however, does not automatically rule out the possibility of toxicity. It is possible that the plant treatment taken up for the clinical trials may lead to some unanticipated/unknown/unrelated side effects e.g. *Psoralea corylifolia* Linn. which is used for treating conditions like psoriasis, leucoderma, and non-healing ulcers and wounds is known to cause hepatosplenomegaly in experimental animals (CHEMEXCIL 1992). Hence it becomes necessary to carry out toxicological studies, both short term and long term.

Toxicological studies should include tests such as acute, subchronic and special toxicology that are impossible to detect clinically such as immunotoxicity, genotoxicity, carcinogenicity and reproductive toxicity (Remirez 2006). These tests help in the identification of possible target organs involved and the toxic

symptoms. Studies of special toxicology such as carcinogenesis are very important if the plants contain compounds with known mutagenic or carcinogenic activities (Chanabra *et al.* 2003).

## **PHYTOCHEMICAL STUDIES**

Phytochemical studies of the plant preparations are necessary for standardization, which helps in understanding the significance of phytoconstituents in terms of their observed activities. Phytochemistry also helps in standardizing the herbal preparations so as to get the optimal concentrations of known active constituents, and in preserving their activities.

Standardization can be carried out by obtaining a chemical fingerprint/profile or through bioactivity guided fractionation. Chemical fingerprints through chromatographic techniques are more commonly used for standardization and are obtained in terms of one or more marker compounds. It would be ideal to use the active constituent in the plant as the marker compound; however, in cases where active constituents are not known the marker compound can be independent of the therapeutic activity. Furthermore, the plant extracts can also be standardized to class of compounds e.g. ginsenosides in ginseng, kava lactones in kava, or oxindole alkaloids in cat's claw (Roman 2001). Such an approach would be suited to situations where though the active constituents are not known they are expected to belong to a particular class of compounds.

According to European Medicines Agency guidelines (EMA 2005), quantification of substances with known therapeutic activity or markers is obligatory. As per the European Pharmacopoeia, marker compounds should be characteristic or unique for the herbal material or herbal preparation, have an established chemical structure, be present in the starting material as well as the finished product in sufficient amounts, be accessible to quantification with common analytical methods such as high-performance liquid chromatography (HPLC) or high-performance thin layer chromatography (HPTLC), be sufficiently stable, and be commercially available or able to be isolated by the company in its own laboratory.

Thin layer chromatography (TLC) and HPLC are the most commonly used methods for obtaining chemical fingerprints and identification of the crude plant extracts. However, there are several possibilities that may arise while using these techniques for standardizing the crude extracts. It is possible that the plant material collected from the same plant in two different seasons can show different phytochemical fingerprint and therefore different biological activity or two plants with identical taxonomy collected under same environmental conditions can show different phytochemical fingerprint but similar biological activity. In such situations comparisons of the phytochemical profiles as an indicator of important constituents can act as a shortcut for identifying biologically active constituents.

DNA fingerprinting is another technique, which though still in its early years, seems to be of immense potential in identification of medicinal plants, particularly when profiling the genotypic differences (Vasudevan 2004). Apart from identifying these genetic variations, it can also aid in identification of germplasms of important or endangered plants for future cultivation or conservation.

Use of isolated compounds can result in better biological activity due to higher concentrations, but it can also lead to potential side effects e.g. the active constituent conessine isolated from *Holarrhena antidysenterica*, a plant commonly used by Ayurvedic practitioners in the treatment of diarrhoea, was found to be toxic to the central nervous system (CHEMEXCIL 1992). More recent studies have also indicated at reduced biological activity with isolated active constituents compared to crude extracts (Kicklighter *et al.* 2003).

The efficacy of crude extracts may be due to the synergism between the different active constituents that may be present in the extract. **Synergism can lead to better activity as well as decrease in potential toxicity of some individual constituents.** Synergism can be due to the individual action of different constituents present in the extract at multiple target sites/parameters. This was observed in a study conducted by us on the antidiarrhoeal activity of *P. guajava* (unpublished data). It was seen that the decoction of the dried leaves of *P. guajava* showed antidiarrhoeal activity by showing antimicrobial activity against five out of the six bacterial strains tested, *Giardia lamblia* and rotavirus. It inhibited adherence to and invasion of the bacterial pathogens to the epithelial cells. It also inhibited production and action of enterotoxins such as *E. coli* labile toxin and cholera toxin. These results suggested that the different constituents present in the decoction could be individually responsible for the different activities observed against these parameters. Another mechanism by which these constituents can show synergism is by having an additive effect against a single target site/parameter. It was observed that the decoction of *P. guajava* leaves was synergistically more active at a dilution of 1% than at 5% against the bacterial adherence to epithelial cells. This effect could be due to the fact that the ratio of constituents achieved at 1% was more optimal for activity than at 5%.

The modern analytical and isolation methods that are used for screening and isolation of plant constituents are the chromatographic and spectroscopic techniques such as TLC, thin layer electrophoresis, HPLC, nuclear magnetic resonance, HPTLC etc. These techniques have proved very useful in isolation and proper identification of the active constituents in the plant extracts.

It is necessary to devise simple techniques for standardization that can be used by the rural community for identifying plants with good biological activity. Use of a class of compounds, as mentioned earlier, as a surrogate marker is a potential approach which can be used by the communities to identify plants with good biological activity. For example, instead of a single polyphenol, tannins can be used as a surrogate marker, which schoolchildren can estimate easily in their laboratories. This approach has been attempted by us in collaboration with the Foundation for Research in Community Health in their field project at Parinche, Maharashtra. Tannin levels were estimated in aqueous decoctions of five different collections of *P. guajava* leaves and compared them with their respective activities against action of cholera toxin. It was observed that the decoctions with ~10.5mg/ml tannin showed good activity with no further significant increase in activity at higher tannin concentrations. However, below this level the activity was significantly poorer (unpublished data). Hence, 10.5mg/ml tannin level may be taken as a cut-off value for differentiating a *P. guajava* plant with good activity from that with poor activity.

## **CONCLUSIONS**

With the tremendous increase in the global use of medicinal plants, several concerns regarding the efficacy and safety of the herbal medicines have also been raised. Hence it has become necessary to standardize the efficacy and safety measures so as to ensure supply of medicinal plant materials with good quality.

After proper botanical identification, WHO guidelines should be followed for collecting plant material in terms of proper season and climatic conditions, correct plant part, practices that are non-destructive and would prevent contamination from soil, toxic weeds or microbes. Post collection, appropriate processing and storage conditions are required to reduce drying time, detoxification to reduce side effects and to enhance therapeutic value of the plant material and to improve its shelf life.

Preclinical biological screening is important not only for establishing the therapeutic efficacy of the medicinal plants but also to validate their historical utilization by traditional healers and herbalists. This is especially important since the plants may have evolved over a period of time leading to changes in their chemical composition and thus the biological activity. Preclinical studies allow comparison of efficacy of different plants and help in designing of rational drug combinations. Toxicity studies need to be done even if the plants have a history of long usage or do not have any documented toxicity, as they can lead to some unrelated toxicity especially during long term treatment for chronic conditions or when used as food supplements and nutraceuticals.

Phytochemical standardization for identification of the plant material can be carried out by obtaining chemical fingerprint through chromatographic techniques in terms of a known marker compound or through bioassay guided fractionation and/or DNA fingerprinting techniques. Chromatographic and spectroscopic techniques have proved very useful in isolation and proper identification of active constituents in the plant extracts.

Though the pharmaceutical industry has been focusing on standardization of plant materials when manufacturing herbal drugs, it is generally believed that standardization is not required when used by rural community for their primary health care. However, irrespective of whether the plant is being used by the industry or by the rural community standardization of plant material is required right from collection of the plant species to the formulation of the herbal drug. It is necessary so that it minimizes batch-to-batch variation and meets standards of quality, safety, and efficacy.

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## REFERENCES

1. Addae-Mensah I. 1992. Towards a Rational Scientific Basis for Herbal Medicine – A phytochemist's two-decade contribution. Accra: Ghana Universities Press.
2. Ahmad M, Khan MA, Zafar M, Sultana S. 2007. Treatment of common ailments by plant-based remedies among the people of district Attock (Punjab) of northern Pakistan. *Afr J Trad CAM*. 4 (1): 112-120.
3. Artuso A. 1997. Drugs of Natural Origin: Economic and policy aspects of discovery, development, and marketing. Pharmaceutical Products Press, New York.
4. Ayensu ES, DeFilippis RA. 1978. Endangered and Threatened Plants of the United States. Smithsonian Institution, Washington DC.
5. Bartram T. 1995. Encyclopaedia of Herbal Medicine. Grace: Dorset.
6. Basso LA, Pereira da Silva LH, Fett-Neto AG, Filgueira de Azevedo Junior W, de Souza Moreira I, Palma MS, Calixto JB, Filho SA, Ribeiro dos Santos R, Soares MBP, Santos DS. 2005. The use of biodiversity as source of new chemical entities against defined molecular targets for treatment of malaria, tuberculosis, and T-cell mediated diseases – A Review. *Mem Inst Oswaldo Cruz, Rio de Janeiro*. 100(6): 575-606.
7. Berry JP, McFerren MA, Rodriguez E. 1995. Zoopharmacognosy: A "biorational" strategy for phytochemical prospecting. *In: Gustine DL, Flores H. (eds.), Phytochemicals and Health, ASPP, Rockville, Pp. 165-178.*
8. Bleicher KH, Bohm HJ, Muller K, Alanine AI. 2003. Hit and lead generation: Beyond high throughput screening. *Nat Rev Drug Discov*. 2: 369-378.
9. Brijesh S, Daswani PG, Tetali P, Rojatkhar SR, Antia NH, Birdi TJ. 2006. Studies on *Pongamia pinnata* (L.) Pierre leaves: Understanding the mechanism(s) of action in infectious diarrhea. *J Zhejiang Univ SCIENCE B*. 7: 665-674.
10. Butcher SP. 2003. Target discovery and validation in the postgenomic era. *Neurochem Res*. 28: 367-371.
11. Chanabra RS, Bucher M, Wolfe abd Portier C. 2003. Toxicity characterization of environmental chemicals by US National Toxicology Program: an Overview. *Int J Hyg Environ Health*. 206: 437-445.
12. CHEMEXCIL. 1992. Selected Medicinal Plants of India, Bhartiya Vidya Bhavan's Swami Prakashanand Ayurveda Research Centre, Bombay.
13. Cragg GM, Boyd MR, Khanna R, Kneller R, Mays TD, Mazan KD, Newman DJ, Sausville EA. 1999. International collaboration in drug discovery and development: the NCI experience. *Pure Appl Chem*. 71(9): 1619-1633.
14. Dove A. 2003. Screening for content – The evolution of high throughput. *Nat Biotechnol*. 21: 859-864.
15. Drews J. 2003. Strategic trends in the drug industry. *Drug Discov Today*. 8: 411-420.
16. Ellenberger A. 1998. Assuming responsibility for a protected plant: Weleda's endeavour to secure the firm's supply of *Arnica montana*. *In: First International Symposium on the Conservation of Medicinal Plants in Trade in Europe, TRAFFIC Europe, Kew, UK. Pp. 127-130.*
17. EMEA. 2005. Guideline on specifications: Test procedures and acceptance criteria for herbal substances, herbal preparations and herbal medicinal

- products/traditional herbal medicinal products. European Medicines Agency (EMA) Committee for Medicinal Products for Human Use (CHMP) Committee for Medicinal Products for Veterinary Use (CVMP), London, UK
18. Ernst E. 1998. Harmless herbs? A review of the recent literature. *Am J Med.* 104: 170-178.
  19. Fabricant D, Farnsworth NR. 2001. The value of plants used in traditional medicine for drug discovery. *Environmental Health Perspectives.* 109: 63-75.
  20. Farnsworth NR. 1993. Biological approaches to the screening and evaluation of natural products. *In: Rasoanaivo P, Ratsimamanga-Urverg S. (eds.) Biological Evaluation of Plants with Reference to the Malagasy Flora, Monograph from the IFS-NAPRECA Workshop on Bioassays. Madagascar, Pp 35– 43*
  21. Farnsworth NR. 1996. Biological and phytochemical screening of plants. *J Pharm Sci.* 55: 225-276.
  22. Farnsworth NR, Bingel AS. 1977. Problems and prospects of discovering new drugs from higher plants by pharmacological screening. *In: Wagner H, Wolff P. (eds.), New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutical Activity. Springer, Berlin, Germany. Pp: 1-22.*
  23. Hamburger M, Hostettmann K. 1991. Bioactivity in plants: The link between phytochemistry and medicine. *Phytochem.* 30: 3864-3874.
  24. Ibanez E, Kubatova A, Senorans FJ, Cavero S, Reglero G, Hawthorne SB. 2003. Subcritical water extraction of antioxidant compounds from rosemary plants. *J Agric Food Chem.* 51: 375-382.
  25. I.U.C.N. 1994. Guidelines on the conservation of medicinal plants. International Union for Conservation of Nature (IUCN), Gland. Pp: 50.
  26. Johnston PA, Johnston PA. 2002. Cellular platforms for HTS: Three case studies. *Drug Discov Today.* 7: 353-363.
  27. Kenakin T. 2003. Predicting therapeutic value in the lead optimization phase of drug discovery. *Nat Rev Drug Discov.* 2: 429-438.
  28. Kinghorn AD. 1994. The discovery of drugs from higher plants. *Biotechnology.* 26:81-108.
  29. Kicklighter CE, Kubanek J, Barsby T, Hay ME. 2003. Palatability and defense of some tropical infaunal worms: alkylypyrrole sulfamates as deterrents to fish feeding. *Mar Ecol Prog Ser.* 263: 299-306.
  30. Knowles J, Gromo G. 2003. Target selection in drug discovery. *Nat Rev Drug Discov.* 2: 63-69.
  31. Kufer J, Forther H, Poll E, Heinrich M. 2005. Historical and modern medicinal plant uses – The example of the Chorti Maya and Ladinos in Eastern Guatemala. *J Pharmacy Pharmacol.* 57:1127-1152.
  32. Kursar TA, Capson TL, Coley PD, Corley DG, Gupta MB, Harrison LA, Ortega-Barría E, Windsor DM. 1999. Ecologically guided bioprospecting in Panama. *Pharmaceutical Biol.* 37: S114-S126.
  33. Lange D. 1998. Europe's medicinal and aromatic plants: their use, trade and conservation. Traffic Europe/International, Cambridge, UK.
  34. Lewis WH, Elvin-Lewis MP. 1995. Medicinal plants as sources of new therapeutics. *Ann Mo Bot Gard.* 82: 16-24.
  35. Lipsky MS, Sharp LK. 2001. From idea to market: The drug approval process. *J Am Board Fam Pract.* 14: 362-367.
  36. Mendonca-Filho RR. 2006. Bioactive Phytocompounds: New Approaches in the Phytosciences. *In: Ahmad I, Aqil F, Owais M. (eds.), Modern Phytomedicine: Turning Medicinal Plants into Drugs. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.*

37. Moore K, Rees S. 2001. Cell-based versus isolated target screening: How lucky do you feel? *J Biomol Screen.* 6: 69-74.
38. Morris BA. 1989. Importance of case reports. *Can Med Assoc J.* 141: 875-876.
39. Nacif de Abreu I, Mazzafera P. 2005. Effect of water and temperature stress on the content of active constituents of *Hypericum brasiliense* Choisy. *Plant Physiol Biochem.* 43: 241-248.
40. Newall CA, Anderson LA, Phillipsen, DJ. 1996. *Herbal Medicines: A Guide for Health Care Professionals*, The Pharmaceutical Press, London, Pp. 296.
41. Prasad R, Patnaik S. 1998. Conservation Assessment and Management Planning (CAMP) workshop for Non Timber Forest products in MP. CAMP workshop briefing book. IIFM, Bhopal (MP), India.
42. Phillipson JD, Anderson LA. 1989. Ethnopharmacology and Western medicine. *J Ethnopharmacol.* 25: 61-72.
43. Ramirez DC. 2006. Update in Pre-Clinical Regulatory Requirements for Phytomedicines in Latin America. *J Compl Int Med.* 3(1): Article 3.
44. Robbins CS. 1998. American ginseng. The root of North America's medicinal herb trade. TRAFFIC North America, Washington DC, USA.
45. Roman M. 2001. The benefits and pitfalls of standardizing botanicals extracts. *Natural Products Insider.*  
([http://www.naturalproductsinsider.com/articles/473/473\\_141labin2.html](http://www.naturalproductsinsider.com/articles/473/473_141labin2.html))
46. Satyavati GV, Gupta AK, Tandon N. 1987. *Medicinal Plants of India*, Vol. 2. Indian Council of Medical Research, New Delhi, India.
47. Sharma GP, Singh JS, Raghubanshi AS. 2005. Plant invasions: Emerging trends and future implications. *Current Science.* 88(5): 726-734
48. Taylor JLS, Rabe T, McGaw LJ, Jäger AK, van Staden J. 2001. Towards the scientific validation of traditional medicinal plants. *Plant Growth Regulation.* 34: 23-37.
49. Vaidya ADB. 2006. Reverse pharmacological correlates of ayurvedic drug actions. *Indian J Pharmacol.* 38: 311-315.
50. Vasudevan H. 2004. DNA fingerprinting in the standardization of herbs and nutraceuticals. *The Science Creative Quarterly.*  
(<http://www.scq.ubc.ca/?p=286>).
51. Vlietinck AJ, Vanden Berghe DA. 1991. Can ethnopharmacology contribute to the development of antiviral drugs? *J Ethnopharmacol.* 32: 141-153.
52. Verkman AS. 2004. Drug discovery in academia. *Am J Physiol Cell Physiol.* 286: C465-C474.
53. Verpoorte R. 2000. Pharmacognosy in the new millennium: lead finding and biotechnology. *J Pharm Pharmacol.* 52: 253-262.
54. Williams M. 2003. Target validation. *Curr Opin Pharmacol.* 3: 571-577.
55. Wink M. 1999. Introduction: Biochemistry, role and biotechnology of secondary products. *In: Wink M. (ed.), Biochemistry of Secondary Product Metabolism.* CRC Press, Boca Raton, FL, USA. Pp 1-16
56. WHO. 2000. General guidelines for methodologies on research and evaluation of traditional medicine. World Health Organization, Geneva.
57. WHO. 2002. WHO Traditional Medicine Strategy 2002-2005. World Health Organization, Geneva.
58. WHO. 2003. WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants. World Health Organization, Geneva.