ROLE OF BIOASSAYS IN DEVELOPMENT OF NATURAL PRODUCTS

INTRODUCTION

The plant kingdom still represents an enormous reservoir of new molecules to be discovered. Among the approximately 250,000 known plant species, only a small percentage has been investigated phytochemically and an even smaller percentage has been submitted to pharmacological screenings. The process that leads from the plant to a biologically or pharmacologically active pure constituent is very long and tedious one, and requires a multi-disciplinary approach. The discovery of promising extracts and the subsequent activity-guided isolation of constituents put specific requirements on the bioassays to be used for the purpose.

They have to be simple, rapid, reproducible and inexpensive in order to be compatible with the large number of assays to be performed. Such assays are few in number. Another important feature for bioactivity-guided fractionation is that the assay should be possible using small quantities of sample (microgram to milligram quantities at best), as it is very difficult and expensive to generate fraction and sub-fractions in higher quantities. This is also the reason for most of the bioassays to be *in-vitro.*

Typically for *in-vivo* experiments one needs 1.5 g to 2 g of sample, which is possible only up to herb or it's extract level. Bioassays have been used to develop high throughput and medium throughput screens using automation and robotized equipment. Drug companies in need to screen several hundred to several thousand samples routinely, have to necessarily rely on bioassays. Use of bioassays for bioactivity guided fractionation and subsequent attempts using isolated moieties as potential drug leads is widely known and well published.

1. CLASSIFICATION OF BIOASSAYS

Bioassays can be classified broadly as General Screening bioassays, Specialized screening and Primary screening bioassays.

General Screening bioassays

These bioassays are non-selective, they simply indicate whether the given sample is biologically active or not. These methods are useful if one is randomly screening many plants for general bioactivity. The plants showing activity in these screens can be later subjected to specialized assays to know specific activity. Active compounds isolated from such assays are referred as biomarkers [Labadie *et* a/, 1990]. Some of the important general bioassays are:

Hippocratic screening

This assay uses intact animals and involves effect of plant extracts or their derivatives on gross behaviour of test animals [Samuelsson *et* a/, 1991]. It is a relatively expensive and time consuming method. Nowadays, it is not considered suitable for bioactivity directed fractionation since it is costly, needs larger amount of sample and is labour intensive.

Isolated organ method using guinea pig ileum (GPI assay)

In this assay effect of the samples on contraction or inhibition of contraction of guinea pig ileum is observed. Two tests are performed for each extract. First, the extracts ability to contract the ileum is tested. In a second test, the effect of the extract on electrically induced contraction of the ileum is tested. A positive response in these tests means presence of active substances in the extracts which are capable of interacting with various receptors on the ileum, with the use of suitable agonists and antagonists, one could characterize the effects of the extracts as adrenergic, cholinergic etc. This method is fairly simple, not expensive and suitable for activity-guided fractionation [Vlietinck *et* a/, 1999].

Brine Shrimp Lethality Test

This bioassay involves testing lethality to brine shrimp nauplii. This assay has been recommended for screening plants for general bioactivity. However, it is widely used for detecting cytotoxicity. BSLT is based on the premise that 'pharmacology is simply toxicology at a higher dose' (or conversely toxicology is pharmacology at a lower dose), thus if one finds toxic compounds, at lower doses these

compounds may elicit useful biological effects [Mclaughlin *et al* 1991]. At present there is not enough data to correlate BSLT with different pharmacological effects. In our laboratory we have shown that nootropic principle, Bacoside A from Bacopa *monnieri* is active in BSLT [D'souza *et al, 2002].* Similarly cerberin, the cardioactive principle from *Cerbera odollam* is also active in BSLT. Cerberin was independently isolated as the active principle when an activity directed fractionation of C. *odollam* was carried out using BSLT and an assay for cardiovascular activity [Venkatarao et *al,* 1973]. Undoubtedly, BSLT could be used as a preliminary screen to detect general bioactivity; the active fractions could later be subjected for specific assays.

Specific Bioassays

These are specialized bioassays and yield information on specific bioactivity, for instance, anti-inflammatory, anti-oxidant activity etc. These assays could be used either as a secondary assay for fractions showing activity in a general bioassay. They could also be used exclusively when one is looking for specific activity. The following table shows examples of some selected specific assays. Specific assays using lower organisms make use of various microbial targets like bacteria, fungi, protozoa, virus, molluscs, helminths etc. Normally, in these assays plant products are tested to know lethality or inhibitory effects on these organisms. Compounds showing activity in these assays can be of chemotherapeutic value. For instance, Artemisin isolated from *Artemisia annua* is useful in malaria. Specific bioassays, which make use of sub-cellular systems, utilize enzymes or receptors as their targets. For instance, inhibition of K-glucosidase and K-amylase can be helpful in obesity and diabetes. Inhibitors of angiotensin converting enzyme and monoamine oxidase are useful in hypertension and depression respectively. These assays are also called molecular assays and can be automated for high throughput screening. Cellular assays utilize various intact cells of human or animal origin to detect various bioactivities like hepatoprotective activity, cytotoxicity, antimutagenecity, immunomodulation, neuroprotection , antioxidant etc.

Table Showing a List of some selected Specific Bioassays

S. No.	Bioassay	Pharmacological Significance	Reference
1	Monoamine oxidase inhibition assay	Anti-depressant effect	Century *etal,* 1968; Tanakaefa/, 1997
2	Trypsin inhibition assay	Anti-viral, Anti-Cancer, anti-inflammatory etc.	Cannell *etal,* 1998
3	Alpha-amylase inhibition assay	Obesity and diabetes	*Berre-Anton et al,* 1997.
4	Caenorhabditis elegans lethality assay	Anthelmintic	Deepak *et al,* 2002
5	Mast cell stabilization assay	Anti-allergy	Rajsekaran *et al,* 1989
6	DPPH scavenging assay	Anti-oxidant	Letitia *et al,* 2002
7	Anti-microbial assays	Infectious conditions	Paxton, 1991 Vanden et a/, 1991
8	DPPH - TLC assay	Anti-oxidant	Marston eta/, 1999
9	K-glucosidase inhibition assay	Anti-diabetic	Prashanth *et al,* 2001
10	Lipid peroxidation assay	Anti-oxidant	Olatund et a/, 2002
11	Phagocytic assay	Immunity, allergy	Wagner et *al,* 1991

12	Pisicidal assay	Pisicidal effect	Marston *et al,* 1991
13	Membrane stabilization assay	Anti-inflammatory	Concepcion et *al,* 1993
14	Angiogenesis assay	Wound healing,toxicity testing	Shukla et a/, 1999
15	Molluscicidal assay	Molluscicidal activity	Marston *et al,* 1991
16	Hepatoprotective assay using primary culture of rat hepatocytes	Hepatoprotective activity	Kiso et a/, 1991
17	Hyaluronidase inhibition assay	Anti-allergy	Kakegawa *et al,* 1985
18	Urease inhibition assay	Uroiithiasis	Hayashi et a/, 1987
19	Tyrosinase inhibition assay	Skin disorders	Isao Kubo *et* a/, 1999
20	Acetycholinesterase inhibition assay	Alzheimer's disease, Nootropic	Vogel et a/, 1997
21	Rat ileal loop assay	Anti-secretory	Shashi Gupta *et* a/, 1993
22	Xanthine oxidase inhibition assay	Uricosuric	Vogel et a/, 1997
23	Effects on isolated GPI	Anti-histamine, anti-cholinergic	Samuelsson *et* a/, 1991
24	5-K-reductase inhibition assay	Anti-androgenic activity	Vogel et a/, 1997
25	Superoxide anion scavenging assay	Anti-oxidant	Yagi *et* a/, 2002
26	Anti-complement assays	Cytotoxicity, anti allergy, anti-inflammatory	Kroes et a/, 1992
27	Ca^{2+} antagonistic activity using rat aorta and Taenia coli .	Ca^{2+} channel antagonistic	Morales era/, 1994
28	Histamine release assay (Peritoneal mast cell)	Anti-allergic	Vogel et a/, 1997
29	Effect on isolated rat uterus	Uterotonic	Ghosh *et al,* 1984; Perry *et al,* 1970
30	*In-vitro* metabolism studies using rat hepatocytes (cyt p 450 estimation)	Drug metabolism	Carlileefa/, 1999
31	Effect on isolated Frog rectus abdominus	Anti-cholinergic activity	Ghosh *et* a/, 1984; Perry *et al,* 1970
32	Effect on isolated Frog heart	Adrenergic & cholinergic activity	Ghosh et a/, 1984; *Perry et al,* 1970
33	Oestrogenic activity using recombinant yeast	Oestrogenic activity	Sampson *et al,* 1998,
34	Proton pump inhibition assay	Anti-ulcer (H^+, K^+, ATPase)	Vogel et a/,"1997
35	Effect on guinea pig tracheal chain	Anti-asthmatic, leucotriene inhibition	Vogel et a/, 1997
36	Aldose reductase inhibition assay	Anti-diabetic	Vogel et a/, 1997

37	Cyclooxygenase inhibition assay	Anti-inflammatory	Wagner et a/, 1991
38	Effect on Rat fundus	Anti 5-HT	Perry et a/, 1970
39	Lipoxygenase inhibition assay	Anti-inflammatory, anti-allergy	Wagner et a/, 1991
40	Nitric oxide inhibition assay	Anti-inflammatory and several others	Takako et *al,* 2000
41	Everted sac technique	Absorption of drugs	Vogel et a/1997
42	Liver perfusion assay	Metabolism studies	Egen-Schwind *et al,* 1992
43	Linoleate peroxidation	Antioxidant	Valenzuela *et* a/, 1987
44	Primary cell culture of rat kidney	Nephroprotective	Kumar *et al,* 2001
45	Intestinal perfusion assay	Absorption studies, Anti-diabetic	Kimura et a/, 1984
46	Insulin release assay *(In vitro)*	Anti-diabetic	Verspohl, 2002
47	Lipolysis assay	Anti-diabetic & anti-obesity	Verspohl, 2002
48	Receptor binding assays	Variety of pharmacological effects	Sweetnam *et al,* 1993

Lastly specialized bioassays make use of isolated organs of vertebrates. These assays usually utilize segments of the gastro-intestinal tract or spirally cut strips of vascular tissue mounted in an organ bath. Since the guinea pig ileum gives contractions to many agonists including acetylcholine, angiotensin, arachidonic acid, bradykinin, histamine, prostaglandin, serotonin and substance P. This could also be used as a specific assay with suitable use of agonists and antagonists. As noted earlier the GPI assay is also useful as a general bioassay. The tracheal spiral from sensitized guinea pigs is often used to represent the large airways; lung parenchyma strips, prepared from the same animals are then utilized to represent peripheral airways. Contractions can be induced with histamine, carbachol, LTD_4, PAF and arachidonic acid, and the effects of plant extracts or tested against these directly. Possible vasodilating effects can be studied using the rabbit central ear artery perfusion model. In the rat mesenteric artery model, the perfusion system is connected to a suitable device for detecting changes in perfusion pressure to indicate vasoconstriction or vasodilation. Cardiotonic properties can be studied by using spontaneously beating right atrium or the stimulated left atrium of the guinea pig (Vlietinck *et al,* 1999).

Primary Screening Bioassays

These are relatively simple bioassays mainly designed for chemists, botanists, pharmacognocists and others, who lack the resources or expertise to carry out advanced bioassays. Active plants/fractions identified in these assays could later be subjected to sophisticated assays. The main objective of these is to discover a large number of bioactive molecules at the phytochemist's laboratory itself, since a vast number of compounds isolated so far by phytochemists are never isolated keeping the bioactivity aspect in to consideration.

Important Primary Bioassays

Type of Assay	References
Brine Shrimp Letality Assay	[Mclaughlin et a/, 1991]
Crown-Gall tumour inhibition test	[Mclauglin *et at,* 1991]
Sea urchin assay	[Ghisalberti *et* a/, 1993]
Assay for anti-bacterial activity	[Vanden berge et a/, 1991]
Assay for antifungal activity	[Paxton, 1991]
Wheat coleoptile bioassay	[Kubo et a/, 1989]
Molluscicidal assay	[Marston *et at,* 1991]
Seed Germination assay	[Stephen *et* a/, 1999]

2. USE OF BIOASSAYS IN QUALITY CONTROL

Recently bioassays have been used/recommended for quality control of herbal products. [Laughlin and Rogers, 1998]. Usually herbal products are heterogeneous due to the presence of mixtures of bioactive components either from the same or from purposefully id botanical sources. Physical methods of analysis like chromatography are of limited since they can be used to detect/quantify a select group of compounds. TLC sprinting of plant extracts can be useful in quality control, but it does not indicate the efficance of various chemical components. Very often a desired biological activity is due to one but a mixture of bioactive constituents and the relative proportions of single detective compounds can vary from batch to batch while the bioactivity still remains within able limits. Thus, the conventional methods of chemical analysis is not fully satisfactory, initializing *in vitro* bioassays, extracts can be tested as a whole and the biological response be expressed as a ED_{50}, IC_{50} etc. (quantal response) with 95% C.I. Deviation from standard range of ED_{50} can be an indication for inadequate quality, for instance, S. angata is widely used in Japan to control obesity and diabetes. Research has shown that it is a potent inhibitor of K-glucosidase activity [Matsada et a/, 1999]. K-glucosidase ends utilization of sucrose in the gut by preventing its breakdown to glucose. Thus, K-Dsidase inhibitors are useful in diabetes and obesity management. Acarbose, a K-jsidase inhibitor is in clinical use. One can use *in-vitro* K-glucosidase assay as a specific ssay to standardize S. angata extracts. We have determined IC_{50} for K -glucosidation activity of S. angata extracts. It was found to be in the range of 100 to 300 : g/ml. ; any deviation from this value can indicate problems in quality of the extract.

Following table shows the IC_{50} values of certain batches of S. *oblongata* extract in K-glucosidase inhibition assay. Note that certain batches have IC_{50} below the standard limit Batch, such a batch could be carefully examined and rejected. As said earlier the sensitivity of the extract could be acceptable or unacceptable irrespective of the marker entrant level. Specific assays used by Anderson ef a/. [Anderson et al, 2000] illustrates this : very effectively. For instance, different batches of *Echninacea* extracts were found to aim acceptable levels of the marker compound, however, when these batches were used for specific bioactivity like TNF-K assay or lipoxygenase inhibition, wide variation were detected. Similarly, several batches of *Hypericum* extracts standardized with reference to Hypericin, showed wide variation in serotonin reuptake inhibition activity.

In attempting to apply bioassay techniques for quality control, one could use them at two levels. At one level non-specific bioassays like brine shrimp lethality could be used to get a measure of general bioactivity of the plant product. For instance 50% alcoholic extract of *Emblica officinalis* extract shows a LC_{50} of 770 g/ml, a deviation from the same could imply variation in bioactivity. At a more advanced level one could use specific bioassays for quality control purposes of extract of polyherbal formulation. The assays should be selected keeping in mind the clinical use of the ingredient or polyherbal formulation.

For instance, MAO inhibition assay could be used for standardization of *Hypericum* extracts, since the Hypericin is used in depression. It has been shown that Brine shrimp lethality test is sensitive enough to detect presence of aflatoxin and pesticide residues, hence herbal products contaminated with these toxins exhibit low LC_{50} values in BSLT than the expected levels. Thus overall there is a need to adopt biological standardization of natural products for routine quality assessment [Jarry et al, 2000].

Table IC_{50} values of S. *oblongata* extract in a-glucosidase inhibition assay

Batch	IC_{50} (µg/ml)
A	99.99
B	170.83
C	832.47
D	246.99
E	304.87

3. USE OF BIOASSAYS TO ASSESS COMBINATIONAL ACTIVITY

Herbal extracts are composed of hundreds of compounds. When an extract shows some bioactivity, it is quite probable that more than one compound may be active. The active compounds in a plant extract may be synergizing or antagonizing the bioactivity. Knowledge of these effects in an extract is useful in formulation and quality control of herbal products [Kiuchi *et* a/, 1993]. Furthermore, in developing polyherbal formulations, one needs to know the compatibility of several herbal ingredients. The compatibility between extracts can be tested using the isobologram technique [Merle ef a/, 1992]. In this technique different concentrations of extracts to be tested are mixed in a checkerboard fashion. Different combinations are then tested in a bioassay, the results are processed to obtain an isobologram. The isobologram indicates 3 possibilities namely, synergy, antagonism and additivity. For an effective formulation one can choose synergistic combination that besides being potent also needs less dose due to synergy. Bioassays are of paramount importance to perform compatibility studies since many combinations have to be tested. Studies on combinational effects have been reported. For instance, K-sanshooil, an alkamide and sesamin have been studied for combinational activity using 5-lipoxygenase-bioassay. Results indicated a synergistic effect in this combination [Bauer *et al,* 2000]. Combinational effects are reported for bisbenzylisoquinoline alkaloids using anti-protozoal activity based on obologram protocol [Phillipson *et al,* 1995], Similarly for those plant drugs whose chemical imposition has been adequately studied and most of the constituents isolated and antified, one can even study and select the constituents desired for a particular bioactivity. any of the constituents present in a single plant extract have been reported to synergize / itagonize the activity of other constituents. As always such studies also demand evaluation a large number of samples for which conventional pharmacological methods are not suitable. Using in-vitro bioassays, recently the constituents of *Atropa belladonna* were analyzed for their combination affects on antihistaminic, anti-muscarinic and urinary retention activities [Capasso ef *al,* 2000]. Such findings demonstrate that standardization is not just an analytical operation and does not end with the identification and assay of the main active principles. Standardization signifies the body of information necessary to guarantee not only a constant chemical composition of herbal medicines, but also an equal efficacy.

4. USE OF BIOASSAYS IN STABILITY TESTING

The ability of a drug is important in any drug development. A drug has to be stable during its shelf life so that it is effective and safe. Stability studies of synthetic drugs is actively easy when compared to herbal drugs due to the differences in complexity, conventionally stability of herbal drugs is done by monitoring parameters like thin layer chromatography profile, assay of markers, physico chemical profile etc. All these methods have limitations because these parameters may remain unchanged during stability studies yet the herbal product could show difference in bioactivity. To overcome such problems,

Standardized bioassays could be used along with chemical assay parameters in stability studies. Both general and specific bioassays could be used. Assays like brine shrimplality can even indicate the production/presence of toxic byproducts formed during the stability study. Very few studies have been reported on stability studies employing bioassays differently, the antj-microbial assays were used to assess the stability of a herbal anti-septic cream. [Shanth et al, 2002]. The stability of a hepatoprotective formula is also reported employing aassay [Mohan ef al, 1993]. Undoubtedly, bioassays can be very useful adjuncts to conventional stability studies.

5. CONCLUSION

Assays thus play a very important role in natural product research laboratories. There are some limitations however which come in the way of their enhanced usefulness. The most important of them is the *in-vitro* nature of most of the assays, which ignores the complex processes of selective absorption and metabolism. Plant extracts showing profound activity in vitro may be totally inactive in-vivo. These are the general limitations of *in vitro* pharmacology. It is due to these reasons that WHO guidelines "Evaluating the Safety and efficacy of Herbal Medicines" insist on performing mutagenicity tests both before and after metabolic activation using S9 mixture [WHO, 1993]. There is an urgent need to develop assays that can serve as models for a variety of diseases. Further work should also aim in bridging the gaps between *in-vitro* and *in-vivo* pharmacology. Several methods of *in vitro* metabolic activation need to be developed in comparison with *in vivo* experiments.

REFERENCE - *GMP for Botanicals*