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ANIMAL SPARING BIOASSAY-AN EMERGING WAY TO INVESTIGATE ANTICANCER COMPOUNDS

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Abstract: Human inquisitive nature compels itself to discover and make things better, easier and more economical. Researchers of anticancer drugs are also not behind. As the new drugs are getting discovered, there is always a need to assay them biologically which involves generally use of many animals. The use of animals for bioassay raises some legal, ethical issues and is not always easy and economically viable too. The present study reviews and summarizes some of the techniques for assaying anticancer/antitumor compounds which do not involves the use of animals such as brine shrimp lethality, the inhibition of crown gall tumors, frond inhibition in *Lemna*; and yet finds them as safe, reliable, accurate and reproducible. Beside this, these methods are inexpensive compared to the conventional anticancer bioassay methods involving animal use which makes it more relevant and acceptable in modern anticancer compounds research field.

Keywords: Bioassay, Anticancer/Antitumor, Brine Shrimp Lethality, Crown Gall Tumors.

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INTRODUCTION

Assay is the estimation of the amount or the activity of an active principle in a unite quantity of the preparation. It can be chemical, biological or immunological [1].

Biological assay is defined as estimation or determination of concentration or potency of a physical, chemical or biological substance (agent) by means of measuring and comparing the magnitude of the response of the test with that of standard over a suitable biological system under standard set of conditions. It is needed in order to compare the properties of different substances, or the same substance under different circumstances. Bioassay can be performed on whole animal either singly or in groups or on isolated tissues or even cells [1-3].

The bioassay compares the test sample with a standard substance. It determines the quantity of test sample required to produce an equivalent or significant biological response to that of standard substance. Standard samples are accepted by expert committee at international level and they represent fixed units of activity [3].

Bioassay is essential for development of new drugs and other scientific monitoring [4] and also to measure the pharmacological activity of new or chemically undefined substances and to investigate the function of endogenous mediators to measure drug toxicity and unwanted effects [2].

BIOASSAY SYSTEMS AND TECHNIQUES [3]

The bioassay systems vary based on the biological system used like animals (mouse, rat, guinea pig, rabbits etc.), plant bioassay (using plant constituents to evaluate a sample like hemolytic activity, microbiological or cell based assay using microbes like bacteria, fungi or cultured cells for antibiotic compound screening etc.

Based on techniques they can be differentiated into two broad types like

a) **In vivo techniques**: These techniques employ a living animal recommended for the purpose of assay. The techniques aim to study the biological effect or response of the compound under screening in a living system directly, examples are use of rodents, rabbits etc.

b) **In vitro techniques**: These techniques employ a cell culture of recommended biological system to study the effect of compound under standard condition not similar to that of living environment. Here the cell culture survives by utilization of the nutrition in the media; examples are use of stem cells, cell culture, microbes (bacteria) etc.

c) **Ex vivo techniques**: These techniques employ a tissue or cells of recommended living system to study the effect of compound under test in suitable conditions within the

stipulated time of organ survival outside the body. This type of technique involves the use of any isolated organ from animals in a glass ware to study the effect of compound within the period of its survival outside the living body with provision of only oxygen, glucose and isotonic salts to maintain cell & cell organelles integrity.

The basis for whole animal bioassays was laid down by Paul Ehrlich in 1894 [5] Genetic models for animals used in bioassay includes hypertensive rats, obese mice, epilepsy prone dogs and mice, rats with deficient vasopressin secretion and many others [2, 6-7].

Cancer is characterized by unregulated growth as well as insufficient and inappropriate vascular supply to the cells and is the leading cause of mortality in the world which claims more than 6 million lives each year [8-10].

Phytochemicals can be valuable source for the discovery and development of unique anticancer drugs. During the last few decades medicinal plants has gained significant importance for the discovery and development of novel drugs [11-13].

Conventional bioassay methods for investigating anticancer properties involves use of either animals or various types of specific cell lines which requires more sophisticated laboratory and are costly methods [14-17].

In the present study an attempt has been made to summarize different anticancer bioassay methods which do not involves use of any animals or cell lines for which ethical clearance is needed, furthermore these methods are simple and cost effective also.

BRINE SHRIMP LETHALITY

Brine shrimp lethality bioassay is widely used and internationally accepted bioassay for screening of antitumor compounds [18-21].

In this method *In vivo* lethality or cytotoxic property of active extracts is used in a simple zoological organism for screening and fractionation in the discovery and monitoring of bioactive natural product [18, 22].

Method

Brine shrimp, *Artemia* species are also known as sea monkeys. These are marine invertebrates about 1mm in size. Freeze dried cysts are used in prawn hatcheries. The cysts remain dormant for several years; they are metabolically active after incubation in artificial sea water for 20-24 hrs. The equipment to grow these nauplii is very simple; a tank with compartments and a light

source is needed. The most important is water quality and oxygen content. The eggs of brine shrimp are readily available in pet shops at low cost and remain viable for many years in the dry state [23].

The eggs of brine shrimp, *Artemia salina* (Leach) are placed in seawater, the eggs hatches within 48 hours providing large numbers of larvae (nauplii) for experimental use.

Vials containing 5 ml of brine and 10 shrimps are taken and test compounds are added usually at initial concentrations of 10, 100 and 1000 ppm (or in g/ml). The test may be performed in triplicate and survivors are counted after 24 hours.

These data were then processed to estimate LC50 values. A number of novel anti-tumor natural products have now been isolated using this bioassay.

Thus, it is possible to monitor fractionations of cytotoxic and active extracts using the brine shrimp lethality bioassay rather than the more tedious and expensive *in vivo* and *in vitro* anti-tumor assays. [23- 24]

Advantages and applications

The brine shrimp assay has advantages of being rapid (24 hours), inexpensive and simple. It easily utilizes a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample. Furthermore, it does not require animal serum as is needed for cytotoxicity [18, 22].

This assay is considered useful in preliminary assessment of toxicity and has been used for the detection of fungal toxins in plant extract toxicity, animal and fish feeds. The brine shrimp lethality assay represents a rapid, inexpensive and simple, reliable bioassay for assessing the bioactivity of medicinal plants for its cytotoxic and anti-tumor properties towards brine shrimp [23, 25].

Fig 1: Brine shrimp nauplii.

POTATO DISC METHOD

Agrobacterium tumefaciens potato disc tumor bioassay is a method to investigate crude extracts for its possible antitumor properties. This method finds its application especially in developing countries where the cost of mammalian tissue culture or experimental animals is high [26- 27].

Crown gall is a neoplastic disease of plants including most dicots, some monocots and some gymnosperms caused by *A*. *tumefaciens*, a Gram negative bacterium, following by the transfer and expression of its Ti plasmids in the plant which eventually transforms normal, wounded plants to autonomous tumor cells [23, 28-31].

After its integration into the plant genome, the T-DNA genes encode enzymes responsible for the uncontrolled synthesis of the plant hormones auxin and cytokinin which account for the appearance of abnormal tissue proliferation and gall formation on the crown, roots and in some cases on stems [32].

The assay is based on the inhibition of *A. tumefaciens* induced tumors (or Crown Gall) in potato discs. The compounds suppress tumors by its antimitotic activity. This method detects a broad range of known and novel antitumor compounds. Development of this simple antitumor prescreen method using a convenient and inexpensive plant tumor system can offer numerous advantages as alternatives to extensive animal testing in the search for new anticancer drugs [22, 26, 33].

Although *A. tumefaciens* is a plant pathogen but it shares similar pathogenicity as that of tumor causing bacteria in human such as *Bartonella henselae* and *Helicobacter pylori*. Many studies have shown relationship and similarities between crown-gall tumors and animal cancer and hence this method is getting popular now a days [30, 34-35].

Method

Antitumor activity can be assessed following the standard procedures. It is a simple method that involves the assessment the ability of test substance to inhibit crown gall tumors on potato discs. Extracts or compounds that inhibit these tumors indicate the probability of having antitumor activity.

Usually *A. tumefaciens* is taken in PBS, Camptothecin may be taken as positive control and red skinned potatoes (*Solanum tuberosum* L.) are chosen to make discs.

After 12 to 21 days, discs are stained with Lugol's solutions (10% KI, 5% I_2), tumors are counted under a stereo microscope. The percentage inhibition of crown gall tumors is calculated and statistical relationships are compared [23, 26, 33, 36].

Fig 2: *A. tumefaciens* induced tumor on potato disc. [37]

Fig 3: *A. tumefaciens* induced crown gall in plant.

DUCKWEED GROWTH INHIBITION TEST

Recently a toxicity bioassay namely Duckweed growth inhibition test (*Lemna gibba* or *Lemna minor* test) has received much attention. The term ''duckweed'' commonly refers to a group of miniature aquatic monocot plants of the family Lemnaceae. These are small, floating and fast-growing plant easy to cultivate. It consists of mother frond with two daughter fronds and a filamentous root which under normal conditions reproduce exponentially with budding of daughter fronds from punches on the sides of mother fronds [38].

These characteristics make it an ideal candidate for toxicity testing. Hence, the duckweed bioassay has become a standard toxicity method and is internationally accepted. Monitoring of the frond proliferation in Lemna *sp* provides a basis for herbicides and plant growth stimulants also [23, 39].

Method

One two-frond colony of *Lemna* plants are placed into vials containing E medium. Test substances at graded concentrations are delivered into the medium and the vials are placed in plant growth chamber at 27 ^{o}C- 29 ^{o}C with 24 hour of fluorescent and incandescent light. After seven days, the number of fronds counted and values are calculated using specially designed program to provide statistical relevance [23, 39-41].

Advantages & Applications

Lemna *sp* are sensitive to a wide range of pollutants, which is an advantage in toxicity evaluation. It has been used extensively for eco-toxicology studies reacting non-specifically to a number of xenobiotics such as herbicides, pesticides and heavy metals. It has shown potent herbicidal and growth stimulating properties, thus fulfilling the commercial need for such natural, biodegradable herbicides and plant growth stimulants [39, 42-43].

Fig 4: *Lemna gibba*

Fig 5: *Lemna minor*

CONCLUSION

Most of the bioassay techniques involve the use of animals in some way to investigate the potency or toxicity of bioactive compounds. Using animals for this purpose may not be always easy as ethical clearance is needed and the experimental procedures are complex and time consuming as well. Compounds which indicate anticancer potential undergo bioassay extensively either by using specific cell lines or some animals which makes it much expensive and only developed countries or economically strong pharmaceutical houses can afford. Scientists have developed some methods with very few limitations to prescreen anticancer potential of compounds. These methods are getting popular especially in developing countries as they are cost effective, highly economical and easy. Further investigations are required to minimize the limitations and upgrade these methods so as to make it acceptable worldwide which in turn will reduce the cost for anticancer compound research and thereby making the cancer treatment more affordable to the common man.

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