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Seed germination: An alternative to animal model to teach bioassay principles

Sir,

Practical training in bioassay methodology is an important exercise for the post-graduate students of pharmacology. Assay of acetylcholine on isolated frog rectus abdominis muscle is the commonest experiment conducted for this purpose.[1] The University Grants Commission (UGC) guidelines prohibit the use of frogs. Recently, Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and People for Ethical Treatment of Animals (PETA) have insisted on the abolition of animal experiments for teaching purposes. Due to the proposed ban on the use of all animals for teaching and learning activities, many medical colleges have stopped using animals for teaching purposes because of the fear of persecution. This has created a vacuum for practical training of post-graduates.

The "three Rs" are the guiding principles for the use of animals in research. They emphasize, replacement of animal methods by non-animal methods, reduction in the number of animals to be used, refinement of experimental method to reduce the pain and suffering of animals used.[2] At present alternatives to animal experiments mainly consists of computer simulation models. However, a live hands-on system is a more exciting and encouraging way for learning biological principles than books and stored media. Inhibitory effect on seed germination has been used to screen cytotoxic and anti-proliferative compounds.[3] Modern tissue culture techniques have replaced such use of seed germination. However, seed germination method offers itself as an opportunity for practical training of post-graduates in the principles of bioassay and biostatistics, especially the statistics part, as the number of samples used can be increased without any restrictions.

An example of "a study of anti-proliferative effect of calcium channel blockers verapamil and diltiazem on seed germination" is given here.

The following materials were procured and used for the experiment:

Best quality green gram seeds of uniform weight (40 ± 1 mg), Petri dishes (10.8 cm diameter), blotting papers (10.8 cm diameter), cotton roll, beakers, distilled water, mercuric chloride (0.1% solution), pure samples of verapamil and diltiazem, measuring scale, and thread.

Green gram seeds were sterilized by soaking in mercuric chloride solution for 2 min and washed with distilled water for five times (prepared seeds). Twenty seeds were allotted for each group ($N = 20$).

The seeds were soaked in 30 mL of distilled water or drug solutions in small beakers, for 48 h. The volume of distilled water/drug solutions was derived by filling Petri dishes up to two-third of their heights, which was found to be 30 mL.

Blotting papers were taken. A cotton layer of approximately 3-mm thickness was sandwiched between two layers of blotting paper. This was placed in sterile Petri dishes (prepared Petri dishes).

The seeds which were soaked for 48 h in the beakers were

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transferred to the prepared Petri dishes with their respective solutions. Each group of distilled water or drug solutions, were made in duplicate. The seeds were allowed to grow for 4 days [Figures 3 and 4]. The root and shoot length were measured with the help of a thread and the results were tabulated.[4] The averages of these two sets were considered to analyze the results. Results [Tables 1 and 2] were expressed as the percentage inhibition of linear growth of the stem and root, calculated by the formula:

Percentage inhibition of growth (PIG) = {mean length of distilled water control (LDWC) − mean length of drug dose (LDD)/LDWC} × 100

Effects of drugs were compared with distilled water control. Student's "*t*"-test was used to calculate probability values. Log dose response curves [Figures 1 and 2] were plotted to obtain the Inhibitory 50% concentration (IC50).

Table 1: Effect of verapamil on seed germination

Study group	Mean growth (mm)	S.E.M	*P* value	Inhibition percentage
Control	68.85	3.888		
Verapamil 125 µg/mL	52.25	5.692	<0.001	25
Verapamil 250 µg/mL	43.45	3.445	<0.001	38
Verapamil 500 µg/mL	35.65	3.581	<0.001	50
Verapamil 1000 µg/mL	2.85	0.493	<0.001	95
Verapamil 2000 µg/mL	0.00	0.00	<0.001	100

Table 2: Effect of diltiazem on seed germination

Study group	Mean growth (mm)	S.E.M	*P* value	Inhibition percentage
Control	56.3	4.415		
Diltiazem 125 µg/mL	40.85	5.739	<0.021	27
Diltiazem 250 µg/mL	36.45	4.484	<0.001	36
Diltiazem 500 µg/mL	22.45	0.596	<0.001	61
Diltiazem 1000 µg/mL	10.65	1.678	<0.001	80
Diltiazem 2000 µg/mL	0.00	0.00	<0.001	100

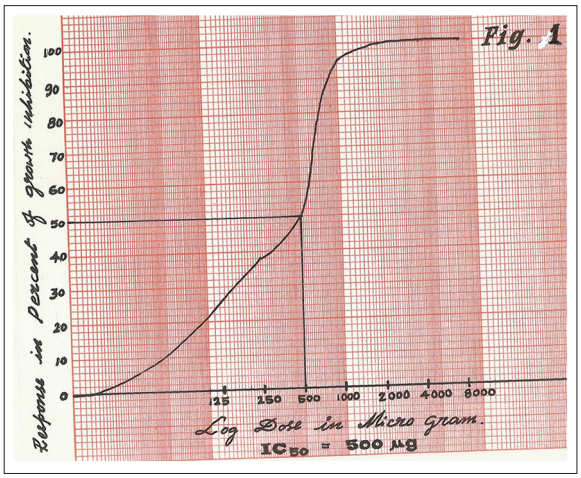

Figure 1: Log dose response curve verapamil

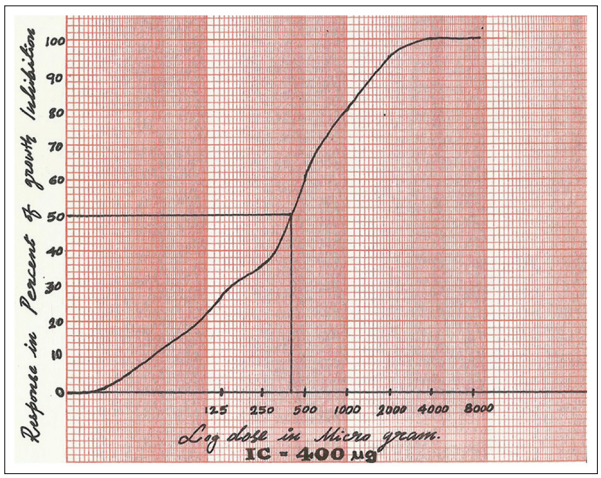

Figure 2: Log dose response curve diltiazem

Figure 3: Seed germination (normal control)I

Figure 4: Inhibition of seed germination (verapamil)

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With 1mg/mL of drug solutions, verapamil showed 58.1% and diltiazem 58.8% inhibition. With 10 mg/mL, there was 100% inhibition with both the drugs. Thus a dose range of 1-10 mg was chosen for the experiment. The doses tested in the main study were 1000, downward 500, 250, and 125 and upward 2000, 4000, and 8000 µg/mL (graded doses, geometric ratio of 2 between doses).

The IC50 was found to be 500 µg for verapamil and 400 µg for diltiazem.

Later the experiment was done using 15 mL of distilled water/drug solutions instead of 30 mL, soaking for 24 h instead of 48 h and allowed to sprout for 2 days instead of 4 days, thus reducing the duration of experiment and the quantity of drugs required. The IC50 for verapamil was 900 µg and for diltiazem it was 600 µg.

"Assay" and "essay" share a common ancestry. Both words came from the French, "essai" which in turn came from the Latin, "exagium" meaning the "act of weighing."An assay is an analysis done to determine the presence of a substance and the amount of that substance. There are several methods of assay; mainly physical, chemical, and biological. Bioassay is the estimation of the presence of a substance and its quantification by virtue of its action on a biological system. The main principle of bioassay consists of, having a known standard of the substance to be assayed, measuring the standard sample's dose response curve and comparing the dose response effect of the unknown with that of the standard and arriving at the amount of the unknown substance. Thus the dose response curve becomes the basic principle of bioassay.[5]

Calcium channel blockers have anti-proliferative effect in mammalian cancer cells.[6] They have inhibitory effect on seed germination and that is the basis of this study.[7] These experiments are financially cheap, easy to conduct, and the results are highly reproducible. Due to long duration of the experiment, it is not suitable for conducting university examinations. However, it can be considered for internal exams.

Main objectives of bioassay today consists of, finding the efficacy and potency of new molecules and bioassay and bio-standardization of molecules like insulin, where contaminants cause variation in response to different batches. One can find out the potency and efficacy of the unknown sample by interpolation from standard dose response curve. It is possible to do "bracketing" and "matching" in an assay on single isolated tissue, where the response to a dose is expected to be same and the response is titrable. Such studies are not possible in this model. We have also worked using weight of the seed, (graded response) or complete inhibition of seed germination (all or none response) as parameters. We can study normal variation in growth, plot frequency distribution curve by germinating hundred or more seeds. Smaller seeds can be used and they can be soaked in the test tubes using a small quantity of water/drug solutions.

Sir Gregor Mendel became the father of modern genetics by using pea plants to demonstrate the "transmission of traits" observed in human beings. Bacteria differ from human cells in that they lack nuclei; yet they have been used for mutagenicity tests,[8] bioassay of vitamins A and B12 and for biosynthesis of human proteins like human insulin by recombinant DNA technology. Plants resemble human cells in that they have nuclei. Therefore, any biochemical similarity in plants and animals can be exploited in favor of the use of plants in lieu of animals for the discovery and development of drugs.

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