

A REVIEW: SRB ASSAY FOR SCREENING ANTICANCER ACTIVITY OF HERBAL DRUGS (IN-VITRO)

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ABSTRACT

W.H.O. reported that Cancer is the second leading cause of death worldwide with 8.2 million deaths so far. Chemotherapy and radiation have many limitations and known adverse effects. Herbal medicine remains an excellent option. *Arbuda* with its therapies is well described in the *ayurvedic* classics like *sushrut samhita*. The herbal drugs needs to be evaluated scientifically for acceptance globally and also for the evaluation of safety. Therefore, there is a need for studying the techniques of screening an anticancer activity of the *ayurvedic* preparations in line with current research methodology. Amongst current available methods for advanced in vitro screening techniques are SRB, MTT, Clogenic, Florescent assays & Dye exclusion test. SRB assay is particularly useful for qualitative analysis. It provides a better linearity with cell number and is highly sensitive. Additionally its staining is not cell dependent. Hence, it is more appropriate assay for screening. Due to its accuracy and feasibility, it is valued by the researchers. For screening anticancer activity on human cancer cell lines, there is need to prepare the hydro-alcoholic (60:40) extract of study drug. Human cancer cell lines are preserved in liquid nitrogen and DMSO at -20 °C. Cell culture is performed in incubators at 37°C. The selected cancer cell lines are then inoculated in 96 well plates. In this study, drug is tested at 4 dose levels at 10, 20, 40, 80 µg/ml. Adriamycin or doxorubicine can be used as a positive control drugs for comparative screening. As per SRB assay protocol, GI₅₀, TGI, LC₅₀ values are calculated as stated by NCI. If percentage growth inhibition is 50 at any of above concentration then it is indicative of effectiveness of the study drug.

Key words: Cancer cell line, In-vitro, SRB, 96 well plates.

INTRODUCTION

Cancer is the second leading cause of death worldwide with 8.2 million deaths so far. According to the most recent survey of W.H.O. conducted in 2012, estimated 12.7 million new cancer cases were reported. The burden of cancer is expected to increase to 22 million new cases each year by 2030. In the classics of *ayurved*, cancer is described as *arbuda*. Its causes, symptoms and therapies is well described since

the period of *sushrut samhita* (i.e. 3rd century)^[1]. Numbers of herbs as well as herbo-mineral formulations are mentioned in the ancient classics. However, according to the modern science, each drug needs to be scientifically, pre-clinically and clinically evaluated for the global acceptance. To study the various techniques of screening, an anticancer activity of the *ayurvedic* preparations by the current researchers is a need of hour. Hence, in vitro screening

techniques exclusively with SRB assay technique are reviewed.

Material and method:

Material: In material along with usual scientific equipment's more specifically human cultured cancer cell lines, cryopreservator, extracts of the study drugs, 96 well plates, colorimeter are used for the study. In the current scenario, the screening of in vitro anticancer activity is done by Adriamycine or Doxorubicin as +ve control drug and solvent used is a vehicle control.

Method of cell culture: For screening the study drug, tumor cell lines derived from several cancer types like lung, colon, melanoma, renal, ovarian, brain can be used. Quality of the cell line is maintained to a suitable growth which shows reproducible profiles for growth and drug sensitivity. The lines are then cryopreserved by using reagents such as DMSO (di methyl sulfoxide) which preserves the cells during freezing. Thawing of cell is done by bringing the frozen ampule to room temperature by slow agitation. The frozen cryovials are plunged into the water bath & is rapidly thawed until they get liquefied. The solution is centrifuged with normal saline for 10 minutes to remove the DMSO. The normal saline is discarded and aliquot is taken for cell counting, cell viability and for sub culturing.

Preparation of extracts: There are two SOPs for the preparation of extracts which are used for screening of the study drug.^[2] For the extraction of herbal drugs, hydro-alcohol in the proportion of 60:40 or the distilled water can be used as a solvent. For the preparation of hydro-alcoholic extract coarse powder of clean and dry material can be used. 40% ethanol should be added to the raw material in a ratio of 4:1 and the mixture should be macerated for 4hrs. The mixture should be refluxed for 2hrs at 80⁰c. The same method should be

repeated 3 times and the residue should be checked for complete extraction. After filtration and concentration of extracts under rotavapour, the alcohol gets evaporated and the extract can be obtained. By following the above said method aqueous extract also can be obtained but the ratio of the distilled water to the coarse powder should be in proportion of 6:1.

SRB assay: According to SRB assay.^[3,4], in vitro testing should be done by using SRB assay protocols. Cell lines should be counted, cultured and inoculated in 96 well plates. In these drugs should be tested at 4 dose levels at 10, 20, 40, 80 µg/ml. Each experiment should be repeated three times. After incubation with different concentrations of test compounds, the cell cultures should be stained with SRB dye. Washing with 1% acetic acid (CH₃COOH) removes the unbound dye and the protein bounded dye gets extracted using Triss-HCl buffer base (100 µl, 0.01 M, pH 10.4). The optical density can be determined by 96-well plate ELISA reader at 540 nm.

Observations and results:

After incubation of study drug on the cultured cell lines the results will be determined in terms of GI50, TGI and LC50 values-

GI50 = Concentration of the drug that produces 50% inhibition of the cell.

TGI = Concentration of the drug that produces total inhibition of the cells

LC50= Concentration of the drug that kills 50% of the cells.

Discussion and conclusion:

Currently available, in vitro screening techniques SRB and MTT assay are the most reliable techniques used to evaluate anticancer activity on the cancer cell lines. Out of which SRB assay is used for qualitative analysis and MTT assay is used for quantitative analysis.^[5] The SRB assay provide a better linearity with cell number

and a higher sensitivity and its staining is not cell dependent. It is known that, in contrast to the MTT assay the SRB assay stains recently lysed cells. Cell debris does not get stained by SRB and therefore the drug enitivity data doesn't get affected.^[6] SRB assay measures whole protein content which is proportional to the cell number. According to cell properties, there are various assays can be used for this purpose such as Dye exclusion test, MTT assay, Florescent assay, SRB assay, Clogenic assay etc. SRB (Sulphorhodamine B) is a bright pink anionic protein staining dye that binds to the basic amino acids of the cellular proteins.

Solvents are needed to extract the herbo-mineral components for in vitro screening. There are three types of solvents- polar, non-polar and mid polar. Water is a universal solvent. Alcohol is the most commonly used solvent for extraction, because of its good polarity & easy penetrating power in cell membranes of plant. Although, Methanol is more polar than Ethanol, due to its cytotoxic nature, it is unsuitable for extraction in certain kind of studies as it may lead to incorrect results.^[7] Acetone, DMSO, Ethers etc. are the other solvents used for extraction depending upon the solubility of herbo-mineral components.

In vitro studies reduce the usage of animals. It helps to test the ability of the compound to kill the cells by taking the advantage of various properties of cell. It is possible to process a larger number of compounds quickly with minimum quantity through in vitro studies. It is a highly cost effective. Also, the range of concentrations are used are comparable to that expected in vivo studies. However, it is difficult to maintain the cell cultures. They show negative results for the compounds which get activated after body metabolism

and vice versa.^[8,9] It is difficult to ascertain the pharmacokinetics of the study drugs and to examine the activity, if compound is insoluble in the solvents.

Anticancer activity is evaluated by plotting the graphs and by the following formulae:

i. GI50: Growth inhibition of 50 % calculated from $[(Ti-Tz)/(C-Tz)] \times 100 = 50$, Drug concentration resulting in a 50% reduction in the net protein increase.

ii. TGI: Drug concentration resulting in total growth inhibition will be calculated From $Ti = Tz$.

iii. LC₅₀: Concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of 50% cells following treatment is calculated from $[(Ti-Tz)/Tz] \times 100 = -50$.

If the compound is pure then GI50 value of 10^{-6} (i.e. 1 μ mole) or 10 μ g/ml is considered is indicative of demonstrable activity and in case of extracts, GI50 value 20 μ g/ml is considered to demonstrate activity. Drug is considered lethal if it causes death of more than 50% of cells i.e. LC50 values more than 50% at any concentration level.

There are number of formulations explained in classics and also in practice that are derived from with tribal masses, folk lore, cowherds, sages, hunters and other forest dwellers which act on *arbuda*, *granthi*, *gandmala*, *apachee* etc. They show promising effect in clinical practice. It is therefore essential that detailed literature reviews and controlled scientific experimentation with correct methods and currently available techniques is carried out to study these in more detail. It is possible to evaluate or to discover new formulations or herbs which show anticancer activity, with the help of advanced techniques of screening in as described here.

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