CHAPTER 1

OPTIMIZATION OF DRUG DOSE (LOG AND LINEAR SCALE)
Abstract
Rapid and accurate assessment of viable cell number and cell proliferation is an important requirement in many experimental situations within in vitro cell culture studies. In vitro techniques for drug screening, determination of the cytostatic potential of different compounds in toxicology testing are very useful. Also it helps to evaluate the cytotoxic, mutagenic and carcinogenic effects of the respective compound. The activation of macrophages in vitro was studied by using BRMs and standard macrophage activator like LPS. Screening of the BRMs and optimization of drug dose was carried out initially on log scale and then to the linear scale by MTT assay.

1.1 Introduction:
Initial morphology of J774A.1 cells was observed during cell culturing. Macrophages are of various shape and size. The cells are irregularly shaped, with surface folds, ruffles, and projections. The cells were surface adherent (Williams Hematology, Part VIII, Chapter 67). Macrophages are oval to spindle shaped and also spreads with the pseudopodia. Macrophages in their sub-confluent phase were treated by different drugs at different concentrations to check their effect on viability (by trypan blue dye exclusion test and MTT assay) and to assess macrophage activation. Generation of nitrite is one of the basic indicator of macrophage activation. The nitrite levels were also estimated from the drug treated and untreated macrophage culture supernatants, by nitrite assay by Greiss reagent at different time intervals.

Fig10: macrophages in culture.
The most convenient assay for determination of cell viability and cell proliferation have been developed in a micro-titer plate or 96 well plates. This miniaturization allows many samples to be analyzed rapidly and simultaneously. It also reduces the amount of culture medium and cells required. It is cost effective and colorimetric assays allow samples to be measured directly in the micro-titer plate with an ELISA plate reader. MTT assay is a colorimetric assay used to check the metabolic activity of viable cells. It is a microtiter assay which uses the tetrazolium salt MTT (succinate dehydrogenase inhibition or SDI test) to quantitate cell proliferation and cytotoxicity (Mosmann, 1983; Vistica, 1991). MTT assay exclusively detect viable cells because tetrazolium salts are cleaved only by metabolically active cells. In the SDI assay MTT is reduced by viable cells to a colored, water-insoluble formazan salt. After it is solubilized, the formazan formed can easily and rapidly be quantitated in a conventional ELISA plate reader.

![Fig11: Reduction of a tetrazolium to a formazan](fig source: biotek.com)

\[\text{note: MTT is cleaved to formazan by the ‘succinate tetrazolium reductase’ system which belongs to the mitochondrial respiratory chain and is active only in viable cells (Slater, 1963). Interestingly however, recent evidence suggests that mitochondrial electron transport may play a minor role in the cellular reduction of MTT. Since most cellular reduction occurs in the cytoplasm and probably involves the pyridine nucleotide co factors NADH and NADPH, (Berridge, 1993) the MTT assay can no longer be considered strictly a mitochondrial assay.}\]

Following BRMs were used in this study:
Gallic acid (A derivative of Emblica Officinalis), Guduchi (Tinospora cordifolia), Canova (a medicament also known as Anbuta), Spirulina (Algal Polysaccharide-Chlorella pyrenoidosa), AOIM-Z (a homeopathic formulation) were used as BRMs.
Also Cisplatin (an anticancer drug) was tested for its effect on macrophage activation and LPS was used as a Positive control.

1.2 Materials and Methods:

1.2 (a) Reagents: Dulbecco’s Modified Eagle Medium (DMEM) with L-glutamine and 25 mM HEPES buffer were purchased from (HiMedia Pvt. Ltd. India.) Fetal bovine serum was purchased from Hyclone (Logan, USA) and heat inactivated at 56°C for 30 min. The drug (guduchi) used was obtained from Himalaya Drug Company products, India. The drugs prepared in incomplete DMEM were tested for endotoxin contamination by limulus amebocyte lysate assay which showed insignificant levels [0.0007ng/mg]. Necessary precautions were taken to avoid endotoxin contamination throughout the investigation, by using endotoxin free buffers, reagents and sterile water. All other chemicals and solvents used in this study were obtained from Sigma Chemical Company (St. Louis, USA) and were of analytical grade or the highest grade available.

1.2 (b) Lab preparations:

1.2 (b).1 Phosphate buffer saline (PBS): PBS is one of the very important solutions required in tissue culture. To maintain the cell culture environment during various assays saline with the constant pH (7.2-7.4) can be applied.

Table 2: The composition for PBS (1L) preparation was as follows:

<table>
<thead>
<tr>
<th>compounds</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.05</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>2.50</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0.40</td>
</tr>
</tbody>
</table>

The saline solution were prepared in sterile conditions. Sterilization was done by autoclaving or by filtering with nitrocellulose filters of 0.22µm diameter. This sterile solution can be used further for preparation or dilutions of several chemicals.

1.2 (b).2 Trypsin (TPVG):

Adherent cells can not be detached simply by hand tapping. To detach the cells from the culture flasks during subculture or assaying they need to treated with trypsin for around 1-2 minutes. TPVG is prepared by adding following compounds in 100ml PBS.
Table 3: The composition for Trypsin (TPVG) preparation is as follows:

<table>
<thead>
<tr>
<th>compounds</th>
<th>Amount g/100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>0.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.05</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.1</td>
</tr>
</tbody>
</table>

After addition of these compounds the pH was checked and adjusted to 7.2-7.4. The solution was then sterilized by filtration.

1.2 (b). 3 Medium preparation:

1.2 (b). 3.1: RPMI 1640:
RPMI 1640 with 10% fetal bovine serum was used for culturing YAC-1 cells. Powdered RPMI medium (16.4 gm) was dissolved in 700 ml sterilized distilled water. The volume then made upto 1 liter after checking the pH (pH was set at 7.2-7.4). Two gm (7.5% w/v) sodium bicarbonate was added to this medium. The medium was then membrane filtered using 0.22 µm membrane filter (Millipore, USA). To these antibiotics were added (streptomycin 100µg/ml, penicillin 100 U/ml, gentamycin 20 µg/ml). Complete medium was prepared by addition of 10% (v/v) heat inactivated fetal bovine serum (FBS) to the filtered medium. FBS was heat inactivated at 56°C for 45 min. The bottles were stored at 4°C temperature for further use. For subculture the temperature raised to room temperature.

1.2 (b). 3.2: DMEM:
DMEM with 10% fetal bovine serum was used for culturing J774A.1 and L929 cells. Powdered DMEM (17.4 gm) was dissolved in 700 ml sterilized distilled water. The volume then made upto 1 liter after checking the pH (pH was set at 7.4). 3.7 gm sodium bicarbonate was added to this medium. The medium was then membrane filtered using 0.22 µm membrane filter (Millipore, USA). To these antibiotics were added (streptomycine 100µg/ml, penicillin 100 U/ml, gentamycin 20 µg/ml). Complete medium was prepared by addition of 10% (v/v) heat inactivated fetal bovine serum (FBS) to the filtered medium. FBS was heat inactivated at 56°C for 45
min. The bottles were stored at 4°C temperature for further use. For subculture the temperature raised to room temperature.

**Note:** No any antibiotics were added to the medium to avoid errors in the microbicidal assay results.

1.2 (c) **Cells:** The macrophage J774A.1 cell line, obtained from National Center for Cell Sciences (NCCS, Pune), was used as source of macrophages (Origin: BALB/c mouse; Nature: Mature), grown and maintained in the DMEM (pH 7.5) enriched with 10% fetal bovine serum, at 37°C and 5% CO₂ environment.

1.2 (d) **Viability assay:** Cell viability was determined by the Trypan blue dye exclusion technique. Equal volumes of cell suspensions were mixed with 0.4% Trypan blue in PBS, and the unstained viable cells were determined. These cells were further used for cytotoxicity assay in 2 x 10⁶ density per ml in the 96 well tissue culture plate.

1.2 (e) **Stimulation of macrophages:** The macrophage cells (cell line J774A.1) from late log phase of growth (subconfluent) were seeded in 96 well flat bottom microtiter plates (Tarsons, India) in a volume of 100μl under adequate culture conditions. Drugs were added in different concentrations in a volume of 100μl in triplicate. The cultures were incubated at 37°C and 5% CO₂ environment. After 24 hr and 48 hr incubation percent viability was checked and culture supernatants were collected and assayed for nitric oxide and lysozyme activity.

1.2 (f) **Cytotoxicity assay:** In order to detect the toxicity of herbal preparation the cytotoxicity assay was standardized by using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at different time intervals for 24 hrs and 48 hrs (Mosmann, 1983) using the drugs at various concentrations. After 24 hrs and 48 hrs of incubation, supernatants were collected and ten microlitres of MTT (3 mg/ml) was added to each well and plates were further incubated for 2 hrs. The enzyme reaction was then stopped by addition of 150μl of dimethyl sulphoxide (DMSO). Plates were incubated for 10 min under agitation at room temperature before the optical density at 570nm was read under an ELISA plate reader. Three independent experiments in
triplicate were performed for the determination of sensitivity to each drug. Cells treated with medium alone were considered as Control.

Percent viability was calculated by the given formula.

\[
\text{Percent viability} = \frac{E}{C} \times 100
\]

Where, \(E\) is the absorbance of treated cells and \(C\) is the absorbance of untreated cells.

1.3 Statistical analysis:

Statistical significance of difference between the control and experimental samples were calculated by Student’s \(t\)-test. All the experiments were done in triplicate samples. Conclusions were drawn from 3 independent experiments.

1.4 Results:

1.4 (a) Viability and proliferation of J774A.1 cells:

J774A.1 cells showed 100% viability before the drug treatment by trypan blue dye exclusion test. The cells were treated with drugs initially on log scale and then on linear scale. J774A.1 cells were incubated in medium alone or drugs for 24 h and 48 h and checked for percent viability as described in Materials and Methods. Guduchi in concentration 80μg/ml, gallic acid at 60μg/ml, spirulina and AOIM-Z at 100μg/ml, canova at 0.1c/ml, and cisplatin and LPS at 10μg/ml each, showed maximum viability of macrophages as compared to medium alone, thereby proving that these drug concentrations were not cytotoxic to the cells. The assay results are mentioned with the respective IC\(_{10}\) values of the drugs (Table 4 and 5). The log scale result explains the effect of drugs at their 10 fold concentrations. It was found that the drugs guduchi, spirulina, canova and AOIM-Z being herbal preparations did not show cytotoxic effect on macrophages (J774A.1). In fact even the high concentrations of these drugs could only reduce the viability upto 10 percent (Table5) and showed negligible cytotoxicity of macrophages (Fig12 and 13). Since the work is in vitro, Optimum drug concentration for guduchi and AOIM-Z was 80μg/ml and for spirulina it was 100μg/ml (Table4). Optimum dose for canova was (0.1c/ml). Gallic acid at the concentration 80μg/ml show 90 percent viability i.e. 10% cytotoxicity, hence the optimum drug dose for gallic acid was 60μg/ml which showed maximum (92%) viability. Cisplatin is an anticancer drug and LPS is the standard positive stimulator of macrophages. Higher concentrations of these compounds are
cytotoxic. Their IC\textsubscript{10} values were 10-15μg/ml and the optimum drug concentration for macrophage activation was 10μg/ml for each.

1.5 Discussion:
One of the most promising recent alternatives to classical antibiotic treatment is the use of immunomodulators for enhancing host defense responses (Tzianobos, 2000). Plant derived immunomodulatory compounds have also been used in traditional remedies for various medical problems and the investigation of these sources has grown exponentially in recent years. India has a rich tradition in the treatment of many diseases by therapy with ‘Rasayans’. In Ayurveda, ‘Rasayans’ are concerned with nourishing body and boosting immunity. They are also modulators of the immune system and one such cell modulated by them is the macrophage. J774A.1 cells are adherent and spread out when cultured in DMEM, however, when cultured in RPMI 1640, the cells are rounded and relatively non-adherent. Different types of tissue culture plates, sera, and media supplements were not responsible for these changes. We examined LPS-induced reactive nitrogen species using the Greiss reagent. J774A.1 cells cultured in RPMI exhibit a 5-fold increase in nitrites in culture supernatants after LPS stimulation whereas those in DMEM do not. In DMEM the cells become more adherent, acquired an amoeboid shape with pseudopods, a more vacuolated cytoplasm and a higher cytoplasmic to nuclear ratio. Interestingly, when the cell is placed back into RPMI, it regains its more ovoid monocytic characteristics. Hence the growth mediums were standardized accordingly and the cells were grown in DMEM culture during this study. (Cohly et. al., 2001). J774A.1 cells showed 100% viability before the drug treatment by trypan blue dye exclusion test. The cells were treated with drugs initially on log scale and then on linear scale. J774A.1 cells were incubated in medium alone or drugs for 24h and 48h and checked for percent viability as described in Materials and Methods. The BRMs with their respective concentrations (guduchi at 80μg/ml, gallic acid at 60μg/ml, spirulina at 100μg/ml, AOIM-Z at 80μg/ml, canova at 0.1c/ml, and cisplatin at 10μg/ml) showed maximum viability of macrophages as compared to medium alone, thereby proving that the drugs were not cytotoxic to the cells. The macrophage cells (J774A.1) were treated with different drugs and the inhibitory concentrations (IC\textsubscript{10}) of the drugs for the cells were estimated by MTT assay and trypan blue dye exclusion test. Then rests of the assays were carried out with the same concentrations.
1.6 Conclusion:
The drugs were observed not to have cytotoxic effect on macrophages as determined by MTT assay.

Fig 12 (a, b, c, d, e, f, g): Results for effect of log scale concentrations of drugs on percent viability of J774A.1 cells.

- **a)** gallic acid

- **b)** guduchi

- **c)** canova

- **d)** AOIM-Z

- **e)** spirulina

- **f)** cisplatin

- **g)** LPS

Fig12: % viability of macrophages (J774A.1) treated with (a)gallic acid, (b)guduchi, (c)canova, (d)AOIM-Z, (e)spirulina, (f)cisplatin, (g)LPS on log scale: The values are mean ± S.D. and are representative of three independent experiments done in triplicate.
Fig13 (a, b, c, d, e, f, g): Results for effect of linear scale concentrations of drugs on percent viability of J774A.1 cells.

a) gallic acid

b) guduchi

c) canova

d) spirulina

e) AOIM-Z

f) cisplatin

g) LPS

Fig13: % viability of macrophages (J774A.1) treated with (a) gallic acid, (b) guduchi, (c) canova, (d) spirulina, (e) AOIM-Z, (f) cisplatin, (g) LPS on linear scale: Bars show SDs of means for triplicate cultures. Data are representative of three separate experiments (*p<0.05).
Table 4: Optimum drug concentrations:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Optimum Concentration</th>
<th>% viability (±S.E.M.) (24 hrs)</th>
<th>% Viability (±S.E.M.) (48 hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium alone</td>
<td>-</td>
<td>99 ± 0.75</td>
<td>99 ± 0.25</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>60µg/ml</td>
<td>92±1**</td>
<td>90±1</td>
</tr>
<tr>
<td>Guduchi</td>
<td>80µg/ml</td>
<td>96±0.89**</td>
<td>94±0.89*</td>
</tr>
<tr>
<td>Canova</td>
<td>0.1 c/ml</td>
<td>98±1*</td>
<td>95±2*</td>
</tr>
<tr>
<td>AOIM-Z</td>
<td>80µg/ml</td>
<td>92±1</td>
<td>91±1*</td>
</tr>
<tr>
<td>Spirullina</td>
<td>100µg/ml</td>
<td>99±0.71**</td>
<td>98±1**</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10µg/ml</td>
<td>91±2*</td>
<td>90±2**</td>
</tr>
<tr>
<td>LPS</td>
<td>10µg/ml</td>
<td>93±1*</td>
<td>92±2*</td>
</tr>
</tbody>
</table>

Table 5: Drug concentrations with their IC<sub>10</sub> value:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (IC10)</th>
<th>% viability (±S.E.M.) (24 hrs)</th>
<th>% Viability (±S.E.M.) (48 hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium alone</td>
<td>-</td>
<td>99 ± 0.75</td>
<td>99 ± 0.25</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>(80µg/ml)</td>
<td>92±1**</td>
<td>90±1</td>
</tr>
<tr>
<td>Guduchi</td>
<td>(125µg/ml)</td>
<td>96±0.89**</td>
<td>94±0.89*</td>
</tr>
<tr>
<td>Canova</td>
<td>(1c/ml)</td>
<td>98±1*</td>
<td>95±2*</td>
</tr>
<tr>
<td>AOIM-Z</td>
<td>(100µg/ml)</td>
<td>92±1</td>
<td>91±1*</td>
</tr>
<tr>
<td>Spirullina</td>
<td>100µg/ml</td>
<td>99±0.71**</td>
<td>98±1**</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>10µg/ml</td>
<td>91±2*</td>
<td>90±2**</td>
</tr>
<tr>
<td>LPS</td>
<td>(15µg/ml)</td>
<td>93±1*</td>
<td>92±2*</td>
</tr>
</tbody>
</table>

Table 4: The culture supernatants were collected from macrophages treated with medium alone or (with LPS, Gallic acid (derivative of Emblica officinalis), guduchi, cisplatin, spirulina, AOIM-Z and canova) and the % viability was checked using MTT assay. Values represent SDs of means for triplicate cultures. Data are representative of three separate experiments (*p<0.05, * *p<0.001).

Table 5: The culture supernatants were collected from macrophages treated with medium alone or (with LPS, Gallic acid (Derivative of Emblica officinalis), Guduchi, Cisplatin, Spirulina, AOIM-Z and Canova) and the % viability was checked using MTT assay. Values represent SDs of means for triplicate cultures. Data are representative of three separate experiments (*p<0.05, * *p<0.001).