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**Evaluation of Effect of Chlorophyllin and Gallic acid
on Macrophage activation**

**Kalpana Pai, Ph.D.
Department of Zoology
Savitribai Phule Pune University
Pune -411007**

ABSTRACT

In the present study we have assessed several BRMs like Gallic acid, Guduchi, Spirulina etc. for macrophage activation potential by some biochemical parameters like production of Nitrite, Cytokines, Lysozyme and percent phagocytosis.

Also assessment of effect of Chlorophyllin and Spirulina on the anti-tumor and anti-leishmanial potential of macrophages was done.

INTRODUCTION

- To overcome disease or disease causing agents, the body initiates an immune response.
- Macrophages are the first line of defense and constitutes important participant in the bi-directional interaction between Innate and Specific immunity.
- Activation of macrophages is a complex phenomenon in which the resident macrophages on interaction with a variety of activating agents, acquire the ability of mediating tumoricidal and microbicidal functions (Hamilton, 2002).
- Analysis of the development of activation is facilitated when the operationally defined stages of activation is characterized using a library of markers for activation (Ma et al, 2003).

- Macrophages are exclusive hosts of *Leishmania donovani* a digenetic protozoan parasite transmitted by sandfly vector.
- The high resistance of the causative agent *Leishmania donovani* to these drugs and the toxicity of the drugs have necessitated the need for discovery of new alternative agents.
- The therapeutic regimens already in clinic are sodium antimony and stibogluconate (SAG & SSG), Amphotericin B and Miltefosine (an oral drug).

- Cancer is one of the foremost killer diseases in both developed and developing countries.
- Since the beginning of this century the role of immune system in controlling the growth of cancer has been widely recognized.
- The potential of the immune system to recognize and kill a wide variety of tumor cells, lays the foundation for the biological therapy, known as the immunotherapy of cancer.
- Plant extracts and plant-derived substances are increasingly becoming popular immunomodulators of choice (Ingolfsson et. al., 1994).

- There are a category of chemicals of diverse origins called as biological response modifiers (BRMs), which modify, host of biological responses in the infected host and help the host in overcoming the deleterious effects caused by *Leishmania donovani*.
- The knowledge of Ayurveda offers immense scope in identifying novel BRMs, present in the Ayurvedic herbs and *Rasayans* (Immuno-modulators), which are useful in human health and disease.

Immunomodulation:

- Various plants identified in the Indian *Ayurvedic* system of medicine display a wealth of pharmacological properties.
- The *Ayurvedic* system of medicine includes various ethnopharmacological activities such as immunostimulation, tonic, neurostimulation, anti-ageing, antibacterial, antiviral, antirheumatic, anticancer, adaptogenic, etc. (Agarwal and Singh, 1999).

MATERIALS AND METHODS

- ***In vitro* culture of macrophages:**

- 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.

- ***In vitro* culture of parasites:**

- Promastigotes of WHO reference strain *Leishmania donovani* Dd8 (MHOM/IN/80/Dd8), kindly provided by Dr. Patole (NCCS, India), were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum at 23°C. For all experiments promastigotes in the late logarithmic phase of growth were used.

- **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.

MATERIALS AND METHODS

- **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 100 μ l under adequate culture conditions. Drugs were added in different concentrations in a volume of 100 μ l in triplicate. The cultures were incubated at 37⁰ 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.
- **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.

Cytokine release:

- PMA-differentiated U937 cells (0.5×10^6 cells/ml) were treated with GA, CHL and SSG for 24 h with LPS as positive control.
- TNF- α and IL-6 concentrations in the culture supernatants was determined using the human TNF- α and human IL-6 ELISA Kits, according to the manufacturer's protocol.

Reactive Oxygen Species (ROS) generation:

- PMA differentiated U937 cells were loaded with 0.02 mM DCFDA, for 30 min and further treated with different drugs for various time points from 0-6 hrs (Dutta *et al.*, 2007).
- DCFDA is hydrolyzed to DCFH in the cell and DCFH is oxidized to form highly fluorescent DCF in the presence of the oxidant.
- ROS production was detected by measuring fluorescence of oxidized DCF at an excitation wavelength of 480 nm and an emission wavelength of 525 nm on a spectrofluorimeter (Powerpack 200; Biotek Instruments, Winooski, Vt.). The DCF fluorescent intensity is directly proportional to ROS production.

- **Superoxide anion (O_2^-) release:**
- Briefly, differentiated 0.5×10^6 cells / ml U937 cells were washed with warm RPMI to remove non-adherent cells. Further, cells were treated with drugs for different time points. Cells were incubated with 0.05% NBT solution in PBS, for 20 min at 37 °C in a humidified chamber.
- The percent of phagocytes with NBT reduced in the cytoplasm was assessed spectrophotometrically in a multi-plate reader measuring the absorbance at 600 nm (Powerpack 200; Biotek Instruments, Winooski, Vt.) (Choi *et al.*, 2006).

- **Effect of Gallic acid and Chlorophyllin on *Leishmania* Promastigotes:**
- Gallic acid (GA) is found in Indian gooseberry: *Emblica officinalis* (Amla). It is a well known anti-oxidant and has been tested for its efficacy against *L. major* amastigotes *in vitro* (Radtke et al., 2004). Though the effect of GA on *Leishmania* amastigotes has been tested, whereas the effect on promastigote forms has not been yet elucidated.
- Chlorophyllin (CHL) is another plant derived immunomodulator; it is a chlorophyll derivative with anticarcinogen and antioxidant activities (Yun et al., 2005). Our results *in vitro* lead us to use GA and CHL as an immunomodulator and an anti-leishmanial compound. This is the first attempt to check the effect of GA and CHL on *Leishmania* promastigotes within administrative doses and determine the mode of action within the promastigotes. In this study, our objective was to evaluate the putative mechanism(s) mediating the leishmanicidal property of GA and CHL.
- Spirulina is an another BRM also showed macrophage activation and yet to be tested for its anti-leishmanial properties.

- **Phagocytic efficiency:**
- FITC labeled *E. coli* K 12 strain BioParticles® were opsonised with 10 % horse serum in PBS for 30 min at 37°C. Differentiated U937 cells were scraped and suspended in RPMI and cell viability was determined using trypan blue dye exclusion. 1×10^6 cells were treated with different drugs for 30 min, 12 hrs. and 24 hrs. at 37°C, after which they were mixed with opsonised FITC-labeled bioparticles in a 1:10 effector to target ratio and incubated at 37 °C for 30 min. Ice-cold PBS with 4% formaldehyde was added to stop phagocytosis. Cells were acquired in a flow-cytometer immediately with trypan blue, to distinguish the fluorescence of surface bound bacteria from phagocytosed bacteria (Coutier *et al.*, 1999). A total of 20,000 U937 cells were acquired for each sample.

- Percent phagocytosis [P%] = macrophages showing phagocytosis x 100%
- Total no. of macrophages
- Phagocytic index [PI] = Total no. of yeast cell phagocytosed x P%
- Macrophages showing phagocytosis

- **Lysozyme Assay:**
- PMA differentiated U937 cells were treated with different drugs for 24 hrs. at 37°C and cell supernatants were collected. To 2.5 ml of dried and killed *Micrococcus lysodeikticus* substrate suspension (0.4 mg/ml) prepared in 0.1 M sodium phosphate buffer (pH 6.2), 0.1 ml cell supernatant was added and absorbance measured at 600 nm on a spectrophotometer. Samples were incubated for 60 min and absorbance measured at 600 nm for the same. The decrease in absorbance was a measure of the enzyme activity and expressed as units/ml culture supernatant/ 5×10^5 cells/ml (Pai *et al.*, 1997).

Tumoricidal assay:

Assay for TNF activity:

- The activity of TNF- α in the culture supernatants of treated and untreated macrophages was measured by a modification of the Mosmann method based on the reduction of MTT (Sigma) to a colored formazan by living cells (Mosmann, 1983).
- Briefly, 2×10^6 L929 fibroblast cells and/or YAC-1 lymphoma cells, in 100 μ l complete medium were grown in wells of a 96 well tissue culture plate in the presence of 1 μ g/ml of actinomycin D and 100 μ l of test culture supernatant. Cell viability was assessed after 24h and 48h of incubation.
- Cells treated with culture supernatants of untreated macrophages were considered as Control. Percent viability and percent cytolysis of these cells was then calculated by the given formula.

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

- Where, E is the absorbance of cells treated with culture supernatants of drug treated and untreated macrophages and C is the absorbance of cells treated with medium alone.
- **Percent cytolysis = (100 - percent viability).**

- **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.

Results

- J774A.1 cells showed 100% viability by Trypan blue dye exclusion test.
- The cells were treated with drugs initially on log scale and then on linear scale.
- Gallic acid at 60 μ g/ml, spirulina and LPS at 10 μ g/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 IC₁₀ values of the drugs.

- It was found that the drugs chlorophyllin being herbal preparations did not show cytotoxic effect on macrophages (J774A.1).
- Their IC_{10} values were 10-15 μ g/ml and the optimum drug concentration for macrophage activation was 10 μ g/ml for each.

Effect of log scale concentrations of drugs on percent viability of J774A.1 cells

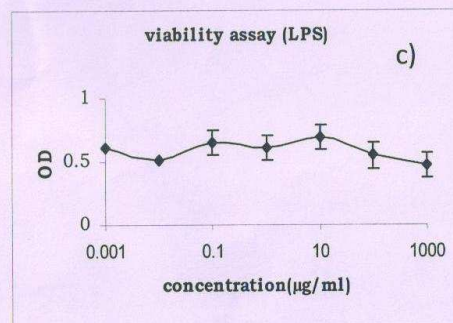
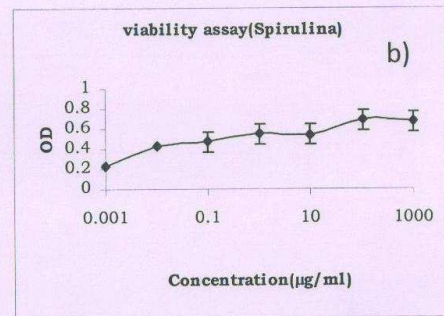
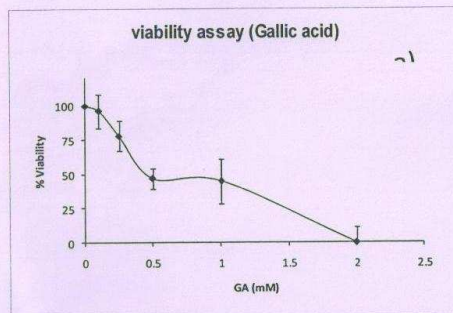


Fig: % viability of macrophages (J774A.1) treated with (a)gallic acid, (b)spirulina, (c)LPS on log scale: The values are mean \pm S.D. and are representative of three independent experiments done in triplicate

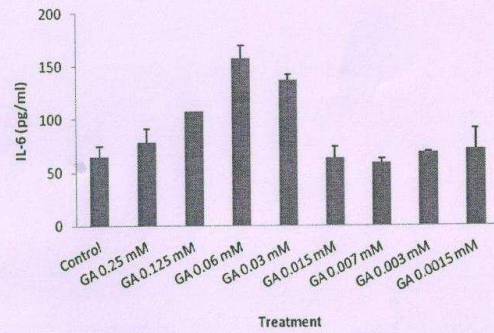
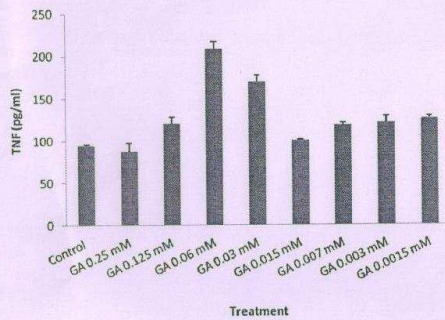
Table: Drug concentrations with their IC₁₀ values

Treatment	Concentration (IC ₁₀)	% viability (±S.E.M.) (24 hrs)	% Viability (±S.E.M.) (48 hrs)
Medium alone	-	99 ± 0.75	99 ± 0.25
Gallic acid	(80µg/ml)	92±1**	90±1
Spirullina	100µg/ml	99±0.71**	98±1**
LPS	(15µg/ml)	93±1*	92±2*

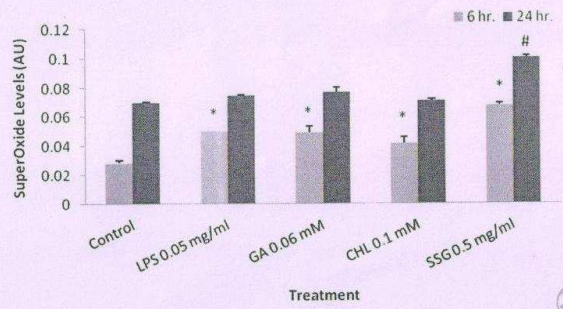
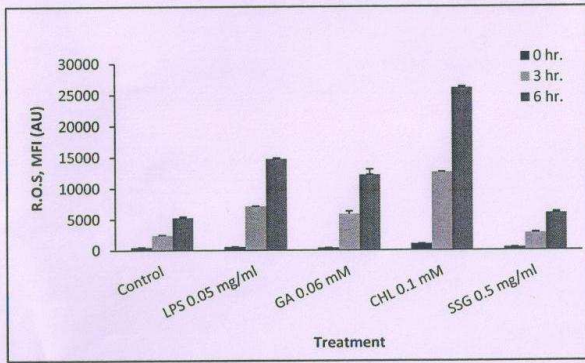
Table: The culture supernatants were collected from macrophages treated with medium alone or (with LPS, Gallic acid (Derivative of *Emblia officinalis*) and Spirulina) and the % viability was checked using MTT assay. Values represent SDs of means for triplicate cultures. Data are representative of three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

Cytokine release:

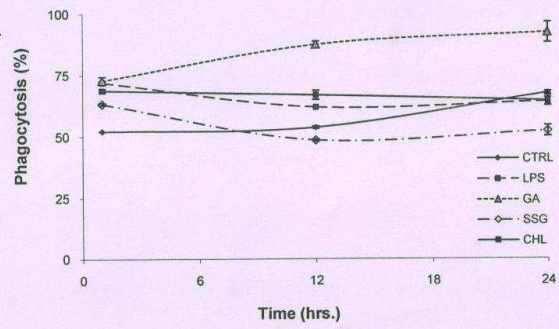
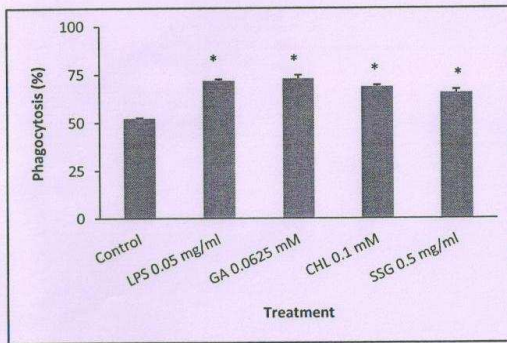
Since the aim of the study was to achieve a Th 1 response, the main cytokines under study were IL-6 and TNF. The levels of these two cytokines in the drug treated supernatants were used as a parameter to determine the optimum drug concentration for macrophage activation. The lowest drug concentrations showing maximum TNF and IL-6 release were selected for all experiments.



R.O.S. & Superoxide Levels



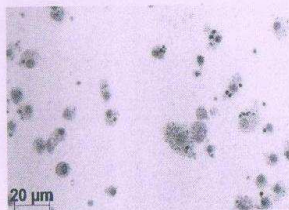
Phagocytosis (% and Index)



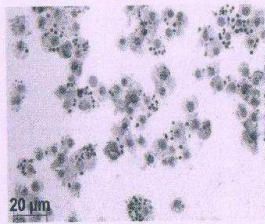
EVALUATION OF CYTOCHEMICAL PARAMETERS OF MACROPHAGE ACTIVATION

Fig:1-6: Phagocytosis with dried and killed yeast cells
Arrows indicating enlarged macrophages with phagocytosed
yeast particles.

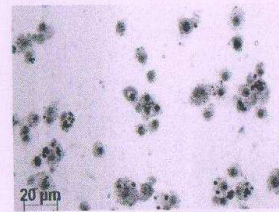
1) medium alone



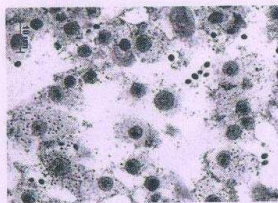
2) guduchi



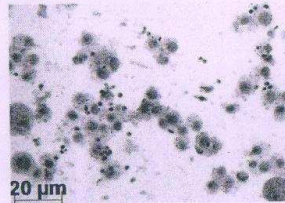
3) LPS



4) gallic acid



5) spirulina



6) canova/anbuta

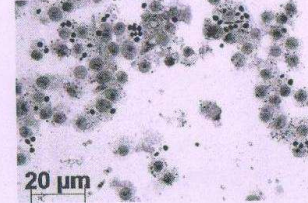


Fig: Effect of log scale concentrations of drugs on nitrite levels of J774A.1 cells

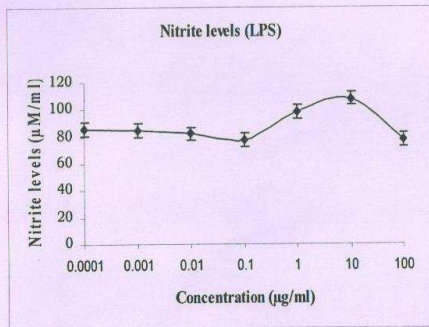
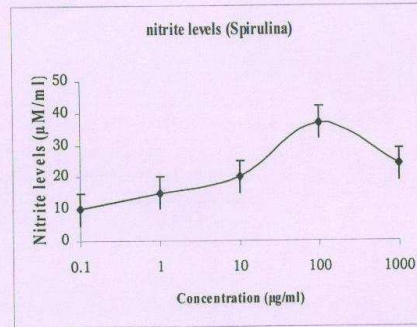
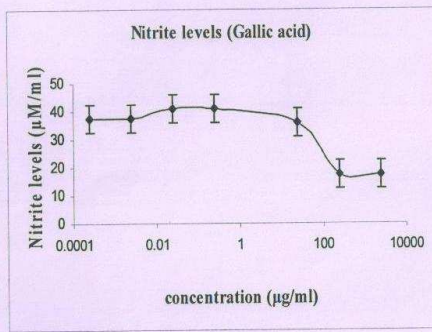


Fig: The culture supernatants were collected from macrophages treated with LPS, gallic acid and spirulina and checked for generation of nitrite. Values represent nitrite contents in μM . Bars show SDs of means for triplicate cultures. Data are representative of three separate experiments. (* $p < 0.05$).

Lysozyme Levels

- The lysozyme levels were measured in units/ml of culture supernatants/2 x10⁶cells/ml. Canova and AOIMZ treatment showed enhanced lysozyme levels especially after 48h treatment.
- Gallic acid, guduchi and cisplatin treated macrophages also showed significantly increased lysozyme levels after 24 and 48h treatment.

Fig18: Macrophages were treated with BRMs, LPS and medium alone for 24h and 48h and then checked for lysozyme release.

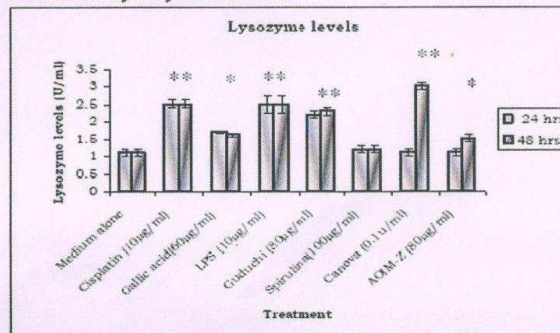


Fig18: Enhanced lysozyme levels were observed as compared to controls (Units/ml of culture supernatant/2 x10⁶ cells/ml. Absorbance measured at 600nm wavelength). (*p<0.05, **p<0.001).

***In vitro* cytotoxicity measurement**

- The inhibitory effect of GA on *L. donovani* promastigotes was verified by the MTT assay, where an increase in the amount of formazan crystals indicates cell viability and the decrease represents cell inhibition.
- The observed IC₉₀ values of GA and HePC for *L. donovani* Dd8 promastigotes were 1 mM and 0.2 mM, respectively.
- The IC₉₀ value of CHL was determined by Trypan blue stain exclusion and found to be 1 mM; the cell cytotoxicity of promastigotes to GA and HePC was also confirmed by Trypan blue stain exclusion (data not shown).

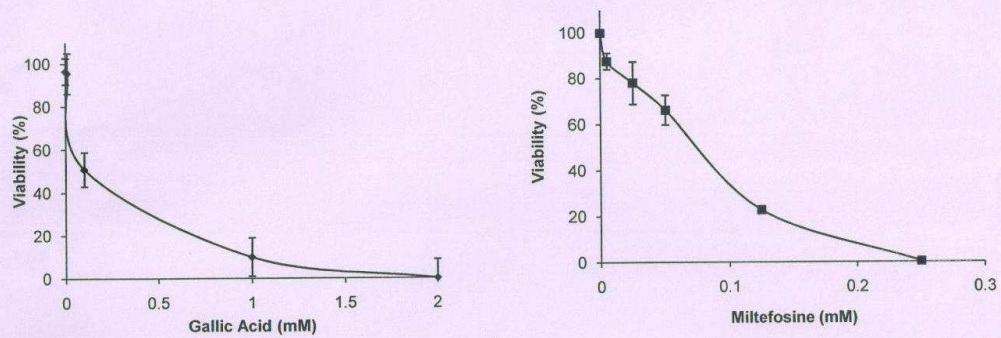


Figure: Antileishmanial activity of GA and Miltefosine (HePC). Promastigotes (MHOM/IN/80/Dd8; 5×10^5 per well) were incubated with increasing concentrations of GA (0–2 mM) or HePC (0–0.25 mM) for 24 h and the MTT assay was performed as described in Methods. Each point corresponds to the mean \pm SE of at least three experiments in triplicate. The cytotoxicity was confirmed by Trypan blue staining (data not shown).

- The log scale result explains the effect of drugs at their 10 fold concentrations. It was found that the drugs chlorophyllin 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 up to 10 percent and showed negligible cytotoxicity of macrophages (Fig).
- Their IC_{10} values were 10-15 μ g/ml and the optimum drug concentration for macrophage activation was 10 μ g/ml for each.

Table: Drug concentrations with their IC₁₀ values

Treatment	Concentration (IC ₁₀)	% viability (±S.E.M.) (24 hrs)	% Viability (±S.E.M.) (48 hrs)
Medium alone	-	99 ± 0.75	99 ± 0.25
Gallic acid	(80µg/ml)	92±1**	90±1
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LPS	(15µg/ml)	93±1*	92±2*

Table: The culture supernatants were collected from macrophages treated with medium alone or (with LPS, Gallic acid (Derivative of *Emblica officinalis*) and Spirulina) 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).

Lysozyme Levels

- The lysozyme levels were measured in units/ml of culture supernatants/2 x10⁶cells/ml.
- Gallic acid, guduchi and cisplatin treated macrophages also showed significantly increased lysozyme levels after 24 and 48h treatment.

Fig18: Macrophages were treated with BRMs, LPS and medium alone for 24h and 48h and then checked for lysozyme release.

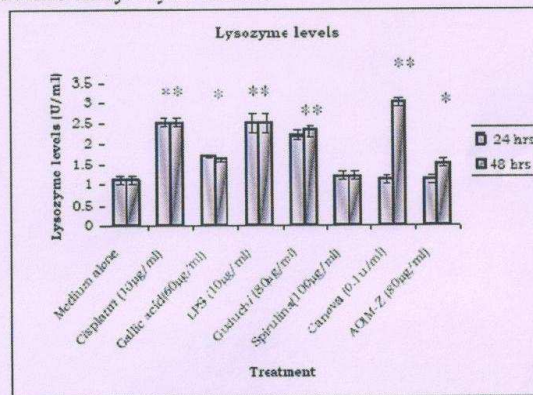
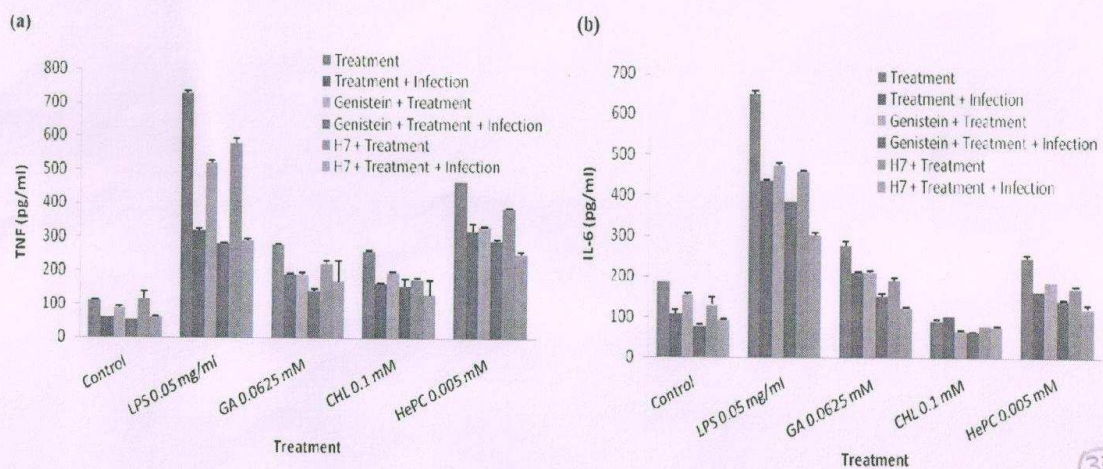


Fig18: Enhanced lysozyme levels were observed as compared to controls (Units/ml of culture supernatant/2 x10⁶ cells/ml. Absorbance measured at 500nm wavelength). (*p<0.05, **p<0.001).

- **Table1:** Effect of BRMs on the percent phagocytosis and phagocytic index of J774A.1 cells.
- The values given in the table are mean \pm standard deviation and represent three independent experiments done in triplicate. (*p<0.05, **p<0.01).

Treatment (conc)	Percent Phagocytosis (P%)		Phagocytic Index (PI)	
	24h	48h	24h	48h
Galic acid 60 μ g/ml)	76 \pm 1*	75 \pm 1*	3.8 \pm 0.25*	3.78 \pm 0.25*
Guduchi(80 μ g/ml)	89 \pm 2**	90 \pm 4**	4.45 \pm 0.12*	5 \pm 0.75**
AOIM-Z(80 μ g/ml)	65 \pm 2*	65 \pm 1*	3.25 \pm 0.15*	3.25 \pm 0.15*
Spirulina(100 μ g/ml)	90 \pm 4**	88 \pm 3**	4.5 \pm 0.1*	4.35 \pm 0.1*
Anbuta(0.1c)	72 \pm 2*	75 \pm 1*	3.6 \pm 0.3*	4.6 \pm 0.3*
Cisplatin(10 μ g/ml)	65 \pm 3*	67 \pm 1*	3.25 \pm 0.25*	3.25 \pm 0.25*
LPS(10 μ g/ml)	90 \pm 2.5**	87 \pm 2**	4.5 \pm 0.37*	4.5 \pm 0.37*
Medium alone	58 \pm 1	55 \pm 2.75*	2.9 \pm 0.2	3.01 \pm 0.2

- Induced TNF levels by GA and CHL that could aid in the clearance of the intracellular parasites.
- Cytokine pattern in presence of enzyme inhibitors. 0.5×10^6 PMA differentiated U937 cells were treated with BRMS for 24 hours, in presence of Genistein and H7. Cells were later co-incubated with GFP transfected *Leishmania* promastigotes for 24 hours. Supernatants collected were tested for TNF and IL-6 release by sandwich ELISA. Data are representative of at least 3 experiments.



Generation of Reactive Oxygen Species (ROS) in promastigotes

- Upon interaction with the reactive oxygen species like H_2O_2 , hydroxyl ions and superoxide ions generated within the cell, the dye gets converted to a non-permeable fluorescent derivative. The fluorescence of H_2DCFDA is proportional to the amount of ROS generated within the cells (Dutta et al., 2007).
- With an increase in time an increase in ROS generation was observed in GA as well as CHL treated *Leishmania* promastigotes, upto 6 hours. However, the ROS measured after 24 hours was not in the detectable range (data not shown). GA is known to possess pro and anti-oxidant properties (Sakagami H and Satoh K, 1997).

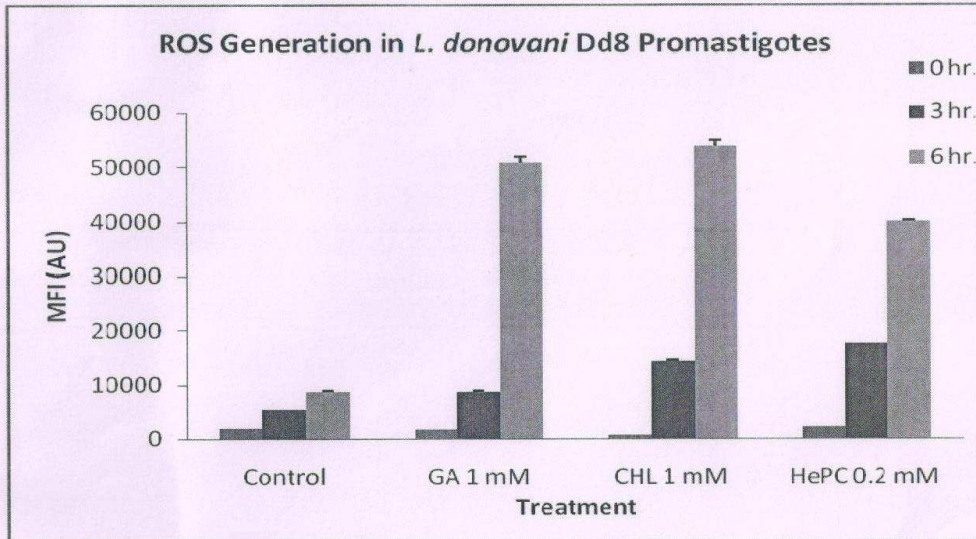


Figure: R.O.S. generation in *L. donovani* Dd8 promastigotes. The R.O.S. activity in *L. donovani* Dd8 promastigotes was measured by the H2DCFDA dye, for 0-6 hours in the absence or presence of 1 mM GA or 1 mM CHL or 0.2 mM HePC. Results are representative of at least 5 independent experiments performed in triplicates. ($p < 0.001$)

DISCUSSION

- 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 & U937 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).
- Macrophages 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 (Gallic acid at 60 μ g/ml, Spirulina at 100 μ g/ml) showed maximum viability of macrophages as compared to medium alone, thereby proving that the drugs were not cytotoxic to the cells. The macrophage were treated with different drugs and the inhibitory concentrations (IC_{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.

- Three Ph.D., one M.Phil, and two M.Sc. Project students were guided .
- The publications are listed below:
- **Title:** Effect of *Aerva sanguinolenta* (Linn.) Blume on *Leishmania donovoni* parasite causative organism of visceral leishmaniasis.
Maheshkumar Kharat, Jagdeep Kour, Kalpana Pai.
Natural Products: Recent Advances, Pages: 257-275.
- **Title:** Gallic Acid induces (ROS) generation and apoptosis like death in *Leishmania donovoni* promastigotes *in vitro*.
Nutan A. Jadhav, T. B. Porval and Kalpana Pai.
(Submitted for publication)
- **Title:** Effect of *Tinospora cordifolia* [guduchi] on the Phagocytic and Pinocytic Activity of Macrophages *in vitro*. (Communicated)
Priti More and Kalpana Pai.
- **Title:** Involvement of tyrosine-specific protein kinase and protein kinase C in J774A.1 macrophage functions activated by *Tinospora cordifolia* (guduchi) *in vitro*. (Communicated)
Priti More and Kalpana Pai.

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