Immunomodulatory effect of Palmitoyl Tuftsin on the antileishmanial efficacy of Miltefosine
CHAPTER 4

IMMUNOMODULATORY EFFECT OF PALMITOYL TUFTSIN ON
ANTILEISHMANIAL EFFICACY OF MILTEFOSINE

4.1 Introduction

In the present work, combination of palmitoyl tuftsin (p-tuftsin) and miltefosine (sub curative dose) was explored for the treatment of experimental visceral leishmaniasis in murine model. Tuftsin, a tetrapeptide (Thr-Lys-Pro-Arg), is an integral part of the Fc portion of the heavy chain of the leukophilic immunoglobulin G (residues 289–292) and is released physiologically as the free peptide after enzymatic cleavage. It is known to augment the phagocytic response of normal and stimulated macrophages assessed both for phagocytosis mediated via non-specific receptors (Bar-Shavit et al., 1979). These interesting features of tuftsin, coupled with its low toxicity, make the peptide an attractive candidate for immunotherapy (Fridkin & Najjar, 1989). It was also able to activate murine peritoneal macrophages to express nitric oxide (NO) synthase and to produce NO which is able to kill the amastigotes of the intracellular protozoan parasite L. major (Cillari et al., 1994). The immunostimulatory activity of tuftsin and its effect against microorganisms is further increased upon its incorporation in liposomes, after attaching a sufficiently long hydrophobic anchor of fatty acyl residue (palmitoyl) using spacer at its C-terminus. This lipopeptide is commonly referred as palmitoyl tuftsin (p-tuftsin) and has been shown to express potent immunostimulatory activity both in vitro as well as in vivo models. The liposomal formulation of p-tuftsin has been found to be superior as compared to its free form. The incorporation of p-tuftsin into liposomes is also quantitative by its lipophilic nature and form stable liposomes. The importance of tuftsin-bearing liposomes in treatment of macrophage-based infections is due to its targeted delivery (Guru et al., 1989; Agrawal and Gupta 2000). In view of these interesting features of p-tuftsin, the potential of a combination therapy using free as well as liposomal p-tuftsin with sub curative dose of miltefosine have been explored. The lower or sub curative dose of miltefosine is used to minimize the toxicity of the drug during treatment. To establish the effect of this
combination therapy on the immune functions of the host, immunological parameters viz. Th1/Th2 cytokines, production of ROS, RNS, H$_2$O$_2$ and phagocytosis were also monitored. Protective efficacy of free p-tuftsin alone and its effect on the therapeutic efficacy of miltefosine at sub-curative was also observed.

4.2 Materials and Methods

4.2.1 Parasite
The WHO reference strain of *L. donovani* (MHOM/IN/80/Dd8) was used in the present study (see Chapter-3, Section-2.1).

4.2.2 Extraction of Peritoneal Exudate Cells from Mouse
Powdered RPMI medium (Sigma Chemical Co., USA) medium was used for the extraction of PECs. Composition and preparation of medium has been already described in Chapter-3, Section-2.1.

4.2.3 Animals
BALB/c mice (18-20 g, age-6 weeks) of both sexes were used for the study. All the experiments were conducted in compliance with the Institutional Animal Ethics Committee guidelines for use and handling of animals. Animals were bred in the National Animal Laboratory Centre, housed in CSIR-CDRI, Lucknow. For experimental studies, animals were kept in plastic cages (38×27 ×13 cms) with husk as bedding. Four to six animals were assigned to each cage to avoid overcrowding. Throughout the study, the animals were housed in climate-controlled (23 ± 2°C; RH: 60%) and photoperiod-controlled (12hr light-dark cycles) animal quarters. They were fed standard rodent pellets diet supplemented with grain and had free access to drinking water.

4.2.4 Compounds

4.2.4.1 Palmitoyl Tuftsin (p-Tuftsin)
Palmitoyl tuftsin obtained from Medicinal Chemistry and Process Division of CSIR-CDRI, Lucknow, India. For *in vivo* experiments, p-tuftsin was dissolved in 100% DMSO and further diluted with deionized water to a final DMSO concentration of 8%-10% prior to administration mice (Wenzler *et al.*, 2009).
4.2.4.2 Miltefosine
Miltefosine was purchased from SynphaBase AG (Switzerland). For the *in vivo* part of the study, miltefosine was dissolved in deionized water.

4.2.4.3 Liposome Preparation of p-Tufts
Unilamellar liposome of p-tufts was prepared from phosphatidylcholine (15 µM), cholesterol (7.5 µM), dissolved in chloroform: methanol (2:1, v/v) in a flat bottom tube. The solvent was removed by slow evaporation under nitrogen in deposition of a thin film of lipid on the tube surface. The tube was dried to remove any trace of solvent. The lipid film was then hydrated and dispersed in a solution of appropriate quantity of p-tufts (7–8% by PC weight) in Tris-buffered saline (10 mM Tris containing 150 mM NaCl, pH 7.4) and processed by probe sonication (W-220) to get the desired preparation of liposomal tufts (Singhal *et al.*, 1984).

4.2.4.4 Estimation of Liposome Intercalated p-Tufts
The amount of p-tufts entrapped in the liposomes was estimated by nanoparticle-based biobarcode amplification assay (BCA method). Briefly, the liposomes (given volume) were lysed with a Triton X-100 solution and incubated with the mixture of solutions A and B of BCA reagent. The absorbance of the colored complex formed was measured at 600 nm, and p-tufts content was determined using a standard curve of p-tufts plotted in the presence of Triton X-100 (Khan *et al.*, 2007).

4.2.5 Procedure used in *in vitro* system
For assessing prophylactic efficacy of immunomodulator against intracellular amastigotes, microscopical Giemsa staining method was used.

4.2.5.1 Antiamastigote Activity in Mice by Microscopical Giemsa Staining Method
On day 1, peritoneal macrophages from pre-treated mice were harvested by lavage in sterile conditions. The abdominal surface of mice was liberally sprayed with 70% ethanol and then 4-5 mL of ice cold RPMI -1640 medium containing 4 µg/mL gentamicin and 5% EDTA was introduced inside the abdominal cavity by 10 ml disposable syringe. Peritoneal fluid was collected and centrifuged in cold centrifuge at 1000 rpm for 10 min. The cells were washed and re-suspended in complete medium (RPMI + 10% FBS). Cells were counted using Neubauer hemocytometer and adjusted with complete medium to get desired
concentration needed for the experiment. More than 95% macrophage cells found to be viable by 0.04% trypan blue dye exclusion method were used for various experiments. A population of $1 \times 10^6$ cells/mL/100µL/well was suspended in complete RPMI medium and layered in 16 well chamber slides (Nunc). Chamber slides were then incubated for 24h in 5% CO₂ incubator at 37°C. On day2 cells were infected with amastigotes harvested from spleen of infected hamsters immediately prior to use as they will not survive for long periods. The protocol used to isolate amastigotes from spleen is described as under.

**Protocol: Harvesting Leishmania Amastigotes from Donor Hamsters**

After terminal anaesthesia, the hamster was liberally sprayed with 70% alcohol until all the fur was soaked. In a sterile cabinet the hamster was placed on its side with the head towards the left so that spleen was on the left hand side of the hamster. An incision was made at a point below the ribcage and the fur was dissected away, without penetrating the body cavity. The exposed area was sprayed with 70% alcohol. The spleen was exposed and carefully removed using blunt-ended forceps and scissors. Care was taken not to drop the spleen on the fur of the hamster. After removing excess connective tissue, the organ was then placed in a sterile tube with a few mL of complete RPMI medium and placed on ice. During the dissection a very small piece was cut at the end, the cut surface blotted and impression smears made on glass slides. The weight of the spleen was determined. When the slides dried impression smears was fixed with 100% methanol and stained with 20% Giemsma stain for 45 minutes. The stained slides were evaluated microscopically using 100X oil immersion. The number of amastigotes per 500 host cell nuclei was counted. The total amastigote burden of the organ was calculated by the Stauber equation (Stauber et al., 1958).

**The Stauber Equation:**

$$\text{Number of amastigotes/organ} = \frac{\text{No. of amastigotes} \times \text{weight of organ (mg)} \times 10^5}{\text{No. of cell nuclei}}$$

In a sterile cabinet weighed spleen was transferred to a sterile, glass homogenizer and pulped to get homogenous solution. The homogenate was transferred to a sterile 50mL centrifuge tube and topped with plain 45mL RPMI 1640. This was then centrifuged, in a
cold centrifuge, at 800-1000 rpm for 10 minutes to separate RBCs and small tissues. The supernatant was transferred to another sterile 50 mL tube and centrifuged at 3100 rpm for 15 minutes. The supernatant was discarded and the resulting pellet was re-suspended in 1mL cold complete RPMI and kept on ice till counting.

These amastigotes were added to each well of 16 well chamber slides in ratio of 1:7 and incubated for 24h in 5% CO₂ incubator at 37°C. After incubation on day3, all slides were fixed with 100% methanol and stained with 20% Giemsa stain for 45 minutes. The number of amastigotes per 500 cell nuclei was counted in each well and the parasitic burden was expressed in terms of the number of amastigote per 100 cell nuclei.

Drug activity (percent inhibition) was determined by comparing amastigote count of treated and untreated wells by the general formula:

\[
\text{Percent Inhibition} = \frac{N-n}{N} \times 100
\]

Where,

N is average number of amastigotes per 100 cell nuclei of untreated well and \(n\) is average number of amastigotes per 100 cell nuclei of treated well.

4.2.6 Procedures used In Vivo System

4.2.6.1 Preparation of Infective Inoculum and Infection in Mice

Hamsters with heavy infection (50-60 days old) were used for harvesting amastigotes and infecting mice. Procedure for preparation of infective inoculum has already been described in Chapter-3, Section-4.2. The inoculum was adjusted to contain \(2 \times 10^7\) amastigotes in 0.1 mL of suspension for giving infection in BALB/c mice intravenously through the lateral tail vein (Yardley et al., 2002)

4.2.6.2 Assessment of Established Infection in BALB/c Mice

Establishment of infection was checked in liver tissue by sacrificing the infected mice after 7 days post infection.

4.2.6.3 Assessment of Parasitic Burden

A small portion (5 mm size) of liver was cut during euthan. Piece was first blotted on filter paper (Whatman No.1) to remove excess of blood and then impression smears were prepared on glass microslides. The smear was quickly air dried, fixed in absolute methanol
and stained with Giemsa dye (20% v/v in PBS, pH 7.2) for 45 min. These stained smears were observed under oil immersion (100X). The number of amastigotes was counted in 500 liver cell nuclei and the parasitic burden was expressed in terms of the number of amastigote per 100 cell nuclei.

4.2.6.4 Biochemical Analysis for Measuring Production of ROS, RNS and H₂O₂
For measuring the production of ROS, H₂O₂ and RNS metabolites in experimental mice, procedures adopted were same as described in Chapter-3, Section-3.2.

4.2.6.5 Phagocytosis Assay
A flow-cytometry based method was used to study the phagocytic activity of macrophages harvested from experimental mice. Procedure was same as described in Chapter-3, Section-3.2.

4.2.6.6 Evaluation of Cytokine Production
Serum samples from animals of treated and untreated control groups were analysed for various cytokines [Tumour Necrosis Factor-α (TNF-α), IFN-γ, IL-12, IL-6, IL-10] by BD OptEIATM ELISA Kits (BD Biosciences) in accordance with the manufacturer’s instructions, where TNF, IFN-γ and IL-12 are Th1 cytokines and IL-10 comes in the category of Th2 cytokine. IL-6 is a pro-inflammatory cytokine (De Larcoet et al., 2001). Each kit contains capture antibody and detection antibody (specific for each cytokine). The wells coated with specific capture antibody were incubated overnight at 4°C. Thorough washing was done at each step. Coated wells were blocked with assay diluent and incubated at room temperature (RT) for 1 hour. The standard and serum sample were added to their respective wells. After incubation for two hours at RT working detector (detection antibody + conjugate) was added to each well and incubation was done for 1 hour at RT. In next step after addition of substrate solution to each well, incubation was done for 30 min in dark at RT. At last stop solution was added to each well. Optical densities of samples were read at 450 nm within 30 min with λ correction 570 nm on microplate spectrophotometer (Power WaveTM X52, BioTek Instruments, USA).
4.2.7 Experimental Plan

*In vivo experiments*

- **Mice**
- Inoculation of 2 x 10^7 amastigotes per mouse intravenously.

Day 7
- Confirmation of infection establishment by sacrificing two mice
- Grouping of animals (5-6 animals per group)
- Initiation of drug treatment

Day 14
- Mice euthanized
- Observation in liver impression smears

4.2.7.1 Antileishmanial Efficacy Evaluation in Mice

Infected BALB/c mice were randomly sorted into groups of 5-6 animals each. Same numbers of mice were kept in untreated control group. Mice were dosed by ip (intra peritoneal) and po (*per os*) routes at 7 days p.i. (post infection) according to individual drug schedule. Animals were sacrificed on day 3 post treatment (day 14 p.i.). Impression smears of livers were prepared, methanol fixed, and stained with 10% Giemsa stain in PBS. The number of amastigotes per 500 liver cell nuclei was determined. The percent inhibition (PI) was calculated for all drug-treated groups in relation to untreated group (Sane et al., 2010).

4.2.7.2 Optimization of p-Tufts in and Miltefosine Dose Regimens against *L. donovani*/Mouse model

For dose optimization, infected animals were divided into different groups. Each group consisting of 5-6 infected animals in two replicates. The p-tufts in was administered in doses viz. 30, 60 and 120 µg/animal by ip route for dose optimization. The dose of 60 µg/animal was found to be most appropriate. Therefore, liposomal encapsulation of this dose of p-tufts in was carried out and when administered, it delivers 60µg of p-tufts in per animal. Miltefosine was given at doses ranging from 1.25 mg/kg to 20 mg/kg for 5 days by po route to select the sub curative doses. The sub curative doses selected were 2.5 and 5 mg/kg for further experiments of combinations and 2.5 mg/kg dose was used for biochemical & immunological assays and in *in vivo* prophylactic studies.
4.2.7.3 Evaluation of Free p-Tufts in and Liposomal Form alone & in Combination with Sub-Curative Dose of 2.5 mg/kg of Miltefosine

Seven groups of mice each consisting of 5-6 animals in two replicates were used for these experiments. Mice of Group I received free p-tufts (60 µg/animal) by ip route, Group II received free p-tufts (60 µg/animal) + miltefosine (2.5mg/kg x 5d), Group III received liposomal p-tufts (60 µg/animal of p-tufts), Group IV received liposomal p-tufts (60 µg/animal of p-tufts) + miltefosine (2.5mg/kg x 5d), Group V and VI received sub-curative (2.5 mg/kg) and curative miltefosine (20 mg/kg) po for 5 days respectively, Group VII receiving placebo served as controls for all the groups. The percent inhibition was calculated for all drug-treated groups in relation to a non-treated group.

4.2.7.4 Evaluation of Liposomal p-Tufts in alone & in Combination with Sub-Curative Dose of Miltefosine (5mg/kg)

Five groups of mice each consisting of 5-6 animals in two replicates were used for these experiments. Mice of Group I received liposomal p-tufts (60 µg/animal of p-tufts) by ip route, Group II received liposomal p-tufts (60 µg/animal of p-tufts) + miltefosine (5mg/kg x 5d), Group III and IV received little higher sub-curative dose (5 mg/kg) and curative dose (20 mg/kg) of miltefosine by oral route for 5 days respectively, Group V receiving PBS served as controls for all the groups. The percent inhibition was calculated for all drug-treated groups in relation to a non-treated group.

4.2.7.5 Prophylactic Studies

The dose of 60 µg/animal for p-tufts in, found to be most appropriate in therapeutic efficacy evaluation, was used in prophylactic studies.

4.2.7.5.1 In Vitro Prophylactic Role of Free p-Tufts in

Mice were divided into 2 groups (2-4 mice/group). Group I was administered with free p-tufts (60µg/animal) by ip route on day-7. Group II receiving only placebo (deionized water) served as control (uninfected mice). On day 0, peritoneal exudates cells (PECs) from animals of both the groups were harvested with sterile RPMI-1640 medium. The cell suspension was centrifuged at 1000 rpm for 10 min. at 4°C and the pellet was suspended in RPMI-1640 containing 10% heat inactivated FBS so as 1 mL contains 1x10^6 cells. Each well of 16-well chamber slides (Nunc, Denmark) was seeded with 100 µL volume of this
cell suspension and slides were incubated at 37°C in a CO₂ incubator. They were infected with amastigotes (extracted from the spleen of infected hamster) in ratio of 1:7 (macrophage: parasites) at day 1. After 24 h of infection (at day 2), chamber slides were washed twice with PBS (pH 7.2 ± 0.2) to remove non-phagocytized amastigotes, air-dried, fixed in methanol, Giemsa stained and examined under 100 X objective of light microscope. The infection was assessed by counting the percentage of infected macrophages and average number of amastigotes in each macrophage in comparison of control (Sharma et al., 2004).

4.2.7.5.2 In Vivo Prophylactic Role of Free p-Tuftsins alone & in Combination with Sub-Curative Dose of Miltefosine (2.5 mg/kg)

For in vivo experiments, mice (18-20 g) were allocated in six groups each consisting of 5-6 mice. On day 0, mice of all the groups were challenged with splenic amastigotes and detailed description of each Group was as follows.

- **Group I** - Free p-tuftsins (60µg/animal) on day -7 of infection (day0)
- **Group II** - Free p-tuftsins on day −7 followed by a booster dose of similar amount on day +7 of infection (day0).
- **Group III** - Miltefosine at 2.5 mg/kg, po for 5 days on day +7 of infection (day0).
- **Group IV** - Free p-tuftsins at day- 7 and miltefosine at day+7 of infection (day0)
- **Group V** - Free p-tuftsins at day-7 and also co-administered a booster dose of similar amount with miltefosine (2.5 mg/kg) on day +7 of infection (day0).
- **Group VI** - Deionized water, served as untreated control.

Animals were sacrificed on day 14 post infection and percent inhibition (PI) was calculated for all drug-treated groups in relation to untreated group.

4.2.7.6 Alterations in Biochemical and Immunological Responses

Following assays were used.

- Reactive oxygen species production assay using inhibitor pentoxifylline (PTx)
- Hydrogen peroxide production assay using inhibitor sodium azide (NaN₃)
- Reactive Nitrogen species production assay using inhibitor N-nitro-L-arginine methyl ester (L-NAME)
- Phagocytosis assay
Evaluation of Th1/Th2 cytokines in *L. donovani*/mouse model

### 4.2.8 Statistical Analysis

Results are presented as mean ± S.D. of two experiments and analysis of data is carried out by Bonferroni’s multiple comparison tests and Dunnett’s multiple comparison tests. Differences with *P* < 0.05 were considered significant. Sub-curative dose was determined by probit analysis (Finney, 1971).

### 4.3 Results

#### 4.3.1 Dose Optimization of p-Tufts in

Of the various doses of p-tufts in tested the best antileishmanial efficacy was witnessed at a dose of 60 µg/animal, single dose, ip (33.90 ± 2.69% inhibition in parasite multiplication). This was followed by gradually decreasing efficacy with 120 and 30 µg/animal doses namely 21.56 ± 3.96 % and 10 ± 5.8% inhibition in parasite multiplication, respectively (Fig.4.1). Based on the results, 60µg/animal, single dose, ip was selected for combination trial.

![Fig.4.1 Dose optimization of p-tufts in *L. donovani*/mouse model. *L. donovani* infection was given to BALB/c mice (2x10^7 amastigotes/animal) intravenously. Mice were dosed at 7 days post infection with single dose of various dosages of p-tufts in by intraperitoneal route. Mice were sacrificed 3 days after the completion of treatment. Mean](image)
P.I. ± S.D. was calculated by comparing parasitic burden of treated groups to control animals.

### 4.3.2 Dose Optimisation of Miltefosine

Miltefosine was evaluated at various doses ranging from 1.25mg/kg to 20 mg/kg for five days by po route to select the sub curative doses. Parasite inhibition observed at 20mg/kg was 97.02 ± 2.10% followed by 88.6 ± 3.70%, 72.10 ± 1.20%, 49.60 ± 2.90% and 33.50 ± 4.10% at 10, 5, 2.5, 1.25 mg/kg doses respectively (Fig.4.2). Sub curative doses for combination trials were selected as 2.5mg/kg and 5mg/kg.

![Graph showing percent inhibition with different doses of miltefosine.]

**Fig.4.2** Dose optimization of miltefosine in *L. donovani/mouse model*. *L. donovani* infection was given to BALB/c mice (2x10^7 amastigotes/animal) intravenously. Mice were dosed at 7 post infection with various dosages of miltefosine by oral route for five days. Mice were sacrificed 3 days after the completion of treatment. Mean P.I. ± S.D. was calculated by comparing parasitic burden of treated groups to control animals.

### 4.3.3 Combination Therapy of p-Tuftsin with Sub-Curative Dose of 2.5 mg/kg of Miltefosine

Results of combination therapy of free and liposomal p-tuftsin with miltefosine have been presented in Fig.4.3. Free p-tuftsin showed an efficacy of 33.90 ± 2.69% which was moderately enhanced to 47.97 ± 3.06% by liposomal encapsulation (*P*<0.05). Parasitic inhibition of miltefosine alone at 2.5mg/kg was 49.60 ± 4.2%. However, when free p-tuftsin given with miltefosine (2.5mg/kg), parasite inhibition increased from 49.60 ± 4.2 % to 65.93 ± 1.30 % (*P*<0.01) and liposomal p-tuftsin further enhanced the efficacy up to
81.42 ± 2.00% ($P<0.001$). Efficacy of the curative dose (20 mg/kg for 5 days) of the miltefosine was 98.80 ± 2.67%.

**Fig. 4.3** Combination therapy of free and liposomal p-tuftsins with sub-curative dose of miltefosine (2.5 mg/kg) in *L. donovani*/mouse model. Mean percent inhibition ± S.D. was calculated by comparing parasitic burden of treated groups to control animals. Significance among different groups was calculated by Bonferroni’s multiple comparison tests. Significance: (I vs II – $P<0.01$; I vs III- $P<0.05$; II vs IV- $P<0.001$), (V vs II – $P<0.01$; V vs IV- $P<0.001$).

### 4.3.4 Combination Therapy of Lipo p-Tuftsins with Sub-Curative Dose of 5 mg/kg Miltefosine

Efficiency of combination was also evaluated with the liposomal p-tuftsins and 5 mg/kg (for 5 days) dose of miltefosine. Alone liposomal p-tuftsins showed 48.00 ± 3.0% whereas alone miltefosine showed 72.10 ± 1.90% parasitic inhibition. Efficacy of miltefosine was markedly increased from 72.10 ± 1.90% to 93.40 ± 2.0% ($P<0.01$) when liposomal p-tuftsins was co-administered with it which is very close of the curative dose (20 mg/kg for 5 days) of the miltefosine (98.80 ± 1.20%) (Fig.4.4).
Fig. 4.4 Combination therapy of liposomal p-tufts in with sub-curative dose of 5 mg/kg of miltefosine. Mean PI ± S.D. was calculated by comparing parasitic burden of treated groups to control animals. Significance among different groups was calculated by Bonferroni’s multiple comparison tests. Significance: (I vs III – P < 0.001; II vs III - P < 0.01).

4.3.5 In Vitro Prophylactic Efficacy of Free p-Tufts in

In vitro prophylactic studies showed that the macrophages activated with free p-tufts in, acquired considerable resistance to Leishmania parasites, as only 58.33 ± 7.9% of the macrophages contracted infection (P < 0.01) as compared to the untreated controls (93.60 ± 11.90%). Besides, the number of parasites in this group were also low (230 ± 5.6 amastigotes/100 macrophages, P < 0.01) in comparison to untreated control (347 ± 11.90 amastigotes/100 macrophages).

Fig. 4.5 In vitro prophylactic efficacy of p-tufts in. Percent infectivity of peritoneal macrophages of mice pre-treated with free p-tufts in (60 µg/animal, single dose, ip), 7 days before to L. donovani infection. The comparisons were made with the untreated group. ■ - Number of amastigotes /100 macrophages. ★★★ - % infected macrophages. Values are mean ± S.D. of two experiments. Significance among different groups was calculated
by Bonferroni’s multiple comparison tests. Significance: (Ia vs Ib – P< 0.01**); (IIa vs IIb - P< 0.01##).

4.3.6 In Vivo Prophylactic Efficacy of Free p-Tufts in alone & in Combination with Sub-Curative Dose of Miltefosine (2.5 mg/kg)

In vivo results of prophylactic potential of p-tufts in (60µg/animal/single dose/ip) have been shown Fig.4.6. Group of mice pre-treated with p-tufts in on day7 prior to challenge with amastigotes resisted the infection to the tune of 28.60 ± 2.68% whereas those having treatment with p-tufts in on day−7 and a booster dose of similar amount on day+7 of infection (day 0), exhibited significant reduction in parasitic load [40.45 ± 1.90% (P<0.05)].

To observe the effect of p-tufts in induced protection on the therapeutic efficacy of miltefosine, the sub-curative dose of 2.5 mg/kg by po route for 5days of later was used. At which miltefosine showed 44.82 ± 4.2% efficacy. The therapeutic efficacy was found significantly enhanced (+12.98% difference, P<0.05) in mice pre-treated with p-tufts in at day-7 (57.80 ± 1.3%). However, animals primed with p-tufts in (day-7) having miltefosine with similar amount of booster dose of p-tufts in at day+7 of infection (day0) showed significantly less establishment of parasites and consequently the therapeutic efficacy of miltefosine was improved showing 77.80 ± 2.5% (P<0.01) inhibition in comparison to the group treated with miltefosine alone.

![Experimental Groups](image-url)

**Fig.4.6 In vivo prophylactic efficacy of p-tufts in.** Efficacy (% inhibition) of p-tufts in administered prophylactically on day −7 and +7 of leishmania challenge (on day 0). Mean PI ± S.D. was calculated by comparing parasitic burden of treated groups to control
animals. Significance among different groups was calculated by Bonferroni’s multiple comparison tests. Significance: (I vs II – $P<0.05$); (III vs IV– $P<0.05$; III vs V – $P<0.01$).

### 4.3.7 Alterations in Biochemical Responses

The results have been displayed in Fig.4.7 (a, b). PECs of mice administered with free p-tuftsins showed a moderately significant NO, ROS and H$_2$O$_2$ production ($P<0.05$; $P<0.05$; $P<0.05$ respectively). Cells of miltefosine treated group also showed significant NO production ($P<0.01$) and a moderate ROS and H$_2$O$_2$ production ($P<0.05$; $P<0.05$ respectively). PECs of the group treated with the combination of free p-tuftsins with miltefosine showed enhanced production of ROS, NO and H$_2$O$_2$ ($P<0.01$; $P<0.001$; $P<0.01$ respectively). Cells of liposomal p-tuftsins treated group also showed significant production of all the metabolites ($P<0.01$). However, when it was combined with miltefosine, remarkable up gradation in the production of NO, ROS and H$_2$O$_2$ was observed in the cells treated with this combination ($P<0.001$; $P<0.001$; $P<0.001$ respectively).

![Biochemical assay for production of ROS and H$_2$O$_2$. Inhibition of ROS and H$_2$O$_2$ in response to different treated groups against untreated (control) animals. Significant difference in inhibition was assessed by Dunnett’s multiple comparison tests. Significance for ROS:(I vs II – $P<0.05$; I vs III – $P<0.05$; I vs IV– $P<0.01$; I vs V– $P<0.01$](image-url)
Significance for $\text{H}_2\text{O}_2$: (I vs II – $P<0.05$; I vs III – $P<0.05$; I vs IV – $P<0.01$; I vs V – $P<0.01$; I vs VI – $P<0.001$).

Fig. 4.7.b Biochemical assay for production of RNS. Inhibition of RNS in response to different treated groups against untreated (control) animals. Significant difference in inhibition was assessed by Dunnett’s multiple comparison tests. Significance for RNS: (I vs II – $P<0.01$; I vs III – $P<0.05$; I vs IV – $P<0.001$; I vs V – $P<0.01$; I vs VI – $P<0.001$).

4.3.8 Phagocytosis Assay

Peritoneal exudate cells of untreated control mice showed lowest phagocytic index (PI, 5.74 ± 2.3). Cells of group treated with miltefosine exhibited moderately increased phagocytic index {PI, 17.75±1.30 ($P<0.01$)}. Cells of mice treated with free p-tufts in also showed phagocytic index of 11.9 ± 1.2 ($P<0.05$). Free p-tufts in when co-administered with miltefosine, enhanced the index to 20.60 ± 1.5 ($P<0.001$) of the cells. Cells of group treated with liposomal p-tufts in gave phagocytic index of 16.75 ± 1.5 ($P<0.01$) and when given with miltefosine there was a remarkable increase in index up to 26.06 ± 1.9 ($P<0.001$), which was almost equal to phagocytic index of cells of normal uninfected animals (PI, 29.32 ± 1.8) (Fig.4.8).
**Fig.4.8 Phagocytosis study in treated, untreated, uninfected normal mice.** The fluorescence of stimulated and un-stimulated cells of each group compared and significance of activity of different treated groups was assessed against untreated (control) animals by Dunnett’s multiple comparison tests. Significance: (I vs II – $P<0.01$; I vs III – $P<0.05$; I vs IV – $P<0.01$; I vs V – $P<0.01$; I vs VI – $P<0.001$; I vs VII – $P<0.001$).

## 4.3.9 Cell Mediated Immune Response

Results have been displayed in Fig.4.9. Serum samples of different treated groups were used for the exploring cell mediated immune response. Two fold rise in TNF-α (Tumour Necrosis Factor-α) & IL-12 production and a moderate rise in IFN-γ production were observed after liposomal encapsulation of p-tuftsins. Free p-tuftsins when combined with miltefosine, a twofold rise in IFN-γ and moderate rise in TNF-α were observed. Significant enhancement in the production of IFN-γ and TNF-α was witnessed in animals co-administered with liposomal p-tuftsins and miltefosine. Level of IL-10 was highest in untreated control group. Briefly, we can say that the combination therapy involving free and liposomal p-tuftsins with miltefosine increased Th1 (TNF-α, IFN-γ, IL-12) cytokine levels and down regulated Th2 (IL-10) cytokine. Animals treated with miltefosine alone showed moderately enhanced Th1 response. Untreated control group, however, did not show any increase in Th1 response whereas production of IL-10 was increased.
Fig. 4.9 Cytokine assay in treated and untreated mice. Serum samples from animals of treated and untreated control groups were analysed for various (TNF-α, IFN-γ, IL-12 and IL-10) cytokines by using anti mouse monoclonal antibodies (specific for respective cytokines). Absorbance was measured on micro-plate reader.

4.4 Discussion
Visceral leishmaniasis (VL) caused by the parasite *L. donovani* is a potentially fatal disease. Available drugs for VL therapy are often associated with toxic side effects and, require prolonged treatment duration and efficacy is also compromised due to suppression of immune function associated during the course of infection. Miltefosine is only promising orally bioavailable anti-leishmanial drug but its efficacy is seriously compromised by teratogenicity as well as a long terminal half life. It has the potential to increase the risk of development of resistance. To overcome these problems efforts are needed to develop combination therapy of miltefosine with effective immunostimulating agent where decrease of parasitic burden and simultaneous enhancement of adaptive immunity can be achieved. Tuftsin is capable of stimulating white blood cells (monocytes, macrophages, and neutrophils) and exhibits a wide spectrum of biological activities; notably enhances phagocytosis, immune response, bactericidal, tumoricidal and antifungal activities (Wardowska *et al.*, 2007).

It is well documented that immune system synergistically aid to the therapeutic efficacy of antiparasitic drugs (Doenhoff *et al.*, 1991; Berger & Fairlamb, 1992). Keeping this in
mind, we have explored the adjunct effect of p-tufts (free and liposomal) on the efficacy of sub curative doses of miltefosine using *L. donovani*/BALB/c model. Results clearly showed that liposomal encapsulation enhanced antileishmanial efficacy of p-tufts. Co-administration of liposomal p-tufts with sub curative doses of miltefosine showed a better inhibitory effect than free p-tufts+ miltefosine, free p-tufts or miltefosine alone. The efficacy of combination of 5 mg/kg dose of miltefosine with single dose of lipo p-tufts was comparable with the efficacy of the curative dose of the miltefosine (20 mg/kg).

Results of immunological assays suggested that p-tufts potentiated the cell mediated immunity, evident from significant rise in Th1 cytokines and down regulation of Th2 cytokine, IL-10. This is in agreement with the earlier reports of Dey *et al* (2007) and Bhattacharjee *et al* (2009) which stated that Th1 responses from cytokines are protective for VL whereas expression of IL-10 increases during Leishmania infection. The reason for carrying out the experiments of immunomodulatory effect of p-tufts in mice is due to non-availability of cytokines of hamsters commercially. In biochemical assays, liposomal p-tufts combined with miltefosine resulted in remarkable production of NO, ROS and H₂O₂. Significant increase in phagocytosis index was also observed which reveals that the cells treated with this combination group deviate towards normal behaviour.

Palmitoyl-tufts also play role in innate immune response and provide a prophylactic approach to prevent VL. This is also reported by Khan *et al* (2005). Since in VL, cure is the combinatorial effect of drug and immune status of the host, the rationale approach towards antileishmanial chemotherapy would be to potentiate the immune functioning of the host to extract desired results. Towards this direction, miltefosine at low dose in combination with the prophylactic treatment of p-tufts was evaluated. The efficacy of miltefosine in mice pre-treated with p-tufts (at day -7 and a booster dose of similar amount on day +7) enhanced significantly.

These findings reveal that the cure of VL is dependent on not only the direct parasite-killing activity as well as Th1- mediated protective cell-mediated immunity which is significantly achieved with the use of low-dose treatment of miltefosine in combination with single dose of liposomal p-tufts. It can also prevent pathogen to attack and establish
them and helpful for miltefosine, providing it low burden of parasite to kill and cure VL effectively.

Thus, the risk of development of drug resistance against miltefosine can be resolved through using low doses of it and liposomal p-tuftsins (single-dose) in combination and also provide a promising alternative for cure of leishmaniasis, with a pronounced transformation of the host immune response.