"Mitigation effect of Leptin and Neem leaf extract in Experimental Visceral leishmaniasis"

A thesis Submitted to University of Hyderabad for the award of

Doctor of Philosophy (Ph.D)

By

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"DECLARATION"

I, **Dayakar Alti**, hereby declare that this thesis entitled **"Mitigation effect of Leptin and Neem leaf extract in Experimental Visceral leishmaniasis"** submitted by me is based on the results of the work done under the guidance and supervision of **Dr. Radheshyam Maurya** at Department of Animal Biology, School of Life Sciences, University of Hyderabad. The work presented in this thesis is original and plagiarism free. I also declare that no part or in full of this thesis has been submitted previously to this University or any other University or Institution for the award of any degree or diploma.

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"CERTIFICATE"

This is to certify that this thesis entitled **"Mitigation effect of Leptin and Neem leaf extract in Experimental Visceral leishmaniasis"** is a record of bonafide work done by **Mr. Dayakar Alti**, a research scholar for Ph.D. programme in the Department of Animal Biology, School of Life Sciences, University of Hyderabad, under my guidance and supervision. The thesis has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

Dr. Radheshyam Maurya

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ABBREVIATIONS	
^{o}C	Degree centigrade/Degree Celsius
µg	Micro gram
µl	Micro litre
µM	Micro molar
µm	Micro meter
nm	Nano meter
mg	Milli gram
ml	Milli litre
ng	Nano gram
pg	Pico gram
%	Percentage
$	Dollar
Ψ_m	Mitochondria membrane potential
λ	Wave length
Φ	Macrophage
ACTH	Adreno-corticotropic hormone
AD	Anno Domini
AIDS	Acquired immunodeficiency syndrome
Akt	Protein kinase B
ATCC	American Type Culture Collection
ANOVA	Analysis of variance
APC	Allophycocyanin
BCG	Bacillus Calmette Guerin
BMDM	Bone marrow derived macrophages
BMI	Body Mass Index
BSA	Bovine serum albumin
B.W	Body weight
CBA	Cytokine bead array
CCAAT/EBP	Multiple C/enhancer binding protein
CD	Cluster differentiation
cDNA	Complementary deoxyribonucleic acid
CL	Cutaneous leishmaniasis
CFSE	Carboxy fluorescein-diaceate succinimidyl ester
CMNR	Committee on Military Nutrition Research
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CO_2	Carbon dioxide
ConA	ConcanavalinA
CREB	Cyclic AMP response element-binding protein
CSF	Cerebrospinal fluid
C_T	Threshold cycle
CTLA-4	Cytotoxic T-lymphocyte antigen-4
C3b	Complement protein 3b
DC	Dendritic cell

DCPP	Disease control priorities project
Dd8	*Leishmania donovani* strain MHOM/IND/80/Dd8
Diet-A	Nutrition diet
Diet-D	Malnutrition diet
DMSO	Dimethyl sulfoxide
DTH	Delayed type hypersensitivity
EAF	Ethyl acetate fraction
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamineacetate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular-signal regulated kinase
FACS	Fluorescence activated cell sorting
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
$FeCl_3$	Ferric chloride
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage- colony stimulating factor
gp63	Glycoprotein63
GRE	Glucocorticoid response element
Grz-A	Granzyme-A
H_2DCFDA	2,7 dichlorodihydrofluoresceindiacetate
H_2DCF	2,7 dichlorofluorescein
H-E	Hematoxylin-Eiosin
HEPES	4-(2-hydroxyethyl)-1-piperazineetahnesulfonic acid
HIV	Human Immunodeficiency Virus
HPA	Hypothalamus-pituitary adrenal axis
HPO	Hypothalamus-pituitary ovarian axis
H_3PO_4	Phosphoric acid
HRP	Horse radish peroxidase
H_2SO_4	Sulphuric acid
IC_{50}	Half inhibitory concentration
iC3b	Inactive complement protein 3b
IFN-γ	Interferon-γ
IFPRI	International Food Policy Research Institute
IgG	Immunoglobulin-G
IL	Interleukin
IL-10R	Interleukin-10 receptor
iNOS	Inducible nitric oxide synthase
IP-10	IFN-γ induced protein 10
ISPF	α-isonitrosopropiophenone
JAK	Janus kinase
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl

	benzimidazolylcarbocyanine
LC-MS/MS	Liquid chromatography-tandem mass spectrophotometry
LDU	Leishman-Donovan Units
LIF	Leukemia inhibitory factor
LH	Luteinizing hormone
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LST	Leishman skin test
M199	Medium-199
MAPK	Mitogen-activated protein kinase
MCL	Mucocutaneous leishmaniasis
MCP-1	Monocyte chemotactic protein-1
mDC	Myeloid dendritic cell
MFI	Mean fluorescence intensity
mg	Milligram
MHC	Major histocompatibility
MNA	Mini-Nutritional Assessment
$MnCl_2$	Manganese chloride
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
myd88	Myeloid differentiation factor 88
m/z	Mass/charge ratio
$NaHCO_3$	Sodium bicarbonate
NIAID	National Institute of Allergy and Infectious Diseases
NK	Natural killer
NO	Nitric oxide
NOS2	Nitric oxide synthase2
NRC	National Research Council
Ob	Obese
Ob-R	Leptin receptor
OD	Optical density
OIE	Office International des Epizooties
PBMCs	Peripheral blood mononuclear cells
PBS-T	Phosphate buffer saline-Tween
PD-1	Programmed death protein-1
PDB	Protein data bank
pDC	Plasmacytoid dendritic cells
PE	Phycoerythrin
PEM	Protein-energy malnutrition
PI	Propidium iodide
PI3K	Phosphatidyl inositol 3-kinase
PKDL	Post kala-azar dermal leishmaniasis
PMA	Phorbol 12-myristate 13-acetate
PSG	Promastigote secreting gel
Q-TOF	Quadrupole time-of-flight

RBC	Red blood cell
RIPA	Radioimmunoprecipitation assay
ROR	RAR-related orphan receptor
RPMI	Roswell park memorial institute
ROS	Reactive oxygen species
RT	Room temperature/retention time
RT-qPCR	Real time-quantitative polymerase chain reaction
SbV	Pentavalent antimonial
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error mean
SLA	Soluble *Leishmania* Antigen
SLC11A1	Solute carrier 11A1
TBS-T	Tris-buffer saline-Tween
TGF-β	Transforming growth factor-β
TGs	Triglycerides
Th	T-helper
THP-1	Human monocytic leukemia cell line
TLR	Toll-like receptor
TMB	Tetra methyl benzene
TNF-α	Tumor necrosis factor-α
Tregs	T-regulatory cells
Tris-HCl	Tris-hydrochloric acid
UNICEF	United Nations International Children's Emergency Fund
US	United states
UV	Ultra violet
VL	Visceral leishmaniasis
WHO	World Health Organization

INTRODUCTION

1.1. History

Leishmaniasis has the history of more than 2000 years which dates back to the first century AD. The name leishmaniasis coined after Dr. William Leishman; a Glasgwegian doctor had been working with the British Army in Calcutta, India. For the first time, he discovered ovoid shaped bodies in the spleen tissue of a British soldier who was already suffering from long-lasting, low-grade fever, anemia, muscular atrophy and swelling of the spleen. He developed a stain in 1901 to detect *Leishmania* amastigotes that reside in the spleen tissue. He called this illness as "Dum Dum fever" based on the town name, and his findings published in 1903. Contemporarily, Charles Donovan also noticed a similar kind of symptoms in other kala-azar patients and published his findings after a few weeks of Leishman discovery. The amastigotes appeared in the tissue smears were officially called as Leishman-Donovan bodies and the causative parasite named as *Leishmania donovani*. Both Leishman and Donovan had been classified the genus *Leishmanias* by linking with kala-azar **(http://leishman.cent.gla.ac.uk./william.htm)**.

1.2. Leishmaniasis

It is a vector-borne infectious disease caused by an intracellular obligate protozoan parasite belongs to the genus *Leishmania*. The disease is being prevailed by the transmission of the parasite via 30 different species of *Phlebotomine* sand flies. These insect vectors transmit infective metacyclic flagellated promastigotes into the mammalian host, and they transform into non-flagellated amastigotes and replicate intracellularly in mononuclear phagocytes **(Pearson and Sousa 1996, Sacks and Kamhawi 2001)**. This amastigotes stage of the parasite associated with disease pathogenesis and various clinical manifestations; ranged from self-healing skin lesions to a life-threatening visceral form that depends on the host immune background and nutritional status. Leishmaniasis is being considered as one of the top neglected tropical diseases, which affects the poor people living in under poverty developing countries. It is endemic in 88 countries, among which 72 are developing countries on the planet. It is mostly prevalent in tropical and subtropical regions of Asia, Africa, the Mediterranean coast, Southern Europe and South and Central America. Approximately, 350 million people are harboring at the risk of developing leishmaniasis and 1.5–2 million new cases are being accounted annually **(Alvar et al., 2012)**. Typically, the parasite transmission is anthroponotic (human to vector to human) in the Indian subcontinent and Asia. However, it is zoonotic (animal to vector to human) in Africa, Europe and the Americas', where the canines and rodents are the parasite reservoirs **(Lipoldová et al., 2006)**.

1.2.1. Cutaneous leishmaniasis (CL)

It is the frequent form of the disease leishmaniasis and being represented for 50-75% new global cases per annum. It is mostly self-curing and asymptomatic. It also referred as "Delhi boil" or "Kandahar sore" or "Lahore sore". There are several species of *Leishmania* have been reported worldwide which causes CL (*L. tropica*, *L. aethiopica*, *L. major*, *L. mexicana*, *L. guyanensis*, *L. amazonensis* and *L. braziliensis*). The annual burden of CL is about 1–1.5 million according to the World Health Organization (WHO), and 90% of CL cases occur only in seven countries such are Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria **(Alvar et al., 2012, Reithinger et al., 2007)**. Despite the variation in clinical features, the development of an ulcerative skin lesion is a hallmark for all CL cases, accounted by numerous parasites in ulcers (Fig.1a and 1b). The variation in the clinical features is because of different *Leishmania* species causes CL. However, the disease begins as an erythematous papule at the site of the sandfly bite, which characterized by inoculation of parasites on exposed parts of the body. Then the papule or nodule slowly expands into a destructive ulcer **(Reithinger et al., 2007)** with the distinctive border. These ulcers seem to be painless until the patients encountered by secondary infections i.e. bacterial or fungal infection. The development of multiple lesions on legs, arms, face and other body parts rationales for severe disability in CL patients which usually incriminated by the nest of sand flies attack at various parts of the body. Despite the cure, it leaves a long-lasting ugly scar, sometimes throughout life.

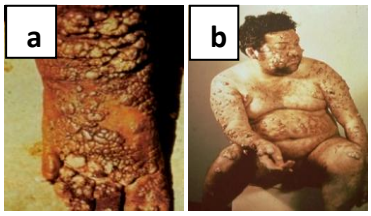


Figure 1

a) http://www.your-doctor.net/dermatology_atlas/rwx/rwx/Disseminated_leishmaniasis.jpg
b) http://www.rainforesteducation.com/terrors/leishmaniasis/cutaneousleishmaniasis2web.jpg

1.2.2. Mucocutaneous leishmaniasis (MCL)

It is the New World disease form, usually caused by *L. braziliensis* and *L. panamensis*. About 90% of MCL cases registered from Bolivia, Brazil and Peru. In MCL, metastasis of the parasites into mucosal tissues can take place by lymphatic or hematogenous dissemination. MCL is an advanced state of the cutaneous lesion that takes a few months to years for this transformation. In MCL, the lesions can lead to the partial or complete destruction of the mucosal tissues that include nasopharyngeal region and adjacent tissues, causes for facial

deformities (Fig.2a and 2b). These devastating features are responsible for the rejection of MCL patients by the society. Despite the established mechanism of pathogenesis of visceral and cutaneous leishmaniasis, it is quite unclear in the MCL. The host genetic background profoundly determines the impact of disease outcome.

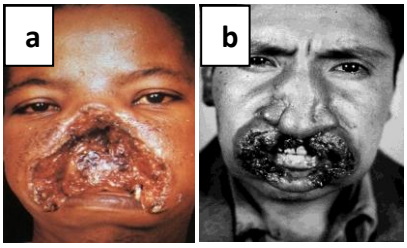


Figure 2

a) http://i.imgur.com/kyeJ28K.png,
b) https://web.stanford.edu/class/humbio103/ParaSites2006/Leishmaniasis/images/MC3.jpg

1.2.3. Visceral leishmaniasis (VL)

It is familiar as kala-azar, most severe clinical form characterized by irregular bouts of fever, substantial weight loss, enlargement of the spleen and liver, cachexia, pancytopenia and anemia (Fig.3a and 3b). If not treated, it causes 100% mortality within 2-years of the first appearance of the disease symptoms **(WHO, 2010)**. Globally, about 500,000 new cases being aroused every year, of which 90% cases only from India, Sudan, Nepal, and Bangladesh **(Desjeux et al., 1998)**. It is mostly prevalent in many tropical and subtropical regions of the world and becomes a serious public health problem in developing countries. According to Office International des Epizooties (OIE), VL is one of the top 10 infectious diseases and in concern for WHO it is top 2 neglected tropical diseases. It is rapidly spreading in sub-Saharan Africa and South America as a co-infection with human immunodeficiency virus (HIV) is another challenge.

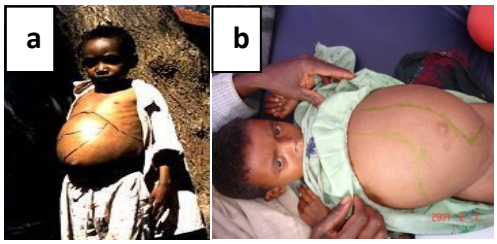


Figure 3

a) http://web.stanford.edu/class/humbio103/ParaSites2003/Leishmania/leishmaniasis_boy2.
b) http://www.who.int/campaigns/world-health-day/2014/photos/leishmaniasis3.jpg?ua=1

1.2.4. Post kala-azar dermal leishmaniasis (PKDL)

It is considered to be as the sequel of the kala-azar since partial treated or untreated VL patients used to develop this clinical form after long-lasting unsuccessful treatment. It also

reported in the patients without VL history and symptoms include; hypopigmented macules, papules, nodules or facial erythema (Fig.4a and 4b). PKDL human patients are assumed to be parasite reservoirs for inter-epidemic periods of VL. Although, *L. donovani* infection manifests it, the pattern of disease is widely varied in India and Sudan patients. In case of Indian variant, it is characterized by the gradual enlargement of nodules on the face and black patches on the skin rather than ulcers in 5-10% of VL patients within 2-3 years, whereas, these nodules often progress to ulceration in 50% of VL cases of African variant within six months. Moreover, the involvement of nerves is often seen in African variety, but rare in Indian patients **(Salotra et al., 2003)**. Histological examinations demonstrated that aggregation of inflammatory cells in PKDL patients is characterized by macrophage infiltrates or epithelioid granulomas **(Rathi et al., 2005)**.

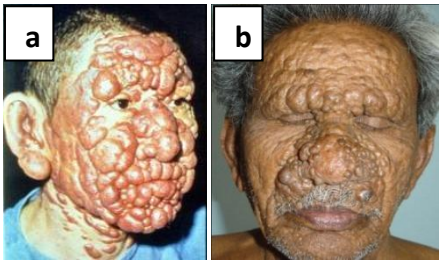


Figure 4

a) http://o.quizlet.com/pYPtmrhl3AEsZ6AQc6uBCA_m.jpg
b) http://www.ijdvl.com/articles/2010/76/2/images/ijdvl_2010_76_2_213_60549_u1.jpg

1.3. Epidemiology of VL

It is endemic in 90 countries of the tropics, subtropics and the Mediterranean basin (Fig.5). It has not reported in Australia and Antarctica. The rural areas located below 600 meters of sea level with heavy annual rainfall, abundant vegetation, high percentage of humidity (~70%), optimal temperature between 38°C-15°C, with a diurnal variation of <7°C; subsoil water, and alluvial soil are the most favorable environmental circumstances for incidence of VL. In South Asia, it is mostly affecting the agricultural field workers and the rural people harboring in houses constructed with mud walls and earthen floors which are close to the other livestock, small bodies of water and vegetation. It could also affect the people living in outskirts of few cities with inadequate sanitation of drinking water. The major risk factor for kala-azar in villages is sleeping on the ground outside the houses. Whereas, sleeping on the cot and using of bed nets was shown to be protective. However, the quality of bed net and its frequency of usage, along with the effectiveness of sandfly repellents all determine the degree of protection. The above mentioned environmental factors are more prone to sandfly survival by facilitating breeding sites, diurnal resting places, and

optimal humidity. Other possible risk factors for kala-azar; prevalence of illiteracy, poor socioeconomic background, lack of hygienic environment and nutrition and the host genetic background **(Blackwell, 1996)** are potentates the infective sandfly bite into the development of life threatening VL. In Brazil, the susceptibility for zoonotic VL in humans and dogs influenced by nutritional status and host genetic background, i.e. the gene locus that has been specified for a default immune settings which increase the risk of infection to kala-azar. These two are assumed to be the high-risk factors for South Asian VL *per* s*e* **(Bern et al., 2010)**.

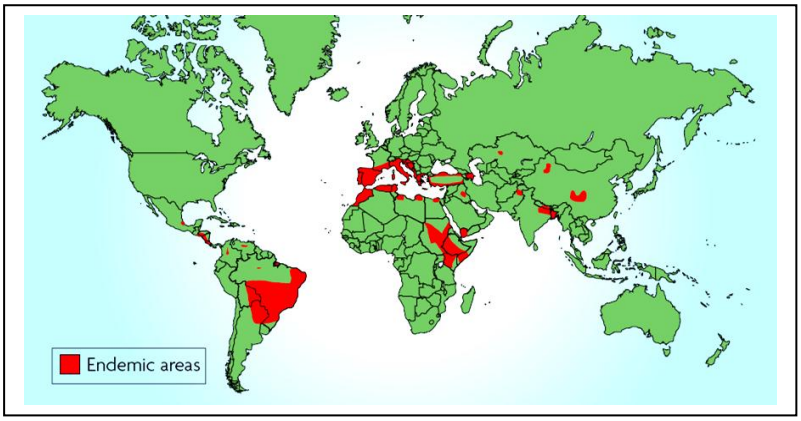


Figure 5: The distribution of VL worldwide. The majority cases occur in just six countries — Bangladesh, Brazil, Ethiopia, India, Nepal and Sudan **(Chappuis et al., 2007)**.

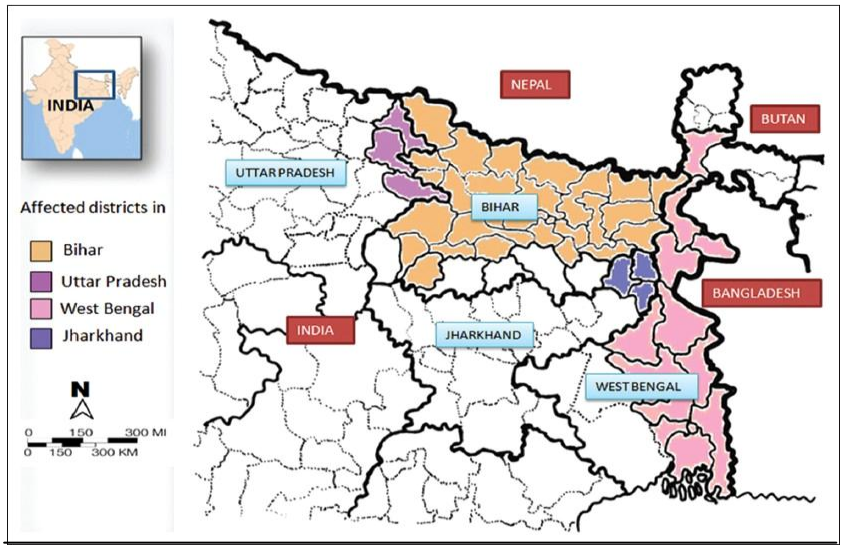


Figure 6: Hot zones of VL in India
http://www.tropicalparasitology.org/articles/2014/4/1/images/TropParasitol_2014_4_1_10_129143_u1.jpg

The VL eradication program is being implemented since 2005 in Bangladesh, India and Nepal with the target of limiting the VL cases to less than 1/10000 populations by 2015. The hot zones of VL in India depicted in Fig.6.

1.4. Classification

Kingdom: Protozoa
Phylum: Sarcomastigophora
Class: Zoomastigophora
Order: Kinetoplastida
Family: Trypanomastidae
Genus: *Leishmania*

1.5. Morphology

The parasite usually exists in digenetic morphology, which accompanied by promastigote and amastigote forms of invertebrate and vertebrate hosts respectively.

1.5a. Promastigote form

Typically, promastigote stage of the parasite develops in the midgut of sand fly. They are motile; spindle shape, having one long flagellum and the size is about 12-20µm × 1.5-3.5µm diameters (Fig.7a). The plasma membrane coated with lipophosphoglycan (LPG) and highly cross-linked sub-pellicular microtubules. A specialized invagination that not subtended by microtubules in the plasma membrane at the anterior end of the parasite is called the flagellar pocket. It is the principal site for exocytosis and endocytosis through the membrane mediated by specialized organelles like Endoplasmic reticulum (ER) and Golgi apparatus localized around the flagellar pocket. The flagellar pocket holds the base of the flagellum and helps in the movement of the parasite anteriorly (Fig.7b left) **(Mc Conville et al., 2002a).**

1.5b. Amastigote form

This form of the parasites is smaller than promastigotes, appears in ovoid shape and also subtended by sub-pellicular microtubules. It has significantly reduced flagellum that enclosed within the pouch, non-motile and proliferated within the phagolysosome of mammalian macrophages (Fig.7b right). During the process of differentiation from promastigotes to amastigotes, the former one undergoes remarkable changes in its secretory and endocytic compartments using their relative abundance and intracellular distribution. In addition, tissue-derived amastigotes piled up with minimalist ER enveloped by a nuclear membrane and Golgi apparatus. Whereas, Golgi apparatus to be dispersed or even tough to detect in serial sections of most of the patients **(Waller and McConville, unpublished data, 1998)**. However, the lysosomal compartment is distinctively organized in the amastigote form of all *Leishmania* species.

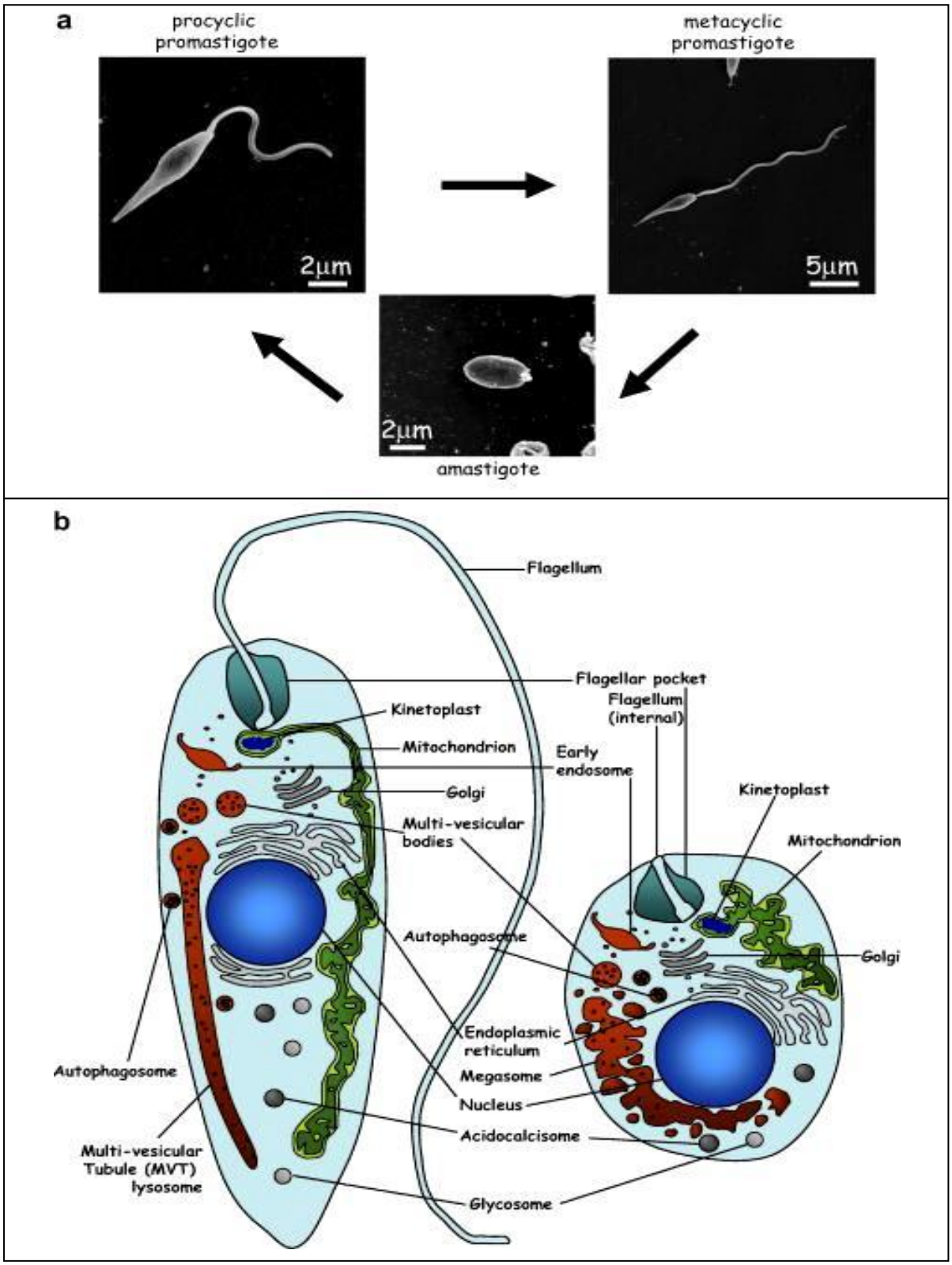
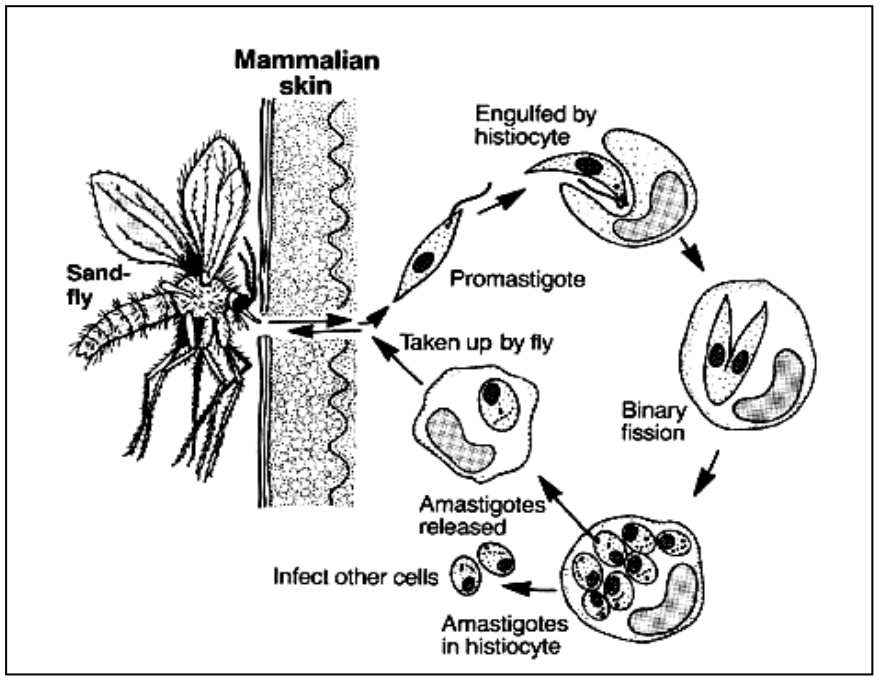


Figure 7: Changes in the parasite shape during the life-cycle of *Leishmania*. (a) Scanning electron microscope images of the *L. major* life-cycle stages; procyclic and metacyclic promastigote, and amastigote (b) Schematic representation of the intracellular organelles in *Leishmania* promastigote (left) and amastigote (right) forms. The flagellar pocket considers the anterior end of the cell (Besteiro et al., 2007).

1.6. Life cycle

Parasite exists in dimorphic forms and cyclically transmits between insect vector and mammalian host. The parasites transmitted via the bite of an infected female sand fly vector of genus *Phlebotomus* (Old World) or *Lutzomyia* (New World) (Fig.8). In the sandfly vector, it takes 8-20 days to complete development after ingestion through blood meal.



http://www.vetnext.com/fotos/Leis1.jpg

Figure 8: The *L. donovani* life cycle. When sandfly (female phlebotomine) takes the blood meal from the mammalian host, it injects the infective metacyclic promastigotes into the host mammalian skin. At the site of infection, various phagocytic cells (dendritic cells and macrophages) can be recruited, and they engulf these promastigotes through phagocytosis. The phagosome fuses with lysosome and forms the phagolysosome, within this phagolysosomal environment the promastigotes differentiate into the amastigotes. These amastigotes replicate through binary fission and eventually leads to the burst of the host cell. Then amastigotes reach the bloodstream and infect other macrophages by disseminating through lymphatic and vascular systems, finally infiltrates into the liver, spleen and bone marrow causes for hepatosplenomegaly and anemia. When sandfly takes the blood meal from these infected hosts, amastigotes can be ingested and transform into procyclic promastigotes followed by infective metacyclic promastigotes **(Lipoldová et al., 2006)**.

The infective stage parasites subsequently invade into different cell types that have encountered. In fact, neutrophils rapidly recruit at the bite site followed by dendritic cells, fibroblasts and mainly the macrophages. Typically, parasites engulfed by different antigen presenting cells (i.e. dendritic cells and macrophages) by a process called phagocytosis. In the parasitophorous vacuole, the metacyclic promastigotes transform into amastigotes followed

by the release of lysosomal content into vacuole that does not affect the amastigotes. During transformation, metacyclic promastigotes turned into ovoid and non-motile amastigotes (3-7 μm in diameter) **(Debrabant et al., 2004)** by losing their flagella. Notably, the amastigotes divide multiple times and attach to the walls of parasitophorous vacuole using its posterior end. The parasitophorous vacuole enlarges in size and occupies the entire cytoplasm that followed by the burst of the cell. Then, amastigotes reach the extracellular space and appear in monocytes and blood stream. From there, parasites metastasize to reticulo-endothelial system by disseminating through the lymphatic and vascular systems and infects other macrophages, thereby infiltrate into bone marrow and visceral organs eventually causes for hepatosplenomegaly and lymphadenopathy **(Chappuis et al., 2007)**. These infected macrophages ingest into sandfly through the fresh blood meal. After ingestion, parasitized cells pass through thoracic midgut to the abdominal midgut. Their excretion could arrest by the secretion of a neuropeptide that prevents the peristalsis of the hindgut. In this new environment, amastigotes form clusters or nest cells which enclosed by the peritrophic matrix, protects from digestive enzymes. Thus, amastigotes transform into the motile, elongated (10-20 μm), and flagellated procyclic promastigote form **(Kamhavi, 2006)** in the midgut of the sand fly. Afterward, the anterior portion of the peritrophic matrix breaks down by the action of chitinase and the parasites released to the midgut epithelium of insect. In the next stage, promastigotes divide by binary fission and attach to the midgut of insect, predominantly using anteriorly postured flagellum. The predominant production of LPG helps in detachment from midgut epithelium. Then, promastigotes migrate towards stomedial valve that localized at anterior midgut and secreted a gel-like substance called promastigote secretion gel (PSG) which obstructs the midgut and pharynx region by forming a plug. During this process, the parasites reduce in their body size and increase the length of the flagellum and become highly motile is called metacyclogenesis, and these parasites referred as infective forms. These metacyclic forms can cause the damage to stomedial valve and interfere with its function and facilitates the efflux of parasites into the proboscis (Fig.9). While taking the blood meal, it injects the bolus of metacyclic promastigotes into the host skin at the bite site by regurgitation process **(NIAID, 2010)**.

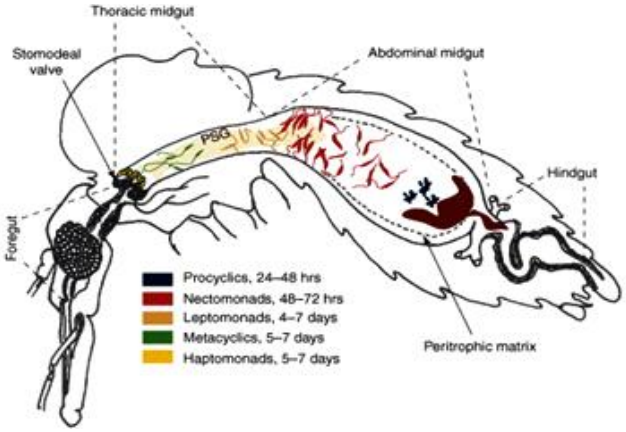


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Figure 9: Developmental stages of *Leishmania* within the sandfly vector

1.7. Pathogenesis of leishmaniasis

In fact, the clinical severity not always correlated with a mode of infection. The ratio of incidence between asymptomatic infection and clinical features differ from one patient to other. Typically, infection always neutralizes by the host specific cell-mediated immunity and VL exacerbates due to the T-cell unresponsiveness to *L. donovani* antigens and abundant production of interleukin (IL)-10. In Indian patients, VL pathogenesis is majorly implicated by IL-10 producing $CD25^{-}$ T-cells **(Nylen et al., 2007)**. The importance of the host specific cell-mediated immune response against VL illustrated by observing its devastation during malnutrition and concomitant immunosuppressive conditions like HIV infection, that increases the incidence of developing clinical illness. In addition, several other risk factors identified for the profound clinical illness of VL that includes childhood, lack of interferon (IFN)-γ production, polymorphism in tumor necrosis factor (TNF)-α promoter gene and IL-4 coding gene etc. In Sudan, the susceptibility for VL pathogenesis is underlying a defect in the solute carrier family 11 A1 (SLC11A1) gene that involve in active macrophage function. It implies for a critical role of innate immunity in driving the host protective adaptive immunity **(Chappuis et al., 2007)**. Sand fly salivary gland proteins suppress the host specific innate immunity; macrophage activation and nitric oxide (NO) production against the parasite, thereby it selectively inhibits parasite killing.

Despite the presence of LPG in different life stages of the parasite, they differ in their quantity of LPG expression on body surface including flagellum. Mostly, the difference can be seen in the carbohydrate moieties those conjugated to LPG. In procyclic forms of the parasite, the LPG molecules are shorter. Whereas, in the infective metacyclic forms the LPG bearing terminal β-galactose capped by α-arabinose residue that helps in the elongation of

LPG moiety of 2-3 folds by repeating the numbers of this disaccharide unit. In spite of LPG mediated classical activation of macrophages, they do not get lysed. After their infection into the host skin along with sandfly saliva **(Awasthi et al., 2004)**, the clinical manifestations usually appear within 12-weeks. The most common symptom is long-lasting low-grade fever, typically occurs during night-time and occasionally double-quotidian and accompanied by tachycardia. Diarrhea and cough also appear; seldom splenomegaly and conspicuously enlarged liver can be seen by the end of the third month. Frequently, the symptoms like hypoalbuminemia, polyclonal hypergammaglobulinemia (IgG and IgM) and antigen-antibody complexes in circulation can appear. Infrequently, glomerulonephritis and interstitial nephritis mediated by immune complexes have been described. The delayed and long-lasting manifestations like edema, cachexia, and hyperpigmentation also been noticed, that reported in PKDL cases.

1.8. Treatment Strategies

In these days, effective treatment for VL mostly relies on available chemotherapy accomplished by specific anti-leishmanial drugs (Fig.10). In addition, concomitant bacterial or parasitic infections, anemia, hypovolemia (decreased blood volume) and malnutrition are under surveillance for reliable treatment against VL. First-line of the drug for the treatment VL is the Pentavalent antimonials (SbV); Sodium stibogluconate and Meglumine antimoniate used for >70 years (Fig.10a). Frequently available cheaper generic forms also equally efficient to the branded products, but these antimonials are found to be highly toxic with adverse side effects include cardiac arrhythmia and acute pancreatitis. Notably, the child patients <2 years and the adults >45 years age found to be more susceptible to develop the above mentioned adverse clinical implications. Also leads to death during an advanced stage of disease and severe malnutrition.

In addition to the toxicity, the emergence of resistant strains hampered the antimonial therapy for VL, convincingly due to the widespread misuse of the drug. Free accessibility and inappropriate dosages cause for resistance development against antimonial drugs in India. In addition, large number of patients (~73%) from rural areas consulting unqualified medical practitioners for the first time and they routinely refer initial small doses that increases with the course of treatment under their common practice. And also been suggesting a course of the interval to control renal toxicity appeared by these drugs. Frequently, they prescripts for the split dosage like two injections per day. Altogether, these malpractices presumably expose the parasites to constant drug pressure that leads to successive retain of tolerance **(Sundar et al., 1994)**.

Beginning in the last decade, antimonial therapy for VL replaced by conventional Amphotericin-B (Fig.10b) as a first-line of the drug in the Bihar state, India, where the antimonials failed to treat >60% of cases. However, infusion-related fever, chills and rigor associated with conventional Amphotericin-B therapy. Also, it frequently causes for life-threatening adverse clinical manifestations such as hypokalemia (low potassium levels in the blood), nephrotoxicity and anaphylaxis. Since, its higher cost, toxicity, the emergence of resistance and complicated treatment regimen, indeed prohibited its usage from treating VL. In Europe and the United States, the liposomal formulated Amphotericin-B is being used as the first-line drug for VL and also been considered as the best existing drug by specialists. In India, Miltefosine (Fig.10c) is the only drug freely accessible since 2002 for a retail price of US$125–200 per VL treatment course. Presently, it is an effective oral drug against VL and was initially used for cancer treatment. Paromomycin (Fig.10d), an aminoglycoside broad spectrum antibiotic tested against VL in India and Africa and got a promising result, and registered as an anti-leishmanial drug in the India in 2006. Despite the several advantages; low price (US$5–10 per treatment) and effectiveness against a broad range of parasitic and bacterial infections, its production was abandoned due to unknown reasons. Sitamaquine (8-aminoquinoline) (Fig.10e) is an oral drug for VL treatment about 20 years ago.

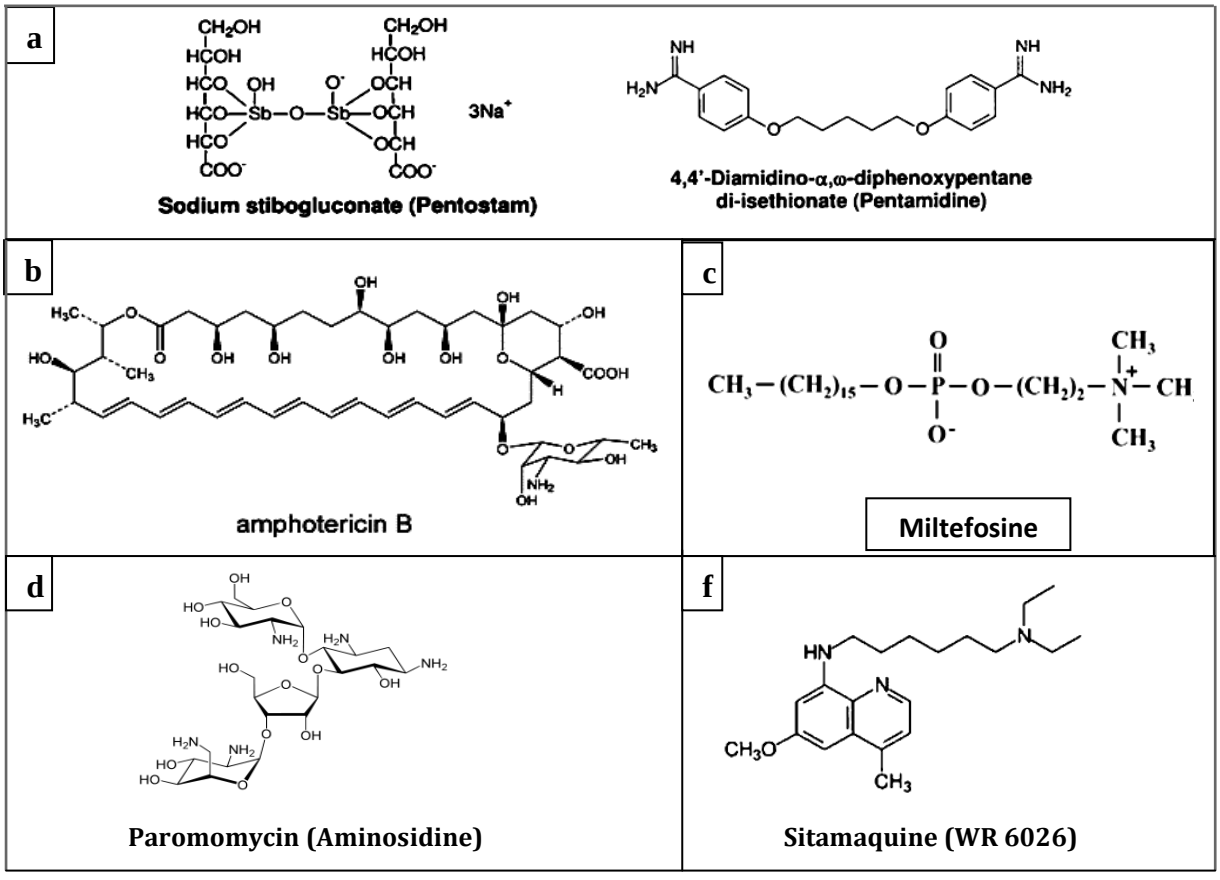


Figure 10: Anti-leishmanial drugs

2. Immunology of leishmaniasis

2.1. Innate immunity

Based on the early innate immune response against *Leishmania* infection, the disease outcome could be predictable. Its functions not only attributed to control the rapid parasite replication/infection dissemination but also to produce the immunoregulatory cytokines. Being an opportunistic pathogen, *Leishmania* adapts several host factors in favor of their survival inside the macrophage phagolysosomes. Innate immunity is a front-line defensive response against any infectious agent, typically mediated by the various lymphocytes; natural killer (NK) cells, mononuclear and polymorphonuclear phagocytes and other factors include TLRs, complement proteins and IL-1α, and myeloid differentiation factor 88 (myd88) **(Hawn et al., 2002)**. After breaching into the skin, parasites can be phagocytized by host dendritic cells and macrophages **(Scharton-Kersten et al., 1995; Sharma and Singh, 2009)** and oppose their proteolytic digestion by phagocytes. The engulfing process of *Leishmania* parasites usually accelerates using complement protein called C3b a potent immune opsonin **(Sharma and Singh, 2009)**. This C3b/opsonin binds to *Leishmania* parasite surface glycoprotein gp63 and converts C3b into inactive form (iC3b) **(Hermoso et al., 1991)**. This conversion helps in the clearance of parasites by phagocytic process rather than lysis, since the intracellular parasites are highly resistant to the degradation. Subsequently, infected macrophages and dendritic cells produce IL-12 via TLR-9 signaling which in turn activates NK cells and produce IFN-γ, thus it drives the subsequent polarization of Th1 response **(Scharton-Kersten et al., 1995; Liese et al., 2008)**. In addition, the activation of NK cells also carried out by various chemokines such as IFN-γ induced protein (IP)-10, monocyte chemotactic protein (MCP)-1 and lymphotactin, and these activated NK cells cytolytic for *Leishmania*-infected macrophages. In detail, the activated NK cell-derived IFN-γ activates the macrophages to kill intracellular amastigotes by producing reactive oxygen and nitrogen intermediates that eventually leads to host protection. Several studies illustrated the importance of NK cells and its mediated IFN-γ production against intracellular parasites. In humans, peripheral blood mononuclear cells (PBMCs)-derived NK cells shown to produce IFN-γ in response to *Leishmania* antigen **(Nylen et al., 2003)**.

In mice, *L. major* challenge leads to reduction in NK cell count, in turn depletion of IFN-γ production and increment in parasite load after 7-days **(Laurenti et al., 1999)**, illuminating the importance of NK cells in innate immune response. TNF-α synergizes with IFN-γ and activates the infected macrophages to produce NO **(Muller et al., 1991)** which is a potent cytostatic and cytotoxic molecule and kills various intracellular pathogens including

Leishmania in a murine model. In addition, activated polymorphonuclear leukocytes also clear the infection, especially through oxidative burst. The anti-leishmanial activity of NO illustrated through the observations like IFN-γ dependent activation of macrophages leads to produce NO and kills the parasite, resistant mice models augments the iNOS expression and NO production and become susceptible after inhibition of iNOS, null mutation of NOS2 gene increases the susceptibility and inhibition of NO production allied with deterioration of functional macrophages during *Leishmania* infection **(Bogdan and Rollinghoff, 1999)**. Accordingly, hepatic *L. donovani* infection exerts the innate immune response via iNOS expression in macrophages **(Murray and Nathan, 1999)** which in contrast to the **Murray et al., 2006** who showed iNOS-independent anti-leishmanial activity. However, a majority of the studies shows anti-leishmanial activity via an iNOS-dependent mechanism in both liver and spleen **(White et al., 2005 and Perez et al., 2006)**. In fact, *L. donovani* infection usually evades the iNOS-dependent killing mechanism in macrophages by inducing the arginase expression that does similar to IL-10 **(Biswas et al., 2011)**. Another notable feature of innate immunity is the FasL-mediated apoptosis in *Leishmania*-infected macrophages **(Huang et al., 1998)**. Thus, macrophages are unique cells engaged with both survival and death of the parasites **(Birnbaum and Craft, 2011)**.

Following macrophages, dendritic cells (DCs) are the key players in the innate immune response. Typically, DCs classified into two types such as myeloid (mDC) and plasmacytoid (pDC). Myeloid DCs produce IL-12 via TLR-9 signaling and exerts anti-leishmanial activity under the influence of IFN-γ producing NK cells. Despite the lack of phagocytic activity, pDCs also able to produce IL-12 imposes its significance during later stages of infection **(Schleicher et al., 2007)**. Active VL patients shows depleted secretion of IL-8 and eotaxin from neutrophils and eosinophils respectively **(Elshafie et al., 2011)** and these exhibits predominant Th2 type response characterized by elevated number of IL-4+ neutrophils and IL-10+ eosinophils and reduced number of IFNγ+ and IL-12+ eosinophils **(Peruhype-Magalhães et al., 2005)**.

2.2. Humoral immune response

Humoral response is a branch of adaptive immunity, that characterized by the production of antigen-specific antibodies. Serological diagnosis of human leishmaniasis is marked by the detection of anti-leishmanial antibodies. Initially, the scientific community believed that the anti-leishmanial antibodies had no role in protection, despite their low levels in CL and abundant levels in VL. Indeed, their role in either protection or pathogenesis is not yet studied in defined level; several studies have reported divergent results. The

immunoglobulin isotypic pattern analysis revealed the presence of *Leishmania* antigen-specific IgG and its subclasses, IgM, and IgE at high titers during active disease **(Ghosh et al., 1995; Atta et al., 1998; Ryan et al., 2002)**. Concurrently, elevated antibody titers marked for the severity of disease in case of VL **(Melby and Anstead, 2001)**. In humans, the importance of humoral immunity during *Leishmania* infection is not well defined. Despite the lack of reports on the exact role of IgG sub-classes, few studies have revealed their relevance in either disease progression or protection. Of these, **Garraud et al., 2003; Caldas et al., 2005** have stated that the elevated titers of IgG1 and IgG3 in human VL correlated with increased IL-10 activity and demolished IFN-γ activity (i.e. decreased IgG2 titers) in contrast to **Ryan et al., 2002**. Despite the lack of solid evidence on the role of IgG4 in parasitic infections, hypothesized that it could play an unfavourable host role and diminish infection clearance **(Jassim et al., 1987; Dafa'alla et al., 1992)**. In accordance, IgG4 titers elevated along with IgG1 and IgG3 in active VL patients **(Shiddo et al., 1996)** and also produced in higher levels along with IgM and IgE in canine VL positively correlated with IL-4 and IL-5 levels **(Iniesta et al., 2005)**. However, IgG2 titers indeed associated with IFN-γ levels thereby implicated its role in host protection **(Garraud et al., 2003; Caldas et al., 2005; Mutiso et al., 2012)**.

2.3. Cell-mediated immune response

Cell-mediated immunity is the characteristic domain of acquired resistance that majorly mediated by T-cells, of these CD4+ and CD8+ T-cell subtypes acts as effector and memory cells respectively in leishmaniasis **(Reiner et al., 1995)**. In contrast, Tregs majorly associated with disease progression influenced by persistent infection **(Belkaid 2002a)**. Majorly, the protection against human cutaneous and visceral infection can be mediated by the Th1 polarization and thus abundant production of IFN-γ. In contrast, higher production of IL-4 and IL-10 leads to the development of mucocutaneous and chronic lesions **(Kemp et al., 2000)**. Following to recovery, their persistence **(Mauel et al., 1987)** leads to prevent the reinfection in endemic and experimental volunteers **(Guirges et al., 1971)**. The active VL directly linked to the predominant levels of IL-10 and sustained levels of IFN-γ rather than IL-4 production **(Kharazmi et al., 1999)**. In addition, IL-10 been implicated in the pathogenesis of all clinical forms of leishmaniasis and consider as a macrophage-deactivating cytokine which in contrast to IFN-γ. The immune dysfunction during active VL caused by *L. donovani* associated with lymphocytes incapability to produce cytokines in response to re-stimulation with *Leishmania*-specific antigen **(Ho et al., 1983)**. Depletion of lymphocytes at T-cell microenvironment in spleen and lymph nodes strengthen the aforementioned statement

about VL patients **(Veress et al., 1977)**. Hence, it strongly evidenced for the importance of cell-mediated immunity and a pivotal role of cytokines in leishmaniasis. In addition, persistent levels of TNF-α **(Ansari et al., 2006)** and imbalance in IL-10 production **(Ghalib et al., 1993; Melby and Anstead, 2001)** typically associated with development of PKDL clinical form. However, pathogenesis of VL associated with elevated *Leishmania*-specific Th2 response and increased number of antigen presenting cells thereby inhibition of protective T-cell response **(Basak et al., 1992)**. During chronic VL, CD4+ T-cell unresponsiveness is apparent due to massive expression of CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4) which leads to the abundant production of TGF-β and helps in infection persistence **(Gomes et al., 2001)**. The resolution of cutaneous infection associated with Th1 response characterized by the abundant production of IFN-γ thus leads to upregulation of iNOS from infected macrophages and NO production, it also routinely allied with IL-12 production. On the contrary, pathogenesis of CL is regulated by IL-4+ Th2 cells **(Sacks 2002)**, being a major hurdle for antigen-specific immunity mediated by lymphocytes in *Leishmania*-infected patients. The restoration of IFN-γ production from T-cells upon anti-IL-10R antibody treatment revealed the relevance of the IL-10 in the suppression of T-cell response during active disease **(Ghalib et al., 1993)**. The production of IL-10 from antigen-stimulated PBMC cultures was significantly higher in acute VL than after cure in *L. chagasi* infected Brazilian patients. The Leishman skin test (LST)/Montenegro test positivity is not directly correlated with the IL-10 production in asymptomatic individuals **(Holaday et al., 1993)**. Several studies reported that IL-10 m-RNA upregulation in bone marrow **(Karp et al., 1993)**, lymph nodes **(Ghalib et al., 1993)**, spleen **(Kenney et al., 1998)** and plasma **(Ghalib et al., 1993; Holaday et al., 1993)** is prominent in active VL patients. In contrast, the disease cure is implicated by the drop in IL-10 mRNA expression **(Ghalib et al., 1993; Karp et al., 1993)**. Indeed, healthy balance between IL-10 and IL-12 is critical for either protection or pathogenesis and chemotherapy **(Sharma and Singh, 2009)**. IL-4, the important classical Th2 cytokine-induced in VL and usually associates with hampered treatment **(Sundar et al., 1997; Dunning, 2009)**. During active VL, the IFN-γ and IL-4 certainly upregulated but significantly declined followed by the cure. In addition, IL-13 is also elevated in active disease but diminished its levels after successful treatment **(Babaloo et al., 2001)**. The disease relapse majorly associated with IL-10 rather than IL-13 and is majorly influenced by IL-10+ IFN-γ+ producing antigen-specific T-cells during human VL **(Kemp et al., 1999)**. The PBMCs harvested from VL cured patients produce IFN-γ or IL-4 in response to *L. donovani* promastigote or amastigote crude antigen. Contrastingly, in response to purified

gp63 antigen, the proliferation capability of these PBMCs was weak and produces either IFN-γ or IL-4 **(Kurtzhals et al., 1994)** that revealed by intracellular staining and demonstrated that more than 80% of CD4+ T-cells habituated for IFN-γ+IL-4+ and few CD8+ T-cells accustomed for IFN-γ+. The role of Th17 subset in human VL is implicated recently by a longitudinal study in Sudan, illustrating the protection against disease progression mediated by *L. donovani*-specific T-cells producing IL-17 and IL-22 **(Pitta et al., 2009)**. In fact, Th17 cells are pleiotropic in nature, responsible for either protection or pathogenesis and frequently associated with neutrophils recruitment. Th17 cells under the influence of IL-27 producing CD4+ T-cells diminish IL-17 and IL-22 secretion **(Murugaiyan et al., 2009)**. Disease progression in Indian VL pre or post treated patients linked with serum IL-27 and splenic IL-27 transcript but not the splenic IL-17 transcript **(Ansari et al., 2011)**. The pathogenic role of IL-27 in active VL linked with suppression of Th17 cytokines production and transcription factors expression. In concordance, IL-27 promotes the parasite dissemination by inducing the antigen specific IL-10+ T-cells differentiation and expansion, and also by inhibiting activation of effector Th17 lineage. Moreover, the host itself negotiate the Th17 response to minimize the pathetic implications of VL driven by the parasite. PKDL associated skin manifestations implicated by the abundant production of IL-10 in keratinocytes. As a sequel, it develops from VL patient who carries rich plasma IL-10 levels **(Gasim et al., 1998)**. Based on the IL-10 levels in the skin and plasma the severity of PKDL pathogenesis and the probability of VL development to PKDL could be predictable respectively. It is evidenced by hyper T-cell response against parasite antigens in active PKDL and also validated by significant production of both Th1 and Th2 cytokines from PBMCs in response to crude *L. donovani* antigens **(El Hassan et al., 1992; Ghalib et al., 1993)**.

Importantly, following parasite invasion into the host skin different chemokines release and they immediately recruit various subsets of leukocytes. Based on the type of chemokines, recruited leukocytes act as either parasite reservoirs or contributors of virulence. Hence, an immunotherapy including antagonists for this chemokine system could help in the disease control **(Bhattacharya and Ali, 2013)**. Interaction between different factors of innate and adaptive immune systems and their possible role in the parasite persistence or infection clearance is depicted in Fig.11.

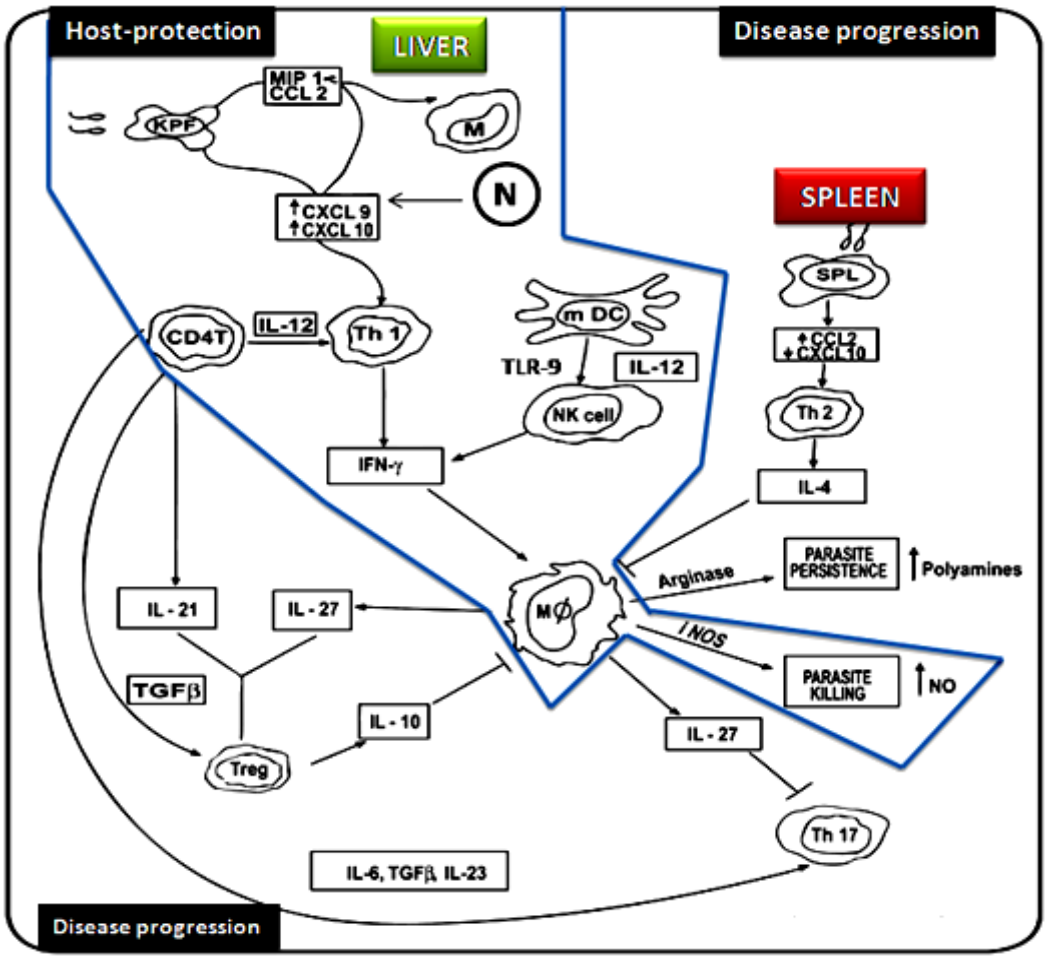


Figure 11: Rapid hepatic accumulation of chemokines CCL2, CXCL9 and CXCL10 that occurs after infection leads to Th1 response through IFN-γ, and its mediated NO production facilitates parasite clearance by macrophages (within blue). In addition, IL-12 produced by mDCs activates NK cells to secrete IFN-γ. However, in the spleen, a consistent expression of CCL2 leads to the dominance of Th2 cytokines (IL-4) and parasite persistence. IL-10 production by Treg is the critical factor in disease pathogenesis that derails the macrophage function. IL-27 produced by defective macrophages leads to suppressing Th17 subset and inhibits the production of IL-22 and IL-17. It also activates Tregs in collaboration with IL-21 produced by CD4 T-cells and induces the secretion TGF-β, which helps in the parasite growth by inducing polyamine biosynthesis **(Bhattacharya and Ali, 2013)**. KPF: Kupffer cells; SPL: Spleen; M: monocyte; MIP-1α: macrophage inflammatory protein-1alpha; CCL: chemokine (C-C motif) ligand; CXCL: chemokine (C-X-C motif) ligand; Mϕ: macrophage; N: neutrophils.

REVIEW OF LITERATURE

1. Malnutrition and Infections

According to Food and Agriculture Organization (FAO) report, 2006, the global burden of malnutrition is ~826 million, of this, ~90% (792 million) only in developing countries and the remaining 34 million in the developed nations. Purging the malnutrition possibly controls the 32% of global disease burden **(Mason et al., 2003)**. However, other reports certified that the above-mentioned figure was roughly estimated only in concern of HIV infection **(Puffer and Serrano, 1973, Pelletier et al., 1993, DCPP, 2007)**. Malnutrition is not the food scarcity and certainly not unique to the poor community. It is also prevalent in developed nations like US **(Chapman, 2006)**.

Malnutrition is a primary risk factor for the development of defective immune response in humans irrespective of age. It also shows a strong affection to infections due to underweight, weak immunity, poor epithelial integrity and inflammation, and causes for the high incidence of infant mortality **(Reuters, 2004)**. The major infectious diseases such as pneumonia, diarrhea, malaria, measles, and acquired immunodeficiency syndrome (AIDS) accounted for more than 50% of deaths in children under the age of 5 years **(UNICEF, 2006)**. Either defective immunity or poor nutritional status alone is not a reliable marker for the high incidence of infections. Until the 1950s, it was believed that the malnutrition accompanied predominantly by a protein-energy deficiency rather than total calorie deficiency due to lack of awareness about the relationship between malnutrition and infections. Since the protein deficiency usually turns off the antibody synthesis and blunts the development of the immune system in infants and children. Later on, the focus has shifted to total calorie deficiency coupled malnutrition and assumed that calories rich diet need to composed with all essential nutrients to meet the metabolic demands. Unless the presence of all nutrients, it also would be considered as malnutrition diet.

The interaction between malnutrition and infection studied extensively and revealed their synergistic, antagonistic, and cyclical relationship (**Keusch, 2003, Scrimshaw et al., 1968)**. In order, WHO published an article in 1968 entitled "Interactions of Nutrition and Infection" illustrates the synergistic relationship between infection and malnutrition **(Scrimshaw et al., 1968)**. Further, the metabolic alterations during infection and the relationship between malnutrition and cell-mediated immunity were elucidated in detail for the first time in 1970 **(Beisel, 1996, CMNR, 1999, Keusch, 2003, Meydani and Wu, 2007, Meydani et al., 2007)**. The emergence of advanced technology in between 1970-80s brought improved tools for insisting the role of humoral and cell-mediated immunity during infection

accompanied by malnutrition. After several studies on humans and better animal models, it clarified that the malnutrition not only confined to children but also affect adults.

Indeed, malnutrition is a more vulnerable factor for infections; subsequently infections also contribute to the development of the malnutrition state in a cyclic fashion. Also, insufficient ingestion of diet leads to weight loss, immune deficiency, mucosal damage, high pathogens raid, default growth and development in children. Typical health complications include diarrhea, malabsorption, low appetite, urinary nitrogen loss, and thus nutrient loss renders the host defense mechanisms. Also, the raised body temperature/fever demands more energy and micronutrients. Hence, malaria and influenza have been causing some deaths that relatively amplified with the severity of malnutrition **(Muller et al., 2003)**.

Among micronutrients, zinc is the most important trace element for several biological functions mediated by various enzymes. It acts as a cofactor for about 1300 enzymes involved in carbohydrate and energy metabolism, protein biosynthesis and degradation, nucleic acids and heme biosynthesis, CO_2 transportation, plasma membrane biosynthesis and immune cell function. Zinc deficiency affects the regular development and function of both innate and adaptive immunity. In a sense, it leads to defective neutrophils/NK cells function, denting of the complement system, and reduced proliferation of lymphocytes, DTH response, cytotoxic activity and antibody production. Abolishment of hepatic vitamin-A release occurs in scarce of zinc that overwhelms the clinical abnormalities like growth retardation, malabsorption, abortion/fetal death. Also, infectious diseases like tuberculosis, Crohn disease, diarrhea and pneumonia also affects the zinc levels in the blood. Its deficiency handles abnormalities in pregnancy (**Fawzi and Msamanga, 2004)** and immune compromisation for several pathetic conditions like alcoholism, kidney disease, burns, inflammatory bowel disease, and HIV infection. Corticosteroids accelerate zinc loss through excretion. Zinc supplementation controls the severity of diarrhea, growth stunt, pneumonia and malaria **(Cuevas and Koyanagi, 2005, Caulfield et al., 2004)**. It limits the infections attack and frequent hospitalization of sickle cell anemia patients by augmenting the production of IL-2 **(Pellegrini Braga et al., 1995, Prasad et al., 1989)**. Its dosage of 70mg per week significantly inhibits the infection incidence and death of pneumonia patients, and also reduces the mortality rate about 85% of diarrhea patients **(Temple and Masta, 2004)**. The constant supplementation of zinc along with potassium and magnesium magnifies the rate of appetite and offers the resistance to infections **(Khanum et al., 1994, Ashworth and Khanum, 1997)**.

Iron is another important trace element and its deficiency been persisting in 20%–50% global population; especially infants, children, and pregnant women **(Patterson et al., 2001)**. It is the only nutrient deficiency that enormously customized in industrialized nations. Its deficiency corroborated by defective cell-mediated immune response and neutrophils function. In fact, the relation between iron deficiency and risk of infection is still unclear, in spite of its associated immune abnormalities. Anemia is the most common symptom manifested by iron deficiency. Since the iron is an indispensable factor of every cell in the body, especially red blood cells (hemoglobin), which carries the oxygen through the blood to all tissues of the body. It is also a critical element for muscular proteins and development of the central nervous system (CNS).

Globally, the enormity of parasitic infection is in large scale. On average, human beings are hostages for 200 different parasites. Despite the evidence for parasite influenced malnutrition, the degree of malnutrition severity is not clear. However, the coexistence of these two factors evidenced by the strong interaction with each other **(Tomkins and Watson, 1989)**. Conventionally, helminthic infections exaggerate the cognitive deficits in school-aged children **(Stoltzfus et al., 2001)** and worm infections causes iron deficit through blood loss **(Stoltzfus et al., 1997)**. It evidenced for the deterioration of child health during parasitic infections without ambiguity. Adequate diet supplementation could thwart the annual deaths of children ~1 million of pneumonia, 0.8 million of diarrhea, 0.5 million of malaria, and 0.25 million of measles **(Reuters, 2004)**.

Recent studies have focused on exploring the dynamics and kinetics of immune system in response to nutritional status, and also trying to find out the reliable markers for mass vaccination. It might be plausible through targeting nutrient entity and its associated immune response. Despite the malnutrition declined sharply among the Indian children, still India has the highest number of underweight children. According to UNICEF, India has occupied 55^{th} rank in 2014 that was slightly improved from 63^{rd} rank in 2013 out of 76 countries. As per International Food Policy Research Institute (IFPRI) report, 2014, the proportion of underweight children falls from 45.1% in 2005-06 to 30.1% in 2013-14 and the proportion of undernourished population falls from 21.5% in 2004-06 to 17% in 2011-13. Globally, 2 billion people are suffering from “hidden hunger” enrolled by the lack of essential vitamins and minerals in their diet (Fig.1).

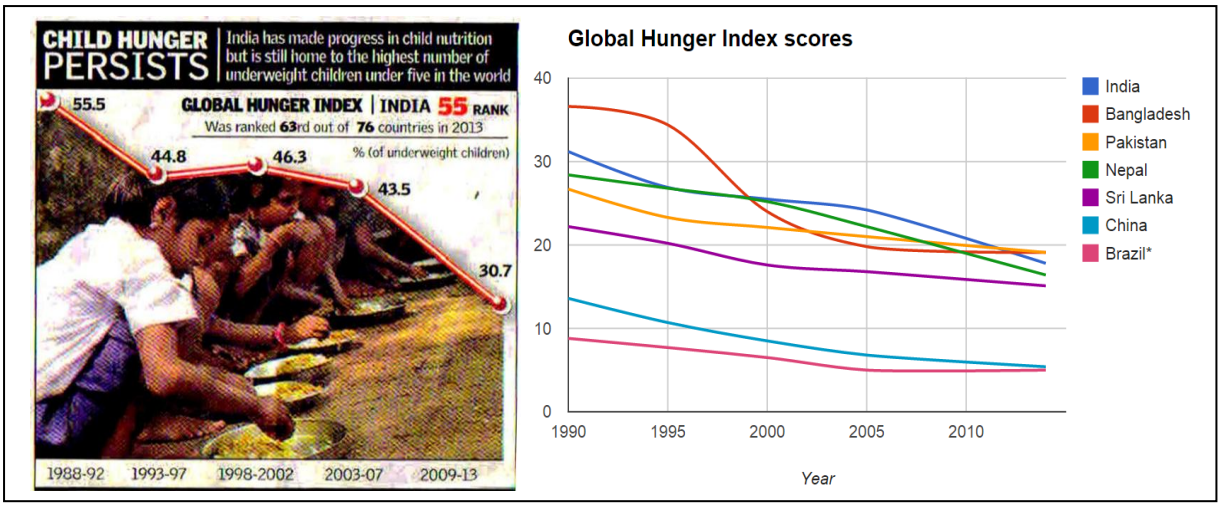


Figure 1: Picture shows the India's rank in % of underweight children and Global Hunger Index scores of different nations.

2. Leptin Biochemistry

Leptin is discovered by **Zhang et al., 1994** and reported as the product of *ob* gene. It is hormone derived from adipocytes **(La cava and Matarese 2004)** in response to the nutritional status, and it signals to the CNS and peripheral organs. It also derives from the placenta and gastrointestinal tract to a lesser extent, while it's role in these tissues, not yet established. Leptin displays the pulsatility and circadian rhythmicity. The circulating plasma leptin concentrations majorly influenced by the total fat mass **(Grinspoon et al., 1996)** and body mass index, and little influenced by metabolic hormones, sex, and body energy demands. The *ob/ob* mice had no functional leptin and authenticated for the obese phenotype. In fact, obesity in animal models is more frequent than human beings by defective leptin signaling; which manipulated by mutations in the leptin or its receptor gene. Obesity in humans is mostly incriminated by overwhelming circulating leptin levels thereafter its resistance. Hence, leptin treatment has been implicated in weight-related disorders such as obesity and anorexia nervosa. The chief function of leptin is metabolic homeostasis that can attain by the delivery of information about the total body fat mass to the hypothalamus that in turn alters the CNS function and regulates food intake and energy balance. Indeed, a minute fluctuation in peripheral leptin concentration manipulates the function of the hypothalamus-pituitary-ovarian (HPO) and hypothalamic-pituitary-adrenal (HPA) axes assisted for reproduction and stress biology respectively. It indicates that leptin or its antagonists can be helpful in disease diagnosis or treatment **(Prolo et al., 1998)**.

2.1. Structure

Leptin is a 167-amino acid containing non-glycosylated protein with a signal peptide of 21-amino acids at NH_2 terminal. The post-translational modifications of pro-leptin take place

in microsomes by removal of signal peptide and subsequently released into the blood stream **(Zhang et al., 1994, Considine and Caro, 1997)** (Fig.2). The circulating leptin is a 146-amino acid-containing functional peptide with a molecular weight of 14-16 kDa. Human leptin has homology with rat (83%) and mouse (84%) leptin **(Zhang et al., 1994)** respectively. The structural elucidation (crystallized protein tertiary structure) of human leptin revealed the presence of four α-helices (Fig.3) similar to that of long-chain helical cytokine family includes IL-6, IL-11, IL-12, Leukemia inhibitory factor (LIF), Granulocyte-colony stimulating factor (G-CSF), Ciliary neurotrophic factor (CNTF) and oncostatin M.

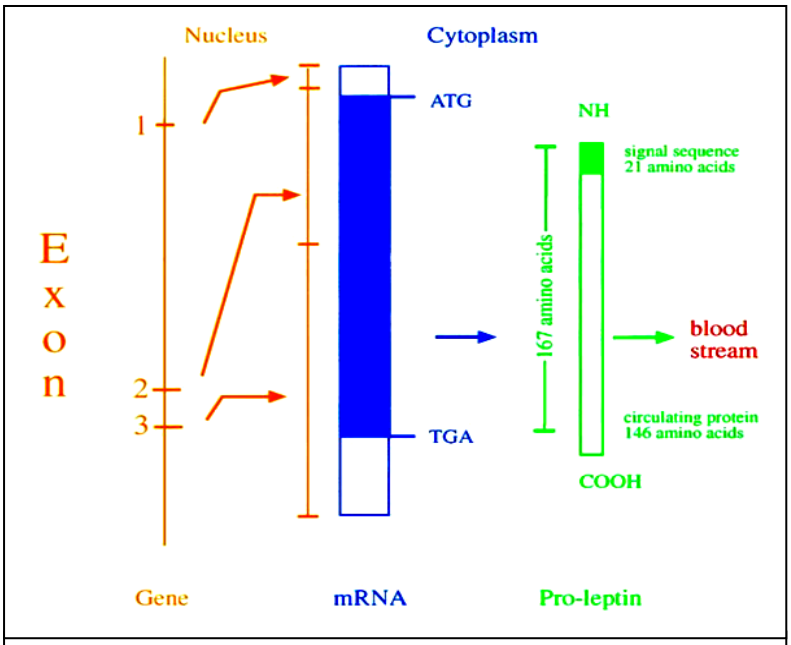


Figure 2: Leptin synthesis. The leptin gene consists of three exons and two introns. The gene (orange) is transcribed into 3.5kb mRNA (blue) that encodes a pro-hormone of 167 amino acids (green). The signal sequence containing 21 amino acids is subsequently removed prior to release of leptin into the circulation (red).

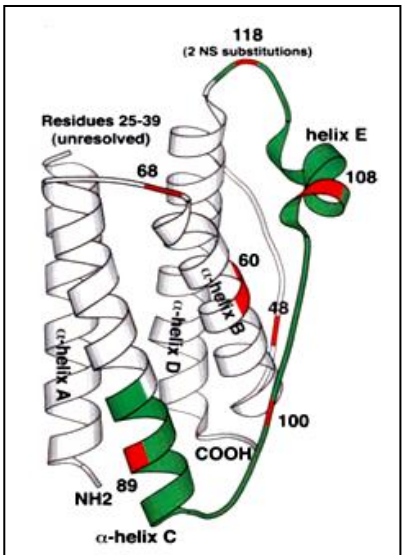


Figure 3: Tertiary structure of human Leptin, PDB accession 1AX8 **(Zhang et al., 1997)**, as rendered with MOLSCRIPT **(Kraulis, 1991)**.

2.2. Synthesis and Degradation

The *ob* gene located on the long arm of the 7th chromosome at position 31.3 (7q31.3) and covers the ~20 kb length accompanied by three exons and two introns. The promoter region is spanned by ~3 kb with the TATA box positioned in a downstream region (-26 to -30 bases), multiple-C/enhancer binding protein (CCAAT/EBP) and SPA sites, a GRE (glucocorticoid response element), and several CREB (cAMP response element-binding protein) sites **(Considine and Caro, 1997)**. The circulatory leptin levels always relatively exist with the leptin mRNA expression in adipose tissue, which influenced by various

metabolic and endocrine factors. Insulin is the one that regulates the leptin mRNA in rodents, but such effect not seen in humans **(Considine and Caro, 1997)**. Also, 17β-estradiol and antagonists of β3-adrenergic receptor up and downregulates the leptin mRNA expression respectively **(Considine and Caro, 1997, Mantzoros et al., 1996)**. In spite of the equal amount of body fat mass, females produce a two-fold higher quantity of leptin than that of males. Since, the circulating leptin directly link to the adipose tissue mass and sex of the individual; mostly females contain huge adipose tissue than men **(Frederich et al., 1995)**. In general, serum leptin levels can be influenced by the changes in energy intake and growth development. In human, the production of leptin is well organized and rise in midnight to early morning. It can secrete in a pulsatile fashion with an average of 32 pulses per day, each pulse lasts for 33 min and involves in circadian rhythmicity **(Licinio et al., 1997 and 1998)**. During pregnancy, the placenta produces a greater amount of leptin than that of adipose tissue and functions as a growth factor that signals the nutritional status of mother to fetus **(Masuzaki, 1997)**. Its half-life is 24.9±4.4 min in circulation that is quite similar in both obese and normal-weight humans **(Klein et al., 1996)** and is majorly influenced by its renal clearance through glomerular filtration.

2.3. Receptors and Signaling

Cloning of the leptin receptor gene **(Tartaglia et al., 1995)** confirmed the presence of five variant splices and a short soluble form. Out of five variables, one has a long intracellular domain that resembles to type I cytokine receptor signaling domain and transducer through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway **(Licinio et al., 1998)**. Despite six isoforms of leptin receptors coded by *db* gene, they share 805 amino acids and 1-14 exons coding extracellular domain (Fig.4). However, most of the biological functions exerted through Ob-Rb signaling (Fig.5) which frequently assigned at a higher rate in the hypothalamus and less in other tissues. It has long cytoplasmic domain pilots the several signaling cascades based on their location and specific metabolic need. The other isoforms such as Ob-Ra and Ob-Rc are more frequent after Ob-Rb and involve in the transportation of leptin across the blood-brain barrier. Ob-Rf also does the similar function at lesser rate comparatively. The Ob-Re considered as a soluble binding protein rather than receptor due to the lack of transmembrane or cytoplasmic domain. It usually binds with plasma leptin and minimizes its loss through glomerular clearance. It does not interfere with the binding of leptin to Ob-Rb, and its infusion enhances the activity of leptin in null *ob/ob* mice. Typically, its overexpression indicates the higher plasma leptin levels without affecting the adipose leptin expression **(Huang et al., 2001)**.

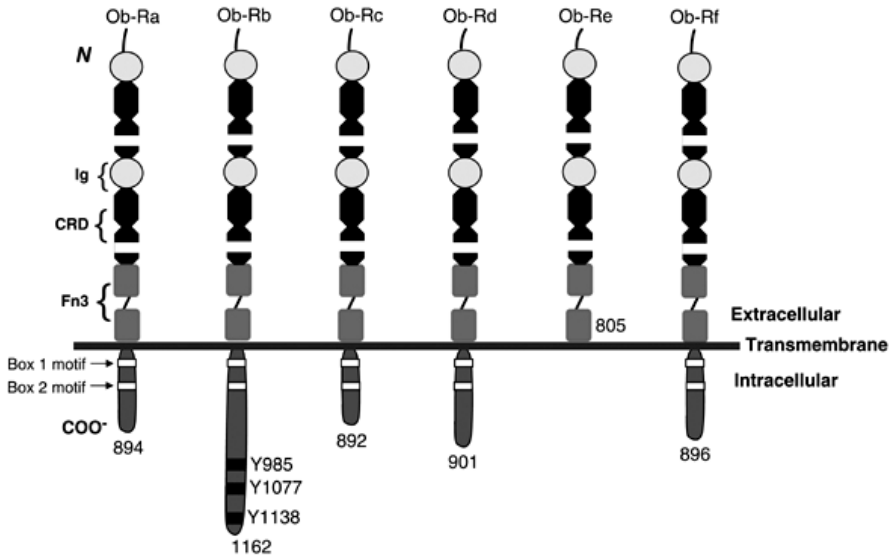


Figure 4: Isoforms of the leptin receptor. Six different isoforms exist, Ob-Ra to Ob-Rf in mice. All share identical extracellular ligand binding domains but differ at C-terminus. Five out of six consists of transmembrane domains, but only Ob-Rb encodes all protein motifs that capable of activating JAK-STAT signaling pathway. Typically, JAKs associated with a conserved box 1 motif (intracellular amino acids 6-17), which is essential for JAK2 activation. In addition, a putative box 2 motif (intracellular amino acids 49-60) is also apparently necessary for maximal activation of JAK2 also identified. Additionally, Ob-Rb has three conserved tyrosine in its cytoplasmic domain, corresponding to positions Y985, Y1077, and Y1138. Later it functions as a docking site for STAT3. Ob-Re truncated before the membrane spanning domain. CRD= cytokine receptor domain; Fn3=fibronectin III domain **(Ceddia, 2005)**.

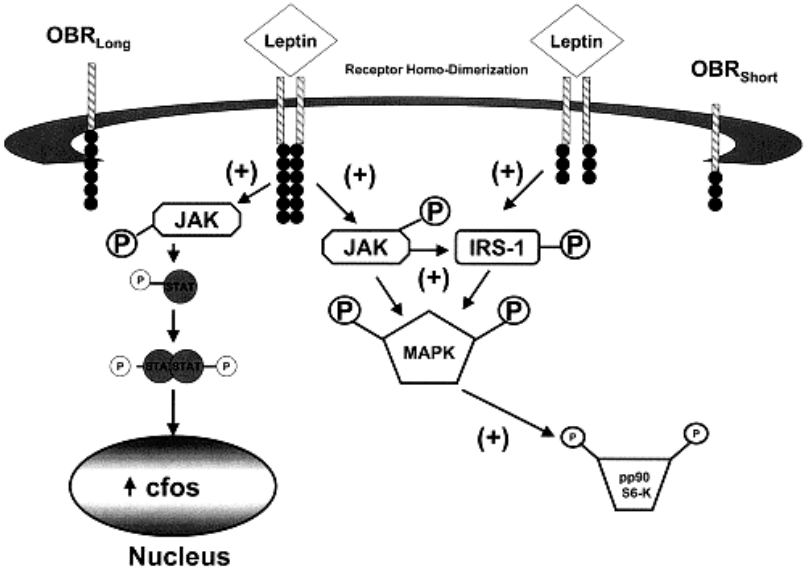


Figure 5: Schematic representation of leptin receptor signaling pathways. Binding of leptin to its receptor leads to homodimerization, in turn the long form of the leptin receptor (OB-Rl) activates JAK/STAT pathways that result in the activation of c-fos. OB-Rl activation may also phosphorylate JAK leading to the activation of insulin receptor substrate-1 (IRS-1) and mitogen- activated protein kinase (MAPK). Upon leptin binding, the short form of the leptin

receptor (OB-Rs) phosphorylates IRS-1 and consequently activates MAPK. The activation of MAPK leads to the activation of pp90 S6-Kinase S6-K). (+, activation; P, phosphorylation) **(Houseknecht and Portocarrero, 1998)**.

2.4. Biological functions

Generally, leptin conveys the metabolic status to the brain and the low or high levels of leptin positively correlated with the significant loss or gain of body weight respectively **(Considine and Caro, 1997, Hebebrand et al., Mantzoro et al., 1997)**. The flier's group has proposed that the starvation or fasting-induced weight loss or cachexia responsible for the alterations in the neuroendocrine system resulted by low circulating leptin levels **(Ahima et al., 1996)**. To accomplishes the hypothalamic functions, leptin has to cross the blood-brain barrier that could attain through a particular transport mechanism. A single gene defect caused for the obese phenotype in animal models. Similarly, a mutation in either leptin or its receptor gene also caused for obesity in children **(Montague et al., 1997)** or sinister obesity and defective gonadal development in adults **(Clement et al., Strobel et al., 1998)**. Biosynthetic leptin replacement therapy could benefit for the individuals suffering from mutated leptin but not its receptor. In spite of the close interaction between leptin and insulin, testing glucose levels during leptin therapy is mandatory in clinical trials. Leptin boosts the sympathetic nerve signals to the kidney, hind limb, and adrenal gland eventually regulates the neuroendocrine function. By acting on HPA axis, leptin negatively governs the release of adrenocorticotropic hormone (ACTH) and cortisol **(Licinio et al., 1998)**. The critical role of leptin in reproduction confirmed by administrating it into the normal and *ob/ob* animals, resulting enhanced gonadal maturation. However, adult humans with a mutated leptin gene suffered from hypogonadism **(Strobel et al., 1998)**; it claims the leptin tropical action on the reproductive system. In addition, leptin acts on HPO axis and regulates the ovarian follicular development during menstruation. Regarding nocturnal leptin high pulsatility, the frequency and amplitude of luteinizing hormone (LH) pulsatility adjust from high to low and low to high respectively. In such fashion, LH synchronizes with leptin levels similar to that of estradiol **(Licinio et al., 1998)**. Thus, leptin signals the nutritional status to the reproductive system since the firm relation with HPO axis. The biological effects of leptin summarized in Fig.6.

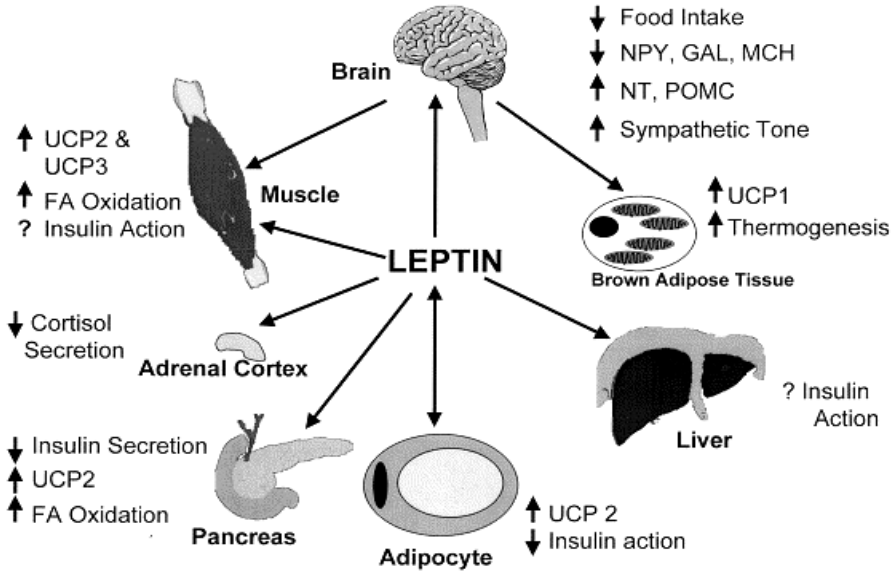


Figure 6: Schematic illustration of leptin coordination of energy homeostasis: central and peripheral mechanisms. The autocrine action of leptin on adipocytes increase the expression of uncoupling protein 2 (UCP2) and suppress insulin action. In fact, leptin's centralized action on the hypothalamus controls the food intake, thermogenesis and insulin by modulating the secretion of multiple neurotransmitters, including neuropeptide Y (NPY), galanin (GAL), and melanin-concentrating hormone (MCH). Leptin also upregulates neurotensin (NT) and proopiomelanocortin (POMC) and increases sympathetic tone. Leptin increases fatty acid (FA) oxidation and upregulates UCP2 and UCP3 in skeletal muscle. Leptin treatment inhibits the synthesis and secretion of cortisol. Leptin inhibits insulin secretion, upregulates UCP2, and increases FA oxidation in the pancreas. In the liver, the role of leptin in insulin action is equivocal. In brown adipose tissue (BAT) leptin upregulates UCP1, leads to thermogenesis. (↑, increase; ↓, decrease; ?, unknown/unclear) **(Houseknecht and Portocarrero, 1998)**.

2.5. Pathophysiology

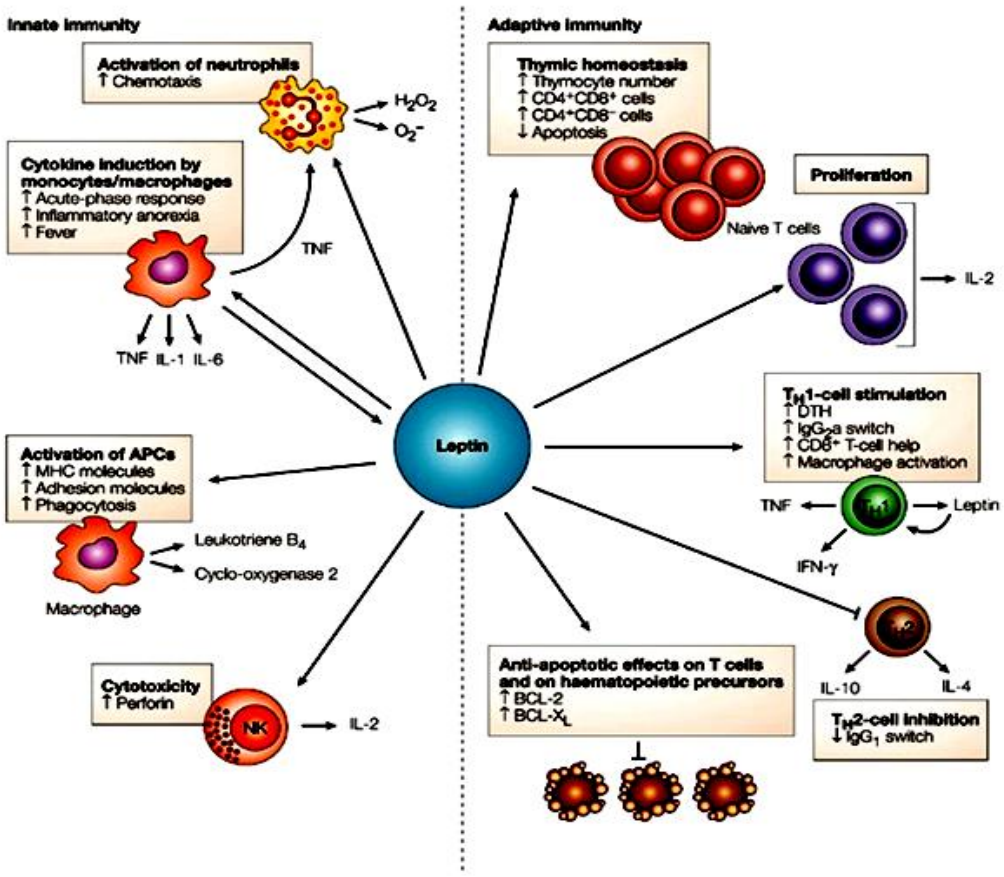
In general, obese individual comprises higher leptin levels in plasma to cerebrospinal fluid (CSF) compared to that of lean beings. The obesity related pathogenesis might be due to defective uptake of leptin is yet to be determined. A rapid accumulation of leptin in the plasma due to its resistance and inadequate transportation to the CSF authorizes for obesity **(Caro et al., 1996)**. In contrast, clinical manifestations like amenorrhea and anorexia nervosa are usually associated with decreased leptin levels **(Hebebrand et al., 1997, Ahima et al., 1996)**. Leptin also plays a critical role in the maintenance of the menstrual system. If systemic circulating leptin levels are >1.85mg/l, that ceases the menstrual cycle. Instead of body mass index (BMI) and body fat mass, leptin being considered as a potent marker for amenorrhea **(Mantzoro et al., 1997)**. It is still unclear that the role of leptin in the pathophysiology of polycystic ovarian syndrome. In Cushing's disease, the two-fold higher

plasma leptin levels corroborated with elevated BMI. A successful trans-phenoidal surgery treated the hypercortisolemia that is a symptom of Cushing's disease but could not regulate the basal or corticotrophin-releasing hormone (CRH)-stimulated leptin levels **(Cizza et al., 1997)**. Leptin role in inflammation related anorexia pathogenesis is indispensable. A Recent study showed that circadian rhythmicity of leptin abducted by a group of sepsis patients, and its levels were 3-folds higher in recovered patients compared to that of non-survivors. Leptin regulates the production of endogenous cortisol by acting on the HPA axis and also regulates the hematopoiesis and induces the macrophage phagocytic activity. In accordance, the leptin role in stress-related activities and healing the critical illness has been predictable **(Bornstein et al., 1998)**. In addition, several studies have reported the leptin protective immune functions or its deficiency associated pathogenesis of various bacterial infections such as tuberculosis **(Wieland et al., 2005)**, pneumonia **(Hsu et al., 2007)**, sepsis **(Tschöp et al., 2010)** and colitis **(Madan et al., 2014)**. It has been also reported that the leptin shown to inhibit oxidative burst in HIV+ monocytes by diminishing the ROS production, is in contrast to its natural ROS inducing activity **(Sánchez-Pozo et al., 2003)**. Leptin signaling impairs during hypertriglyceridemia, insulin resistance, and elevated SOCS3 expression **(Martin et al., 2008)** in obesity. It's desensitization in obesity diminishes vaccine-specific antibody response following influenza, tetanus, and hepatitis-B vaccination **(White et al., 2013)**. Leptin is been an effective mucosal vaccine adjuvant against *Rhodococcus equi* **(Cauchard et al., 2011)** and *Helicobacter pylori* **(Wehrens et al., 2008)**, its signaling in mucosal gut epithelial cells offers resistance against amebiasis caused by *Entameoba histolytica* infection **(Vedantam and Viswanathan, 2012)**. Further studies are needed to implicate its role in the pathophysiology and treatment of various human diseases.

2.6. Leptin as a cytokine

Leptin is a pleiotropic molecule, functions as a hormone and as a cytokine (adipokine). Normal development of hematopoiesis, angiogenesis, and innate and adaptive immunity are the characteristic features of adipokine action **(La cava and matarese 2004, Loffreda et al., 1998, Santos-Alvarez et al., 1999, Martin-Romereo et al., 2000, Matarese et al., 2005)**. Leptin's peripheral administration does not affect either food intake or body weight shown in previous studies reviewed by **Janet M. Bryson, 2000**. Also a thesis (Topically Applied Leptin Accumulates in the Eye and Hypothalamus but does not Influence Food Intake in Rats) submitted by Paul Raymond Mayo II, The New England College of Optometry, 2008 has shown that subcutaneous administration of leptin did not affect the food intake in rats, it

increases the serum leptin but not hypothalamic leptin. Studies on *ob/ob* mice revealed the critical relevance between leptin and inflammation or autoimmunity. In detail, *ob/ob* mice secreted the low amount of IL-2, IFN-γ, and the IL-18 and massive amount of Th2 cytokines (IL-4 and IL-10). Leptin regulates thymic homeostasis and induces the Th1 response by increasing IFN-γ and TNF-α production thus leads to activation of monocyte/macrophages and dendritic cells **(Loffreda et al., 1998, Santos-Alvarez et al., 1999, Martin-Romereo et al., 2000, Matarese et al., 2005, Zhang et al., 1997, Mattioli et al., 2005)**. Leptin directly activates the DCs to secrete IL-12, which is a key cytokine that facilitates the shifting of T-cells towards the Th1 phenotype **(Spencer and Daynes 1997)**. Acute leptin levels during infection and inflammation may be a protective component of the host response to inflammation **(Sarraf et al., 1997)**. Leptin induces the phagocytic activity of macrophages and prevents the apoptosis of various immune cells involved in innate and adaptive immune response by delaying the cleavage of *Bid* and *Bax*, and the mitochondrial release of cytochrome-c, and the activation of both caspase-8 and caspase-3 **(Bruno et al., 2005)**. Interestingly, leptin induces the proliferation of naive T-cells (CD4+CD45RA+) but inhibits proliferation of memory T-cells (CD4+CD45RO+) in mice model **(Lord et al., 1998)**. Leptin facilitates a survival signal to CD4+CD8+ and CD4+CD8− T-cells during maturation **(Howard et al., 1999)**. T-cells polarization towards a Th1 response by leptin seems to be mediated by inducing the synthesis of IL-2, IL-12, and IFN-γ and the inhibition of the production of IL-10 and IL-4 **(Martin Romereo et al., 2000, Napoleone et al., 2007)** (Fig 7). Therefore, leptin may be considered as a therapeutic target in some clinical aspects, such as proinflammatory states or immune deficiencies to improve the impaired immune response. Leptin associated immune aspects from different cell types of innate and adaptive immunity summarized in Fig.7.



http://www.nature.com/nri/journal/v4/n5/fig_tab/nri1350_F2.html

Figure 7: Immunoregulatory role of leptin. In innate immunity, leptin modulates the activity and function of neutrophils by increasing chemotaxis and the secretion of oxygen radicals such as hydrogen peroxide (H_2O_2) and superoxide (O_2-) through direct and indirect mechanisms. In mice, leptin seems to activate neutrophils directly. In humans, the action of leptin seems to be mediated by TNF secreted by monocytes. Leptin increases phagocytosis by macrophages and enhances the secretion of pro-inflammatory mediators of the acute-phase response and the expression of adhesion molecules. On NK cells, leptin increases cytotoxic ability and the secretion of perforin and IL-2. In adaptive immunity, leptin affects the generation, maturation and survival of thymic T-cells by reducing their rate of apoptosis. On naive T-cell responses, leptin increases proliferation and IL-2 secretion through the activation of MAPK and phosphatidylinositol 3-kinase (PI3K) pathways. On memory T-cells, leptin promotes the switch towards Th1 cell-mediated immune response by increasing IFN-γ and TNF secretion, the production of IgG2a by B-cells and DTH response. This process sustained by an autocrine loop of leptin secretion by Th1 cells. Finally, leptin has anti-apoptotic effects on mature T-cells and hematopoietic precursors.

Recently, the combinational therapy approved as effective against VL. Perhaps, this can minimize the emergence of drug resistance, treatment regime, and price. In clause of this, standard doses of Sodium stibogluconate in a combination of Paromomycin **(Chunge et al., 1990; Neal et al., 1995; Thakur et al., 2000)** and IFN-γ with SbV tested against VL and claimed it inefficient **(Jha, 2006)**. Other drug combinations include liposomal Amphotericin B and Miltefosine, being tested in India. Certainly, the most promising approach for the VL

eradication is immunotherapy, which involves the exogenous Th1 response stimulating cytokine or vaccine in a combination with the potent chemotherapeutic agent. Accordingly, the immune candidates like Bacillus Calmette-Guerin (BCG), Alum, IFN-γ and antigen-stimulated dendritic cells, etc. have been tested for VL and PKDL treatment in a combination with currently available drugs. Also, the antagonists of IL-10, TGF-β, IL-13 have also been tested against experimental VL in a combination of Pentavalent antimonials **(Murray, 2001)**. For socioeconomic reasons, taking of prevention measures is always more desirable than therapy. Despite the lack of vaccine, the considerable protection was achieved by chemotherapy with the implementation of successful prophylactic action against VL.

3. Plant-based natural therapy

Traditionally, plants and their derivatives or products become a part of our routine life. In which, herbal medicines achieved more importance in the treatment of both infectious and non-infectious diseases. In order, several reports have been tested the anti-leishmanial activity of different regional plants and identified promising effect **(Fournet et al., 1992; Singha et al., 1992; Torres-Santos et al., 1999; Delorenzi et al., 2001; Plock et al., 2001; Ferreira et al., 2002; Salvador et al., 2002; Khalid et al., 2004; Luize et al., 2005; Mishra et al., 2005; Singh et al., 2004; Sharif et al., 2006; Lakshmi et al., 2007; Bafghi et al., 2008; El-On et al., 2009; Getti et al., 2009; Kivcak et al., 2009; Yousefi et al., 2009; Biswas et al., 2010)**. The medicinal plants showing anti-leishmanial activity against VL also listed in Table.1. Among various traditional medicinal plants, Neem or *Azadirachta indica* is superlative and renowned as “One tree pharmacy” belongs to Meliaceae family. In Sanskrit, it being called as 'Arishtha' meant for a reliever of sickness. In Indian pharmacy, it engraved a unique place since more than 2000 years. Neem is a perennial tree cultivated in tropical and subtropical nations and also various regions of the Indian subcontinent.

Till date, more than 135 compounds identified in different fractions of Neem and divided into 2 major groups such as isoprenoids (diterpenoids, azadirone, gedunin, nimbin, salanin and azadirachtin) and non-isoprenoids (proteins, carbohydrates, sulphurous compounds, polyphenols: flavonoids and aliphatic compounds) **(Biswas et al., 2002)**. Neem seed oil extracts been using in manufacture of soaps and cosmetics and twigs been using as toothbrushes for several centuries. Various crop products being protected from insect pest attack by storing with Neem plant parts **(NRC, 1992, Brahmachari, 2004, Ogbuewu et al., 2011)**. It's selective insect pest-controlling property and low toxicity to mammals been attracting the scientists in a plethora of science.

Plant species (Family)	Active part or constituents	*In vitro* activity (IC_{50})	*In vivo* activity (ED_{50}) or % inhibition	References
Aloe vera (Liliaceae)	Leaf exudates	100-180 µg/ml- P 6.0 µg/ml- Ax	nd	Dutta *et al.* 2007a Dutta *et al.* 2007b
Alstonia scholaris (Apocyanaceae)	Stem bark	nd	66.2 ± 1.5 at 1.0 g/kg × 5, oral	Singha *et al.* 1992
Artemisia annua (Asteraceae)	Artemisinin	160 µM- P 22 µM- Am	nd	Sen *et al.* 2007a
Campnosperma panamense (Anacardiaceae)	Lanaroflavone	7.2 µM- Ax	nd	Wenigera *et al.* 2006
Cassia fistula (Fabaceae)	Clerosterol	10.03 µg/ml- P 18.1 µg/ml- Am	nd	Sartorelli *et al.* 2007
Croton cajucara (Euphorbiaceae)	Linalool-rich essential oil	8.3 ng/ml- P 8.7 ng/ml- Am	nd	Rosa *et al.* 2003
Diospyros montana (Ebenaceae)	Diospyrine	40-90% lethality at 2.5 µg/ml- P	nd	Hazra *et al.* 1987
Ginkgo biloba (Ginkgoaceae)	Ginkgetin Isoginkgetin	2.8 µM- Ax 1.9 µM- Ax	nd nd	Wenigera *et al.* 2006 Wenigera *et al.* 2006
Glycyrrhiza spp (Fabaceae)	Licochalcone A	nd	>96% activity at 20 mg/kg × 6, i.p.; 65-85% activity at 5-150 mg/kg × 6, oral.	Chen *et al.* 1994; Zhai *et al.* 1999
Maesa balansae (Maesaceae)	Triterpenoid saponin extract	1.0 µg/ml- Am	95% activity at 0.4 mg/ml by s.c. route	Maes *et al.* 2004
Myristica malabarica (Myristicaceae)	Ether extract	31.0 µg/ml- P	nd	Sen *et al.* 2007b
Nyctanthes arbortristis (Oleaceae)	Leaves extract Arbortristoside A	nd 64 % at 100 µg/ml- m	72.3 at 1.0 g/kg × 5, oral 79.7 at 10 mg/kg × 5, i.p.	Singha *et al.* 1992 Tandon *et al.* 1991
Peganum harmala (Peganaceae)	Harmine	25 µg/ml- P	79% activity at 1.5 mg/kg in nanoparticles	Lala *et al.* 2004
Picrorrhiza kurroa (Scrophulariaceae)	Iridoid glucosides Picroliv	nd	66-70% inhibition alone 74-85% Picroliv+SSG	Puri *et al.* 1992 Mittal *et al.* 1998
Swertia chirata (Gentianaceae)	Amarogentin Whole plant extract	nd nd	2.5 mg/kg × 1 79.8 ± 6.4 at 1.0 g/kg × 5, oral	Medda *et al.* 1999 Singha *et al.* 1992
Syzygium arnotianalum (Mynaceae)	Whole plant extract	nd	76.2 ± 5.2 at 1.0 g/kg × 5, oral	Singha *et al.* 1992
Taxus baccata (Taxaceae)	10-Deacytylbaccatin-III	70 nM- Am	nd	Georgopoulou *et al.* 2007
Tephrosia purpurea (Fabaceae)	Ethanol extract n-Butanol fraction	nd nd	61.1 at 500 mg/kg × 5, oral 90.3 at 100 mg/kg × 5, oral	Sharma *et al.* 2003 Sharma *et al.* 2003
Tibouchina semidecandra (Melastomaceae)	Whole plant extract	nd	74.7 ± 5.8 at 1.0 g/kg × 5, oral	Singha *et al.* 1992
Tinospora cordifolia (Menispermaceae)	Stem extract	nd	80.3 ± 1.7 at 1.0 g/kg × 5, oral	Singha *et al.* 1992
Virola surinamensis (Myristicaceae)	Neolignan	100 µM- P	100 mg/kg × 5 days	Barata *et al.* 2000

nd = not described; P = promastigotes; Am = amastigotes; Ax = axenic amastigotes

Table.1: Different medicinal plants tested against VL

Till date, many bioactive compounds identified from different parts of the Neem plant. In which, nimbin (anti-inflammatory), nimbidin (anti-bacterial & anti-ulcerative activities), nimbidol (anti-tubercular & anti-protozoal), gedunin (anti-malarial & anti-fungal), sodium nimbinate (diuretic & anti-arthritic), and salanin (insect repellent) exerts respective activities **(Brahmachari, 2004**, **Xuan et al., 2004, Subapriya and Nagini, 2005, Bhattacharyya et al., 2007, Gahukar, 2012)**. Despite the number of biological activities, few toxic effects also found to be exerted by the different species of Neem; perhaps mostly associated with salanin, melzatriol, nimbin, cardiac glycosides, tannins, alkaloids and saponins **(Biswas et al., 2002, Biu, 2008)**. However, till date no single report illustrated the Neem cytotoxicity in

macrophages. The IC_{50} <1µg/ml and selectivity index >20 are highly safe for macrophages and are best predictors of anti-parasitic activity (**Nwaka and Hudson, 2006)**. List of the various parts of Neem plant and its medicinal uses in Ayurvedic treatment shown in Table.2.

	Ayurvedic uses of Neem
Part	**Medicinal use**
Leaf	Leprosy, eye problem, epistaxis, intestinal worms, anorexia, biliousness, ulcers
Bark	Analgesic, alternative and curative of fever
Flower	Bile suppression, elimination of intestinal worms and phlegm
Fruit	Piles, intestinal worms, urinary disorder, epistaxis, phlegm, eye problem, diabetes, wound and leprosy
Twig	Cough, asthma, piles, phantom tumor, intestinal worms, spermatorrhoea, obstinate urinary disorder, diabetes
Gum	Scabies, wounds, ulcers, skin diseases
Seed	Leprosy and intestinal worms
Oil	Leprosy and intestinal worms

Table.2: List of the various parts of Neem plant and its medicinal uses in Ayurvedic treatment (**http://www.infinityfoundation.com/mandala/images/Neem_table.jpg)**.

OBJECTIVES

Undernourished children in endemic regions of developing countries are very vulnerable for VL due to immune dysfunction, which is also accompanied by low circulating leptin levels. Since the depletion of leptin levels in these children could be a possible high-risk factor for VL. In assumption, adequate levels of leptin might oppose the infection dissemination and disease progression using its immunoregulatory functions. It may become a new strategy in the treatment of infectious diseases associated with immunodeficiency. Recent failure of chemotherapy due to the unresponsiveness of host immunity or HIV/VL co-infection, change in the sensitivity of parasites, alteration in the pharmacokinetics of the drugs and rapid emergence of clinical resistance strains stipulates for the advanced approach of treatment. Hence, the combinational chemotherapy includes the liposomal Amphotericin-B and Miltefosine studied in the Indian subcontinent. However, in current circumstances, the most promising approach is immunotherapy that comprises the Th1 response stimulating exogenous cytokine/vaccine alone or in a combination with the potent chemotherapeutic agent for VL eradication. In fact, as a cytokine leptin could induce the secretion of Th1 cytokines and regulate the immune homeostasis. Hence, we assumed that leptin perhaps functions as an adjuvant and could minimise the treatment regimen thereby emergence of resistance and toxicity of currently recommended drugs. Indeed, the drawbacks of current chemotherapy against VL are also affirming the importance of safe and cost-effective remedy that could originate from plant-based natural products. Therefore, the Neem plant considered for our study that is notorious as "one tree pharmacy". It has anti-bacterial, anti-cancerous, anti-fungal, anti-inflammatory, anti-parasitic and immune-stimulatory roles. Based on this brief literature, we have tested the therapeutic efficacy of leptin and Neem leaf extracts against experimental VL. To address this, we have designed two primary objectives.

1. To decipher the Immunomodulatory activity of Leptin against Experimental Visceral leishmaniasis

(A) Leptin induces the phagocytosis and protective immune response in *Leishmania donovani* infected THP-1 cell line and human PBMCs

(B) Leptin augments the host protective immune response during experimental visceral leishmaniasis in malnourished BALB/c mice

2. Evaluation of Anti-leishmanial and Immunomodulatory properties of Neem leaf extract in Experimental Visceral leishmaniasis

CHAPTER 1

To decipher the Immunomodulatory activity of Leptin against Experimental Visceral leishmaniasis

1. Introduction

Visceral infection is the most severe clinical form of leishmaniasis characterized by the systemic disease to vital lymphoid organs. Fortunately, it is curable in most of the cases, but if left untreated, it ultimately leads to death. It has reported that about 67% of global VL cases are harboring in the Indian subcontinent alone, and about 200 million people are at the risk of developing VL **(Chappuis et al., 2007)**. The global burden of VL is about 4 lakh new cases and 60,000 deaths per year **(Alvar et al., 2012)**. During human VL, the protective immune response against *Leishmania* infections is accompanied by IFN-γ producing $CD4^+$ cells **(Kharazmi et al., 1999)**. On the other hand, the pathogenic response is predominantly mediated by Th2 type cytokines characterized by IL-10 production and absence of IFN-γ in *Leishmania* antigen-activated PBMCs **(Ghalib et al., 1995)**. Recently, it has been reported that in active VL cases, PBMCs could fail to produce cytokines in response to *Leishmania*-antigen stimulation *in vitro*, suggesting the immune dysfunction **(Kumar and Nylén 2012)**. Among the VL cases, malnutrition is major risk factor, since default immune setting and low circulating leptin levels.

Malnutrition affects both innate and acquired immunity **(Schaible and Kaufmann, 2007, Woodward, 1998)** and also affects the ratio of CD4+/CD8+ T-cells **(Chandra et al., 1991)**. Protein-energy deficiency is a primary concern of malnutrition, globally affecting 826 million people, of which 95.9% only in developing countries **(Katona, Katona-Apte, 2008)**. It mostly affects the children under 5-years old and causes for 2.2 million annual deaths worldwide **(Black et al., 2008)**. It is associated with immune suppression **(Faggioni et al., 2000)** and low leptin levels **(Sánchez-Margalet et al., 2003)** thereby highly susceptible to infections due to defective cytokine production **(Rodríguez et al., 2007)**. VL is one such disease that majorly affects undernourished children in tropical and subtropical countries. Although VL is asymptomatic at an early stage, protein-energy deficiency increases the risk of rapid development of symptomatic VL. Typically, leptin deficiency leads to increased susceptibility to various infectious diseases via dysregulation of the cytokine production or immunosuppression **(Faggioni et al., 2001)**. In humans leptin deficiency is associated with increased production of IL-4 and IL-10 and depletion of IFN-γ, IL-2, IL-12 and TNF-α production **(Napoleone et al., 2007)** which correlated to VL pathogenesis **(De Medeiros et**

al., 1998). Leptin modulates CD4+ and CD8+ T-cell activation towards a Th1 phenotype by stimulating the synthesis of IL-2 and IFN-γ **(Rodríguez et al., 2007)**.

Based on the previous reports, we had hypothesized that leptin could be helpful in the maintenance of favorable host environment by regulating the immune dysfunction appeared in VL patients **(Dayakar et al., 2011**). Recently, leptin was shown to augments the protective immune response and Miltefosine efficacy in mouse macrophages (J774.1 cell line) against experimental VL **(Shivahare et al., 2015)**. In the current study, initially we analyze the immunomodulatory activity of leptin by measuring the relative expression of cytokine mRNA profile in *L. donovani* infected human macrophages both *in vitro* and *ex vivo*. We also studied macrophage stimulation activity of leptin by measuring phagocytic index, intracellular ROS production and phosphorylation of signaling molecules. Later on, we evaluated leptin associated protective immune response in malnourished BALB/c mouse model.

(1A) Leptin induces the phagocytosis and protective immune response in *Leishmania donovani* infected THP-1 cell line and human PBMCs

1. Abstract

To determine the cytokine role of leptin in VL, we tested the leptin associated Th1/Th2 cytokine profile at mRNA level from *L. donovani* infected human monocytic leukemia cell line (THP-1) and PBMCs. We observed that leptin-induced Th1 specific response by upregulation of IL-1α, IL-1β, IL-8 and TNF-α in THP-1 and IFN-γ, IL-12 and IL-2 in PBMCs. We also noticed that the Th2 type cytokine downregulation i.e. IL-10 in THP-1 and unaltered expression i.e. TGF-β, IL-10 and IL-4 in PBMCs. In addition, leptin stimulates the macrophages by inducing phosphorylation of extracellular-regulated kinase1/2 (Erk1/2) and protein kinase-B (Akt) which usually dephosphorylated in *L. donovani* infection. In concordance, leptin also induces the macrophage phagocytic activity by enhancing the intracellular reactive oxygen species (ROS) generation that helps in phagolysosome formation and oxidative killing of the parasite. In compilation, leptin can maintain the defensive environment against *L. donovani* infection through the classical macrophage activity.

2. Methodology

2.1. Parasite culture

The promastigotes of *L. donovani* (Dd8) WHO reference strain (MHOM/IN/80/Dd8) obtained from ATCC (American Type Culture Collection, U.S.A) and cultured in Medium-199 supplemented with15% heat-inactivated fetal bovine serum (FBS), 20mM HEPES, pH7.4, 4mM $NaHCO_3$, 100U/ml of penicillin and 100mg/ml of streptomycin at 25±1^oC.

2.2. THP-1 &PBMCs maintenance

THP-1 suspension cells obtained from National Center for Cell Science (Pune, India) and PBMCs isolated from healthy adult humans on 30% Ficoll-Histopaque density gradient. Mononuclear phagocytes were purified from PBMCs by adherence to 8-well chamber slides at 37^oC and 5% CO_2 for overnight. Non-adherent cells harvested at 400×g for 10 min and added back at the time of treatment after completion of infection to adherent macrophages. Cells cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 4.5g of glucose/liter, 10mM HEPES, 1mM sodium pyruvate and 10% (v/v) FBS and maintained in 5% CO_2 incubator at 37^oC.

2.3. Phagocytic activity

Prior to the infection, THP-1 cells were seeded in 8-well chamber slides and treated with PMA (10ng/ml) and incubated at 37^oC and 5% CO_2 for 24h to transform into macrophage-like phenotypes. Macrophages (3×10^4/ml) of both THP-1and PBMCs cocultured with 10:1 ratio of parasites to macrophage and different concentrations (0, 25, 50, and 100ng/ml) of recombinant human leptin (Sigma) for 6h at above-mentioned conditions. Non-internalized parasites were thoroughly washed off with phosphate buffer saline (PBS) and incubated for next 18h in fresh medium, and the nonadherent subpopulation of cells added back in case of PBMCs. After incubation, slides were stained with Giemsa (Himedia). Each time, the percentage of infected macrophages and the number of engulfed parasites were counted out of total 500 macrophages using the light microscope (Leica). Experiment was performed in triplicates. The phagocytic index was calculated using formulae:

$$\frac{\text{Number of engulfed parasites} \times \text{Number of infected } \varphi}{\text{Total number of counted } \varphi} \times 100$$

2.4. Determination of intracellular ROS

2,7-dichlorodihydrofluoresceindiacetate (H_2DCFDA) is a cell permeable dye was used to monitor the intracellular ROS levels from the PMA-activated THP-1 (3×10^4 cells/ml). Here, we measured the ROS from different experimental variables such as uninfected,

infected, and post-infection with leptin (100ng/ml), and lipopolysaccharide (LPS) treated (100ng/ml) cells after 6h. Briefly, cells were washed with PBS and incubated with H_2DCFDA dye (10µM) in PBS for 15 min in dark at room temperature (RT). Green fluorescence of 2,7-dichlorofluorescein (H_2DCF) was measured using a flow cytometry (FACS Calibur) and mean fluorescence intensity (MFI) represented as bar graphs.

2.5. Analysis of signaling molecules phosphorylation status

Briefly, the phosphorylation status of Akt and Erk signaling moieties during *L. donovani* infection in PMA-activated THP-1 (2×10^6 cells/ml) was determined by western blot analysis. The whole cell lysates prepared after 30 min of incubation from different experimental variables (infected, post-infection with leptin (100ng/ml), and LPS treated (100ng/ml) using radioimmunoprecipitation assay (RIPA) buffer (contain 1% proteinase inhibitor cocktail). Aliquots of cell lysates containing 25µg of total protein resolved by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto membranes (Millipore) by electro-blotting. The membranes were blocked for 1h at RT in TBS-T buffer with 5% non-fat dried milk and incubated with primary antibodies (1:1000) overnight at 4°C. The blots were washed three times for 20 min with TBS-T buffer and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000) for 1h at RT. After washing as mentioned above, the blots were developed using enhanced chemiluminescence reagents (ECL, Pierce Biotechnology, Inc., Rockford, IL) and visualized using versa doc instrument (Bio-Rad). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Bands scanned for the normal and phosphorylated signals of both Akt, Erk1/2 using their specific antibodies.

2.6. Gene expression analysis

Leptin, associated cytokine gene expression analysis, was performed by using real-time quantitative polymerase chain reaction (RT-qPCR). Briefly, the total RNA isolated from PMA-activated THP-1 and PBMCs (2×10^6 cells/ml) of different experimental variables such as uninfected, infected, post-infection with leptin-treated (100ng/ml) and LPS treated (100ng/ml) cells after 6h using Nucleospin RNA kit protocol. RNA was quantified, and 0.53µg of the template used for complementary DNA (cDNA) preparation using kit (Takara) protocol. The cDNA amplified for our following targeted genes using SYBR Premix Ex Taq (2X) (Takara) with their respective primers (Table.1). The reaction programmed for 2 min at 50^oC, 10 min at 95^oC, 40 cycles of 15 sec at 95^oC, 1 min at 60^oC (ABI Prism 7300 Sequence Detector). Results were analyzed using StepOnePlus™ software, Applied Biosystems. The

relative fold expression ($2^{-\Delta\Delta ct}$) of the target gene calculated by normalization with GAPDH constitutive gene threshold cycle (C_T) values.

Primer name	Sequence (5'-3')
IL-8	FP: 5'-CAGCCTTCCTGATTTCTGCAGCTC-3' RP: 5'-GGTCCACTCTCAATCACTCTCAGTTC-3'
IL-1β	FP: 5'-CCCACAGACCTTCCAGGAGAATGA-3' RP: 5'-GGAGCGTGCAGTTCAGTGATCGTA-3'
IL-1α	FP: 5'-CGCCAATGACTCAGAGGAAGA-3' RP: 5'-AGGGCGTCATTCAGGATGAA-3'
IL-2	FP: 5'-AACTCACCAGGATGCTCACATTTA-3' RP: 5'-TCCCTGGGTCTTAAGTGAAAGTTT-3'
IL-12p40	FP: 5'-TGGAGTGCCAGGAGGACAGT-3' RP: 5'-TCTTGGGTGGGTCAGGTTTG-3'
IFN-γ	FP: 5'-TCAGCTCTGCATCGTTTTGG-3' RP: 5'-GTTCCATTATCCGCTACATCTGAA-3'
TNF-α	FP: 5'-TCTTCTCGAACCCCGAGTGA-3' RP: 5'- CCTCTGATGGCACCACCAG-3'
IL-10	FP: 5'-GTGATGCCCCAAGCTGAGA-3' RP: 5'- CACGGCCTTGCTCTTGTTTT-3'
IL-4	FP: 5'-CCACGGACACAAGTGCGATA-3' RP: 5'- CCCTGCAGAAGGTTTCCTTCT-3'
TGF-β	FP: 5'-CAGCAACAATTCCTGGCGATA-3' RP: 5'- CTGCTGGCACCCAGCGACTCG-3'
iNOS	FP: 5'-TGCAGACACGTGCGTTACTCC-3' RP: 5'-GGTAGCCAGCATAGCGGATG-3'
GAPDH	FP: 5'-CCCATGTTCGTCATGGGTGT-3' RP: 5'-TGGTCATGAGTCCTTCCACGA-3'

Table 1: Human gene specific primers for several cytokines have been tabulated and were used to amplify the target mRNA by RT-qPCR.

2.7. Statistical analysis

Results expressed as mean ± standard deviation (SD) values and significance were calculated using two-tailed unpaired t-test (GraphPad Prism 5). Statistical significance considered as $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ and represented by *, **, and *** respectively.

3. Results

3.1. Leptin induces parasite uptake by macrophages of both THP-1 and PBMCs

We observed the increased percentage of phagocytised macrophages in THP-1 (~52%) and PBMCs (~56%) upon leptin (100ng/ml) treatment compared to untreated THP-1 (~33%) and PBMCs (35%) respectively. Moreover, the amastigotes count per macrophage in THP-1 (~2.3) and PBMC (~1.3) was not significantly altered to the untreated THP-1 (~2.1) and PBMCs (~1.3) respectively (data not shown). Likewise, we observed the increased phagocytic indexes; 113.75±20.29, 141±34.91, 189.71±24.32, and 312.08±42.7 respectively in THP-1 (Fig.1A) and 79.19±3.20, 97.23±6.25, 109.29±9.42, and 204.16±31.85 respectively in PBMCs (Fig.1B) with increased leptin 0, 25, 50 and 100ng/ml respectively, substantiated the early report on *L. major* infection **(Gainsford et al., 1996)**.

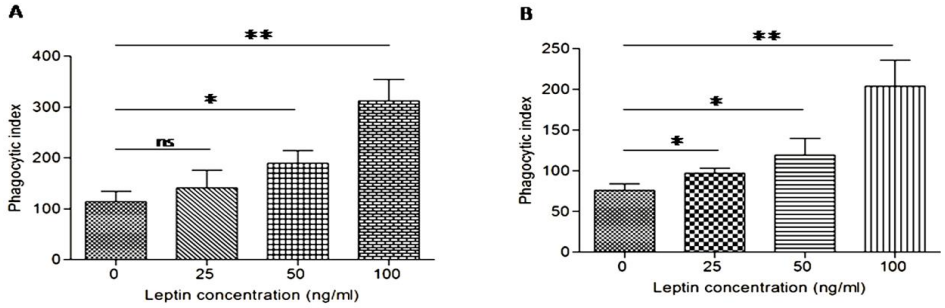


Figure 1: Macrophages phagocytic index versus leptin concentration (ng/ml). (A) THP-1 and (B) PBMCs were showing the proportional increase in phagocytic activity of macrophages with an increase in the leptin concentration (0, 25, 50 and 100ng/ml). Significance represented by *$p \leq 0.05$ and **$p \leq 0.01$ respectively.

3.2. Leptin upregulates the ROS generation in infected THP-1 cell line

In comparison between the infected and its leptin-treated THP-1 cells, the intracellular ROS production or MFI was significantly induced in leptin-treated cells (247.41±4.24). On other hand, the MFI of infected cells (212.38±5.62) was lesser than uninfected cells (233.33±5.73), which might be due to the dephosphorylation of Akt kinase during *L. donovani* infection (Fig.2). It implies for delayed phagolysosome formation **(Gueirard et al., 2008)**, which affects the parasite uptake and its oxidative killing by host cells **(Laufs et al., 2002)**.

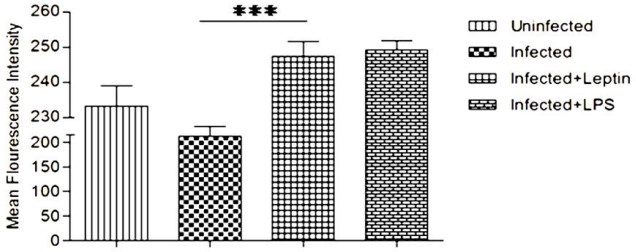


Figure 2: Intracellular ROS production (MFI) in THP-1. In comparison between the infected and its leptin-treated cells, the MFI was significantly increased in leptin-treated cells. Uninfected cells considered as normal, and LPS treated cells were a positive control. Significance represented by ***$p \leq 0.001$.

3.3. Leptin enhances the phosphorylation of Akt and Erk1/2 signaling

During *L. donovani* infection, the dephosphorylation of signaling cascade proteins such as Akt kinase **(Dey et al., 2007)** and Erk1/2 **(Nandan et al., 1999 and Kar et al., 2010)** is responsible for immunosuppression. Accordingly, in our study, we have noticed the induced phosphorylation signal of both Akt and Erk1/2 in leptin-treated THP-1 infected cells compared to its control suggesting its protective role against *L. donovani* infection. However, the normal Akt and Erk1/2 signal molecule expression was found to be unaltered among different experimental variables (Fig.3).

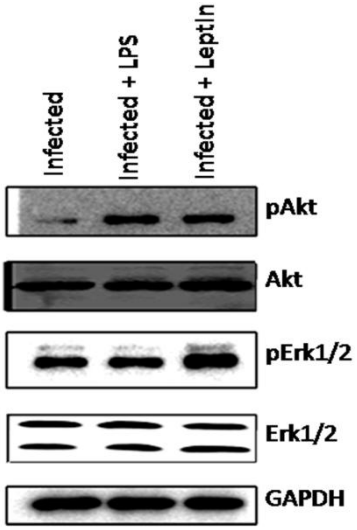
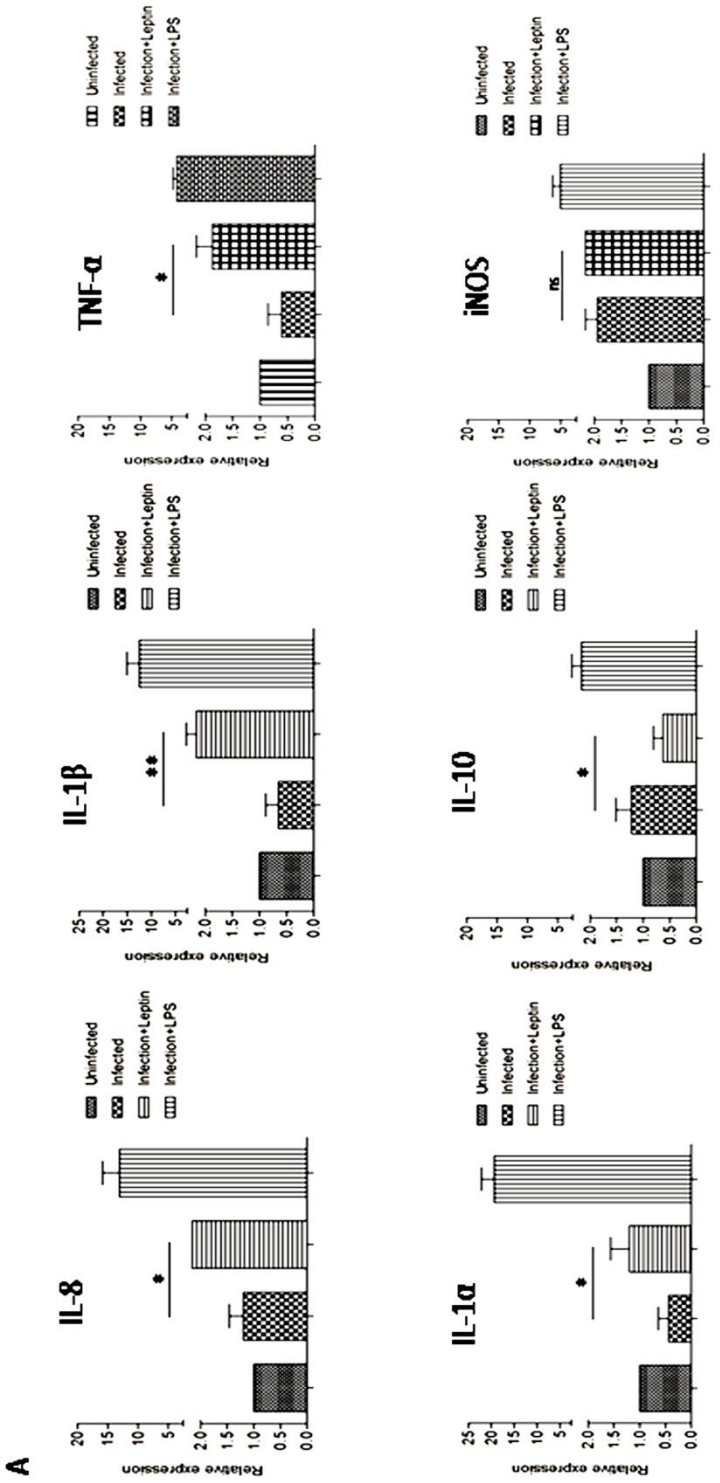


Figure 3: Phosphorylation status of Akt and Erk1/2 in THP-1. In comparison between the infected and its leptin-treated cells, the phosphorylation signal of Akt and Erk1/2 was induced in leptin-treated cells. The normal status of both signaling molecules were unaltered between the infected and its leptin-treated cells. GAPDH used as constitutively expressed loading control and LPS treated cells were a positive control.

3.4. Leptin enhances proinflammatory cytokine response against *L. donovani* infection

In comparison between the infected and its leptin-treated cells, the Th1 type cytokines namely, TNF-α, IL-8, IL-1α, and IL-1β were significantly upregulated and the Th2 type cytokine IL-10 expression was significantly downregulated in leptin-treated THP-1 cells but the inducible nitric oxide synthase (iNOS) expression was found to be unaltered (Fig.4A). In PBMCs, the Th1 cytokines such as IFN-γ, IL-2, and IL-12 expression was significantly upregulated and TNF-α was found to be unaltered along with Th2 cytokines such as IL-10, IL-4 and TGF-β in leptin-treated cells (Fig.4B). However, the relative expression of Th1 to

Th2 cytokines was higher in leptin-treated THP-1 and PBMCs suggests that leptin could be a protective agent against *L. donovani* infection.



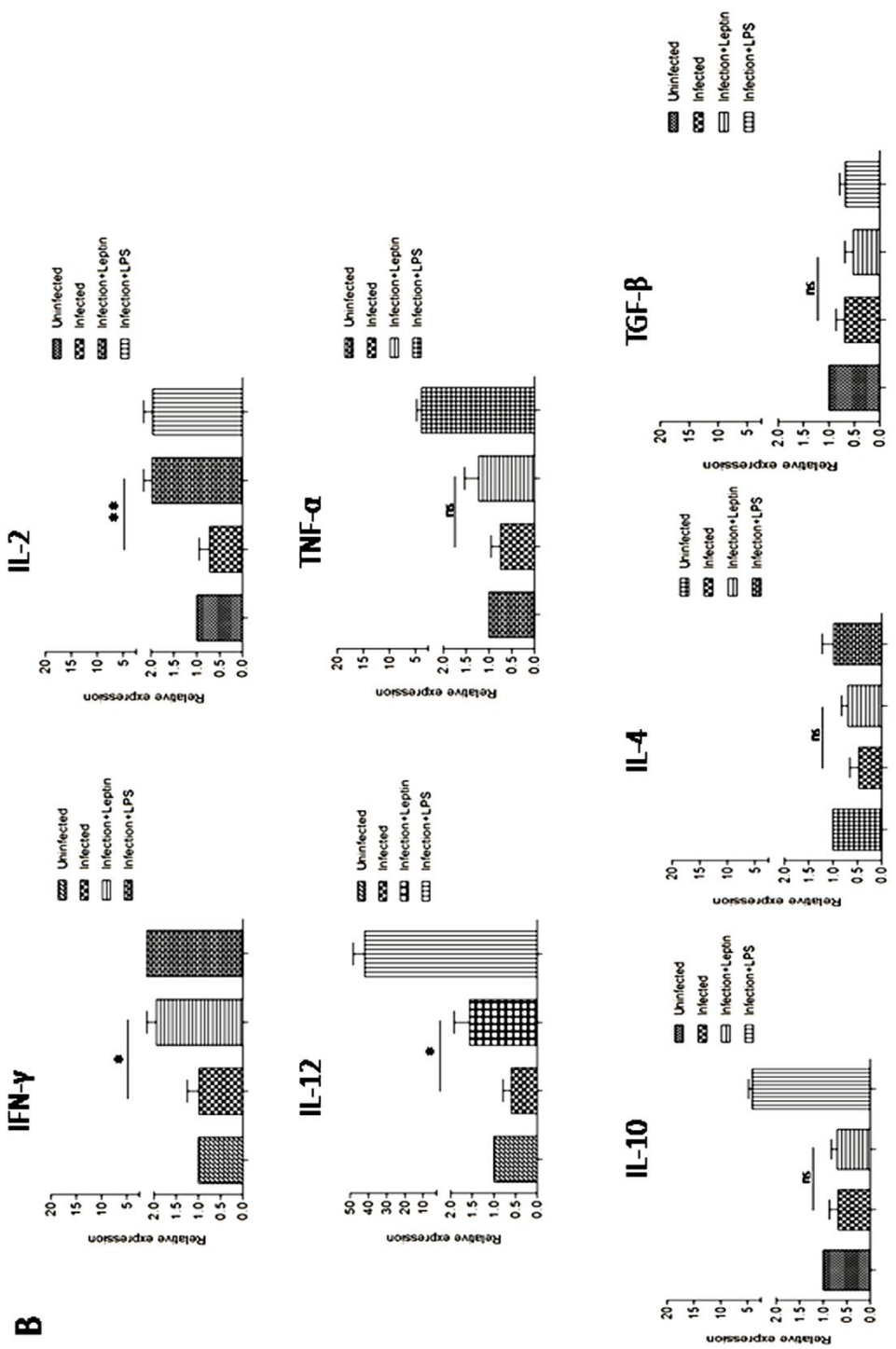


Figure 4: RT-qPCR analysis of cytokine mRNA relative folds expression. In comparison between the infected and its leptin-treated cells, (A) In THP-1, the IL-8, IL-1β, IL-1α, and TNF-α upregulated, and IL-10 was downregulated in leptin-treated cells. In addition, iNOS expression was unaltered between these two experimental variables (B) In PBMCs, the IFN-γ, IL-2 and IL-12 upregulated in leptin-treated cells and TNF-α, IL-10, IL-4 and TGF-β were unaltered between these two experimental variables. Uninfected cells considered as normal, and LPS treated cells were a positive control. Significance represented by *$p \leq 0.05$ and **$p \leq 0.01$ respectively.

4. Discussion

Despite the several studies on the role of immunoregulatory activities of leptin, a limited research is being focused on the fate of leptin during the infections and its associated immunity. Of which, the bacterial infections such as pulmonary tuberculosis and pneumococcal pneumonia infection have implemented the protective role of leptin **(Wieland et al., 2005, Hsu et al., 2007)**. Most recently, its protective action was also established *in vitro* experimental VL **(Shivahare et al., 2015)** based on our earlier hypothesis **(Dayakar et al., 2011**). In order to this, the current study has proved the immunomodulatory activity of leptin for the first time during protozoan (*L. donovani*) infection in human macrophages. We have noticed an apparent Th1 polarization by leptin at mRNA level in both THP-1and PBMCs as reported in the earlier studies **(Mattioli et al., 2005, Loffreda et al., 1998, Santos-Alvarez et al., 1999, Martin-Romereo et al., 2000, Matarese et al., 2005, Zhang et al., 1997)**. It has been noticed that leptin was able to induce the phagocytic activity of macrophages **(Bruno et al., 2005)** as shown in an earlier report on *L. major* infection in mice model **(Gainsford et al., 1996)**. Concurrently, it induces the phosphorylation of Akt kinase and intracellular ROS production from infected THP-1 as already been established in Sodium antimony gluconate treatment of VL **(Mookerjee et al., 2006)**. The abundant intracellular ROS could help in the rapid internalization of the parasite by host cells through inducing the fusion of phagosome with lysosome **(Gueirard et al., 2008)** for oxidative killing **(Laufs et al., 2002)**. A weak phosphorylation signal of Akt kinase in infected THP-1 cells could be one of the possible implications for immune dysfunction in *L. donovani* infection as reported in viral infection **(Avota et al., 2001)**. In *Leishmania* infection, inhibition of Erk1/2 signaling diminishes the iNOS mRNA expression **(Nandan et al., 1999 and Kar et al., 2010)**. In contrast, we could not find such an inhibition in infected THP-1, but with leptin-treated cells, the iNOS mRNA expression was unaltered in spite of a stronger Erk1/2 phosphorylation signal. Altogether, as a cytokine, it is playing a host protective role against *L. donovani* infection *in vitro*. Further, it is needed to evaluate the circulating leptin levels and its immunomodulatory role in experimental and clinical VL.

(1B) Leptin augments the host protective immune response during experimental visceral leishmaniasis in malnourished BALB/c mice

1. Abstract

Protein-energy malnutrition (PEM) influenced low serum leptin is a deleterious factor for the rapid development of VL. According to Mini-nutrition assessment (MNA) tool, low serum leptin is considered as a good biomarker for malnutrition. In concordance, we also noticed a drastic fall in serum leptin during PEM is evidenced for compromised immune response for VL. Interestingly, the low serum leptin during *L. donovani* infection in well-nourished mice also strengthens our idea of proving the leptin as a protective component in VL. In this report, leptin influences the host protection for some extent by inducing VL competent immune response during PEM. Elaborately, leptin restoration in serum correlates with reduced parasite load in visceral organs such as spleen and liver. Interestingly, in spleen it increases the functional CD8+T-cell population (i.e. upregulation of Granzyme (Graz)-A, and downregulation of PD-1 and CTLA-4. On the other hand, it decreases the CD4+ Th2-phenotypic cell population (i.e. downregulation of IL-10, IL-4 and TGF-β expression). Simultaneously, it also induces apparent Th1 polarization by stimulating the production of IFN-γ, IL-2 and TNF-α in SLA-stimulated splenocytes. It regulates the defective activation and proliferation of monocytes which mediated by granulocyte macrophage-colony stimulating factor (GM-CSF). In the liver, it induces granuloma formation (i.e. size and count) to clear the infection and controls the hepatic degeneration/necrosis at the centrilobular region. Further, it decreases the arginase activity in bone marrow-derived macrophages (BMDM), which deprives the L-arginine at microenvironment required for efficient T-cell function. It also induces serum IgG2a and the ratio of IgG2a/IgG1, which positively correlates with host protective Th1 phenotypic response. Altogether it suggests that leptin could be the key component during malnutrition to maintain competent host immunity against VL.

2. Methodology

2.1. Parasite culture

Promastigotes strain (Dd8) of *L. donovani* was cultured at 25^{o}C±1 as mentioned earlier in chapter 1A, methods section 2.1.

2.2. Experimental diets

Experimental diets obtained from National Centre for Laboratory Animal Sciences (NCLAS, Hyderabad). In our study, we have used two diets such as diet-A and diet-D, majorly differed in their protein content and micro-elements like zinc and iron **(Anstead et al., 2001)**. The diet-A (nutritional diet) consists of 21% of protein and sufficient zinc and iron, and the diet-D (malnutrition diet) consists of 1.25% of protein, deficient in zinc and iron. Usually, the zinc deficiency implies the PEM **(Filteau and Woodward 1982)** and the iron deficiency is more prevalent in developing countries.

2.3. Ethical license and Experimental design

Animal experiments performed according to Institutional ethical committee guidelines (UH/IAEC/2014/RM/14) University of Hyderabad, Hyderabad. In our study, female BALB/c mice with 8-weeks of age and 28-32g of average body weight were used. Our experimental animals (*N*=23) were divided into 5 groups, of which, uninfected (normal) group of each diet (*n*=4), infected group of diet-A (*n*=4) and diet-D (*n*=5), and infected diet-D supplemented with leptin (*n*=6). Each group fed for 6-weeks with 3.8g/mouse/day of the respective diet. Infected groups of each diet received the stationary phase promastigotes (2×10^{7}) through tail vein injection after 3-weeks of feeding. Leptin (5µg/day) was supplemented to diet-D group by subcutaneous injection for 3-weeks during the period of infection. After 6-weeks, body weight of each mice group was recorded, and then post-prandial blood glucose was monitored using the ACCU-CHEK® kit by tail vein puncture. Then the mice were euthanized; blood withdrawn through retro-orbital puncture and serum was separated. The visceral organs (i.e. spleen and liver), and the bone marrow also collected and used in further experiments. The percentage of visceral organ weight to body weight was calculated separately for both the spleen and liver.

2.4. Pre-assessment of malnutrition

In a different set of experiment, it has been pre-assessed for the malnutrition state after 3-weeks of feeding. In order to this, we measured the serum leptin followed by other important parameters like body weight, serum triglycerides (TGs) and serum cholesterol and blood glucose. Here, the experimental animals (*N*=8) were divided into two groups (*n*=4) based on their diet.

2.5. Quantitation of serum leptin

Serum leptin was estimated by using Mouse Leptin enzyme-linked immune sorbent assay (ELISA) kit protocol (Sigma). Briefly, 10µl of serum diluted with 90µl of assay buffer was loaded into appropriate wells and incubated at RT for 2:30h and the solution were discarded and then washed with 1x wash buffer for four times. After this, 100µl of 1x biotinylated detection antibody was added and incubated for 1h at RT with gentle shaking followed by repeated washes as mentioned above. In the next step, 100µl of Horseradish peroxidase (HRP)-Streptavidin was added and incubated for 45 min at RT followed by repeated washes. Finally, 100µl of 1x tetramethyl benzene (TMB) reagent was added and incubated for 30 min at RT in the dark with gentle shaking, and the reaction was terminated by adding 50µl of Stop solution. The color intensity was read at λ_{450nm} using a microplate reader (TECAN), and leptin concentration was calculated by using standards.

2.6. Quantitation of serum TGs

Serum TGs were quantified by an enzymatic method using Prism Diagnostics kit protocol. Briefly, 10µl of serum mixed thoroughly with 1ml of enzyme reagent and incubated for 15 min at RT. The color intensity was measured at λ_{505nm} using spectrophotometer (HITACHI U-2910). Double-distilled water (10µl) mixed with enzyme reagent used as blank. The amount of TGs was calculated by comparing the optical density (OD) of their respective standards.

2.7. Quantitation of serum IgG1 and IgG2a

Serum IgGs' quantified by the standard indirect ELISA method. Briefly, 250ng/well of SLA was coated into appropriate wells and incubated for overnight at 4^oC. Next day, the solution was discarded and washed for 3-times with1x phosphate buffer saline-tween20 (PBS-T) followed by blocking for 2h at RT with 150µl of 1% bovine serum albumin (BSA) prepared in PBS. After this, the solution was discarded, and washes were repeated. In the next step, serum (1:100 in dilution buffer) was loaded into appropriate wells and incubated for 1h at 37^oC followed by repeated washes. After this, 1:10000 dilutions of anti-mouse IgG1 and IgG2a antibodies (Abcam®) added to their respective wells and incubated for 1h at 37^oC. After the incubation solution discarded and washes were repeated with 5 min incubation. Finally, 100µl of 1x TMB substrate reagent was added and incubated for 30 min in the dark at RT. The reaction was terminated by adding 50µl of Stop solution (1N H_2SO_4), and the color intensity was measured at λ_{450nm} using a microplate reader (TECAN).

2.8. Quantitation of parasite burden

Parasite burden in visceral organs was determined by limiting dilution assay. Briefly, spleen and liver tissues were homogenized in complete Medium-199 (cM199) by using 70µm cell strainers (BD Biosciences). Total homogenates were spun down at 400×g for 5 min. The red blood cells (RBCs) of the spleen were lysed by incubating with RBC lysis buffer for 10 min at RT followed by a wash with fresh medium at 400×g for 5 min. Then, splenocytes and hepatocytes were resuspended in 2ml and 5ml of fresh cM199 respectively. From there, 50 µl of each tissue homogenate was loaded separately into 1st well of 96-well plates that preloaded with 200µl of fresh medium in all the wells. After this, limiting dilution was performed by transferring 50µl from 1st well to 2nd well and so on till 12th well in a row. After 10-days of incubation at 25^{o}C, the plates were read under the light microscope for detection of motile parasite in positive highest dilution. The total parasite burden was calculated by using the formula:

$$\text{Total tissue parasite load} = \frac{\text{Parasite positive highest dilution containing tissue homogenate volume}}{\text{Dilution factor}} \times \text{Total homogenate volume}$$

2.9. Histopathological studies

Liver tissue sections were collected from different mice groups and placed in 10% formalin saline buffer and then processed for histopathological observations. After fixation, tissues were embedded in paraffin and sliced into 4µm thick sections and mounted on microscopic slides. These slides stained with haematoxylin-eosin (H-E) using standard laboratory protocol. Inflammatory foci/granuloma formation and degenerative/necrosis changes examined under the light microscope (Zeiss Axioplan 2 imaging microscope, AxioVision 3.1software). The number of granulomas was counted using a light microscope (Leica).

2.10. Gene expression analysis

For gene expression analysis, total RNA was isolated from spleen tissue and RT-qPCR was performed using 500ng of template cDNA as mentioned earlier in chapter 1A, methods section 2.6. Gene-specific primers of mouse origin were designed and tabulated (Table.1).

Primer name	Sequence (5'-3')
IFN-γ	FP: 5'-TCAAGTGGCATAGATGTGGAAGAA-3' RP: 5'-TGGCTCTGCAGGATTTTCATG-3'
IL-12p40	FP: 5'-GGAAGCACGGCAGCAGAATA-3' RP: 5'-AACTTGAGGGAGAAGTAGGAATCG-3'
IL-10	FP: 5'-GGTTGCCAAGCCTTATCGGA-3' RP: 5'-ACCTGCTCCACTGCCTTGCT-3'
IL-4	FP: 5'-ACAGGAGAAGGGACGCCAT-3' RP: 5'-GAAGCCCTACAGACGAGCTCA-3'
TGF-β	FP: 5'-TGACGTCACTGGAGTTGTACGG-3' RP: 5'-GGTTCATGTCATGGATGGTGC-3'
Granzyme-A	FP: 5'-CAT TGG AGG AGA CAC GGT TGT TCC-3' RP: 5'- CTC TTT CCC ACG TTA CAG TGG GC-3'
CTLA-4	FP: 5'-GGACTTGGCCTTTTGTAGCCCT-3' RP: 5'-ATT CAC ATG GAAAGC TGG CGA CAC-3'
PD-1	FP: 5'-CCTGGTCATTCACTTGGGCTGTG-3' RP: 5'- GGT GGC ATT TGC TCC CTC TGA-3'
GM-CSF	FP: 5'-GCCATCAAAGAAGCCCTGAA-3' RP: 5'- GCGGGTCTGCACACATGTTA-3'
GAPDH	FP: 5'-CAAGGCTGTGGGCAAGGTCA-3' RP: 5'-AGGTGGAAGAGTGGGAGTTGCTG-3'

Table 1: Mouse gene specific primers for several cytokines have been tabulated and were used to amplify the target mRNA by RT-qPCR.

2.11. Quantitation of cytokines

Splenocytes (5×10^6/ml) were seeded into a 12-well plate and stimulated by incubating with SLA (60µg) for 72h in RPMI-1640 medium supplemented with 10% heat-inactivated FBS. After incubation, the supernatant was collected and stored at -80^{o}C until use. At the time of experiment, samples were processed according to BD™ CBA mouse kit instructions. Briefly, 50µl of capture bead mixture of different cytokines (IFN-γ, IL-2, TNF-α, IL-4 and IL-5), 50µl of culture supernatant and 50µl of Phycoerythrin (PE) detection reagent were mixed and incubated for 3h in dark at RT, then spun down at 200×g for 5 min, gently discarded the supernatant and pellet washed and resuspended in wash buffer (300µl) and analyzed by using a flow cytometer (BD LSRFortessa™, FCAP Array™ v3. 0 software). The cytokine concentration in the culture supernatant was quantified by comparing with their standards (ranged between 1-6000 pg/ml).

2.12. Cell surface staining

Splenocytes stained with fluorescence-labeled cell-surface markers such as Fluorescein isothiocyanate (FITC)-CD3, PE-CD4 and Allophycocyanin (APC)-CD8 (eBiosciences) to

analyze T-cells and its subpopulation. Briefly, splenocytes (5×10^6/ml) were washed with cold 1x PBS and resuspended in 100µl PBS containing 1µg FITC-CD3, 0.5µg PE-CD4, and 0.5µg APC-CD8, and incubated for 30 min on ice in the dark. After incubation, 500µl fresh cold PBS was added and spun down at 400×g for 5 min, the supernatant discarded. Finally, cells were resuspended in 500µl PBS and analyzed by flow cytometer (BD LSRFortessa™, FACSDiva™ software).

2.13. Measurement of arginase enzyme activity

Arginase enzyme activity measured in BMDM cell lysates. Briefly, bone marrow flushed with RPMI-1640 complete medium and spun down at 400×g for 5 min and then resuspended in fresh medium. The heterogeneous population of bone marrow cells were cultured by stimulating with 25ng/ml macrophage colony-stimulating factor (M-CSF) for 7-days in the CO_2 incubator. Of which, BMDM (5×10^5/ml) were seeded into a 24-well plate and stimulated by incubating with SLA (60µg) for 72h. Next, cell lysates prepared by using 100µl RIPA lysis buffer (contains 1% proteinase inhibitor cocktail). 100µl of cell lysates (25µg) incubated with 10µl $MnCl_2$ (10mM) at 56^{o}C for 10 min to activate the arginase enzyme. The hydrolysis of 100µl L-arginine (0.5M, pH-9.7) performed by incubating with activated lysates at 37^{o}C for 20 min. The reaction terminated with 900µl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1v/3v/7v) followed by incubation with 40µl of 9% α-isonitrosopropiophenone (prepared in 100% ethanol) at 95°C for 30 min. The color intensity measured at λ_{540nm} using spectrophotometer (HITACHI U-2910).

2.14. Statistical analysis

Data analysis was performed using one-way ANOVA and significance was calculated by Newman-Keuls multiple comparison tests and results are expressed as standard errors mean (SEM). Few experimental results expressed as mean ± SD using unpaired t-test (GraphPad Prism 5). Statistical significance considered as $p\leq0.05$, $p\leq0.01$, and $p\leq0.001$ and represented by *, **, and *** respectively.

3. Results

3.1. Malnutrition state approved with diet-D based on the reduction in serum leptin, TGs and body weight

We have noticed a significant reduction in body weight ($p \leq 0.01$), serum leptin ($p \leq 0.05$), serum TGs ($p \leq 0.01$) in the diet-D group compared to diet-A after 3-weeks (Fig.1). Based on this, we have approved the malnutrition status of our experimental animals. In our study, the loss of body weight was about 30%, which represents the moderate malnutrition state as shown by the previous report **(Cuervo-Escobar et al., 2014)**. In addition, blood glucose and serum cholesterol levels were not significantly varied between both the diets.

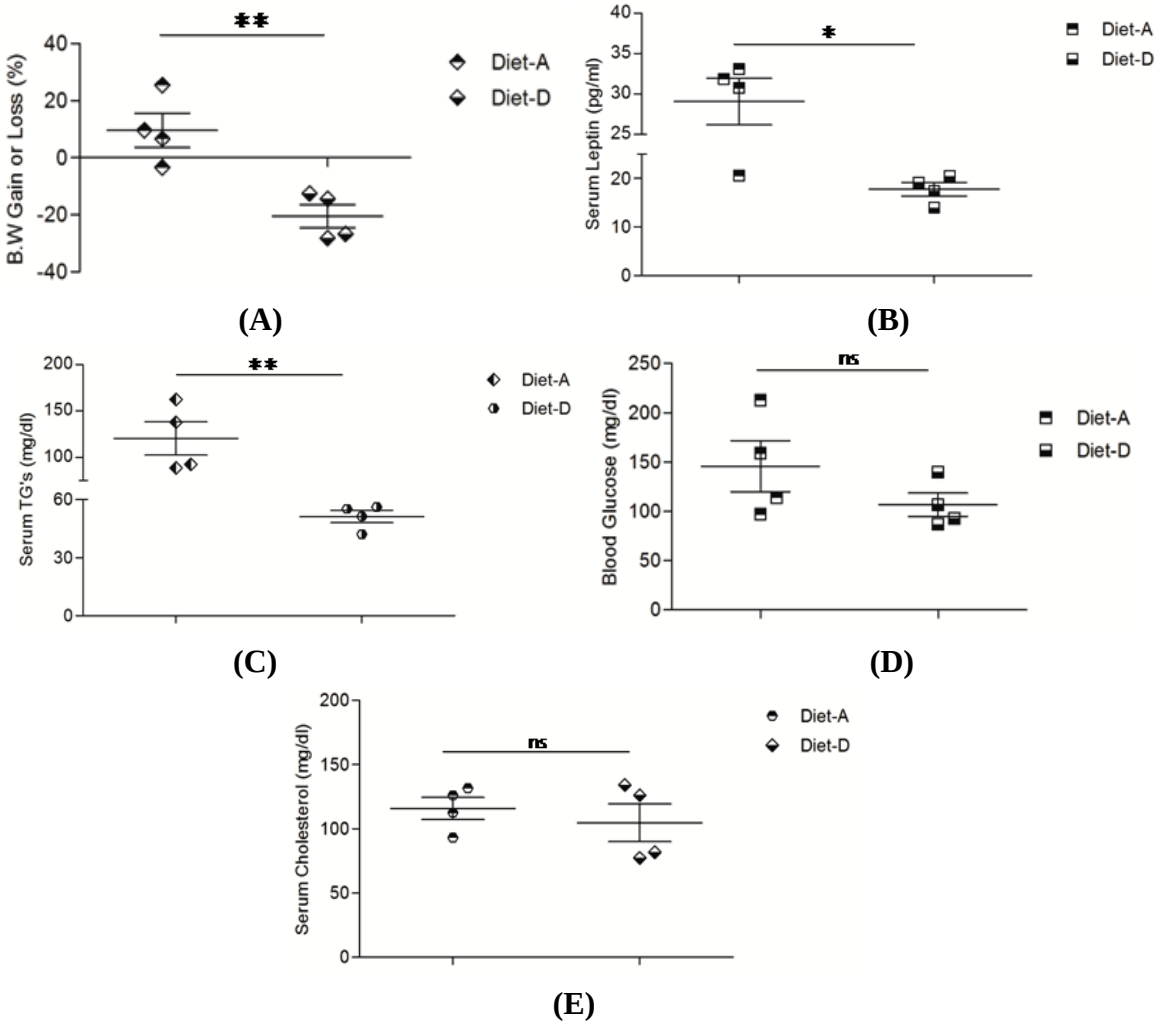


Figure 1: Pre-assessment of malnutrition status after 3-weeks. (A) percentage of body weight gain or loss (B) Serum leptin (pg/ml) and (C) Serum TGs (mg/dl) significantly reduced in diet-D compared to diet-A. (D) Blood glucose (mg/dl) and (E) Serum cholesterol (mg/dl) were found to be unaltered in both the diets. Results expressed as SEM and analysis of variance was done using one-way ANOVA, Newman-Keuls multiple comparison test calculated significance and $p \leq 0.05$ and $p \leq 0.01$ represents * and ** respectively. One of the two experiments with similar results is shown.

3.2. Leptin regulates the rapid loss of body weight

Percentage of body weight gain or loss calculated for each mice group of both the diets. During malnutrition, the body weights were significantly reduced ($p \leq 0.001$) in both the normal and infected groups compared to respective diet-A groups. But leptin-treated diet-D infected group showed a significant regulation in the weight loss ($p \leq 0.01$) compared to its counter (diet-D infected without leptin treatment) group (Fig 2).

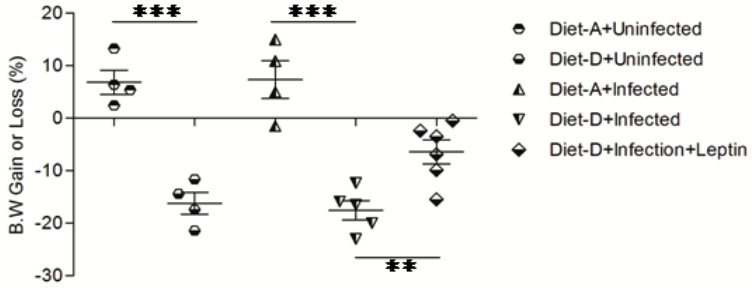


Figure 2: Percentage of body weight (B.W) gain or loss with SEM. In comparison, between the uninfected groups and between the infected groups of both the diets, the body weight was significantly reduced with diet-D. In comparison, between the infected diet-D and its leptin-treated group, the loss of body weight significantly regulated in leptin-treated group. Significance indicated by $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

3.3. Leptin has no effect on the blood glucose levels

The blood glucose is an important energy source to the lymphocytes for their survival and proper function **(MacIver et al., 2008)**. In our study, the post-prandial blood glucose concentration was found to be unaltered in both the diets during the normal and in the case of *L. donovani* infection, which coincide the early report **(Ghosh et al., 2013)**. Even though its metabolism influenced by either leptin or its receptor deficiency **(Friedman and Halaas, 1998),** we could not find such a difference between the infected diet-D and its leptin-treated group (Fig.3).

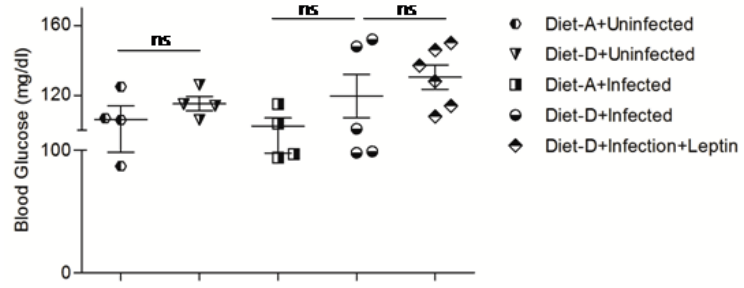
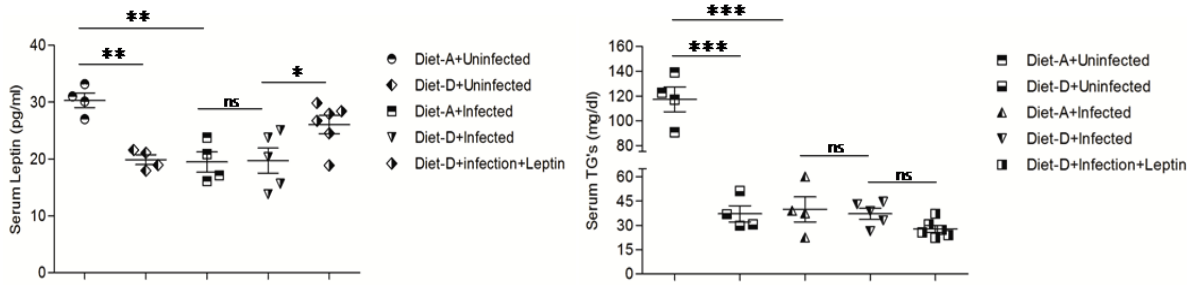


Figure 3: Post-prandial blood glucose (mg/dl) levels with SEM. Here, we could not find a significant difference in blood glucose levels, in comparison between the uninfected groups and between the infected groups of both the diets or in comparison between the infected diet-D and its leptin-treated group.

3.4. Malnutrition and *L. donovani* infection affect the serum leptin and TG levels

In the normal groups, serum leptin ($p \leq 0.01$) and TGs ($p \leq 0.001$) were significantly reduced with diet-D compared to diet-A. Interestingly, we also noticed a significant reduction in serum leptin ($p \leq 0.01$) and TGs ($p \leq 0.001$) during the infection in diet-A compared to its normal group, strengthens our idea of proving that leptin as a protective component in VL. However, this effect was not observed during the infection in diet-D because the serum leptin and TGs have been already at minimal concentrations in the normal group. Hence, it has been noticed that there was no significant difference between the diet-A and diet-D infected groups. In order to leptin subcutaneous injection, we noticed the significant restoration of serum leptin in the diet-D infected group ($p \leq 0.05$) compared to its counter group but the TGs were found to be unaltered (Fig.4).



(A) **(B)**

Figure 4: The concentration of serum leptin in pg/ml (A) and serum TGs in mg/dl (B) with SEM. In comparison, between the uninfected groups of both the diets, the serum leptin and TGs were significantly reduced with diet-D. We also noticed a drastic reduction of these two serum parameters in the infected diet-A group compared to its uninfected group. Hence, it was shown to be non-significant in comparison between the infected groups of both the diets. Whereas, in comparison between the infected diet-D and its leptin-treated group, the serum leptin levels were significantly restored, and TGs were unaltered in leptin-treated group. Significance indicated by *$p \leq 0.05$, **$p \leq 0.01$, and *** $p \leq 0.001$.

3.5. Leptin induces the IgG2a titers but not IgG1

The type of CD4+ Th-subset mounted response determines the class and quantity of serum IgGs, which is also influenced by disease susceptibility or resistance **(Ebrahimpoor et al., 2013)**. In our study, the serum IgG1 and IgG2a levels were found to be unaltered in both the diets during the normal and infection. Even though IgG1 was unaltered, the IgG2a ($p \leq 0.001$) and the ratio of IgG2a/IgG1 ($p \leq 0.05$) were significantly increased in leptin-treated diet-D infected group compared to its counter group (Fig.5).

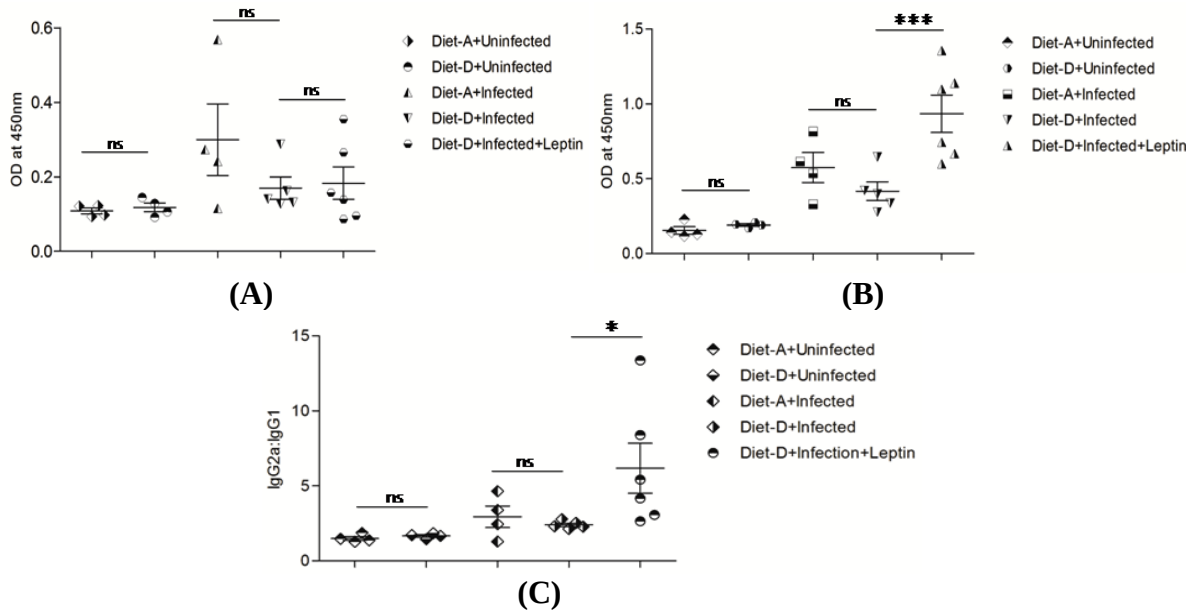


Figure 5: Serum IgG1 and IgG2a titers (OD at λ_{450nm}) and also the ratio between IgG2a/IgG1 with SEM. In comparison, between the uninfected groups and between the infected groups of both the diets, the IgG1and IgG2a titers and also the ratio of IgG2a/IgG1 were found to be unaltered. Whereas, in comparison between the infected diet-D and its leptin-treated group, the IgG1 titers were found to be unaltered, but the IgG2a titers and also the ratio of IgG2a/IgG1 significantly induced in leptin-treated group. Significance indicated by *$p\leq0.05$ and ***$p\leq0.001$.

3.6. Leptin controls the rapid growth of the parasites in visceral organs

The intravenous injection of stationary phase promastigotes could affect the visceral organs by infecting the tissue macrophages and dendritic cells, thereby spread the infection by devastating the host immune response. In our study, the total parasite load in the spleen ($p\leq0.05$) and liver ($p\leq0.001$) of diet-D were significantly higher compared to diet-A. Whereas, in leptin-treated diet-D group the parasite load was significantly lesser in both the spleen ($p\leq0.01$) and liver ($p\leq0.05$) compared to its counter group (Fig.6).

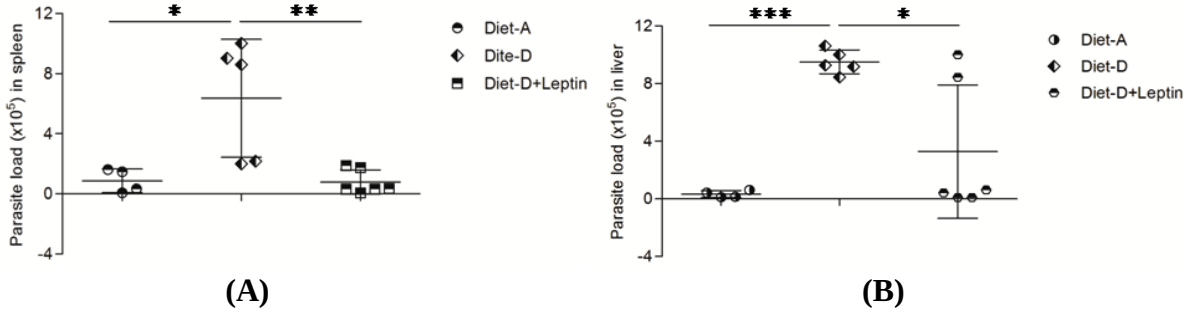
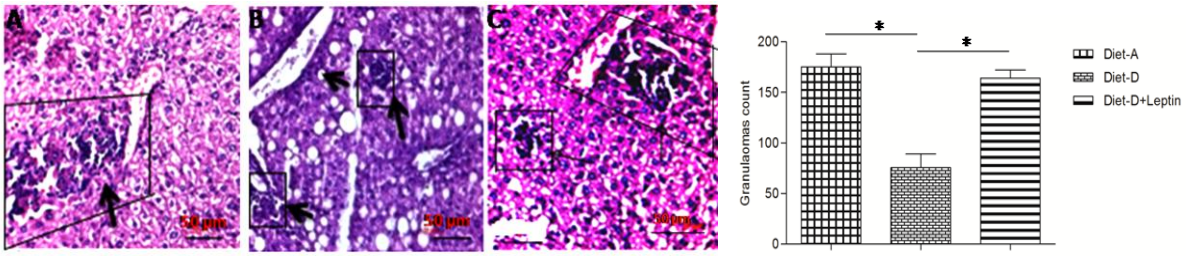


Figure 6: Parasite load in visceral organs with SEM. In comparison between both the diets, the parasite load was significantly higher in both (A) Spleen and (B) Liver of diet-D. Whereas, in comparison between the diet-D and its leptin-treated group the parasite load was significantly reduced in both visceral organs of the leptin-treated group. Significance indicated by *$p\leq0.05$, **$p\leq0.01$ and ***$p\leq0.001$.

3.7. Leptin induces the hepatic granulomatous response to clear the infection

Histological studies on hepatic tissue confirmed that the size and number ($p \leq 0.05$) of the granulomas significantly reduced in diet-D compared to diet-A. But leptin-treated diet-D infected group showed a significant increase in size and number ($p \leq 0.05$) of granulomas compared to its counter group (Fig.7A & 7B respectively). Here, we also focused on the integrity of the granulomas, which showed 50-60% of granulomas in diet-D were diffused and their size was also too small. Even though, the number of granulomas increased in leptin-treated diet-D group, still 20-30% of granulomas were found to be diffused, and 50% of granulomas size was small (data not shown). Discretion of intracellular amastigotes with H-E stain was found to be difficult at granulomatous inflammatory foci.



(A) **(B)**

Figure 7: (A) Granulomas in H-E stained liver sections. Panel-A shows diet-A infected group with large and well-organized granulomas, Panel-B shows diet-D infected group with unorganized/diffused type granulomas and Panel-C shows leptin-treated diet-D infected group with better granulomas compared to panel-B but its size and integrity too less compared to diet-A. Black arrows indicate the granulomas. (B) In comparison, between both the diets, the granulomas count/50 focal fields (mean ± SD) significantly reduced with diet-D. Whereas, in comparison between the diet-D and its leptin-treated group, the granulomas count was significantly higher in leptin-treated group. Significance indicated by *$p \leq 0.05$.

3.8. Leptin controls the hepatic degeneration

H-E staining of liver sections shows that the moderate to severe vacuolar degeneration noticed in the diet-D infected group. Whereas, it was significantly healed in leptin-treated diet-D infected group, and mild hepatic degeneration confined to few places of centrilobular regions. Interestingly, we also noticed a mild hepatic degeneration at centrilobular region and proliferation of fibrous tissue at Peribiliary region in the diet-A infected group. It suggests that the *L. donovani* infection itself causing hepatic degeneration. Normal groups of both the diets appeared normal in all the regions of liver sections (Fig.8).

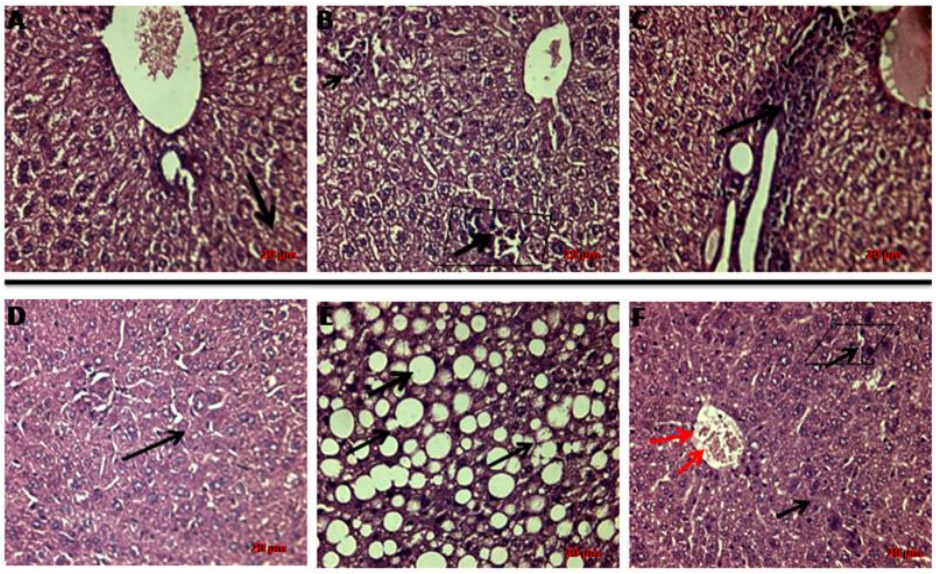


Figure 8: Hepatic tissue degenerative changes. (A) In diet-A uninfected, hepatocytes appeared normal–black arrow, portal, and periportal regions along with bile duct appeared normal–red arrow. (B) In diet-A infected, mild hepatic degeneration at centrilobular region was observed (C) In diet-A infected, the proliferation of fibrous tissue at Peribiliary region also noticed. (D) In diet-D uninfected, hepatocytes appeared normal-black arrow, portal vein, a periportal region along with bile duct appeared normal–red arrow, and no necrosis or degenerative changes were observed. (E) In diet-D infected, moderate to severe vacuolar degeneration was noticed at periportal and centrilobular region–black arrow. (F) In leptin-treated diet-D infected, most of the hepatocytes appeared normal, portal, and periportal region along with bile duct appeared normal–red arrow. Mild hepatic degeneration noticed in few places of the centrilobular region-black arrow.

3.9. Leptin upregulates Th1 downregulates Th2 cytokines mRNA expression in spleen

Results showed that during the infection, the relative expression of Th1 cytokines such as IFN-γ significantly downregulated ($p \leq 0.05$) and IL-12p40 was unaltered in diet-D compared to diet-A. On other hand, the relative expression of Th2 cytokines such as IL-10 ($p \leq 0.01$), IL-4 ($p \leq 0.01$) and TGF-β ($p \leq 0.001$) was significantly upregulated in diet-D compared to diet-A. Leptin treated diet-D showed a significant upregulation of IFN-γ ($p \leq 0.001$) but no effect on IL-12p40, and also showed significant downregulation of Th2 cytokines ($p \leq 0.001$) compared to its counter group (Fig.9). In the normal groups, the relative expression of IFN-γ was unaltered but IL-12p40 ($p \leq 0.05$) was significantly downregulated in diet-D compared to diet-A. On other hand, the relative expression of IL-10 ($p = 0.09$), IL-4 ($p \leq 0.05$) and TGF-β ($p = 0.076$) was upregulated in diet-D compared to diet-A (Fig.10).

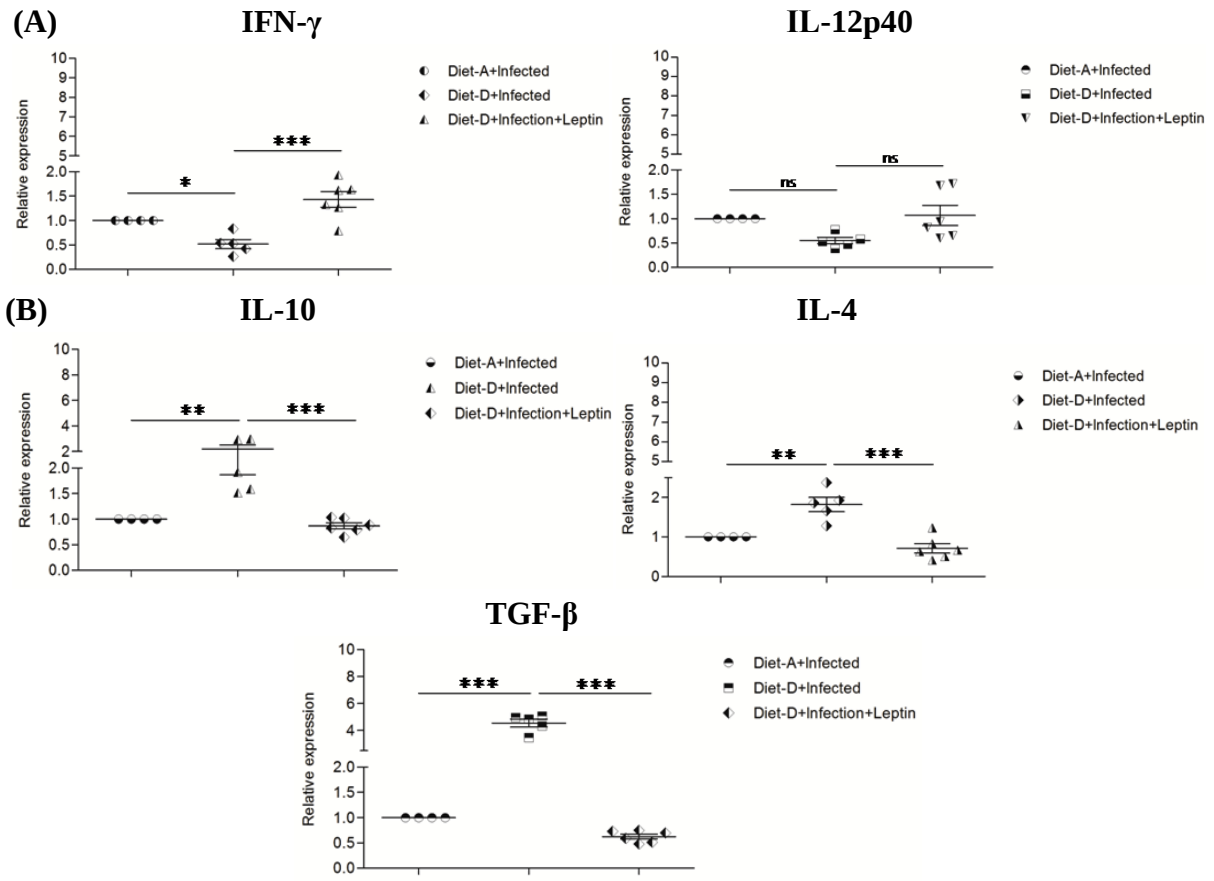
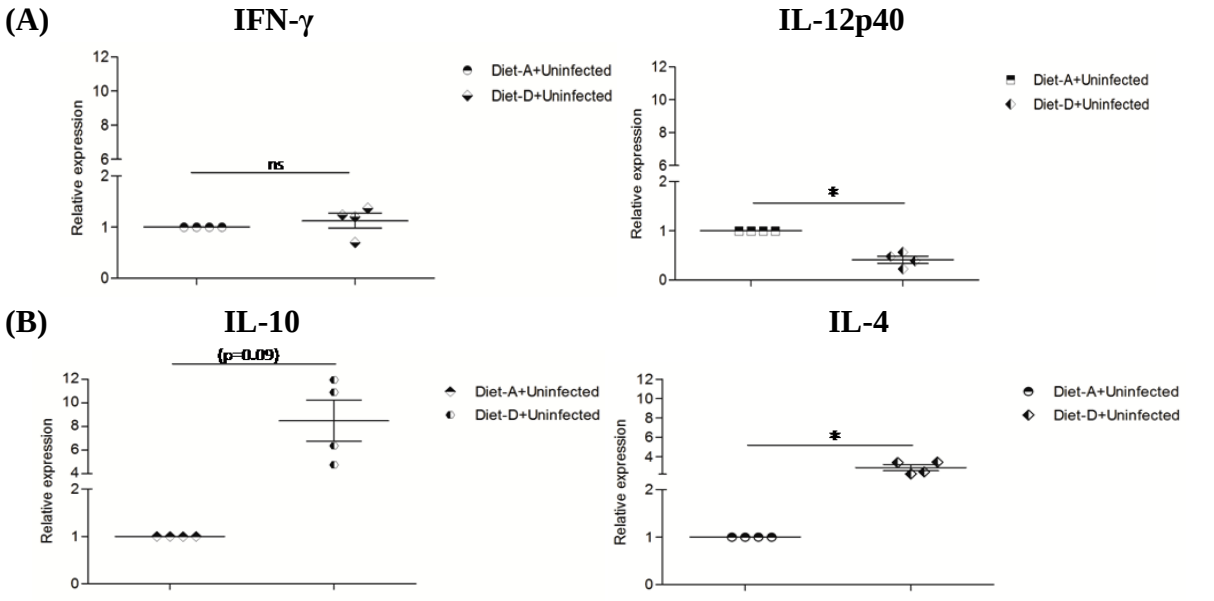


Figure 9: Real-time qPCR analysis of Th1 (A) and Th2 (B) cytokines relative expression with SEM. In comparison, between the infected groups of both the diets, the Th1 cytokines such as IFN-γ significantly downregulated, and IL-12p40 was unaltered, and the Th2 cytokines such as IL-10, IL-4 and TGF-β were significantly upregulated in diet-D. Whereas, in comparison between the infected diet-D and its leptin-treated group, the IFN-γ was significantly upregulated, and IL-12p40 was unaltered, and the Th2 cytokines were significantly downregulated in leptin-treated group. Significance indicated by *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.



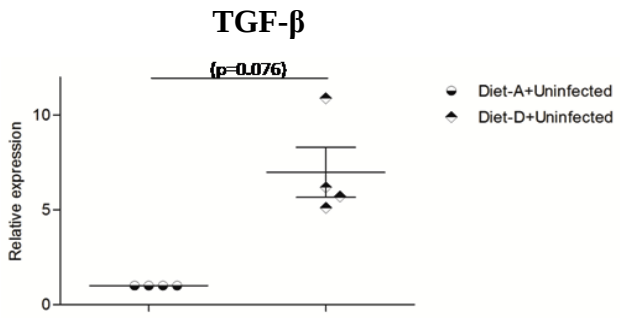


Figure 10: Real-time qPCR analysis of Th1 (A) and Th2 (B) cytokine relative expression with SEM. In comparison, between the uninfected groups of both the diets, the IFN-γ was unaltered, and IL-12p40 significantly downregulated, and the IL-10 (p=0.09), IL-4 and TGF-β (p=0.076) were upregulated in the diet-D. Significance indicated *$p\leq0.05$.

3.10. Leptin induces the secretion of Th1 cytokines into SLA-stimulated splenic culture supernatant

Results showed that during the infection and normal the Th1 cytokines (IFN-γ, IL-2 and TNF-α) production into culture supernatants was drastically reduced ($p\leq0.05$) upon SLA-stimulation in diet-D compared to diet-A group except IL-2 between the normal groups of both the diets. Whereas, the leptin-treated diet-D infected group shown and to produce an abundant amount of Th1 cytokines IFN-γ ($p\leq0.001$), IL-2 ($p\leq0.05$) and TNF-α ($p\leq0.05$) compared to its counter group. On the other hand, during the normal or infection the Th2 cytokine such as IL-4 not produced in diet-D, but it was too low in diet-A and leptin-treated diet-D infected group. Other Th2 cytokine, IL-5 production was insignificant between the diet-D infected group and its leptin-treated group, but it was not produced in the normal groups of both the diets and diet-A infected group (Fig.11).

3.11. Leptin induces the CD8+ T-cell and reduces the CD4+ T-cell population in spleen

During the infection, the percentage of the CD4+ population was significantly increased ($p\leq0.01$) and the CD8+ population was significantly reduced ($p\leq0.01$) in the diet-D compared to diet-A. During the normal, the similar effect was observed in the diet-D ($p\leq0.001$) compared to diet-A. However, in leptin-treated diet-D infected group this effect was reversed significantly ($p\leq0.01$) compared to its counter group. The ratio of CD4+/CD8+ significantly increased during the normal ($p\leq0.001$) and infection ($p\leq0.01$) in the diet-D groups compared to respective diet-A groups. But in leptin-treated diet-D infected group the ratio was significantly reduced ($p\leq0.001$) compared to its counter group. The percentage of the CD4+CD8+ double positive cell population was found to be unaltered among different groups either in both the diets of normal and infected or in leptin-treated diet-D (Fig.12).

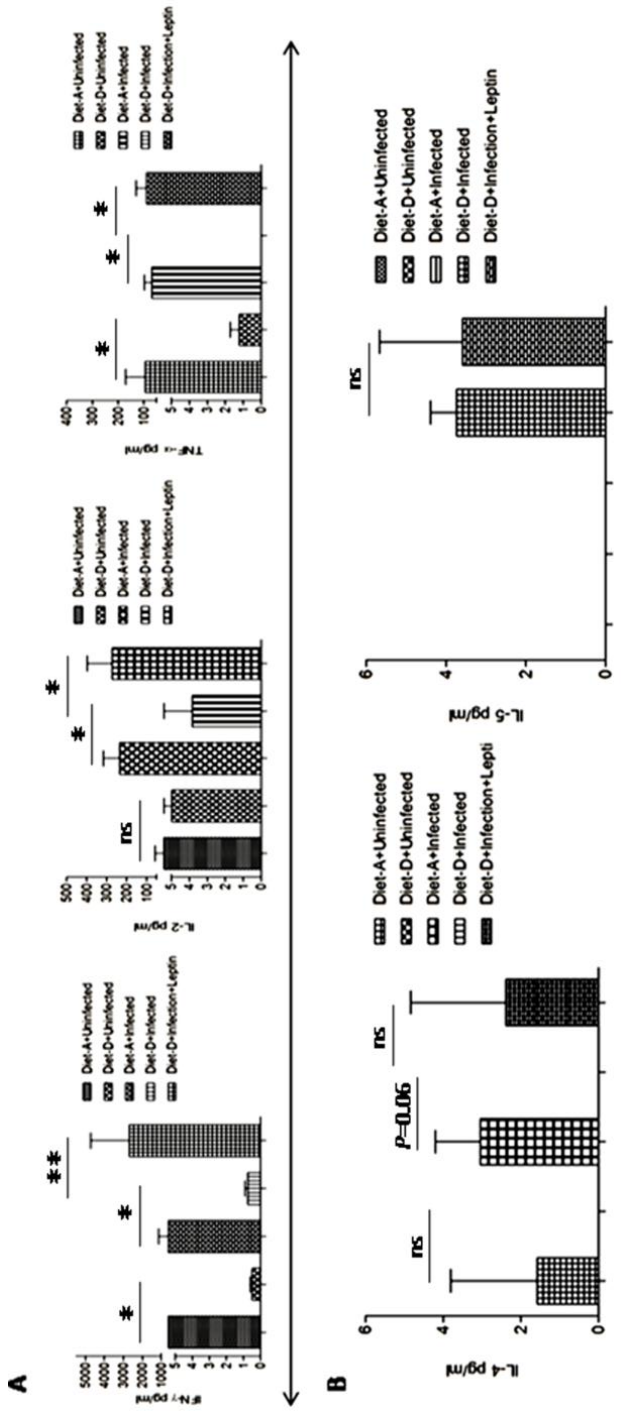
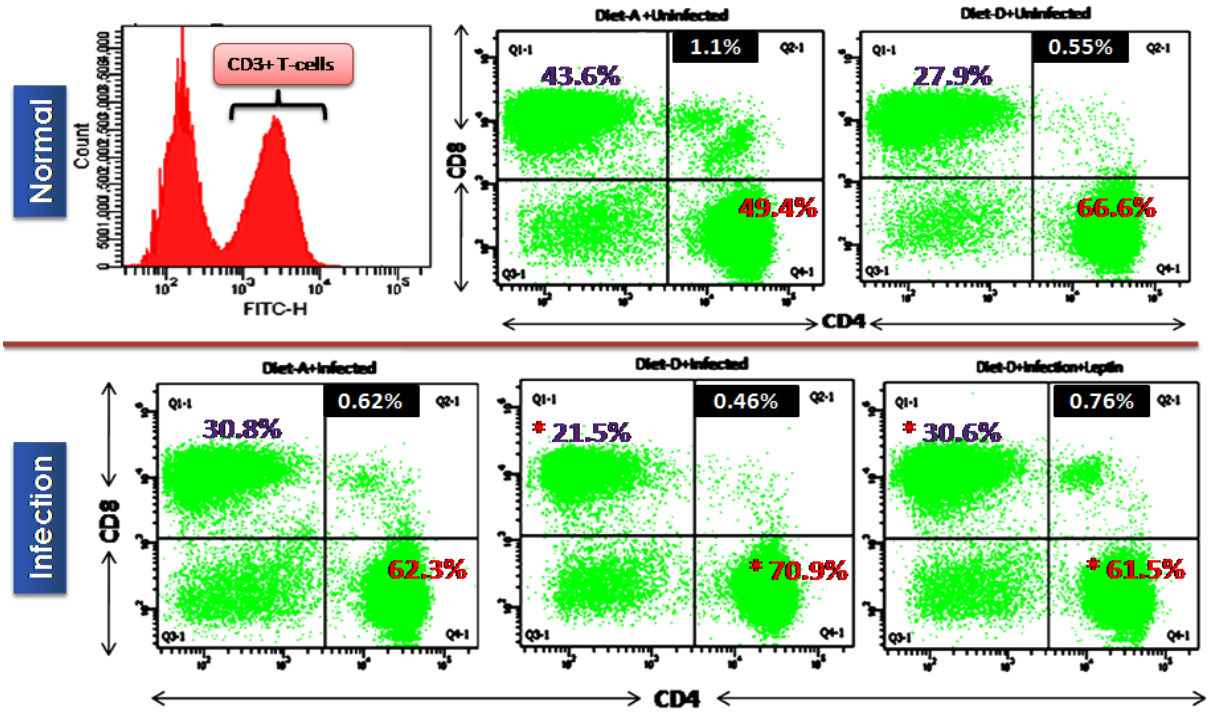


Figure 11: Flow cytometry analysis of Th1 (A) and Th2 (B) cytokines with mean ± SD in SLA-stimulated splenocytes culture supernatant. In comparison, between the uninfected groups of both the diets, the Th1 cytokines (IFN-γ, IL-2 and TNF-α) produced in lesser quantity, and the Th2 cytokines (IL-4 and IL-5) produced at negligible amount in the diet-D. In comparison, between the infected groups of both the diets, the IFN-γ and IL-2 produced in lesser quantity, TNF-α and IL-4 produced at negligible amount, and IL-5 induced at a lesser amount in the diet-D. Whereas, in comparison between the infected diet-D and its leptin-treated group, the IFN-γ, IL-2 and TNF-α were produced in abundant quantity, IL-4 produced in very lesser quantity, and IL5 was unaltered in leptin-treated group. Significance indicated by *$p \leq 0.05$, and ** $p \leq 0.01$.



The raw file of this picture is showing the CD3+ T-cells peak in FITC-H channel (red) and the quadrants showing the percentages of the CD4+ T-cells (PE-labelled in Q4-1), CD8+ T-cells (APC-labelled in Q1-1) and double positive (CD4+CD8+ in Q2-1). The above panel is showing the normal groups and below panel is showing the infected groups of both the diets along with the leptin-treated diet-D infected group.

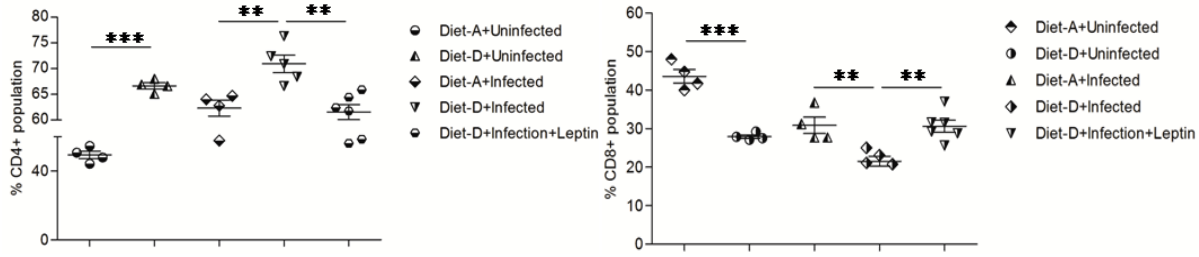


Figure 12: Percentage of T-cell subpopulations with SEM. In comparison between the uninfected groups and between the infected groups of both the diets, the CD4+ population was significantly increased, and the CD8+ population significantly reduced in the diet-D. Whereas, in comparison between the infected diet-D and its leptin-treated group, the CD8+ population was significantly increased, and the CD4+ population significantly reduced in leptin-treated group. Significance indicated by ** $p \leq 0.01$, and *** $p \leq 0.001$.

3.12. Leptin downregulates the anergic CD8+ T-cell markers and upregulates the Graz-A mRNA expression in the spleen

During the infection, the relative expression of CTLA-4 ($p \leq 0.001$) and PD-1 ($p \leq 0.05$) was significantly upregulated, and Graz-A was unaltered in diet-D compared to diet-A (Fig.13A). In the normal groups, the similar effect was observed (except for PD-1 ($p \leq 0.01$)) in the diet-D compared to diet-A (Fig.13B). Whereas, in leptin-treated diet-D infected group

the relative expression of CTLA-4 ($p \leq 0.001$) and PD-1 ($p \leq 0.01$) significantly downregulated, and Graz-A ($p \leq 0.05$) was significantly upregulated compared to its counter group (Fig.13A).

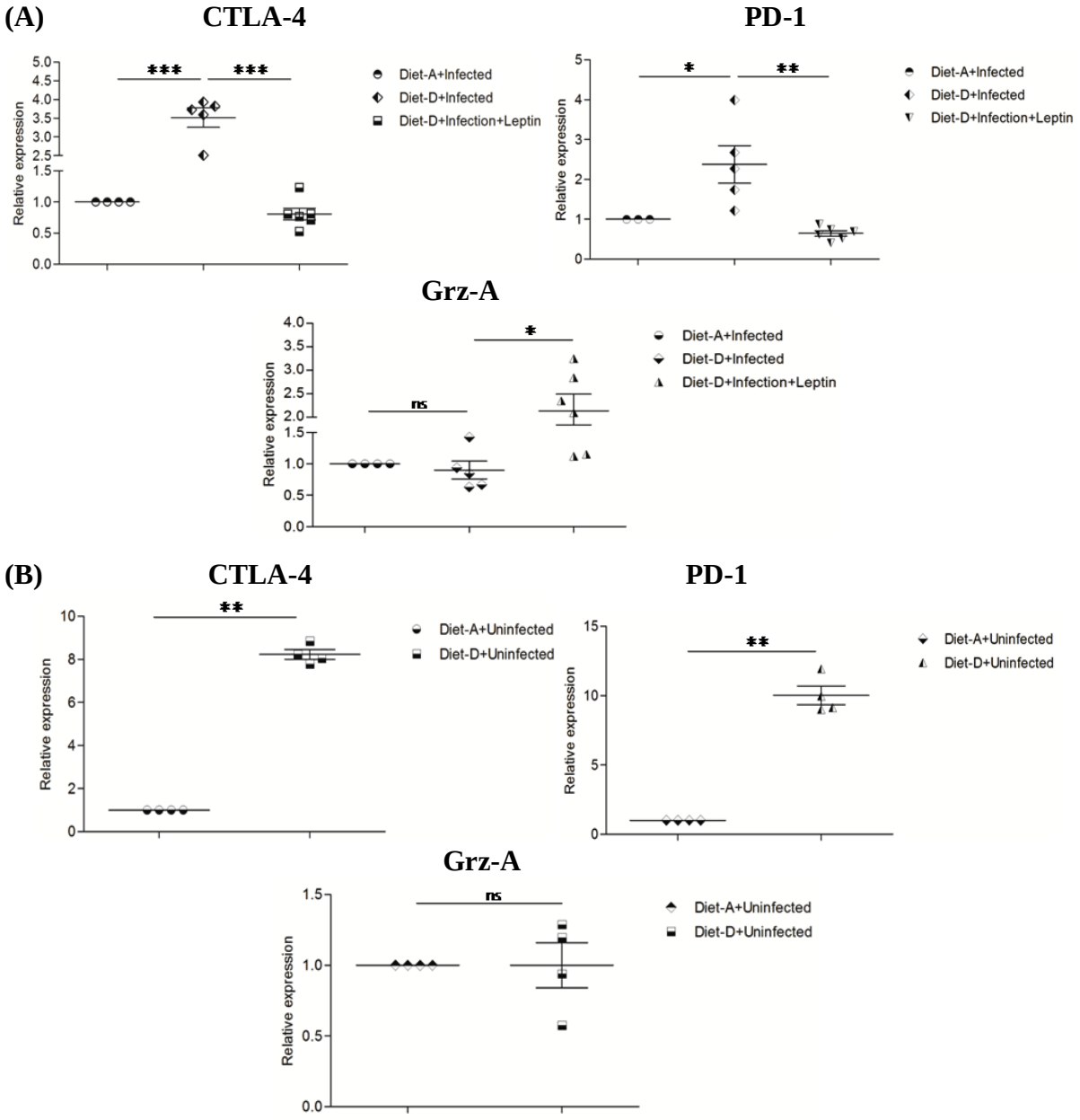


Figure 13: Real-time qPCR analysis of anergic CD8+ T-cell markers and Graz-A relative expression with SEM. (A) In comparison, between the infected groups of both the diets, the anergic CD8+ T-cell markers such as CTLA-4 and PD-1 were significantly upregulated, and Graz-A was found to be unaltered in diet-D. Whereas, in comparison between the infected diet-D and its leptin-treated group, the both anergic markers were significantly downregulated, and Graz-A was significantly upregulated in leptin-treated group. (B) In comparison, between the uninfected groups of both the diets, the similar effect was observed in diet-D as we found in the infected groups. Significance indicated by *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

3.13. Leptin downregulates the splenic GM-CSF mRNA expression

During the infection, the relative expression of GM-CSF was significantly upregulated in diet-D ($p \leq 0.001$) group compared to diet-A. However, in leptin diet-D infected group ($p \leq 0.01$) it was significantly downregulated compared to its counter group (Fig.14). It substantiates the early report on hepatic GM-CSF mRNA expression during *L. donovani* infection **(Murray et al., 1995)**.

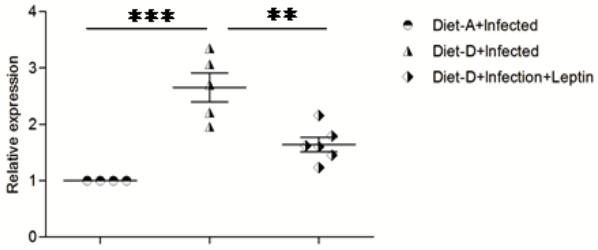


Figure 14: Real-time qPCR analysis of GM-CSF relative expression with SEM. In comparison, between the infected groups of both the diets, the GM-CSF expression was significantly upregulated in the diet-D. Whereas, in comparison between the infected diet-D and its leptin-treated group, the GM-CSF was significantly downregulated in leptin-treated group. Significance indicated by $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

2.15. Leptin reduces the arginase activity in BMDM

Arginase mediated catabolism of L-arginine led to produce ornithine, which further catabolized to polyamines, the essential components of host cell division and also for the parasite survival. The increased arginase activity during malnutrition and several pathological conditions depletes L-arginine at microenvironment which affects the T-cell response **(Munder, 2009, Nagaraj and Gabrilovich 2010, Bronte and Zanovello, 2005, Ochoa et al., 2007)**. In our study, during the infection the arginase enzyme activity was increased in diet-D ($p \leq 0.01$) compared to diet-A. In leptin-treated diet-D infected group ($p \leq 0.01$), the arginase activity was significantly reduced compared to its counter group (Fig.15).

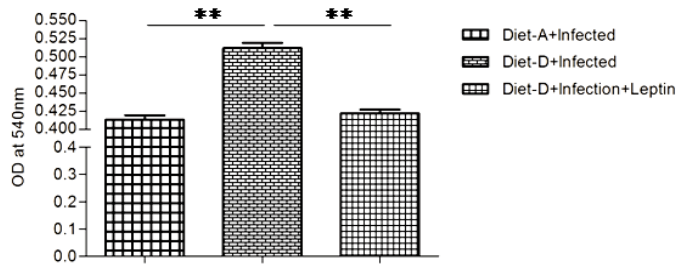


Figure 15: Arginase enzyme activity in BMDM with mean OD (λ_{540nm}) ± SD. In comparison, between the uninfected groups and between the infected groups of both the diets, the arginase enzyme activity was significantly induced in the diet-D. Whereas, in comparison between the infected diet-D and its leptin-treated group, the arginase activity was significantly downregulated in leptin-treated group. Significance indicated by $^{**}p \leq 0.01$.

4. Discussion

According to the MNA tool, serum leptin accompanied by body weight and serum TGs are the optimal biomarkers for the prediction of malnutrition **(Amirkalali et al., 2010)**. Our study also substantiates the above statement of malnutrition by detecting the low serum leptin with respect to body weight loss and serum TGs. Interestingly, a drastic fall in serum leptin during *L. donovani* infection in diet-A strongly correlating with serum TGs but not body weight as shown in tuberculosis **(Crevel et al., 2002)**. It suggests that *L. donovani* infection affects the host lipid profile **(Ghosh et al., 2013)** and its associated low leptin may contribute to T-cell unresponsiveness as proved in tuberculosis **(Crevel et al., 2002)**. Nevertheless, both diet groups has drastic fall in the serum leptin during *L. donovani* infection; the disease severity was more in diet-D compared to diet-A, exploring the importance of other nutritional factors. Hence, the supplementation of vitamin-E **(Isermann et al., 1999)** and/or zinc could help in leptin production **(Mantzoros et al., 1998)** which is more cost-effective rather than direct administration of leptin which is not much feasible in developing countries.

In our study, the protein-restricted diet affects the percentage of visceral organs to body weight accompanied by tissue atrophy **(Cuervo-Escobar et al., 2014)** which might be due to the defective cell proliferation and/or apoptosis or necrosis-like death **(Morrot et al., 2011)**. Even though, leptin treatment does not restore the visceral organs weight; it regulates the drastic loss of body weight and lymphoid atrophy **(Howard et al., 1999)**. Splenic infection of diet-D might accelerate the maturation of double positive T-cells inclined towards CD4+ population **(Cuervo-Escobar et al., 2014)** rather than CD8+. Hence, we could find decreased CD8+ and increased CD4+ T-cell population, which correlate the early report on leptin-deficient mice during pulmonary tuberculosis **(Wieland et al., 2005)**. Here, we hypothesized that the increased CD4+ population would be Th2 phenotype, which strongly correlated by upregulation of IL-10, IL-4, and TGF-β mRNA expression. Interestingly, we also noticed upregulation of CTLA-4 and PD-1 expression which suggests the defective activation **(Chen, 2004)** and exhaustion/anergy of CD8+ T-cells **(Day et al., 2006, Kaufmann, 2007, Joshi et al., 2009)**. Blockade of these anergic markers **(Murphy et al., 1998, Joshi et al., 2009)** and production of IFN-γ followed by cure implies the contribution of CD8+ T-cells in control of *L. donovani* infection **(Gautam et al., 2014)**. Leptin could retain the effector T-cell function evidenced by induced production of IFN-γ, IL-2 and TNF-α into splenocytes culture supernatant upon SLA-stimulation, which limits the parasite replication. It substantiates an earlier report on infected malnourished children showed to induce the IL-2 and IFN-γ producing CD4+ and CD8+ T-cells **(Rodríguez et al., 2007)**. The increased CD8+ T-cell

population and Graz-A production entails the disease heal **(Kaushal et al., 2014)** mediated by leptin. The type of CD4+ T-cell response influences the B-cell differentiation and IgG class switching. Of which, the overall Th1 response correlates to serum IgG2a was induced by leptin treatment and Th2 response coincides with serum IgG1 **(Coffman et al., 1993, Snapper et al., 1987)** was found to be unaltered.

Infection or parasite dissemination in hepatic tissue is controlled by granulomatous response which mediated by T-cells **(Murray et al., 1992, Engwerda et al., 1996, Stern et al., 1988)** and monocytes influenced by Th1 cytokine environment **(Cervia et al., 1993, Engwerda et al., 1998, Tumang et al., 1994, Murray et al., 1993, Squires et al., 1989)**. But this response was not apparent in diet-D. Here, the granulomas size was too small, mostly organized in diffused manner and have poor integrity. We also noticed a moderate to severe hepatic vacuolar degeneration at periportal and centrilobular regions during the infection in diet-D, as previously shown with 4% protein diet **(Cuervo-Escobar et al., 2014)**. But leptin supplementation has restored the granulomatous response (i.e. increases the number of granulomas but their size and integrity was not too apparent as observed in diet-A) as found in pulmonary tuberculosis in leptin-deficient mice **(Wieland et al., 2005)** and also prevents the tissue degeneration, this might be through its tissue repair and angiogenesis activities. The increased arginase activity in BMDM of diet-D evidenced for the severity of disease as it is associated with several pathological conditions such as HIV co-infection of VL **(Takele et al., 2012)** and leishmaniasis **(Abebe et al., 2012 and 2013)**. It results in the deprivation L-arginine at the microenvironment impairing the T-cell response **(Munder, 2009, Nagaraj and Gabrilovich 2010, Bronte and Zanovello, 2005, Ochoa et al., 2007)**. But leptin supplementation has shown to reduce the arginase activity in diet-D strengthens its role in T-cell activation. Elevated GM-CSF expression is evidence of spreading of infection by inducing the defective activation and proliferation of monocytes to exploit the host purine nucleotide machinery that lacks in *Leishmania* **(Mock et al., 2012)**. Here, we hypothesized that the *Leishmania*-infected macrophages would be immature or alternatively activated and might lack the microbicidal activity. In spite of this, few studies are indirectly evidence for that the alternatively activated macrophages emerge into a Th2 environment that facilitates the parasite dissemination in the host **(Vieira et al., 1994)**. Leptin might regulate the defective activation of monocytes (viz. downregulation of GM-CSF) that helps to control the infection. In conclusion, this is the first report on leptin protective role in any parasitic infection, and L. donovani infection influenced low leptin levels also an indispensable factor employed in VL associated immune suppression.

CHAPTER 2

Evaluation of Anti-leishmanial and Immunomodulatory properties of Neem leaf extract in Experimental Visceral leishmaniasis

1. Introduction

Till date, there is no prophylactic vaccine available for human VL, and the treatment relies on the anti-leishmanial drugs. Oral Miltefosine is a currently recommended drug in the Indian subcontinent. However, its higher cost, teratogenic potential and rapid emergence of resistance are the major drawbacks **(Sundar et al., 2007)**. VL co-infection with HIV is an emerging challenge in developing nations **(Alvar et al., 2008)**. Thus, there is an immediate necessity of efficient, safe, resistance surpassable and low-cost drugs to combat this disease. Plant-based products may play a pivotal role in search of a better anti-leishmanial compound. Conventionally, a wide range of plant-derived products have been tested in treatment of protozoan diseases **(Weiner and Weiner, 1994)**, this could help in the finding of new therapeutic agents against different clinical forms of *Leishmania* infection.

A. indica is one such plant with abundant medicinal values commonly called as Neem **(Biswas et al., 2002)**. It is a traditional medicinal plant growing profusely in tropical countries where the leishmaniasis is endemic. All kinds of Neem plant parts sourced for many therapeutic agents. It has anti-inflammatory, immunomodulatory and anti-carcinogenic properties **(Ray et al., 1996; Subapriya et al., 2006; Dholi et al., 2011)**. It functions as an immune booster by inducing the humoral and cell-mediated immunity, phagocytic activity of macrophages and NO production **(Abhishek et al., 2009)**. Notably, Neem oil can kill multi-drug resistant bacteria species from human infections **(Jain et al., 2013)**. In addition, it has been used in the treatment of ulcers, leprosy, gum and dental diseases **(Aggrawal et al., 1995)**. Thus, there has been much interest in recent years regarding the use of various Neem products in ayurvedic and herbal medicines.

The anti-parasitic activity of *A. Indica* studied on trypanosomatids and also *L. major* **(Khalid et al., 2005; Mbaya et al., 2010)**. In which, terpenes appeared as an active group of compounds, mostly azadirachtin found to be effective, usually exist abundantly in all parts of the plant. In comparison, seeds comprise in the higher concentration than other parts **(Forim et al., 2010)**. Azadirachtin induces the resistance against reinfection by *T. cruzi* in the insect vector **(Garcia et al., 1991)**. Despite the anti-*Plasmodium falciparum* activity **(Tahir et al., 1999)**, azadirachtin was found to be inefficient against *L. amazonensis* promastigotes. However, other terpenes of *A. indica* would also have the anti-leishmanial activity. The other

group of compounds rich in *A. indica* are limonoids (especially gedunin, dihydrogedunin, nimbolide and nimbidin) also tested against *P. falciparum*, and offered significant activity *in vitro* **(Shumutterer, 2002; Roy and Saraf, 2006)**. In addition, other limonoids (2, 6-dihydroxyfissinolide, fissinolide and 3β-acetoxy-6-hydroxy-1-oxomeliac-14-enoate) from *Khaya senegalensis* have shown moderate anti-protozoal activity on *P. falciparum* and *L. major* **(Khalid et al., 1998)**. *A. indica* is able to activate both innate and adaptive immunity, induces the production of IFN-γ and TNF-α **(Mukherjee et al., 1999)**, which synergistically acts on macrophages to produce NO, thereby induces the clearance of *Leishmania* infection **(Bogdan et al., 1996)**. This study was carried out with the objective of evaluating the anti-leishmanial and immunomodulatory activities of Neem leaf extract during *L. donovani* infection *in vitro* and *in vivo*.

2. Abstract

The toxicity and emergence of resistance to available chemical drugs against visceral leishmaniasis are evoking to explore herbal treatment. One such attempt with the Neem is being reported here. The current study primarily focused on evaluating the anti-leishmanial effects of Neem leaf extracts. Among which, ethyl acetate fraction (EAF) alone was found to exhibit leishmanicidal effect validated through cytotoxicity assay and estimated its IC_{50} to be 52.4µg/ml on promastigote stage of the parasite. In addition, propidium iodide (PI) staining of dead cells substantiated the aforementioned activity of EAF. Further, carboxyfluorescein diacetate succinimidyl ester (CFSE) staining of the promastigotes has affirmed the anti-proliferation activity of EAF. The characteristic features of DNA fragmentation, reduced mitochondrial membrane potential, increased sub-G_0/G_1 phase parasites and increased ROS production in EAF treated promastigotes indicate the apoptosis-like death. In addition, the reduced parasite burden both *in vitro* (viz. ~45% in THP-1 and ~50% in PBMCs) and *in vivo* (spleen and liver) provides the evidence for its anti-leishmanial activity on amastigote stage. The increase in ROS levels in THP-1 and NO production from J774.1 cell line upon EAF treatment evidenced for the oxidative killing of the intracellular amastigotes. Furthermore, the active immunomodulatory activity at mRNA level (viz. upregulation of Th1 cytokines and down-regulation of Th2 cytokines) both *in vitro* and *in vivo* was also shown to be exhibited by EAF. Finally, liquid chromatography- tandem mass spectrometry (LC-MS/MS) analysis of EAF revealed the presence of 14 compounds **(Dayakar et al., 2015).**

3. Methodology

3.1. Parasite culture

Promastigotes strain (Dd8) of *L. donovani* was cultured at 25^{o}C±1 as mentioned earlier in chapter 1A, methods section 2.1.

3.2. Preparation of Neem leaf extract

Neem leaves collected during November 2011 at the University of Hyderabad Campus, Hyderabad, Andhra Pradesh. The leaves were dried in the shade and powdered for extract preparation. Extracts have prepared from 30g of dry leaf powder using a soxhlet apparatus with solvents (each 500ml) like hexane, ethyl acetate, alcohol and water according to increasing the order of the polarity. The solvents were evaporated using a rotary vacuum evaporator and stored at room temperature **(Chandrasekaran et al., 2013)**.

3.3. *In vitro* studies on promastigotes

3.3.1. Anti-leishmanial activity

Cytotoxicity was performed using the colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay. In which, mitochondrial reductase of viable cells reduce the MTT into purple colour formazan crystals where the dead cells can't do the same. Exponentially growing log phase promastigotes (2×10^5/well) were incubated in a 96-well microtiter plate with the crude extracts pre-dissolved in dimethyl sulfoxide (DMSO) at different concentrations ranged from 500 to 0µg/ml in a serial dilution (final conc. of DMSO ≤0.1% in all treated variables). After 48h of treatment, 20µl of MTT (5mg/ml) was added to each well and incubated for 4h at 37^{o}C. The optical density was measured using a microELISA reader (Biotek) at λ_{540nm} after dissolving the formazan crystals in DMSO (150µl).

3.3.2. PI dye exclusion assay

Typically, PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye molecule per 4-5bp of DNA. PI is impermeable and generally exclude by viable cells. PI routinely used for identifying the dead cells in a population as a counter-stain in multicolour fluorescent technique. Parasites (1×10^6 cells/ml) were collected after 24h and 48h of EAF treatment, washed with PBS and resuspended in 1µg of PI dissolved 1ml PBS solution. Untreated cells used as a control. The acquisition was done using FACS Calibur to differentiate PI^+ dead cells from PI^- viable cells.

3.3.3. CFSE proliferation assay

CFSE is a colourless and nonfluorescent dye passively enters into the cells. The

intracellular esterases remove the acetate group and make it highly fluorescent and coloured dye. The succinimidyl ester group of this activated dye react with amine groups of the proteins and retains its fluorescence for a longer period (i.e. seven generations). Promastigotes (1×10^6 cells/ml) labeled with 10µM CFSE prepared in PBS for 5 min at 26^{o}C. Labelling was quenched by adding six volumes of chilled M199 medium and washed twice with PBS and treated with EAF for 24h and 48h. After treatment, promastigotes were resuspended in PBS with 10µg/ml of PI to exclude dead cells. The analysis was done using ModFit software.

3.3.4. Genomic DNA fragmentation assay

Genomic DNA isolated from both untreated and EAF treated promastigotes after 48h using phenol-chloroform method. Briefly, promastigotes treated with sarcosyl detergent buffer (50mM Tris, 10Mm Ethylenediamineacetate (EDTA), 0.5w/v sodium lauryl sarcosine pH7.5) and 0.1µg/ml of proteinase-K (10µg/µl) and vortex followed by incubation at 50^{o}C overnight. Next day, RNase-A (0.3mg/ml) was added and incubated at 37^{o}C for 1h. Then, lysates were extracted with phenol: chloroform: isoamyl alcohol (25:24:1) by spun at 16000×g for 5 min. Further, upper layer carefully separated and treated with 0.1 volume of 3M sodium acetate pH5.5, followed by treated with two volumes of ice-cold 100% ethanol and incubated for overnight at -20^{o}C to precipitate the DNA. Next day, spun at 16000×g for 10 min and supernatant was discarded followed by wash with 70% ethanol. Finally, DNA was allowed to air dry and resuspended in 0.5M Tris-EDTA. 530ng of DNA was loaded separately onto the 1% agarose gel and run for 1h at 100 volts, and the bands visualized under UV light. Miltefosine (25µM) treated promastigotes used as positive control.

3.3.5. Flow cytometric analysis of sub G_0/G_1 phase of cell cycle

Promastigotes were synchronized using 5mM hydroxyurea for 12h and then washed twice with PBS. Further, resuspended in fresh media and allowed to progress through their cell cycle. Both untreated and EAF treated promastigotes were harvested at different time points (6, 12, 24, and 48 h) and washed thrice with PBS. Then, fixed in 70% ethanol followed by a wash, subsequently resuspended in 500µg/ml RNase-A prepared in PBS after 15 min of incubation repeated the wash. Finally, promastigotes were incubated with 25µg/ml of PI prepared in PBS for 45 min in the dark. Further, they were processed and analyzed using BD^{TM} FACS Calibur and CellQuest Pro software.

3.3.6. Measurement of mitochondrial membrane potential (Ψ_m)

In general, changes in mitochondria that the release of caspase activator (cytochrome-c) and alteration in electron transport chain leads loss of membrane potential. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl carbocyanine iodide) is a lipophilic cationic dye, selectively enters into mitochondria and reversibly changes from red to green as the Ψ_m decreases. In healthy cells with high Ψ_m, JC-1 can spontaneously form complexes known as J-aggregates with intense red color. On the other hand, in apoptotic or unhealthy cells with low Ψ_m, JC-1 remains in a monomeric form that shows only green fluorescence. The red to green fluorescence is purely depends on Ψ_m but not on other factors like size, shape and density. Promastigotes were harvested after 48h of EAF treatment and washed with PBS. Then, resuspended in 10µg/ml of JC-1 dye prepared in Hank's balanced salt solution (HBSS) and incubated for 10 min in dark at 37^{o}C. Further, repeated the wash and resuspended in 1ml HBSS and analysis was done by flow cytometry. MFI (the ratio of red (590nm) and green (530nm) fluorescence) of the JC-1 dye represented as a bar graph that gives the indication of Ψm. DMSO (0.1%) treated promastigotes used as a vehicle control.

3.3.7. Measurement of ROS production

The ROS levels measured in both untreated and EAF treated promastigotes for 24h and 48h as mentioned earlier in chapter 1A, methods section 2.4. The acquisition was done using flow cytometry and green fluorescence of H_2DCF measured as MFI. DMSO treated promastigotes used as a vehicle control.

3.4. *In vitro* and *In vivo* studies on intracellular amastigotes

3.4.1. THP-1 and PBMCs maintenance

THP-1 and PBMCs were harvested and maintained as mentioned earlier in chapter 1A, methods section 2.2. Briefly, cells cultured in RPMI-1640 medium containing 4.5g of glucose/liter, 10mM HEPES, 1mM sodium pyruvate and 10% (v/v) FBS and maintained in 5% CO_2 incubator at 37^{o}C.

3.4.2. MTT assay on THP-1 cell line

PMA activated THP-1 macrophages (3×10^5/ml) were treated with EAF concentrations ranged from 10, 20, 40 and 60µg/ml for 48h in 24-well polystyrene plate. The experiment performed as mentioned in section 2.4.1. The percentage of viability of THP-1 cells plotted against the compound concentration. Miltefosine (Concentration 0, 2.5, 5, 10, 20, and 40µM) cytotoxicity on THP-1 also tested by MTT assay and calculated its IC_{50}.

3.4.3. Infection and treatment

THP-1 cells were seeded in 8-well chamber slides and treated with PMA (25ng/ml) for 24h at 37^{o}C and 5% CO_2 to transform into macrophages. Macrophages (1×10^4/ml) of THP-1 and PBMCs infected for 6h by adding 10:1 ratio of the parasite to macrophage. Non-internalized parasites were washed off, and infected cells treated with 10µg/ml EAF for 48h. Miltefosine (2.5µM) treated cells were a positive control.

Female BALB/c mice (5-6 weeks age) divided into four groups (uninfected, infected, EAF treated, and Miltefosine treated), and each group consists of 5 mice. The infection has given through a tail vein by injecting metacyclic promastigotes (1×10^8). After 28 days of post-infection, EAF treatment (100mg/kg body weight) was given through oral administration for seven times (on every 3rd day for three weeks). Next week, mice were sacrificed, and tissues were collected. Miltefosine (5mg/kg body weight) treatment was as a reference drug control. Animals maintained in the Animal House, School of Life Sciences, University of Hyderabad, under standard guidelines of Indian Council of Medical Research, India.

3.4.4. Light microscopy

For the light microscopic observation, slides were stained with Giemsa (1volume of stain: 9 volumes of distilled water) for 20 min. *In vitro*, the infection rate was calculated by counting the number of amastigotes present per 100 macrophages. *In vivo* infection rate was measured from both spleen and liver tissue smears i.e. Leishman-Donovan Units (LDU) = Number of amastigotes/1000 nucleated cells × tissue weight (grams).

3.4.5. Gene expression analysis

In vitro, total RNA was isolated as mentioned earlier (chapter 1A; methods section 2.6) from 1×10^6 cells/ml of both THP-1 and PBMCs of different experimental variables (uninfected, infected, LPS (100ng/ml) and EAF (10µg/ml) treated cells) after 6h using Nucleospin RNA kit protocol. *In vivo*, spleen tissue from different experimental mice groups (uninfected, infected, EAF treated, and Miltefosine treated) was collected and disrupted in liquid nitrogen using motor pestle. RNA isolation and cDNA preparation performed as mentioned above. For RT-qPCR analysis 900ng of cDNA template was used for all the experimental variables. RT-qPCR also performed as stated earlier (chapter 1A; methods section 2.6) with gene specific primers designed using Primer Express Software **(Overbergh et al., 2003)** of both human and mouse origin and tabulated (Table.1).

Primer name	Sequence (5'-3')
IL-8	FP: 5'-CAGCCTTCCTGATTTCTGCAGCTC-3' RP: 5'-GGTCCACTCTCAATCACTCTCAGTTC-3'
IL-1β	FP: 5'-CCCACAGACCTTCCAGGAGAATGA-3' RP: 5'-GGAGCGTGCAGTTCAGTGATCGTA-3'
IFN-γ	FP: 5'-TCAGCTCTGCATCGTTTTGG-3' RP: 5'-GTTCCATTATCCGCTACATCTGAA-3'
TNF-α	FP: 5'-TCTTCTCGAACCCCGAGTGA-3' RP: 5'- CCTCTGATGGCACCACCAG-3'
IL-10	FP: 5'-GTGATGCCCCAAGCTGAGA-3' RP: 5'- CACGGCCTTGCTCTTGTTTT-3'
iNOS	FP: 5'-TGCAGACACGTGCGTTACTCC-3' RP: 5'-GGTAGCCAGCATAGCGGATG-3'
GAPDH	FP: 5'-CCCATGTTCGTCATGGGTGT-3' RP: 5'-TGGTCATGAGTCCTTCCACGA-3'

Table 1A: Human gene specific primers

Primer name	Sequence (5'-3')
IFN-γ	FP: 5'-TCAAGTGGCATAGATGTGGAAGAA-3' RP: 5'-TGGCTCTGCAGGATTTTCATG-3'
IL-12p40	FP: 5'-GGAAGCACGGCAGCAGAATA-3' RP: 5'-AACTTGAGGGAGAAGTAGGAATCG-3'
TNF-α	FP: 5'-CATCTTCTCAAAATTCGAGTGACAA-3' RP: 5'-TGGGAGTAGACAAGGTACAACCC-3'
IL-10	FP: 5'-GGTTGCCAAGCCTTATCGGA-3' RP: 5'-ACCTGCTCCACTGCCTTGCT-3'
IL-4	FP: 5'-ACAGGAGAAGGGACGCCAT-3' RP: 5'-GAAGCCCTACAGACGAGCTCA-3'
TGF-β	FP: 5'-TGACGTCACTGGAGTTGTACGG-3' RP: 5'-GGTTCATGTCATGGATGGTGC-3'
GAPDH	FP: 5'-CAAGGCTGTGGGCAAGGTCA-3' RP: 5'-AGGTGGAAGAGTGGGAGTTGCTG-3'

Table 1B: Mouse gene specific primers

3.4.6. Measurement of ROS in THP-1

The ROS levels measured in THP-1 cells (1×10^6/ml) of all experimental variables (uninfected and infected, and EAF treated (10µg/ml) after 6h as mentioned earlier in the chapter 1A, methods section 2.4. LPS (500ng/ml) treated cells were a positive control. MFI represents the production of ROS levels measured by FACS Calibur.

3.4.7. Griess reaction

J774.1 cells (1×10^6/ml) seeded in a 12-well plate and infected for 6h by adding 10:1 ratio of the parasite to macrophage. Non-internalized parasites were washed off, and infected cells treated with EAF (10 µg/ml). LPS treated cells used as positive control. After 72h of treatment, supernatants were collected for NO estimation by Griess assay. Briefly, 100µl of culture supernatant mixed with 100µl of Griess reagent (equal volumes of 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthyl ethylenediamine-HCl) and incubated at room temperature for 10 min, and then the absorbance at λ_{548nm} was measured in a microplate reader (TECAN). The amount of NO_2^-(µM) in the samples was calculated from a sodium nitrite standard curve.

3.5. LC-MS/MS analysis

The active EAF subjected to LC-MS/MS analysis for identification of compounds and the fractionation of various compounds achieved on a 4.6×150mm C18 column with a pore size of 3.5µm on an Agilent Technologies 6520 accurate mass Q-TOF LC-MS machine. The solvent gradient used in the ratio of 10mM ammonium formate in water (40): acetonitrile (60) for 40 min at a flow rate of 0.4ml/min.

3.6. Statistical analysis

Statistical analysis performed using two-tailed unpaired t-tests. Significance with $p\leq0.05$, $p\leq0.01$ and $p\leq0.001$ represented as *, ** and *** respectively.

4. Results

4.1. EAF induces the death in promastigotes

Results demonstrated that only EAF exhibits anti-leishmanial activity after 48h of treatment. We observed that there was a dose-dependent decrease in the percentage of viability. The graph shows the percentage of promastigotes viability versus EAF concentration. The IC_{50} of EAF on promastigotes found to be 52.4µg/ml (Fig.1) considered for following *in vitro* experiments on promastigotes. The other crude fractions (i.e. hexane, alcohol and water) were found to be ineffective (data not shown).

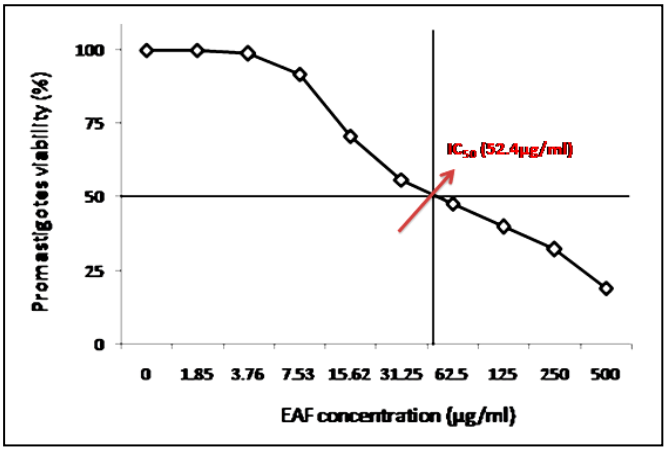


Figure 1: Percentage viability of promastigotes versus EAF (µg/ml). The graph used to determine the IC_{50} of EAF is 52.4µg/ml. As the EAF concentration increases (0, 1.8, 3.9, 7.8, 15.6, 31.2, 62.2, 125, 250 and 500µg/ml) the promastigotes viability proportionally inhibited.

4.2. EAF increases the PI positive dead promastigote population

PI binds to DNA in dead cells due to their leaky membranes, which readily allows the dye to enter the nucleus. The increase in the fluorescence will give an indication of the amount of death in the parasites. There was a significant increase in the percentage of PI-positive promastigotes (48.14±0.92) and (75.51±2.27) upon EAF treatment compared to untreated promastigotes (14.41±0.18) and (18.13±0.35) at 24h and 48h respectively (Fig.2). 0.1% DMSO treated promastigotes used as a vehicle control. It clearly shows that EAF induces death in promastigotes.

4.3. EAF inhibits the proliferation of promastigotes

The (inhibition of) parasite proliferation represented in terms of proliferation index by using ModFit software. CFSE staining demonstrated a decrease in the proliferation index of EAF treated promastigotes after 24h and 48h compared to untreated promastigotes. The proliferation indices of untreated promastigotes were found to be 4.45 and 4.56 for 24h (4 generations) and 48h (5 generations) respectively. Whereas, these values being 1.46 and 1.92

in case of EAF treated promastigotes after 24h and 48h respectively (Fig.3A & 3B). It shows that EAF inhibits the promastigotes proliferation and in turn causes death.

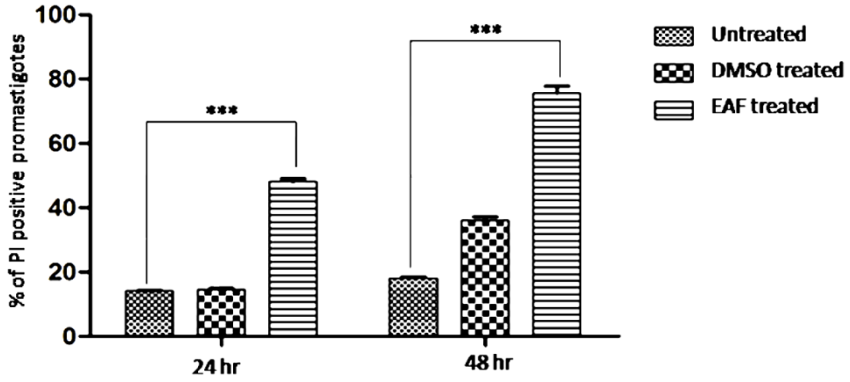


Figure 2: Propidium iodide dye binds to DNA in dead promastigotes. The increase in the fluorescence has correlated the % of death in the parasites at 24 and 48h time points of treatment. There was a significant ($p \leq 0.001$) increase in the percentage of PI-positive promastigotes after EAF treatment. DMSO treated cells used as a vehicle control. Asterisk denotes the significance calculated by two-tailed unpaired t-test.

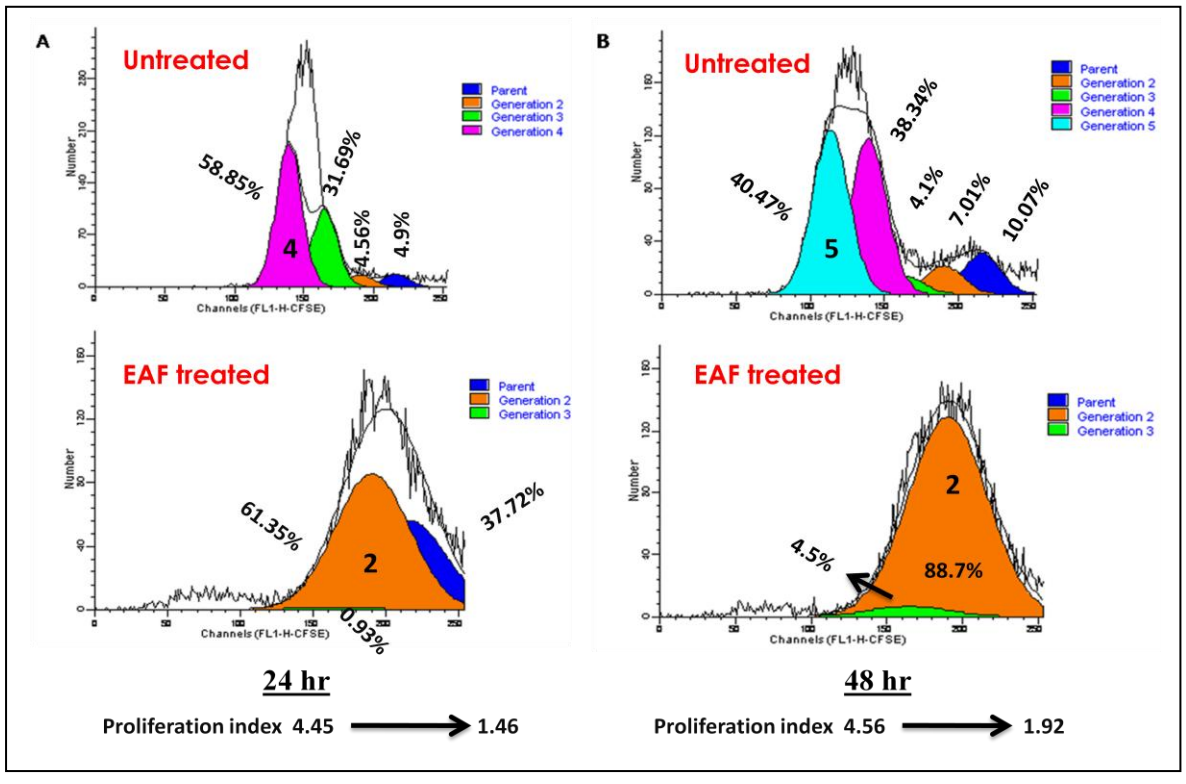


Figure 3: FACS data is showing the promastigotes count on FL1-H-CFSE channel. Promastigotes proliferation was arrested significantly in the second generation with EAF treatment. (A) After 24h (4 generations) the proliferation index of untreated promastigotes was 4.45 and it significantly decreased to 1.46 in EAF treated promastigotes (B) after 48h (5 generations) the proliferation index of untreated promastigotes is 4.56, and it significantly decreased to 1.92 in EAF treated promastigotes. The results were analyzed using ModFit software.

Each peak represents the one generation (shown in colors), shifts towards the left as the promastigotes proliferation increases in time dependent.

4.4. EAF induces the DNA fragmentation

DNA fragmentation by endogenous nucleases is one among the characteristic features of apoptosis. We observed the DNA fragmentation in EAF treated promastigotes after 48h (Fig.4) which suggests that it affects the promastigotes survival.

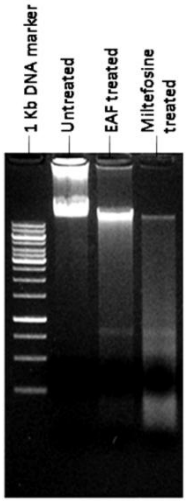


Figure 4: Pattern of genomic DNA fragmentation (Lane 3) in EAF treated promastigotes after 48h on 1% agarose gel. Lane 1: 1kb DNA ladder, Lane 2: Untreated promastigotes DNA, Lane 4: Miltefosine treated promastigote DNA.

4.5. EAF arrests the cell cycle at sub G0/G1 phase of promastigotes

Growth arrest at the sub G0/G1 phase of the cell cycle is an indication of apoptosis. The bar graph denotes the time-dependent increase in the percentage of promastigotes at this stage. EAF treated promastigotes were found to be (0.28±0.07), (2.25±0.18), (5.14±0.51) and (9.18±0.44) at 6, 12, 24 and 48h respectively (Fig.5).

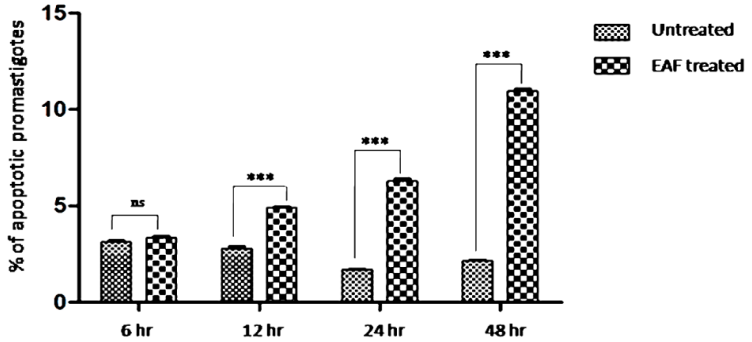


Figure 5: The bar graph represents the percentage of sub G0/G1 or apoptotic promastigotes at given time points (viz. 6, 12, 24 and 48h). It shows that there was a time-dependent increase in the percentage of apoptotic promastigotes upon EAF treatment. The asterisk represents the significance ($p \leq 0.001$) comparison between untreated and EAF treated at

different time points. P>0.05 shown as non-significant (ns).

4.6. EAF depolarizes the Ψ_m of promastigotes

Results demonstrated that there is a significant reduction of Ψ_m in EAF treated promastigotes (9.21±1.1) compared to that of untreated promastigotes (50.52±2.3) after 48h (Fig.6). It clearly shows that EAF causes depolarization of mitochondrial membrane potential in promastigotes, which is a key event in apoptosis.

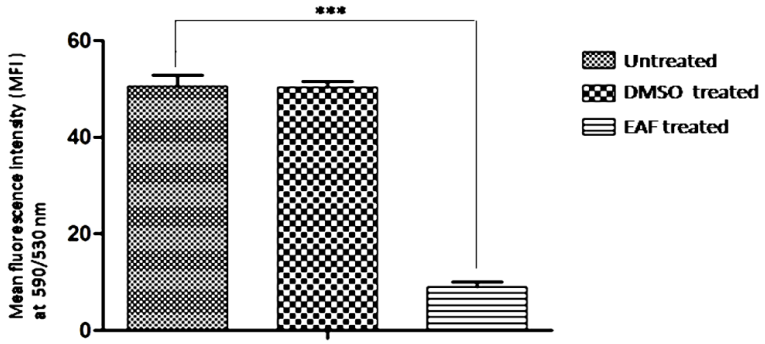


Figure 6: Relative mitochondrial membrane potential determined by the ratio of mean fluorescence intensity at 590/530 nm. The bar graph shows a significant (p≤0.001) reduction in Ψm of EAF treated promastigotes compared to untreated promastigotes. DMSO treated promastigotes considered as a vehicle control.

4.7. EAF induces the oxidative killing of promastigotes through ROS production

The ROS production was estimated by using FACS Calibur, and the bar graph (Fig.7) represents MFI. We found a considerable increase in MFI of EAF treated promastigotes with the values of (51.81±1.67) and (55.3±1.41) at 24h and 48h respectively. And the MFI of untreated promastigotes was shown to be (18.27±1.41) and (20.25±0.87) at 24h and 48h respectively. Our results indicate that EAF has the capability to induce ROS during the apoptosis-like death in promastigotes.

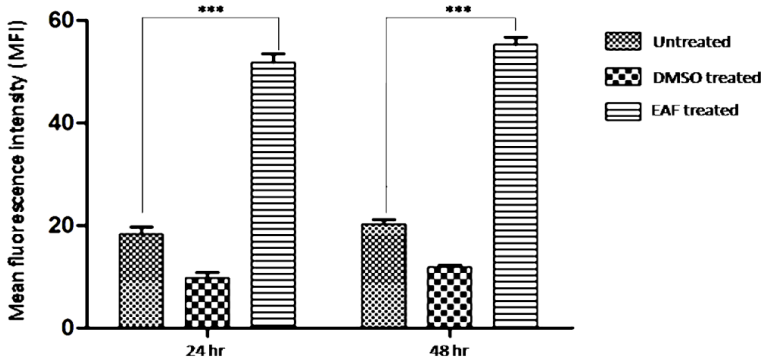


Figure 7: ROS levels were estimated by measuring the mean fluorescence intensity on flow cytometry using CellQuest Pro software. The bar graph represents a significant (p≤0.001) increase in MFI (ROS production) in EAF treated promastigotes compared to untreated promastigotes after 24h and 48h. DMSO treated promastigotes were vehicle control.

4.8. EAF induces the macrophages death at higher concentrations

Results demonstrated that the EAF was considerably toxic to THP-1 cell as the concentration goes up from 10 to 60µg. Only 10µg/ml of EAF was showing >90% (93.5±2.46) viability of host macrophages whereas, other concentrations (20, 40 and 60µg/ml) were showing (74.71±3.457, 49.096±3.355 and 42.33±3.798 respectively) ≤80% viability (Fig.8A). Therefore, following *in vitro* experiments on cell lines were progressed with only 10µg/ml of EAF. Miltefosine is also showing the death of THP-1 as the concentration goes up. At 2.5µM concentration, it has shown ~80% viability considered for the positive control treatment (Fig.8B).

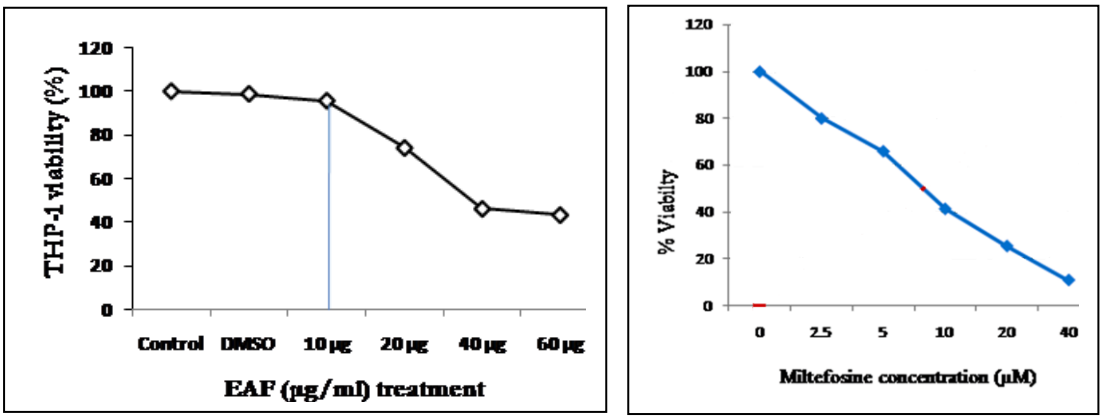


Figure 8: Percentage of THP-1 viability versus different experimental variables (i.e., Control (untreated), DMSO (0.1%) and EAF (10, 20, 40 and 60µg/ml) treated cells. EAF treated macrophage viability was reduced gradually as the concentration goes up from 10, 20, 40 and 60µg/ml. Only the 10 µg/ml was showing >90% of viability others were showing ≤75% viability. Percentage of THP-1 viability versus Miltefosine concentration (0, 2.5, 5, 10, 20, and 40µM) and the IC_{50} of Miltefosine on THP-1 is about 7.9µM.

4.9. Microscopic depiction of intracellular amastigotes in spleen and liver

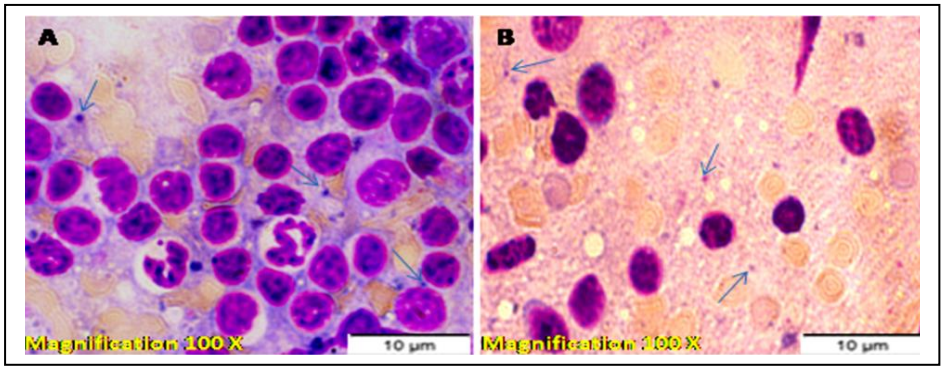


Figure 9: Images showing *in vivo* infection in the spleen (A) and liver (B) tissues and intracellular amastigotes marked with arrows.

4.10. EAF reduces intracellular amastigotes growth

In vitro results demonstrated that the growth or multiplication of intracellular amastigotes in the EAF treated macrophages reduced by ~45% in THP-1 (Fig.10A) and ~50% in PBMCs (Fig.10B) relative to untreated macrophages suggesting that IC_{50} of EAF on amastigotes was ~10µg/ml. *In vivo*, LDU was significantly reduced in both spleen (98.69±10.62) and liver (1354.62±193.99) of EAF treated mice compared to untreated mice, which showed LDU as (243.5±22.86) and (4923.75±355.80) in spleen and liver respectively (Fig.11A and 11B).

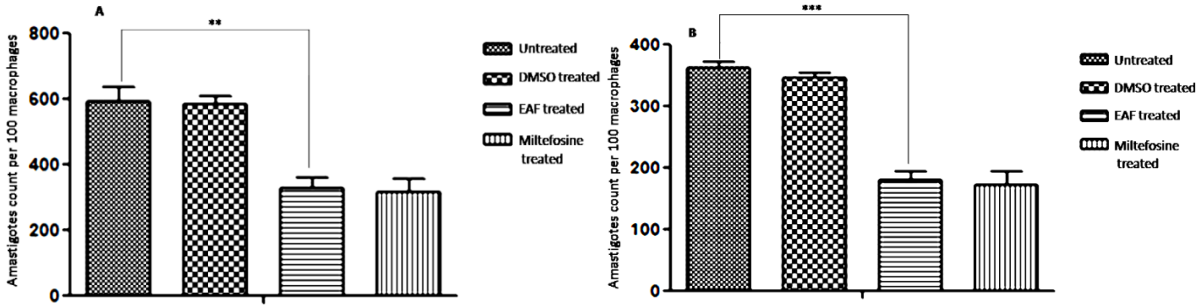


Figure 10: The bar graph shows the microscopic study of an average number of amastigotes per 100 macrophages. Results show the infection rate in EAF treated cells was significantly reduced ($p \leq 0.01$) in THP-1 (A) and ($p \leq 0.001$) in PBMCs (B) compared to untreated cells. Miltefosine treated cells considered as positive drug control. DMSO treated cells were a vehicle control.

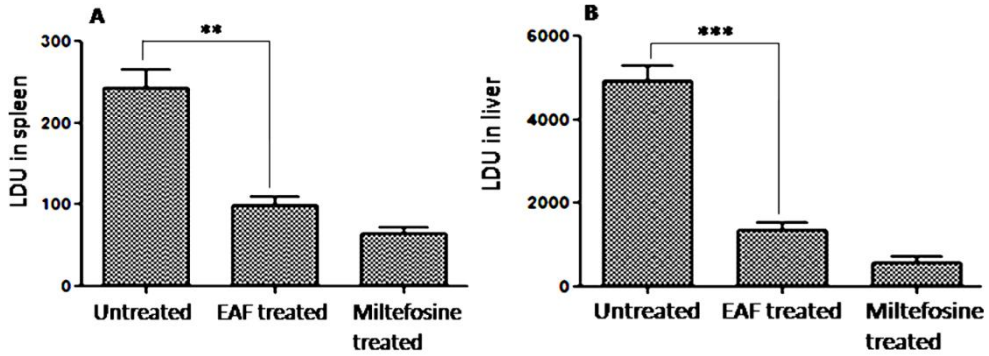


Figure 11: The bar graph shows the microscopic count of Leishman-Donovan units. Results showing the significant reduction of the intracellular amastigotes burden (A) spleen ($p \leq 0.01$) and (B) liver ($p \leq 0.001$) in EAF treated mice compared to untreated mice. Miltefosine treated mice used as positive control.

4.11. EAF upregulates the Th1 and downregulates Th2 cytokines mRNA expression

In THP-1 cells, the Th1 cytokines namely, TNF-α, IL-8 and IL-1β were significantly upregulated (Fig.12A) and the Th2 cytokine, IL-10 expression was unaltered (Fig.12B) upon EAF treatment. The relative expression of Th1 cytokines was higher than Th2 cytokine in EAF treated cells. However, the iNOS expression was unaltered (Fig.12C). In PBMCs, the Th1 cytokines (TNF-α, IFN-γ) expression was significantly induced (Fig.13A) along with

Th2 cytokine, IL-10 (Fig.13B). However, the relative expression of IFN-γ was higher than IL-10 in EAF treated cells. Altogether, *in vitro* results suggest EAF induces a protective immune response. *In vivo* results demonstrated that in the spleen tissue the Th1 cytokines, TNF-α, IFN-γ expression was unaltered, but the IL-12 expression was upregulated (Fig.14A) by EAF. On the other hand, Th2 cytokines (IL-10, IL-4 and TGF-β) expression was significantly downregulated (Fig.14B). However, the relative expression of IFN-γ was higher than IL-10 in EAF treated mice suggesting the host protective immune polarization.

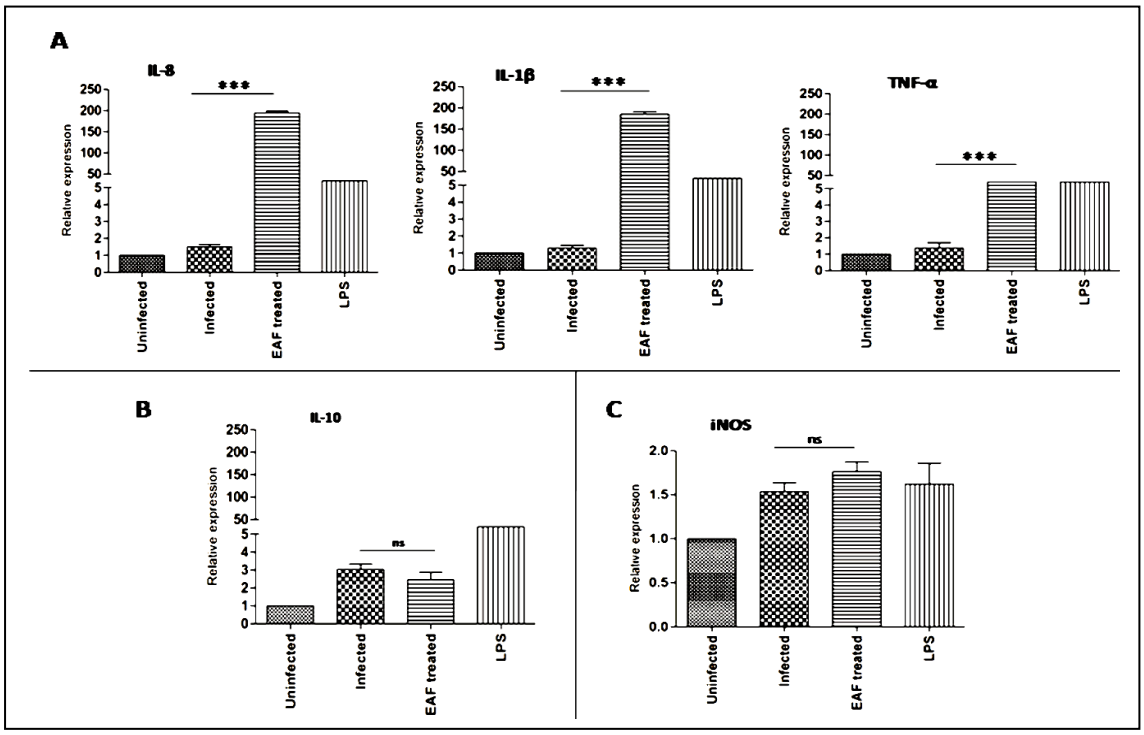


Figure 12: The relative expression of cytokines at mRNA level using RT-qPCR in THP-1. (A) THP-1 associated Th1 cytokines (TNF-α, IL-8 and IL-1β) were significantly ($p \leq 0.001$) upregulated and (B) Th2 cytokine (IL-10) expression was unchanged in EAF treated cells. (C) iNOS expression was unaltered by EAF. Uninfected cells used as normal control. LPS treated cells were a positive control. Significance considered between infected and EAF.

4.12. EAF induces the oxidative killing of amastigotes ROS production in THP-1

Results demonstrated that ROS production was significantly induced by EAF (230.33±3.89) during the infection in THP-1 (Fig.15). The ROS levels in infected cells were low (207.54±4.37) compared to uninfected cells (223.93±3.92). It is an adaptive mechanism lead by the parasite to escape from oxidative killing by host cells.

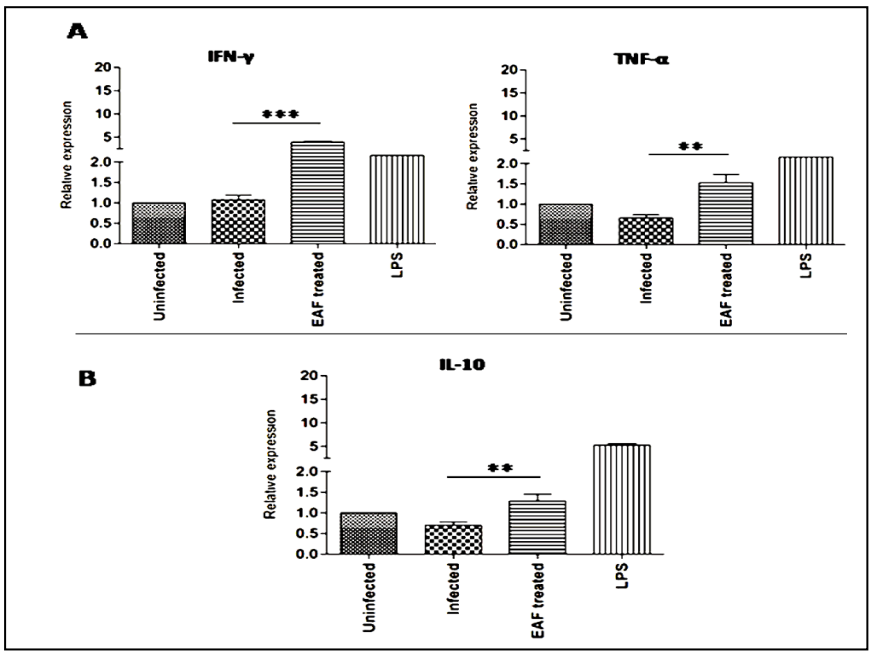


Figure 13: The relative expression of cytokines at mRNA level using RT-qPCR in PBMCs. (A) Th1 cytokines, TNF-α ($p \leq 0.01$), and IFN-γ ($p \leq 0.001$) were significantly upregulated along with (B) Th2 cytokine IL-10 ($p \leq 0.01$) in EAF treated cells. LPS treated cell were a positive control. Uninfected cell used as normal control. Significance considered between infected and EAF treated.

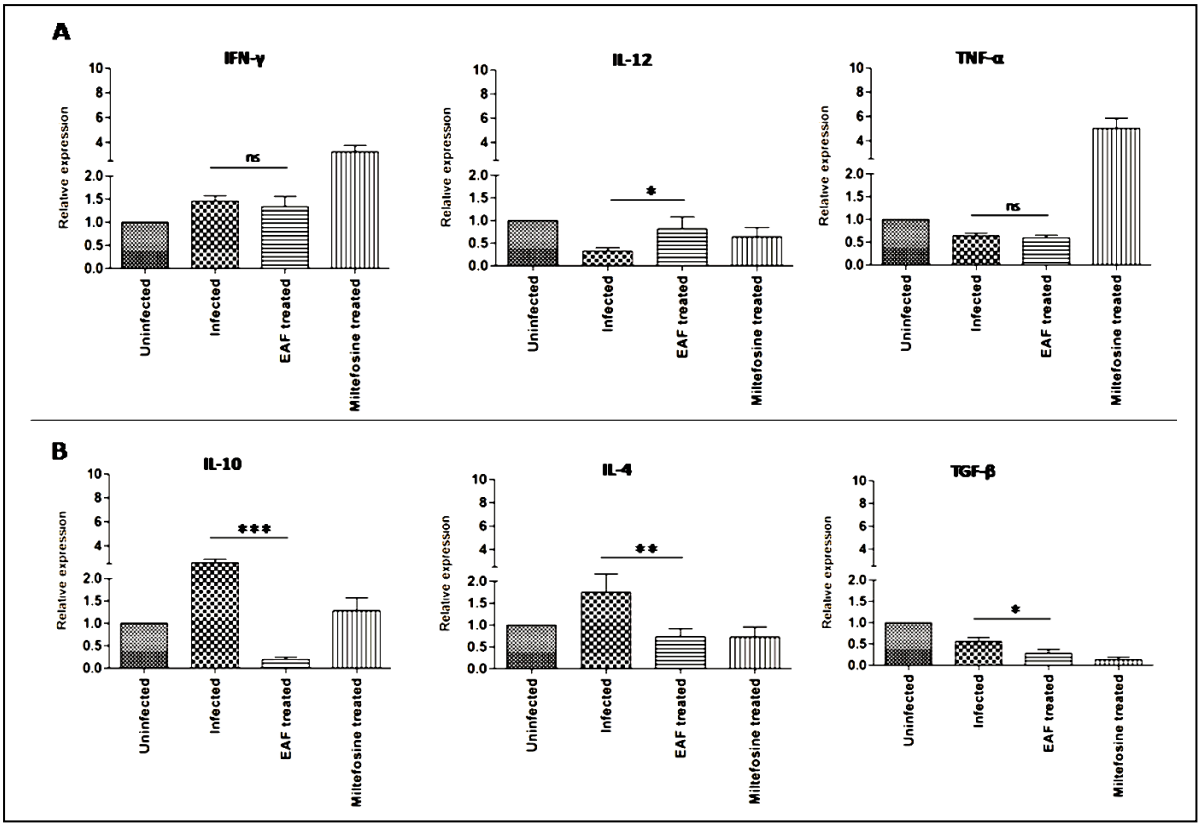


Figure 14: The relative expression of cytokines at mRNA level using RT-qPCR in the spleen. (A) Th1 cytokines, TNF-α and IFN-γ expression unaltered while IL-12 significantly

(p≤0.05) upregulated, on the other hand (B) Th2 cytokines, IL-10 (p≤0.001), IL-4 (p≤0.01) and TGF- β (p≤0.05) expression significantly downregulated in EAF treated mice. Miltefosine treated mice group was a positive control. Uninfected mice were a normal control. Significance considered between infected and EAF treated.

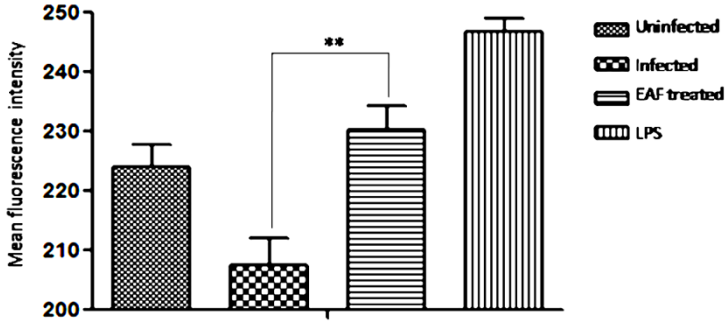


Figure 15: The bar graph is showing a significant increase ((p≤0.01)) of ROS production (MFI) in THP-1 upon EAF treatment. Significance considered between infected and EAF treated cells. LPS treated cell were considered as positive control. Uninfected cells were normal control.

4.13. EAF induces the oxidative killing of amastigotes via NO production in J774.1

Results demonstrated that EAF treatment was considerably induced the NO production (9.2±1.05μM) compared to infected cells (2.2±0.5μM). In uninfected resting macrophages, the NO levels were to be (3.13±0.25μM). LPS considered as a positive stimulator of NO (29.96±2.43μM) (Fig.16).

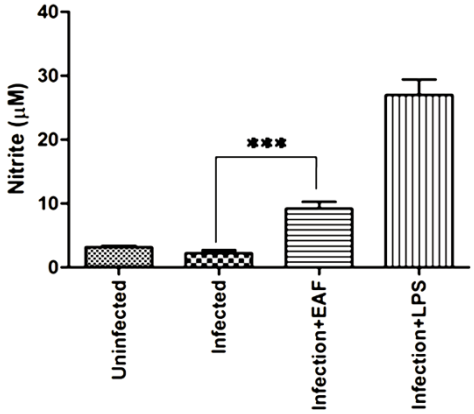


Figure 16: The NO_2^- levels (μM) versus different experimental variables (Uninfected, Infected, EAF treated, and LPS). The NO production was significantly (p<0.001) induced by EAF after 72h. Uninfected cells were normal control and LPS was a positive inducer of NO. Significance considered between infected and EAF treated group.

4.14. Active EAF contains different compounds

LC-MS/MS fractionation of EAF has revealed the presence of 14 compounds by comparing the masses of these compounds with standard masses provided in PubChem

library. The list of these compounds was tabulated (Table.2).

Compound name	RT	Mass	m/z	Molecular formula
Margocinolide	3.883	484.209	485.2163	$C_{27}H_{32}O_8$
Iso-azadirolide	11.607	586.2761	587.2834	$C_{32}H_{42}O_{10}$
Deacetylnimbin	14.476	498.226	499.2333	$C_{28}H_{34}O_8$
Nimbolide	14.766	466.2012	467.2085	$C_{27}H_{30}O_7$
Azadiradione	14.959	450.2412	451.2484	$C_{28}H_{34}O_5$
Deacetylsalannin	15.158	554.289	555.2963	$C_{32}H_{42}O_8$
7-deacetyl gedunin	15.574	440.2204	441.2277	$C_{26}H_{32}O_6$
Nimbin	15.948	540.2353	541.2425	$C_{30}H_{36}O_9$
1, 2 diepoxy-azadiradione	16.949	482.2307	483.2379	$C_{28}H_{34}O_7$
Salannin	17.009	596.2977	597.3049	$C_{34}H_{44}O_9$
Gedunin	17.19	482.2302	483.2375	$C_{28}H_{34}O_7$
Vepinin	18.396	452.2571	453.2644	$C_{28}H_{36}O_5$
Meldenin	18.987	454.2722	455.2795	$C_{28}H_{38}O_5$
Azadirone	19.023	436.2617	437.269	$C_{28}H_{36}O_4$

Table 2: LC-MS/MS fractionation of EAF has identified the presence of 14 compounds by comparing the masses of these compounds with standard masses given in PubChem library. The table depicts the compound name, retention time (RT), mass, mass/charge (m/z) ratio and a molecular formula of each compound.

5. Discussion

Previous reports on herbal treatment for leishmaniasis have been encouraged to explore an effective remedy with affordable cost and minimal side effects. Neem leaf crude fractions tested for anti-leishmanial activity on *L. amazonensis* addressed a promising role in disease control **(Carneiro et al., 2012)**. Neem, effective against multi-drug resistant bacteria **(Jain et al., 2013)**, might also be effective against drug-resistant strains of *L. donovani*. The IC_{50} of EAF on the promastigotes was comparatively less than that of water extract as shown by **Singh et al., 2011**. Cleavage of DNA into its nucleosomal components by introducing nicks **(Verma and Dey, 2004)** and ROS production **(Chipuk and Green, 2005)** evidence for apoptosis-like death in protozoan parasites as exhibited by EAF. Other characteristic features exhibited by EAF such as a reduction in proliferation index, Ψm, and sub-G_0/G_1 phase cell cycle arrest suggest the apoptosis-like death of *Leishmania* **(Chandrasekaran et al., 2013)**. This study provides comprehensive evidence for the anti-leishmanial activity of Neem.

In vitro and *in vivo* studies also strengthen the EAF leishmanicidal activity on intracellular amastigotes by reducing the parasite load in cells and tissues. More than 90% viability of host macrophages also indicates the selective inhibition of amastigotes by EAF. Our study substantiates the previous reports **(Singh et al., 2011, Carneiro et al., 2012)** by showing ~5 fold less IC_{50} of EAF on amastigotes compared to promastigotes. This selective inhibition of amastigote proliferation and the low IC_{50} concentration recommend this extract as an anti-leishmanial drug and also exclude the toxicity that has been the major hindrance in the present chemotherapy.

During *Leishmania* infection, the host protection or disease progression is lead by cell-mediated immune response majorly characterized by the cytokine types. Based on their levels in culture supernatants of human PBMCs, the response is classified into either Th1 or Th2 type. The predominant Th2 response in case of active VL is characterized by higher levels of IL-10 rather than a lack of Th1 cytokine, IFN- γ **(Murphy et al., 2001)**. In contrast, lack of IFN-γ activity may be related to the simultaneous presence of elevated levels of IL-10 seems to be the main macrophage deactivating cytokine in human leishmaniasis **(de Medeiros et al., 1998)**. In accordance with above reports, we also observed the Th2 skewing during *L. donovani* infection *in vivo.* However, this Th2 shift was not consistent during infection in human macrophages (i.e. IL-10 expression elevated in THP-1 but unaltered in PBMCs) coincide with the statement i.e. Th2 shift is not apparent in human VL **(Nylen et al 2007)**. The relative higher expression of IFN-γ to IL-10 with EAF is being a key protective

factor both *in vitro* and *in vivo*. Similarly, the relative higher expression of Th1 to Th2 cytokines in THP-1 also substantiates the aforementioned statement.

In addition, TNF-α can activate macrophages to clear the intracellular amastigotes **(Titus et al., 1989)** and has been found to be significantly upregulated *in vitro* by EAF. It also protects the host by the activation of macrophages to produce NO and thereby suppressing the infection and visceralization **(Wilhelm et al., 2001)**. EAF has failed to induce iNOS expression at mRNA level and also failed to produce NO in culture supernatants of THP-1 cell line (data not shown) as they are the poor source of NO production unlike mouse macrophages **(Perez et al., 2006)**. It might be due to lack of other blood components and IFN- γ mediated activation. However, EAF induces a low amount of NO production from mouse macrophages and ROS production in THP-1, strongly recommends its anti-leishmanial activity by the oxidative killing of the intracellular parasite (**Bogdan et al., 1996)**. Similar to TNF-α, IL-1 is also a major proinflammatory cytokine secreted by macrophages, and it mediates adaptive T–cell immune response limiting the parasite dissemination **(Kostka et al., 2006)**. It was found to be upregulated significantly in THP-1 by EAF also suggesting its potent immunomodulatory activity. The role of IL-8 in leishmaniasis is less understood but in case of *L. major* infection it promotes the recruitment of human neutrophils at lesion sites **(van et al., 2002)**, whose levels were also significantly upregulated by EAF in THP-1. Altogether, EAF therapy seems to be a potent alternative for VL. Few purified compounds of this fraction have already been tested for their anti-bacterial **(Sharma et al., 2009)**, anti-protozoal and anti-carcinogenic activities **(Rochanakij et al., 1985, Gupta et al., 2011)** and reported that it could induce all features of apoptosis. In conclusion, the active purified compounds of this fraction have to be further explored to treat experimental VL.

This study is limited to the only non-polar solvent fraction of crude Neem leaf extract. Despite the lack of toxicity studies; it could be more toxic than aqueous extract, which cannot be used directly to treat clinical disease. This study also lacks the reference leishmanicidal plant-derived compound testing against crude fraction activity. The safety index (SI) of neem extracts was <10, which is not calculated in this study. It seems that the SI would be ~3.75 on THP-1 macrophages. However, the oral administration of EAF 100mg/kg b.w dosage for 7 times in 21 days might not be toxic, unless it extremes 500 mg/kg b.w for 7 days e.g. ethanol extract causes genotoxicity **(Awasthy et al., 1995 and 1999)**. Over dosage or longer period of treatment (≥21 days) cause reproductive toxicity in rodents **(Boeke et al., 2004)**.

SUMMARY

In the conclusion of the chapter: 1, this is the first report on immunotherapeutic activity of leptin in any parasitic infections. In our study, the reduction in serum leptin and TGs during *L. donovani* infection in well-nourished animals is also an indispensable factor to address the fate of leptin during VL. It could be one of the best clinical implications for several complications associated with VL, in spite of immune suppression. Overall results from our study suggest that the leptin can drive the VL compromised host immunity to VL competent host immunity during malnutrition. Therefore, leptin alone could be helpful in the immunotherapy of VL or a combination of chemotherapy as an adjuvant. Further studies are needed to prove its fate in clinically confirmed, disease relapsed and treated patients. It is necessary to investigate the leptin associated immune response during *L. donovani* infection in well-nourished BALB/c mice. In addition, identifying the T-cell subset source (CD4+ or CD8+) for the significant proportion of Th1 cytokines production is another requisite to conclude the track of leptin action in cell-mediated immunity during VL. In our study, leptin was claimed to induce the CD8+ T-cell and reduce the CD4+ T-cell population. Based on this we raised the question is that the increased CD8+ T-cells are the source of which type of cytokines (Th1/Th2)? And the reduced CD4+ T-cells are whether Th2 phenotypic or not? Leptin associated tissue repair activity is to study in detail at the molecular level. The reduced GM-CSF mRNA expression with leptin is also to be analyzed, whether it belongs to defective monocytes or not? And the involvement of Graz-A in host protection is also to be negotiated during malnutrition associated VL.

In the conclusion of the chapter: 2, the toxicity and emergence of resistance to available chemical drugs against VL are evoking to explore plant based natural therapy. To this, our study has claimed that the Neem EAF has a potential anti-leishmanial activity and also augments Th1 protective immune response *in vitro*, *ex vivo* and *in vivo*. Therefore, Neem derived compounds could be better alternatives for the treatment of VL. Further, it is necessary to test the toxicity and to decipher the anti-leishmanial and immunomodulatory properties of EAF derived purified compounds in experimental VL. Few compounds of EAF have already tested for their different activities i.e. nimbidin (anti-bacterial & anti-ulcer), and nimbidol (anti-tubercular & anti-protozoan), etc. But none of them has tested for the anti-leishmanial activity. So that, we are highly fascinated to explore such compounds in the treatment of VL directly or at least as adjuvant (i.e. would be helpful in minimizing the current chemotherapy regimen, toxicity or adverse effects and emergence of resistance).

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- Zinc deficiency in sickle cell disease. Nutr Rev 1975, 33:266–7.

PUBLICATIONS

List of published articles

1) **A. Dayakar**, S. Chandrasekaran, J. Veronica, R. Maurya. Role of leptin in Human Visceral leishmaniasis? Medical Hypotheses 77 (2011) 416–418.

2) **Alti Dayakar**, Sambamurthy Chandrasekaran, Vijay Kumar Prajapati, Jalaja Veronica, Shyam Sundar, Radheshyam Maurya. A rapid method to assess the stage differentiation of *Leishmania donovani* by flow cytometry. Experimental Parasitology 132 (2012) 495-500.

3) **Alti Dayakar**, Sambamurthy Chandrasekaran, Jalaja Veronica, Shyam Sundar, Radheshyam Maurya. *In vitro* and *in vivo* evaluation of anti-leishmanial and immunomodulatory activity of Neem leaf extract in *Leishmania donovani* infection. Experimental Parasitology 153 (2015) 45-54.

4) Radheshyam Maurya, **Dayakar Alti**, Sambamurthy Chandrasekaran. A risk of visceral leishmaniasis in case of helminthes co-infection in endemic regions. IJMHS (2012) 47-50.

5) Sambamurthy Chandrasekaran, **Alti Dayakar**, Jalaja Veronica, Shyam Sundar, Radheshyam Maurya. An *In vitro* study of apoptotic like death in *Leishmania donovani* promastigotes by withanolides. Parasitology International 62 (2013) 253–261.

Manuscripts Under review

1) **Alti Dayakar**, Sambamurthy Chandrasekaran, Jalaja Veronica, Radheshyam Maurya. Leptin induces the phagocytosis and protective immune response in *Leishmania donovani* infected THP-1 cell line and human PBMCs (EP-15-297).

2) **Alti Dayakar**, Sambamurthy Chandrasekaran, Jalaja Veronica, Vadloori Bharadwaja, Radheshyam Maurya. Leptin augments the host protective immune response during experimental visceral leishmaniasis in malnourished BALB/c mouse model (Nutrition & Metabolism).

Manuscripts Under preparation

1) Sambamurthy Chandrasekaran, Jalaja Veronica, **Alti Dayakar**, Shyam Sundar, Radheshyam Maurya. Ex vivo and in vivo studies on withanolides showing ROS mediated anti-leishmanial and Th2 suppressing immunomodulatory activity.

2) Suresh K. Kalangi, **Dayakar Alti**, Gangappa. D, R. S. Maurya, D. Narayana Rao, R. Sathyavathi. Biocompatible silver nanoparticles reduced from *Anethum graveolens* leaf extract augment the anti-leishmanial efficacy of miltefosine.

REPRINTS OF PUBLICATIONS

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Role of leptin in human visceral leishmaniasis? ☆

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ABSTRACT

Human visceral leishmaniasis (VL) is frequently found in poor population who are suffering from malnutrition in endemic areas. Therefore, obviously they may have reduced levels of leptin due to reduction in number of adipocytes which are major source of leptin production. Human pathogenesis of VL and reduced levels of leptin both are associated with increase in Th2 type immune response, characterized by secretion of cytokines such as IL-4 and IL-10. Whereas, the protective immune response during visceral leishmaniasis is associated with effective Th1 type immune response characterized by secretion of IFN-γ, IL-2 and IL-12, which correlates with leptin induction of T cells polarizing to Th1 population and secretion of proinflammatory cytokines, and also inhibition of Th2 type response. Therefore, we hypothesized that leptin might be effective in treatment of visceral leishmaniasis alone or VL patients who have co-infection with other immune deficiency syndromes such as AIDS/diabetes/autoimmune disorders by regulation of Th1/Th2 homeostasis.

Introduction

Kala-azar or human visceral leishmaniasis (HVL) is caused by protozoa belongs to the genus *Leishmania* which infect host macrophages. In India, *L. donovani* is responsible for this disease which is characterized by parasite invasion of lymphoid tissues without causing clinical symptoms [16]. The disease is spread by the bite of certain species of sand fly (subfamily–Phlebotominae). The genera that transmit this disease to humans are *Lutzomyia* in the New World and *Phlebotomus* in the Old World [1]. HVL is mainly affects poor people who are suffering from malnutrition, and is also a serious public health problem in many tropical and subtropical regions of the world. It is endemic in 88 countries of the world including tropics, sub-tropics and the Mediterranean basin (WHO, 1984) among which 16 are developed countries and 72 are developing countries. HVL is one of the top 10 diseases of concern to the Office International des Epizooties (OIE) and top 2 tropical parasitic diseases according to World Health Organization, is rapidly spreading, often in association with HIV infection, especially in sub-Saharan Africa and South America. Recently, it was estimated that 12 million people were infected and 350 millions were at risk of acquiring infection. HVL has emerged as an opportunistic infection in HIV-positive patients. The incidence of HIV infection has been rapidly increasing, which may lead to increasing number of Kala-azar patients. Signs and symptoms include fever, weight loss, mucosal ulcers, fatigue, anemia and substantial swelling of the liver and spleen.

Leptin is a non-glycosylated, 167 amino acids containing protein with molecular weight of 16-kDa, encoded by the obese (*ob*) gene, which is located on human chromosome 7. White adipose tissue is the major source of leptin production [2]. Leptin functions as a hormone as well as a cytokine (adipokine). As a cytokine, it attributes to various functions such as hematopoiesis, angiogenesis, and innate and adaptive immunity [2–6]. Leptin belongs to a family of class I cytokines, which are characterized by a four α-helix bundles [7].

Th1/Th2 polarization in HVL

During visceral leishmaniasis in humans the immune response is predominantly Th2 type, with absence of IFN-γ to *Leishmania* antigens [17,18] and the production of IL-10 and IL-4. IL-10 producing $CD25^-$ T cells were recently implicated in the pathogenesis of HVL in India [9]. Protective immunity in *Leishmania* infections is generally attributed to a Th1 immune response that generates IFN-γ and IL-2 producing $CD4^+$ cells. IL-12 plays an important role in the activation of Th1 cells to secret IFN-γ and IL-2 [21,22].

Leptin induces protective Th1 response

Studies on *Ob/ob* (leptin deficient) mice appeared to show an incredible role of leptin in inflammation and autoimmunity. Leptin deficient mice have reduced secretion of IL-2, IFN-γ, and IL-18 and increased production of Th2 cytokines (IL-4 and IL-10). Leptin

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A rapid method to assess the stage differentiation in *Leishmania donovani* by flow cytometry

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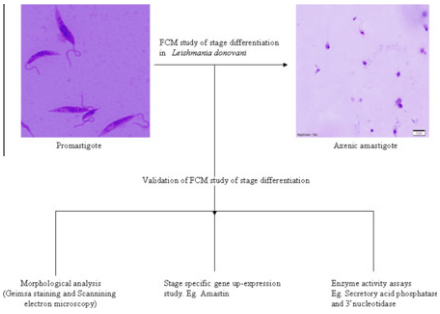
HIGHLIGHTS

- FCM is a non-flourimetric tool to study stage differentiation in *Leishmania donovani*.
- FCM was validated using different molecular and enzymatic assays.
- Partial CDS sequence (472 bp) of amastin gene was submitted in GenBank (HQ840734).
- Successful differentiation and propagation of LEM 138 were established.

GRAPHICAL ABSTRACT



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ABCA3
ATP binding cassette A3
SAcP
Secretory acid phosphatase
3′-Nucleotidase.

ABSTRACT

In this study we describe a rapid and novel method to assess the morphological stage differentiation in *Leishmania donovani* by flow cytometry (FCM). FCM is fast, accurate, and inexpensive to study the stage differentiation of promastigote into *L. donovani* axenic amastigote (LdAxAm). The non-flourimetric FCM method is easy to perform; with requirement of little expertise, and provides unambiguous results. It is an advanced tool, requires minimal time, and no fluorescent dyes. The gradual increase of differentiation and reduction in size from promastigote stage to LdAxAm leads to peak shifting from right to left on histogram. Earlier reports assessed the stage differentiation of *Leishmania* by studying the expression of stage specific markers like surface or secretory proteins and genes. For validation, conventional methods like microscopic analysis are used. These methods are quite expensive, laborious and time consuming. Non-flourimetric morphological parameters were further validated by conventional methods like optical and scanning electron microscopy. Additionally, differential expression of stage specific genes (e.g. upregulation of amastin and ATP binding cassette A3 (*ABCA3*) transporter gene transcripts) and differential activity of enzymes (down regulation of secretory acid phosphatase (SAcP) and 3′-nucleotidase enzyme activity) in LdAxAm suggest stage differentiation. Therefore, we believe that our method is an alternative tool for high reproducibility and reliability in assessment of stage differentiation.

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Full length article

In vitro and *in vivo* evaluation of anti-leishmanial and immunomodulatory activity of Neem leaf extract in *Leishmania donovani* infection

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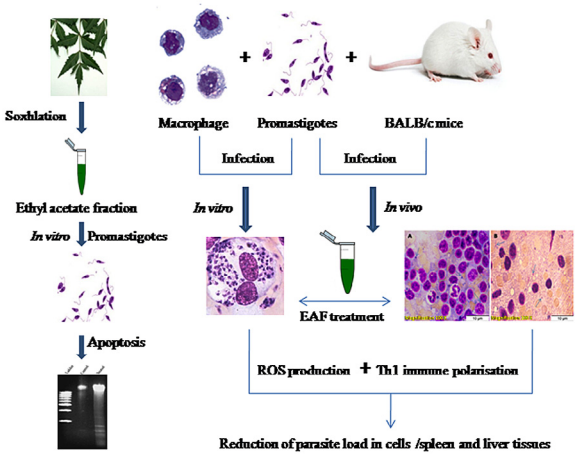
HIGHLIGHTS

- Anti-leishmanial activity of Neem leaf ethyl acetate fraction on promastigotes.
- Anti-proliferation activity and apoptosis like death in promastigotes.
- Anti-leishmanial activity on intracellular amastigotes both *in vitro* and *in vivo*.
- Th1 polarization and Th2 downregulation at m-RNA level by real-time PCR analysis.
- Increased production of reactive oxygen and nitrogen species from macrophages with extract treatment.

GRAPHICAL ABSTRACT



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ABSTRACT

The toxicity and emergence of resistance to available chemical drugs against visceral leishmaniasis is evoking to explore herbal treatment. One such attempt with the Neem is being reported here. The current study is primarily focused to evaluate the anti-leishmanial effects of Neem leaf extracts. Among which, ethyl acetate fraction (EAF) alone was found to exhibit leishmanicidal effect validated through cytotoxicity assay and estimated its IC_{50} to be 52.4 μg/ml on the promastigote stage. Propidium iodide (PI) staining of dead cells substantiated the aforementioned activity. Carboxy fluorescein–diaceate succinimidyl ester (CFSE) staining of promastigotes has affirmed its anti-proliferation activity. The characteristic features such as DNA fragmentation, reduced mitochondrial membrane potential, increased sub G_0/G_1 phase parasites and increased reactive oxygen species (ROS) production in EAF treated promastigotes indicate the apoptosis like death. In addition, the reduced parasite burden both *in vitro* (*viz.* ~45% in human monocytic leukemia cell line (THP-1) and ~50% in peripheral blood mononuclear cells) and *in vivo* (spleen and liver) provides the evidence for its anti-leishmanial activity on amastigote stage. The increase of ROS levels

Abbreviations: EAF, ethyl acetate fraction; VL, visceral leishmaniasis; THP-1, human monocytic leukemia cells; PBMCs, peripheral blood mononuclear cells; LC–MS/MS, liquid chromatography–tandem mass spectrometry; ROS, reactive oxygen species; Ψ_m, mitochondria membrane potential; MFI, mean fluorescence intensity; LDU, Leishman–Donovan units; NO, nitric oxide.

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A RISK OF VISCERAL LEISHMANIASIS IN CASE OF HELMINTHS CO-INFECTION IN ENDEMIC REGIONS.

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ABSTRACT

People in the endemic region are more prone to helminth infections due to the poor hygienic conditions and their genetic susceptibility may be a factor for *Leishmania* infection. The acute worm infections may not cause fatal outcome to host but it may facilitates the infection of other intracellular pathogens which cause higher rate of morbidity. In case of *Leishmania donovani* infection, helminth infections mainly elicit Th2 type protective host immune response characterized by secretion of IL-4, IL-13, IL-5, IL-9 and IL-10, which may suppress the Th1 protective host immune response. Pre-immune polarized (Th2/Th1) individuals due to the helminth infections and their recovery from infection after treatment may have higher levels of serum IL-4 and IL-13 are highly susceptible for visceral leishmaniasis in endemic regions. These Th2 type cytokines, IL-4, IL-13 and IL-10 have immunosuppressive activity, help in the parasite survival by inhibiting the macrophage induced IFN-γ production and oxidative burst mechanism, thereby enhancing disease progression in chronic visceral leishmaniasis. In addition, IL-5 dependent eosinophilia in helminth infections may cause inflammation in visceral organs leading to tissue damage hence these individuals might be susceptible for parasite attack. Therefore, we hypothesize that the Th2 type cytokine milieu of helminth infection might be increase rate of susceptibility to VL occurrence in endemic regions.

INTRODUCTION

Billions of people and their domesticated animals worldwide are constantly affected by the parasitic helminths, or worms, which come under a diverse group of metazoan organisms [1] causing great morbidity, increased susceptibility to other infectious agents and, in some cases, it may leads to death. Majority of individuals infected with parasitic worms are usually asymptomatic or less symptoms compared to those infected with acute viral or bacterial infections and very few will have life-threatening consequences. Good hygienic conditions and health care can minimize the helminthic parasite infections, but in endemic regions immunological intervention can be an effective option of treatment. Till date, there have been no vaccines or other effective immunotherapies for helminth infections and the understanding of the immune response to these important pathogens remains at a very early stage.

Visceral leishmaniasis (VL), also known as kala-azar, black fever, and Dumdum fever [2], is the most severe form of leishmaniasis. It is the second-largest parasitic killer in the world (after malaria), responsible for an estimated 500,000 cases each year worldwide, with 90% of cases caused by the transmission of *Leishmania donovani* in India, Sudan, Nepal, and Bangladesh. [3]. Every year, more than 100,000 cases of VL occur in India alone and in the state of Bihar accounting for more than 90% of these cases, followed by West Bengal and Eastern Uttar Pradesh [5]. It is endemic in 88 countries of the world including tropics, sub-tropics and the Mediterranean basin (WHO, 1984) among which 16 are developed countries and 72 are developing countries. The parasite migrates to the internal organs such as liver, spleen (hence '*visceral*') and bone marrow without causing clinical symptoms [4].

Host immune response to helminth infection

The primary cause of disease in many helminth infections is due to the under development of immune system after encountering the pathogens which lives longer and causes chronic infections. Helminth pathogens differ based on the presence of different glycoconjugates, which contain unusual sugars [15, 21, 24], thought play a role in Th2 response development [22]. These features of helminth Ag are likely to be recognized by Toll receptors and/or other pattern recognition receptors [6]. This extensive organismal complexity, in the majority of cases evokes Th2-like immune response against the worms with the production of a significant quantity of IL-4, IL-5, IL-9, IL-10, and IL-13 and thereby developing a strong immunoglobulin E (IgE), eosinophil, and mast cell responses. Among these cytokines, IL-4 and IL-10 may play a crucial role in reducing the severity of acute disease and

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An *in vitro* study of apoptotic like death in *Leishmania donovani* promastigotes by withanolides

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ABSTRACT

The aim of this study was to isolate and evaluate the withanolides in inducing apoptotic like death in *Leishmania donovani in vitro*. Withanolides were fractionated and isolated from the leaves of *Withania somnifera* and LC-MS/MS analysis of two fractions namely, F5 and F6 of ethanolic extracts, obtained through column chromatography with silica gel, was performed. The antileishmanial effect of withanolides on *L. donovani* promastigotes was assessed *in vitro* using PI dye exclusion test. The effect of withanolides on promastigote morphology was determined by scanning electron microscopy. To understand their mode of action against *L. donovani*, DNA fragmentation, quantification of parasites at sub G_0/G_1 phase, determination of phosphatidylserine externalization, measurement of reactive oxygen species (ROS) and mitochondrial membrane potential (Ψ_m) were done. Results showed that LC–MS/MS analysis confirmed the presence of withanolides in isolated fractions. Treatment with withanolides resulted in morphological alterations from spindle to round shape and loss of flagella/cell integrity in promastigotes. Moreover, it induced DNA nicks, cell cycle arrest at sub G_0/G_1 phase and externalization of phosphatidylserine in dose and time dependent manner via increase in ROS and decrease in Ψ_m. Results of this study indicate that withanolides induce apoptotic like death through the production of ROS from mitochondria and disruption of Ψ_m in promastigotes of *L donovani*.

1. Introduction

Leishmaniasis is an endemic disease in 98 countries worldwide with 200 to 400 thousands new cases each year [1]. It is caused by the various species of the genus *Leishmania*. *L. donovani* is a causative agent of visceral leishmaniasis (VL) or "Kala-azar" in India. The parasite completes its life cycle in two hosts, namely sand fly and humans [2]. VL is the most severe form of leishmaniasis which is fatal, if left untreated. Approximately, world's 50% VL cases are from Indian subcontinent [3]. Till date, there has been no effective vaccine against leishmaniasis and the treatment relies exclusively on chemotherapy. Pentavalent antimonials have been the mainstay of therapy for all forms of leishmaniasis for last seven decades, however, its efficacy has declined in recent years with the result that only about one third of patients respond to it [4,5]. Efficacy of miltefosine, the drug chosen for the Elimination programme in the Indian subcontinent, has declined over the years as well [6]. Hence, there is an urgent need for the development of effective, cheaper and safer drugs to combat this disease.

Traditionally, plants have been used for the treatment of protozoan diseases [7] and plant products may play a significant role in the search of a new antileishmanial compounds. *Withania somnifera* (WS), also known as Ashwagandha is being used as an important medicinal herb for over 3000 years [8]. It has been used for various purposes ranging from an antioxidant to antibacterial [9–13]. The biological activity of WS leaves is mainly attributed to withanolides which comprises withaferin A, withanolides A-y, withanone, *etc.*, which are C_{28} steroidal lactone triterpenoids with ergosterane framework [14,15]. Mainly, much of the pharmacological activities are attributed to the two withanolides namely, withaferin A and withanolide D.

In this study, we evaluated the antileishmanial role of the withanolides isolated from the ethanolic extract of WS leaves through column chromatography with silica gel. Two fractions named as F5 and F6, at a dosage of 60 μg/ml and 15 μg/ml respectively, induce apoptotic like death in the promastigote stage of the *L. donovani in vitro*. The antileishmanial effect was found to be exerted through morphological alterations like cell shrinkage, DNA fragmentation, externalization of phosphatidylserine, induction of ROS ultimately leading to the loss of mitochondrial membrane potential. These observations clearly indicate that withanolides induce apoptotic like death in the promastigote stage of the parasite.

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Mitigation Effect of Leptn And Neem Leaf Extract in Experimental Visceral Leishmaniasis

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Annexure-I

Evaluation Report 1:

1. A paper by Awasthi e al., 2004 has been extensively cited wherein information regarding the transformation of metacyclic promastigotes to non motile amastigotes. The paper deals with immune responses and accordingly, a more relevant paper should be cited.

Ans: In page 9, line 3, the reference Awasthi et al., 2004 is replaced by Debrabant et al., 2004 and in line 16, it is replaced by Kamhavi, 2006.

2. In the Review of Literature, the presentation has to be improved as also references inserted in the correct format. As for e.g. on Page 19, a reference has been stated as 'John Mason and colleagues...' where it should have been given as Mason et al., 2003. In addition, several references have been stated as (i) DCPP, 2007 (ii) UNICEF, 2006 (iii) CMNR, 1990 (iv) NRC 1992 to name a few. The links may be provided as these references are not in the reference section and therefore giving these abbreviations are meaningless.

Ans: In page 19, line 4, Manson and colleagues, 2003 is corrected as Manson et., 2003. Other references already present in old version of the thesis and their abbreviations also provided in relevant section at earlier pages. In page 20, line 28, the reference ILSI, 1975 is removed and its full reference was not provided in bibliography section of original thesis.

In the context of improving presentation in the review of literature, we have revised paragraph 1 in page 19 and incorporated "Globally" instead of "On this planet" in page 21, paragraph 2, line 1. In the same page paragraph 3 is well revised for better presentation. In the page no 28: lines 13-21 are incorporated in revised version for better presentation about leptin fate in infections as shown below and the references are included in bibliography.

"It has been also reported that the leptin shown to inhibit oxidative burst in HIV+ monocytes by diminishing the ROS production, is in contrast to its natural ROS inducing activity (Sánchez-Pozo et al., 2003). Leptin signaling impairs during hypertriglyceridemia, insulin resistance, and elevated SOCS3 expression (Martin et al., 2008) in obesity. It's desensitization in obesity diminishes vaccine-specific antibody response following influenza, tetanus, and hepatitis-B vaccination (White et al., 2013). Leptin is been an effective mucosal vaccine adjuvant against *Rhodococcus equi* (Cauchard et al., 2011) and *Helicobacter pylori* (Wehrens et al., 2008), its signaling in mucosal gut epithelial cells offers resistance against amebiasis caused by *Entameoba histolytica* infection (Vedantam and Viswanathan, 2012)".

In page 28, 2.6. Leptin as a cytokine; line 5-9 and in page 29, line 1 is incorporated in revised version for better presentation about leptin route of administration and effect.

"Leptin's peripheral administration does not affect either food intake or body weight shown in previous studies reviewed by Janet M. Bryson, 2000. Also a thesis (Topically Applied Leptin Accumulates in the Eye and Hypothalamus but does not Influence Food Intake in Rats)

submitted by Paul Raymond Mayo II, The New England College of Optometry, 2008 has shown that subcutaneous administration of leptin did not affect the food intake in rats, it increases the serum leptin but not hypothalamic leptin".

3. References should be correctly stated as for e.g. in the reference of Bogdan et al., 1995 and 1999, the name of Rollinghoff is incorrectly stated in both references. It is important that authors are correctly stated, as this is a public document.

Ans: "Rollinghoff" name is corrected in below two references in page 90.

Bogdan C, Gessner A, Solbach W, Rollinghoff M. Invasion, control and persistence of Leishmania parasites. Curr Opin Immunol 1996, 6:517–525.

Bogdan C, Rollinghoff M. How do protozoan parasites survive inside macrophages? Parasitol Today 1999, 15:22–28.

4. The definition for Phagocyte index appears inadequate as the numbers are not stated. How many infected macrophages were counted as also what was the total number of macrophages counted?

Ans: In page 38, 2.3. Phagocytic activity, lines 9-11 are revised shown below.

"Each time, the percentage of infected macrophages and the number of engulfed parasites were counted out of total 500 macrophages using the light microscope (Leica). Experiment was performed in triplicates".

In page 41, Phagocytic index results are revised by furnishing full details; the percentage of infected macrophages and number of engulfed parasites per macrophage is shown below.

"We observed the increased percentage of phagocytised macrophages in THP-1 (~52%) and PBMCs (~56%) upon leptin (100ng/ml) treatment compared to untreated THP-1 (~33%) and PBMCs (35%) respectively. Moreover, the amastigotes count per macrophage in THP-1 (~2.3) and PBMC (~1.3) was not significantly altered to the untreated THP-1 (~2.1) and PBMCs (~1.3) respectively (data not shown). Likewise, we observed the increased phagocytic indexes; 113.75±20.29, 141±34.91, 189.71±24.32, and 312.08±42.7 respectively in THP-1 (Fig.1A) and 79.19±3.20, 97.23±6.25, 109.29±9.42, and 204.16±31.85 respectively in PBMCs (Fig.1B) with increased leptin 0, 25, 50 and 100ng/ml respectively, substantiated the early report on *L. major* infection (Gainsford et al., 1996)".

5. The statistical data is incorrectly represented in several figures. As for example in Page 40-41, Figure 2, the figure shows*** but in the figure legend it states **p<0.001. This mistake has been repeated in several figures and needs to be addressed. It is also stated as 'picture depicts'. It should simply state the facts, and these words are redundant.

Ans: In page 42, figure 2, the mistake is corrected in legend by incorporating ***$p \leq 0.001$ in revised version of thesis. In page 78, line 1, $p \leq 0.05$ is corrected to $P > 0.05$ shown as non-significant (ns)". "Picture depicts" is removed in figure legends.

6. Page 41, the statement does not match with the figure and must be restated.

Ans: It is revised by deleting unmatched explanation to the figure 3, in page 42 of revised thesis.

"During *L. donovani* infection, the dephosphorylation of signaling cascade proteins such as Akt kinase **(Dey et al., 2007)** and Erk1/2 **(Nandan et al., 1999 and Kar et al., 2010)** is responsible for immunosuppression".

7. In Chapter 1, why were a minimum of 6 animals not included in each group (Page 41) to ensure a statistical analysis which requires at least 6 data in each group. I am assuming that data was collected in duplicates or triplicates to ensure statistical accuracy. This needs to be stated. It may be noted that ICMR does not approve animal studies, instead CPCSEA does, a letter of approval may be provided.

Ans: It might be page 51, because studies on animals were shown here only but not shown in page 41. It was performed in duplicates and incorporated the below sentence in figure legend, in page 52 of revised thesis.

"One of the two experiments with similar results is shown". CPCSEA certificate, we will provide at the time of viva voce.

8. In Chapter 2, the labeling of legends needs to be corrected. As for example, there is no need to state what is the x and y axis. It is also important that the limitations of the study be included, in view of the fact that the safety index of the neem extract was <10.

Ans: In chapter 2, labelling of legends for figure 1 in page 75, figure 2 and 3 in page 76, figure 4 in page 77, figure 6 in page 78, and figure 8 in page 79 is corrected and the limitations of this study is included in page 86, last paragraph is shown below. The coated references incorporated in bibliography.

"This study is limited to the only non-polar solvent fraction of crude Neem leaf extract. Despite the lack of toxicity studies; it could be more toxic than aqueous extract, which cannot be used directly to treat clinical disease. This study also lacks the reference leishmanicidal plant-derived compound testing against crude fraction activity. The safety index (SI) of neem extracts was <10, which is not calculated in this study. It seems that the SI would be ~3.75 on THP-1 macrophages. However, the oral administration of EAF 100mg/kg b.w dosage for 7 times in 21 days might not be toxic, unless it extremes 500 mg/kg b.w for 7 days e.g. ethanol extract causes genotoxicity **(Awasthy et al., 1995 and 1999)**. Over dosage or longer period of treatment (≥21 days) cause reproductive toxicity in rodents **(Boeke et al., 2004)**".

Saftey index of neem extract was <10, is included as it suggested by reviewer.

9. For cell viability experiments, the data is either represented as % death or % viability and both are not needed as done in Figure 8, page 78.

Ans: It is corrected and shown only % viability in figure 8, page 79.

Evaluation Report 2:

1. Chapter2, Fig 10A: Calculation of IC50 of EAF against amastigotes is not clear. It appears that antileishmanial activity of EAF was studied at 10ug/ml concentration and looking at 50% reduction in amastigote count per 100 cell nuclei, conclusion was suggested. On the other hand, it has to be studied at several concentrations.

Ans: In fact, we have not at all focused on calculating the EAF IC_{50} on amastigotes; rather we had interest in looking at the rate of infection in macrophages (host cells) with EAF treatment. For that initially we have performed cytotoxicity on THP-1 macrophages with 10, 20, 40, and 60µg/ml EAF. There we found >90% viability of macrophages only with 10µg, the other concentrations of EAF (20, 40, and 60µg/ml) were showing <75% viability of macrophages. Based on the host cells viability criteria, we have selected only 10µg/ml EAF for further studies on macrophages and eliminated other EAF concentrations. In the study of rate infection; we observed about 45 to 50% reduction of amastigotes load in macrophages with 10µg/ml EAF, it happened by chance. Therefore, we assumed that this ~10µg/ml could be the plausible IC_{50} on amastigotes.

2. SI index also need to calculated against both THP1 and PBMCs for the suggested EAF

Ans: Due to the lack of knowledge in exact calculation of SI index and the insufficient data availability with us, could not measure the SI index of EAF exactly on cell lines as well as animals. However, we assumed the SI index of EAF on THP-1 macrophages is about 3.75, which is included in page 86 as the limitations of study.

3. It is always interesting to study the in vivo antileishmanial activity of EAF in hamster model of visceral leishmaniasis.

Ans: Though, hamsters are most reliable model for human visceral leishmaniasis, at present, we do not have permission to work on hamster model in our University, if we get in future, our laboratory will definitely address your suggestion.

Mitigation effect of lepin and neem leaf...

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