COMPARARTIVE ANALYSIS OF BIOLOGICALLY AND CHEMICALLY SYNTHESIZED IRON OXIDE NANOPARTICLES AGAINST *LEISHMANIA TROPICA*

MS Thesis

By

Qurat-ul-ain
20/FBAS-MSNS/F-15

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Faculty of Basic and Applied Sciences,
International Islamic University,
Islamabad,

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Iron oxide

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Dated: 29/9/17

FINAL APPROVAL

It is certified that the contents and form of the thesis entitled **"Comparative Analysis of Biologically and Chemically synthesized Iron Oxide Nanoparticles against *Leishmania tropica*"** submitted by Qurat-ul-ain have been found satisfactory for the requirement of the degree of MS in BioScience.

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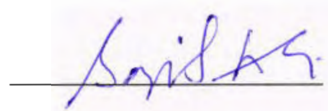

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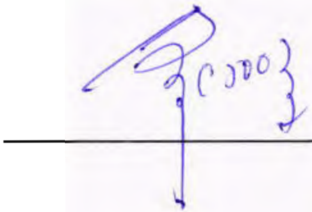

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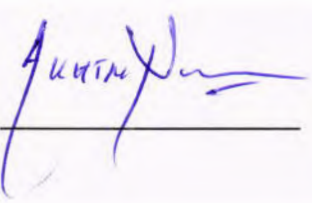

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A thesis submitted to Sulaiman bin Abdullah Aba-Al khail- Centre for Interdisciplinary Research in Basic Science (SA-CIRBS) Faculty of Basic and Applied Sciences International Islamic University, Islamabad as a partial fulfillment of requirement for the award of the degree of MS.

DEDICATION

"Dedicated to my parents whose support, appreciation, and prayers always kept me steadfast to achieve my aims and goals and to be on that position what they dreamt of."

Title
Acknowledgement

Abbreviation

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LIST OF ABBREVIATIONS

Acronym	Abbreviation
nm	Nanometer
XRD	X- Ray Diffraction
SEM	Scanning Electron Microscopy
WHO	World Health Organization
ROS	Reactive oxygen species
LPG	Lipophosphoglycan
PDT	Photo dynamic therapy
Inos	Inducible nitric oxide synthase
Nzvi NPs	Nanoscale zero-valent iron nanoparticles
FeO-NPs	Iron oxide nanoparticles
NP	Nanoparticles
MRI	Magnetic resonance imaging
FBS	Fetal bovine serum
EDTA	ethylene diamine tetra acetic acid
DPBF	1, 1-diphenyl-2-picryl-hydrazyl
PBS	Phosphate buffer solution
FWHM	Full width at half maximum
FT-IR	Fourier transform infrared spectroscopy
LED	Light emitting diode
KBr	Potassium bromide
VL	Visceral leishmaniasis

CL	Cutaneous leishmaniasis
MCL	Muco-cutaneous leishmaniasis

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Abstract:

The applications of nanomaterials in the field of medicines are increasing rapidly owe to their targeting specialized cells and lower toxicity .This research is based on developing more reliable, effective and efficient therapeutic approaches against the fatal leishmaniasis. The current study include synthesis of iron oxide nanoparticles by chemical and green routes, their characterization, and further comparative analysis of chemo-synthesized and bio-inspired nanoparticles as antileishmanial agent. The bio-inspired synthesis was performed by employing secondary metabolites of *Trigonella foenum-graecum* plant. Synthesized nanomaterials were characterized by UV-spectrophotometer, X-ray powder diffraction (XRD) and fourier transform infrared spectroscopy (FTIR). Antileishmanial efficacy was assessed in time and dose dependent manner against both promastigotes and amastigote both in light and dark conditions. Maximum suppressive effects were observed after 72 hours by light exposed green nanoparticles treated groups by exhibiting 50% inhibitory concentration (IC_{50}) 0.001572±0.02μg/ml and 0.011408±0.02μg/ml for promastigotes and amastigotes, respectively. The inhibitory mechanism of nanoparticles is linked with light due to generation of reactive oxygen species (ROS) which gave a quantum yield 0.15 and 0.26 by chemically synthesized and green synthesized iron nanoparticles, respectively. By using different ROS scavengers (mannitol and sodium azide) hydroxyl radical and singlet oxygen were considered as main moieties for cell death. The photo-responsive nanoparticles were found biocompatible on human red blood cells (RBCs).

Chapter 1
Introduction

1. Introduction

1.1. Leishmaniasis:

Leishmaniasis is a vector born protozoal disease that is caused by parasite belonging to genus *Leishmania*. It is worldwide infectious disease, causing increase rate of mortality and morbidity in different region mainly in tropics and subtropics areas (Alvar, Yactayo *et al.* 2006, Alvar, Vélez *et al.* 2012). *Leishmania* pathogens can cause infections in vertebrates ,after transmission into host blood while taking blood meal by the female sand-fly , belonging to *Lutzomyia* or *Phlebotomus* genus (Ali, Khalil *et al.* 1997). World Health Organization (WHO) reported it as one of serious most parasitic diseases and ranked second after malaria.

1.2. Classification:

Leishmania belongs to Eukaryota,

- Kingdom Protista
- Subkingdom Sarcomastigophora
- Phylum Protozoa
- Class Zoomastigophora.
- Order kinetoplastida,
- Family Trypanosomatidea.

There are 22 different species of *Leishmania*, that are responsible for causing leishmaniasis, including the *L. mexicana* complex including 3 major species (*L. amazonensis, L. mexicana* and *L. venezuelensis*); *L. donovani* complex including 2 main species (*L. infantum, L. donovani* also called as *L. infantum*); *L. tropica ,L. major;L. aethiopica*; and the subgenus *Viannia (V)* with four major species *L. (V.) panamensis, L. (V.) braziliensis, L. (V.) guyanensis* and *L. (V.) peruviana* (Chappuis, Sundar *et al.* 2007, Santos, Coutinho *et al.* 2008). These species vary according to their geographical location and also effect the form of leishmaniasis which they exhibits. Morphologically these species can be distinguished through different mechanisms like molecular methods, isoenzymes analysis, and monoclonal antibodies (Chappuis, Sundar *et al.* 2007). Theses

pathogens remain in nature by transmission between mammalian hosts through infected female sand-fly bite.

1.3. Epidemiology:

According to an estimation about 12 million individuals become the victim of leishmaniasis annually and leading to70,000 death rate (Reithinger, Dujardin *et al.* 2007). Worldwide, currently there are almost 350 million individuals are threatened to leishmaniasis (Gour, Srivastava *et al.* 2009) . This disease is more common in tropical and moderate areas. It is estimated by World Health Organization (WHO) (2010), there are 1-2 million new cases appear each year. Leishmaniasis is endemic in 88 countries including 72 developing countries.

1.4. Morphology:

Most of *Leishmania* species share same morphology. Under light microscopy, it has central nucleus, rod shaped kinetoplast and a flagellum. The plasma membrane of pathogen has coating of various surface glycoproteins that play an important role in protection of pathogen from attack of host immune system. *Leishmania* specie involve two hosts in their life cycle, a vertebrate and an invertebrate, the sand-fly. It exits mainly in two distinguished forms: amastigote exist intracellular with in the host (vertebrate), and the promastigote, exist as the extracellular form within the sand-fly (Handman and Bullen 2002). (Figure 1.1)

1.4.1. Amastigote:

The amastigote is intracellular, non-motile, and non-flagellated form reside within the macrophages phagolysosomes of the host. It gets divided through longitudinal binary fission at $37^{O}C$. In size intracellular amastigotes ranges from 1.5-3.0 µm in width and 3-6 µm in length. The amastigote is not entirely devoid by flagella, but its protrusion outside of the cell surface does not occur so its visualization through light microscopy is not possible. Nucleus is relatively larger than promastigotes and situated centrally.

1.4.2. Promastigote:

It is also called as flagellar form. The promastigote is an extracellular, motile having an anterior flagellum on the body surface (liu and uzonna 2012). In size it is ranges from 5mm

in width and 15-30 μm in length. Promastigotes get divides by longitudinal binary fission at 27ºC in the sand-fly. Nucleus is small in size and located in the middle of the cell or along the sides of cell wall. Promastigotes can be culture in vitro at 23 ºC to 26ºC temperature in culture medium.

Plasma membrane's exterior of parasite have coating of various surface glycoproteins that are responsible for protecting the pathogen from the immune attack of host. These surface proteins also facilitate its adhesion and entrance to macrophages, manipulating the macrophage environment and functions in its favor.

1.5. Life cycle of Leishmaniasis:

The female sand-fly transmit the pathogen from humans to humans, mostly from animals to humans and sometimes from humans to animals. In humans, blood transfusion, congenital routes, coitus and various other sources of contacts are consider as major factors responsible for the transmission of disease. The life cycle of parasitic protozoans is complex and characterized by three major developmental stages; promastigotes, metacyclic-promastigotes and amastigotes. The non-motile amastigote stage develops intracellular in the mammalian host (Ullah, Nadhman *et al.* 2016). The motile promastigote form resides in the alimentary tract of arthropod vector, where these pathogen grow in the gut of sand-fly for 8-20 days. When this infected sand-fly bites to healthy organisms it delivers these parasites (metacyclic-promastigotes) into blood of that organism. The metacyclic-promastigotes inoculated to ruptures skin where it is phagocytized by macrophages. These promastigotes multiply and turn to amastigotes and infect many other major tissues which gradually express the disease. (Figure 1.2). During blood meal, these infected cells in the tissue or circulation are again taken up by female sand-fly.

1.5.1. Life cycle in human:

Female *phlebotomine* sandflies are responsible for transmission of promastigote form into skin surface. The infected sand-fly injects metacyclic promastigotes into healthy person while taking blood meal. After transmission, dendritic cells and macrophages in the dermis layer internalized the parasites, where the fusion of phagosome containing promastigote

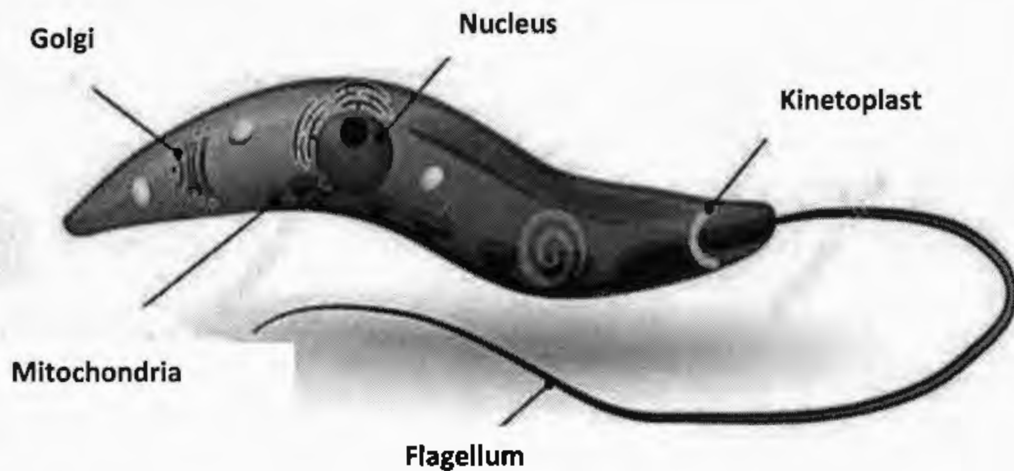
with the lysosome occur. The promastigotes transform to round shape amastigote form by losing their flagella. The amastigotes get multiply, destroying the host cell and survive in the toxic environment of phagolysosomes. It starts division through asexual binary fission and stay in macrophages until it gets ruptured release the amastigotes. The amastigotes circulate within vascular and lymphatic system, finally get enters into other main body organs like bone marrow, liver, and spleen. Depending on the effectiveness of host's immune system, the *Leishmania* infected macrophages differ in their location. For example, *L.donovani* can survive at high temperature deep body organs like liver, bone marrow, spleen, and lymph nodes while *L.major* remains in macrophages of external tissues. However, the mechanism facilitating the survival of parasite in the harsh environment of phagolysosome is still under debate.

1.5.2. Life cycle in sand-fly vector:

Female sand-fly ingests the intracellular amastigotes while taking blood meal from infected person. In the midgut, the round shape amastigotes transformed back into flagellated and elongated promastigote forms.

These promastigotes get attach to the midgut or hindgut wall of the sand-fly and multiply by asexual binary fission. Then promastigotes changes into metacyclic promastigotes by various biochemical modifications and morphological alterations of *Leishmania* surface (Al-Amer 2008). Finally, these metacyclic promastigote get move towards the pharynx and reside there until they get a new host.

Promastigote



Amastigote

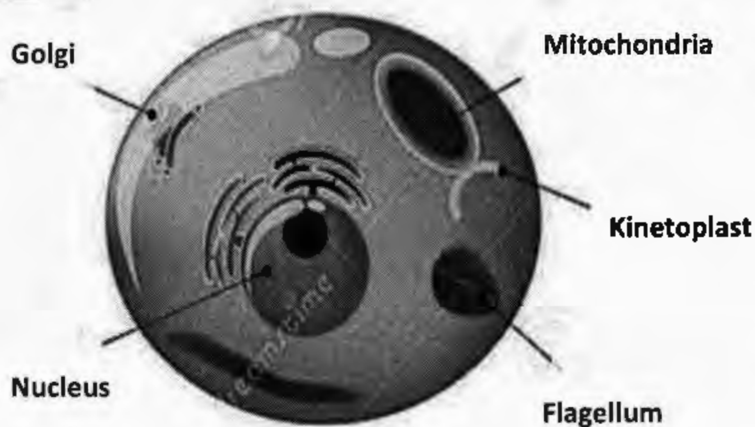


Figure 1.1. Promastigote and Amastigote forms

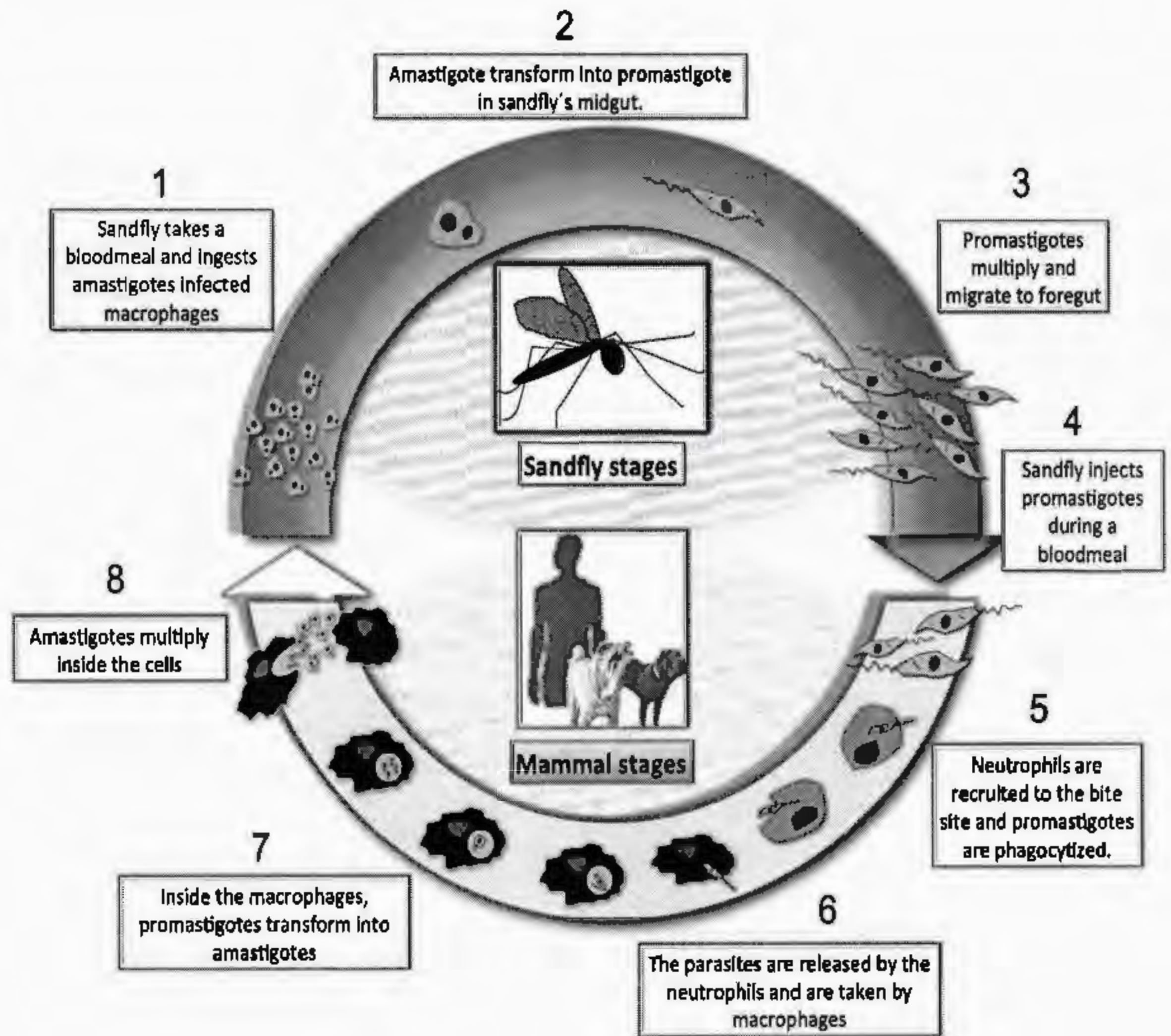


Figure1.2. Cell Cycle of Leishmaniasis(Gutiérrez, Seabra *et al.* 2016)

1.6. Clinical Symptoms:

On the basis of parasite species and cellular immune system of patient, this disease includes three types of clinical manifestations:

1. Cutaneous leishmaniasis,
2. Muco-cutaneous leishmaniasis (espundia)
3. Visceral leishmaniasis (kala-azar) (Gutiérrez, Seabra *et al.* 2016).

1.6.1. Cutaneous Leishmaniasis:

In many developing area, cutaneous leishmaniasis is very serious social and public health issue. It can severely affect the mucous membranes and skin. Worldwide it is prevalent in almost 70 countries, of which 90% cases occurs in Pakistan, Afghanistan, Peru, Syria , Brazil ,Algeria and Saudi Arabia (Yücel, Günaşti *et al.* 2013). Cutaneous leishmaniasis, is mostly triggered by *Leishmania* species comprising *Leishmania major and Leishmania tropica* (Shah, Khan *et al.* 2014), *L braziliensis,* *L mexicana, L (L) amazonenesis, (V) guyanensis* and *L (V) panamensis* (Jebali and Kazemi 2013). In this manifestation, the infection is typically start with small erythema. The erythema transformed into a papule, which progressively develops single or multiple ulcers or nodule over the time period of two weeks to six months to produce the lesion in the patient's skin. These sores spontaneously get heal but gradually in immunodepressent patient causing disfiguring skin scars (González, Pinart *et al.* 2008) . There are two main types of cutaneous leishmaniasis.

• Zoonotic leishmaniasis: the vector gets transmitted from different animal's reservoir to humans. Its frequency is greater in rural areas and geographically, it is mainly distributed in China, Middle East, and North Africa. Zoonotic cutaneous leishmaniasis is caused by various *Leishmania* species but mainly by *L. major*. Although it gets spontaneously heal in the time period of three to four months, but sometimes it persists for almost four to six years.

• Anthroponotic leishmaniasis, the pathogen transmission is occurring from infected person to healthy person. As compare to zoonotic leishmaniasis, it is most common in urban areas and is geographically prevalent in the Middle East and Western Asia. *L. tropica* is mainly

responsible for anthroponotic cutaneous leishmaniasis. Ulcer remains for 5 to 16 months and may cause significant disfiguration.

1.6.2. Muco-cutaneous leishmaniasis:

Muco-cutaneous leishmaniasis also called as "espunda." *Leishmania braziliensis* is primary agent of causing muco-cutaneous leishmaniasis (Hussain, Al-Harrasi *et al.* 2014). In this syndrome individual suffer from gradual ulceration of the mucosa, spreading from the nose, mouth, and throat. When the naso-oropharyngea mucosa macrophages become colonized then these ulcers are unable of self-healing, most probably appear within few months to years after occurrence of cutaneous leishmaniasis, and cause extensive damage and disfiguration. *L. tropica, L.infantum* and *L. major* are responsible for muco-cutaneous leishmaniasis.

1.6.3. Visceral leishmaniasis:

Visceral leishmaniasis also known as kala azar, is chronic leishmaniasis syndrome. It particularly affects the internal organs like liver, spleen, lymph node, bone marrow. Sometimes it's may prove fatal, if left untreated. Affected individual have prominent symptoms like weight loss, fever, swellings of spleen, liver infection and anemia. Sometime also causes decrease in platelet count and white blood cell count (González, Pinart *et al.* 2008).

L. donovani complex and *L.infantum* are responsible for causing visceral leishmaniasis. It may exist in the form of opportunistic infection in individuals with the defected immunological defenses (Bern, Maguire *et al.* 2008) Depending upon their transmission characteristics, it mainly consists of two types: anthroponotic visceral leishmaniasis, vector is transmitted from human to human, whereas in zoonotic visceral leishmaniasis, vector is transmitted from animals to human. In anthroponotic visceral leishmaniasis, humans act as occasional host and different animals (dogs and cats) serve as the reservoir of the pathogen (Guerin, Olliaro *et al.* 2002).

1.7. Establishment of infection and intracellular survival:

Leishmania have extraordinary ability to survive in alternative forms in mammalian macrophages and sand-fly guts. During leishmanial infection, the parasites are phagocytized by the macrophages of host individual as a result generation of reactive oxygen species (ROS) occurs. ROS like superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radicals have ability to produce oxidative stress by causing mutations in macromolecules including proteins, lipids, and nucleic acids thus effecting the cell viability , integrity and making the cell wall fragile (Palmieri and Sblendorio 2007). The *Leishmania* pathogens are very sensitive to these oxygen derived moieties as they are highly reactive and unstable. In order to cope with these reactive oxygen species, *Leishmania* produces enzymes such as acid phosphatase, peroxiredoxins, and lipophosphoglycan which act as scavenger of ROS release by macrophages (Allahverdiyev, Abamor *et al.* 2013). This problem can be overwhelming by releasing more ROS extracellulary to the extent that it can cause the death of pathogens, while safer for human cells. *Leishmania* exhibit the ability to subvert the some important functions of macrophages by distorting the signaling pathway of host cells (Gregory and Olivier 2005). Understanding the basic mechanisms that how *Leishmania* cells have ability of manipulation and survival within the host that provide favorable conditions for their transmission and replication, is very important to generate novel drugs and therapeutic approaches for the treatment of disease. There are various mechanisms adopted by *Leishmania* cells to tolerate the host immune attack. To tolerate the hostile environment of phagolysosomes, LPG (Lipophosphoglycan) on promastigotes prevent the fusion of phagosomes and lysosomes, thus delay, or inhibit the endosomal maturation. By preventing this fusion, parasite protect itself from the degradation by acidic enzymes produce by macrophages (Liu and UZONNA 2012). *Leishmania* also produce an enzyme, acid phosphatases on its surface that prevent it from oxidative burst.

In *Leishmania*, peroxiredoxin and trypanothion peroxidase contribute as the main defense line against the microbicidal oxidative metabolites produce by mammalian host. The parasite possesses glutathionyl-spermidine conjugates in the form of trypanothione. Redox reactions of these complex molecules are consider as important for the conservation and

maintenance of cellular redox potential. In addition it has ability to down regulate the iNOS (Inducible nitric oxide synthase) expression and NADPH oxidase expression that suppresses the production of NO and ROS respectively (Gupta, Oghumu *et al.* 2013).

1.8. Treatment of Leishmaniasis:

In endemic areas, currently practiced means of diagnosis, vector control, and chemotherapies have become outdated because of ineffectiveness. There are various complications associated with currently available modalities(Pentostam, Glcantime ,pentamidine and amphotericine B) to control leishmaniasis (Yasinzai, Khan *et al.* 2013).These drugs are overwhelmed because of possible side effects, complicated administration and not cost effective as well (Shah, Khan *et al.* 2014). The primary treatment against leishmaniasis comprises the use of pentavalent antimonial, mostly N-methylglucamine antimoniate and sodium stibogluconate (Berenguer, Gómez-Campderá *et al.* 1998, Rath, Trivelin *et al.* 2003). In the case of drug resistance, paromomycin pentamidine and amphotericin are used as a second option, although they exhibit great toxicity to the host (Santos, Coutinho *et al.* 2008). In treating some immune-depressed individuals, the conventional drugs become less efficient and require long period treatment with higher dose.

Due to the increasing complexities in our natural ecosystems by the interference of man and global environmental changes the pathogens are becoming more resistant, which has resulted in life-threatening consequences like failure of treatment. Mechanisms of drug resistance are mostly associated with faster drugs metabolism, increased efflux, and uptake of lower drug dose and over-expression of drug transporters. Taking into account the above mentioned complications there is utmost need of modern era to explore natural products as an alternative synthetic antibiotic agents (Gleiter 2009). By understanding the nature of parasite interaction with the mammalian host and the biology behind the cellular and molecular mechanism responsible for parasitism is expected to be helpful in rational development of more effective and efficient therapies

To date, various nanomaterial including metal and metal oxide nanoparticles have been reported to possess antimicrobial properties. Silver nanoparticles, zinc oxide nanoparticles,

titanium dioxide nanoparticles and gold nanoparticles have been reported to have antimicrobial efficacy (Shrivastava, Bera *et al.* 2007, Jebali and Kazemi 2013). Even though there is no exact mechanism have been reported for antimicrobial properties of nanoparticles but binding of cell components and proteins, catalytic oxidation and release of ions are some proposed mechanisms (Lodge and Descoteaux 2006). One of well-known mechanism of antiprotozoal efficiency of metallic nanoparticles is the generation of reactive oxygen species (ROS).

Generic names of drugs	Mode of action	Limitations
Pentavalent antimonials (Pentostam)	Show effects on the macrophages and get activated in amastigote form	Renal insufficiency anemia, cardio-toxicity, pancreatitis vomiting, and headache on long term administration.
Pentamidines(Mepacrine,pentamidine isethionate)	After binding to tRNA, it inhibits aminoacylation, and translation of replicating parasites.	Drug resistance may emerge especially in HIV co-infections, hypotension, leucopenia, hypoglycemia cardiac arrhythmia, mild renal failure, and thrombocytope nia.
Amphotericin B (polyene antibiotics)	Bind to the ergosterols of pathogen's cell membrane and form binary complex with the sterols of the membrane causing membrane permeability and change ionic balance leading to cell death of pathogens.	May cause cellular dysfunction by reacting with mammalian cell membrane and Poor gastro-intestinal absorption.

Paromomoycin	Cause impairment to the synthesis of macromolecules and changes the membrane properties of *Leishmania.*	Has limited potential to treat visceral leishmaniasis
Miltefosine	Mode of action is uncertain, inhibition of synthesis phosphatidylcholine	Emergence of quick drug resistance

Table 1.1. Conventional anti-leishmanial drugs, mode of action and possible sides effects

1.9. Photo dynamic therapy (PDT):

In some cases, certain nanoparticles are activated by photo dynamic therapy (PDT), where ROS production is stimulated under the exposure of photon particles (light) (Matějka and Tokarský 2014).The three main factors contributes to PDT are photosensitizer ,oxygen and light .Individually, none of these is dangerous but collectively they initiate the series of photochemical reactions leading to production of highly reactive oxygen species. Photo dynamic therapy is being most commonly used for the treatment of cancer. Tumor cell is administrated with photosensitizer, followed by irradiation with photon particles of specific wavelength. Basically two steps are involve in PDT that includes:

administration of any photosensitizing material (Mroz, Huang *et al.* 2010),and further activation of the nanomaterial with non-thermal light. For instance titanium dioxide nanoparticles, under the exposure of UV light ability to cause lipid peroxidation which leads to cell death by causing respiratory dysfunction (Beyth, Houri-Haddad *et al.* 2015).

1.10. Nanotechnology:

The term "nanotechnology" was introduced by the scientist named Norio Taniguchi in 1974. Nanotechnology is branch of science that deals with the synthesis, use of products of both chemical and biological nature at nanoscale i.e.1 to 100 nm (Ferrari 2005, Nikalje

2015). In recent conditions, it is generally believing that nanotechnology is opening new promising paths by introducing novel drug delivery systems. It plays important role in wide range of applications in different fields of life such as energy production, waste management, mechanics, electronics, optics, plastics, cosmetics, and health sciences etc (Allahverdiyev, Abamor *et al.* 2011).

1.10.1. Metal oxide nanoparticles:

Nanoparticles are particles, whose size is measured in nanometer (nm) scale. The "Nanos" means dwarf or extremely small. The reason why scientists all round the globe are allured by nanoparticles is because of their immaculate properties which include high surface area, small size, easily soluble in liquids, ability to deeply access the cells and organelles and unique physiochemical properties. Surface area of nanoparticles is large as compare to volume, because of this property their reactivity increases that facilitates their application as catalyst to speed up various chemical reactions. Nanoparticles have appearance of variety of shapes like spherical, cubical, circular, triangular, tubular, or irregular shape. They can be in fused, aggregated or in agglomerative form. The word agglomerative means gathered into rounded shape.

In medical research, the applications of nanotechnology and nanomaterials are growing rapidly. In the field of medicine, nanomaterials are being widely used for gene and drug delivery (Pantarotto, Partidos *et al.* 2003, Han, Ghosh *et al.* 2007), proteins detection (Nam, Thaxton *et al.* 2003), tissue engineering (De La Isla, Brostow *et al.* 2003), hyperthermia treatment for the destruction of tumors and cancer cells (Hergt, Dutz *et al.* 2006), separation and purification of cells and molecules (Salata 2004). Nanoparticles based drug delivery have many advantages over conventional therapeutics delivery systems. It allows targeted delivery to minimize the side effects, improve solubility of drug, amplify therapeutic effects, release the drug in time dependent and environmentally responsive manner thus reduce the frequency of administration, prolongs the drug's half-life, increase biocompatibility and reduce the immunogenicity (Zhang, Gu *et al.* 2008). Gold nanoparticles have been recognized as to play vital role in drug delivery systems, it evades the clearance mechanisms by encapsulating the drugs and also increase its solubility.

1.11. Fabrication approaches for nanoparticles synthesis:

Nanoparticles can be synthesized by two common fabrication approaches that are:

1. Top- Down Method
2. Bottom- up Method

1.11.1. Top- down fabrication method:

- Top down approach is design to generate smaller material by using bulk material.
- Referred as destruction.
- Top down methods involve:
  - Gas condensation
  - Lithography
  - High energy ball milling

1.11.2. Bottom-up fabrication method:

- Bottom up approach involve the arrangement of small size components into cluster form to generate complex assemblies.
- Referred as Construction
- Bottom up methods include:
  - Sol gel processing
  - Inert gas condensation
  - Physical vapor deposition
  - Lithography
  - Pyrolysis

1.12. Nanoparticles Synthesis Methods:

There are basic three techniques used for synthesis of nanoparticles:

1) Physical method
2) Chemical method
3) Biological method.

1.12.1. Physical Methods:

- Ball Milling
- Thermal Evaporation
- Lithography
- Vapor phase

1.12.2. Chemical Methods:

- Sol-gel Processing
- Solution-based synthesis
- Co-precipitation method

1.12.3. Biological Methods:

(Production of nanoparticles using certain living organisms that have innate ability to produce nanoparticles.)

- Bacteria
- Fungi
- Yeast
- Plant

1.12.4. Chemical verses green synthesis of nanoparticles:

Chemical route of synthesis involves the use of highly toxic and hazardous chemical as the reducing and capping agents followed by generation of hazardous byproducts. Their applications in the field of nanomedicine is highly limited because it may cause some harmful effects. Hence, in the field of nanotechnology, there is critical need to introduce most reliable, effective, and eco-friendly approaches to synthesized nanoparticles. To fulfil this need, the use of natural products like biological systems to synthesize nanoparticles is

good alternative. The biological synthesis protocols is more preferable as compare to conventional chemical methods used for synthesis of nanoparticles:

- As via green route, there is no use of toxic chemicals, so it recommended as safe and ecofriendly.
- It is cost effective because the active metabolites like proteins, vitamins, and terpenoids act as capping, reducing, and stabilizing agents.
- Uniform shape and small size nanoparticles production is possible.
- No specific external conditions like high energy and pressure is required (Jain, Daima *et al.* 2009).
- Have safer use in the field of nano-medicines and medical research.

1.12.5. Microbes mediated synthesis of nanoparticles:

A very wide range of active biological components like yeast, bacteria, fungi ,viruses and algae can be used as reducing and stabilizing agents for synthesis of nanoparticle (Li, Shen *et al.* 2007). The different enzymes from the microbes are mostly accountable not only for reduction of metal compounds into their corresponding nanoparticles but also act as stabilizing agents. Some microorganisms have ability to synthesize the nanoparticles by having interaction with metals via their cell surface. The cell- metal interaction is a complex process. Some microorganisms have ability to separate the metal ions. *Pseudomonas stuzeri Ag259* is a bacterium most commonly found in silver mines. It can accumulate silver ions inside to their cell wall (Slawson, Van Dyke *et al.* 1992). *Bacillus* species are also capable of producing metal nanoparticles (Nair and Pradeep 2002). Microbes mediated synthesis of silver nanoparticles of different shape, size, and morphology have been reported. Bacterial strain (CS 11) of *Bacillus* spp. isolated from contaminated soil have been reported to have ability to reduce metal ions into their respective nanoparticles. The biological routes for nanoparticles synthesis is considered as cost effective, environment friendly and simplest approach as compare to chemical routes. *Actinobacter* species have ability to synthesized magnetite nanoparticles, when it is treated with aqueous solution of any suitable iron precursors, in the presence of complete aerobic

conditions. Consequently, the synthesized nanoparticles exhibit good magnetic properties (Bharde, Wani *et al.* 2005)

However, this approach also has some drawbacks, the foremost is the need of control aseptic conditions which also demand trained staff and raises the cost.

1.12.6. Plant mediated synthesis of nanoparticle:

In green synthesis of nanoparticles plants phytochemicals can be used as reducing and capping agents, it refers as more effective and less harmful approach. This process is not accountable for any hazardous effect on the environment. Reduction of metal ions via plant extract is occur in short period of time as compare to microbes. Depending upon types of phytochemical, the nanoparticles synthesis is possible in time ranging from few minutes to hours while microbes mediated synthesis require longer time. The plant mediated synthesis of silver nanoparticles (Ag-NPs) by using the extract of *Pelargonium graveolens* has been reported (Shankar, Ahmad *et al.* 2003). Upon comparative analysis with the earlier study based on the microbes mediated silver nanoparticles synthesis, it was found that silver ions reduction rate by plant extract was more frequent. Time required for the reduction of ions by microbes ranges from 24 to 120 hours, while plant assisted reaction completed within 2-4 hours.

Commonly plants are always a source of ease and cost effective medication, whether in the form of traditional preparations or as in form of pure active principle agents. Plant extracts are of great importance due to their medicinal value and their property against different pathogens for controlling disease. The some important bioactive elements of plants are phenolic compounds, alkaloids, tannins, flavonoids, proteins, sugars and vitamins etc. Besides these ascorbic acids, reductases, flavonoids, and dehydrogenases play significant role by acting as powerful reducing agents for metal ions.

1.12.6.1. Fenugreek (*Trigonella foenum-graecum*):

Fenugreek (*Trigonella foenum-graecum*) is a self-pollinating leguminous bean which belongs to *Fabaceae* family. It is commonly known as methi (Acharya, Thomas *et al.* 2006). It is one of the most commonly used ancient medicinal herbs. Fenugreek have profound hypocholesterolaemic, hepatoprotective effects, hypoglycemic (Dixit, Ghaskadbi *et al.* 2005) and antioxidant potency (Bordia, Verma *et al.* 1997) . Fenugreek seeds are the most useful and important part of the plant. They are rich source of polysaccharide galactomannan. The metabolic profile of the seed extract of fenugreek plant has been reported to contain proteins, terpenoids, vitamins, carotenoid ,flavonoids (Kharissova, Dias *et al.* 2013), lignin curcumins, saponin, lignin and plant sterol (Bhanger, Bukhari *et al.* 2008). Flavonoids act as powerful reducing agents ,it's also reported to exhibit anti-leishmanial properties (Polonio and Efferth 2008).

1.13. Iron oxide nanoparticles:

Magnetic iron oxide nanoparticles have emerged as novel class of promising nanoparticles as they possess many exceptional properties like superparamagnetism. Owe to their outstanding chemical, physical ,mechanical and thermal properties ,FeO-NPs are the only type of magnetic nanoparticles approve by Food and Drug Administration, for the clinical uses (Wilczewska, Niemirowicz *et al.* 2012). Iron oxides of different types naturally occurs in liver, spleen and heart (Grassi-Schultheiss, Heller *et al.* 1997), which shows their biocompatibility and reduced the toxicity at acceptable concentration. In biomedical applications, Iron oxide nanoparticles have wide applications like target drug delivery (Pisanic, Blackwell *et al.* 2007), tissue repair, hyperthermia treatment for cancer (Thanh and Green 2010) and magnetic resonance imaging (MRI) (Awwad and Salem 2012, Bull, Madani *et al.* 2014).(Fig 1.4)

For iron nanoparticles, although physical and chemical methods of synthesis are more common, but these methods, have several limitations such as the following.

1) Nanoscale zero-valent iron nanoparticles (nZVI) synthesized by physical and chemical methods are highly reactive in nature and tend to form aggregates, which ultimately results in loss of reactivity (O'Carroll, Sleep *et al.* 2013).

2) The magnetic nanoparticles synthesized by conventional methods cannot be used in biomedical applications because the use of nonpolar organic solvents.
3) Upon the exposure of air, synthesized iron oxide nanoparticles lose their magnetism and dispersibility (Wang, Jin *et al.* 2014).

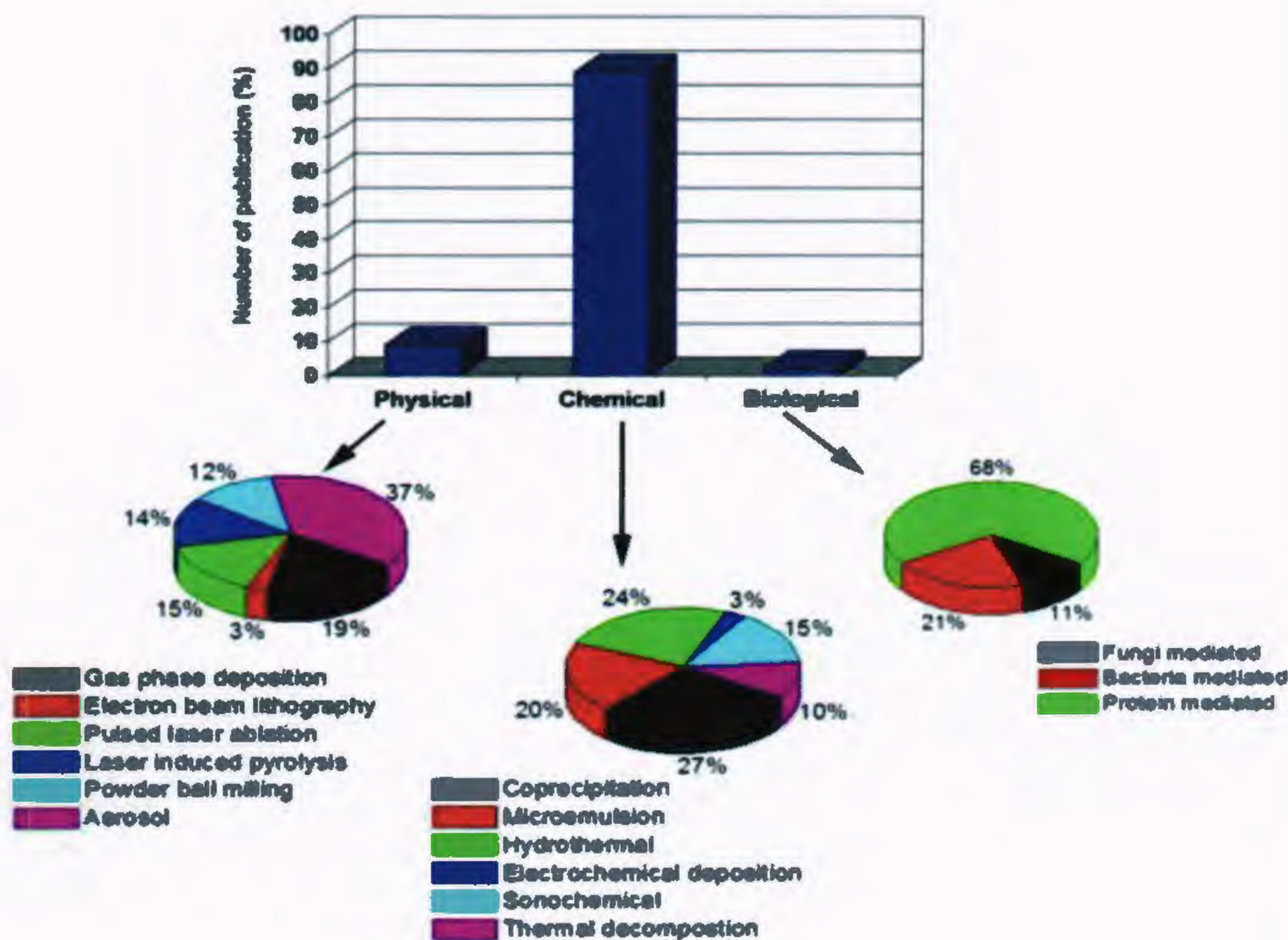


Fig.1.3 A comparative analysis of published work on the, iron oxide nanoparticles synthesis by three different methods (Mahmoudi, Sant *et al.* 2011)

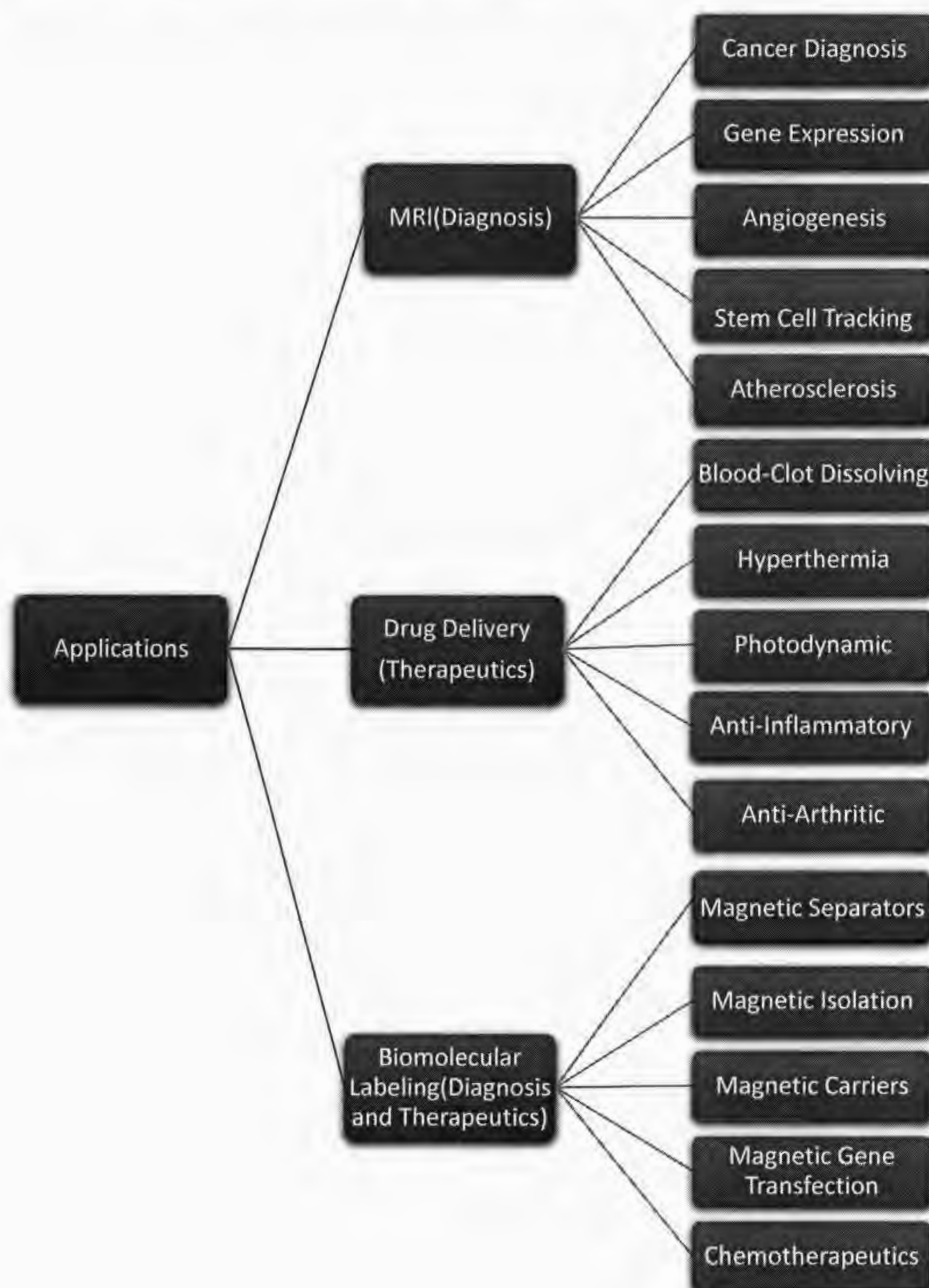


Fig.1.4. Applications of iron oxide nanoparticles in medical research

To overcome these limitations green synthesis of iron nanoparticles is known as eco-friendly, less toxic and cost effective approach.

1.13.1. Antimicrobial properties of FeO-NPs:

There are several studies that demonstrate the bactericidal effects of iron oxide nanoparticles. Antibacterial activity of iron oxide nanoparticles against *Staphylococcus aureus, Salmonella enterica, Escherichia coli* and *Proteus mirabilis* have been reported (Naseem and Farrukh 2015). It is known that production of reactive oxygen species (ROS) is main mechanism that is responsible for the antibacterial properties of FeO-NPs. Reactive oxygen species like superoxide radicals (O2), hydrogen peroxide(H_2O_2) and singlet oxygen cause oxidative stress that cause damage to macromolecules including proteins and nucleic acid in bacteria.

Antibacterial, antifungal, antiviral, and anti-carcinogenic efficacy of iron oxide nanoparticles have been observed and reported but to date, there is no reported study that demonstrate their anti-leishmanial efficiency on *Leishmania* parasite. It is the preliminary study that investigate the antileishmanial effects of iron oxide nanoparticles under different conditions (dark and LED exposures).

Iron oxide nanoparticles also have profound role in environmental remediation circles. As it removes both of organic and inorganic heavy metal pollutants from polluted water.

1.14. Biocompatibility and toxicity evaluation of nanoparticles:

The toxicity and biocompatibility of nanoparticles are another important issues to take into account for the sake of their different biomedical applications. According to Williams, biocompatibility is define as capability of a bio-component to accomplish its preferred functions with regard to medical treatment, without causing any unwanted systemic or local effects to the recipient individual but promoting the most desirable beneficial tissue or cellular responses (Williams 2008).Toxicity refer to the efficiency of biomaterial to cause adverse effects to the normal physiology of individuals and also effect the structure of main organs and tissues. It is generally believed that toxicity significantly dependent on various physical and chemical parameters like particle shape, size, composition, and surface

charge. Although, metal and metal oxide nanoparticle have outstanding potentials to combat with resistant strains and several diseases, their applications in drug delivery is restricted owing to their possible toxic effects (Donaldson, Poland *et al.* 2010). Various studies reported that metallic nanoparticles, at higher concentrations have shown toxic effects on liver cells, fibroblast, macrophages and kidney (Arora, Jain *et al.* 2009). The effects of nanoparticles may cause genotoxicity, teratogenicity, and carcinogenicity. Certain nanoparticles have ability to cross blood-brain barrier, and can have sever toxic effects on brain (Ai, Biazar *et al.* 2011).

To explore the biomedical applications of iron nanoparticles including drug and gene delivery, MRI and magnetic hyperthermia, stringent evaluation of toxicity is required for biological biosafety of nanomaterial. Nanoparticles are used as a vector in gene and drug delivery, where they directly contact with the blood. It's very important to estimate the blood-compatible nature of nanoparticles. For in vitro conditions hemolysis assay, blood cell coagulation and aggregation behavior experiments have been conducted to assess the blood compatibility of nanomaterials. However, hemolysis assay is considered as most reliable and simple measure to estimate the blood compatibility of nanoparticles (Lee, Powers *et al.* 2004). In general, superparamagnetic iron oxide nanoparticles are considered as biocompatible, screening no hazardous effects for in vitro or in vivo experiments (Müller, Skepper *et al.* 2007). Earlier studies reveal that there are no immunomodulatory effects were observed for primary human macrophages, when the cells were treated with 30 nm dextrane-coated superparamagnetic iron oxide nanoparticles (Siglienti, Bendszus *et al.* 2006).

1.15. Aims and Objectives:

1.15.1. Aim of study:

The present study is aim to synthesize, optimize and characterize the iron oxide nanoparticles (NP), by both chemical and biological methods. Further this study involves the comparison of both methods and their invitro application as anti-leishmanial activity by annihilation of the amastigotes and promastigotes both in the presence and absence of LED light.

1.17.2. Objectives:

i. Synthesis and characterization of iron nanoparticles by both chemical and biological method.
ii. Anti-leishmanial activity of iron nanoparticle.
iii. Biocompatibility of iron nanoparticles on human blood cells.
iv. Quantification, and qualitative analysis of iron nanoparticles against leishmanial DNA.
v. Evaluation of cell death mechanism of *Leishmania* by the synthesized nanoparticles.
vi. Comparison of chemically and biological synthesized iron nanoparticles.

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Chapter 2

MATERIALS AND METHODS

2. Materials and methods:

2.1. Materials:

2.1.1. Chemicals:

Iron chloride hexahydrate ($FeCl_3.6H_2O$), sodium hydroxide (NaOH), sodium bicarbonate, fetal bovine serum (FBS), HEPES, ethylenediaminetetraacetic acid (EDTA), sucrose, iron chloride tetrahydrate ($FeCl_2.4H_2O$), sodium dodecyl sulphate, Tris-HCL, triton X-100, proteinase K solution, phenol, chloroform, DPBF.

2.2. Chemical Synthesis of iron oxide nanoparticles:

Preparation method greatly affects the shape, size, and surface distribution of nanoparticles. There are various methods have been proposed to chemically synthesized the nanoparticles. Iron oxide nanoparticles were synthesized by using co-precipitation method. Nucleation and crystal growth were two fundamental pathway involve in the co-precipitation method. When the concentration of the species gets critical supersaturating, the formation of small nuclei occurs. These nuclei can grow uniformly through diffusion from solution to their surface and it is followed by the growth of the crystal. During crystal growth, the solutes get diffuse to the crystal's surface and the process is measured by the mass transport.

Iron chloride hexahydrate ($FeCl_3.6H_2O$) and iron chloride tetra hydrate ($FeCl_2.4H_2O$) salts were dissolved in 100 ml of dist.H_2O, by using the molar concentration 0.2M and 0.1M, respectively. To get homogenous solution, mixture was stirred with magnetic stirrer for 5 minutes. Afterward, the solution was heated at 70°C for 30-35 minutes in water bath, followed by the drop wise addition of 10ml 0f 1M sodium hydroxide (NaOH) solution. Immediately formation of black precipitate occurred by base addition. After completion of reaction, black precipitates were separated by centrifugation at 10,000 rpm for 10 minutes. The precipitates were washed for 4-5 times with dist.H_2O to remove all the impurities. The iron oxide nanoparticles were harvested by drying the precipitates in oven (Lab Tech) at 60°C for overnight. Further the powdered form of nanoparticles was calcinated at 400°C for 3hrs. (Figure 2.1)

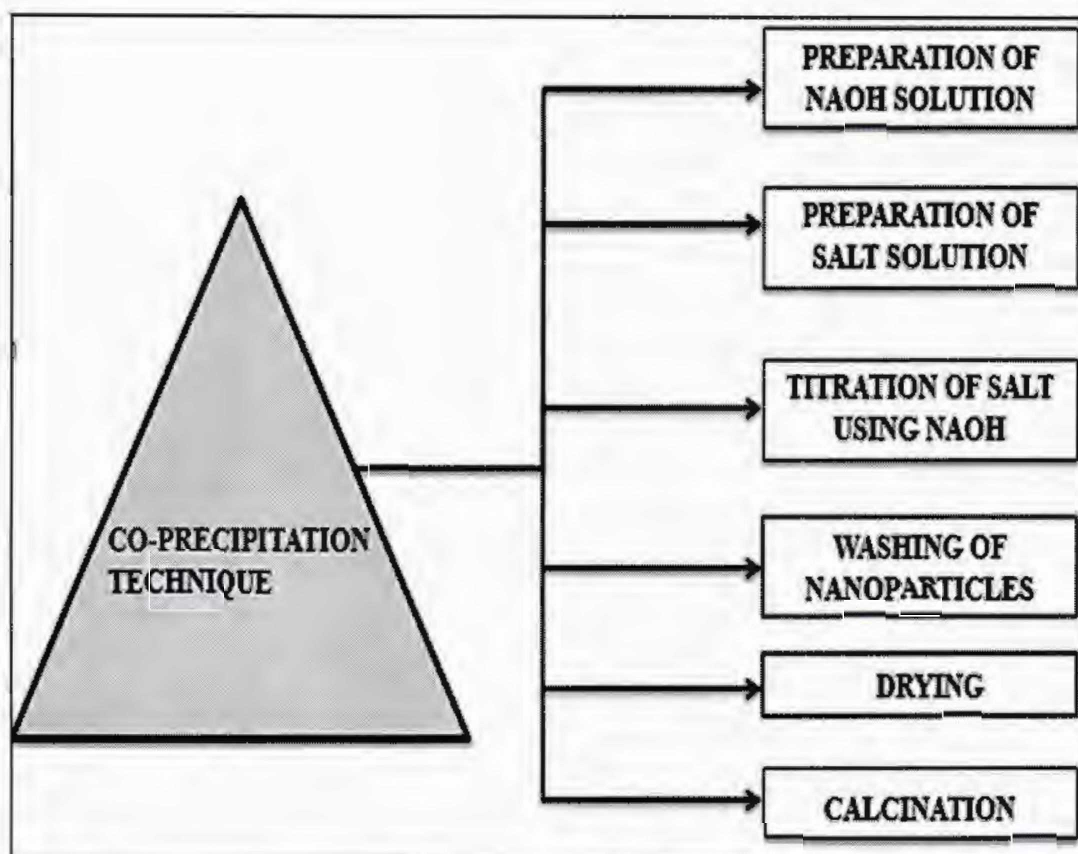


Fig 2.1. CO-Precipitation Method for Nanoparticles synthesis

2.3. Green synthesis of iron oxide Nanoparticles:

In this study seeds extract of *Trigonella foenum-graecum* (fenugreek) plant was used as reducing and capping agents to synthesized iron oxide nanoparticles. Fenugreek seeds brought from local market have been washed with distilled water for 2 to 3 times. Five gram of seeds were boiled in 50ml of distilled water for 15 minutes (Fig.2.3). Then filtered through Whatman filter paper (pore size 90mm) to get plant extract. Then this solution was used for the reduction of iron ions to iron oxide nanoparticles. Four different molarities of ferric chloride salts solution were used for reaction, 1mM, 2.5mM, 5mM, and 7.5mM. Plant extract was added to 1mM solution of ferric chloride in 1:2 volume ratio and boiled at 85°C for 45minutes under vigorous stirring. Visual observation of color change from pale yellow to dark brown was indicative of -reduction of ferric ions. The experiment was carried out at varying pH from 4 to 7 respectively. At the end of the reaction, colloidal solution was subjected to centrifugation at 10,000 rpm for 15 minutes. The precipitates obtained were washed for 3-5 times with deionized water. The synthesized nanoparticles were dried in an oven at about 70°C for 24 hours. The dried nanoparticles sample was stored in an air-tight container and subjected for characterization. (Fig.2.2) Whole experiments were conducted at ambient temperature to avoid agglomeration.

2.4. Characterization of FeO-NPs:

Characterization of iron oxide nanoparticles was carried out by UV–visible spectrometer, XRD and Fourier Transform Infrared Spectroscopy to study the size, morphology, and elemental analysis.

Fig.2.2. Green synthesis route for the synthesis of Nanoparticles

Fig.2.3 Plant mediated synthesis of Iron Oxide Nanoparticles

2.5. *Leishmania* cell culture:

L.tropica KWH23 was incubated at 23°C in M199 media (pH 7.2) supplemented with sodium bicarbonate, 10% fetal bovine serum (FBS) and 25mM HEPES. For all experiments, concentration of cells was $2X10^7$ cells/mL. Culture was maintained at 22°C-25°C for promastigotes growth and proliferation.

2.6. Time dependent Anti-promastigote assay:

Stock solution of FeO-NPs (chemical and green) was prepared by suspending nanoparticles (1mg) in 1 ml of distilled water. Nanoparticles were mixed by vortexing for 1-2 minutes and in order to completely disperse the aggregates, solution was subjected to sonication for 10 mints. Medium M199 containing culture promastigotes were suspended to yield 1×10^6 cell/ml in each well of 96 wells plate. Afterwards, drug nanoparticles were added serially (10μg/ml, 5μg/ml, 2.5 μg/ml, 1.25 μg/ml, 0.625 μg/ml, 0.3125 μg/ml, 0.15625 μg/ml, 0.078125 μg/ml) in each well. The final volume was attuned to 100 μl with M199 media, for each well of 96 well micro plate in all experiments. It was further divided into two group. First group was exposed to LED light for 10 mints at 25°C and followed by incubation in the dark for overnight at 25°C-27°C while second group was directly incubated in the dark. Control leishmanial culture was not treated with nanoparticles solution or any irradiation. Viability of promastigotes was analyzed by Neubauer chamber after the time period of 24, 48, and 72 hours of incubation. All the reactions were performed in triplicate. The reactions were proceeded from mid of November to end of September. When the external temperature was 23°C-26°C that is favorable for the growth of *Leishmania*.

2.7. Anti-leishmanial activity against amastigotes:

FeO-NPs (chemical and green) that were found to annihilate *L.tropica* promastigotes were further tested to evaluate their leishmanicidal activities against amastigotes. Stock solution of FeO-NPs (chemical and green) was prepared by suspending nanoparticles (1mg/ml) in distill water. For axenic amastigote, pre-established culture of *Leishmania* promastigote were grown in MI99 media at pH 5, afterward incubated at 33°C for 7 days. This variation in temperature and pH transformed the promastigotes into axenic amastigotes. Medium M199 containing culture of amastigotes was suspended in the wells of a 96-well microtiter

plates. They were treated serially (10µg/ml, 5µg/ml, 2.5 µg/ml, 1.25 µg/ml, 0.625 µg/ml, 0.3125 µg/ml, 0.15625 µg/ml, 0.078125 µg/ml) with FeO-NPs solutions. Similar to promastigotes, reaction was performed in two conditions. First was proceeded by the exposure to LED light for 10 minutes and then incubated in the dark while second was carried out in complete darkness. After 72 hours of incubation, viable cells were counted on the Neubauer chamber by staining with trypan blue and percent inhibition was calculated.

2.8. Biocompatibility of FeO-NPs:

Hemoglobin get release by destroying the external membrane of erythrocytes. Thus it is possible to estimate the quantity of destroyed erythrocytes by measuring the amount of hemoglobin in a sample.

Hemolysis assay was conducted to evaluate the cytotoxicity of synthesized nanoparticles on human blood. Fresh human blood was collected from healthy volunteer (O, A+ and B+ group) in vacutainer. Blood was centrifuged at 3000 rpm for 3 minutes to separate the erythrocytes, afterward washed with phosphate buffer for 2-3 times.

Erythrocytes solution was diluted 10:90 with PBS. 100µl of this was added to each eppendorf and treated with serial solutions of nanoparticles (chemical and green). Red blood cells suspended /'in phosphate buffer, without treated with nanoparticles was taken as negative control. For positive control, red blood cells were lysed with 0.1% triton X-100. Reaction was performed in two groups; one group was photo-irradiated with LED for 10 minutes while other was performed in complete dark conditions. After treatment with nanoparticles both groups were incubated at 37°C for 3 hours. Then, centrifugation at 6000rpm for 10 minutes was performed to separate the hemoglobin. Finally, absorbance of hemoglobin was measured at 576nm by UV-visible spectrophotometry. These experiments were performed in triplicates.

$$\% \text{ Hemolysis} = \frac{\text{(OD of 576nm in FeO-NPs test sample)}}{\text{(OD at 576nm in negative control i.e. 0.1\% triton X-100)}} \times 100$$

2.9. ROS Quantification:

To detect the ROS production, solution of DPBF was prepared by dissolving 0.15mM of DPBF in ethanol. Stock solution of nanoparticles was prepared by dissolving 1mg/ml of FeO-NPs in ethanol. For ROS evaluation, mixed 20µg/ ml of nanoparticles solution in 1.5ml of 0.15mM DPBF solution, in a quartz cuvette, and exposed to LED light. The absorbance was measured after every 30 minutes by UV/VIS Spectrophotometer at 410nm for up to time period of 350 seconds. The absorbance was decreased continuously, actually it was because of photo-bleaching effect of DPBF. The natural logarithm of absorbance values was calculated. Graph of natural logarithm was plotted against irradiation time to get the decaying rate of photosensitizing process. Methylene blue was used as a standard, it was taken at the same concentration as that of nanoparticles, and absorbance was measured at 410nm.

To evaluate main ROS moieties, ROS scavengers like 0.1mM sodium azide and 0.1 mM mannitol were used.

2.10. Qualitative and quantitative analysis of FeO-NPs against *Leishmania* DNA:

DNA extraction from *Leishmania* (treated and untreated) was performed by adding 100µl lysis buffer (2 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 0.3 sucrose, 10 Mm sodium dodecyl sulphate, 10 mM Tris-HCL (pH 7.5), 1% triton X-100, 8.5 µl proteinase K solution), afterward incubated at 60°C for 2 h in water bath. After incubation, equal ratio of phenol: chloroform (1:1) was added to the above reaction, followed by centrifugation at 10,000 rpm for 10 minutes. Supernatant, the aqueous layer containing the DNA was separated, 1ml of chilled isopropanol was added. The resulted solution was again centrifuged at 10,000 rpm for 10 min. The pellet containing the DNA was washed with 70% ethanol and centrifuged for 2 min. The precipitated DNA was suspended in 50 µl of Tris-EDTA buffer (0.5 M EDTA, 1 M -HCL). For qualitative analysis, reaction was performed in two groups. In first group, *Leishmania* culture was treated with FeO-NPs under light and dark conditions, while in second group, isolated *Leishmania* DNA was treated with nanoparticles, under the dark and light exposed conditions.

For qualitative analysis DNA was extracted from the treated *Leishmania* (LED light and dark) by the above method. All the DNA samples including treated *Leishmania* DNA and extracted *Leishmania* DNA (treated with nanoparticles) were electrophoresed by using 1% agarose gel at 100V. For DNA visualization, 0.7μg/ml of ethidium bromide was used.

Quantitative analysis was done by nanodrop: the nanodrop determine the quantity and purity of the DNA based on the absorbance at A260.

2.11. Apoptosis, necrosis, and membrane permeability evaluation:

L.tropica KHW23 cells were treated with synthesized nanoparticles (chemical and green) in two groups. First group was exposed to LED light for 15 minutes at temperature 27°C, and then incubated for 24 hours in dark. Second group was directly incubated in the dark. After incubation period, both the groups were washed with phosphate buffer solution (PBS) and centrifuged at 6000 rpm for 10 minutes. The pellet was further suspended in the PBS and divided the both groups further into two groups. First group was stained with acridine orange (100μg/mL) .Second group was stained with acridine orange mixed with ethidium bromide in 3:1 (Nadhman, Nazir *et al.* 2014). Florescent microscope was used to measure the fluorescence.

To investigate the membrane permeability *L.tropica* promastigotes were treated with FeO-NPs just like in apoptotic and necrotic evaluation and then washed with HBSS. SYTOX green dye was used for membrane permeability assessment. It has high affinity for nucleic acid, it cannot cross the live cell membranes but can penetrate through compromised plasma membrane. The nanoparticles treated cells were stained with 1μM sytox dye for 10 to 15 minutes under dark condition. Then fluorescent was measured through fluorescent microscope. Triton X-100 (0.1%) was taken as a control to cause complete permeability.

2.12. Analysis of data and statistics

In current research, all the experiments were performed in triplicates and results are presented as mean ± standard deviation (SD). For the evaluation of significant differences, the Student's t-test was done by using SPSS software and P-values < 0.05 were measured as statistically significant difference.

Chapter 3

RESULTS

3. Results:

3.1. Characterization of iron oxide nanoparticles:

3.1.1 UV-Vis spectrophotometer:

UV-Vis spectroscopy is one of most commonly used technique for the structural characterization of nanomaterial. Synthesis of iron oxide nanoparticles (green and chemical routes) was physically observed by color change from yellow to black and confirmed by UV-Vis spectrophotometer. This color change was because of Surface Plasmon Resonance phenomena. The metallic nanoparticles contain free electrons, which shows SPR absorption bands upon the vibration of these free electrons in resonance with the photon waves. This technique specifies the rate of metal reduction as well as progress of nanoparticle synthesis. Fig.3.1 shows the absorbance spectra of FeO-NPs synthesized from chemical and green route. Characterized surface Plasmon resonance for green and chemically synthesized FeO-NPs shows sharp spectral band at 267 nm and 268 nm, respectively.

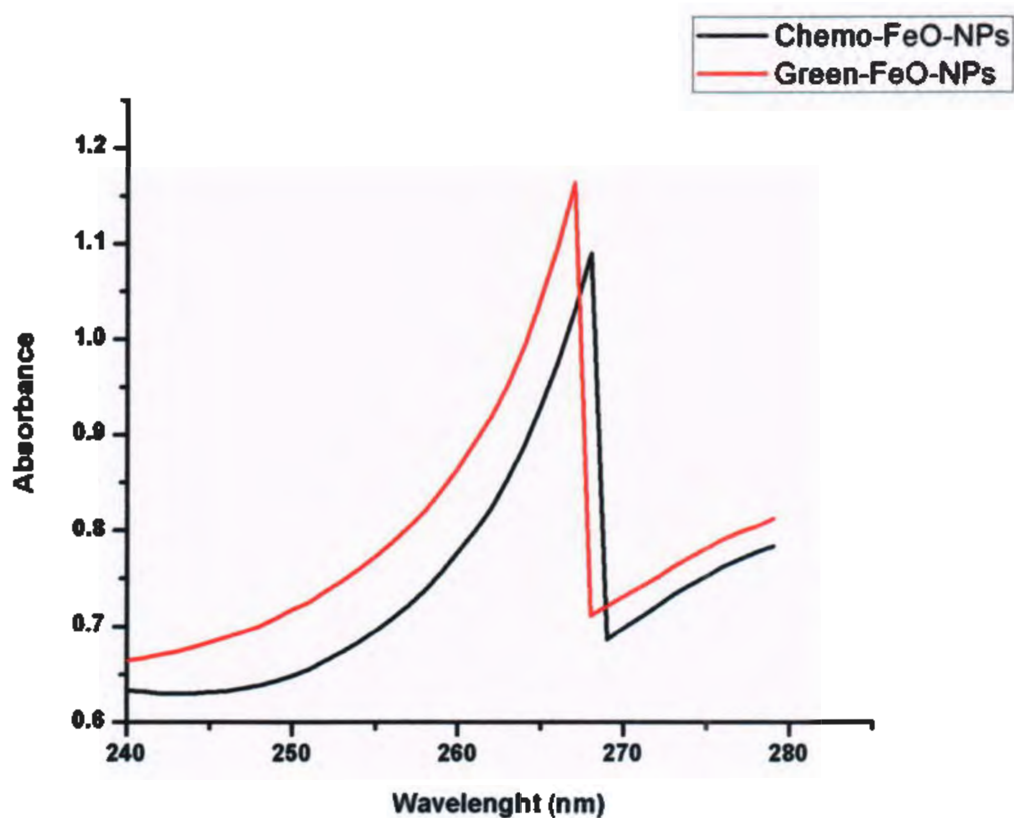


Fig.3.1. UV spectra of synthesized iron oxide nanoparticles

3.1.2. FT-IR analysis of Iron oxide nanoparticles:

FT-IR analysis of FeO-NPs nanoparticles was carried out to identify the interaction between different species and for determination of basic functional groups responsible for the reduction of iron precursor to nanoparticles and further capping and stabilizing of the reduce iron oxide nanoparticles. *Trigonella foenum-graecum* seeds extract and synthesized green iron oxide nanoparticles were analyzed by FT-IR spectra. Infrared absorption peaks were obtain by using IRTracer-100 fourier transforms infrared Spectrophotometer in the range of 300 cm^{-1} to 4000 cm^{-1}. Fig.3.2 (a) shows the absorption bands of green synthesized FeO-NPs, peaks were observed at 572 cm^{-1}, 617 cm^{-1}, 1122 cm^{-1}, 1382 cm^{-1}, 1635 cm^{-1}, 2366 cm^{-1}, 2846 cm^{-1}, 3016 cm^{-1}, 3448 cm^{-1}, 3500 cm^{-1}, 3600 cm^{-1}, 3734 cm^{-1}.

The peaks laying with in the region of 400 cm^{-1} 650 cm^{-1} are considered as corresponding to FeO-NPs. The two significant peaks at 572 cm^{-1} and 617 cm^{-1} show the Fe-O stretching of iron oxide nanoparticles, which confirm the formation of FeO-NPs. Absorption bands 3448 cm^{-1} and 3500 cm^{-1} correspond to O-H group of phenolic compounds and amine N-H stretches, respectively. The bands 3600 cm^{-1} and 3734 cm^{-1} is assigned to the alcohol O-H stretches and amide N-H stretches, respectively. The band 2846 cm^{-1} show the presence of carboxylic acids groups and 3016 cm^{-1} correspond to C-N stretches of aliphatic amines. The bands at 2366 cm^{-1} indicate the N-H stretching vibration and 1635 cm^{-1} associated with N-H bending of amide groups. (Figure 3.1.2).

Fig. 3.2 (b) shows the spectra of chemically synthesized FeO-NPs. The prominent absorption bands were observed at 551cm^{-1} 619.14 cm^{-1}, 775.86 cm^{-1}, 1107.41 cm^{-1} , 1382.96 cm^{-1}, 1641.42 cm^{-1}, and 2069.28 cm^{-1} .The bands at 619.14 cm^{-1}, 775.86 cm^{-1}, 1107.14 cm^{-1} correspond to O-H stretching vibration, carboxyl group, alkenyl C=C stretch respectively. The characteristic IR vibrations of FeO-NPs at 551 cm−1 relates to the bending and stretching vibrations of Fe−O bond, which confirms the synthesis of FeO nanoparticles.

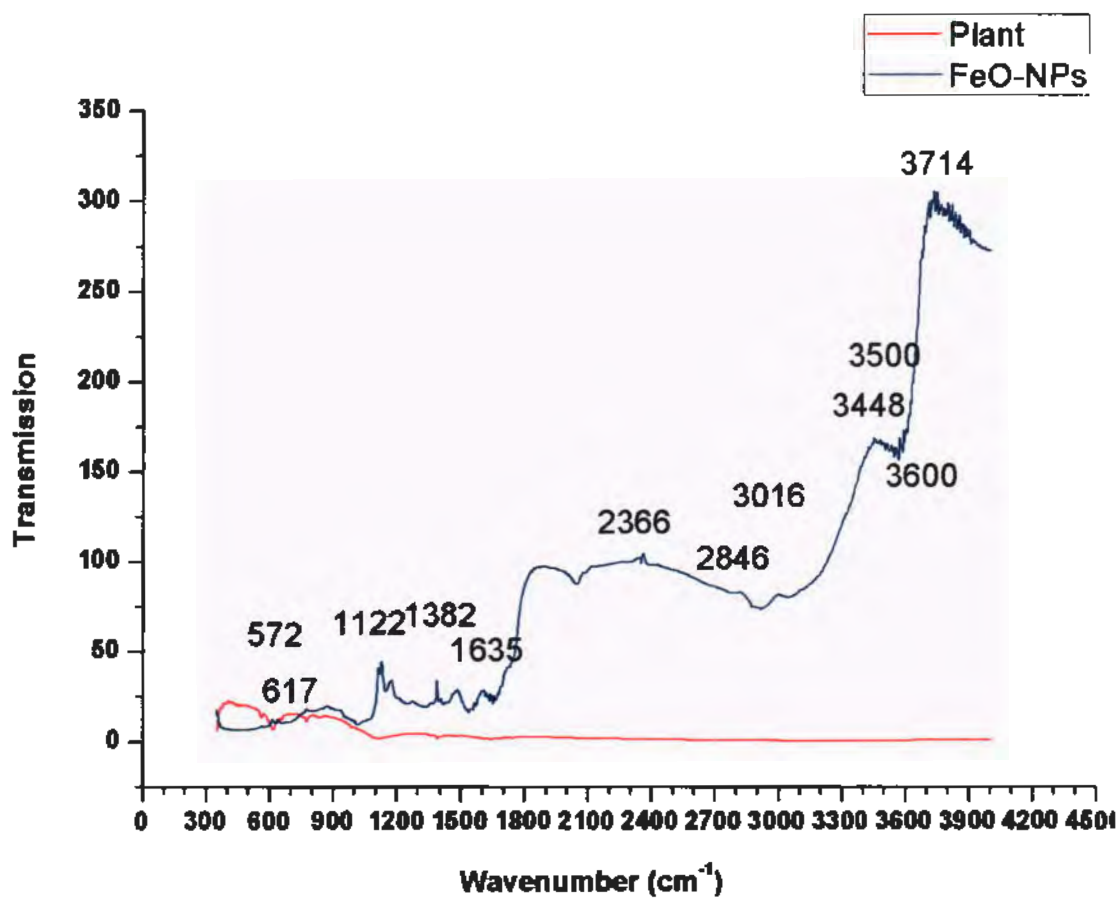


Fig. 3.2 (a). FTIR Analysis of Green synthesized iron oxide nanoparticles

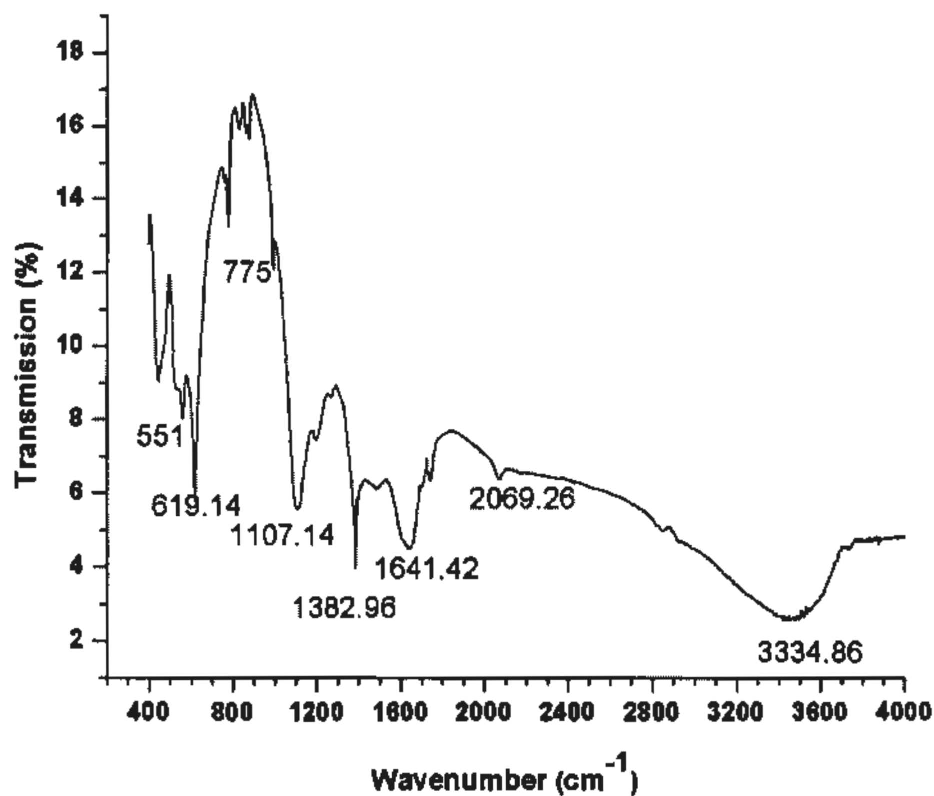


Fig. 3.2 (b). FTIR Analysis of Chemically synthesized iron oxide nanoparticles

3.1.3. XRD analysis of FeO-NPs:

The structural feature and purity of FeO-NPs were determined from X- ray diffraction (XRD) data. Fig.3.3 (a), (b) shows XRD pattern of FeO nanoparticles synthesized from green and chemical method, respectively. The data was collected in the 2 θ range. Green synthesized FeO-NPs showed peaks at angle of 30.08°, 33.34°, 33.74°, 34.04°, 35.30°, 62.71° and 76.55°, which respectively corresponds to 220, 112, 104, 106, 113, 200, 220, 440, and 311 planes. The narrow peaks for chemically synthesized FeO-NPs were found in a diffractogram at an angle of 36.22°, 43.20°, 56.64°, 64.32° associated to 311, 222, 440, 331 planes. The XRD results shows that green synthesized FeO-NPS has increased crystalinity than chemical route.

According to full width at half maximum (FWHM) the average crystallite size of the FeO nanoparticles were determined from the XRD peaks using Debye-Scherrer equation.

$$D = K\lambda/(\beta \cos \theta)$$

D is the crystallite size, k is a constant taken as 0.9, λ is the wavelength of X-Ray, β is the full width half maximum, and θ is the Bragg diffraction angle. Calculated crystallite size of FeO-NPs synthesized by green and chemical route were found to be 32.14 nm and 37.61 nm respectively. The decrease particle size was observed for green synthesized FeO-NPs. It is expected that the biosolvent facilitate the formation of FeO nuclei and allow growing to some extent leads to reduction in the crystallite size.

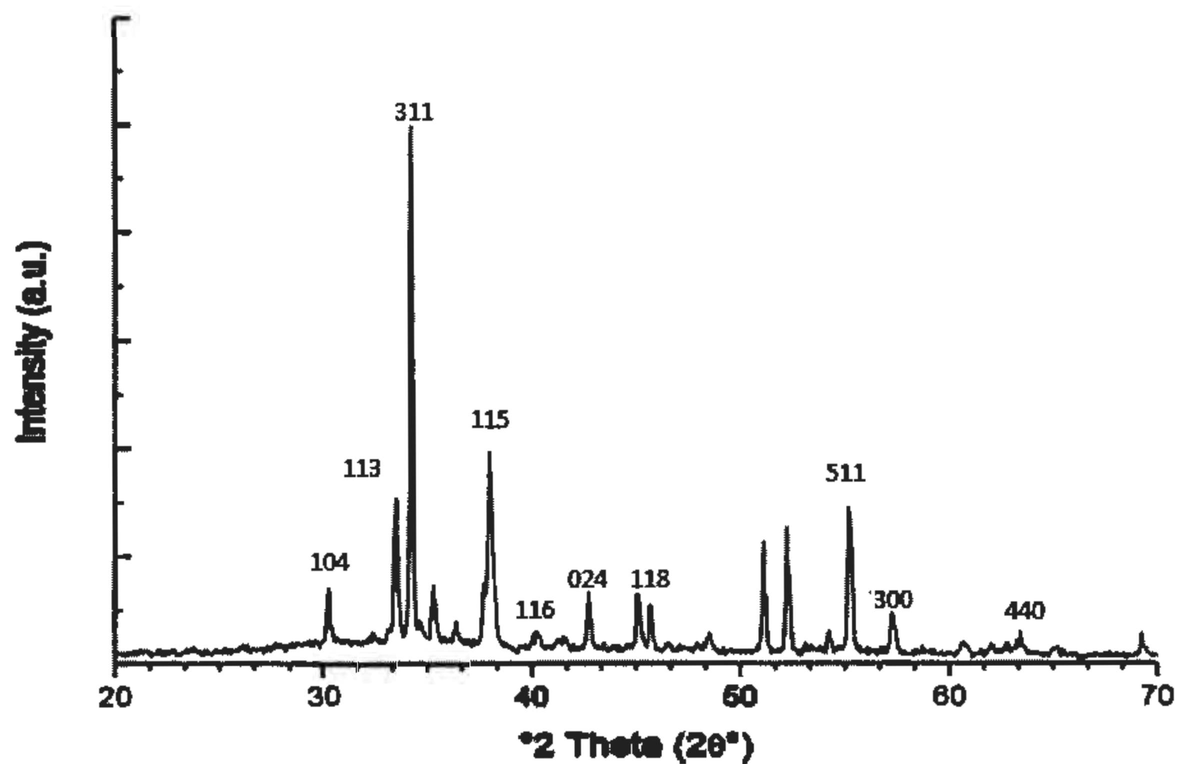


Fig.3.3 (a). XRD spectra of Green synthesized FeO nanoparticles

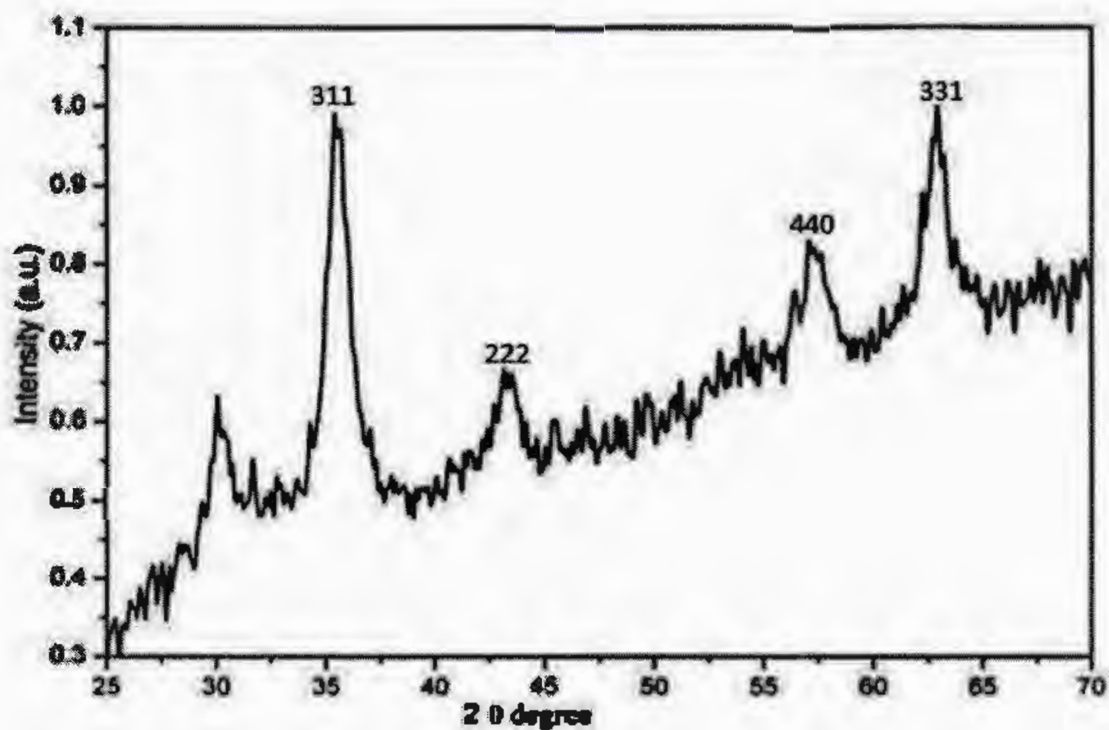


Fig.3.3 (b). XRD spectra of chemically synthesized FeO nanoparticles

3.2. Dose- time dependent *in-vitro* anti-promastigote activity:

L.tropica KWH23 strain was used to analyze the antileishmanial efficacy of synthesized nanoparticles (chemical and green) at different concentrations in the dark and light conditions. *Leishmania* promastigotes cells were treated with serial dilutions of nanoparticles (green and chemical) under light and dark conditions. In order to make determination, the viability of promastigotes was checked in both control and test groups at various time intervals (24h, 48h, and 72h). In figure 3.4. Significant decrease in viability was seen in the treated groups as compare to control. This effect was examined during all analyzed incubation time periods. Beside this determination, there was a considerable decrease in promastigotes survival was observed in LED exposed, NPs (chemical and green) treated groups as compare to without LED exposed NP' s treated groups. Fig.3.4 and fig.3.5.clearly shows, the effects of FeO-NPs on the viability of promastigotes, under the LED light were more significant than they were in the dark conditions.

Beside these determinations, there was significant increase in mortality rate was observed in green synthesized FeO-NPs treated groups. This assessment was further confirmed by estimating the ROS production. Reactive oxygen species can cause oxidative stress by causing biochemical and morphological changes. The maximum mortalities were observed in LED exposed, green synthesized nanoparticle treated group, after 72h of incubation at highest dose concentration with IC_{50} value of 0.001572±0.022 µg/ml. Further IC_{50} values after 24 and 48h was 0.020274±0.015 µg/ml and 0.018577±0.016 µg/ml, respectively. While under dark condition, nanoparticles (green) treated groups show IC_{50} values 0.022512±.014 µg/ml, 0.02070±.014 µg/ml, and 0.001512±0.017 µg/ml at incubation period of 24h, 48h, and 72h respectively.IC_{50} values for chemo-nanoparticles treated groups, under dark conditions are, 0.025385±.014, 0.022522±.014, 0.001938 ±0.016 at incubation period of 24h, 48h, 72h respectively. While for LED exposed groups, IC_{50} values are 0.022705 ±.015, 0.018324 ±.015, 0.001938 ±0.017, respectively. (Fig.3.6.)

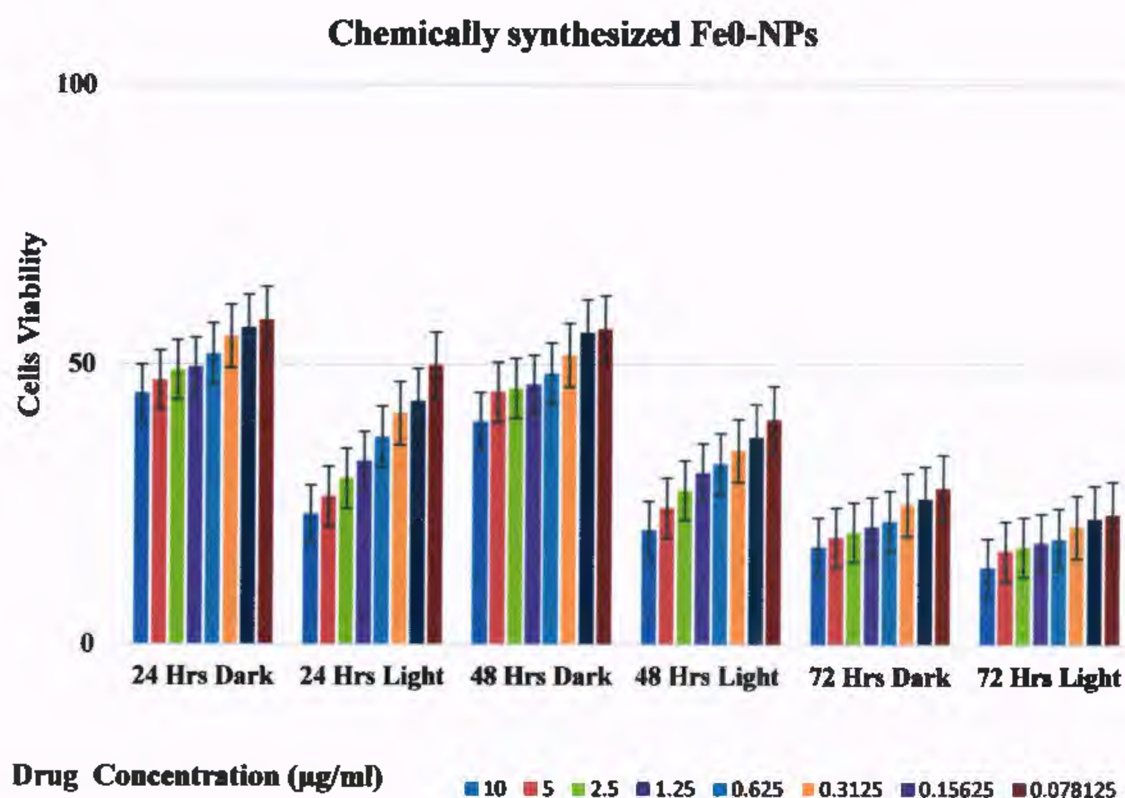


Figure 3.4. Anti-leishmanial activity against promastigotes of *Leishmania tropica* of chemically synthesized FeO nanoparticles

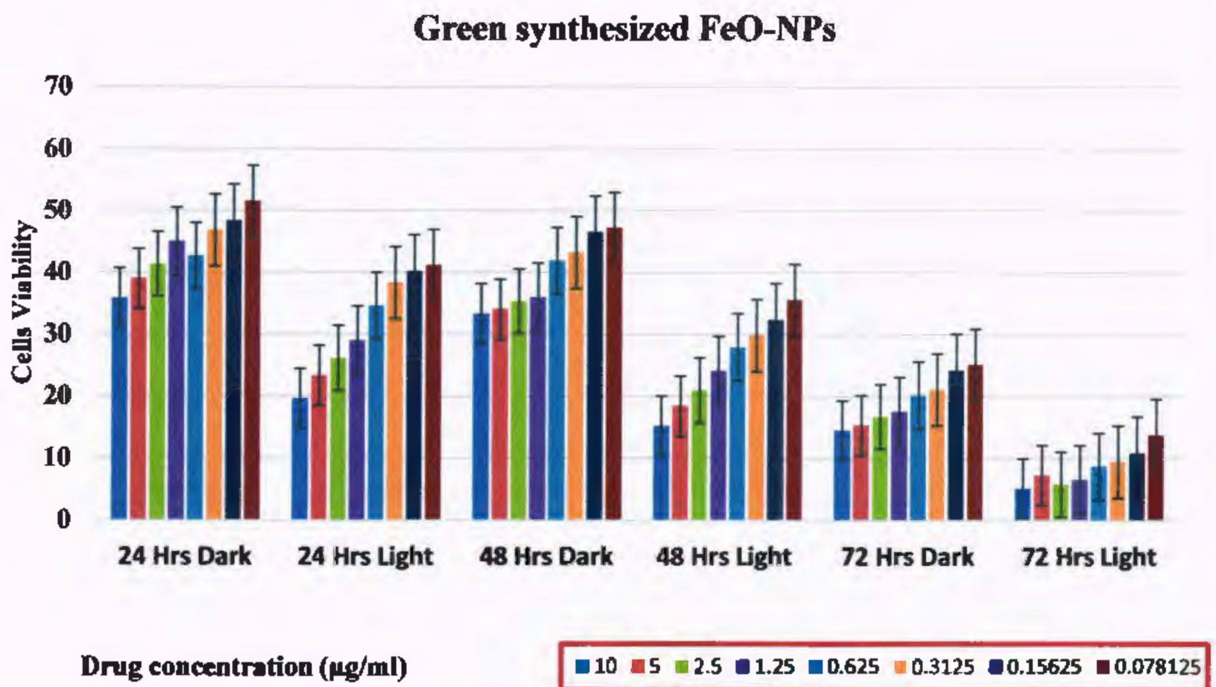


Fig.3.5. Anti-leishmanial activity against promastigotes of *Leishmania tropica* of Green synthesized FeO-NPs

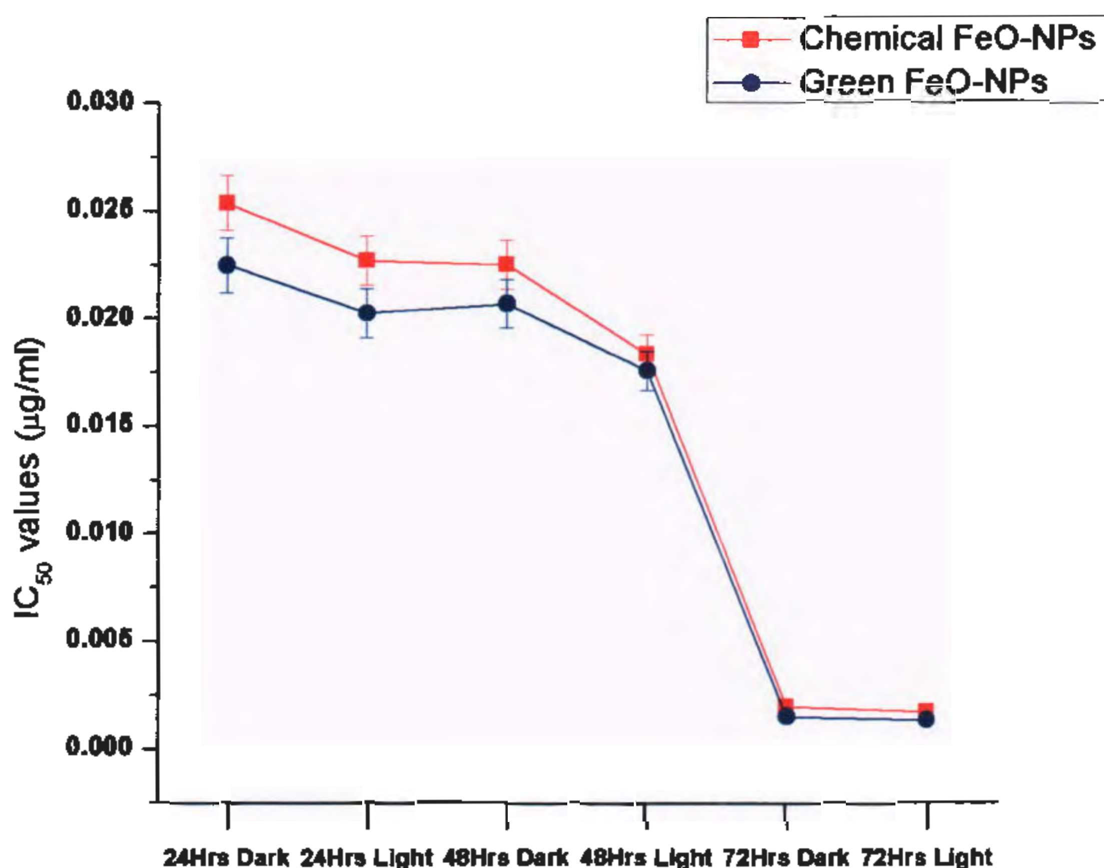


Fig.3.6. Statistical comparison of IC_{50} of Green and Chemically synthesized Fe-NPs against *Leishmania tropica* promastigotes

Thus these findings clearly conclude that FeO-NPs at lower dose can significantly kill the *Leishmania* cells and it may serve as favorable candidate for the treatment of fatal leishmaniasis.

3.3. Anti-leishmanial activity against amastigotes of *Leishmania tropica:*

Leishmanial amastigotes were incubated with FeO-NPs (chemical and green) at differnet dilutions for 72hrs. Similar to promastigotes, the reaction was performed in two groups ,one was exposed to LED light for 10 minutes while other was proceeded under dark conditions. Reduced survival of amastigotes was observed in both LED exposed and non LED exposed treated groups. Significant killing of cells was observed in light exposed, NPs treated groups. The green synthesized nanoparticles cause increased generation of ROS, as a result higher killing ability of amastigotes was observed,as compare to chemo-synthesized nanoparticles.

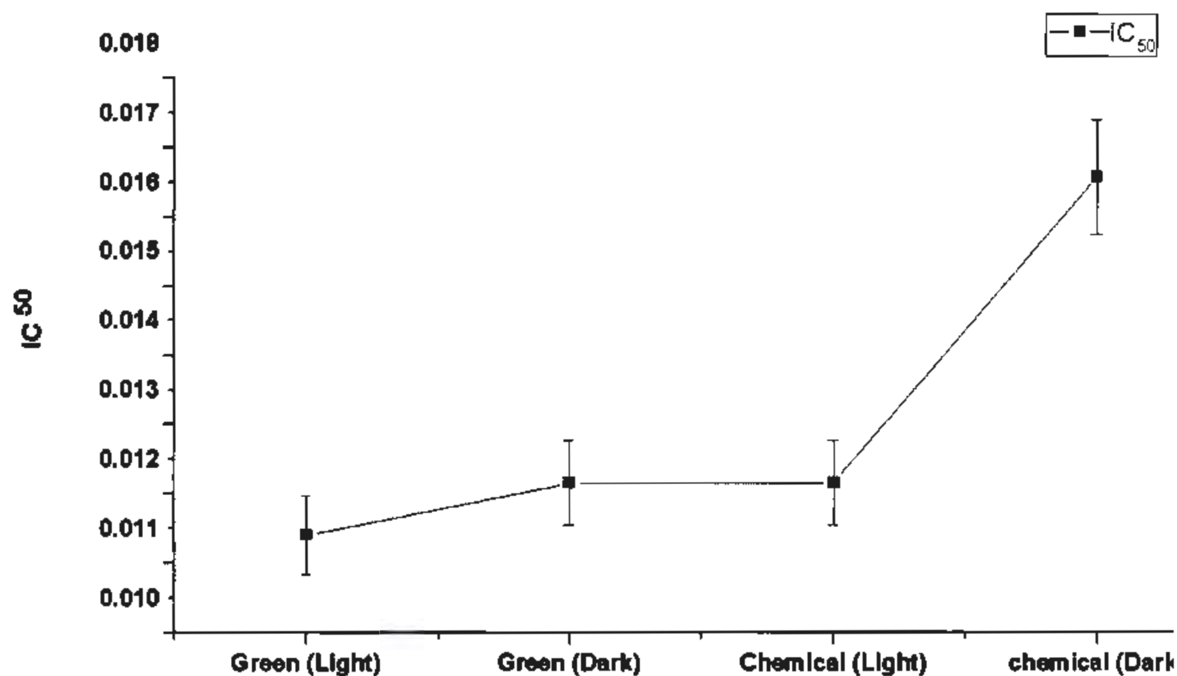


Fig.3.7. Statistical comparison of IC50 of Green and Chemically synthesized Fe-NPs against *Leishmania tropica* amastigotes

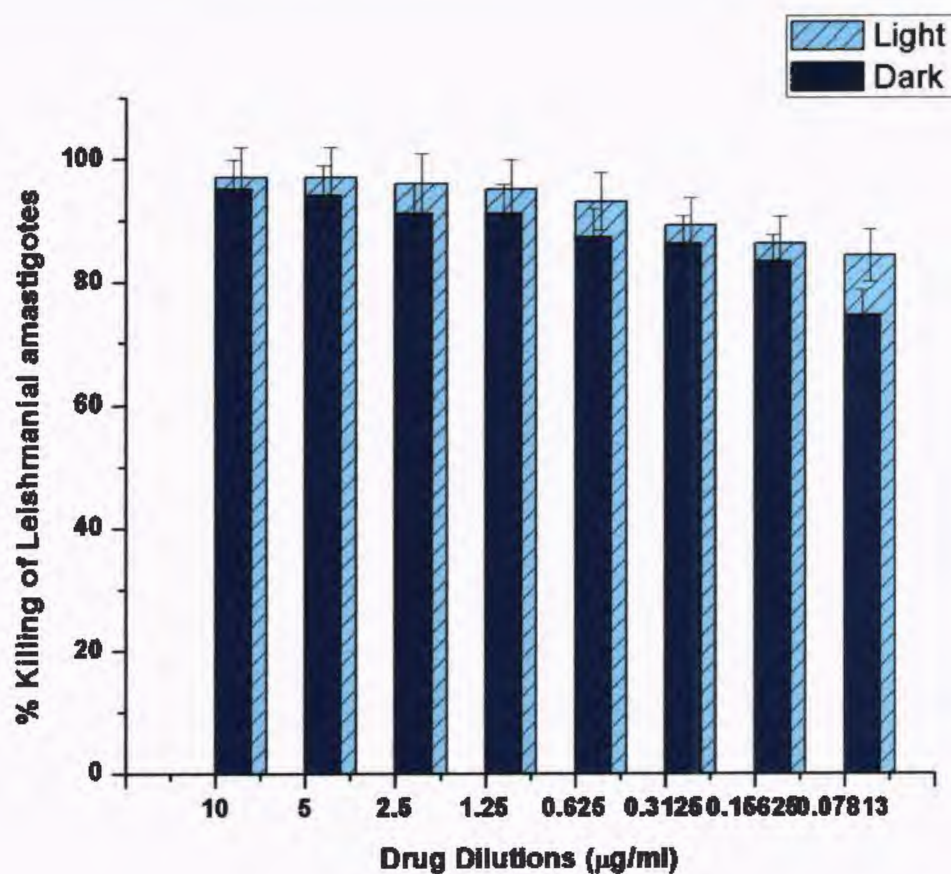


Fig.3.8. Activity of iron oxide nanoparticles (Green) against amastigotes of *Leishmania tropica*

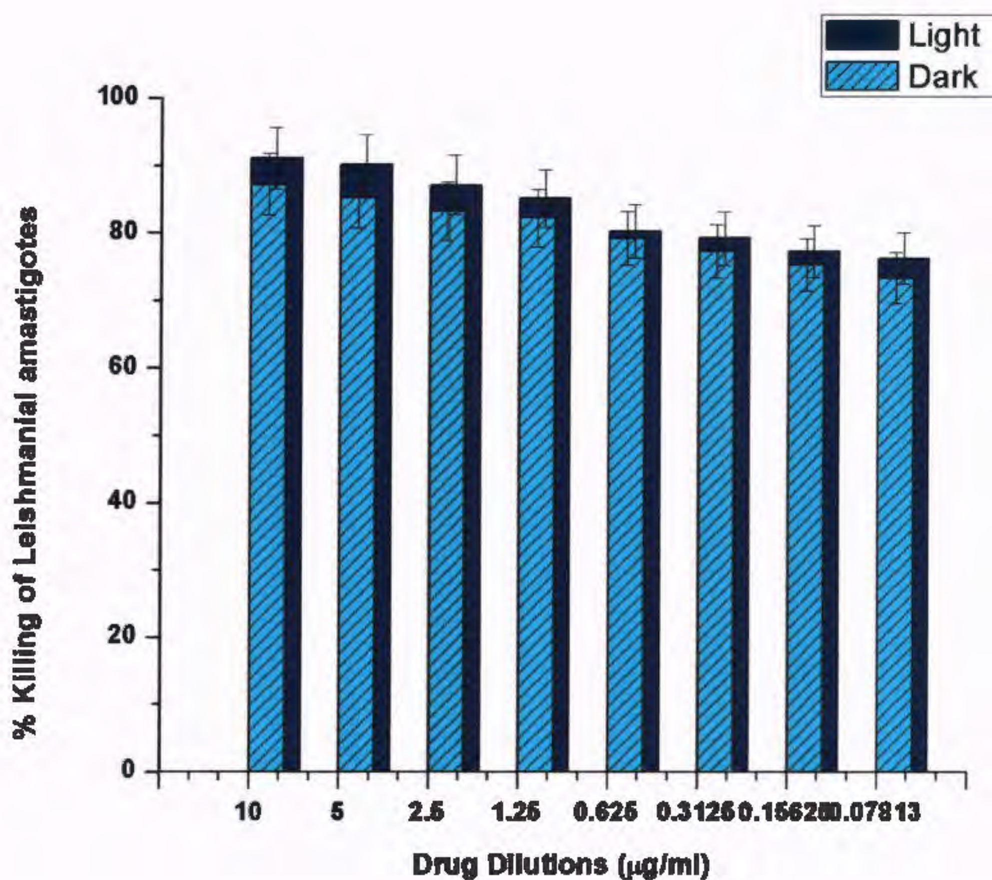


Fig. 3.9. Activity of iron oxide nanoparticles (chemical) against amastigotes of *Leishmania tropica*

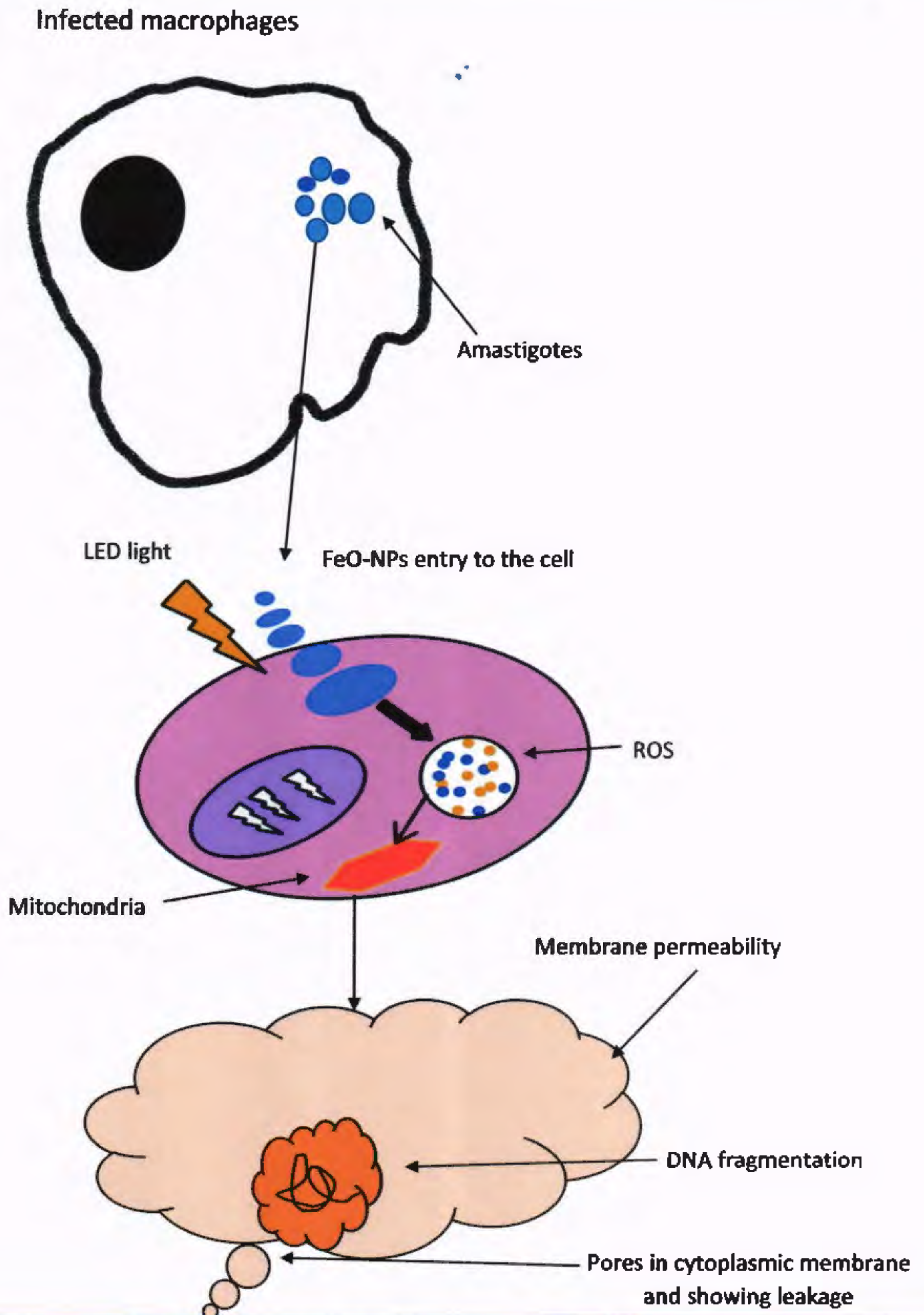


Fig.3.10 Cell showing infection of macrophages with amastigotes

3.4. Biocompatibility of iron nanoparticles

Iron oxide nanoparticles are promising candidate possessing broad spectrum antimicrobial properties. However, it is necessary to evaluate the biocompatibility of these nanostructures on normal cells to ensure their safer use. The FeO-NPs (chemical and green) were evaluated for their biocompatibility against human macrophages in dose dependent manners under dark and light (LED) exposure. The result clearly indicated that green synthesized nanoparticles in light exposure show biocompatibility at low concentrations, by exhibiting LD_{50} value 2659μg/ml. The experiment under dark conditions indicated that current photo-catalytic nanoparticles can be toxic at higher concentration. (Fig.3.13. Lethal dose concentrations (LD)

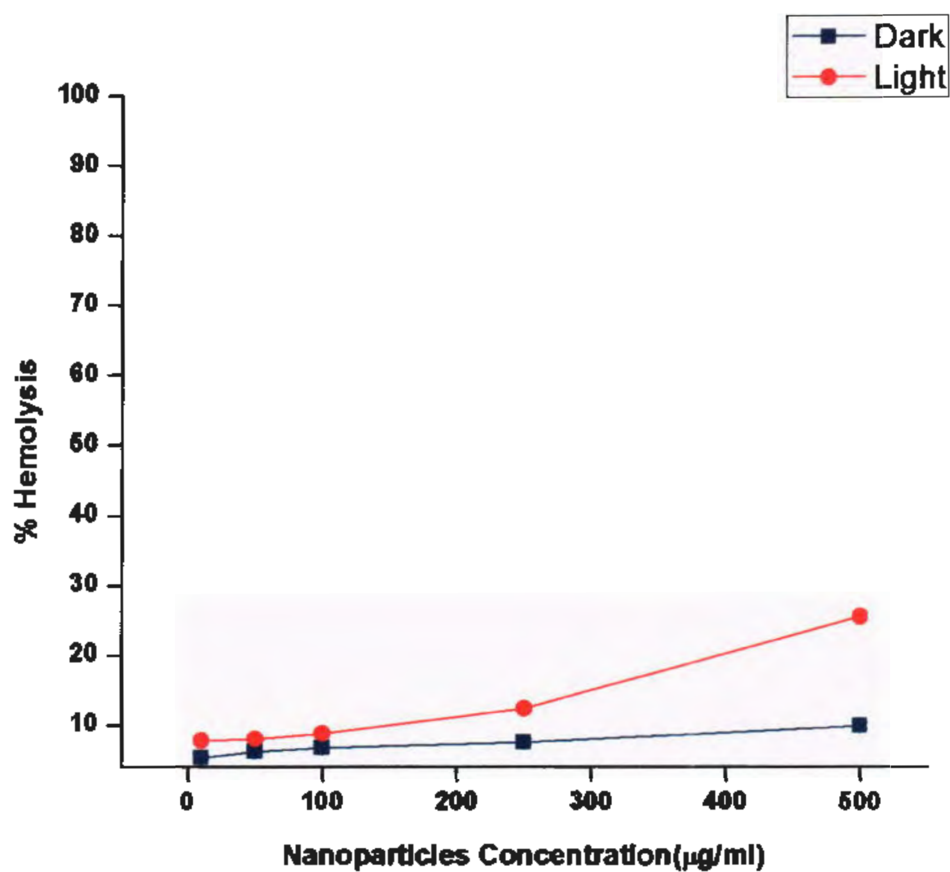


Fig.3.11. Biocompatibility of Green synthesized FeO-NPs against human blood

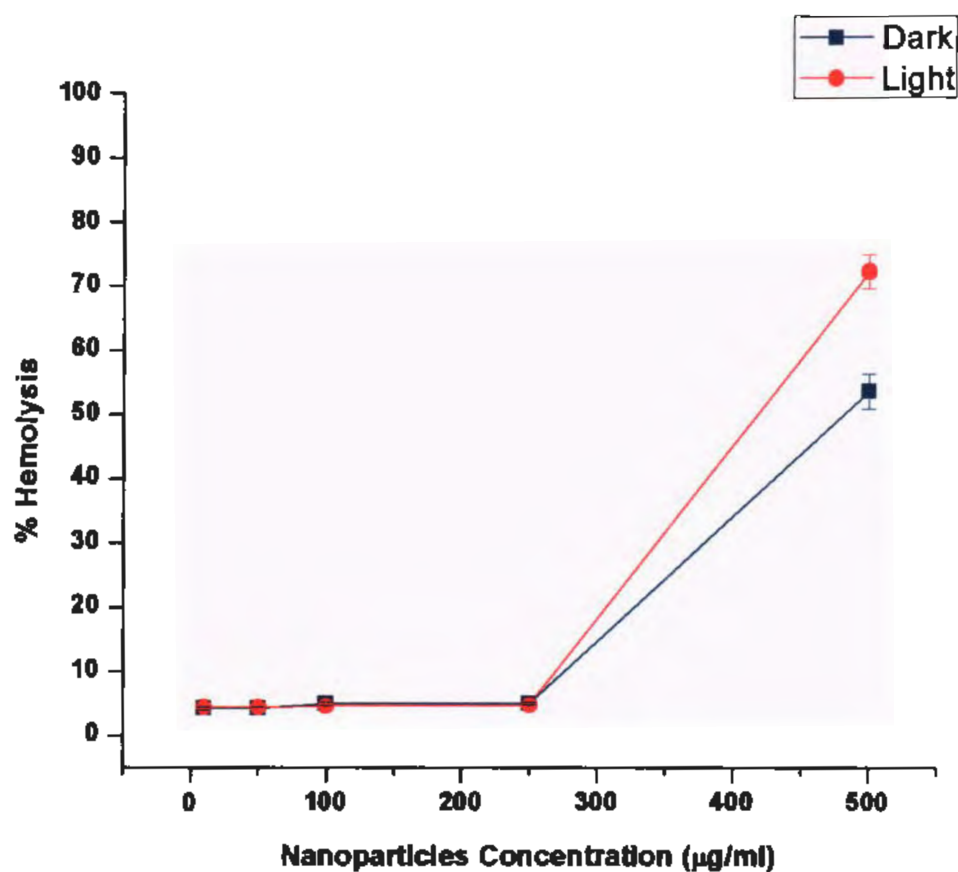


Fig.3.12. Biocompatibility of chemically synthesized FeO-NPs against human blood

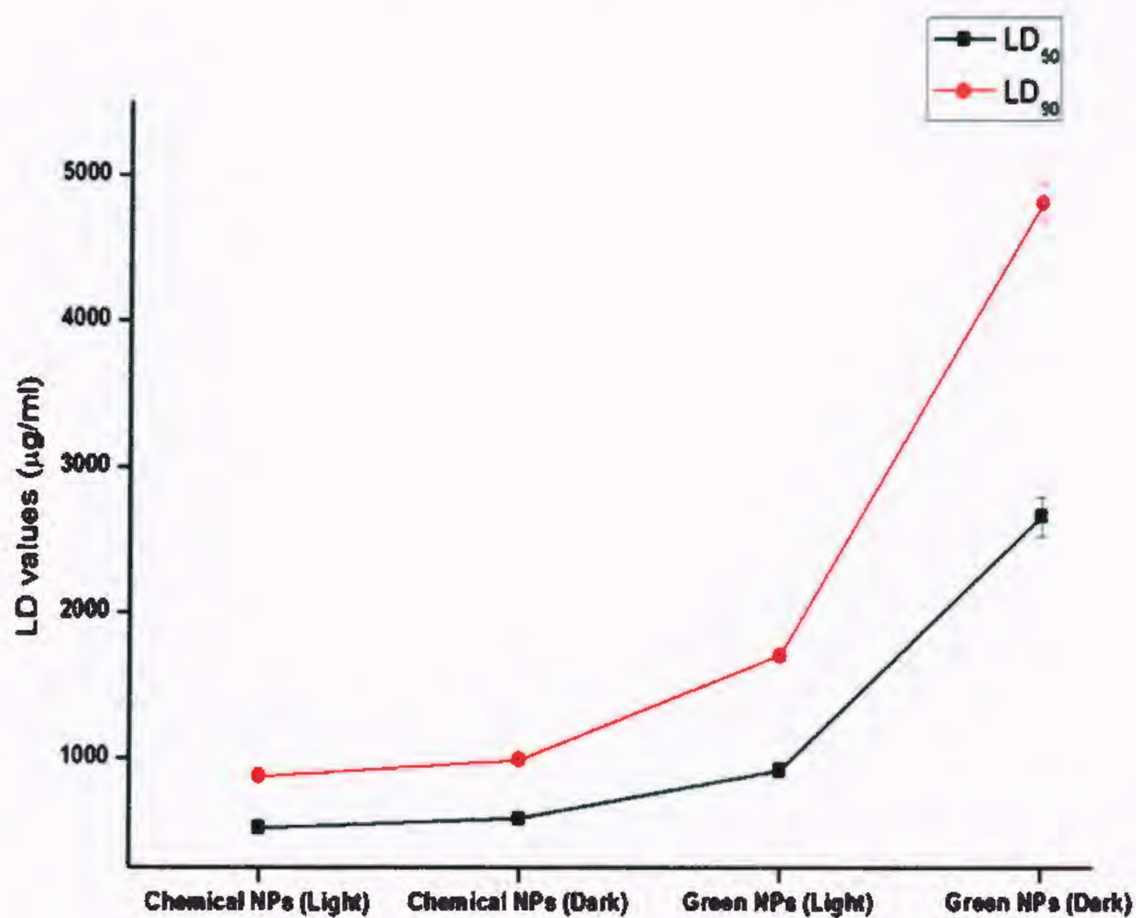


Fig.3.13. Lethal dose concentrations (LD) of Fe-NPs (green and chemically synthesized) against macrophages

3.5. ROS quantification :

Photodynamic effect of iron oxide nanoparticle was evaluated by the quantification of ROS produced. DPBF is fluorescent probe. It reacts rapidly with singlet oxygen and form colorless intermediate endoperoxide which can be detected as decrease in DPBF absorption at 410nm (Vankayala, Lin *et al.* 2014) . In current study, the generation of singlet oxygen and hydroxyl group by photo-excited iron oxide nanoparticles (chemical and green) in ethanol solution was detected by using 1, 3-diphenylisobenzofuran (DPBF). Quantum yield for ROS production by iron oxide nanoparticles (chemical and green) was quantified by using comparative method. Quantum yield was calculated by comparison with standard photosensitizer i.e. methylene blue (Xiao, Gu *et al.* 2011) .

$$\Phi_{FeO} = \Phi_{MB} (K_{FeO} / K_{standard})$$

Φ_{MB} is the quantum yield of standard methylene blue while Φ_{FeO} is the quantum yield of photo-excited iron oxide nanoparticles. K_{FeO} and $K_{standard}$ are the rate constant for the degradation of DPBF by photo-excited iron oxide nanoparticles and by standard photosensitizer, respectively. By evaluating photoactive green and chemically synthesized nanoparticles with their ability to generate ROS, the quantum yield was estimated 0.26 and 0.15, respectively as compare to standard methylene blue (0.52). Thus green synthesized nanoparticles enhance the ROS production ($P<0.05$).

By using ROS scavengers like sodium azide and mannitol, it was evaluated that singlet oxygen and hydroxyl ions were main ROS moieties responsible for the significant annihilation of *Leishmania* parasites.

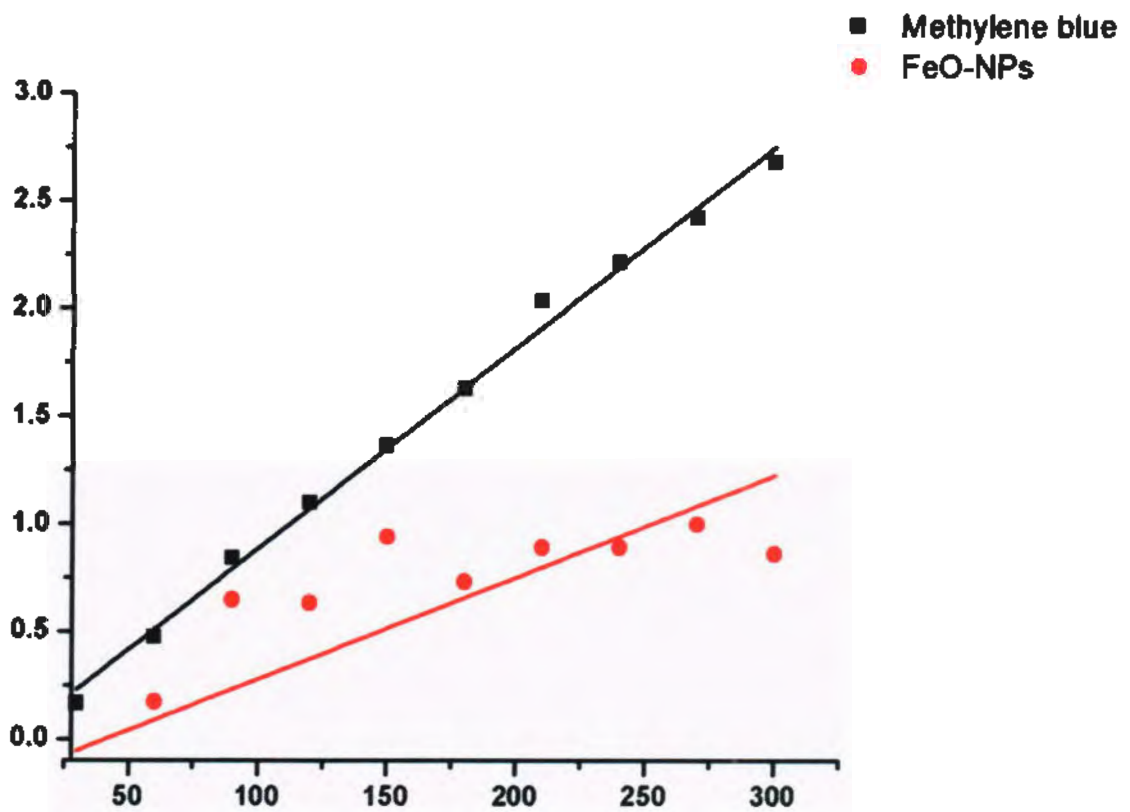


Fig. 3.14. ROS quantification of Green synthesized FeO-NPs

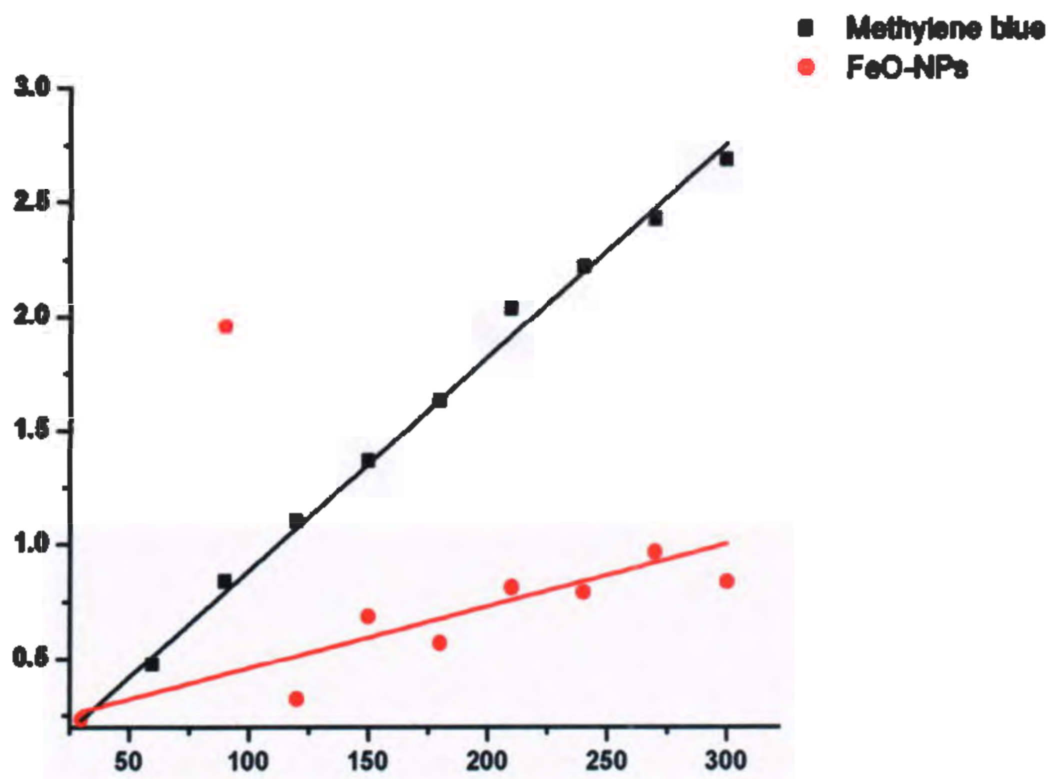


Fig.3.15. ROS quantification of chemically synthesized FeO-NPs

3.6. Qualitative and quantitative analysis of *Leishmania tropica* DNA:

From ROS quantification it was found that both chemically and green synthesized nanoparticles have the ability to produce good quantum yield of ROS i.e. 0.15 and 0.26, respectively. Thus photoactive FeO-NPs have the ability to damage the *Leishmania* DNA in the presence of light. Figure 3.16 (a) show there is no DNA degradation under the dark conditions. Figure 3.16 (b) clearly indicate that upon light exposure FeO-NPs (green and chemical*)* have ability to degrade DNA by producing reactive oxygen species (ROS).

For quantitative analysis, the extracted *Leishmania* DNA, without any exposure to nanoparticles was quantified as 618.3 ng/µl through nanodrop spectrophotometer .Then this extracted DNA was treated with FeO-NPs (green and chemical) followed by incubation for 24 hrs. To determine degraded effects of nanoparticles after incubation period, the samples were again quantified. It was estimated that nanoparticles have significant DNA degrading ability and DNA quantified as 154.9 ng/µl and 304.42 ng/µl respectively for green and chemically synthesized FeO-NPs.

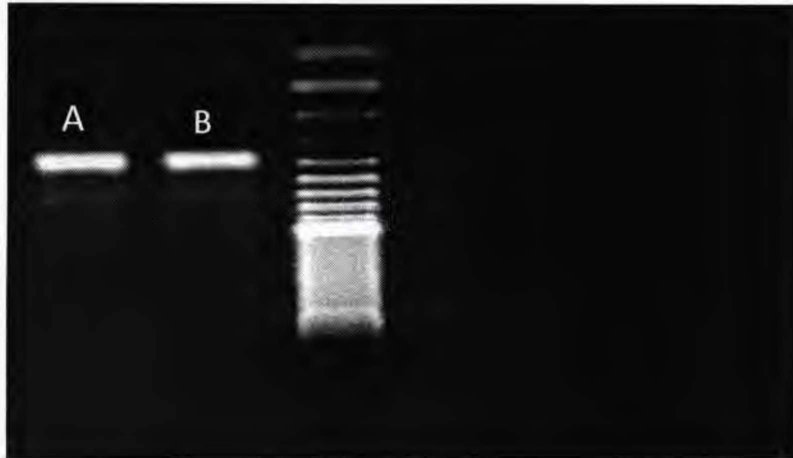


Fig. 3.16 (a): *Gel electrophoresis of Leishmania DNA. (A) Control (untreated) Leishmania DNA; (B) treated Leishmania under dark conditions and its DNA.*

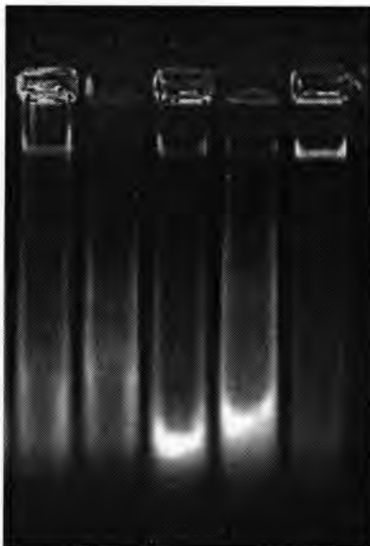
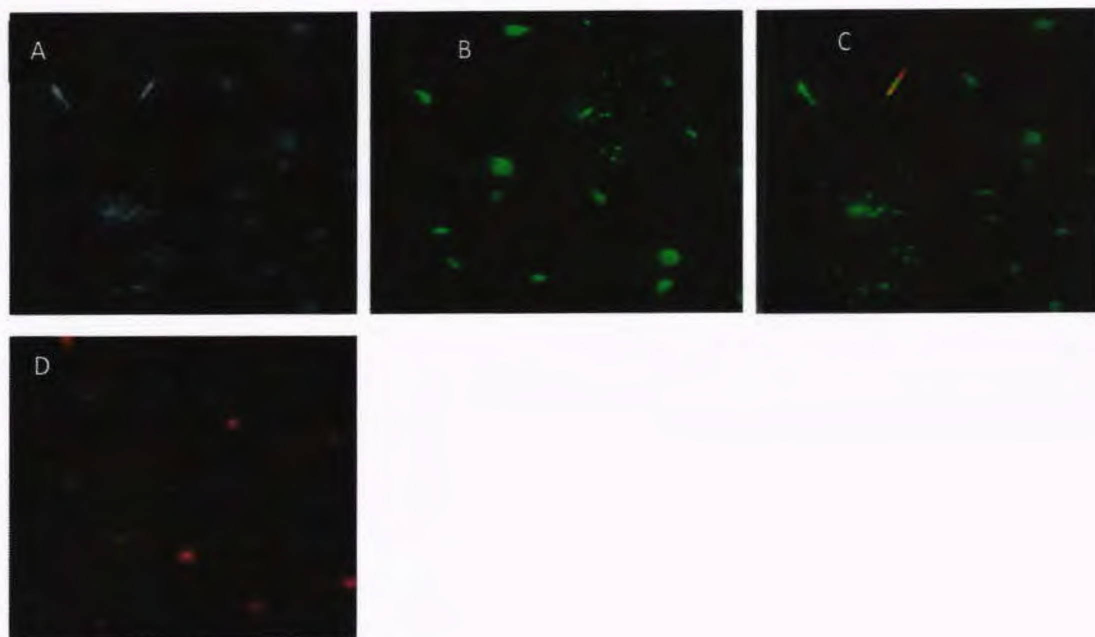


Fig. 3.16 (b): *Notes: (C) (D) DNA extracted from treated Leishmania cells with FeO-NPs (green and chemical), on light exposure; (E) (F) DNA extracted from Leishmania cells and then treatment with FeO-NPs (green and chemical) and exposed to light.*

3.7. Apoptosis, necrosis, and membrane permeability evaluation:

Cellular death by apoptosis is complex and multistep mechanism, which occurs in every cell (Wong, Recht *et al.* 2006). To study the various biological pathways, microscopic visualization of cells stained with suitable dye, is most appropriate technique. Ethidium bromide and acridine orange staining was performed to evaluate morphological evidences of apoptosis on the FeO-NPs treated cells. Upon entry to the cell, both dyes get attach to the nucleic acid in the nucleus of the cell, and emit the green and orange color. In the first hour of FeO-NPs exposure, light green nuclei showed the early apoptosis started in *Leishmania* cells as the result of condensation of chromatin material. The main features of apoptosis are the production of apoptotic bodies by the condensation and fragmentation of cellular bodies in the nucleus and cytoplasm. After few hours of NPs exposure necrotic bodies formed and ethidium bromide entered into the *Leishmania* cells, radiating an orange color. Upon fluorescent microscopic visualization, it was observed that both necrotic and apoptotic bodies formed, some showed early and late apoptosis. In the first few hours of incubation, about 80%-90% of the *Leishmania* cells were apoptotic, emitting green color, while after the incubation period, more than 90% cells were necrotic, emitting orange color.

By using SYTOX green dye, the membrane permeability was assessed, which entered the cell and got attached to the nucleic acid by emitting green fluorescent. Thus it confirmed the permeability of membrane cause by reactive oxygen species.



Apoptotic and necrotic bodies of Leishmania cells after treatment with FeO-NPs for 24 hours. Notes: (A) Bright field image; (B) After few hours of cells exposure to FeO-NPs, apoptotic bodies formed within the cells; (C) Necrotic and apoptotic bodies (early and late apoptosis); (D) after 24 hours of treatment of cells with FeO-NPs, about 90-95% cells are necrotic while remaining are apoptotic (early and late apoptosis).

Fig 3.17. Apoptotic and necrotic bodies

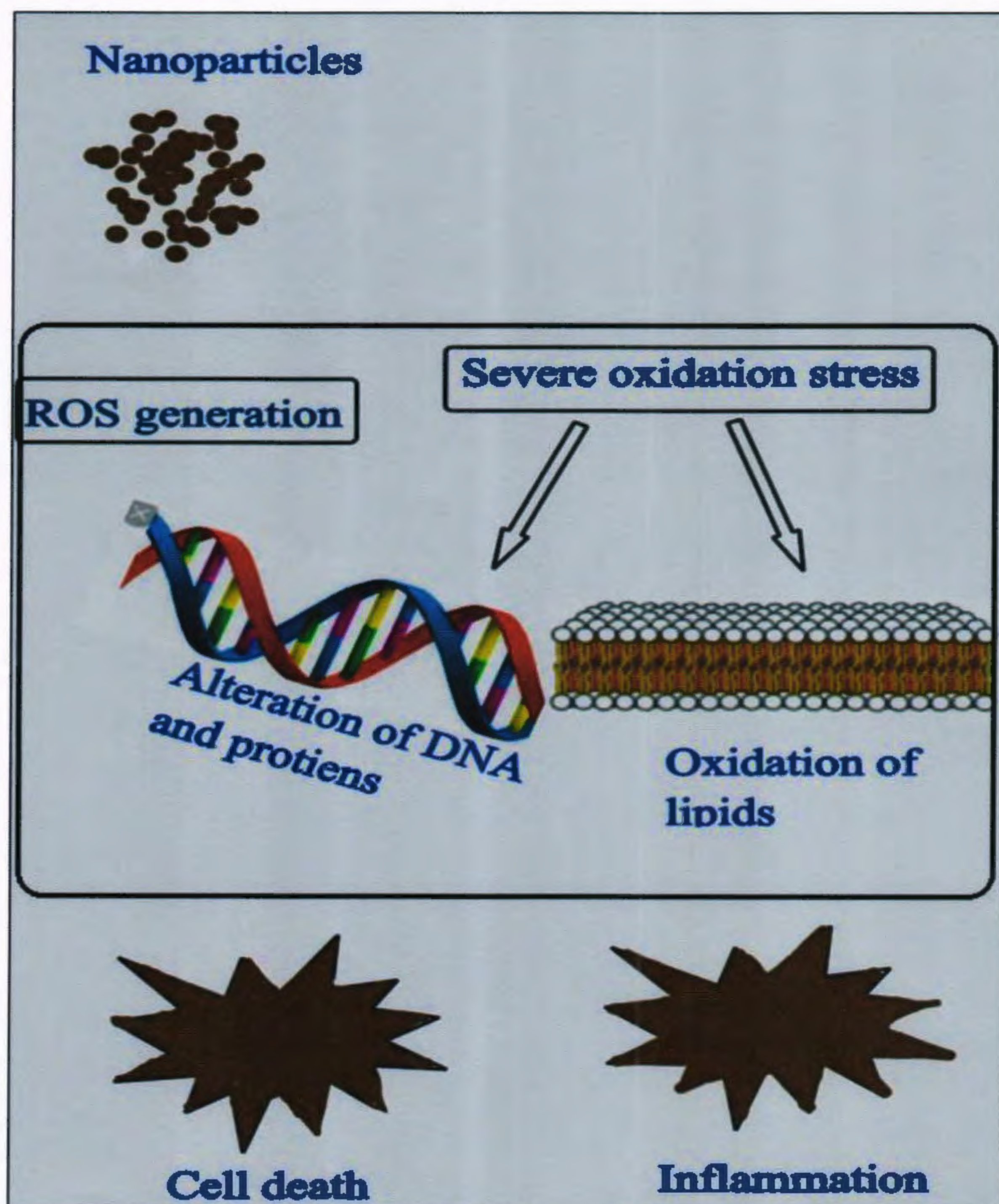


Fig.3.18. Mechanism of cell death by metallic nanoparticles

4. Discussion:

All the practice modalities for leishmaniasis lack appropriate safety, efficacy, and affordability. One of the most important issue associated with currently available leishmanicidals is the resistance by pathogens. There is need to develop more efficient candidates for fatal leishmaniasis. The current study is the first to demonstrate the comparative analysis of antileishmanial effects of UV-exposed and non-UV-exposed of both chemically and green synthesized FeO-NPs on promastigotes and amastigotes forms of *L.tropic*a.

Green synthesis is best alternative remedy to synthesize eco-friendly metallic nanoparticles that avoids the use of toxic chemicals. Plant extracts contain certain important functional groups including citric acid, cyclic peptides, tannins, gallic acids and retinoic acid that not only causes bio-reduction but also act as capping and stabilizing agents (Tippayawat, Phromviyo *et al.* 2016). Plant mediated synthesis seems most reliable, facile, cost effective and eco-friendly route for metallic nanoparticles synthesis.

It was evaluated that both visible light-exposed and non-light exposed FeO-NPs possess antileishmanial properties by annihilating biological activities like metabolic activities and viability of parasites.

The small size of iron oxide nanoparticles facilitate their ability to cross cellular barriers followed by oxidative stress. Lee et al demonstrated that the inactivation of *Escherichia coli* by zero-valent iron nanoparticles (Lee, Kim *et al.* 2008) could be because of the penetration of the small particles (sizes ranging from 10–80 nm) into *E. coli* membranes. Iron oxide nanoparticles then reacts with intracellular oxygen, and cause oxidative stress and eventually causing disruption of the cell membrane. Chatterjee *et al.* has reported that iron oxide nanoparticles possess antimicrobial properties against *E. coli*, and the activity increases by increasing the concentration of FeO-NPs (Chatterjee, Bandyopadhyay *et al.* 2011)

Despite there being various studies regarding antibacterial, antifungal, antiviral efficacy of FeO-NPs have shown encouraging results. However, to date, in literature, there is no reported study regarding leishmanicidals effects of FeO-NPs. This study is the first to show

the synergistic effects of FeO-NPs and LED light together demonstrating antileishmanial effects. In previous research, it has been demonstrated that various metal oxide nanoparticles like silver nanoparticle, ZnO NPs, and gold nanoparticles significantly reduced the survival rate of *Leishmania* cells (Davies 2003, Nadhman, Sirajuddin *et al.* 2016). Production of reactive oxygen species (ROS) following light illumination known as most appropriate mechanism responsible for the antileishmanial and antimicrobial activities (Nadhman, Khan *et al.* 2016).

Nanoparticles have ability to cross biological barriers own to their small size. They are highly reactive and their transparency increases with exposure to LED light. This characteristic increases the delivery efficacy of nanoparticles and also cellular uptake(Nadhman, Nazir *et al.* 2014). It has been reported that photocatalytic processes significantly increase the cell- killing ability of such nanoparticles (Nadhman, Nazir *et al.* 2014). The basic mechanism behind this mortality rate is production of reactive oxygen species (ROS) (Kohanski, Dwyer *et al.* 2007). Following PDT, the metallic nanoparticles absorbs light energy and transfer it to molecular oxygen and water molecules in biological enviroment, initiatig the production of ROS. In previous study it is demonstrated that the extensive antileishmanial properties of various nanoprticles along with UV light have been seen. Butkus et al eh explained for the first tme the synergistic efficcacy of silver nanoaprticles and UV light together on the RNA viruses.He explained that just UV irrradiation have no effects on RNA ,but UV irradiated siver nanoparticles showed significant effects on RNA viruses (Butkus, Labare *et al.* 2004). By following this study,Kim et al (Kim, Lee *et al.* 2008) demonstrated the synergistic effects of Ag-NPs and UV light on *E.coli*. He demonstrated that antibacterial effects of silver nanopartices significantly incresed upon UV irradiation. A similar research was carried out by Keenan et al in which Fe^{2+} reacted with oxygen to create hydrogen peroxide (Keenan and Sedlak 2008). This H_2O_2 consequently reacted with ferrous irons via the Fenton reaction and produced hydroxyl radicals which are known to damage biological macromolecules.

The previous knowledge, regarding the sensitivity of *Leishmania* parasite against the ROS, encouraged to explore leishmanicidals properties of FeO-NPs for *L.tropica*, and comparative analysis of their efficacy under dark and light irradiation conditions. As it is

estimated, LED irradiated, FeO-NPs exhibits significant antileishmanial properties against all promastigotes and amastigotes. It is estimated that this enhanced inhibitory effect under LED exposure, is directly related to generation of ROS by irradiating the nanoparticles.

Certain nanoparticles have ability of accumulation in mitochondria, thus severely effects the functions performed by mitochondrial enzymes. By inhibiting the mitochondrial enzymes, the synthesis of an important energy molecule, adenosine triphosphate ATP get decreases. This inhibition of ATP contents, after nanoparticle treatment could significantly effects the DNA repairing mechanism. ATP is vital in regulating the cascade of events required for phosphorylation of various proteins involved in reparation of DNA damage (Wong, Recht *et al.* 2006). The cell death is initiated by the activation of death receptors that causes various biochemical changes. These variations started by the condensation of chromatin material and nucleus shrinkage which led to the fragmentation of DNA, followed by the formation of apoptotic bodies eventually to the cell death (AshaRani, Low Kah Mun *et al.* 2008). Therefore, it is known the antibacterial properties are due to inhibition of ATP synthesis.

However, it is very important to develop more effective and less toxic agents to treat particular pathological condition. Cytotoxicity is an important matter which must be dealt with before iron oxide nanoparticles can be considered as good modality for the treatment of fatal disease.

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