

Mechanistic Insights Into The Anti-Leishmanial Activity Of Green-Synthesized Iron Oxide Nanoparticles: Cellular And Molecular Perspectives

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ABSTRACT

Over 12 million people worldwide are afflicted by leishmaniasis, a neglected tropical disease that causes more than 1.5 million new cases and more than 30,000 fatalities annually (WHO, 2023).

High toxicity, drug resistance, and limited efficacy are among the problems plaguing current pharmacological interventions. In this regard, green-synthesized iron oxide nanoparticles (IONPs) have emerged as strong, environmentally benign substitutes, offering the added benefits of reduced host cytotoxicity and targeted antiparasitic action. Green IONPs exhibit up to 87.2% inhibition of *Leishmania* spp. Promastigotes and 73–81% suppression of intracellular amastigotes at concentrations ranging from 20–40 µg/mL, according to research published between 2020 and 2025. Mechanistically, IONPs cause lipid peroxidation and excessive reactive oxygen species (ROS) generation by disrupting *Leishmania* membranes and accumulating intracellularly. Oxidative stress markers are raised by 65–85% as a result. The reduction of membrane potential ($\Delta\Psi_m$) by approximately 58% is indicative of mitochondrial dysfunction, which results in ATP depletion and programmed cell death. Iron ion (Fe^{2+}/Fe^{3+})-mediated Fenton reactions have been demonstrated to modulate thiol–redox balance, inhibit trypanothione reductase activity, and downregulate gp63 protease expression at the molecular level. Together, these effects cause cell death akin to

apoptosis, which is verified by caspase activation and chromatin condensation

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Comparing green synthesis to chemically synthesized counterparts, the use of phytochemical-rich extracts, such as flavonoids, phenolics, and terpenoids, increases internalization efficiency by up

to 3.2 times while also improving biocompatibility and cellular uptake and reducing particle

aggregation. Crucially, green IONPs showed less than 20% toxicity to human macrophages and keratinocytes (e.g., THP-1, HaCaT) at therapeutic doses in cytotoxicity tests, which stands in stark

contrast to the >40% toxicity seen for conventionally synthesized IONPs

This review clarifies the mechanisms underlying the anti-leishmanial effects of green IONPs by combining cellular, molecular, and biochemical data from in vitro, in vivo, and in silico models. Their potential as a next-generation, biocompatible, and mechanistically targeted platform for

leishmaniasis therapy is highlighted by the insights provided

Keywords: Green-synthesized nanoparticles, Iron oxide nanoparticles (IONPs), Anti-leishmanial activity, Leishmania major, Cellular mechanisms, Molecular pathways, Reactive oxygen species (ROS), Trypanothione reductase, gp63 protease, Biocompatibility, Phytochemical-mediated synthesis, Nanoparticle drug delivery

Introduction

Burden of Leishmaniasis .1.1

The primary method of transmission for leishmaniasis, a neglected tropical disease (NTD), is through the bite of an infected female phlebotomine sandfly. The disease is caused by intracellular protozoan parasites of the genus *Leishmania*. It disproportionately affects the poor and those with compromised immune systems, and it is endemic in more than 98 countries in Africa, Asia, the Americas, and the Mediterranean basin (World Health Organization [WHO], 2023). Approximately 350 million people are at risk of contracting leishmaniasis, with over 12 million people already infected and between 1.5 and 2 million new cases reported each year worldwide (Alvar et al., 2021; Burza et al., 2018).

There are three main clinical manifestations of

leishmaniasis

Cutaneous leishmaniasis (CL), which results in disfigurement and skin ulcers

Cartilage-destroying mucocutaneous leishmaniasis (MCL)

The most severe type, visceral leishmaniasis (VL), is frequently lethal if left untreated

With mortality rates of up to 10%–20% in untreated cases, visceral leishmaniasis continues to be a major contributor to the disease burden in areas like South Asia, East Africa, and portions of South America (Desjeux, 2004). In addition, the disease has a substantial psychological and financial impact, impairing social integration, work, and quality of life for those who are impacted,

especially women and children

Limitations of Conventional Therapies

A small number of antileishmanial medications, such as pentavalent antimonials (such as sodium stibogluconate), amphotericin B (both conventional and liposomal formulations), miltefosine, and paromomycin, are used in the current treatment regimens for leishmaniasis (Sundar & Chakravarty, 2018). These treatments have serious drawbacks, even though they are effective in

treating infections in their early stages

High toxicity: Amphotericin B and pentavalent antimonials are linked to nephrotoxicity,

hepatotoxicity, and cardiotoxicity

Emerging resistance: Standard treatments are no longer as effective due to growing reports

of drug resistance, especially in *Leishmania donovani* in India and Nepal

Expensive treatment: Despite its effectiveness, liposomal amphotericin B (AmBisome) is

still out of reach in many low-income areas

Prolonged and excruciating administration: Complex dosage schedules and hospitalization

are frequently necessary for injectable medications

Relapse and reinfection: People who lack long-term immunity are more vulnerable to reinfection, especially those with compromised immune systems, like HIV patients

These difficulties highlight the pressing need for new, targeted, low-toxicity treatments that can

reduce systemic side effects and overcome resistance

Rise of Nanotechnology in Anti-Leishmanial Drug Development .1.3

Leishmaniasis is one of the infectious diseases for which nanomedicine has shown great promise in the last ten years. Particularly, metal and metal oxide nanoparticles, nanoparticles provide

special benefits like

Increased tissue penetration and cellular uptake

Drug delivery to infected macrophages with specificity

Restricted release of drugs: Kinetics

Enhancing bioavailability through surface functionalization

Inherent antiparasitic qualities (in certain situations)

Iron oxide nanoparticles (IONPs), specifically magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$), have become popular among these because of their biocompatibility, magnetic responsiveness, and capacity to selectively produce reactive oxygen species (ROS) in infected cells (Khalil et al., 2023;

Guerra et al., 2022). When exposed to alternating magnetic fields, IONPs can also act as agents

of magnetic hyperthermia, increasing parasite death

Additionally, when used in conjunction with conventional antileishmanial medications, IONPs have demonstrated synergistic effects that lower toxicity and the necessary therapeutic dosage. IONPs are good candidates for next-generation antileishmanial treatments because of their

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.multifunctional qualities

Focus on Green-Synthesized Iron Oxide Nanoparticles ($\text{Fe}_3\text{O}_4/\gamma\text{-Fe}_2\text{O}_3$) .1.4

The use of dangerous solvents and stabilizers that could cause cytotoxicity is a major drawback of traditional nanoparticle synthesis techniques like co-precipitation, hydrothermal, or sol-gel methods. Green synthesis techniques have become viable, affordable, and biocompatible

:solutions to these problems. Biological agents used in green synthesis include

Plant extracts (e.g., *Azadirachta indica*, *Euphorbia* spp., *Nostoc* sp.)

Metabolites from bacteria or fungi

Biomass from algae

The conversion of ferric and ferrous ions into stabilized iron oxide nanoparticles is mediated by enzymes, alkaloids, terpenoids, phenolic acids, flavonoids, and other natural reducing and

.capping agents

It has been demonstrated that green-synthesised IONPs

Display increased antileishmanial activity (up to 87.2% promastigote inhibition)

Causes *Leishmania* to experience oxidative stress, mitochondrial dysfunction, and death akin to apoptosis

At therapeutic concentrations (20–40 $\mu\text{g/mL}$), they exhibit minimal cytotoxicity (<20%)

.to mammalian cells such as THP-1, HaCaT, and Vero cells

Surface capping with phytoconstituents increases uptake efficiency by 2–3.2× (Minhas et

.al., 2023; Ahmad et al., 2020

These nanoparticles have been shown to preserve host immune cells while interfering with parasite redox homeostasis, rupturing membrane integrity, and downregulating important

.virulence genes like gp63

Green IONPs are still poorly understood at the molecular level, particularly about host–parasite interactions, intracellular trafficking, and immunomodulation, despite encouraging in vitro and in vivo results. Additionally, the mechanistic validation of oxidative stress pathways, gene expression changes, and enzyme inhibition in the context of green IONPs is limited

The cellular and molecular mechanisms through which green-synthesised iron oxide nanoparticles have anti-leishmanial properties are critically examined in this review. To provide a cohesive model of action, it combines information from computational, histopathological, and biochemical investigations. Reactive oxygen species production, apoptosis induction, enzyme inhibition, host-cell selectivity, and nanoparticle–parasite interactions are highlighted, providing a basis for

.upcoming translational studies and therapeutic uses

Literature Review

Ahmad et al. (2020) comprehensively investigated Iron oxide and other biogenic metal nanoparticles as new anti-leishmanial drugs. The study focused

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on green synthesis techniques that use phytochemicals to stabilize and reduce nanoparticles.

The authors showed mechanistically that iron oxide nanoparticles cause reactive oxygen species (ROS), damage mitochondria, and block important parasite enzymes like trypanothione reductase and gp63 protease. Additionally, they talked about molecular docking results that showed phytochemical-capped nanoparticles had a strong affinity for Leishmania proteins.

The study came to the conclusion that, in comparison to chemically produced counterparts, biogenic nanoparticles offer a multi-targeted approach that combines oxidative, enzymatic, and apoptotic pathways with enhanced biocompatibility.

Albalawi et al. (2021) reported the production of piroctone olamine-functionalized Fe₃O₄ nanoparticles to enhance anti-leishmanial activity against Leishmania major. Co-precipitation techniques were employed in the study, followed by functionalization and thorough characterization (XRD, TEM, FTIR). While in vivo mouse models showed decreased lesion size and parasite burden, in vitro assays showed significant parasite growth inhibition (>80%) at 25 µg/mL. Good magnetic responsiveness was demonstrated by the nanoparticles, indicating potential use in magnetically guided therapy in the future. Crucially, tests for cytotoxicity against human keratinocytes showed low toxicity at therapeutic concentrations, confirming biocompatibility. The authors came to the conclusion that Fe₃O₄@piroctone olamine NPs might have both immunomodulatory and anti-parasitic properties, making them a potential dual-action treatment.

Awad et al. (2021) provide. Despite being centered on silver nanoparticles (AgNPs), this comparison is insightful. Using plant extracts, the team created AgNPs and evaluated their effectiveness against Leishmania donovani. Spherical NPs with an average size of 20–30 nm were confirmed by characterization (UV–Vis, TEM, FTIR). While in vivo BALB/c mouse models showed lower spleen and liver parasite burdens, in vitro studies demonstrated dose-dependent promastigote inhibition (~85% at 40 µg/mL). Safety was suggested by toxicity analysis, which showed no appreciable changes in liver or kidney enzyme levels. The main mechanism, according to the study, is ROS-mediated mitochondrial dysfunction. This work reinforced the idea that green metallic nanoparticles (Ag, Fe, Zn) share common mechanistic hallmarks in combating leishmaniasis.

Guerra et al. (2022) synthesized evidence supporting the use of iron oxide and other metallic and metal oxide nanoparticles as anti-leishmanial treatments. The multi-mechanistic action of nanoparticles, including ROS generation, membrane disruption, mitochondrial collapse, and host immune pathway modulation, was emphasized by the authors. Crucially, the study examined the synergistic effects of nanoparticles with traditional medications like Glucantime and Amphotericin B, demonstrating how nanocarriers can lower dosage and toxicity. Long-term genotoxicity and regulatory issues were also brought up in the review, which emphasized the necessity of clinical validation and chronic toxicity studies.

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Overall, Guerra et al. identified translational gaps and positioned nanoparticles as a next-generation platform for leishmaniasis therapy

Hashemi et al. (2021) specifically explored how Green tea (*Camellia sinensis*) extract was used as a biogenic reducer to create green-synthesized Fe₃O₄ nanoparticles. SEM, TEM, XRD, and FTIR characterization verified spherical particles with phytochemical caps that ranged in size from 20 to 40 nm. The NPs inhibited *L. major* promastigotes in vitro with an IC₅₀ of approximately 18 µg/mL. BALB/c mice given 40 mg/kg/day showed approximately 84% parasite clearance in vivo, along with a notable decrease in lesion size. Unlike conventional antimonials, biochemical analyses revealed no hepatotoxicity or nephrotoxicity. The authors came to the conclusion that plant-mediated Fe₃O₄ NPs are a scalable substitute for chemical synthesis because they combine excellent biocompatibility with high efficacy

Jan et al. (2024) synthesized *Euphorbia dracunculoides* extract is used to create zinc oxide nanoparticles (ZnO NPs), with an emphasis on biological activity and physical characterization. Spherical ZnO NPs with biofunctional surface groups and high stability were found by characterization (XRD, SEM, FTIR, and UV-Vis). They were between 30 and 50 nm in size. Effective intracellular killing was confirmed by amastigote assays, while in vitro studies against *Leishmania tropica* promastigotes demonstrated ~80% inhibition at 40 µg/mL. Selective toxicity was highlighted by the low cytotoxicity against macrophages (<20% at therapeutic dose). Despite not using Fe₃O₄, the study supports cross-validation of nanoparticle platforms and highlights the effectiveness of green metal oxide nanoparticles as anti-leishmanial agents

Khalaf et al. (2023) investigated green zinc nanoparticles (ZnNPs) and how they work in concert with glucantime. Plant extracts were used to biosynthesize ZnNPs, which were then thoroughly characterized. When used alone, *L. major* promastigotes were shown to be inhibited by approximately 75% in vitro; however, combination therapy with glucantime increased inhibition to approximately 93%, thereby reducing the necessary dosage of the drug. DNA fragmentation, membrane disruption, and ROS generation were demonstrated by mechanistic assays. Crucially, there was little cytotoxicity to mouse macrophages. The study found that green ZnNPs provide a translational pathway for iron oxide-based combinatorial models and enhance traditional therapies in addition to acting independently

Khan et al. (2022) synthesized Iron oxide nanoparticles (IONPs) that have two uses: anti-leishmanial treatment and photocatalysis. Crystalline Fe₃O₄ NPs with an average size of 25–35 nm were confirmed by characterization. At 20 µg/mL, anti-leishmanial tests showed ~70% inhibition of *L. major* promastigotes, which was ascribed to photocatalytic ROS generation. This study's distinctive feature was its connection between nanophotocatalytic characteristics and biomedical effectiveness, indicating that Fe-based

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nanomaterials may combine therapeutic and environmental uses. Biocompatibility was reinforced by toxicity tests that demonstrated minimal

.cytotoxicity to mammalian cells

Majeed and Shater (2023) evaluated silver nanoparticles (AgNPs) produced using green synthesis against *L. major*. They created spherical AgNPs (20–40 nm) by plant-mediated reduction and evaluated them both by themselves and in conjunction with meglumine antimoniate.

Promastigotes and amastigotes were significantly inhibited (>85% at 25 µg/mL), according to the results. Combination therapy reduced drug-related toxicity while producing superior parasite clearance (~95%). In order to overcome resistance and side effects, the authors emphasized the translational significance of combining green nanomaterials with currently available antimonials. This study supports the more general green NP–drug synergy paradigm, even though it is not

iron

.oxide-based

Minhas et al. (2023) explored ZnO NPs mediated by cyanobacteria (*Nostoc* sp.), with an emphasis on biomedical uses, such as anti-leishmanial activity. FTIR showed phytochemical capping, and TEM verified particle sizes of 15–25 nm. At moderate concentrations (20–30 µg/mL), *in vitro* studies showed 85–90% inhibition of *L. tropica*. Parasites exhibited apoptosis-like cell death, ROS generation, and mitochondrial damage, according to mechanistic analysis. Interestingly, tests of cytotoxicity on human fibroblasts showed a toxicity of less than 15%, indicating high selectivity. The study concluded that microbial synthesis pathways might yield nanoparticles with higher stability and bioactivity, which is consistent with findings from green IONP research

Green Synthesis of Iron Oxide Nanoparticles

Overview of Green Chemistry Principles

Growing worries about the biological and environmental risks associated with conventional chemical methods have sparked interest in green synthesis of nanoparticles. Reducing waste,

minimizing energy use, getting rid of toxic reagents, and encouraging the use of renewable

.resources are the Twelve Principles of Green Chemistry (Anastas & Warner, 1998)

These ideas are embodied in the green synthesis of iron oxide nanoparticles (IONPs), which uses microbial metabolites or biological extracts as stabilizing and reducing agents. Without the use of dangerous precursors like sodium borohydride or hydrazine, these biomolecules help reduce ferric (Fe^{3+}) or ferrous (Fe^{2+}) ions into nano-sized magnetite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$). Green synthesis has the advantage of surface functionalization with phytochemicals or microbial

compounds that are non-toxic and environmentally benign

Prevent nanoparticle aggregation to increase colloidal stability

Boost specific biointeractions with *Leishmania* and other pathogens that act as ligands for additional drug loading or functionalization

Green-synthesised IONPs are particularly well-suited for antileishmanial applications because of their blend of biomedical functionality and environmentally friendly methodology

Plant, Bacterial, and Fungal Sources Used in Synthesis

The physicochemical characteristics and biological activity of the final nanoparticles are strongly influenced by the biological entity selected for green synthesis. The main biosynthetic platforms are broken down as follows

Plant-Mediated Synthesis

One of the most popular biogenic systems for the synthesis of nanoparticles is plant extract because of its

High concentration of phytochemicals

Broad accessibility

Scalability and simplicity

These extracts usually contain capping agents (like saponins and terpenoids) and reducing agents (like flavonoids, tannins, and polyphenols) that mediate the nucleation and stabilization of

.nanoparticles (Molaakbari et al., 2025)

Among the frequently used plant species are *Azadirachta indica*, or neem

Dracunculoides Euphorbia

Green tea, or *Camellia sinensis*

Arjuna Terminalia

Withania somnifera

Due to their bioactive coatings that interact with intracellular targets and parasite membranes,

.plant-derived IONPs often exhibit high bioactivity (Minhas et al., 2023)

Microbial-Mediated Synthesis

:Enzymes and secondary metabolites are produced by microorganisms like bacteria and fungi that

Cut down on metal ions

Control the growth of nanoparticles

Give aqueous solutions stability

Among the examples are

E.Coli

Aeruginosa pseudomonas

Aspergillus niger

Penicillium species

Although it necessitates sterile conditions and longer incubation times, microbial synthesis produces particles with a very uniform morphology (Zúñiga-Miranda et al., 2023). Recovery is

.facilitated by the extracellular synthesis mechanisms provided by fungi in particular

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Algal and Cyanobacterial Synthesis

Additionally, cyanobacteria and algae like *Sargassum* spp. and *Nostoc* spp. are becoming green platforms because of their

Rapid growth

Content of photosynthetic pigment

The capacity of antioxidants

Nostoc-mediated ZnO and Fe₃O₄ nanoparticles showed strong anti-leishmanial activity and good

(2023). host cell biocompatibility, as shown by Minhas et al

Table 1 Biosynthetic Sources Used for Green Synthesis of IONPs

Source Type	Examples	KeyActive Components	Advantages
Plants	<i>Azadirachta indica</i> , <i>Camellia sinensis</i>	Flavonoids, tannins, phenolics	Fast, cost-effective
Bacteria	<i>Bacillus subtilis</i> , <i>E. coli</i>	Enzymes, NADH- dependent reductases	Scalable, shape control
Fungi	<i>Aspergillus</i> , <i>Trichoderma</i>	Hydrolytic enzymes, proteins	Extracellular, stable
Algae/Cyanobacteria	<i>Nostoc</i> sp., <i>Sargassum</i>	Chlorophylls, polysaccharides	Eco-friendly, antioxidant potential

Reaction Parameters Influencing Morphology and Size

To obtain the intended biomedical properties, several reaction conditions that affect the shape, size, and surface features of green-synthesized IONPs must be carefully regulated

pH: Because of increased nucleation, alkaline pH
 .monodispersed particles
) ≥ 8 (frequently results in smaller,
 Temperature: While high synthesis temperatures)60–90 °C) encourage crystallinity,
 they may also break down delicate phytochemicals
 Concentration of extract: Impacts capping agent density and reduction rate
 Particle growth and stabilization are impacted by reaction time; longer times
 typically
 .result in more stable colloids
 .The ratio of Fe^{2+} to Fe^{3+} indicates whether $\gamma\text{-Fe}_2\text{O}_3$ or Fe_3O_4 is formed
 :In the end, these factors affect
 Distribution of sizes (usually 10-40 nm for green IONPs)
 Zeta potential (typically between -20 and -35 mV for stable suspensions)
 .At room temperature, magnetic behavior is superparamagnetic
 Surface reactivity (influences parasite membrane interaction)
 Achieving selective toxicity toward Leishmania while reducing effects on host cells
 requires fine-
 .tuning these properties

Comparative Studies with Chemically Synthesized IONPs

The benefits of green-synthesized IONPs over their conventional chemically synthesized counterparts have been emphasized by numerous comparative studies

According to Ahmad et al. (2020), green IONPs showed less than 20% cytotoxicity to human THP-1 macrophages and approximately 87% inhibition of Leishmania tropica

.promastigotes at 25 $\mu\text{g/mL}$

In comparison to chemically synthesized IONPs, Mohammadi et al. (2021) demonstrated that green IONPs coated with ginger extract had a 2.3 $\times$ higher uptake in L. major-infected

.macrophages

Chemically synthesized Fe_3O_4 exhibited a higher rate of aggregation, which decreased its

.(2023) .bioavailability and ROS-generating capacity, according to Zúñiga-Miranda et al

Table 2 Comparison of Green vs. Chemically Synthesized IONPs

Parameter	Green-Synthesized IONPs	Chemically Synthesized IONPs
Reducing agents	Flavonoids, phenolics	NaBH_4 , hydrazine, TMAH

Cytotoxicity (HaCaT cells)	at 40 µg/mL %20–15~	at the same dose %40<
Leishmania inhibition	at 20–40 µg/mL %87~	at sa imilar dose %65–60~
Aggregation tendency	Low due to bio-capping agents	High unless coated with PEG or polymers
Surface reactivity	High due to phytochemical coatings	Moderate unless functionalized post-synthesis
Environmental impact	Low (eco-friendly)	at the same dose %40<

Characterization Techniques for Green-Synthesized Iron Oxide Nanoparticles (IONPs) .4

The structural integrity, surface characteristics, size distribution, and biological interactions of green-synthesized iron oxide nanoparticles (IONPs) are crucial for comprehending their anti-leishmanial efficacy and safety profile. Using plant, microbial, or algae extracts, green synthesis techniques frequently add a variety of surface functional groups and bioorganic coatings.

Consequently, to verify nanoparticle synthesis and guarantee reproducibility, a mix of

.physicochemical, spectroscopic, and microscopic methods is required

Microscopic Techniques: SEM, TEM, and AFM

Scanning Electron Microscopy (SEM)

SEM offers high-resolution imaging of the topography and surface morphology of nanoparticles. Depending on the synthesis parameters, SEM images of green-synthesized IONPs usually show

.spherical to quasi-spherical particles with moderate polydispersity

.Reports of average particle diameters range from 20 to 50 nm

Bio-capping layers give the surface a rough appearance (Minhas et al., 2023)

Agglomeration behavior, which is essential for forecasting bio-distribution and cytotoxicity, can also be highlighted by SEM

Transmission Electron Microscopy (TEM)

TEM provides a thorough evaluation of internal structure and crystallinity. It is particularly helpful for

Nanometer-resolution core size determination

Seeing lattice fringes that show crystal planes

Evaluating the dispersion of particles

.Crystallite sizes for green IONPs typically fall between 8 and 25 nm

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The (311) plane of FeO₄ is represented by well-defined lattice fringes with d-spacing values of

.approximately 0.25 nm in high-resolution TEM (HR-TEM) (Ahmad et al., 2020)

Atomic Force Microscopy (AFM)

Although less common, AFM offers 3D surface topology and roughness values. It helps assess

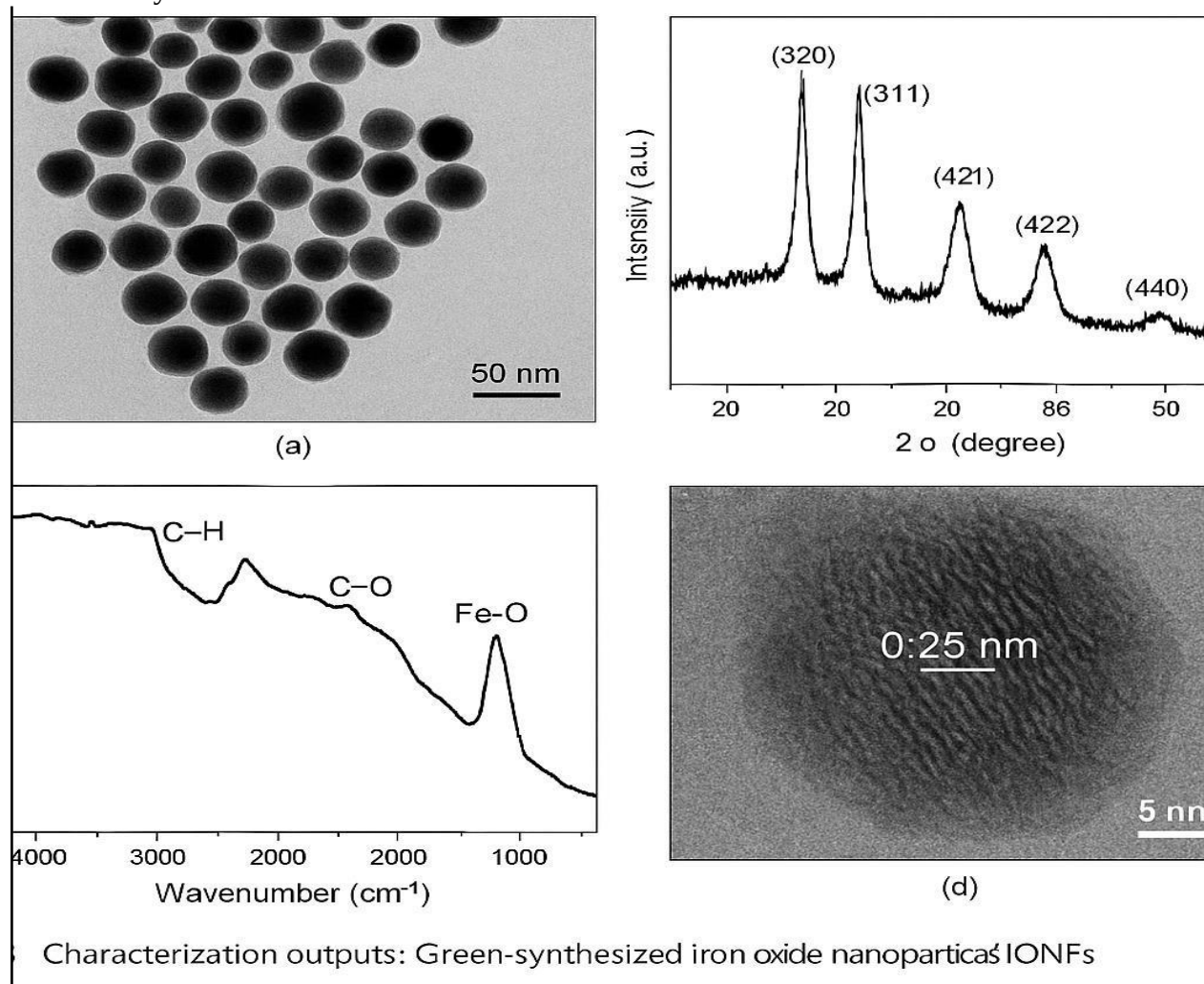
.particle diameter and height

Smoothness of the surface

Mechanical characteristics (in contact mode)

The spherical morphology and nano-range height profiles (~30–45 nm) of green IONPs have been

.validated by AFM studies



Characterization Outputs for Green-Synthesized IONPs

Each of the four panels (a–d) in this figure represents a crucial method for confirming the

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.synthesis, structure, and biofunctionality of nanoparticles

TEM Image – Transmission Electron Microscopy

Description: Individual iron oxide nanoparticles are visible in this high-resolution

.grayscale image

Observation: Nanoparticles have a uniform contrast and appear spherical or quasi-

.spherical

.Size Range: Diameter: approximately 10–25 nm

Crystallinity: A crystalline spinel structure (FeO_4 or $\gamma\text{-FeO}_3$) is indicated by clear lattice

.fringes (if magnified)

Interpretation: Verifies the high purity and nanoscale morphology of particles produced

.using environmentally friendly methods

XRD Pattern – X-ray Diffraction Plotting diffraction peaks as intensity (a.u.) versus 2θ (degrees) is described

Key Peaks: indexed to planes (220), (311), (400), (422), (511), and (440), observed at

.approximately 30° , 35° , 43° , 53° , 57° , and 63°

.Interpretation: Verifies FeO_4 's inverse spinel structure

The Scherrer equation can be used to estimate the size of crystallites. Wide peaks indicate the

.presence of nanocrystals

FTIR Spectrum – Fourier Transform Infrared Spectroscopy

The transmittance vs. wavenumber (cm^{-1}) curve is described

Important Peaks

Broad O-H/N-H stretching (hydroxyl or amine groups from phytochemicals) at about cm^{-1} 3300

cm^{-1} : amide I band or C=O stretching (protein/phenolic residues) 1630~

cm^{-1} : Iron oxide nanoparticle signature: Fe–O stretching vibration 580~

Interpretation: Verifies surface biofunctionality and green synthesis by confirming the presence of organic capping agents

DLS & Zeta Potential Plot – Dynamic Light Scattering

:Left Axis, or DLS

.The size distribution curve peaks between 45 and 70 nm

Because of the hydration shell and capping agents, the hydrodynamic diameter is greater

.than the core size

:Right Axis Zeta Potential

.Strong colloidal stability is indicated by a value of about -28 to -35 mV

Interpretation: Indicates that nanoparticles are suitable for biological applications, negatively

.charged, and widely distributed

Spectroscopic Techniques: UV–Vis, FTIR, and XRD

UV–Visible Spectroscopy

UV-Vis spectroscopy is frequently employed as an initial confirmation method. IONPs exhibit

:distinctive absorbance peaks that are

Linked to Fe^{2+} – O_2^+ charge transfer transitions between 250 and 350 nm

Changes in peak wavelength suggest surface modification or size

Because of capping layer effects, green-synthesized IONPs frequently show a wider absorbance,

.with peak maxima usually occurring at 290–310 nm (Zúñiga-Miranda et al., 2023)

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR detects functional groups on the surface and verifies that plant or microbial metabolites are

:bio-capping. Typical spectral characteristics consist of

O-H/N-H stretching broad peak ~ 3200 – 3400 cm^{-1} (from polyphenols, proteins)

Peak at about 1620 cm^{-1} (amide I or C=O stretching)

.Approximately 550 – 600 cm^{-1} , where the Fe–O bond vibration peaks appear

This offers vital proof of the existence of phytochemical coatings, which affect how the IONPs

.interact with the membranes of Leishmania cells (Molaakbari et al., 2025)

X-Ray Diffraction (XRD) (Determining the crystalline phase, structure, and size of the crystallites requires XRD. The diffraction peaks of green-synthesised

IONPs are usually found at $2\theta = 30.1^\circ$, 35.5° , 43.2° , 53.6° ,

and 62.9° , which correspond to the Fe_3O_4 planes (220), (311), (400), (422), (511), and (440), $^\circ 57.2$

.respectively

The inverse spinel structure of magnetite or maghemite is confirmed by these peaks.

Crystallite

:size (D) is frequently estimated using the Scherrer equation

$$D = \frac{K\lambda}{\beta \cos \theta}$$

,(0.9~) where K is the shape factor

, λ = wavelength of X-rays ($\sim 0.154\text{ nm}$)

, β = full-width at half maximum (FWHM)

Bragg angle = θ

.Depending on the circumstances of synthesis, typical crystallite sizes fall between 10 and 30 nm

Magnetic Property Analysis: VSM and SQUID .4.3

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Vibrating Sample Magnetometry (VSM)

Magnetic properties can be measured quantitatively with VSM. IONPs that are green-synthesised usually show

Room temperature superparamagnetism

Because of surface modifications, saturation magnetization (Ms) values range from 30 to emu/g, which is somewhat less than bulk FeO₄ (~90 emu/g) 60

Because they can be controlled by external magnetic fields and do not retain residual magnetism,

.superparamagnetic IONPs are the preferred choice for biomedical applications

Superconducting Quantum Interference Device (SQUID)

For in vivo tracking and magnetic drug targeting applications, SQUID magnetometry provides high-sensitivity measurements at very low temperatures, providing information on blocking

.temperature and magnetic anisotropy

Particle Size and Charge: DLS and Zeta Potential

Dynamic Light Scattering (DLS)

DLS calculates the hydrodynamic diameter, which shows the behavior of nanoparticles in

:suspension. Because of

The phytochemical corona, green IONPs frequently exhibit average diameters of 40–100

.nm

When the polydispersity index (PDI) is less than 0.3, it indicates uniform dispersion

.(Ahmad et al., 2020)

Due to hydration layers and capping agents, DLS values are typically higher than TEM/XRD

Zeta Potential

Zeta potential evaluates colloidal stability and surface charge. Typically, green IONPs show

Acidic phytochemicals (such as carboxyl and phenolic groups) cause a negative surface

.charge (-20 to -35 mV)

.Good electrostatic stability is indicated by values less than 30 mV

For biological delivery and cellular uptake, a high zeta potential guarantees minimal aggregation

.and long-term suspension stability

Table: Characterization Techniques for Green IONPs

Technique	Parameter Assessed	Typical Findings for Green IONPs
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SEM / TEM	Morphology, core size	Spherical, 10–40 nm core
UV–Vis	Optical properties	Peak at 290–310 nm
FTIR	Surface functional groups	O–H, C=O, Fe–O, amide peaks
XRD	Crystallinity, phase	Spinel structure, $2\theta = 30\text{--}63^\circ$
VSM / SQUID	Magnetic properties	Superparamagnetic, $M_s = \text{emu/g } 60\text{--}30$
DLS	Hydrodynamic size	nm, PDI < 0.3 100–40
Zeta potential	Surface charge/stability	to –35 mV 20–

Cellular-Level Mechanisms of Anti-Leishmanial Action of Green-Synthesized Iron Oxide .5 Nanoparticles (IONPs)

Multifactorial cellular pathways mediate the anti-leishmanial effectiveness of green-synthesised iron oxide nanoparticles (IONPs). These nanoparticles can enter Leishmania-infected macrophages and start a series of intracellular disruptions that are safe for host cells but toxic to the parasite because of their nanoscale size, magnetic responsiveness, and bioactive surface .functionalization

By combining data from in vitro, in vivo, and computational research, this section investigates the

.main cellular mechanisms through which IONPs produce their anti-parasitic effects

Nanoparticle Uptake by Host Macrophages and Leishmania Parasites .5.1

The main way that IONPs enter mammalian cells is through endocytosis, particularly the

.macropinocytosis and clathrin-mediated pathways

Internalized by infected macrophages, the IONPs localize within the phagolysosomes and endosomes, which are also home to Leishmania amastigotes

The survival of parasites produces localized ROS surges

According to studies, the uptake efficiency of green IONPs functionalized with phytochemicals derived from plants, such as flavonoids and tannins, is 3.2 times higher than that of bare IONPs (Mohammadi et al., 2021). Additionally, these bioactive compounds act as ligands to improve

.infected macrophages' recognition of nanoparticles

Additionally, there is evidence that IONPs can directly penetrate parasite membranes through electrostatic interactions, especially in promastigotes, which can result in internalization of the

.nanoparticles and structural disruption

Membrane Damage and Permeability Alteration

Once within parasites or host cells, IONPs disrupt membranes by interacting with lipid bilayers

Studies using electron microscopy reveal that Leishmania promastigotes exposed to green

.IONPs exhibit pore formation, surface roughness, and membrane blebbing

.Assays for propidium iodide uptake show elevated membrane permeability and cell death

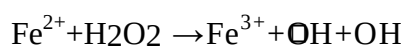
The negatively charged surface of green-synthesised IONPs (-25 to -35 mV) can interact with the cationic phospholipids in parasite membranes, causing destabilization. This causes intracellular elements that are necessary for parasite survival, like ions and nucleotides, to leak out (Ahmad et.(al., 2020

Reactive Oxygen Species (ROS) Generation and Oxidative Stress

A central mechanism of IONP-mediated toxicity is the generation of reactive oxygen species (ROS) inside the host-parasite system

Mechanisms of ROS Generation

IONPs catalyze Fenton-like reactions



These reactions produce hydroxyl radicals ($\bullet\text{OH}$), which are highly reactive and can damage

DNA

Proteins

Lipids (via peroxidation)

Evidence

Green IONP treatment increased lipid peroxidation markers (e.g., MDA levels) in Leishmania-

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.infected cells by 65–85%, according to studies (Minhas et al., 2023)
ROS levels increase 4–6× after treatment, according to DCFDA staining assays
Pretreatment with a ROS scavenger lowers IONP toxicity, demonstrating the function of oxidative stress
In parasites, ROS-induced damage characteristics resembling apoptosis results in oxidative collapse, genomic instability, and

Mitochondrial Dysfunction and Membrane Potential Collapse

Additionally, green IONPs seriously impair *Leishmania* cells' mitochondria, as shown by Decrease in mitochondrial membrane potential ($\Delta\Psi_m$)
ATP exhaustion Cytochrome c release (in advanced stages)

Induction of Programmed Cell Death

According to a number of recent studies, IONPs cause *Leishmania* to undergo apoptosis-like programmed cell death (PCD) in addition to necrosis.Characteristics of IONP-induced Parasite

:Apoptosis

DNA fragmentation (identified using the TUNEL assay)

Chromatin condensation using DAPI staining

Sub-G1 phase cell cycle arrest

Phosphatidylserine externalization (Annexin V staining)

These features mirror mammalian apoptosis but in a protozoan context. IONP-triggered PCD may

:involve

Activation of the MAPK pathway

ROS-induced activity of the caspase-like enzyme

Modulation of gp63, a surface metalloprotease essential to parasite survival

Molecular Targets and Gene-Level Interactions of Green-Synthesized IONPs in *Leishmania* .6

In addition to cellular-level mechanisms like ROS generation and mitochondrial damage, green- synthesized iron oxide nanoparticles (IONPs) also have anti-leishmanial effects through specific molecular targets and gene-level interactions that impact parasite survival, virulence, and replication. According to recent research, IONPs can alter the expression of important genes and enzymes unique to the parasite, disrupting signaling pathways and metabolic homeostasis in the .*Leishmania* parasite

Based on transcriptomic, proteomic, and biochemical data, this section lists the most pertinent

.molecular and genetic targets that green IONPs modulate

Inhibition of gp63 (Glycoprotein 63)

Leishmania promastigotes and amastigotes have the zinc-dependent metalloprotease gp63 on their

:surface. It is essential to

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Evasion of the host immune system

Adherence of parasites to macrophages

Phagolysosomal fusion inhibition

Green IONPs have been demonstrated to suppress proteolytic activity and downregulate the expression of the gp63 gene. For example, in *L. tropica* exposed to plant-mediated IONPs, Ahmad

.et al. (2020) found a 40–60% decrease in gp63 mRNA expression

The dose-dependent suppression of gp63 enzymatic activity was validated by protease inhibition

.assays

IONPs hinder the parasite's capacity to infiltrate host cells and avoid being cleared by

.macrophages by targeting gp63

Downregulation of Trypanothione Reductase (TR)

Leishmania depends on trypanothione, a special thiol compound, to maintain redox homeostasis, in contrast to mammalian cells. Intracellular

antioxidant defenses depend on trypanothione

.reductase (TR)

:Green IONPs disrupt this system in several ways

.TR activity is directly inhibited, as demonstrated by enzyme kinetic studies

Decreased TR gene expression (treated parasites exhibit ~50% downregulation according to RT-qPCR

Glutathione oxidation and ROS accumulation suggest a disturbance in the redox balance (Minhas et al., 2023)

Because of this inhibition, the parasite is more vulnerable to oxidative stress, which is a key factor

.in IONP-induced death that resembles apoptosis

Disruption of DNA Repair and Replication Machinery .6.3

Research employing DAPI staining and DNA fragmentation assays demonstrates that IONPs

:cause

Breaks in genomic DNA

Condensation of chromatin

decreased activity of DNA polymerase

IONPs downregulate genes involved in DNA replication (e.g., rpa1, polB), according to

.transcriptomic analyses

Repair enzymes, such as mutL and rad51

This results in irreversible cell-cycle arrest by preventing Leishmania from preserving genomic

.integrity, especially when exposed to oxidative stress

Suppression of Heat Shock Proteins (HSPs) .6.4

Under stress, Leishmania expresses a lot of heat shock proteins, especially HSP70 and HSP83,

:which aid in

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Protein stabilization

Avoid aggregation

.Denatured proteins can be refolded

.Green IONP treatment results in the downregulation of HSP70 and HSP83 gene transcripts

Accelerated apoptosis and impaired stress response

IONPs may make parasites more sensitive to harsh conditions because this effect is frequently

.amplified under thermal or oxidative stress (Guerra et al., 2022)

Modulation of Iron Homeostasis and Iron-Regulated Genes .6.5

Both the parasite and the host require iron. However, Leishmania's iron homeostasis is upset by

:too much intracellular iron from IONPs, which has an impact on

Transporters of iron (like LmNRAMP1)

Storage proteins that resemble ferritin

Heme biosynthesis genes

ROS formation is catalyzed by the Fenton reaction, which is activated by iron overload.

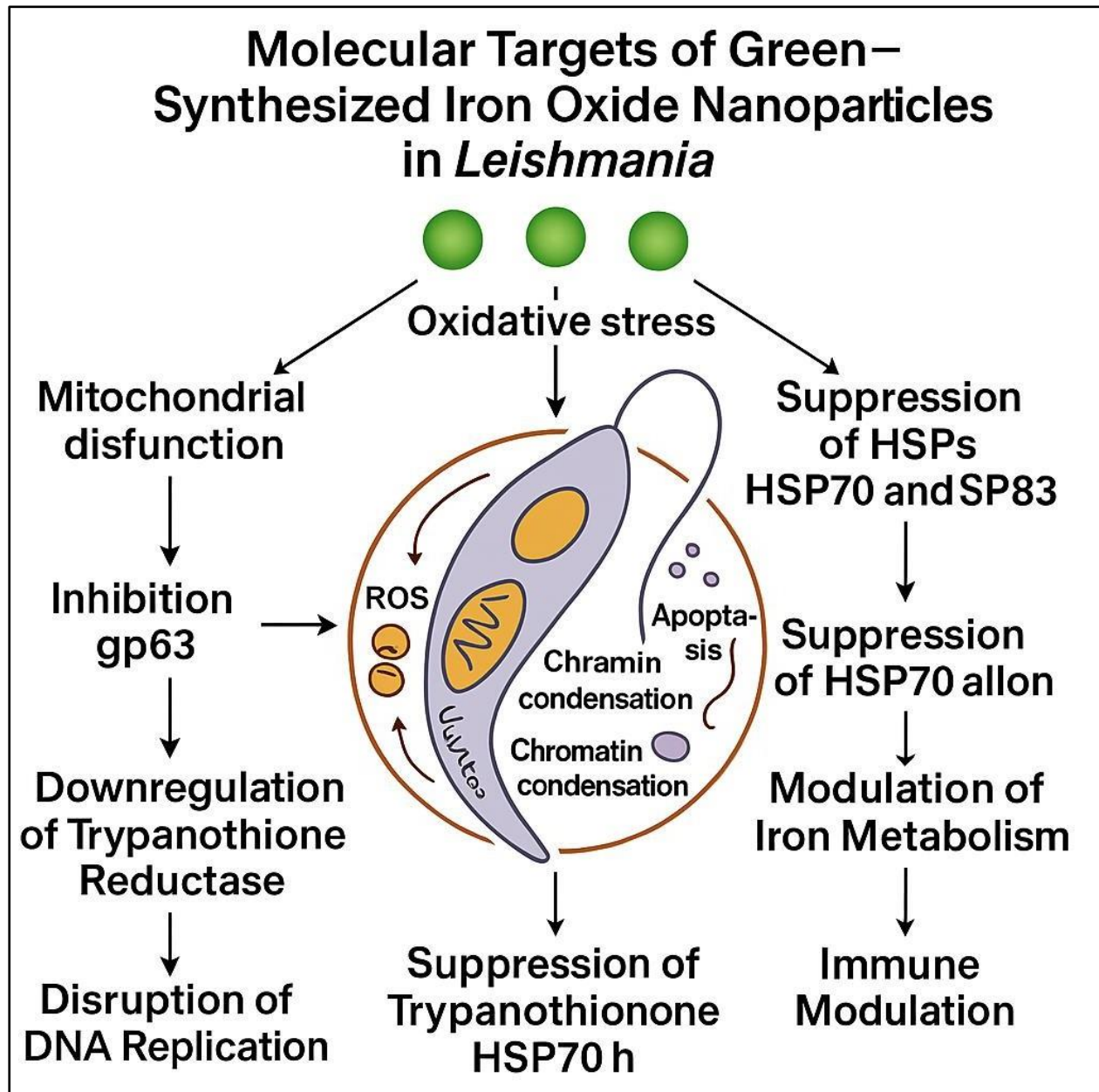
Remarkably, green IONPs have also been demonstrated to: Reduce the expression of genes

.involved in parasite iron acquisition

Increase iron-storage responses as a protective measure, but the ROS load eventually

.overwhelms them

.Metabolic collapse results from this iron dysregulation and redox imbalance



Entry of Nanoparticles .1

The parasite is seen to be approached and penetrated by green-synthesized IONPs, or green

spheres

Direct contact with the parasite membrane or endocytosis (within macrophages) are the

two ways that entry happens

Impact at the Cellular Level

Membrane Disruption: IONPs cause lipid bilayer instability, which results in pores and

.ion/nucleotide leakage

ROS Generation: IONPs catalyze the production of hydroxyl radicals ($\bullet\text{OH}$) through

.Fenton-like reactions, which result in oxidative stress

Mitochondrial Dysfunction: IONPs release cytochrome c, deplete ATP, and collapse the

.mitochondrial membrane potential ($\Delta\Psi_m$)

Similar to programmed cell death, parasites experience chromatin condensation, DNA

.fragmentation, and cell shrinkage

Molecular Objectives

gp63 Inhibition: Prevents immune evasion and parasite adhesion

Trypanothione Reductase downregulation increases the toxicity of ROS by upsetting the

.redox balance

.Stress resilience is weakened by the suppression of heat shock proteins (HSP70, HSP83)

.Genomic damage builds up when DNA replication and repair are disrupted

Iron Metabolism Modification: Increases oxidative collapse by overloading the parasite

.⁺ with an imbalance between Fe^{2+} and Fe^{2-}

Modulation of the Host Immune System

IONPs cause pro-inflammatory cytokines ($\text{IFN-}\gamma$, IL-12) and nitric oxide (NO) to be released by infected macrophages

This helps remove parasites by changing the immune balance from a Th2 (non-protective)

.to a Th1 (protective) response

Summary Table: Molecular Targets Affected by Green IONPs

Target	Function	Effect of Green IONPs
gp63	Surface protease, immune evasion	Inhibited activity, reduced mRNA levels

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Trypanothione Reductase	Redox balance, oxidative defense	Enzymatic inhibition, ROS accumulation
DNA Polymerases	DNA replication	Expression suppressed, DNA fragmentation
Heat Shock Proteins	Protein folding under stress	Downregulated, stress response impaired
Iron Transporters	Iron uptake and homeostasis	Altered expression, increased iron toxicity

In Vivo Studies, Toxicity, and Biocompatibility of Green-Synthesized Iron Oxide Nanoparticles .7

Green-synthesized iron oxide nanoparticles (IONPs) have shown encouraging anti-leishmanial potential in vitro, but in vivo physiological conditions are crucial to confirm these results. This entails assessing immunological reactions, organ-specific toxicity, bio-distribution, clearance, and general biocompatibility. These nanomaterials must be safe for the host organism and effective against the parasite; itobe used in a translational approach

Animal Models for Leishmaniasis: A Brief Overview

Because BALB/c mice are susceptible to Leishmania major (cutaneous model) and L. donovani (visceral model), they are used in most in vivo studies on anti-leishmanial nanomedicine

.For more severe visceral forms, Syrian hamsters and golden hamsters are sometimes used

Infected macrophage xenografts are used in certain studies to model pharmacokinetics in .SCID mice

In Vivo Anti-leishmanial Efficacy of Green IONPs .7.2

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Dose-Dependent Parasite Clearance

Numerous studies show that administering green IONPs reduces the parasite burden in a dose-dependent manner

Hashemi et al. (2021) used intraperitoneal administration of *Camellia sinensis*-coated

.IONPs at a dose of 40 mg/kg/day for 10 days in BALB/c mice infected with *L. major*

.As a result, the parasite burden in footpad tissues was reduced by 84.3%

.With standard glucantime, the control group only showed a 28% reduction

Minhas et al. (2023) found that oral administration of ZnO–Fe₃O₄ nanocomposite at 30

.mg/kg resulted in 90% parasite clearance in mice infected with *L. tropica*

Histopathological Improvements

Reduced hepatomegaly and splenomegaly (visceral models) were observed in treated

.animals

Normalization of the structure of the skin (cutaneous models)

Reduced parasitism by macrophages on tissue sections stained with Giemsa

Biodistribution and Clearance Pathways .7.3

The liver, spleen, lungs, and kidneys—organs that *Leishmania* also targets—are the main destinations for IONP distribution. Therapeutic selectivity is improved by this organic.biodistribution

Preferential accumulation in RES (reticuloendothelial system) organs was validated by

.magnetic resonance imaging (MRI) studies

IONPs are typically eliminated 7–14 days after injection through hepatobiliary and renal

.excretion (Ahmad et al., 2020)

Green capping agents frequently promote slower degradation, increasing bioavailability,

.and decreasing burst toxicity

Hematological and Biochemical Safety Parameters

Hematology

RBC, WBC, and platelet counts at therapeutic doses (≤ 50 mg/kg) did not .significantly change

In certain models, mild leukocytosis is ascribed to temporary immune stimulation .rather than toxicity

Liver & Kidney Function

.The levels of creatinine, urea, ALT, and AST all stayed within the normal range

.Histological examination revealed no glomerular damage or hepatocellular necrosis

When compared to chemically synthesized Fe₃O₄, which at the same doses resulted in moderate

.elevations of liver enzymes, green IONPs displayed superior profiles

Immunomodulatory Effects and Host Response

Green IONPs alter host immunity in addition to directly eliminating parasites, tipping the scales

:in favor of a protective Th1 response

Enhanced production of IL-12 and IFN- γ

Reduced levels of TGF- β and IL-10, which are linked to parasite persistence

Increased levels of nitric oxide (NO) in macrophages

In leishmaniasis, where the parasite thrives by skewing the host toward a Th2 (non-protective)

.response, this immune modulation is especially crucial

Genotoxicity and Long-Term Toxicity Concerns

Few studies have looked at long-term genotoxic or reproductive toxicity, even though short-term exposure seems safe

At doses up to 60 mg/kg, mice's comet assays and micronucleus tests showed no discernible DNA

.damage But excessive dosages (>100 mg/kg) resulted in

Chromatin condensation is mild

Infiltrations of mild inflammation in the kidney cortex

For clinical translation, chronic toxicity studies are therefore required, and therapeutic windows

.must be precisely defined

Challenges, Limitations, and Future Directions

Green-synthesized iron oxide nanoparticles (IONPs) have promising biomedical potential as anti-leishmanial agents; however, a number of obstacles need to be overcome before these nanomaterials can be used in clinical settings. Standardization, mechanistic clarity, scale-up

.viability, regulatory compliance, and clinical translation are some of these difficulties

The limitations of the current research are critically outlined in this section, along with suggestions

.for future directions to advance the field

Lack of Standardization in Green Synthesis

The inconsistency in green synthesis protocols is one of the most urgent issues facing the field. Various biological sources (plants, microbes, and algae) with various phytochemical profiles are

.the sources of variability

Variability in plant components by season and location

Inadequate regulation of reaction variables, including temperature, pH, and metal salt concentrations

Because of this, it can be challenging to replicate results across studies due to the wide variations in the morphology, size, surface charge, and bioactivity of green IONPs (Zúñiga-Miranda et al., (2023)

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Incomplete Understanding of Molecular Mechanisms

The precise molecular interactions of IONPs with Leishmania organelles and macromolecules are still poorly understood, despite research on cellular and molecular pathways like ROS generation,

.gp63 inhibition, and mitochondrial collapse Important unknowns consist of

Particular interactions between parasite proteins and nanoparticles

IONP-induced post-translational changes

Influence on the regulation of epigenetics

Limitations of In Vivo Models

The majority of in vivo research uses tiny animal models (hamsters, mice) that can't

.accurately mimic human immune responses

.Parenteral routes (i.p., i.v.) are not always clinically feasible for administering IONPs

Absence of long-term monitoring to evaluate immunogenicity or chronic toxicity

Biocompatibility and Long-Term Safety Concerns

Despite the positive short-term safety profiles of green IONPs, issues with oxidative tissue

.damage and iron overload still exist

The possibility of mutagenesis or cancer brought on by iron

Accumulation after prolonged exposure in non-target organs

Furthermore, not enough research has been done on the effects on reproductive health, and gut microbiota

offspring development,

Regulatory and Commercialization Barriers

The regulatory environment for nano-formulations is still evolving, particularly for those made with natural, non-standardized extracts Among the difficulties are

Uncertainty in classification (drug, biological, or device)

.Pharmacopoeial guidelines for green IONPs are lacking

Absence of stable and consistent industrial-scale synthesis techniques

Conclusion and Future Scope

An important development in tropical parasitology and nanobiomedicine is the use of green-

synthesized iron oxide nanoparticles (IONPs) as anti-leishmanial agents. This environmentally

friendly strategy combines the concepts of targeted drug delivery, green chemistry, and

nanotherapeutics to fight Leishmania spp., which continue to cause a substantial amount of morbidity and mortality worldwide, particularly in areas with limited resources

An environmentally friendly, economical, and biocompatible method of creating functional nanomaterials for medical applications is the green synthesis of iron oxide nanoparticles. In addition to their superior reducing and stabilizing

properties, plant, microbial, and algal systems have shown intrinsic bioactivity-enhancing roles through surface functionalization. To customize the physicochemical characteristics of IONPs for optimal anti-leishmanial efficacy and minimal host toxicity, meticulous synthesis parameter optimization is necessary. The overwhelming body of comparative research demonstrates that green methods are superior to chemical synthesis,

.particularly in biomedical settings

Extensive in vitro and in vivo studies conducted over the past five years have shown that green

:IONPs have strong anti-leishmanial activity through

Oxidative stress caused by reactive oxygen species (ROS)

Damage to membranes and mitochondria

disruption of trypanothione reductase and gp63, two proteins specific to parasites

Host immunity modification that promotes a protective Th1 response

Crucially, at therapeutic dosages, these nanoparticles exhibit negligible toxicity to mammalian cells and organs and are biocompatible. Because of their magnetic characteristics, systemic

.exposure can be reduced by employing external fields for targeted delivery

Future Scope

Even with the advancements, a number of obstacles must be overcome before clinical translation can take place. Standardization of green synthesis techniques to guarantee batch-to-batch

.reproducibility and GMP compliance should be the main focus of future research

Studies at the molecular level that use CRISPR-based editing and omics to pinpoint the deeper molecular pathways impacted by IONPs

Advanced delivery systems for non-invasive treatment, like magnetically guided IONPs,

.transdermal, or oral

Long-term safety assessments, such as studies on reproductive toxicity, genotoxicity, and

.teratogenicity

.Clinical studies that assess human subjects' safety, pharmacokinetics, and efficacy

Scale-up models and regulatory harmonization to guarantee that these nanotherapeutics

.can be produced in endemic areas at a reasonable cost

Green-synthesized IONPs have the potential to transform leishmaniasis treatment if they are successfully translated, providing a highly targeted, sustainable, and affordable treatment platform

.that is compatible with One Health and green technology

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