Bioinspired synthesis and activity characterization of iron oxide nanoparticles made using Rhamnus Triqueta leaf extract

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Abstract

The present study reports a facile, green, low cost and ecofriendly route for the formation of IONPs using Rhamnus triqueta (RT) leaves extract. The color change of the reaction mixture from dark brown to reddish black determined the formation of IONPs which was further confirmed by the presence of an absorbance peak at 300 nm. The IONPs were further characterized using various characterization techniques; SEM, TEM, XRD, DLS, FT-IR, EDX and Raman. The bioactive functional groups available in the leaves extract of RT involved in reduction and stabilization of IONPs were determined by FTIR analysis. The IONPs were further evaluated for anticancer activity against HepG2 cancer cells (IC50: 11.2 μg ml⁻¹). Dose-dependent cytotoxicity assays were performed against Leishmanial amastigotes (IC50: 25.09 μg ml⁻¹) and promastigotes (IC50: 11.54 μg ml⁻¹). Additionally, cytotoxicity assay was determined against brine shrimps (IC50: 35.37 μg ml⁻¹). Disc-diffusion method displayed significant antibacterial and antifungal activities. Significant antioxidants and enzyme inhibition potentials were revealed. The biocompatibility tests were done against human RBCs and macrophages. Overall, IONPs are non-toxic and shown potential role in multiple biological application. We hope, that the present study could be a helpful contribution and will open new avenues for diverse fields including drug design, pharmaceutical and biomedical industries.

1. Introduction

Nanotechnology is a multidisciplinary technology that uses biosynthetic and eco-friendly methods for the formation of nanoparticles (one billionth part of a meter) (10⁻⁹) [1, 2]. It deals with manipulations and strategies to develop particles ranging from 1 – 100 nm. Nanoparticles (NPs) possess numerous applications in diverse areas such as biomedicine, diagnostics, therapeutics, bioremediation, cosmetics, materials engineering and drug-delivery [3]. NPs have attracted special attention due to their fascinating properties; physical, mechanical, optical, magnetic, electronic, sensing and considerably vary in many aspects to those of their bulk counterparts [4–7]. The scientific community is involved in developing different metal and metal oxides NPs; cobalt, nickel, silver, gold, iron, copper, bismuth, palladium, iron oxide and zinc oxide etc [8, 9]. These different metal nanoparticles (MNPs) nanoparticle have their own unique functional properties based on their size, shape, nature and the way it is synthesized [10]. Among the various MNPs, IONPs is the most appealing multifunction material and has significantly attracted the attention of scientific community [11]. IONPs possess broad range application in various commercial areas; biomedicine, diagnostics, radiology, cosmetics, sensors, pathogens, enzymes, vaccines [3, 12]. These wide range potentials are due to their unique characteristics; high chemical stability, low toxicity, high biocompatibility, magnetic nature, high surface area and small band gap. IONPs are...
present in different forms such as hematite ($\alpha$-Fe$_2$O$_3$), magnetite (Fe$_3$O$_4$), magmatic ($\gamma$-Fe$_2$O$_3$) and goethite (FeO (OH)) [13].

Generally, IONPs are fabricated by number of physical and chemical methods [3, 14, 15]. However, synthesis by conventional methods face numerous challenges such as hazardous, non-ecofriendly, use expensive metal salts, harsh reducing agents, organic solvent and require high temperature and pressure [2, 16]. These physicochemical methods are not only expensive at large scale industrial level, but cannot be employed in designing medicines due to having toxic effects on human health [16, 17]. To overcome these challenges, new ways and methods must be explored that are sustainable, eco-friendly, nontoxic and cost-effective [3, 18]. Therefore, synthesis of NPs using plants has significantly attracted the attention of scientific community due to their simplicity, bioavailability and being green. These methods are cost-effective, eco-friendly, fast and scalable [1]. Plant extracts are comprised of different bioactive clusters; phenolic, alkaloids, flavonoids, polysaccharide, vitamins etc [19–21]. These plant-extracts may function as a potential reducing and stabilizing agents, eliminates multiple steps, reduces cost and avoid use of noxious chemicals agents [2]. IONPs have been prepared from different medicinal plants [3, 22–24]. In current study, Rhamnus triquetra leaves extract were used to prepare iron oxide nanoparticles [25]. R. triquebra is a multipurpose plant used for different remedies. The plant is comprised of high contents of flavonoid; kaempferol (Kaempferol-4-O-methyl ether, Kaempferol-7-O methyl ether), quercetin, emodin, gluside, Physsicon and have shown significant antioxidant, deobstrucent, anti-inflammatory potentials. In traditional medicines, R. triquetra is used for the treatment of dysentery, diarrhea and malarial fever and to cure intestinal worms [26, 27]. The present work reports for the first time green synthesis of IONPs from R. triquetra leaves extract without using any kind of surfactant, organic/inorganic solvent. Further, characterization was done using different spectroscopy and microscopy techniques. The IONPs possessed all properties of active nanoparticles. In addition, multiple biological applications were performed to determine their bio-potentials.

2. Materials and methods

2.1. Rhamnus triquetra leaves extract preparation

The medicinal plant Rhamnus triquetra (Rhamnaceae) was collected from upper mountainous area of Pir Sohawa, Islamabad, Pakistan and was taxonomically identified by Dr Sayed Afzal Shah Department of Plant Sciences, Quaid-i-Azam university Islamabad. The plant leaves were properly washed using distil water to remove dust and other particulate material, shade dried for ~15–20 days to remove water content. The dried leaves were carefully grounded into fine powder and were stored in airtight container in the Lab. Furthermore, 30 gram leaves powder of R. triquetra was suspended in 300 mL of distil water. The suspension was continuously stirred and heated at 80 °C for ~100 min. The solutions obtained were cooled and filtered 3–4 times with the help of Whatman filter papers to remove any kind of coarse materials and impurity. The clear leaves extract obtained was used for the biosynthesis of nanoparticles.

2.2. Green synthesis of the targeted IONPs

The iron oxide nanoparticles were synthesized by adding 3 gm of ferric acetate basic (hydrous) into 50 mL of filtered R. triquetra leaves extract. The reaction mixture was placed on hot plate and heated at ~70 °C for 2 hr with constant stirring to ensure maximum reactions. After 2 hr, reaction was cooled and pH was checked, hence determined as 8.53. Centrifugation was done for obtained solution at 3000 rpm for 30 min. The pellet comprised of IONPs was pipetted 3-4 times with distil water followed by centrifugation to remove iron ions and R. triquetra leaves extract residues. The resulting powder, considered as IONPs were incubated at 85 °C for 2 hr. Furthermore, IONPs were calcined in open air furnace at 400 °C for 2 hr to obtain pure IONPs. Later on, IONPs were placed in dry, cool and dark place and were extensively characterized using different techniques. Figure 1 shows study plane of IONPs.

2.3. Physico-chemical characterization of IONPs

The bioinspired IONPs were done with various characterization techniques (UV, FTIR, XRD, SEM, TEM, EDS, Raman, DLS). Ultraviolet visible (UV–vis) spectrophotometer (UV–4000 spectrophotometer, Germany) was used to investigate the reduction rate of metal ions and progress in biosynthesis of IONPs. Aliquots taken from synthesized IONPs were scanned in the range of 200–600 nm wavelength while the spectrum background was corrected against DMSO. The FTIR spectra were recorded using FTIR Spectrometer (Alpha, Bruker, Germany) to characterize the possible biomolecules responsible for reduction, effective stabilizations and capping of IONPs via KBr pellet method in the range 500–5000 cm$^{-1}$. The spectrum was analyzed using Origin Pro 8.5 software. Further, vibrational properties of IONPs were investigated using Raman spectroscopy.
The XRD analysis was done for IONPs on x-ray diffractometers (PANalytical, Netherland) armed with Cu radiations source at voltage of 45 kV and of 40 mA current and mean crystalline size was recorded via Scherrer approximation. The EDX analyses was studied to investigate other elements associated with IONPs. The EDX values reflect the atomic structure of biogenic NPs and shows the atomic content of nanoparticles. SEM (EM (NOVA-FEISEM-450 linked to EDX-detectors)) was utilized to image and study the size and morphology of IONPs. In addition, TEM analysis was done to exactly calculate the size dispersities and shape of IONPs. The zeta potential, polydispersity index (PDI), hydrodynamic size distribution were investigated by DLS using Zeta-sizer equipment (Malvern Zetasizer Nano). For this purpose, 200 μg of IONPs were suspended in 2 mL of deionized water (Milli-Q) by bath sonication (Elmasonic E-30 H Germany) for 40 min and suspension was transferred to a folded capillary cell for zeta potential and conductivity measurements.

2.4. Preparation of test sample
The colloidal suspension of biosynthesized IONPs was prepared as a test sample (10 mg IONPs dissolved in 99% DMSO to the final concentration of 10 mg ml⁻¹) for the investigation of different in vitro biological applications. Moreover, stability of colloidal suspensions was explored by adding 10 mg IONPs into 10 ml of DMSO. The slightly turbid colloidal suspensions was obtained. The suspension was further filtered and allowed to stand. The UV-spectra were observed after regular time intervals for 60 hr to check their stability.
2.5. Bio-potentials of IONPs

2.5.1. Antileishmanial potential

The antileishmanial potential of R. triqueta mediated iron nanoparticles against L. tropica’ KWH23 strain’ (collected from Khyber Medical Hospital, Peshawar Pakistan) (promastigote and amastigote) was evaluated by MTT cells viability assay [2, 28]. For this purpose, both types of parasites promastigotes and amastigotes were cultured on M199 media containing 10% FBS. Different concentrations of IONPs were tested against L. tropica’ KWH23 ranging from 3.91 – 500 μg ml⁻¹ to investigate the antileishmanial potentials. The reaction mixture (200 μl) is composed of 100 μl of the standardized culture, 50 μL fresh media and 50 μl of colloidal nanoparticles suspension in DMSO. The amphotericin B was employed as positive and DMSO as negative controls. The 96-well plate containing iron oxide nanoparticles were incubated at 24 °C / 72 hr in 5% carbon-dioxide incubator. After treatment and incubation with IONPs, readings were recorded at 540 nm using micro-plate readers. Median lethal concentration (IC50) was calculated using GraphPad software while percent inhibition was calculated using the following formula;

\[
\text{%inhibition} = \frac{1 - \text{sample absorbance}}{\text{absorbance of control}} \times 100
\]

2.5.2. Anticancer potential

HepG2 (Hepatocellular carcinoma) (RCB1648) cancer cells were used for the determination of anticancer potential of bioinspired IONPs by the MTT assay in DMEM media loaded with 10% FBS, streptomycins (1%) and penicillin (1%) [1]. The HepG2 cells were cultivated in 5% carbon-dioxide incubator at 37 °C. The MTT assay was carried out in micro-plate using various doses of IONPs (9.375 – 1200 μg ml⁻¹) for ~48 hr. For this purpose, 20 μl MTT solutions was added in each well of the 96-well microplate and was shifted in incubator for 3 hr. The DMEM media was replaced with DMSO (100 μl) and was further incubated for 25 min. The quantity of formazans produced by living cells was determined by plate reader at 570 nm and reference wavelength of 630 nm, respectively. The GraphPad software was used to calculate the IC50 value. Untreated cells were considered as control, while % inhibition was calculated as:

\[
\text{%inhibition} = \frac{1 - \text{OD of sample}}{\text{OD of control}} \times 100
\]

2.5.3. Brine shrimp cytotoxicity (BSC)

For assessing cytotoxic potency of R. triqueta mediated IONPs, Artemia salina eggs were incubated for ~24 h / 30 °C in artificial sea water (3.8 g l⁻¹) in the presence of light to get mature nauplii. 30 nauplii were transferred into glass vial through Pasteur pipette having sea water and test sample. Various doses of IONPs ranging from (3.91–500 μg ml⁻¹) were used. The glass vials comprised of sea water and vincristine sulphate and mature nauplii were served as positive controls while vial with sea water, DMSO and mature nauplii served as negative controls. The vials were kept in incubator for 24 hr at 30 °C and dead shrimps were carefully counted in each vial. The IC50 values were recorded for IONPs via GraphPad software to determine their cytotoxicity.

2.5.4. AA (Alpha amylase) inhibition (antidiabetic potential)

For the analysis of AA inhibition potentials, the reaction mixture was prepared by using 25 μl alpha amylase, 40 μl starch solution and 15 μL phosphate-buffer saline, 10 μl IONPs and was added stepwise into micro-plate [29]. The micro-plate with all components was incubated for 30 min at 50 °C. The 90 μl iodine solution and 20 μl (1 M HCL) were added to the mixture after incubating micro-plate for 20 min. The acarbose was served as positive and DMSO as negative controls. The micro-plate reader was used to record the optical density at 540 nm. Median lethal concentration (IC50) was calculated using GraphPad software while percent inhibition was calculated using the following formula;

\[
\text{%inhibition} = \frac{\text{sample absorbance} - \text{absorbance of negative control}}{\text{absorbance of blank} - \text{absorbance of negative control}} \times 100
\]

2.5.5. PK (Protein kinase) inhibition

For the determination of PK inhibition potential, 100 μl of inoculum of Streptomyces 85E strain was cultivated in ISP4 media to get uniform lawns [3, 30]. 6 mm sterilized filter disc impregnated with IONPs (10 μl) were cautiously kept on microbial lawns and incubated at 30 °C / 72 hr to target the growth of Streptomyces 85E strains. After 72 hr, different bald and clear zones appeared around the discs. These zones determine the inhibition of spores and formation of mycelia in Streptomyces 85E strain. Vernier caliper was used to measure the zone of inhibition (ZOI) in millimeter. The surfactin was served as positive and DMSO as negative controls.
2.5.6. Antifungal activity of IONPs
Various fungal strains were used for the investigation of antifungal potentials of IONPs by disc diffusion method on the surface of autoclaved SDA media [16]. To cultivate the fungal strains, 100 μl broth culture was spread on SDA media with the help of autoclaved cotton swab. Different concentrations of IONPs were utilized ranging from 37.5 – 1200 μg ml⁻¹. Filter discs were laden with 10 μL of IONPs, incubated at 37 °C/ 24 hr and ZOI were observed after 15 hr. The Amp B was served as positive and DMSO as a negative controls.

2.5.7. Antibacterial activity of IONPs
In *vitro* antibacterial activities of *R. triquetra* mediated IONPs were assessed against different bacterial strains. The bacterial strains were spread on nutrient agar using cotton swabs. Disc-diffusion method was used to determine the antibacterial potentials. The filter disc loaded with 10 μL of each IONPs (with dilution 37.5 – 1200 μg ml⁻¹) were placed on bacterial lawns and were incubated at 37 °C/ 24 hr. Oxytetracycline was served as positive control and were observed with regular intervals for the appearance of ZOI. MIC values of the nanoparticles were recorded with producing ZOI.

2.5.8. Biocompatibility of IONPs with human macrophages
Human macrophages were used to examine the biocompatibility of IONPs. To achieve this purpose, Ficoll –Gastrografin mechanisms was used to extract human macrophages. The biocompatibility assay was performed gradually by dissolving 5.7 gram of Ficoll into 95 ml of distil water with 5 ml of Gastrografin. The isolated blood cells were diluted with hank’s buffer salt solutions and were layered on Ficoll –Gastrografins and centrifugation was done at 400 × g for 30 min. The cells purification was done utilizing percoll gradient adjusted with distil water. The cells were grown in RPMI media with 10% FBS, 25 mM Hepes, antibiotic (Penicillins: 100 U ml⁻¹, streptomycins: 0.1 mg ml⁻¹) and incubated in 5% CO₂ incubator. The % inhibition of IONPs was calculated against human macrophages employing the formula below.

\[
\% \text{ inhibition} = \frac{1 - \text{Abs sample}}{\text{Abs control}} \times 100
\]

2.5.9. Biocompatibility of IONPs with human RBCs
The hemocompatibility assay was done to confirm the biosafe nature of IONPs with human RBCs [3, 31]. In order to perform biocompatibility assay, 90 μl of freshly obtained human RBC were taken in EDTA tube and were centrifuged at 13 000 rpm for 10 min. Then the obtained pallet was washed three times with PBS (phosphate-buffer salines). The erythrocytes suspensions was prepared by mixing 200 μl erythrocytes into 9.8 mL PBS. The 100 μl of erythrocyte suspension was added to various concentration of IONPs, incubated for 1 hr at 35 °C and were centrifuged at 10 000 r pm/10 min. The supernatant was shifted into 96-well plate and hemoglobin release was studied at 540 nm employing micro plate reader. The Triton X-100 was employed as positive while DMSO as negative controls. The results are calculated as % hemolysis produced by different concentration of IONPs and can be calculated employing the formula below:

\[
\% \text{ hemolysis} = \left[ \text{Sample}_{\text{abs}} - \text{Neg.control}_{\text{abs}} \right] / \text{Pos.control}_{\text{abs}} \times 100
\]

2.5.10. Antioxidant capacities of IONPs
The spectrophotometric method was employed to evaluate the free radical scavenging potency of iron oxide nanoparticles. For this purpose, 2.4 mg DPPH was added into methanol (25 ml) as a free radicals and was properly vortexed. Next we used different concentrations of IONPs in the range of 3.91 – 500 μg ml⁻¹ to explore the free radicals quenching potential. The ascorbic acid (AA) was served as positive and the DMSO as negative controls. Moreover, 20 μL of IONPs and 180 μL of reagent solution was loaded in 96-well plate and incubated in dark for 1 hr. The readings were recorded at 517 nm using microplate reader. The formula below was used to calculate the % scavenging of DPPH.

\[
\% \text{ DPPH scavanging activity} = 1 - \frac{\text{Abs sample}}{\text{Abs control}} \times 100
\]

In addition, total reducing power of test sample (IONPs) was examined using previously described [32, 33]. The AA served as positive while DMSO as negative controls. The microplate reader was used to record the absorbance capacity at 630 nm. The result of reducing power are shown in terms of μg ascorbic acids equivalent per mg (μg AA/mg) of nanoparticles. Further, the total antioxidants capacity (TAC) was evaluated utilizing phosphomollybdenum standard method [32]. The absorbance was calculated at 695 nm utilizing microplate reader. The AA was employed as positive while DMSO as negative controls.
control to better compare total antioxidant potential of IONPs. The results are expressed as number of ascorbic acid equivalents in microgram per milligram of the sample, in other words, \( \mu g \text{AAE/mg} \).

### 3. Results and discussion

The plant mediated synthesis of NPs initiates, once precursor salt ferric acetate basic \((C_{4}H_{10}FeO_{5})\) was introduced into \( R. \) *triquetra* leaves extract. The gradual color change of \( \text{Fe(C}_{2}H_{3}O_{2})_{2}/R. \) *triquetra* solution at 60 °C from dark brown to reddish black determine the indication of formation iron oxide nanoparticle. This color change of plant extract is because of surface plasmon vibrations, an optical property exclusive to noble metals [33]. The biosynthesis of iron oxide nanoparticles was further evaluated using different characterization techniques such as UV, SEM, TEM, XRD, EDX, FT-IR, Raman, zeta size and zeta potential.

The production of the IONPs in aqueous environment was further determined by the UV-visible spectroscopy. Figure 2(a) illustrates UV–vis spectra of IONPs was scanned at every 1 hr between 200−600 nm. This maximum absorbance peak showed the reduction of salt to IONPs, which is within the range of surface plasmon resonance (in the range of 318 nm−608 nm) of IONPs (35). A stable reading was recorded at 300 nm until 48 hr. The reduction in the absorption peak after 48 hr corresponds to the settlement of IONPs at the bottom of the falcon. The UV–vis spectral analysis depicts valuable information regarding the size, shape and stability of NPs [34].

FTIR spectrum was used to analyze the presence of possible biomolecules responsible for the synthesis and effective stabilizations of iron oxide nanoparticles. Figure 2(b) shows FTIR spectra of biosynthesized IONPs. FTIR spectra of IONPs revealed significant peaks at 3741 cm\(^{-1}\), 3028.20 cm\(^{-1}\) and 1106.51 cm\(^{-1}\) which are attributed to stretching vibration of OH, CH, and CN bonds. However, the peak appearing at 526.51 cm\(^{-1}\) represents Fe–O bond vibrations from hematite phase \( \alpha-\text{Fe}_{2}O_{3} \). Furthermore, the Raman spectra revealed the position of major modes of raman at 227.04 cm\(^{-1}\) (A\(_{g}\)), 273.31 cm\(^{-1}\) (Eg), 391.33 cm\(^{-1}\) (Eg), 497.02 (A\(_{g}\)) and 606.31 (Eg). The intense peak at 1306 cm\(^{-1}\) is assigned to two magnon scattering which arise from the interaction of two magnons created on antiparallel close spin sites. No other peaks were detected, indicating that
this simple method yields only $\alpha$-Fe$_2$O$_3$ free from other iron oxides and iron oxyhydroxides. Our results of Raman spectra are in line with previous reports of green IONPs thus confirm the biosynthesis of IONPs [35]. Figure 2(c) illustrated the Raman peaks of IONPs. Moreover, the position of Raman mode or Raman peak may change with the difference in synthesis protocol and distributions of vacancies in the unit cell [31].

XRD analysis was done to confirm the crystalline nature of bioinspired IONPs. The XRD pattern of synthesized IONPs (figure 3(a)) indicates the diffraction bands at 24.37, 33.22, 35.71, 40.96, 43.82, 49.52, 54.08, 57.67, 62.51 and 64.16 representing (012), (104), (110), (113), (202), (024), (116), (018), (214) and (300) crystallographic planes respectively. The observed Bragg peaks of $R$. triqueta mediated IONPs are in agreement with the pure phase hematite IONPs with JCPD card no: 079–1741. The sharp peaks show high crystalline nature of IONPs. The mean size of IONPs was calculated $\sim$21 nm via Scherer’s approximation. No other peaks relating to other impurities were observed indicating single crystalline phase of IONPs. The XRD analysis of IONPs is in line with previously published reports [36–38].

EDX analyses was studied to confirm elemental compositions of IONPs. The EDX peaks indicated strong signals at 0.3 KeV for both iron and oxygen. The appearance of ‘carbon’ is attributed to the grid support while no other significant element has been found in the EDS spectrum apart from ‘Fe’ and oxygen ‘O’ which relates the single phase purity of the NPs. The EDX spectra are indicated in figure 3(b). The reduction of precursor ferric acetate basic occurred frequently upon the contact of ferric acetate with the leaves extract of $R$. triqueta. Several researchers have identified different flavonoid compounds Kaempferol-7-O methyl ether, quercetin, Kaempferol-4-O-methyl ether, emodin, gluside, Physicon 8 B-D gluside etc) in the leaves extract of $R$. triqueta.
These compounds present in the water extract of *R. triquetra* bind on the surface of metal ions and play a significant role in stabilizing NPs.

Figures 4(a)–(c) shows the SEM images of *R. triquetra* leaves extract stabilized IONPs at different magnifications, which has confirmed that biogenic IONPs are spherical in shape. The fabricated IONPs were further characterized by TEM to determine size and morphology of nanoparticles. After TEM analysis, the average particle size of the bioinspired IONPs was found to be ~21 nm. The TEM analysis determines the synthesis of IONPs which were found to be spherical in shape (figure 4(d)).

The size distributions, PDI and zeta potential of *R. triquetra* mediated IONPs were analyzed through DLS analysis. The DLS analysis results have indicated large particles aggregates of 293.4 d.nm. The average zeta potential and PDI of IONPs were -16.4 mV and 1.00 (figures 5(a), (b)). Our results corroborate with earlier reports by other researchers [39, 40]. DLS is mainly used to determine the size of nanoparticles in colloidal suspension at nanosize scale and PDI values that suggest moderate polydispersity. The ζ-potential is based on particles movement under an electric field. The surface charge and local environments of the nanoparticles also play a promising role in measuring ζ-potentials [16].

### 3.1. Antileishmanial activity

The current drugs present in the market are not enough and are often costly, toxic and less effective. Therefore, significant research work has been done to design different nanoparticles that can be assess for their cytotoxic potential against *L*. parasites [1, 16, 41]. However, green IONPs are rarely studied to determine cytotoxic potential against *L. tropica*. In recent work, antileishmanial potential of green IONPs were investigated against *L. tropica* as shown in figure 6(a). The leishmania parasites were treated with different doses of IONPs (3.91 – 500 μg ml⁻¹) for 72 hr and showed concentration-dependent response. The antileishmaniasis potency was increasing as concentrations of IONPs was increasing. The IONPs showed significant potentials against *L. tropica* promastigote (IC₅₀: 11.54 μg ml⁻¹). Similarly, antileishmanial potential of IONPs was also reported against *L. tropica* amastigote (IC₅₀: 25.09 μg ml⁻¹). Our results are in line with previous reports of green IONPs using *S. thea* [32]. The dose-dependent response and lower IC₅₀ value for IONPs demonstrate that these particles may be used for targeted drug delivery in nano-pharma industries for the treatment of leishmaniasis.
3.2. Anticancer activity
Cancer is a deadly disease around the globe and rate of incidence of cancer is constantly increasing and is predicted to be ∼21 million by 2030 [3, 42]. The scientists are working hard to overcome this menace by formulating novel drugs with strong therapeutic potentials [43, 44]. For this purpose, anti-hepatocellular carcinoma potential of IONPs was explored against HepG2 cells line. The HepG2 cells were exposed to different doses of IONPs ranging from 9.375−1200 μgm l−1 for ∼24 hr and caused dose-dependent inhibitions against HepG2 cells. Our results of R. triquetra mediated IONPs have indicated strong anticancer potency against HepG2 cancer cell line. The IONPs has reported 90% mortality at 1200 μgm l−1, while anticancer effect was reducing as concentrations of IONPs was reducing. The IC50 value recorded was 11.2 μgm l−1 and showed strong potency. Our results are consistent with earlier studies of biosynthesized IONPs from Rhamnus virgata using HepG2 cancer cells [3, 45, 46]. The results are shown in figure 6(b).

3.3. Brine shrimp cytotoxicity (BSC)
The BSC assay was determined to confirm the cytotoxic potency of IONPs against freshly hatched A. salina. BSC is most suitable screening test to confirm the biological potentials of compounds [47]. The cytotoxicity activity of IONPs were investigated using Artemia salina. Figure 6(c) indicates % mortality of IONPs at different concentrations ranging from 3.91−500 μgm l−1. The BSC assay of IONPs has shown dose-dependent response. However, none of the tested IONPs concentration has given % inhibition greater than Vincristine sulphate (positive control). The IC50 for IONPs was calculated as 35.37 μgm l−1 while 1.976 μgm l−1 for vincristine sulphate. Our test sample (IONPs) is considered to be in the category of general cytotoxicity with IC50 values of 35.37 μgm l−1. Our results of R. triquetra IONPs are consistent with earlier studies of IONPs using Sageretia thea and Rhamnus virgata [3, 32].

3.4. Enzyme inhibition potential
The ability of IONPs to inhibit alpha amylase (AA) have been investigated. The IONPs has shown significant inhibition potential ranging from 37.5−1200 μgm l−1 while maximum inhibition (35%) was recorded at 1200 μgm l−1. The AA enzyme perform its functions by converting carbohydrate into glucose [48], thus, blocking function of AA can stop glucose level which is an important area of diabetic research [49]. The IONPs were explored for their AA inhibition potentials. The results have investigated that IONPs were found to be...
potent by inhibiting the AA. The rate of inhibition gradually decreasing with decrease in concentrations of IONPs. However, none of the tested IONPs concentration has given % inhibition greater than positive control (i.e. Surfactin). Figure 7(a) indicate AA inhibition potency of IONPs. Our results of green IONPs corroborate with the Sageretia thea and Rhamnus virgata mediated IONPs [3, 32].

Protein kinase are enzymes that play significant role of adding phosphate group to serine-threonine and tyrosine amino acids which plays a critical role as signalling cascade in the metabolism, cellular differentiation and apoptosis. The deregulated phosphorylations can result in genetic problems and can cause cancer progression. So, any product with potential to inhibit PK enzyme can be of great importance in oncology [48]. Protein kinase phosphorylation play important role in developing hyphae in Streptomyces fungal strain and similar mechanism can be used to explore PK inhibition potentials. The strain served as model to confirm the biomedical value of compounds to determine PK inhibition potentials [50].

Figure 7(b) shows significant inhibition of potential of IONPs against PK enzyme. The PK inhibition potentials was determined using disc-diffusion method. The PK inhibition activity was carried out in sterile condition using different doses of IONPs (37.5 – 1200 μg ml⁻¹). The surfactin was served as positive controls. The ZOI was 25 mm at concentration of 1200 μg ml⁻¹, which show significant PK inhibition potency of IONPs. The study has shown concentration dependent response for IONPs. Our results of IONPs corroborate with the earlier studies where Rhamnus virgata IONPs has shown potential results against PK inhibition and can be further researched for their potential role in cancer therapeutic [3].

3.5. In vitro antimicrobial activities
The new advancements in nanobiotechnology are opening new avenues to develop new substances with special antimicrobial potential to reduce the risk of development and spread of these diseases [51]. For this purpose, antifungal potency of IONPs was studied against Aspergillus flavus (FCBP 0064), Fusarium solani (FCBP 0291), Aspergillus niger (FCBP 0918), Candida albicans (FCBP 478), Mucor racemosus (FCBP 0300) (figure 8(a) using different concentrations of IONPs ranging from 37.5 – 1200 μg ml⁻¹. The Amp B was served as positive controls. Extensive research studies have been done on antibacterial activities of IONPs while limited antifungal assays have been reported. This novel report is for the first investigating antifungal potential of R. triquetra assisted IONPs. Our antifungal activity for IONPs have shown dose-dependent response against different fungal
Figure 7. (a) Inhibition potential of IONPs against alpha amylase (b) Inhibition potential of IONPs against protein kinase.

Figure 8. (a) Antifungal potential of biogenic IONPs (b) Antibacterial potential of biogenic IONPs.
strains. Among the different fungal strains, A. flavus and C. albicans were the least susceptible strains with MIC value of 75 μg ml\(^{-1}\) while A. niger was the most susceptible (MIC: 37.5 μg ml\(^{-1}\)). The biogenic IONPs have shown significant antifungal potential at all tested concentration. However, none of the tested IONPs concentration has given % inhibition greater than Amp B (positive control). Among these different tested strains, Fusarium solani, Mucor racemosus and Aspergillus niger were inhibited at all types of concentration. Lower concentration of test sample (IONPs) was found to be ineffective against Aspergillus flavus and Candida albicans. Earlier research studies reported that ROS and interference of IONPs with fungal spores/hyphae may also result in fungal growth arrest. The literature review and earlier reports have shown significant dose-dependent response for IONPs against different fungal strains [3] and is consistent with the present study.

Figure 8(b) shows inhibition potentials of IONPs against various pathogenic bacterial strain. For this purpose, disc diffusion method was used in the concentrations range of (37.5 – 1200 μ/mL). The gram positive bacteria used were Bacillus subtilis (ATCC 6633), Staphylococcus aureus (ATCC 25923) and gram negative are E. coli (ATCC15224), Pseudomonas aeruginosa (ATCC9721), Klebsiella pneumoniae (ATCC4617). The results determined that most bacteria strains were susceptible to IONPs and shown strong inhibition potential. The IONPs were more effective against B. subtilis and S. aureus (MIC: 37.5 μg ml\(^{-1}\)) and least effective against E. coli and K. pneumoniae (MIC 75 μg ml\(^{-1}\)). The pure oxytetracycline (10 μg) was served as positive control. No single concentrations was found more effective than that of the positive control. In nutshell, we have determined significant antibacterial activity for bioinspired IONPs. The antibacterial properties of IONPs have been already well reported in the earlier reports using different medicinal plants [3, 52]. In addition, our research study determined that antibacterial potency was increasing as the increase in IONPs concentrations. The different fungal and bacterial strains MIC values are provided in figures 8(a), (b).

The researchers have explained different mechanisms for antibacterial property of IONPs. Among these mechanisms, ROS production is the most important mechanism that provide antimicrobial potential to IONPs. In addition to ROS production, some other factors like surface defects in symmetry of NPs, membrane injury due to NPs adsorption on the surface cause cell damage [53]. Furthermore, we consider the importance of biomolecules attached on the surface of nanoparticles from the leaves extract might involve in the stabilization and capping of IONPs as a result they play potential role in inhibiting different bacterial strains.

3.6. Biocompatibility assays

The biocompatible and toxicological nature of IONPs was evaluated against human RBC and macrophages. The bio-safe nature of IONPs was confirmed by performing MTT cytotoxic assay against human macrophages. Research study has concluded dose-dependent inhibition response. The results indicated that IONPs at 500 μg ml\(^{-1}\) inhibited growth of macrophages by ~34% which confirmed the non-toxic behavior of IONPs. Usually macrophages have developed mechanisms to combat reactive oxygen species produced from external sources. Thus, ROS at lower quantity is not toxic to human erythrocytes and macrophages unless its concentration increases beyond the limit [54]. The IC\(_{50}\) was recorded as 865.5 μg ml\(^{-1}\).

The biocompatible and toxicological nature of IONPs was also evaluated against human RBC. The hemolysis was determined as hemoglobin releases by rupturing of RBC after treatment with IONPs. The biocompatibility nature of IONPs was investigated against human RBCs using hemolytic assay in the range of 3.91–500 μg ml\(^{-1}\). The current research study has confirmed that green IONPs are not hemolytic at lower concentrations of 7.81 μg ml\(^{-1}\) while showed hemolysis at greater than 62.5 μg ml\(^{-1}\). The results of R. triquetra mediated IONPs were found to be consistent with the earlier reports of IONPs using R. virgata and Sageretia thea [2, 35]. The IC\(_{50}\) value calculated as >2872 μg ml\(^{-1}\). Our study concluded that green IONPs are non-hemolytic at low concentration against RBCs confirming biocompatibility and nan-hazardous nature of IONPs. The biocompatibility assays results are given in figure 9(a).

3.7. Antioxidant activities of IONPs

Figure 9(b) illustrate the different antioxidant potentials of R. triquetra IONPs. The antioxidants activities were carried out for IONPs in concentration ranging from 3.91–500 μg ml\(^{-1}\). Maximum values of total antioxidants in terms of ascorbic acid equivalents/mg was 56.81% at 500 μg ml\(^{-1}\). These values were determined from the regression equation obtained from standard ascorbic acid curve (y = 0.0413 × + 0.3565). The TAC has revealed high antioxidant nature of IONPs towards ROS. Since in this study, R. triquetra leaf extract was used as a strong reducing and capping agents. It is assumed, that some flavonoids quench ROS which are available on the surface of IONPs.

The presence of adsorbed antioxidant species on IONPs were further confirmed using total reducing power (TRP) assay. This mechanism was developed to explore reductones involved in antioxidant potentials of IONPs by providing H-atom and may cause damage to free radical chain [35]. The R. triquetra mediated IONPs showed strong antioxidant potential and was found to be concentration dependent. The reducing power was decreasing...
as the concentrations was decreases. The maximum reducing power (50.93%) was recorded at 500 μg ml⁻¹. Strong DPPH radical scavenging potentials of IONPs (79.83%) was reported at 500 μg ml⁻¹. From the results indicated in figure 9(b), it can be deduced that some antioxidants compound may be involved in reducing and stabilizing IONPs using leaves extract of R. triquetra. Our results of IONPs corroborate with earlier reports of S. thea and R. virgata mediated IONPs [32, 56]. The range of variations and disagreement with other research studies might be due to experimental condition (seasonal variations, humidity and location), route of NPs synthesis, medicinal plant used and size of NPs [3].

4. Conclusion and future outlook

The current study showed an eco-friendly and nontoxic method for the synthesis of IONPs using Rhamnus triqueta leaves extract without using any toxic chemical. This method is simple, cost-effective, energy efficient and biocompatible for the formation of IONPs. Furthermore, IONPs were characterized using various spectroscopic and microscopic techniques to elucidate stability and functionality of IONPs. In addition, IONPs were exposed to different biological evaluations (antibacterial, antifungal, anticancer, antileishmanial, biocompatibility assays and antioxidant activities). However, it was determined that therapeutic potentials of IONPs varied with different concentration of IONPs used. In future, considering the evolution of disease resistance to currently available drugs, it is expected that biogenic IONPs could become a potential antimicrobial and cytotoxic agents alone or in combination with other FDA approved drugs. In summary, further in vivo studies are recommended on the toxicity aspects in different animal models.

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