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Original Article

ONE STEP SYNTHESIS OF IRON OXIDE NANOPARTICLES VIA CHEMICAL AND GREEN ROUTE– AN EFFECTIVE COMPARISON

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ABSTRACT

Objective: Magnetic Iron oxide nanoparticles (IONP) are of potential use in the field of biomedical, bioengineering particularly *in vivo* applications like tissue repair, drug delivery. However, biocompatibility of the nanoparticles is of great concern. Hence in this manuscript, we compare suitability using IONP prepared by two different routes namely chemical and green, for the biological applications.

Methods: In the green route, *Desmodium gangeticum* root extract was used as the reducing agent with no specific capping agent for the synthesis of nanoparticles unlike chemical route, where propylene glycol was used. The synthesised nanoparticles were characterized and compared by UV-Vis spectrophotometry, X-ray Diffractometry (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Zeta analyser and Vibrating Sample Magnetometry (VSM).

Results: The results were similar except for that the size of green synthesised IONP was reduced and possesses even distribution (i.e. mono dispersed). Biological activity, as assessed by its free-radical scavenging potential and anti-microbial effect was found to be better in the case of green IONP. Toxicity studies using LLC-PK1 cell line shows relatively low toxicity of green synthesised nanoparticles.

Conclusion: Biologically synthesized IONPshow significant antioxidant effect,retains magnetic behaviour and found to be less toxic, therby proving its compatible nature as required in biomedical applications.

Keywords*: Desmodium gangeticum*, VSM, Antioxidant, Antimicrobial, Cytotoxicity.

INTRODUCTION

The last decade has seen an increasing interest in developing materials at nanoscale. The progress made in nanotechnology has made the scientists to characterize nanomaterial with excellent properties [1]. Magnetic nanoparticles are explored for various biomedical applications such as targeted drug delivery, cell sorting, contrast agents for magnetic resonance imaging (MRI), and hyperthermia [2].

Iron oxide nanoparticle (IONP) based delivery systems have many advantages over others. They are biodegradable, biocompatible and magnetic and thus controllable by an external magnetic field [3]. These attributes add to their prospective biomedical and healthcare applications. However, the increased exposure to these nanoparticles could be potentially hazardous to human systems. Risk assessment is the major area of research, at present. The chemicals used for the synthesis of nanoparticles increase the toxicity issue [4]. A recent review of magnetic IONP-induced toxicity studies at the cellular level in animal and human cells indicated that the nanoparticles can penetrate the cellular system by both passive diffusion and endocytosis, causing several toxic effects through the alteration of genes expressions and the generation of oxidative radicals [5]. Hence, a nano delivery system needs to be developed in order to reduce the ROS generation and thereby, cellular damage.

Desmodium gangeticum (Fabaceae family) (DG) is a small shrub found in tropical countries, is widely used in the indigenous system of medicine and also reported to contain flavones and isoflavanoid glycosides [6]. Alkaloids, terocarpnoid, flavonoid, saponins and isoflavonoid glycoside were in the extract and report to have high reducing property. DG extract is widely used as the therapeutic agent in many Ayurvedic preparations due to its significant antioxidant potential [7]. It has high antioxidant potential and anti-apoptotic activity [8]. Hence, could be used to quench the free radicals generated at the time of cellular injury.

The main aim of this study is to compare and contrast the structural characterisation, biological effects and toxicity of IONP synthesized via chemical and green route.

MATERIALS AND METHODS

Materials

Iron (III) chloride ($FeCl_3$), Iron (II) chloride ($FeCl_2$), Ammonia solution (NH_3), Propylene glycol ($C_3H_8O_2$), DPPH (2, 2-diphenyl-1-picrylhydrazyl), Folin-Ciocalteu (FC) reagent, Sodium carbonate (Na_2CO_3), Potassium hexacyanoferrate ($Na_4Fe(CN)_6$), Trichloroacetic acid (TCA), Sodium cyanide (NaCN), Nitroblue tetrazolium (NBT), Riboflavin, ethanol were purchased from Sigma Aldrich, India. LLC-PK1 cell line was purchased from NCCS Pune, India. All the chemicals were used without any further purification.

Preparation of aqueous DG extract

Roots of *Desmodium gangeticum* (Linn) DC were collected from St. Berchmans College Botanical garden, Changanassery, Kerala, India and were authenticated by HOD, Botany Department, SB College. The voucher of the specimen was kept for reference. Dried roots were washed thrice thoroughly with tap water and dried in the shade. Dried roots were cut into small pieces and ground coarsely using pulverizer. Coarse root powder was extracted using distilled water in the Soxhlet apparatus. The crude extract obtained was dried, lyophilised to powder form that was stored in the refrigerator for further use.

Synthesis of iron oxide nanoparticles

IONP was prepared by chemical (I) and green (II) routes.

(I) IONP was synthesised by an improved chemical co-precipitation method [9]. A solution of $FeCl_3$ (0.1M) and $FeCl_2$ (0.1M) was mixed in molar ratio 1:2 in 150 mL distilled water with 10 mL propylene glycol. Under constant agitation, a concentrated solution of ammonia was added dropwise slowly until pH reached 9.5. The solution was stirred continuously at 40 °C for 30 minutes. The black solid precipitate was washed a dozen times until pH was 7 and dried at 40 °C for 48 hours to obtain magnetic iron oxide nanoparticles.

(II) IONP were prepared by adding 10 mM Iron (III) chloride and Iron (II) chloride solution to the aqueous DG root extract (2.5 mg/ml) in 1:1 and 1:2 ratio [10]. The mixture was heated under continuous stirring for 30 minutes at two different temperatures (40, 80) and 80 °C was found to be appropriate with respect to the formation of black coloured product. The nanoparticles synthesized in 1:2 ratio was found to be better to compensate for the lack of inert gas environment and the use of surfactants. Then, the particles were dried and further characterization was carried out.

Characterization

Preliminary examination of the synthesised nanoparticles was carried out by UV-visible spectrophotometer. FTIR spectral analysis

was carried out in the Perkin Elmer spectrum 1 in diffuse reflection mode operating at a resolution of 4 cm-1, recorded between 4000 and 400 cm^{-1}. XRD measurement was carried out with Rigako instrument that was operated at 40 kV voltage and 30 mA current with CuKα radiation (1.5405 Å). The average hydrodynamic particle size and potential was measured by Malvern Zeta Sizer. The Malvern Zetasizer measures particle size from below a nanometer to several microns using dynamic light scattering. Measurements were done at 25 °C after the dilution of samples in deionized water (RI 1.330, Viscosity 0.8872). Zeta potential was analyzed at 25 °C using electrophoretic light scattering technique. Magnetic measurements were carried out on a Vibrating Sample Magnetometer (Make and Model: Lakeshore, USA; Model 7404) using maximum magnetic field of 2.17 T (0.64" air gap); Dynamic moment range: 1x10e-7 emu–10e3 emu with moment & field accuracy of 1 %.

Antioxidant assays

IONP due to their magnetic behaviour are widely used in the biological systems for drug delivery and is very important to understand its scavenging activity and its behaviour with biomaterials. Radical scavenging assays are studied for the synthesised IONP by DPPH (2, 2-Diphenyl-2-picrylhydrazyl) assay [11] and superoxide radical scavenging assay [12]. The synthesized IONP by both green and chemical route was taken in different concentrations (1000 to 125 µg/ml) along with the precursors (iron chloride and DG extract) for the study. The absorbance was measured at 518 nm and 560 nm respectively for both the assays. The phenolic content was also studied for the nanoparticles by Folin-Ciocalteu assay whose absorbance was measured at 765 nm.

Toxicology

Cytotoxicity of IONP was determined by lactate dehydrogenase activity (LDH) in the culture medium. This assay determines the release of cytoplasmic enzyme lactate dehydrogenase to the culture media due to its leakage from the damaged cells in the presence of the nanoparticles. Cells were seeded in 96 well plates and were treated with synthesized Fe NP, along with the precursor separately. After 24 hrs of treatment, a small aliquot of the medium was collected and incubated with a reaction mixture containing NAD+, lactate and phosphate buffer (0.2 M, pH 7.4), each 50 µl. The LDH catalysed conversion results in the reduction of NAD+to NADH, which was measured at an absorbance of 340 nm.

Statistical analysis

In the biological evaluation assays like DPPH, reducing potential, total phenol and cytotoxicity, the data signify the mean±SD of three samples and are representatives of three independent experiments. The data were subjected to analysis of variance (ANOVA) by graph pad prism 5.

RESULTS AND DISCUSSION

Physiochemical properties

The end product obtained after the chemical and green synthetic routes was black IONP, confirmed initially by UV-Vis Spectroscopy. Research suggests that when the size of a nanoparticle decreases the absorption edge is shifted to shorter wavelengths [13]. IONP prepared by green route showed an absorption maximum at 303 nm (fig. 1A) while nanoparticles synthesised by chemical route exhibited a surface plasmon resonance at 523 nm (fig. 1B). The difference in scale could be attributed to the relatively less particle size of IONP synthesised via the green route.

X-ray diffraction patterns have been widely used to characterize critical features such as a crystal structure, crystallite size, and strain. The XRD pattern of IONP synthesized from plant extract showed sharp diffraction peaks at 2θ angles of 24.2 °, 32.2 °, 54.1 °, 40.9 °, 49.5 °, 57.3 °, 62.5 ° and 64.0 ° corresponding to the hkl values (fig. 2A) from (012), (104), (116), (113), (024), (122), (214), (300) crystal planes respectively (JCPDF # 089-2810). Chemically synthesized IONP showed peaks at 28.34, 31.74, 33.04, 35.53, 40.51, 49.48, 54.01, 62.52, 63.98, 66.31, 75.57, 71.88 (fig. 2B) corresponding to (012), (104), (116), (113), (024), (122), (214), (300) crystal planes respectively (JCPDF # 089-2810). The results represents rhombohedral crystalline phase of IONP [14]. The particle size, as calculated by Debye-Scherer equation was found to be 6.30 nm in case of green method and 9.05 nm for chemical IONP.

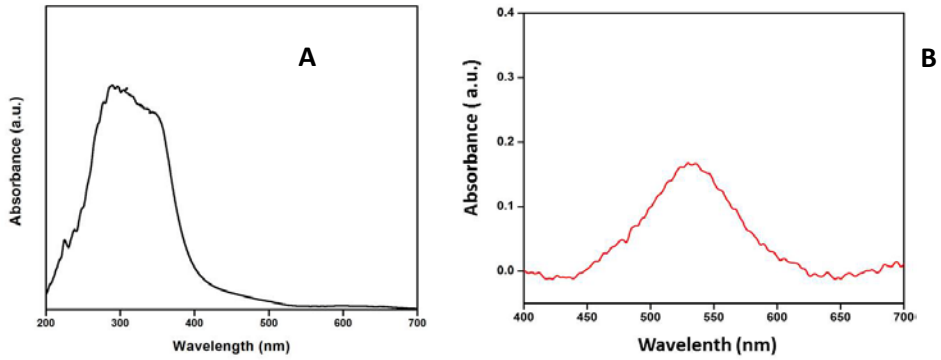

Fig. 1: Characterization of IONP by UV
(A) UV-Visible spectrum of Green synthesised IONP were measured at a range of 300-800 nm and showed broad peak at 303 nm (B) UV-Visible spectrum of chemical IONP showed a broad peak at nm

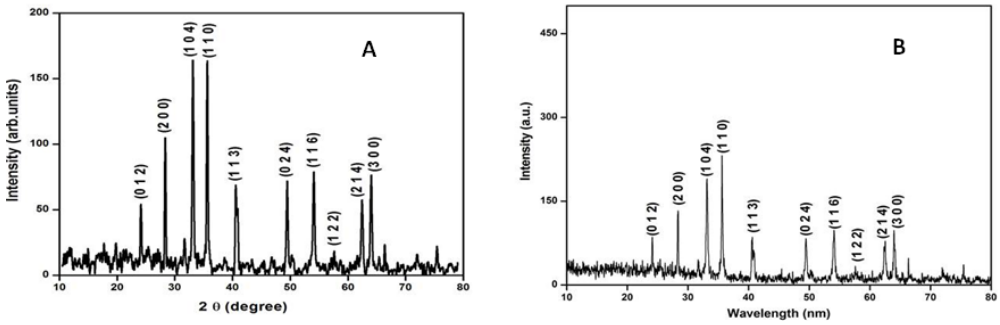

Fig. 2: X-Ray Diffractometry of IONP

A & B X-Ray Diffractometry of IONP (Rigako instrument) synthesised by green and chemical were measured and showed sharp diffraction peaks at 2θ angles of 24.2 °, 32.2 °, 54.1 °, 40.9 °, 49.5 °, 57.3 °, 62.5 ° and 64.0 ° corresponding to the hkl values from (012), (104), (116), (113), (024), (122), (214), (300) crystal planes respectively.

FTIR analysis was performed to identify the interaction between different species and changes in chemical composition of the mixture. For the green IONP, the broad peaks at 3399.98 cm^{-1}, 1630.09 cm^{-1}, 1404.49 cm^{-1}, 1063.80 cm^{-1}, 680.49 cm^{-1}refers to O-H stretch, alkenyl C=C stretch, carboxyl groups, C-O is stretching vibration and alkene C-H bend respectively (fig. 3A). The formation of IONP is characterized by the peak at 571.14 cm^{-1} that correspond to Fe-O stretching bond. Fig. 3B shows the spectra of IONP synthesized by chemical route. Broad peaks at 3793.61, 3394.32, 2929.69, 1625.85, 1400.32, 1048.53, 920.19, 630.94 in case of chemical IONP indicates O-H stretch, alkyne C-H stretch, C-H bend, C=C stretch, carboxyl groups respectively.

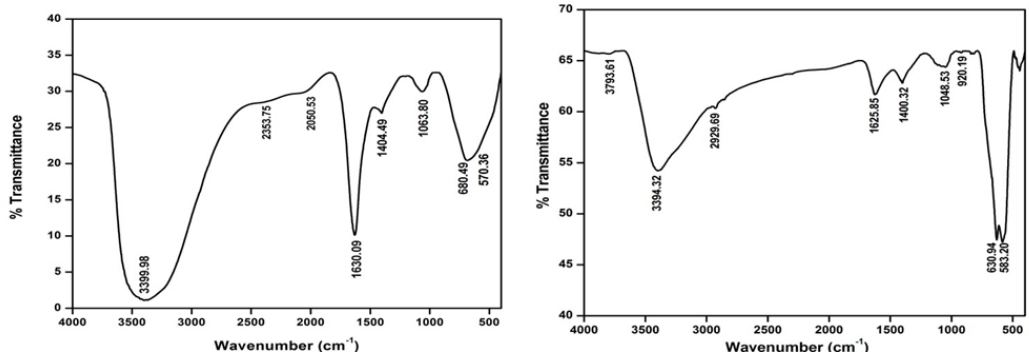

Fig. 3: Fourier Transform Infrared Spectra of IONP

(A) Fourier Transform Infrared Spectroscopy of green IONP (Perkin Elmer spectrum 1) were measured between 400-4000 cm^{-1}showing broad peaks at 3399.98 cm^{-1}, 1630.09 cm^{-1}, 1404.49 cm^{-1}, 1063.80 cm^{-1}, 680.49 cm^{-1} (B) Fourier Transform Infrared Spectroscopy of chemical IONP (Perkin Elmer spectrum 1) was measured between 400-4000 cm^{-1}showing peaks at 3394.32 cm^{-1}, 2929.69 cm^{-1}, 1625.85 cm^{-1}, 1404.32 cm^{-1}, 583.20 cm^{-1}

Magnetic properties

The magnetic property of IONP was measured by vibrating sample magnetometer (VSM). The magnetization measurement obtained for IONP synthesized by green and chemical route from VSM technique measured at room temperature is shown in fig. 4. The highest saturation magnetization was achieved at 0.51emu/g for green synthesized IONP and 60 emu/g for chemical. The maximum amount of useful work that can be performed by the magnet can be determined by energy product (BH) max. The magnetic remanence and the coercivity determined by the magnets' microstructure is widely used to measure energy product [15]. In this case, the intrinsic coercivity was observed as 1348 Oe and 7.83 Oe and remanence 0.12 emu/g, 1.23 emu/g for green and chemical IONP respectively (fig. 4). Coercivity is a measure of ferromagnetic materials to withstand an external magnetic field. It measures the resistance of a ferromagnetic material to becoming demagnetized. High coercivity attributes to hard magnetic nature of green IONP that arises due to the large magnetocrystalline anisotropy of the materials with a non-cubic structure. Magnetic materials with higher coercivity are potential candidates for magnetic recording medium [16]. Higher coercivity in case of green IONP highlights its use as the permanent storage device.

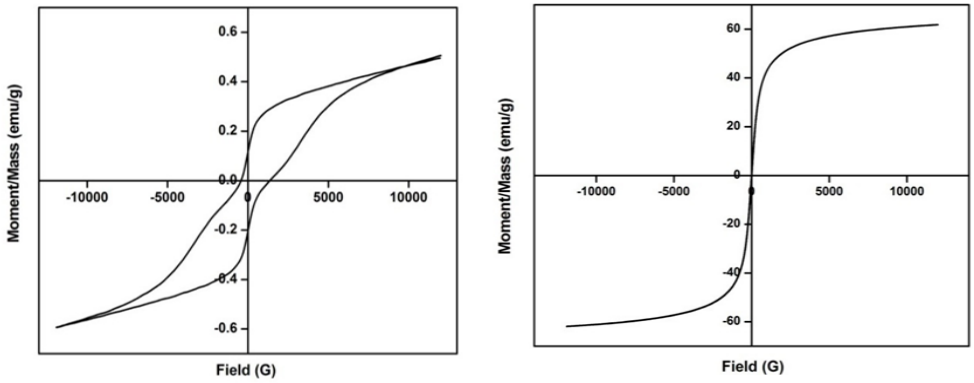

Fig. 4: Vibrating sample magnetometry
(A) VSM of green IONP shows highest saturation magnetism at 0.51 emu/g (B) VSM of chemical IONP shows highest saturation magnetism at 60 emu/g

Surface properties

The surface charge of nanoparticles gives an indication of their colloidal stability. Zeta potential is a technique for determining the surface charge of nanoparticles in solutions. The particles have a surface charge that attracts a layer of ions of opposite charge. Nanoparticles having high positive or negative zeta potential show dispersion stability, as a result, do not agglomerate on storage. The hydrodynamic diameter was found to be 58.77 nm with PDI of 0.693 in case of green IONP and 331.1 nm with 1 PDI in case of chemical (fig. 5A). Zeta potential of green IONP (fig. 5B) was-1.37 and chemical was+9.05. Due to Vander Waal inter-particle attractions, dispersions with a low ze ta potential value will eventually aggregate. However, lesser size and PDI of green synthesised IONP promises its use in various applications as lesser PDI implies uniform distribution.

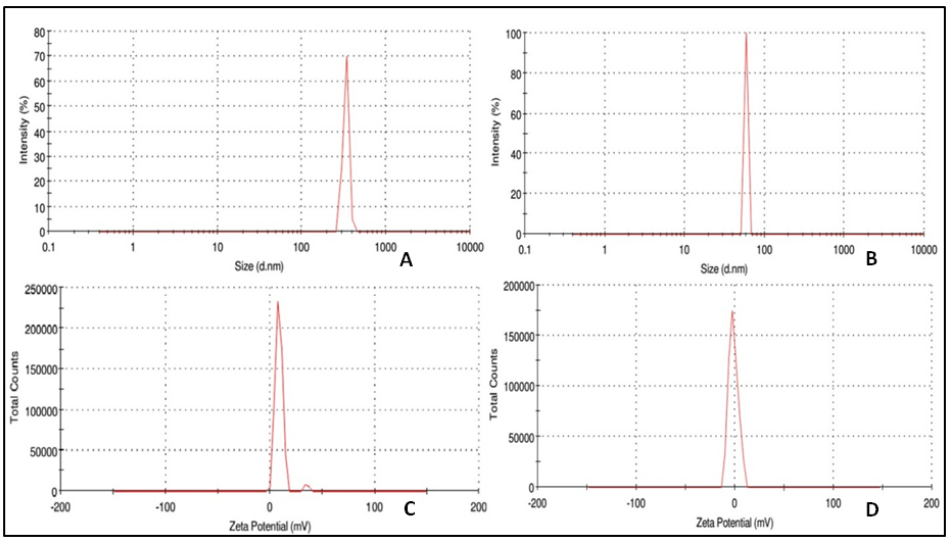

Fig. 5: Zeta potential analysis spectra of IONP
(A) Size distribution of chemical IONP showing average size as 331.1 nm (B) Zeta potential analysis of chemical IONP showing maximum at+9.05 (C) Size distribution of green IONP with average size as 58.77 nm (D) Zeta potential analysis of green IONP showing maximum at-1.37

Antioxidant assays

Free radicals, particularly reactive oxygen species (ROS) have a greater impact on humans both from within the body and the environment. During metabolism, ROS such as superoxide (O^{2-}), hydroxyl (OH) and hydrogen peroxide (H_2O_2) can arise normally or sometimes the immune cells create them purposefully to neutralize the foreign bodies. These ROS can damage essential proteins, DNA and lipids and cause various human diseases like atherosclerosis, cancer, liver injury, cardiovascular disease, neurodegenerative disorders and rheumatism as a result of 'oxidative stress' [17]. DPPH is widely used to monitor chemical reactions involving radicals as it gives the violet colour to DPPH solution in its radical form and the colour will change to pale yellow when a hydrogen atom is donated to the molecule. Hence, the high DPPH scavenging of green IONP shows that it can neutralize the generated free radicals more efficiently. This property could be attributed to the presence of phenolic groups in the green IONP that was verified by measuring the total phenol content. In order to confirm the radical scavenging activity, we carried our Superoxide radical scavenging activity. The scavenging activity was found to be significantly higher for green IONP (fig. 6).

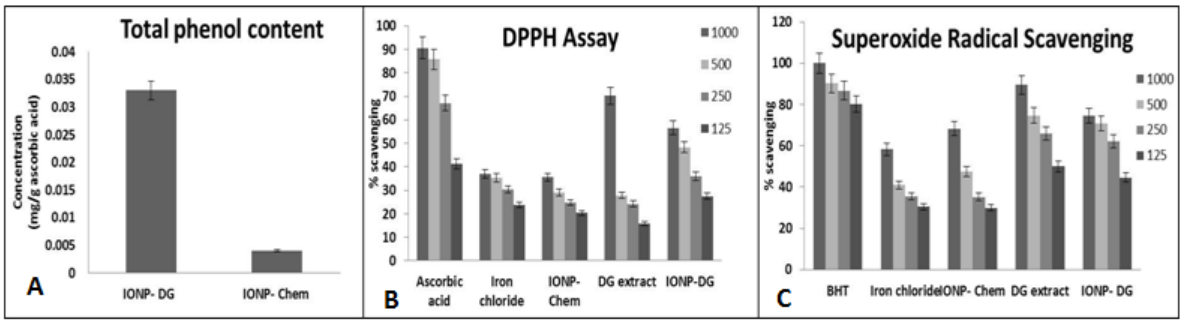

Fig. 6: Free radical scavenging activity and total phenol content of IONPs
(A) Total phenol content of chemical and green IONP (B) DPPH assay showing comparative antioxidant potential of chemical and green IONP, Iron chloride, DG extract, and ascorbic acid (Positive control) (C) Superoxide Radical Scavenging assay showing comparitive antioxidant potential of chemical and green IONP

Toxicity study

Nanoparticles have the ability to enter, translocate within, and damage living organisms. This ability results primarily from their small size, which allows them to penetrate physiological barriers, and travel within the circulatory systems of a host. The toxicity of IONP in LLC-PK1 cell line was assessed by LDH based cytotoxicity assay. Upon damage of the plasma membrane, lactate dehydrogenase, a stable cytoplasmic enzyme will be released into the cell culture supernatant and its measurement is used as the injury index. LLC PK1 cells (80% confluent, in 96 well plates) was incubated with NP for 24 hrs showed relatively very less damage to the cell caused by green IONP as compared to the chemical IONP, precursor (iron chloride) or even DG extract itself, measured by LDH activity (fig. 7).

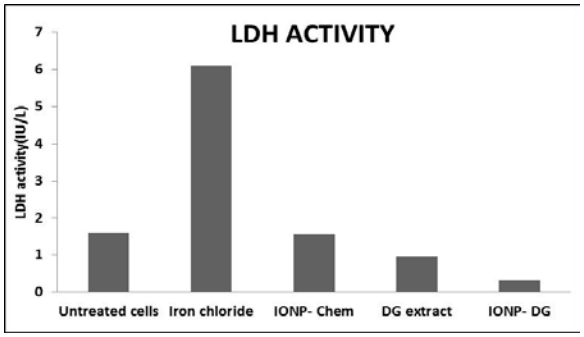

Fig. 7: LDH release in culture medium of LLC-PK1 cells

The graph shows LDH activity of untreated cells, Iron chloride, chemical IONP, DG extract, green IONP. Treatments were made once the seeded cells reached 80% confluence. The results are expressed as mean SD of n=4-6 independent assays, $p<0.05$, statistically different from the normal control group.

CONCLUSION

A comparative study of magnetic IONP synthesized by chemical and green routes was presented. Though the results of characterization were more or less similar, the antioxidant, anti-microbial assays suggests green synthesized IONP as potential candidate for biomedical applications. Also, lesser toxicity of green IONPs reduces the chances of potential damage to vital organs. Magnetic studies indicate higher coercivity for green synthesized IONP highlighting its use as the storage device.

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CONFLICT OF INTERESTS

Declared None

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