Synthesis of Iron oxide Nanoparticles,

Characterization and Biological Applications

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I, Humera Bashir, declare that all work presented in this thesis is the result of my own work. Where information has been derived from other sources, I confirm that this has been mentioned in the thesis. The work here in was carried out while I was postgraduate student at Atta-ur-Rahman school of Applied Biosciences NUST under the supervision of Dr. Rumeza Hanif

Humera

Humera Bashir

Dedicated to

My Beloved Parents

The reason of what I become today

Your affection, great support, encouragement

and prayers has made me able to

achieve this success

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LIST OF ACRONYMS

α-Fe2O3	Hematite
Ag	Silver
Al2O3	Aluminum Oxide
AuNPs	Gold Nanoparticles
CdSe QDs	Cadmium Sulphide Quantum Dots
CdS,	Cadmium Sulphide
CdZn	Cadmium Zinc
CNT	Carbon Nanotubes
CQDs	Carbon Quantum Dots
CTC	Circulating Tumor Cell
CTX	Cefotaxime
CuO,	Copper oxide
DLS	Dynamic Light Scattering
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl hydrate
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
Fe	Iron
Fe2O3	Iron Oxide
Fe3O4	Magnetite
FeCl3 .6H2O	Iron (III) Chloride Hexahydrate
FeSO4.7H2O	Ferrous Sulphate
FMN	Fluorescence magnetic nanoparticles
FTIR	Fourier Transformed Infrared Spectroscopy
γ-Fe2O3	Maghemite

GMO	Genetically Modified Organism
Hr	Hour
IONPs	Iron oxide Nanoparticles
KBr	Potassium Bromide
keV	Kilo Electron Volt
Kg	Kilogram
LB	Luria broth
mA	milliampere
MDR	Multi Drug Resistant
MCF-7	Michigan Cancer Foundation-7
MDA-MB-231	Triple Negative Breast Cancer Cell line
mg	Milligram
MHA	Mueller- Hinton Agar
mM	Millimolar
mm	Millilitre
MNPs	Magnetic Nanoparticles
MRI	Magnetic resonance imaging
MTT	3-(4,5-Dimethylthiazole-2yl)-2,5-Diphenyltetrazolium Bromide
NA	Nutrient Agar
NaCl	Sodium Chloride
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaOH	Sodium Hydroxide
NCDs	Nitrogen-doped carbon quantum dots
nm	Nanometer
NMR	Nuclear magnetic resonance
NPs	Nanoparticles
PEG	Polyethylene Glycol

rpm	Revolutions per minute
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SEM	Scanning Electron Microscopy
SiO2	Silicon Oxide
SLN	Solid Lipid Nanoparticles
SPIONS	Superparamagnetic Iron oxide Nanoparticles
TEM	Transmission Electron Microscopy
TiO2,	Titanium dioxide
μm	Micrometer
UV/Vis	Ultraviolet/Visible
XPS	X-Ray Photoelectron Spectroscopy
XRD	X-Ray Diffraction
ZnO	Zinc oxide

ABSTRACT

Nanotechnology is the fastest rising technology with great economic outputs due to its usage in a large range of fields including biomedicine, bio-analysis, bio-detectors, drug delivery systems, therapeutics, diagnostics, medical implants analysis, and materials sciences. Iron oxide nanoparticles (IONPs) have gained interest due to a wide range of biomedical applications from probing to therapeutics. In the present study IONPs were synthesized through co-precipitation method by using Iron (III) Chloride Hexahydrate ($FeCl_3.6H_2O$) and ferrous sulphate ($FeSO_4.7H_2O$) which resulted in the formation of black colored precipitates. IONPs synthesis was further verified using UV-Vis spectroscopy which showed the characteristic peak at the 306nm. X-Ray Diffraction (XRD) was performed to check the crystallinity of the IONPs. Their size and morphology was determined by Scanning Electron Microscopy (SEM) which showed that IONPs are spherical in shape and their sizes are in range of 42-78nm. The FTIR spectrum analysis showed that nanoparticles have hydroxyl group (O-H), alkane group (C-H), carbonyl group (C=C), alcohol group (C-O) and metal and oxygen group (Fe-O) on their surface. MTT assay for cytotoxic analysis of IONPs against MCF-7 and MDA-MB-231 cell lines revealed that they have dose dependent manner of cytotoxicity. As the concentration of IONPs was increased, the cell viability has been decreased. Antimicrobial assay was evaluated against *Escherichia coli, Stapylococcus Aureus* and *Klebsiella pneumonia,* which revealed that IONPs have good antibiotic potential against *Escherichia coli* and *Stapylococcus Aureus* but have low antibiotic activity against *Klebsiella pneumonia.* DPPH antioxidant assay showed that IONPs have free radical scavenging activity of 51% at 800μg/ml but not significant enough as compare to other standard natural antioxidants like ascorbic acid. These nanoparticles can be used in biomedical application after further optimization such as circulating tumor detection.

INTRODUCTION

Nanotechnology is the branch of science in which materials are manipulated at nano-scale (M. A. Ali et al., 2014). These may be natural or synthetic (polymeric particles) and biodegradable (lipid particles). Their size varies between 1-100 nm (Agrawal et al., 2018). The late Nobel Prize winning physicist in 1959 named Richard P. Feynman hypothesized the potential of nano-size materials (Feynman, 1992). Because of the high surface to volume ratio of nanoparticles, they have different physiochemical properties which make them an excellent candidate for various biomedical applications at nano scales (Mody, Siwale, Singh, & Mody, 2010). Nanoparticles associated drug delivery system seems to be very attractive carrier system which have ability to target the specific site by maintaining its integrity (Sahni et al., 2011).

NPs vary in shapes and sizes which include nano-spheres, polymeric micelles (Pu et al., 2019) nanotubes (Ibrahim, 2013), dendrimers, nano-capsules, biodegradable nanoparticles, liquid base NPs, cages, nano-composites, nano rods (Cartaxo, 2010) and quantum dots (Chan & Nie, 1998). NPs have been classified into four nano-systems. They can be bio-metallic or alloy NPs, magnetic NPs, metallic NPs (Hasan, 2015) and metal oxide NPs (Auffan et al., 2009)**.** NPs are divided intro organic, hybrid and inorganic NPs (E. C. Wang & Wang, 2014). Inorganic NPs are mostly metallic in nature (Mody et al., 2010).

In recent years, nanotechnology has been growing interest in many applications of biomedical science such as drug delivery, photo-ablation therapy, therapeutics, bio-conjugation, biosensors, hyperthermia based killing of tumor cells and bio-imaging (McNamara & Tofail, 2015). There are many nanotechnology based therapeutic drugs almost over two dozen have been approved for clinical use (Wagner, Dullaart, Bock, & Zweck, 2006). Nanotechnology improved the poor water soluble drug delivery, targeted drug delivery, large macromolecules delivery to intracellular sites, combinational therapy, imaging and efficacy of therapeutics (Farokhzad et al., 2006). NPs have wide applications in pharmaceutical technology that mainly emphasizes on formulation of drug in

biocompatible nano-forms for drug delivery (Moghimi, Hunter, & Murray, 2001). NPs give us best way of time controlled and site specific delivery of the drug as well as bioactive agents (Hamidi, Azadi, & Rafiei, 2008).

In spite of many innovative technology and strategies developed for the effective detection and treatment of different cancers all over the world, the cases of cancers are increasing due to the delay in diagnosis of cancer. Use of NPs in the field of cancer diagnosis and treatment is gaining interest by scientists due to their quantum dimensions which change the internal energy of NPs and their other properties (Thoidingjam & Tiku, 2017). Early detection is the major challenge in cancer therapy. NPs have greatly enhanced the properties like sensitivity as well as specificity of cancer cells for the detection and treatment purpose. Fluorescent magnetic nanoparticles (FMN) are proved to have the greater ability to detect the cancer cells at early stages (Fu et al., 2012). Surface coated NPs are very useful to achieve active targeted site in cancer cells and enhance the biocompatibility of NPs. They stimulates a wide range of nano-sized particles which are able to recognize, visualize and remove the tumors at targeted site at early stages of cancer (Barreto et al., 2011). Multimodal fluorescent-magnetic based nanomaterials have been used as diagnostic to facilitate the diagnosis of cancer on early stages (Serrano García, Stafford, & Gun'ko, 2018).

Iron oxide nanoparticles (IONPs) stand out among other nanoparticles due to their biocompatibility, low cost, biodegradability and super-paramagnetic properties (Shaw, Murthy, & Pradhan, 2010). Many scientists have been focused on the biomedical applications of IONPs in bio-imaging, bio sensing, drug and gene delivery due to their better biological, chemical and magnetic properties such as non-toxicity, high saturation magnetization, chemical stability, biocompatibility and high magnetic susceptibility (C. S. Kim, Tonga, Solfiell, & Rotello, 2013).

IONPs have different magnetic phases, among which maghemite (Fe_2O_3) and magnetite (Fe_3O_4) are mostly used in bio-medical applications and known to be biocompatible. They have been vigorously investigated for guided targeted drug delivery, treatment of cancer, analysis of DNA and manipulation, gene therapy and also their use in magnetic resonance imaging (MRI) (Hasan, 2015). In cancer therapy they are being used because of their magnetite properties which simply

guided to a targeted site. IONPs are the only approved metal oxide which can be used in MRI (Morteza Mahmoudi, Sant, Wang, Laurent, & Sen, 2011). IONPs having metal core attached with the antibody against breast cancer specific receptor ERBB2 have been reported which can image and target the breast cancer cells (Artemov, Mori, Okollie, & Bhujwalla, 2003).

Magnetic nanoparticles (MNPs) having magnetic elements like iron binds to receptors of specified tumor and can be detected under the influence of a magnetic field. IONPs might need protective coating of inorganic materials or surfactant polymers (Lu, Salabas, & Schüth, 2007). Biocompatible and high magnetite property of IONPs can be synthesized through argon/nitrogen gas by means of chemical method for the selective separation and detection of breast cancer receptors. Antibody against specific breast cancer receptor can also be attached to the NPs. (Fakayode, Tsolekile, Songca, & Oluwafemi, 2018). IONPs imparts their high magnetic property through which they are helpful in detection of cancer. IONPs based nano-carriers are excellent source of targeted drug delivery. They are proved very specific and effective in reducing the toxic effects on the normal tissues of the body (Ajinkya et al., 2020). Moreover, multifunctional NPs have been proved to be used as potent therapeutic material because of the modification of their surfaces with different functional polymers or biomolecules. Surface modifications of nanoparticles have gained great interest by the researchers in the targeted therapy. Most of the NPs have good antimicrobial properties and low toxicity, thus are suitable to be used in therapeutics (S. Parveen, R. Misra, & S. K. Sahoo, 2012). IONPs are non-toxic, low coast and will be ideal of model imaging and therapy of cancerous cells(Hui Wang, 2014). IONPs with specific ligand can be beneficial for clinical doctors to get real-time noninvasive imaging for early detection of cancer. So they can be used as therapeutic, diagnostic as well as for theranostic purposes for cancer therapy in clinical field (Shi et al., 2015).

IONPs can ne synthesized by means of chemical method. IONPs are precipitated out when sodium hydroxide (NaOH) is added in the reaction mixture of iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) and ferrous sulphate ($FeSO_4.7H_2O$) (Ansari et al., 2019). To evaluate the different physical properties of newly synthesized IONPs, they are subjected to standard characterization techniques. These standard characterization techniques include Ultraviolet/visible (UV/Vis)

spectrophotometry, Fourier transformed infrared (FTIR) spectroscopy, X-Ray Diffraction (XRD) and Scanning Electron Microscopy (SEM) (A. Ali et al., 2016).

The application of IONPs has verified one of the utmost significant transition metals oxide-based medications in nano technological improvements and biological uses due to enriched biocompatibility of iron. The bactericidal result of the IONPs is due to its small particle size (Arakha et al., 2015). After characterization technique, the antibacterial activity of the newly synthesized IONPs is evaluated through well diffusion assay against *Escherichia coli* (Gram-negative), *Stapylococcus Aureus* (Gram positive) and *Klebsiella pneumonia* (Gram-negative) (Jagathesan & Rajiv, 2018). Antioxidant activity is also examined through DPPH assay (Sulaiman et al., 2011)

IONPs are non-toxic, low cost and will be ideal of model imaging and therapy of cancerous cells (Hui Wang, 2014). IONPs with specific ligand can be beneficial for clinical doctors to get real-time noninvasive imaging for early detection of cancer. So they can be used as therapeutic, diagnostic as well as for theranostic purposes for cancer therapy in clinical field (Shi et al., 2015).

- **HYPOTHESIS**

Breast cancer can be treated by its early detection using nano-particles. Our research is designed to synthesize the biocompatible IONPs and apply them for the detection of cancerous cells due to their distinct properties. IONPs have also been reported to demonstrate antimicrobial and antioxidant properties so well diffusion assay and antioxidant assay are also designed to check the properties of IONPs.

- **OBJECTIVES**

The particular objectives of our study are following:

- Synthesis of IONPs from Iron (III) Chloride Hexahydrate ($FeCl_3.6H_2O$) and Ferrous sulphate ($FeSO_4.7H_2O$) by degassing through Argon/Nitrogen
- Characterization of newly synthesized IONPs
- Evaluation of cytotoxicity of IONPs through cell viability assays
- Well diffusion assay to determine the antimicrobial properties of IONPs
- DPPH assay to evaluate antioxidant properties of IONPs

LITERATURE REVIEW

2.1. Nanotechnology and Nanoparticles

Nobel Laureate, Richard P. Feynman introduced the term "Nanotechnology" for the first time in his famous lecture held in 1959 entitled, "There's Plenty of Room at the Bottom" (Feynman, 1992). "Nano" word comes from a Latin word which means "dwarf". The NP corresponds to one thousand millionth of a precision unit, so the nanometer is one thousand millionth of a meter as $1nm = 10^{-9}$ m (Bhatia, 2016). Nanotechnology is the knowledge of the strategy, properties, manipulation, and uses of shapes, instrument, and arranged by handling shapes and dimensions at nano level. So, this technology is not latest but, the term was firstly described in 1960s, so when the term came up, it can be said that materials researchers and chemists were working in the field of nanotechnology. In the past, nano-atoms were utilized about two thousand years ago in the era of Roman glass, in 10^{th} century, AuNPs bundles were used to promote vibrant colors and many varieties in ceramics industries and glass production. In fact, massive usage was started of NPs from the twentieth century, when manipulation of carbon black began and ultimately fumed silica was formed in 1940. The finding of C_{60} in 1985 and carbon nano-tubes (CNT) in 1991 provided good initiation to the production of nanoparticles and for the use of advance material (Pitkethly, 2004).

The alteration of the characteristics of the nanostructured materials is primarily the result of the following factors:

1. The amount of atoms in the limits of the grains is higher as 40–50% than in the traditional materials with polycrystalline structure

2. The look of quantic effects that activate to marked within this dimension range (Vida-Simiti, Jumate, Chicinas, & Batin, 2004).

NPs can be classified in a variety of range on the basis of patterns, from spheres to scales and platelets, to dendritic structures, rods and tubes etc. (Pitkethly, 2004). The variation in the physical and chemical attributes of nanomaterial can be recognized by its high surface to volume ratio. Because different biological pathways occur at nanometer scales, there are exceptional candidates for biomedical use because of these special attributes (Mody et al., 2010).

2.2. Significance of Nanotechnology

Nanotechnology is the fastest rising technologies with great economic outputs because of its wide range of uses in fields comprising bio-medicine, bioanalysis, bio-detectors, drug delivery systems, medical implants analysis, food packaging, cosmetics, catalysis, bio-remediation, electronics and materials sciences (NelA, 2006). Recently, the progress in nanotechnology has shown that NPs have an excessive potential as drug carriers in many biomedical applications. Size reduction techniques and various tools give nanostructures of various types that show special physicochemical and biological characters. All of these techniques make the nanostructure material suitable for many biomedical usage and thus have gained the significant position in the field of pharmaceutical sciences (Ghaffari & Dolatabadi, 2019). Moreover, these techniques support in reduction of toxicity, improving release, enhancing solubility and bio-accessibility and give well formulations for drugs used in various applications. Nanotechnology also deals with drugs that range within the nanometer size, and it increases the quality in a different dosage types. Different advantages of nano size include low fasted changeability, low patient-to-patient variability, increase water solubility, enhance oral bio-availability, enhance allowance of dissolve, enhance surface vicinity, low quantity of dose required and excessive onset of remedial action (Bhatia, 2016).

2.3. Classification of Nanoparticles

NPs categorized depends on various factors, their chemical composition, origins, formation, their size, shape and properties (Ealia & Saravanakumar, 2017). On the basis of their chemical configuration some types of NPs are mentioned here:

2.3.1. Carbon-based Nanoparticles

Carbon-based nanostructures, prepared of refined carbon which are categorized as:

i. Fullerenes:

Fullerenes consist of nano-material which might be organized of globular unfilled cage such as allotropic shape of carbon. They have generated remarkable commercial attention just because of their electrical conductivity, high firmness, structure, electron affinity, and flexibility. C_{60} and many other groups with a range carbon atom were generated by carbon vaporization from graphite into a high-density helium flow consuming a pulse vaporization laser (Astefanei, Núñez, & Galceran, 2015).

ii. Carbon nanotubes (CNTs):

Fullerene, C60, a ball which is made up of combination of 60 carbon atoms in a shape that is referred to as "buckminsterfullerene". C60 is a round molecule in which carbon atoms are organized on the tips of a truncated icosahedron structure (Heymann, Chibante, & Smalley, 1995). The special features of CNTs are their low weight, small size, good tensile strength, and appropriate conducting property, which make them relevant as fillers in different substance including polymers, metallic surfaces and ceramics. CNTs have lot of uses in the areas of transistors, nano-medicine, detectors, actuators, membranes, and capacitors (Ibrahim, 2013). CNTs have some other usage such as: natural organic matter, elimination of pathogens, and cyano-bacterial toxins from aquatic life just because of high adsorption abilities. The fibrous

shape and massive outside surface vicinity can be opened by biological impurity (Upadhyayula, Deng, Mitchell, & Smith, 2009).

iii. Carbon quantum dots:

CQDs have arisen to address another class of carbon-based optical nanomaterials with splendid and vivid fluorescence discharges, attracting much consideration as of late in cell imaging, photoacoustic imaging, and photodynamic treatment and focused on drug conveyance.

2.3.2. Organic Nanoparticles

i. Lipid based nanoparticles

The first NP platform was the liposomes. Liposomes were first named in 1965 as a model of cell layers. Since the liposomes have stimulated from a model in biophysical exploration to be valuable for quality and medication delivery. Liposomes are round structured vesicles that comprise a single or many bi-layered structures of lipids that compile in watery systems. The special benefits reported by liposomes are various compositions range, their ability to transport and maintain different biomolecules types, biocompatibility, and biodegradability (Scott, Wilson, & Crooks, 2005). Lipids with desirable features can self-arrange into nano-structure or nano-film, micelles or liposomes. Lipids molecules in nano-structure interact with other molecule to perform different function in human body via soft or hard nano-devices by special linkage (Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2013). Solid lipid nanoparticles (SLN) show a special to old-style colloidal carriers, in particular emulsions, polymeric and liposomes nanoparticles. Many hydrophobic and hydrophilic drugs have been combined into SLN. SLN seem as a capable vehicle for up-to-date ocular direction of tobramycin (Cavalli, Gasco, Chetoni, Burgalassi, & Saettone, 2002).

ii. Dendrimers:

A dendrimer is morphologically categorized by a branched shape developed from one or more fundamental key. The size of these NPs is easily organized by the number of generations that permitted to create on these cores. Dendrimers show problems concerning drug combination and liberate, being their production is time taking (Cartaxo, 2010). There are some good useful characteristics of dendrimers. First, it is easy to handle the chemical configuration of the peripheral groups on the dendrimer, and this help a source for attaching dendrimers to support and for dissolving them in any solvent. Second, the dendrimer periphery can role as a size-and-shape specific molecular filter, therefore giving a source for selectivity to intrinsically non-specific metal catalysts. Third, the dendrimer in some cases, give a means for handling product selectivity (Scott et al., 2005).

iii. Polymeric Micelles:

When polymeric molecules voluntary link with aqueous solution to form core shell structures polymeric micelles are formed. The inner most layer of a micelle is hydrophobic and is surrounded or covered with hydrophilic polymers, like PEG. Its hydrophobic layers help as a source of poor water solubility, and its hydrophilic coating helps in stabilization of the core, prolonging the circulation time in the blood, and also helpful in increasing the aggregation in tumor tissues. Thus, larger types of drug molecules are assembled into polymeric micelles through physical encapsulation method or covalent attachment. (Bamrungsap et al., 2012).

2.3.3. Inorganic Nanoparticles:

i. Metallic Nanoparticles:

Metal NPs are made up of the metal atoms. It includes some of the inorganic NPs which are mostly composed of noble elements (Ag, Au), and also include some transition metals such as Zn, Fe, along with other uses such as optical sensor, catalysis, solar panels, carrier, bioremediation, surgery, biomolecule detection (da Silva et al., 2011). Due to their enhanced

optical character, metal NPs discover the uses in numerous experimentation areas. Gold plated NPs are extensively used in SEM specimen to improve the sporting electronic flow in attaining high quality SEM images (Khan, Saeed, & Khan, 2019). AgNPs have specific anti-microbial actions just because of close interaction between the nucleus of Ag and cell walls that affect their degradation. Their use has developed AgNPs in the nanomaterial class that is massively and very rapidly growing (Shahverdi, Fakhimi, Shahverdi, & Minaian, 2007). Colloidal gold that is also known as AuNPs, is a suspension of small sized gold fragments. Remarkable optical character of these AuNPs is just because of their special inter-activity with light. If the oscillating electromagnetic light field is generated, the free electrons of the metal NPs rely on an oscillation compared to the metal lattice (Mody et al., 2010). AuNPs are usually working in optical sensors. AuNPs have gained importance as an important target to act as tags in proteomics. They can be combined with biomolecules which link their functional groups and thus can help in anchoring as chemical anchors (Wu et al., 2011).

ii. Quantum dots (QDs):

QDs are auto fluorescent semi-conductor nano crystals that work for a broad range of in vivo biomedical imaging (Larson et al., 2003). QDs exhibit special and attractive optical characteristics such as symmetrical emission spectra and sharpness, high photo-stability, unique chemical character, and high quantum efficiency. The modest chemical parts of QDs, CdS, CdSe, CdZn etc. are broadly used in biological labeling in various animal cells (Chan & Nie, 1998). Other QDs can be created with mixtures like ZnS-CdSe core-shell nanocrystals work as a bioactive fluorescent probe in imaging analysis, immune-assays, detection, and have many other diagnostic uses (Mattoussi et al., 2000).

iii. Metal oxide Nanoparticles:

This group of NPs includes a number of transient metal oxides such as TiO2, CuO, ZnO, Fe2O3 and CeO and SiO2, which combine the unique characteristics of these elements with the characteristics of the NPs like high reactivity. They are also used in a wide range of consumer

products like use in cosmetics such as sunscreens, creams, catalysts, and in biomedicine (Auffan et al., 2009). The special and unique super-paramagnetic characteristic of IONPs have been broadly applied in various in vivo uses like magnetic resonance imaging contrast improvement, detoxification of biological fluids, tissue repair, immunoassay, drug delivery etc.(Gupta & Gupta, 2005). On account of their ultrafine size, biocompatibility and magnetic characteristics, super-paramagnetic IONPs appeared as encouraging candidates for several biomedical functions, like targeted drug delivery and imaging, enhanced resolution contrast agents for MRI, gene therapy, stem cell tracking, hyperthermia, molecular tracking, magnetic separation technologies for early detection of inflammatory, cancer, atherosclerosis and diabetes. Super paramagnetic NPs look like magnificent imaging probes to be used as MRI contrast agents (Mody et al., 2010).

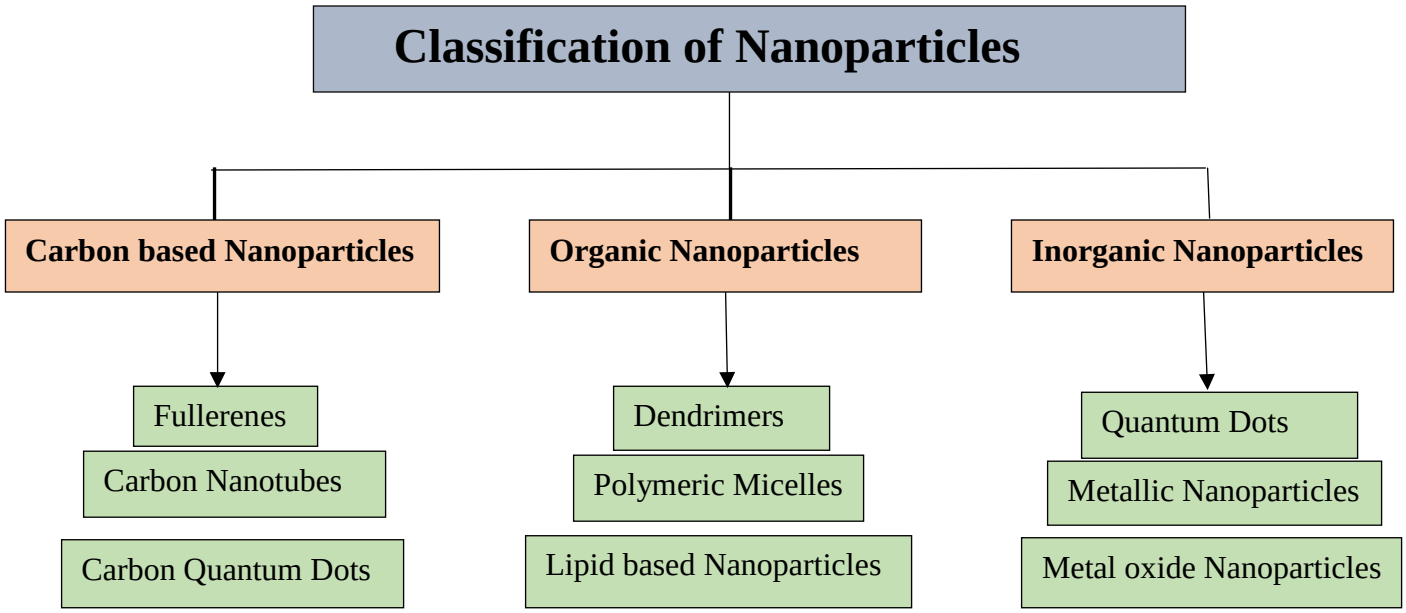

Figure 2.1: Classification of NPs based on their chemical configuartion

2.4. Magnetic Nanoparticles:

MNPs have involved much attention because their characters frequently differ greatly from those of bulk materials and they can therefore be employee to prepare materials and devices with new properties (Mattoussi et al., 2000). MNPs such as Fe_3O_4-magnetite and Fe_2O_3-maghemite are most broadly used MNPs and known to be biocompatible. They have been vigorously examined for drug delivery, targeted cancer treatment, DNA analysis, gene therapy, stem cell sorting, manipulation and MRI (Hasan, 2015). These NPs exhibit size-dependent super-paramagnetism, which permits them to develop magnetized with the use of an external magnetic field and show zero net magnetization upon elimination of the magnetic field. IONPs have been used as T2-weighted MR contrasts agents to track and monitor cells (Sowers et al., 2014).

MNPs as SPIONs in the nano-size, providing high potentials in a wide range of applications in bare or naked form or coated with a specialized and specific surface coating and functional groups which can be choose for special uses (Teja & Koh, 2009). MNPs such as Fe3O4-magnetite and Fe2O3-maghemite are most broadly used MNPs and known to be biocompatible. They have been vigorously examined for guided or targeted drug delivery, stem cell sorting and manipulation, DNA analysis, gene therapy, targeted cancer treatment and MRI (Hasan, 2015). Every MNP has a single magnetic domain which displays superparamagnetic character of MNPs when there is blocking temperature. Such type of individual NP has a great constant magnetic moment and act as a giant paramagnetic atom which have ability of fast reaction in many useful magnetic fields. These characters mark SPIONS very pretty useful for a wide range of many biomedical because their risk of formation of agglomerates is very insignificant at room temperature (Lu et al., 2007).

2.5. Biomedical Applications of Nanoparticles:

2.5.1 Biological Labels:

Because of the small size of NPs and fluorescent characters, mainly QDs, they can be used as biological labels. The core of nano-biomaterials is made by NPs and they interrelate or interact with the biological target with the help of their noncovalent contacts. To synthesize these nano-biomaterials the methods are adopted which are based on their biocompatibility, shape recognition, fluorescent signaling and antigen detection. They have also wide applications in the bacterial detection (Chan & Nie, 1998). Nitrogen-doped carbon quantum dots (NCDs) with γ-Fe_2O_3 (NCDs/γ-Fe_2O_3) has been stated for imaging and also to control the cellular motility. It is due to their photo-stability, biocompatibility, efficient uptake, potential selective affinity and low toxicity (Kumar et al., 2018).

2.5.2 Drug and Gene Delivery:

There is large number of drugs that limited due to low solubility, huge toxicity, and high amount of dosage and accumulation property. Due to all these reason these are degraded and shorten their half-lives and low circulating ability. By the introduction of nanotechnology is seen have great effect on drug delivery field and have very high potential uses in the field of clinical medicine and research area. NPs have been seen to deliver drugs, vaccine protein, and recombinant protein and advance nucleotides. NPs and some colloidal drug-export way change body's delivery, kinetics and discharging belong to drugs (Suphiya Parveen, Ranjita Misra, & Sanjeeb K Sahoo, 2012). NPs have to suggest to deliver the drug as due to delivery potential that develop the diagnostic system and therapeutic tool in the coming years(Gupta & Gupta, 2005). NPs are a colloidal carrier system, which has been exposed to progress the efficacy of the compressed drug by over-coming drug resistance as well as by giving sustained drug effect.

2.5.3 Drug Targeting:

Target the specific site in the cells is an important field of research. NPs are useful for the best delivery of oligonucleotides and drugs in the targeted tumor cells (Brigger, Dubernet, & Couvreur, 2002). There is a requirement of nucleus for drug-delivery as all the information of the cell and the transcription system presents the nucleus. Selected NPs delivery of therapeutic molecules has the probable to provide safer and more active therapies for cancer uses. Active targeting exploits the over appearance of surface receptors on cancer cells by helping targeting ligands that can involve these receptors (Thorpe, 2004). Selected nuclear delivery is fascinating work if a nuclear probe has or satisfies these points:

- There must be entrance in the cell
- There must be let out by endosomal ways
- There must be hold on nucleus localization signal to link with the nuclear-core complex
- The size of probe must be small as can cross membrane (Katz & Willner, 2004).

2.5.4 Therapeutics:

The medicinal applications of NPs are not same as from cancer medicine, gene delivery, vaccine delivery, antimicrobial and target specific, it eliminate the undesired effects of present medicine. Usually drugs like Paclitaxel, Carboplatin, Etoposide, and Doxorubicin etc., have been progressively inserted with NPs and these NPs systems are highly fast for numerous cancers. On the other side, NPs having large variety of functions with surface functional biomolecule are made and use as medicinal agents. The NPs are also use for the target gene silencing process and gained lot of attraction in the research field for researchers. Many NPs are favorable for the medicinal use due to their antimicrobial characteristics (S. Parveen et al., 2012). This prospect give us to trust as we can use nanoparticles in diagnostic and drug delivery therapy in the future in order to treat many human diseases and cancer (Maeng et al., 2010).

2.5.6 Detection and Monitoring/Diagnostics

Early recognition of cancer is the time taking process and use costly equipments. To find out the solution of these limitations, the colorimetric assay have been introduced for the direct detection of diseases or cancerous cells. In this assay, aptamer-conjugated AuNPs was applied by combining the sensitivity and targeting properties of aptamers with AuNPs which allow the sensitive detection of cancer cells (Medley et al., 2008). By the use of DNA aptamer and NPs, researchers are going to manipulate new bioassay for the recognition of small targeting molecules such as adenosine. The AuNPs can powerfully recognize the two small DNA molecules through the plasmon resonance color change. By using this technique, adenosine can be recognize to low molecular weight molecule, which could be used for many small targeting molecule (Li et al., 2009). A unique approach to recognize and detect the mammary cancer cells at early stage of cancer in a noninvasive way is through MNPs. Scientist found the applications of MNPs in the many areas of medical fields such as magnetism based drug and gene delivery, MRI agent, heating mediators for cancer treatment, and target selective biomolecules (Corsi et al., 2009).

Semiconductor QDs are NPs having steady and strong fluorescence which can be used to recognize or detect 10 to 100 cancerous biomarkers on the surface cancerous tissue or in the blood assay. These NPs give the special feature to the recognition and detection for the cancer marker in biological specimens (S. Parveen et al., 2012). These NPs are used to be applied for the detection and targeting the selective molecules. In the presence of specific viral particles, the conjugation of virus surface specific antibodies with the mono disperse MNPs in the presence of specific viral proteins makes a supra molecule having special magnetic characteristics for the detection which can be detected through resonance methods. The varying magnetic relax-ability on the induction of viral assembly allows the highly sensitive selection of virus in biological media (Perez, Simeone, Saeki, Josephson, & Weissleder, 2003).

2.5.7 Imaging:

The development of imaging and tracking of any nano therapeutics at sub cellular level is and essential thing in the treatment of many diseases. In the diagnostic and imaging process, NPs progressively interact with fluorescent markers. FNPs overcome the complexities toward clinical applications by its application in the tumor imaging and disease in vivo (S. Parveen et al., 2012). In the cancer imaging, silica NPs have gained lot of interest due to its inertness, chemistry and optical properties recently. Silica NPs have ability to overcome the non-specificity in linkage and in vivo aggregations of silica NPs. Silica NPs have an interesting photo-stability after loading the various kinds of fluorescent color. The stability of photo is due to silica layer around the NPs. Photochemical oxidation is minimal by this protection of fluorescent color (Nurunnabi, Cho, Choi, Huh, & Lee, 2010). From this idea, we can get benefit for the drug delivery and imaging to cure the human cancer and many other disease by making nano medicine (Maeng et al., 2010).

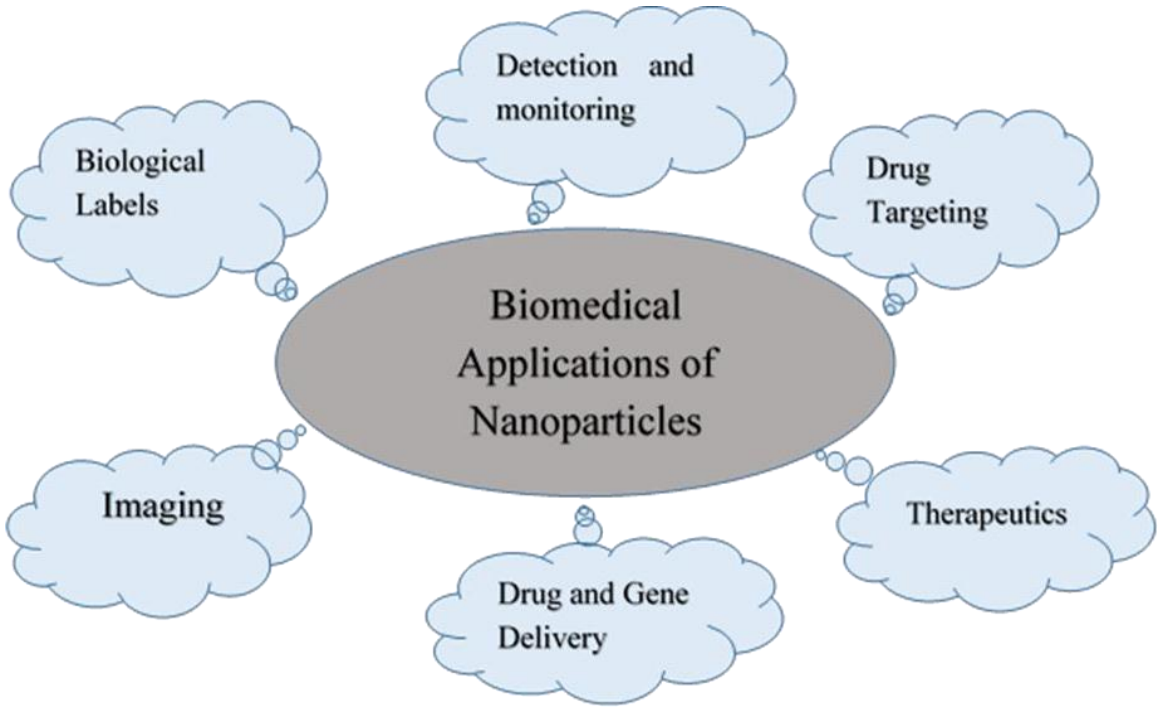

Figure 2.2: Biomedical Applications of NPs

2.6. Iron oxide Nano particles:

Amongst several kinds of MNPs, IONPs are the utmost popular and broadly help in the area of biomedicine due to their ease in surface alteration, production, and small toxicity (G. Liu, Gao, Ai, & Chen, 2013). In this era, IONPs have attained special attention, in particular for their uses in the biomedical field due to their increasing attention because their exclusive features such as their capability to react to magnetic fields (Torres-Lugo & Rinaldi, 2013). The application of IONPs for magnetic drug targeting (MDT) was first designed by Frei. It includes the combination of anticancer agents and IONPs, inoculation of the nano vehicle into the blood stream and its regulation to the tumor area applying an externally applied magnetic field. Previous study has verified the effectiveness of this method and its capability to decrease side effects (Frei, 1969). Iron (III) oxide is an inorganic molecule with the formula (γ-Fe_2O_3) maghemite and it is a type of the three main oxides of iron; the other two are hardly available iron oxide (FeO) (Serrano García et al.) and naturally happening iron (II, III) oxide (Fe_3O_4) magnetite (Katikaneani, Vaddepally, Reddy Tippana, Banavath, & Kommu, 2016). For the greater power in surface alteration and greater magnetic character, IONPs like Fe3O4 and γ-Fe2O3 are supposed to be best magnetic candidate in the improvement of drug delivery systems. Hematite (α-Fe2O3) is also applied in several uses (W. Gao et al., 2012). IONPs also has involved extensive interest as new contrast agents for biomedical imaging due to their ability of deep-tissue imaging, noninvasiveness and low toxicity (Maeng et al., 2010).

Moreover, their super paramagnetism provides MNPs a clear benefit. When the size of the NPs is lesser than a critical value e.g. 30 nm, the single NP acts like a giant paramagnetic atom with a single magnetic domain, showing super paramagnetic act. SPIONs act quickly to an applied magnetic field, but show negligible residual magnetism away from the magnetic field, focusing special attention in advanced biomedicine (El-Boubbou, 2018). IONPs i.e., Fe_3O_4 or Fe_2O_3 are known due to their good biocompatibilities. Different toxic heavy metal consisting NPs that are hard to be degraded or excreted from the body, MNPs have been proved for clinical application with minimal cytotoxicity (J. S. Kim et al., 2006).

2.7. Coating of IONPs:

The power of MNPs stems from the intrinsic character of their magnetic cores joined with their drug loading ability and the biochemical feature that can be granted on them by means of a favorable coating (Arruebo, Fernández-Pacheco, Ibarra, & Santamaría, 2007). Functionalization of MNP surfaces with special biomolecules i.e., peptides, proteins, antibodies, enzymes, nucleic acids, gene, imaging agents, carbohydrates, etc. for specific biomedical uses have been stated. The target biomolecules can be special labeled with fluorescent dyes, probes, tags on surface or the functionality of the NP surface can be designer to link the relevant probes (El-Boubbou, 2018). To improve the biocompatibility and the quality of MPs, their size regulation, their surface and shape different coating techniques have been used (Gupta & Gupta, 2005). The properties of MNP in drug delivery and imaging can powerfully be applied in targeted drug therapy to sort out the problems related to other cancer treatments and chemotherapy. MNP design can provide long lasting against for MRI and can be applied in combination with hydrophobic anticancer chemical for drug delivery system. Conjugated MNPs can also be modified as multimodal imaging character as drug targeting to improve cancer treatment, offering constant diagnosis and controlling therapeutic effects in tumors long time (Foy et al., 2010). This general methodology has different advantages over the other methods such as

- MNPs less than 10nm in diameter giving good performance because of their great surface/volume ratios and easy connection to bacteria
- Multivalent linkage at the dimension of 10nm, similar Immunoglobulin M (IgM), offer extraordinary affinity
- The joint effect of magnetic manipulation and fluorescence eliminated the need for costly or complex instruments (J. Gao et al., 2006).

Bare NPs usually exhibit unwanted features in biological systems. These NPs are usually hydrophilic or hydrophobic susceptible to oxidation and agglomeration which are the highly challenging related in biomedical uses can be lower or restricted (Munasinghe, Aththapaththu, & Jayarathne, 2019). Non-toxic FMNPs are noticeable multifunctional imaging probes by displaying

best MR sensitivity as well as also great fluorescent intensity with high signal amount under short contact time of Ultra Violet light from the great imaging examination of in vivo and ex vivo using orthotropic and xenograft mice models (Lim et al., 2010). A fluorescent monomer can be linked on the surfaces of MNPs directly (J. Liu et al., 2011). Deposition of conjugated polyelectrolytes above the negatively charged SPIONS used in efficient drug delivery (Howes et al., 2010).

IONPs coated with a silica and water-soluble CdSe−ZnS QDs were gathered collected by the linkage of an SH group (Xu et al., 2010). The production of CdSe QDs on Fe3O4 NPs process Fe3O4−CdSe NPs with two kind features, fluorescence and super paramagnetism (J. Gao et al., 2008). AgNPs were placed onto the surface of Fe3O4 nanospheres (Chen et al., 2013). Organic dyes express poor photo stability and strong toxicity. The formation of these magnetic fluorescent hybrid NPs includes complex multistep synthetic procedures (Cheng et al., 2012). For selective treatment, HER2 antibody was linked to glycerol mono-oleate covered MNPs. MNPs exhibit increase absorbance in human breast carcinoma cell line (MCF-7) (Dilnawaz, Singh, Mohanty, & Sahoo, 2010). Herceptin was conjugated with SPIONs for the identification of tumor cells by MRI procedure (Shamsipour et al., 2009).

2.18. Early Diagnosis of Cancer:

Although, several innovative technology and methodology made for the active identification and treatment of different cancers throughout the world, the cases of cancers are rising due to the delay in diagnosis of cancer. From all the cancers treated or diagnosed, breast cancer is till now on the fifth more causing of morbidity and mortality (Ferlay et al., 2015). The application of MNPs in biomedical science has been suggested in different ways like MRI, targeted drug delivery to a specific site, malignant cell treatment through hyperthermia application etc. The main problem of using NPs in therapy is their targeted delivery i.e. targeted to particular tumor site inside the body. This problem can be sort out by using MNPs in which specific magnetic gradient is created to a specific localized site so that MNPs can reach to the specific targeted site and then easily eliminate whenever the therapy is completed (Berry & Curtis, 2003). There is an important synthetic

advantage which enhance the biocompatibility and active targeting. It can be done by modification of the surface of NPs by using different polymers and other molecules. This modification leads to the synthesis of diverse types of nanomaterial which can help in the identification of the cancer cells, to deliver the drugs to tumor site, to visualize the cancer site and thus treated the cancer cells at early stage with the combination of many therapeutic methods (Barreto et al., 2011).

Early identification and targeted therapy are 2 main dares in order to treat the cancer. There is need to do advancements in imaging material and site specific targeted techniques so that their sensitivity and selectivity to cancer cells can be enhanced in theranostics. FMN and synthesized magnetic micro-meshes are bio-friendly which greatly enhance the targeting to cancer cells magnetically in the living body. The specific targeted drug is conjugated on to the surface of the MNPs which proved to be helpful in therapy (Fu et al., 2012). A new approach has been developed for noninvasive imaging of Her-2/neu receptors was examined in several cellular and artificial systems and was seems to offer high sensitivity, with the smaller limit of recognition (Artemov et al., 2003). Synthesis of multimodal FMNPs consider the specific properties of the nanomaterials so that they can be used in diagnostics and targeted drug delivery to diagnose and treat the cancer at early stages (Serrano García et al., 2018). Magnetic cell parting is greatly famous for the parting of CTC from clinical blood samples applying antibody linked magnetic beads. Aptamer-conjugated membranes stated, there have special power for the initial diagnosis of diseases that are now being identified by source of cell capture approaches on initial stage (Viraka Nellore et al., 2015). Conventional chemotherapy has many restrictions which can be minimized by the application of nanoparticles due to their properties to target the cancer sites only, avoid off targeted delivery and no or less harm to the healthy cells of the body. If NPs are planned accurately and modified then they have the ability to target the cancer cells through active or passive means and also enhance the cytotoxic effects of attached antitumor molecules or drugs (Pillai, 2014).

2.9. Cellular Cytotoxicity:

IONPs are the best candidate in biomedical uses for diagnostics and therapeutics. So it is significant to examine the whole toxicity linked with them. IONPs are coated with an appropriate biocompatible material for enhancement in stability, water dispersibility and biocompatibility (Patil et al., 2018). MTT test has been widely used method to check the cytotoxicity of IONPs with respect to other in-vitro cytotoxicity assays. MTT test which is colorimetric and also non-radioactive method, can be used to measure the cellular cytotoxicity or viability, the proliferation of IONPs in the cells, their biocompatibility, their reproducibility, cell survival and growth (M Mahmoudi, Simchi, Milani, & Stroeve, 2009). Decrease of MTT can also be facilitated by NADH or NADPH within the cells and also outside of mitochondria. Thus only energetic mitochondria comprise these enzymes; therefore, the reaction only happens in living cells (Mosmann, 1983). In another assay named necrosis-apoptosis assay, a fluorescent dye named Hoechst-33342 also called Roche is used as an indicator and to mark the apoptotic cells. The other cells are marked with another dye named propidium iodide. Hoechst-33342 do not need the cell-membrane permeability and moves inside the intact cells helps in the identification of the apoptotic cells (Naqvi et al., 2010). The cell membrane injury initiated by nanoparticles can be examined applying the lactate dehydrogenase assay which is depends on the quantity of lactate dehydrogenase activity in the extracellular medium. The assay is general method of calculating cellular viability (Thorat, Khot, Salunkhe, Ningthoujam, & Pawar, 2013).

Intracellular uptake of nanoparticles can be counted by cell counting chamber (Malvindi et al., 2014). Toxicity of IONPs is verified to be concentration based and it also depends on exposure time like in the case of high dose exposure, the particles may activate cellular stress and changed response (Patil et al., 2018). Different studies have been done to investigate the cytotoxicity of the bare IONPs and modified with different polymers on their surface i.e. surface coating. Research findings showed that IONPs have low cytotoxicity when used in lower concentration but on exposure of high concentration of IONPs (>100 µg/ml) then they show higher level of cytotoxicity (Singh, Jenkins, Asadi, & Doak, 2010).

2.10. Antimicrobial Property of IONPs

Antibiotic resistance is a well understand concept which has got noteworthy importance with respect to the public health when its effects has been amplified several times due to the negligence and mistreatment of human beings. The appearance of greatly resistant bacterial strains and the decreased changes to conventional antibiotics has aroused interest in the scheme of antibiotic carrier nano systems (Hussein-Al-Ali, El Zowalaty, Hussein, Ismail, & Webster, 2014). MNPs have many exceptional properties like magnetism, mechanical, physicochemical and thermal properties. They have been applied in many different fields which include analytical chemistry, diagnosis of antigens, hyperthermia, identification of the pathogens and tissue repair. Because of the nano-sized structure of MNPs i.e. less than 100nm which give them ability to connect with the microbial cells to interact, can be used in biomedicines. Because of the external magnetic field, the MNPs can be directed to the targeted specific cancer site where they become concentrated and also can be easily eliminated when the therapy has been completed (Lee et al., 2008). The newly synthesized IONPs possess good antimicrobial property against many pathogens including bacterias. The drug loading capacity of nanomaterials like IONPs and their antibacterial activity can be greatly enhanced against micro-organisms (Arokiyaraj et al., 2013).

IONPs has proved to be one of the best thing to use as metal oxide medications. Because of the high biocompatibility of IONPs, they have wide applications in many nano-technological techniques and biomedical sciences. Because of the small size of IONPs, they have the bactericidal property. The IONPs interact with the cell wall of bacterias which results in the deactivation of the cellular enzymes and thus distract the permeability of plasma membrane. The ROS is discharged when plasma membrane has been disrupted. Because of this, DNA and proteins of bacteria has been destroyed which ultimately leads towards the death of the cells (Saqib et al., 2019). In another study modified IONPs have been used to enhance the antibacterial activity of IONPs and it showed that IONPs have good antibacterial property against different bacterial strains (Arakha et al., 2015).

2.11. Antioxidant Property of IONPs

Nanotechnology has emerged in biomedical advancements to give prediction, identification and treatment of many infectious diseases. The potential of nanotechnology in biomedical applications can be evaluated by the ability of NPs to scavenge or remove free radical through DPPH (1,1-diphenyl-2-picrylhydrazyl) assay (Bhattacharya, Gogoi, Buragohain, & Deb, 2014). Antioxidants are widely present in biological systems or cells and scavenge free radicals are generated inside a cell as a byproduct of a biochemical process (Serpen, Capuano, Fogliano, & Gökmen, 2007). The interaction of the IONPs inside a cell can be understand with the study of their antioxidant property. (Paul, Saikia, Samdarshi, & Konwar, 2009). There are many NPs have been reported as a potent antioxidants and free radical scavengers. There are many infectious microorganisms are reported who can cause infection but their ability of making free radicals which ultimately damage the cells. But many nanoparticles have ability to counteract the effect of these infectious microorganisms (Elswaifi, Palmieri, Hockey, & Rzigalinski, 2009).

IONPs are found to have some antioxidant potency. To check the antioxidant activity of nanoparticles DPPH assay is mostly used. The antioxidant property is due to the transfer of an electron which ultimately neutralizes the free radical DPPH (Naik et al., 2003). IONPs antioxidant property can also be examined by DPPH method. The DPPH reducing ability of IONPs can be visually examined by change of the color from violet to pale yellow or also by spectrophotometer through the absorbance analysis at 517nm. The scavenging ability is increased with increasing the concentration of NPs (Paul et al., 2009).

MATERIALS & METHODS

This research is focused on the synthesis, characterization and applications of IONPs, followed by the coating of carbon quantum dots. The study was carried out in the Cancer Biology Laboratory of Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad. The method and materials used during research are explained below:

3.1 Chemicals

Table 3.1: Chemical Compositions used in IONPs Synthesis

Chemicals	Concentrations
1M FeSO4.7H2O (Merck, Germany)	10.002g
1M FeCl3.6H2O (Merck, Germany)	5.001g
0.5M NaOH (Sigma Aldrich, UK)	1g
70% Ethanol	120ml
Distilled water	100ml
Argon gas	2 balloons of Argon gas filled

3.2 Synthesis of Iron oxide Nanoparticles (IONPs)

IONPs synthesized by chemical means through co-precipitation method. To synthesize IONPs, 10.002g solution of Iron (III) Chloride Hexahydrate ($FeCl_3.6H_2O$) (Merck, Germany) and, 5.001g solution of ferrous sulphate ($FeSO_4.7H_2O$) (Merck, Germany) and 1g solution of Sodium hydroxide (NaOH) (Sigma Aldrich, UK) were used as the chemical source followed by the degassing through argon gas balloon as explained in flow chart in Figure 3.1.

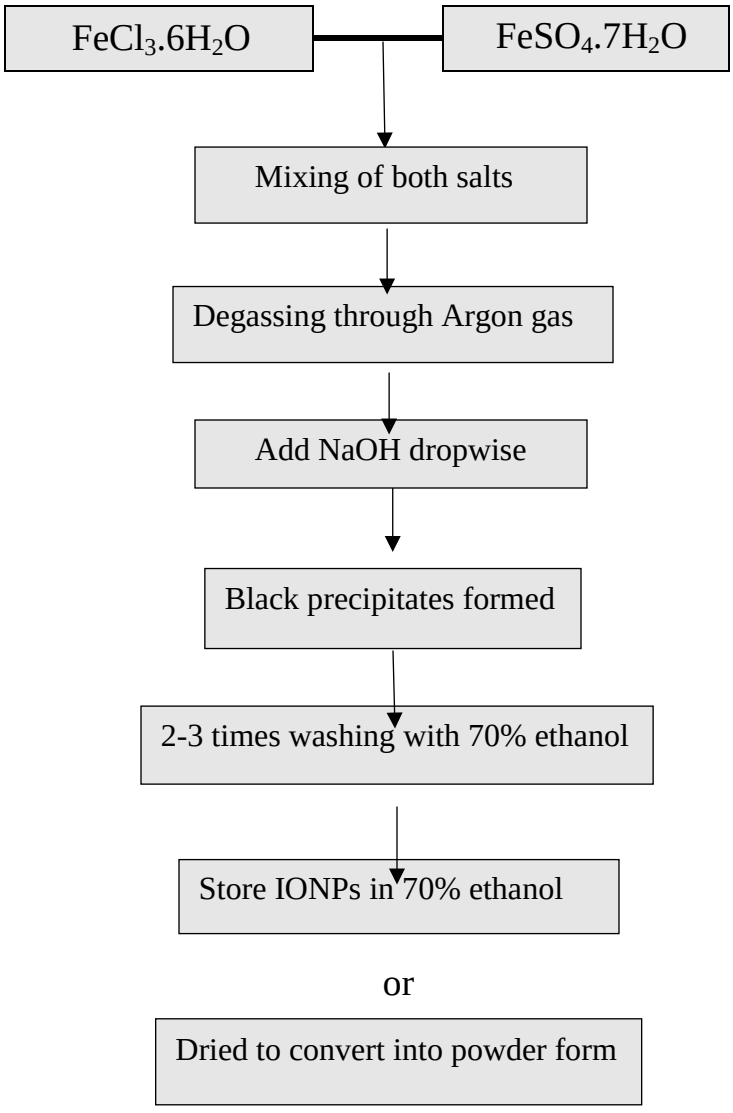

Figure 3.1: Schematic illustration of synthesis of IONPs through co-precipitation method

3.2.1. Solution Preparation

1M $FeCl_3.6H_2O$ (Merck, Germany) and 1M $FeSO_4.7H_2O$ (Merck, Germany) was taken in 2:1 ratio. 10.002g of 1M $FeCl_3.6H_2O$ was dissolved in 37ml distilled water and 5.001g of 1M $FeSO_4.7H_2O$ was dissolved in 18.5ml distilled water with the help of magnetic stirrer. 50ml of 0.5M NaOH solution was also prepared by dissolving 1g NaOH (Sigma Aldrich, UK) into 50ml distilled water.

3.2.2. Assembling of Procedure

Both solutions were mixed in a double neck round bottom flask. One neck was fixed with the cork along with a syringe needle and the other neck of the flask was fixed with an Argon balloon in such a way that argon gas from the balloon moved into the flask and oxygen moved out from the cork fitted neck of the flask. After that 50ml of 0.5M NaOH solution was added into the reaction mixture drop wise through 50ml syringe. Upon adding NaOH, the brown colored reaction mixture was converted into a black colored suspension due to the formation of IONPs. It was the visible indication of the formation of IONPs.

3.2.3. Storage of Nanoparticles

IONPs suspensions were taken in 50ml falcon tubes. These falcons were then centrifuged at 6000 rpm for 15 minutes. After centrifugation supernatant was discarded and pellet obtained was washed thoroughly with 70% ethanol. To wash IONPs pellet with ethanol, the pellet was suspended in 35.5ml of 70% ethanol, vortex and centrifuge at 6000 rpm for 15 minutes. This washing step with ethanol was repeated 2 times. After that obtained IONPs were stored in 70% ethanol solution.

3.3 Characterization of Iron oxide Nanoparticles (IONPs)

3.3.1 UV/Vis Spectrophotometry

Newly synthesized IONPs were then subjected for characterization step by Ultraviolet-Visible spectrophotometry (UV/Vis). It was done by Ultraviolet-Visible spectrophotometer model AE-S90-2D (A & E Lab, UK). IONPs interact with light at very specific wavelength represents the unique optical properties of the NPs. By evaluating the absorbance spectra of aliquots of reaction mixture obtained through UV-Vis spectrophotometer in range of 200-800nm.

3.3.1.1 Sample Preparation of UV/Vis Spectrophotometry

To obtain the UV-Vis spectra of IONPs, a 3/4th of cuvette was filled with IONPs solution and was loaded in spectrophotometer, where the IONPs absorbed the photons of wavelength and then an absorption spectra of IONPs was recorded between the ranges of 200-800nm wavelength. 70% ethanol was used as blank for the correction of spectrophotometer's base line. The recorded spectral data was then stored and analyzed by using UV/Vis spectral analysis software. The obtained numerical data was then plotted on graphs for comparison.

3.3.2. Scanning Electron Microscopy (SEM)

SEM analysis was performed by using SEM (Jeol JSM-6490LA, Japan) to evaluate the NPs size and their morphology. 10kV accelerating voltage, 15mm working distance and 11 beam intensity of microscope range for each sample was set to perform SEM.

3.3.2.1. Sample Preparation for SEM

To prepare the sample for SEM, 10µl of colloidal IONPs solution was added to 10ml of 70% ethanol in glass vial. The glass vial was then placed in ultra-sonicator (Cole-Parmer) for ultra-sonication process for 1 hour at 37^{o}C to break the agglomerates of NPs and also to evenly distribution of particles throughout dispersion. Then 1 cm^2 cut glass slides were taken and a drop of sonicated homogenized sample was placed on it with the help of micropipette. Then allowed it

to dry under lamp for 30 minutes. This step was then followed by gold-palladium sputter coating of sample with the help of Sputter coater model no. (Jeol JFC-1500, Japan) to make their surface conductive by sputter coater. After coating of sample, it was placed on stubs of conductive tape and then sample was subjected for SEM analysis. The SEM images of IONPs were obtained at different magnifications and resolutions which were then analyzed.

3.3.3 Fourier Transform Infrared (FTIR) Spectroscopy

To determine the functional groups on IONPs FTIR was done by using Perkin-Elmer Spectrum-100 spectrometer (United States) by scanning at the wavelength range of 350-4000cm^{-1} and at resolution of 4cm^{-1}.

3.3.3.1. Sample Preparation for FTIR

Air dried samples are used in FTIR analysis. As the IONPs samples were in colloidal solution form, so firstly it was centrifuged to obtain the pellets. After that the samples were dried by placing them in incubator at 49^{o}C temperature in eppendorff tube. To remove any extra water molecule present in the sample, the air dried sample was then mixed with KBr because of its hygroscopic properties. Hydraulic press was then applied to form the pellet of IONPs and KBr. The pellet was then placed under infrared waves and scanned at the range of 350-4000cm^{-1} and at resolution of 4cm^{-1}. Then the obtained spectra from FTIR was plotted with wave number (cm^{-1}) on X-axis and transmittance (%) on Y-axis. The obtained spectral peaks was compared with standard functional group charts by method of manual peak picking.

3.3.4 X-Ray Diffraction (XRD) Spectroscopy

XRD is assumed to be one of the most proficient technique which can be used to identify the crystalline phases present in the materials. The instrument used in XRD analysis was X-ray generation device, model D8 Advance with DAVINCI design (BRUKER, Germany).

3.3.4.1. Sample Preparation for XRD

Air dried sample casted on microscopic glass slides was used in XRD analysis. This was done by placing a drop of IONPs solution on a microscopic glass slides and allowed it to dry in oven for 5-10 minutes. This step was repeated 4-5 times. Then sample was directed towards the X-ray generation device and a spectra was recorded. It was optimized at 40kV and 40mA with Cu-Kα radiation (λ= 1.58Å). Sample was scanned with an increment of 0.02^{O}/0.1 sec interval at 2θ range of 20-80^{O} at 6^{O}/min. Debye-Scherrer equation was used to measure the average crystallite diameter.

$$D = 0.9\lambda / \beta\cos\theta$$

In this, D (Feynman) is the size, λ (Feynman) is the wavelength of Cu-K radiation, θ (radians) is the half of the Bragg angle and β (λ= 1.58Å) is the full width at half maximum (FWHM).

3.4 Cytotoxicity of IONPs

Cytotoxicity analysis was done through cell viability assay MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide) to check the effect of different concentrations of IONPs on 2 different cell lines.

3.4.1 Culturing of Cell Lines

Human breast epithelial estrogen receptor positive cell lines, Michigan Cancer Foundation-**7** (MCF-7) and triple negative breast cancer (MDA-MB-231) were cultured in RPMI-1640 (Sigma Aldrich USA) which was supplemented with 1% antibiotic (Penicillin-Streptomycin) and 10% Fetal Bovine Serum (FBS) (Sigma Aldrich USA).

3.4.2 Sub-culturing of MCF-7 and MDA-MB-231 Cell Lines

MCF-7 and MDA-MB-231 cell lines were grown cell culture flaks of 250cm^2 and their confluency was evaluated under the inverted microscope. They were subjected to splitting when their confluency reached to 80%. For splitting of the cells, the media of the cells was removed and the

cells were washed out with the use of 2ml of autoclaved 0.01M Phosphate Buffered Saline (PBS) (Sigma Aldrich USA). PBS was removed from the cells after washing and 2ml of Trypsin-ethylenediaminetetraacetic acid (EDTA) solution (Sigma Aldrich USA) was added into it to detach the cells. Then they were incubated at 37°C for 5 minutes. As both cell lines were adherent so the cells were checked under the microscope of detachment which were then gently tapped so that all adherent cells scrap off from the surface of the flask. Then to obtain the pellet, the cells were centrifuged at 13,000 r.p.m for 3 minutes and supernatant was discarded. The cells were then re-suspended in RPMI media at 200 r.p.m for 2 minutes. To ensure the mono cell distribution of the cells, the cell were mixed with the use of pipette. Then the cells were seeded in flasks of 250cm^2 at 1 X 10^5 cells/ml density and were incubated at 37°C temperature and 5% CO^2 concentration.

3.4.3 Dilutions of Iron oxide Nanoparticles

For the cytotoxic evaluation of IONPs, different formulations were prepared. 0.1µg/ml, 0.5 µg/ml, 1 µg/ml, 1.5 µg/ml, 2 µg/ml and 2.5 µg/ml for IONPs were prepared and labelled as A, B, C, D, E and F respectively. Deionized water as positive and nor formulations except media as negative control labelled as X and Y respectively. All the dilution were performed in three replicates. The 200µl of each dilution was added into the plates and then covered it with aluminium foil without disturbing the wells. Labelled the plates with cell lines and placed them at incubator at 37°C temperature and 5% CO^2 concentration (Mahmoudi, Simchi, Milani, & Stroeve, 2009).

3.4.4 MTT Assay

The MTT assay is the widely and most frequent form of cell viability assay to investigate the cytotoxicity of the NPs or drug. MTT is a dye used to evaluate the cytotoxicity of IONPs. For MTT assay, 5mg/ml of MTT solution was made in PBS (Sigma Aldrich USA), filter sterilized and then stored at 4°C temperature. In each well of 96 well plate, 1.92 X 10^4 cells were added which were further incubated for 24 hours. Above prepared formulations of IONPs were added to these wells of 96 well plate and then incubated for 48-72 hours for 37°C. after that 15µl of MTT (5mg/ml) dye was added in each well and again incubated for 3 hours at 37°C C. after that 150µl

of solubilizing solution (DMSO) was added into each well after removal of MTT without creating any disturbance for formazon crystals. The incubation was conducted at room temperature for several minutes and on the other hand, to lysed the cells and to dissolve the purple crystals the material in each well were pipetted up and down. To protect against light, the plate was wrapped with aluminum foil. Absorbance was recorded at 550nm wavelength in the spectrophotometer. The reaction was conducted in three replicates and average value was determined (Almaki et al., 2016). The analysis was done through Graph-Pad Prism statistical software.

3.4.5 Evaluation of % Cell Survival & % Inhibition

Following formula was used to evaluate the MTT assay results:

$$\% \text{ Cell Survival} = \frac{(\text{Absorbance of Sample} - \text{Absorbance of Blank})}{(\text{Absorbance of Control} - \text{Absorbance of Blank})} \times 100$$

$$\% \text{ Cell Inhibition} = 100 - \% \text{ Cell Survival}$$

3.5 Antibacterial Activity of IONPs:

Antibacterial activity assay is very important and can lead to the epidemiology, drug discovery and the also lead to the prediction of therapeutic outcomes. Antibacterial activity is one of the property of IONPs. To evaluate the antibacterial activity, well diffusion assay was performed (C. S. Kim et al.). Well diffusion assay is basically is the method to quantify the ability of antibiotics or the sample to inhibit the growth of the bacterias. Well diffusion assay was performed for three different bacterial strains i.e. *Escherichia coli* (Gram-negative), *Staphylococcus Aureus* (Gram positive) and *Klebsiella pneumonia* (Gram-negative).

3.5.1 Preparation of Mueller-Hinton Agar (MHA) Media

Mueller-Hinton Agar (MHA) media was prepared as prescribed on the bottle by 38g/1000ml of Mueller-Hinton agar (HiMedia Laboratories, India) dissolved in 1000ml of distilled water and then sterilized the media by autoclaving at 121°C temperature, 15psi pressure for 45 minutes. Cool that liquid media to 40-50°C and then poured it into autoclaved petri dishes in laminar flow hood to avoid any contamination. After that allow the media in petri dishes to solidify. Then sealed all petri dishes and incubated at the temperature of 37°C to maintain the sterility of petri plates before doing experiment.

Table 3.2: Composition of Mueller-Hinton Agar Media

Ingredients	Concentration
Beef Extract (HiMedia Laboratories, India)	2g/L
Starch (HiMedia Laboratories, India)	1.5g/L
Acid Hydrolysate Casein (HiMedia Laboratories, India)	17g/L
Agar (HiMedia Laboratories, India)	17g/L

3.5.2 Preparation of Nutrient Agar (NA) Media:

Nutrient Agar (NA) media was prepared as prescribed on bottle by dissolving 28g/L of nutrient agar (HiMedia Laboratories, India) dissolved in 1000ml of distilled water. After that media was sterilized by autoclaving at 121°C. Then poured the sterilized nutrient media into autoclaved petri plates and allow them to solidify to grow the bacterial isolates.

Table 3.3: Composition of Nutrient Agar Media

Ingredients	Concentration
Yeast Extract (HiMedia Laboratories, India)	1.5g/L
Beef Extract (HiMedia Laboratories, India)	1.5g/L
Sodium Chloride (NaCl) (HiMedia Laboratories, India)	5g/L
Agar (HiMedia Laboratories, India)	15g/L
Peptone (HiMedia Laboratories, India)	5g/L

3.5.3 Preparation of Luria Broth Media

Luria Broth (LB) Media was prepared to culture the bacteria in falcon tubes. Appearance of turbidity is the sign of growth of bacterias. To prepare LB media, 10g of tryptone (Merck, Ponda,

Goa), 5g of yeast extract (Merck, Ponda, Goa) and 10g of sodium chloride (Sigma Aldrich, USA) was dissolved in 1000ml of distilled water. Mixture was heated to dissolve all the ingredients. After that it was autoclaved at 121°C. Then the media was poured into15ml falcon tubes to grow the bacterial cultures**.**

Table 3.4: Composition of Luria Broth Media

Ingredients	Concentration
Tryptone (Merck, Ponda, Goa)	10g/L
Yeast Extract (Merck, Ponda, Goa)	5g/L
Sodium Chloride (NaCl) (Sigma Aldrich, USA)	10g/L

3.5.4 Collection of Bacterial Isolates:

Bacterial isolates of *Escherichia coli* (Gram-negative), *Staphylococcus Aureus* (Gram positive) and *Klebsiella pneumonia* (Gram-negative) were provided by Dr. Fazal Adnan ASAB, NUST, Islamabad. These bacterial isolates were grown on nutrient agar plates prior to perform the well diffusion assay.

3.5.5 Inoculation of Bacterial Isolates

All 3 bacterial isolates taken with inoculation wire loops from pure cultures. These isolate were used to inoculate the LB broth present in 15ml falcon tubes with the help of wire loops. After that these 3 inoculated falcon tubes were incubated at 37°C. The appeared turbidity was the sign of growth of bacteria.

3.5.6 Preparation of IONPs Concentrations

Pellet of IONPs was dispersed in 1ml of deionize water through vortexing at high speed. This step is followed by sonication for 1 hour. Different concentrations of IONPs were made in eppendorff tubes. These concentrations were 35µg/ml, 45µg/ml and 55µg/ml. These dilutions made to test the antimicrobial property of IONPs.

3.5.7 Well Diffusion Assay

Well diffusion assay was used to evaluate the antibacterial activity of the IONPs. This assay was performed in laminar flow hood. To avoid any contamination, before using laminar flow hood, switch on the UV light for 15 minutes. Petri plate having solidified MHA were used to this assay. Marked the plates with 4 sections, 3 sections for 35µg/ml, 45µg/ml and 55µg/ml IONPs dilutions and 1 for negative control i.e. deionized water for all three bacterias. 1 agar solidified petri dish was labelled separately in 2 sections for positive control i.e. Cefotaxime (CTX) antibiotic (Oxoid Ltd., UK) and Ciprofloxacin (CIP) antibiotic (Oxoid Ltd., UK). These labelled petri dishes were cultured by dipping the sterilized cotton swab in LB broth having bacterial isolates and then spread it over the surface of MHA plate to inoculate the entire surface of the agar on petri plates. After that a hole with a diameter of 6-8 mm was punched on the solidified agar plate aseptically with a sterile tip. Then prepared dilutions of IONPs i.e. 35µg/ml, 45µg/ml and 55µg/ml were poured into the wells and deionized water was poured in the well of negative control. The CTX and CIP were also added in positive control labelled agar solidified plate. Then the petri plates were incubated at 37°C for 24 hours and evaluate the zone of inhibitions appeared on the petri plates after incubation. This experiment was perform in triplicates (Balouiri, Sadiki, & Ibnsouda, 2016).

3.6. Antioxidant Activity of IONPs

The free radical scavenging activity of synthesized IONPs was measured through DPPH (2,2-di-phenyl-2-picryl hydrazyl hydrate) assay (Sulaiman et al., 2011). It is quantified in terms of the inhibition percentage of free radicals formed by the antioxidants. This assay based on the principle that an antioxidant i.e. ascorbic acid acts as a hydrogen donor. When DPPH reagent is added to a solution then it has ability to interact with antioxidant and do its reduction. DPPH is a quantitative and calorimetric substance whose reduction can be determined by the observation of visual change of color from purple to yellow due to the formation of yellowish picrylhydrazine molecule when DPPH is reduced on the acceptance of a hydrogen from the donor. Its reduction can also be monitored by spectral analysis through UV-Vis spectrophotometer at wavelength of 517nm.

3.6.1 Preparation of IONPs Dilutions:

Different concentrations of IONPs, 200µg/ml, 400µg/ml, 600 µg/ml and 800µg/ml were taken. These concentrations were further diluted in methanol to get the final concentrations ranging from 100µl-1000µl.

3.6.2 Preparation of Standard Solution (Ascorbic Acid):

The standard solution (1mg/ml) of ascorbic acid (Sigma Aldrich, Germany) was prepared by dissolving 1 mg of ascorbic acid in 1 ml methanol. The solution in eppendorff was wrapped in aluminium foil to shield it from light. This solution was further diluted with methanol to get the final concentrations ranging from 100µg/ml-1000µl/ml i.e. 200µg/ml, 400µg/ml, 600µg/ml and 800µg/ml.

3.6.3 DPPH Free-Radical Scavenging Assay

To perform DPPH assay, 0.1mM DPPH solution was prepared by dissolving 3.9mg fresh DPPH (Sigma Aldrich, USA) was dissolve in 100ml of methanol. The solution was covered with aluminium foil and kept in dark to protect it from light. 2 sets of foil covered eppendorff was

labelled, 1 set for IONPs and other for standard solution i.e. ascorbic acid. 1 ml was taken from 3 prepared IONPs dilutions and 3 prepared ascorbic acid dilutions, which were then added into aluminium foil covered eppendorffs having 1ml DPPH solution. Mixed these vigorously and then incubated at 37°C for 30 minutes in the dark. After that absorbance was measured at 517nm, taking methanol as blank. Percentage inhibition of DPPH radical or percentage of scavenging radical by ascorbic acid and IONPs was evaluated through this formula:

% scavenging radical = [Absorbance $_{(Control)}$ – Absorbance $_{(Test\ Sample)}$ / Absorbance $_{(Control)}$] X 100

Where,

Absorbance $_{(Control)}$: Absorbance of methanolic DPPH solution

Absorbance $_{(Test\ Sample)}$: Absorbance of sample (ascorbic acid/IONPs) + DPPH solution

This experiment was performed in triplicates and Graph-Pad Prism software was used to analyze and plot the data to evaluate the antioxidant property of IONPs at different concentrations.

RESULTS

4.1 Observation of color change as initial confirmation of IONPs synthesis

Reaction was carried out between $FeCl_3.6H_2O$ and $FeSO_4.7H_2O$ on the dropwise addition of NaOH, followed by degassing through argon gas. After the dropwise addition of NaOH solution in reaction mixture, the color of final reaction mixture i.e. black color was compared with the color of starting reaction mixture of solutions of $FeCl_3.6H_2O$ and $FeSO_4.7H_2O$ i.e. light brown color. A color change from light brown color to black color gave the initial confirmation of the synthesis of IONPs as shown in Figure. 4.1. After 24 hours black colored pellet was settled down. It was the first step in the visual confirmation of synthesis of IONPs in the reaction mixture.

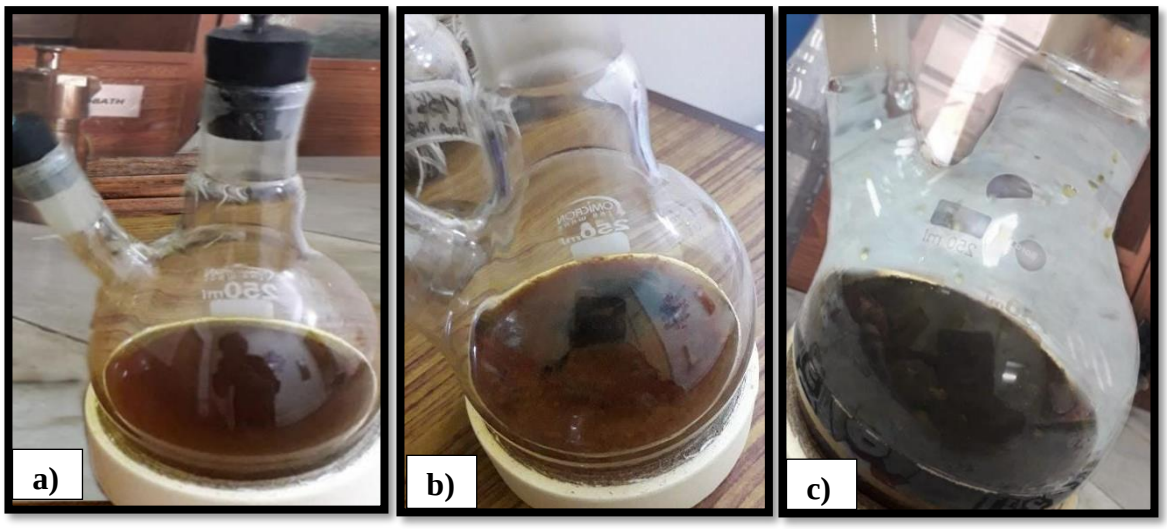

Figure 4.1: Comparison of color change during IONPs formation. **a)** Reaction mixture ($FeCl_3.6H_2O$ + $FeSO_4.7H_2O$) shows light brown color at start of the reaction, **b)** Black color precipitates start to form on the dropwise addition of NaOH, **c)** Black color solution at the end of the reaction illustrates the IONPs synthesis

4.2 Visual Analysis of Magnetic Property of IONPs:

To visually analyze the newly synthesized IONPs, the colloidal dispersion of newly synthesized magnetic IONPs was taken in the glass vial. A magnet was placed externally near to the glass vial having IONPs colloidal solution. The IONPs moved towards the direction of the magnet as shown in Figure. 4.2. It showed the visual confirmation of magnetic property of newly synthesized IONPs.

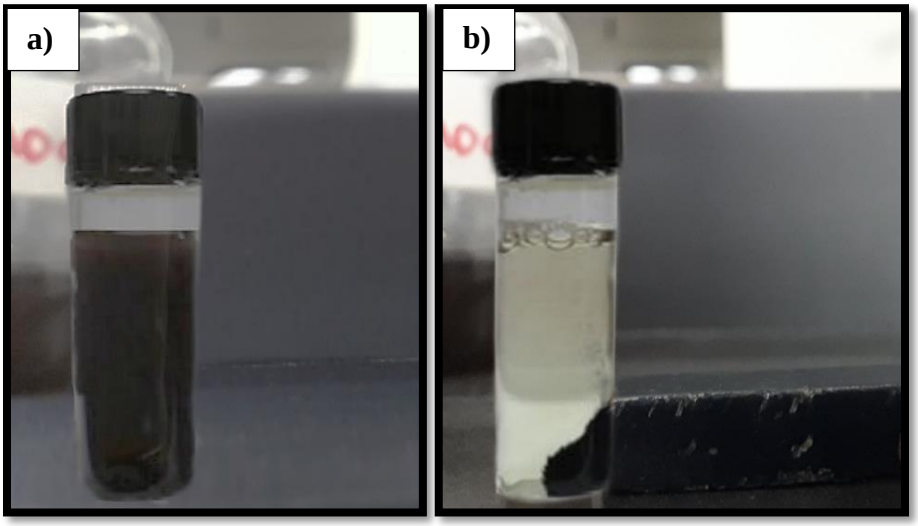

Figure 4.2: Visual Analysis of Magnetic Property of IONPs. **a)** Colloidal dispersion of magnetic IONPs without the magnet**, b)** Colloidal dispersion of magnetic IONPs in the presence of magnet, as IONPs move towards the magnet shows their synthesis

4.3 Characterization of IONPs

4.3.1. UV/Vis Spectral Analysis

The spectral analysis of IONPs was performed using UV/Vis spectrophotometer. It was done to confirm the synthesis of IONPs. Absorbance peak for IONPs sample was observed between the ranges of 200-800nm as reported in the literature (Justin, Samrot, Sahithya, Bhavya, & Saipriya, 2018), which confirmed the synthesis of IONPs. Figure 4.3 shows the UV-Vis spectra of synthesized IONPs as a result of reaction between $FeCl_3.6H_2O$ and $FeSO_4.7H_2O$ in the presence of NaOH. In Figure 4.3 the absorption peak can be clearly observed in the range of 299-310nm which confirms the formation of IONPs as per reported in the literature (Karpagavinayagam & Vedhi, 2019).

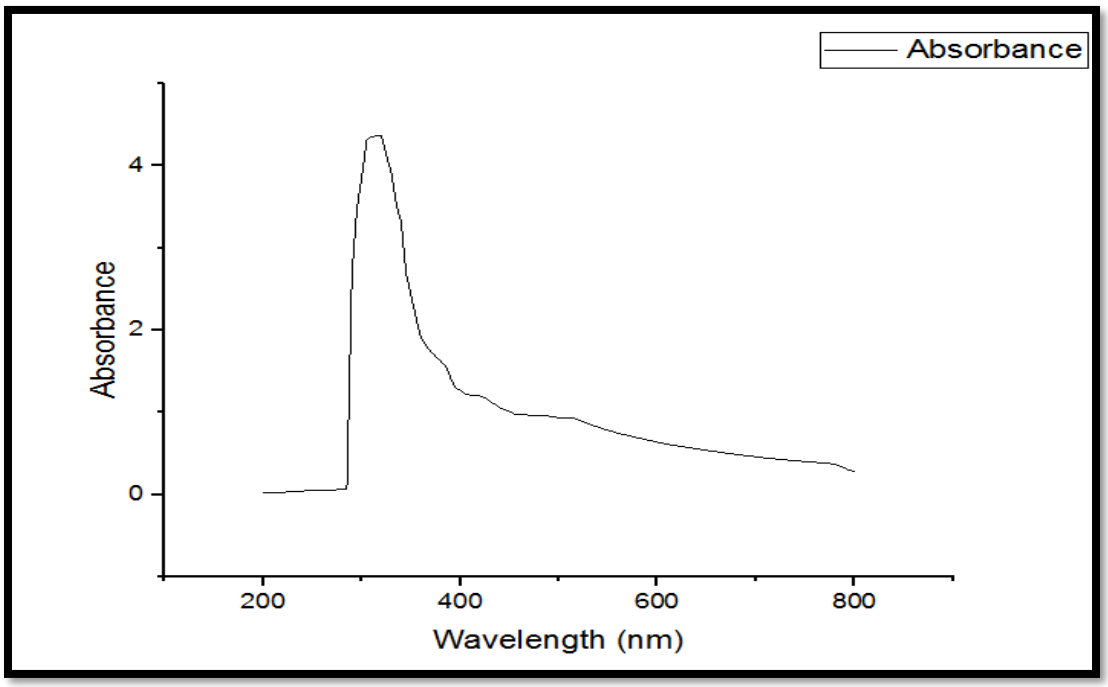

Figure 4.3: UV/Vis spectra of IONPs indicating peak at 306nm

4.3.2. Fourier Transformed Infrared (FTIR) Analysis

FTIR analysis was performed to determine and evaluate the potential of functional group forming properties of NPs present in the sample. Presence of functional groups and their activity is essential for the synthesis of NPs. FTIR of IONPs sample was performed through Perkin-Elmer Spectrum-100 FTIR spectrophotometer that was scanned at a range of 350-4000cm^{-1} and at a resolution of 4cm^{-1}. FTIR spectrum was obtained which can be seen in Figure 4.4.

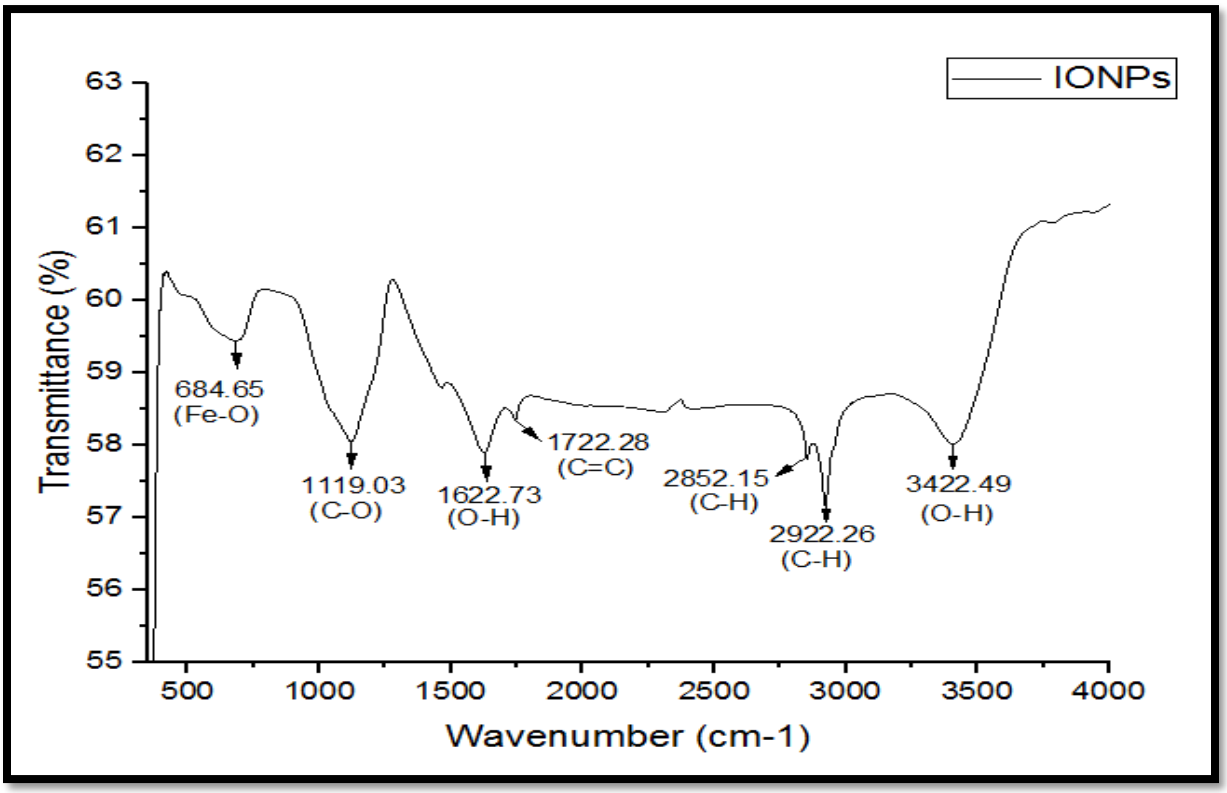

Figure 4.4: Infrared Spectrum of IONPs at the range of 350-4000cm-1. Spectral peaks at 684.65, 1119, 1622, 1722, 2852, 2922 and 3422 depicts the presence of different bands.

The bands at 3422.49cm^{-1} was due to the stretching of hydroxyl group (O-H) on the surface of IONPs. Two peaks were observed at 2922.26cm^{-1} and 2852.15cm^{-1}. These bands showed the presence of aliphatic and aromatic C-H stretching respectively. The peak at 1722.28cm^{-1} was the sign of presence of carbonyl group represents the C=C stretching. The band at 1622.73cm^{-1} determined the O-H group. 1119.03cm^{-1} peak corresponded the alcohol group (C-O) stretching present on IONPs. Characteristic bond between metal and oxygen was observed in the region of 350-800cm^{-1}. Peak at 684.65cm^{-1} of the sample showed the association of bands with metal and oxygen Fe-O stretching vibration on the surface of IONPs (Karpagavinayagam & Vedhi, 2019). These linkages provide the stability to the IONPs (Fatemi, Mollania, Momeni-Moghaddam, & Sadeghifar, 2018).

Table 4.5 Functional Groups of FTIR Spectra of IONPs and their Characteristic Group Frequency

Functional Groups	Wavenumber (cm^{-1})	Assignment
O-H	3422.49 (Broad)	Hydroxyl group, H-bonded OH stretch
C-H	2922.26 (Sharp)	Alkane group C-H stretch
C-H	2852.15 (Sharp)	Alkane group C-H stretch
C=C	1722.28 (Sharp)	Carbonyl group C=C stretch
O-H	1622.73 (Sharp)	Hydroxyl group O-H stretch
C-O	1119.03 (Sharp)	Alcohol group C-O stretch
Fe-O	684.65 (Broad)	Metal and oxygen group Fe-O stretch

4.3.4 X-Ray Diffraction Analysis

XRD was performed to check the phase and crystallinity nature of the IONPs. The diffracted intensity of IONPs was recorded from 0° to 80° 2(θ). The observed characteristic peaks of IONPs sample at 2θ = 30.35°, 35.81°, 43.39°, 53.88° and 63.09° were indexed to (220), (311), (400), (422) and (440) lattice plane of Bragg's reflection respectively as shown in Figure 4.5. The sharpest peak was corresponded to (311) which is the characteristic peak of IONPs (Jadhav & Patil, 2014). XRD results represented the spinal cubic centered and crystalline nature of IONPs. These results are very similar to many reported studies (Lal & Verma, 2017) (Rwei, Wang, & Chen, 2013).

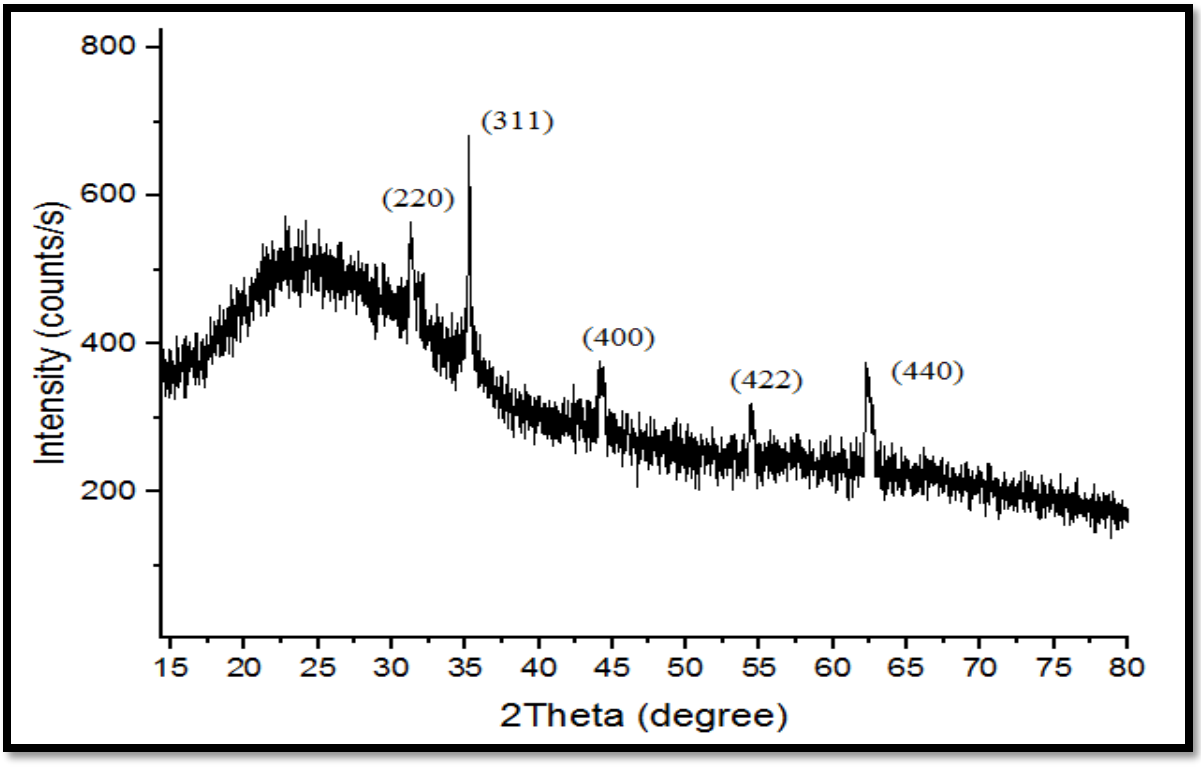

Figure 4.5: X-Ray Diffraction Pattern of IONPs

4.3.5 Scanning Electron Microscopy (SEM) Analysis

SEM analysis was done to magnify and determined the characteristic size and morphology of IONPs. The images obtained by SEM showed that synthesized IONPs were spherical in shape and distributed in aqueous colloidal solution. However agglomerates of IONPs were also showed in SEM images. It was because of the delay of time between sonication process and SEM procedure or may be due to the magnetic attraction of IONPs with each other caused agglomeration. According to the SEM analysis, the observed average size of all IONPs were in nano range which was 42-78nm as shown in Figure 4.7 and 4.8.

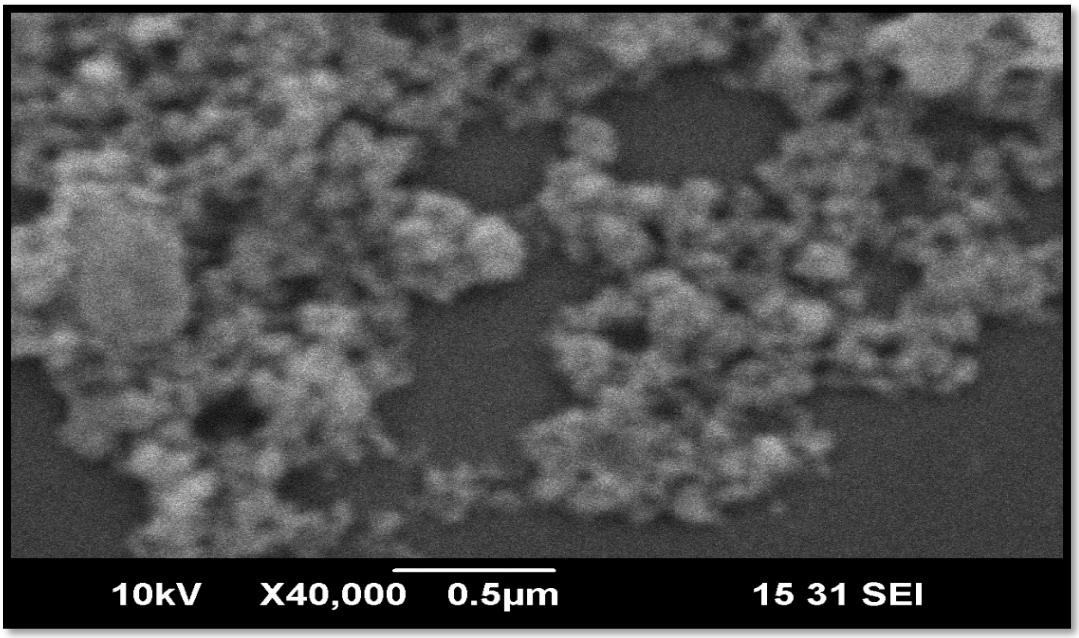

Figure 4.6: SEM image of IONPs at 0.5µm resolution (×40,000)

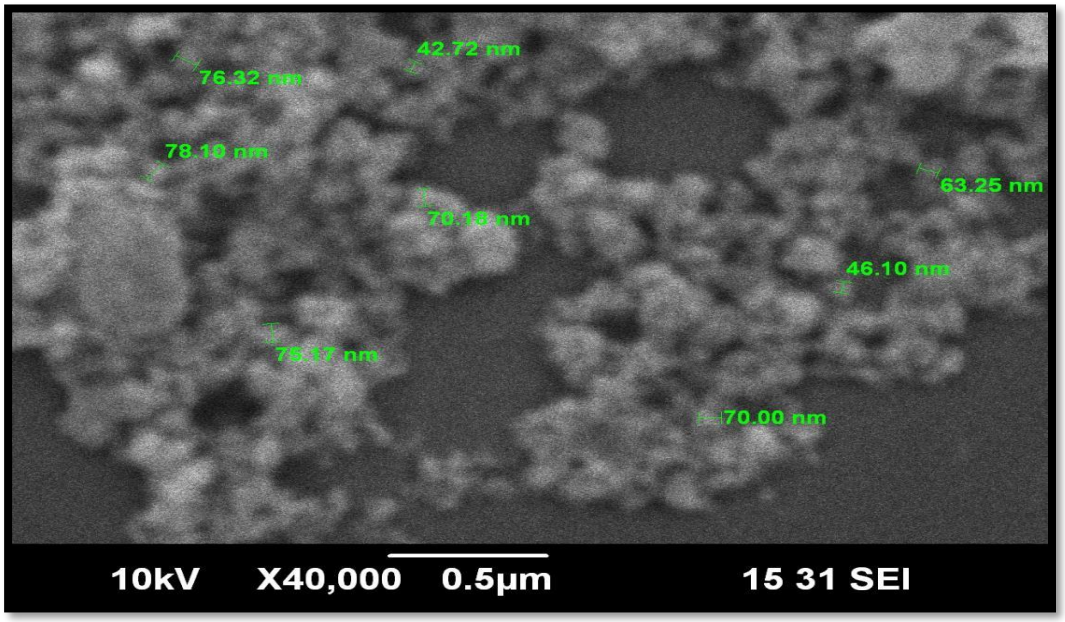

Figure 4.7: SEM image of IONPs at 0.5µm resolution with particles identification (×40,000). The image illustrates the different sizes of IONPs ranging from 42-78nm.

4.2.6 Cytotoxicity Analysis of IONPs

Human breast epithelial estrogen receptor positive cell lines (MCF-7) and triple negative breast cancer cells (MDA-MB-231) were exposed to different concentrations of IONPs to measure the cytotoxicity. Both cell lines were exposed at 0.1, 0.5, 1, 1.5, 2 and 2.5µg/mL concentrations of IONPs. The results revealed the dose dependent manner of cytotoxicity in both MCF-7 and MDA-MB-231 cell lines. IONPs were effective against cancer cell lines in lower concentration. As the concentration of IONP was increased, the cytotoxicity was also increased and cell viability was decreased as shown in Figure 4.8 and 4.9. The results were similar to previous studies which explained the dose dependent cytotoxicity of IONPs, as higher concentration of IONPs resulted into low cell viability (Kanagesan et al., 2013). It is expected that high concentration or dose

dependent cytotoxicity may be due to an increase of release of lactate dehydrogenase enzyme (LDH) which cause the damage of cell membrane (Alarifi, Ali, Alkahtani, & Alhader, 2014).

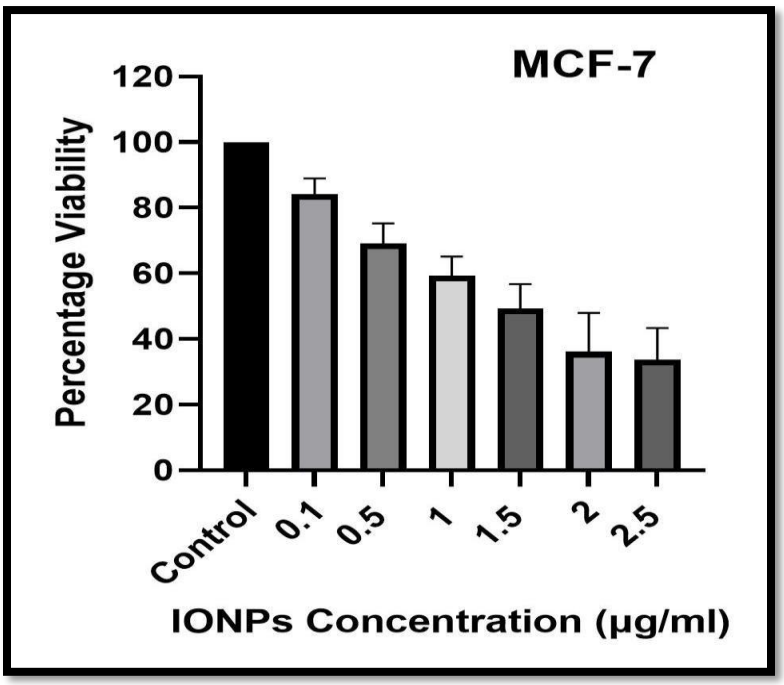

Figure 4.8: The graph illustrates the cytotoxic activity of IONPs against MCF-7 cell line at different concentrations (P<0.0001). Graph bars of MCF-7 cell viability becomes lower with an increase in concentrations of IONPs from 0.1ug/ml to 2.5ug/ml depicts the dose dependent cytotoxicity of IONPs

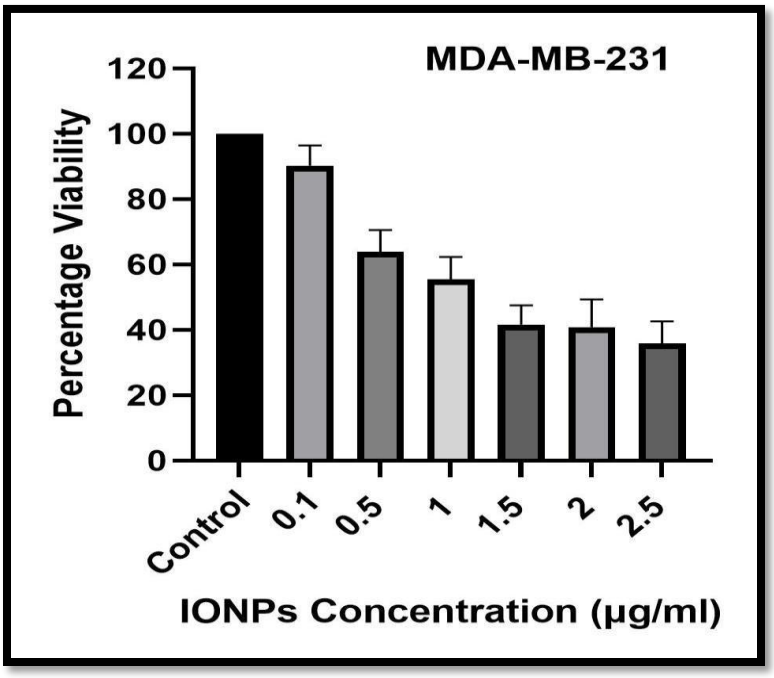

Figure 4.9: The graph illustrates the cytotoxic activity of IONPs on MDA-MB-231 cell line (P<0.0001). Graph bars of MDA-MB-231 cell viability becomes lower with an increase in concentrations of IONPs from 0.1ug/ml to 2.5ug/ml depicts the dose dependent cytotoxicity of IONPs

4.2.7 Analysis of Antibacterial Assay for IONPs

To check the antibacterial activity of IONPs, well diffusion assay was performed (Jagathesan & Rajiv, 2018). Well diffusion assay is basically is the method to quantify the ability of antibiotics or the sample to inhibit the growth of the bacterias. Well diffusion assay was performed for three different bacterial strains i.e. *Escherichia coli* (Gram-negative), *Staphylococcus Aureus* (Gram positive) and *Klebsiella pneumonia* (Gram-negative). 3 different concentrations of IONPs dilutions, 35µg/ml, 45µg/ml and 55µg/ml were taken for this purpose. After well diffusion assay, results were obtained which showed the dose dependent antibacterial activity of the IONPs because the diameter of zone of inhibition was increased as increasing the IONPs concentration. Different zones of inhibitions for all these 3 bacterias are shown in Figure 4.11.

The results showed that IONPs are effective against all three bacterial strains with maximum diameter of zone of inhibition of 22mm for *Escherichia coli*, 18mm for *Staphylococcus Aureus* and 12mm for *Klebsiella pneumonia.* The minimum zone of inhibition was observed by *Klebsiella pneumonia*. The minimum zone of inhibition or least antibacterial activity of IONPs was observed for *Klebsiella pneumonia*. No zone of inhibition was observed in negative control as it was filled with deionized water. 2 antibiotics named Cefotaxime (CTX) antibiotic and Ciprofloxacin (CIP) antibiotic were used in this assay as positive control. The experiment was repeated in triplicates and values were analyzed through Graph-Pad Prism software. The obtained results can be observed in Figure 4.10.

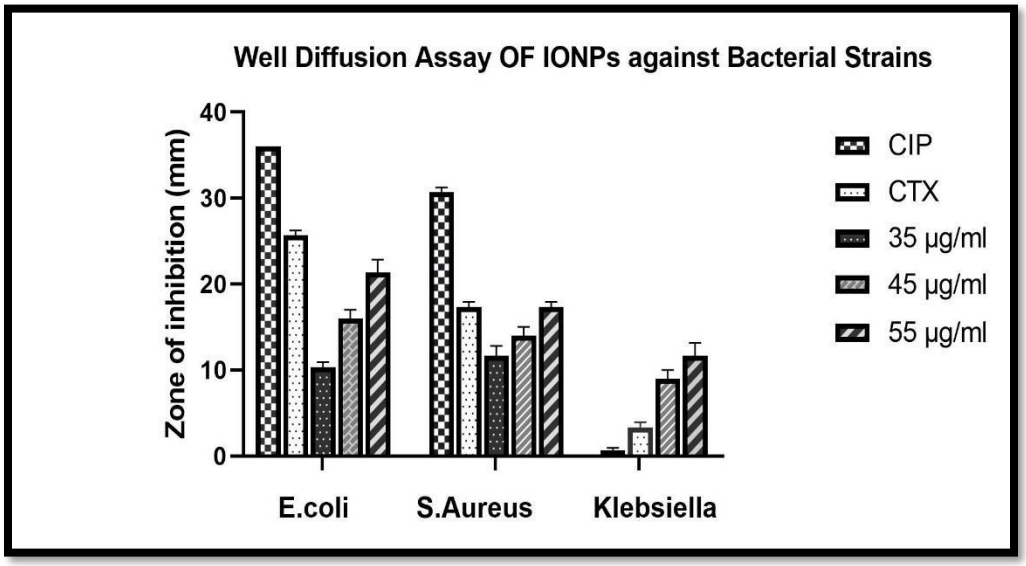

Figure 4.10*:* Comparison of zone of inhibition of 3 bacterial isolates *E.coli. Staphylococcus Aureus* and *Klebsiella pneumonia* after treatment with 35, 45 and 55ug/ml concentrations of IONPs and antibiotics CIP and CTX used as controls ($P < 0.001$).

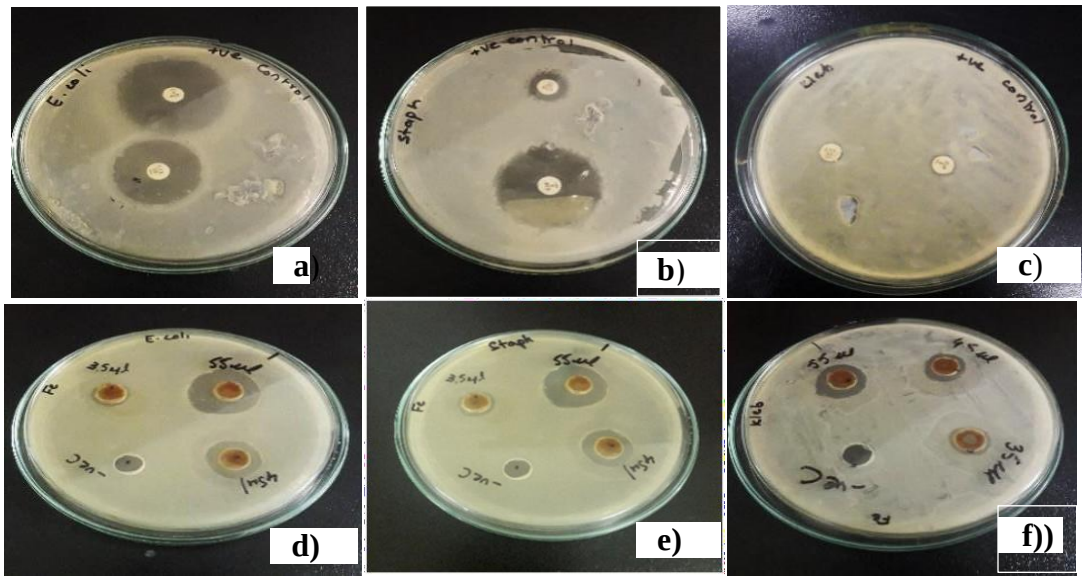

Figure 4.11: Inhibition zones of bacterial strains after treatment with 35, 45 and 55ug/ml concentrations of IONPs. **a)** *Escherichia coli* treated with positive controls**, b)** *Staphylococcus Aureus* treated with positive controls, **c)** *Klebsiella pneumonia* treated with positive controls, **d)** *Escherichia coli* treated with 35, 45 and 55ug/ml concentrations of IONPs, **e)** *Staphylococcus Aureus* treated with 35, 45 and 55ug/ml concentrations of IONPs, **f)** *Klebsiella pneumonia* treated with 35, 45 and 55ug/ml concentrations of IONPs

4.2.8 Analysis of Antioxidant Activity of IONPs

Antioxidant activity or free-radical scavenging activity of IONPs was evaluated by DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) assay (Alavi & Karimi, 2019; Sulaiman et al., 2011). When DPPH was dissolved in methanol to form solution then it gave purple violet color, but when an antioxidant was added then it caused its reduction, resulted in change in color from purple to pale yellow or colorless. Ascorbic acid was used as positive control in DPPH assay.

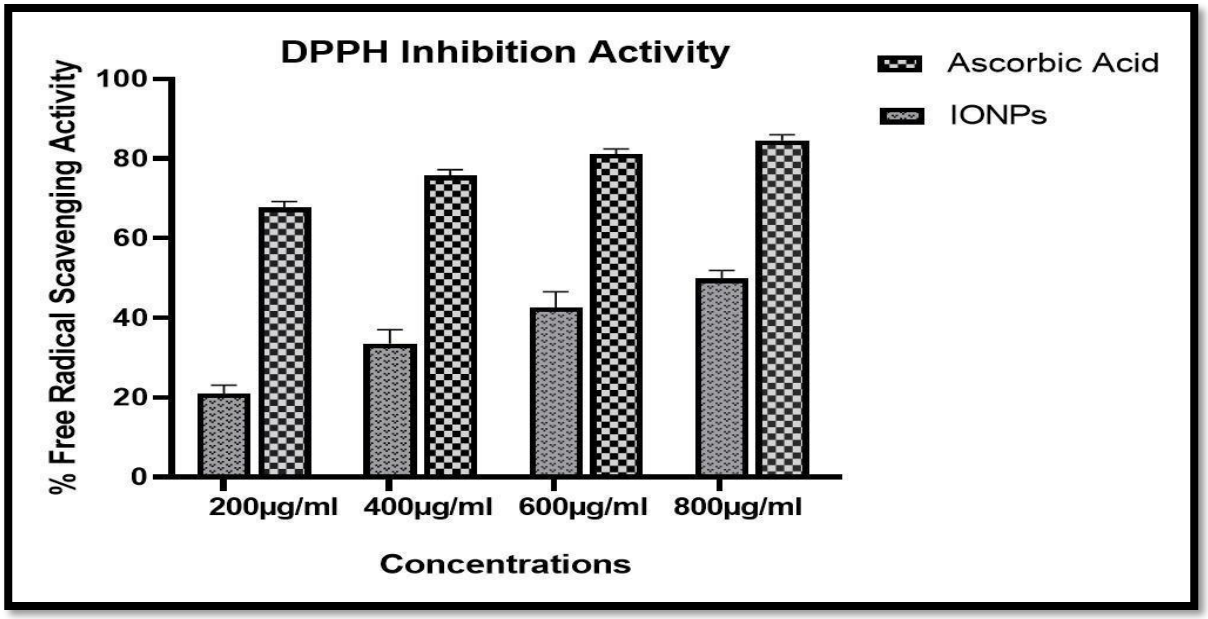

Figure 4.12: Comparison of free-radical scavenging activity of IONPs with standard ascorbic acid as control at different concentrations of 200, 400, 600 and 800ug/ml by DPPH assay ($P < 0.0001$). Graph illustrates the low % free-radical scavenging activity of IONPs than ascorbic acid.

The percentage inhibition of DPPH or free-radical scavenging activity was calculated through this formula,

% scavenging radical = [Absorbance ($_{Control}$)] – Absorbance $_{(Test\ Sample)}$ / Absorbance ($_{Control}$)] X 100

Where,

Absorbance ($_{Control}$): Absorbance of methanolic DPPH solution

Absorbance $_{(Test\ Sample)}$: Absorbance of sample (ascorbic acid/IONPs) + DPPH solution

Then the calculated percentage inhibition of DPPH was compared with positive control. It can be seen in Figure 4.12. In Figure 4.12, it can be observed that ascorbic acid showed maximum percentage inhibition of 86% at and IONPs showed maximum percentage inhibition of 51% at

same concentration of 800µg/ml. the lowest observed percentage inhibition for ascorbic acid and IONPs were 69% and 21% respectively at same concentration of 200µg/ml The obtained results revealed that IONPs have scavenging ability but is not significant to compare with other standard natural antioxidants because there is higher difference between percentage inhibition of control and IONPs at same concentrations (Alavi & Karimi, 2019).

DISCUSSION

Nanotechnology has a wide range of application in fields including biomedicine, bio-analysis, bio-detectors, drug delivery systems, medical implants analysis (NelA, 2006). The use of nanotechnology in the medicines has been revolutionized the way of our thinking about the disease to diagnose and also in their treatment for the humans (Doane & Burda, 2012). Among all the metallic NPs, IONPs have gained the prominent place in nanotechnology because of ease in the synthesis of IONPs and also in the modification of their surfaces (G. Liu et al., 2013). IONPs have gained the attraction because of its low toxicity, low retention time, its biodegradability and biocompatibility. IONPs are known for their excellent biocompatibilities as compare to other heavy metal based nanoparticles which are very difficult to degrade and are approved for medical use with low cytotoxicity level (J. S. Kim et al., 2006). The main feature of IONPs is their property of magnetism i.e. their ability to response to the magnetic field, so that the IONPs can be localized to the targeted site which play a very important role in the diagnosis and treatment. Their magnetism property helps to located lesion with the help of external magnetic field (Xie et al., 2010). They are also used in combination with drugs to enhance it activity and functionality and avoid many side effects of chemotherapy. IONPs have many other applications in drug delivery, drug targeting, detection, imaging, biological labels and targeted therapy (Patil et al., 2018).

Despite of many innovations in cancer diagnosis and treatment in the world, the detection of cancer on early stage and targeted drug delivery are still very difficult task in diagnosis of cancer. One of the main hurdle of using NPs in therapy is that the NPs are not able to reach the specific cancer site where they have to target it. But with the use of magnetic gradient field the IONPs can be attracted towards the chosen cancer site until the treatment of diagnosis is done (Berry & Curtis, 2003). Magnetic IONPs have the ability to diagnose the cancer at early stage in combination with tracing agents. Thus, IONPs can help in image guided drug therapy due to its efficacy in drug delivery and imaging (Farokhzad et al., 2006).

The properties of IONPs can be modified to enhance their effectiveness. It can be done by changing their surface, size distribution, their shape and coating with different polymers which enhance their effectiveness in terms of drug delivery, imaging, visualization of tumor and biocompatibility (Gupta & Gupta, 2005). The use of IONPs in drug targeting with increased effectiveness and low side effects was first given by Frei who used the coupling mechanism of IONPs with anticancer agents which was injected in blood stream and reached to the targeted cancer site with the help of external magnetic field (Frei, 1969).

IONPs can be synthesized from various ways. One of them is co-precipitation method because of its easy instrumentation, low labor and low cost. Upon the dropwise addition of NaOH in the mixture of $FeCl_3.6H_2O$ and $FeSO_4.7H_2O$ after degassing of the solution through argon gas then black color precipitates were formed (Ansari et al., 2019). The initial visual confirmation of the synthesis of IONPs was the change of the color of reaction mixture from light brown to black color which is the feature of IONPs (F Hasany, H Abdurahman, R Sunarti, & Jose, 2013). The synthesis of IONPs was further confirmed by placing the magnet adjacent to the glass vial having IONPs which resulted in the attraction of newly synthesized IONPs towards the magnet. It is because of the magnetic property of IONPs (Kharisov et al., 2014). The magnetic property of IONPs make them an effective source for the targeted drug delivery to the cancer site and have reduced side effects or off-targeted drug delivery. Thus IONPs is an excellent approach in diagnostics and therapeutics (Xie et al., 2010).

Bare NPs may show undesirable or less effective results in biological systems. But due to the coating with other polymers may greatly enhance their effectiveness. IONPs have advantages because they have large surface/volume ratio, multivalent interactions with other polymers and high binding affinity (J. Gao et al., 2006). In a study, it was reported that the conjugation of magnetic IONPs and fluorescence CQDs makes hybrid NPs having magnetic property of IONPs and photoluminescence and photo-stability property of CQDs which will be an excellent replacement of other devices in diagnostics and therapeutics (H. Wang et al., 2014).

The different physical properties of IONPs were evaluated through different standard characterization techniques. These characterization techniques include UV/Vis spectrophotometry, SEM, FTIR spectroscopy and XRD (Justin et al., 2018). Synthesis of IONPs was verified through these standard characteristics techniques. To know about the functional groups present or adsorbed on the surface of the IONPs, FTIR analysis is used. The obtained FTIR spectrum of air dried sample of IONPs showed different bands present on them. In Figure 4.4, stretching of hydroxyl group (O-H stretch), alkane group (C-H stretch), carbonyl group (C=C stretch), alcohol group (C-O stretch) and metal and oxygen group (Fe-O stretch) can be observed present on the surface of the IONPs. These groups give stabilization to the newly synthesized IONPs and also provides the capping sites for other molecules (Fatemi et al., 2018). Studies have reported that surface of IONPs has many covalent and electrostatic interactions. These interactions are helpful in coating and conjugation of IONPs with other polymers or nanoparticles. Through FTIR we can analyze the expected linkages (Munasinghe et al., 2019).

NPs have unique and different optical properties. They are sensitive to different states like their size, concentration, and way of method, shape, agglomeration and the surface of NPs. So the UV-Vis spectrum analysis is best to confirm the synthesis of IONPs. In UV-Vis analysis of IONPs at the wavelength of 200-800nm, the characteristic peak of newly synthesized IONPs was observed at the range of 299-310nm as shown in the Figure. 4.3. The UV-Vis peak of IONPs at the range of 299-301nm was also reported in another study (Karpagavinayagam & Vedhi, 2019).

The main function of XRD analysis is to check the phase and crystallinity of the IONPs. XRD diffractogram of air dried sample of IONPs was obtained which showed the characteristic peaks of IONPs sample at 2θ = 30.35°, 35.81°, 43.39°, 53.88° and 63.09°were indexed to (220), (311), (400), (422) and (440) lattice plane of Bragg's reflection respectively as shown in Figure 4.5. The sharpest peak was corresponded to (311) which is the characteristic peak of IONPs (Jadhav & Patil, 2014). XRD results represented the spinal cubic centered and crystalline nature of IONPs. These results are very similar to many reported studies. In one study, cubic phased IONPs at 2θ = 30.103°, 35.451°, 43.088°, 53.516°, 56.998°, 62.657° and 74.098° indexed to the (220), (311),

(400), (422), (511), (440) and (533) planes respectively was reported (Lal & Verma, 2017). In other studies, similar XRD patterns were explained (Rwei et al., 2013) (Mishra & Sardar). SEM was performed to know about the shape, size, contamination and any fracture on the surface of IONPs through high resolution imaging analysis (Saqib et al., 2019). High resolution SEM images of IONPs showed that IONPs were spherical in shape and did not lose their shapes even after intense sonication, purification and centrifugation. The sizes of IONPs were ranging from 42-78 nm as per shown in Figure 4.7.

NPs have different properties according to their size, shape, surface and concentration. They behave differently in bulk or in trace amount. So it is better to check their overall cytotoxicity so that they can be efficiently use for biomedical applications. IONPs are the best candidate for diagnostics and therapeutics purpose in biomedical science (Patil et al., 2018). MTT method is most widely, efficient and calorimetric method which is used to check and measure the cellular cytotoxicity, cell viability and proliferation quantitatively. It is also used to check the growth and biocompatibility of the cells under specific conditions (M Mahmoudi et al., 2009). Several studies have been reported on the cytotoxic potential of many different types of IONPs. They have found that there is no or low cytotoxicity is associated with IONPs. But have adverse effects if the concentration of IONPs will rise from 100µg/ml. It means that IONPs have concentration dependent cytotoxicity (Singh et al., 2010). The cytotoxicity of IONPs also depends on time of exposure. High dose exposure may results in causing cellular stress which may alter the response (Patil et al., 2018). Our MTT analysis for the evaluation of cytotoxicity on MCF-7 cell lines and MDA-MB-231 cell lines against IONPs revealed that IONPs have dose dependent cytotoxicity which increased as the concentration of dose of IONPs has been increased from 0.1µg/ml to 2.5µg/ml as shown in Figure 4.8 and 4.9. These results are similar to previously reported studies (Fatemi et al., 2018). Kanagesan et al., studied the cytotoxic effect of IONPs on MCF-7 cell line and explained the dose dependent cytotoxicity of IONPs, as higher concentration of IONPs resulted into low cell viability (Kanagesan et al., 2013). It is expected that high concentration or dose dependent cytotoxicity may be due to an increase of release of lactate dehydrogenase enzyme (LDH) which cause the damage of cell membrane (Alarifi et al., 2014).

IONPs have also gained attention from cancer diagnosis and its treatment to their antimicrobial and antioxidant activity (Patra, Ali, Oh, & Baek, 2017). Drug resistance in microbes is one of the major problem in medical sciences. It was observed that people having minor surgery resulted due to the microbial infections which cannot be treated with the help of antibiotics, even if infection gets serious then it also may result in the death of a person. The antimicrobial resistance is a catastrophic threat for humans which need an immediate attention to do some improvements in this field. There is need of new and novel ways to developed to efficient destruction of these microbes so that these circumstances can be avoided in the future (Vallabani & Singh, 2018).

Nano-medicines have contributed a lot in this regard. NPs as antimicrobial agents have many advantages as compare to the traditional used antibiotics. It is expected that microbes may don't have the ability to develop resistance against nanomaterials or NPs. It means that even after many dosages, the antimicrobial activity of nanoparticles against microbes may would remain same. In this scenario, studies have been reported to explore about an effective antimicrobial activity of the IONPs (Nehra, Chauhan, Garg, & Verma, 2018). In a reported study, it was revealed that IONPs had exhibited the antibacterial activity when a well diffusion assay was performed against *S. epidermidis* (Groiss, Selvaraj, Varadavenkatesan, & Vinayagam, 2017). In another reported study it was observed that IONPs have good antibacterial activity against S. *aureus, P. fluorescens and E.coli* (Jagathesan & Rajiv, 2018). *Staphylococcus aureus, Shigella dysentry* and *Escherichia coli* had also demonstrated the antibacterial activity of IONPs (Saqib et al., 2019). Newly synthesized IONPs were also tested on *S.aureus* who developed a clear zone of inhibition against this bacteria. This may be due to the oxidative stress because of the electromagnetic interactions and also may be due to the production of ROS species which cause disruption in membrane and ultimately leads to the death (Das et al., 2020). Ismail et al. also tested the antimicrobial activity against *Staphylococcus aureus* and demonstrated that IONPs with the help of magnetic field can capture the *Staphylococcus aureus* (Ismail, Sulaiman, Abdulrahman, & Marzoog, 2015).

Well diffusion assay of IONPs against *Escherichia coli* (Gram-negative), *Stapylococcus Aureus* (Gram positive) and *Klebsiella pneumonia* (Gram-negative) revealed the antibacterial property of

IONPs. As in Figure 4.11, *Escherichia coli*, *Staphylococcus Aureus* and *Klebsiella pneumonia* have shown the maximum zone of inhibition of 22mm, 18mm and 13mm respectively at 55 μg/ml concentration. The antibacterial activity of IONPs may be due to the disruption of cell wall, disruption in plasma membrane and also by deactivation of the cellular enzymes of the microbe. It ultimately cause the ROS production which leads to the damage of DNA and death of microbes. (Arias et al., 2018)

Antioxidants are present in our biological systems and cells and generate scavenge free radicals inside a cell as a result of a biochemical process (Serpen et al., 2007). IONPs have been found to possess some antioxidant potency. To examine the antioxidant activity of nanoparticles DPPH assay is widely used. The antioxidant ability is due to the transfer of an electron which neutralizes the free radical DPPH (Naik et al., 2003). To check the antioxidant activity of IONPs, DPPH antioxidant assay was performed. The DPPH reducing ability of IONPs was visually examined by observing change of the color from violet to pale yellow. It was also analyzed by spectrophotometer. The absorbance values at 517 nm were obtained. As in Figure 4.12, it can be observed that the free radical scavenging ability of IONPs is increased with increasing the concentration of IONPs. The observed lowest free radical scavenging activity of IONPs was 21% at 200μg/ml concentration and highest free radical scavenging activity was 51% at 800μg/ml concentration. But in case of standard ascorbic acid the observed highest free radical scavenging activity was 86% and lowest free radical scavenging activity is 69%. The findings show that IONPs have potent antioxidant activity but is not significant enough to be compare with other natural standard antioxidants like ascorbic acid (Alavi & Karimi, 2019).

CONCLUSION & FUTURE PROSPECTIVES

The present study is majorly emphasized on the synthesis of IONPs through co-precipitation method in which black color precipitates of IONPs were formed on the dropwise addition of NaOH in the mixture of $FeCl_3.6H_2O$ and $FeSO_4.7H_2O$, followed by the degassing of the solution through Argon gas. To check the characteristics and the detailed analysis of newly synthesized IONPs they were subjected to different standard characterization techniques which include UV-Vis, SEM, FTIR and XRD. The obtained results of SEM of IONPs revealed that IONPs are spherical in shape and their sizes are in range from 42-78 nm. FTIR revealed that they have hydroxyl, carbonyl group, carboxyl group and metal groups present on the surface of the IONPs which helps in forming the different linkages and conjugation with other nanoparticles or polymers to enhance the effectiveness and protection. The sharpest peak was indexed to $2\theta = 35.81^o$ at (311) plane in XRD analysis which revealed the crystalline nature and cubic structure of IONPs as per reported in literature which gives them stability. To evaluate the cytotoxicity of the IONPs on MCF-7 and MDA-MB-231 cell lines, MTT assay was performed. The obtained results of cytotoxicity revealed that IONPs have dose dependent cytotoxicity, as the concentration of IONPs increased the % cell viability has been decreased. Antibacterial activity of IONPs against 3 different strains was evaluated by well diffusion assay to provide an alternative way to antibiotics. It was revealed that IONPs have good antibacterial activity against *Escherichia coli* and *Stapylococcus Aureus*, but have low antibacterial activity against *Klebsiella pneumonia*. DPPH assay was performed to evaluate the antioxidant activity which revealed that IONPs have free radical scavenging ability but it is not significant enough to be compared with other standard natural antioxidants such as ascorbic acid. The IONPs hold a progressive future in biomedical applications with respect to early diagnosis of cancer, deep cellar and tissue imaging, targeted drug delivery and therapeutics. After the extensive analysis of the obtained results in this research study, it is suggested to further characterize the IONPs with more sensitive and elaborative characterization techniques which includes EDS, TEM, AFM, and Zeta potential so that the further information about characteristics

of IONPs can be found. Other methods for the synthesis of IONPs should be focused which use the source of natural means in their synthesis to further increase their effectiveness and biocompatibility. Presently, IONPs are successfully used in many biomedical applications like as a diagnostic probe for the detection of disease and cancer in different organs of human body include brain, blood vessels, heart, liver etc. But there is need of more research on IONPs. Multifunctional modalities and advancements in IONP synthesis and applications can bring better ways in the field of biomedical science. Coating and conjugation of IONPs with other nanoparticles or polymers like CQDs conjugation with IONPs needs to gain attention because the combination of highly magnetic property of IONPs and photoluminescence property of CQDs is an excellent approach in early diagnosis of cancer and other diseases. It will also helpful in targeted drug delivery and used as carriers. Strategies should be focused and developed to conjugation IONP@CQD hybrids with an antibody to enhance the effectiveness of nanoparticles by avoiding off target drug delivery. The interaction of IONPs with cancer cells and other human cells needs to be evaluated. Cell cycles occur inside the body in cancer cells can be a source of information about their molecular pathway and how these nanoparticles can be modified to target and treat the cancer cells with minimal cytotoxicity. The interaction of IONPs with microbes is needed to be modified to enhance the antimicrobial activity of the IONPs as the replacement of the resistance causing antibiotics. The attempt of using IONPs in theranostics would be able to make diagnosis and therapeutics much easier, simpler and less invasive. Multifunctional IONPs would be an effective and attractive strategy in biomedical applications to make the human's life easier.

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