CHAPTER V

ANTIBACTERIAL APPLICATIONS OF IRON AND SILVER NANOPARTICLES

5.1 INTRODUCTION

The emergence of nanoscience and nanotechnology in the last decade presents opportunities for exploring the bactericidal effect of metal nanoparticles. Nanoparticles possess increased surface area and therefore have increased interactions with biological targets (such as bacteria) compared with conventional, micron particles. In addition, nanoparticles are much more likely to enter cells than micron particles. As a result, nano crystalline antibacterial particles are likely to exert stronger effects on bacteria than their micron-counterparts. Nanoparticles are special and interesting because their chemical and physical properties are different from their macro counterparts. Nanoparticles have unique properties due to their small size. All nanoparticles regardless of their chemical constituents have surface area to volume ratios that are extremely high. This makes the physical properties of nanoparticles to be dominated by the surface atoms present on the nanoparticles surface. The high surface area to volume ratio in nanocrystals can lead to unexpected properties.

Nanoparticles have been studied for a wide range of medical applications. The advantages of nanoparticles include their high surface-to-volume ratios and their nano scale sizes. The high surface areas of nanoparticles allow for more active sites for interacting with biological entities such as cells. The higher surface area of nanoparticles compared with conventional micron-size particles also offers more sites for functionalization with other bioactive molecules, such as anticancer and antibacterial drug molecules. The nanoscale sizes of nanoparticles provide valuable properties that are not available in micron particles.

A particle with a high surface area has a greater number of reaction sites than a particle with low surface area, and thus, results in higher chemical reactivity. The change in properties of materials is due to increased surface area to volume ratio. Reactions take place at the surface of a chemical or material; the greater the surface for the same volume, the greater the reactivity. The link to nanotechnology is that as particles get smaller; their surface area to volume ratio increases dramatically.

5.2 ANTIBACTERIAL ASSAY

A cell wall is present outside the bacterial cell membrane and it is essential to the survival of bacteria. It is made from polysaccharides and peptides named peptidoglycan. There are two different types of cell wall in bacteria, called gram-positive and gram negative. The names originate from the reaction of cells to the gram stain, a test long-employed for the classification of bacterial species. Gram-positive bacteria possess a thick cell wall containing many layers of peptidoglycan. In contrast, gram-negative bacteria have a relatively thin cell wall consisting of a few layers of peptidoglycan. Surfaces of copper nanoparticles affect / interact directly with the bacterial outer membrane, causing the membrane to rupture and kill bacteria. Nanoparticles rely on an entirely different mechanism of antibacterial activity when compared to traditional antibiotics, and hence is an encouraging alternative.

The bactericidal property of nanoparticles depends on their size, stability, and concentration added to the growth medium, since this provides greater retention time for bacterium nanoparticles interaction. In general, bacterial cells are in the micron-sized range. Most bacterial cells have cellular membranes that contain pores in the nanometer range. A unique property of crossing the cell membrane can potentially be attributed to the entering of synthesized nanoparticles through such bacterial pores. However, to make this possible, it is important to overcome challenges and prepare/design nanoparticles which are stable enough to significantly restrict bacterial growth while crossing the cell membrane.

Therefore, the objective of this study is to examine the antibacterial effect iron (Fe) and silver (Ag) nanoparticles against two Gram-negative bacteria

(*Escherichia coli and Pseudomonas aeruginosa*) and two Gram-positive bacteria (*Bacillus subtilis and Staphylococcus aureus*).

To realize the potential of iron (Fe) and silver (Ag) nanoparticles to act as antimicrobial agents, Fe and Ag nanoparticles were synthesized by chemical precipitation method. Furthermore, the antibacterial activities of different quantities of Fe and Ag nanoparticles against two Gram-negative bacteria (*Escherichia coli and Pseudomonas aeruginosa*) and two Gram-positive bacteria (*Bacillus subtilis and Staphylococcus aureus*) were investigated.

5.3 ANTIMICROBIAL SUSCEPTIBILITY TESTING BY KIRBY-BAUER DISK DIFFUSION

Kirby-Bauer disk diffusion is the least expensive screen for antimicrobial susceptibility testing. These tests are useful for screening isolates to categorize them as susceptible, intermediate, resistant, or non-susceptible for several antimicrobials. Either 150mm or 100mm plate can be used for Kirby-Bauer disk diffusion depending on the number of antimicrobial agents to be tested per isolate. Clinical and Laboratory Standards Institute (2012), CLSI guidelines state that not more than two disks can be used on a 100-mm plate and up to five disks can be used on a 150-mm plate (Figure 5.1).

Isolates to be tested should be sub cultured and incubated in a CO_2-enhanced atmosphere (5% CO_2 in a CO_2-incubator or candle-extinction jar) at 35±2°C for 20-24 hours prior to testing. If the organism is frozen, it should be sub-cultured twice when it is removed from the freezer before proceeding with susceptibility testing.

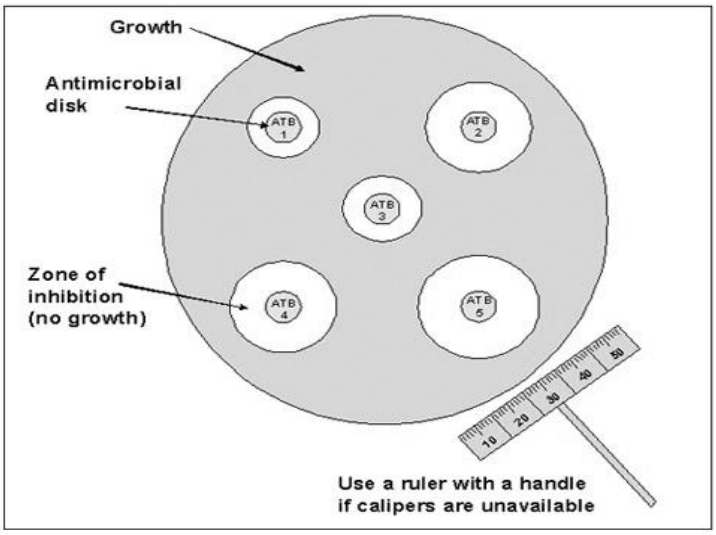

Figure 5.1 The antimicrobial susceptibility disk diffusion test: approximate disk placement and measurement of inhibition zone diameters.

ATB1 = antibiotic 1, ATB2 = antibiotic 2

Remove agar plates from the refrigerator and allow them to come to room temperature (25°C) before inoculating. Warm the cation-adjusted Mueller-Hinton broth (CAMHB) to 35°C before using. Allow the antibiotic disks that will be used in the batch of testing to warm to room temperature (25°C).

Using a sterile cotton-tip applicator, touch the surface of one to four morphologically identical, isolated colonies. Immerse the applicator into a tube containing sterile CAMHB. Rub the applicator against the wall of the tube slightly to release a small amount of growth into the liquid. Cap the tube and mix the cells using a vortex to form a suspension; be careful not to allow formation of froth or bubbles in the suspension when mixing the cells. Adjust the turbidity of the inoculum to that of a 0.5 McFarland turbidity standard (approximately 1-4 x 10^8 CFU/ml). If the turbidity of the inoculum is greater than the standard, dilute it with CAMHB to equal the turbidity of the standard. This suspension must be used within 15 minutes. Perform regular colony counts to verify that the density of the inoculum suspension is correct. It is not necessary to perform colony counts on every isolate tested.

Immerse a sterile cotton-tipped swab into the adjusted inoculum. Remove excess liquid by pressing the swab tip against the inside of the tube. Inoculate the entire surface of a Müller-Hinton broth three times with the same swab of inoculum, rotating the plate 60 degrees after each inoculation to ensure even distribution of the inoculum and confluent growth of the bacteria. Use a single swab of inoculum and do not return the swab to the broth after each rotation. Allow the inoculum to dry on the surface of the plate (which should take approximately 5-10 minutes). Be sure the plate is entirely dry before proceeding, but do not exceed 15 minutes.

When the surface of the inoculated plate is dry and the disks are at room temperature, place the disks onto the agar with an applicator or sterile forceps. Make sure that the disks are spaced enough distance apart on the agar so the zones of inhibition do not overlap (Figure 5.1). Press down on the disks to ensure complete contact with the agar surface. Alternatively, a mechanical disk dispenser can be used. Once applied, it is important to not to move the antibiotic disks as the antibiotic will begin to diffuse immediately upon contact with the plate.

Incubate the plates in an inverted position in a 5% CO_2 atmosphere or candle jar for 18-24 hours at 35±2°C. After overnight incubation, measure the diameter of each zone of inhibition with a ruler or callipers (Figure 5.2). Measurements should be performed in a biosafety cabinet, if possible. Use either callipers or a ruler with a handle attached for these measurements, holding the ruler over the centre of the surface of the disk when measuring the inhibition zone (Figures 5.1 and 5.2).

In the case of an isolate completely resistant to the antimicrobial, simply measure the diameter of the disk: 6 mm (left). When there is a zone of inhibition, measure the diameter as shown: 16 mm (right). Care should be taken not to touch the disk or surface of the agar. Decontaminate the ruler occasionally to prevent transmission of the bacteria.

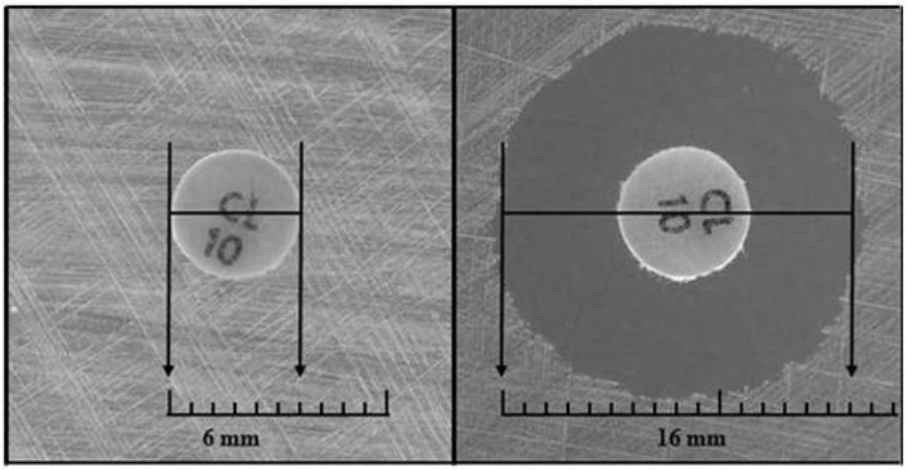

Figure 5.2 Images showing proper measurement of zone diameter

In all measurements, the zones of inhibition are measured as the diameter from the edges of the last visible colony of the unaided eye. Record the results to the nearest millimetre (mm).

5.4 CULTIVATION OF BACTERIA

For the cultivation of bacteria, a common medium nutrient broth is used; a liquid which contains proteins, salts and growth enhancers that supports bacterial growth were used. To solidify the medium, agar (a polysaccharide) was added and solidified. This medium is called as nutrient Agar. Presently this medium was used for the cultivation of bacteria.

5.4.1 Preparation of Nutrient Broth Medium

In a conical flask accurately weighed chemical ingredients Peptones 15.0 g, yeast extract 3.0 g, sodium chloride 6.0 g and D(+)glucose 1.0 g of nutrient broth to which agar-agar 12.0 g added as a solidifying agent were dissolved in 100 ml distilled water. The chemical content of conical flask was heated with slight agitation to dissolve the added ingredients. Distilled water was added to the conical flask to make the volume of solution to 1 litre. The pH of the broth was measured by using a pH meter and it was adjusted to pH 7 by adding the NaOH solution in drop wise. The prepared 1litre broth was distributed into 10 ml broth to each culture tube to prepare the agar slants. The mouth of the broth tubes were covered tightly by

cotton plugs with aluminum foil. Then the broth tubes are placed inside the autoclave and sterilized at 121˚C for 30 minutes and it is stored at room temperature for further use. The process of preparation for the nutrient broth medium is depicted through the flow chart given in Figure 5.3.

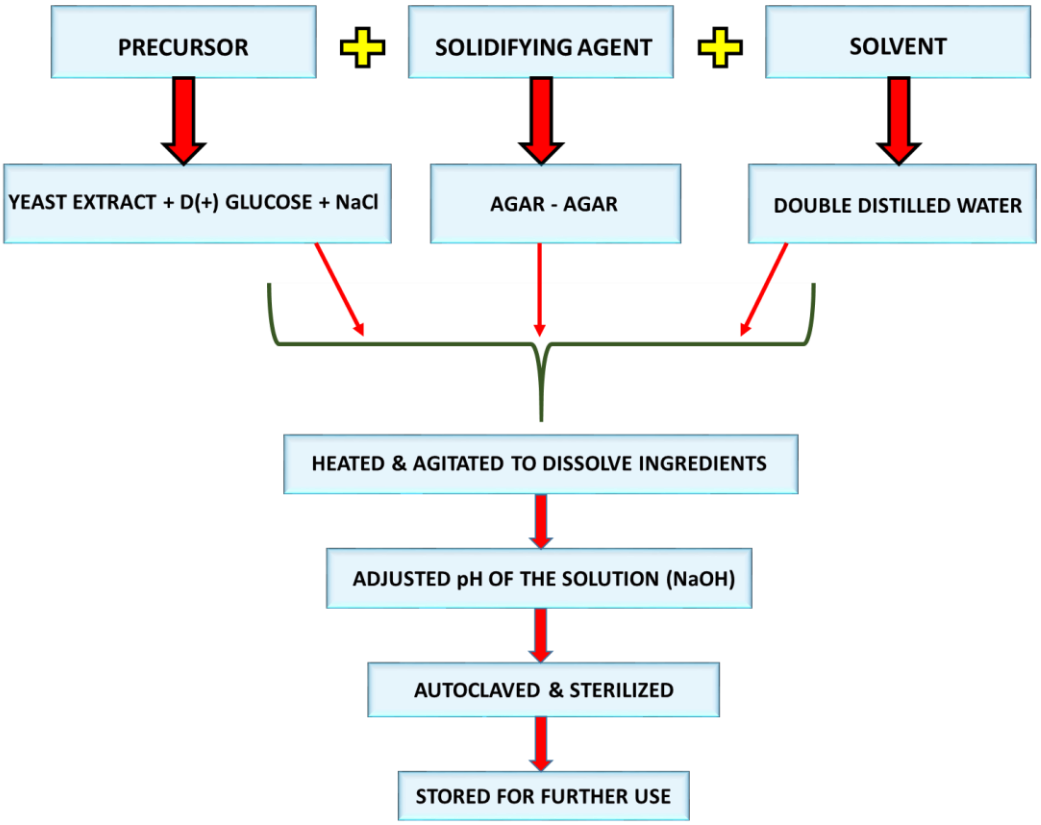

Figure 5.3 Flow chart for the preparation of nutrient broth medium

5.4.2 Culturing of bacteria in agar plates for disc-diffusion method

The antibacterial effect iron(Fe) and silver(Ag) nanoparticles against two Gram-negative bacteria (*Escherichia coli and Pseudomonas aeruginosa*) and two Gram-positive bacteria (*Bacillus subtilis and Staphylococcus aureus*) were studied by following a modified Kirby Bauer disc diffusion method. In brief, the bacteria were cultured in Müller-Hinton broth at 35°C ± 2°C on an orbital shaking incubator (Remi, India) at 160 rpm. A lawn of bacterial culture was prepared by spreading 1 mL culture broth of each test organism on solid Müller-Hinton agar plates. The plates were allowed to stand for 10-15 minutes to allow for culture absorption.

The 5 mm size discs/wells were punched into the agar with the head of sterile micropipette tips.

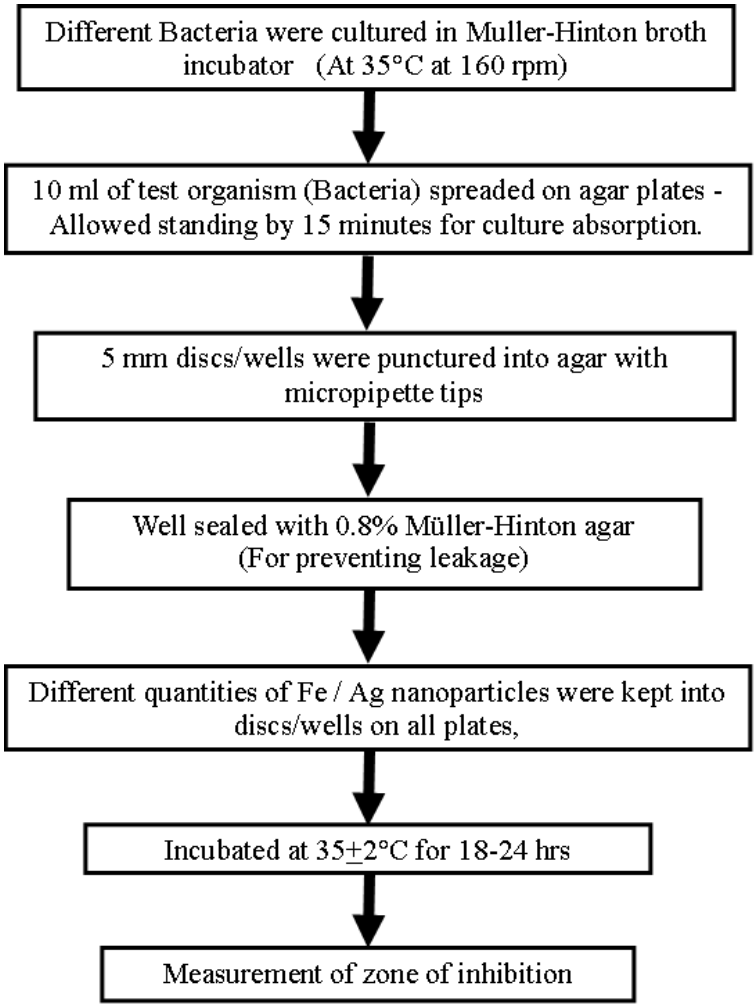

Figure 5.4. Flow chart for the culturing of bacteria in agar plates.

Wells were sealed with one drop of molten agar (0.8% Müller-Hinton agar) to prevent leakage from the bottom of the plate. Using sterilized micro pipette different quantities (20, 40, 60, 80 µg/disc) of synthesized iron and silver nanoparticles were kept into each of the wells on the respective plates. The petri plates were incubated at 35±2°C for 18-24 hours for culturing bacteria. After incubation, the sizes of the zone of inhibition were measured. A solvent blank was run as a control. The Culturing of bacteria in agar plates for disc-diffusion method is depicted through the flow chart given in Figure 5.4.

A Zone of inhibition is a circular zone around a disc in which the growth of bacteria susceptible to the antibiotic is inhibited. The antibiotic giving rise to the largest diameter zone is the most effective against that bacterial infection.

5.4.3 Disc Diffusion Test of Iron (Fe) and Silver (Ag) Nanoparticles

The effect of iron (Fe) and silver (Ag) nanoparticles against two gram negative (i.e., *Escherichia coli, Bacillus subtilis*) and two gram positive (i.e., *Pseudomonas aeruginosa, Staphylococcus aureas*) bacteria was studied. In the present study different concentrations (20, 40, 60, 80 μg/disc) of the prepared nanoparticles were kept into each of the wells on the respective plates. The diameter of the inhibition zones of the as-prepared nanoparticles was measured and tabulated. The diameter of inhibition zones for silver was observed as larger than that of iron nanoparticles.

5.5 RESULTS AND DISCUSSION

5.5.1 Antibacterial test - Zone of inhibition for Fe nanoparticles

The antibacterial effect of as-prepared iron (Fe) nanoparticles against two gram negative (ie., *Escherichia coli, Pseudomonas aeruginosa*) and two gram positive (ie., *Bacillus subtilis, Staphylococcus aureas*) bacteria is studied in detail.

Two gram negative (ie., *Escherichia coli, Pseudomonas aeruginosa*) and two gram positive (ie., *Bacillus subtilis, Staphylococcus aureas*) bacteria were inoculated to the culture media in the different petri plates. In each petri plate 5 wells / discs are made. The macro-iron powder (not to inhibit the bacterial growth) was used as control (C) in the petri plate, its diameter 5 mm and was given in Table 5.1. Different concentrations (20, 40, 60, 80 μg/disc) of the prepared iron nanoparticles were kept into each of the wells on the respective plates.

The diameter of the dark circle / well / disc increases due to the inhibition effect of the particular sample nature. Larger the darker circle, greater is the inhibition effect and greater is the destruction of the bacteria.

The diameter of the inhibition zones of the as-prepared iron nanoparticles is measured and given in Table 5.1 and illustrated by a respective bar diagram in Figure 5.5.

Table 5.1 Zone of inhibition for Fe nanoparticles with different concentrations against two Gram-negative bacteria (A1) *Escherichia coli* (A2) *Pseudomonas aeruginosa* and two Gram-positive bacteria (B1) *Bacillus subtilis* (B2) *Staphylococcus aureas*.

Organisms		Zone of inhibition (Diameter, mm)				
		Control	Concentration of Fe Nanoparticles (µg/disc)			
			20	40	60	80
A1	*Escherichia coli*	5	5.3	5.6	7.8	9.3
A2	*Pseudomonas aeruginosa*	5	5.9	6.1	8.5	9.9
B1	*Bacillus subtilis*	5	6.4	7.2	10.2	10.7
B2	*Staphylococcus aureas*	5	7.0	8.4	10.8	11.2

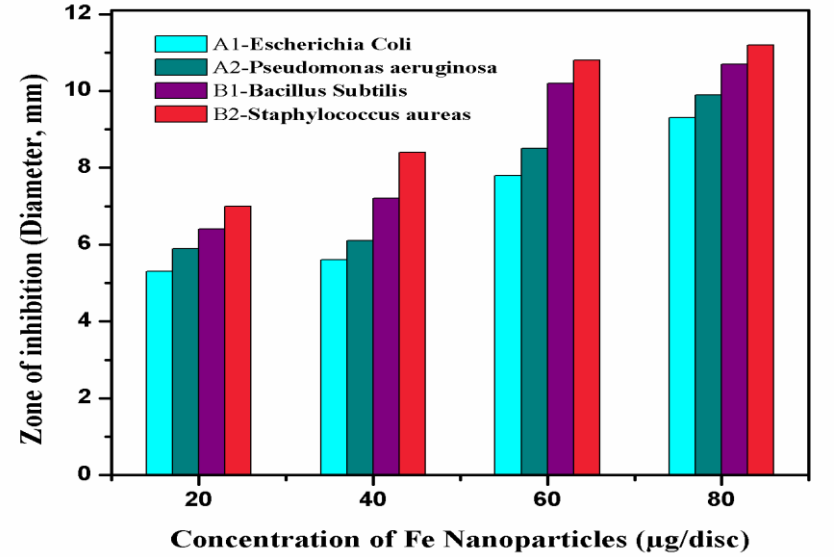

Figure 5.5 Zone of inhibition for Fe nanoparticles with different concentrations against two Gram-negative bacteria (A1) *Escherichia coli* (A2) *Pseudomonas aeruginosa* and two Gram-positive bacteria (B1) *Bacillus subtilis* (B2) *Staphylococcus aureas*.

5.5.1.1 Zone of inhibition on Gram-Negative Bacteria

In the petri plate in which *Escherichia coli* bacteria is inoculated, different concentrations of the as prepared, iron (Fe) nanoparticles start to inhibit the growth of *Escherichia coli* bacteria. The inhibited area of each sample is represented as the zone of inhibition. Except the dark circle of the control point 'C', the other circles are increased in size due to the inhibition of the bacterial growth. The zone of inhibition was measured in millimeters and the diameter of the inhibited zone for 20, 40, 60, 80 μg/disc concentrations of the as-prepared iron (Fe) nanoparticles was measured as 5.3mm, 5.6mm, 7.8mm and 9.3mm respectively.

In the petri plate in which *Pseudomonas aeruginosa* bacteria is inoculated, different concentrations of the as prepared, iron (Fe) nanoparticles start to inhibit the growth of *Pseudomonas aeruginosa* bacteria. The inhibited area of each sample is represented as the zone of inhibition. Except the dark circle of the control point 'C', the other circles are increased in size due to the inhibition of the bacterial growth. The zone of inhibition was measured in millimeters and the diameter of the inhibited zone for 20, 40, 60, 80 μg/disc concentrations of the as-prepared iron (Fe) nanoparticles was measured as 5.9mm, 6.1mm, 8.5mm and 9.9mm respectively.

5.5.1.2 Zone of inhibition on Gram-Positive Bacteria

In the petri plate in which *Bacillus subtilis* bacteria is inoculated, different concentrations of the as prepared, iron (Fe) nanoparticles start to inhibit the growth of *Bacillus subtilis* bacteria. The inhibited area of each sample is represented as the zone of inhibition. Except the dark circle of the control point 'C', the other circles are increased in size due to the inhibition of the bacterial growth. The zone of inhibition was measured in millimeters and the diameter of the inhibited zone for 20, 40, 60, 80 μg/disc concentrations of the as-prepared iron (Fe) nanoparticles was measured as 6.4mm, 7.2mm, 10.2mm and 10.7mm respectively.

In the petri plate in which *Staphylococcus aureas* bacteria is inoculated, different concentrations of the as prepared, iron (Fe) nanoparticles start to inhibit the growth of *Staphylococcus aureas* bacteria. The inhibited area of each sample is

represented as the zone of inhibition. Except the dark circle of the control point 'C', the other circles are increased in size due to the inhibition of the bacterial growth. The zone of inhibition was measured in millimeters and the diameter of the inhibited zone for 20, 40, 60, 80 μg/disc concentrations of the as-prepared iron (Fe) nanoparticles was measured as 7.0mm, 8.4mm, 10.8mm and 11.2mm respectively.

5.5.1.3 Discussion of antibacterial activities of Iron nanoparticles

The rate of inhibition is also shown in the form of bar graph given in Figure 5.5. In this graph four different plots are plotted against two Gram-negative (A1) *Escherichia coli* (A2) *Pseudomonas aeruginosa* and two Gram-positive (B1) *Bacillus subtilis* (B2) *Staphylococcus aureas* bacterial inhibition growths by the different concentrations of as prepared iron (Fe) nanoparticles. The different concentration (20, 40, 60, 80 μg/disc) of the prepared iron (Fe) nanoparticle was taken in the x-axis and the zone of inhibition (mm) was taken in the y-axis. Thus, the graph was plotted against different concentration of iron nanoparticles versus zone of inhibition.

The present study clearly indicates that the prepared Fe nanoparticles show preferably good antibacterial activity against both gram negative and positive organism, showing inhibition effect significantly compared with the control sample. As much as the concentration of Fe nanoparticles increases, there is no such significant increase in the antibacterial activity. In this study, even the lowest concentration of Fe Nano particle shows measureable inhibition effect.

The presence of an inhibition zone clearly indicates the mechanism of the biocidal action of nanoparticles involved disrupting the membrane. Extending of inhibition depends on the concentration of nanoparticles as well as on the initial bacterial concentration. The reason could be that the smaller size of the particles which leads to increased membrane permeability and cell destruction (**Ankanna and Savithramma (2011))**. The inactivation of bacteria by iron nanoparticles could be because of the penetration of the small particles into membranes of the bacteria.

It is reasonable to state that binding of the nanoparticles to the bacteria depend on the surface available for interaction. Smaller particles having large surface area available for interaction will give more bactericidal effect than the larger particles (**Panacek et al., (2006))**.

Because of the large surface area of the nanoparticles, it could be tightly adsorbed on the surface of the bacterial cells so as to disrupt the membrane, which would lead to the leakage of intracellular components, thus killing the bacterial cells (**Qi et al., (2004))**. There is also potential for multiple adverse interactions such as oxidative stress and inflammatory responses (**Wei et al., (2009))**.

Iron nanoparticles could then react with intracellular oxygen, leading to oxidative stress and eventually causing disruption of the cell membrane and disturb its power function such as permeability and respiration functions followed by dysfunction of metabolic pathways including, and they can preferentially interact with nucleic acids.

One possible explanation of the antibacterial effect is that the ions released by the nanoparticles may attach to the negatively charged bacterial cell wall and rupture it, thereby leading to protein denaturation and cell death (**Lin et al., (1998)).**

Nanoparticles and their ions (Iron and silver) can produce free radicals, resulting in induction of oxidative stress (i.e., Reactive Oxygen Species (ROS)) **(Sies (1997))**. The main mechanism by which antibacterial drugs and antibiotics work is via oxidative stress generated by Reactive Oxygen Species which include superoxide radicals(O^{2-}), hydroxyl radicals (- OH), hydrogen peroxide (H_2O_2), and singlet oxygen (1 O_2), causing damage to proteins, membrane, mitochondria and DNA in bacteria (**Park et al., (2009))** and resulting in bacterial death.

Fe^{2+} reacts with oxygen to create hydrogen peroxide, this H_2O_2 consequently reacts with ferrous iron via the Fenton reaction and produces hydroxyl radicals which are known to damage biological macromolecules (**Touati (2000))**.

Hence there is a potential candidate for multiple adverse interactions such as oxidative stress and inflammatory responses. Such cellular processes may lead to cell death via cell necrosis or apoptosis.

The present study is useful towards authenticating the Fe nanoparticles to be potent antibacterial agents with several advantages such as low cost, easy preparation, and high reactivity compared to other metal nanoparticles.

5.5.2 Antibacterial test - Zone of inhibition for Ag nanoparticles

The antibacterial effect of as-prepared Silver (Ag) nanoparticles against two gram negative (ie., *Escherichia coli, Bacillus subtilis*) and two gram positive (i.e. *Pseudomonas aeruginosa, Staphylococcus aureas*) bacteria is studied in detail.

Two gram negative (ie., *Escherichia coli, Pseudomonas aeruginosa*) and two gram positive (ie., *Bacillus subtilis, Staphylococcus aureas*) bacteria were inoculated to the culture media in the different petri plates. In each petri plate 5 wells / discs were made. The macro-silver powder (not to inhibit the bacterial growth) is used as control (C) in the petri plate, its diameter 5 mm and is given in Table 5.2. Different concentrations (20, 40, 60, 80 μg/disc) of the prepared silver nanoparticles were kept into each of the wells on the respective plates.

The diameter of the dark circle / well / disc increases due to the inhibition effect of the particular sample nature. Larger the darker circle, greater is the inhibition effect and greater is the destruction of the bacteria.

The diameter of the inhibition zones of the as-prepared iron nanoparticles is measured and given in Table 5.2 and illustrated by a respective bar diagram given in Figure 5.6.

Table 5.2 Zone of inhibition for Ag nanoparticles with different concentrations against two Gram-negative bacteria (A1) *Escherichia coli* (A2) *Pseudomonas aeruginosa* and two Gram-positive bacteria (B1) *Bacillus subtilis* (B2) *Staphylococcus aureas*.

Organisms		Zone of inhibition (Diameter, mm)				
		Control	Concentration of Ag Nanoparticles (µg/disc)			
			20	40	60	80
A1	*Escherichia coli*	5	6.2	6.6	10.3	12.2
A2	*Pseudomonas aeruginosa*	5	7.1	7.4	11.2	13.3
B1	*Bacillus subtilis*	5	8.1	8.6	11.9	14.1
B2	*Staphylococcus aureas*	5	8.5	9.7	12.1	15.6

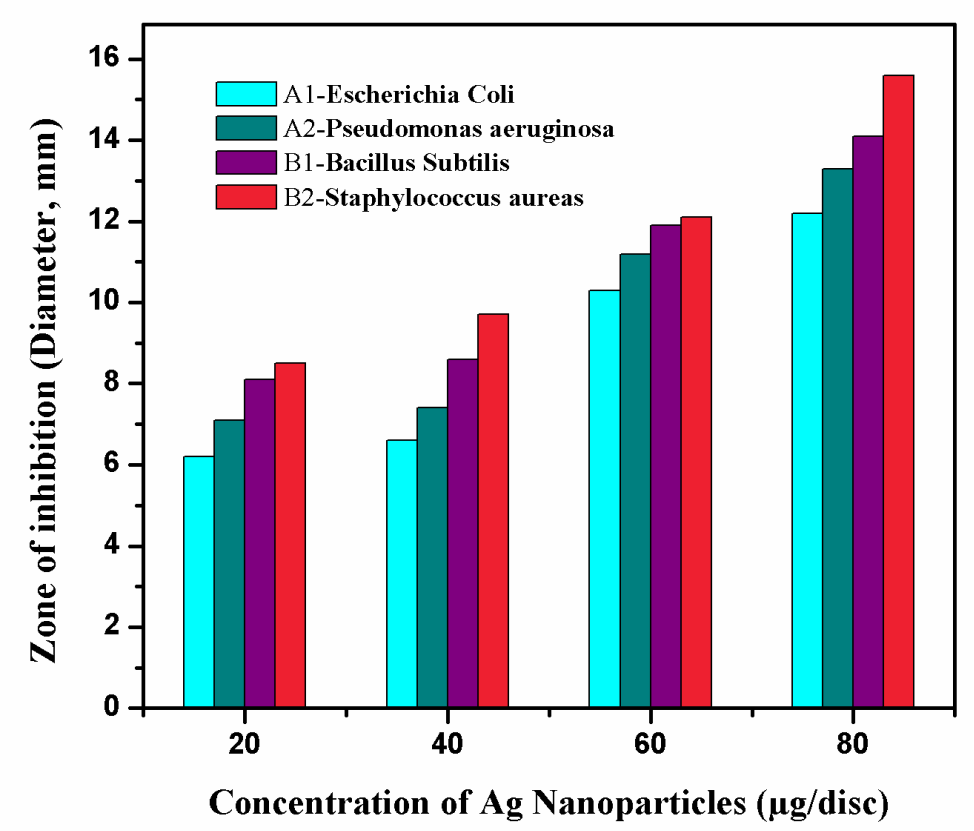

Figure 5.6 Zone of inhibition for Ag nanoparticles with different concentrations against two Gram-negative bacteria (A1) *Escherichia coli* (A2) *Pseudomonas aeruginosa* and two Gram-positive bacteria (B1) *Bacillus subtilis* (B2) *Staphylococcus aureas*.

5.5.2.1 Zone of inhibition on Gram-Negative Bacteria

In the petri plate in which *Escherichia coli* bacteria is inoculated, different concentrations of the as prepared, silver (Ag) nanoparticles start to inhibit the growth of *Escherichia coli* bacteria. The inhibited area of each sample is represented as the zone of inhibition. Except the dark circle of the control point 'C', the other circles are increased in size due to the inhibition of the bacterial growth. The zone of inhibition was measured in millimeters and the diameter of the inhibited zone for 20, 40, 60, 80 μg/disc concentrations of the as-prepared silver (Ag) nanoparticles was measured as 6.2mm, 6.6mm, 10.3mm and 12.2mm respectively.

In the petri plate in which *Pseudomonas aeruginosa* bacteria is inoculated, different concentrations of the as prepared, silver (Ag) nanoparticles start to inhibit the growth of *Pseudomonas aeruginosa* bacteria. The inhibited area of each sample is represented as the zone of inhibition. Except the dark circle of the control point 'C', the other circles are increased in size due to the inhibition of the bacterial growth. The zone of inhibition was measured in millimeters and the diameter of the inhibited zone for 20, 40, 60, 80 μg/disc concentrations of the as-prepared silver (Ag) nanoparticles was measured as 7.1mm, 7.4mm, 11.2mm and 13.3mm respectively.

5.5.2.2 Zone of inhibition on Gram-Positive Bacteria

In the petri plate in which *Bacillus subtilis* bacteria is inoculated, different concentrations of the as prepared, silver (Ag) nanoparticles start to inhibit the growth of *Bacillus subtilis* bacteria. The inhibited area of each sample is represented as the zone of inhibition. Except the dark circle of the control point 'C', the other circles are increased in size due to the inhibition of the bacterial growth. The zone of inhibition was measured in millimeters and the diameter of the inhibited zone for 20, 40, 60, 80 μg/disc concentrations of the as-prepared silver (Ag) nanoparticles was measured as 8.1mm, 8.6mm, 11.9mm and 14.1mm respectively.

In the petri plate in which *Staphylococcus aureas* bacteria is inoculated, different concentrations of the as prepared, silver (Ag) nanoparticles start to inhibit

the growth of *Staphylococcus aureas* bacteria. The inhibited area of each sample is represented as the zone of inhibition. Except the dark circle of the control point 'C', the other circles are increased in size due to the inhibition of the bacterial growth. The zone of inhibition was measured in millimeters and the diameter of the inhibited zone for 20, 40, 60, 80 μg/disc concentrations of the as-prepared silver (Ag) nanoparticles was measured as 8.5mm, 9.7mm, 12.1mm and 15.6mm respectively.

5.5.2.3 Discussion of antibacterial activities of Silver nanoparticles

The rate of inhibition is also shown in the form of bar graph in Figure 5.6. In this graph four different plots are plotted against two Gram-negative (A1) *Escherichia coli* (A2) *Pseudomonas aeruginosa* and two Gram-positive (B1) *Bacillus subtilis* (B2) *Staphylococcus aureas* bacterial inhibition growths by the different concentrations of as prepared silver (Ag) nanoparticles. The different concentration (20, 40, 60, 80 μg/disc) of the prepared silver (Ag) nanoparticle was taken in the x-axis and the zone of inhibition (mm) was taken in the y-axis. Thus, the graph was plotted against different concentration of iron nanoparticles versus zone of inhibition.

Even though the antibacterial activity Elemental silver and silver compounds have been used as antimicrobial agents from ancient times, Silver nanoparticles are excellent antibacterial agents, because of their area of contact with bacteria is higher as nanoparticles have larger surface area.

The present study clearly indicates that the prepared silver nanoparticles show good antibacterial activity against both gram negative and positive organism. As much the concentration of Ag nano particle increases there is no such significant increase in the antibacterial activity. In this study, the lowest concentration of Ag nanoparticles 20 mg/mL shows measureable cell growth on agar plates.

Some researchers have reported that the antimicrobial effect of silver nanoparticles on Gram-negative bacteria was dependent on the concentration of Ag

in the nanoparticles and was closely related to the formation of "pits" in the cell walls **(Amro et al., (2000) and Sondi and Salopel (2004)).**

The major mechanism through which silver nanoparticles manifest antibacterial properties was either by anchoring or penetrating the bacterial cell wall, and modulating cellular signaling (**Shrivastava et al., (2007)).** However, the general mechanism of antibacterial activity of silver nanoparticles was proposed by many researchers, but the detailed mechanism remains to be understood.

Recently, it was reported that the antibacterial activity of Ag nanoparticles is related to the formation of free radicals (**Kim et al., (2007)).** Under certain conditions, high levels of Reactive Oxygen Species (ROS) can increase the oxidative stress in cells. Oxidative stress can not only cause damage to the cell membrane, but also cause damage to the proteins, DNA, and intracellular systems such as the respiratory system.

The bacterial growth was inhibited by silver ions, which accumulated into the vacuole and cell walls as granules. Silver nanoparticles may attach to the surface of the cell membrane and disturb its power function such as permeability and respiration functions followed by dysfunction of metabolic pathways; silver ions can interact with nucleic acids they preferentially interact (**Maribel et al., (2009)).** It is reasonable to state that the binding of the particles to the bacteria depends on the surface area available for interaction. Smaller particles having the larger surface area available for interaction will give more bactericidal effect than the larger particles.

Moreover the present study also proved to have potential antibacterial activities with the synthesized silver nanoparticles. This might be due to denaturation of the bacterial cell wall and leaking the sugars from the cell wall. This may lead to the blocking of bacterial respiration, destabilization of outer membrane and depletion of intracellular ATP (**Vivekanandhan et al., (2009)).**

The antibacterial activity is probably derived, through the electrostatic attraction between negative charged cell membrane of microorganism and positive charged nanoparticles. Further the variation in the sensitivity between the gram positive and gram negative against the nanoparticles varies greatly. This might be due to the membrane permeability (**Ravikumar et al., (2010)).**

The inhibitory effect of silver on microorganisms tested is effected via two possible mechanisms First, is the electrostatic attraction between the negatively charged cell membrane of the microorganisms and the positively charged Ag, and second, is the formation of 'pits' in the cell wall of bacteria related to Ag concentration (**Sondi and Salopel (2004)).**

The differences observed in the diameter of the zone of inhibition may be due to the difference in the susceptibility of different bacteria to the prepared silver nanoparticles. The differential sensitivity of gram negative and gram positive bacteria towards silver nanoparticles possibly depends upon their cell structure, physiology, metabolism and their interaction with the charged silver nanoparticles (**Bindhu and Umadevi (2015)).**

Effective antibacterial agents should be toxic to different pathogenic bacteria with the ability to be coated as antimicrobial coating on variety of surfaces like wound dressings, medical appliances, biomaterials, purifying and purity testing devices, textiles, biomedical and food packaging, consumer products and so on.

This study suggests that the synthesized nanoparticles can be effectively used as an alternative to antibiotic of bactericides against skin wounds by reducing antimicrobial activities. This synthesis of silver nanoparticles can prove as potential candidates for medical applications.

5.6 ASSESSMENT OF ACTIVITY INDEX AND FOLD INCREASE

According to **Singariya et al., (2012)** the assessment of activity index was obtained by comparing the resultant inhibition zones of nanoparticles with the standard reference using the formula,

$$\text{Activity index (AI)} = \frac{\text{Inhibition zone of the sample}}{\text{Inhibition zone of the standard}}$$

Increase in fold area was assessed by calculating the mean surface area of the inhibition zone generated by the standard reference and nanoparticles (**Fayaz et al., (2010))**. The fold increase area was calculated by the equation,

$$\text{Fold increase (\%)} = (b-a)/a \times 100$$

where 'a' and 'b' refer to the inhibition zones of the reference and nanoparticles respectively.

Activity index (AI) and percentage fold increase for Fe and Ag nanoparticles are given in Table 5.3 and Table 5.4 respectively.

Table 5.3 Activity index (AI) of iron (Fe) and silver (Ag) nanoparticles with reference to standard against gram negative and gram positive bacterial pathogens

Bacterial Pathogens		**Activity Index of the nanoparticles**								**Average Activity Index of nano particles**	
Type	**Organism**	**20 µg/disc**		**40 µg/disc**		**60 µg/disc**		**80 µg/disc**			
		Fe	**Ag**	**Fe**	**Ag**	**Fe**	**Ag**	**Fe**	**Ag**	**Fe**	**Ag**
Gram Negative	*Escherichia coli* (A1)	1.06	1.24	1.12	1.32	1.56	2.06	1.86	2.44	**1.40**	**1.77**
	Pseudomonas aeruginosa (A2)	1.18	1.42	1.22	1.48	1.7	2.24	1.98	2.66	**1.52**	**1.95**
Gram Positive	*Bacillus subtilis* (B1)	1.28	1.62	1.44	1.72	2.04	2.38	2.14	2.82	**1.72**	**2.14**
	Staphylococcus aureas (B2)	1.4	1.70	1.68	1.94	2.16	3.03	2.24	3.12	**1.87**	**2.45**

Table 5.4 Percentage fold increase of iron (Fe) and silver (Ag) nanoparticles with reference to standard against gram negative and gram positive bacterial pathogens

Bacterial Pathogens		Fold Increase % of the nanoparticles								Average Fold Increase % of nano particles	
Type	Organism	20 µg/disc		40 µg/disc		60 µg/disc		80 µg/disc			
		Fe	Ag	Fe	Ag	Fe	Ag	Fe	Ag	Fe	Ag
Gram Negative	*Escherichia coli* (A1)	6	24	12	32	56	106	86	144	**40**	**76.5**
	Pseudomonas aeruginosa (A2)	18	42	22	48	70	124	98	166	**52**	**95**
Gram Positive	*Bacillus subtilis* (B1)	28	62	44	72	104	138	114	182	**72.5**	**113.5**
	Staphylococcus aureas (B2)	40	70	68	94	116	142	124	212	**87**	**129.5**

Shrivastava and Dash (2009) have reported that the antimicrobial activity of silver nanoparticles against bacterial pathogens was dose dependent and hence the inhibition zone increased significantly with the increase in the dosage of the nanoparticles. Silver nanoparticles having small particle size and large surface area available for interaction will give more bactericidal effect than larger particles. (**Panacek et al., (2006))**

It shows that all the tested bacteria exhibited excellent antibacterial activity than the standard reference. Among the tested strains, Fe nanoparticles demonstrated the highest average percentage fold increase (87%) and Ag nanoparticles demonstrated the highest average percentage fold increase (129.5%) against the pathogen *Staphylococcus aureas.*

5.7 CONCLUSION

Bacteria inhibition zone was correlated to antimicrobial properties of iron (Fe) and silver (Ag) nanoparticles. It is believed that DNA loses replication ability and cellular proteins become inactive on these metal ion treatment. Table 5.1 and Table 5.2 clearly indicate that the iron(Fe) and silver (Ag) nanoparticles inhibit the

growth of both Gram-negative and gram-positive bacteria and the zone of inhibition increased as the concentration of the prepared nanoparticles were increased.

It was found that the inhibition of bacteria was better for silver (Ag) nanoparticles than iron (Fe) nanoparticles under the same conditions. These results demonstrate that excellent antimicrobial behavior was exhibited for silver (Ag) nanoparticles than iron (Fe) nanoparticles synthesized at low temperature.