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RESEARCH ARTICLE

Antibacterial activity of Metal Based Nanoparticles–A Comparative view

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ABSTRACT

The emergence and spread of antibiotic resistance is an alarming concern in clinical practice. This would lead to the development of new antibacterial agents from natural and inorganic substances. In the current scenario, one of the most promising and novel therapeutic agents are the nanoparticles. Hence the present study has been made an attempt to find out the potential metal nanoparticles for the management of human bacterial diseases. Metal nanoparticles (MeNPs) were synthesized by simple, cost effective, chemical reduction method. The synthesized particles were further characterized by X-ray Diffractogram (XRD), Scanning Electron Microscopy (SEM), and Energy Dispersive Spectroscopy (EDS) techniques to analyze size, morphology of the nanoparticles, and quantitative information of elemental metals respectively. Average crystalline size of the particle ranged from 17.85 to 44.87 nm. Bactericidal effect of metal nanoparticles was examined by agar well diffusion technique. Metal nanoparticles showed excellent activity against selected bacterial pathogens. Different classes of bacteria exhibited different susceptibilities to nanoparticles. All the experimental strains depicted highest sensitivity to silver nanoparticles compared to other metal based nanoparticles. Our results suggest that chemically mediated metal nanoparticles could act as an effective alternative for the development of new antibacterial agent to combat resistant problems.

INTRODUCTION

Resistance to antibiotics is a ubiquitous and relentless clinical problem that is compounded by a dearth of new therapeutic agents (Boucher *et al.,* 2009). Therefore, there is an immediate need to develop new approaches to handle this problem. One of the promising approaches for overcoming bacterial resistance is the use of metallic nanoparticles (Chaloupka *et al.,* 2010). Owing to their small sizes and higher surface-to-volume ratio, metallic nanoparticles have an enlarged contact area with a microorganism. This feature enhances biological and chemical activity of the nanoparticles with high antibacterial efficacy. Metallic nanoparticles with antimicrobial activity, when embedded and coated on to surfaces can find immense applications in water treatment, synthetic textiles, biomedical and surgical devices, food processing and packaging (Gutierrez *et al.,* 2010). Another important property of metallic nanoparticles is their ability to target different bacterial structures (Gordon *et al.,* 2010). Nanoparticles can disturb functions of cell membranes such as permeability and respiration. In addition, after penetrating into bacterial cell, nanoparticles can also disturb the functions of sulfur-containing proteins and phosphorus-containing compounds such as DNA effectively reacting with them (Singh *et al.,* 2008). Complex action mechanisms of metals decrease the probability of bacteria developing resistance to them (Chopra, 2007). Owing to their high antibacterial properties, nanoparticles of silver, oxides of zinc, titanium, copper, and iron are the most commonly used nanoparticles in antimicrobial studies. Furthermore, these nanoparticles have been used to deliver other antimicrobial drugs to the site of pathological process (Jong and Borm, 2008). Bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio, which allows them to interact closely with microbial membrane (Morones *et al.,* 2005). The presence of nanoparticles in suspension would ensure continuous release of ions into the nutrient media. 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). There is also potential for multiple adverse interactions such as oxidative stress and inflammatory responses (Wei *et al.,* 2009). Such processes may lead to cell death *via* cell necrosis or apoptosis (programmed cell death). Hence the aim of this work was to fabricate and characterize various metal based nanoparticles and to investigate the bactericidal activity of such nanoparticles against both gram positive and gram negative bacterial strains.

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MATERIALS AND METHODS

Materials

Silver nitrate ($AgNO_3$), Trisodium citrate ($Na_3C_6H_5O_7$), Gold (III) chloride trihydrate ($HAuCl_4.3H_2O$), Copper sulfate pentahydrate ($CuSO_4.5H_2O$), Sodium borohydride ($NaBH_4$), Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), Ethanol and Standard antibiotic discs were purchased from Himedia (P) Ltd, Mumbai and used as starting materials without further purification. Milli-Q water was used for the fabrication of metal nanoparticles.

METHODS

Preparation of metal nanoparticles

Silver (AgNPs)

The silver nanoparticles were prepared using chemical reduction method (Fang *et al.,* 2005).

Gold (AuNPs)

Gold nanoparticles were prepared by reducing $HAuCl_4.3H_2O$ with trisodium citrate according to the description of Grace and Pandian (2007).

Copper (CuNPs)

The preparation of copper nanoparticles was followed the method of Samim *et al.* (2007).

Zerovalent iron (Fe^0NPs)

The preparation of Fe^o nanoparticles was followed the method according to He and Zhao (2005) with slight modifications. In brief, the preparation was carried out in a 250 ml flask attached to a vacuum line. Before use, deionized (DI) water was purged with purified N_2 gas for 15 min to remove dissolved oxygen (DO). In a typical preparation, a stock solution of 0.21 M $FeSO_4.7H_2O$ was prepared right before use. Fe concentration used in this study was 0.1 g/L. The Fe^{2+} ions were then reduced to Fe^o by adding a stoichiometric amount of $NaBH_4$ aqueous solution at a BH_4^-/Fe^{2+} molar ratio of 2.0 to the mixture with magnetic stirring at 230 rpm under ambient temperature. The ferrous ion was reduced to zero-valent iron according to the following reaction:

$$Fe(H_2O)_6^{2+}+2BH_4^-\rightarrow Fe^o\downarrow+2B(OH)_3+7H_2\uparrow$$

The resultant black particles were separated from the solution by centrifugation at 4000 rpm for 5 min and washed with N_2 saturated deionized water and at least three times with 99% absolute ethanol. Finally, the synthesized Fe^o nanoparticles were dried in an oven at 60°C.

Characterization of metal nanoparticles

Visual inspection

The reduction of metal ions was roughly monitored by visually observing the change of color in the reaction solution.

X-ray diffractogram

The phase purity and crystalline structure of the prepared MeNPs were identified and characterized by X-ray diffraction pattern. X-ray diffraction patterns of synthesized MeNPs were recorded with an X'pert PROPAN analytical instrument operated at 40 kV and a current of 30 mA with Cu α radiation (λ=1.54060 Å). A continuous scan mode was used to collect 2θ data from 10.08° to 79.93°. The diffraction intensities were compared with the standard JCPDS files. The information of the particle size was obtained from the full width at half maximum (FWHM) of the diffracted beam. Crystalline size of the nanoparticles was calculated from the line broadening of X-ray diffraction peak according to the Debye-Scherer formula (Huang and Tang, 2005).

$$D = k\lambda/ \beta \cos\theta,$$

Where D is the thickness of the nanocrystal, 'k' constant, 'λ' wavelength of X-rays, 'β' width at half maxima of reflection at Bragg's angle 2θ, 'θ' Bragg's angle.

Scanning electron microscopy

Surface morphology and size distribution of the MeNPs were examined by Scanning Electron Microscope (SU 1510) operated at 5kV, magnification x10k. Thin films of the sample were prepared on a carbon coated copper grid by just dropping the suspension of nanoparticles in water on the grid, extra solution was removed using blotting paper and then, the film on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min. The sample surface images were taken at different magnifications.

Energy dispersive spectroscopy

The quantitative information and distribution of the elemental metals (Silver, Gold, Copper, Iron) were investigated by EDS analysis (JSM 35 CF JEOL) in a resolution of 60 Å, magnification of 5 k. The operating conditions were 15.0 kV accelerating voltage and 15 mm working distance under high vacuum mode.

Antibacterial studies of metal nanoparticles

Bacterial culture

The following bacterial pathogens namely *Bacillus cereus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus epidermis* were procured from the Microbial Type Culture Collection (MTCC), Chandigarh, India. All the cultures were grown on nutrient agar plates and maintained in the nutrient agar slants at 4°C. Overnight culture in the nutrient broth was used for the present experimental study.

Antibacterial activity

The antibacterial activity of chemically fabricated MeNPs against the pathogenic bacteria was examined by agar well diffusion method. Pure cultures of each bacterial strain were sub cultured in nutrient broth for 24 h at 37°C. After 24 h, the inoculum was spread with sterile cotton swab on Mueller Hinton agar (MHA) plates. Wells of 6 mm diameter were made using sterile cork borer and 50 µl of nanoparticles suspension were poured onto each well on all plates. The plates were left at 37°C for 24 h and results were recorded by measuring the diameter of inhibition zone (mm). To determine the comparative efficacy of MeNPs with antibiotics, each commercial antibiotic disc namely *Chloramphenicol, Tetracycline, Vancomycin, Kanamycin* (30 mcg/disc), *Erythromycin* (15 mcg/disc), *Gentamycin, Penicillin, Ampicillin, Streptomycin, Tobramycin* (10 mcg/disc), and *Rifampicin* (5 mcg/disc) was placed onto the MHA medium inoculated with test organisms. These plates were incubated overnight at 37°C. After incubation, results were recorded by measuring the inhibitory zone diameter (mm).

Assessment of increase in fold area

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

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

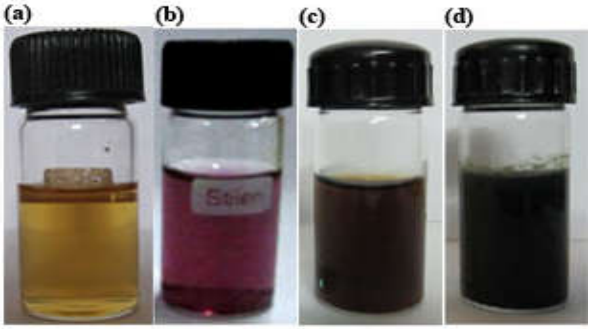
where a and b refer to the zones of inhibition for antibiotics and metal nanoparticles respectively.

RESULTS AND DISCUSSION

Characterization of metal nanoparticles

Visual inspection

The appearance of pale yellow color colloidal solution for silver nanoparticles, wine red for gold, dark brown for copper and black for zerovalent iron colloids in the reaction mixture indicateed the formation of respective MeNPs (Fig. 1a-1d). The formation of color in the reaction solution arises from excitation of surface Plasmon vibration in the metal nanoparticles (Shahverdi *et al.,* 2007)



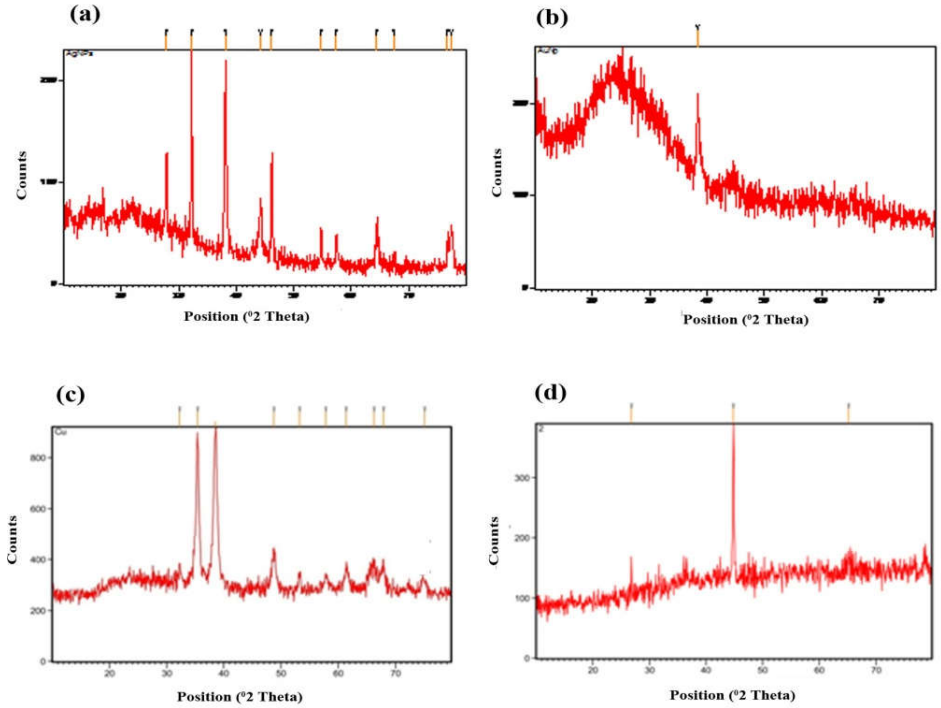
a - Silver nanoparticles; b- Gold nanoparticles; c – Copper nanoparticles; d- Iron nanoparticles

Fig. 1. Visual inspection of metal nanoparticles

Table 1. Comparative efficacy of antibacterial susceptibility test for metal nanoparticles with commercial antibiotics and their level of inhibition zone

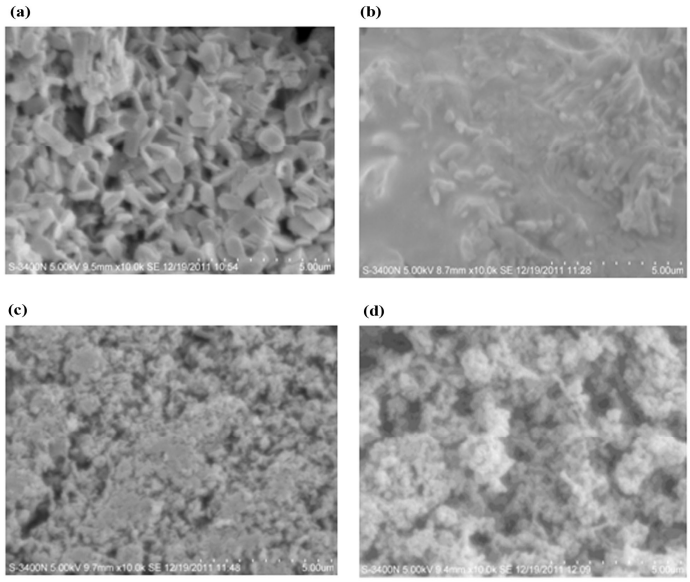
Antibiotics / Metal Nanoparticles	Zone of inhibition (mm) Bacterial pathogens					
	Bacillus cereus	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Streptococcus epidermis*
Erythromycin	12	11	15	10	10	15
Rifampicin	8	10	11	15	8	9
Gentamicin	10	14	8	12	15	18
Choloramphenicol	18	23	19	20	16	25
Tetracycline	10	11	6	13	14	17
Vancomycin	7	7	6	10	8	6
Penicillin	7	6	10	7	6	6
Ampicillin	10	7	13	8	8	11
Kanamycin	12	10	6	16	14	11
Streptomycin	23	12	6	6	24	14
Tobramycin	10	11	8	11	9	12
AgNPs	24	20	21	13	18	15
AuNPs	12	16	15	10	12	20
CuNPs	13	13	12	16	14	15
Fe^{o}NPs	20	15	19	13	12	15

AgNPs – Silver nanoparticles; AuNPs – Gold nanoparticles; CuNPs – Copper nanoparticles;
Fe^{o}NPs – Zerovalent iron nanoparticles



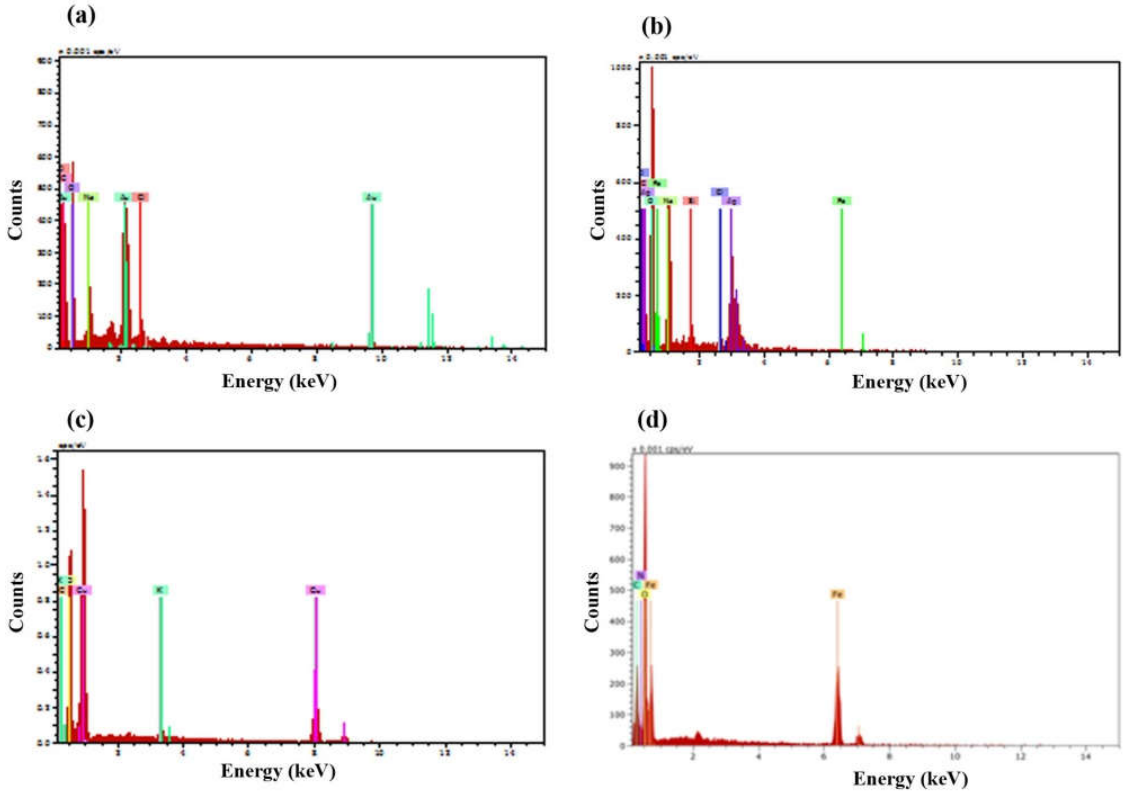
a - Silver nanoparticles; b- Gold nanoparticles; c – Copper nanoparticles; d- Iron nanoparticles

Fig. 2. X-ray diffraction pattern of metal nanoparticles



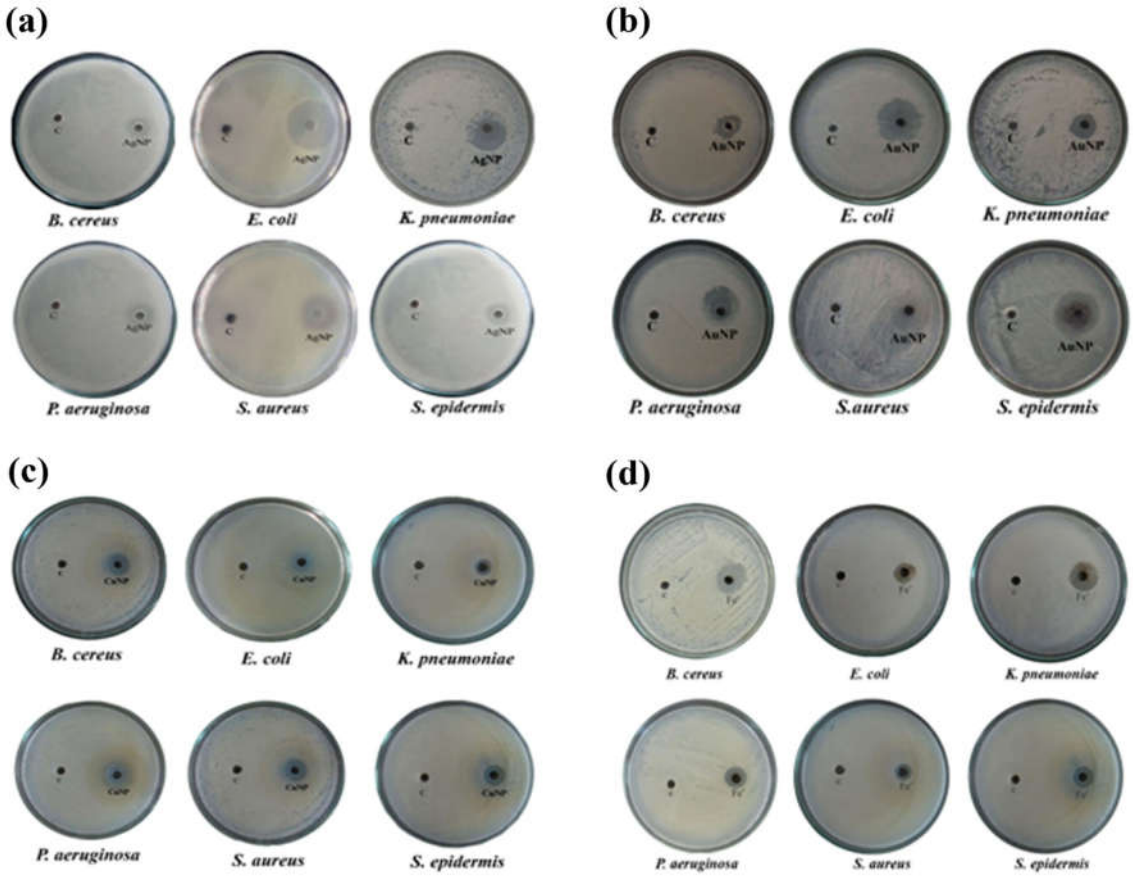
a - Silver nanoparticles; b- Gold nanoparticles; c – Copper nanoparticles; d- Iron nanoparticles

Fig. 3. Scanning electron micrograph of metal nanoparticles



a - Silver nanoparticles; b- Gold nanoparticles; c – Copper nanoparticles; d- Iron nanoparticles

Fig. 4. Energy dispersive spectrum of metal nanoparticles



C – Control; AgNP – Silver Nanoparticles; AuNP – Gold Nanoparticles; CuNP – Copper Nanoparticles; Fe^{o} – Zerovelent Iron Nanoparticles

Fig. 5. Inhibitory effect of metal nanoparticles against selected human bacterial pathogens

X-ray diffractogram

The X-ray diffraction patterns recorded for MeNPs prepared in the present study are shown in Fig. 2a-2d. The X-ray diffraction pattern shows that the synthesized MeNPs are in amorphous stage and in tetragonal system. The XRD pattern clearly showed the crystalline nature of MeNPs. The diffraction peaks at 2θ of 38.18°, 38.38°, 38.51°, and 44.8° correspond to the (111), (111), (111) and (311) reflections planes of silver, gold, copper and iron respectively. The different peaks in XRD pattern are indexed and the corresponding values of interplanar spacing "d" are calculated and compared with standard JCPDS-ICDD, PDF, Files. The XRD study confirms that the resultant particles are face centered cubic (fcc) metal nanoparticles.
The obviously broadened diffraction peaks suggest that the resultant nanoparticles should have a very small crystalline size and its size is found to be 29.26 nm (silver), 21.96 nm (gold), 17.85 nm (copper), and 44.87 nm (zerovalent iron).

Scanning electron microscopy

Scanning electron micrograph of the synthesized MeNPS is presented in Fig. 3a-3d. The micrograph shows that the appearance of the particles is spherical in shape. Synthesized particles do not appear as discrete one but form much larger particles. The observations of such larger nanoparticles are composed of van der Waals clusters of smaller entities and magnetic interactions among the particles.

Energy dispersive spectroscopy

EDS micrograph explains the surface atomic distribution and chemical composition of MeNPs. In our analysis, we confirmed the presence of elemental metal signals. Strong signals from the silver (48.13%), gold (75.08%), copper (97.07%), and iron (72.11%) atoms are observed, while weaker signals from K, C and O are also observed (Fig. 4a-4d).

Antibacterial activity

Due to overuse of antibiotics and a growing problem of antibiotic resistance, nanoparticles are being researched as an alternative antibacterial agent. The inhibitory activity of the MeNPs was evaluated against pathogenic bacteria and their potency was assessed qualitatively by the presence of inhibition zones (Fig. 5a-5d). Diameter of inhibition zone (DIZ) reflects the magnitude of susceptibility of the microbes. The strains susceptible to nanoparticles exhibit larger DIZ, whereas resistant strains exhibit smaller DIZ. Bacterial sensitivity to nanoparticles was found to vary depending on the microbial species. MeNPs showed excellent antibacterial activity against all the bacterial strains used in the present experimental study. The presence of nanoparticles in suspension would ensure continuous release of ions into nutrient media and it cross the cell membrane without hindrance and deposit over the cell organelles impairing the intracellular transport and nutrient uptake. This nanoparticle diffuses through the matrix of the agar in a circular formation. As the bacteria attempted to grow over this circle, there is an interaction with the nanoparticles which inhibits the growth. The diameter of inhibition zone (mM) among metal nanoparticles and antibiotics against test strains is given in Table 1. It shows that all tested bacteria are resistant to more than one antibiotic. The maximum inhibition zone (24 mm) was obtained against *B. cereus* for silver nanoparticles which are followed by *S. epidermis* (20 mm) for gold, *B. cereus* (20 mm) for iron, and *P. aeruginosa* (16 mm) for copper nanoparticles. The results obtained in the present study revealed that the metal nanoparticles possess potential antibacterial activity on the corresponding strains as well as broad spectrum antibiotic resistant strains. The important observation from this study is that the antibacterial activity of colloidal silver against the tested strains which exhibit superior effect compared with other antibiotics. Silver nanoparticles showed an excellent bactericidal effect against all the experimental strains than other metal based nanoparticles used in this study. Calculated fold increase is ranged from 8.33% to 216.67% times among silver nanoparticles and antibiotics. Extend of inhibition depends on the concentration of nanoparticles as well as on the initial bacterial concentration. 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). In specifically, the nanoparticles bind to the building elements of the outer membrane causing structural changes, degradation and finally cell death (Grace and Pandian, 2007). The exact mechanism behind bactericidal effect of MeNPs is not clearly known and needs to be studied further. It is supposed that microorganisms carry a negative charge while metal nanoparticles carry a positive charge. This creates an "electromagnetic attraction" between the microbe and particles surface. Once the contact is made, the microbe is oxidized and dead instantly (Roy *et al.,* 2010). Generally, it is believed that nanomaterials release ions, which react with the thiol group (-SH) of the proteins present on the bacterial surface. Such proteins protrude through the bacterial cell membrane, allowing the transport of nutrients through the cell wall. Nanomaterials inactivate the proteins, decreasing the membrane permeability and eventually causing cellular death (Zhang and Chen, 2009).

Conclusion

Nanobiotechnology is an upcoming and developing field with potential application of human welfare owing to its small size and volume ratio to fight against antibiotic resistant pathogens. MeNPs used in this work have great promise as antibacterial agents and it exhibits the broad spectrum of antibacterial activity. Results from this study showed that these non-toxic nanomaterials, which can be prepared in a simple and cost-effective manner, may be suitable for the formulation of new types of bactericidal materials and may be solve the problem of the emergence and spread of *in vitro* antibiotic resistance. Meanwhile an additional detailed research is needed to better understand the exact mechanism behind the bactericidal efficiency of MeNPs.

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