7.1 Therapeutic Applications of Gold Nanoparticles

It includes Anti-microbial, Biosensor, Diagnosis, Drug delivery & HIV/AIDS applications.

Anti-Microbial:

One of the basic prerequisites for using gold nanoparticles in biomedical application is that they are non-toxic and biocompatible to both in vivo and in vitro environments. For biomedical applications that require lower concentrations of gold nanoparticles, it is crucial that dilution of gold nanoparticles does not change the properties. Although silver has a long history of being used as an antimicrobial, in recent years gold has also become a good rival for silver. For example gold nanoparticles can fight against '*E. coli*' bacteria.

Bio Sensors:

The primary principle involved in the design of a biosensor based on gold nanoparticles is that the gold nanoparticles are functionalized with a thiolated biomolecule which upon recognizing the perfecting biomolecule brings about change in the optical absorption of gold nanoparticles. Variety of sensors uses gold nanoparticles. For example, a calorimetric sensor based on gold nanaoparticles can identify if foods are suitable for consumption [Ali *et al.* (2012)].

Diagnosis and Therapy:

Gold nanoparticles are used to detect biomarkers in the diagnosis of heart diseases, cancers and infectious agents [Ogi *et al.* (2010)].

In cancer research, colloidal gold can be used to target tumors and provide detection using SERS (Surface Enhanced Raman Spectroscopy) bin vivo. These gold nanoparticles is surrounded with Raman reporters which provide light emission that are over 200 times brighter than quantum dots. It was found that the Raman reporters were stabilized when the nanoparticles when encapsulated with a thiol-modified polyethylene glycol coat. This

allows for compatibility and circulation in vivo. To specifically target tumor cells, the **peg-ylated gold nanoparticles** are conjugated with an antibody (or an antibody fragment such as scFv), against e.g. Epidermal growth factor receptor, which is sometimes over expressed in cells of certain cancer types. Using SERS, these peg-ylated gold nanoparticles can then detect the location of the tumor [Qian (2008)].

The main problem with many currently available cancer treatments is that they cannot be precisely targeted. As it is very hard to get an effective drug, such as **paclitaxel**, directly to the tumor, large doses are needed in the hope that enough of the drug will reach the diseased cells where it is needed. Recently gold nanoparticles have found a role to deliver drug easily.

Cancer therapy has various routes such as chemotherapy, photo-thermal therapy and radiotherapy. Gold nanoparticles have been investigated for potential candidates to aid in photo-thermal therapy and radiotherapy. It is important to understand the difference between normal and cancerous tissue to efficiently improve hybrid nanoparticles in cancer diagnosis and treatment. Optical and electronic properties of gold nanoparticles can be used to improve the contrast in molecular imaging for the detection of cancer at early levels.

Chronic Lymphocytic Leukemia (CLL), a cancer caused due to the overproduction of lymphocytes. Chronic leukemia starts in the bone marrow but would spread to other organs also. It was reported that as gold nanoparticles posses the ability to inhibit the function of heparin based growth factor, gold nanoparticles alone can inhibit the function of factors secreted by CLL cells and induce apoptosis [Bhattacharya *et al.* (2008), Bhattacharya *et al.* (2007), Zent *et al.* (2006)].

Drug Delivery:

Functionalized gold nanoparticles represent highly attractive and promising candidates in the applications of drug delivery owing to their unique dimensions, tunable functionalities

on the surface and controllable drug release [Han G. et. al. (2007)]. Gold-based technologies are also provide a unique needle-free delivery system, a technique that used gold nanoparticles and allowed vaccines to be delivered through the skin making use of the fact that small particles can pass through gaps between cells while large ones cannot. Therapeutic agents can also be coated on to the surface of gold nanoparticles. The large surface area-to-volume ratio of gold nanoparticles enables their surface to be coated with hundreds of molecules (including therapeutics, targeting agents and anti-fouling polymers).

The development of targeted vehicles for systemic drug delivery relies on optimizing both the cell-targeted legand and the physicochemical characteristics of the nanoparticle carrier [Bergen *et al.* (2006)]. Gold nanoparticles provide non-toxic carriers and drug and gene delivery applications. With these systems, the gold core imparts stability to the assembly, while the monolayer allows tuning of surface properties such as charge and hydrophobicity. An additional attractive feature of these is their interactions with thiols, providing an effective and selective means of controlled intracellular release [Ghosh *et al.* (2008)]. Gold nanoparticles are being investigated as carriers for drug such as Paclitaxel [Gibson *et al.* (2007)]. The administration of hydrophobic drugs requires molecular encapsulation and it is found that nano sized particles are particularly efficient in evading the reticuloendothelial system. Plasmon resonance with light can be harnessed to release payload molecules of therapeutic importance.

HIV/AIDS:

One of the most efficient usages of gold nanoparticles in recent years is detecting and fighting against HIV [Rad *et al.* (2011)].

AuNP is used as non-conventional desorption ionization promoters in laser desorption ionization mass spectrometry (LDI-MS), as bioanalytical applications [Pilolli *et al.* (2012)]. By using low power for imaging and destroying cancer cells no side effects due to

destruction of healthy tissues can occur. This is the key concept in curing cancer by nanoparticles. Even to image certain parts of body like in dentistry, bones etc. nano phosphors are being used. Drugs can be encapsulated in nanocapsules and targeted towards desired parts of body. Drug can then be fast or slow delivered, as desired, by opening the capsule using some external stimulus like magnetic field or infrared light or under some physiological conditions. There is considerable nanotechnology based research going on to help diabetic and HIV affected patients. The body implants should be strong and biocompatible.

AuNPs with facile preparation, multifunctional modifications, electrical, controlled geometrical, optical and surface chemical properties, and good biocompatibility are the subject of intensive studies and application in biology and medicine. The physiochemical properties of AuNP at nano scale, like size, shape, surface chemistry, and neat field effects are rendering AuNP potent candidates in biomedicine. The use of gold and gold compounds, as well as their potential therapeutic applications, in ancient and contemporary medicine has been reviewed periodically over the years [Tiekink (2002)]. Such particles are being explored as gold-based pharmaceuticals [Evans et al., (2000), Okada *et al.* (1993), Tiekink (2002)] and as agents in biohydrogen production [Zhang *et al.* (2007)]. Even quick, reliable and field detection of viruses, *E. Coli*, DNA, proteins, antibodies etc. is extremely important. [Kulkarni (2009)].

7.2 Therapeutic Applications of Silver Nanoparticles

Its includes Anti-Microbial, Pro-Healing, Anti-Inflammatory & Anti-Viral applications.

Anti-Microbial:

Silver nanoparticles have been widely used as an effective microbial agent against bacteria, fungi and viruses. The medical properties of silver have been known for over 2,000 years. Since the nineteenth century, silver-based compounds have been used in many

antimicrobial applications. Nanoparticles have been known to be used for numerous physical, biological and pharmaceutical applications. Silver nanoparticles are being used as antimicrobial agents in many public places such as railway stations and elevators in China, and they are said to show good antimicrobial action.

Silver ions and silver based compounds are highly toxic to microorganisms which include 16 major species of bacteria [Sanghai *et al.* (2010), Zhao *et al.* (1998)]. Their effect was recognized already in ancient times. Ag and its compounds have long been used for the disinfection of the medical devices and water purification. In medicine, Ag compounds are commonly applied to treat burns, dental work, catheters, wounds and a variety of infectious diseases [Aditya *et al.* (2013), Avalos *et al.* (2014), Elliott (2010), Crabtree *et al.* (2003)].

AgNPs were applied in a wide range of applications from disinfecting medical devices and home appliances to water treatment [Bosetti *et al.* (2002), Cho *et al.* (2005), Gupta (1998), Jain *et al.* (2005); Li *et al.* (2008)].

The antimicrobial efficacy of Ag, as of other metals and metal oxide nanoparticles, was reported to be size dependent [Pal *et al.* (2009)]. Silver is generally used in the nitrate form to induce antimicrobial effect, but when silver nanoparticles are used, there is a huge increase in the surface area available for the microbe to be exposed to. Though silver nanoparticles find use in many antimicrobial applications.

It has been shown that **hybrids** of silver nanoparticles with amphiphilic hyper branched macromolecules exhibited effective antimicrobial surface coating agents [Ayomnier *et al.* (2002)]. These inorganic nanoparticles have a distinct advantage over conventional antimicrobial agents. The most important problem caused by the chemical antimicrobial agents is the development of multidrug resistance. Ag ions or salts have only limited usefulness as an antimicrobial agent for several reasons, including the interfering effects of

salts. In contrast, these kind of limitations can be overcome by the use of silver nanoparticles.

Silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes in the cell membrane like the permeability of cell membrane and death of the cell. There is formation of 'pits' on the cell surface, and there is accumulation of the nanoparticles on the cell surface [Shaligram *et al.* (2009)].

The formation of free radicals by the silver nanoparticles may be considered to be another mechanism by which the cells die. There have been electron spin resonance spectroscopy studies that suggested that there is formation of free radicals by the silver nanoparticles when in contact with the bacteria, and these free radicals have the ability to change the cell membrane and make it porous which can ultimately lead to cell death [Danilcauk *et al.* (2006)].

Pro-Healing:

Although silver is an electrocolloidal form, had been reported to improve the healing of indolent wounds in the early 20^{th} century, that finding disappeared with the use of silver salts and complexes. Recently there have been several reported studies of improved re-epithelialization rates across partial thickness wounds with silver in the nanocrystalline form. The mechanism, although unknown at present, does not appear to be due to silver's antimicrobial action.

Anti-Inflammatory:

Increased wound inflammation not only accentuates pain but markedly impairs healing. Several heavy metals have been reported to decrease surface inflammation, the most recognized being gold. Wound surface inflammation has been reported to be decreased with the use of nanocrystalline silver. Excess matrix metalloproteases (MMP) are known to increase inflammation by both increasing inflammatory cell exudates and also leading to a

non-healing chronic wound. A characteristic of this type of wound is excess surface MMP activity, decreased inhibitory MMP activity and degradation of growth factors by the MMP's. Nanocrystalline have been shown both in vitro and in vivo to decease but not prevent MMP activity as some activity is needed to remove devitalized tissue. The mechanism for this action also remains unknown. Decreasing the necessary zinc activity required for MMP's is one possibility. The other is an effect on the expression or release of pro-inflammatory cytokines.

Anti-Viral:

Silver nanoparticle undergoes size-dependent interaction with HIV-1, and that the bound particles exhibit regular spatial relationships. These observation lead to the suggestion that the nanoparticles undergo preferential binding with the gp 120 subunit of the viral envelope glycoprotein. Silver nanoparticles inhibit the HIV-1 virus infectively in vitro, which also supports the proposal regarding preferential interaction with gp 120 [Kuhn *et al.* (2006)].

The interactions of inorganic nanoparticles with bio-systems are just beginning to be understood, and potential applications are being discovered at an increasing rate. However, in order to realize the future promise of nanoscience, it is imperative that the toxicity and long term health effects of exposure to nanomaterials be fully explored. The flexibility of nanoparticle preparation methods, the multitude of fictionalization techniques, and facile incorporation of nanoparticles into a variety of media provide the incentive for future research.

7.3 Therapeutic Applications of Bimetallic (Gold-Silver) Nanoparticles

Properties of BMNPs are influenced by both the metals; they provide excessive ordinary metallic NPs, which is an advantage [Mohl *et al.* (2011), Huang *et al.* (2006)]. The properties of alloy NPs can be extremely different from those of the elemental monometallic nanoparticles [Mohamed *et al.* (2000)].

Its applications includes Anti-Microbial & Photo thermal Cancer Therapy.

Anti-Microbial

Individual metallic nanoparticles of gold and silver posses antimicrobial activities. When these nanoparticles are used in bimetallic forms, they shows enhanced antimicrobial activities. Au–Ag Bimetallic Nanoparticles (BMNPs) shows high-quality antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Klebsiella pneumoniae*. BMNPs at the concentration of 1:3 (Au:Ag) exhibit areas of inhibition against the pathogenic bacteria, for example, *Staphylococcus aureus* and *Klebsiella pneumonia* [Ramakritinan *et al.* (2013)]. The bimetallic nanocomposite hydrogel has shown a significant antibacterial activity on *Bacillus* [Reddy *et al.* (2012)].

In the **Au-Ag core-shell nanoparticles**, the Au NPs act as the seeds for continuous deposition of silver atoms on its surface. The core-shell BNPs showed antibacterial activity against both gram-negative and gram-positive bacteria at low concentration of silver present in the shell; TEM and flow cytometric studies showed that the core shell BNPs attached to the bacterial surface cause membrane damage which leads to cell death. The enhanced antibacterial properties of Au-Ag core-shell BMNPs were possibly due to the more active silver atoms in the shell surrounding gold core due to high surface free energy of the Ag atoms, owing to shell thinness in the bimetallic NP structure [Banerjee *et al.* (2011)].

Photo thermal Cancer Therapy

The Au-Ag nanostructures (aptamer-Ag-Au) directly target the surface of human breast cancer cells (MCF-7) which have a high affinity and specificity. Aptamer Ag-Au nanostructures have a high capability of adsorbing near-IR (NIR) irradiation and able to perform photo thermal therapy of MCF-7 cells at a very low irradiation ($0.25 W/cm^2$) without destroying the surrounding cell and tissue [Wu *et al.* (2012)].

7.4 Antimicrobial Application of Gold, Silver and Bimetallic (Gold-Silver) Nanoparticles

Au–Ag BNPs can be used as nanomaterials in several in vitro and in vivo cancer therapies, Raman scattering development, and catalytic reactions using extremely well-organized photo thermal ablation, large resistive heat generation, and tunable near-IR absorption [Liu *et al.* (2014)]. Ru-Shi Liu et al. found that oral cancer cells without NPs were not hurt after exposure to laser irradiation for 5 min. On the contrary, cells with added NPs experienced significant cell death after laser exposure for 1 min.

In the burn healing, bacterial contamination is the biggest problem. This infection hinders the wound healing. It is responsible for 75% of all death in patients with burns exceeding 40% of the total surface area. Gram positive bacteria such as *Staphylococcus aureus* and gram-negative bacteria like *Escherichia coli* and *Pseudomonas aeruginosa* are commonly found in burnt surfaces. The presence of bacteria, mainly multi resistant organisms and bacterial biofilms, in the wound burn delays the wound healing process because of competing with host cells for nutrients and oxygen. The burns treatment is difficult, uncomfortable for the patients and expensive for health system. Due to antimicrobial properties of nanoparticles, these particles can avoid bacterial infection in wound and accelerate the wound healing. Furthermore, the complexation of nanoparticles with antibiotics may improve the healing process of lesions due to **anti-inflammatory** and **angiogenic** activity.

Certain bacteria can display resistance to one or more antibiotics. Determining bacterial antibiotic resistance – whether a bacterium can survive in the presence of an antibiotic – is a critically important part of the management of infectious diseases in patients. The **Kirby-Bauer (K-B) disk diffusion test** is the most common method for antibiotic

resistance/susceptibility testing. The K-B disk diffusion testing results helps physicians in choosing which antibiotic to use when treating a sick patient [Nunez *et al.* (2009)].

The development of metal nanoparticles (NPs) and nano-structured materials are attracting attention in recent research because of their extensive properties which enhance the biomedical properties [Pal *et al.* (2009)] such as drug delivery and wound dressing properties. Generally, the inertness of the noble bulk metallic fabrics such as Au & Ag is useful in macroscopic level but in nano scales level they are useful in plasmonics related fields, drug delivery, as well as wound dressing [Qiu *et al.* (2001)]. Nanoparticles have an important role in the pharmaceutical and biotechnology industries.

Wound Infection

Skin collects information from environment and helps in maintaining body temperature. It acts as a protective barrier to the human body organs. Wound causes a break in the integrity of skin and various tissues which may be a superficial cut, burns, puncture or scratch [Peng *et al.* (2009)].

Types of Wound Infections

I. Infection on outer layer of skin

II. Infection through bites

III. Infections after surgery

IV. Infections through burns

Microorganisms involved in wound infections on human skin.

Table 7.1: Pathogenic microorganisms and their corresponding frequency

Pathogens	**Frequency (%)**
Staphylococcus aureus	20
Coagulase Negative *Staphylococci*	14
Enterococci	12
Escherichia coli	8
Pseudomonas auroginosa	8
Enterobacter species	7
Klebsiella pneumoniae	3
Streptococci	3
Candida albicans	3
Other gram positive aerobes	2

Of which, ***Staphylococcus aureus*** has highest frequency for wound infections on human skin.

Purpose:-

The purpose of KB test is to determine the sensitivity or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antimicrobial compounds in order to assist a physician in selecting treatment options for the patients. The pathogenic organism is grown in MH agar in the presence of various antimicrobial impregnated filter paper disks. The presence or absence of growth around disks is an indirect measure of the ability of that compound to inhibit that organism.

7.5 Mechanism Behind Antimicrobial Activity of Nanoparticles

Product that exhibits antibacterial properties can be more efficient and interesting to burn treatment. Being a noble metal, silver is an inert metal but its ions have been known to have strong inhibitory and bactericidal effects as well as wide spectrum of anti-microbial activities. Silver ions work against bacteria in number of ways: they interact with thiol groups of the enzymes and proteins that are important for the bacterial respiration and transport of substances across the cell membrane and within the cell [Cho *et al.* (2005), Ivan *et al.* (2004)] and silver ions are bound to the bacterial cell wall and outer bacterial cell thereby altering the function of the bacterial cell membrane. In this way, silver metal and its compounds were very effective in preventing the infection of the wound [Wright *et al.* (1999)].

The gold nanoparticles conjugated antibiotics were seen to be more stable than their free forms. Most of antibiotics were dependent on temperature and solutions from which they are taken out and thereby affecting their stability [Rao *et al.* (2000)].

The antimicrobial properties of nanoparticles were investigated using *Escherichia coli* (four strains), *Bacillus subtilis* & *Staphylococcus aureus* (three strains). Bacteriological tests

were performed in MH agar medium on solid agar plates and in liquid systems supplemented with different concentrations of nano sized silver particles. These particles were shown to be an effective bactericide.

Certain bacteria can display resistance to one or more antibiotics. Determining bacterial antibiotic resistance – whether a bacterium can survive in the presence of an antibiotic – is a critically important part of the management of infectious diseases in patients. The Kirby-Bauer disk diffusion test is the most common method for antibiotic resistance/susceptibility testing. These test results help physicians in choosing which antibiotics to use when treating a sick patient.

Synergistic Effect is an effect arising between two or more agents, entities factors, or substance that produces an effect greater than the sum of their individual effects. These bimetallic NPs offer the versatility of combining the antimicrobial silver activity with the presence of gold, stabilized with biomolecule.

Bimetallic nanoparticles comprising of gold and silver have been reported to possess greater antimicrobial activity than monometallic ones [Ramakritinan (2013), Reddy *et al.* (2012), Ghosh *et al.* (2015)]. Ag-Au bimetallic nanoparticles were synthesized by a chemical method and assayed for their antibacterial activity against *S. aureus* [Bahrami *et al.* (2014)]. Ag-Au alloy nanoparticles antibacterial activity was increased when combined with penicillin G and piperacillin.

Bimetallic Au-Ag bimetallic nanoparticles were synthesized by using *Ocimum basilicum* aqueous leaf and flower extracts and revealed a high antibacterial activity against many pathogenic microorganisms [Malapermal *et al.* (2015)]. Our previous work was limited to the production of gold nanoparticles by different fungal species like *Fusarium oxysporum, Trichoderma reesei, Verticillium luteum, Aspergillus niger, Aspergillus versicolor etc*. and their varied applications [Karmakar *et al.* (2010)].

We were now interested to utilize the potential of mold strain for simultaneous bio-reduction of metal salts to form bimetallic nanoparticles. Therefore, the potential of *Trichoderma reesei* was used to form gold-silver bimetallic nanoparticles. Daptomycin, an anti-MRSA antibiotic which treats complicated skin and skin structure infections was combined with non-functionalized bimetallic nanoparticles which were biologically synthesized and are non-toxic [Ramakritinan *et al.* (2013), Reddy *et al.* (2012)]. The antimicrobial activities of the Daptomycin, bimetallic nanoparticles and the combination of antibiotic and nanoparticles were then evaluated against methicillin-resistant *S. aureus, S. epidermis* and *M. luteus* strains. Thus, promising its potentiality for therapeutic purposes, especially; skin infection treatments in future endeavors.

7.6 Kirby-Bauer (K-B) Disk Diffusion Test

Lysogeny Broth (LB) Media for subculturing of bacteria

- Tryptone: 10.0 g
- NaCl: 10.0 g
- Yeast Extract: 5.0 g
- Distilled water: 1L

Mueller Hinton (MH) Agar Media for KB disk diffusion test:

- Beef Extract: 2.0 g
- Casein hydrolysate: 17.5 g
- Starch: 1.5 g
- Agar: 17.0 g
- Distilled water: 1L

pH: 7.2±0.2

Heat with frequent agitation and boil it for one minute to completely dissolve the components. The petriplates must be poured to a depth of 4.0 mm (Approx. 25 ml of liquid agar for 100-mm plates and 60 ml of liquid for 150-mm plates). Plates that are too shallow will produce false susceptible results as the antimicrobial compound will diffuse further than it should, creating large zones of inhibition. Conversely, plates poured to a depth >4.0 mm will give fast resistant results.

Sterilize in Autoclave at 121°C for 15 minutes. Allow to solidify at room temperature, and then store it at 4-8°C. This media is approximately stable for 70 days. If the pH is <7.2 certain drugs will appear to lose potency (aminoglycosides, quinolones, macrolides), while other agents may appear to have excess activity (tetracycline). If the pH is >7.4, the opposite results may occur.

MH agar is considered as the best medium to use the routine susceptibility testing of non fastedious bacteria for the following reasons:

- It shows acceptable batch-to-batch reproducibility for susceptibility testing.
- It is low in sulfonamide, trimethoprim and tetracycline inhibitors
- It supports satisfactory growth of non fastedious pathogens.
- A large body of data and experience has been collected concerning susceptibility tests performed with this medium

Please note that use of media other than MH agar media may results in erroneous results. Also note that only the aerobic or facultative bacteria that grow well on un supplemented MH agar should be tested using this protocol. Fastidious organisms require MH agar supplemented with additional nutrients.

Antibiotic Susceptibility disks

Antimicrobial disks purchased from Remel or BD BBL. They are packaged in spring-loaded cartridges 25 disks and ordered as packages of 10 cartidges. Proper storage of these disks is essential for reproducible results.

Sealed cartridges should be stored as either 8°C or frozen at -14°C in a non-self-defrosting freezer. Allow disk come to room temperature prior to removing the protective plastic packaging. Once opened, store the cartridge in a storage container containing desiccant for no more than one week.

7.7 Antimicrobial Assay of Daptomycin and Daptomycin conjugated Nanoparticles (obtained from *Trichoderma reesei* NCIM 992) in *Staphylococcus aureus*

To determine the antibacterial activity of synthesized gold nanoparticles, standard disk diffusion method was carried out against Multi Drug Resistant Strain (MRSA) *Staphylococcus aureus.* The agar plates having suitable nutrient media (LB/MH media) was prepared, sterilized and allowed to solidify. After solidification, the agar plates were inoculated with bacterial cultures. 6 mm disks were impregnated with required quantity of antibiotic and nanoparticles in petri-plates containing suitable nutrient agar medium seeded with 120 µL of 36 h of each pathogen. 20 µg/ml (Minimum Inhibitory Concentration (MIC) of Daptomycin antibiotic, 20 µg/ml (equal amount as that of antibiotic) of nanoparticle and a mixture of both Daptomycin and lyophilized nanoparticle was formulated. 150 µl of each of these was impregnated on the corresponding disks and incubated at 28°C for two days. The diameter of zones of inhibition was measured using a ruler and mean value was recorded for each pathogen and expressed in millimeter (mm). All the above experiments were carried out in duplicate.

The Kirby-Bauer (K-B) utilizes small filter disks (around 6 mm) impregnated with a known concentration of antibiotic. The disks were placed on Agar plate that was inoculated with

the test microorganism. During incubation, antibiotic diffuses from the disk into the surrounding agar. If susceptible to the antibiotic, the test organism will be unable to grow in the area immediately surrounding the disk, displaying a **zone of inhibition**. Size of that zone depends on various factors such as the sensitivity of microorganism to the antibiotic, the rate of diffusion of the antibiotic through the agar and the depth of the agar. Microorganisms that are resistant to an antibiotic would not show a zone of inhibition (growing right up to the disk itself) or display relatively small zone [Nunez *et al.* (2009)].

The anti-bacterial activity was performed by agar disk diffusion method against *Staphylococcus aureus* bacteria which is a gram positive bacterium. Results showed that Nanoparticles have a discrete antibacterial activity at different combinations. Further study is to be done to find out the MIC and anti-bacterial activity against various drug resistant wound infections causing pathogen.

Zone of inhibition (in mm) for Daptomycin and Daptomycin combined with synthesized nanoparticles (Gold, Silver and Bimetallic).

Fold change percentage is a measure describing how much a quantity changes going from an initial to a final value. Thus, if the initial value is A and final value is B the fold change is

$$\frac{B-A}{A} \times 100$$

Table 7.2 Synergistic Effect of Daptomycin and Daptomycin conjugated Gold, Silver and Bimetallic (Gold-Silver) Nanoparticles synthesized from *Trichoderma reesei* NCIM 992

Different Nanoparticles used for anti-microbial activity on *Staphylococcus aureus* bacterial culture	**Zone of Inhibition (mm) Antibiotic (Daptomycin) (A)**	**Zone of Inhibition (mm) Antibiotic (Daptomycin) Conjugated Nanoparticles (B)**	**Fold increase (Percent)**
Gold Nanoparticles	7.5±0.32	9.41±0.2	25.5 %
Silver Nanoparticles	8.1±0.3	10.8 ± 0.12	33.3 %
Bimetallic Nanoparticles	8.67 ± 0.45	12.32 ± 0.20	42.0 %

It had been found that, there exists a synergistic effect of Daptomycin and synthesized nanoparticles which were yielding enhanced antimicrobial activities.

According to the Fold number percentage, it was clear that the Au/Ag Bimetallic nanoparticles showed highest percentage i.e. 42.0 %, as compared to the Gold Nanoparticles (25.5 %) and Silver nanoparticles (33.3 %).

With the help of fold number percentage, it was clear that the Au/Ag Bimetallic Nanoparticle showed maximum synergistic effect as compared to Gold and Silver nanoparticles. Au/Ag Bimetallic nanoparticles have the maximum capability to enhance the anti-bacterial activities as compared to other Nanoparticles.

7.8 Antimicrobial Assay of Daptomycin and Daptomycin conjugated Bimetallic (Gold-Silver) Nanoparticles in different Bacterial Strains

To determine the antibacterial activity of synthesized gold nanoparticles, standard disk diffusion method was carried out against Multi Drug Resistant Strain (MRSA) *Staphylococcus aureus.* The agar plates having LB media was prepared, sterilized and allowed to solidify. After solidification, the agar plates were inoculated with with different bacterial cultures. 6 mm disks were impregnated with required quantity of antibiotic and nanoparticles in petri-plates containing suitable nutrient agar medium seeded with 120 µL of 36 h of each pathogen. 20 µg/ml (Minimum Inhibitory Concentration (MIC) of Daptomycin antibiotic, 20 µg/ml (equal amount as that of antibiotic) of nanoparticle and a mixture of both Daptomycin and Gold-Silver Bimetallic nanoparticles were formulated. 150 µl of each of these was impregnated on the corresponding disks and incubated at 28°C for two days. The diameter of zones of inhibition was measured using a ruler and mean value was recorded for each pathogen and expressed in millimeter (mm). All the above experiments were carried out in duplicate.

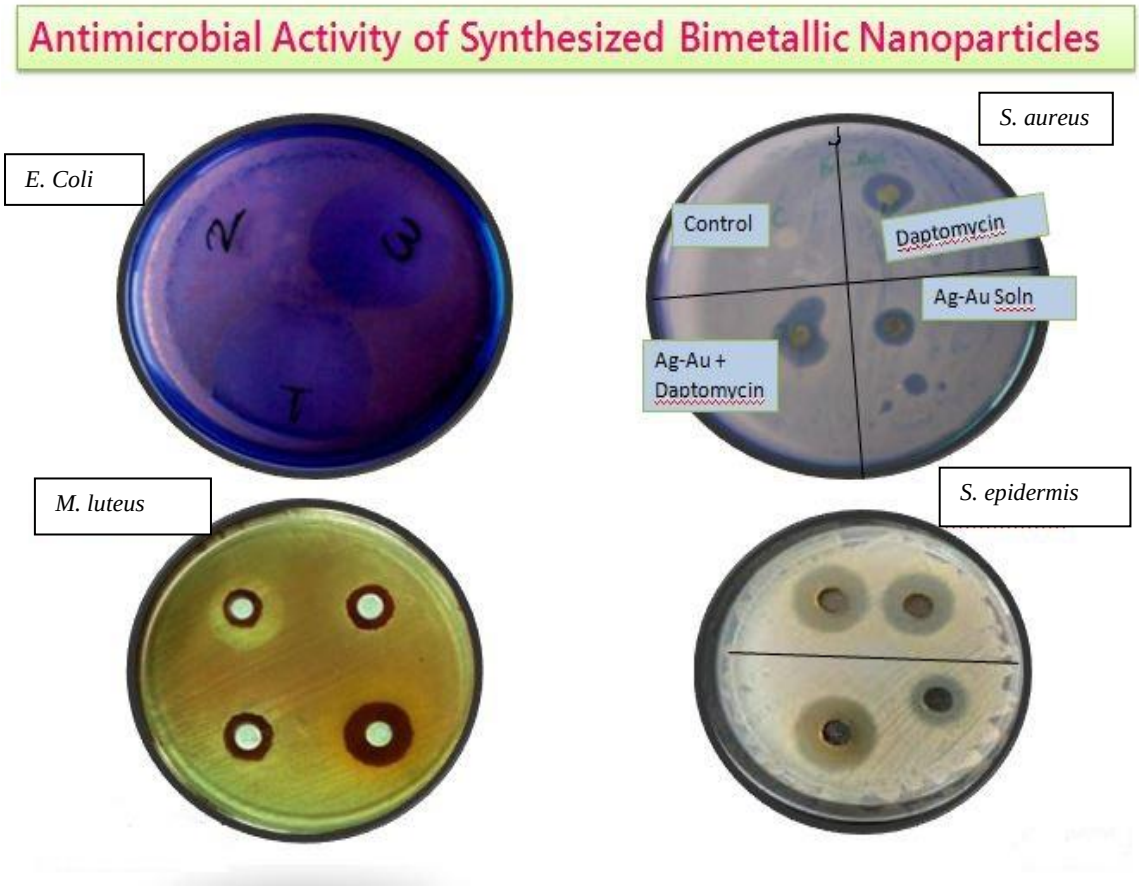

Figure 7.1: Zone of inhibition in mm of Control, Daptomycin, Gold-Silver solution and Daptomycin conjugated in Bimetallic (Gold-Silver) in *E. coli, S. aurius, M. luteus* and *S. epidermis*.

Table 7.3: Synergistic Effect of Daptomycin and Daptomycin conjugated Bimetallic (Gold-Silver) Nanoparticles in different Bacterial Strains

Name of the Bacterial strain	**Zone of Inhibition (mm) Antibiotic (Daptomycin) (A)**	**Zone of Inhibition (mm) Antibiotic (Daptomycin) Conjugated Nanoparticles (B)**	**Fold increase (Percent)**
Staphylococcus aureus	8.67 ± 0.45	12.32 ± 0.20	42.0 %
Micrococcus luteus	9.45 ± 0.1	12.95 ± 0.43	37.0 %
Staphylococcus epidermis	10.34 ± 0.2	13.5 ± 0.12	30.0 %

Bimetallic nanoparticles have already proved their mettle as good antimicrobial compounds in previous studies, over the monometallic compounds [Bahrami *et al.* (2014), Malapermal (2015)]. Now, the *in vitro* bactericidal activity of Daptomycin (antimicrobial agent for skin infections) was combined with bimetallic nanoparticles. This combined therapeutic agent was found to be more effective as compared to their respective free forms. Daptomycin combined with bimetallic nanoparticles and free Daptomycin were tested on bacterial strains by comparing the corresponding zone of inhibition (mm) as shown in Table 8.3.

In the comparative study of the Gold-Silver Bimetallic synergistic effect in different strains of bacteria. It is found that as compared to *Micrococcus luteus* (37.0 %) and *Staphylococcus epidermis* (30.0 %) bacterial strains, in *S. aureus* (42%) strain, Au-Ag Bimetallic Nanoparticle showed maximum synergistic effect.

Zone of inhibition is absent in *Escherichia coli*, as it is a gram negative bacteria. Daptomycin is an antibiotic which acts only on gram positive bacteria. It do not act on gram negative bacteria. It is used to check the nature of Daptomycin.