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HIGHLY BIOACTIVE ULTRA SMALL VANCOMYCIN DOPED SILVER NANOPARTICLES GROWN BY SOL-GEL METHOD

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Keywords:

Sol-gel method, Silver nanoparticles, Drug Doping, Anti bacterial activity, XRD, TEM

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ABSTRACT: Highly bioactive ultra-small vancomycin doped silver nanoparticles were synthesized by the sol-gel method. The samples were characterized by UV, FR-IR, XRD, Photoluminescence, and TEM analysis. Continuous stirring helped in preparing ultra-small drug conjugate silver nanoparticles. Observation of weak (broadened peak) in XRD diffractograms indicates the formation of very small size drug conjugate sliver nanoparticles. Doping of Vancomycin drug improves the antibacterial activities of silver nanoparticles in the serial dilution method and results in strong yellow, green, and blue luminescence peaks. Antibacterial assay showed that sliver nanoparticles conjugation with vancomycin drug enhanced the antibacterial activity against a variety of gram-positive bacteria like *E. coli*, *S. aureus*, and *Enterococcus faecalis*. These findings suggest that modifying clinically approved drugs using nanotechnology is a feasible approach in our work for effective antibacterial molecules. The results of this study revealed that the potential therapeutic applications of silver nanoparticles in combination with vancomycin antibiotic had been increased drastically. Nanosilver with antibiotic is a promising candidate for the development of future antibacterial therapies because of its wide spectrum of activity.

INTRODUCTION: Now-a-days, infectious diseases are a huge burden on society, driven largely by socio-economic, environmental, and ecological factors [1]. The burden of morbidity and mortality falls very heavily on people in developing countries [2]. Drug-resistant microorganisms are a major cause for causing microbial re-emergence [3]. Among the variety of Gram-positive and gram-negative bacteria E. coli, S. aureus, P. aeruginosa, Bacillus species, K. pneumoniae are important pathogens contributing to urinary tract infection, gastro-enteritis wound and skin diseases, food poisoning and nosocomical infection [4-10].

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The overdose of antibiotics has developed bacterial resistance resulting in a decreased efficacy of available marketed antibiotics. During the last four decades, nanomedicines have shown great potential due to the effectiveness of various drug nanoconjugates against pathogenic micro-organisms [11]. Now-a- days nanomaterials have been used as effective coatings on antibiotic to present bacterial adhesion to surfaces as well as bactericidal agents [12].

Dai and He *et al*., reported the development of self-defensive and antibacterial adhesion surface casting based on bilayer hydrogel, which can promote cell adhesion and proliferation [13-14]. From ancient times, silver has been known to possess antibacterial properties [15,] but the insoluble characteristics properties of sliver metal and its salts like $AgNO_3$ limit it impractical in many clinical applications. According to researchers [16-17] it is not only facile to synthesize silver nanoparticles of required size [18-19]

and shapes [20-21] dispersed in aqueous and non-aqueous phases but also suitable to make films with the composite of these particles using numerous applications in the field of medical diagnosis and therapy. The silver nanoparticles is used in material modifications in various fields like clothing, semiconductors, nanomedicines, and preparation of nanocomposite materials with improved performance has been reported as silver nanoparticles have been successfully coated or medical devices for infection-free transplantations [22-23]and on various fabrics [24-25]. The coating of silver nanoparticles on fabrics not only the metallic feature to the fiber rendering the textiles conductive but also provide the antibacterial property to the textile, especially socks. These research studies suggest that it is possible to have extended action of AgNPs based antibacterial activities. Moreover, according to the literature, it can be expected that the high specific surface area and a high fraction of surface atoms of vancomycin doped sliver nanoparticles will lead to high antimicrobial activity as compared to bulk sliver metal [26]. With the limited discovery of novel anti bacterial agents, a unique and feasible approach is to modify clinically approved drugs to enhance their efficacy and drug repurposing to expedite the discovery of effective formulation of novel antibacterial agents.

MATERIALS AND METHODS:

Synthesis & Spectroscopic Characterization of Nanoparticles: Silver nanoparticles and vancomycin doped silver nanoparticles were obtained by modified sol-gel chemical route. The silver nitrate (0.5mM) was mixed with ethanol and magnetically stirred for 60 min at room temperature. The sol thus was kept in ultrasonic chamber at 40 KHZ at 60 °C for the next 2 h. For Vancomycin doping, pure Vancomycin drug was added in the ratio 0.01 M. Mixture was magnetically stirred and heated to 60 °C for 4-6 h to obtain homogenous solution. The gel was obtained by again sonicating for another 3 h at 60 °C followed by the aging of 24 h. Nanoparticles were obtained by centrifuge the gel, which was self-assembled on the sides of the tubes. The samples were characterized by XRD (x-ray diffraction), UV -Vis transmission, Photoluminescence, TEM, and FT-IR spectroscopy. The transmission spectra were obtained using Perkin Elmer Lemda 25 in the range of 200 to 800 nm. The PL spectra were obtained by Perkin Elmer LS55 with excitation at 200nm of Xe lamp. The X-ray diffractograms were obtained in the 2θ range from 20° to 80° with Cu-k α radiation of wavelength 1.546 A° using Bruker D8 advanced XRD. The FT-IR spectra using spectrums were measured at room temperature using KBr pellets.

***In-vitro* Antibacterial Activity of Nanoparticles:** Bacterial strains and their relevant attributes are mentioned in **Table 1**. Strains were received from PGI Chandigarh, India, and maintained in a sterile broth medium. To determine the Minimum Inhibitory Concentration (MIC) of synthesized compounds were grown in Mueller Hinton Broth with aeration at 37 °C for 24 h.

TABLE 1: BACTERIAL STRAINS USED IN OUR STUDY

S. no.	Bacterial Strains	Strain no.
1	*Escherichia coli*	MTCC-1563
2	*Stayphylococcus aureus*	MTCC- 3160
3	*Enterococcus faecalis*	MTCC-439

The effects of synthesized compounds on individual bacterial isolates were determined according to the following method [27]. The minimum inhibitory concentration (MIC) was determined by the microdilution method with serially diluted samples. The samples were diluted to get a series of concentrations from 0.19 mg/ml to 100 mg/ml in sterile nutrient broth. The bacterial inoculums were prepared by adjusting the turbidity to 0.5McFarland (1.5×10^8 CFU/ml) standard. The microorganism suspension of 50 microlitres was added to the broth dilutions. These were incubated for 18 h at 37 °C. Then 10 microlitre of each suspension was added to the solution to obtain a final concentration. The lowest concentration of antibiotic preventing the appearance of turbidity is considered to be UV Spectrophotometer at 660 nm [28].

RESULTS AND DISCUSSION:

UV-VIS Spectroscopic Characterization of Synthesized Silver Nanoparticles and Vancomycin Doped Silver Nanoparticles: Silver nitrate solution has turned dark brown in color, which indicated the formation of silver nanoparticles. This color change is due to the property of quantum confinement, which is a size-dependent property of nanoparticles which affects the optical property of nanoparticles. The color change in silver nitrate solution is treated with a Schiff base. The resulting

color change of aqueous silver nanoparticles solution may be due to the quantum confinement of silver nanoparticles. The synthesis of silver nanoparticles by the reduction of silver nitrate was followed by UV-VIS spectroscopy in the presence of a reducing agent (Schiff base ligand) and starch as a capping agent. It is well known that silver nanoparticles exhibit a pale yellowish color in aqueous solution due to the surface plasmon resonance (SPR) of metal nanoparticle [29]. UV-VIS spectra of the aqueous silver nanoparticles solution synthesized being recorded after completion of the reaction. The UV-VIS spectrum of silver nanoparticles synthesized at room temperature clearly shows an intense surface plasmon resonance (SPR) band at 425 nm (visible region) which confirmed the formation of silver nanoparticles. Vancomycin shows an absorption peak around at 290 and 320 nm (UV region), respectively. After the addition of vancomycin to AgNPs solution, the absorption peak shifts to a shorter wavelength (blue shift) up to 30 nm due to charge transfer between silver nanoparticles & Vancomycin doped silver nanoparticles [30]. However, in case of vancomycin drug a significant blue shift of around 30 nm in absorption peaks was observed. The cause of blue shift could be explained on the basis of either nucleophilic reaction (charge transfer) or electronic transitions between different orbitals.

The nucleophilic substitution reaction takes place between lone pair of Schiff base ligand and a hydrogen atom of the amine group of the drug. So, Schiff base ligand acts as a nucleophile in this reaction, which increases the electron density and causes hypsochromic shift in absorption peak. Further, there could also be the possibility of electronic transitions occurring between bonding or non-bonding orbital to antibonding orbital. In the visible region, two main electronic transitions are possible: (1) bonding to antibonding orbital ($\pi \rightarrow \pi^*$) and (2) non-bonding to antibonding orbital ($n \rightarrow \pi^*$). The first one occurs at low energy (higher wavelength), and second one requires high energy (lower wavelength). Due to the presence of lone pair on PVP, the transition from $n \rightarrow \pi^*$ might be more dominating than $\pi \rightarrow \pi^*$, causes a significant blue shift. Hence, Schiff base ligand acts as a bridge between silver nanoparticles and Vancomycin doped silver nanoparticles surface and this blue shift attributed to the attachment of drug with nanoparticles surface. Applied UV radiations cause the excitement of surface plasmon on the periphery of silver nanoparticles, which leads to the occurrence of the phenomena called surface plasmon resonance. The UV absorption apex range of synthesized compounds is 450 nm.

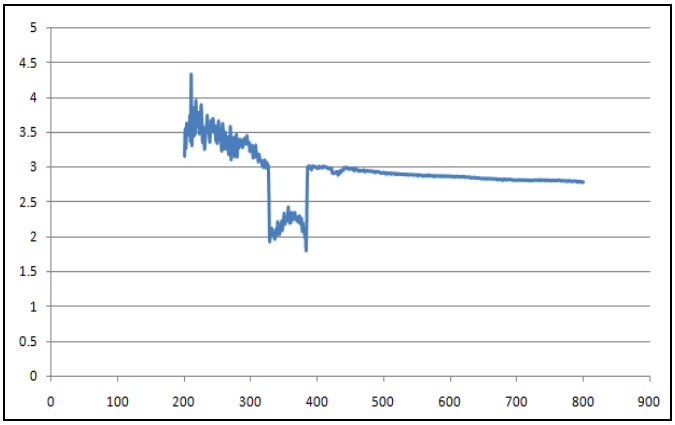

FIG. 1: UV SPECTRA OF SILVER NANOPARTICLES PREPARED FROM SOL-GEL METHOD

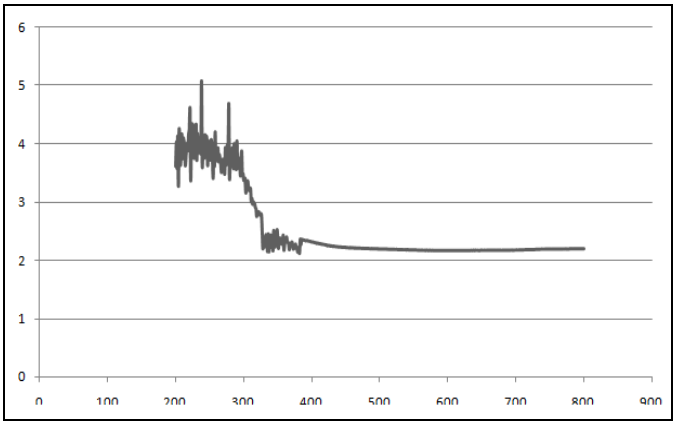

FIG. 2: UV SPECTRA OF VANCOMYCIN DOPED SILVER NANOPARTICLES

Fourier Transform Infrared Absorption Studies of Silver Nano Particles and Vancomycin Doped Silver Nano Particles: The infrared spectra of silver nanoparticles and vancomycin doped silver nanoparticles were recorded in the region 4000-400 cm^{-1}. FTIR is a characterization technique used to quantify the vibrational frequencies of the bonds in the molecule, which can be seen in the figure. FTIR analysis is performed to understand the vibrational kinetics of atoms, molecules and to identify the possible functional groups responsible for the reduction and as well as capping of reduced silver nanoparticles along with the nature of surface adsorbents. The surface modification by functional

groups may generate different properties. The FTIR spectra, due to the presence of various functional groups over the surface of the nanoparticles thus shows a number of absorption peaks, each peak showing the presence of particular functional groups in the compounds. From FT-IR data, it is thus possible to understand the oxidation levels of synthesized nanoparticles, prepared at a different partial pressure. To determine the coupling of the Vancomycin drug to the silver nano-particles nanoparticles FTIR spectroscopy has been used to shows the interaction between silver nanoparticles and Vancomycin drug. Vancomycin drugs have various amino and hydroxyl groups, so mainly two types of bonding take place between drugs and metal nanoparticles. One is amino bonding (through amine or amide group) and thiol bonding. The free vancomycin has vibrational bands at 3286, 1640, 1505, 1399, 1229, and 1062 cm^{-1} corresponding to stretching –OH– bond, bending vibrations of N–H group of amines and amides, –CN– group of amines, and C–O group of ethers and esters, respectively. In case of Vancomycin doped silver nanoparticles , there was no significant shift in frequency peaks except the peaks at 1632 and 1640 cm^{-1} corresponding to N–H group of secondary amines or amides, which were shifted to higher wavenumbers. Hence, the FTIR data infers that it might be the hydrogen atom of amines or amides that binds to the precursor, which was free for the attachment on the surface of the silver.

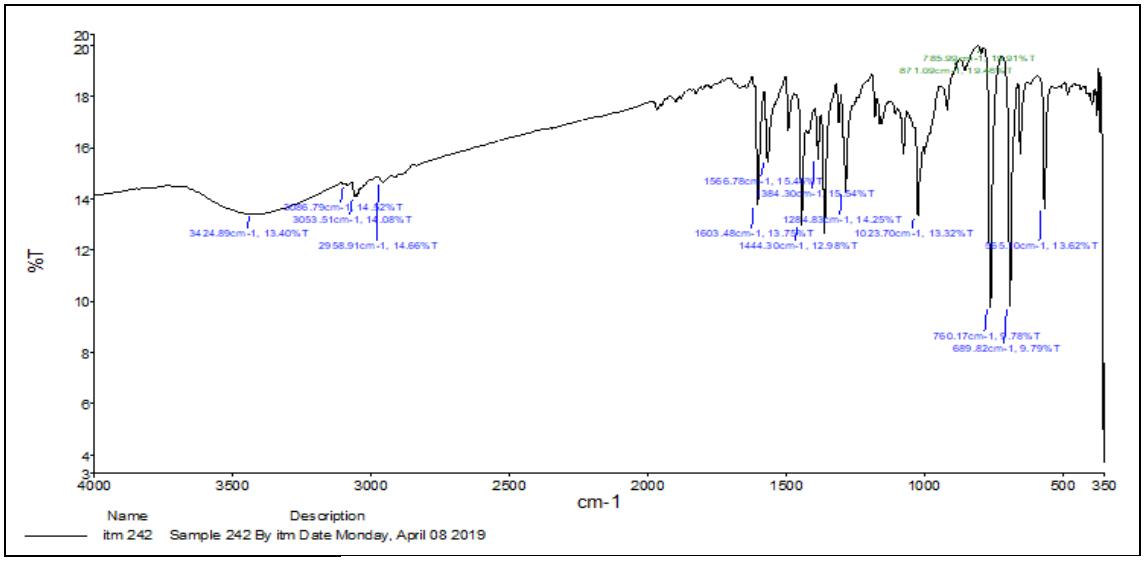

FIG. 3: FT-IR SPECTRA OF SILVER NANOPARTICLES SYNTHESIZED *VIA* SOL-GEL METHOD

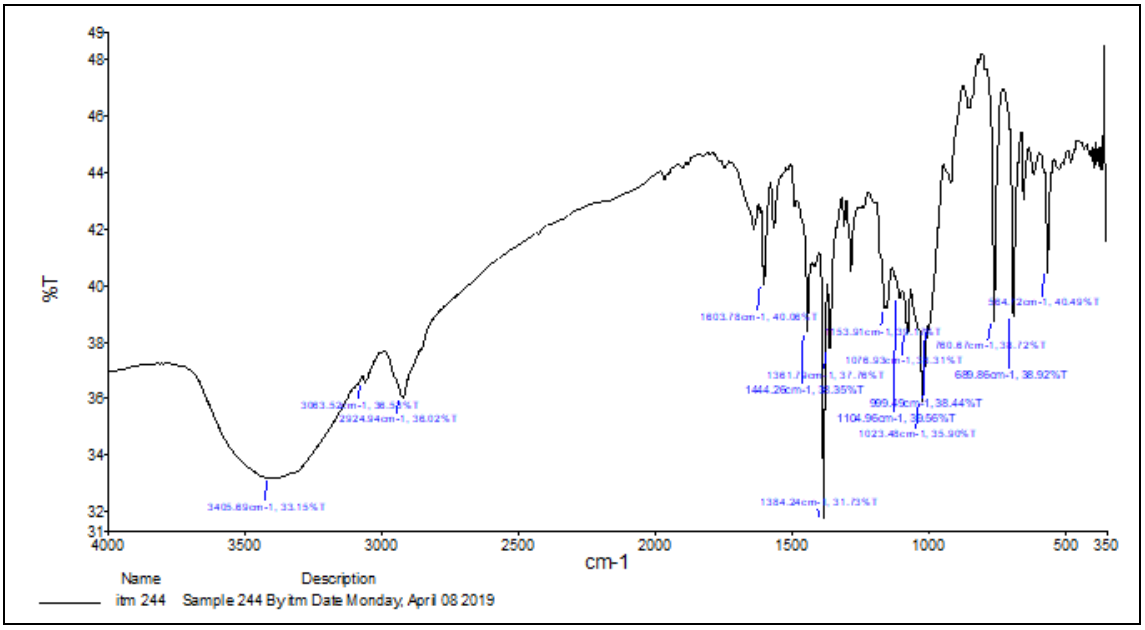

FIG. 4: FT-IR SPECTRA OF VANCOMYCIN DOPED SILVER NANOPARTICLES

Particle Size Distribution of Synthesized Silver Nanoparticles & Vancomycin Doped Silver Nanoparticles: The Shape and size morphology of synthesized silver nanoparticles & Vancomycin doped silver nanoparticles were characterized using particles size analyzer and transmission electron microscopic (TEM) study as shown in **Fig. 5, 6, 7, 8** which demonstrated the formation of slightly spherical AgNPs and irregularly shaped its drug conjugate. The size of the AgNPs & its drug conjugate is 0.2 µm and 100 nm, respectively.

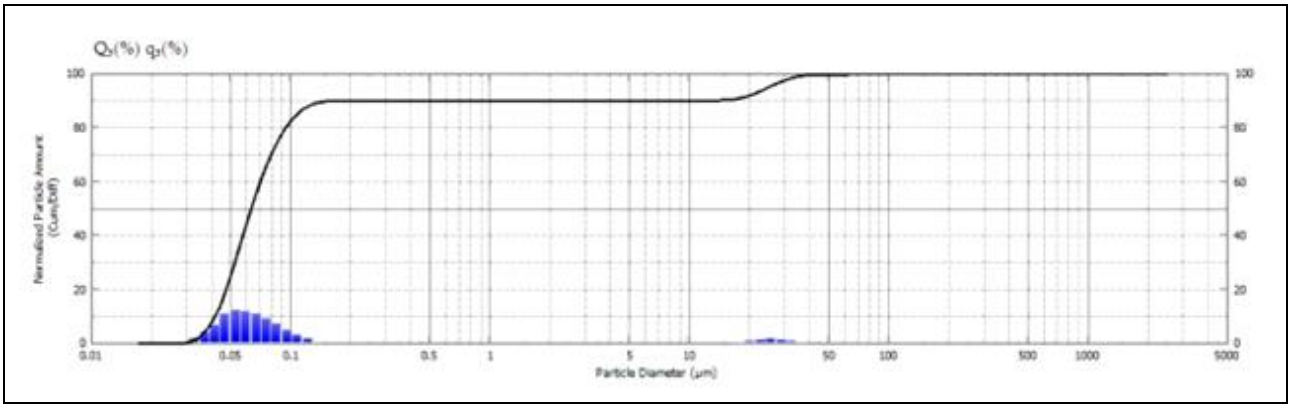

FIG. 5: PARTICLE SIZE DISTRIBUTION OF SILVER NANOPARTICLES

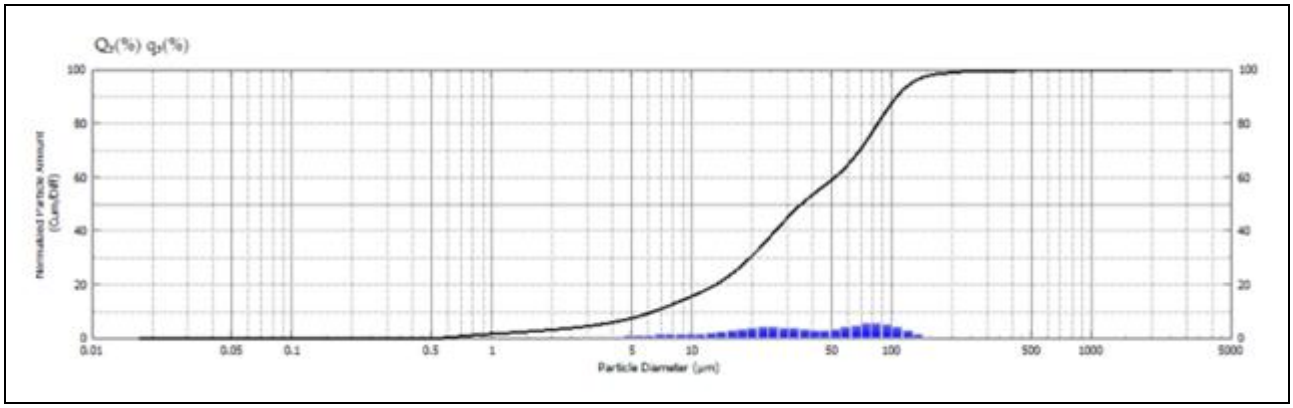

FIG. 6: PARTICLE SIZE DISTRIBUTION OF VANCOMYCIN DOPED SILVER NANOPARTICLES

Transmission Electron Microscopic Examination of Silver Nanoparticles & Vancomycin Doped Silver Nanoparticles: Transmission electron microscopy (TEM) has been used to study the size, shape and surface morphology of AgNPs and vancomycin doped AgNPs synthesized by sol-gel method. The corresponding micrographs are shown in **Fig. 7 & 8**.

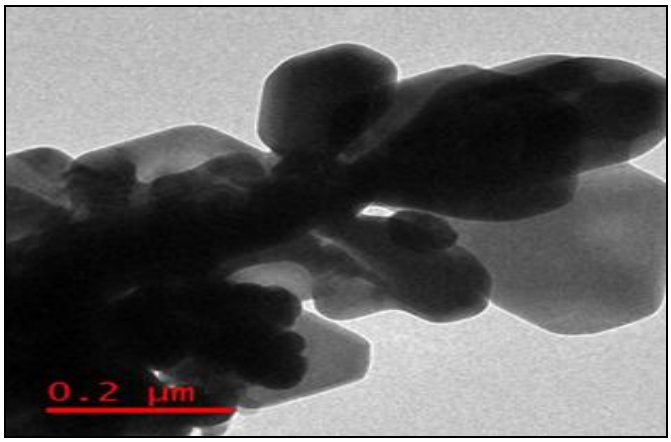

FIG. 7: TEM IMAGE OF SYNTHESIZED SILVER NANOPARTICLES VIA SOL-GEL METHOD

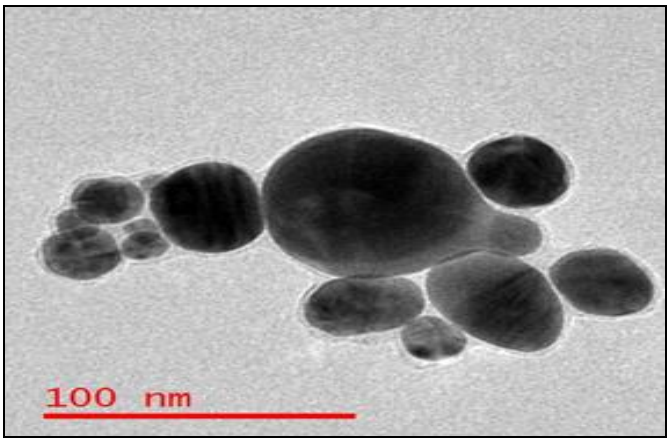

FIG. 8: TEM IMAGE OF SYNTHESIZED VANCOMYCIN DOPED SILVER NANOPARTICLES *VIA* SOL-GEL METHOD

The morphology of AgNPs and Vancomycin doped silver nanoparticles were found spherical, and the size of AgNPs and its drug conjugate are 0.5 µm & 100 nm. The diameter of AgNPs observed in TEM was lower as compared to DLS because by DLS, wet and extended diameter of AgNPs suspension has been measured whereas TEM micrographs show the dry and shrunk configuration of AgNPs.

X-ray Diffraction Analysis of Synthesized Silver Nanoparticles and Vancomycin Doped Silver Nanoparticles: The X-ray diffraction patterns of AgNPs & Vancomycin loaded AgNPs are showing in **Fig. 6, 7, 8**. Single crystals of the compounds could not be isolated from any solvent. The powder XRD patterns of Cu (II) complex show shape crystalline peaks indicating its crystalline peaks nature. The crystallite domain size was calculated using the Debye-Scherer formula:

$$D = 0.94 * \frac{\lambda}{\beta} * \cos\theta$$

Where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the wavelength of $Cu_{k\alpha}$, β is the full width at half maximum, and θ is the Bragg diffraction angle. Using the full width at half maximum intensity of the patterns, the average size of AgNPs & Vancomycin doped AgNPs at around 0.445 nm & 0.2411 nm. To confirm the crystalline nature of synthesized silver nanoparticles **Fig. 9**. For silver nanoparticles, the XRD reflection lines were observed at 38.10°, 44.09°, 64.20°, and 77.42°, ascribed to (111), (200), (220) and (311) respectively, the reflections of the face-centered cubic structure of metallic silver. The estimated crystallite size for AgNPs was around 445 nm. The lattice constant '*a*' worked out to be 4.098 Å, which is in good agreement with the standard data file JCPDS no 04. After doping of silver nanoparticles with Vancomycin drug, there was a slight shift in the major peak of AgNPs at 37.75° and 37.30°. There was also a minor shift in small peaks (2θ values) from 43.90°, 64.02° and 77.24° respectively. The different reflection peaks for drug doped silver nanoparticles nanoparticles confirmed their crystalline nature and face-centered cubic symmetry.

There was no notable change in crystallite size observed in the case of vancomycin doped silver nanoparticles. The shift in XRD reflection lines indicates the presence of lattice strain that is resulted from either compressive stress or tensile stress. Moreover, the shift in reflection lines towards the lower angle implies the presence of compressive stress. Therefore, XRD results also suggest the interaction of Vancomycin drug with silver nanoparticles which were synthesized via sol-gel method.

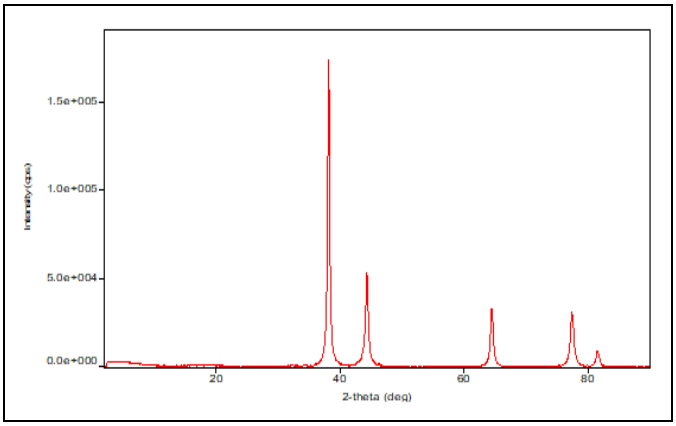

FIG. 9: XRD SPECTRA OF SILVER NANOPARTICLES SYNTHESIZED *VIA* SOL-GEL METHOD

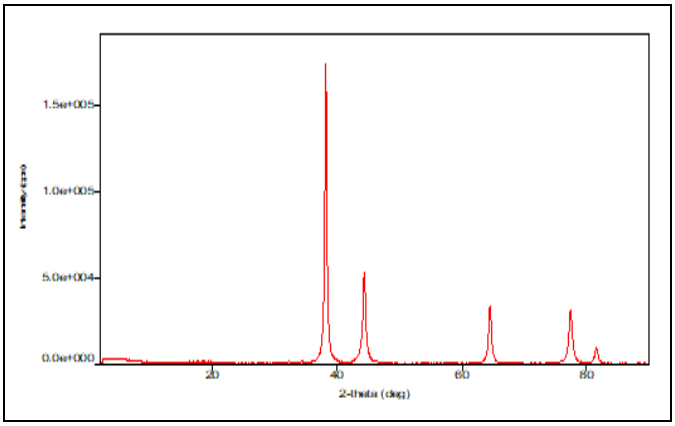

FIG. 10: XRD SPECTRA OF VANCOMYCIN DOPED SILVER NANOPARTICLES

Fluorescence Spectral Analysis of Silver Nanoparticles and Vancomycin Doped Silver Nanoparticles: The emission spectra of silver nanoparticles & Vancomycin doped silver nano-particles have been measured in the liquid state at room temperature. DMSO: Ethanol in ratio 1:2 was used as solvent for obtaining all fluorescence spectra. Fluorescence emission and excitation spectra of synthesized compounds were recorded at room temperature in very dilute solution (10^{-3} to 10^{-5} mol). Synthesized silver nanoparticles showed an intense emission bands at 425 nm upon

photoexcitation at 350 nm & Vancomycin doped silver nanoparticles showed an intense emission band at 495 nm upon photoexcitation at 325 nm. The fluorescence intensity of Silver AgNPs & Drug conjugated silver AgNPs increased drastically due to the excitement of surface Plasmon on the surface of AgNPs and drug conjugated AgNPs, which leads to the occurrence of the phenomena called surface plasma resonance.

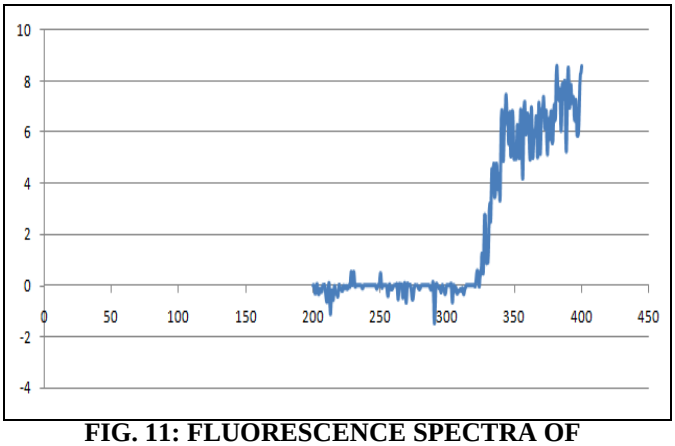

FIG. 11: FLUORESCENCE SPECTRA OF SYNTHESIZED SILVER NANOPARTICLES *VIA* SOL-GEL METHOD

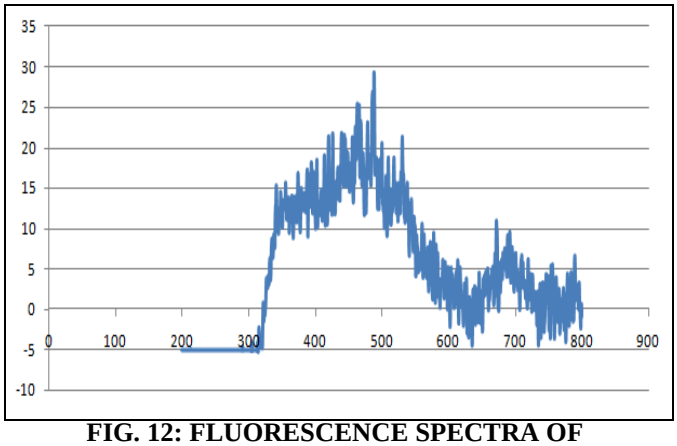

FIG. 12: FLUORESCENCE SPECTRA OF SYNTHESIZED VANCOMYCIN DOPED SILVER NANO PARTICLES

***In-vitro* Antibacterial Activity of Nanoparticles:** Bacteriological examination was performed in sterile nutrients for both liquid systems. The conjugates exhibited antibacterial activity against MDR bacterial strains. The MIC of pure vanco-mycin, silver nanoparticles and vancomycin doped silver nanoparticles for these bacteria was calculated as the lowest concentration at which bacterial growth was inhibited [31]. The synergetic effect is due to the efficient delivery of Vancomycin attached to AgNPs to bacterial cell membrane which is hydrophobic in nature. As Vancomycin is hydrophilic in nature, and AgNPs are hydrophobic. Therefore, AgNPs can easily interact with the cell membrane, and the drug attached to AgNPs delivered to the bacterial cell more efficiently [32]. Subsequently, the surface charge on AgNPs also contributes to the synergy as negatively charged AgNPs disrupt the permeability of the cell membrane, which in turn affects cellular respiration severely [33]. Due to disrupted permeability, the drug can easily damage the bacterial cell and inhibit the cell wall and protein biosynthesis [34-35]. It has also been reported that as compared to bare AgNPs, the nanoconjugates -AgNPs will enhance the release of silver ion, which also attribute to the synergetic effect [36-39].

TABLE 2: ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES, VANCOMYCIN AND VANCOMYCIN DOPED AND THEIR ZONE OF INHIBITION (mm) AGAINST *E. COLI, S. AUREUS* AND *ENTEROCOCCUS FAECALIS*

S. no.	Bacterial Strains	Synthesised silver nanoparticles	Vancomycin drug	Vancomycin doped silver nano particles
1	*Escherichia coli* (MTCC -1563)	0.176µg/ml	≥3.2µg/ml.	0.007 µg/ml ,
2	*Stayphylococcus aureus* (MTCC-31620)	0.187 µg/ml	≥3.2µg/ml.	0.063 µg/ml
3	*Enterococcus faecalis* (MTCC- 439)	0.042 µg/ml	≥3.2µg/ml.	0.029 0 µg/ml

The MIC of pure Vancomycin, AgNPs and Vancomycin doped AgNPs for these bacteria was calculated as the lowest concentration at which bacteria growth was inhibited. The MIC of pure vancomycin for gram-negative strains was ≥3.2 µg/ml. When pure AgNPs were employed, the MIC was 0.176 µg/ml, 0.187 µg/ml & 0.042 µg/ml. Furthermore, when NPs are used as a doping agent in combination with vancomycin, drug, MIC values decreased drastically. In this condition, the MIC were 0.007 µg/ml, 0.063 µg/ml & 0.0290 µg/ml. The MIC corresponded to the MBC in all bacterial species. The results are shown in **Table 2**. The research reported that AgNPs with larger surface areas provide a better contact with microorganisms [40]. Thus, these particles may penetrate the bacterial cell membrane or attach to the bacterial surface and inhibit their replication [41-42.] In our experiment, Ag NPs have

been found to be most effective against *Enterococcus faecalis*.

It has been reported that antibacterial efficiency is increased by lowering the particle size [43]. Usually, NPs attach on the cell wall of bacteria and damage membrane and respiration system leading to cell death [44]. The toxicity of smaller NPs was greater than those of larger ones because the smaller ones can easily adhere to bacterial cell wall [45].

Bio-Mechanism of Silver Nanoparticles: Silver ions penetrate into cytoplasm; denature the ribosome leading to the suppression of enzymes and proteins, which eventually arrest their metabolic function resulting in apoptosis of bacteria. Bactericidal activity is due to silver ions released from AgNPs as a consequence of their interaction with microbes [46]. However, four possible mechanisms of antibacterial activity of AgNPs have been proposed (a) interference during cell wall synthesis (b) suppression during protein biosynthesis (c) disruption of transcription process and (d) disruption of primary metabolic pathways [47]. Each mechanism involves structural changes, biochemical changes and charges on both the silver ions and biomolecules in the microbial cells. Ag NPs also inhibit the proliferation of cancer cell lines by different modes of action. They mediate and amplify the death signal by triggering the activation of the Caspase-3 molecule. The DNA splits into fragments by Caspase-3. AgNPs may interfere with the proper functioning of cellular proteins and induce subsequent changes in cellular chemistry. Sometimes AgNPs alter the function of mitochondria by inhibiting the catalytic activity of lactate dehydrogenase. AgNPs may also cause the proliferation of cancer cells by generating ROS, which ultimately leads to DNA damage.

CONCLUSION: The lack of development and approval of new and effective antibacterial, as well as growing MDR microbes, presents a major challenge in our ability to counter bacterial infections [48]. It offers great promise in the field of biomedicines, especially the diagnosis and drug delivery. It offers opportunities for therapeutic agent delivery to specific cells and receptors. Nanomaterial-based drug delivery systems have the potential to improve pharmacokinetics and pharma-codynamics of the drugs [49].

The small size of nanoparticles provides them a greater surface area for maximum drug loading as well as high accessibility for specific targets.

Recently, various drug-conjugated nanoparticles are being developed against infections caused by resistant microbes [50]. The most common metal carriers for nanoparticle-based drug delivery systems include gold, silver, and iron oxide due to their inertness and biocompatibility. Though the mode of action of silver nanoparticles on the bacteria has been suggested to affect morphological and structural changes in the bacterial cells, the large surface area, provides better uptake by microorganisms [51]. Hence, silver nanoparticles have the ability to anchor to the bacterial cell wall and subsequently penetrate it, thereby causing structural changes leading to increased permeability of the cell membrane and cell death. In addition, the formation of free radicals by the silver nanoparticles has the ability to damage the cell membrane and make it porous, resulting in bacterial cell death [52].

The bacterial membrane contains sulfur-containing proteins, and the AgNPs interact with these proteins in the cell as well as with the phosphorus-containing compounds. When AgNPs enter the bacterial cell, it forms a low molecular weight region in the center of the bacteria to which the bacteria conglomerate, thus protecting the DNA from silver ions. Also, it generates reactive oxygen species, which are produced to attack the respiratory chain, cell division, and finally leading to cell death [53].

Interaction of Vancomycin drug with AgNPs surface was confirmed from the shift in UV-VIS absorption peak and XRD reflection lines. The calculated values of *Z*-average after drug incorporation also infer the successful loading of drugs on AgNPs surface. Also, FTIR studies attributed to the hydrogen bonding between amino group of drug and oxygen atom of Schiff base ligand. Further, it was shown that the Vancomycin doped silver nanoparticles have a profound synergetic antibacterial efficacy against *S. aureus* and *E. coli* test strains, and this synergetic effect was augmented. The results showed that Vancomycin doped silver nanoparticles were more effective against *E. coli*.

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CONFLICTS OF INTEREST: There are no conflicts to declare.

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