IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

DEVELOPMENT OF NANO FORMULATION FOR THE ADMINISTRATION OF ANTIMICROBIAL PEPTIDE

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ABSTRACT:

The current global crisis of antibiotic drug resistance is driving the search for novel treatment approaches. Antimicrobial peptides (AMPs) are small molecular weight proteins with a varying number of amino acids found in both eukaryotes and prokaryotes which have recently been targeted as novel antimicrobial agents with the potential to treat multiple-drug resistant infections. Their conjugation with various classes of materials such as antibiotics, polymers, DNA, salts, and phenolic derivatives, and their delivery via nanocarrier systems are strategies being used to enhance their therapeutic efficacy. The potential of AMPs and their conjugates and encapsulation into nano-delivery systems for improving activity are also identified.

Silver nanoparticle (AgNPs) was synthesized by dissolving silver nitrate as a salt of silver in ethanol, a non-polar solvent system when certain surfactants are also present, and kept for long hours to one day. Due to the oxidation of oxyethylene groups, the yellow color of the silver nanoparticle was confirmed under UV visible spectrophotometry. Then freshly prepared silver nanoparticle solution was transferred into an antimicrobial peptide (VR-18) solution and powdered was dissolved into the suitable solvent system and sonicated for 5-10 min, temperature was maintained from 15-19 degrees centigrade by adding ice cubes. In the meantime, the stearic acid solution was prepared to maintain a similar solvent medium. Next, the AgNPs-VR-18 conjugated solution was transferred to a stearic acid solution and sonicated mildly. Thereafter a beaker containing 50 ml of double distilled water was taken and placed on the magnetic stirrer. Then the conjugated mixture was transferred into the beaker water and set the RMP 1000-1130 for 3-4 hours until the ethanol had evaporated out. Then the sample needs to be separated, which can be done by cooling the centrifuge machine at 4ºc and 9000 RPM speed for 20 minutes and collecting the soup and precipitate separately. Further characterized by FTIR, PXRD, and UV-scanning

Keywords: Silver nanoparticle, antimicrobial peptides, AMP, FTIR, PXRD

INTRODUCTION

In developed countries, 90 percent of documented infections in hospitalized patients are caused by bacteria, which are single-celled ubiquitous microorganisms that lack a nuclear membrane, are metabolically active, and /or divide by binary fission. Bacteria are classified based on their growth responses in the presence and absence of oxygen. Aerobic bacteria grow in the presence of oxygen such as *Bordetella pertussis*. Anaerobic bacteria such as *Clostridia* can grow in the absence of oxygen (1). These can be classified as Gram-positive or Gram-negative based on the characteristics of their cell wall as seen under a microscope after stains have been administered, a procedure called Gram staining. Medically they are a major cause of bacterial infection and disease transmitted to humans through air, water, food, or living vectors then spreads readily throughout the body via the bloodstream and can cause infection of the lungs, abdomen, heart valves, and almost any other site. These cases probably reflect only a small percentage of the actual number of bacterial infections occurring in the general population, and usually represent the most severe cases. Various microorganisms hold medical significance, including bacteria, viruses, fungi, and parasites. Antibiotics are compounds that target bacteria and, thus, are intended to treat and prevent bacterial infections (2). Antibiotics include destroying the bacterial cell by either preventing cell reproduction or changing a necessary cellular function or process within the cell. Antimicrobial agents are classically grouped into 2 main categories based on their in vitro effect on bacteria: bactericidal antibiotics "kill" bacteria and bacteriostatic antibiotics "prevent the growth" of bacteria. To accurately define each category, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) must be understood. The lowest concentration that inhibits visible bacterial growth at 24 hours is the MIC. The MBC is the concentration of an antibiotic that reduces bacterial density by 1000-fold at 24 hours.

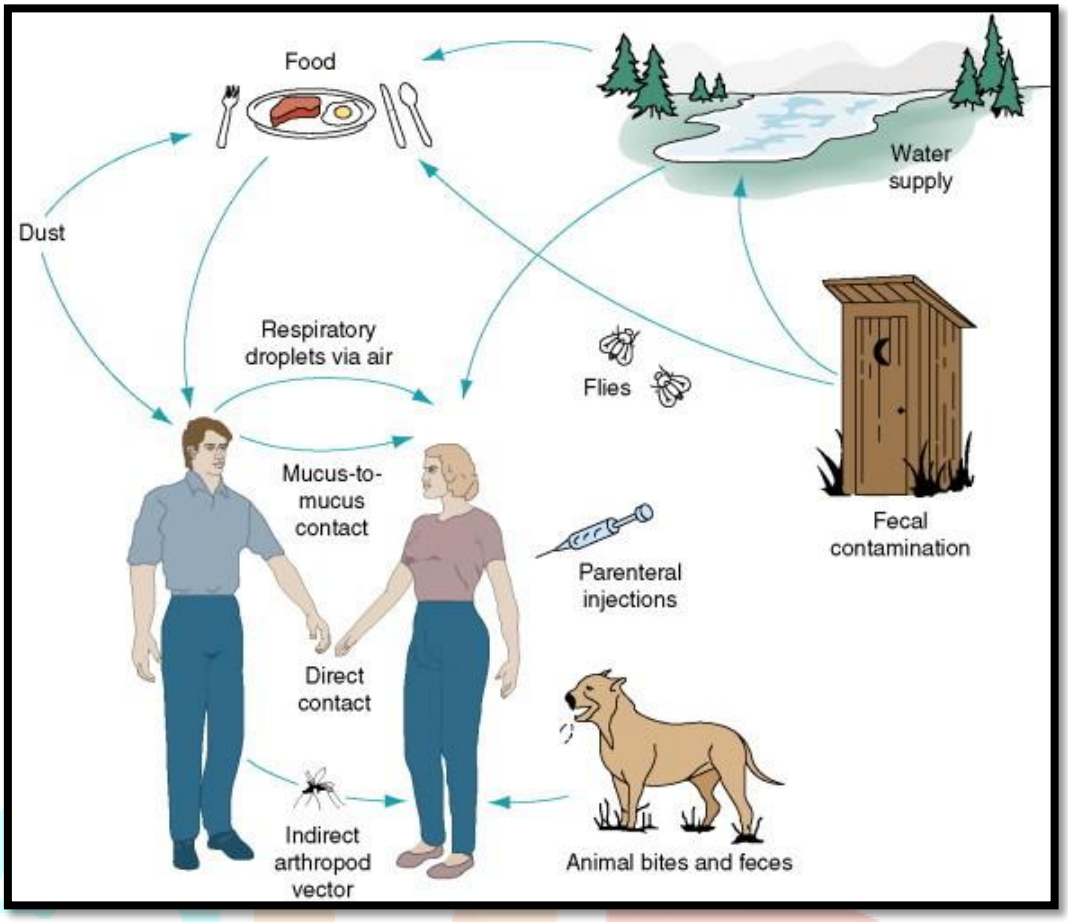


Fig: 1 Source of bacteria in an environment which spread due to the contamination from environment to eucaryotic and as a result world suffers life-threatening infections leads to severe disease conditions

The current global crisis of antibiotic drug resistance is driving the search for novel treatment approaches antimicrobial peptides (AMPs), which can be considered natural antibiotics produced by animals, plants, protozoa, fungi, and bacteria contain 5–50 amino acid chains and are generally composed of L-amino acids defined in secondary structures formed by α-helices, β-sheets or both (2,3). The first reported AMP of animal origin is phagocytic, which was isolated from rabbit leukocytes in 1956. In the following years, other AMPs such as bombing (1962) from the orange speckled frog Bombina variegate, and lactoferrin from cow milk was reported. Contemporarily, other AMPs were identified in the lysosomes of human leukocytes AMPs emerge as a new antimicrobial therapeutic, and resistance to available antibiotics has become a worldwide problem. Thus, as favorable possibilities, the use of AMPs has been studied not only against susceptible pathogens but also against persistent bacteria and fungi. In addition to their direct (although sometimes weak) antimicrobial activities, AMPs have additional antimicrobial effects as they can suppress biofilm formation and induce the dissolution of existing biofilms, chemotactically attract phagocytes and mediate non-opsonic phagocytosis. The main interesting property of AMPs is their ability to selectively disrupt bacterial membranes without affecting mammalian cells, thus being safe Some natural AMPs, like porcine protegrin, exhibit strong antimicrobial effects but in general, the activity of AMPs is improved by their increased concentrations in phagocyte granules, the crypts of the intestine and near degranulating phagocytes. Notably, the development

of resistance against AMPs has occurred to a much lesser degree as they usually work by attacking multiple hydrophobic and/or polyanionic targets. Thus, it is difficult to obtain mutants that are resistant to AMPs, and training methods, for example, multiple passages with half the MIC of AMPs are usually required for the development of any resistance.

Antimicrobial peptides (AMPs), a crucial component of the innate immune system, are tiny molecules that may exhibit antibacterial, antifungal, antiparasitic, and antiviral activity According to the size, shape, and makeup of the amino acids, these molecules typically contain 10 to 50 amino acid residues and are organized into several groups Cationic peptides, which are classified into three classes and comprise the biggest group.

AIM AND OBJECTIVE

1.1 Synthesis of blank nanoparticle

1.2 Synthesis of silver nanoparticles (AgNPs)

1.3 Conjugation with Anti-microbial peptide (AMP)

1.4 Encapsulate the (AgNPs- AMP) conjugation within the lipid nanoparticle.

2. PHYSICOCHEMICAL CHARACTERIZATION

2.1 Characterization of blank nanoparticles

2.1.1 Particle Size Distribution by Dynamic Light Scattering (DLS)

2.1.2 Spectroscopy studies

2.1.3 Determination of surface charge (zeta potential)

2.1.4 Particle size determination by Transmission electron microscope (TEM)

2.2 characterization of silver nanoparticles

2.2.1 Particle Size Distribution by Dynamic Light Scattering (DLS)

2.1.2 Spectroscopy studies

2.1.3 Determination of surface charge (zeta potential)

2.1.4 Particle size determination by Transmission electron microscope (TEM)

2.3 Conjugation with Anti-microbial peptide (AMP)

2.3.1 Particle Size Distribution by Dynamic Light Scattering (DLS)

2.3.2 Spectroscopy studies

2.3.3 Determination of surface charge (zeta potential)

2.3.4 Particle size determination by Transmission electron microscope (TEM)

2.4. Encapsulate the (AgNPs- AMP) conjugation within the lipid nanoparticle.

2.4.1 Particle size distribution by Dynamic Light Scattering (DLS)

2.4.2 Spectroscopy studies

2.4.3 Determination of surface charge (zeta potential)

2.4.4 Particle size determination by Transmission electron microscope (TEM)

3. ANTIMICROBIAL METHODS (IN-VIVO)

3.1 Membrane disruption activity

3.2 Scanning electron microscopy (SEM)

3.3 Cytotoxicity assay

3.4 Fluorescence-activated cell sorting (FACS)

3.5. Zone of inhibition

RATIONALITY OF WORK

1. Antibiotic/antimicrobials have several side effects such as nausea, hypersensitivity gastrointestinal, intolerance and the development of bacterial resistance, headaches, abdominalpain, and many more. To avoid these side effects nanoparticles can be more suitable due to theirsmall and controllable size, large surface area to mass ratio, and functionalized structure.

2. The antibiotics develop antibiotic resistance in pathogenic organisms so to avoid these problems conjugated nanoparticles are used to improve the activity against multidrug-resistant bacteria dueto their small size, large surface area to mass ratio, and structure.

3. Antimicrobial Peptides have been found to have antibacterial activity against a broad spectrumof bacteria even antibiotic-resistant bacteria as compared with conventional antibiotics.

4. Antimicrobial peptides are easily available as they had been obtained from prokaryotic to eucaryotic and as a naturally obtaining possess few limitations.

5. Silver Nanoparticle has multiple uses in the field of nanotechnology and acts as metallic drug- carrier with antimicrobial activities.

6. The rationality of our work is to stabilize the AgNPs-AMP conjugated product to protect it from proteolytic enzyme degradation.

7. Stearic acid is a biodegradable, non-toxic lipid that has been chosen to encapsulate the AgNPs-AMP conjugated product.

Materials

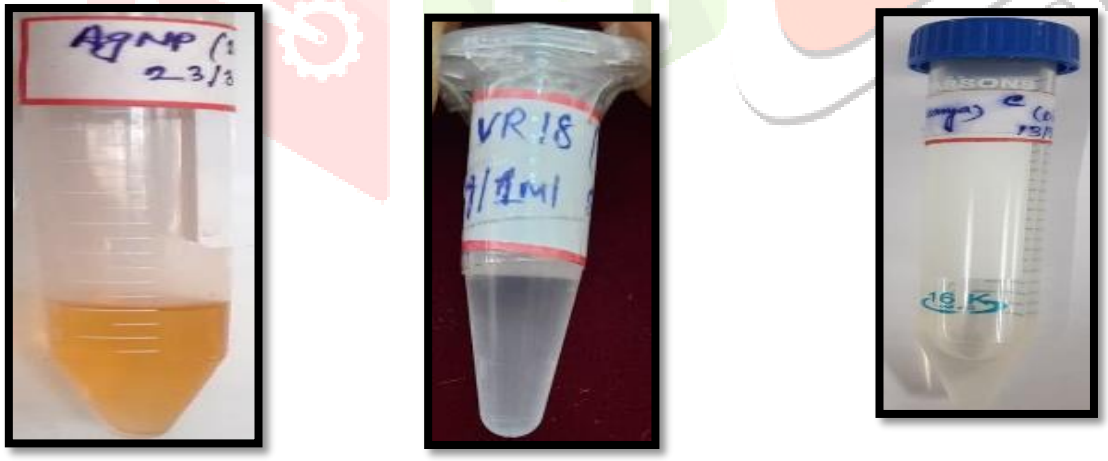
Silver Nitrate
Stearic acid
Polysorbate 20
VR18
Hot air oven
Cyclo mixer
Magnetic stirrer
Sonicator
UV-vis Spectroscopy

Methodology

Silver nanoparticles (AgNPs): is prepared by dissolving silver salt (silver nitrate) in a non-polar solvent (ethanol) system in the presence of a non-ionic surfactant (Tween-20).

Anti-microbial peptide (AMP) (VR18): is weighed (1mg) dissolved into ethanol (1ml) and marked a stock solution (1mg/ml) in an Eppendorf tube.

Stearic acid (Lipid) solution: is prepared by adding stearic acid into ethanol and tween-20 as a surfactant and dissolving it properly (cyclomixer).



a b c

Fig:5 Development of methods. (a) Silver nanoparticle, (b) Antimicrobial peptide solution, (c) Stearic acid solution

Method in detail

Silver nanoparticle (AgNPs) was synthesized by dissolving silver nitrate as a salt of silver in ethanol, a non-polar solvent system when certain surfactants are also present and kept for long hours to one day. Due to the oxidation of oxyethylene groups, the yellow color of the silver nanoparticle was confirmed under UV visible spectrophotometry. Then freshly prepared silver nanoparticle solution was transferred into an antimicrobial peptide (VR-18) solution and powdered was dissolved into the suitable solvent system and sonicated for 5-10 min, temperature was maintained from 15-19 degrees centigrade by adding ice cubes. In the meantime, the stearic acid solution was prepared to maintain a similar solvent medium. Next, the AgNPs-VR-18 conjugated solution was transferred to stearic acid solution and sonicated mildly. Thereafter a beaker containing 50 ml of double distilled water was taken and placed on the magnetic stirrer. Then the conjugated mixture was transferred into the beaker water and set the RMP 1000-1130 for 3-4 hours until ethanol has evaporated out.

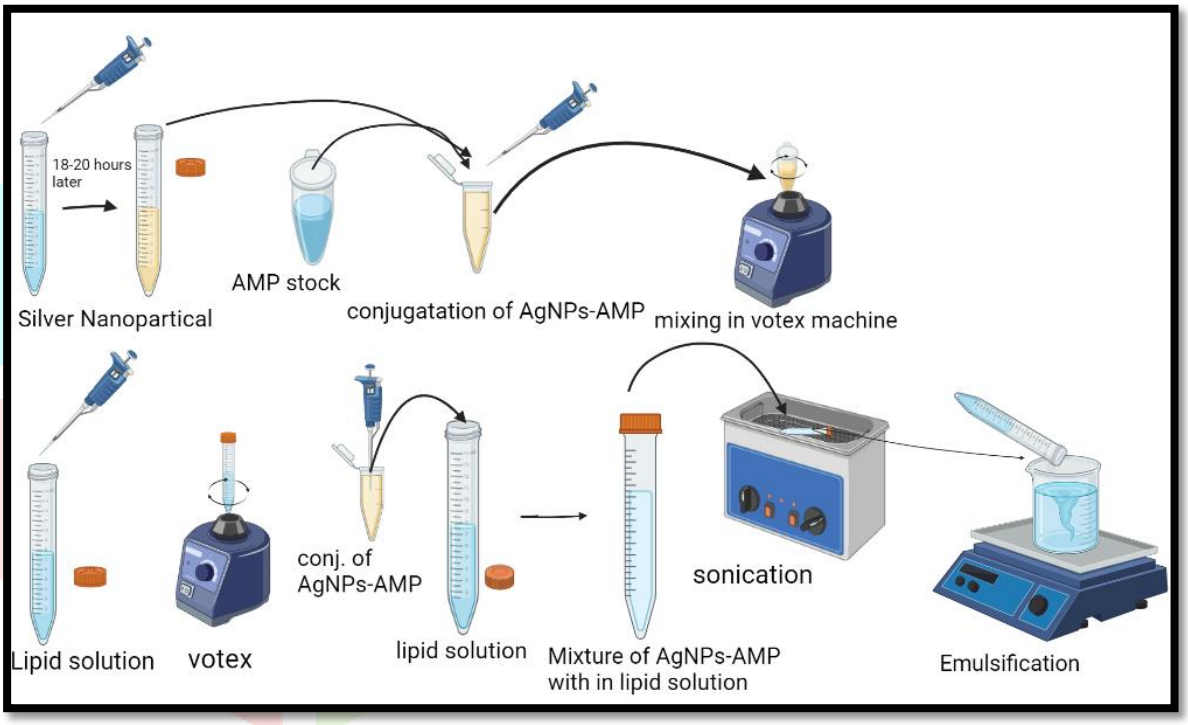


Fig:6 Graphical representation of method of synthesis. (a) Synthesis of silver nanoparticle after 18 hours the dark yellow color change confirms the formation. (b) Preparation of antimicrobial peptide solution. (c) synthesis of Stearic acid solution. (d) silver nanoparticle was mixed with vr18 solution by taking small amount of their stock. (e) transferred the mixture into stearic acid solution.

Sample separation by Centrifugation

Centrifugation is the process of separating two or more liquids in a mixture by rotation in a container so that the lighter density liquid rises to the top. It is occasionally necessary to centrifuge samples in refrigerated conditions to ensure sample integrity.

After preparation the sample need to be separated, which can be done by cooling the centrifuge machine at 4ºc and 9000 RPM speed for 20 minutes and collecting the soup and precipitate separately.

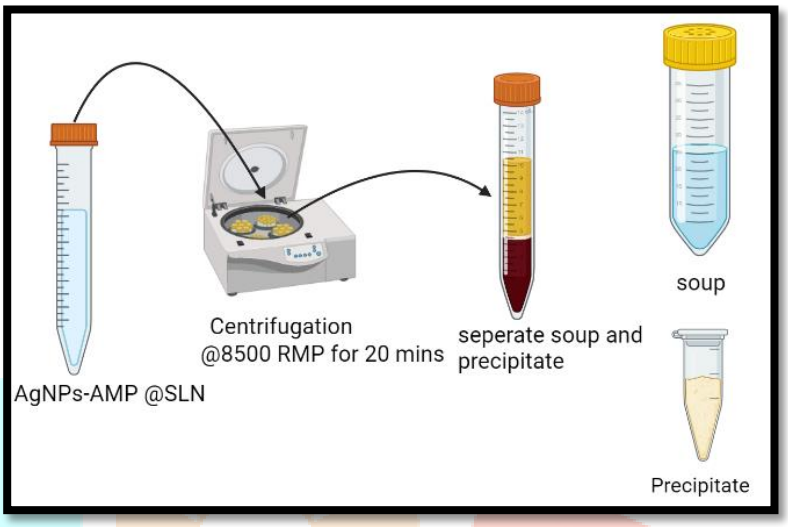


Fig:7 Separation of Silver NPs-AMP conjugated lipid encapsulated sample.

Sample Lyophili

Add calculated (5 times higher) amount of D-sorbitol in 2ml of filtered water and dissolved it and volume make up to 5 ml by adding filtered water. This sucrose solution was then transferred to the liquid precipitate which was collected after separation by a cooling centrifuge machine. Then store the mixture at 4ºc and after freeze drying the liquid sample become solid and stability gets increase.

Fig: 8 Sample lyophilization. (a) Precipitate was collected after centrifugation. (b) D- sucrose solution was transferred into the precipitate and volume gets up. (c) after lyophilization the liquid sample turns in to powdered form which further stored at 4°c

Characterization of Silver nanoparticles and stearic acid (16, 17)

Particle size distribution study by Dynamic Light Scattering (DLS) technology

50µl of the sample was dissolved in 1ml Double distilled water. The diluted sample was placed and evaluated by the Dynamic Light Scattering (DLS) technique usingMalvern Zetasizer NanoS (1000S, Malvern Instruments, UK)

Ultraviolet spectroscopic analysis

UV-VIS spectral analysis was recorded by Jasco-V670 manufactured by JASCO, USA.Properly diluted samples were scanned within the range of 200–800 nm. Ethanol was taken as Blank. The data were analyzed. After scanning the sample at the range between 200 nm-800 nm there were several peaks were recorded. To solve this problem, the deconvolution technique was used and with the help of this technique, the correct peak was recorded at 437nm for silver nanoparticles and 258nm for antimicrobial peptides (VR18)

Determination of surface crystallinity

Powder X-ray diffraction (PXRD) measures the diffraction pattern of crystalline material PXRD can be used as a qualitative and sometimes quantitative assessment of the degree of crystallinity. The disorder leads to peak broadening in the powder pattern, and eventually an amorphous “halo.” This also means that conversion to amorphous phases in APS studies is not detectable by PXRD. Only a powdered sample is needed (14).

Fourier-Transform Infrared Spectroscopy

The Fourier-transform infrared spectroscopy analysis of every raw material, silver nitrate, and stearic acid wasdone with FTIR Spectrophotometer (Perkin Elmer, 1000 Spectrum). Dried Conjugated samples were converted into fine powders before the preparation of the palette with potassium bromide. The FT-IRspectra were analyzed with Spectrography Version 1.2 Software.

Particle Size determination study

Particle size determination can be done under high resonance Transmission electron microscopy. For the TEM study, silver NPs-AMP conjugation did not separate. A freshly prepared sample was loaded on a glass cover and placed under a Transmission electron microscope (TEM) using carbon-coated copper grid(#300 mesh) and got an average size of 20 nm.

6.2.3 Antimicrobial assay

The antibacterial properties of the synthesized silver nanoparticle conjugated Antimicrobial peptides were evaluated by a qualitative method against the microorganisms. Gram-positive and Gram-negative bacteria were cultured on an agar medium. For Gram-positive bacteria
S. aureus and for gram-negative bacteria, E. coli was taken. D mannitol and EMB agar media was prepared for S. aureus and E. coli respectively (18,16)

6.2.3.1. Preparation of Mannitol Salt Agar media

11.18 gm of mannitol salt agar media was suspended in 100 ml of distilled water. Then it was Boiled to dissolve the media completely. After boiling the media was Autoclaved at 15lbs pressure (121°C) for 15-20 minutes. After that, the media was Cooled to 45-50°C and poured into Petri dishes in aseptic condition.

6.2.5.1 Preparation of EMB agar media

3.596 grams in 100 ml distilled water was suspended. It was mixed until the suspension is uniform.After that, it was boiled to dissolve the medium completely. Then it was sterilized byautoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and shake the medium poured into Petri dishes in aseptic condition.

6.2.5.2 Preparation

The D mannitol and EMB agar media were prepared and sterilized by autoclaving under aseptic conditions for S. aureus and E. coli respectively for fully free from contamination. Then it waspoured onto disposable sterilized Petri dishes and allowed to solidify to form a thick gel. The surfaces of the solidified agar plates were allowed to dry in the incubator before streaking microorganisms onto the surface of the agar plates. 100 µL of the microbial culture suspensionin broth was streaked over the dried surface of the agar plate and spread uniformly using a sterilized glass rod and allowed to dry before applying the loaded disks. Three cups of bores were dug out in each Petri plate containing agar media. It was dug out at an equal distance from each other. The Silver NPs-AMP conjugation encapsulated sample was allowed. The Silver NPs-AMP within lipid encapsulated compound were suspended on the surface of the seeded agar plates using sterile forceps. The experiment was carried out in triplicate and the diameters of the zones of inhibition were measured after 24 hours of incubation at 37°C**.** The zone of inhibition was measured using a scale (19,20).

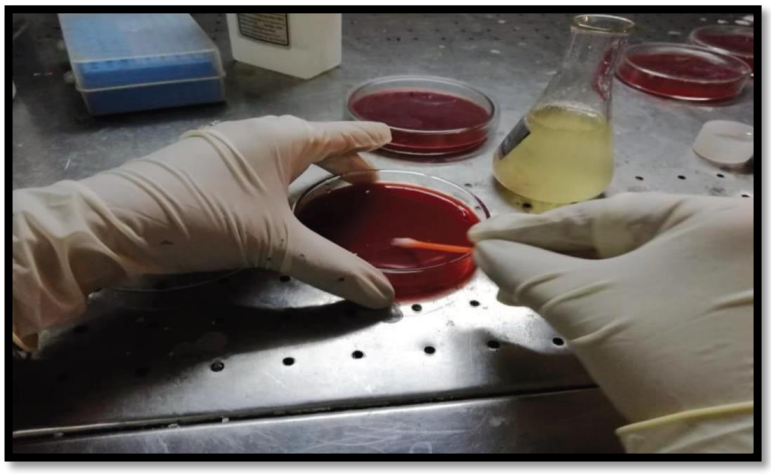


Figure 9: Streaking of *S. aureus* on the surface of the agar plate

RESULTS

Particle size distribution studies of the silver nanoparticle-AMP conjugated stearic acid encapsulated sample.

The particle size distribution of silver NPs-AMP conjugated lipid encapsulated nanoformulation was assessed by DLS analysis shown in DLS size distribution analysis.20µl of each sample were taken and add filtered water up to 2 ml so, dilution of 100 times was checked and was found to be 292nm for stearic acid (blank) nanoparticle and 199nm for Silver NPs-AMP conjugated.

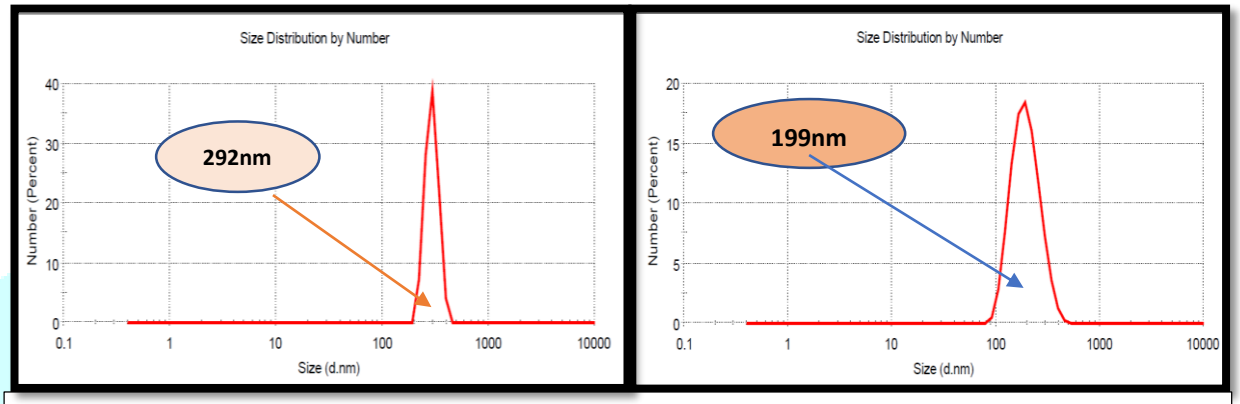


Fig: 10 Results of particle size distribution by Dynamic light scattering (DLS) method. (a) Stearic acid containg blank nanoparticle was diluted 100 times and was found to be 292nm. (b) Silver NPs-AMP conjugated compound was diluted 100 times and was found to be 199nm.

7.2 UV-VIS Spectroscopy studies (21, 13).

For the optical property analysis of the silver nanoparticle conjugated antimicrobial peptide,UV-visible spectral studies were carried out. The color silver nanoparticle in the solution gradually changed from pale yellow toreddish brown color. Similarly, AMP solution was prepared, diluted, and checked at 200-500 nm and was found to peak at 258nm. In the case of silver NPs-AMP conjugation within lipid (stearic acid) solution we got the pick for peptide as well as for silver nanoparticle also and the baseline was checked by using a common solvent system ethanol

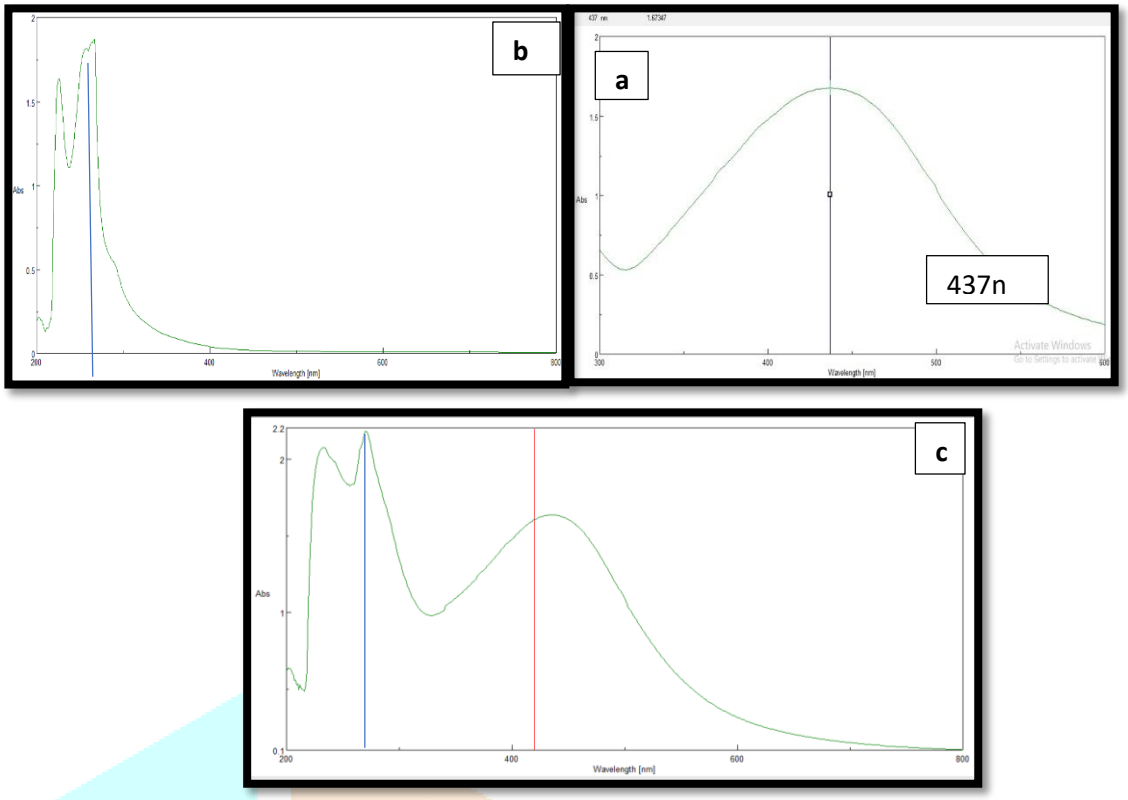


Figure 11: Results of UV-vis spectrophotometry study(a) Shows the release of Silver nanoparticle and found at 437nm.(b) For Antimicrobial peptide vr18 was found to ba at 258nm and (c) for silver NPs-AMP conjugated sample was found to be peak at 268nm and 430 nm

7.3. Fourier Transform Infrared Spectroscopic Studies.

The FTIR spectrum of below. In the caseof silver, NPS-AMP conjugated nanoparticles, we got the characteristic peaks of 2917 cm−1, 1704 cm−1, 1464 cm−1, 1297 cm−1, 935 cm−1, and 720 cm−1. 2917 cm−1 is for carboxylic acid. 1704 cm−1 is for amines, 1464 cm−1 is for alkanes, and 1297 cm−1 is for amines.

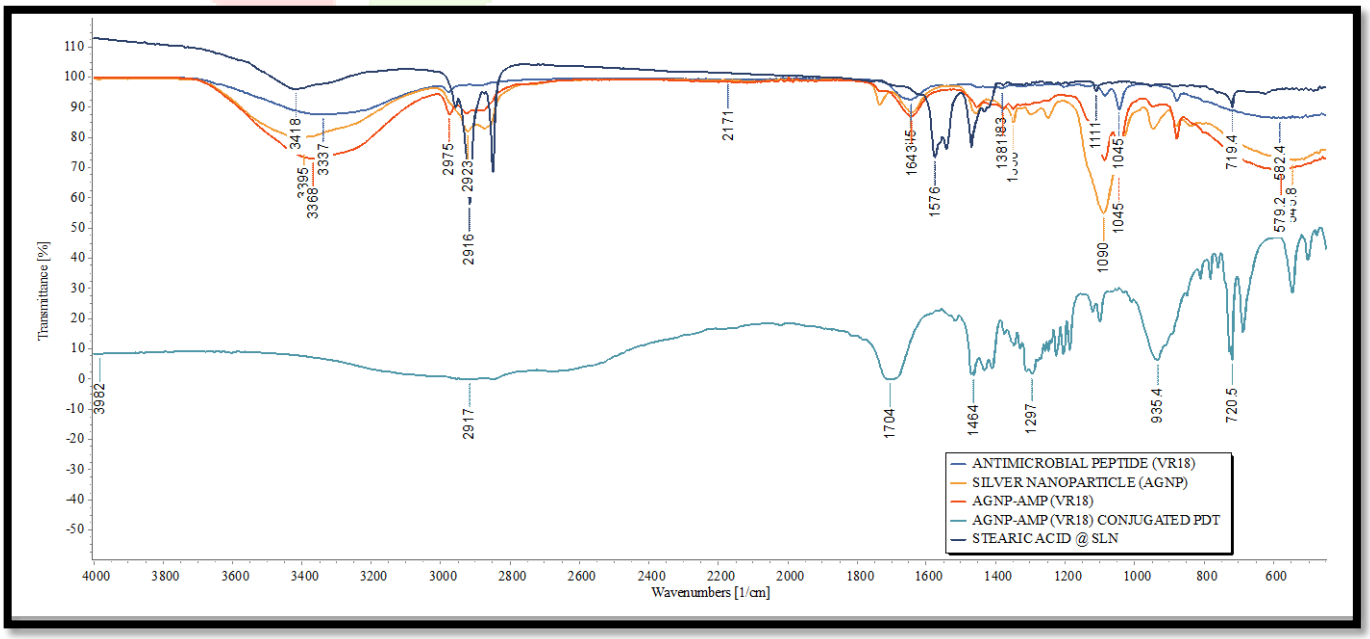


Fig: 12 Results of FTIR study. Five different samples were ploted i) Antimicrobial peptide, ii) Silver nanoparticle (AgNPs), iii) silver NPs-AMP conjugation, iv) AgNPs-AMP conjugated within stearic acid encapsulation and v)Stearic acid as blank nanoparticle.Each sample shows peaks which indicates which chemical group is present.

7.4 Particle size determination

The Particle size determination was further continued by the TEM. At higher magnification, the size range of silver NPs-AMP conjugated samples can be easily determined. The size of the silver nanoparticle-AMP conjugated sample is average 20 n

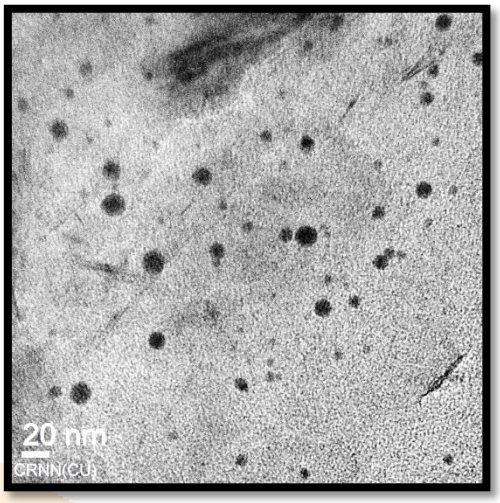


Fig:13 Result of Transmission electron microscopy image (TEM) and the silver nanoparticle-AMP conjugated sample is average 20 nm in size

7.4. Antimicrobial activity studies

An in-vitro antimicrobial study was performed as mentioned in the methodology on *S. aureus* organisms. With silver NPs-AMP conjugated sample of 2.5 $\times 10^{15}$ mol was checkedfor antimicrobial efficacy against gram-positive *S. aureus.* The zoneof inhibition was observed against *S. aureus* Fig. 7. The zones of inhibition of prepared formulations were 2.13 cm for *S.* The study indicates that the prepared silver NPs-AMP conjugated lipid encapsulated nanoparticles retained their antibacterial activity. Even at the lowest dose studied, the nanoparticle was equally effective to inhibit the growth ofGram +ve bacteria. There was a dose-dependent increase in the diameter of the zone of inhibition. With the increase in the concentration of nanoparticles the zone of inhibition increased. The zone of inhibition for both gram-positive bacteria is shown below.

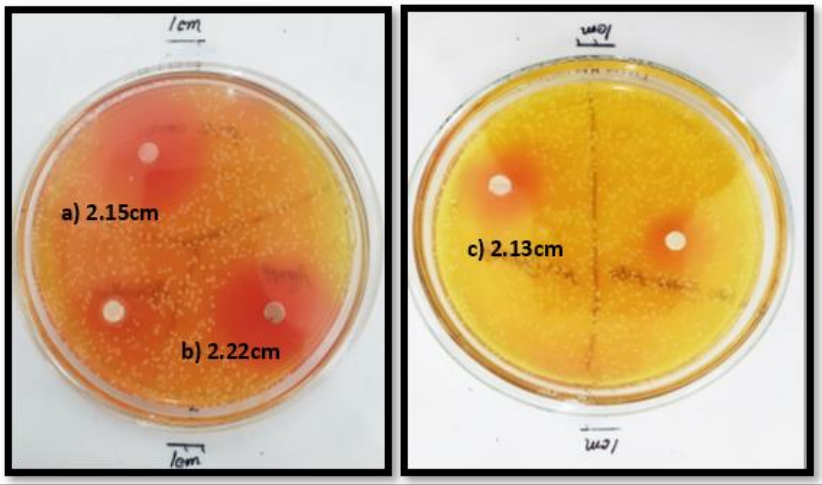


Fig: Result of antimicrobial activity study and zone of inhibition was mentioned as compare to dose calculation (a) Anti-microbial assay of ciprofloxacin as positive control against *s. aureus* Zone of Inhibition is 2.15cm, (B):Silver NPs $6*10^{11}$ no./l and zone of inhibition is 2.22cm, (C): silver NPs-AMP conjugated lipid encapsulated compound where no of AMP was $2.497*10^{-15}$ mol and zone of inhibition was 2.13 cm.

DISCUSSION

To create silver nanoparticles (AgNPs), silver nitrate, a salt of silver, was dissolved in ethanol, a non-polar solvent solution, for several hours up to a day. The silver nanoparticle's yellow hue was confirmed by UV visible spectrophotometry as a result of the oxidation of oxyethylene groups. The antimicrobial peptide (VR-18) solution was then added to the freshly prepared silver nanoparticle solution, which was then transferred. The powdered substance was then dissolved in the appropriate solvent system and sonicated by adding ice cubes. To maintain a comparable solvent medium in the interim, the stearic acid solution was made. The AgNPs-VR-18 conjugated solution was then added to the stearic acid solution and briefly sonicated.

After preparation of the sample separate it and lyophilized it to get a stable powdered sample and carried out characterization by using the lyophilized sample and get the positive results which were also mentioned in previous pages.

Then we plan antimicrobial experiments and get the zone of inhibition also mentioned.

The goal of this study was to develop a silver nanoparticle-AMP conjugated product as a problem was related to degradation followed by enzymatic activities so, lipid mainly stearic acid has been used here to encapsulate. Then lipid encapsulated conjugated nanoformulation becomes stable, free form degradation, and controlled in its release pattern. : Result of the Transmission electron microscopy image (TEM) and the silver nanoparticle-AMP conjugated sample is an average of 20 nm in size.

Under UV-spectrophotometry the silver nanoparticle gets a peak at 437 nm and the Antimicrobial peptide gets at 250nm and the developed conjugated sample gets 2 peaks at 268nm and 430nm so, the data represents positively that the Silver NPs-AMP conjugation within lipid has been done.

In the FTIR study, nanoparticle and polymer compatibility was observed. No new peak could be seen

on the spectrograph which indicated that there was no interaction occurred between them which suggests that encapsulation has been done successfully.

The result of the antimicrobial activity study and zone of inhibition was mentioned as compared to the dose calculation done. Anti-microbial assay of ciprofloxacin as a positive control against *s. aureus* Zone of Inhibitionis 2.15cm. Silver NPs $6*10^{11}$ no./l and zone of inhibition is 2.22cm, (C): silver NPs-AMP conjugated lipid encapsulated compound was no of AMP was $2.497*10^{-15}$ mol and zone of inhibition was 2.13 cm.

CONCLUSION

Silver nitrate was dissolved to create silver nanoparticles (AgNPs). The ongoing hunt for new strategies to address antimicrobial medication resistance is a result of the constant evolution of dangerous bacteria. Infection control and prevention are seriously threatened by the emergence of resistance to novel antibiotic derivatives and the inability of antibiotics to reach infection sites at an effective concentration. New methods, such as the development and use of AMPs singly or in conjugates, as well as their delivery via nano-carriers, however, present interesting alternatives to conventional antibiotics. Model membranes and QSAR in the molecular dynamics of AMPs. The final creation of novel AMPs, including their conjugates and nano delivery systems, depends on methods to discover or comprehend descriptors in AMPS for activity.

Up to now, AMPs have been successfully encapsulated into lipid with improved activity and sustained release. Only the AMPs in the helical group have so far been investigated for distribution using nanocarriers. Again, the characterization of these trials is restricted, which is crucial to guarantee safety, quality, and efficacy for regulatory approval. Future research should concentrate on experimental layouts for formula optimization. Determining the solid phase transformation properties, release kinetics, physicochemical stability, cell uptake mechanisms, and in vivo efficacy and toxicity testing in animal models should all be the focus of detailed characterization studies.

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