

Green Synthesis and Characterization of Gelatin-PVA Silver Nanocomposite Films for Improved Antimicrobial Activity

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ABSTRACT

The present study deals with the in-situ production of gelatin-poly(vinyl alcohol)-silver nanocomposite films in view of their growing applications as antimicrobial packaging/container, wound dressing and antibacterial materials. Silver in the form of silver nanoparticles has made an amazing comeback as a prospective antimicrobial agent. The use of silver nanoparticles is also significant, as several pathogenic bacteria have developed resistance against various antibiotics. A unique, nontoxic, simple, lucrative and ecofriendly technique was used to synthesize green silver nanoparticles (AgNPs). The AgNPs were synthesized using *Lupulus amarus* extract as a reducing agent for silver nitrate salt (AgNO₃). The particle size distribution of AgNPs was examined by Dynamic Light Scattering (DLS) and the concentration was examined by UV-VIS Spectrophotometer. The stable dispersion of silver nanoparticles was added slowly in gelatin-PVA solution and was crosslinked using glutaraldehyde (cross-linker). The Gelatin-PVA Silver nanocomposite solution was casted in a petri dish and dried to form a film. The nanoparticles encapsulated within polymer chains were characterized by X-ray diffraction (XRD) and Scanning Electron Microscopy (SEM). The green AgNPs nanocomposite film exhibited significant antimicrobial activity against both Gram-negative bacteria, and Gram-positive bacteria. Therefore, the present study clearly provides an approach to develop novel antimicrobial films which are possibly useful in preventing/treating infections and can be used as antibacterial container or in food packaging.

Key words: Nanocomposites, Crosslinking, Polyvinyl alcohol, Nanoparticles

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1. INTRODUCTION

The nanomaterials can be produced by diverse approaches which include chemical, physical, and biological methods. The advance of new chemical or physical methods has resulted in environmental adulteration since the chemical trials involved in the production of nanomaterials produce a large quantity of dangerous and hazardous byproducts [1]. Thus, there is a necessity for “green nanotechnology” that embraces an uncontaminated, safe, environment-friendly, and non-toxic method of nanoparticle production, without the use of high pressure, energy, temperature, and toxic chemicals [2]. The biological approaches comprise production of nanomaterials from the extracts of plant, bacterial, fungal species [3].

Mankind is often infected by micro-organisms such as bacteria, virus, yeast, mold, etc [4]. Silver and silver ion based resources are extensively recognized for their bactericidal and fungicidal activity. Their antimicrobial outcome is owing to obstruction of respiratory enzyme pathways, modification of microbial DNA and the cell wall [5]. Thus, silver and silver ion containing materials are being constantly in use in the field of medical biotechnology [6]. Current studies indicate that silver nanoparticles are very effective as an antimicrobial agent compared to bulk silver or silver ions [7]. The therapeutic effectiveness of silver nanoparticles is numerous folds greater than conventional silver



compounds. The nanoparticles employ their antimicrobial property by interacting with the Sulphur comprising proteins existing in the bacterial cell membrane in addition to with the phosphorous containing DNA [7]. Other than that, silver nanoparticles based antimicrobials have added advantages due to their thermal firmness, and Eco friendliness [8]. Hence, the usage of silver based marketable products including topical ointments, bandages, augmentation devices; tissue scaffolds, antimicrobial filters, and gels have amplified for improving public health care [9, 10]. Some commercially existing silver-containing purification arrangements such as Aqua pure, Kinetico and QSI-Nano have shown to eliminate 99.99% pathogens present initially after purification.

Silver nanoparticles (AgNPs) forms composites with polymers such as polyvinyl alcohol, polypyrrole, polyvinylidene fluoride, chitosan, and cellulose as reported in number of research reports [10, 11]. The development of polymer-silver nanocomposites desires that the size of nanoparticles in the polymer matrix be controllable and that their dispersal within the polymer matrix is even [11]. Many prior attempts to form polymer-silver nanocomposites have involved mixing of a nanoparticle solution into the polymerization mixture. These polymer-silver nano composites can have a wide range of application in biomedical fields, such as surgical gloves, antibacterial clothes, sheets and towels, and anti-infectious urinary catheters [12]. They also can be integrated into antiseptic coverings for plastic surgery, shocking wounds, leg sores, skin grafts, cuts, and abrasions. Further, they can be used in numerous household applications such as textiles disinfection in water treatment, food storage containers, home appliances and in medical devices [13].

The idea of the present study was a green synthesis of AgNPs by chemical reduction of silver nitrate using *Lupulus amarus* extract as a reducing agent and the preparation of AgNPs/Gelatin-PVA nanocomposite film and to study the antimicrobial potential of a silver-Gelatin-PVA nanocomposite system. To the best of our knowledge, this is the first study describing the preparation of silver nanoparticles using *Lupulus amarus* extract and their composite with Gelatin-PVA along with a cross linker.

2. MATERIALS AND METHODS

2.1 Materials

Silver Nitrate (Sigma), PVA with a molecular weight of 115000 kDa, gelatin (purified) and 25 % Glutaraldehyde Solution was purchased from Merck, *Lupulus amarus* pallets, Ethanol, D.D Water.

2.2 Green Synthesis of Silver Nanoparticle

2.2.1 Extract Preparation

A 100 mg portion of *Lupulus amarus* Pallets where crushed into fine powder by a Mortar Pestel and was then added to 100 mL (40% Ethanol) solution and was stirred continuously for 1 h in a Magnetic stirrer and was kept at 4 °C for 24 h for maceration. The Mixture is then concentrated using a Rotary Evaporator and finally the concentrated sample was filtered using a Buchner funnel to obtain the final extracted solution (Hops extract).

2.2.2 Synthesis of Silver Nanoparticles

18mL of 0.1mM Solution of silver nitrate was added slowly to 2mL of plant extract with continuous stirring at room temperature for 6 h until the color changes to reddish/brown.

2.3 AgNP–Polymer Film Synthesis

18mL of 0.1mM AgNO₃ solution was added to 2mL of Hops Extract and was mixed thoroughly for 6h until silver Nano particles are formed. The formation of silver nanoparticle was confirmed from the change of the color of the solution from pale yellow to dark reddish brown. From the solution 10mL solution is taken and mixed thoroughly with 5mL of 5 % Gelatin solution and 10 mL of 5% PVA solution and the solution was stirred in a Magnetic Stirring Plate (REMI) at a temp of 40-60 °C for 30min. To the homogenized solution, 1mL of 2 % Glutaraldehyde was poured dropwise and the

solution was again stirred for another 30mins at a speed of 650 rpm. The final solution was then solvent casted at 40°C for 24 h to obtain the desired membrane. A Gelatin-PVA film was also prepared in similar manner without the nanoparticle.

2.4 Characterization

The Synthesized nanoparticle was characterized using DLS –Zetasizer and using a UV-VIS (carry-60) Spectroscopy to confirm the production of silver nanoparticle. The nanoparticles were scanned from 300 to 800 nm in the UV-VIS spectroscopy.

Morphology of the films and nanoparticles are observed by SEM (Inspect F50 SEM). To image the film samples (surface or cross-sections) were coated with a thin layer of palladium gold alloy after mounting on a double sided carbon tape.

The FTIR spectra of the Nanocomposite films are recorded on Bruker Alpha-Eco ATR. To record the FTIR spectra of films, the samples were completely dried in an oven at 60 °C for 12 h. These samples were read between 500 and 4000 cm⁻¹ using ATR mode.

Thermal studies of the films were carried out using Perkin Elmer TGA 4000 System, 100-240V/50-60Hz at a heating rate of 10°C/min and the temperature range was 40-450°C

The wetting property was analyzed by measuring the contact angle of nanocomposite film with respect to Glycerin.

The Shore D hardness of the nanocomposite material was measured using a SHORE-D meter.

2.5 Hemocompatibility Test

Estimation of Hemocompatibility of the Composites is performed through Hemolysis Studies using fresh human blood, collected in a EDTA tube was diluted with normal saline solution (2 mL blood + 2.5mL normal saline). A standard sample without sharp edges was kept in a centrifuge tube containing 10 mL of normal saline and was kept in an incubator at 37 °C for 30 min. To this was added 0.2 mL of the diluted blood which was then mixed gently and incubated for 60 min. For the positive control, 0.2 mL of diluted blood was taken in 10 mL of 0.1% sodium carbonate solution and for negative control; 0.2 mL of diluted blood was taken in 10 mL of normal saline solution and incubated for 60 min at 37 °C. In a similar way, sample material was incubated for 60 min at 37 °C. After 60 min of incubation, all the test tubes were centrifuged for 5 min at 4000 rpm and the supernatant was carefully removed and transferred to the cuvette for readings at 545 nm wavelength and percentage hemolysis was calculated [14]. Percentage hemolysis is calculated based on average of three replicates.

$$\% \text{Hemolysis} = \frac{\text{OD}_{\text{test}} - \text{OD}_{\text{negative}}}{\text{OD}_{\text{positive}} - \text{OD}_{\text{negative}}} \times 100$$

1. Highly hemocompatible (<5% hemolysis)
2. Hemocompatible (within 10% hemolysis)
3. Non Hemocompatible (>20% hemolysis)

2.6 Antimicrobial Activity

The antimicrobial activity of the developed nanocomposite films were tested by disc diffusion method using *E. coli* (gram negative) and *S. aureus* (gram positive).

For disc diffusion method, the films were cut into a disc shape with 5 mm diameter, sterilized by autoclaving for 30 min at 120 °C, and placed on different cultured agar plates. The plates were incubated overnight at 37°C in an incubator and the inhibition zone was then measured.

2.7 Cytotoxicity experiment

MTT assay was applied to determine the cytotoxicity of prepared sample film on PBMC normal Cell line. The PBMC cells were isolated from blood of a normal human who donated the blood voluntarily. Briefly, PBMC cells were dispensed in 24-well culture plates incubated at 37 °C. After 24 h, the nanocomposite film and PBS was introduced to the grown cell. The medium containing only PBS was used as a control. Each group was analyzed in triplicate. After 24 h, 50 µl MTT (5 mg/mL) was added to the medium which was then incubated for another 4 h at 37 °C. The formazan crystals in the cells were solubilized with stock DMSO solution (100 µl/well). The optical density (OD) value was then measured at 590 nm using spectrophotometer. The % viability of is calculated by the following equation [15]:

$$\% \text{Viability} = \frac{\text{OD value of samples}}{\text{OD value of negative control}} \times 100$$

And cytotoxicity was assessed according to cytotoxicity grading criteria as indicated in Table 1.

Table 1: Cytotoxicity grading criteria

Cell Viability (%)	Cytotoxicity grading criteria
100	0 (non-poisonous, qualification)
75–99	1 (light poisonous, qualification)
50–74	2 (moderate poisonous, disqualification)
25–49	3 (severe poisonous, disqualification)
1–24	4 (disqualification)
0	5 (disqualification)

3. RESULTS AND DISCUSSION

At the macro scale, silver always looks like silver. But solutions of silver nanoparticles can have many colors. The main reason behind this color change is the Surface Plasmon resonance [16]. In the silver nanoparticles, electrons oscillate collectively. These oscillations affect how light interacts with the nanoparticles. The specific oscillations depend on the particles' size and shape, so particles of different sizes have different colors. Color change indicates particle size. Solution color gives an approximate idea of the particle size. The color we see is basically an integration of the absorption spectra. Nanoparticle size could be monitored more accurately by taking absorption spectra.



FIGURE 1a : Color change during production of Silver Nanoparticle with time.

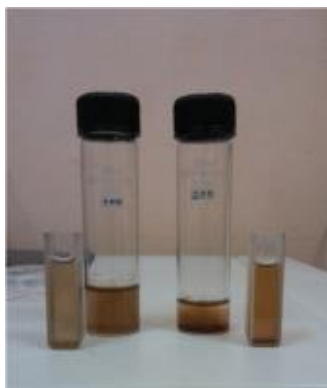


FIGURE 1b : Final color of the solution after 3 h of the reaction with silver nanoparticle precipitation

The absorbance value of reacting mixture measured at the end i.e 3h of reaction in order to verify the concentration of production of silver nanoparticle which is presented in the figure 3. Reduction of silver ions present in the aqueous solution of silver complex during the reaction with the ingredients present in the extract have been evaluated through UV-Vis spectrograph and has been recorded as a function of time with water as reference.

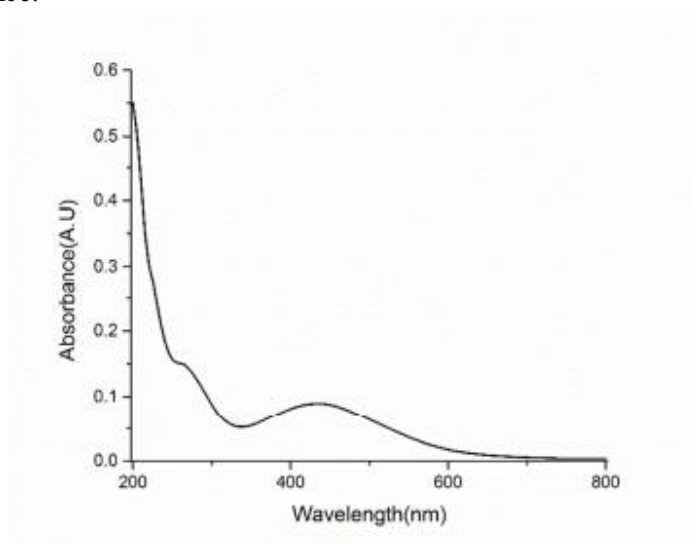


FIGURE 2 : UV Vis spectra of diluted solution of AgNPs

The results of DLS Zetasizer showed very homogenous distribution of AgNPs with average particle size of 86.55 nm as observed from the Figure 4. This denotes to monodispersity of nanoparticles which provides very high stability of nanoparticles for a long time. In addition, the Poly Dispersity Index (PDI), which is 0.302, specifies high stability and uniformity of the resulting AgNPs. The zeta potential of the synthesized Nanoparticle was also found to be -23.3 mV concluding that the nanoparticle is moderately stable [17].

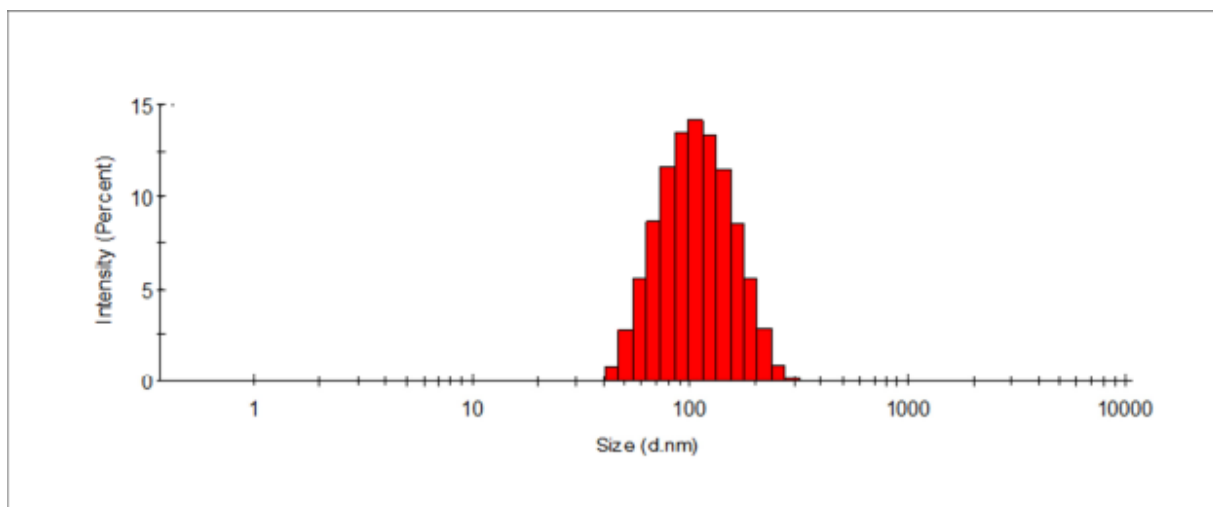


Figure 3: DLS analysis of the nanoparticle

SEM analysis (Figure 4) indicates high-density AgNPs synthesized by the extract. Image indicates moderately spherical and uniform AgNPs were formed with diameter of 15 to 80 nm. The SEM image also illustrated the interactions of hydrogen bond and electrostatic interactions between the bioorganic and phytochemical capping molecules bound to the AgNPs. The nanoparticles were not in direct contact even within the aggregates, demonstrating stabilization of the nanoparticles by the capping agent. The bigger silver particles may be due to the aggregation of the smaller ones.

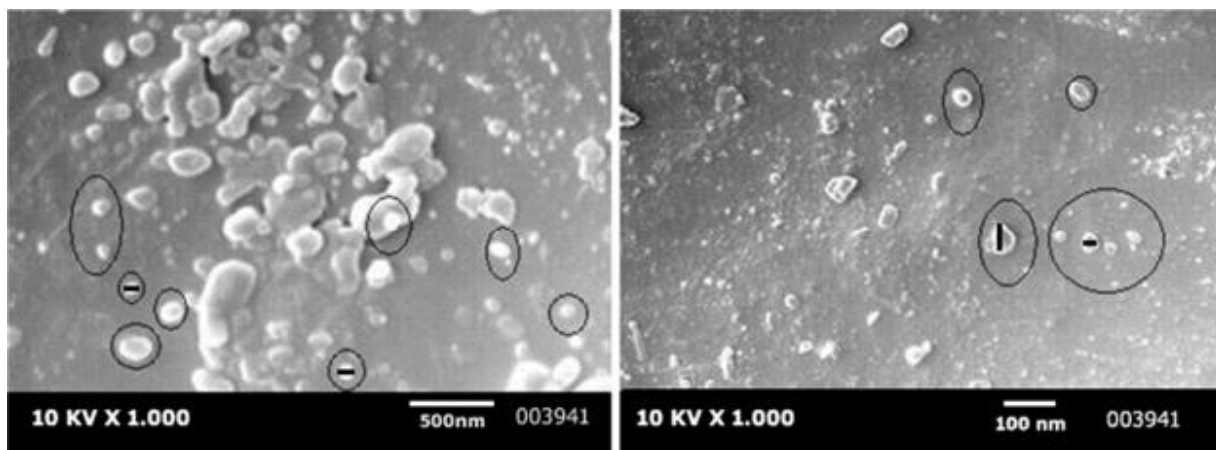


Figure 4: SEM of Nanoparticle

The SEM analysis of the silver nanoparticles loaded Gelatin-PVA films are shown in **Figure 5**. The nanocomposite film has exhibited a dense and uniform plain microstructure. The nanocomposite film also shows the presence of defined nanoparticles in the film. The cross-sectional view of the film indicated a uniform structure with a width of 53.73 μm confirming the formation of thin film.

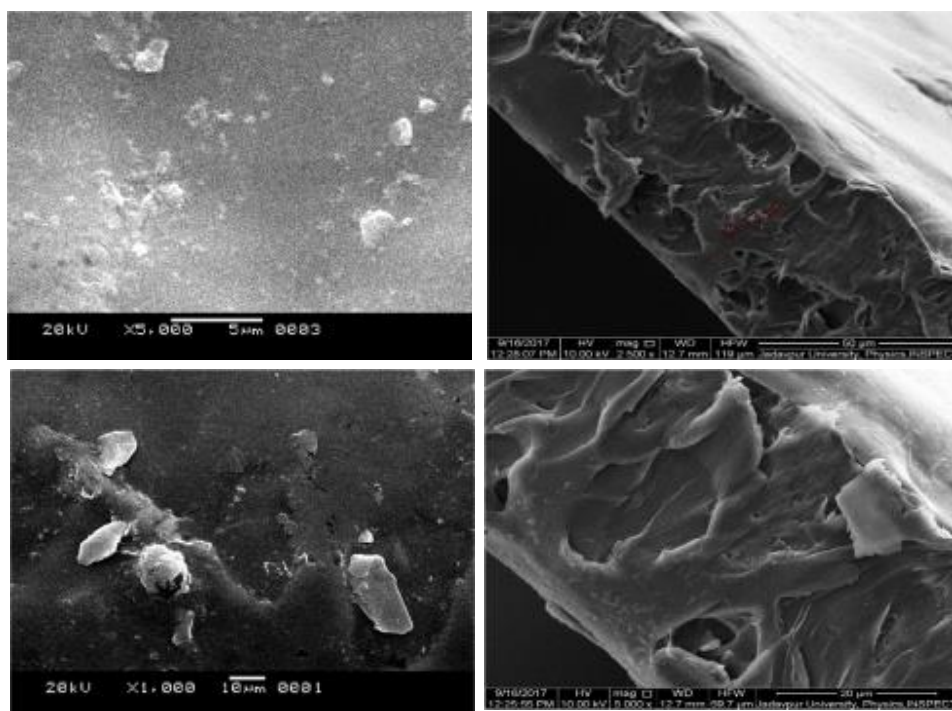


Figure 5: SEM analysis of nanocomposite film; a. surface view, b. cross-sectional view

The interfacial interaction between AgNPS and Gelatin –PVA nanocomposites was confirmed by FT-IR spectra (Figure 6). The infrared spectrum of film features band at 3702 cm^{-1} and 3717 cm^{-1} indicating presence of Amine (N-H stretching) which is for gelatin. Two major peaks at 3397 cm^{-1} and 3311 cm^{-1} indicates the presence of O-H stretching or this is majorly due presence of alcohol which is PVA in this case. Bands at 3084 cm^{-1} & 2927 cm^{-1} due to the stretching of the (C-H) group. The peaks at 1641 cm^{-1} , 1534 cm^{-1} , 1449 cm^{-1} , 1335 cm^{-1} , 1229 cm^{-1} , and 1086 cm^{-1} could be attributed to the presence of aromatic C=C bonds stretching vibrations. In FTIR spectra, the bands at 674 cm^{-1} and 624 cm^{-1} confirm the presence of a skeletal vibration of C-C groups.

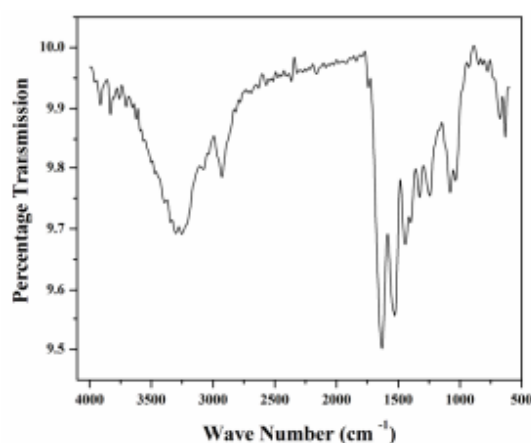


Figure 6: FTIR Spectrum of the Nanocomposite films

at about 160°C , indicating melting of nanocomposite chains and finally the film shows the greatest weight

100°C) observed in the films is due to loss of moisture present in the films. ; The second weight loss starts

and partial breaking of the molecular structure. Overall the film showed a smooth degradation curve. Thermal degradation starts at around 275 °C. So it will be stable during the sterilization needed before medical application.

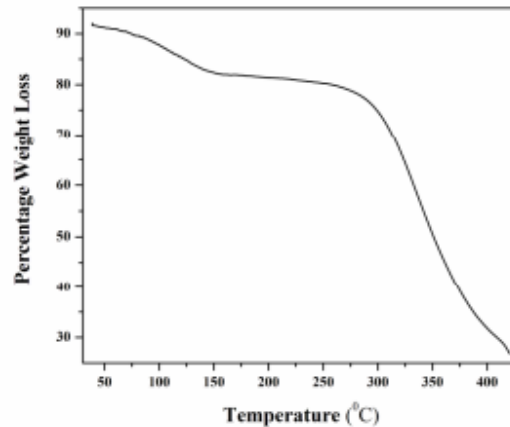


Figure 7: TGA of the Gelatin-PVA Nanocomposite films

The contact angle was measured using glycerin and was found to be 41.4 ° which indicates that the nanocomposite films have a good wetting property or else it can be interpreted that it is highly hydrophilic. As water and Glycerin have near values of surface energy so the contact angle with glycerin and water are likely to same i.e below 90° [18].

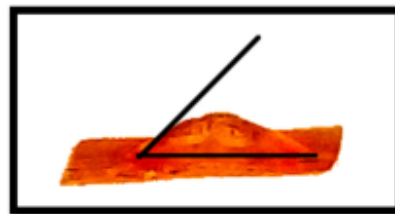


Figure 8: Contact angle with respect to Glycerin

The hardness of the polymer nanocomposite film was tested using Shore D meter and the hardness of the film is 6 in the Shore D measurement scale. This indicates that the material is extra soft and can be used for biological purpose [19].

Table 2: Hemocompatibility Test (Human Blood)

Sample	O.D at 545 nm	% Hemolysis	Remarks
+ve Control	0.6629		-
-ve Control	0.0038		-
Gelatin-PVA Silver Nanocomposite	0.0384	5.25%	Hemocompatible

It can be seen from Table 2 that the Nanocomposite films are highly compatible with human blood as the percentage hemolysis is around 5 % which is regarded as hem compatible range. It is reported earlier that Silver nanoparticles alone are highly toxic [20] but here in when it is used as a nanocomposite its toxicity is highly reduced as a total system. But this was only a preliminary test and vigorous biocompatibility tests need to be done with further experiment in animal model.

The antibacterial activity is a demonstration of the release of silver nanoparticles from the polymer network. Silver nanoparticles exhibit relatively large surface area, thus increasing their contact with bacteria. Silver nanoparticles show powerful bactericidal activity by binding with microbial DNA, thereby preventing bacterial replication. The use of Ag-containing Gelatin-PVA films as functional wound dressings is assessed by observing their antimicrobial activity (based on the disc diffusion method) against some common bacteria like *Escherichia coli* & *Staphylococcus aureus*.

Antimicrobial activity of pure, film and encapsulated silver nanocomposite films, were evaluated from their capacity to inhibit bacterial cultures along with one standard anti-bacterial medicine, Streptomycin (100 µg/mL) available in market was kept as control. The Minimum inhibition concentration of Streptomycin form *E. Coli* is 32 µg/ml, and that for *S. aureus* is 42 µg/ml [21]. Figure 9 shows the disc diffusion technique result for different bacterial culture. The experiment contained to sample to confirm the activity.

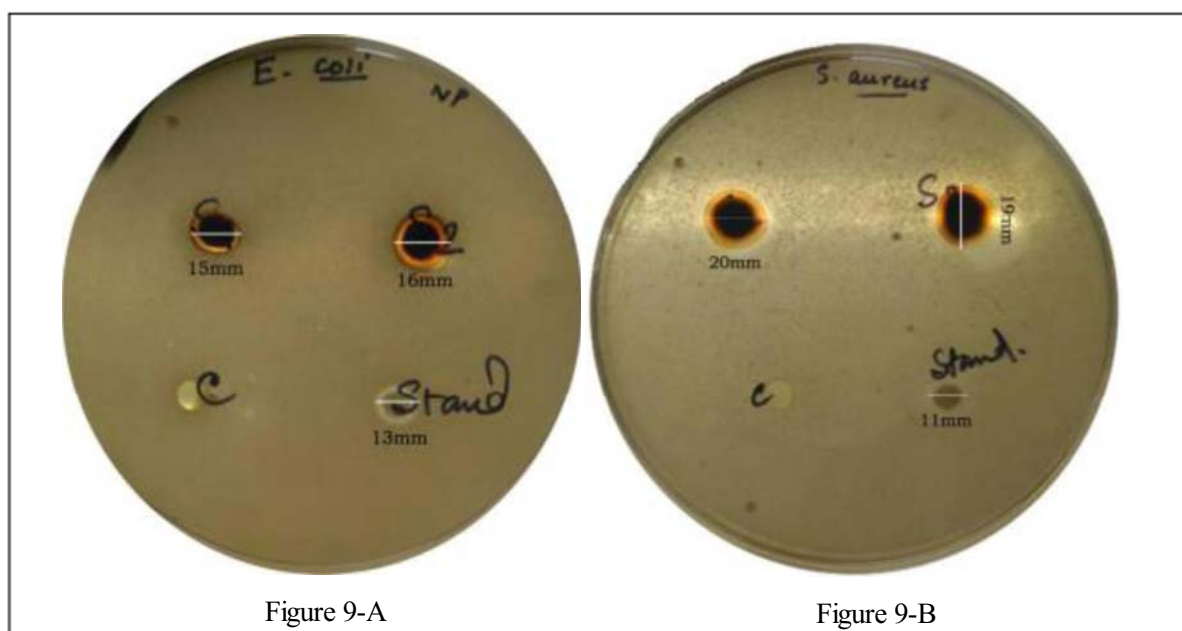


FIGURE 9: Anti-Bacterial Assay using Silver Nanocomposite Gelatine/PVA films

The Ag nanoparticles synthesized and encapsulated in Gelatin /PVA films showed inhibition zone against all test organisms. The Zone of inhibition (ZOI) of AgNP for *Escherichia coli* (16mm) was greater than that of the standard antibiotic streptomycin (13mm). (Table 3) (Fig 9).

TABLE 3: Zone of Inhibition of AgNPs–Gelatin-PVA films synthesized from Hops extract

Bacterial strains	inhibition(AgNPs)	Zone of inhibition (Standard Drug)
<i>Staphylococcus aureus</i>	20mm	11mm
<i>Escherichia coli</i>	16mm	13mm

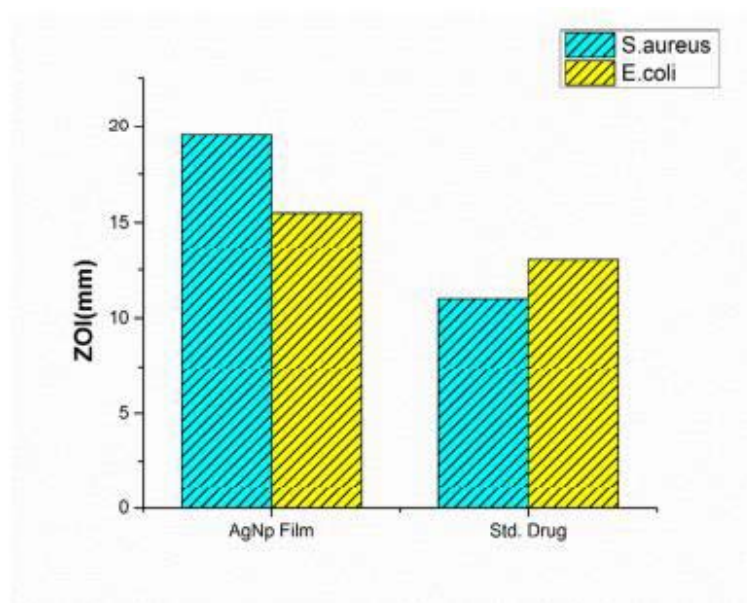


Figure 10: Antibacterial assay Results

The % Cell Viability of PBMC cells on the nanocomposite film was 82.14% (Figure 11) and the cytotoxicity grades of the nanocomposite films was 1 as indicated in Table 1. According to the cytotoxicity grading criteria, the nanocomposite film qualifies as a non-cytotoxic biomaterial.

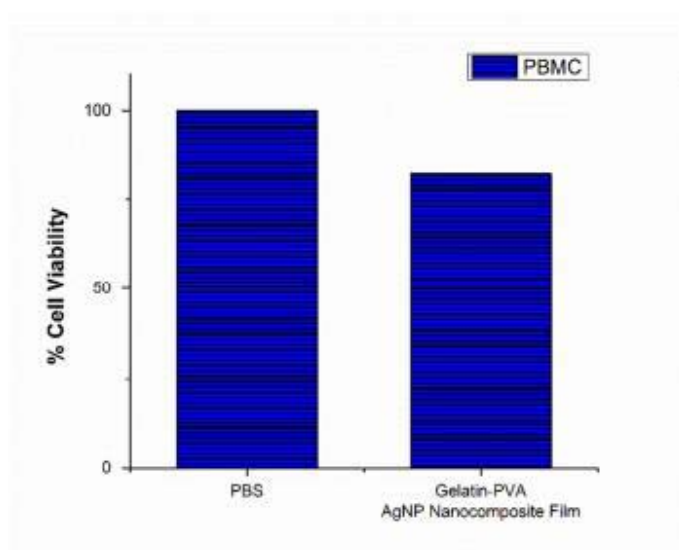


Figure 11: Percentage cell viability assay

4. CONCLUSIONS

The present work demonstrates a simple method for producing novel chitosan-PVA silver nanocomposite films. The developed silver nanocomposite films have exhibited fairly good mechanical strength and superior hemocompatibility properties. Further, the current work demonstrates a promising method to combine silver Nano-composites with a natural compound (Lupulus). The synthesis of Gelatin PVA silver nanocomposite film showed substantial antibacterial activity on both Gram-positive and Gram-negative bacteria. Indicates a potential use of the nanocomposite in the pharmacological, biomedical and industrial fields, such as bandages, wounds

dressings, anti-bacterial gloves and dental tools. In addition, the applications include also nutrition food and water storage container as well as wastewater treatment.

5. REFERENCES

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