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FORMULATION, CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY EVALUATION OF COLLAGEN BASED SILVER NANOPARTICLE

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ABSTRACT: Nanoparticles are widely used in different technological fields, one of which is medicine. Collagen is used in several types of wound dressing. A silver nanoparticle is used as an antimicrobial agent to reduce microbial infection. In this research work, we have synthesized silver nanoparticles with collagen and studied for antibacterial activity against *aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*). The silver content and cell line study results reveal that the formulation contains less concentration of silver (0.109 µg /ml), and it is not toxic in 3T3 cell lines. Antibacterial activity of collagen-based silver nanoparticle formulation (F7) was found to be high against *S. aureus, E. coli, P. aeruginosa* when compared with silver nanoparticles alone.

INTRODUCTION: A wound bed delivers an ideal condition for the growth of microorganisms owing to its moist, warm, and nutrition environment [1, 2]. Therefore, it is necessary to provide better protection against microbial infection, which enables faster regeneration of wound tissue. Silver was widely used for the treatment of infected wounds. The silver cream was topically applied to wounds for reducing infection severity due to its anti-microbial properties [3, 4]. Pure silver has been reported as a broad-spectrum antibiotic due to its antibacterial and anti-inflammatory properties [5]. The latest literature documents suggested that silver nanoparticles showed strong antibacterial efficacy [6, 7]. Collagen is the major structural component of the extracellular matrix found in connective tissue. It is the single most abundant protein in the animal kingdom found in dermis, tendons, and bones [8]. Tissue disruption following injury requires collagen for the repair and restoration of structure and function [9, 11]. The aim of the present work is to develop topical collagen-based silver nanoparticles for getting synergistic antibacterial activity.

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MATERIALS AND METHODS:

Materials: Eggshell membrane collagen was collected as a gift sample from Microcore Research Laboratories, Erode. Silver nitrate (Sigma Aldrich), Trisodium citrate (Himedia), Polyvinyl alcohol (Hi –Pure, Chennai) were purchased.

Synthesis of Collagen Based Silver Nanoparticle: Silver nitrate was used as a starting material, Trisodium citrate was used as a reducing agent, and Polyvinyl pyrrolidone (PVP) was used as a stabilizer for the preparation of silver nanoparticle. The silver colloid was prepared by using a chemical reduction method. About 85 mg of silver nitrate and 5 mg of PVP were dissolved in 50 ml of distilled water and heated to boil.

5 ml of 50 mg trisodium citrate was added dropwise into the silver nitrate solution and stirred for 10 min until the solution color becomes pale yellow. Then 50 ml of collagen-containing (85 mg) was added slowly to the silver nanoparticle solution and stirred until the color becomes yellowish-brown.

Characterization of Collagen Based Silver Nanoparticle:

Fourier Transform Infra-Red Studies: All the excipients and silver nitrate was subjected for FT-IR spectroscopy to check the compatibility.

UV–Visible Spectroscopy: The prepared nanoformulation was analyzed in UV–Visible spectroscopy (UV 1700 Shimadzu spectrometer, Shimadzu Corp., Tokyo, Japan), to confirm the collagen silver nanoparticle formation in the desired wavelength [12].

Particle Size and Zeta Potential Analysis: The prepared nanoformulation was analyzed in Malvern zeta sizer to find out the particle size and zeta potential [13].

Atomic Force Microscopy: The prepared nano-formulation was scanned under atomic force microscopy to analyze the morphology of the sample in 2D and 3D images [14].

Atomic Absorption Spectroscopy: The prepared sample was analyzed using atomic absorption spectroscopy to quantify the silver content present in the formulation. [12]

X-ray Diffraction: Powder X-ray diffraction was carried out on freeze-dried samples in order to determine the phase nature of silver present in the nanoparticles [14].

Determination of Minimum Inhibitory Concentration: The determination of MICs for silver nanoparticle with collagen were carried out using a two-fold microdilution method, according to Clinical and Laboratory Standards Institute guidelines. Concentrations of twofold serial-diluted solutions in Mueller-Hinton broth used for determining MIC for formulation.

Anti-bacterial Activity: The antimicrobial activity was carried out using clinical bacterial strains *Staphylococcus aureus (S. aureus), Escherichia coli (E. coli) and Pseudomonas aeruginosa (P. aeruginosa)* by disc diffusion method. This strains were swabbed on petriplates containing nutrient agar. The prepared formulation was added in a separate sterile disc and dried in an incubator for 24 hours. Then, the bacterial petri plates were incubated at 37 °C for 24 h. The sensitivity of the test organism towards formulation was indicated by a clear zone of inhibition around the disc, and the diameter of the zone of inhibition was measured [14].

Cell Culture: The normal 3T3 fibroblast cells were procured from the National Center for Cell Sciences (NCCS), Pune, India. The cells were maintained in Dulbecco's modified eagles medium (DMEM) supplemented with 2 mM 1-glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/l Na_2CO_3, 0.1 mM nonessential amino acids, 1mM sodium pyruvate, 2 mM 1-glutamine, 1.5 g/l glucose, 10 mM (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum (GIBCO, USA). The cells were maintained at 37 °C with 5% CO_2 in a humidified CO_2 incubator.

Statistical Analysis: All experiments were performed in triplicates. Statistical analysis was performed using Prism software (version 5). Statistical significance was calculated using ANOVA (Nonparametric), and the value of $P<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION:

FT-IR Studies: The characteristic peaks for collagen CH stretching are 1346 cm^{-1}, C-OH – stretching for PVP is 1656 cm^{-1}, O-bending for stearyl alcohol is 1463 cm^{-1}, N=O stretching for silver nitrate is 2300 cm^{-1}. Peaks with minor wave number changes were observed in the IR spectra of the physical mixture of collagen and excipients, as shown in **Fig. 1**. This confirms that the formulation under study has not undergone any major structural changes or incompatibility issues with the selected excipients. This also demonstrated that the physicochemical properties of silver and excipients were reserved in the physical mixture.

The synthesized collagen-based silver nanoparticles were characterized by UV-visible spectroscopy. The colloidal dispersion of silver prepared by

citrate reduction exhibited a surface plasma absorption band with absorption maxima at 423 nm, indicating the presence of silver nanoparticles. The particle size results showed that the particle size of prepared formulations was 50 nm - 3447 mm in size may due to change in concentration of trisodium citrate and PVP.

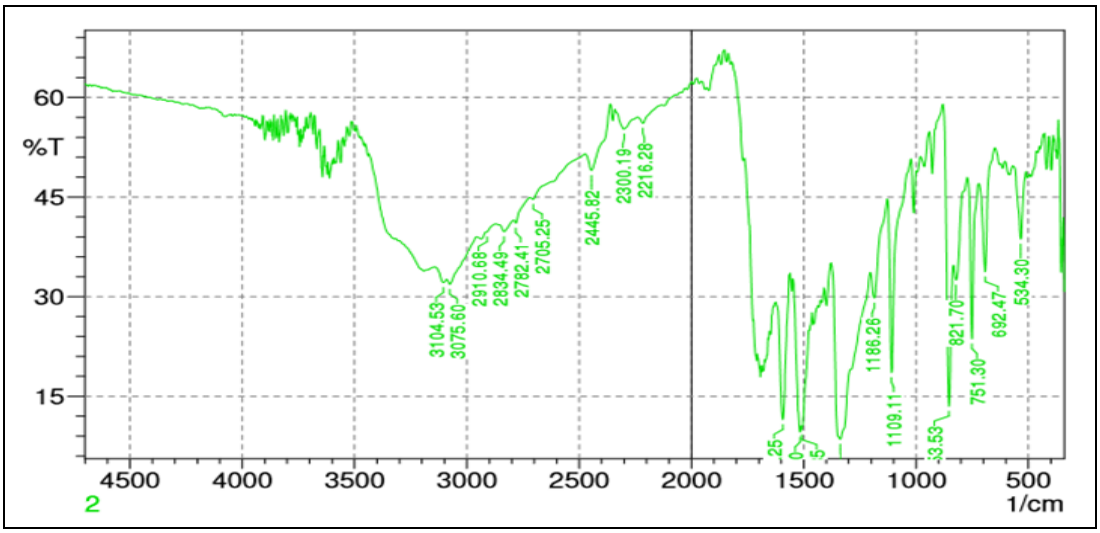

FIG. 1: FT-IR SPECTRUM OF COLLAGEN AND EXCIPIENTS

While increasing the concentration of trisodium citrate with low concentration of PVP in (F3, F6, F9), the particle size was high, and it is not in the nanosize range. Another observation in this study was when the PVP concentration was increased, the particle size got reduced (F6, F9) **Table 1**.

TABLE 1: PARTICLE SIZE AND ZETA POTENTIAL FOR COLLAGEN BASED SILVER NANOPARTICLE FORMULATIONS

Formulation	Particle Size (nm)	PDI	Zeta Potential (mV)
F1	216	0.243	-28.3
F2	85.16	0.505	-35.8
F3	3447	0.376	-27.4
F4	598.7	0.759	-27.7
F5	167.7	0.413	-49.9
F6	2496	0.169	-31.4
F7	51.04	0.569	-40.8
F8	153.2	0.245	-31.7
F9	1274	0.234	-2.47

The study was done by Cardosa *et al.*, 2016 [12] reveals the silver nanoparticle size prepared by chemical reduction method without stabilizer was in the range of 140.7 nm to 148 nm and another study report by Akturk *et al.*, 2016 [14] states that particle was in size range of 25 – 55 nm. Zeta potential in the formulation was in the range (-2.47 to -49.9); the difference may be due to change in the PVP concentration. The four formulations with two high and two low sizes were subjected for further evaluation.

Minimum Inhibitory Concentration: MIC was carried out using a broth microdilution method. The minimum inhibitory concentration of F7 formulation was found to be 25 µg/ml for *Staphylococcus aureus*, 50 µg/ml for *Escherichia coli* and 50 µg/ml for *Pseudomonas aeruginosa.*

Zone of Inhibition Test: The zone of inhibition test was carried out for determining the antibacterial effect of silver nanoparticle and collagen-based silver nanoparticle formulation (F7) using the disk diffusion method. The measured zones were mentioned in **Table 2**.

TABLE 2: ZONE OF INHIBITION

Organism	AgNPCOL	AgNP
Staphylococcus aureus	16	11
Escherichia coli	11	9
Pseudomonas aeruginosa	15	11

Statistically, a significant difference between the antibacterial efficacy of collagen-based silver nanoparticle *vs.* plain silver nanoparticle in *Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa* were analyzed by one way ANOVA test using prism software (version 5). The results revealed that collagen-based silver nanoparticles have significantly ($P<0.001$) increased antibacterial activity than a silver nanoparticle. Nanoparticle with different particle sizes is active against both gram-negative and gram-positive bacteria.

The increased antibacterial effect may be due to their small size and high surface-to-volume ratio, which will help in the close interaction with microbial membranes.

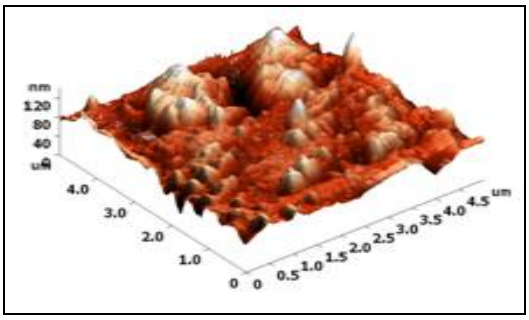

FIG. 2: AFM IMAGE OF SILVER NANOPARTICLE WITH COLLAGEN

Atomic Force Microscopy: The 3D AFM image for the nanoparticles shows the spherical shape, and some particles showed elongation due to the presence of collagen **Fig. 2**.

Atomic Absorption Spectroscopy: Silver content was analyzed in the prepared silver nanoparticle with collagen, and the silver was found to be 109.53 mg/l. This study confirms the silver content in the formulation was (109.53 mg/l) was less and less than the toxic range (4.35µg/ml) reported by Cardosa *et al.*, (2016) [14].

X-ray Diffraction Study: The prepared silver nanoparticle with collagen was found to be crystalline in nature. The silver peak was found in the XRD graph **Fig. 3**. The graph represents peaks with high intensity, and narrow base width were related to crystalline materials, whereas wide base peaks were related to amorphous substances.

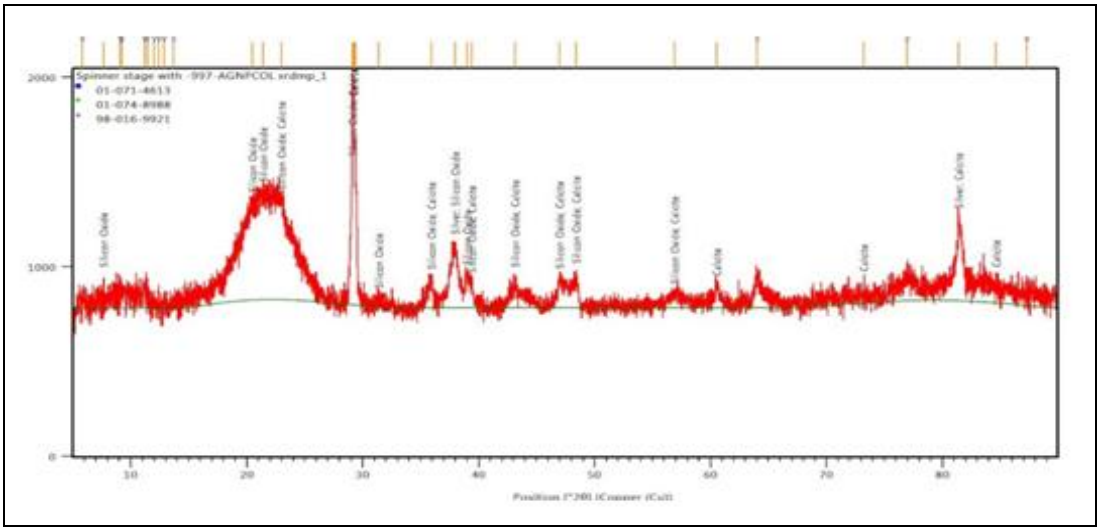

FIG. 3: XRD IMAGE OF SILVER NANOPARTICLE WITH COLLAGEN

Cell Line Studies: The MTT assay was tested in the normal 3T3 fibroblast cell line for silver nanoparticle and collagen with silver nanoparticles **Fig. 4**. The results showed that there is no activity on the collagen with silver nanoparticles up to (50 µm/ml) when compared to the silver nanoparticle (50 µm/ml).

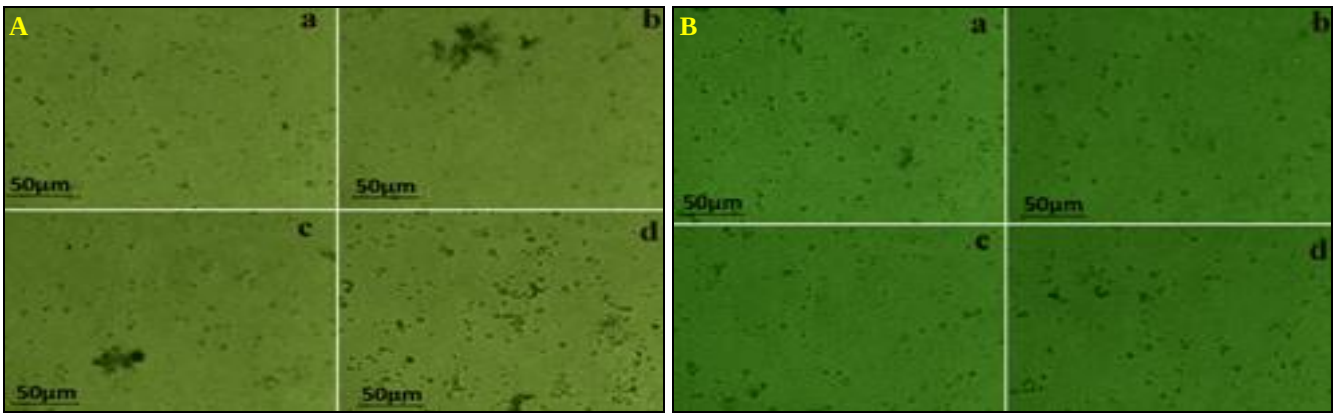

FIG. 4: CELL LINE STUDY IMAGE. A. SILVER NANOPARTICLE, B. SILVER NANOPARTICLES WITH COLLAGEN

The more viability observed in collagen with silver nanoparticles indicating it is non-toxic when compared to silver nanoparticles. Although the cytotoxicity reduced because of nanosize, the antibacterial activity increased. The study was done by cardosa *et al.*, 2016 [14] showed that the silver nanoparticle with collagen was found to be non-toxic in fibroblast cells and also found to be toxic in MV3 cancer cells.

Another study report by Gautam rath *et al.*, 2015 [12] also states that the silver nanoparticles with collagen have good antibacterial activity compared to plain collagen.

CONCLUSION: It is a challenge to design drug carriers that maximize antimicrobial activity and minimize cellular toxicity. In the present work, silver nanoparticle was prepared by the chemical reduction method. Antibacterial activity of collagen-based silver nanoparticles was found to be very effective against gram-positive bacteria. Collagen-based silver nanoparticles showed synergistic antibacterial activity when compared to silver nanoparticles. More importantly, the collagen-based silver nanoparticle exhibited an improved antibacterial activity against common pathogens invading burn wounds. Further in vivo experiments should be conducted in the future to better compare the antibacterial activity of collagen-based silver nanoparticle in this study with that of commercial products.

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CONFLICTS OF INTEREST: Nil

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