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Formulation Development of Mupirocin Adsorbed Collagen Stabilized Silver Nanoparticles to Enhance Synergistic Wound Healing Activity

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Abstract

Aim

The research aim is to formulate, characterize and evaluate the effect of mupirocin adsorbed collagen stabilized silver nanoparticles.

Methods

Mupirocin adsorbed collagen stabilized silver nanoparticles were synthesized by chemical reduction method and characterization for the formulations was done.

In vitro drug release studies were carried out using dialysis membrane.

In vitro skin permeation study was conducted in pig ear skin and % permeation was studied.

In vitro scratch assay was carried out to determine the wound healing activity in fibroblast cell lines.

Results

Silver nanoparticles was synthesized by chemical reduction method with the particle size range of 44 nm and 64 nm and the morphological characterization by Transmission Electron Microscopy (TEM) showed spherical and triangular shaped particles. Silver concentration in the formulation was recorded using Energy dispersive x-ray Analysis (EDAX) spectrum , Nanocrystalline nature of the formulation was confirmed by Selected Area Electron Diffraction (SAED) studies. Silver concentration in the formulation was analysed in Atomic absorption spectroscopy (AAS) and it was 12.75 mg/l for AgNP Col prepared using sodium borohydride , 33.60 mg/l for AgNP Col formulations prepared using trisodium citrate and tannic acid . *In vitro percentage* drug release for AgNP Col formulated using sodium borohydride was (87.5 ± 0.763) and AgNP Col formulated using trisodium citrate and tannic acid was (90 ± 2.00) . Adsorption efficiency of AgNP Col formulation with sodium borohydride was found to be 93.3% . FT IR studies was carried out for the drug and excipients with the physical mixtures and no incompatibilities was observed. *In vitro* skin permeation study was carried out using pig ear skin and the % permeation was found to be (72 ± 1.322) , (84.66 ± 2.516) for AgNP Col (TSC TA) and AgNP Col ($NaBH_4$) formulations at the end of 6 hours. The migration rate of fibroblast cells at each time point was compared by visual comparison and AgNP Col formulations showed their ability to close the scratch wound in the cells up to 24 hours. Mupirocin adsorbed AgNP - Col prototype formulation confirms the wound healing activity in fibroblast cells .

Keywords : Wound healing , Silver nanoparticles, Mupirocin, Collagen, Sodium borohydride Trisodium citrate, Tannic acid .

1.INTRODUCTION

Nanotechnology is an emerging technology extensively used to study various medical applications. [1] Silver nanoparticles (AgNPs) have a large surface area, resulting in large quantities of silver ions being released and potentially penetrating the skin, especially the damaged skin. Numerous reports indicate that AgNPs promote wound healing and exhibit antimicrobial properties against a wide range of bacteria including *pseudomonas aeruginosa*, *Escherichia coli and staphylococcus aureus.* [2]

Collagen components, such as fibroblast and keratinocytes, are a major part of skin development. Collagen may be harvested from a variety of sources including living and nonliving bovine, porcine, and equine skin. Collagen possess low allergenicity, low antigenicity and high biocompatibility. It is bioabsorbable, hemostatic, biodegradable, nontoxic, synergistic with bioactive components and compatible with natural and synthetic polymers. It is highly tensile and has a high affinity for water. The increase in the application of collagen as a biomaterial is due to its natural abundance and the diversity of ways by which it can be molded.[3]

Mupirocin or pseudomonic acid A is a natural antibiotic produced by Pseudomonas Fluorescens which was successfully used in the treatment of wound infections as a topical agent. The research was undertaken to study the wound healing effect of collagen-stabilized silver nanoparticles loaded with topical antibiotic mupriocin.[4]

2.MATERIALS AND METHODS

2.1. Materials

Mupirocin was a gifted sample from Fourts (India) Laboratories Pvt. Limited, Chennai. Silver nitrate -Sigma Aldrich, Trisodium citrate, Qualigens fine chemicals,- Mumbai ,Tannic acid, HiMedia Laboratories Pvt. Ltd- Banglore , Sodium borohydride, Sigma Aldrich, Glacial acetic acid, Loba chemie Pvt ltd,- Mumbai and Collagen from Microcore Research laboratories, Erode.

2.2. Synthesis of Collagen Stabilized Silver nanoparticles

Silver nanoparticles was synthesized by chemical reduction method using Sodium borohydride, Trisodium citrate and Tannic acid as reducing agents. Silver nitrate at a concentration of 0.74 mM solution was heated to boil with vigorous stirring at 60˚C .Collagen at a concentration of 0.1mg/ml was prepared by dissolving in 0.5 M acetic acid solution and the mixture was agitated and homogenized for 10 minutes (15,000 rpm) using high speed homogenizer and centrifuged at 3600 rpm for 15

minutes (temperature 25˚C) and separated supernatant. The final solution was added to the silver nitrate solution and allowed to mix and agitate in a magnetic stirrer for few minutes. Trisodium citrate at a concentration of 6.8 mM and tannic acid at a concentration of 24 µm solution was prepared and heated separately at 60˚C and the citrate – tannic acid solution was added dropwise to the silver salt solution. Finally the colour of the mixture slowly turned from colourless to yellow indicating the reduction of Ag^+ ions and the solution was removed from the heating mantle and to cool ed in dark place. Similarly, using Sodium borohydride as a strong reducing agent, silver nanoparticles was synthesized without employing heating process. [3,5]

2.3. Surface Adsorption of Mupirocin on AgNP-Col

Mupirocin is adsorbed to AgNP-Col surface by electrostatic interaction method which is a non-covalent bond occurring between two oppositely charged particles. Mupirocin 40 mg was added to the 40 ml (1 mg in 1 ml) of AgNP-Col solution and gently stirred in a magnetic stirrer at room temperature for 24 hours. Lambda max and spectrum of the formulation after drug adsorption was confirmed by UV-double beam spectroscopy.

2.4. Adsorption efficiency

About 2 ml of the formulation was taken in an eppendorf tube and centrifuged at 13000 rpm for 15 minutes at 4˚C and 1 ml of the supernatant was collected, diluted with water and the absorbance was measured in UV Spectrophotometer at 222 nm wavelength, then the amount of drug adsorbed and adsorption efficiency was calculated according to the formulae given below [6]

Amount of drug adsorbed
= Initial drug loaded – unabsorbed drug
Adsorption efficiency (%)
= [Amount of drug adsorbed / Initial drug loaded] × 100

2.5.Characterization of AgNP-Col

The plasmon resonance spectrum of AgNP-Col formulation was measured to verify the reduction of silver ions in the solution and ƛmax was scanned in the wavelength range from 300 – 800 nm using glass cuvette with distilled water as the blank in UV- double beam spectrophotometer (UV- 1601 PC Shimadzu spectrophotometer, Japan). The Mean particle size diameter, Zeta potential and PDI (Poly dispersity index) were measured by dynamic light scattering (DLS) using Zetasizer [7] (Nano ZS 90, Malvern Instruments, united kingdom)

2.6.Morphology of AgNP-Col

Transmission Electron Microscopy (TEM 200 Kv ; T12 Fei, Teenai G2 Spirit TWIN **) w**as performed to analyze the surface morphology of AgNP-Col formulations. The sample was placed in a copper grid and kept for air dried at room temperature. The dried sample in the grid was loaded in the loading point and scanned under 200 Kv, then the beam was passed through the sample and the images were scanned under microscope at different nanometer scales.

Energy dispersive x-ray Analysis (EDAX) (T12 Fei, TecnaiG2 spirit TWIN) was also carried out to find the Inorganic elements present in the formulation and to confirm their electrostatic attraction. EDAX Spectrum of the AgNP-Col formulations was observed and spotted as a spectrum peak graph. Selected Area (electron) Diffraction (SAED) is a crystallographic experimental technique which was performed inside the TEM to confirm nature of the formulations.[7,8]

2.7.Atomic Absorption Spectroscopy

The silver concentration in the AgNP-Col formulation was determined with the Flame method using AAS technique. [Thermo Scientific ICE 3000, USA] and the silver content was measured in terms of Ag in mg/l .[8,9]

2.8. Compatibility Studies

FT-IR Studies was carried out for the drug, excipients and physical mixtures of the formulation by the KBr press pellet technique. [9]

2.9. *In Vitro* release studies

About 2 ml of the AgNP-Col liquid formulation was taken in an dialysis bag and closed on both the sides with HI – Media closure clips to prevent the leakage of formulations. Then the dialysis bag was placed inside a beaker containing 50 ml phosphate buffer pH 7.4 at room temperature and allowed to stir gently at 100 rpm using magnetic stirrer. Samples were withdrawn at a periodic time intervals from [1-8 hours] and replaced with equal volume of buffer, then sample was analysed in uv for absorbance at 222 nm after making suitable dilutions. [9]

2.10. *In Vitro* skin permeation study

In vitro skin permeation study was performed on open end cylindrical tube using pig ear skin. Skin was excised from the domestic pig ears obtained from a local commercial supplier for human feeding. No animal was killed for the purpose of this study and full thickness skin with a surface area of 3.5 cm^2 was used.

The pig ear was cleaned under running tap water after excision and skin hair from the outer region was removed by applying hair removal cream (FEM), then skin was carefully separated from cartilage using a scalpel. Subsequently adipose, subcutaneous tissue was removed by scrapping the layers with spatula, finally skin layer was rinsed and washed with 0.9% Nacl saline, a thickness of 2 mm was controlled with a vernier.

The skin was tied and fixed at the bottom of cylindrical tube between the donor and receptor compartments. Sink conditions was maintained in the receptor compartment with phosphate buffer saline (PBS) at pH 7.4 with a volume of receptor fluid for 50 ml , and donar compartment was maintained with a sample volume of 2 ml. and the cylindrical tube was clipped in the burette stand and receptor fluid containing beaker was placed in a magnetic stirrer at 32˚c with 100 rpm stirring, samples were withdrawn at a periodic time intervals up to 6 hours with the same volume of buffer replacement and uv determination was carried out at 222 nm wavelength, and percentage drug release across the skin membrane was determined. [10]

2.11 *In vitro* scratch assay

Scratch assay was carried out to observe the wound healing activity of the formulation in cell lines. Fibroblast cells were grown in a six well plates to a confluent monolayer. The cell monolayer was scraped in a straight

line with a 200-µL pipette tip. Cell debris was removed by washing the cells three times with phosphate buffer saline (PBS). Then cells were immediately cultured with the AgNP-Col formulations and keeping 0.2% FBS as control. The cells were incubated at 37˚c for 24 hours and the image of the scratch acquired was observed under a phase contrast microscope at 0, 12 and 24 hours. [11,12]

2.12 Statistical analysis

All *in vitro*, particle size, PDI and Zeta potential measurements were analyzed in triplicate and the results were expressed as mean ± standard deviation of three separate experiments. The results were considered statistically significant when $p < 0.05$. Datas were evaluated by two-way analysis of variance (ANOVA) and unpaired [t] test using Graph pad prism software.[3]

3. RESULTS AND DISCUSSION

3.1 Characterization of AgNP-Col

Collagen stabilized silver nanoparticles were synthesized by chemical reduction method with two sets of reducing agent using sodium borohydride and trisodium citrate – tannic acid. A bright yellow colour change in the formulation indicates the formation of silver nanoparticles. The spectrum peak widely ranging from 300 to 450 nm wavelength indicates the formation of silver ions in the formulation. Mean average particle size, zeta potential, and poly dispersity index (PDI) values indicates the nanoparticle confirmation. (Table a).

We found that concentration of sodium borohydride and trisodium citrate – tannic acid in this study is sufficient to overcome the repulsive forces and enable controlled aggregation of similar ions to produce stable colloidal silver dispersion with a narrow size distribution. Zeta potential of optimized silver nanoparticle turn out to be -18.1 and -29.8 mv revealing the presence of a compact surface layer of trisodium citrate – tannic acid and sodium borohydride on the colloidal silver. (Table a).

Particle size distribution, PDI, and zeta potential measurements are compared with similar AgNP Col datas [4] reporting particle size range of 140 ± 7.8 nm and zeta potential of + 20.1±0.7 are not similar to our study.

After adsorption of mupirocin, the average particle size, Zeta potential, and PDI of AgNp Col (TSC TA) F_1, AgNP Col ($NaBH_4$) F_4 formulation was compared and the p value found to be significant ($p < 0.001$). The particle size of mupirocin adsorbed AgNP Col was higher than the AgNP Col formulation because of the adsorption of mupirocin to AgNP- Col and there is no aggregation and morphological changes observed after drug adsorption (Table a).

The resonance wavelength of AgNP Col formulations in UV depends on the particle size and shape. If the particle size becomes larger the plasmon peak shifts to the longer wavelength and broadens. AgNP Col (TSC TA) formulation have a strong UV spectrum absorption peak at 441 nm and for AgNP Col ($NaBH_4$) at 415 nm and this confirms the reduction of Ag^+ to AgNP Col. (Table b).

Table a): Particle size, PDI and Zeta potential characterization for AgNP COL formulations

Formulation	Particle size (nm) Before drug adsorption	PDI Before drug adsorption	Zeta potential (mV) Before drug adsorption	Particle size (nm) After drug adsorption	PDI After drug adsorption	Zeta potential (mV) After drug adsorption
AgNP-Col (TSC-TA)	39.74 ± 0.801	O.654 ± 0.11	-23.4 ± 1.05	44 ± 1.015	0.460 ± 0.0995	-18.1 ± 1
AgNP-Col ($NaBH_4$)	56 ± 1.0	0.339 ± 0.096	-17.5 ± 1	64 ± 1.06	0.585 ± 0.1015	-29.8 ± 1

Table b) : Peak Absorbance, Adsorption efficiency, *In vitro drug* release, silver concentration and permeation for AgNP-COL formulation

Formulations	Absorbance	Wavelength (nm)	Adsorption Efficiency	*In vitro* drug release	Silver Concentration(mg/l)	Permeation
AgNP COL (TSC-TA)	0.249	441	93.6 %	90.0 % ± 2.00	12.75	72 % ± 1.322
AgNP COL ($NaBH_4$)	0.609	415	93.3 %	87.5 % ± 0.763	33.60	84.66 % ± 2.516

Table c): In vitro scratch assay measurements for different samples

Sample	0 hrs (mm)	6 hrs (mm)	12 hrs (mm)	24 hrs (mm)
AgNP	12	9	7	2
AgNP Col ($NaBH_4$)	12	10	8	6
AgNP Col (TSC- TA)	12	10	8	5
Collagen	12	7	5	0

3.2 Morphology of silver nanoparticles

Surface morphology of AgNP Col formulations was carried out in TEM .TEM micrographs shows the formulations are spherical, triangular and rod in shaped. (Fig i). EDAX Spectrum confirms the presence of silver (Ag) in the formulation (Fig. ii) .Copper grid was used for the sample analysis hence Cu and C are detected in the spectrum

SAED (Selected area electron diffraction pattern) (Fig.iii) shows small bright spots making up a circular rings that each spot arise from bragg reflection of an individual crystallite which confirms the formulations are nanocrystalline in nature.

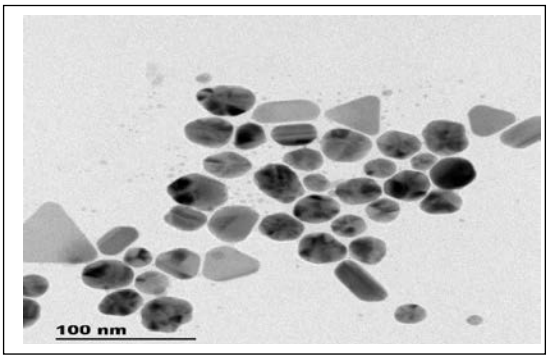

Fig. i): TEM Micrographs

3 Adsorption Efficiency and *In vitro* drug release study

Adsorption efficiency of mupirocin loaded AgNP Col (TSC TA) and AgNP Col ($NaBH_4$) formulations after surface adsorption of the drug was found to be 93.3 % and 93.6 % (Table b) and there was no colour change observed in the formulation after drug adsorption. *In vitro* drug release of AgNP Col (TSC TA), AgNP Col ($NaBH_4$) fomulations at the end of 8 hours study shows a maximum release of 87.5% and 90% as listed in (Table b). All the measurements was made in triplicate.

In vitro release in AgNP Col (TSC TA) F_1 formulation was compared with the AgNP Col ($NaBH_4$) F_4 and there is no significant difference in release ($p > 0.05$) between two formulations .

3.4 Atomic absorption spectroscopy

Silver content in the formulation containing collagen was measured in atomic absorption spectroscopy and silver concentration was determined.

The silver concentration of both the AgNP Col formulations was found to be less than the toxic range (222-362 mg Ag-day) reported for silver (Table b)

3.5 FT-IR Studies

3.5.1 FT- IR Data of Mupirocin

The characteristic peaks readed for O- H Stretching 3306.10 cm^{-1}, C-H Symmetrical stretching 2976.26 cm^{-1}, C = O Stretching 1715.74 cm^{-1} and CH_3 bending 1256.67 cm^{-1} which is evident that 4 peaks confirms the presence of mupirocin.

3.5.2 FT IR Data of Collagen

The characteristic peaks readed for C = O Stretching 1685.84 cm^{-1}, C-H Symmetric Stretching 2963.72 cm^{-1}, and CH_3 bending 1476.56 cm^{-1} which is evident that peaks confirms the presence of collagen

3.5.3 FT- IR Data of Physical Mixture 1 (AgNP Col – $NaBH_4$)

The characteristic peaks readed without major changes with O-H Stretching 3274.27 cm^{-1}, C=O Stretching 1714.77 cm^{-1} for mupirocin and collagen. Na Stretching 890.18 $cm^{-1}$for tri sodium citrate, sodium borohydride and CH_3 bending 1278.85cm^{-1} for mupirocin confirms 4 peaks observed in the IR Spectrum of the physical mixture 1 confirming AgNP Col ($NaBH_4$) not having any incompatability with the excipients (Fig. iv)

3.5.4 FT IR Data of Physical Mixture 2 (AgNP Col TSC TA)

The characteristic peaks readed with O-H Stretching 3288.74 cm^{-1} for mupirocin C-H asymmetrical Stretching 3227.98 cm^{-1} for tannic acid, C-H Symmetric Stretching 2931.90 cm^{-1}, C=O Stretching 1722.49 cm^{-1} for mupirocin and collagen, CH_3 bending 1258.56 cm^{-1} for mupirocin, collagen, tannic acid and Na Stretching 887.28 cm for tri sodium citrate which is evident that resulting 6 peaks were observed in the IR spectrum of the physical mixture 2 confirming AgNP Col (TSC TA) confirming the presence of drug and excipients without in compatability. (Fig. v)

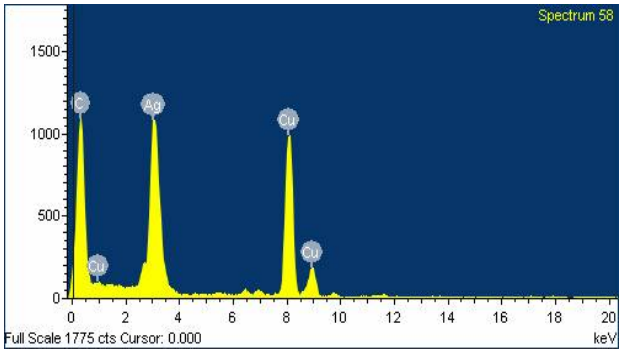

Fig ii): Energy Dispersive X-ray Analysis Spectrum of AgNP COL – $NaBH_4$ Formulation

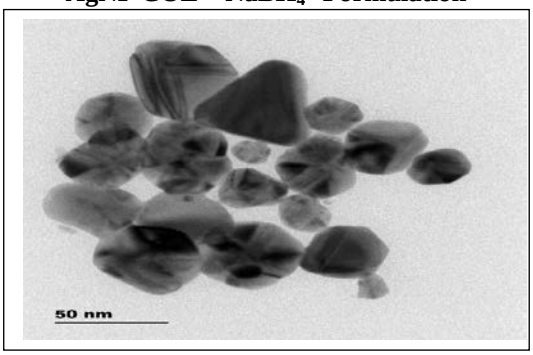

Fig ii(a). AgNP (Silver nanoparticles)

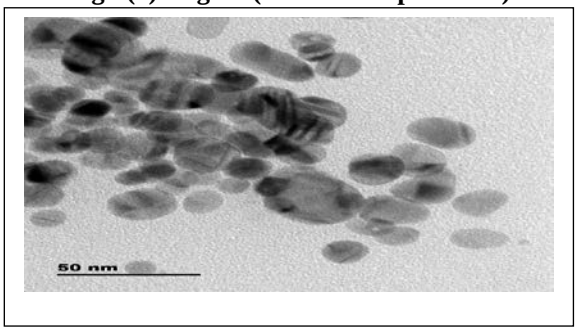

Fig ii(b). AgNP Col (TSC – TA)

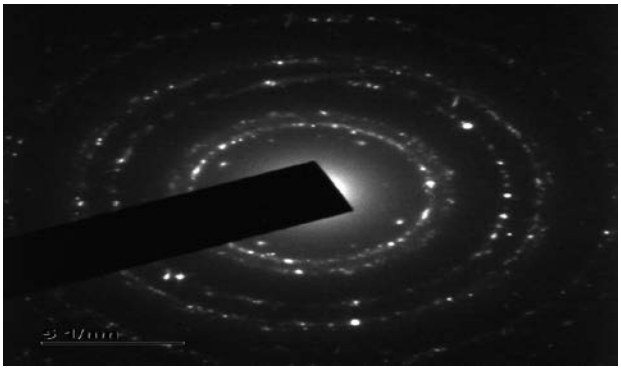

Fig iii): Selected Area Electron Diffraction Pattern of AgNP Col – TSC TA Formulation

3.6 *In vitro* skin permeation study

Skin permeation study was conducted across the pig ear skin on open end cylindrical tube set up with AgNP- Col formulations and the estimated permeation study on the skin was carried out up to 6 hours and results are reported in (Table b).

In vitro skin permeation release of AgNP Col (TSC TA) F_1 and AgNP Col ($NaBH_4$) F_4 was compared and the p value results ($p< 0.0015$) which was observed statistically significant**.** *In vitro* skin permeation release of AgNP Col (TSC TA) F_1 formulation showed maximum permeation release of 84.66 % ± 2.516 than the AgNP Col ($NaBH_4$) F_4 formulation**.** Both the formulations of AgNP Col showed good release in the pig skin. The % permeation from pig skin was not similar to the dialysis membrane method for AgNP Col (TSC TA), AgNP Col ($NaBH_4$). High permeation of drug was observed through pig skin and dialysis membrane in AgNP Col (TSC TA) formulation may be due to the higher amount of surface drug adsorption. The results of the skin permeation displayed the ability of AgNP col to diffuse through skin layers and to the region below the skin, allowing applications to local injury.

3.7 *In vitro* scratch Assay

The image, distance between one side of scratch and the other was measured from time 0 hrs to the last time point (24 hrs). The distance of each scratch closure on the basis of the distance was measured and listed in (Table c). Restoration of the full cellular density of the mesothelium in fibroblast cells was faster in the AgNP Col group than the control group. AgNP Col promoted the migration rate of fibroblasts obviously at each time point which was confirmed through the scratch cell distance closure. Cells closure was rapid in collagen alone followed by in silver nanoparticle samples. It may be due to rapid migration of fibroblast cells. But the closure was slow and steady in AgNP-collagen formulation (Fig.vi). No synergistic effect was observed in the AGNP-collagen formulation

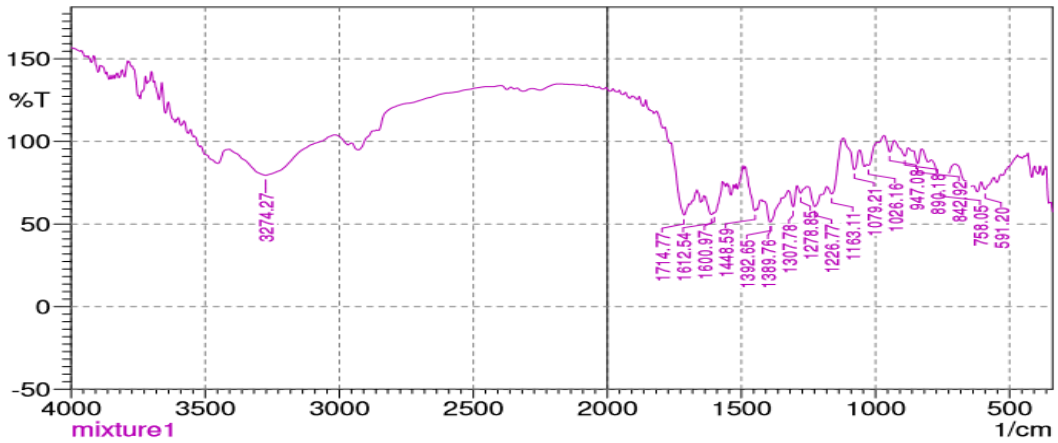

Fig. iv): FT- IR Spectrum of Physical Mixture 1 (AgNP Col – $NaBH_4$)

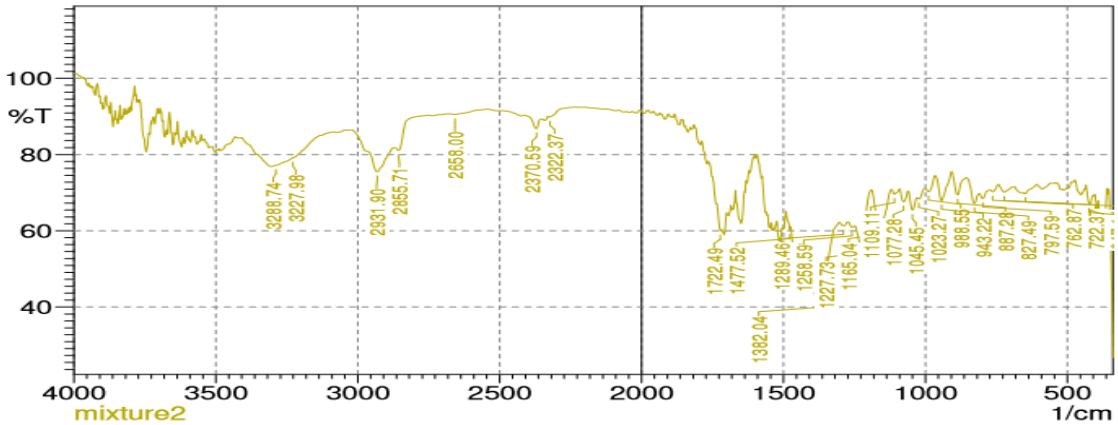

Fig v): IR Spectrum of Physical Mixture 2 (AgNP Col – TSC TA)FT-

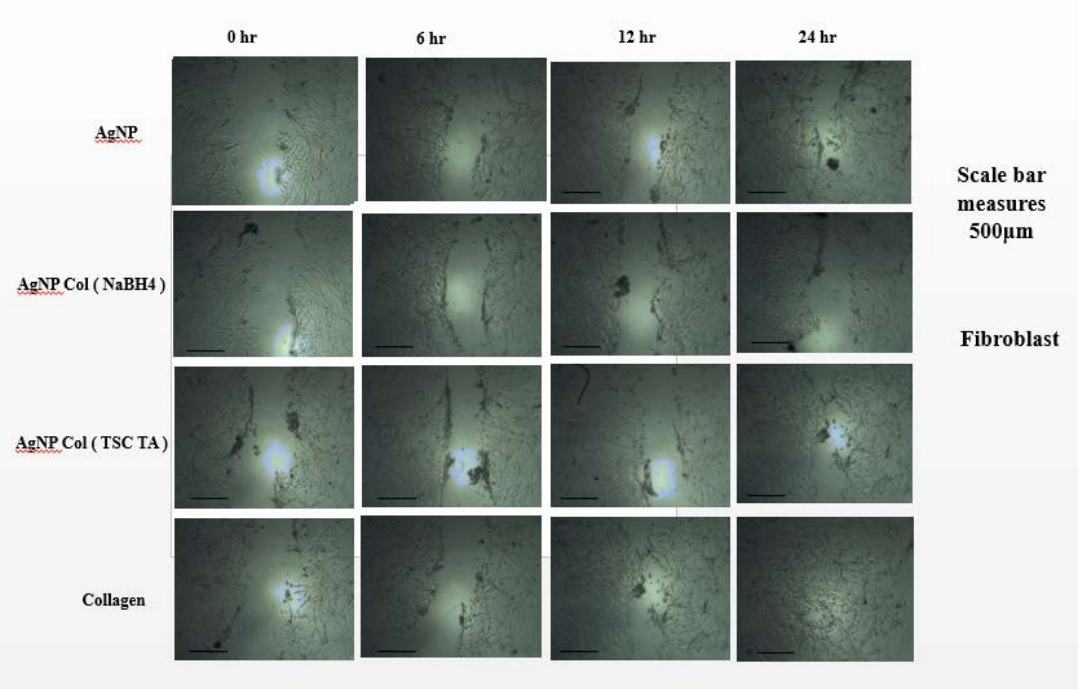

Fig.vi): *In vitro* scratch assay reports of different samples in fibroblast cell lines

SUMMARY AND CONCLUSION

In the present work collagen stabilized silver nanoparticles were synthesized by chemical reduction method using sodium borohydride, tri sodium citrate and tannic acid as reducing agents and drug mupirocin was surface adsorbed to the formulation. AgNP Col formulations showed their maximum ability to close the scratch wound in the cells up to 24 hours. Thus Mupirocin adsorbed AgNP - Col prototype formulation confirms the wound healing activity in cells. AgNPcol can be used as a viable alternative to improve the repair process, thereby making it necessary to study this perspective further.

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