International Research Journal of Engineering and Technology (IRJET) e-ISSN: 2395-0056
Volume: 09 Issue: 06 | June 2022 www.irjet.net p-ISSN: 2395-0072

Development and Characterization of Rejuvenate Cellulose and Fibrin Bio-complex Infused with Silver Nanoparticles as Tissue Engineering Applications

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ABSTRACT:

In the present study, a composite wound dressing material containing fibrin (F) and regenerated cellulose (RC) impregnated with silver nanoparticles (AgNPs) was developed. The AgNPs was prepared by green synthesis process from $AgNO_3$ solution through the extract from the leaves of plant Atlanta monophyla which have been used in traditional medicine such as Ayurvedic system in India. The average particle size was found to be 50 nm for silver nanoparticles. From the UV Spectroscopy, the formation of stable nanoparticles was confirmed between the 300nm to 700 nm. The size of AgNPS and different elements in sample composition was confirmed by TEM and EDX respectively. Antibacterial activity of synthesized silver nanoparticles was carried out in liquid growth medium against four pathogens (Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Escherichia coli) was conducted. The bio composites were prepared by using conventional techniques and its invitro cytotoxicity was evaluated by MTT. We found that the RC-F-Ag bio composite prepared from plant Atalantia monophylla was consider may be a potential candidate as a wound dressing material and may be tried on the clinical wounds of animals before being applied on humans.

Key words: Regenerated cellulose, Silver nanoparticles, *Atlantia* monophyla, wound healing, anti-bacterial,

INTRODUCTION

Nanotechnology is one of the most exciting discoveries in contemporary materials engineering. Based on particular properties such as size, disruption, and morphology, nanomaterials new and improved properties. Because of their unique antimicrobial properties, metallic nanoparticles have recently piqued the interest of researchers for use in wound healing as nanotechnology advances. Because of their remarkable properties, Ag-NPs have piqued the interest of researchers in recent decades. They have a large surface area, making them a strong contender for antimicrobial effect due to increased contact with micro - organisms and potential noxiousness [1]. A high-efficiency wound closure material that creates an optimal environment for epidermal regeneration while also providing a barrier against water loss and infection of the chronic wound is required for proper wound treatment. Infomercial dressings are commonly used for wound repair, but these components are certainly dry and can attach to the wound's exterior, causing physical disquiet when excluded. Instead of traditional dressings, new natural wound care products made from bio-companionable polymeric sources (such as collagen, elastin, chitosan, and alginates and hyaluronic acid) are now used [2]. These polymeric compounds have the capability to issue bioenergetic ingredients and aid in the rehabilitation progression. Traditional wound bandages are incapable of meeting wound care requirements. Because of their unique properties, electro spun nano-fiber mats, hydrogels, and nanocomposites, as the new generation of wound dressing, hasten curative and avert pathogenic bacteria. Nanofibrous synthesis with morphometric and architectural possessions comparable to the natural extracellular medium has also been empowered by modern improvements in nanoscience. Nano-fibers, hydro-gels, and nano-composites have high permeability, a large superficial area-to-volume ratio, and excellent gas permeation. The properties mentioned above improve cell respiration, skin and moisture regeneration, hemostasis, and purulent removal. Similarly, many nosocomial pathogens are drug-resistant, posing a high risk to patients afflicted with them. As more bacterial strains become resistant to antibiotics, new strategies for combating these pathogens are being developed. The primary wound curative strategy is to combine different non-traditional antibacterial agents (such as nanoparticles) to overcome microorganism resistance and gain complementary antimicrobial effect [3]. A composite wound dressing material containing fibrin (F) and regenerated cellulose (RC) impregnated with silver nanoparticles (AgNPs) was developed in this study. Green synthesis was used to create

Ag-NPs from AgNO3 solution using extracts from the leaves of three different plants, *Chloroxylon swietnia*, Atlanta monophyla, and *Tarenna asiatica*, all of which have been used in traditional medicine such as the Ayurvedic system in India. We discovered that the RC-F-Ag bio matrix planned from the plant *Atalantia monophylla* is a potential candidate as a wound management material and should be tested on clinical injuries in animals before being used on humans [4].

MATERIALS AND METHODS:

Materials

Untainted and systematic rating chemicals were used in experiment together with fusion of silver nanoparticles, media groundwork for progress of bacterial cells. silver nitrate (AgNo3) were obtained. The bacterial cultures of *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa*were obtained and antibiotics (Gentamicin). Peptone, Sodium chloride, Yeast extract, Beef extract, Agar-agar. Vero (African green monkey kidney normal cell line) was obtained from the national center for cell sciences (NCCS), Pune, India. Cell were maintained in the logarithmic phase of growth in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS),100µg/ml streptomycin [5].

Methodology

Preparation of plant extracts:

Weighing 2 g of plant (*Atalantiamonophylla*) was meticulously eroded in distilled water, dehydrated, cut into fine pieces and smashed into 50 ml distilled water and filtered through Whatmann No.1 filter paper and further filtered. The extract was stored at 4°C for further experiments [6].

Fabrication of silver nanoparticles from *Atalantiamonophylla*

The aqueous solution of 1 mM silver nitrate (AgNo3) was prepared and used for silver control and test. 10 ml of *Atalantiamonophylla* extract was added into 90 ml of aqueous solution of 1mM silver nitrate incubation at RT for 15 hours Ag-NP formation.

Characterization techniques

Ag nanoparticles were characterized in a Perkin-Elmer UV-VIS Spectrophotometer, Lambda-19 to know the kinetic behavior of Ag nanoparticles. The Scanning range for the samples was 300-700 nm at a scan speed of 480 nm/min [7]

Transmission Electron Microscopy (TEM)

TEM analysis was done to visualize the shape and size of synthesized Ag-NPs. The sample was dispersed in double distilled water. A drop of dispersion was placed on a "staining mat". Carbon coated copper grid was inserted into the drop with the coated site upwards after about 10 minutes, the grid was removed and air dried, then screened in JEOL, JEM 2100 transmission electron microscope.

Energy Dispersive X-ray (EDX) Analysis

EDX analysis was conducted in above instrument attached with thermo EDX to confirm the presence of different elemental composition of the sample. The synthesized Ag-NPs were isolated by centrifugation for 20 min at 10,000rpm. The pellets were collected and dried in the in the oven at 50°C to remove any excess water, cooled to room temperature and observed for EDX analysis [8].

Antibacterial Activity

The mechanism of cellular toxicity exhibited by metal nanoparticles is through the release reactive oxygen species (ROS) (Nel *et al.*,2009). The antibacterial properties of silver nanoparticles are associated with slow oxidation and liberation of Ag+ ions to the environment making it an ideal biocidal agent. Silver nanoparticles are found to penetrate into the bacterial cells causing membrane damage and ultimately the death of the organism [9].

Media Preparation

Table 1. Nutrient agar medium of 150ml

S.no	Name of the materials	Quantity
1.	Peptone	0.75g
2.	Yeast extract	0.45g
3.	Beef extract	0.45g
4.	Nacl	0.45g
5.	Agar-Agar	3.5g

The antibacterial activities of the synthesized Ag-NPs obtained from the leaf extract of *Atalantiamonophylla* was tested for its antibacterial potent against Gram positive

bacteria, such as *Bacillus subtilis, Staphylococcusaureus* and Gram-negative bacteria, Such as *Pseudomonas aeruginosa, Escherichia coli* were determined by following the agar plate well diffusion method [10]. An incubated antibiotic assay by the agar well diffusion method. The agar plates were seeded with freshly prepared different pathogens. Agar wells with diameter of 6 nm were made with the help of a sterile stainless-steel core borer. The wells were labeled as A, B, C, D. The wells A and B were loaded with 80µL aqueous plant extract of *Atlantiamonophylla* and AgNO3: C well was loaded with 80µL contain 20µg of the gentamicin. The D well was loaded with 80µL of Ag-NPs. The plates were incubated at 37ºC for 24 hours and the zone of inhibition (ZOI-:mm) appearing around the wells was recorded. The microbial activity of Ag-NPs was recorded by the determination of minimum inhibitory concentration (MIC). The selected four different bacterial suspensions were prepared and seeded on the agar medium represented in Table1. Then 0µl, 20µl, 40µl, 60µl, and 80µl of Ag-NPs were loaded to the respective wells. All the plates were incubated at 37ºC for 24 hours, and the MIC concentration was recorded [11].

Preparation of Cellulose Fiber

Scaphiumsp. wood samples were obtained from furnishing shop kajang, Selangor. The sample first been dried directly to sunlight and cut into a small piece. Next, the small pieces of wood chips were ground into powder using grinder. Then sawdust was dried overnight in vacuum oven at 100ºC before using. Then the small pieces of wood chip have been ground and prepared for drying process [12].

Preparation of Film:

Saw dust extract has been prepared by taking 20g of saw dust dissolved in 150ml of distilled water boil it for 20 minutes. After that filter it through filter paper to remove undissolved residue in which 25ml of saw dust extract were dispersed into 5g of PVA in a 100ml beaker. The mixture was magnetic stirred on a hot plate at 50ºC in the open atmosphere. All the mixture has been stirred with speed of 3 and duration in 1.5 hours. A dark and viscous wood suspension with about 5wt % wood concentration were obtained. After the dissolution process, the brownish liquid is obtained. Then the liquid was poured in Petri plate for both control and test. In control only extract of saw dust and PVA. In test extract of saw dust, PVA and bio synthesized AgNPs were added [13].

Preparation of AgNPs Loaded PVA Film:

10 wt.% PVA (Polyvinyl alcohol) solution was used for film formation prepared by stirring using magnetic stirrer (1KA C-MAG HS10 digital).2.5g of PVA in 25 ml of distilled water are used as control. 2.5g of PVA in 5ml of AgNPs and 20ml of distilled water as test. All the prepared solution is casted on the 10×10cm sheet plate and allowed to dried for 24hrs and dried AgNPs loaded PVA film is used for further characterization and wound healing [14].

Antibacterial Test for Prepared Films:

20 ml of prepared agar is poured in Petri plate. After solidification agar surface is streaked with bacterial strain by cotton swab. Then the prepared films are placed on the surface of agar and incubated at 37ºC for 72 hours and observed for zone of inhibition [15].

Invitro Cytotoxicity

Cell Culture Maintenance

Vero (African green monkey kidney normal cell line) was obtained from the national center for cell sciences (NCCS), Pune, India. Cell were maintained in the logarithmic phase of growth in Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS),100µg/ml streptomycin. They were maintained at 37ºc with 5% CO2 in 95% air humidified incubator [16].

Cytotoxicity Effect

MTT (3-(4.5-dimethylthialzol-2-yl)-2,5)–diphenyl-terazolium bromide assays were used to assess the sample's cytotoxicity against the Vero cell line (Mossman,1983). The cell was implanted in microplate (1 10 cell/well) and incubated at 37 ^{o}C for 48 hours in a 5% Co2 incubator, allowing it to grow to 70-80% confluence [17]. The medium was then supplemented, and the cells were incubated for 24 hours with different concentrations of sample (20, 40, 60, 80, and 100 g/ml). After 24 hours, the morphological changes of untreated (control) and treated cells were captured on camera under a digital inverted microscope (40x magnification). The cells were then washed with phosphate buffer saline (PBS, Ph-7.4) and 20l of MIT solution (5mg/ml) in PBS was got to add to each well. The plates were then stranded in the dark at 37oC for 2 hours. The formazan crystals were dissolved in 100l DMSO, and the absorbance at 570nm was measured spectrophotometrically. The equation was used to determine the proportion of cell viability [18].

Cell viability (%) = (absorbance of sample /absorbance of control) ×100.

In-vitro scratch wound healing assay:

This experiment was performed according to the previously reported and standardized protocol (Liang et al., 2007). Vero cell were seeded in 6-well plates (8 × 10^5 cells/well) and grown until reached a confluence of 90-95%, in the optimum culture conditions [19]. In the middle of cell monolayer, a scratch was made by a P10 pipette tip, to mimic a wound, and cell debris were removed by washing with fresh medium. The wound was exposed with mouthwash sample (50µg/ml) for 24-48 h at 37°C in a humidified atmosphere of 5 % CO_2. The negative control cells were maintained without any treatment. Scratch wound closure was analyzed in two modalities [20]. Under the digital inverted microscope, by taking digital images at time zero (T0), 24 hours (T2), and 48 hours (T3) (static imaging). Using the ImageJ processing software, the difference between the wound widths at T0 and T1/T2 was used to quantify the scratch closure. The scratch closure rate (SCR) was calculated using the formula described by Felice et al. (2015): SCR = ((T0 – T1/T2) / T0) 100, where T0 represents the scratch area at time zero and T1/T2 represents the scratch area at 24 and 48 hours [21].

RESULTS & DISCUSSION

Synthesis of Ag nanoparticles by green synthesis process (from Atalantiamonophylla):

Because of the excitation of surface plasmon resonance, Ag nanoparticles in aqueous solution have a yellowish-brown color. When the extract is mixed with an aqueous solution of Ag ion complex. The colorless to yellowish brown transition was observed [22]. It was caused by a decrease in Ag+, indicating the formation of Ag represented in Fig 1.

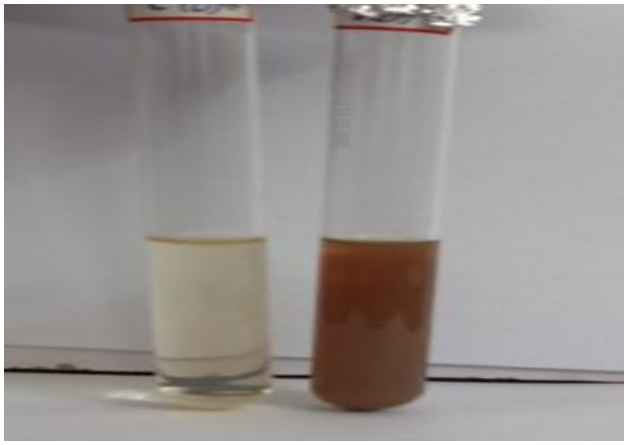


Fig 1. Synthesis of Silver Nanoparticles

UV –Visible Spectroscopy

Aqueous extract treated with 1mM silver nitrate changed colour from pale yellow to dark brownish. This was due to AgNPs activating surface plasmon resonance (SPR). The UV-visible spectra confirm this [23]. The contrast enhancement of the peak in the absorption spectra of AgNPs at 430nm after 24 hours of incubation indicated that the particles are monodispersed represented in Fig 2.

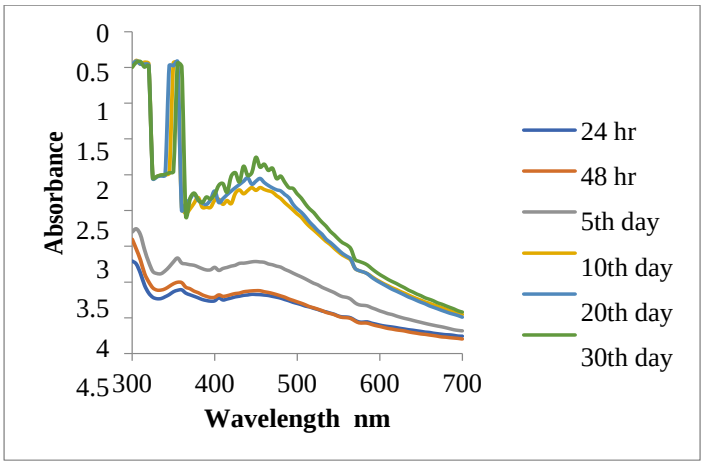


Fig 2. UV-Spectroscopy peaks indicating the formation of monodispersing of AgNPs

Stability of Silver nanoparticles (AgNPs)

The stability of the formed nanoparticles was also studied using UV-visible spectroscopy at various time intervals (0 hours- 1 month) [24]. It was discovered that as contact time is increased, the peak becomes shaper. Within 6 hours, the reaction had begun, and the SPR showed a peak at 420 nm. Following that, the peak moved from 426 nm at 12 hours to 428 nm at 18 hours. After 24 hours, the reaction ended with an absorption peak at 430 nm mentioned in Table 2. The sharper peak indicated the formation of monodisperse nanoparticles from *Atalantiamonophylla* extract [25].

Table 2. Represents the different peaks of AgNPs up to 14^{th} day

S.no	Time	Wavelength (nm)
1	0 hrs.	0
2	24hrs.	430
3	48 hrs.	440
4	5^{th} day	460
5	14^{th} day	450

Transmission Electron Microscopy (TEM)

The particles size, distribution, shape and morphology of silver nanoparticles (AgNPs) were characterized using Transmission electron microscopy (TEM).The TEM analysis revealed that the synthesized AgNPs were mostly spherical in shape and well dispersed in nature with a particle size range of 30 to100nm represented in Fig 3.TEM provides the accurate size of the metallic part of the nanoparticles [26].

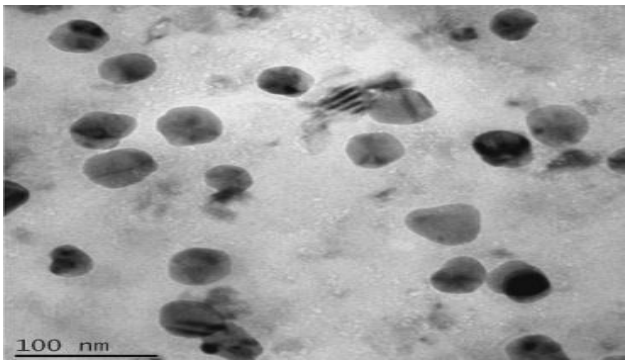


Fig 3. The TEM analysis of AgNPs

Energy Dispersive X-ray Analysis:

The reduced silver nanoparticles were subjected to EDX analysis with an optical adsorption characteristic peak at 3KeV represented in Fig 4 [27].

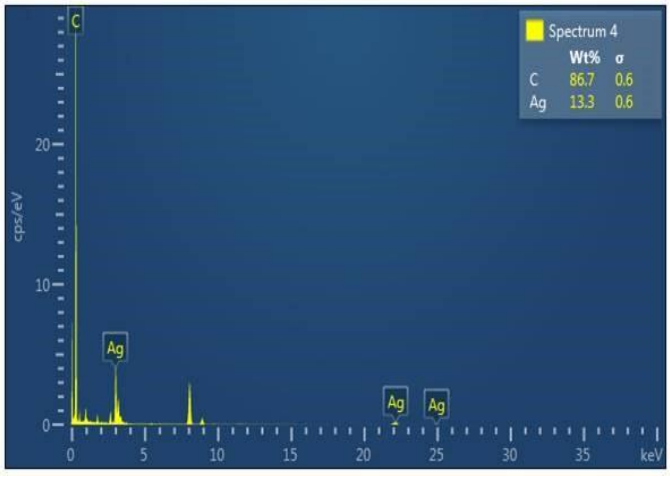
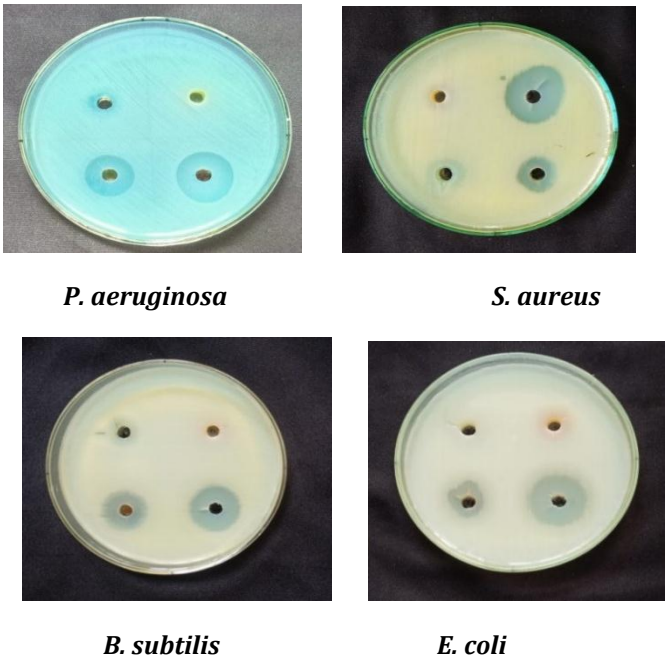


Fig 4. Represents the EDX of AgNPS

Antibacterial Activity of Synthesized AgNPs:

Biosynthesized AgNPs were studied for their antibacterial activity against different pathogenic bacteria by following standard method, and ZOI was recorded among the pathogens. Anti-bacterial tests against gram positive bacteria namely *Bacillus subtilis, Staphyloccocusaureus* and gram-negative bacteria namely Escherchia *coli and Pseudomonas aeruginosa*all. In the well plates, sample was injected plates were kept at 37 ºC for incubation 24 hrs. [28]. Then the zone of inhibition is measured for four pathogens is shown in Table 3 and Fig 5.



P. aeruginosa ***S. aureus***

B. subtilis ***E. coli***

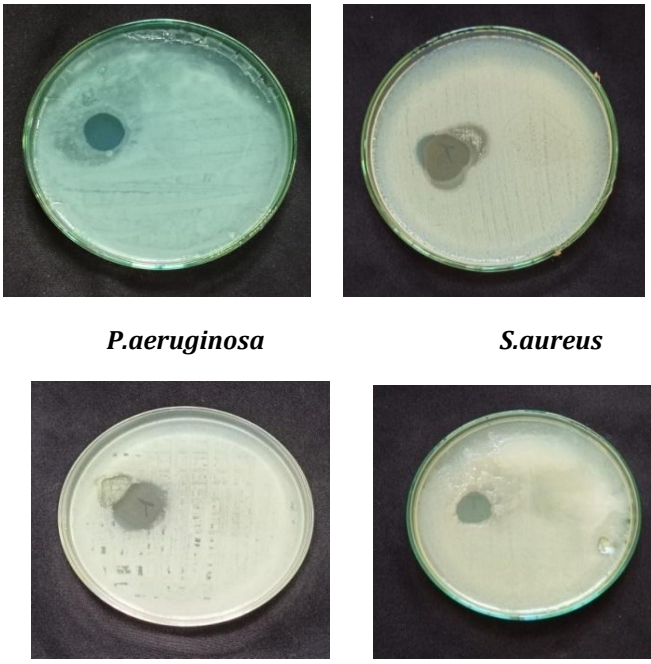
Figure 5. Zone of Inhibition Antibacterial Activity of Synthesized AgNPs

Table3: Antibacterial activity of fabricated AgNPs

S.no	Name of the organism	Zone of inhibition (mm in diameter)			
		AgNPs	plant extract	Positive control	AgNO3
1.	*Staphylococcus aureus*	1.7	_	_	2.6
2.	*Psedomonasaeruginosa*	2	_	_	2.3
3.	*Bacillus subtilis*	2	_	_	2.2
4.	*Escherichia coli*	1.8	_	_	2.2

Antibacterial activity for Impregnated silver nanoparticles:

Then the prepared films are placed on the surface of agar and incubated at 37ºC for 72 hours and then zone of inhibition is measured and represented in Fig 6 and Table 4 [29].



P.aeruginosa ***S.aureus***

B.subtilis ***E.coli***

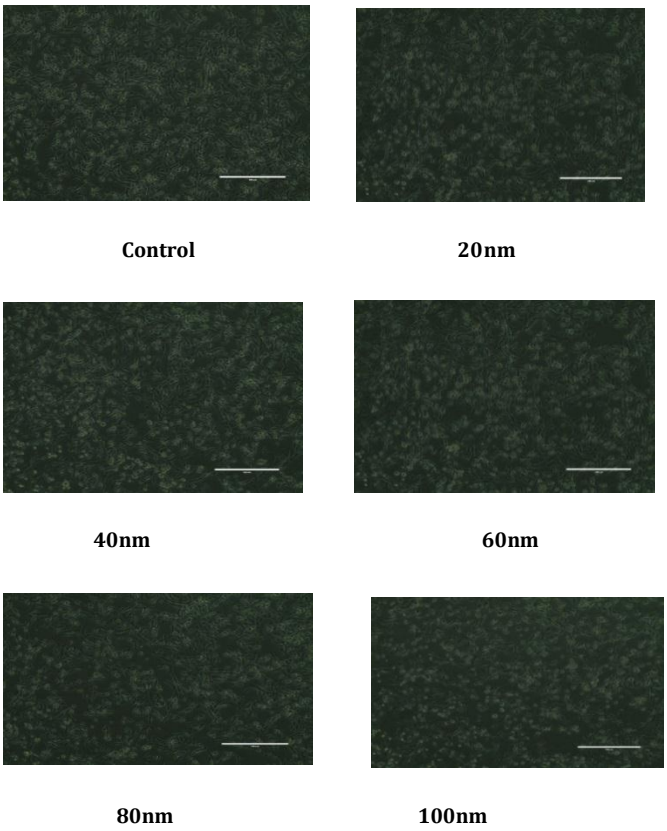
Fig 6. Zone of Inhibition Antibacterial Activity of Impregnated silver nanoparticles

Table 3. Antibacterial activity for Impregnated Silver Nanoparticles

S.no	Human Pathogenic bacteria	Zone of inhibition (mm in diameter)	
		Control	Test
1.	Bacilussubtilis	_	2.6
2.	Staphylococcus aureus	_	2.3
3.	Eschericha coli	_	2.2
4.	Pseudomonas aeruginosa	_	2.2

Cytotoxicity Against 3T3 Fibroblast Cells

The cytotoxicity effect of AgNPs was tested against 3T3 fibroblast cell line by MTT. The concentrations taken for control,20,40,60,80,100 for the cell viability is shown below Fig 7, Fig 8 and Table 4 [30].



Control **20nm**

40nm **60nm**

80nm **100nm**

Fig 7. Cytotoxicity against 3T3 fibroblast cells against different sizes of AgNPs

Table 4. Cytotoxicity at Different Concentrations:

S.no	Concentrations (µg/mL)	Absorbance			Average	% cell viability
1.	Control	0.745	0.742	0.767	0.751	100
2.	20	0.741	0.748	0.747	0.745	99.201
3.	40	0.679	0.683	0.687	0.683	90.905
4.	60	0.625	0.621	0.626	0.624	83.052
5.	80	0.578	0.569	0.573	0.573	76.308
6.	100	0.523	0.531	0.536	0.53	70.541

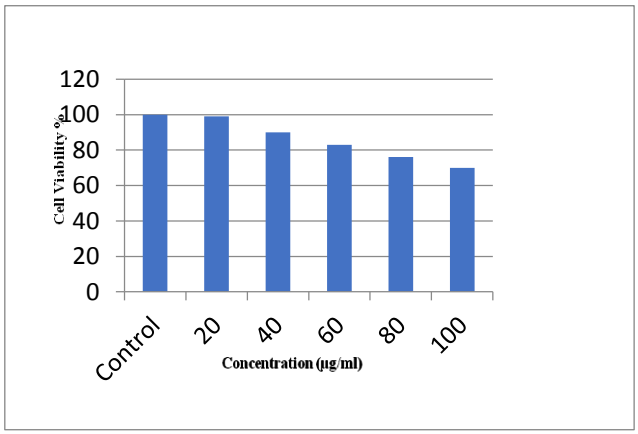


Fig 8 - Cytotoxity Concentration and Cell Viability %

CONCLUSION

In the present investigation, The AgNPs was prepared by green synthesis process from $AgNO_3$ solution through the extract from the leaves of plant *Atlanta monophyla* and impregnated the silver nanoparticles with cellulose fibers for wound healing activity. From the UV Spectroscopy, the formation of stable nanoparticles was confirmed between the 300nm to 700 nm. It was found that *Atlanta monophyla was* stable showing the absorbance of 430nm. The size of AgNPS and different elements in sample composition was confirmed by TEM and EDX respectively. Antibacterial activity of synthesized silver nanoparticles and impregnated AgNPs was carried out in liquid growth medium against four pathogens conducted and it was found to be more antibacterial effect when compare to control. The bio composites were prepared by using conventional techniques and its invitro cytotoxicity was evaluated by MTT in 3T3 fibroblast cell line was found to be 99.20 % viability with a concentration of 20µl/ml. Finally, we conclude the RC-F-Ag bio composite prepared from plant *Atalantia monophylla* was consider may be a potential candidate as a wound dressing material for treating the wound**.**

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