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Green Synthesis of Camellia Sinensis-Mediated Silver Nanoparticles Incorporated Hyaluronic Acid, Poly Vinyl Alcohol and its Antimicrobial Activity Against Oral Pathogens and its Biocompatibility Through Embryonic Toxicology Evaluation, Cytotoxic Effect and Cell Line Study - An in Vitro Study

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KEYWORDS

Plants, Herbs, Spice, Embryonic toxicology, silver nanoparticles

ABSTRACT:

The study investigates the synergistic potential of a novel nanocomposite combining green tea extract, silver nanoparticles (AgNPs), and hyaluronic acid (HA) for its antioxidant, anti-inflammatory, and antimicrobial properties. Green tea, rich in polyphenols, offers potent antioxidant and anti-inflammatory effects, while silver nanoparticles are renowned for their broad-spectrum antimicrobial activity. Hyaluronic acid, a biocompatible polymer, serves as a stabilizing and bioactive agent, enhancing the biocompatibility and efficacy of the nanocomposite. The synthesis of the green tea-AgNP-HA nanocomposite was optimized using a green synthesis approach, ensuring eco-friendliness and minimizing toxicity. Comprehensive in vitro assays demonstrated significant antioxidant activity, as indicated by DPPH radical scavenging, along with notable anti-inflammatory effects in LPS-stimulated macrophage models. Additionally, the nanocomposite exhibited robust antimicrobial efficacy against common oral pathogens, including Streptococcus mutans and Candida albicans. These findings suggest that the green tea-AgNP-HA nanocomposite holds promise as a multifunctional therapeutic agent in the management of oral diseases, with potential applications in preventing and treating infections, reducing inflammation, and promoting tissue regeneration. Further in vivo studies are recommended to explore its clinical efficacy and safety.

1. Introduction

Several plants, herb and spice extracts, as well as essential oils have shown antimicrobial activity. It has been demonstrated that different doses of these culinary herb and spice extracts in the culture medium suppress the growth of certain bacterial strains. Alkaloids, phenolics and polyphenols, terpenoids and essential oils, lectins and polypeptides are some of the groups of phytochemicals that have antimicrobial properties(1). Comparing phenolic and polyphenols to other phytochemical classes reveals that they have several antibacterial modes of action. They have the ability to combine with nucleophilic amino acids in proteins to produce an irreversible complex that inactivates the amino acids and causes bacteria to stop functioning(2). It has been observed that phenolic and polyphenols damage microbial membranes, deactivate microbial enzymes, and boost macrophage activity to initiate an

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immunological response(3). Green tea is a widely consumed beverage, particularly in Asian nations, but it's renown is spreading over the world. The Advantages of green tea for health come from the leaves of the It is only recently that scientists have started to investigate the possible use of green tea for infection prevention and antimicrobial therapy(4). The specific characteristics of the tea's catechins have demonstrated potential as antibacterial agents. (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), and (-)-epigallocatechin-3-gallate (EGCG) are the four primary catechins (polyphenols) present in green tea(5). Diverse antibacterial processes have been demonstrated by these catechins. Studies on the antibacterial properties of green tea have demonstrated that there is promise for both therapeutic and preventive uses. Nanoparticles (NPs) have a lot of catalytic activity, which is useful for chemical processes in both industry and research purposes. Energy conversion and storage, chemical manufacture, biological applications, and environmental technologies are just a few of the sectors where nanoparticles are used(6). Hyaluronic acid ,a naturally occurring carbohydrate polymer, possesses excellent bioadhesive properties, which is an ex- cellent material mostly used in medicine and cosmetic industry. As a natural carbohydrate polymer generally present in the extracellular matrix, HLA exhibits outstanding biocompatibility in comparison to synthetic polymers. It has been reported that HLA based biomu- coadhesive microparticles possess the enhanced bio mucoadhesive property in vitro and much longer pulmonary retention and reduced systemic exposure in vivo(7). And hydrogels formed by HLA can adhere well to the skin surface, enhance the skin hydration and improve drug permeation time. Therefore, it may be an interesting idea, that HLA can be used as an alternative matrix of nanocrystals-based hydrogel (NC-gel). Nanotechnology delivers several novel approaches for regenerative medicine Recently, numerous biocompatible self-assembling nanoparticles were developed(8). NPs boost deferred wound recovery and injury treatment. The metal NPs, such as zinc oxide, gold, and silver, have displayed favorable properties, such as less in vivo toxicity and bacte-riostatic and bactericidal activities.

2. Materials and Methods

Green tea (AgNPs)

Preparation of Plant Extract

1 g of green tea powder was weighed and mixed with 100 mL of distilled water. The plant extract was kept in the heating mantle for boiling the extract with 50°C for 15 - 20 mins. After the boiling extract was filtered using muslin cloth and the filtered extract was separated. The plant extract was used to synthesize nanoparticle solutions.

Figure 1: Depicts the weighting of green tea powder and placement in the orbital shaker

Preparation of Silver Nanoparticles

2 mM of silver nitrate was weighed and mixed with 80 mL of distilled water. The silver nitrate solution was mixed with 20 mL of plant extract. Both the solutions were mixed and placed in the orbital shaker for 48 hrs in 400 rpm. The synthesized silver nanoparticle was centrifuged at 8000 rpm for 10 mins. After the completion of centrifugation, the pellet was collected in the separate sterile tubes for the use of further biomedical applications.

Figure 2: Depicts the preparation of green tea-Silver nanoparticles along with addition of hyaluronic acid and Poly vinyl Alcohol

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Preparation of Hyaluronic acid + AgNPs

500 mg of Hyaluronic acid was weighed and mixed with 25 mL of distilled water. The solution was mixed and kept in the clear solution. 5 mL of hyaluronic acid solution and 5 mL of AgNPs was mixed well. The solution was placed in the sonicator for 30 mins. After the completion of the sonication process, the AgNPs and hyaluronic acid solution was kept in the orbital shaker for 24 hrs. The AgNPs and hyaluronic acid solution was used for further biomedical application.

Figure 3: Depicts the prepared Green tea - AgNP - Hyaluronic acid - Polyvinyl Alcohol

Procedure

The antimicrobial activity of the green synthesized silver nanoparticles was evaluated using the agar well diffusion technique. Mueller Hinton agar plates were prepared and sterilized using an autoclave at 121°C for 15- 20 minutes. After sterilization, the medium was poured onto the surface of sterile Petri plates and allowed to cool to room temperature. The bacterial suspension (Streptococcus mutans, Lactobacillus sp, Staphylococcus aureus, Enterococcus faecalis, Candida albicans) was spread evenly onto the agar plates using sterile cotton swabs. Wells of 9 mm diameter were created in the agar plates using a sterile polystyrene tip. The wells were then filled with different concentrations (25, 50, 100 µg/mL) of AgNPs. An antibiotic (e.g., Bacteria-Amoxyrite, Fungi-Fluconazole) was used as a standard. The plates were incubated at 37°C for 24 hours and 48 hours for fungal cultures. The antimicrobial activity was evaluated by measuring the diameter of the inhibition zone surrounding the wells. The diameter of the zone of inhibition was measured using a ruler and recorded in millimeters (mm) and the zone of inhibition was calculated.

Time-Kill kinetic Analysis:

A time-kill curve assay was conducted to assess the bactericidal properties and concentration-dependent relationship between Greentea-mediated silver nanoparticles and the net growth rate of and Staphylococcus aureus, Streptococcus mutans, Lactobacillus species, Enterococcus recalls and Candida albicans over regular time intervals. The assay involved culturing the three wound pathogens in Mueller Hinton Broth supplemented with varying concentrations of silver nanoparticles (25, 50 and 100 µg/ml), followed by time-kill curve analysis. An antibiotic (e.g., Bacteria-Amoxyrite, Fungi-Fluconazole) was used as a standard. After a pre-incubation period of four hours in a medium devoid of any antimicrobial agents, growth curves were carried out before the test to ensure that all pathogens had reached a stable early-to-mid log phase. An inoculum consisting of 0.5 McFarland of each pathogen was created in sterile phosphate-buffered saline. This inoculum was collected from cultures that had been cultivated on Mueller Hinton agar plates at 37 °C for 18–20 h. After that, 30 µL of the inoculum was diluted in 15 mL of antimicrobial-free Mueller Hinton Broth medium that had been pre-heated to 37 °C, and 90 µL of the resultant mixture was distributed evenly over each well of a 96-well ELISA plate. To each well containing 90 µL of pre-incubated wound pathogens, 10 µL of Green tea - mediated silver nanoparticles at five different concentrations was added, along with the untreated control**.**

3. Results

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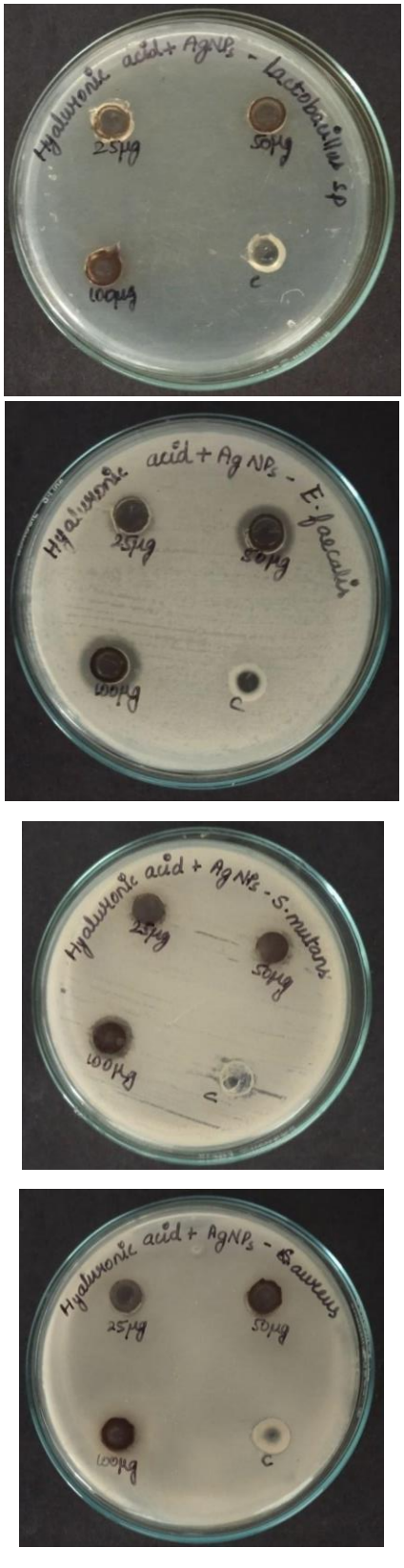

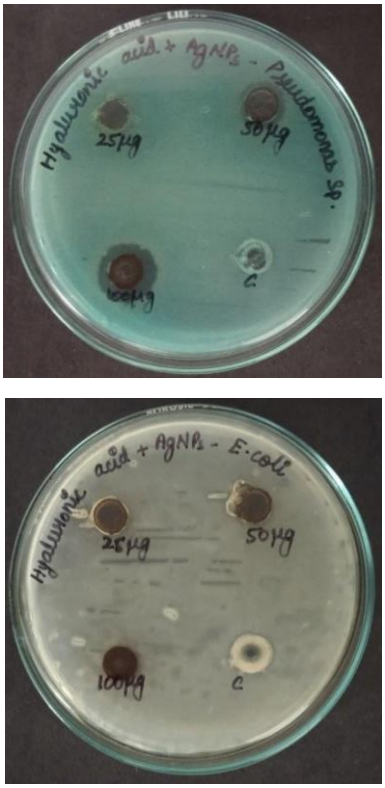

Figure 4: Petri dish showing zone of inhibition of Hyaluronic acid, polyvinyl alcohol and Green tea induced with Silver nanoparticles in different antimicrobial organisms

Table 1 depicts the anti microbial activity by the values of zone of inhibition of Hyaluronic acid, poly vinyl alcohol and Green tea induced with Silver nanoparticles in different organisms

Organism	25 µg/mL	50 µg/mL	100 µg/mL	Control
S. aureus	9	9	9	9
S. mutans	9	10	12	9
E. faecalis	11	14	16	9
Lactobacillus sp.	9	9	9	9
C. albicans	9	9	9	9
E. coli	9	9	9	9

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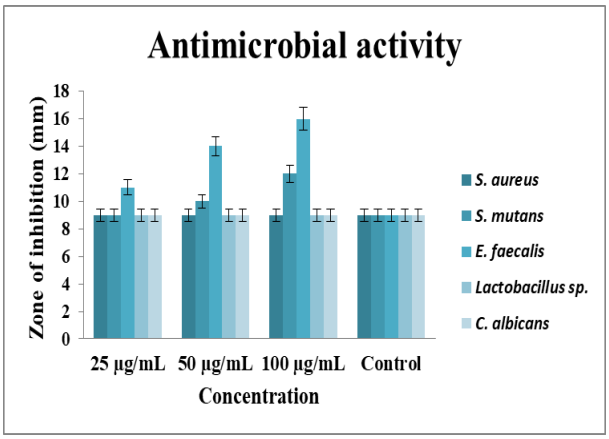
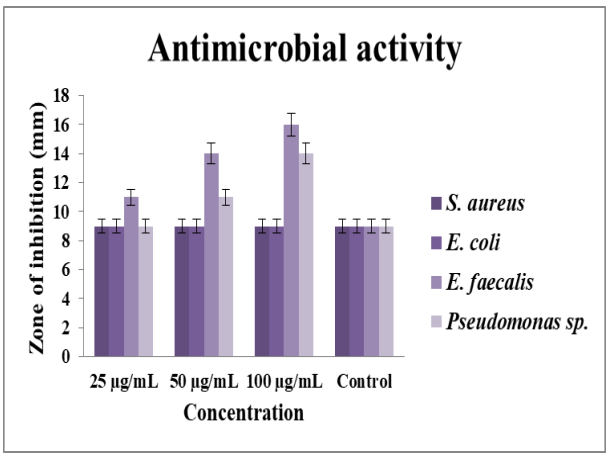
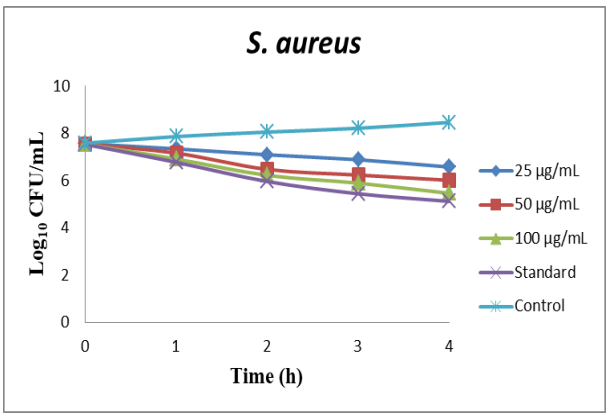
Pseudomonas sp.	**9**	**11**	**14**	**9**

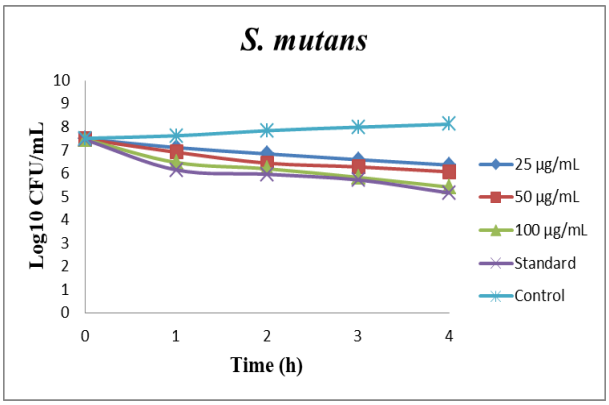


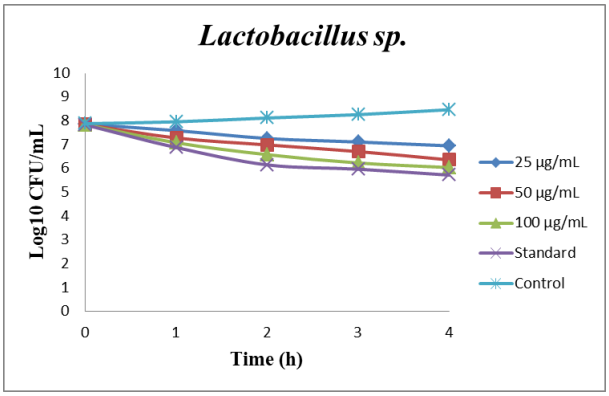


Graph 1 and Graph 2 depicts the Antimicrobial activity of hyaluronic acid, poly vinyl alcohol and green tea induced in silver nanoparticles

From the Figure4, Table1 and Graph1 it is shown that E. faecalis has more zone of inhibition in 100 µg/mL of inoculation. Staphylococcus aureus has 9mm of zone of inhibition in 25 µg/mL, 50 µg/mL, 100 µg/mL and control. There is the same level of zone of inhibition under different concentrations of green synthesized silver nanoparticles. Streptococcus mutans have 9mm of zone of inhibition in 25 µg/mL, 10mm in 50µg/mL, 12mm in 100µg/mL and 9mm in control. So S.mutans has a high zone of inhibition of 12mm in 100µg/mL inoculation of plant extract with silver nanoparticles. Enterococcus faecalis has 11mm of zone of inhibition in 25 µg/mL, 14mm in 50µg/mL, 16mm in 100µg/mL and 9mm in control. Enterococcus faecalis has good antimicrobial activity among all organisms. Lactobacillus species Candida albicans and E.coli have 9mm of zone of inhibition in 25 µg/mL, 50 µg/mL, 100 µg/mL and control. There is the same level of zone of inhibition under different concentrations of green synthesized silver nanoparticles. Pseudomonas species have 9mm of zone of inhibition in 25 µg/mL, 11mm in 50µg/mL, 14mm in 100µg/mL and 9mm in control. Enterococcus faecalis and Pseudomonas species have good antimicrobial activity among all organisms.







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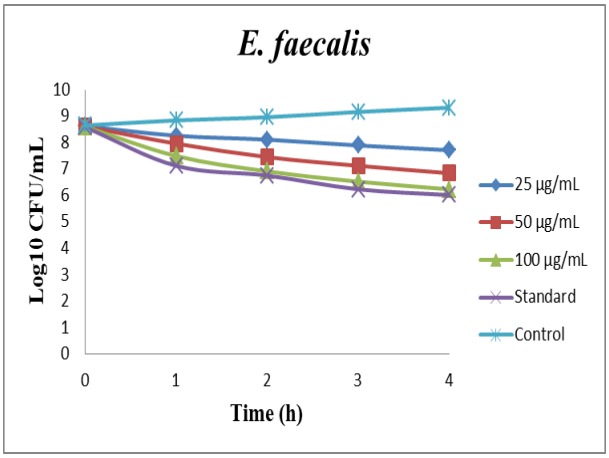

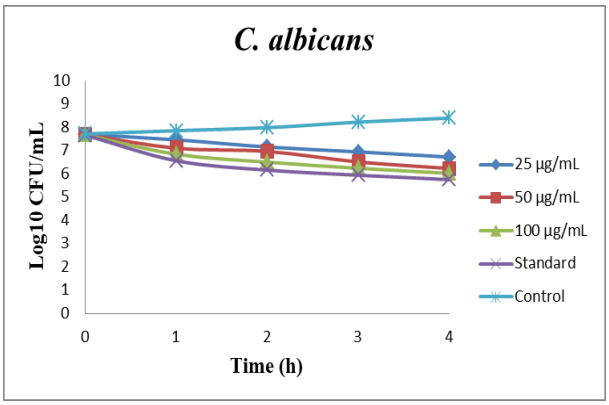

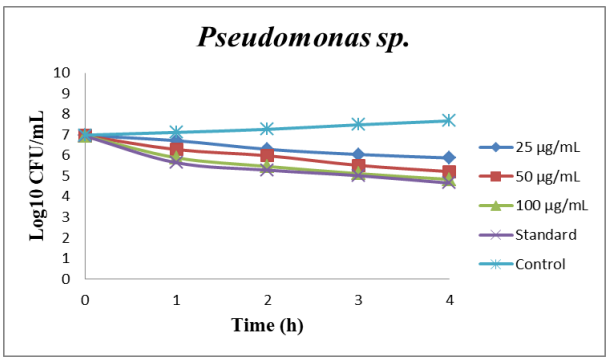

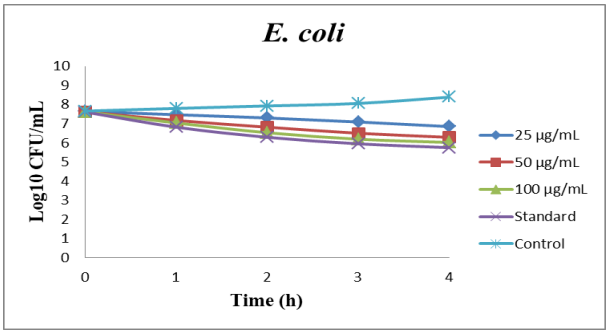

The above mentioned graph shows the Time-Kill kinetic Analysis of hyaluronic acid, poly vinyl alcohol and green tea induced in silver nanoparticles against individual species.

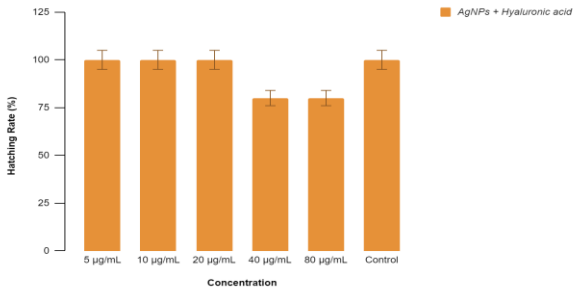

Table 2 Depicts the compares the hatching rate of live nauplii in the Hyaluronic acid, PVA and Green tea induced with Silver nanoparticles combination.

AgNPs + Hyaluronic acid		
	Viability rate (%)	
	Concentration	Viability rate (%)
	5 µg/mL	100
	10 µg/mL	100
	20 µg/mL	100
	40 µg/mL	100
	80 µg/mL	80
	Control	100

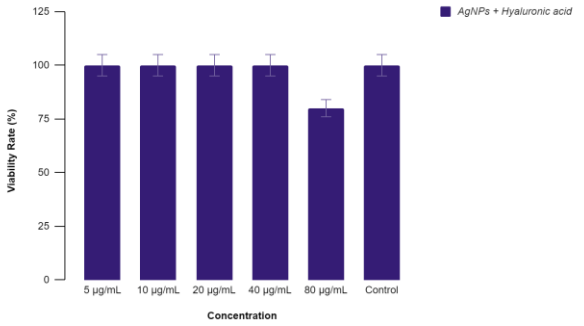

Table 3 Depicts the compares the viability rate of live nauplii in the Hyaluronic acid, PVA and Green tea induced with Silver nanoparticles combination.

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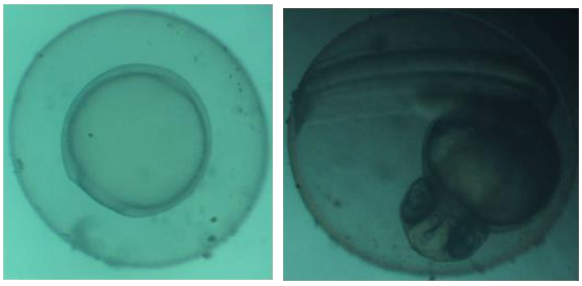


Day 1 **Day 2**

Day 3

Figure 5: Depicts the embryonic growth of live nauplii on day 1, day 2 and day 3 respectively.

Cytotoxic Effect

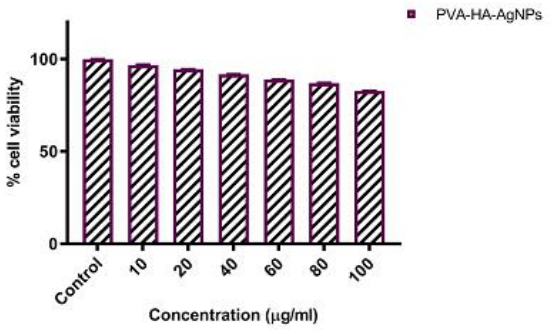
Concentration	Day 1 (% of live nauplii)	Day 1 (% of live nauplii)	Day 2 (% of live nauplii)
5	**100**	**100**	**100**
10	**100**	**100**	**100**
20	**100**	**100**	**100**
40	**100**	**100**	**100**
80	**100**	**100**	**100**
Control	**100**	**100**	**100**

Table 4 Depicts the cytotoxic effects of live nauplii of different concentrations against the Hyaluronic acid, PVA and Green tea induced with Silver nanoparticles combination

Concentration (g/ml)	% Cell Viability
Control	99.960.21
10	96.660.49
20	94.410.20
40	91.700.21
60	88.940.0
80	86.920.32
100	82.730.22

Data expressed in terms of MeanSEM (n=3)

Table 5 showing the percentage cell viability of 3T3L1 fibroblast cells treated with different concentrations of PVA-HA-AgNPs gel



Data expressed in terms of MeanSEM (n=3)

Bar graph showing the percentage cell viability of 3T3L1 fibroblast cells treated with different concentrations of PVA-HA-AgNPs gel

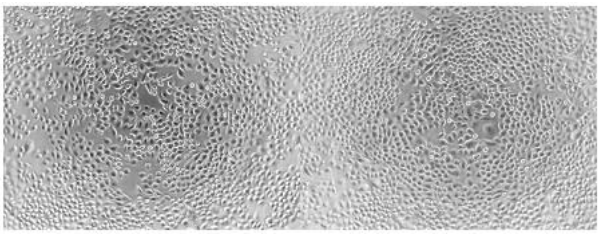


Figure 6 represents the microscopic images showing morphological changes in the 3T3L1 fibroblast treated with different concentrations of PVA-HA-AgNPs gel, magnification 10x

Biocompatibility Study

Cytotoxicity Evaluation by MTT Assay

3T3-L1 mouse fibroblast cells, sourced from the National Centre for Cell Science (NCCS), Pune, were cultured in 25 cm^2 vented flasks under controlled conditions of 37°C and 5% CO_2 within a humidified

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incubator. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and 1% penicillin-streptomycin (Life Technologies, USA)(15). Once the cells reached approximately 70–80% confluence, they were harvested and seeded into 96-well plates at a density of 3×10^3 cells per well. These cultures were incubated for 24–48 hours to allow for adhesion and the establishment of a confluent monolayer. A stock solution of the PVA-HA-AgNPs gel (100 mg/mL, w/v) was prepared using dimethyl sulfoxide (DMSO) and diluted in DMEM to achieve final concentrations ranging from 10 to 100 µg/mL. Untreated cells served as the control group. Following the adherence phase, fibroblasts were treated with different concentrations of the test solution and incubated for 24 hours. After incubation, the treatment media was removed, and 50 µL of MTT solution (5 mg/mL) was added to each well(16). The plate was then incubated at 37°C for 2 hours to allow formazan crystal formation. Subsequently, 150 µL of DMSO was added to each well to dissolve the crystals, and absorbance was measured at 490 nm using a TECAN microplate reader. Additionally, to assess cytotoxic effects, morphological changes in fibroblasts were examined under a phase-contrast microscope, and images were captured to document any structural alterations in the treated cells.

Results

The biocompatibility of the PVA-HA-AgNPs gel was evaluated using the MTT assay in 3T3L1 fibroblast cells, with cell viability assessed at different concentrations (10–100µg/mL). The results indicate a concentration-dependent decline in cell viability, suggesting a mild cytotoxic effect at higher concentrations. At 10 µg/mL, cell viability remained high at 96.66 ± 0.49%, showing minimal toxicity. As the concentration increased, a gradual reduction was observed, with viability decreasing to 94.41 ± 0.20% at 20 µg/mL and 91.70 ± 0.21% at 40 µg/mL, still indicating good biocompatibility. At higher concentrations, a more noticeable decline was observed, with 88.94 ± 0.20% at 60 µg/mL, 86.92 ± 0.32% at 80 µg/mL, and 82.73 ± 0.22% at 100 µg/mL, suggesting a mild cytotoxic effect at elevated doses. However, cell viability remained above 80%, indicating that the PVA-HA-AgNPs gel maintains good compatibility with fibroblast cells, especially at lower concentrations. Overall, these findings suggest that the PVA-HA-AgNPs gel exhibits favorable biocompatibility, with only a moderate reduction in cell viability at higher concentrations. These results support its potential for biomedical applications, with lower concentrations (<40 µg/mL) being the most biocompatible for fibroblast cells.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8 software (San Diego, USA). Experimental results are expressed as the Mean ± Standard Error of the Mean (SEM).

4. Discussion

The time-kill kinetic analysis has shown that the Enterococcus faecalis and Pseudomonas species have good antimicrobial activity among all organisms against the AgNP-Green tea-HA-PVA combination. The cytotoxic effects against the live nauplii in the embryonic stage shows good growth of the species with almost equal amount of hatching and viability rate(9). Green tea extracts include unique bioactive components including EGCG and catechins, which have strong bactericidal and inhibitory activity; they demonstrated effective anti-microbial activity against a variety of gram positive strains and fungal strains. Because of their higher surface-to-volume ratio, AgNPs are more powerful at lower doses, reducing their toxicity(10). Silver Nano composites can be combined to create an effective wound dressing for treating microbial infections based on endogenous triggers such as pH, temperature, enzymes, and toxins released by bacteria. Several studies have shown that silver nanoparticles have positive effects in biocompatible and nano- structured materials and devices. Nano particles have an amazing drug carrying ability, hence a great number of new medications have been loaded onto them(11). However, the mechanism of interaction between silver nanoparticles and bacteria, as well as clinical and toxicological investigations, require further investigation. Engagement, alignment, and collaboration on Antimicrobial stewardship among wound care experts, related teams, and governments can help to accelerate progress in the battle against antibiotic-resistant infections in wound care.

When green tea, hyaluronic acid, and silver nanoparticles are combined, they can work synergistically to enhance

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antimicrobial activity. Green tea can aid in the penetration of silver nanoparticles into microbial cells, while hyaluronic acid can provide a stable medium for the delivery of silver nanoparticles, potentially increasing their efficacy(12). This combination could be particularly useful in developing antimicrobial coatings, wound dressings, or skincare products aimed at reducing bacterial load and preventing infections.

Enhanced wound Healing by reducing microbial load, minimizing inflammation, and supporting tissue repair. Reduced Risk of Infection, this combination offers a comprehensive antimicrobial strategy that targets multiple types of pathogens, reducing the risk of postoperative infections(13). The use of natural components like green tea and hyaluronic acid can help reduce potential cytotoxic effects associated with silver nanoparticles, making the formulation safer for application on surgical incisions.Overall, the combination of green tea, hyaluronic acid, and silver nanoparticles presents a multifaceted approach to managing surgical incisions, offering both antimicrobial protection and enhanced healing properties(14).

5. Conclusion

Antimicrobial resistance is a developing global concern, involving wound care and issues connected to wound biology and treatment. Infections causing non-healing wounds continue to be a severe concern, and clinical data show the existence of biofilm and its relationship to wound chronicity.The green tea extract incorporated with silver nanoparticles, poly vinyl alcohol and hyaluronic acid showed a marked zone of inhibition against gram positive bacterial species and fungal species such as S.mutans , S. aureus, E faecalis, Lactobacillus species,Candida albicans and especially Pseudomonas species and also shows negative embryonic toxic effects and potential at lower concentrations.

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