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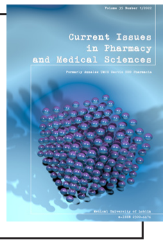

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Acetyl amine xanthan gum-based silver and molecular Iodine nanocomposite for wound healing therapy

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ABSTRACT

Wound healing is the process of repairmen and restoration of structure and function of an injured tissue or skin. It is a complex process and requires safe and effective treatment modalities. Various plant and chemical entities are used in wound management. Iodine is one of them, and a Povidone-Iodine formulation is commonly applied for healing wounds. Silver Nanoparticles have been found to be a powerful weapon against various bacteria, therefore it is also used in wound therapy.

In this research, modified xanthan gum (MXG) based hydrogels of molecular iodine and nano silver were prepared and applied on rats in order to measure wound healing activity. Marketed Iodine-povidone gel was employed as positive control and placebo was negative control. The excision wound model as per OECD guideline 434 was implemented in this study. By tracing the wound on different days, the wound closure rates were assessed.

According to our experimental results, the iodine-silver nanocomposite treated group was found to show significant wound closure activity on day 16, and there was no significant differences observed in wound healing activity between marked Iodine-povidone gel and MXG based hydrogel of iodine. However, significant differences between negative control group and test groups animals were observed. Wound closure of the negative control group was observed on day 24, whereas the group containing only iodine showed closer on day 20. We also noted that the nanocomposite demonstrated significant antimicrobial activity against gram –ve bacteria and gram +ve bacteria. Thus, the gel formulation revealed significant wound healing and antibacterial activity due to the additive effect of molecular iodine & Ag^{++} nanoparticles.

In this experiment, synthesis of Ag^{++} nanoparticles and its gel with iodine using modified xanthan gum was successfully formulated. Iodine-Ag NPs gel are more sensitive to gram –ve bacteria and our work demonstrated that this showed faster wound repair and shorter period of epithelization. It can be concluded that modified xanthan gum based hydrogel of molecular iodine and nano silver can be effectively used in the treatment of wounds.

INTRODUCTION

The disruption of the anatomic & cellular continuity of skin tissue caused by physical, thermal, chemical or microbial agents are called 'wounds' or 'injuries'. Statistically, at any given moment, people around the world experience approximately 100 million traumatic & 300 million chronic wounds [1]. Because the skin is an effective barrier against the external environment and continuance of this barrier is crucial, rapid healing of wound tissue after injury is essential. Wound healing is the replacement of damaged tissue with new living tissue [2]. There are four basic phases of regeneration of wounded tissue – coagulation activation, inflammation phase, proliferation phase and tissue remolding [3]. Wound site coverage with suitable dressings is required for protection from external risk and to accelerate healing processes [4].

Generally, infection and sepsis of wound will be observed due to contamination with pathogenic microbial flora or because the wound has been colonized with microorganisms which disrupt the healing process [5]. Therefore, for wound treatment, various antimicrobial substances are considered. Out of these, iodine has continued to be broadly

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used [6-7]. Iodine has a broad spectrum of activity and its employment comes with several advantages, among others, lack of associated resistance, low cytotoxicity and good tolerability. Iodine also has anti-inflammatory properties along with the ability to penetrate biofilms and has no negative effect on wound healing processes [8].

To develop efficient strategies to accelerate the wound healing, various research groups have worked on different concept. In that, nanotechnology has become an important area of interest due to its unique properties. The size of nano particles typically ranges from 10 nm to 1000 nm, which is much smaller than that which the targeted pathogen presents in damaged cell. Nanoparticles also have high surface to volume ratio which result in high therapeutic efficacy [9]. Ag, Au, Zn and Cu are some of the nanoparticles used in nanotechnology.

In recent years, Ag NPs have been recognized as a powerful weapon against various infectious bacteria [10]. Nano silver can be molded in to desired shapes and bears unique properties such as better permeability by pH and dissolved ions, as compared to routine metal [11]. Ag-NPs are preferred to overcome bacterial resistance with improved antibacterial activity. Ag-NPs adhere to cell walls and by producing free radicles which degrade cell membranes ultimately leading to bacterial cell death.

Various types of hydrogels are being widely studied as wound dressings, but most are inefficient for treating infected and severe chronic wounds or for use in long term patient care, particularly for those with large wound areas [12-13]. Thus, a multifunctional wound healing hydrogel has been proposed for defect therapies.

In this study, the effect of a gel containing iodine -nano silver composites was studied and compared to a standard formulation. Such a combination therapy can lead to the synergistic and more effective response as compared to the individual drugs. Acetyl amine modified xanthan gum was synthesized by treating xanthan gum with chloroacetyl chloride which resulted in an intermediate to which ammonia was reacted. This MXG was prepared for enhancing the gelling property of parent xanthan gum.

MATERIAL AND METHODS

All synthetic chemicals were procured from local distributors and were of Loba Chemie pvt. Ltd, Mumbai, India.

Synthesis of silver nanoparticles

Silver nitrate (170 mg, $AgNO_3$) was dissolved in 1000 ml water. Subsequently, 0.2 ml solution of sodium borohydrate (50 mM, $NaBH_4$) was added drop wise. The mixture was then stirred for further 30 min by centrifugation through 5000 rpm for 20 min, the residual metals were, afterwards, removed. The supernatant solution was then taken for further study [14].

Preparation of Acetyl amine Xanthan gum

Xanthan gum (10 gm) was dispersed in 100 ml of pyridine. Chloro acetyl chloride (5 ml) was subsequently added slowly with shaking. The reaction mixture was then stirred for 2 hours and kept overnight for digestion. The solid product was filtered, washed with alcohol and dried to give chloro acetyl xanthan gum (10 gm). Following this, an ammonia solution (10 ml) was added with shaking. The flask was stirred for 2 hours then filtered and washed with 50 ml alcohol. The solids were collected, dried and used for the preparation of the hydrogel [15].

Preparation of Iodine gel

25 mg potassium iodide was dissolved in 10 ml water. In this resulting solution, 200 mg Iodine crystals was added with stirring. Modified xanthan gum (100 mg) was then brought in, along with 0.2 ml glycerin with stirring to form gel.

Preparation of Iodine – silver nanocomposites

25 mg potassium iodide was dissolved in 10 ml water. In this solution, 200 mg Iodine crystals was then added with stirring. Silver nano particle solution (10 ml) was then introduced into the iodine solution. Modified xanthan gum (100 mg) was subsequently added, along with 0.2 ml glycerin with stirring to form gel.

Characterization

UV visible spectroscopy

To confirm the formation of silver NPs in the solution, it was subjected to UV-Visible spectral analysis in the wavelength range of 200-800 nm.

Particle Size Analysis (PSA) and zeta potential

Particle size and zeta potential of Iodine Ag-NPs composite were determined via HORIBO SZ-100, Particle Size Analyzer.

Physicochemical evaluation of gel

Viscosity

Viscosity of gel was measured using a Brookfield Viscometer (spindle L4 at 100 rpm).

Spreadability

Formulated gel (10 mg) was placed between two slides, with a predefined weight placed on the outside of the top slide. The amount of jell spread was measured so as to determine distance of spread, with spreadability index then calculated [16]:

$$\text{Spreadability} = \text{Weight tied} \times \frac{\text{Length of silde}}{\text{Time in second}}$$

Antimicrobial evaluation

The antimicrobial activity of modified xanthan gum based Iodine-Ag^{++} NPs containing gel was evaluated as per reported method against *E. coli* (*Gram –ve*) and *Bacillus* (*Gram +ve*) bacteria. The bacterial strains were obtained from Department of Microbiology, GIPER, Limb, India. In this study, Ciprofloxacin (5 µg/ml) was used as positive control. The measurements of antimicrobial activity of gel were accomplished by following the agar diffusion method. Under sterile condition, gram +ve and gram –ve bacteria were grown on agar plates. All solutions (0.1 ml) were place in the agar plate cups and the plate were incubated at 37°C for 24 hours. After incubation, the plate were observed for microbial growth. All readings were done in triplicate and an average diameter in the form of zone of inhibition was calculated [17].

Wound healing property of Iodine – AgNPs hydrogel

Wistar albino Rats having weight 150±5 mg were anaesthetized prior to and during creation of the wounds. The excision wound method was used. The excision wound of circular area 500 mm^2 and 0.2 cm depth was created using surgical blade, and pointed scissors on the dorsal fur of rats. The wound was left open for a day. The animals were divided into four groups of six each. Group 1 was negative control, whereas group 2 were topically treated with marketed Iodine-povidone cream as a standard. Group 3 were Iodine-MXG gel (without Ag NPs) treated animals, while group 4 animals were topically treated with MXG based gel of Iodine-silver nanocomposite. The wound closure rate was assessed by tracing the wound on days 0, 2, 4, 8, 12, 16, 18, 20, 22 and 24 using transparency sheets and a permanent marker and calculated by [18]:

$$\% \text{ closure} = 1 \left(\frac{\text{Wound area on corresponding days}}{\text{Wound area on day zero}} \right) \times 100$$

RESULTS

UV visible spectroscopy

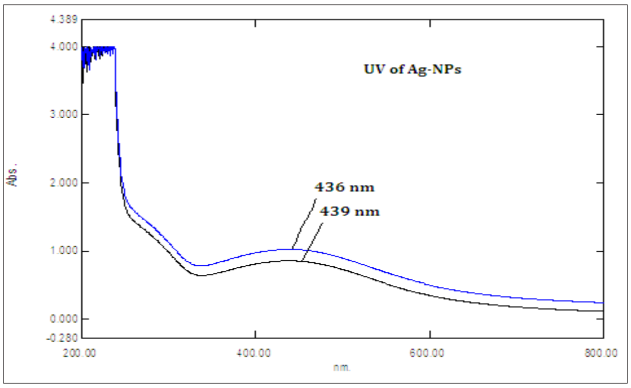

Figure 1. Two UV Visible spectra of silver nanoparticle indicating formation of nanoparticle

Particle size analysis

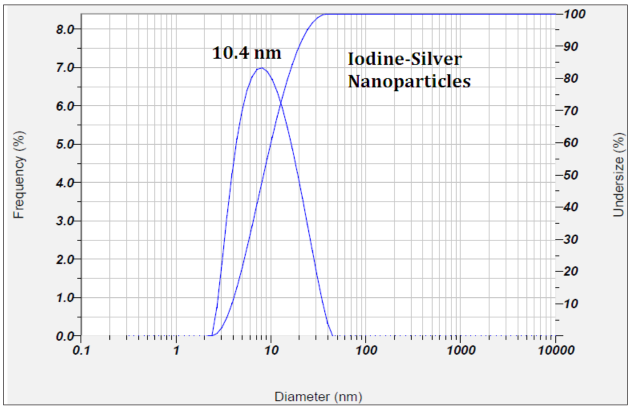

Figure 2. Particle size analysis spectra of Iodine-silver nanoparticle

Zeta potential

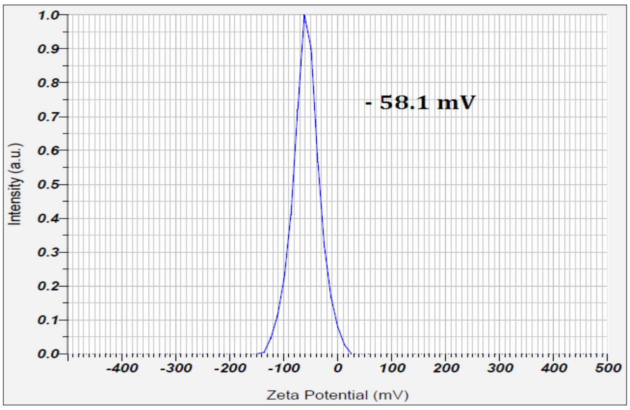

Figure 3. Zeta potential spectra of Iodine-silver nanoparticle

Physiochemical evaluation

Aclear appearance of gel was observed. The color was brownish yellow. The viscosity of nano composite gel was found to be 600±25 cp (L4 spindle). The spreadability of gel formulation was found to be 23.3±0.5.

Antimicrobial activity

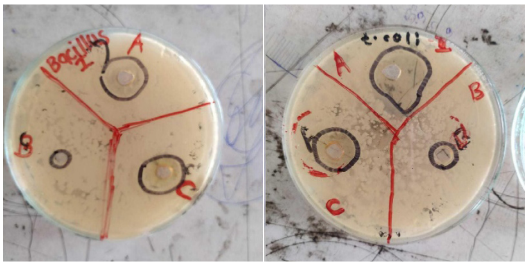

Figure 4. Zone of inhibition of Ciprofloxacin standard (A), Blank (B) & Iodine- Ag^{++}NPs hydrogel (C)

Table 1. Antibacterial activity of Iodine- Ag^{++}NPs hydrogel with respect to *Bacillus* and *E. coli*

Compounds	*Bacillus (gram +ve)*	*E coli (gram –ve)*
Iodine-Ag NPs gel (C)	25±3	27±2
Ciprofloxacin (A)	28±2	30±1
Blank (B)	0	0

Wound healing activity

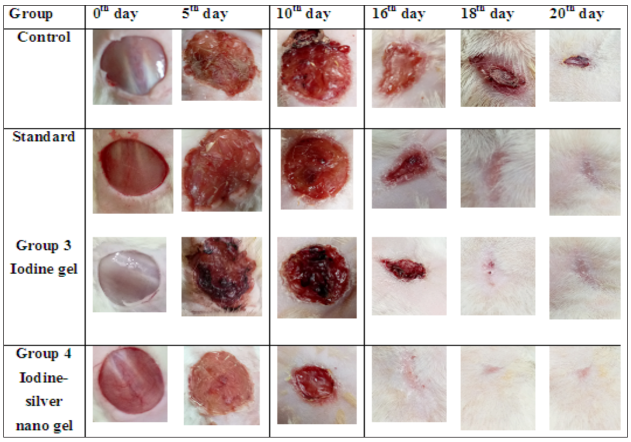

Figure 5. Photographical representation of contraction rate on different days. Healing activity of Iodine-Ag^{++}NPs gel, as compared to marketed iodine-povidone gel as standard, Iodine gel and control

DISCUSSION

There are several ways of characterizing NPs. The most convenient way is through color change of solution. The conversion to a white foggy colour of solution indicate the formation of nanoparticles. No further colour change was observed after several hours of preparation.

UV visible spectroscopy

The formation of Ag-NPs was further validated by UV-Visible spectroscopy. The UV-visible spectrum of Ag NPs solution showed absorption at 436 nm (Figure 1). The position of the λmax may be shifted due to the size and shape of the synthesized silver nanoparticles. Two spectra indicate slight variation in the λ-max due to size of nano particles. However, the λ-max in the UV-visible spectra in between 417 to 440 nm and indicates silver nanoparticle formation. A strong absorption peak is noticed at 438 nm due to the change in the surface plasmon resonance, which is in good agreement with reported data in the literature [19-20]. Due to the Surface Plasmon Resonance in the interaction of electrons and electromagnetic radiation in the conduction band around the nanoparticles, an optical absorption band of λ max value is a typical characteristic of the absorption of metallic Ag-NPs because of the SPR, revealing the presence of Ag-NPs in the solutions [21].

Particle size analysis

Particle size analysis is an important tool for determining nano size in any system. The particle size spectra of the synthesized AgNPs and molecular iodine system reveals a particle size at 10.4 nm, indicating the formation of silver and molecular iodine at nano size (Figure 2).

Zeta potential

Zeta potential explains the stability, dispersion and surface charge of the nanoparticles. A negative sign suggests stability against the aggregation potential. The Zeta potential of the prepared AgNPs- iodine solution was found to be -58.1 mV (Figure 3). This value showed that the prepared AgNPs-iodine system have sufficient charge and mobility to inhibit aggregation.

Physiochemical Evaluation

The nanogel formulation was found to be brownish yellow in nature, and to have good consistency, while the appearance was transparent or clear. Viscosity measurements were carried out using a Brookfield viscometer and ranged between 600-650 cps for 100 rpm (L4 spindle). The viscosity mean and mean error of the created nanogel was found to be 600±25 cps. These viscosity values meet the requirements for gels to be acceptable for marketing. The spreadability value indicate that the derivative gel is easily spreadable. The spreading diameters of the prepared gel were found to be in the range of 23.3±0.5.

Antimicrobial Activity

The result of antibacterial activity testing showed that while Ciprofloxacin was more effective against both gram +ve and gram –ve bacteria, the synthesized Iodine-AgNPs gel had efficient activity against both gram +ve and gram –ve bacteria (Figures 4 and 6, Table 1). The fabricated Iodine-AgNPs gel, however, showed less sensitivity against gram +ve bacteria. The lower sensitivity of silver nanoparticles against gram +ve bacteria is primarily due to the thickness of peptidoglycan layer, which may prevent the transport of those nanoparticles through the bacterial cell wall [22].

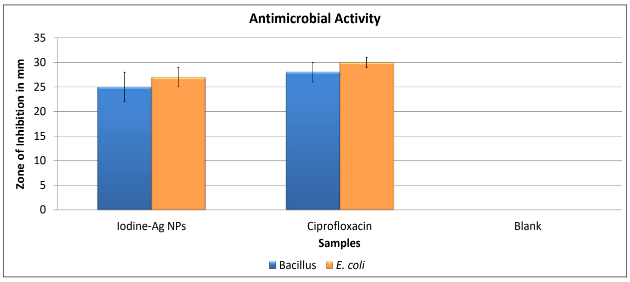

Figure 6. Graph showing antibacterial activity of synthesized nanogel against *Bacillus* (gram +ve) and *E. coli* (gram –ve) strains (n = 3±SD)

The antibacterial mechanism is still not very clear whether the killing is due to any one individual mechanism or due to the combination of more than one mechanism. In this research, it may be due to the combination of the interaction of Iodine-Ag NPs with the bacterial cell wall and simultaneous penetration of Ag^{++} ions inside the bacterial cells. We theorize that the Ag^{++}NPs joined to the microbial cell surface and enter inside, where intracellular target including respiratory catalysts are disturbed [23]. Li *et al.* [24] researched AgNPs bacterial cell membrane permeability and respiratory function and demonstrated that this leads to cell death. However, AgNPs may not only interacted with the membrane surface but might also penetrate inside the structure of bacteria, resulting in a disruption of adenosine triphosphate (ATP) production, DNA replication and activation of reactive oxygen species (ROS), which will lead to cell damage and death [25]. We think that the significant antibacterial activity against bacteria displayed by our fabricated gel could be attributed due to the presence of iodine and silver NPs in the formulation.

Wound healing activity

As per procedure described in methodology, all formulation were applied on the wound, and wound healing processes was studied for 24 days. The rate of healing was evaluated with respect to negative and positive control.

The result showed that by applying AgNPs-iodine hydrogel, rate of healing increased significantly. Figure 5 shows photos of wound healing in comparison with standard iodine- povidone gel and only iodine-MXG gel. Necrosis and haemorrhage from the wounds of some animals was observed in the control group. However, no any evidence of necrosis/sepsis or haemorrhage were indicated in the animals of standard group 3 and group 4. We observed that from 5th day onwards, visible wound healing had begun in groups 2, 3 and 4, and about 90-95% wound size reduction was seen in groups 2 and 4 on day 16. After 16 days, the AgNP-iodine base hydrogel of XG showed complete wound closure, but the positive control (standard iodine- povidone) gel revealed complete wound closure only after 18 days (Figure 7), whereas the negative control required more than 24 days for complete healing.

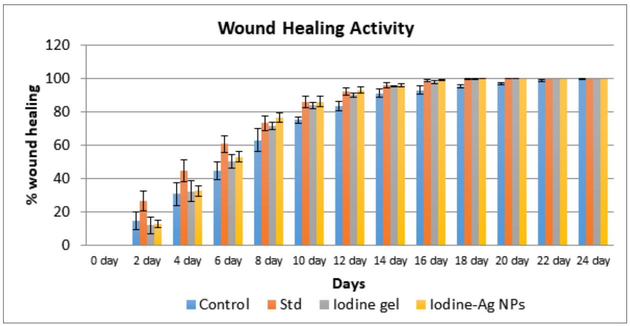

Figure 7. Graphical presentation of wound healing activity of Iodine-Ag NPs gel as compared to standard & control form Day 0 to 24 days. The values are Mean±SD of six animals

The developed AgNPs-iodine hydrogel of MXG demonstrated several advantages:

1. The iodine nanoparticles inhibited bacterial exotoxins.
2. It showed release of AgNPS and iodine.
3. It was applicable directly to the wound surface.
4. The hydrogel rapidly increased the rate of wound healing.
5. Synergetic activity was indicated.

CONCLUSION

The nano silver and molecular iodine hydrogel we prepared by using MXG is safe and effective in all types of wound management and gives significant efficiency and faster wound recovery, as compared to traditional or other topical antiseptic formulations. The physicochemical characterization studies indicated that the prepared formulation is a nanocomposite of molecular iodine- AgNPs. While the formulation showed significant antibacterial activity against both *E. coli* (gram –ve) and *bacillus* (gram +ve), it demonstrated less antibacterial activity against E coli (gram –ve). The fabricated molecular iodine-AgNPs hydrogel brought about accelerated wound healing activity as compared to negative control. The shorter period of epithelialization and faster wound area contraction could be due to the additive effect of the derived MXG-based nanocomposite of molecular nano-iodine & silver nano-particles. Therefore, It can be concluded that modified xanthan gum based hydrogel of molecular iodine and nano silver can be safely and effectively used in the treatment of wounds.

ETHICAL APPROVAL

All the experimental procedures in this study were conducted in accordance with the Institutional Animal Care guidelines of NC3Rs and approved by the Research Ethics Committee of GIPER, Limb, Satara, Maharashtra, India (ethical code: **GIPER/IEC/2020-21/03** dated **27/01/2021**). All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

LIST OF ABBREVIATIONS

Ag NPs – Silver nanoparticles
MXG – Modified xanthan gum
XG –Xanthan gum

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