OPEN ACCESS
Saudi Journal of Biomedical Research
Abbreviated Key Title: Saudi J Biomed Res
ISSN 2518-3214 (Print) |ISSN 2518-3222 (Online)
Scholars Middle East Publishers, Dubai, United Arab Emirates
Journal homepage: https://saudijournals.com

Original Research Article

Synergistic Wound Healing Properties of Silver-nanoparticles and Gentamicin in Ointment base: An *in Vivo* Study on Rat Excision Wound Model

Ezealisiji Kenneth Maduabuchi[1*], Xavier Siwe-Noundo[2]

[1]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Port Harcourt, Nigeria
[2]Departments of Chemistry, Rhodes University, Grahamstown, South Africa

DOI: 10.36348/sjbr.2022.v07i12.008 | **Received:** 27.05.2022 | **Accepted:** 01.07.2022 | **Published:** 31.12.2022

***Corresponding author:** Ezealisiji Kenneth Maduabuchi
Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Port Harcourt, Nigeria

Abstract

Objective: Metal based nanomaterials has been implicated in the treatment and healing of antibiotic – resistant bacteria infected wounds such as found in patients with tuberculosis, human immunodeficiency virus (HIV) and diabetes. Current research investigates the wound healing potentiation of green synthesized silver nanoparticles in the presence of Gentamicin in Wistar rats. ***Methods:*** The reaction of phytochemical extracts of *Anonna muricata* and aqueous solution of silver nitrate afforded the silver nanoparticles used in this study. The silver nanoparticles produced were subsequently characterized using advanced spectroscopic techniques including; Transmission electron microscopy, Ultra-violet visible spectroscopy and Photon correlation spectroscopy (DLS). Adult Wistar rats were divided into five (5) groups of three rats each. Excision wound (3.0 mm diameter) were made on the dorsal part of each rat under anesthesia. Topical application of various test formulation were administered on the excision wound twice daily and wound healing parameters such as % wound contraction, re-epithelialization time and scare formation were monitored over a period of 12 days . Group 1 received simple ointment base, group 2 received 0.01 % w/w penicillin in ointment base, group 3 received 0.01 %w/w gentamicin in ointment base only, group 4 received 1.0 % w/w silver nanoparticles in ointment base, while group 5 received a combination of 1.0 % w/w silver nanoparticles and 0.01 % w/w gentamicin. ***Results:*** The mean particle size of the bio-synthesized silver nanoparticle were found to be 24 ± 1.0 nm. There are observed synergy in wound healing effect amongst the combination of silver nanoparticles and gentamicin after 12 days with respect to the % wound contraction, re-epithelialization time and scare formation when compared to single formulation and controls. ***Conclusion:*** Improved wound healing could be achieved using nanotechnology.

Keywords: Immunodeficiency; silver nanoparticles; Transmission electron microscopy; nanomaterials; bio-synthesized.

INTRODUCTION

Wounds, whether accidental or intentional in etiology can occur as a result of perturbation of the integrity of skin [1]. The largest organ of the body has been recorded to be the skin [2-4]. Crucial roles of the skin include maintaining sensory functions, homeostasis, control of temperature and providing cover against trauma and other pathogenic organisms. Unsatisfactory outcome of wound healing can occur whenever there is any disruption of either the external or internal factors such as homeostasis, inflammation [5-7] or the proliferation or epithelization stage of wound healing. Interferences in wound healing are known to be rampant with the colonization of contaminating pathogens or secondary infections. These pathogenic bacteria including biofilm formers are known to retard wound healing process hence influencing the initial phases of wound reconstruction. Pseudomonas aeruginosa and Escherichia coli as well as Staphylococcus aureus are commonly implicated in wound infections [8-10]. Silver nanoparticles (AgNPs) are also used as artificial implantations, coating for implantable medical devices as it helps in preventing infections [11, 12]. They (AgNPs) can also be used as antibacterial agents to control bacterial infections [13-15].

EXPERIMENTAL SECTION

Materials

Gentamycin Sulfate and penicillin used were of pure grade and are products of Drug field Pharmaceuticals (Nigeria).

Silver nitrate, ointment base and other reagents used were of analytical grade obtained from Merck,

Germany and Oxoid, Hampshire, UK. The phytochemical extract from A. muricata employed in the synthesis of the silver nanoparticles (AgNPs) were procured from the Pharmacognosy garden of the Faculty of Pharmaceutical Sciences, University of Port Harcourt, Nigeria.

Plant Material

The root bark of A. muricata was harvested from the plant garden of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, University of Port Harcourt, Nigeria. The Sample was identified and authenticated by Associate Professor Osuala, Ngozi of the above department. A voucher specimen (UPHC 205860) of the sample was deposited in the Departmental herbarium.

Instrumentation

The average particle size and morphology of the AgNPs were characterized by transmission electron microscopy (TEM) (VEGAimu GmbH, Germany). UV-Visible spectrophotometer (Perkin Elmer Lambda 35) were used to ascertain the surface plasmon resonance absorption while the polydispersity index and zeta potential were determined using photon correlation microscope (Malvern Nano ZS, ZS290 and UK).

Annona muricata root extract preparation

The root bark of Annona muricata were harvested, washed, dried and pulverized followed by boiling in double distilled water for 45min at 100°C. The extract was filtered through a cotton cloth mesh to eliminate insoluble fractions and macromolecules. The resulting filtrate was further filtered using a 0.45µm sintered glass funnel and the final extract were stored safe in refrigerator at 4°C until use. The extract afforded the basis for the reducing and stabilizing agent.

Synthesis of Silver Nanoparticles using Annona muricata root bark aqueous extract

Silver nitrate in deionized water yielded the silver ions necessary for the progression of the reaction. 200ml of the root bark aqueous extract was mixed with 150ml of silver nitrate solution (1.00mM). The above mixture was incubated in the dark at the temperature of 25°C to avoid photochemical activation of silver nitrate. The observation of brown color at the end 30min indicates the formation of AgNPs. The product formed was washed severally with deionized water, rinsed and centrifuged with double distilled water, dried and stored away from light until used. A. muricata extract alone was kept aside as control. All experiment were carried out in triplicate,

Characterization of Synthesized Silver Nanoparticles

The absorption spectral maxima for the silver nanoparticles synthesized were taken at 180-610nm using a UV-Visible spectrophotometer. This affords the maximum point of production of silver nanoparticles. Methanol was used as blank. The particle size and morphology of silver nanoparticles were determined by transmission electron spectroscopy (TEM). Sample were processed for TEM analysis by depositing a drop of aqueous silver nanoparticles suspension on a carbon-coated copper grid and allowed to dry at room temperature. The Transmission electron micrographs were produced and analyzed for particle size and morphology.

The size distribution by intensity, mean particle size and poly dispersity index were determined by injecting 1:20 dilution of the aqueous silver nanoparticle solution into the photon correlation microscope via the U-shaped glass cuvette.

Preparation of 1.0% $^{W}/_{W}$ Silver Nanoparticle in Ointment Base

To one gram (1.0g) silver nanoparticle in a clean glass mortar was added 90g of pure ointment base, the product were carefully triturated and final product were adjusted to 1.0%$^{W}/_{W}$ following additional pure ointment base. Similar method were used to prepare other treatment products.

Excision Wound Model

Prior to wound infliction on the dorsal part of the animals, a shaving machine was used to remove all the hairs around the study area. The wounds were inflicted under anesthesia using 1 ml Lignocaine HCl (2%, 100mg/5ml) as a local application. The wound was treated following the method reported by Singer AJ and Clark 1999 [16] with slight modification. Wound was inflicted using a round seal of 3cm diameter to create a round mark before excision with surgical blade. Homeostasis was ensured by carefully wiping the wound with cotton swab soaked in normal saline. The animals were housed in different cages and allowed free access to feed and water.

Wound Area Measurement

The wound surface area was traced using a transparent graph paper from which the wound contraction and hence the surface area was evaluated on days 0, 4, 8, 10 and 12. Evaluated wound surface was then used to calculate the percentage of wound concentration taking the initial size of the wound as 100%. The re-epithelization period was also observed for each of the treatment group. Percentage wound concentration was determined from the mathematical relation below:

$$\% \text{ Wound Contraction} = \frac{wound\ area\ on\ day\ 0 - wound\ area\ on\ N^{th}\ day}{wound\ area\ on\ day\ 0} \times \frac{100}{1}$$

N^{th} day= 4, 8, 10 and 12 (post wounding days)

Statistical Analysis

The data herein presented as mean ± SEM of at least triplicate determination (n=3). A one-way

Analysis of Variance (ANOVA) were employed to demonstrate statistical significance of data using Graph pad prism 5 software. (Graph Pad software, Inc. San Diego, CA) followed by Dunnetts Post-hoc test. Differences between test and control treatment are considered significant at P= 0.05.

In Vivo Wound Healing Evaluation

Experimental Animals

White Wistar rats were procured from the Department of Pharmacology animal house, at the University of Port Harcourt, Nigeria. The rats were randomly divided into 5 groups of 3 rats each. Adult male and female rats with weight between 200-220 g were selected for the experiment. The animals were fed on standard feed and water. Internationally accepted best practices as approved by the use of animal and handling ethics of the Local University Committee of our institution were ensured with approval (EAU 1986: 86/609/EEC/UPN)

Administration of Test Preparations

Group 1 received simple ointment base, group 2 received 0.01% w/w penicillin in ointment base, group 3 received 0.01 %w/w gentamicin in ointment base only, group 4 received 1.0% w/w silver nanoparticles in ointment base, while group 5 received a combination of 1.0% w/w silver nanoparticles and 0.01% w/w gentamicin. 1.0g topical application were administered twice daily for twelve days after which the wound healing parameters were monitored and evaluated.

RESULTS AND DISCUSSION

The biosynthesis of crystalline silver nanoparticles were readily achieved using aqueous root bark extract of *Annona muricata* with good product yield and high level of simplicity [17]. Phytochemicals are implicated in the capping and stabilization of nanoparticles as reported in the previous work of Muna *et al*., 2022 [18]. These polyphenol rich molecules are known to effect the reduction of Ag^{+} to Ag^{0}. The root bark of *A. muricata* is rich in Antioxidants hence their use in biosynthesis of silver nanoparticles.

Spectrophotometrical Characterization of AgNPs

One pot synthesis using aqueous silver nitrate and root bark aqueous extract of *A. muricata* afforded a silvery brown precipitate which marked the formation of silver nanoparticles. The UV-Visible spectral data showed an absorption surface plasmon peak at the region of 420nm after 10h (see Fig 1).

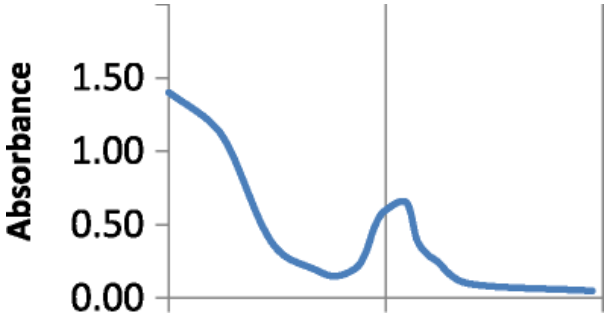

Fig 1: UV-visible spectroscopic micrograph Showing the spectral absorbance of *A. muricata* stem bark-synthesized AgNPs at 420 nm

Transmission Electron Microscopy Micrograph of AgNPs

A closer view on the TEM spectral data of AgNPs gave a revealing impression on the mean particle size and size distribution of the AgNPs and were observed to be monodispersed and generally discreet but spherical in nature (Fig 2) with size range between 18.02 and 34.22nm. The average particle size was found to be 24±10nm.

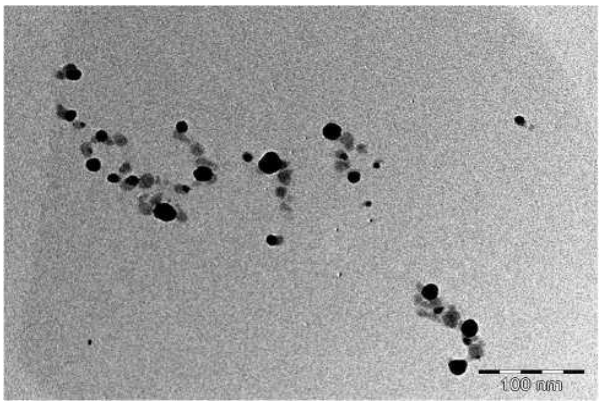

Fig 2: TEM micrograph revealing the topography, size and monodispersed AgNPs

Zeta-potential and Poly dispersity index

The zeta potential as revealed by Photon correlation microscopy analysis is -28.40±0.21m with poly dispersity index of 0.43±0.02 while the average particle size was 280.20±14.20nm as shown in Figure 3. Fewer particle size were recorded below 100nm while on the average, some were found between 200 and 500nm though few particles were in the micrometer size range. The hydration layer around nanoparticles coupled with the presence of phytochemical stabilizing agents accounts for larger particle size as most often recorded by the DLS [19].

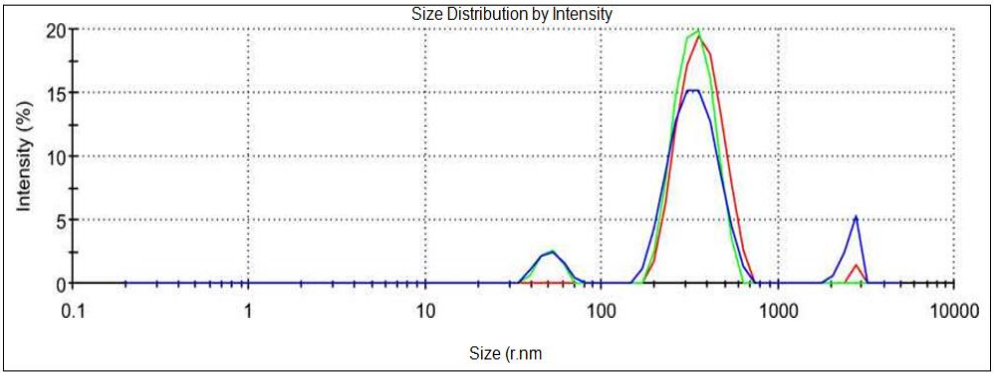

Fig 3: Size distribution by intensity for synthesized AgNPs

Wound Healing Ability

The wound healing potential of AgNPs and Gentamycin combination as well as controls is shown in Figures 4.1 – 4.5. A quick but progressive wound closure pattern was observed in rats treated with AgNPs and Gentamycin combination (group 5), within 12 days. Compared to the control group that received gentamycin and penicillin alone and respectively. Significant reduction in wound area were observed from day 4 and down to day 10 for all the rat group that received the test samples except for the control group that received the ointment base alone. Significant wound contraction were observed in group 5 from day 6 (AgNPs + Gentamycin Groups) with every other group showing significant contraction from day 10. Considering the reepithelization period in days, the positive standard group gave reepithelization time of 10 days while the negative control gave reepithelization period of 12 days. The reepithelization period for group 5 (AgNPs + Gentamycin) were higher than those of positive control; gentamycin and penicillin alone. The test group 5 healed perfectly after 12 days without scar formation while the negative control group produced healing with minor scar formation.

Table 1: Percentage wound contraction. Data represented in mean ± SEM

Groups	Day 0	Day 4	Day 6	Day 8	Day 10	Day 12	ANOVA (p-value)
[1] Ointment base only	0	42.22±5.87	58.20±3.32	64.00±4.20	68.20±1.00	90.00±1.28	0.0001*
[2] Penicillin ointment	0	60.21±4.42	65.74±2.10	83.20±2.10	84.80±2.60	92.21±2.78*	0.0001*
[3] % w/w Genta. ointment	0	51.11±8.89	60.08±4.26	82.32±4.00	86.34±4.10*	90.28±4.00	0.0001*
[4] 1% w/w AgNPS ointment	0	53.32±6.66	62.20±4.02	84.44±2.22*	90.66±2.00*	96.36±0.88*	0.0001*
[5] 0.01% w/w Genta. + 1% w/w AgNPS	0	62.21±4.40	74.32±1.92*	89.99±1.92*	96.88±1.92*	99.66±0.66*	0.0001*

**Difference across the groups is statistically significant*

Table 2: Dunn's post Hoc Test of Day of Exposure

	Ointment base only	Penicillin ointment	0.01 % w/w Genta. ointment	1% w/w AgNPS ointment	0.01% w/w Genta. + 1% w/w AgNPS
Day 4 vs Day 6	0.014*	0.0001*	0.004*	0.005*	0.006*
Day 4 vs Day 8	0.006*	0.004*	0.003*	0.003*	0.004*
Day 4 vs Day 10	0.001*	0.002*	0.004*	0.008*	0.001*
Day 4 vs Day 12	0.0001*	0.002*	0.002*	0.006*	0.005*
Day 6 vs Day 8	0.004*	0.001*	0.004*	0.007*	0.009*
Day 6 vs Day 10	0.002*	0.003*	0.007*	0.002*	0.009*
Day 6 vs Day 12	0.001*	0.0006*	0.003*	0.002*	0.003*
Day 8 vs Day 10	0.003*	0.0002*	0.008*	0.004*	0.006*
Day 8 vs Day 12	0.0006*	0.0031*	0.007*	0.002*	0.005*
Day 10 vs Day 12	0.0001*	0.002*	0.008*	0.009*	0.006*

**Difference across the groups is statistically significant*

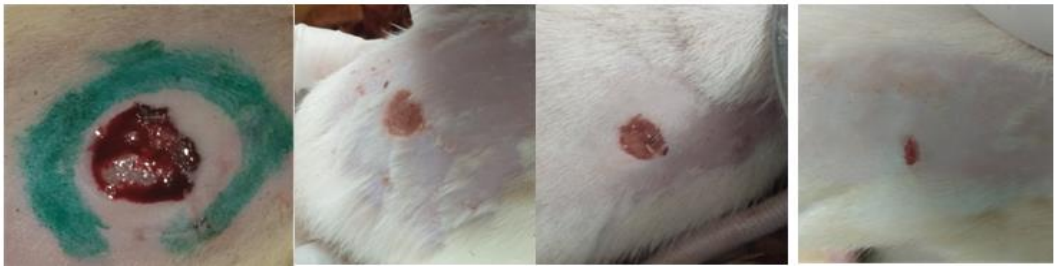

Fig 4.1: group 1, Ointment base only. Day 0, 4, 8 and 12

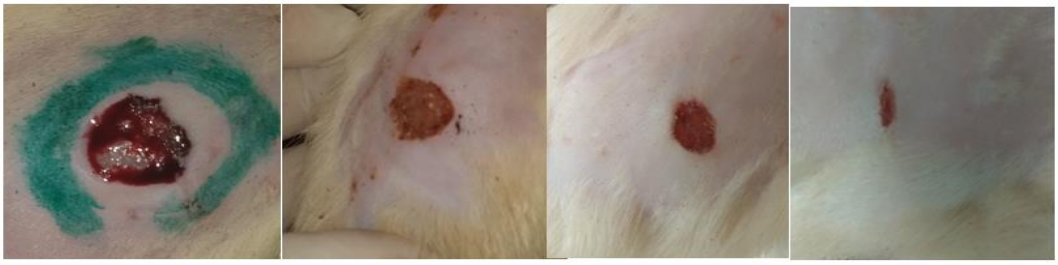

Fig 4.2: group 2, Penicillin Ointment. Day 0, 4, 8 and 12

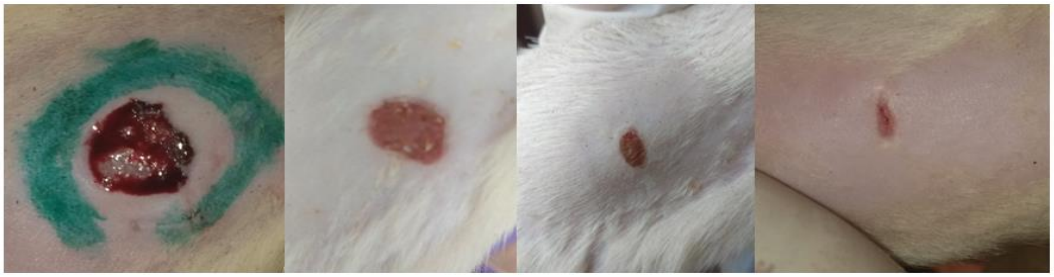

Fig 4.3: group 3, Gentamicin Ointment. Day 0, 4, 8 and 12

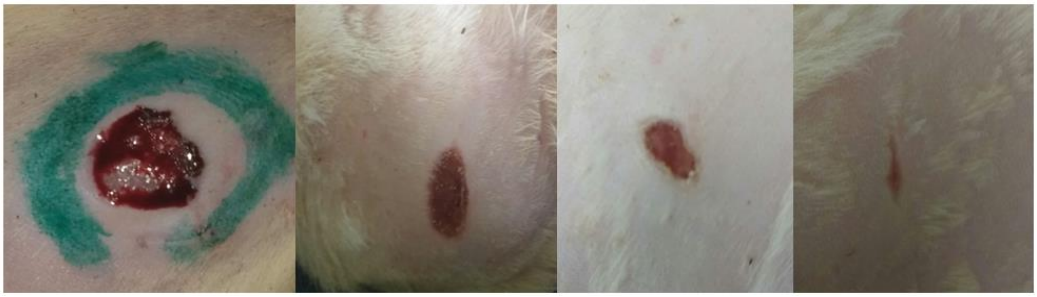

Fig 4.4: group 4, 1.0 % AgNPs. Day 0, 4, 8 and 12

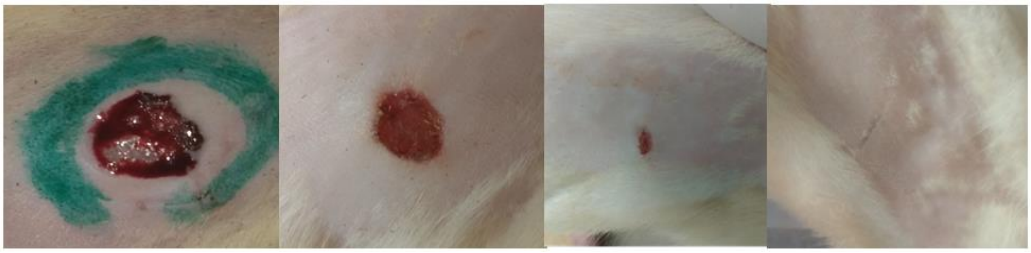

Fig 4.5: group 5, 0.01% Gentamicin + 1.0% w/w AgNPs, Day 0, 4, 8 and 12

CONCLUSION

The wound healing results observed, following topical application of Silver-nanoparticles in combination with gentamicin was in agreement with previous work of Thirumurugan G *et al.*, 2012 [20] which affirms that nanoparticles in ointment was found to be an excellent wound healing agent for biomedical application.

Silver nanoparticles have made its way as a very vital component of wound management plans in advance burn clinics and centers where patients

normally present with different degrees of burns. The research herein has bridged several knowledge gaps by revealing novel wound healing formulation that can allow wounds to heal with minimum or no scare formation.

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