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Gentamicin nanogel films based on Carrageenan-*Prosopis africana* for improved wound healing

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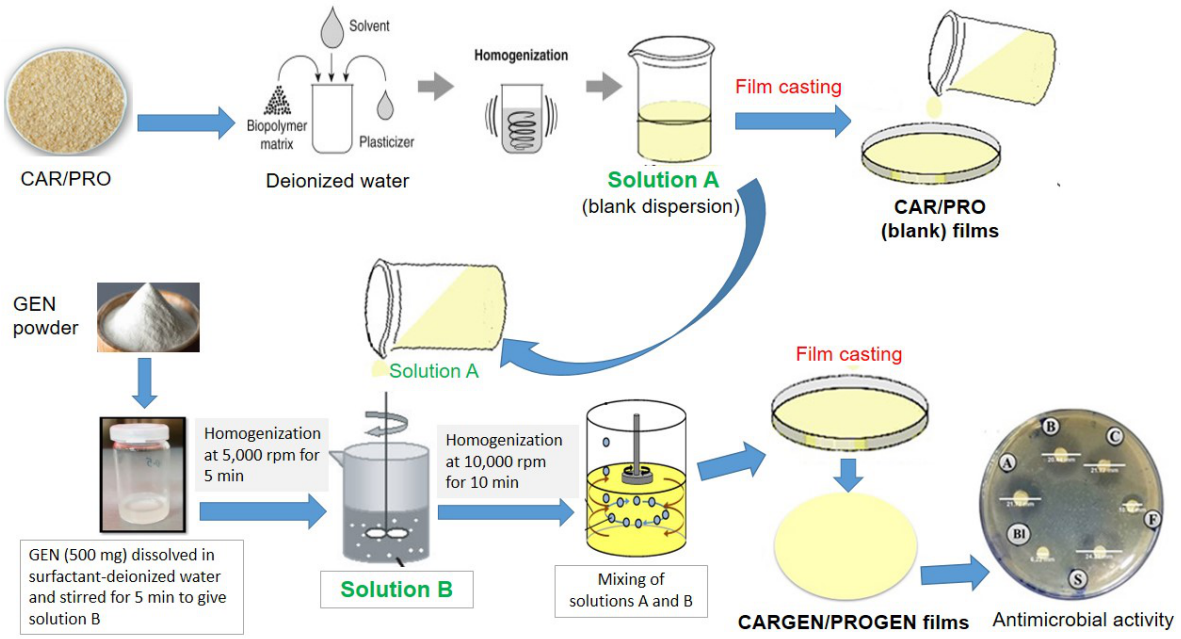
Graphical abstract



Abstract

Dermal injuries (e. g., trauma, surgical incisions, and burns) are burdensome health care issues in the world. The delayed healing process can be caused by aerobic and anaerobic bacteria infections, including *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus and Enterococcus species, Pseudomonas aeruginosa, Peptostreptococcus, and Coliforms*. Therefore, reduction of bacterial burden to an acceptable level promotes healing. The rationale for this innovative wound dressing relies on good hydration as the single most important external factor responsible for optimal

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wound healing. Objective: To study the wound healing potential of GEN nanogel films based on natural polymers using carrageenan (CAR) and *Prosopis africana* (PRO), a local African plant. Method: Nanogel dispersions and films were characterized by their physical and technological properties, microenvironment, Zeta sizer, Differential Scanning Calorimetry, FTIR spectroscopy, *in vitro* drug release, and efficacy of wound healing in the rat in a full-thickness excisional model. Results: GEN was dispersed in the polymers, with PROGEN films appearing smoother than CARGEN. Wound closure rate was 88, 76, and 72 % with PROGEN, CARGEN, and GENTA cream, respectively, whereas drug release was sustained in the order of PROGEN (~99 % at 8 h), CARGEN (83 % at 10 h), and pure GENTA powder (81% at 1 h). Topical administration of GEN nanogel-films significantly accelerated (< 15 days) wound closure with no scabbing observed compared to the commercial GENTA cream used. Conclusion: GEN-loaded PROGEN and/or CARGEN films have the potential for developing an improved and affordable topical treatment for cutaneous wound healing.

Keywords:

Wound dressing; Carrageenan; *Prosopis africana;* Gentamicin; Tissue repair

Purpose and Rationale

Gentamicin (GEN) is a polar, water-soluble compound with poor dermal and intestinal permeability. Injection GEN is an aqueous solution of gentamicin sulfate in vials or ampoules. GEN has a narrow therapeutic index. Like other aminoglycosides, it is potentially ototoxic and nephrotoxic; excreted almost entirely unchanged by the kidney, mostly by glomerular filtration, and has a short plasma elimination half-life (2−3 h) in adults with normal renal function. This design aimed to produce low-dose topical nanogel films of GEN using cheap bioadhesive polymers to treat cutaneous wounds.

Introduction

The skin is protective for the body, and when injured, subcutaneous tissues and organs are threatened by pathogens and excessive water loss. Dermal injuries (e.g., trauma, surgical incisions, and burns) are burdensome healthcare issues globally [1]. A burn injury can occur in any person, age, race, and place with different severity. Most severe lesions require intensive care and surgery, depending on the depth and size of the body surface area [2]. The method of burn wound management can significantly influence the healing time; hence, faster healing minimizes the risk of infection. Wound repair is a complex dynamic process requiring the interplay of soluble mediators, blood cells, extracellular matrix, and parenchymal cells [3]. Numerous growth factors, such as epidermal growth factor (EGF), transforming growth factor-beta (TGF-β), fibroblast growth factor, and platelet-derived growth factor, are some critical regulators of the wound healing process [4-6]. Impaired chronic wound healing is associated with decreased secretion of endogenous growth factors. As seen in diabetic patients, impaired immune response to injury frequently affects granulation tissue formation that is a prerequisite for epithelialization or complete skin healing [7, 8]. Foot ulceration affects 15–20% of diabetic patients and precedes amputation in about 85% of cases in these patients, thereby reducing their quality of life [7]. Therefore, providing exogenous growth factors at precisely timed intervals would induce faster re-epithelialization and minimize the risk of infection [9-11]. Current guidelines recommend using pressure-relieving devices and wound dressings to promote healing and prevent infection and, when appropriate, debridement, drainage, and revascularization [8]. Commonly, delay in wound healing may occur depending on poor patient compliance to treatment regimens, scarcely controlled glycemic levels, and poor tissue oxygenation [8]. The delayed healing process can also be caused by aerobic and anaerobic bacteria infections, including *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus* and *Enterococcus species, Pseudomonas aeruginosa, Peptostreptococcus,* and *Coliforms* [7, 8]. However, reduction of bacterial burden to an acceptable level promotes healing.

Gentamicin (GEN), an aminoglycoside antibiotic, is used to treat severe Gram-positive and Gram-negative microbial infections due to its rapid bactericidal activity and comparatively low levels of resistance in most community hospital-associated Gram-negative pathogens [12]. This broad-spectrum hydrophilic bactericidal antibiotic inhibits protein synthesis

after binding to specific 30S-subunit ribosomal proteins. Like other aminoglycosides, GEN is toxic to the ear's sensory cells and causes nephrotoxicity by inhibiting protein synthesis in renal cells, causing necrosis in the proximal tubule (acute tubular necrosis) which can lead to acute renal failure [13]. In addition, it accumulates in the tissues, and there may be considerable inter-subject variation in the pharmacokinetics. GEN belongs to Class III drugs (Biopharmaceutics classification system, BCS), which are well soluble in water (100 mg/mL, Log P = −3.1). However, it has limited permeability across biological membranes, resulting in poor oral bioavailability [12] with low plasma half-life (2-4 h), long terminal elimination phase of several days [14, 15], and poor oral absorption but rapidly absorbed after IM injection. It is also absorbed systemically following topical application to wounds; hence, it is commonly administered topically (to wounds), intramuscularly, intravenously, and subcutaneously [12, 15]. In normal subjects, it is rapidly excreted in the urine as an unchanged drug, up to 80% of a dose being excreted in 24h and may be detected in serum and urine for several days after cessation of treatment. These properties make it a beneficial empirical drug when rapid control of a serious infection is required [12].

On the other hand, nanotechnology offers the possibility to overcome the above-listed drawbacks (poor solubility, low bioavailability, and extremely short half-life *in vivo*) [12, 16]. Our earlier work demonstrated the possibility of administering GEN as a fast-release topical agent (microgels) using an optimized nanostructured lipid carrier (NLC) regimen with excellent microbial growth inhibition [12]. Therefore, innovative wound dressing relies on good hydration as the single most important external factor responsible for optimal wound healing [17-19]. Healing under wet and moist environments has been clearly demonstrated to be significantly faster than under dry conditions [18]. The optimum wound healing environment is intermediate gelatinous between moist and dry, as seen in highly vapor-permeable dressings [1, 20]. Some mechanisms attributed to this improved wound healing include increased partial pressure of oxygen, preferred migration of epidermal cells over moist wound surfaces than under dry scabs, preservation of growth factors, and presence of proteinases in fluid exudates [19]. Increased moisture impedes capillary activity and reduces hyperemia and collagen deposition. It is proven that keratinocytes need a moist environment to down-regulate fibroblast collagen deposition [20-22] and that hydration enhances collagenolysis [21, 22]. Given these, the present study investigates the potential to develop GEN-loaded films based on nanogels with such favorable properties as cutaneous wound dressing based on some natural polymers, locally available at low costs in Africa.

In skin delivery, flux across the skin is usually dependent upon skin hydration, partitioning, transport, and concentration gradient across the skin [12, 16-19, 23, 24]. Hydrogels are cross-linked hydrophilic polymers, highly swollen but not soluble in the surrounding medium [12, 16-19], while films and/or patches are dressings that release the drug from a reservoir through a microporous membrane into the skin [25]. To strike a reasonable balance between the benefits of the breadth of activity of gentamicin and its rapid bactericidal activity, especially in bloodstream infections, versus the toxicity limitations with prolonged use, we report here an alternative low dose gentamicin regimen for cutaneous wound dressing. This formulation employed k-carrageenan (CAR), a standard polymer, and *Prosopis africana* gum (PRO), a cheap local polymer [16]. PRO polymer is obtained from the tropical plant *Prosopis africana* (Fam. Leguminosae) and is a generally regarded as a safe (GRAS) polymer for different uses, including extended-release polymer [26], nanogels [16], bio/mucoadhesive systems [27]; solubility enhancer, plasticizer, binder for tablets, film/patches [27, 28], food supplement [29], antidiabetic [30], antimicrobial [31], and water treatment [32], etc. Prosopis gum is a natural polysaccharide consisting of glucose, fructose, galactose, and xylose as the monosaccharide units, as determined by thin-layer chromatography and complete acid hydrolysis analysis [27]. The gum has been reported to be made up of highly branched polysaccharides with chain structure formed when monosaccharides condense with the elimination of water molecule(s) [27], and the structural components of its rich amino acid constituents have been implicated in solubility enhancement due to abundant hydrogen

bonding groups [28] as well as feed supplement [30]. In addition, it is a well-known biodegradable and biocompatible bioadhesive agent which, due to its anionic nature, which is more effective in mucosal and/or tissue binding [16, 27, 33, 34].

Additionally, it is a sought-after food condiment for the Nigerian people due to its pleasant flavor, biosafety, and probiotic properties. This makes it an ideal low-cost bioadhesive agent for developing effective drug films to treat some tropical diseases to ensure sustainability and affordability. In the light of the above, the current study utilized two tropical polymers (CAR and PRO) to disperse GEN nanoparticles and use the resultant drug films as low-cost and sustainable wound dressing, taking advantage of the prolonged GEN-release properties.

Materials and methods

Materials

Gentamicin was a gift from Ipca Laboratories Ltd. India. Other ingredients included k-carrageenan and absolute ethanol (Sigma Aldrich, Germany), propylene glycol, sodium saccharine, and peppermint oil (Merck, Germany). In addition, gentamicin sulfate cream USP, 0.1% (Perrigo Bronx, New York, USA), was used as a commercially available topical gentamicin cream. All other reagents and solvents were analytical grade and were used as procured.

Preparation of Prosopis polymer

Prosopis seed was procured from Orba market, Udenu Local Government Area of Enugu State, Nigeria. *Prosopis africana* peel powder (PAPP) was prepared according to earlier methods (26, 28, 34), with slight modifications. *Prosopis africana* peel is an agro-waste product among the local Nigerian people who process this plant seed as food condiment and/or feed it to domestic animals (e.g., goats). Briefly, Prosopis seeds were boiled overnight. After the inner fermentable seed (used for soup flavoring in Nigeria) was separated from the peels, there was a further separation of the peel into two parts: (1) fleshy white mesocarp (gum containing portion) and (2) hard pericarp (outermost part). Next, the white mesocarp (gum) was washed (thrice) with deionized water, sun-dried to constant weight, and ground into powder using a laboratory grinding machine (Hammermill SG 50 UK). Finally, the powder was sieved with a laboratory sieve of known mesh size to obtain fine powders used for this study, stored in an air-tight container.

Purification/Isolation of polysaccharide

The method of Nwokocha and Williams 2015 [35] was slightly modified. Approximately 10 g of the resultant powder was placed in deionized water (500 mL) and kept for overnight hydration at room temperature to avoid a high-temperature effect on viscosity. This was poured into centrifuge cups and centrifuged at 2500 rpm for 2 h. The supernatant was decanted, and the residue was reconstituted in deionized water and centrifuged again. The pooled supernatant was centrifuged again, and the supernatant was treated with excess isopropanol to precipitate the polysaccharide. The precipitate was collected, centrifuged to expel trapped solvent, reconstituted in a small amount of deionized water, and freeze-dried. The yield of polysaccharides based on the initial powder was 54 %. The polysaccharide powder was stored in a dry container.

Preparation of nanogel films

Before final preparations, all parameters, including concentration (polymer, drug, and plasticizer), stirring time, and drying temperature, were optimized before final preparations. In order to determine the optimal drying time, equal amounts of dispersion were poured into Petri dishes (diameter 3.4 cm^2) and weighed every 24 h up to 72 h until a constant weight was obtained. An impermeable backing membrane/layer was prepared from ethanol (E), ethylcellulose (EC), and soybean oil (SBO) to represent 4 and 10 % w/v respectively of SBO and EC in absolute E. The mixture was stirred until a homogeneous solution was obtained. This solution (10 mL) was poured into a Petri dish (3.4 cm) and dried in the oven at 30 °C for 3 h. An aqueous polymer (CAR and PRO) solution was prepared by adding 1 g of CAR or PRO to 25 mL of deionized water and stirring at 5000 rpm for 5 min. Next, propylene glycol (10 mL) as plasticizer was added and stirred (1 min), followed by the addition of peppermint oil (1 mL) with final stirring for another 1 min to give solution A. For the blank films (without drug), solution A was cast into Petri dishes (diameter 3.4 cm) containing dried

backing layers and covered with an inverted funnel for gradual drying at room temperature to give CAR and/or PRO films. For drug-containing films, an optimized concentration of GEN (500 mg) was first dissolved in deionized water containing Tween® 80 (2 %w/w) and stirred for 5 min to give solution B. Solution A was separately added to solution B, and the mixture was stirred at 10,000 rpm for 10 min before casting as earlier described. Formulations were made for each batch in triplicates. After drying at room temperature, the films were stored in desiccators until used. GEN films formulated with CAR were coded as CARGEN, while those formulated with PRO were referred to as PROGEN.

Physical evaluation of nanogel dispersions

The nanogel formulations were physically examined for color, homogeneity, consistency, and pH before casting. The pH was also re-evaluated (before each use) to ensure that it was stable within the skin pH of 5.5–6.5.

Microenvironment pH and particle characterization

The microenvironment pH of carefully measured film sizes (2 cm^2) was determined to evaluate possible skin irritation. The films were allowed to swell for 8 h in milliQ-water (10 ml) of pH 6.8 contained in small beakers. The pH of the aqueous film solution was determined using a pH meter (L1 127 Elico, India). Triplicate measurements were done per batch of formulation for the validity of the statistical analysis.

The mean diameter (Z-ave) and polydispersity index of the above aqueous nanogel dispersions (10 mL) obtained from CARGEN and PROGEN films (100 mg) were measured after appropriate dilutions (to 25 ml) using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) equipped with a 10 mW He–Ne laser employing the wavelength of 633 nm and a backscattering angle of 173 ° at 25 °C. All samples were diluted with sufficient milliQ-water to obtain a suitable scattering intensity before photon correlation spectroscopic analysis and were then placed in a 10 mm diameter cell. Particle size analysis was performed using Mie theory.

The zeta potentials of drug films were determined via electrophoretic mobility measurements using a Zetasizer Nano-ZS (Malvern Instruments, Worcester, UK). The zeta potential was calculated applying the Helmholtz–Smoluchowski equation (n = 3).

Morphology and texture analysis

The morphology of the film samples was analyzed using a scanning electron microscope (SEM). The dry films were gold-coated to about 5 µm thickness using a coater unit under a high vacuum. SEM analysis of films (PRO, PROGEN, CAR, and CARGEN) was performed on a Philips XL-30M scanning electron microscope instrument.

Technological evaluation of the films

The thickness of the film was measured with a micrometer screw gauge (Mitutoyo, Kawasaki, Japan). Measurement was taken at five different areas of the film: four around the edges and one in the center. The mean and standard deviation of the five measurements was calculated for each film. Ten films from each CARGEN and PROGEN batches were individually weighed for weight variation. Variations in weight were determined in triplicates. The folding endurance (FE) of the formulated films was determined by repeatedly folding the films of uniform cross-sectional area (4 × 4 cm^2) and thickness at the same place until they broke. The number of times each film was folded at the same place without breaking gave the value of FE. Finally, triplicate determinations were done for the validity of the statistical result.

Bioadhesion

The bioadhesive properties of the formulated films were determined using a texture analyzer (TA.XT2, Stable Micro System, UK) and a fresh chicken pouch as a model tissue as previously described [36] with slight modification. The calculated work of adhesion was used to measure the bioadhesive property of the films.

Differential scanning calorimeter (DSC)

DSC experiments (TA Instrument, USA) were performed to detect the phase transition of pure compounds and their mixtures and investigate any possible interaction between the initial compounds used to prepare the films. In order to investigate the transition of the drug molecule from crystalline to amorphous form during the formulation process, a sufficient quantity of PROGEN and CARGEN films

enough to contain at least 3–5 mg of GEN was weighed in aluminum pans, heated from 25 to 350 °C at 10 °C/min under constant flushing with nitrogen (10 mL/min). The DSC parameters were generated, such as temperature onset, maximum peak, and enthalpy.

Fourier transform infrared spectroscopy (FTIR)

FTIR study was carried out using a BIO-RAD FT-IR 3000 instrument (BIORAD Company, USA). The FT-IR spectra of PROGEN and CARGEN films, GEN, PRO, and CAR polymers were obtained using KBr discs in the region of 4000–500 cm^{-1}.

Thermogravimetric analysis (TGA)

TGA was used to measure the water content within the films. Samples weighing between 3 and 10 mg were packed into an aluminum pan and placed in a high-resolution TG 2950 instrument (Crawley, UK). The TGA was programmed to heat the sample from 25 to 150 °C at 10 °C/min. The weight loss was noted and used to calculate the water content of the films.

Drug content analysis

Accurately weighed 100 mg of drug film (CARGEN and/or PROGEN) was selected and transferred to a 100 ml volumetric flask. The drug content was determined by dissolving each film in 100 ml of the casting solvent deionized water. Gentamicin sulfate concentration was determined spectrophotometrically (Shimadzu UV-1601 UV/Vis double beam spectrophotometer, Japan) after derivatization with o-phthaldialdehyde reagent [25]. Briefly, the o-phthaldialdehyde reagent was formulated by adding 2.5 g of o-phthaldialdehyde, 62.5 mL methanol, and 3 mL 2- mercaptoethanol to 560 mL sodium borate in distilled water solution. The reagent was stored in a brown bottle in a dark chamber for at least 24 h before use. This reagent could be used only for up to three days. Gentamicin sulfate solution, o-phthaldialdehyde reagent, and isopropanol (to avoid precipitation of the products formed) were mixed in similar proportions and stored for 30 min at room temperature. The homologous aromatic dialdehyde, o-phthaldialdehyde, is essentially non-fluorescent until it reacts with a primary amine of gentamicin in excess sulfhydryl such as 2-mercaptoethanol to yield a fluorescent isoindole whose absorbance was then measured at 332 nm [25].

Drug dissolution

The drug dissolution rate was determined using the magnetic stirrer method. Accurately weighed 100 mg of drug film (CARGEN and/or PROGEN) was transferred to a 100 mL beaker to which 50 ml of the dissolution medium (deionized water) was added. The o-phthaldialdehyde reagent was formulated as described above, stored in a brown bottle in a dark chamber for at least 24 h before use, and used only for up to three days. Gentamicin sulfate solution, o-phthaldialdehyde reagent, and isopropanol (to avoid precipitation of the products formed) were mixed in similar proportions and stored for 30 min at room temperature to derivatize the fluorescent isoindole, whose absorbance was then measured at 332 nm [25], as earlier described.

Biopharmaceutical evaluation of drug films

The antimicrobial activity of the drug films was tested against different isolates using the agar diffusion technique [25, 36-38]. This method depends on the diffusion of GEN from holes on the surface of the seeded microbial agar. Molten nutrient agar (20 mL) was inoculated with 0.1 mL of *Staphylococcus aureus* broth culture. It was mixed thoroughly, poured into sterile Petri dishes, and rotated for even distribution of the organism. The agar plates were allowed to set, and a sterile cork-borer (8 mm diameter) was used to bore four cups in the seeded agar medium. A definite size of a batch formulation was placed into the holes Using a sterile instrument. The plates were allowed to stand at room temperature for 15 min to enable the samples to diffuse into the medium before incubating at 37±1°C for 24 h. The experiment was repeated for *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella typhi,* and *Klebsiella pneumoniae*. Three replicate tests were performed in each case. Growth was examined after incubation, the diameter of each inhibition zone was measured, and the average was determined.

Animal care and use protocols

Clinically normal male albino Wistar rats weighing 205 ± 10 g, procured from the animal breeding center, Department of Pharmacology and Toxicology, Faculty of Pharmaceutical

Sciences, University of Nigeria, Nsukka, and housed in a regulated environment (25 ± 2 °C, 12 h light/dark cycle, a light period was starting at 7 am). The rats were then acclimatized for 1 week before the study and provided free access to standard rodent pellets (Guinea feeds Ltd Nigeria) and water in clean glass water bottles *ad libitum*. All animal experiments complied with the ARRIVE guidelines and were performed following the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. Cage-side clinical observations of the rats were made throughout the study period. The study protocols were approved by the Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences (ethical approval number: FP/PT/019/A/010).

In vivo wound healing assay

Full-thickness excision wounds of 2×2 cm^2 were created under ketamine hydrochloride (60 mg/kg body weight) anesthesia. A total of thirty-six (36) rats were used. The rats were randomly assigned to one of the following groups (6 rats per group): GENTA commercial cream sample, CARGEN, and PROGEN films containing equivalent GEN doses of 8 mg/kg body weight each; then CAR, PRO, and NS (as negative controls). The wounds were topically treated with a single application of the formulations mentioned above and covered with a piece of Tegaderm® (3M, Maplewood, MN, USA) to prevent the rats from removing the treatments. Subsequent measurement of wound area was taken on different days (0, 3, 5, 6, 9, 10, 12, and 15 post-wounding, and wound closure was calculated according to Equation 1:

$$Wound\ closure\ \% = \frac{Ao-At}{Ao}\ x\ 100 \quad (1)$$

where A0 was the original wound area on day 0, and At was the wound area on day t post-wounding.

Statistical analysis

All the data generated were expressed as mean ± standard deviation. A one one-way analysis of variance (ANOVA) with duplication was applied for group comparisons. Statistical significance was determined using a student t-test, with $P<0.05$ considered statistically significant.

Results

Pharmaceutical characterization of nanodispersions and films

Table 1 shows the properties of the formulation. PROGEN dispersions and films were more homogenous, more viscous, moderately transparent, and smoother than CARGEN films (Figure 1). CAR (−51 mV) and CARGEN (−14 mV) nanogels had the highest zeta potential compared to PRO and PROGEN (−10 mV and −9 mV, respectively). Plain CAR particles (~569 nm, PDI ~0.8) were larger and not as monodispersed as drug containing CARGEN particles (~191nm, PDI ~0.2), whereas this difference was not so pronounced for the somewhat larger PRO (799 nm, PDI 0.9) and PROGEN (~758 nm, PDI 0.5) nanogels.

The drug-containing films had higher thickness and water content than the corresponding drug-free polymer films, which, however, invariably had higher adhesivity than the drug-containing films. In addition, FE indicated that the films would not break and could maintain their integrity with general skin folding when applied [36]. The implication is that such film could be placed in a part of the body involved in the movement, and that part of the body would have been moved approximately 300 times before the film could crack/break. This was considered to provide sufficient film adhesion to the cutaneous wound surface, which was also the case for drug contents, weight uniformity, and pH values of all films prepared.

The thermal properties of the nanogel films and polymer films shower lower enthalpy values compared to pure GEN. This confirms that GEN was a crystalline drug and its formulation into nanogel films lowered its crystallinity. In other words, lower enthalpy values of the nanogel films ensure the molecular dispersion of GEN nanoparticles in the polymer matrices [16, 25]. The water content of drug films was higher than those of polymers with uniform weight variation.

Table 1: Pharmaceutical properties of tested formulations

Parameters	Formulation Codes ± SD				
	CAR[ab]	CARGEN[ab]	GEN powder	PROGEN[ab]	PRO[ab]
Z-Ave. (nm) (± SD)	568.50 ± 10.91	190.8 ± 6.85	-	757.9 ± 15.27	798.9 ± 19.24
PDI (± SD)	0.80 ± 0.19	0.197 ± 0.01	-	0.54 ± 0.16	0.92 ± 0.23
Zeta potential (mV) (± SD)	−50.5 ± 3.42	−14 ± 1.18	-	−9.0 ± 0.55	−9.6 ± 0.52
Thickness (mm) (± SD)	0.08 ± 0.01	0.18 ± 0.04	-	0.28 ± 0.13	0.19 ± 0.02
Bioadhesive activity (g/mm) (± SD)	19.24 ± 0.11	0.22 ± 0.01	-	0.14 ± 0.01	17.24 ± 0.80
Melting peak (°C)	242.1	129.6	249.1	128.1	312.6
Enthalpy (J/g)	−3.16	−0.9382	−4.27	−2.43	−0.79
Folding endurance (± SD)	300 ± 4.17	316 ± 5.30	-	327 ± 3.89	307 ± 5.02
Water content (%)	18.00 ± 1.06	22 ± 1.21	-	16 ± 0.99	10.14 ± 0.66
Weight variation (g) (± SD)	0.98 ± 0.05	1.48 ± 0.07	-	1.49 ± 0.05	0.98 ± 0.02
pH	7.0 ± 0.17	6.5 ± 0.15	-	5.9 ± 0.03	6.2 ± 0.11
Drug content (%) (± SD)	-	90.21 ± 2.62	95.60 ± 1.57	98.90 ± 2.32	-

[a]Mean±SD, [b]n=3, Z. Ave., PDI, CAR, PRO, GEN, CARGEN, PROGEN, are average particle size, polydispersity index, k-Cargeenan, *Prosopis africana* polymer, gentamicin powder, and gentamicin-loaded films CARGEN and PROGEN.

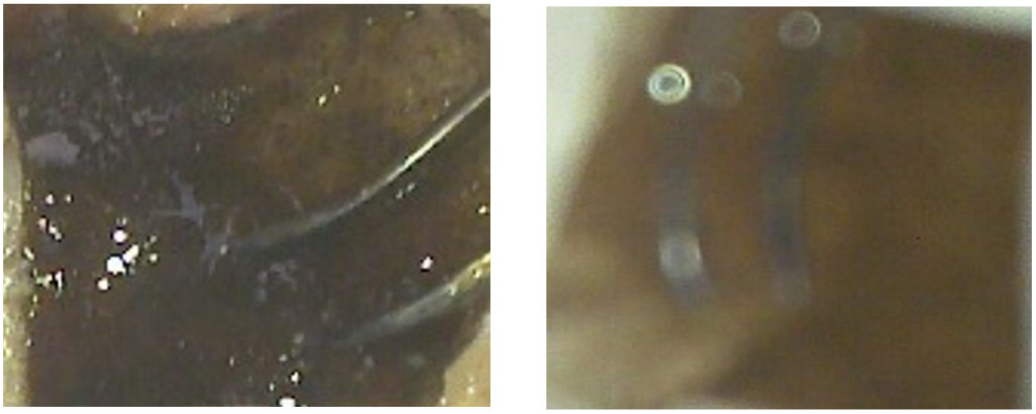

Figure 1: Original photos of films: CARGEN (A) and PROGEN (B)

The pH was acceptable, i.e., within the skin pH of 5.5-6.5. Drug content was slightly higher in PROGEN (98.9 %) than in CARGEN (90.2 %).

Additionally, SEM images (Figure 2, next page) suggest that GEN films (CARGEN (B) and PROGEN (D)) were amorphous, somewhat flaky (ash-like), and fluffy compared to the plain polymers (A and C) CAR and PRO, respectively, which had varied shapes. FTIR spectra (Figure 3) show that a characteristic peak of GEN around 3700–3900 cm^{-1} was also

recorded in the spectrum of CARGEN, which suggests that GEN was successfully encapsulated in CAR polymer. The interaction bands at 3900–3560 cm^{-1} were due to the N-H bond from GEN and 1800–-1600 cm^{-1} due to the C=O bond from CAR. In the PROGEN spectrum, there was complete disappearance of GEN peak except for an augmented reaction at 3356 and 3541 cm^{-1} due to hydroxyl group (O-H stretching vibration, intermolecular H-bonding). This disappearance has been reported due to the molecular dispersion of GEN in the PRO polymer [12, 14-19].

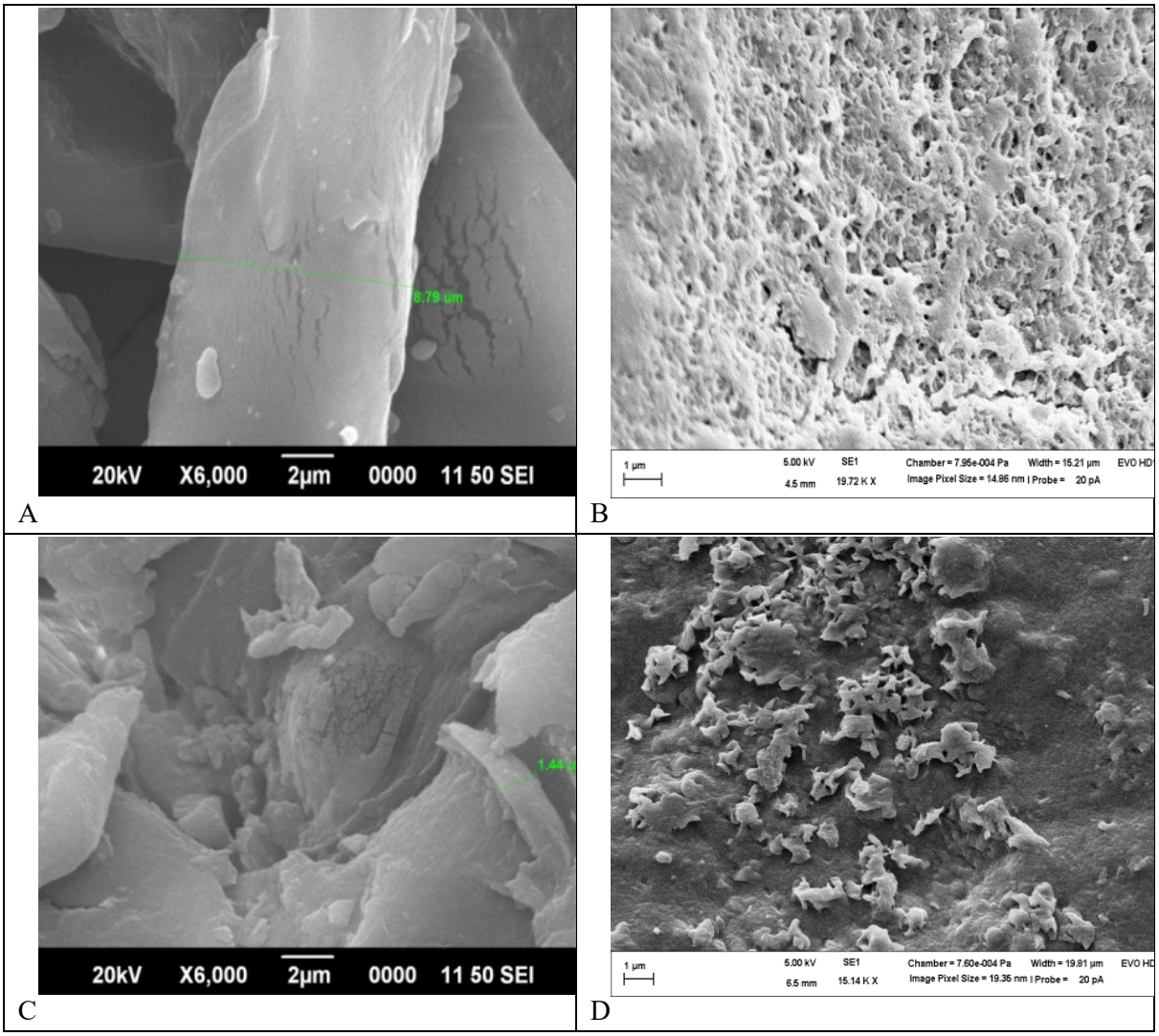

Figure 2. Morphology and internal structure of CAR (A); CARGEN film (B); PRO (C), and PROGEN film (D).

Figure 4 shows that PROGEN films released ~98 % GEN at 8 h, more than CARGEN (83 % at 10 h) and GEN powder (~81 % at 1 h) *in vitro*. The results of formulation effect on growth inhibition zone diameter using the agar plate diffusion method are presented in Figure 5. The drug containing films (PROGEN>CARGEN>GEN) gave good zones of growth inhibition against (A) *Escherichia coli*, (B) *Staphylococcus aureus,* (C) *Salmonella typhi,* and (D) *Pseudomonas aeruginosa* while (E) *Klebsiella pneumoniae* was insensitive to actions of GENTA cream but susceptible to drug films.

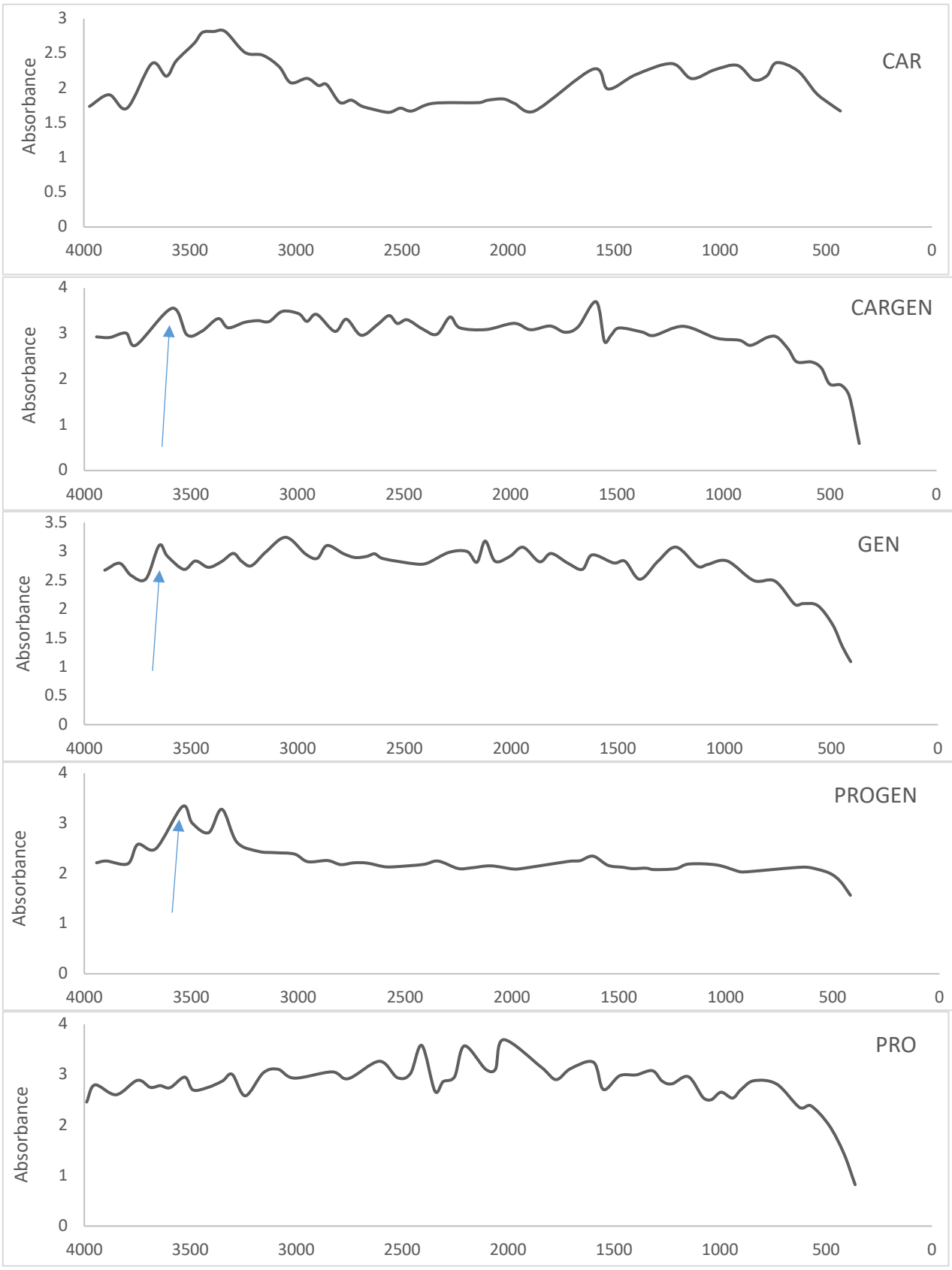

Figure 3. FTIR plots. Legends: CAR refers to Carrageenan; CARGEN means Gentamicin-loaded Carrageenan film; GEN means Gentamicin; PROGEN means Gentamicin-loaded Prosopis africana film; PRO refers to Prosopis africana

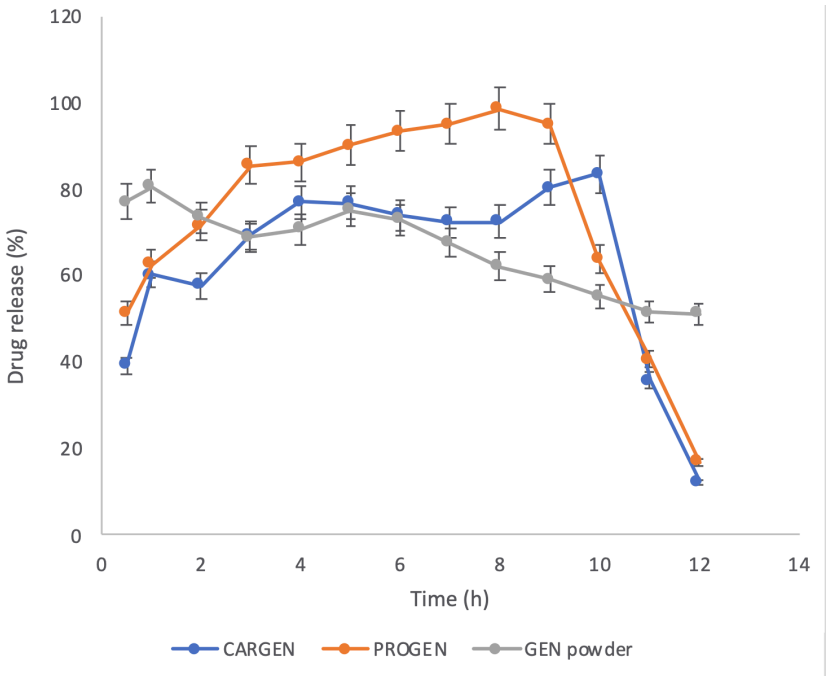

Figure 4. In vitro drug release study (n=6). Legends: CARGEN: Gentamicin-loaded Carrageenan film; GEN: Gentamicin powder; PROGEN: Gentamicin-loaded Prosopis africana film

There was a significant difference (P<0.0001) upon multiple comparisons (One-way ANOVA) of the various treatment efficacies against organisms generally as well as on each studied microorganism (Figure 5). First, the PROGEN formulation had the best growth inhibition on all microorganisms (P<0.0001), followed by CARGEN (P<0.0001) against the commercial sample (GENTA Cream). PRO alone equally had significant growth inhibition (P<0.0001) against A (*E. coli*), C (*Sal. typhi*), and D (*Klebsiella pneumonia*) but insignificant effects on B (*Staph. aureus*, *ns* $p > 0.7586$) and D (*Pseud. aeruginosa,* $p > 0.1855$) when compared to GENTA Cream. GEN alone also demonstrated significant growth inhibition against A, B, C ($^{****}p < 0.0001$) and E ($^{***}p = 0.0001$) but insignificant effect on D (*ns* $p > 0.0877$). Secondly, the susceptibility of *Klebsiella* to gentamicin alone, either as free drug (GEN) E ($^{***}p = 0.0001$) or as a commercial product (GENTA Cream) alone, was clearly less than all other microorganisms ($^{****}p < 0.0001$). Surprisingly, however, *Klebsiella* showed some susceptibility for PRO ($^{****}p < 0.0001$), which was even stronger than GEN alone ($^{***}p = 0.0001$), whereas CAR alone did not show any growth inhibition (data not shown). The gentamicin containing PROGEN nanogel films showed the strongest growth inhibition for *Klebsiella pneumonia* (E) compared to all other tested formulations, in agreement with other tested organisms (A, B, C, and D). In other words, it was clear that the combinations of PROGEN and CARGEN were always better than GEN alone. Figure 5 shows that PROGEN is somewhat better than CARGEN on all tested five organisms (A-E). Meanwhile, there was a slight but significant inhibition by PRO alone on ACE microorganisms compared to GENTA Cream. This inherent antibacterial activity of PRO may have augmented the performance of GEN in PROGEN nanogel films, hence its outstanding performance.

In vivo wound healing assay

Cutaneous administration of drug films significantly accelerated wound closure and healing with no scabbing observed in contrast to the commercial GENTA cream sample used (Figure 6, next page).

Measurements of wound closure for all treatment groups at 0, 3, 5, 6, 9, 10, 12, and 15-days post-wounding are shown in Figure 7 (n=6). On day 3 post-injury, the NS group showed signs of hemorrhage and inflammation in the wounds ($^{*}p < 0.0223$). PROGEN films had no measurable effect (0–3 days) until day 5, and the action exerted on this day 5 became comparable to CARGEN, which has a quantifiable impact from day 3, and GENTA cream,

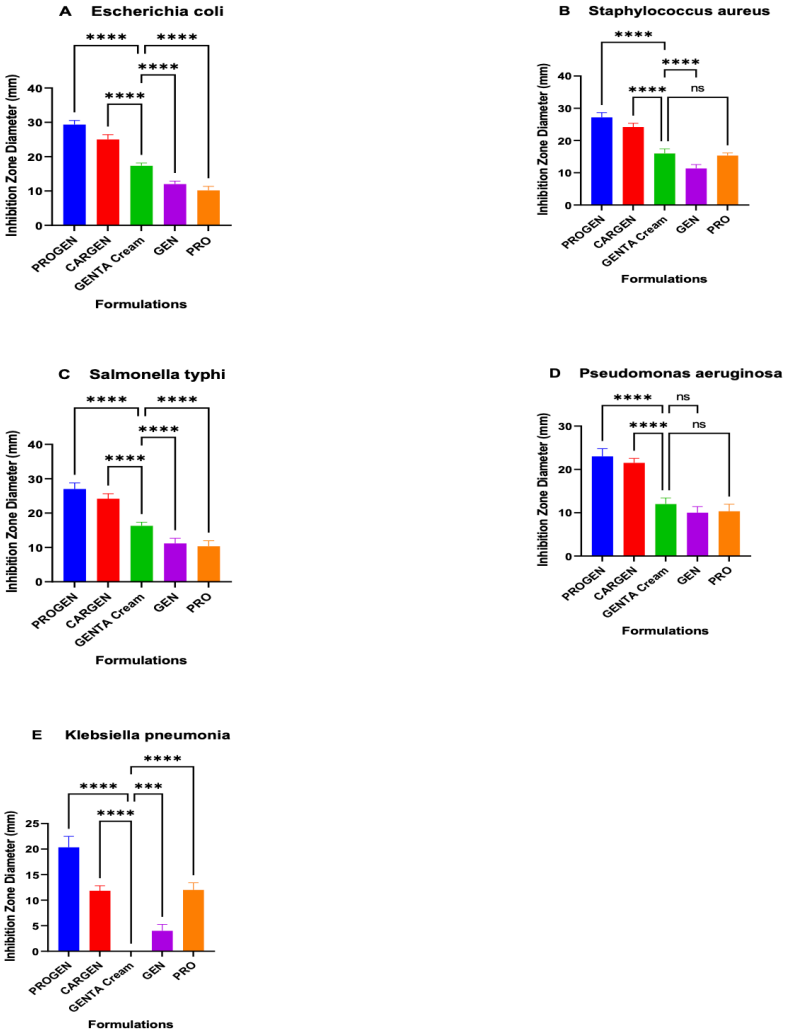

*Figure 5. Effect of tested formulations on bacterial growth. One-way analysis of variance (ANOVA), Dunnett multiple comparisons test: A (****$p < 0.0001$); B (****$p < 0.0001$; ns $p > 0.7586$); C (****$p < 0.0001$); D (****$p < 0.0001$; ns $p > 0.0877$; $p > 0.1855$); E (****$p < 0.0001$; ***$p = 0.0001$). $N \geq 25$ of ≥ 5 individual experiments, (**** and *** imply significant difference) and ns = not significant.*

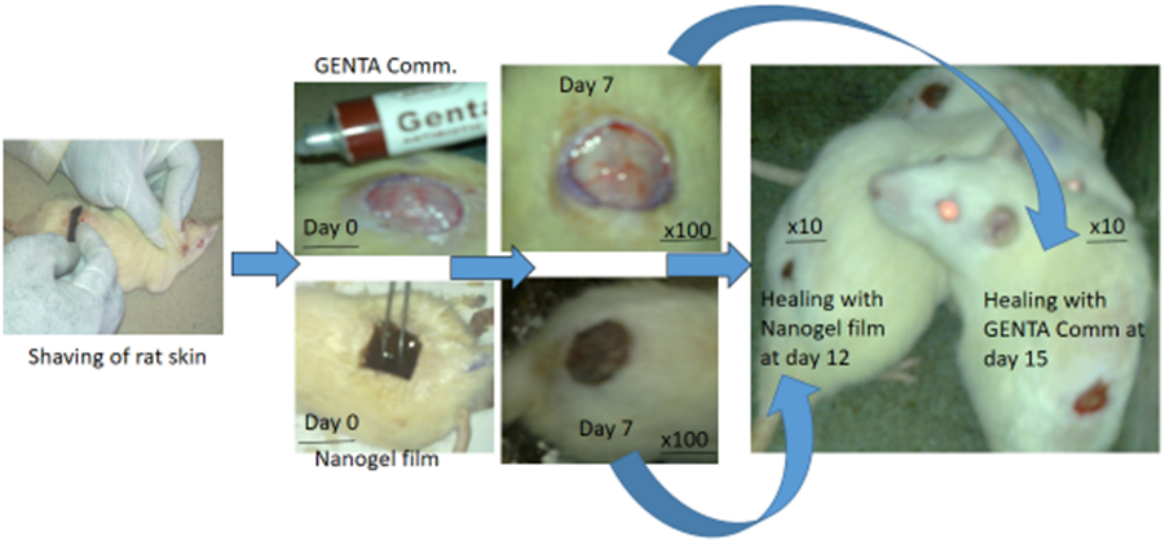

Figure 6. Images of wound closure during treatment.

which acted from day 0. In other words, GENTA cream was faster acting (between days 0-6) than the GEN nanogel films within the first 6 days. However, on day 9, PROGEN films demonstrated significantly faster (P<0.013) but controlled diametrical wound closure (0.6 cm) than CARGEN (1 cm) and GENTA cream (0.9 cm); which, respectively, on day 15 gave closure diameters of 0.19, 0.35 and 0.50 cm (P<0.010). The overall statistics of the healing process from day 0 to 15 agree that a significant effect was measurable from day 9 ($^{**}p < 0.0044$) to day 15 ($^{****}p < 0.0001$).

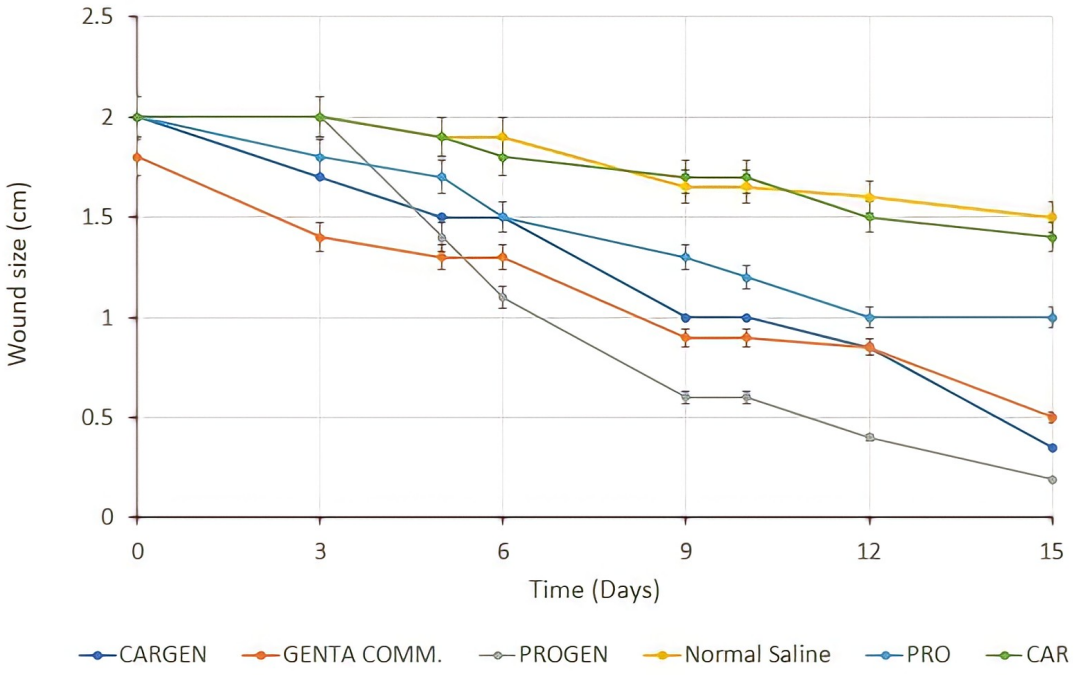

Figure. 7: Wound healing properties of the formulations. *PROGEN means Gentamicin-loaded Prosopis africana nanogel film; CARGEN means Gentamicin-loaded Carrageenan nanogel film; Genta Comm is the commercial cream sample of GEN; PRO refers to Prosopis africana; CAR refers to Carrageenan; Normal Saline served as control.*

PROGEN films had the highest percentage of wound-healing (90.50% ± 4.12 %, *P*, 0.001), significantly higher than CARGEN film (82.50 ± 3.92 %, *P*, 0.011), GENTA commercial cream (72.22 % ± 4.62 %), PRO (50 % ± 2.02 %), NS groups (25 % ± 2.93 %), and CAR (30 % ± 1.02 %) as summarized in Table 2.

Table 2: Wound healing of the rat in the full-thickness excisional model (n=6)

Wound healing time (days)	Samples ± SD					
	CARGEN	GENTA COMM.	PROGEN	Normal Saline	PRO	CAR
0	2 ± 0.05	1.8 8 ± 0.03	2 ± 0.04	2 ± 0.06	2 ± 0.05	2 ± 0.06
6	1.5 ± 0.01	1.3 ± 0.01	1.1 ± 0.01	1.9 ± 0.03	1.5 ± 0.02	1.8 ± 0.04
9	1 ± 0.01	0.9 ± 0.01	0.6 ± 0.01	1.65 ± 0.02	1.3 ± 0.02	1.7 ± 0.03
15	0.35 ± 0.01	0.5 ± 0.01	0.19 ± 0.01	1.5 ± 0.02	1 ± 0.01	1.4 ± 0.02
Wound healing (%)	82.50 ± 3.92	72.22 ± 4.62	90.50 ± 4.12	25.00 ± 2.93	50.00 ± 2.02	30.00 ± 1.02

Discussion

CAR and PRO polymers were used in the present study to deliver GEN as drug films for wound healing (Figure 1). The two polymers could be easily mixed with the water-soluble drug (gentamicin), forming nanogels that could be dried to films suitable as potential wound dressings. As shown in Figure 2, the dispersions contained larger particles of plain polymers, which tended to be smaller when interspersed with GEN. Bio-pharmaceutical properties (e.g., drug content, pH, thickness, water content, bio-adhesivity, stability, etc., (Table 1) appeared suitable for open wound dressing, so all films were considered for further biopharmaceutical testing. It is imperative to cover an injury with an effective dressing as early as possible to remove exudate, inhibit exogenous microorganism invasion, and initiate rapid skin regeneration [39, 40]. It is widely accepted that a warm, moist wound healing environment could optimize healing rates, and various wound care products such as hydrogels [17-19], films [25], sponges [28], and nanofibers are designed to provide these conditions [12, 17, 18, 39, 40]. Additionally, biocompatible hydrogels with *in-situ* gel-forming properties have raised great interest in wound dressing due to many desirable advantages such as the provision of a moist environment, the flexibility of loading growth factors or drugs, proper adherence without wrinkling or fluting in the wound bed, ease of application and improved patient compliance [40].

PROGEN and CARGEN films similarly appeared to provide an adequate environment for moist wound healing without the need for cumbersome, elaborate, or expensive secondary dressing, yet containing high contents of the antibiotic drug (98 and 90 % respectively); releasing high amounts of GEN over extended periods with PROGEN (~98 % at 8 h) as the best (Table 1), followed by CARGEN (83 % at 10 h) then the commercial GENTA cream (81 % at 1 h). PROGEN release curve depicted an ideal sustained-release regimen (better than CARGEN) and GENTA cream (n=6). This observation agrees with the high GEN content of other film delivery systems that employed PURASORB® polymers [25].

PRO and CAR interacted differently with GEN (Figure 3). GEN entrapment in PROGEN showed high peak intensity at 3055 cm^{-1} due to hydrogen bonding (O-H group) which was not seen for PRO alone. On the other hand, GEN encapsulation in CARGEN resulted in increased peak intensities at 3772 and 1882 cm^{-1} (free N-H and C=O stretch). Additionally, CARGEN, which involved reaction with nitrogen from GEN (N-H and C-N groups), could have offered steric hindrances by the groups on nitrogen, hence may be responsible for the longer duration of GEN release (10 h). On the other hand, PROGEN may have additionally taken advantage of its natural sulfur-containing amino acid (methionine), which naturally works well on the skin, hair, and as a chelator [28], to proffer controlled *in vitro* and *in vivo* release of GEN as the overall best formulation (Figure 4). Figure 5 shows the zone of bacterial growth inhibition generally in agreement with the order of PROGEN>CARGEN>GENTA Cream>GEN powder against all organisms (A) *Escherichia coli*, (B) *Staphylococcus aureus,* (C) *Salmonella typhi,* and (D) *Pseudomonas aeruginosa* ($P<0.0001$). Only *Klebsiella pneumoniae* (E) was resistant to the activity of GENTA cream, represented by no growth inhibition at all (Figure 5E). PRO polymer inherently had mild antibacterial properties, and this observation agreed with some reports [39, 40], but CAR had none in consonance with the literature [40, 41]. The wound closure rate seemed to invariably reflect the drug release rate, which corroborates that both polymer nanogel-films released GEN in a sustained manner due to molecular interactions with the CAR and/or PRO polymers.

GEN was released slowly, which means the films could have acted as a wound dressing and a sustained drug depot. Table 2 displays the wound healing capabilities of the rat full-thickness excisional model and indicates that there is a much faster recovery of the wounds treated with PROGEN ($P<0.001$) and/or CARGEN (<15 days) ($P<0.013$) than with GENTA cream (>15 days). PRO is a natural polymer with proof of skin and wound healing properties [28, 40, 41], and this property may have augmented the superior performance of PROGEN films over the other films. In addition to accelerated closure, the wounds displayed much faster wound maturation and re-epithelialization, more well-formed granulation

tissue, and more compact and denser collagen alignment without scabbing. Optimum healing of wounds requires a complex physiological response process involving four distinct but overlapping phases: hemostasis, inflammation, proliferation, and remodeling [2, 42, 43]. Efficient wound repair is essential to protect the body against debris and foreign pathogens, while any disturbances in the process may impair the restoration of tissue architecture and function, resulting in chronic wounds or even uncontrolled wound healing [44, 45].

Concerning these basic principles, any dressing that does not provide the necessary moisture for wound healing should be disregarded except probably when dealing with infected wounds in which a moist environment could exacerbate the infection. While there are many products, devices, and dressing materials to aid in wound management, PROGEN and/or CARGEN nanogel films could make a significant difference by delivering gentamicin as an antibiotic wound dressing (Figures 5-7). Despite the intrinsic skin and wound healing effect of PRO, which synergistically improved the performance of GEN in PROGEN film above all, these film formulations, however, circumvented the difficulties inherent in conventional GENTA topical administration (as powder or cream), enabling sustained delivery and wound healing without scar formation [45-47] (Figure 6). The healing processes observed with drug-containing nanogel films have shown good drug content, microbial growth inhibition and/or release, and significant enhancement of wound healing rate due to suitable particle sub-division and environment provided by the film-forming polymers. Based on sustainable and affordable natural polymers, PROGEN and/or CARGEN have the potential to serve as novel topical treatments for cutaneous wound healing. This formulation appears promising for possible scale-up. The ingredients are available and affordable, and the methods are reproducible and feasible for large-scale production.

Conclusion

PROGEN and/or CARGEN nanogel films positively affect wound healing. The intrinsic skin and wound healing effect of PRO synergistically improved the performance of GEN in PROGEN nanogel film. Our study has demonstrated the ease of application and practicality of these drug films in providing the needed moist conditions for optimal healing compared to currently available GENTA cream. Containing the antibiotic GEN as submicron-sized nanogels, these films appear ideally suitable for treating established wounds by topically delivering adequate antibacterial activity to maintain open wounds in a healthy, non-infected condition. The drug films also improved cosmetic appearance and prevented pathologic scar formation. In an outlook also, it may be imperative to explore further cellular-level effects, such as the production and release of different cytokines and other mediators, proven lately to be essential in determining the outcome of wound healing.

Conflict of interest

The authors declare no conflict of interest in the work done.

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