

Research Article

Zukhra Abbasi, Bushra Uzair*, Barkat Ali Khan, Farid Menaa, Mohd Saeed, Irfan Ahmad, and Amjad Islam Aqib*

Tracking success of interaction of green-synthesized Carbopol nanoemulgel (neomycin-decorated Ag/ZnO nanocomposite) with wound-based MDR bacteria

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Abstract: Multidrug-resistant wound infections are a global health threat and a leading cause of death, persisting despite available treatments due to antibiotic resistance, biofilms, and ineffective drug delivery systems. The aim of this study is to (i) formulate an innovative nano-drug delivery system (NDDS) based on a Carbopol nanoemulgel (NEG) co-loaded with neomycin-silver/zinc oxide nanocomposite (NC) that could fight clinical MDR and treat biofilm-forming wound pathogens through topical application, and (ii) assess its *in vivo* wound-healing potential. The silver/zinc oxide (Ag/ZnO) NC was synthesized by co-inoculating the metabolites of *Aspergillus welwitschiae* and *Meyerozyma guilliermondii*. The synthesized NC was then conjugated with neomycin and loaded into a Carbopol NEG for efficient topical delivery. The resulting Neo-Ag/ZnO NEG was characterized physicochemically (e.g., UV-visible [UV-Vis] spectrophotometry, field emission scanning electron microscopy, X-ray diffraction, and Fourier transform infrared [FTIR] spectroscopy), biologically (e.g.,

in vitro antimicrobial, antibiofilm, and hemolytic activities), and pharmacologically (e.g., drug content, *ex vivo* drug release behavior, and *in vivo* wound-healing potential). The physicochemical analysis confirmed the successful mycosynthesis of the Carbopol NEG-loaded Neo-Ag/ZnO NC. SEM depicted a crystalline polyhedral shape of the small NC (average particle size of 38 nm). FTIR studies showed a slight interaction with the drug and other bioactive moieties in the Carbopol NEG. The Neo content in the Carbopol NEG was as high as 98%, and a maximum release of 81% for Neo, Ag, and ZnO ions was noticed after 12 h. The NDDS appeared hemocompatible and displayed a minimal inhibition concentration of 0.002 µg/mL with the greatest antimicrobial potential against *S. aureus* (an inhibition zone of 46 mm) compared to other tested wound microbes ($p < 0.05$). Statistically significant wound-healing activity was found for NDDS ($p = 0.0001$) in comparison to the control at a concentration of 100 mg/mL. The results showed that this newly developed Carbopol NEG-loaded neo-Ag/ZnO NC appeared promising for controlling resistant skin infections and boosting wound regeneration.

Keywords: green synthesis, Carbopol nanoemulgel, Neo-Ag/ZnO nanocomposite, antimicrobial activity, wound healing

* **Corresponding author: Bushra Uzair**, Department of Biological Sciences, International Islamic University, Islamabad 44000, Pakistan, e-mail: bushra.uzair@iiu.edu.pk

* **Corresponding author: Amjad Islam Aqib**, Department of Medicine, Cholistan University of Veterinary and Animal Sciences, 63100, Bahawalpur, Pakistan, e-mail: amjadislamaqib@cuvas.edu.pk

Zukhra Abbasi: Department of Biological Sciences, International Islamic University, Islamabad 44000, Pakistan

Barkat Ali Khan: Drug Delivery and Cosmetics Lab (DDCL), GCPS, Faculty of Pharmacy, Gomal University, Dera Ismail Khan 29050, Pakistan

Farid Menaa: Department of Internal Medicine and Nanomedicine, California Innovations Corporation, San Diego, CA, 92037, United States of America

Mohd Saeed: Department of Biology, College of Sciences, University of Hail, Hail, Saudi Arabia

Irfan Ahmad: Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, Abha, Saudi Arabia

Abbreviations

Ag	silver
ANOVA	analysis of variance
BLAST	basic local alignment search tool
B-NEG	blank nanoemulgel
CTAB	cetyl trimethyl ammonium bromide
DIW	deionized water
EDX	energy-dispersive X-ray spectroscopy
FESEM	field-emission scanning electronic microscopy

FTIR	Fourier-transformed infrared (spectroscopy)
ITS	internal-transcribed spacer
GC-MS	gas chromatography-mass spectrometry
MDR	multidrug resistance
MIC	minimum inhibition concentration
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
NC	nanocomposite
NEG	nanoemulgel
Neo	neomycin
Neo-Ag/ZnO NEG	neomycin-loaded Ag/ZnO NEG
NDDS	nano-drug delivery system
NIST	National Institute of Standard and Technology
PBS	phosphate buffer solution
PEG	polyethylene glycol
RBCs	red blood cells
RP-HPLC	reverse-phase high-pressure liquid chromatography
UV	ultraviolet
Vis	visible
WTC	wound total closure
ZnO	zinc oxide

1 Introduction

Untreatable microbial infectious diseases, especially those associated with MDR bacteria, remain the second leading cause of mortality in developing countries and the third leading cause globally, despite several developments in the medical field, as per a 2019 World Health Organization report [1]. Infection is a complication in both acute and chronic wounds. Common resistant pathogens associated with wound infections are *S. aureus*, *P. aeruginosa*, and *C. albicans* [2]; all three impede the regenerative process, leading to complex amputations and sepsis due to biofilm formation [3,4].

The effectiveness of existing treatments such as Ceftaroline, Fosfomycin, Dalbavancin, and Ceftobiprole against MDR bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA), as well as Ceftolozane-Tazobactam, Aztreonam-Avibactam, and Ceftazidime-Avibactam against *P. aeruginosa* is diminishing due to the emergence of resistance mechanisms as stated by Giurazza *et al.* [5]. Likewise, antifungal medications, including fluconazole, chlorhexidine, nystatin, and amphotericin, become less effective as microbes develop resistance mechanisms, which can include the production of enzymes (lipases,

proteinases) [6]. Additionally, the injudicious administration of antibiotics, as highlighted by Ahmed *et al.* [7], exacerbates the problem, underscoring the urgent demand for innovative antimicrobial approaches [7]. Notably, earlier studies point out the existing gap between the escalating antimicrobial resistance (AMR) and the development of new antibiotics, as elucidated by Pammi *et al.* [8]. In this context, recent research has shown promising avenues in nanomaterial-based antimicrobials, as bacteria exhibit reduced resistance to metal nanoparticles (NPs) compared to conventional antibiotics [9]. For instance, Ag NPs exhibit broad-spectrum antimicrobial activity, effective against MDR strains [10], but have concerns over cytotoxicity and agglomeration, which can be alleviated by capping Ag with less toxic metal oxide NPs, such as ZnO or polymers [11]. ZnO NPs, approved by the FDA, are widely used in medicine due to their affordability and diverse therapeutic benefits, including anti-inflammatory, antimicrobial, and wound-healing activities [12]. Therefore, Ag–ZnO nanocomposite (NC) could offer a new way of preventing infection and favoring wound healing. In addition, green synthesis methods for metallic NPs have emerged as viable approaches, reducing toxicity and offering significant benefits to the pharmaceutical industry [13]. The fungus-mediated synthesis of metal oxide NPs is novel and more advantageous than synthesis mediated by bacteria or plants [14]. Endophytic fungal strains residing within medicinal plants produce NPs of different sizes, stability, and shapes enhancing their biomedical applications. This is attributed to their ease of handling, ability to produce a large amount of biomass, high metal binding strength, tolerance to metals, and secretion of metabolites [15]. Furthermore, what sets our study apart is the utilization of a fungal consortium for NPs synthesis, which enhances the efficiency and scalability of production while minimizing environmental impact.

The establishment of antibiotic(s) synergism with NPs further enhances the spectrum of antibacterial activity while reducing toxicity by targeted drug delivery [16,17]. Neo (neomycin sulfate), generally recognized as a safe topical antibiotic, acts by disrupting bacterial DNA and protein synthesis, leading to reactive oxygen species (ROS)-induced cell death [18]. Applying this combined approach with Ag/ZnO NC could effectively address wound pathogens resistant to conventional treatments.

Currently, topical DDS are a leading choice for favoring the healing of infected wounds; this increasing interest is due to their high therapeutic index and minimal non-target organ toxicity compared to other routes of drug administration (*e.g.*, systemic [intravenous] and *peroral* [oral]) [19]. Nanoemulgels (NEGs) are the preferred option among topical drug delivery systems (emulsions, gels, or emulgels) for their effective protection of drugs from enzymatic degradation, enhancement

of emulsion stability through increased viscosity, and reduction of surface tension. This results in superior therapeutic efficacy in enhancing drug permeability, bioavailability, and skin diffusion, making NEG a promising option for topical drug delivery [20–22]. Moreover, these nano-drug delivery systems (NDDSs) provide a controlled and sustained delivery of topical drugs to the targeted site [23]. Recently, a study by Rehman *et al.* showed the enhanced wound-healing effect of eucalyptol (EU) due to improved permeability and bioavailability provided by the nanocarrier (NEG) [24]. Further, polymeric substances are widely used as matrices in topical formulations, including for wound healing [25–27], to enhance or confer unique properties like stability. In this regard, the synthetic polymer Carbopol is considered safe and widely used as a gelling agent in topical pharmaceutical formulations for sustained release [28]. Hence, the co-delivery of neomycin (topical antibiotic) and green synthesized antimicrobial Ag/ZnO NC using Carbopol NEG (NDDS) would result in a promising *in vitro* antimicrobial potential against resistant wound pathogens by eradicating biofilm and *in vivo* wound-healing efficacy due to increased bioavailability, permeability, and sustained release of bioactive moieties at the wound site.

Herein, we aimed to formulate an original Carbopol NEG-loaded Neo-Ag/ZnO NC (Green nanoantibiotic) using a fungal consortium of two strains (*i.e.*, *Meyerozyma guilliermondii* and *Aspergillus welwitschiae*), with the hope of enhancing the efficacy to prevent (resistant) infections in wounds and provide a better topical wound healing. The physicochemical characterization, *in vitro* antimicrobial activity against resistant microbes, and *in vivo* wound-healing efficacy of the NDDS were also investigated. To the best of our knowledge, this is the first investigation reporting such a promising topical NDDS.

2 Materials and methods

2.1 Chemicals

Cetyl trimethyl ammonium bromide (CTAB), mercaptoethanol, proteinase K, sodium dodecyl sulfate, chloroform, isoamyl alcohol, isopropanol, and triethanol buffer were used for DNA (desoxyribonucleic acid) extraction and purification. Silver nitrate (AgNO_3 , 99.9%) and ZnO salts (99.5%) were used to synthesize the NC. Tween 80, polyethylene glycol (PEG) 400, Carbopol 940 (99.99%), and triethanolamine (99.0%) were used to formulate the emulgel. Acetic acid and India ink were used to prepare the fungal stain. All these chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Pure (HPLC-grade) neomycin (see datasheet in Suppl. Mat. File) and olive oil

were purchased from the local market. All the reagents used were of analytical grade and did not require further purification.

2.2 Strains

The three microbial strains, *i.e.*, *P. aeruginosa*, *S. aureus*, and *C. albicans*, were isolated from infected wounds of human patients to check their antibacterial and antifungal activity. They were kindly gifted from the National Institute of Health (Islamabad, Pakistan). These skin microbial strains were maintained on their respective growth media at 4°C and were refreshed after 2–3 months.

2.3 Isolation, purification, and genomic identification of fungal strains

Leaf discs of fresh and healthy *Madagascar periwinkle* and *Cupressus* plants were prepared. Their surfaces were rinsed with distilled water (dH_2O) and ethanol; subsequently, they were dried before being cultivated on potato dextrose agar plates. Plates were sealed with a sealing film and incubated at 26°C for 4 days. The fungi that grew on the leaf discs were sub-cultured on potato dextrose agar plates several times to obtain a pure isolate (single species); the isolates were then preserved at 4°C [29]. The fungal strains IIUI313 (the purified strain from the *Madagascar periwinkle*) and IIUI314 (the purified strain from the *Cupressus* plant), which had rapid growth and large biomass production, were selected to synthesize the NC.

The isolated fungal species were identified phenotypically based on colonial and cellular morphology [30,31], followed by molecular characterization for genotypic identification. A former CTAB method [32] with certain modifications was used to extract the genomic DNA. Nuclear ribosomal internal-transcribed spacers, *i.e.*, forward ITS1 5' TCCGTAGGTGAACCTGCGG-3' (19mer) and reverse ITS4 5'-TCCTCCGCTTATTGATATGC-3' (20mer), obtained from Macrogen (Seoul, South Korea) were amplified using the polymerase chain reaction (PCR) thermal cycler (BIOER, LifeECO, Shanghai, China) in a final reaction volume of 25 μL [32]. The PCR end-product was run on gel electrophoresis (1.5%) at 100 V for 40 min to separate DNA and confirm the amplicon size. The amplified PCR product was sequenced by Macrogen (Seoul, South Korea) using the Sanger sequence technology, and the obtained internal-transcribed spacer-related data of both strains were analyzed using NCBI's (National Center

for Biotechnology Information) BLAST for strain identification [6].

2.3.1 Identification of reducing agents in fungal extracts using GC-MS

Gas chromatography-mass spectrometry (GC-MS QP5050 with a mass detector, Shimadzu, Tokyo, Japan), using helium as the carrier gas, was used for the quantitative analysis of metabolites (including potential reducing and capping agents) in the fungal methanolic extract (20 μ L). The metabolic products present in the fungal extracts (Figures S1 and S2) matched with standard chemical compounds available from the National Institute of Standards and Technology (NIST) 17 library on a mass-spectrum basis [6].

2.4 Mycosynthesis of the Ag/ZnO NC

The Ag/ZnO NC was synthesized using the method described previously [33]. It was modified by mixing cell-free culture extracts from two fungal strains, as shown in Figure 1. Briefly, the NC was prepared by co-inoculation of the fungal extracts *M. guilliermondii* (IIUI313) and *A. welwitschiae* (IIUI314). Both species were isolated as endophytes and grown separately in 250 mL of Sabouraud dextrose broth using 500 mL flasks. Flasks with spores were incubated at $25 \pm 2^\circ\text{C}$ under shaking (120 rpm) for 4 days in dark conditions to produce biomass. This fungal biomass was isolated from the liquid media by physical separation and washed with deionized water (DIW) to remove all unwanted substances. Fresh, clean biomass (24 g) from each strain was placed in two separated 500 mL flasks containing 250 mL of sterile DIW and incubated in a

rotatory shaker under the same previously described conditions. A cell-free filtrate was extracted by filtering the suspension with Whatman filter paper # 1. Then, sterile aqueous salt solutions of 20 mL of 1 M AgNO_3 and 2 M ZnO were mixed with 50 mL of the fungal extracts (25 mL each), followed by continuous stirring (200 rpm) and heating (40°C) on a magnetic hot plate for 3–4 h. The percentage-to-weight ratio of Ag:Zn was 1:1, and the ratio of salts to extract was 4:5 v/v. The reduction of salts was visualized by the color change and the precipitate formation. A light-brown aggregate at the bottom of the flask was separated by centrifugation at 4,000 rpm, followed by a double washing with sterile dH_2O and a single washing with ethanol to remove all unreacted molecules. The particles were eventually dried, calcined at 400°C , and stored at room temperature (RT) until use.

2.4.1 Conjugation of neomycin drug with Ag/ZnO NC

Conjugation of the antibiotic drug Neo in the synthesized Ag/ZnO NC was adapted from a formerly reported method [34]. Briefly, the NC and Neo solutions were made in dH_2O at a concentration of 0.1 g/mL (w/v) each. The NC solution was then added to the Neo solution, stirred constantly (250 rpm) for 2 h at RT, and incubated for 24 h at RT. After 24 h, the suspension was centrifuged (4,000 rpm) for 20 min at RT to obtain a pellet of Neo-conjugated NC.

2.4.2 Encapsulation efficiency (EE) of neomycin in Ag/ZnO NC

Neo-Ag/ZnO NC was assessed for interactions between NPs and antibiotics using a UV-Vis spectrophotometer

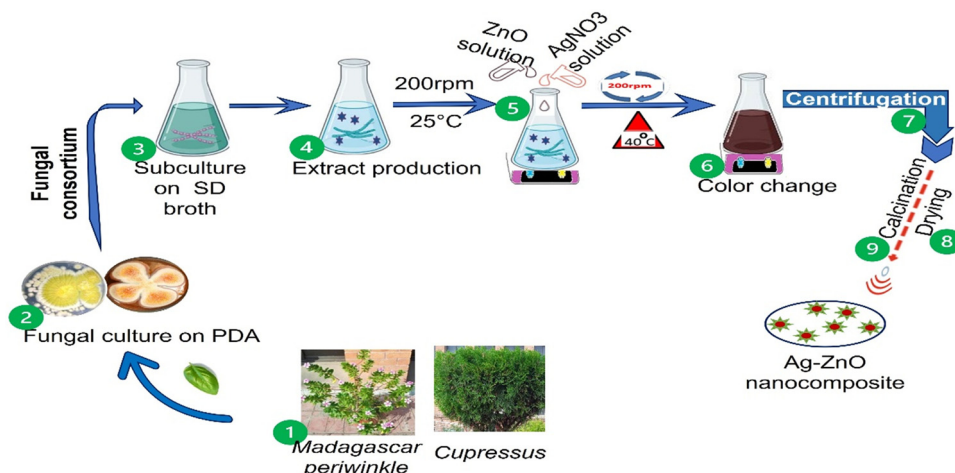


Figure 1: Illustration of the fungal consortium-based synthesis of Ag/ZnO NC.

(HITACHI U 2800, Tokyo, Japan) at the UV-wavelength of 300 nm. The amount of Neo that reacted with the NC was estimated by collecting a volume of supernatant (5 mL) obtained after centrifugation of the neo-conjugated NC and 1/20 dilution in DIW (with 95 mL of DIW). The drug EE was calculated using the following formula [35]:

$$EE(\%) = \frac{\text{Total amount of drug} - \text{Amount of free drug}}{\text{Weight of the obtained NPs}} \times 100.$$

2.5 Preparation of NEG formulations

The synthesis of the NEG is a two-step process, which was based on the method published earlier [36]. It was slightly modified by using pure olive oil in the organic phase and drug dissolution in the aqueous phase (Table S1). Three Carbopol NEGs were prepared (Table S2) and named as follows: (i) blank Carbopol NEG, Formulation 1 (blank NEG, F1); (ii) Carbopol NEG-loaded Ag/ZnO NC, Formulation 2 (Ag/ZnO NEG, F2); and (iii) Carbopol NEG-loaded Neo-Ag/ZnO NC, Formulation 3 (Neo-Ag/ZnO NEG, F3). F3 was selected after optimization using various experimental conditions, as shown in Figure S3.

2.5.1 Step 1. Preparation of nanoemulsions

In this first step, the high-shear homogenization technique was used to mix the organic phase (O) and aqueous phase (W) to synthesize the W/O nanoemulsion. The organic phase was prepared by mixing olive oil and PEG (co-surfactant). Tween 80 (surfactant) was mixed with DIW to prepare an aqueous phase. The NC and the Neo-conjugated NC (50 mg) were added to the aqueous phase and kept in a water bath (GYROMAX 939XL, Amerex Instruments, Inc.) at 70°C for 1 h. Subsequently, the oil phase was added in a dropwise manner to the aqueous phase with constant stirring, initially at 800 rpm (low speed) with an electric stirrer for 8 min to avoid surfactant dissociation. As the formulations cooled to RT, the stirrer speed increased to 1,600 rpm (middle speed) for 8 min. Then, the speed of the stirrer was reduced again to low speed to obtain uniform and homogenized emulsions. In the end, the emulsion was exposed to high-shear homogenization (Daihan Scientific, Wonju, Kangwon-do, South Korea) at 10,000 rpm for 10 min to obtain a clear isotropic nanoemulsion.

2.5.2 Step 2. Preparation of gelling solutions

In this second step, a gelling solution was prepared by dispersing 1 g of Carbopol 940 in 99 g of dH₂O (w/w). This was followed by electrical stirring (WiseStir Direct Driven

HS-120A, Witeg Labortechnik GmbH, Wertheim, Germany) to obtain a homogenized gel matrix. The gel solution was kept overnight to stabilize it and before its incorporation into the W/O nanoemulsion formulation. Eventually, the nanoemulsion was dispersed (uniformly) into the Carbopol gel matrix at a ratio of 1:1 with constant stirring for 24 min. The whole formulation was subjected to high-shear homogenization at 10,000 rpm to obtain the NEG, as illustrated in Figure S4.

2.6 Physicochemical characterization of the mycosynthesized nanoformulations

2.6.1 Physical analyses

UV-Vis spectrophotometry, field emission scanning electron microscopy (FESEM), X-ray diffraction (XRD), and Fourier-transform infrared (FTIR) spectroscopy were used as the routine state-of-the-art techniques for the physical characterizations of nanoformulations [37]. UV-Vis spectrophotometry (HITACHI U2800, Tokyo, Japan) in a wavelength range of 200–800 nm was used to obtain the spectrum of each NC. The synthesis of NEG was confirmed by DLS using a zeta sizer (Nano ZS 90, Malvern instruments, UK) [38]. FTIR spectroscopy (Tensor 27 MRS, Bruker, Bremen, Germany) was used for infrared spectral analyses of the NEGs, mainly the characterization of their active functional groups and interactions. FESEM (TESCAN MIRA3 XMU, Bremen, Germany) was used to analyze the topography and particle size (PS) of the NCs. Finely grounded NCs were mounted on the microscope stub and Cu–C sputter coated (sputter coater CCU-010 HV, Safematic GmbH, Zizers, Switzerland). FESEM, equipped with an energy-dispersive X-ray (EDX) spectrometer, was also used to analyze the elemental composition of the NEGs. X-ray diffractometry (D8 ADVANCE Bruker, Bremen, Germany) integrated with a LynxEye at 60 kV and 60 Ma allowed to obtain the XRD pattern of dried and grounded NEGs. Information about the phase formation, crystalline morphology, and purity of the NCs were provided. The PS was calculated from the very sharp peak obtained with diffraction analysis by applying the following Debye–Scherrer formula [37,39]:

$$D = 0.9\lambda/\beta \cos \theta.$$

2.7 Organoleptic properties

2.7.1 Stability tests

The prepared NEGs (Neo-loaded Ag/ZnO NEG *versus* blank NEG) were inspected for changes in color, pH, viscosity,

phase separation, odor, and spreadability at 0 h, 24 h, 72 h, 1 week, 2 weeks, and 4 weeks, following methods previously described [27].

2.7.2 Spreadability

The spreadability of the biosynthesized NEG was determined by using a previously reported slip-and-drag method [27]. Each prepared NEG (2 g) was placed on a stationary glass slide and fixed with an upper movable slide weighing 70 g. The time (in s) taken by the upper slide to cover 8 cm was noted. The same protocol was performed thrice, and the spreadability of the NEG was calculated by the following equation:

$$S = M \times L/T,$$

where S was the spreadability, M was the weight (in g) placed on the top slide, L was the length (in cm²) of the glass slides, and T was the time (in s) needed by the upper movable slide to separate from the stationary slide.

2.7.3 *In vitro* drug release behavior

The release study for the Neo drug, as well as the Ag and ZnO ions from the Neo-Ag/ZnO NEG, was conducted on a Franz diffusion cell apparatus with an artificial cellulose acetate membrane having a pore size of 0.8 µm. The outer jacket temperature was set at 37°C, and the inner compartment was filled with acetate buffer solution (pH 5.5) to mimic *in vivo* conditions (*i.e.*, human skin pH). One gram of NEG was diluted with 1 mL of acetate buffer and placed in the donor compartment. About 4 mL of aliquot was withdrawn using a sterile syringe from the receptor cell at time intervals of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 h, and the receptor cell was then refilled with 4 mL of fresh acetate buffer solution to maintain sink conditions. Samples were collected in test tubes and quantified by measuring their respective absorbance using a UV-Vis spectrophotometer (HITACHI, Model U 2800, Tokyo, Japan) at the UV-wavelength of 280 nm for Neo, 300 nm for ZnO, and 395 nm for Ag. The standard absorbance was found to be 1.250 for Neo, 0.587 for ZnO, and 1.265 for Ag ions. The percentage of nano-antibiotic released from the NEG overtime was calculated using the following formula [36]:

$$\text{Drug release(\%)} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the standard}} \times 100.$$

2.7.4 RP-HPLC determination of neomycin content in the Neo-Ag/ZnO NEG

The amount of Neo in the Neo-Ag/ZnO NEG was quantified using the reversed-phase high-pressure liquid chromatography (RP-HPLC) technique [40] with a C18 column and methanol as the diluent. A standard neomycin sulfate solution and test neomycin solution (analyte) at a concentration of 20 µL were injected separately into the HPLC injection chamber, and the effluents were examined at the UV wavelength of 265 nm. The peak area for the Neo (sample plus standard) was calculated from the calibration curve obtained by analyzing the reaction for 10 min. At a peak retention time of 5.611 min, the Neo content was determined in the conjugate-based NEG. The quantity of Neo in the Neo-Ag/ZnO NEG was calculated using the following equation:

$$\begin{aligned} \text{Quantity(\%)} \\ = \frac{\text{Average area of the sample} \times \text{Claimed quantity}}{\text{Average area of the standard}} \times 100. \end{aligned}$$

2.8 Biological and safety characterization of the mycosynthesized Neo-Ag/ZnO NEG

2.8.1 *In vitro* antimicrobial assays using DDM

The antimicrobial potential of the Neo-Ag/ZnO NEG (50 µg/mL) was screened for the wound infection-causing pathogens *S. aureus*, *P. aeruginosa*, and *C. albicans*, using the disc diffusion method (DDM) in agar-agar [41,42]. Commercially available Neo ointment was used as a positive control and blank disk was used as a negative control. Inoculums from freshly prepared overnight culture strains of *S. aureus* and *P. aeruginosa* in nutrient broth and *C. albicans* in potato dextrose broth having 10⁵ colony-forming units (CFU) were swabbed over solidified agar plates. About 10 µL from a stock solution (50 µg/mL) of Ag/ZnO NC, Neo-Ag/ZnO NC, and Ag/ZnO NEG were also used as internal controls to impregnate discs. All the dried discs were kept on inoculated agar plates and incubated overnight at 37°C to measure the size of the ZI (in mm). The values were calculated thrice, and the results were averaged.

2.8.2 *In vitro* antimicrobial assay by minimal inhibition concentration (MIC)

MIC assay is used to determine the lowest concentration of an antimicrobial agent that prevents the visible growth of a microorganism. The agar-well diffusion method based on

a previously published work [43], albeit with a slight modification in the dilution factor, was used to analyze MIC of Neo-Ag/ZnO NEG against MRSA isolated from a wound site of a patient. MRSA suspension was prepared in Mueller–Hinton broth (MHB), and turbidity was visually compared to a 0.5 McFarland standard, followed by swabbing onto a Mueller–Hinton agar plate. About 50 μL of five different dilutions having serial concentrations of 0.16, 0.12, 0.08, 0.04, and 0.02 mg/mL of the prepared NEG formulation was added to each well in the inoculated agar plate. The ZI (mm) around each well following overnight incubation was measured to determine the MIC of the NEG.

2.8.3 Biofilm disruption assay against MRSA

Neo-Ag/ZnO NEG was assessed for its antibiofilm potential against MRSA by biofilm diffusion assay [44]. A suspension of MRSA with an approximate cell density of 1×10^8 CFU/mL was maintained in MHB. An MRSA suspension of 100 μL was added to 10 mL of MHB in a sterilized test tube and incubated for 24 h under static conditions. The biofilm was stained with crystal violet, and the excess stain was rinsed off with dH_2O . This was followed by the addition of a 30% acetic acid solution. In addition to the tube containing cells treated with Neo-Ag/ZnO NEG, the assay also included a negative control in a tube containing only the medium, and a positive control in a tube containing untreated cells. The content from the tubes was analyzed at the Vis-wavelength of 595 nm by spectrophotometry (HITACHI, Model U 2800, Tokyo, Japan).

2.8.4 *In vitro* hemocompatibility

The *in vitro* hemolytic studies of the as-synthesized NEG followed the protocol reported in previous studies [45]. A freshly acquired 5% blood sample in an EDTA tube was obtained from a healthy volunteer after written consent (ICF20221011-001) and following the guidelines of the Declaration of Helsinki. The blood was poured into a nutrient agar medium at RT and mixed thoroughly. Neo-Ag/ZnO NEG (200 μL) was transferred to a well and incubated at 37°C for 24 h to evaluate hemolysis. Triton X-100 (positive control, 200 μL), 1 $\times$ PBS (negative control, 200 μL), and neomycin (standard, 200 μL) were also employed to optimize the experiments.

2.9 *In vivo* wound-healing potential

2.9.1 Animal selection

White albino mice (6 males and 6 females, $N = 12$) of 14 weeks with an average weight of 250 g were selected to

evaluate the *in vivo* wound-healing efficacy. The mice were housed in cages in a well-ventilated area at 25°C and fed *ad libitum* prior to the experiment [40].

2.9.2 Experimental protocol

The mice were anesthetized with Ketamine HCl injection (50 mg/kg) and shaved. Then, a wound area of 400 mm^2 was created on the back using a biopsy punch. In the set-up experiment, 4 mice (2 males and 2 females) were used as control (untreated group) and 8 mice (4 males and 4 females) were tested with Neo-Ag/ZnO NEG (treated group) to assess its *in vivo* wound-healing activity. The Neo-Ag/ZnO NEG (100 mg) was applied to the mouse twice a day for 30 min, and the total wound closure, which is attributable both to contraction and to re-epithelialization [46], was measured at 4-day intervals using the following formula [47]:

$$\text{WTC}(\%) = \frac{\text{WA}^0 - \text{WA}^t}{\text{WA}^0} \times 100,$$

where WA^0 represents the wound area at day 0, and WA^t represents the wound area at time point t .

2.10 Statistical analysis

The one-way ANOVA, repeated measures (RM), one-way ANOVA, and unpaired *t*-test were performed using Graphpad Prism 8.0.2 (San Diego, CA, USA). Experiments were performed thrice, and the obtained values were presented as mean $\pm$ SD. *p*-values <0.05 were considered as statistically significant.

Ethical considerations: Animal experimentations were approved on 12/27/21 by the ethical review board (ERB) of the International Islamic University, Islamabad, Pakistan (Reg #470-FBAS/MSBT/F20). The experiments were conducted according to the recommendations and guidance of this ERB. All precautions have been undertaken to minimize the suffering of animals.

3 Results and discussion

With approximately 100 million people suffering from acute wounds and 300 million from chronic wounds worldwide, addressing these conditions poses a substantial economic burden [3,48]. However, despite the availability of various therapeutic wound-healing agents, the challenge of effectively treating infectious wounds persists due to microbial resistance to antibiotics. To overcome this hurdle, metallic NPs have emerged as a promising alternative, as

microbes exhibit less resistance to them. Nonetheless, the cytotoxicity associated with metallic NPs could be mitigated by encapsulating them with polymers, thus enhancing their therapeutic potential in wound-healing applications [11]. Hence, current research focused on the production of a new and potent broad-spectrum green nano-antibiotic, namely Neo-Ag/ZnO NEG, to combat MDR wound pathogens while favoring wound healing. Topical antibiotic “neomycin” was incorporated with Ag/ZnO NC to increase its antimicrobial potential. Further, this combination was loaded into NDDS for maximum therapeutic effect on resistant pathogens on wound beds while reducing toxicity. In this pioneered study, a selected fungal consortium produced Ag/ZnO NC in a large amount (4 g) within a short incubation time (3–4 h), which might be due to the secretion of metabolites (including reducing and capping agents) in substantial amounts, as indicated by GC-MS analyses.

3.1 Identification of fungal strains

Morphological features of major fungal strains, respectively, found in *M. periwinkle* and *Cupressus* plant leaves,

were examined. The colonial and cellular morphology revealed with India ink and without India ink (control) is shown in Figure 2; the findings were confirmed by molecular characterization. The IIUI313 strain found in the *M. periwinkle* leaves was *M. guilliermondii* (GenBank accession number: OP216058), and the IIUI314 strain found in the *Cupressus* leaves was *A. welwitschiae* (GenBank accession number: OP216102). A phylogenetic tree of these identified fungal strains was constructed using Mega-7 software by the neighbor-joining method with 700 bootstrap replications, as represented in Figure 3.

3.2 Phytochemical analysis of the mycoextracts by GC-MS

Myriads of metabolic products were detected and analyzed by GC-MS in the extracts of *M. guilliermondii* (Figure S1, Table S3) and *A. welwitschiae* (Figure S2, Table S4). The main chemical compounds found in *M. guilliermondii* were squalene, di (1,2,5-oxadiazolo) [3,4-*b*:3,4-*E*] pyrazine, stearic acid, 10(*E*),12(*Z*)-conjugated linoleic acid, and dibutyl phthalate, whereas 1,2-benzenedicarboxylic acid, palmitic acid, phthalic acid, stearic

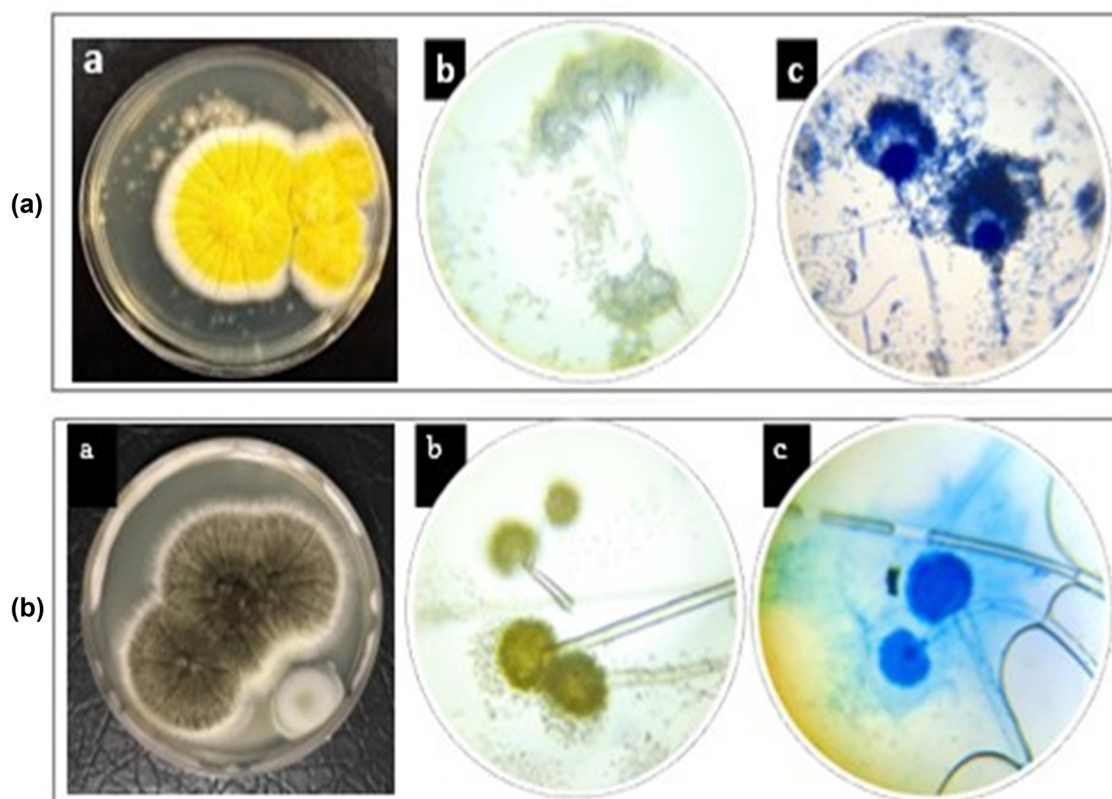


Figure 2: Phenotypic identification by optical microscopy of *M. guilliermondii* (a) and *A. welwitschiae* (b): ((a) colonial morphology; (b) cellular morphology without staining; (c) cellular morphology with India-ink staining. Magnification $\times 40$).

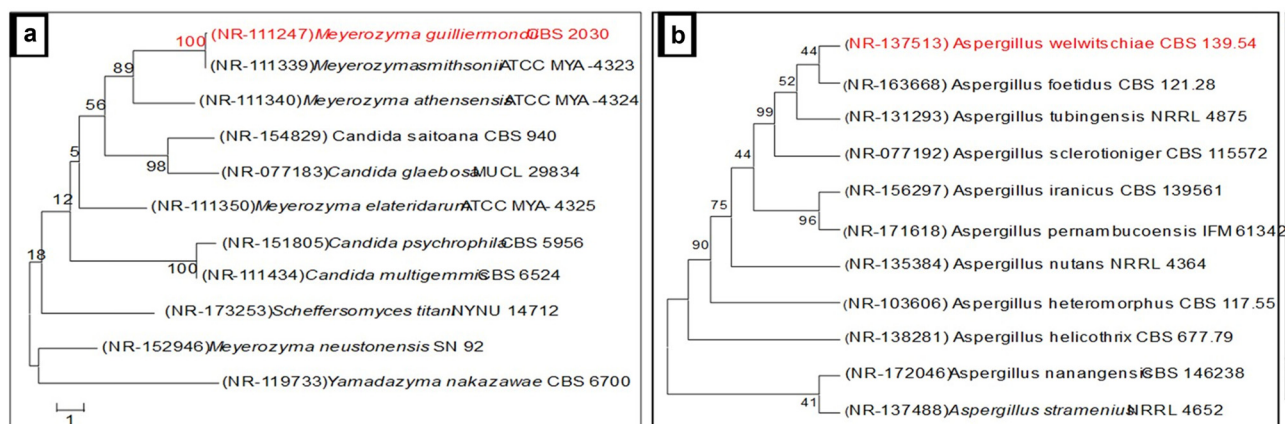


Figure 3: Genotypic identification of *M. guilliermondii* (a) and *A. welwitschiae* (b).

acid, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl-phenol] were detected in *A. welwitschiae*. These metabolites can not only contribute to the identification of the fungal strain but also, like phytochemicals, might act as reducing and capping agents during the production of NC (e.g., Ag/ZnO NC). Thereby, these metabolites most likely reduced the salts Ag^{2+} to Ag^0 and Zn^{2+} to Zn^0 since a color shift was noticed from white to dark brown during the synthesis of Ag/ZnO NC. The selected strains produced a relatively high yield of Ag/ZnO NC (i.e., 4 g) in a relatively short time (within 3–4 h). Moreover, this metabolic diversity enhances the properties of the NPs, including stability and variations in shape. Similar to the varying reducing powers of different agents, as reported by Yoo *et al.* [49], the distinct bioactive compounds and enzymatic activities present in each fungal extract play a crucial role in influencing the synthesis of NPs.

3.3 Physicochemical characterizations of the mycosynthesized nanoformulations

The EE of Neo (100 mg/mL) in Ag/ZnO NC analyzed using a UV-visible spectrophotometer was calculated as 81%. The high encapsulation efficiency of the drug in the Ag/ZnO NC was probably due to the high-level solubility of Neo in the solvent (H_2O). The results were compatible with a recent study that reported the encapsulation efficiency of azithromycin to range between 50 and 87% in ZnO NPs using drug concentrations in the range of 200–500 mg [50].

The strong and broad absorbance peaks at 370 nm for ZnO, 410 nm for Ag, and 415 nm for Neo-Ag/ZnO NC are shown in Figure 4a observed during diffused reflectance spectroscopy analysis. The band gap values were calculated as 3.4 eV for Zn, 3.02 eV for Ag, and 2.95 eV for Neo-Ag/ZnO NC using the formula $E_g = 1,240/\lambda_{\text{abs}}$. Diffused reflectance spectroscopy confirmed the nanoscale and

absorbance range of AgNPs [51–53] and ZnO NPs [54–56]. The absorbance shifts toward a longer wavelength confirmed the successful conjugation of the Neo drug to Ag/ZnO NC [57]. The agglomeration of NPs with the antibiotic, as well as the decrease in the energy gap between the excited state and the ground state, might be the reason for this red shift in UV [58]. The synthesized particles (Ag/ZnO NC) with the aforementioned band gap values can be employed as antimicrobial agents because of the easy penetration of nano-size particles into bacterial cells [59,60].

Figure 4b shows the FTIR spectra of Ag/ZnO NC, Neo-Ag/ZnO NC, blank NEG (pure Carbopol NEG), Ag/ZnO NEG, and Neo-Ag/ZnO NEG. The capping of NPs by functional groups present in fungal extracts was confirmed by the presence of the amide group shown in FTIR spectra. Enzymes or proteins present in the cell-free culture filtrate can bind to NPs through their $\text{C}_3\text{H}_7\text{NO}_2\text{S}$ cysteine or free amine group and provide stability to NPs, as reported earlier [61,62]. The fingerprint region of Ag/ZnO NC and Neo-Ag/ZnO was observed below $1,500\text{ cm}^{-1}$. The peak at $1,336\text{ cm}^{-1}$ might be due to the bending vibration of ZnO. The small absorption signal at $1,600\text{ cm}^{-1}$ was assigned to the secondary amide ($-\text{NHR}$) in Ag/ZnO NC. The FTIR spectrum of Ag/ZnO NC displayed a weak intensity peak due to the incorporation of Ag^+ with Zn^{2+} . When Neo was conjugated with Ag/ZnO NC, the bending vibrational bands shifted to a lower wave number, at $1,033\text{ cm}^{-1}$, and an extra peak at $3,000\text{ cm}^{-1}$ confirmed the conjugation of Neo with Ag/ZnO NC.

The FTIR spectrum of blank NEG (unloaded Carbopol nanocarrier) showed a strong, broad O–H stretching vibration at $3,307\text{ cm}^{-1}$, and this indicated the presence of a carboxylic acid group. The adsorption band at $2,884\text{ cm}^{-1}$ could be attributed to the weak U-shaped aliphatic C–H stretching. The spectral region at $1,639\text{ cm}^{-1}$ showed medium C=C stretching and weak C–O–C stretching at $1,350$ and

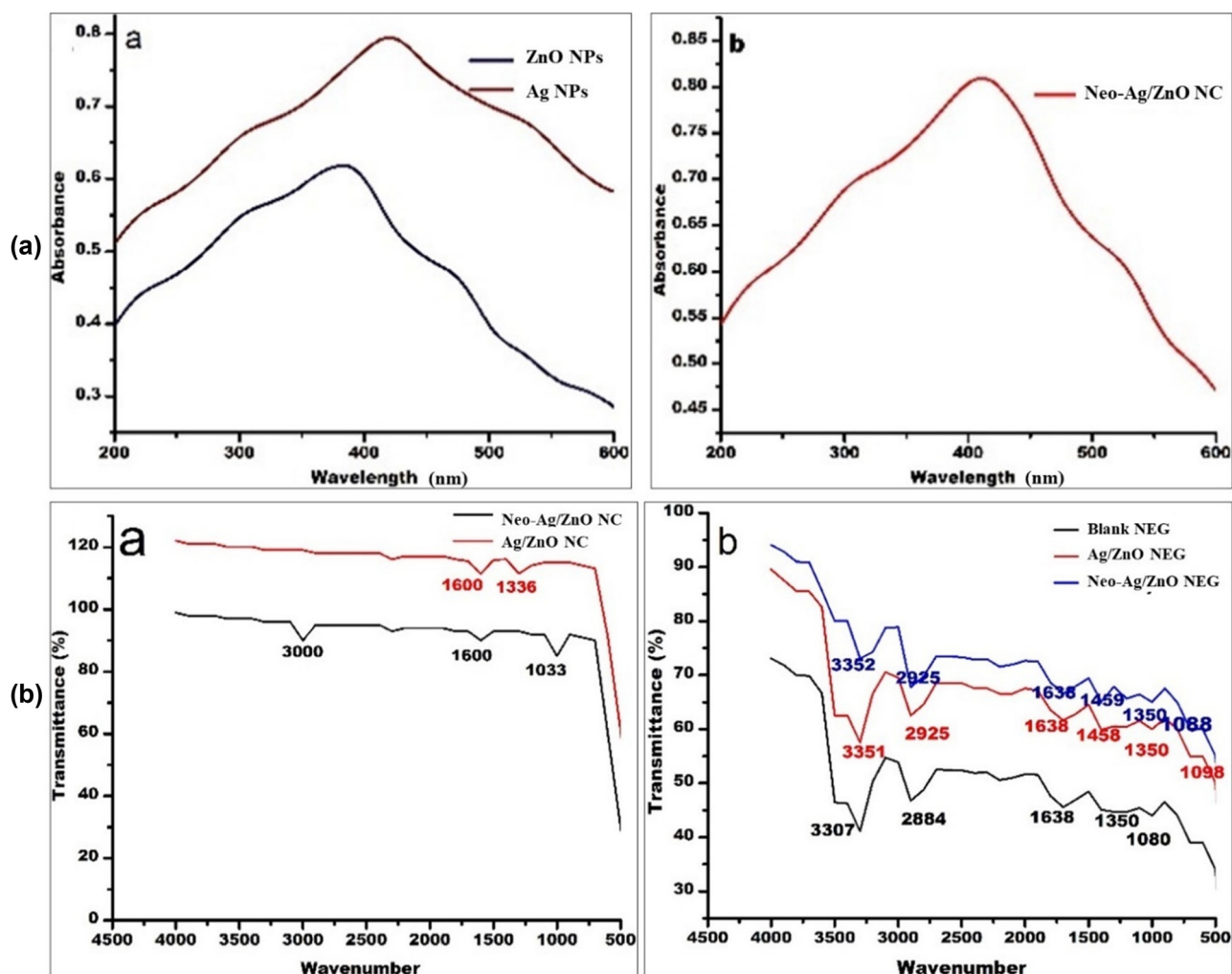


Figure 4: (a) Diffused reflectance spectra of mycogenic nanoformulations: ((a) Ag NPs and ZnO NPs and (b) Neo-Ag/ZnO NC). (b) FTIR spectra of mycogenic nanoformulations: ((a) Ag/ZnO NC and Neo-Ag/ZnO NC and (b) blank NEG, Ag/ZnO NEG and Neo-Ag/ZnO NEG).

1,080 cm^{-1} owing to the presence of alkene and alkane groups in the Carbopol NEG. The FTIR spectrum of the Carbopol NEG (Blank NEG) is characterized by the presence of carboxylic acid, alkene, and alkane groups [24,63]. An additional peak is observed at 1,458 cm^{-1} for Ag/ZnO NEG and at 1,459 cm^{-1} for Neo-Ag/ZnO NEG. The presence of an additional peak and a band shift toward a longer wavelength after decoration with Ag/ZnO NC and Neo-Ag/ZnO NC indicated some physical interactions between the nanomaterial, the drug, and the Carbopol NEG, but this interaction did not affect the permeation of the nanomaterial, as indicated by the *in vitro* release study. Our data agree with a recent study that showed EU interaction with Carbopol NEG [24].

XRD analysis revealed sharp diffraction peaks, which indicates the crystallinity nature of the Neo-Ag/ZnO NC. A similar diffraction pattern was observed in a recent study

[10]. In accordance with JCPDS files (010891397 for ZnO, 030652871 for Ag), the XRD patterns of Ag/ZnO NC and Neo-Ag/ZnO NC showed lattice plane indices of 100, 002, 101, 111, 200, 102, 110, 103, 220, 112, and 201 related to the 2θ values 31.65, 35.0, 36.1, 38.0, 47.55, 56.55, 62.6, 67.3, 68.1, 69.7, and 78.1, respectively. Neo-Ag/ZnO NC showed similar peaks with a slight reduction in the peak intensity at planes 111 and 200 compared to that of Ag/ZnO NC; this revealed the amorphous nature of the Neo drug and the maintenance of the original NC structure (Figure 5a). Nevertheless, the dominant peaks associated with Ag/ZnO NC are of significantly higher magnitude, which may mask the presence of neomycin in the XRD pattern. This phenomenon underscores the challenge of detecting minor components in samples dominated by more abundant phases. Similar results were found in a study by El-Banna *et al.* [64], where they observed the absence of neomycin peak in the XRD

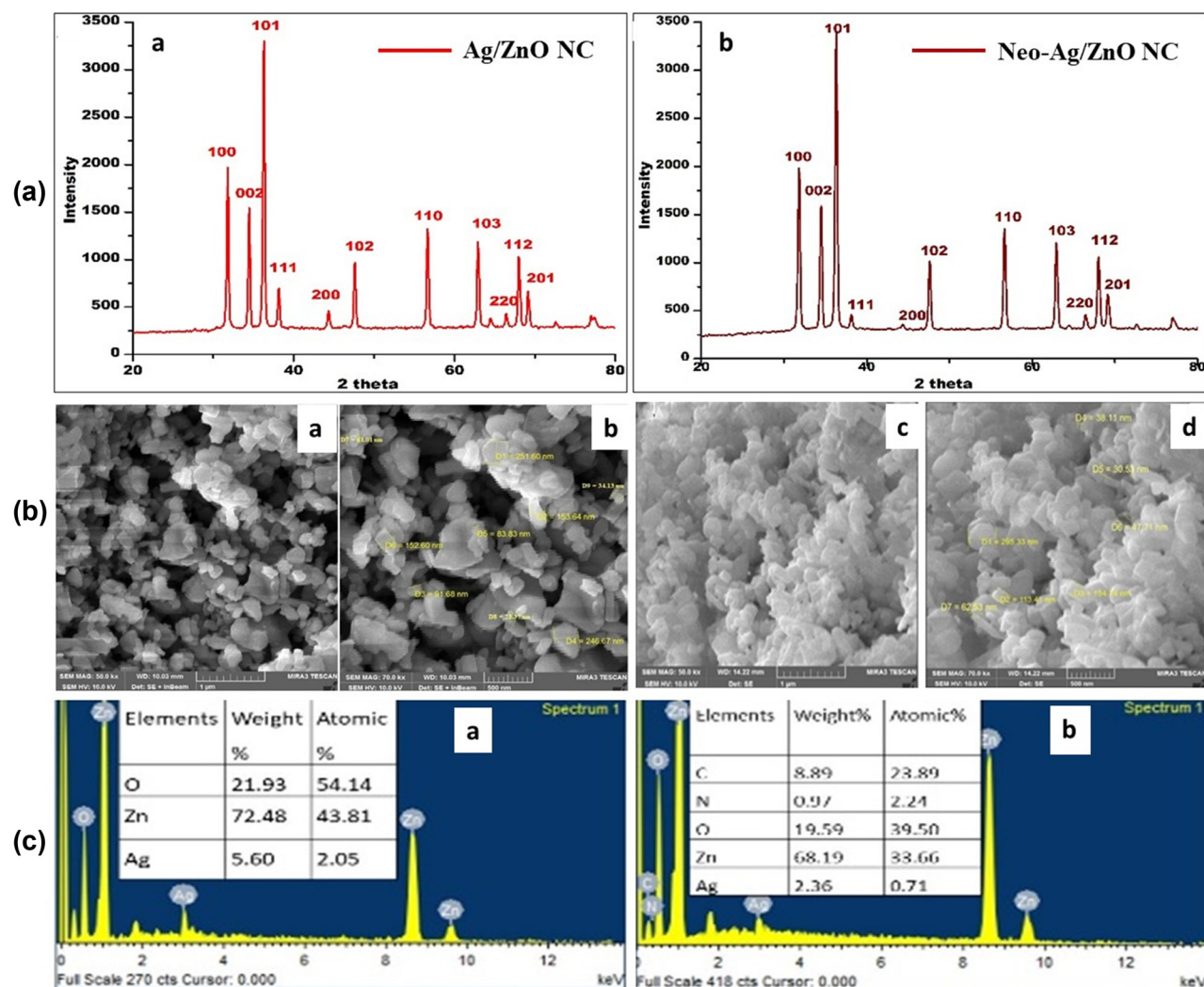


Figure 5: (a) XRD spectra of mycogenic nanoformulations: ((a) Ag/ZnO NC) and (b) Neo-Ag/ZnO NC). (b) FESEM micrographs: ((a) Ag/ZnO NC at 1 µm, (b) Ag/ZnO NC at 500 nm), (c) Neo-Ag/ZnO NC at 1 µm, and (d) Neo-Ag/ZnO NC at 500 nm. (c) EDX spectra: ((a) Ag/ZnO NC) and (b) Neo-Ag/ZnO NC).

spectra of neomycin-silver nanocomposite gel. The average crystalline size of pure NC (Ag/ZnO NC) was 38 nm. It was 40 nm for the drug-conjugated NC (Neo-Ag/ZnO NC) calculated from the very sharp peak obtained with diffraction analysis corresponding to the plane (101) by applying the Debye–Scherrer formula. The size calculated by the Debye–Scherrer formula was quite similar to the size of the Ag/ZnO NC reported in previous studies [54,65]. Ag–ZnO NC with a size of less than 45 nm is less toxic to the epidermal cells of the skin, as reported by Das *et al.* and Majhi *et al.* [66,67]; hence, they can be used in topical wound care as antimicrobial agents.

Remarkably, the FESEM images (Figure 5b) depicted a diversity of shapes (rectangular, triangular, pentagonal, and sheet-like crystalline shapes) owing to the use of two different fungal strains and the significant release of many

different secondary metabolites that might act as reducing agents and ultimately impact NP properties. This observation is in line with the findings reported recently; indeed, two distinct morphologies for ZnO NPs with two different fungal strains were reported [68]. The average crystalline size of the pure NC (Ag/ZnO NC) and the drug-conjugated NC (Neo-Ag/ZnO NC) was 38 nm (range of 23–251 nm) and 40 nm (30–295 nm), respectively. The porosity increased with the increase in the PS, which is evident in the comparison between Neo-Ag/ZnO NC and pure Ag/ZnO NC. This increase in porosity was found to enhance the antimicrobial properties of Neo-Ag/ZnO NC against resistant strains, attributed to the higher surface area-to-volume ratio. This finding aligns with a study conducted by Bhosale *et al.*, which demonstrated similar results [69]. They observed enhanced antibacterial and photocatalytic applications of

Table 1: Physical stability parameters of the mycosynthesized nanoformulations over 4 weeks

Time	Color change	Homogeneity	Phase separation	Consistency	Odor
Fresh	–ve	++++	–ve	++++	–ve
12 h	–ve	++++	–ve	++++	–ve
24 h	–ve	++++	–ve	++++	–ve
7 days	–ve	++++	–ve	++++	–ve
14 days	–ve	++++	–ve	++++	–ve
28 days	–ve	+++	–ve	+++	–ve

(++++), excellent; (+++), good; and (–ve) negative.

ZnONPs after doping with AgNPs, attributing this enhancement to an increase in porosity and surface area as PS increased [69]. Additionally, EDX spectra revealed the purity of the synthesized NPs as no extra peak was observed (Figure 5c). These data indicated the successful conjugation and absorption of the Neo drug in the NC. The Neo-Ag/ZnO NEG with an average PS of 215 nm and a polydispersity index (PDI) of 0.019 suggests both uniform PS distribution and homogeneous dispersion of the active pharmaceutical agent. Figure S9 shows a PS of 215 nm, within the range favorable for topical applications, facilitating enhanced drug penetration and release. Additionally, the low PDI value indicates consistent PS distribution, further supporting the formulation's suitability for efficient drug delivery in topical applications [38]

3.4 Organoleptic and stability features of the myconanoformulations

All the mycosynthesized nanoformulations (blank-NEG, Ag/ZnO NEG, and Neo-Ag/ZnO NEG) were inspected visually for organoleptic and stability features (Table 1). They were time-independent and similar in color compared to that of the freshly prepared Blank-NEG at different times: the color was creamy white for the Blank-NEG, light brownish for Ag/ZnO NEG, and greyish for Neo-Ag/ZnO NEG. The homogeneity and consistency were observed to be excellent; no differences in odor were observed, and no phase

separation (sedimentation) was seen upon centrifugation for the period of 28 days. Therefore, all the synthesized formulations were considered stable.

Statistically significant differences ($p < 0.05$) were observed in the pH values of fresh Ag/ZnO NEG (F_2) and Neo-Ag/ZnO NEG (F_3) in comparison with the fresh Blank-NEG (F_1) after 12 h, as analyzed by one-way RM ANOVA (Table 2); this was due to the addition of triethanolamine. Also, F_2 and F_3 were slightly acidic ($p > 0.05$) compared to F_1 , and this may be due to the acidic nature of the nanomaterials added to F_2 and F_3 . No other significant changes ($p > 0.05$) in pH from 12 h to day 28 were found in F_1 , F_2 , or F_3 , which denotes an overall pH stability. Overall, the pH values recorded were within the skin pH range of 5–6 and, thus, suitable for topical application [36]. All the values are expressed as mean $\pm$ SEM.

Also, the viscosity determination is a fundamental step in evaluating emulgel formulations because it determines the permeability and bioavailability of bioactive molecules in topical DDS [36]. The unpaired t -test showed significant changes ($p < 0.05$) in the F_1 , F_2 , and F_3 viscosities at low (6 rpm) and high (12 rpm) shear rates over the period of 28 days following non-Newtonian fluid (viscosity change with applied stress). F_1 was significantly more viscous than F_2 and F_3 on different days (Table 3) since it was slightly acidic. The calculated spreadability value was 9.8 ± 1.20 s for F_1 , while it was 10 ± 1.25 s for F_2 and 12.25 ± 1.21 s for F_3 . The spreadability of F_1 was lower compared to that of F_2 and F_3 due to its higher viscosity; conversely, the spreadability of F_3 was higher compared to that of F_1 and F_2 due

Table 2: pH values of the mycosynthesized nanoformulations over 4 weeks

Formulations	pH					
	Fresh	12 h	24 h	7 days	14 days	28 days
F_1 (standard)	5.37 ± 0.0046	5.51 ± 0.0045	5.52 ± 0.005	5.53 ± 0.0046	5.53 ± 0.0051	5.53 ± 0.0046
F_2 (control)	5.35 ± 0.0045	5.49 ± 0.0051	5.50 ± 0.005	5.51 ± 0.005	5.51 ± 0.0046	5.51 ± 0.005
F_3 (test)	5.33 ± 0.005	5.47 ± 0.0046	5.46 ± 0.0051	5.47 ± 0.0051	5.47 ± 0.005	5.47 ± 0.0046

Table 3: Viscosities (cP) of the mycosynthesized nanoformulations over 4 weeks

Shear rates (rpm)	Viscosity (in cP)					
	Fresh	12 h	24 h	7 days	14 days	28 days
F1 (Blank-NEG)						
6	5,598 ± 0.5	5,595 ± 0.454	5,593 ± 0.51	5,591 ± 0.51	5,590 ± 0.045	5,590 ± 0.51
12	5,587 ± 0.046	5,583 ± 0.51	5,582 ± 0.046	5,581 ± 0.045	5,581 ± 0.51	5,580 ± 0.046
F2 (Ag/ZnO NEG)						
6	5,487 ± 0.51	5,487 ± 0.454	5,486 ± 0.045	5,485 ± 0.046	5,484 ± 0.454	5,483 ± 0.046
12	5,473 ± 0.046	5,471 ± 0.51	5,471 ± 0.51	5,470 ± 0.046	5,470 ± 0.51	5,469 ± 0.5
F3 (Neo-Ag/ZnO NEG)						
6	5,410 ± 0.045	5,409 ± 0.046	5,409 ± 0.51	5,407 ± 0.046	5,407 ± 0.51	5,406 ± 0.5
12	5,396 ± 0.51	5,396 ± 0.5	5,395 ± 0.046	5,393 ± 0.045	5,391 ± 0.451	5,390 ± 0.51

to its lower viscosity. Herein, the rheological study showed that the synthesized nanoformulations followed a pseudo-plastic behavior based on a recent study [70]. A decrease in viscosity at a high shear rate results in easier spreadability and improved pharmacodynamics after topical applications [71]. Viscosity of $F1 > F2 > F3$ ($p < 0.05$). Viscosity in $F1$ or $F2$ or $F3$ was overall stable ($p > 0.05$), as analyzed by unpaired t -test. All values are expressed as mean ± SEM.

3.5 *In vitro* drug release behavior, drug content, hemocompatibility, and antimicrobial activity of Neo-Ag/ZnO NEG

The most efficient nanoformulation, Neo-Ag/ZnO NEG (#F3), was assessed for its *in vitro* release and drug content. The *in vitro* study of Neo, ZnO, and Ag ions released from Neo-Ag/ZnO NEG showed a slow and sustained release for 12 h. The cumulative release (Neo, ZnO, Ag) increased to 81% after 12 h (Figure S5). The *in vitro* release study showed a good pharmacokinetic profile of the mycosynthesized NEG; this was due to the optimum viscosity of 1% Carbopol 940 [72] and PEG, used as the permeation enhancer [73]. The release of a drug from an emulsion is controlled by the interaction of a drug with the emulsion's surfactant and co-surfactant; formulations with sustained release for a long time are preferred for topical delivery [36]. Any pharmaceutical dosage form must have an even distribution of pharmaceuticals to achieve a good interaction within the body, and this can be determined by finding the percentage of drug content. The drug content in Neo-Ag/ZnO NEG (F3) was as high as 98.70% obtained by RP-HPLC (Figure S6). The drug content in the F3 was found to be within the standard limit, and a relatively

good LC of the Carbopol emulgel with minimum drug loss during formulation was observed [36].

Besides, Neo-Ag/ZnO NEG (test, F3) exhibited no cytotoxicity against human erythrocytes (red blood cells – RBC) since no halo was observed (Figure S7). This was also the case for Neo (standard). A negligible ($p > 0.05$) RBC lysis zone (1 mm) was observed with 1× PBS (negative control). F3 was negligible (1 mm) compared to that of Triton-X 100 (positive control), which was 23 mm ($p < 0.0001$). The antimicrobial effects of the synthesized nanoformulations (*i.e.*, Ag/ZnO NC, Neo-Ag/ZnO NC, blank NEG, Ag/ZnO NEG, and Neo-Ag/ZnO NEG) against most of the prevalent MDR wound pathogens, such as *P. aeruginosa*, *S. aureus*, and *C. albicans*, were assessed by DDM (Figure 6a and Table 4) as well as by MIC (Figure 6b) and biofilm inhibition assay (Figure 6c). From DDM, B-NEG (Blank NEG) did not exhibit zones against selected strains. Prominent inhibition zones were observed for Ag/ZnO NC and the Neo-Ag/ZnO NEG, whereas Neo-Ag/ZnO NEG showed the greatest antimicrobial potential against *S. aureus*, with an inhibition zone of 46 mm compared with other tested strains ($p < 0.05$) and compared to that of other tested nanoformulations ($p < 0.05$). The relative efficiency could be ordered as follows: Neo-Ag/ZnO NEG > Neo-Ag/ZnO NC > Ag/ZnO NEG > Ag/ZnO NC >> Neo (pure and validated antibiotic – see datasheet suppl. mat. file) >>> B-NEG when tested against *P. aeruginosa*, *S. aureus*, and *C. albicans* (Figure 6a and Table 4). F3 manifested superior antimicrobial properties compared to that of the Neo ointment (used as control) and the other mycosynthesized nanoformulations against selected microbial strains; this is most likely due to the successful incorporation and release of Neo, Ag⁺, and Zn²⁺ from NEG and its small (nano) size [36]. Such a nanoformulation could easily interact with the cell wall surface of microorganisms, resulting in DNA disruption, enzyme inactivation, and inhibition of protein synthesis by ROS

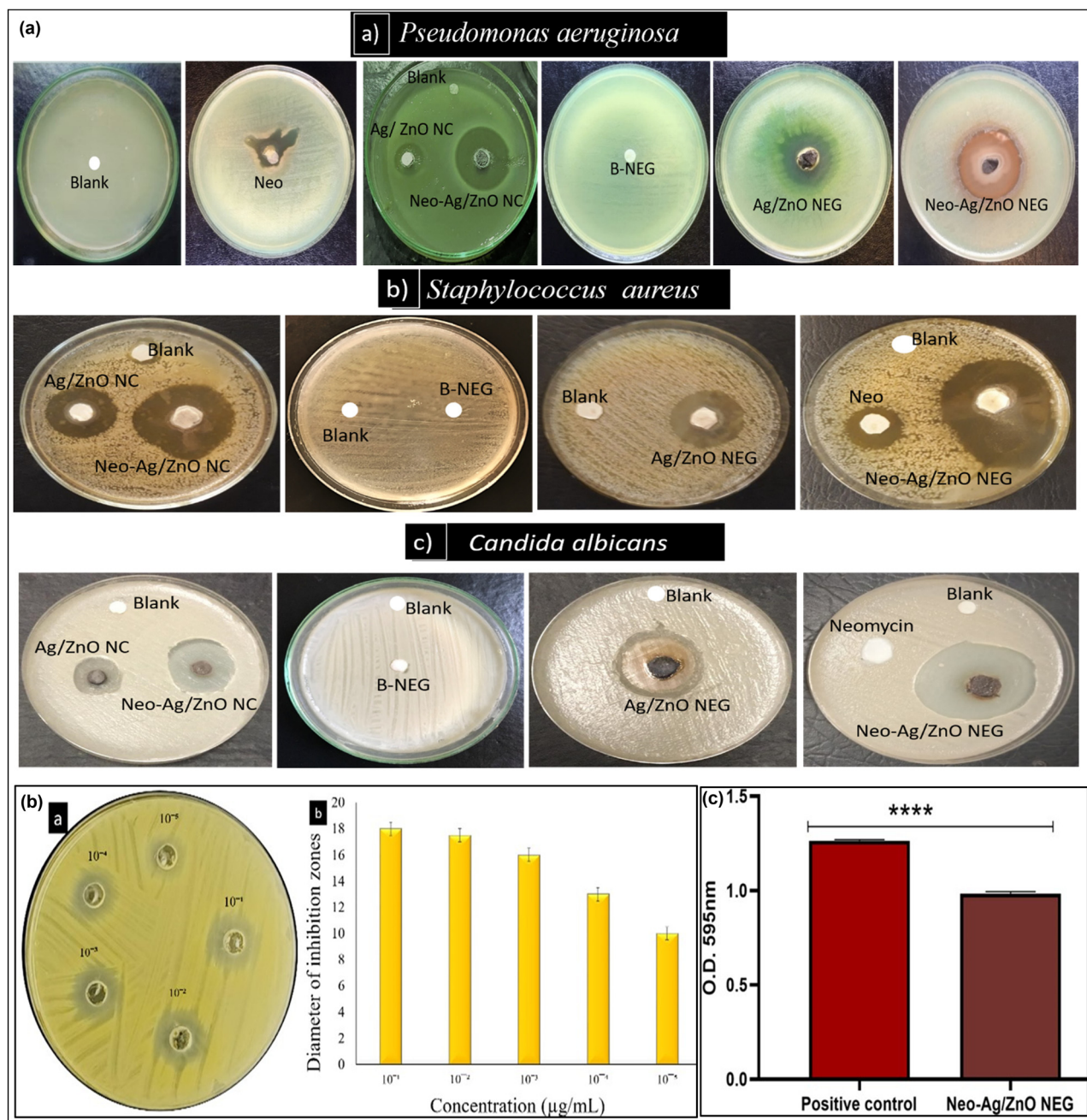


Figure 6: (a) Antimicrobial activity of samples against ((a) *P. aeruginosa*, (b) *S. aureus*, and (c) *C. albicans*). (b) MIC of Neo-Ag/ZnO NEG against MRSA: ((a) agar well diffusion plate and (b) graphical representation). (c) Antibiofilm activity of Neo-Ag/ZnO NEG against MRSA. Positive control: untreated microorganisms. Neo: neomycin; NC: ananocomposite; B-NEG: Blank NEG; NEG: nanoemulgel; Ag/ZnO: silver/zinc oxide.

formation [42]. These findings are in accordance with a recent study that used NEG for the topical delivery of Neo and reported an increase in the efficacy of Neo in terms of antibacterial activity after incorporating it into a tea tree oil-based nanoemulsion [74]. The MIC of Neo-Ag/ZnO NEG was 0.002 μg/mL against MRSA, indicating that this test formulation (F3) was active at low concentrations (Figure 6b). The percentage of MRSA biofilm inhibition by Neo-Ag/ZnO NEG was 22%

(Figure 6c and Figure S8). Significant differences ($p < 0.0001$) were observed in the antibiofilm potential between the positive control (i.e., untreated MRSA cells) and in Neo-Ag/ZnO NEG-treated MRSA cells. The small size and broad-spectrum activity of Neo, Ag, and ZnO led to an excellent biofilm inhibition potential against MRSA at MIC. Biocompatibility studies against human erythrocytes showed that Neo-Ag/ZnO NEG was safe for *in vivo* application in an animal model following the 3R

Table 4: Antimicrobial activity of mycosynthesized nanoformulations

Strains tested	Diameter of growth inhibition zones (mm)						
	Formulation codes						
	Blank	Neo	Ag/ZnO NC	Neo-Ag/ZnO NC	B-NEG	Ag/ZnONEG	Neo-Ag/ZnO NEG
<i>P. aeruginosa</i>	—	9 ± 0.5	15 ± 0.46	27 ± 0.46	—	24 ± 0.45	31 ± 0.51
<i>S. aureus</i>	—	13 ± 0.54	28 ± 0.51	39 ± 0.54	—	30 ± 0.46	46 ± 0.45
<i>C. albicans</i>	—	—	20 ± 0.51	25 ± 0.46	—	32 ± 0.51	42 ± 0.45

Neo: neomycin; NC: nanocomposite; B-NEG: Blank NEG; NEG: nanoemulgel. All values are expressed as the mean ± SEM.

principles (*i.e.*, Replacement, Reduction, and Refinement) in animal research.

3.6 In vivo wound healing

Neo-Ag/ZnO NEG had excellent wound-healing activity in mice when compared with the control (untreated wound).

Indeed, the WTC achieved by Neo-Ag/ZnO NEG was found to be markedly higher (100%) compared to that of the control (55%) on day 12. Because of skin contraction and fast skin re-epithelialization, WTC was observed from day 4 onward with a concentration as low as 0.01% of 100 mg Neo-Ag/ZnO NEG. Treated mice showed a significant ($p = 0.0001$) increase in wound contraction either on day 4, day 8, or day 12 in comparison to the control. No itching,

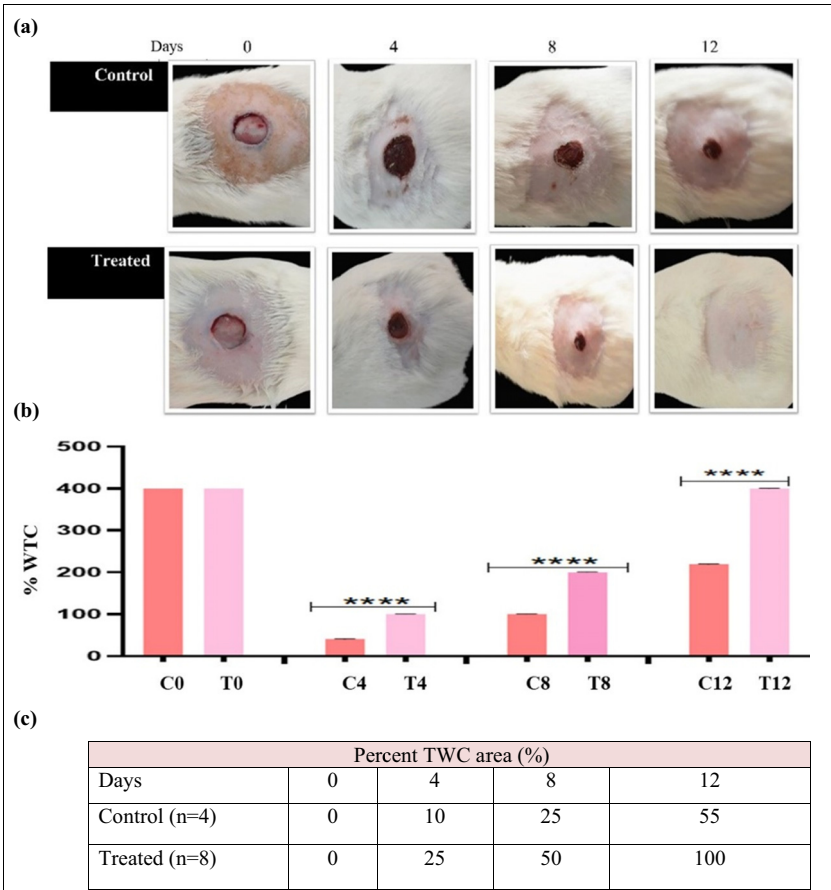


Figure 7: In vivo (in mice) wound-healing potential of Neo-Ag/ZnO NEG over a period of 12 days. (a) Pictures were taken at 4-day interval after treatment with Neo-Ag/ZnO NEG. (b) Graphical representation of the Neo-Ag/ZnO NEG effect on wound area (mm²) at indicated days. Data are presented as the mean ± SEM. $N = 12$ (**** $p = 0.0001$). (c) Tabulated data. WTC: wound total closure.

irritation, or inflammation was observed. Pictures, graphical representation, and tabulated data representing wound closure on various days are summarized in Figure 7a–c, respectively.

3.7 Hypothetical wound infection and healing mechanism

When the skin's integrity is compromised, whether through surgery or physical trauma, microbes from the skin surface invade the wound site, initiating colonization and releasing toxins, eventually forming a biofilm. This impedes immune cells like macrophages and neutrophils from effectively eliminating the microbes, leading to persistent inflammation and disrupting collagen synthesis [75] as shown in Figure 8a. Oral antibiotics are not recommended as microbes build tolerance against antibiotics [76]. Hence, our synthesized Neo-Ag/ZnO NEG offers a solution by gradually releasing nano-antibiotics from the Carbopol NEG at the wound site as shown Figure 8b, effectively eradicating biofilms and killing microbes due to its broad-spectrum antimicrobial properties by ROS production. Moreover, the NEG prevents microbial entry, mitigates infection, promotes collagen deposition and maintains

optimal moisture levels for wound contraction. This facilitates immune cell migration to the wound site with neoangiogenesis and skin cell formation, promoting early wound healing [36]. In a recent research, Majhi *et al.* stated that Ag/ZnO NPs stimulate the production of antimicrobial peptides and enhance the migration and antibacterial abilities of keratinocytes, which can potentially aid in wound healing and combating bacterial infection [67]. In comparison to prior studies, our research findings align with previous evidence indicating that Ag/ZnO NPs stimulate the production of antimicrobial peptides and enhance the migratory and antibacterial capabilities of skin cells (keratinocytes) against *S. aureus*. This consistency reinforces the potential therapeutic utility of Ag/ZnO NPs in promoting wound healing and combating bacterial infections. However, it is noteworthy that our study contributes novel insights by specifically highlighting the effectiveness of Carbopol NEG.

4 Conclusions

We report a novel and cost-effective fungal consortium-based synthesis of an Ag/ZnO NEG, which could be used as an effective topical antibiotic drug nanocarrier to

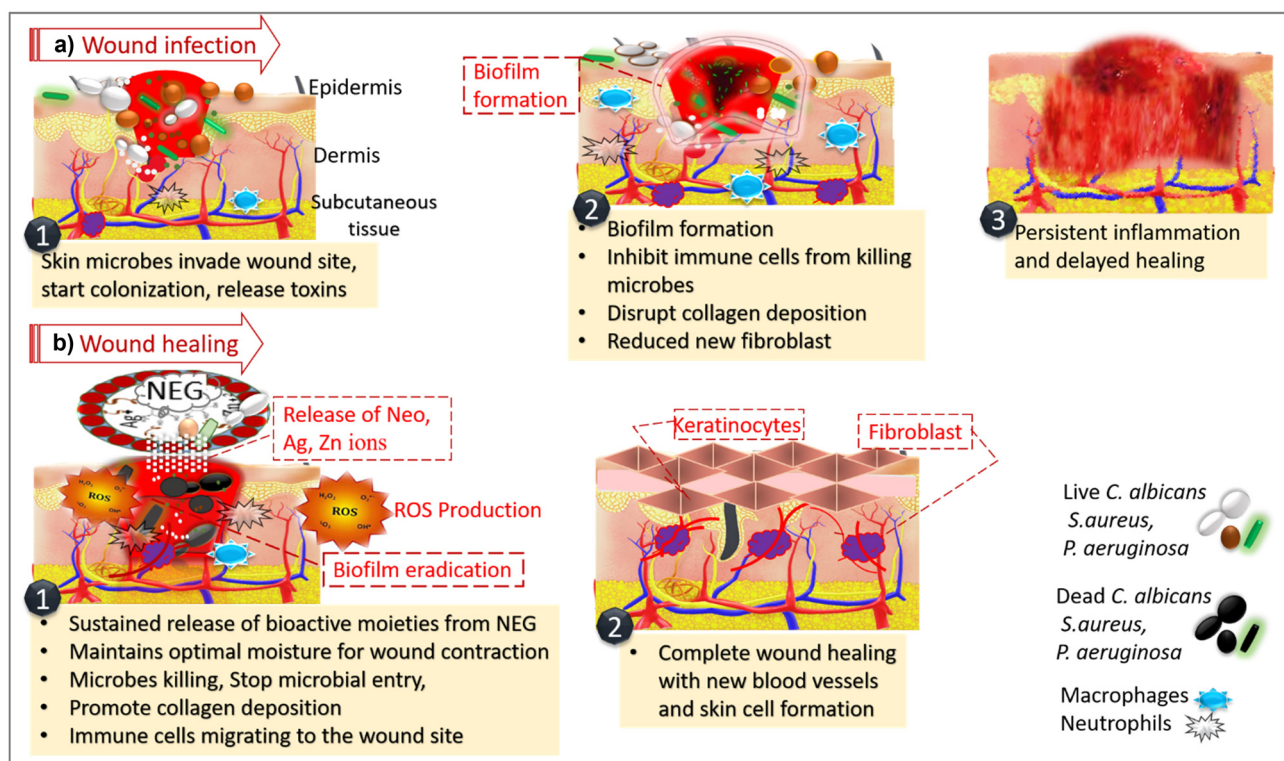


Figure 8: Illustration of putative skin wound infection mechanism by microbes (a). Neo-Ag/ZnO NEG-induced wound healing mechanisms (b).

overcome resistant skin infections and boost the skin healing process. To the best of our knowledge, this is a pioneered study. We have shown that it is possible to produce a relatively high yield of Ag/ZnO NC and stabilize it with the polymer matrix Carbopol after encapsulation with Neo. FTIR studies confirmed the incorporation of the drug and NPs (Ag and ZnO) into the prepared NEG. The release profile, pH, viscosity, spreadability, and hemocompatibility tests revealed that the Neo-Ag/ZnO NEG was suitable for topical delivery. Remarkably, Neo-Ag/ZnO NEG showed broader-spectrum activity against MDR wound pathogens *in vitro* compared to that of Neo and Neo-Ag/ZnO NC. Neo-Ag/ZnO NEG showed the highest activity (46 mm) against *S. aureus* and was prominent against MRSA. Importantly, Neo-Ag/ZnO NEG elicited a huge and safe wound-healing potential in mice, most likely due to its small size, stability, and sustained release of the NPs. We propose that the current Neo-Ag/ZnO NEG formulation is a promising topical NDDS for infected wound management due to its sustained and targeted release of bioactive nanomaterials.

5 Future directions

In future research, exploring the potential of the green fabricated nanoparticle-embedded GEL (NEG) could involve investigating its antimicrobial, anti-inflammatory, and antioxidant properties through *in vivo* studies. Additionally, conducting further cytotoxicity assays on animal models and cell lines is recommended to ensure long-term safety, biocompatibility, and potential adverse effects of the NEG (NDDS), thereby paving the way for subsequent clinical trials. Furthermore, mechanistic studies elucidating its mode of action in facilitating wound healing would offer valuable insights for clinical translation. Pursuing these avenues may offer significant advancements in wound management and infection control. Concurrently, investigating the compatibility of this NDDS with other antimicrobial agents for combination therapy could offer enhanced treatment strategies against a broader spectrum of microbial challenges and mitigate AMR.

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Author contributions: Conceptualization: Z.A., B.U.; writing – original draft preparation: Z.A., F.M.; methodology, software, validation, formal analysis, investigation, data curation: Z.A., B.U., B.A.K., F.F.; S.M.A., F.M.; visualization, supervision, and project administration: B.U.; resources and funding acquisition: B.U.; writing-review and editing, and submission: F.M. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Conflict of interest: The authors state no conflict of interest.

Ethical approval: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of the Department of Biological Sciences Ethics and Biosafety Committee of International Islamic University Islamabad, Pakistan (protocol code Reg #470-FBAS/MSBT/F-20, date of approval 12/27/21). The blood sample was provided by a healthy human with written informed consent and kept confidential throughout the experiment. All precautions have been undertaken to minimize the suffering of animals.

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