

Antibiofilm and wound healing effects of Triphala polyphenol-loaded silver nanoparticles hydrogel through regulation of MMP-9, inflammatory cytokines, and collagen deposition

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Abstract

Deviation in the normal wound healing process often results in the development of chronic wounds, frequently worsened by ongoing inflammation, bacterial presence, particularly biofilms. The aim of this study was to investigate the antibiofilm and wound healing potential of synergistic therapeutic effects of polyphenol-rich methanolic extract of triphala and silver nanoparticles and to study the underlying mechanisms. Triphala, an ayurvedic herbal preparation rich in polyphenols, exhibits antioxidant, anti-inflammatory, and matrix metalloproteinase (MMP) regulating properties. Various polyphenolic compounds such as gallic acid, ellagic acid, quercetin and β -sitosterol were identified and quantified in the triphala powder extract (TPE) using LC-MS/MS. TPE-loaded SNPs were synthesised and found to be small, spherical and stable. TPE-loaded SNPs exhibited greater biofilm inhibition against *Staphylococcus aureus* than *Pseudomonas aeruginosa*. TPE-loaded SNPs hydrogel was formulated and evaluated. *In vivo* wound healing studies in wistar rats demonstrated TPE-loaded SNPs gel significantly accelerated wound contraction and achieved complete wound closure with minimal scarring, outperforming plain SNPs, TPE alone, and a marketed formulation. Biochemical estimations revealed increased hydroxyproline content, indicating enhanced collagen deposition. The TPE-loaded SNPs group also showed significantly reduced levels of inflammatory markers (IL-6 and TNF- α) and lower oxidative stress (SOD levels) compared to control and other treatment groups, suggesting effective modulation of the inflammatory response. Furthermore, MMP-9 levels were estimated, providing insight into extracellular matrix remodeling. These findings highlight the promising antimicrobial, antibiofilm, and wound healing properties of TPE-loaded SNPs, suggesting their potential as an effective therapeutic strategy for wound management.

1. Introduction

A wound constitutes disruption of the epithelial barrier of the skin, often involving damage to the underlying tissues. Rapid restoration of skin integrity after wound formation is essential, as it plays a critical role in homeostasis and barrier function. The physiological process of wound healing comprises a tightly regulated cascade involving four overlapping phases: hemostasis, inflammation, proliferation, and remodeling. However, deviations from the healing process can result in either impaired healing (e.g., chronic wounds) or excessive healing (e.g., hypertrophic scars and keloids) [1]. Chronic wounds exhibit delayed healing owing to persistent inflammation, ischemia, and microbial colonization [2]. Effective management of wounds demands not only symptomatic care but also targeted treatment of these underlying etiologies [1]. Clinical decision-making in wound management should be guided by the wound's etiology, depth, contamination level, and the patient's comorbid conditions to optimize outcomes and minimize complications [3]. Topical wound healing medications encompass a range of therapeutic agents, such as wound dressings, antiseptics, and antibiotics. However, they have disadvantages, such as toxicity to host tissues and the development of the resistance [4, 5, 6].

Biofilms are communities of microorganisms that adhere to surfaces and significantly contribute to chronic bacterial infections. Biofilms are complex in nature and create a protective environment for the embedded bacteria [7, 8]. Biofilms usually form on the outer layer of wounds, but some like *P. aeruginosa*

biofilms, are also embedded in the deep layers of a wound. Bacterial biofilms have various effects, such as decreased penetration of antimicrobial agents and the ability to concentrate bacterial enzymes that render antibiotics inactive [9, 10, 11]. Compared to an acute wound, which is usually not associated with a biofilm, a biofilm-related wound heals slowly [12].

Plant polyphenols are secondary metabolites that exhibit antibacterial and antibiofilm properties by disrupting bacterial cell structures and influencing regulatory mechanisms [13, 14]. Thus, biofilm inhibition can be a novel therapeutic strategy for targeting biofilm-related infections, an area still lacking specific clinical treatments [15, 16]. In Ayurvedic medicine, Triphala is a herbal preparation. It combines three indigenous Indian plants: bellerica myrobalan (*Terminalia bellerica*), black myrobalan (*Terminalia chebula*), and Indian gooseberry (*Emblica officenalis*). It is a rich source of polyphenols, mainly flavonoids, tannins, phenolic acids, and glycosides [17]. According to the published literature, Kirubanandan S. et al. [18], polyphenols in Triphala offer antioxidant and anti-inflammatory effects, regulate Matrix Metalloproteinases (MMPs), and support interconnected healing stages, facilitating effective tissue regeneration and closure. The potential of triphala has been explored in different ways by a few researchers, viz. Kumar M. M. et al., 2008 [19], Yadav S. et al., 2016 [20], Singh et al., 2017 [21], Malik and Madan, 2020 [22], and Lakshmi et al., 2022 [23]. Earlier reports from Somayaji et al., 2014 [24] and Ranjani et al., 2020 [25] indicated that Triphala significantly inhibited *Enterococcus faecalis* and *Klebsiella pneumoniae* biofilms, respectively, and it can be potentially used as a natural alternative to combat drug-resistant infections. Thus, it can be concluded that triphala combined with nanosilver may improve wound healing efficacy while reducing cytotoxicity and microbial resistance concerns [26].

Silver nanoparticles (SNPs) have garnered significant attention in biomedical research owing to their small size, large surface area, unique optical and electrical properties, and ability to eliminate pathogenic microbes that can hinder the normal stages of wound healing. Their mechanism of action involves the release of silver ions, disruption of microbial cell membranes, generation of reactive oxygen species, and interference with cellular processes, leading to microbial death [27]. In addition to their antimicrobial effects, they promote wound healing by enhancing fibroblast proliferation and collagen synthesis. Moreover, their biocompatibility and minimal cytotoxicity make them attractive for incorporation into dressings and topical formulations [28–32]. Additionally, methanolic extract of triphala, which is rich in phenolic compounds, exhibit antibacterial and antibiofilm activities. These phenolic components are known to disrupt bacterial cell membranes [33–35], suggesting that the synergistic mechanism between triphala and silver nanoparticles may enhance antibiofilm activity, further supporting wound healing efforts [36, 37].

Thus, authors have made an attempt to study the synergistic therapeutic potential of triphala powder extract (TPE) in combination with the SNPs for wound healing activity. First, a triphala extract was prepared, followed by its tandem-mass spectrometric (LC-MS/MS) analysis to identify the polyphenolic compounds present in it. Subsequently, TPE-loaded SNPs were synthesized and characterized using different techniques. The antimicrobial and antibiofilm potential of SNPs against *Staphylococcus aureus* and *Pseudomonas aeruginosa* were studied. A hydrogel of TPE-loaded SNPs was formulated and

evaluated for its application. The anti-inflammatory, antioxidant, MMP-9 inhibition, and *in vivo* wound healing effects of TPE-loaded SNPs were assessed in wistar rats.

2. Methods

2.1 Reagents and Chemicals

Silver nitrate (AgNO_3), trisodium citrate (TSC, $\text{Na}_3\text{C}_6\text{O}_5\text{H}_7$), carbopol 934, glacial acetic acid, dimethyl sulfoxide, and methanol were purchased from Loba Chemicals Pvt. Ltd., India. Glycerin and triethanolamine used in the gel formulation were purchased from Sisco Research Laboratories Pvt. (Mumbai, India). Triphala powder (batch no. 9021) was purchased from Shree Baidyanath Ayurved Bhawan Pvt. Ltd. (Nagpur, India). Mueller hinton medium was purchased from HiMedia Laboratories (India). Bacterial strains of *Pseudomonas aeruginosa* (MTCC741) and *Staphylococcus aureus* (MTCC 96) were used for antimicrobial studies. The species were obtained from the National Center for Cell Science, Pune, India, and stored in the Department of Microbiology, Yashwantrao Mohite College of Arts, Science and Commerce, Pune, India. Phosphate-buffered saline (PBS) and crystal violet staining solutions used for antibiofilm activity were sourced from Loba Chemie Pvt. Ltd. The formulation available in the market used in the *in vivo* research was Cipladine (Povidone-Iodine Ointment USP). Wistar rats used in the animal studies were procured from Global Bioresearch Solutions Pvt. Ltd., Pune, India. Loba Chemie Pvt. Ltd. supplied formalin, alcohol, and paraffin for histopathological investigations, whereas Spectrochem Pvt. Ltd., Mumbai, India, supplied xylene. The pathologist graciously provided hematoxylin and eosin (H and E) and Masson's trichrome (MT) staining. Histopathological slides were examined at 40x magnification using an optical microscope. Different ELISA kits employed in the measurement of the levels of IL-6, TNF- α , hydroxyproline, and MMP 9 were used to measure the levels in serum samples by ELISA kits (Make: GENELISATM) as per the following lot numbers: IL-6 (Lot No: R160525), TNF- α (Lot No: RTA0621), MMP-9 (Lot No: RMMP90824), and Hydroxyproline (Lot No: RHYP0729).

2.1 Preparation of Triphala powder extract (TPE) [38]

Twenty-five gms of triphala powder was made into a wet slurry by adding 25 ml of distilled water and left to rest for three hours. The mixture was then macerated overnight after the addition of 250 ml of acidic methanol. The mixture was allowed to settle for 24 h at room temperature. The supernatant was collected, filtered, and evaporated using a rotary evaporator to obtain a dark brown mass. The extract yield was 14.5%. The extract was stored in an amber vial under refrigeration until further use.

2.2 LC-MS studies on Triphala powder extract (TPE)

LC-MS analysis was performed using a triple quadrupole LC-MS/MS system (Agilent 6460, Agilent Technologies, USA) in conjunction with an Agilent 1260 series LC system. The detector was operated in the heated electrospray ionization mode, both positively and negatively. High-purity nitrogen was used as both the source and collision gas. The settings for all substances were as follows: capillary voltage,

3500 V; gas temperature, 250°C; gas flow, 11 L/min; nebulizer gas pressure, 55 psi; sheath gas temperature, 350°C; sheath gas flow, 11 L/min; and nozzle voltage, 500 V. Separation was performed using an Agilent Eclipse Plus C18 silica column (150 mm× 4.6 mm, 5 µm) with a stepwise gradient technique involving 0.1% formic acid in water and acetonitrile as mobile phase solvents. Quantification was performed in the MRM mode. The column was maintained at 25°C, with a total run time of 15 min and an injection volume of 10 µL. The flow rate was set to 0.8 mL/min. Data were processed using the Agilent MassHunter Workstation Data Acquisition software (Agilent Technologies, USA).

2.3 Preparation of agar plates, well formation, and antimicrobial susceptibility of TPE [39, 40]

The cup plate method was employed to determine the antimicrobial susceptibility of TPE against the selected species. Molten Mueller-Hinton agar media were prepared. Two milliliters of bacterial cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* were mixed separately in the previously cooled agar media. The agar media were then aseptically poured into sterile Petri dishes. To ensure a uniform distribution of bacteria, the agar plates were gently swirled immediately after pouring. Using a sterile cork borer, wells with a diameter of 9 mm were created in the solidified agar. Each well was filled with 50 µL of the respective test solution using a sterile micropipette under sterile conditions. The wells were filled with 50 µL of TPE at varying concentrations (2%, 4%, 6%, and 8%) prepared in DMSO. The plates were incubated at 37°C for 24 h. After incubation, the zones of inhibition around the wells were measured. The concentration of the TPE that exhibited visible bacterial growth inhibition comparable to that shown by the positive control was considered for the comparative antimicrobial activity, as well as for loading onto the plain nanoparticles. Streptomycin and amoxicillin served as positive controls for *Pseudomonas aeruginosa* and *Staphylococcus aureus* species, respectively.

2.4 Synthesis of plain and TPE-loaded silver nanoparticles (TPE-loaded SNPs) [41]

Silver nitrate was used as the precursor for the synthesis of silver nanoparticles (SNPs). Trisodium citrate served as a reducing and capping agent. In short, 50 ml of 1 mM silver nitrate (AgNO₃) solution was heated using a hot plate magnetic stirrer. Once boiled, 5 ml of a 10 mM solution of trisodium citrate was added drop-wise at a rate of 1 drop/s with continuous stirring until the colorless solution turned yellowish-brown. This batch of SNPs was referred to as plain SNPs. TPE-loaded SNPs were prepared by dropwise addition of 8% TPE solution while heating continuously and stirring for 15 min to a 50 ml batch of plain SNPs using the following three volumes: (0.25, 0.5, and 1.0 ml). We refer to these batches as TPE-loaded SNPs.

2.5 Characterization of plain and optimized batch of TPE-loaded SNPs

The stability of the plane and three batches of TPE-loaded SNPs was studied through surface plasmon resonance (SPR) analysis using a Lab India UV-visible spectrophotometer by scanning from 800 to 200

nm against plane SNPs. The optimized batch, wherein 0.5 ml of 8% TPE of triphala was loaded onto SNPs, was further characterized to confirm its composition and stability. Infrared spectra were scanned on FT-IR spectrophotometer (Jasco 4100, Japan) using the KBr pellet method to understand the chemical composition of the loaded SNPs. The surface morphology of the SNPs was observed using field-emission scanning electron microscopy (FESEM). The synthesized SNPs were spread on a glass slide and subjected to X-ray diffraction crystallographic studies. The XRD patterns were verified using an X-ray diffractometer (D8 Advanced Bruker, UK) with copper as the anode material, Lynx Eye as the detector, and Ni as the filter. The scanning speed was maintained at a rate of 4°/min, and the samples were analyzed by varying the 2θ angle from 05 to 60°. The diffraction images were analyzed using Origin 8 software.

2.6 Development of TPE-loaded SNPs gel [42]

To formulate the TPE-loaded SNPs gel, 1% carbopol 934 and glycerine were gently mixed with the TPE-loaded SNPs solution (50 ml) and agitated until the carbopol was hydrated. After a day, the pH of the SNPs was maintained at 6.5 using 10% w/v triethanolamine until the solution gelled. The physical characteristics of the gel, such as color, homogeneity, consistency, phase separation, and spreadability, were studied. A digital pH meter (Systronics Limited) was used to measure the pH of the gel. The 'spreadability' was assessed by transferring 0.5 g of the formulation onto a glass slide, which was then placed onto another glass slide inside a 1 cm diameter circle that had been previously marked. A 500 g weight was left to rest on the upper glass plate. The increase in the diameter of the sample after spreading was measured. A Brookfield viscometer (RTV, USA) was used to measure the viscosity of the gel.

2.7 Comparative antimicrobial efficacy

The comparative antimicrobial efficacy of plain SNPs, TPE, and TPE-loaded SNPs against '*Pseudomonas aeruginosa*' and '*Staphylococcus aureus*' was evaluated. The wells prepared in the petri dish were filled with 50 µL of the test samples (plain SNPs, TPE, and TPE-loaded SNPs). Control wells containing antibiotics (Streptomycin for *Pseudomonas aeruginosa* and amoxicillin for *Staphylococcus aureus*) at known concentrations were included to validate the results. The plates were incubated at 37°C for 24 hours. Following incubation, the diameters of the visible zones of bacterial growth inhibition were measured in triplicate.

2.8 Antibiofilm activity [43]

The antibiofilm activity of the test groups: plain SNPs, TPE, and TPE-loaded SNPs was evaluated using a crystal violet assay in a 96-well microtiter plate against both *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Streptomycin and Amoxicillin served as positive controls while, wells with no test solution served as negative controls. Additionally, wells containing Mueller-Hinton broth medium without any bacterial culture were used to account for background staining. Overnight cultures of bacterial strains were grown in sterile Mueller-Hinton broth. Aliquots of 200 µL of these cultures were dispensed into each well of a sterile 96-well microtiter plate, followed by the addition of 20 µL of the test compound.

The plates were incubated at 37°C for 24 h to allow biofilm formation. After incubation, the wells were gently washed twice with phosphate-buffered saline (PBS) to remove non-adherent cells. The remaining biofilms were stained with 0.1% crystal violet solution for 15 min. Excess stain was rinsed off with distilled water, and the bound crystal violet was solubilized with 200 µL of 33% acetic acid solution. The absorbance of the resulting solution was measured at 590 nm using a microplate reader. Biofilm inhibition was quantified by comparing the absorbance values of the treated wells with those of the control wells. All experiments were performed in triplicate, and the results were averaged.

2.9 Wound healing activity [44], biochemical parameters [45], and histopathological studies in wistar rats [46]

The *in vivo* wound healing efficacy of various test groups was compared using an excision wound model in healthy male wistar rats weighing approximately 250 g. On arrival, the rats were kept inside plastic cages in groups at optimal temperature (24 ± 2°C) and (45 ± 3%) relative humidity with a 12:12 h daily light-dark cycle. The animals had free access to food and water. Rats were anesthetized using ketamine hydrochloride (60 mg/kg body weight), dorsal hair was shaved, and a circular wound of 2 cm diameter was excised on the dorsal surface of each animal. The rats were randomly distributed into five groups. Each group comprised six animals as follows: Group I, disease control; Group II, plain SNPs gel; Group III, TPE-loaded SNPs gel; Group IV, triphala powder extract (TPE); and Group V, marketed formulation (Cipladine ointment). The treatments were applied topically once daily for 21 days.

2.9.1 Percentage wound contraction [42]

The percentage of wound contraction was computed as a decrease in the initial wound area. On the 0th, 3rd, 7th, 14th, and 21st days post-injury, the area of the wound was measured by tracing it on a clear sheet and expressing the result in square millimeters (mm²). The following equation was used to calculate the percentage of wound closure:

$$\text{Wound Closure (\%)} = ((A_0 - A_t) / A_0) \times 100 \text{ eqn.... (A)}$$

Where A₀ is the original wound area and A_t is the wound area at a specific time point. The wound healing process was photographed to observe wound closure. The percentage of wound closure was calculated based on the initial wound size, and the results were statistically analyzed to determine the efficacy of each treatment.

2.9.2 Estimation of various biochemical parameters

To support the wound contraction studies, a few biochemical markers, such as hydroxyproline, TNF, IL-6, superoxide dismutase, and matrix metalloproteinase-9, were estimated from rat serum samples. The estimations were performed at Preclinbio Solutions LLP laboratory (Pune, India) using commercial Enzyme-Linked Immunosorbent Assay (ELISA) kits as per the manufacturer's instructions. Blood

samples from each group were collected, transferred into clot activator tubes, and allowed to clot. Further samples were centrifuged at 3000 rpm for 15 min. at 4 °C to separate the serum.

2.9.2.1 Hydroxyproline

On days 7 and 21, hydroxyproline levels were estimated by following the manufacturer's instructions. The hydroxyproline content in the samples was determined by comparing them to a standard curve and expressed as ng/mL hydroxyproline.

2.9.2.2 Inflammatory markers: TNF and IL-6

The levels of inflammatory cytokine, IL – 6 (day 3 and 7) and TNF- α (day 7) were estimated by following the manufacturer's instructions. The concentrations of IL 6 and TNF- α in the samples were expressed in pg/mL.

2.9.2.3 Antioxidant enzyme - superoxide dismutase (SOD)

On day 21, the antioxidant activity of the treatment groups was determined by estimating the enzyme superoxide dismutase (SOD) across the different tissue groups by following the manufacturer's instructions. The results are expressed as U/mg protein.

2.9.2.4 Matrix Metalloproteinase-9 (MMP – 9) [47]

The MMP-9 content of each group was determined on day 21 using an ELISA procedure according to the manufacturer's instructions. A standard calibration curve was used to estimate the MMP-9 concentration in the samples and the SOD levels are expressed as ng/mL.

2.9.3 Re-epithelization and collagen distribution studies

Animals from each group were sacrificed on the 3rd, 7th, 14th, and 21st days of the study. For histopathology, the wounded skin was promptly collected from each rat. The samples were fixed in 10% buffered formalin, dehydrated in alcohol, and cleared in xylene. After embedding in paraffin, the tissue slices were sliced into 5 mm thick pieces and placed on glass microscope slides. Hematoxylin and eosin (H and E) and Masson's trichrome (MTS) staining were used to analyze the samples. To assess the tissue structure and collagen deposition, two slides per group were viewed at 10x and 40x magnification.

2.10 *In silico* studies

The protein model for matrix metalloproteinase 9 inhibitor, viz. 1GKC was downloaded from protein databank [<https://www.rcsb.org/>]. The protein structure was refined and energy-minimized using the energy minimization features of ChemBioDraw Ultra 14.0 and ChemBio3D Ultra 14.0. The ligand structures (gallic acid, ellagic acid, quercetin, and β -sitosterol) were prepared using the MMFF94s force field. The grid was produced according to several atom types present in the ligands for the protein. Supplementary Material (Supplementary File 1) contains the dimensions of the grid box for the selected protein target. Molecular docking was performed using the AutoDock Vina software. The docking results

were visualized and analyzed using Discovery Studio Visualizer – 2019 [48, 49, 50] and PyMOL to examine the key interactions between the ligands and the active site residues.

2.11 Statistical Analyses

Statistical analyses were performed using GraphPad Prism (version 10.2.3 (347), GraphPad Software Inc., San Diego, California, USA). One-way and two-way analyses of variance, followed by comparison tests, were applied for the analyses. All quantitative data are expressed as mean $\pm$ standard error (SEM). Statistical significance was set at $p < 0.05$.

3. Results

3.1 Extraction and characterization of TPE using LC-MS/MS

The TPE yield was 14.5%. LC-MS/MS analysis of TPE revealed the presence of four polyphenolic compounds: gallic acid, ellagic acid, quercetin, and β -sitosterol, at retention times of 3.02, 6.28, 7.77, and 9.95 min, respectively. The mass spectrum was scanned in the positive ionization mode for β -sitosterol and in the negative ionization mode for gallic acid, ellagic acid, and quercetin. The observed m/z values for these compounds were 169.1 for gallic acid, 300.9 for ellagic acid, 301.1 for quercetin, and 415.0 for β -sitosterol. These were found to closely match the actual molecular mass values. The results of the LC-MS analysis of the triphala powder extract are presented in Table A in the Supplementary Information. The mass spectra of gallic acid, ellagic acid, quercetin, and β -sitosterol are provided in Figure A in the supplementary information.

3.2 Synthesis and characterization of Plain SNPs [40]

After the synthesis of silver nanoparticles, a color change was observed from colorless to yellowish-brown with a distinct surface plasmon resonance (SPR) peak at approximately 420 nm. For the synthesis of silver nanoparticles, a chemical reduction method employing trisodium citrate as a stabilizing and reducing agent was chosen because it offers several advantages, including controllable particle size and shape, high stability because of citrate's stabilizing effect, and the capacity to customize the nanoparticle properties [51]. Plain SNPs had a particle size of 45.1 ± 2.35 nm and zeta potential of $-45 \text{ mV} \pm 2.70$, indicating a stable colloidal system with sufficient electrostatic repulsion to prevent particle aggregation. The breadth of the size distribution is measured using the polydispersity index (PI). It ranges from 0–1. A lower PI value indicates greater nanoparticle stability. The PI of the plain SNPs was 0.467 ± 0.031 .

3.3 Synthesis, characterization and optimization of TPE-loaded SNPs

Three batches of TPE-loaded SNPs were synthesized by loading 0.25, 0.5, and 1 ml of 8% TPE. After loading TPE onto the SNPs, the color of the nanoparticle solutions changed to dark brown. The TPE-

loaded SNPs exhibited a surface plasmon resonance peak at 450 nm. The red shift observed in the SPR band compared to that of plain SNPs suggests the effective loading of phytochemicals in TPE onto the nanoparticle surface. Each batch was carefully monitored for consistency, ensuring that the addition of the extract did not destabilize the nanoparticles and that the final batch maintained its desired properties [52]. The optimization of TPE-loaded SNPs was conducted by comparing the data of particle size, PI, zeta potential values, and SPR bands for three trial batches. Among the three batches wherein 8% TPE solution was loaded, a batch with 0.5 ml of TPE loaded onto Plain SNPs was considered optimal, as it produced desirable results across all measured parameters. The particle size of the TPE-loaded SNPs was approximately 62 nm. The polydispersity index of the TPE-loaded SNPs batch was 0.381, and the zeta potential of the TPE-loaded SNPs was -28.3 mV. This indicated that the synthesized TPE-loaded SNPs were stable colloidal solutions with sufficient electrostatic repulsion. These findings demonstrate that the use of trisodium citrate in the reduction process, coupled with the loading of triphala extract, resulted in stable and uniform SNPs. As a result, the batch containing 0.5 ml of 8% TPE was ultimately optimized for additional analytical characterization, antibacterial and antibiofilm assessment, and *in vivo* animal studies.

3.4 Evaluation of Plain and TPE-loaded SNPs by using FT-IR, XRD and FE-SEM studies

FT-IR spectroscopic analysis revealed significant differences between the TPE and TPE-loaded SNPs, indicating the successful loading of TPE onto the SNPs. The FT-IR spectrum of TPE (Fig. 1A) showed peaks at 3499.2 cm^{-1} and 3173 cm^{-1} corresponding to O-H stretching vibrations, characteristic of hydroxyl groups present in polyphenols and other bioactive compounds, respectively. In the FT-IR spectrum of the TPE-loaded SNPs, these peaks were narrowed, suggesting an interaction between TPE and the silver nanoparticles, possibly through hydrogen bonding or van der Waals forces. However, the peak at 1736.58 cm^{-1} in the spectrum of TPE, associated with the C = O stretching vibrations of carbonyl groups, disappeared in the spectrum of TPE-loaded SNPs. This disappearance indicates the involvement of the carbonyl group in the interaction with the AgNPs, confirming the successful loading of TPE onto the nanoparticles. Together, the shift and disappearance of these peaks show that TPE was loaded and adsorbed on the SNP surface. Figure 1B shows the XRD analysis results for the plain and TPE-loaded SNPs when the samples were scanned with 2θ values ranging from 5° to 60° . The resulting XRD patterns showed broad diffraction peaks rather than sharp ones. These broad peaks suggest that the Ag silver nanoparticles have an amorphous structure. This is because the rapid chemical reduction and stabilization during their synthesis prevented the formation of a well-defined crystalline structure [52]. Figures 1C and 1D show the histogram plots of the plain and TPE-loaded SNPs, respectively, from the SEM studies. SEM analysis was performed to understand the topology and size of the SNPs. This indicates that the size (diameter) of the plain and TPE-loaded SNPs ranged from 20 to 40 nm and 30–59 nm, respectively. The SNPs were spherical or oval in shape and polydispersed. Elemental analysis was performed to confirm the presence of silver in the SNPs. As seen in Figs. 1E and 1F, elemental Ag was

present in plain SNPs as well as in SNPs loaded with TPE. The reduction of silver was clearly demonstrated by the sharp peak of silver.

3.5 Anti-microbial studies

3.5.1 Anti-microbial susceptibility and comparative anti-microbial activity

The antimicrobial efficacy of 2, 4, 6, and 8% concentrations of triphala polyphenolic extract (TPE) was evaluated against *Pseudomonas aeruginosa* (gram –ve species) and *Staphylococcus aureus* (gram +ve species) using the pour plate technique, as per the procedure described in Section 2.3. All the concentrations created clear zones of inhibition, indicating areas where bacteria could not grow. The 2% concentration was effective enough to stop the growth of bacteria; hence, it was considered the minimum inhibitory concentration (MIC). Furthermore, TPE at 8% concentration demonstrated similar inhibitory effects on bacterial growth as shown by the standard antibiotics. Solvent dimethyl sulfoxide served as a negative control, whereas antibiotics amoxicillin and streptomycin served as positive controls for *Pseudomonas aeruginosa* and *Staphylococcus aureus* study groups, respectively. Figures 2A and 2B show the antimicrobial susceptibility testing results for different concentrations of TPE against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

3.5.2 Comparison between antimicrobial properties of TPE, Plain and TPE-loaded-SNPs

The antimicrobial activities of plain SNPs, TPE, and TPE-loaded SNPs were evaluated against *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains using zone of inhibition studies. The results are shown in Figs. 2C and 2D. The results were compared with those of Amoxicillin and Streptomycin as positive controls for *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacterial strains, respectively. In the case of *Pseudomonas aeruginosa*, the TPE-loaded SNPs demonstrated superior antimicrobial activity ($p < 0.001$) compared to plain SNPs and TPE (Fig. 2E). In the case of *Staphylococcus aureus*, the TPE-loaded SNPs exhibited significantly greater antimicrobial activity than plain SNPs and TPE, with a statistically significant difference ($P < 0.001$) (Fig. 2F). These findings suggested that incorporating TPE into SNPs enhances the antimicrobial effect, highlighting the potential of TPE-loaded SNPs as an effective treatment strategy against these bacterial strains.

3.6 Antibiofilm studies

The antibiofilm activity of various test samples against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was evaluated using the crystal violet assay, revealing significant differences in their efficacy in inhibiting biofilm formation. In the case of *Pseudomonas aeruginosa*, the TPE-loaded SNPs demonstrated superior antibiofilm activity with $66 \pm 7\%$ inhibition. Plain SNPs, TPE, and marketed

formulations showed $35 \pm 2\%$, $52.2 \pm 84\%$, and $57.1 \pm 7\%$ biofilm inhibition, respectively. The % inhibition of biofilm formation was compared with that of the control group, in which no biofilm was inhibited. Statistical analysis confirmed that TPE-loaded SNPs exhibited significantly greater biofilm inhibition than as compared to TPE as well as plain SNPs ($p < 0.001$) and an equal inhibition as compared to the standard antibiotic (Fig. 3A). In case of *Staphylococcus aureus*, the TPE-loaded SNPs demonstrated $72.8 \pm 2.22\%$ biofilm inhibition, while plain SNPs, TPE, and marketed formulations showed $66 \pm 5\%$, $53.2 \pm 48\%$, and $22.6 \pm 07\%$ inhibition, respectively. Statistical analysis revealed that TPE-loaded SNPs exhibited significantly greater inhibition of biofilms than TPE and the standard antibiotic ($p < 0.001$) (Fig. 3B). These findings demonstrate the potential of TPE-loaded SNPs as a successful treatment for *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilm-associated wound infections. The probable reason for enhanced biofilm inhibition in the TPE-loaded SNPs group can be attributed to the various polyphenolic compounds present in the triphala powder, which act as antibiofilm agents by disrupting bacterial membranes and causing cell death (13, 14). Figure 3C shows the formation of biofilms, staining of biofilms using crystal violet, and inhibition of biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* using test samples.

3.7 Formulation and evaluation of TPE-loaded SNPs gel

Gel has high tissue compatibility and offers controlled release of medicaments, which makes them a perfect topical formulation. Therefore, the carbopol polymer was dispersed in the SNPs solution to formulate the gel. Carbopol, a cross-linked polymer, was utilized to form a gel matrix suitable for dermal use because it offers gel-forming and bio-adhesive properties, which help prolong the gel's retention at the application site. The TPE-loaded SNPs gel exhibited a uniform and homogeneous consistency. The pH of the formulation was 6.8 ± 0.042 , which closely matched the physiological pH of the skin. The gel demonstrated good spreadability, recorded at 6.4 ± 0.057 g cm/s, indicating ease of application with minimal shear forces. Viscosity, a critical parameter influencing drug release from topical systems, was found to be 2933 ± 115 poise for the TPE-loaded SNPs gel.

3.8 *In-vivo* wound healing activity

3.8.1 Percent wound contraction

The study of wound contraction in the excision wound model, following the application of TPE-loaded SNP gel, plain SNP gel, TPE gel, and a commercially available wound healing formulation, revealed notable differences in healing efficacy. Photographs were systematically captured on days 0, 3, 7, 14, and 21 to track the healing process in each group. By day 3, the TPE-loaded SNPs gel group demonstrated statistically significant ($p < 0.01$) wound contraction compared to the other groups, suggesting that the TPE-loaded SNPs gel more effectively expedited the wound healing process than the other test groups. By day 14, the TPE-loaded SNPs gel group achieved complete wound closure with minimal scarring, which was statistically significant ($p < 0.001$) (Fig. 4A), compared to the other groups that had not yet reached full healing. This finding highlights the superior efficacy of the TPE-loaded SNPs gel in

promoting wound healing. The control group exhibited the slowest healing with minimal progress. The plain SNPs group exhibited some healing improvement, but it did not match the recovery level of the TPE-loaded SNPs group. The TPE-treated group showed moderate wound contraction but did not achieve the complete closure observed with the TPE-loaded SNPs gel. The marketed formulation also progressed in healing but was outpaced by the TPE-loaded Ag NP group in terms of speed and quality of closure. These results indicate that the TPE-loaded SNP gel not only accelerates the healing process but also results in superior outcomes, such as faster closure and reduced scarring, making it a potentially more effective topical treatment than plain silver nanoparticles and triphala extract. By day 21, all groups achieved complete wound closure. Statistical comparison of %wound contraction for normal control, plain SNPs gel, TPE gel, TPE-loaded SNPs gel, and marketed formulation is shown in Fig. 4B.

3.8.2 Biochemical estimations

3.8.2.1 Hydroxyproline Content

This estimation was aimed to assess collagen deposition in the tissue, as hydroxyproline, a non-proteinogenic amino acid located in collagen fibers, acts as a quantifiable marker of collagen content. On days 7 and 21, the hydroxyproline levels in the groups treated with the TPE gel, plain SNPs gel, TPE-loaded SNPs gel, and marketed formulation were significantly elevated as compared to those in the control group ($p < 0.001$) (Fig. 5A). The data demonstrated a synergistic interaction between plain SNPs and TPE, which amplified collagen deposition and tissue regeneration beyond the effects of the marketed formulation, TPE gel, and plain SNPs gel. Earlier published literature [53, 54] has also supported the fact that triphala stimulates the production of collagen in wound tissues, which leads to better tissue structure and strength.

3.8.2.2 Effect of TPE, Plain SNPs and TPE-Loaded SNPs on inflammatory markers IL-6 and TNF- α

Interleukin-6 (IL-6) is a key inflammatory cytokine integral to wound healing, facilitating the early inflammatory response and cellular proliferation. On day 3 and 7, the IL-6 levels were assessed in all groups. The IL-6 levels in the groups treated with plain SNPs, TPE, TPE-loaded SNPs, and marketed formulations were significantly lower than those in the control groups ($p < 0.001$). Importantly, treatment with the TPE-loaded SNPs gel resulted in a significant reduction in IL-6 levels compared to the TPE ($p < 0.001$) (Fig. 5B). These diminished levels of IL-6 on the third day of wound healing in the treatment group suggest a transition from the initial inflammatory phase to the subsequent phase of wound healing, promoting tissue repair, remodeling, and angiogenesis.

By the seventh day, a significant elevation in TNF- α levels was observed in the control group. In contrast, the experimental groups demonstrated a notable restoration of TNF- α levels to baseline, as evidenced by the application of plain SNPs, TPE-loaded SNPs, and the marketed formulation ($p < 0.001$), compared to the control group (Fig. 5C). The group treated with TPE-loaded SNPs showed the lowest TNF- α levels

among all other groups, indicating that a combination of TPE and SNPs effectively modulates inflammation, promotes tissue regeneration, and supports a balanced cytokine environment, thereby contributing to more efficient wound healing.

3.8.2.3 Antioxidant enzyme (SOD) estimation

At day 21, a statistically significant elevation ($p < 0.05$) in the levels of superoxide dismutase (SOD) was observed in the control group. In contrast, SOD levels significantly decreased following treatment in the plain SNPs ($p < 0.05$), TPE ($p < 0.001$), TPE-loaded SNPs ($p < 0.001$), and marketed ($p < 0.001$) groups. The findings are shown in Fig. 5D. On the 21st day, the group treated with plain SNPs had SOD concentration of 140.90 U/mg, while other experimental groups treated with TPE, TPE-loaded SNPs, and marketed formulation exhibited enzyme levels of 118.04, 101.56 U /mg and 130.20 U /mg, respectively. Thus, it can be concluded that the SOD enzyme concentration in the TPE-loaded SNP-treated group was minimum, which suggested that TPE along with SNPs showed the lowest oxidative stress compared to the control, plain SNPs, TPE, and marketed groups.

3.8.2.4 Estimation of MMP-9 in wound tissue homogenates

Matrix metalloproteinase 9 (MMP-9) is crucial for wound healing. It is primarily involved in the degradation of the old extracellular matrix (ECM) to create a new scaffold for tissue repair and remodeling. However, persistently high levels of MMP-9 contribute to prolonged inflammation, disrupt the delicate balance of ECM degradation and synthesis, and interfere with the normal re-epithelialization process [55–57]. The quantification of MMP-9 levels was conducted on the 21st day in the control group, yielding a value of 320.86 ng/mL. In contrast, the plain AgNPs, TPE, TPE-Loaded AgNPs, and marketed formulation groups exhibited MMP levels of 294.67, 222.52, 203.95, and 229.67 ng/mL, respectively. The group treated with TPE-loaded SNPs resulted in the statistically low ($p < 0.001$) values for MMP-9 levels as compared to all other groups (Fig. 5E). This suggests that TPE-loaded SNPs maintain an optimal level of MMP-9, which is essential for breaking down the damaged extracellular matrix and facilitating cell migration and tissue remodeling, thereby aiding in the restoration of the epidermis. This could be probably due to the MMP-9 inhibition activity offered by the triphala [58] as supported by the already published literature.

3.9 *In-silico* studies for molecular interactions

Protocol validation was performed by redocking the co-crystallized ligand, NFH (N-[(2R)-2-{[formyl(hydroxy)amino]methyl}-4-methylpentanoyl]-N,3-dimethyl-L-valinamide), and calculating the root mean square deviation (RMSD) between its docked conformation and the co-crystallized pose. Docking validation of the standard inhibitor of MMP-9 (PDB ID: 1GKC) showed a root mean square deviation value of 0.843 Å, which is well within the acceptable range, confirming the reliability of the docking protocol. The native ligand (NFH) exhibited a binding energy of -5.3 kcal/mol and key interactions with the protein, including a conventional hydrogen bond with Ala189, π -sigma interactions with Phe110 and His405, alkyl

interaction with Leu187, and π -alkyl interaction with His411. Among the selected phytoconstituents, quercetin exhibited the strongest binding affinity for the target protein, with a binding energy of -9.5 kcal/mol. It formed conventional hydrogen bonds with Ala189, Tyr420, and Leu418, π - π stacking interactions with His401, π -alkyl interactions with Leu188 and Val398, and a π -sigma interaction with Tyr423. Ellagic acid displayed a binding energy of -7.3 kcal/mol and formed hydrogen bonding with Glu402, a π - π T-shaped interaction with His411, a π -sigma interaction with Leu187, and a π -alkyl interaction with Leu188. Gallic acid exhibited a binding energy of -6.4 kcal/mol, forming hydrogen bonds with Glu402, Ala189, Pro421, and Met422; π - π stacking with His401; π -alkyl interactions with Leu188 and Val398; and a π -sigma interaction with Tyr423. β -Sitosterol exhibited a moderate binding energy of -7.1 kcal/mol and displayed conventional hydrogen bonding with Glu111, carbon hydrogen bonding with His411, alkyl interactions with Pro180 and Leu187, and π -alkyl interactions with Phe110 and Tyr179. However, unlike the native NFH, β -sitosterol did not form direct hydrogen bonds with the key catalytic amino acid residues of the active site, suggesting fewer specific and desirable interactions with the protein. The docking interactions and binding energies of quercetin, ellagic acid, gallic acid, and β -sitosterol with the MMP-9 inhibitor (PDB id 1GKC) are shown in Table no. 1.

Table 1

Docking interactions and binding energies of Gallic acid, Ellagic acid, Quercetin and β -sitosterol with matrix metalloproteinase 9 inhibitor (PDB id 1GKC)

Ligands with Inhibitor	Binding energy (Kcal/mol)	Interactions
Co-crystallised standard ligand (NFH): N ~ 2~-(2R)-2-[[formyl(hydroxy)amino]methyl]-4-methylpentanoyl]-N,3-dimethyl-L-valinamide	- 5.3	Ala189a, Phe110f, His405f, Leu187e, His411d
Gallic acid	-6.4	Glu402a, Ala189a, Pro421a, Met422a, His401c, Leu188d, Val398d
Ellagic acid	-7.3	Glu402a, His411g, Leu187f, Leu188d
Quercetin	-9.5	Ala189a, Tyr420a, Leu418a, His401c, Leu418d, Val398d, Tyr423f
β -sitosterol	-7.1	Glu111a, His411b, Pro180e, Leu187e, Phe110d, Tyr179d
<i>Note: ^a Conventional hydrogen bond, ^b Carbon hydrogen bond, ^c π-π interaction, ^d π-alkyl, ^e alkyl, ^f π-σ, ^g π-π T-interaction</i>		

The images of the docked ligands superimposed over the co-crystallised ligand (6A), 3D interaction of quercetin, ellagic acid, gallic acid, and β -sitosterol with MMP – 9 inhibitor (PDB id – 1GKC), (6-B, C, D, E) and 2D interaction of quercetin, ellagic acid, gallic acid, and β -sitosterol with MMP – 9 inhibitor (PDB id –

1GKC), (6-F, G, H, I) are shown in Fig. 6. The various docking parameters used for grid generation are listed in Table B in the Supplementary Information.

3.10 Histopathological investigations

3.10.1 Hematoxylin and eosin staining for re-epithelialization

On day 7, the control group (a) showed disrupted epithelium with poor healing (Fig. 7). The marketed formulation (m) and TPE gel (g) demonstrated mild epidermal thickening; however, the TPE-loaded SNPs gel (j) displayed greater thickening accompanied by reduced neutrophil infiltration, suggesting an advanced healing stage. On day 21, histopathological analysis revealed limited wound healing in the control group (C), characterized by a disturbed epidermal structure and elevated neutrophil infiltration. In contrast, the group treated with TPE-loaded SNPs (I) and the marketed formulation (o) showed significant epidermal thickening, regeneration of skin appendages, and reduced inflammation. The TPE-loaded SNP-treated group exhibited the lowest neutrophil infiltration and the highest number of epidermal cells, indicating superior wound healing compared to the TPE gel. Overall, TPE-loaded SNPs demonstrated the most effective wound healing, characterized by enhanced regeneration and reduced inflammatory responses.

3.10.2 Masson's trichome staining collagen distribution

As shown in Fig. 8, after 7 days, the groups treated with the marketed formulation (i), plain SNPs gel (c), TPE gel (e), and TPE-loaded SNPs gel (g) exhibited greater collagen regeneration than the control group (a). After 21 days, collagen abundance was assessed by measuring blue coloration intensity. The control group (b) showed small vessels and minimal collagen regeneration. The control group (b) displayed small vessels and minimal collagen regeneration; in contrast, wounds treated with the marketed formulation (j), plain SNPs gel (d), TPE gel (f), and TPE-loaded SNPs gel (h) showed collagen bundles with irregular patterns, indicative of enhanced healing. Notably, the TPE-loaded SNPs gel demonstrated collagen uniformity and distribution comparable to that of the marketed formulation, suggesting its significant wound-healing potential.

4. Discussion

Delayed wound healing represents a significant clinical concern characterized by a prolonged healing process that deviates from the typical stages of repair. This delay is often attributable to underlying patient health disorders, persistent infections, a high microbial load, and biofilm formation, alongside chronic inflammation and reduced wound contraction. Triphala, an indian ayurvedic formulation is a combination of three herbs *Terminalia bellirica*, *Terminalia chebula*, and *Phyllanthus emblica*, which possess antibacterial, antioxidant, and anti-inflammatory properties. In this study, identification and quantification of the polyphenolic compounds in the methanolic extract of the triphala powder extract

was performed using a highly sensitive LC-MS/MS tool. Four polyphenolic compounds, gallic acid, ellagic acid, quercetin, and β -sitosterol, were identified and quantified. Silver nanoparticles were synthesized by employing a chemical synthesis method using trisodium citrate as a stabilizing and reducing agent, which produced highly stable and controlled-sized nanoparticles. Small silver nanoparticles are important because they exhibit a significantly high surface area-to-volume ratio, which enhances their antimicrobial activity and ability to penetrate biological membranes, essential for maximizing efficacy in wound care.

The antibiofilm activity of the test samples against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was evaluated using the crystal violet assay. TPE-loaded SNPs inhibited biofilm formation by the most common wound pathogens, *S. aureus* and *P. aeruginosa*. Thus, it can be concluded that TPE-loaded SNPs can effectively treat biofilm-associated wound infections because of the polyphenolic compounds in triphala powder extract that disrupt bacterial membranes and cause cell death. In these studies, the authors formulated a hydrogel using TPE-loaded SNPs. Hydrogel keeps the wound moist, which is crucial for encouraging the formation of new tissue and keeping it from drying up. Additionally, it can reduce pain and inflammation while softening the wound area. They also create a barrier that helps protect the wound from contamination and additional damage.

The results of the *in vivo* excision wound model showed that TPE-loaded SNPs significantly produced wound contraction compared to the other test groups. It was observed that TPE-loaded SNPs gel significantly improved hydroxyproline levels in the wound tissue. This proved that enhanced hydroxyproline levels strengthen collagen deposition and tissue regeneration. The lowest levels of inflammatory cytokines, TNF- α and IL6, suggest that the combination of TPE and SNPs effectively modulates inflammation and supports a balanced cytokine environment, thus facilitating improved wound healing. The authors observed that the developed hydrogel formulation balanced oxidative stress in the wound, thereby controlling the inflammatory stage of healing. This prevents tissue damage and promotes the transition to the healing phase.

Earlier studies have suggested the role of plant polyphenols in the modulation of Matrix Metalloproteinase-9 levels. Excessive MMP-9 leads to uncontrolled extracellular matrix degradation, resulting in a delayed phase 4 of wound healing. This study demonstrated that the polyphenols in Triphala powder extract directly decreased the catalytic activity of MMP-9. In addition to promoting re-epithelialization (skin regeneration) and angiogenesis (new blood vessel development), this regulation prevented excessive tissue degradation. Polyphenols in the Triphala powder extract modulated the levels of MMP-9, thus balancing the inflammatory environment of the wound, which further resulted in enhanced tissue regeneration.

In the current study, histopathological changes in the wound tissue were studied on days 7 and 21. The effects of TPE-loaded SNPs on re-epithelialization, inflammatory cell infiltration, and collagen fiber organization were evaluated. Results indicated that the TPE-loaded SNP-treated group performed better in terms of healing than all other groups. This improvement was evidenced by a notable reduction in

neutrophil infiltration and a marked increase in epidermal cell proliferation, indicating a more regulated inflammatory response. Furthermore, collagen fibers in SNP-treated wounds appeared denser and exhibited a more uniform orientation. This transition from a disorganized matrix to a well-structured fiber network underscores the strong regenerative capacity of Triphala-loaded nanoparticles in restoring epidermal integrity.

Previous studies have demonstrated that triphala has been used to study its wound healing potential in different animal models. This study further elucidated the underlying mechanisms for enhanced wound healing by studying the key polyphenolic compounds present in the triphala powder extract and correlating their antioxidant, anti-inflammatory, and MMP-9 inhibition properties. These observations suggest that polyphenols reduce oxidative stress, decrease inflammation, inhibit biofilms, and inhibit the activity of MMP-9, which is also supported by *in-silico* studies.

5. Conclusion

In conclusion, triphala is a powerful polyherbal formula used in clinical ayurvedic medicine. The polyphenolic compounds present in the triphala powder extract exhibited antibiofilm activity against the targeted bacterial strains. The integration of triphala extract with silver nanoparticles enhanced the therapeutic properties of the nanoparticles by leveraging the bioactive compounds present in triphala. Triphala powder extract along with silver nanoparticles balanced inflammation, promoted tissue regeneration, stimulated collagen production in wound tissues, and maintained the lowest oxidative stress, thereby contributing to more efficient wound healing. A mechanistic approach was proposed for greater wound closure in the group treated with the TPE-loaded SNPs gel. Molecular docking studies indicated that quercetin, gallic acid, and ellagic acid had more prominent and specific interactions with the matrix metalloproteinase-9 inhibitor protein. Thus, it can be concluded that the polyphenolic compounds present in TPE maintain optimum levels of MMP-9, which is necessary for cell migration and tissue rebuilding, which repairs the epidermis. The observed effect could also be linked to the inhibitory action of the compound on MMP-9, as documented in previous reports.

Abbreviations

MMP: Matrix Metalloproteinase, **SNPs**: Silver nanoparticles, **TPE**: Triphala powder extract, **PBS**: Phosphate buffered saline, **H and E**: hematoxyline and eosin, **M and T**: Masson's trichrome, **IL-6**: Interleukin 6, **TNF- α** : Tumor Necrosis Factor-alpha, **SOD**: superoxide dismutase, **LC-MS/MS**: Liquid chromatography-tandem mass spectrometry, **SPR**: Surface plasmon resonance, **FT-IR**: Fourier transform-infrared spectrophotometer, **ELISA**: enzyme-linked immunosorbent assay, **SEM**: Scanning electron microscopy, **TPE-loaded SNPs**: triphala powder extract-loaded silver nanoparticles, **XRD**: X-ray diffraction, and **FE-SEM**: Field Emission-Scanning Electron Microscopy.

Declarations

Ethical approval

This study was approved by the Institutional Animal Ethical Committee of Poona college of Pharmacy approved the protocol for animal studies (approval number-PCP/IEAC/2024/1-24). All experimental procedures complied with the national and institutional guidelines for the care and use of laboratory animals.

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Figures

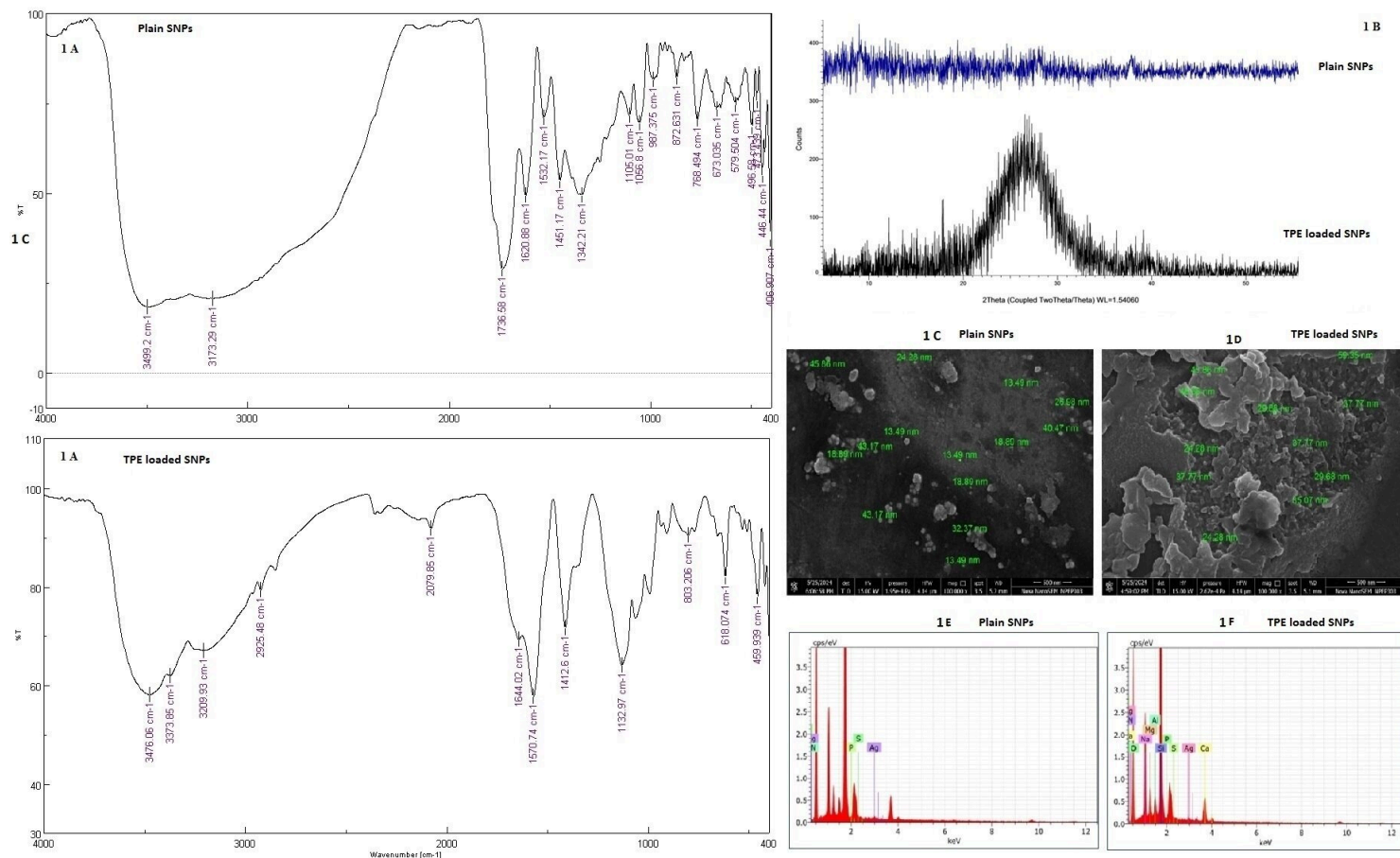


Figure 1

FT-IR spectrum, XRD patterns, Field emission SEM images and elemental detection images SEM studies

FT-IR spectra of TPE (1A) and TPE-loaded SNPs (1B), XRD patterns of plain SNPs and TPE-loaded SNPs (1C), field-emission SEM images of plain SNPs (1D) and TPE-loaded SNPs (1E), and elemental detection using SEM studies for plain SNPs (1F) and TPE-loaded SNPs (1G).

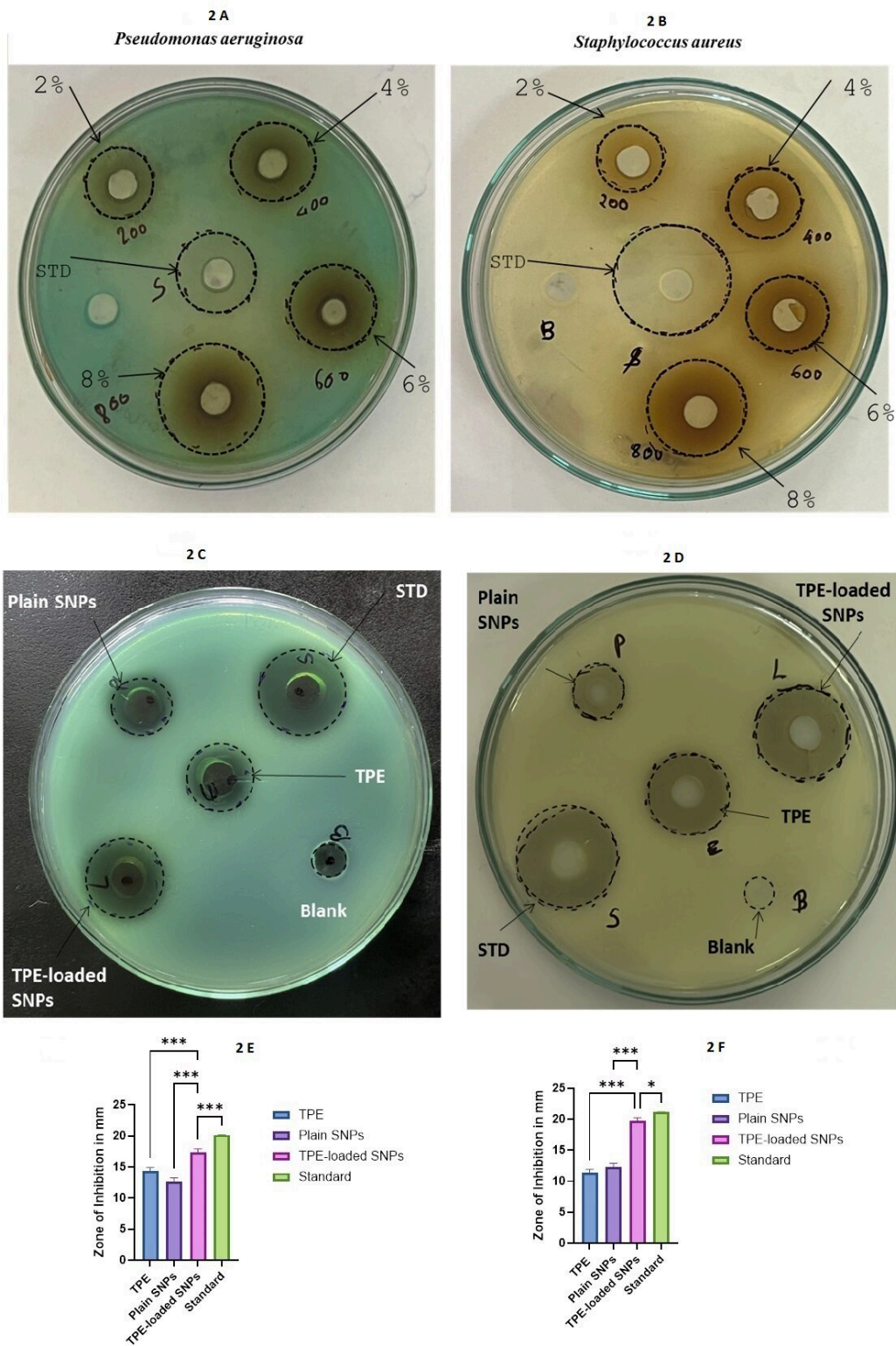


Figure 2

Antimicrobial susceptibility testing of different concentrations of TPE, Comparison between antimicrobial properties and statistical analysis of TPE, Plain and TPE-loaded-SNPs against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Comparison of antimicrobial properties of TPE, Plain, and TPE-loaded-SNPs against *Pseudomonas aeruginosa* (2A) and *Staphylococcus aureus* (2B), antimicrobial susceptibility testing of TPE, Plain, and TPE-loaded-SNPs against *Pseudomonas aeruginosa* (2C) and *Staphylococcus aureus* (2D), and statistical comparison of antimicrobial properties of TPE, Plain, and TPE-loaded-SNPs against *Pseudomonas aeruginosa* (2E) and *Staphylococcus aureus* (2F). Data are expressed as mean $\pm$ SE ($n = 3$), *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$. (One-way ANOVA followed by Tukey's comparison test).

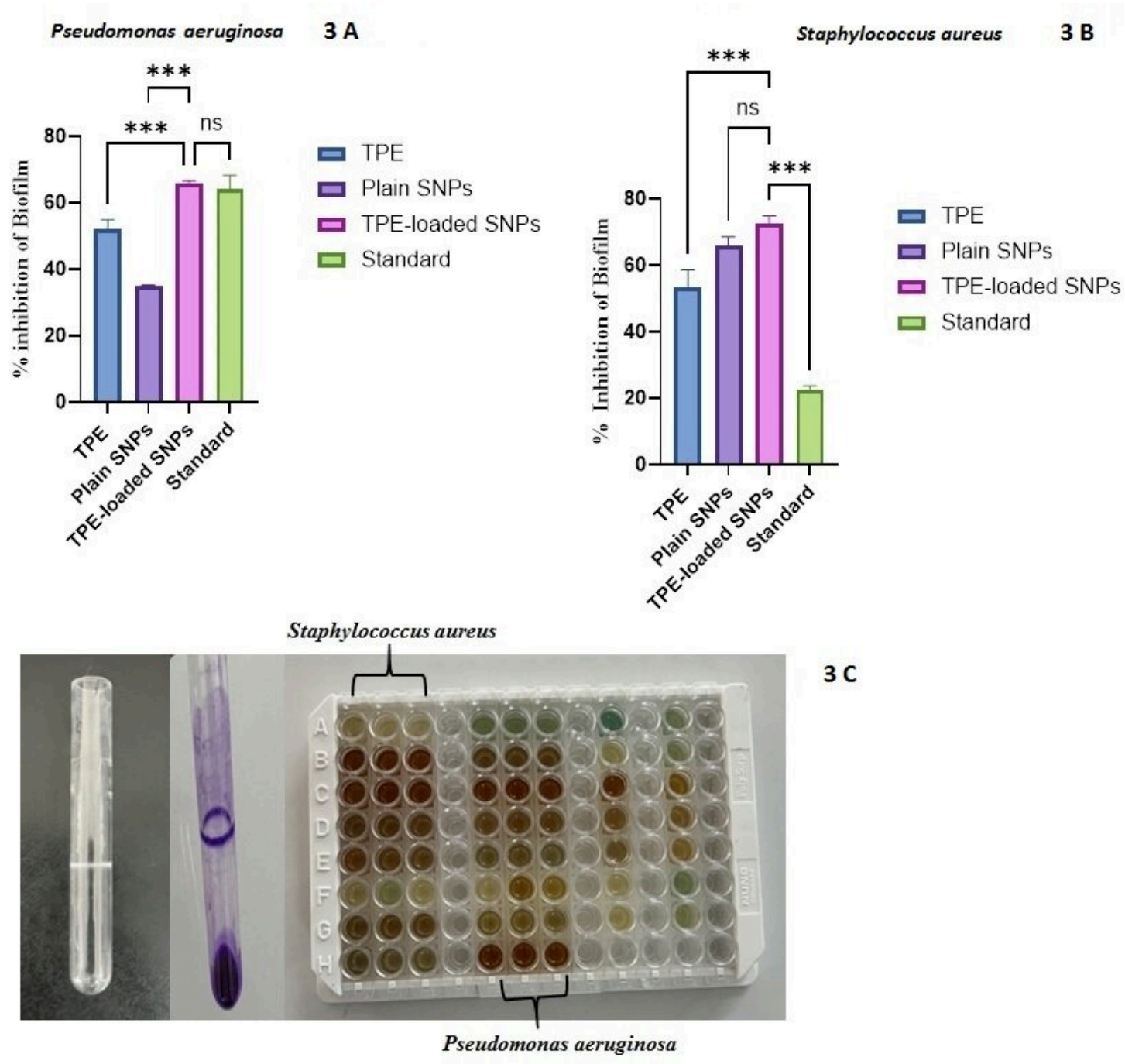
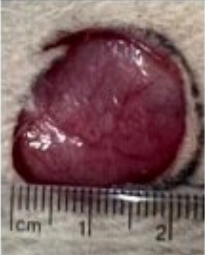
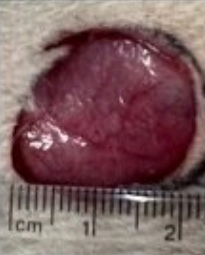
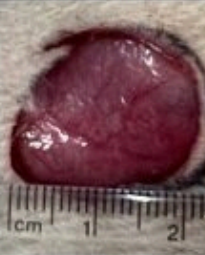
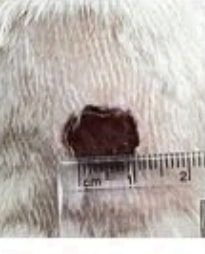
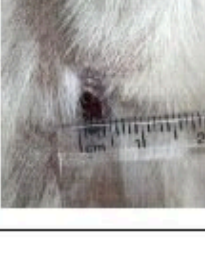


Figure 3

Antibiofilm activity of various test samples against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

% biofilm inhibition of various test samples against *Pseudomonas aeruginosa* (3A) *Staphylococcus aureus* (3B). Formation of biofilm, staining of biofilm using crystal violet, and inhibition of biofilm images (3C). Data are expressed as mean $\pm$ SE (n = 3), *** p<0.001, ** p<0.01, *p<0.1. (One-way ANOVA followed by Tukey's comparison test).

4 A	Normal control group	Plain SNPs Gel	TPE Gel	TPE-loaded SNPs Gel	Marketed Formulation
0 th day					
3 rd day					
14 th day					

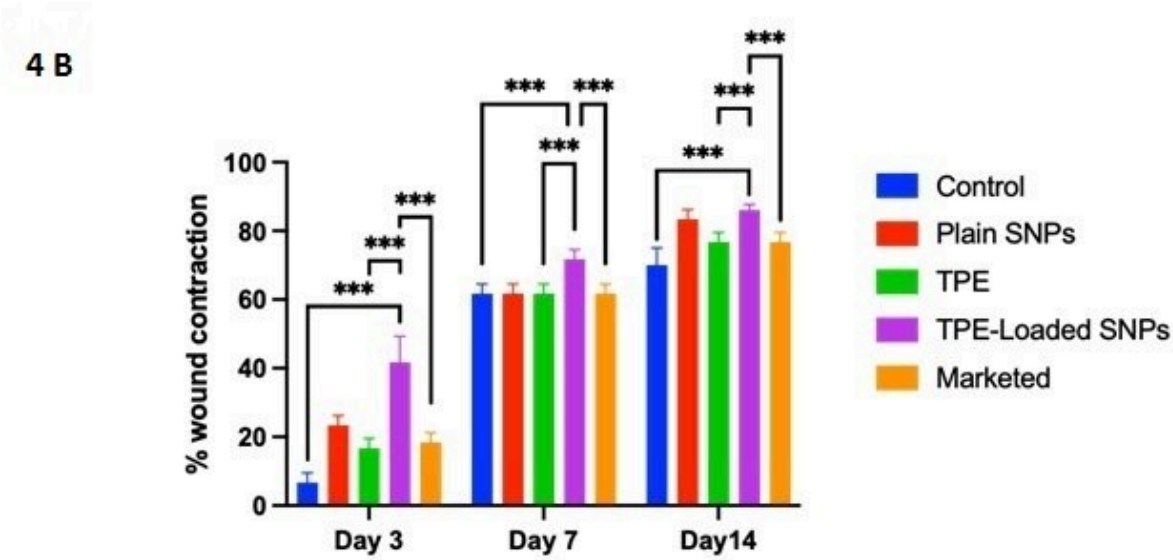


Figure 4

(4A) Photographic images of wounds at 0th day, 3rd day and 21st day: Normal control, plain SNPs gel, TPE gel, TPE-loaded SNPs gel, Marketed formulation

(4B) Statistical comparison of % wound contraction for normal control, plain SNPs gel, TPE gel, TPE-loaded SNPs gel, and marketed formulation. Data are expressed as mean $\pm$ SD ($n = 3$), *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$. (Two-way ANOVA followed by Bonferroni Comparison test)

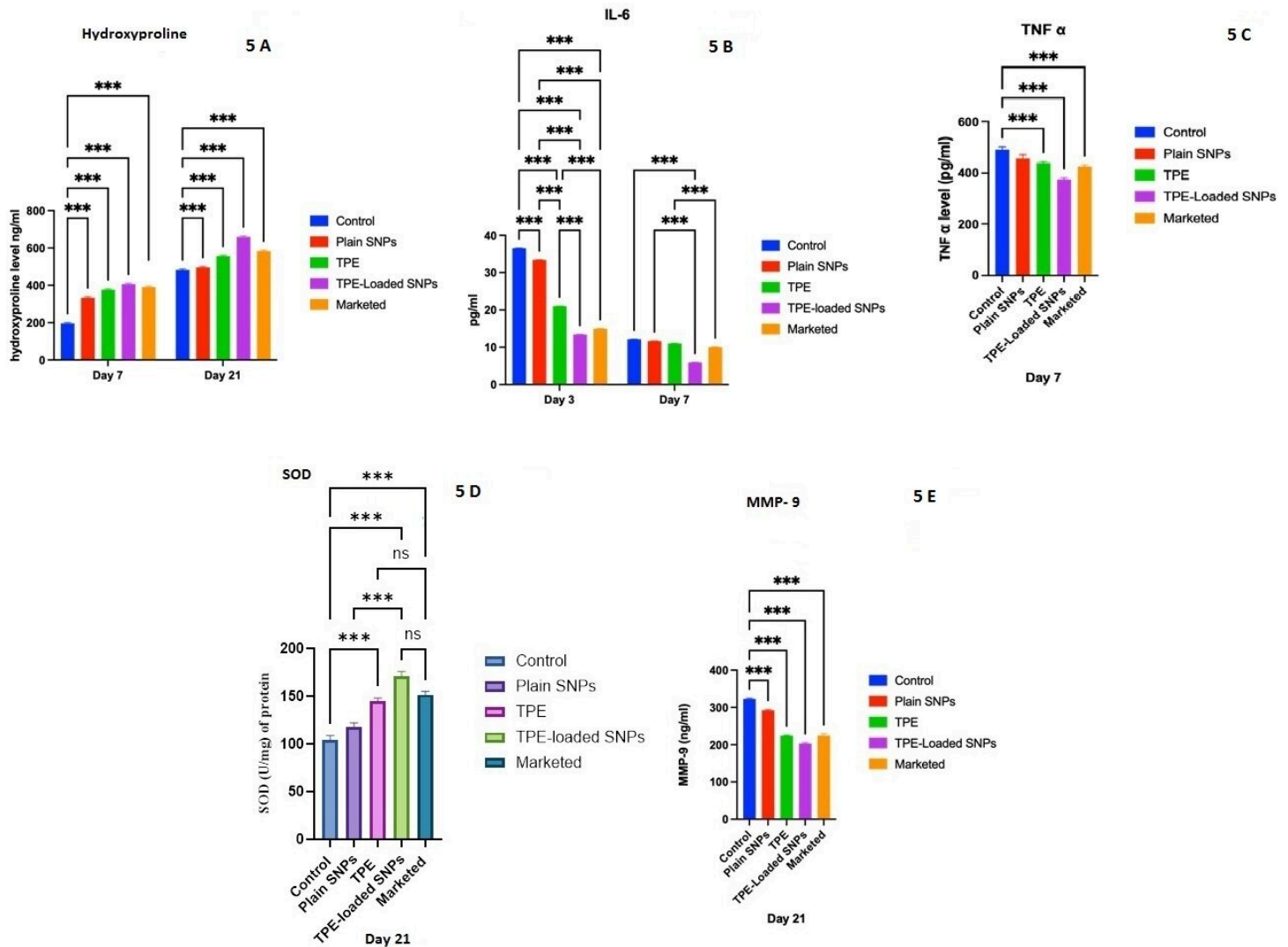
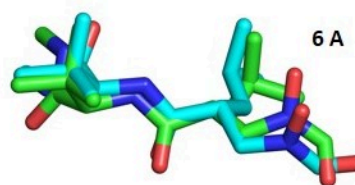


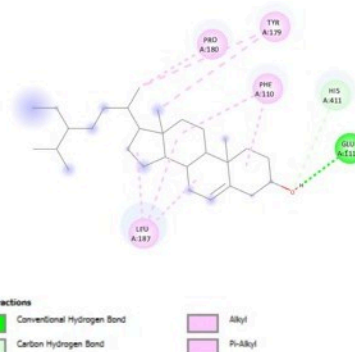
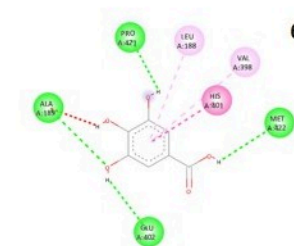
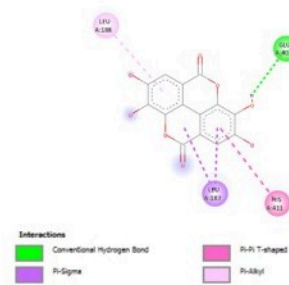
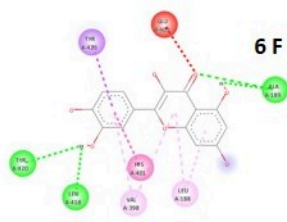
Figure 5

Statistical comparison of hydroxyproline levels on days 7 and 21 (5A), IL6 levels on day 7 (5B), TNF- α levels on day 7 (5C), superoxide dismutase (SOD) enzyme levels on day 21 (5D), and matrix metalloproteinase 9 levels on day 21 (5E) among groups treated with the TPE gel, plain SNPs gel, TPE-loaded SNPs gel, and marketed formulation.

*Note: Data are expressed as mean $\pm$ SD ($n = 3$) for all tests. Statistical tests applied: hydroxyproline and IL6 levels - two-way ANOVA followed by Bonferroni Comparison test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.1$; TNF- α*



RMSD = 0.843 Å



Superimposition of the docked ligands over co-crystallized ligand NFH (6A), 3D interaction of Quercetin, Ellagic acid, gallic acid, and β -sitosterol with MMP – 9 inhibitor (PDB id - 1GKC), (6 - B,C,D,E) and 2D interaction of Quercetin, Ellagic acid, gallic acid and β -sitosterol with MMP – 9 inhibitor (PDB id - 1GKC), (6 - F,G,H,I).

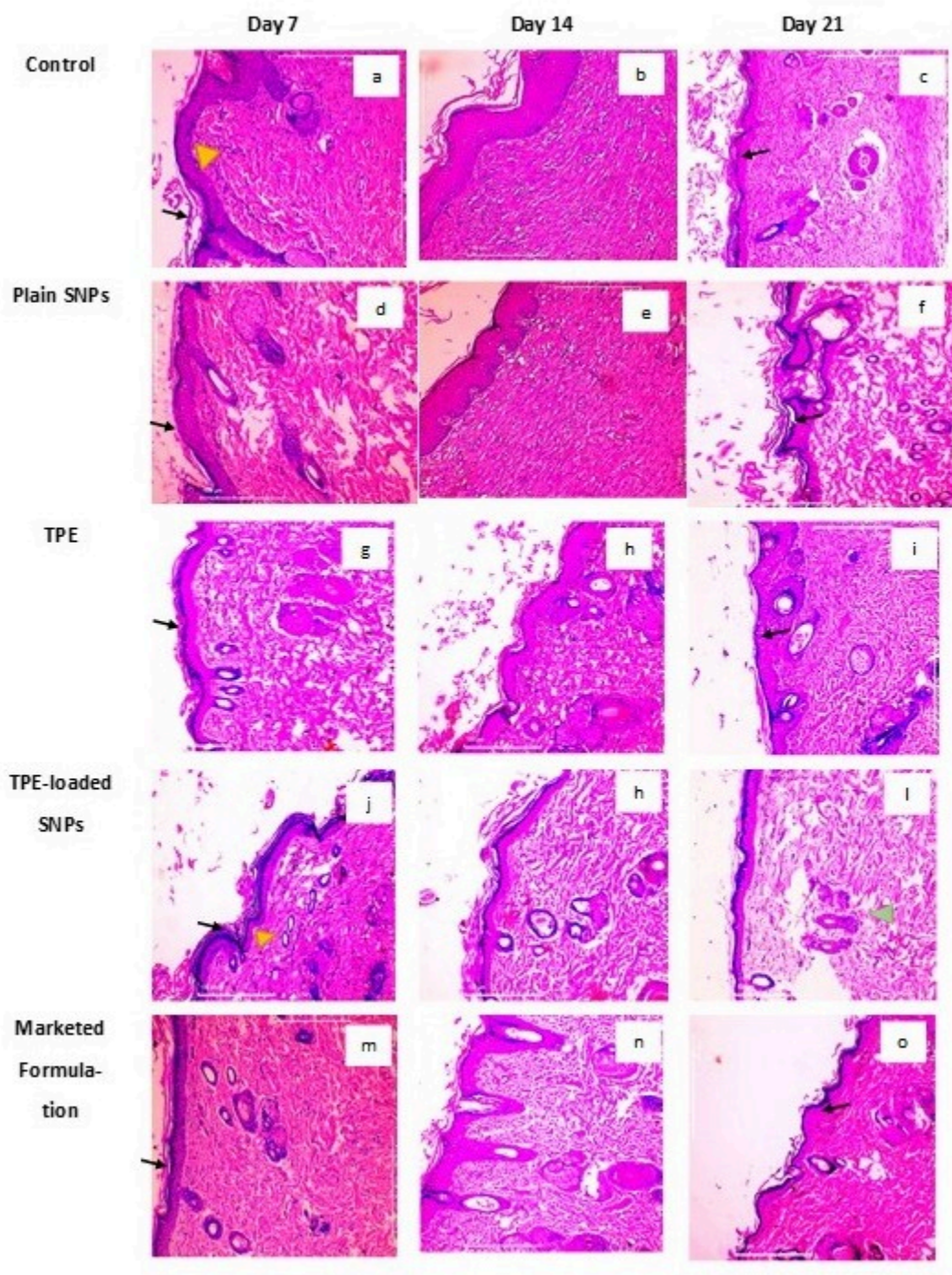


Figure 7

Hematoxylin and eosin histopathological images of wound tissue samples: control group (a,b,c), plain SNPs gel (d,e,f), TPE gel (g,h,i), TPE-loaded SNPs gel (j,k,l) and marketed formulation (m,n,o) on day 7th, 14th and 21st, respectively showing Epithelial thickness (black arrow), neutrophil infiltration (Yellow triangle), regeneration of skin appendages (Green triangle)

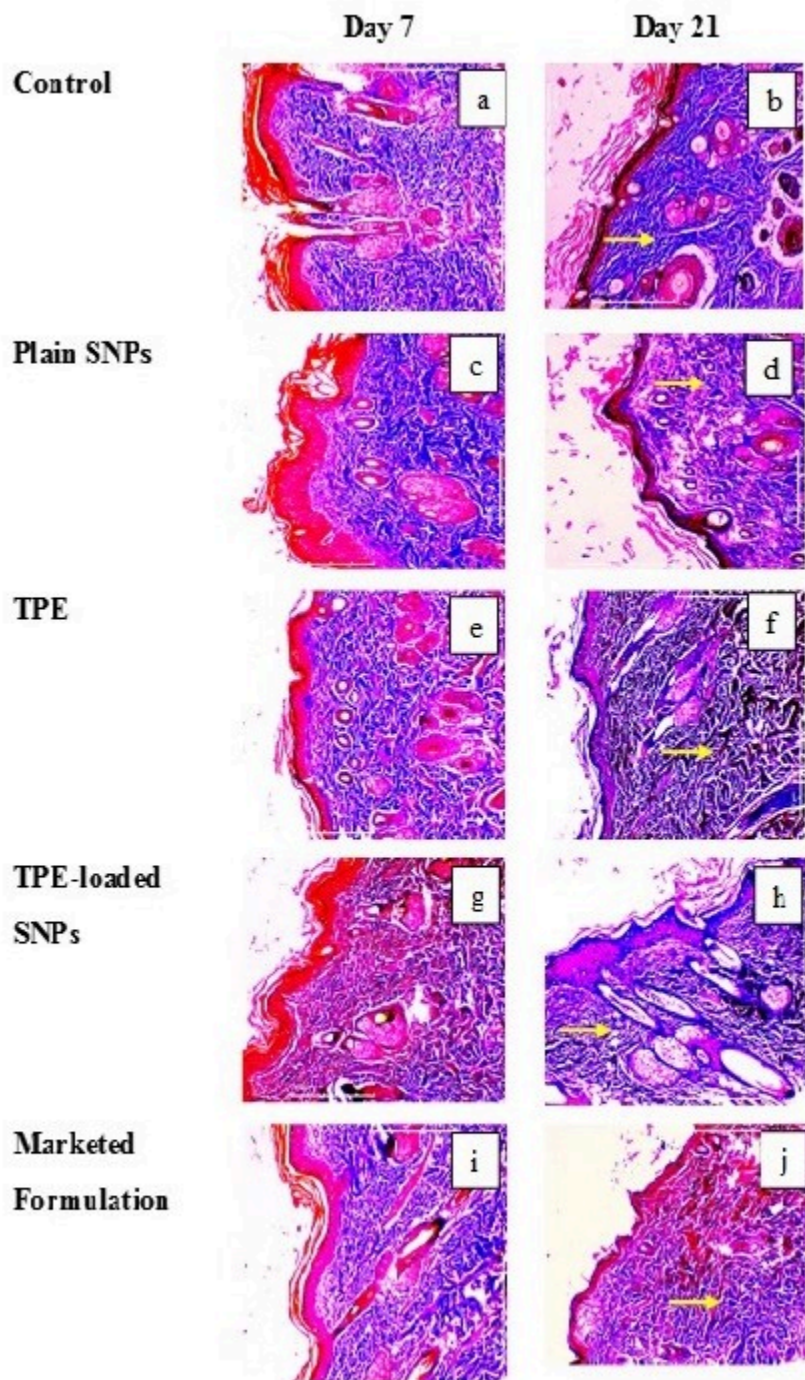


Figure 8

Masson's trichrome staining histopathological images of wound tissue samples: control group (a, f), plain SNPs gel (b, g), TPE gel (c, h), TPE-loaded SNPs gel (d, i), marketed formulation (e, j) on day 7 and 21, respectively showing collagen regeneration (Yellow arrow)

Supplementary Files

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