1 INTRODUCTION

Resistance to currently available antibiotics is one of the fastest-growing challenges to global health. The overprescription of antibiotics, inadequate control of infection in hospitals, incompletion of the entire course of antibiotics by patients, overuse of antibiotics in the medical and non-medical sectors, sanitation deficiency, and poor hygiene are the prime factors of building resistant strain. According to the 2019 AR threat report by the Centers for Disease Control and Prevention (CDC), overall about 2.8 million infections with antibiotic resistance occur in the U.S. each year, resulting in more than 35,000 deaths (Church *et al.*, 2006a). According to estimates by Christopher Murray and colleagues, the worldwide toll of AMR in the year 2019 alone would be 495 million (362-657), with 127 million of those fatalities (with a 95% confidence range of 0.911-1.71) attributed to bacterial AMR. At the regional level, the rate of all-age mortality related to resistance was highest in western sub-Saharan Africa, where it was estimated to be 27.3 deaths per 100,000 (20.9-35.3), and lowest in Australasia, where it was projected to be 6.55 deaths (4.3-9.4) per 100,000 (Murray *et al.*, 2022). According to The National Institutes of Health-Fogarty International Center (NIH-FIC), India has one of the highest rates of AMR in the world (matters, 2022). India has a high burden of mortality due to infectious diseases (417/100,000 persons) where bacterial infections appear to be the leading cause of death in children as well as adults (Gunasekaran, 2022).

Globally, burns are the fourth most frequent type of injury, after traffic accidents, falls, and physical violence (Greenhalgh, 2019; Markiewicz-Gospodarek *et al.*, 2022). Burn represents one of the most severe and debilitating traumas and the infection of which results in severe complications in patients with burns. Although more patients with burns die of pneumonia than of burn wound infection, burn wound infection remains an

important infectious complication in the burn population (Sartelli *et al.*, 2021). According to World Health Organization (WHO), approximately 3,00,000 deaths are caused each year mainly due to fire burns, with additional deaths attributed to heat and other causes of burns e.g., electric devices, chemical materials, radioactive rays, etc. More than 95% of burn injuries occur in developing countries (Kumar *et al.*, 2022). The Data from the National Center for Injury Prevention and Control in the United States show that approximately 2 million fires are reported each year which result in 1.2 million people with burn injuries where 50% percent of all deaths from thermal injuries were believed to be due to infection. Every year in India about seven million people are burned and 1.4 lakhs perish and 2.4 lakhs are suffered from a disability (Khan *et al.*, 2020). Additionally, biofilms are thought to be present in most wounds and are known to be comprised of multiple bacterial species which further delays the wound-healing process (DeLeon *et al.*, 2014; Ferry *et al.*, 2022; Lashtoo Aghaee *et al.*, 2022). Medical procedures may introduce bacterial biofilms via healthcare-provider interaction, patient skin bacteria, and more. Infections due to the *Staphylococcus aureus (S. aureus), Klebsiella pneumoniae (K. pneumoniae), Pseudomonas aeruginosa (P. aeruginosa), and Acinetobacter baumannii (A. baumannii)* are the most common in burn wound whereas MDR resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* is known as the most common co-isolated cause of life-threatening poly bacterial infection in burn patients (Jassim *et al.*, 2012; Nasser *et al.*, 2003; Rose *et al.*, 2014).

Mother Nature has provided a solution for these resistant strains in the form of Bacteriophage which are the "bacteria eating viruses" that infect and replicate within bacteria and are effective even against resistant bacterial strains. Bacteriophage therapy has been emerging as a better alternative to antibiotics against all kinds of bacterial infections due to the increasing prevalence of antibiotic-resistant bacteria. Interestingly, bacteriophages are specific against single or closely related bacterial strains without

affecting the natural microflora of the host organism. In 2017, Geoff Watts succinctly defined how phage therapy works: "Either bacteriophages kill the bacteria or they won't, but the worst that can happen is nothing they are not going to harm so it's worth trying" (Watts, 2017). Recently, FDA approved the emergency Investigational New Drug allowance of adaptive phage therapeutics to administer the phase therapy to 30 COVID-19 critical patients where the standard antibiotics failed to show any improvement (Wu *et al.*, 2022).

Numerous experimental studies have shown the efficacy of phage treatment as a potent antibacterial agent capable of effectively combating a diverse array of bacterial illnesses. Nevertheless, the task of actualizing these ideas is difficult within the present circumstances. Bacteriophage therapy is a ray of hope after surging the AMR yet, lack of regulatory guidelines, phage formulation development, phage stability during formulation processing and following the entrapment into the formulation, maintaining lytic activity during complete therapy, determining the minimum effective dose, etc. are some of the issues which need to be addressed before the phage therapy becomes the clinical reality. Based on the literature review, we focused on overcoming the bacteriophage delivery stability limitations by maintaining bacteriophage titer in the formulation and throughout treatment. In the current project, we have proposed a bacteriophage-loaded microparticles-laden topical gel to overcome the biofilm-mediated AMR in burn wounds.

The Lytic bacteriophage against the most prominent MDR bacteria *viz. S. aureus, K. pneumoniae, P. aeruginosa, and A. baumannii* causing wound infection were isolated and evaluated for morphological class identification, host specificity, and growth curve in the host. We used both single phage and mixed phage, for the treatment of bacterial wound infections targeting mono-bacterial and poly bacterial infections. These phages

were encapsulated inside chitosan and trehalose matrices to protect them from external and internal environmental factors, thereby preventing their destruction and inactivation. Further microparticles were integrated into the glycerol and SEPINEO™ P 600 gel, to ensure the preservation of their structure, lytic activity, and ease of application to wound areas. Chitosan is a biopolymer macromolecule that is derived from crustaceans. The degree of acetylation and deacetylation, as well as surface modification and molecular weight, determine the biological and chemical properties of chitosan (Aranaz *et al.*, 2021). Additionally, chitosan is a biocompatible, nontoxic polymer with antibacterial properties, making it an appropriate drug delivery vehicle. (Yan *et al.*, 2021). Chitosan has antibacterial activity by selectively adhering to the negatively charged bacterial cell wall, resulting in compromising cellular integrity. This disruption leads to alterations in membrane permeability, followed by the attachment of chitosan to bacterial DNA. Consequently, chitosan inhibits DNA replication, ultimately leading to bacterial cell death (Ke *et al.*, 2021). Additionally, the versatility of chitosan makes it a suitable polymeric material for wound dressings and drug delivery systems (Liu *et al.*, 2018; Meng *et al.*, 2021). The use of chitosan as a biomaterial for microencapsulating bacteriophages has been demonstrated in previous publications (Abdelsattar *et al.*, 2019; Ma *et al.*, 2008a; Rahimzadeh *et al.*, 2021; Rotman *et al.*, 2023). There are some grades of chitosan (low degree of deacetylation), which has low water solubility, that require 0.1-2 % acetic acid or formic acid for solubilization. In such an acidic environment, a bacteriophage cannot survive. In this research, we have used chitosan (chitosan oligosaccharide, water-soluble grade, extra pure, >90% degree of acylation), which is biodegradable, biocompatible, and non-toxic with potential for pharmaceutical applications (Mehata *et al.*, 2019). Further, we have used trehalose to protect BP (viral capsid) from desiccation and thermal stress by providing structural and conformational stability to the proteins via direct hydrogen bond formation or

vitrification, thus limiting the protein's mobility (Vandenheuvel *et al.*, 2014). We have also used glycerol as one of the formulation components that prevents skin dryness and helps to preserve BP, during their storage (Xiao *et al.*, 2022). Additionally, SEPINEO™ P 600 (3-in-1 polymer: thickening, emulsifying, and stabilizing agent) ready-to-use gel, which improves stability, applicability, and texturizing of BP microparticles. One other benefit of the SEPINEO™ P 600-based gel's manufacturing process is the absence of highs, which makes gel formation simple with mild glass rod stirring only (Baghel *et al.*, 2020b).

Further, the microparticles particle size, polydispersity index, Zeta potential, and entrapment efficiency were identified. Additionally, the morphology of microparticles and gel was determined. Furthermore, the antibacterial, antibiofilm potency, and in vivo wound healing were evaluated. Also, we have traced *in vivo* wound healing process in rats by ultrasound and photoacoustic imaging. A 3D-Mode of Ultrasound (USD) was employed to collect the area (two-dimensional data) and depth-volume (three-dimensional data) of the wound. Photoacoustic imaging (PA-Mode) was used to determine saturated oxygen percentage (sO_2%). PA imaging is ideally suited to monitor local angiogenesis, perfusion, and oxygen saturation which are the key parameters for wound healing. Additionally, a gel occlusion bio-imaging study was performed on burn wounds in rats by *in vivo* bioimaging system.