# 1.0

# INTRODUCTION

"**The enemy was the microbial world, and over the centuries, it has killed more people than all of man's wars combined**"

Tess Gerritsen

Infectious diseases have become a prominent priority at the global level for public health due to the enhanced rate of mortality. Recent data estimated that more than 10 million sepsis-associated infections are caused by microbial infections that take ~20% of the deaths globally. Among the microbial infections, bacterial pathogens have been remarkably involved in the global infection death reports. According to the Global Burden Sepsis Survey, 13.6% of global deaths and 56.2% of sepsis-associated deaths were reported due to bacterial infections in the patients (Wang *et al.,* 2024a; Baker *et al.,* 2022).

Microbes can enter the host by impairing the first-line defenses especially, skin and mucous membranes to cause infections. Wound infection is described as an invasion of bacterial pathogens into the dermal and subdermal tissues, resulting in damage to the host cells. It poses a significant threat to the global community that largely influences economic development and quality of life. These infections lead to numerous fatalities, which enhances the economic crisis in the healthcare system. According to the World Health Organization's findings endorsed by the Guidelines Review Committee, surgical wound infections are the second most prevalent healthcare-associated infection globally. Approximately 11% of patients who undergo surgery become infected in developing and underdeveloped countries (WHO, 2016). According to the International consensus, the microbes proliferate in greater numbers at the site of infection and invoke local or systemic responses in the host; thus resulting in extensive local tissue damage and impeded wound healing processes. Wound infections have been initiated by the

contamination of bacteria at the wound site through the colonization process to cause local infection followed by systemic infections. These worsening conditions are highly influenced by the enhanced microbial loads, virulence and the degree of pathogenicity (Kulayta *et al*., 2024; Wörner *et al*., 2023; Ikuta *et al*., 2022).

There are two kinds of wounds such as acute and chronic wounds. The uncomplicated acute wounds can be repaired through a regular phase of the healing process depending on the clinical signs of erythema, the nature of the injury, warmth, swelling and purulent discharges. Acute wound infection occurs in ~5.6-26% of wounds and the top three most common acute wounds prone to microbial infections are burn wounds, surgical site infections and traumatic wounds. In comparison, burn wound infections are responsible for more than 75% mortality rate and various pathogenic bacteria are identified during post-burn injury. During the surgery, microbial infections can occur on sutures or other prostheses that can induce superficial and deep wound infections. Traumatic wounds such as lacerations and abrasions generally affect extensive tissue bone and integral organs. The most prominent bacterial pathogens responsible for acute wound infections are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus* spp., *Proteus mirabilis*, *Klebsiella pneumoniae*, *Staphylococcus epidermis* and *Escherichia coli* (Alhumaid *et al*., 2024).

Paradoxically, chronic wounds are more complicated than acute wounds due to the delayed healing process. They are more often manifested in patients with diabetes, obesity, vascular diseases, malnutrition and elderly people. The prevalence of chronic wound infections in India is estimated to be 10.55 per 1,000 people due to developing resistance in microbes to several antibiotics (Las Heras *et al*, 2020). Post-surgical chronic wound infections are common in India, with rates ranging from 3–12%. Polymicrobial attacks are the major perpetrators in most chronic wounds that almost delay wound repair by promoting infection susceptibility and inflammation in the host. *Staphylococcus aureus* is the most dominant infectious pathogen in chronic wound infections followed by *Pseudomonas aeruginosa* (Khalid *et al*., 2024; Johnson *et al*., 2022; Puca *et al*., 2021).

One of the protruding factor of bacterial infections in the wound site is the biofilm formation potential of microbes. Biofilms are the three-dimensional (3D) matrix that provides protection and consistency for the growth of microbes at wound sites. Biofilm formation is a dynamic and complex process that follows several steps including, surface adherence, proliferation, microcolony formation, growth and differentiation. In general, the host immune system can easily remove the bacterial cells under normal physiological conditions to promote wound healing processes. During the dysregulation and dysfunctional status of the host immune system, microbes are more likely to attach to the wound area through biofilm processes. The two key players of the immune system such as macrophages and neutrophils are effectively fighting against bacterial pathogens and protecting the innate immune system of the host. Nevertheless, the pathogenic bacteria encased inside the biofilm structures have not been easily prone to neutrophils and macrophages through phagocytic attacks. It is observed that the bacteria can form a biofilm on the wound bed within 24 hours of infection. Once the biofilm has become mature, the host immune system is ineffective in fighting against bacterial infections and it is also responsible for worsening and delayed wound healing processes (Liu *et al*., 2022; Bandy *et al*., 2022; Ding *et al*., 2022).

Biofilms can be categorized into two major divisions as commensal and pathogenic. Under various parameters, pathogenic biofilms differ from commensal biofilms including the occurrence of the more upregulated genes. Those genes can promote the degradation of enzymes (matrix metalloproteinases (MMPs)), extracellular polymeric substances (EPS) endurance, enhanced bacterial proliferation and propagation to promote wound infections. The enormous growth of biofilms by the pathogenic bacterium may upregulate the immune responses that lead to chronic wounds. In general, the biofilm matrix is formed by the successful adherence of bacteria on the surface of the wounds and the induction of biofilm phenotype is greatly influenced by the complex intracellular signals that alter the gene expression profiles of planktonic bacteria. During the alteration in the gene expression patterns, bacteria can form microcolonies that ultimately become biofilms in conjunction with quorum sensing systems. Quorum sensing is

a cell-cell communication that is responsible for bacterial communication and is largely controlled by signaling molecules that bind to the receptors (response regulators). Further, the microcolonies matured into an EPS matrix and the inflammatory materials from the wounds are surrounded by normal skin in the form of a cellular matrix (Soni *et al*., 2024; Darvishi *et al*., 2022).

The volume of biofilm is ultimately dominated by EPS which accounts for 90% of the total volume and bacteria accounts for the remaining 10%. The matured/developed biofilm forms a characteristic structure of mushroom towers or stalk-like structures or water channels. Those structures are involved in the transfer of nutrients from the host to the lower layer of the biofilms and carry away waste materials from the lower layer to dispose off. The EPS system consists of exopolysaccharides, extracellular DNA, extracellular proteins and extracellular lipids. This EPS provides a protective barrier against the host-immune responses and facilitates the adhesion, aggregation, and stabilization of biofilms. Furthermore, it provides an interactive environment between bacteria to form polymicrobial biofilms (Diban *et al*., 2023; Goel *et al*., 2023).

The bacterial biofilm communities of the pathogenic microbes are formed from single or multiple bacteria. Compared to the single-species biofilms, the polymicrobial biofilms have exhibited a new type of characteristics via interspecies interactions which include as follows: (i) increased bacterial cell count; (ii) enhanced biofilm mass; (ii) increased metabolic activity of community members (bacteria); (iv) changes in structural organization and spatial arrangements; and (v) enhanced antimicrobial resistance. There are various steps or processes involved between bacteria to form polymicrobial biofilms which include synergy, cooperation, antagonism, mutual benefit, competition and utilization. Due to the competition or cooperation behavior with other microbes, the polymicrobial biofilms showed a different set of characteristic behaviors compared to their planktonic state. Wound-related biofilms are greatly governed by a heterogeneous polymicrobial population which includes the widespread opportunistic pathogens of *Staphylococcus aureus* and *Pseudomonas aeruginosa* with a reported prevalence of 93.5% and 52.2%, respectively. These two bacteria commonly occur

in both acute and chronic wound infections through their dynamic biofilm phenotypes (Zhao *et al*., 2023; Razdan *et al*., 2022).

*Staphylococcus aureus* is a Gram-positive, cocci-shaped pathogenic bacterium responsible for skin and soft-tissue infections which further leads to the development of various invasive diseases including osteomyelitis, endocarditis and pneumonia. It has been associated with various diseases in hospital-acquired and community-acquired settings. Since it is a versatile pathogen, it can adapt to multiple niches that lead to various infections in humans. The structural dynamics and population heterogeneity highly influence the persistence of *Staphylococcus aureus* in the skin during infectious processes. This pathogen has the ability to sense environmental signals effectively to change its adaptive nature to the surrounding environments. The virulence factors secreted by *Staphylococcus aureus* play a prominent role in wound dehiscence, tissue damage and delayed wound healing (Gehrke *et al*., 2023; Chelkeba and Melaku, 2022).

Another prominent pathogen in wound infection is *Pseudomonas aeruginosa*, a Gram-negative rod-shaped, heterotrophic, motile bacterium that belongs to the class of γ-proteobacteria and the family of Pseudomonadaceae. It is a difficult-to-treat bacterium and is responsible for causing cystic fibrosis, pulmonary diseases, sepsis, cancer, traumas, ventilator-associated infections. The biofilm formed by *Pseudomonas aeruginosa* may survive in harsh environments including a hypoxic atmosphere. By producing a series of virulence factors, it can adapt to adverse environments which contributes to the successful persistence and pathogenesis of this pathogen. The regulation of all the secreted virulence factors responsible for *Pseudomonas aeruginosa* infections is highly influenced by the quorum sensing system (a mass communication system). It can enter into the host cells during the impaired host-immune system and loss of skin integrity. The recent report also demonstrated that *Pseudomonas aeruginosa* can form fully grown biofilms at the wound site within 48 to 72 hours (Sathe *et al*., 2023; Qin *et al*., 2022; Rubio-Canalejas *et al*., 2022).

The primary goal of wound management is to maintain a clean and moist environment around the site of infections to support the physiological wound healing processes and prevent wound infections. Three major steps are followed in wound infection treatment to reduce the count of pathogens such as debridement, cleansing and antimicrobial administration. The removal of foreign bodies or necrotic tissues around the infected areas is done by the debridement method to reduce the bacterial loads. The wound healing process can be resumed with the remaining viable tissues. Therapeutic cleansing also removes the problematic or excessive infected exudate from the wound-infected area. The cleansing solution has the ability to disrupt the biofilms and kill the pathogenic bacteria with low cytotoxic. The common cleansing solution used on the infected area is potable tap water, sterile saline solution and surfactants. Antimicrobials (antiseptics and antibiotics) have the capacity to inhibit and kill bacteria. Antiseptics are a non-selective agent that exhibits a wide range of antimicrobial activity against bacteria, viruses and fungi, while antibiotics exert their activity against bacterial pathogens (Zhang *et al*., 2023; Kaiser *et al*., 2021).

Antibiotic therapy for wound infections follows both topical and systemic administrations. Chloramphenicol, aminoglycosides, polypeptide antibiotics, sulphonamides, fusidic acid, metronidazole, retapamulin and mupirocin are commonly employed antibiotics for topical applications at the site of wound infections. One prominent antimicrobial agent with a broad spectrum of action against both Gram-positive and Gram-negative bacterial pathogens is chloramphenicol. It is mainly applied for skin and wound infections. It can maintain its viable activity against a wide range of bacterial pathogens with a broad range of bacteriostatic activity. Chloramphenicol has the ability to specifically target protein synthesis (Roska *et al*., 2022; Losito *et al*., 2022; Zakhour *et al*., 2022).

Even though, front-line antibiotics effectively target bacterial pathogenesis, the formation of pathogenic biofilms complicates the treatment strategy, thus economic and humanitarian interest lies in the development of new antimicrobial approaches. There is a wide range of antimicrobials including antiseptics and antibiotics commonly employed for treating wound infections, which are associated

with relevant demerits. Bacterial pathogens show an enhanced resistance profile against many selective antibiotics due to overuse or misuse of antibiotics in order to achieve sufficient concentrations at the target site of infection. Moreover, *Pseudomonas aeruginosa* is found to be intrinsically resistant to many antibiotics owing to its limited outer membrane permeability. Likewise, *Staphylococcus aureus* also quickly develop and evolve resistance to nearly all antibiotics employed to kill it. The organisms seem to exhibit a wide range of resistance profiles against erythromycin, tetracyclines, methicillin, cloxacillin, oxacillin, nafcillin and dicloxacillin (Sanya *et al*., 2023; Esposito *et al*., 2023a).

Chronic wound infections can increase the longer stay in the hospital which probably influences the chances of long-term and indiscriminate use of antibiotics. The inappropriate medications and dosages trigger genetic changes in the bacteria, thus ultimately reducing the effectiveness of many classes of antibiotics. This phenomenon resulted in the emergence of antimicrobial resistance (AMR). Eventually, there is an alarming rise in wound infections due to multi-drug resistance (MDR) bacterial pathogens which complicate the management and treatment strategies. MDR is driven by various mechanisms, such as efflux pumps, enzymatic degradation, and biofilm formation. Primary mechanisms that lead to the development of antimicrobial resistance include enzymatic breakdown of antibacterial medications, modification of bacterial proteins targeted by antimicrobials, and alterations in membrane permeability to antibiotics. In recent decades, the proliferation of antibiotic-resistant strains has been prompted which heightened the awareness of AMR endurance and insisted on the adoption of antibiotic stewardship programs (Muteeb *et al*., 2023; Yaacoub *et al*., 2022; Uddin *et al*., 2021).

According to the WHO, AMR is a major threat to humanity and is estimated to cause 10 million deaths annually by 2050. Generally, MDR strains survives for a longer period and their proliferation can take place in the host even under the low-nutrient conditions. The epidemiological profile also has an impact on AMR and MDR status of wound-associated bacterial pathogens. Recent studies have iterated that the presence of AMR and MDR mitigate the treatment efficacy against common wound infections in developing countries. Thus, the emergence of AMR

and MDR leads to therapeutic failure, lavishness in treatment cost, prolonged hospitalization, mortality rate and spread of MDR pathogenic bacteria. Concerning the severity of wound infections, the World Health Organization has classified *Pseudomonas aeruginosa* and *Staphylococcus aureus* as high-priority MDR pathogens and there is a pressing need for the development of new antimicrobial strategies for eradicating their persistence in wounds (Yassin *et al*., 2023; Wong *et al*., 2022).

Herbal medicine has a strong history that spans more than 3000 years of potential applications and acts as a foundation to treat various illnesses. According to the WHO, many developing nations including India, China, and Africa follow sought-after treatment by 3.5 to 4 billion people. Medicinal plant extracts are used directly to treat many diseases and disorders in ancient times. Due to the excessive abundance of natural compounds in almost all parts of the plants, it can be exploited as the starting material for the development of lead compounds to cure targeted diseases. Among 35,000 plant species utilized for medicinal purposes, only 20% of them undergo phytochemical analysis and 10% of them are evaluated for their bio-therapeutic applications. According to the WHO, 25% of the drugs have been derived from plant materials. Drugs from medicinal plants are popular due to their enormous benefits in terms of efficacy, quality, safety and less or no adverse reactions (Israyilova *et al*., 2024; Breijyeh and Karaman, 2024; Albahri *et al*., 2023).

Among various medicinal plants, *Couroupita guainensis* Aubl. (common name: Cannonball) is a plant which belongs to the Lecythidaceae family resided in South India, Malaysia and the Amazon rainforest in South America. This plant has been used traditionally for various therapeutic purposes, such as preparing it in the form of decoction or combining it with other medicinal plants. Reports have demonstrated the efficacy of whole plants in wound healing applications and most of the ancient people exploited whole parts of the plants to treat a wide range of human illnesses. Many diseases and disorders including skin diseases, scabies, microbial infections, stomach problems, allergies, inflammation, ulcers, hypertension, malaria, piles, kidney problems, dysentery and toothaches have been cured by

leaves, flowers and bark of *Couroupita guianensis* Aubl. The significant antioxidant activities of the flower extract of this plant were noted (Esposito *et al*., 2023b; Logambal *et al*., 2023; Sheba *et al*., 2023). Literature has scientifically proved that the leaves and flowers of *Couroupita guianensis* Aubl. contains various phytocomponents including alkaloids, sterols, flavonoids, glycosides, triterpenes, tannins, indirubin, isatin, α-amirin, couroupitine, and β-amirin. Isatin is an alkaloid that has the documented properties against tumor, inflammation, wounds, toothaches, microbial infections, stomachaches, skin diseases and hypertension. Tripathi and Sonawane (2013) successfully isolated isatin from the flower parts of *Couroupita guianensis* Aubl. and various studies have iterated their potential antimicrobial activities against several pathogens (Kavitha *et al*., 2024; Sheba and Anuradha, 2019; Guo, 2019; Kavitha *et al*., 2013).

Due to the therapeutic efficacy, low toxicity and non-adverse reactions of existing interventions, natural compounds are highly demanded by pharmaceutical industries. The secondary metabolites or active compounds in the plants are present in smaller quantities; most of them are hydrophobic and poorly soluble components which include, terpenoids, alkaloids and phenolic compounds. The bioactive metabolites have been subjected to pre-mature degradation during the process of formulation, storage and other viable reactions in the host which largely influences their biological and functional properties (Patra *et al*., 2018).

However, concerns were raised with the biocompatibility, degradation before action, drug self-tapering, controlled release at the target site to exert their complete mechanism and the formidable challenge of utilizing natural compounds as such in the drug development process. In addition, many natural compounds have not cleared the clinical trial phases due to the above-mentioned issues. Therefore, there is a need for a new trend of utilizing a specific drug delivery system and the choice of drug delivery material is also crucial. With the help of a drug delivery system, the bioactive components and their efficacy can be protected and the system may direct the natural compounds to exert a targeted activity on the specific site of body parts and would be an alternative option to solve the critical issues (Ramesh *et al*., 2024; Khezerlou and Jafari, 2020).

These critical shortcomings in pharmacotherapy can be avoided by utilizing a framework of supramolecular chemistry. The division of supramolecular chemistry is a versatile area to develop dynamic, multifunctional and stimuli-responsive structures. There is a lot of paid attention to the supramolecular host-guest complexes for targeted drug delivery. It is considered as one of the intensively growing fundamental directions that is completely devoted to the encapsulation of different bioactive compounds of different natures in supramolecular self-assembling systems. It utilizes multiple non-covalent interactions that contribute to their dynamic properties including reversibility, predictability, tunability and specificity which make them ideal candidates for drug delivery. Various non-covalent interactions such as metal chelation, π–π stacking, hydrogen bonding, *van der Waals* interaction and hydrophobic interactions are involved in specific recognition on the molecular level. Significantly, the molecules are arranged and self-assembled through non-covalent interactions in the specific solvent system that give rise to the formation of supramolecular materials for targeted drug delivery (Li *et al*., 2024; Zakharova *et al*., 2023; Hao *et al*., 2021).

Crown ethers, cyclodextrins, cucurbit[n]urils and calix[n]arenes are commonly used host molecules for the formation of host-guest complexes. The macrocyclic compounds are involved in the process of capturing the guest molecules in their cavities by exploiting various physical interactions. In general, the host-guest complexation event occurs in the solution environment where both the molecules (host and guest) are completely dispersed in solution and the complexation between the host and guest occurs when they meet each other. Withholding all these characteristics, pillar[n]arenes are a new type of host molecules in the stream of host-guest chemistry. The pillar[n]arenes are macrocyclic host molecules with the characteristic features of cylindrical and rigid pillar-like structure and hold promising avenues in host-guest chemistry (Zuilhof *et al*., 2021; Ogoshi *et al*., 2019).

Pillar[n]arenes generally consist of n hydroquinone monomers that have been covalently linked with the methylene bridges at their para positions. Among pillar[n]arenes, pillar[5]arenes and pillar[6]arenes have attained a greater interest as the process of formation is relatively easy compared to others. The X-ray

crystallographic analysis exhibited that the pillar[5]arene mainly comprises five repeating units of 1,4-dialkoxybenzene linked with the methylene bridges at the 2$^{nd}$ and 5$^{th}$ positions (para-positions). Pillar[5]arene is found to be in highly symmetric pillar-shaped architecture owing to the presence of para-bridge linkages. It displayed a 5-fold symmetry with a cavity diameter of about 4.7 Å. In this context, pillar[5]arenes have been frequently exploited in host-guest chemistry to capture various organic drug molecules as guests through various effective and multiple non-covalent C–H···π interactions (Zhu *et al*., 2023; Shi *et al*., 2023; Liu *et al*., 2021).

In order to form host-guest complexation, an interaction between the molecules is crucial and essential. Almost all organic molecules (guest) probably contain C-H groups which may interact with the pillar[5]arene molecule via π-π interactions to form a complexation of host-guest and this kind of interaction is generally exploited by pillar[n]arenes due to the formation of multiple C–H···π interactions in their systems. Pillar[n]arenes bear 10 alkoxy groups in their structure, which were identified through electron density mapping analysis. In general, the alkoxy groups are electron-donators that contribute to the electron-rich benzene rings in the units; thus ultimately results in the formation of a cavity to form multiple C–H···π interactions with the hydrophobic small molecules (Ohtani *et al*., 2022).

The size of the guest molecule and the host cavity also plays a major role in the formation of host-guest complexation. Due to the symmetrical and modifiable rims in pillar[n]arene structures, the cavities of pillar[n]arenes can be easily modified and controlled to provide unique structures with enhanced properties. A difunctionalized pillar[4]arene[1]quinone derivative with ethanolamine unit can be modified at the 5$^{th}$ position of pillar[5]arene and it is referred to as BEA. The resultant derivative may exhibit a superior binding affinity towards guest molecules due to the occurrence of ethanolamine units in the macrocyclic structures during the modification process. The developed supramolecular host molecule is a strong hydrogen-bond donor owing to the presence of ethanolamine units in their cavities (Yang *et al*., 2024a).

A variety of small molecules and other therapeutic compounds of a hydrophobic nature can be a suitable guest molecule to complex with host molecules. The strong hydrophobic drug molecules have acted as proton-donating agents to fit in the electron-dominating cavities of pillar[5]arenes. The resulting host-guest complexation superiorly achieved molecular-level protection of the drug from premature degradation and deactivation. Once the guest molecules formed an inclusion complex with host molecules, the hydrophobic drug molecules achieved greater solubility and stability inside the host to exert their targeted activities. The host motif has also played a major role in penetrating into the biological membranes and it might become an alternative choice to deliver a drug in the form of oral administration, topical creams, topical ointments, nasal sprays and eye drops (Jin *et al*., 2019; Jiang *et al*., 2018).

Overall, the dynamic and reversible phenomena of the host-guest complexation display stimuli-responsive characteristics that allow the controlled release of guest (drug) molecules in response to environmental stressors including temperature, pH and enzymatic activity. The adaptive and tunable nature of the host molecules ensures the controlled therapeutic potential that prominently mitigates the dosage forms and frequency to treat various ailments. Considering all these phenomenal features of supramolecular host-guest chemistry, the hydrophobic drug (isatin) could be complexed with the superior host molecules (pillar[n]arenes). This will provide new insight into the proposed host-guest complexes for the enhanced therapeutic applications of isatin.

With this background of known facts, the objectives of the current study were framed as follows:

- ✓ To synthesize and characterize the pillar[n]arene-isatin inclusion complexes
- ✓ To assess the antimicrobial action of pillar[n]arene-isatin inclusion complexes against clinical pathogens
- ✓ To determine the antibiofilm potential of pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*
- ✓ To investigate the drug release kinetics and *in vitro* wound healing potential of pillar[5]arene-isatin inclusion complexes based ointment formulations.