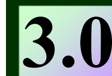


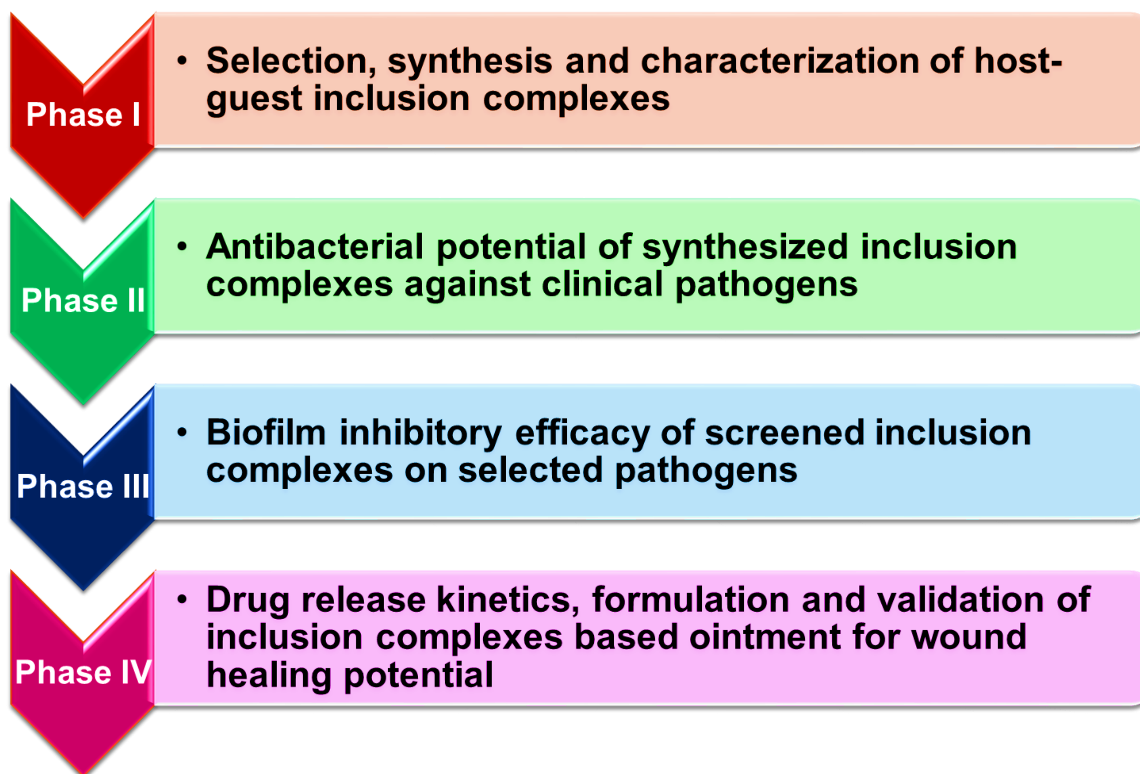
METHODOLOGY

Wound infections are the most common and devastating conditions leading to global health and economic burden. If it is inadequately diagnosed or managed, this would result in secondary complications and incur a loss of life. Wound infections play a crucial role in the progression of chronification of wounds thus delaying the wound healing processes. In definition, a wound is a break in epithelial integrity with a plausible accumulation of microbial load to worsen the wound healing stages. Bacterial pathogens potentially worsen the wound conditions by forming biofilms on the surfaces to promote microbial colonization. There are various strategies available to manage wound infections to prevent the devastating conditioning of wounds. Plenty of antibiotics are on the frontline to treat wound infections to prevent the delay of wound healing. Due to the development of resistant microbial strains at the wound site, the antibiotics could be difficult to play their action effectively (Masson-Meyers *et al*., 2020). Therefore, an effective strategy is strongly required to combat wound infections by promoting the wound-healing processes.

With this background, the present study has been focused on the **'Biological Evaluation of Pillar[5]arene-Isatin Inclusion Complexes to Combat Wound Infections'**. The research study was carried out in four phases. Phase I was involved with the selection of drugs (Isatin) and drug carriers (Pillar[n]arenes) and validation of their pharmacokinetic properties to determine their drug-likeliness. Followed by, the synthesis of pilla[n]arenes-isatin inclusion complexes and their characterization was performed to identify the binding stoichiometry of the inclusion complexes. Phase II was dealt with the assessment of the antibacterial efficacy of pillar[n]arenes-isatin inclusion complexes and their mechanism of action against the prominent bacterial pathogens responsible for wound infections. Phase III of the work was focused on determining the antibiofilm

potential of selected pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In Phase IV, the drug release kinetics and *in vitro* wound healing potential of formulated ointment loaded with inclusion complexes was evaluated using L929 human fibroblast cell lines.

The methodology adopted in each phase of the study is discussed in detail in this chapter. The overall experimental design of the study is depicted below.



PHASE I

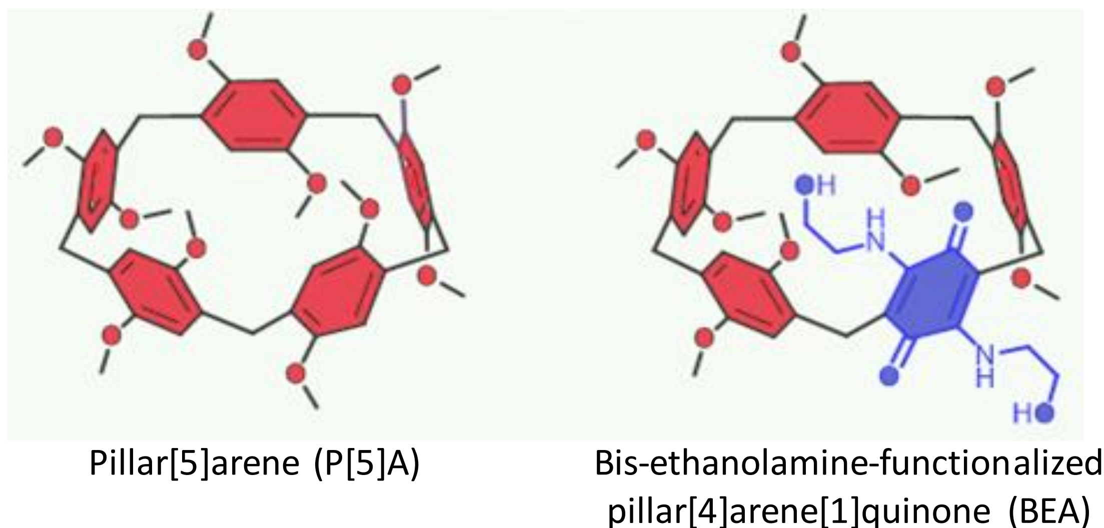
3.1. Selection, Synthesis and Characterization of Host-Guest Complexes

Phase I encompassed the selection of drug (Guest) and drug carriers (Host) for the synthesis of host-guest complexes and their pharmacokinetic profile was initially analyzed using pkCSM software. The formation of host-guest inclusion complexes was confirmed with various spectrophotometric methods including ^{1}H-NMR and UV-visible Spectroscopy.

3.1.1. Selection of drug carriers (hosts)

In recent decades, a supramolecular host-guest based drug-delivery system has held a specific place in biomedical applications due to its stimuli-responsive release of encapsulated drug molecules. Over the past decades, the pillar[n]arenes have been attracted by many fields by exhibiting host-guest interactions, controlled drug release, cancer therapy and drug delivery systems. In this era of antibiotic resistance endurance, pillar[n]arenes are used as a transport system to deliver the antibiotics to the target sites. The pillar[n]arene based drug delivery system and targeted drug delivery has been following two main strategies; one, host-guest complex will be formed by direct formation with small drug or pro-drug and next the formation of micelles or vesicles after forming amphiphilic pillar[n]arene based drug delivery systems (Zyryanov *et al*., 2023).

Controlled drug targeted release and efficient therapy to treat infections lies in the utilization of the pillar[n]arene based self-assembly as drug carriers or hosts (Liu *et al*., 2021). Among the various macrocyclic supramolecular hosts, pillar[5]arene is a cyclic oligomer composed of five repeating units with unique host-guest chemistry. It has a rigid, tubular and symmetric architecture with electron-rich internal cavities which enables them for the formation of inclusion complexes with various guest molecules (Kiruthika and Arunachalam, 2022). A difunctionalized pillar[4]arene[1]quinone derivative named as BEA is also considered as the potential supramolecular host. It is anticipated that BEA could exhibit superior binding affinity towards guest molecules due to the presence of ethanolamine units in the macrocycle which is a strong hydrogen bonding donor. Therefore, two supramolecular hosts or drug carriers were selected for the present study namely pillar[5]arene and pillar[4]arene[1]quinone derivative (BEA). Plate 1 depicts the structure of selected drug carriers (hosts).



Pillar[5]arene (P[5]A)

Bis-ethanolamine-functionalized pillar[4]arene[1]quinone (BEA)

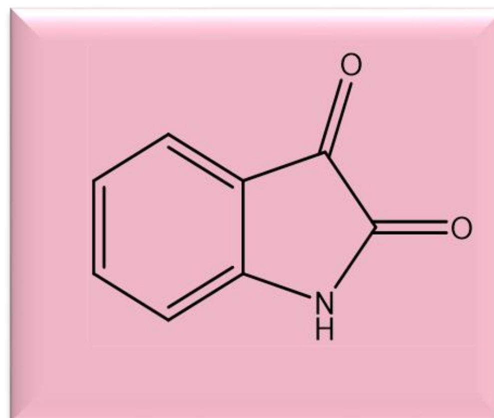
Plate 1: Structure of pillar[n]arenes (hosts)

3.1.2. Selection of drug (guest)

Medicinal plants have been used traditionally for various purposes from the beginning of human civilization. Herbal plants play a prominent role in several medicinal systems including Unani, Siddha, and Ayurvedha. Nearly, more than 50,000 plants have been recorded for their therapeutic potential across the globe. For the past few decades, medicinal plants have been well known to produce compounds from their various parts like root, stem, leaf, flower, seed, and fruits and it plays a crucial role in pharmacology and drug discovery. Most of the developing countries still depend on traditional medicinal plants which contain therapeutic compounds as an active principle to treat various diseases and disorders (Chaudhary *et al*., 2023; Anusha *et al*., 2022; Bhardwaj and Thakur, 2022). *Couroupita guianensis* is a popular tree with potential medicinal value among tribal communities. Earlier studies have reported that the phytoconstituents present in the leaves and flowers of *Couroupita guainensis* are alkaloids, sterols, flavonoids, glycosides, triterpenes, tannins, indirubin, isatin, α-amirin, couroupitine, and β-amirin. Among them, isatin is one of the major alkaloids that possess several medicinal properties including, antioxidant, antimicrobial, antitumor, and anti-inflammatory. These components in the plant system may act as a driving force for the pharmacological and therapeutic properties of the plant,

Couroupita guainensis (Augusco *et al*., 2023; Muthulakshmi *et al*., 2022; Shwetha *et al*., 2020; Sheba and Anuradha, 2019). Our laboratory has extensively studied the biotherapeutic potential of isatin against various microbial pathogens. However, there is a gap in the commercialization of isatin due to premature degradation of drugs and uncontrolled drug release. Hence, isatin could be selected as a drug for the synthesis of inclusion complexes with the host molecules to enhance its bioavailability. The structure of isatin is provided in Plate 2. Isatin was commercially purchased (Sigma-Aldrich).

Couroupita guianensis Aubl.



Isatin

Plate 2: *Couroupita guianensis*. Aubl and structure of isatin (guest)

3.1.3. Pharmacokinetic profiling of selected drug (isatin) and drug carriers (pillar[n]arenes)

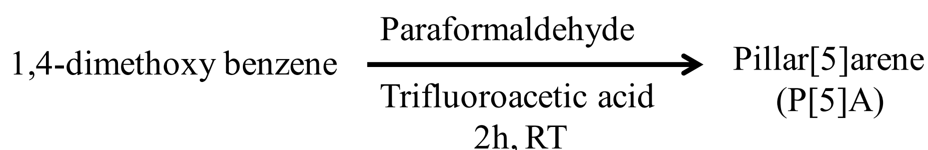
The development of new drugs are facing a lot of challenges including risky endeavors, costly, and low success rates. Identifying the interaction between the pharmacokinetics and toxicity profile is crucial for developing novel/new drugs. Evaluating the ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicological properties) profile can minimize the later-stage failure of lead compounds during clinical trials. Experimental determination of the pharmacokinetic profiles of the lead compounds is laborious, expensive and time-consuming. Exploiting the computational approaches effectively optimizes the pharmacokinetic and toxic properties and may enhance the quality and

success rate (Flores-Holguín *et al*., 2021; Pires *et al*., 2015). pkCSM is an online web tool that can characterize the pharmacokinetic profiles of prospective therapeutic compounds. Here, the ADMET profile of the two selected hosts (drug carriers) and guest (drug) namely, pillar[4]arene[1]quinone derivative (BEA), pillar[5]arene, isatin and chloramphenicol were studied by pkCSM (http://biosig.unimelb.edu.au/pkcsm/prediction#) online server. Chloramphenicol was the standard antimicrobial drug to compare the efficiency of selected drugs and drug carriers. Initially, all the molecular structures of the selected hosts and guest were downloaded from PubChem databases. The SMILES (Simplified Molecular Input Line Entry Specification) was obtained from CHEMDRAW and it was imported to the web page of pkCSM. After running the programme, the data were downloaded and interpreted with the manual provided by pkCSM. Appendix 1 explains the data interpretation manual of the pkCSM web tool.

3.1.4. Synthesis of supramolecular hosts (drug carriers)

Major advantages in synthesizing the pillar[n]arenes include the process and the raw/starting material for the synthesis are very straightforward, electron-rich cavity to hold small molecules and their versatile functionalization. In this, the two selected supramolecular hosts such as pillar[5]arene and bis-ethanolamine functionalized pillar[4]arene[1]quinone (BEA) were synthesized by two major steps such as condensation and oxidation. The detailed procedure for the synthesis of selected host molecules are discussed below.

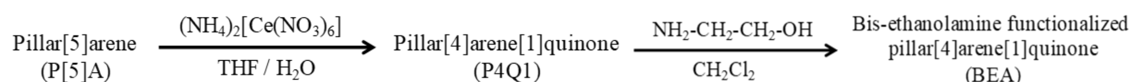
3.1.4.1. Synthesis of decamethoxy pillar[5]arene (P[5]A)



The selected supramolecular host, pillar[5]arene possesses a rigid pillar-like columnar structure with an electron-rich interior cavity owing to the presence of π-electrons rich aryl building blocks. Pillar[5]arenes can encapsulate electron-deficient guests within its cavity primarily through non-covalent

interactions. P[5]A was synthesized by the modified protocol of Boinski and Szumna, (2012). A reaction mixture was prepared by combining 1,4-dimethoxybenzene (1.4 g, 10 mmol) and paraformaldehyde (0.3 g, 10 mmol) in 1,2-dichloroethane (95 ml). Trifluoroacetic acid (5 ml) was then introduced to the solution. The mixture was refluxed for 2 hours, after which it was cooled to room temperature and poured into methanol, leading to the precipitation of the target compound. The precipitate was collected, and an additional fraction was obtained by filtering the reaction mixture, evaporating the filtrate, and re-precipitating with methanol. To purify the crude product without chromatography, it was dissolved in chloroform, and acetone was added in a 1:1 volume ratio. Colorless crystals of decamethoxypillar[5]arene upon crystallization was obtained in good yield.

3.1.4.2. Synthesis of bis-ethanolamine functionalized pillar[4]arene[1]quinone (BEA)



Another supramolecular host selected for the study was Bis-ethanolamine functionalized pillar[4]arene[1]quinone (BEA), which is a fascinating host molecule with an internal cavity to encapsulate small molecules for their enhanced biological activities. In this regard, the BEA was synthesized according to the protocol mentioned by Kiruthika *et al*. (2021) and Han *et al*. (2012). Initially, the pillar[4]arene[1]quinone (P4Q1) was synthesized by partial oxidation of P[5]A using ceric ammonium nitrate in a solvent mixture of tetrahydrofuran and water. P[5]A (1.5 g, 2.00 mmol) was dissolved in tetrahydrofuran (THF, 50 ml) and placed in a 100 ml round-bottom flask equipped with a stirrer. An aqueous solution of ceric ammonium nitrate, $(NH_4)_2[Ce(NO_3)_6]$ (1.10 g, 2.00 mmol), was added dropwise to the reaction mixture under constant stirring. The mixture was stirred at room temperature for 24 hours to ensure the reaction proceeded to completion. THF was removed under reduced pressure and the aqueous layer was extracted with dichloromethane (25 ml × 3) to recover the organic components. The combined dichloromethane extracts were washed sequentially with water

(50 ml) and a saturated sodium chloride (NaCl) solution (50 ml) to remove any water soluble impurities. The organic phase was then dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and concentrated under reduced pressure to yield a crude residue. The crude product was purified using silica gel column chromatography with a gradient solvent system of petroleum ether and dichloromethane (v/v, 4:1 to 2:1). This purification process yielded a red solid powder which was identified as P4Q1.

A solution of 0.30 g (0.42 mmol) of synthesized pillar[4]arene[1]quinone (P4Q1) in 3 ml of DCM was prepared, and 150 µl (2.4 mmol) of ethanolamine was added. The contents in the RB flask was continuously stirred for 48 hours, with the progress monitored by thin-layer chromatography (TLC). After the reaction, the mixture was poured into 3 ml of cold water, and the organic layer was separated and washed three times with water. The organic layer was then evaporated, resulting in a dark brown precipitate. The crude product was purified by column chromatography, using a 9:1 ethyl acetate: acetone (v/v) mixture as the mobile phase.

3.1.5. Synthesis of pillar[5]arene-isatin inclusion complexes (host-guest complexes)

Supramolecular chemistry has burgeoned in the modern research area due to its potential contributions to the spectrum of key applications including drug delivery. In general, the interactions between the host molecules and the low-molecular-weight guest molecules can be made via non-covalent interactions (Burkhanbayeva *et al*., 2024; Butkiewicz *et al*., 2024). In this context, pillar[n]arenes possess electron-rich cavities to encapsulate cationic drug molecules to form host-guest complexes with enhanced biological activities. A 1:1 inclusion complex of decamethoxypillar[5]arene (P[5]A) and isatin was synthesized by dissolving 1 mmol (750.34 mg) of P[5]A in 5 ml chloroform in a clean round bottom flask. The solution was stirred at room temperature until the compound was completely dissolved. In a separate beaker, isatin (1 mmol, 147.13 mg) was dissolved in 5 ml of acetone. The resulting isatin solution was then

added dropwise to the P[5]A solution under constant stirring. The reaction was allowed to proceed at room temperature for 12 hours to facilitate the formation of the host-guest complex. To obtain the orange solid complex, the reaction mixture was concentrated by removing the solvent under reduced pressure. Dried the final product under vacuum at room temperature for 2 hours to ensure complete removal of residual solvent.

3.1.6. Synthesis of bis-(ethanolamine) functionalized pillar[4]arene [1]quinone-isatin inclusion complexes

A 1:1 inclusion complex of BEA and isatin was prepared by dissolving 1 mmol (839.38 mg) of BEA in 5 ml of acetone in a clean round-bottom flask. The solution was stirred at room temperature until the BEA was completely dissolved. In a separate container, 1 mmol (147.13 mg) of isatin was dissolved in 5 ml of acetone. The prepared isatin solution was slowly introduced dropwise into the BEA solution with constant stirring. The mixture was allowed to stir at room temperature for 12 hours to ensure thorough host-guest complexation. Subsequently, the solvent was removed under reduced pressure to concentrate the reaction mixture, yielding the complex as a purple solid. The product was further dried under vacuum at room temperature for 2 hours to eliminate any residual solvent completely.

3.1.7. Characterization of synthesized pillar[n]arene-isatin inclusion complexes

Isatin inclusion complexes of P[5]A and BEA were thoroughly characterized. The structural properties of the synthesized isatin inclusion complexes have been elucidated in detail by using a combination of analytical techniques, including nuclear magnetic resonance (NMR) spectroscopy and UV-visible spectroscopy.

3.1.7.1. Determination of binding constant for the formation of inclusion complexes by Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is recognized as a powerful and unique methodology to analyze the supramolecular host-guest systems in terms of the structure and behavior of inclusion complexes. Here, ^{1}H NMR spectroscopy was performed to determine the interactions between the hosts and the guest for a better understanding of the behavior of supramolecular systems in solution.

Avance III HD Nanobay 400 MHz NMR spectrometer was used for ^{1}H NMR spectroscopic analyses. The instrument was equipped with advanced capabilities for high-resolution NMR experiments. To calibrate chemical shifts accurately, tetramethylsilane (TMS) was used as the internal standard. TMS was commonly used as a reference due to its distinctive peak in the ^{1}H NMR spectrum at 0 ppm. The pillar[5]arene-isatin inclusion complexes were dissolved in 1:1 v/v mixture of $CDCl_3$: acetone-d_6 to obtain a concentration suitable for NMR analysis. The choice of solvent was based on its compatibility with the inclusion complex and its ability to dissolve the sample effectively. The raw NMR data obtained from the spectrometer were processed using MestReNova NMR software. The software was employed for baseline correction, phasing, and Fourier transformation to convert the time-domain NMR signals into frequency-domain spectra. The chemical shifts of the peaks in the ^{1}H NMR spectrum were determined in parts per million (ppm) relative to the TMS serving as the reference. The NMR titration experiments were performed between the synthesized host-guest complexation and the detailed preparation of the stock solutions of both isatin and hosts (P[5]A and BEA) are given in Appendix 2.

^{1}H NMR titration experiment was conducted to determine the complexation efficiency of P[5]A and BEA with isatin. The titration experiments were performed by titrating host solution with varying concentrations of isatin. The NMR titration data were analyzed using the WINEQNMR2 program, a specialized software tool for fitting binding isotherms and calculating binding constants. The program

employs nonlinear regression analysis to derive the equilibrium binding constants and provide insights into the host-guest interactions. The detailed methodology of the determination of the binding constant and stoichiometry for the host-guest complexation is provided in Appendix 3.

3.1.7.2. Evaluation of host-guest complexation by UV-visible spectroscopy

UV-visible spectroscopy is a valuable technique for probing host-guest complexation in solution. UV-visible spectroscopy can detect absorbance changes resulting from electronic transitions in the molecules involved in the host-guest complexation. When a host molecule forms a complex with a guest molecule, the electronic environment of the guest may change, leading to shifts in absorbance peaks or the appearance of new peaks in the UV-visible spectrum. The formation of a host-guest complex may induce shifts in the absorption peaks of the molecules involved. These shifts can be attributed to changes in the electronic structure of the guest molecule upon complexation. By comparing the UV-visible spectra of the host, guest, and host-guest complex, one can identify shifts in absorbance peaks, providing evidence for complex formation. Changes in the intensity of absorption peaks can also indicate host-guest complexation. The intensity of absorption bands may increase or decrease upon complex formation due to changes in the molar absorptivity of the molecules involved. Monitoring these intensity changes can provide insights into the binding affinity and stoichiometry of the host-guest interaction. UV-visible spectroscopy can also be used to construct Job plots, which involve plotting the absorbance at a specific wavelength as a function of the mole fraction of one component in a series of mixtures. Job plots can help determine the stoichiometry of the host-guest complex and the binding constants associated with the interaction. The UV-visible titration experiments were performed between the synthesized host-guest complexation and the detailed preparation of the stock solutions of both drug and drug carriers are given in Appendix 4.

PHASE II

3.2. Mechanism of antibacterial action of synthesized pillar[n]arenes-isatin inclusion complexes on clinical pathogens

Phase II of the research study consisted of the determination of the antibacterial efficacy of isatin, pillar[n]arenes, and synthesized inclusion complexes against the prominent clinical pathogens. Chloramphenicol was used as the standard antibiotic control throughout the study. The antibacterial mechanism of the synthesized pillar[n]arenes-isatin inclusion complexes was performed using various techniques namely bacterial time kill kinetics, membrane disintegration assays and confirmation of membrane damage by scanning electron microscopy.

3.2.1. Procurement of bacterial strains

The antibacterial potential of the synthesized supramolecular systems has to be determined against a spectrum of bacterial strains to become potential therapeutic applications. Hence, the major bacterial pathogens responsible for wound infections namely, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella paratyphi* A were procured from PSG Institute of Medical Sciences and Research, Coimbatore, Tamil Nadu, India. All the bacterial strains were subcultured and subsequently, the glycerol stock was prepared and stored at -20ºC. The culture was thawed and used for the subsequent assays. The consent to carry out the research work on clinical pathogens to determine the therapeutic potential of pillar[n]arenes-isatin inclusion complexes was approved by the Review Committee on Genetic Manipulation (RCGM) and the IBKP UAC Number is AVIRDIP50236 (Annexure I).

3.2.2. Determination of antibacterial efficacy of synthesized pillar[n]arenes-isatin inclusion complexes

The identification of the susceptible pattern of the selected bacterial pathogens towards the synthesized pillar[n]arenes-isatin inclusion complexes are

crucial parameters to validate their antibacterial properties. Hence, the antibacterial efficacy of the synthesized pillar[n]arenes-isatin inclusion complexes was determined by agar well diffusion method, minimum inhibitory & minimum bactericidal concentration (MIC & MBC) and the synergistic potential.

3.2.2.1. Assessment of bacterial susceptibility profile of the selected pathogens treated with pillar[n]arenes-isatin inclusion complexes

The susceptible or resistant profile of bacterial pathogens towards the tested compounds can be analyzed by the agar well diffusion method. The diffusion method relies on overlaying the pathogenic culture on the media and the growth of the pathogens has been inhibited by the diffused compounds in the agar wells, which can be visually observed by measuring the zone of inhibition (ZoI) (Puca *et al*., 2021). Hence, the agar well diffusion method was carried out to identify the antibacterial potential of the selected hosts, guest and their inclusion complexes against selected pathogenic Gram-positive and Gram-negative bacteria including, *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella paratyphi* A (CLSI, 2012).

Inoculums of the test pathogens were prepared from overnight cultures grown in nutrient broth. 0.5% McFarland turbidity was prepared to standardize the bacterial inoculum suspensions to determine the bacterial susceptibility. Bacterial susceptibility testing was conducted using the swab method to evenly spread the adjusted bacterial inoculums across the entire agar plate surface. A sterile pipette tip was then used to aseptically create wells with a diameter of 6–8 mm on the agar surface. Stock solutions of P[5]A (15 mg/ml), BEA (17 mg/ml), isatin (3 mg/ml), P[5]A-isatin inclusion complexes (18 mg/ml), and BEA-isatin inclusion complexes (20 mg/ml) were prepared in DMSO. Subsequently, 20 µl of each antimicrobial agent was introduced into the wells. The plates were covered and incubated at 37°C for 24 hours. After incubation, the diameters of the zones of inhibition were measured and recorded in millimeters for each sample. The detailed procedure for determining the antibacterial susceptibility are provided in Appendix 5.

3.2.2.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of pillar[5]arene-isatin inclusion complexes

MIC is defined as the lowest concentration of test compounds that inhibit the growth of microbes. In this concern, the MIC and MBC of the synthesized pillar[5]arene-isatin inclusion complexes were performed using the broth dilution method according to the protocol of the Clinical and Laboratory Standards Institute – NCCLS (CLSI, 2012). The bacterial inoculum was prepared and standardized using 0.5% McFarland standard. Stock solutions of each sample at a concentration of 20 mM were combined with 100 μl of bacterial suspension in a 96-well plate to achieve final concentrations ranging from 20 mM to 0.039 mM. The plates were then incubated at 37 °C for 24 hours. Following incubation, 20 μl of resazurin sodium solution (1 mg/ml), a blue-colored oxidation-reduction indicator, was added to each well. The plates were further incubated at 37 °C for 3 hours in the dark. Dimethyl sulfoxide (DMSO) served as the solvent control, while chloramphenicol was used as the antibiotic control. The reaction was indicated by the color change of resazurin from blue to pink because the metabolically active cells of microbes reduce blue resazurin to the pink product resorufin. The color change confirms the growth of bacteria in the well. The inhibitory profile of the compounds was recorded by observing the color change of the medium. (Haji *et al*., 2022; Urnukhsaikhan *et al*., 2021; Kowalska-Krochmal and Dudek-Wicher, 2021)

The Minimum Bactericidal Concentration (MBC) is defined as the lowest concentration of a drug or compound that completely inhibits bacterial growth, resulting in no visible colonies on the agar plate after 24 hours of incubation. The MBC of pillar[5]arene-isatin inclusion complexes against the tested bacterial pathogens was determined by inoculating the bacterial suspension treated with the supramolecular host, guest and inclusion complexes (where there is no bacterial growth in the wells of the MIC experiment) onto LB (Luria-Bertani medium) plates. The plates were incubated at 37°C for 24 hours to evaluate the bactericidal activity of isatin, P[5]A and their inclusion complexes.

3.2.2.3. Assessment of the synergistic antibacterial potential of isatin and pillar[5]arene

Recently, combinatorial drug treatments have gained much attention owing to their synergistic activity to reduce the poor efficacy of single-drug administration for targeted drug therapy. The checkerboard method has emerged to evaluate the synergistic or additive or antagonistic effect of two or more drugs. The method works on the principle of microdilution method by comparing the cumulative efficacy of tested combinations in order to determine the lower concentration of tested drug combination i.e., the resulting activity occurs at a lower concentration compared with the sum of MICs of two drugs. The determination of the synergistic activity may have resulted in reduced toxicity and adverse effects with enhanced efficacy along with the lowest therapeutic dosage forms.

Here, the fractional inhibitory concentration index (FICI) can be used to determine the efficacy of tested drug combinations in terms of synergism, partial synergism and antagonism against specific pathogens. FICIs can be calculated by comparing the MICs of individual drugs with them in combinations (Zhou *et al*., 2020; Costa *et al*., 2019). Hence, the checkerboard method was employed to identify the synergistic potential of isatin and pillar[5]arene for their biological activities according to the protocol followed by Bellio *et al*., 2021. The concentrations of P[5]A were varied within a range of 7.5 mg/ml to 7 µg/ml and isatin was varied from 3 mg/ml to 50 µg/ml, all prepared in 96-well plates. Isatin and P[5]A individually served as controls. The fractional inhibitory concentration index (FICI) was determined by comparing the minimum inhibitory concentration (MIC) values of isatin and P[5]A individually with the MIC values of their combination, calculated using the formula:

$$\text{FIC index} = \frac{\text{MIC}_a \text{ in combination}}{\text{MIC}_a \text{ alone}} + \frac{\text{MIC}_b \text{ in combination}}{\text{MIC}_b \text{ alone}}$$

$$\text{FIC index} = \text{FIC}_a + \text{FIC}_b$$

where,

FIC_a = (MIC of isatin in combination)/(MIC of isatin alone)

FIC_b = (MIC of P[5]A in combination)/(MIC of P[5]A alone).

Interpretation of combination therapy based on FICIs is as follows:

FICI ≤ 1.0 is considered as synergy,

FICI=1 as an additive or partial synergism,

1 < FICI ≤ 4 is indifferent

FICI>4 is antagonistic.

3.2.3. Mechanistic action of pillar[5]arene-isatin inclusion complexes against selected bacterial pathogens

The antimicrobial compounds should target a variety of bacterial targets or processes as their unique mechanism of action. They are as follows: (i) interference with the cell wall and cell membrane synthesis, (ii) inhibition of protein synthesis, (iii) inhibition nucleic acid (DNA & RNA) synthesis and (iv) interference of metabolic pathways (Álvarez-Martínez *et al*., 2021; Eshboev *et al*., 2024). Considering the facts, the mechanism of action of the synthesized pillar[5]arene-isatin inclusion complexes were evaluated through various techniques such as bacterial time-kill kinetics, membrane disintegration assays and confirmation of membrane damage by scanning electron microscopy.

3.2.3.1. Bacterial time kill kinetics of pillar[5]arene-isatin inclusion complexes treated *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Time kill curves have been used to determine the kinetics of bacterial killing *in vitro* to ensure whether an antibacterial agent is bacteriostatic or bactericidal. The assay can be useful in distinguishing whether bacterial killing is concentration-dependent and/or time-dependent. The lethal activity can be expressed as the rate of killing at a given concentration of antimicrobials under controlled conditions. The bacterial time-kill kinetics of P[5]A, isatin and

P[5]A⊃isatin were compared with the standard drug, chloramphenicol. The time-kill kinetics was determined by estimating the inactivation time required to inhibit the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in growth medium supplemented with or without supramolecular host, guest and their inclusion complexes. The treated and untreated bacterial cultures were withdrawn from the medium with constant time intervals (0 minutes to 180 minutes) and then it was plated on the nutrient agar to determine the viable colonies. The detailed methodology of bacterial time-kill kinetics is provided in Appendix 6.

3.2.3.2. Action of pillar[5]arene-isatin inclusion complexes on cell membrane integrity of selected bacterial pathogens

The cell wall of the bacteria is a prominent structure that is required to maintain the integrity for their survival. The cell wall of the bacteria is the outermost layer that is made up of two major constituents including sugar and protein molecules. The integrity change was monitored by estimating the glucose and protein content in treated and untreated bacterial pathogens. Another important target of the antibacterial compounds is the bacterial cell membrane since it can be easily differentiated from the mammalian cell membrane. Hence, most of the antimicrobials target bacterial cell membranes for their killing efficacy which increases the permeability of the cell membrane and leads to lysis of the outer membrane. The disintegration of bacterial cell membrane integrity was measured by evaluating the leakage of cellular contents such as DNA and RNA (Li *et al*., 2022; Talapko *et al*., 2022). The synthesized pillar[5]arene-isatin inclusion complexes were tested to determine the antibacterial mechanisms by analyzing their interference in the cell wall and cell membrane integrity of the prominent bacterial pathogens responsible for wound infections.

3.2.3.2.1. Determination of cellular leakage of bacterial cell wall components of *Staphylococcus aureus* and *Pseudomonas aeruginosa* treated with pillar[5]arene-isatin inclusion complexes

The integrity of the cell membrane of a pathogenic strain could be evaluated by the release of cell wall constituents (protein and glucose). The protein and glucose concentrations in the supernatant of both treated and untreated bacterial samples were quantified using the Lowry method and the Anthrone method. Logarithmic growth phase was estimated for bacterial pathogens treated with MICs of selected compounds during the incubation period of 18 hours at 37ºC. Then, the culture was centrifuged at 10,000 g for 5 minutes at 4ºC. The cytoplasmic proteins and glucose molecules in treated and untreated supernatants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were colorimetrically quantified. The detailed methodology of evaluating the release of cellular constituents by protein and glucose is provided in Appendix 7 and 8, respectively.

3.2.3.2.2. Morphological characterization of bacterial cell membrane damage using scanning electron microscopy

The scanning electron microscopy is commonly employed to analyze the morphological damage of the bacterial strains by the antimicrobial compounds. The possible mechanism of action of pillar[5]arene-isatin inclusion complexes on the treated and untreated bacterial cell membrane was evaluated using SEM. Initially, *Staphylococcus aureus* and *Pseudomonas aeruginosa* cultures were treated with the MICs of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes for 2 hours. The treated culture was centrifuged at 1000 rpm for 5 min. Finally, the cells were fixed on a glass slide with the help of 2.5% glutaraldehyde in a phosphate buffer solution. The adhered cells were treated with 30–100% ethanol stepwise to dry and the Scanning Electron microscopic images were taken after 2 days at different magnifications. The detailed procedure has been given in Appendix 9.

PHASE III

3.3. Antibiofilm efficacy of Pillar[5]arene-Isatin Inclusion Complexes against Prominent Bacterial Pathogens

In this phase, it was focused on revealing the antibiofilm activity of isatin, pillar[5]arene, and their inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The mechanism underlying the formation and development of biofilms in bacterial pathogens was also targeted. The 1/2 MIC, 1/4 MIC and 1/8 MIC of all the selected compounds were subjected to determine the biofilm inhibition and eradication profiles.

3.3.1. Determination of biofilm inhibitory potential of pillar[5]arene-isatin inclusion complexes against the bacterial pathogens

The antibiofilm potential of the synthesized pillar[5]arene-isatin inclusion complexes were tested against prominent bacterial pathogens responsible for wound infections. Initially, various concentrations such as 1/2 MIC, 1/4 MIC and 1/8 MIC of isatin, P[5]A and their inclusion complexes were tested for their antibiofilm efficacy against the *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms and were assessed by crystal violet method. Crystal violet is a dye that stains the microorganisms responsible for biofilm formation. The changes can be measured by observing the absorbance at 495 nm. A 96-well plate was prepared by adding 100 µl of bacterial suspensions in tryptic soy broth (TSB) supplemented with 1% sucrose to each well. The plate was incubated at 37°C for 24 hours to facilitate biofilm formation. After incubation, the supernatant was carefully removed, and the attached biofilms were washed three times with sterile phosphate-buffered saline (PBS, pH 6.8) to remove non-adherent cells. Subsequently, 100 µl of the test compounds at varying concentrations (1/2 MIC, 1/4 MIC, and 1/8 MIC) were added to the wells, followed by an additional 24-hour incubation at 37°C. The detailed procedure of the crystal violet assay to determine the biofilm inhibition is provided in Appendix 10.

3.3.2. Evaluation of biofilm eradication efficacy of pillar[5]arene-isatin inclusion complexes against the bacterial pathogens

Most of the clinically important pathogenic bacteria exploit biofilm formation as the prominent virulence mechanism to exert their pathogenesis in the host. It is very difficult to remove the pre-formed biofilms by pathogenic bacteria and it requires a strong and efficient strategy to completely eradicate them. Hence, the synthesized pillar[5]arene-isatin inclusion complexes were analyzed for their efficacy in eradicating the pre-formed biofilms by prominent pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa.* To target preformed biofilms at the maturation stage (48-hour biofilms), plates were incubated for 48 hours, with the medium refreshed after 24 hours. Various concentrations of compounds were then introduced and incubated for an additional 24 hours. Untreated biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa* served as controls. For the experiment, plates were initially incubated for 24 hours at 37°C to facilitate biofilm attachment and growth. The following day, non-adherent cells were removed, and the attached biofilms were rinsed twice with PBS to remove residual planktonic cells. The eradication of biofilms was assessed by crystal violet assay and the detailed methodology is provided in Appendix 10.

3.3.3. Investigation of sub-inhibitory concentrations of pillar[5]arene-isatin inclusion complexes on the motility behaviour of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

The motility behaviour of bacteria is an important parameter to rapidly enter and colonize the niches to propagate their survival and pathogenesis. The major characteristics for adapting and surviving the nature of the bacteria in the external environment largely depend on how they are moving and growing in the solid, semi-solid or liquid environment. The motility behaviour of the bacteria is strongly required for the formation and development of biofilms from persister cells. The motility nature can be closely associated with the functions of host-microbe interactions. For instance, the planktonic bacteria can swim close to the biofilm-attached surface by rotating their flagella during the early stage of biofilm

formation. The motility behaviour acts as a key factor in exacerbating skin inflammatory response and endurance of bacterial resistance (Zegadło *et al*., 2023; Kim *et al*., 2022). Hence, the 1/2 MICs of the pillar[5]arene, isatin and their inclusion complexes were analyzed for their efficacy in reducing the motility behavior of the tested bacterial pathogens.

3.3.3.1. Swimming motility behaviour of *Pseudomonas aeruginosa* treated with pillar[5]arene-isatin inclusion complexes

The swimming motility of bacterial pathogens is one of the major characteristics of survival adaptation under unfavorable conditions to exert their pathogenesis. Swimming motility is described as individual cell movement assisted by flagella rotations in an aqueous environment. Only a fraction of bacteria associated with the host are known to have a motile nature, but they are responsible for easy colonization and virulence characteristics of such bacteria. The swimming motility behavior of the isatin inclusion complex of pillar[5]arene treated and untreated *Pseudomonas aeruginosa* was observed following the protocol described by Packiavathy *et al*., 2014. Swimming agar medium (tryptone (1.0%), NaCl (0.5%), and agar (0.3%)) was point inoculated with the overnight grown culture of *Pseudomonas aeruginosa,* which was previously diluted in the ratio of 1:100. The medium was incorporated with the 1/2 MIC of each compound. The inoculated plates were kept for 24 hours at 37°C under static conditions. The swimming zone diameter was measured using a transparent ruler in millimeters (mm).

3.3.3.2. Swarming motility behavior of *Pseudomonas aeruginosa* treated with pillar[5]arene-isatin inclusion complexes

In general, bacteria can swarm in a crowded environment and get jammed frequently, but they are plotting a plan to escape the traps with the help of a head-tail movement. This kind of swarming motility of the bacteria helps to invade the host cell, thereby it forms a sessile form of the biofilm community. Flagellated bacteria under solid surfaces utilize swarming motility by adopting their locomotion machinery to accomplish specialized flagellum-driven motility. In other words,

swarming motility is well characterized by the multicellular movement of pathogenic bacteria and has the ability to migrate under solid surfaces in a group of tightly bound cells. Hence, the swarming motility behavior of the pillar[5]arene-isatin inclusion complexes treated and untreated *Pseudomonas aeruginosa* was assessed according to the protocol of Packiavathy *et al*., 2014. The swarming motility experiment was carried out by inoculating the overnight culture (diluted 1:100) of *Pseudomonas aeruginosa* treated with and without compounds at the center of the swarming medium (peptone (1.0%), NaCl (0.5%), agar (0.5%), and filter sterilized glucose (0.5%)). The inoculated plates were kept for 24 hours at 37°C, and the swarming zone diameter (mm) was measured using a transparent ruler.

3.3.3.3. Swarming and hitchhiking motility behavior of *Staphylococcus aureus* treated with pillar[5]arene-isatin inclusion complexes

Staphylococcus aureus is typically classified as a non-motile organism due to the absence of flagella and pili, it can exhibit movement on soft agar surfaces through spreading (swarming). Hence, the effect of pillar[5]arene-isatin inclusion complexes on the swarming motility behavior was assessed according to the protocol of Packiavathy *et al*., 2014. This kind of swarming movement provides an opportunity for *Staphylococcus aureus* to interact with neighboring microbes, especially *Pseudomonas aeruginosa*, and it is referred to as hitchhiking motility. In this interaction, teichoic acids from *Staphylococcus aureus* interact with lipopolysaccharides of *Pseudomonas aeruginosa* to facilitate hitchhiking motility. Determination of hitchhiking motility of *Staphylococcus aureus*, the protocol described by Liu and Lin, (2023) was followed. Tryptic soy agar (TSA) plates containing 0.4% agarose were prepared for the experiment. A 2 µl sample from a mid-logarithmic growth phase culture of *Staphylococcus aureus*, either individually or mixed with *Pseudomonas aeruginosa*, was placed in the center of a tryptic soy agar (TSA) plate. The plate was then incubated at 37°C for 4 hours to enable *Pseudomonas aeruginosa* swarming activity. Bacteria at the periphery of the swarm were retrieved using a sterile toothpick and transferred onto mannitol salt

agar for further analysis. The viable cell count method was employed to quantify *Staphylococcus aureus* cells present in the bacterial suspension.

3.3.4. Morphological characterization of pillar[5]arene-isatin inclusion complexes treated *Staphylococcus aureus* and *Pseudomonas aeruginosa* by FE-SEM

FE-SEM is the commonly employed conventional method to describe the biofilm morphology using high-resolution and high-magnification images. The architecture and structure of *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilm in the untreated and treated conditions were observed by field emission scanning electron microscopy (FESEM). *Staphylococcus aureus* and *Pseudomonas aeruginosa* were cultured on glass slides immersed in LB broths with and without sub-inhibitory concentrations of the compounds. They were allowed to form biofilms by keeping the slides for 48 hours at 37°C. Thereafter, glass slides with biofilms were initially prefixed by overnight incubation (4°C) with glutaraldehyde (2.5%). After fixation with glutaraldehyde, they were dehydrated using an ethanol series gradient (10%-95%) for 10 minutes at each concentration. The dried slides were then sputter-coated with gold and observed under FESEM (Sigma Carl Zeiss, Jena, Germany).

PHASE IV

Isatin release from the host molecules was calculated using the direct addition method and it was validated with various mathematical models. Following this, the wound healing ointment was prepared with pillar[5]arene-isatin inclusion complexes with an ointment base. Subsequently, the physiochemical and cytotoxicity of the developed pillar[5]arene-isatin inclusion complexes based wound healing ointment was assessed. Further, the antibacterial activities of the developed wound against the topmost pathogens causing wound infections were evaluated. The wound-healing activity of the developed ointment was carried out in human fibroblast L929 cells.

3.4. Drug release kinetics of isatin (guest) from pillar[5]arene (host)

Embedding the active principles in the supramolecular systems has promising applications in the release of drugs in a controlled manner. The rate and the extent of availability of the active metabolites at the site of action with absorption profile determine the safety and efficacy of the drugs.

3.4.1. Determination of isatin release from pillar[5]arene-isatin inclusion complexes by UV and HPLC techniques

The direct addition method has been widely used for analyzing the controlled release of drugs from the delivery systems with simple operational procedures. Hence, the drug release kinetics of isatin from pillar[5]arene-isatin inclusion complexes was determined by the direct addition method followed by Paswan and Saini, (2021). The P[5]A⊃isatin complex was directly added to the dissolution media (phosphate buffer saline with a pH of 7.4). 2 mg of isatin without P[5]A was used as a control. The experimental solution contains 2 mg of isatin and 8.8 mg of P[5]A in 100ml of drug release media and was stirred at 100 rpm on a magnetic stirrer. 5ml aliquot of dissolution fluid was withdrawn at different time intervals (1hour intervals up to 4 hours) and refilled with the new dissolution media. The withdrawn samples were centrifuged for 15 minutes at 4000rpm at 4ºC. The centrifuged supernatant was filtered using a 0.22 micrometer polyvinylidene fluoride membrane and the filtrate was measured at 275nm in a UV spectrophotometer.

The release of isatin from the pillar[5]arene-isatin inclusion complexes by the direct addition method was further confirmed by High Performance Liquid Chromatographic (HPLC) (SHIMADZU Lab Solutions) analysis. The release of isatin from the inclusion complexes to the dissolution media (PBS) was collected at an interval of 0 h, 2 h and 4 h. The 20 µl volume of the samples from each period was injected into the sample injection portal in the HPLC system and allowed to pass through the 0.45 mm filter. The obtained filtrate was subjected to chromatographic conditions to detect the presence of isatin from pillar[5]arene-isatin inclusion complexes. The chromatographic analysis was carried out using c-8 column at ambient temperature with the mobile phase of methanol: water (60:40,

v/v). The detection wavelength was set to be 290 nm with the run time of 10 minutes. Simultaneously, a pure isatin (Standard) was run in the similar manner to compare the release kinetics of isatin from pillar[5]arene-isatin inclusion complexes.

3.4.2. Analysis of dissolution profile of isatin from the drug carriers using mathematical models

The prediction of release kinetics of active small metabolites is an essential phenomenon to become a successful drug. The main goal of controlled drug release is to maintain the concentration of drugs at the target sites as long as possible. Usually, the drug delivery systems may release the drug at a part of the concentration to attain the therapeutic effect. The drug release kinetics would allow us to understand the behavior of the drug release in order to attain the desired concentration of drug molecules. Mathematical models generally define the dissolution profile of drug-loaded delivery systems. Once an appropriate function has been selected, the evaluation of the dissolution profile can be carried out and hence the drug release profile can be correlated with drug release kinetic models.

Various mathematical models are employed to understand drug release kinetics including, the zero-order model, first-order model, Higuchi model and Korsemeyer-Peppas model. The zero-order model has been used to determine the drug dissolution profile for the modified pharmaceutical dosage forms, especially for transdermal systems. In the case of the first-order model, it has been used to describe the absorption or elimination of certain drugs. The Higuchi model has been applied to analyze the release of drugs from the proposed system. Korsemeyer-Peppas model has been derived to determine the mechanism of drug release from the system. The different mathematical models for the data obtained from the direct drug release method for pillar[n]arenes-isatin inclusion complexes were plotted. The detailed methodology of the mathematical models to validate the drug release mechanisms is provided in Appendix 11.

3.4.3. Development of wound healing ointment with pillar[5]arene-isatin inclusion complexes

There is a need to develop a better strategy to enhance the process of wound healing. One such strategy is the development of topical applications including topical ointments or creams to accelerate the wound healing process and mitigate major inflammatory reactions. Hence, the pillar[5]arene-isatin inclusion complexes was incorporated into the ointment base to develop a wound-healing ointment to subside wound progression at the target site. The ointment composition and their formulations containing pillar[5]arene-isatin inclusion complexes are provided in Appendix 12.

3.4.4. Physiochemical characterization of the developed ointment loaded with pillar[5]arene-isatin inclusion complexes

A vast number of dermatological products have been available to treat infections on the skin. The base of the ointment may also determine the performance of the ointment to treat wound infections. Various parameters ought to be analyzed to characterize the prepared formulations at physical, chemical and biological levels. The formulated ointments loaded with the pillar[5]arene-isatin inclusion complexes should possess the properties of good appearance, odor, color, pH and better spreadability profile in order to utilize as topical applications.

3.4.4.1. Organoleptic property of the developed ointment loaded with pillar[5]arene-isatin inclusion complexes

Organoleptic property may influence the overall acceptability and applications of the developed topical ointments. The interactions of the developed ointment loaded with pillar[5]arene-isatin inclusion complexes were analysed to observe the shape, texture, color and odour. These parameters have a huge impact on the function of the ointment at the target site.

3.4.4.2. pH of the developed ointment loaded with pillar[5]arene-isatin inclusion complexes

pH of the ointment plays a crucial role in the topical application. High or low pH could possibly cause irritation and other inconveniences to the host at various levels. Hence, this parameter should be measured for the developed ointment formulations with bioactive principles loaded polymeric system. The pH of the

ointment was measured by universal pH paper which was dipped in 0.5 g of ointment with distilled water of 5 ml. The observation of pH was recorded.

3.4.4.3. Assessment of the spreading potential of pillar[5]arene-isatin inclusion complexes based ointment on the surfaces

The spreadable nature of the ointment needs to be evaluated for its application on skin surfaces. This was performed by weighing 0.5g of the developed ointment which was placed in the middle of the microscopic slide. After that, the top was covered with another microscopic slide or other transparent material. The entire setup was not to be disturbed for about 2 to 5 seconds. Then, the diameter of the spreading nature of the developed ointment was measured by a ruler at each additional load when the preparation stopped spreading.

3.4.5. Determination of cytotoxicity of the pillar[5]arene-isatin inclusion complexes based ointment by MTT reduction assay

Cytotoxicity of the formulated topical applications should be tested to ensure the safety profile for human use. It is very important to obtain accurate and reliable results from the *in vitro* tests that may influence the success rate during the preclinical stage. In this regard, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay can be performed to decipher cell toxicity and it becomes the gold standard protocol for determining cell viability. The assay determines cell viability by evaluating the enzymatic conversion of a tetrazolium compound into water-insoluble formazan crystals, a reaction catalyzed by mitochondrial dehydrogenase enzymes in metabolically active cells. Hence, the cytotoxicity of the formulated wound healing ointment was determined using an MTT assay. Cell viability was assessed by evaluating the formation of formazan crystals from MTT following treatment with the developed ointment at varying concentrations (500–2500 µg/ml). Whole blood cells were initially seeded into a 96-well plate, and the ointment was added to the designated treatment wells. The plates were incubated for 24 hours at 37°C in a humidified environment containing a CO_2/95% air mixture. Afterward, MTT was introduced to each well, and incubation continued for 3 hours to allow formazan crystal formation. Dimethyl sulfoxide (DMSO, Himedia)

was then added to dissolve the crystals completely. Wells containing MTT and lymphocyte cells served as negative controls, while blanks were prepared by adding MTT and phosphate-buffered saline (PBS). The absorbance of each well was measured at 570 nm using an ELISA reader. The detailed protocol to determine the cytotoxicity using an MTT assay is provided in Appendix 13.

3.4.6. Determination of antimicrobial efficacy of pillar[5]arene-isatin inclusion complexes based ointment against prominent bacterial pathogens

The formulated topical ointment has to be validated for its profound antimicrobial activity. The ointment has to possess the property of eradicating the persistence of microbial load at the wound site. The antimicrobial potential of the developed ointment loaded with pillar[5]arene-isatin inclusion complexes was analyzed against selected prominent wound-causing pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The activity was performed by the disk diffusion method to assess the sensitivity or susceptibility or resistant pattern of the ointment against the selected pathogen. The samples were placed on the Mueller-Hinton agar with respective pathogenic cultures streaked onto it. The plates were incubated at 37ºC for 24 hours. The zone of inhibition in diameter was measured after incubation. The entire experiment was performed by following the detailed procedure provided earlier.

3.4.7. Determination of wound healing potential of pillar[5]arene-isatin inclusion complexes based ointment on L929 fibroblast cells

The *in vitro* scratch assay is a cost-effective and widely used method to assess the wound healing potential of topical formulations. When skin is injured, various cells, including fibroblasts, macrophages, keratinocytes, and immune cells, migrate to the wound site, contributing to the complex and prolonged healing process. Fibroblast cells, in particular, play a pivotal role in wound healing, as they perform essential functions during the healing process which include producing extracellular matrix components and collagen structures necessary for tissue homeostasis and breaking down fibrin clots. Due to these critical roles, fibroblast-based scratch assays are recognized as a reliable and economical

model for evaluating the wound healing properties of therapeutic formulations (Bolla *et al*., 2019).

In this study, the wound healing efficacy of the developed ointment containing an pillar[5]arene-isatin inclusion complexes was investigated using an *in vitro* scratch assay on L929 fibroblast cells to examine cell migration (Bolla *et al.,* 2019). L929 fibroblast cells (2×10^5 cells/ml) were seeded into 96-well plates and cultured overnight. The following day, cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS), and a scratch was introduced using a sterile 200 µl pipette tip. Detached cells and debris were removed by washing with DPBS. The cells were then treated with 500 µg/ml of the developed ointment and 500 µg/ml of Cipladine, a standard drug known for its wound-healing properties, serving as the positive control. Untreated cells were used as the negative control. The cultures were incubated for 24 hours, and cell migration, as well as morphological changes, was monitored using an inverted microscope equipped with a digital camera. All experiments were conducted in triplicates. The scratch width and wound closure percentages at 0, 24, and 48 hours were quantified using ImageJ software. The wound closure by the developed ointment was calculated using the formula as follows:

Percentage of Wound Healing Scored = (Initial area-Final area)/Initial area *100