4.0

RESULTS AND DISCUSSION

Wound and wound infections pose a significant threat at the global level thus enhancing the mortality rate and economic burden. In general, skin acts as a front-line defense barrier to prevent the entry of various invading microbes into the wound-affected area. The wounds are more susceptible to microbial infections due to the skin and dermal appendages losing their barrier functions. This process can accelerate the endurance of bacterial colonization in the wound area. The major wound microbiome contains both bacteria and fungi which may interact with other microbial communities to promote wound infections, thus also enhancing antimicrobial resistance. The wound microbiota has a significant impact on each stage of the repairing process and promotes impaired wound healing processes (Uberoi *et al*., 2024).

Apart from the other major factors for wound infections, one of the major issues of impaired wound healing is biofilm formation. The biofilm producing bacteria present a formidable challenge from the treatment perspective. Of note, it becomes less susceptible to human immune defense mechanisms and also uses sophisticated mechanisms to develop resistance. Bacteria encased in biofilms are 1000 times more resistant to conventional antibiotics, thus, an effective strategy is required to eliminate the biofilm forming capabilities of the pathogens responsible for wound infections (Kaiser *et al*., 2021; Munyeshyaka *et al*., 2021).

A formidable challenge in the development of an effective treatment strategy is the preclinical parameters to optimize the absorption, efficacy and safety of new antimicrobials in the infected wound environment. To circumvent the problems and challenges associated with proper treatment, a suitable drug delivery system is of paramount importance to address the growing incidence of chronic wounds and associated complications (Kaiser *et al*., 2021).

The use of macrocyclic hosts for the encapsulation of drugs is recommended

by various scientific literature. 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). On the other hand, isatin is a well-known alkaloid compound and that has been successfully isolated from the flower extracts of *Couroupita guianensis* Aubl. The plant has been popular from ancient times to now in the context of its medicinal values. It has been used to treat various human illnesses, including skin infections (Sheba and Anuradha, 2019). Our laboratory has explored the potential antimicrobial activities of various parts of *Couroupita guianensis*. Aubl and their active component (isatin) against various bacterial and fungal pathogens (Kavitha ***et al***., 2013).

In the present study, the drug (guest) and drug carriers (hosts) were selected and their pharmacokinetic profile was evaluated for the drug-likeliness properties. The selected drug and drug carriers were synthesized and characterized. Further, the drug, drug carriers and their inclusion complexes were analyzed for their antimicrobial efficacy against a wide range of clinical pathogens. The inclusion complex with good antimicrobial efficacy has been taken for further studies. The *in vitro* mechanistic action of the selected host-guest inclusion complexes was performed against the prominent pathogens followed by the evaluation of biofilm inhibitory potential for the host-guest inclusion complexes. The *in vitro* drug release potential was assessed and validated through various mathematical models. Finally, the selected inclusion complex loaded wound healing ointment was developed and their wound healing potential was elucidated. The cytotoxicity of the developed ointment was also carried out to determine the safer applications at the wound sites.

PHASE I

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

4.1.1. Selection of drug and drug carriers for host-guest complex synthesis

Couroupita guianensis. Aubl was reported to have many secondary metabolites namely, indirubin, isatin, α-amirin, couroupitine, and β-amirin. Earlier, various secondary metabolites such as alkaloids, flavonoids and phenolic fractions of *Couroupita guianensis*. Aubl had exhibited a high antimicrobial potential against various pathogenic microbes, among which the alkaloids fractions were found to possesses outstanding antimicrobial actions (Kavitha *et al*., 2013). Isatin is a red-orange powder and its biotherapeutic potential has been highly appreciated since from ancient era. Since, isatin is a hydrophobic drug that shows poor solubility and bioavailability, it is limited to exert its targeted action at the disease sites. Due to these properties, it might be prone to premature degradation by various environmental conditions and cannot be released at the target sites in a controlled manner. Hence, there is a need for the appropriate drug carriers to encapsulate isatin to promote its bioavailability and controlled release at the target sites.

Supramolecular-based drug delivery systems have been observed with the burgeoning interest in encapsulating the hydrophobic drugs in to their electron rich cavities to enhance their bioavailability and targeted activity. Among the various macrocyclic supramolecular hosts, pillar[5]arene is a cyclic oligomer composed of five repeating units with unique host-guest chemistry. Bis-ethanolamine functionalized pillar[4]arene[1]quinine (BEA) is a derivative of P[5]A. It is anticipated that BEA could exhibit superior binding affinity towards isatin compared to P[5]A due to the presence of ethanolamine units in the macrocycle which is a strong hydrogen bonding donor. Henceforth, pillar[5]arene and BEA were selected as the host molecules (drug carriers) to encapsulate isatin (guest/drug) into their electron-dominant cavities through various interactions to promote the targeted and controlled action of isatin at the target sites.

4.1.2. Drug-likeliness profile of drug and drug carriers

Many drugs fail in the drug development process due to their poor pharmacokinetics and toxicity problems. Many drawbacks that have arisen during the drug development could be addressed at the early stage in the pipeline of this process. Drug-likeliness profiles such as ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties of the compounds can be assessed in the initial stages to avoid later-stage failures in clinical phases. Generally, conventional methods of predicting pharmacological properties are time-consuming and expensive. Hence, computational prediction of pharmacokinetic properties may provide precise and accurate results with reduced cost and time factors involved, thus enhancing the quality and success rates. In this study, the ADMET properties of the selected drug (isatin) and drug carriers (pillar[5]arene and difunctionalized pillar[4]arene[1]quinine derivative), were carried out to determine their pharmacokinetic profiles.

4.1.2.1. Absorption

A lead compound must be absorbed to reach its target site to exert the mechanism of action. Many factors can hinder the process of absorption including, poor solubility, gastric emptying time, permeability to the intestinal walls, intestinal transit time and instability of the drug components. Absorption properties of the lead compounds ultimately determine their bioavailability in the host (Pires *et al*., 2015). The results of the absorption parameters of the selected compounds are given in Table 1.

From the results, the selected compounds were observed to have a moderate solubility in water ranging from -0.948 to -3.105 log mol/L. Since isatin is a hydrophobic alkaloid, it showed a relatively low solubility profile. Most of the compounds lie in the moderate Caco-2 intestinal permeability and skin permeability. The moderate permeability profiles have directed them to employ as topical antimicrobial applications. These compounds were found to have good gastrointestinal absorption with greater than 70%. P-glycoprotein has the ability to extrude toxins and other compounds out of the cells (efflux mechanisms). The drug

(isatin) and drug carrier (pillar[5]arene) were not found to be a p-glycoprotein substrate. In addition, isatin was not observed as p-glycoprotein inhibitors, suggesting that the transportation and secretion of drugs were following p-glycoprotein-independent pathways.

Table 1: Absorption characteristics of the selected drug and drug carriers

Pharmacological properties	Predicted value			Optimal range
	Isatin	Pillar[5]arene	BEA	
Water solubility	-0.948	-2.903	-2.947	Not higher than 6 log mol/L
Caco2 permeability	1.17	0.792	0.576	> 0.90 log Papp in 10^{-6} cm/s (high)
Intestinal absorption (human)	89.943	100	100	<30% (poor absorption)
Skin permeability	-2.51	-2.35	-2.735	>-2.5 log Kp (low skin permeability)
P-glycoprotein substrate	No	No	Yes	Yes/No
P-glycoprotein I inhibitor	No	Yes	Yes	Yes/No
P-glycoprotein II inhibitor	No	Yes	Yes	Yes/No

4.1.2.2. Distribution

The distribution parameters including, VDss (volume of distribution at steady state), fraction unbound (Fu), blood-brain barrier (BBB) permeability, and CNS (central nervous system) permeability were analysed for the selected compounds such as isatin, P[5]A and BEA. These parameters provide insights into the tissue distribution and central nervous system accessibility by the selected drug and drug carriers. Table 2 provides the significant distribution profiles of isatin and pillar[n]arenes.

The VDss values of the selected compounds demonstrated their uniform distribution in the tissues. Comparatively, isatin has a higher VDss than the selected compounds, suggesting their good distribution properties in the infected areas. The permeability of substances to the blood-brain barrier and central nervous system (CNS) is an important parameter to reduce toxicity and side effects. All the selected compounds exhibited moderate BBB and CNS permeability, indicating their applications in the topical areas.

Table 2: Bioavailability and permeability prediction of selected drug and drug carriers

Pharmacological properties	Predicted Value			Optimal range
	Isatin	Pillar[5]arene	BEA	
VDss (human)	0.314	-1.076	-0.704	>0.45 log L/kg (high)
Fraction unbound (human)	0.509	0.517	0.416	Equals to fraction unbound in plasma
BBB permeability	0.154	0.278	0.174	log BB>0.3 (high permeability)
CNS Permeability	-2.213	-3.028	-3.171	< -3 log PS (low penetration)

4.1.2.3. Metabolism

Human cytochrome (CYP) systems are known for their metabolic and excretory activity toward drugs. It is based on identifying their roles as substrates or inhibitors of the major key CYP enzymes like CYP2D6, CYP3A4, CYP1A2, CYP2C19, and CYP2C9 (Table 3). From the results, it was revealed that none of the compounds was found to be a substrate or inhibitor of CYP2D6. However, pillar[5]arene and BEA serve as substrates for CYP3A4, which is a major drug-metabolizing enzyme in humans. Pillar[5]arene was noted with the inhibitory action on CYP2C19 and CYP2C9 which indicated the possible metabolism of pillar[5]arene through this pathway.

Table 3: Interaction of the selected drug and drug carriers with major human cytochrome-mediated metabolic systems

Human cytochromes	Predicted Value		
	Isatin	Pillar[5]arene	BEA
CYP2D6 substrate	No	No	No
CYP3A4 substrate	No	Yes	Yes
CYP1A2 inhibitor	No	No	No
CYP2C19 inhibitor	No	Yes	No
CYP2C9 inhibitor	No	Yes	No
CYP2D6 inhibitor	No	No	No
CYP3A4 inhibitor	No	No	Yes

4.1.2.4. Excretion

The drug clearance has been usually evaluated by a combinatory action of hepatic and renal clearance parameters (Table 4). Compared to the isatin, the pillar[n]arenes such as pillar[5]arene and BEA were found to be quickly excreted. This phenomenon explained the prolonged exposure of isatin at the target site compared to the macromolecules.

Table 4: Pharmacological excretion characteristics of the selected drug and drug carriers

Pharmacological Properties	Predicted Value			Optimal range
	Isatin	Pillar[5]arene	BEA	
Total Clearance (log/ml/min/kg)	-0.066	0.966	1.167	Equals to CLtot (predicted total clearance log)
Renal OCT2 substrate	No	No	No	Yes/No

4.1.2.5. Toxicity

The toxicity parameters provide a comprehensive overview of the potential adverse effects of isatin, pillar[5]arene and BEA on various parameters. The toxicity properties of isatin, pillar[5]arene and BEA are presented in Table 5. From the results, all the selected compounds did not exhibit mutagenic properties, immunotoxicity, hepatotoxicity and skin sensitization. None of the compounds were found to be hERG I inhibitors. The predicted toxicity results indicated that isatin, pillar[5]arene and BEA were found to be non-carcinogenic. Furthermore, the isatin and pillar[5]arene were considered nontoxic, implying no significant adverse effects on the liver. Additionally, the maximum tolerated doses of isatin, pillar[5]arene and BEA were suggested to be safe, indicating that these compounds can be administered at relatively high doses without causing toxicity.

Table 5: Toxicological parameters of the selected drug and drug carriers

Pharmacological properties	Predicted Value			Optimal range
	Isatin	Pillar[5]arene	BEA	
AMES Toxicity	No	No	No	Yes/No
Max. tolerated dose (human)	0.139	0.346	0.299	>0.477 log mg/kg/day (high)
hERG I inhibitor	No	No	No	Yes/No
hERG ii inhibitor	No	Yes	Yes	Yes/No
Oral rat acute Toxicity (mol/kg)	2.158	2.318	2.706	Equals to LD50
Oral rat Chronic Toxicity (log mg/kg_bw/day)	1.641	0.278	3.281	Equals to low adverse effects level (LOAEL)
Hepatotoxicity	No	No	Yes	Yes/No
Skin Sensitisation	No	No	No	Yes/No
Minnow toxicity	2.068	1.23	-2.786	< -0.3 log mM (high acute toxicity)

Several scientific studies have predicted the pharmacokinetic profile of selected compounds to assess their druggable nature.

Ornithine derivatives synthesized using the methodology of supramolecular-based chemistry have been found to possess good characteristics of bioavailability, drug-likeliness and low toxicity for utilizing the derivatives in biomedical applications (Bojarska *et al*., 2020).

Majumdar *et al*. (2023) have demonstrated the desired characteristics of possessing no skin irritability, eye sensitivity and mutagenicity in the synthesized metals (Hg/Cu/Pb) in complexes with M-SCN (thiocyanate) supramolecular structures. Further, it had elaborated on the possible functions for the successful development of medications.

Benzo-fused seven-membered heterocyclic compounds are of particular interest in the process of drug discovery and the ADMET properties revealed their druggable nature of the compounds to become a potential drug candidate for the treatment of various illnesses (Frimayanti *et al*., 2020).

The insulin and the supramolecular host of cucurbit[7]uril have demonstrated their superior pharmacokinetic profiles to turn out to be a potential therapeutics to treat a wide spectrum of diseases (Maikawa *et al*., 2021).

All these data supported the findings of the pharmacokinetic profiles of the selected supramolecular hosts (pillar[5]arene and BEA) to become a potential drug carriers for the treatment of various ailments. Further, the synthesis of hosts and host-guest inclusion complexes were performed.

4.1.3. Synthesis of host molecules (P[5]A and BEA)

Pillar[5]arene (P[5]A) and BEA were successfully synthesized at our laboratory (Figure 14). Trifluoroacetic acid (TFA) was used as a Lewis catalyst which facilitated the reaction between 1,4 - dimethoxybenzene and paraformaldehyde in 1,2-dichloroethane (DCE). The direct condensation of 1,4-dimethoxybenzene and paraformaldehyde in the presence of TFA yielded crystalline pillar[5]arene (P[5]A) with a 60% yield. The formation of the cyclopentamer P[5]A is favored due to its

presumed thermodynamic stability, leading to its preferential production. BEA was prepared from pillar[4]arene[1] quinine (P4Q1) which was synthesized using decamethoxy pillar[5]arene upon reaction with ceric ammonium nitrate ($Ce(NH_4)_2(NO_3)_6$), a known oxidizing agent. Following the synthesis of P4Q1, it underwent a subsequent reaction known as a Michael addition with 2-aminoethanol. BEA was formed as a white solid with a 73% yield as an outcome of this reaction. The Michael addition reaction, a fundamental organic transformation, involves the nucleophilic addition of an enolate or enamine to a Michael acceptor, resulting in the formation of a new carbon-carbon bond.

Pillar[n]arenes were functionalized through the conversion of alkoxy groups located on the rims of the pillar[n]arene cavity, as well as the modification of methylene bridges between arene units and the arene units themselves. Huang *et al.* (2017). pioneered the synthesis of pillar[4]arene[1]quinone (P4Q1) and achieved the difunctionalization of pillar[5]arene through partial oxidation of decamethoxy pillar[5]arene. Strutt *et al*. (2014) described the conjugate addition of amines to P4Q1, resulting in the synthesis of amino-functionalized pillar[5]arene and the fabrication of one-dimensional nanotube oligomers. Recently, Huang *et al*. (2017) showcased the physical adsorption followed by Michael addition reactions of aliphatic amines with P4Q1, highlighting its non-porous adaptive characteristics. Additionally, Han *et al*. (2019) reported the synthesis of difunctionalized pillar[5]arene with hydroxyl and amino groups at A1/A2 positions.

The results have exhibited that the pillar[n]arenes such as P[5]A and BEA were successfully synthesized and could be analyzed for their drug-likeliness profile to carry out the biological evaluations.

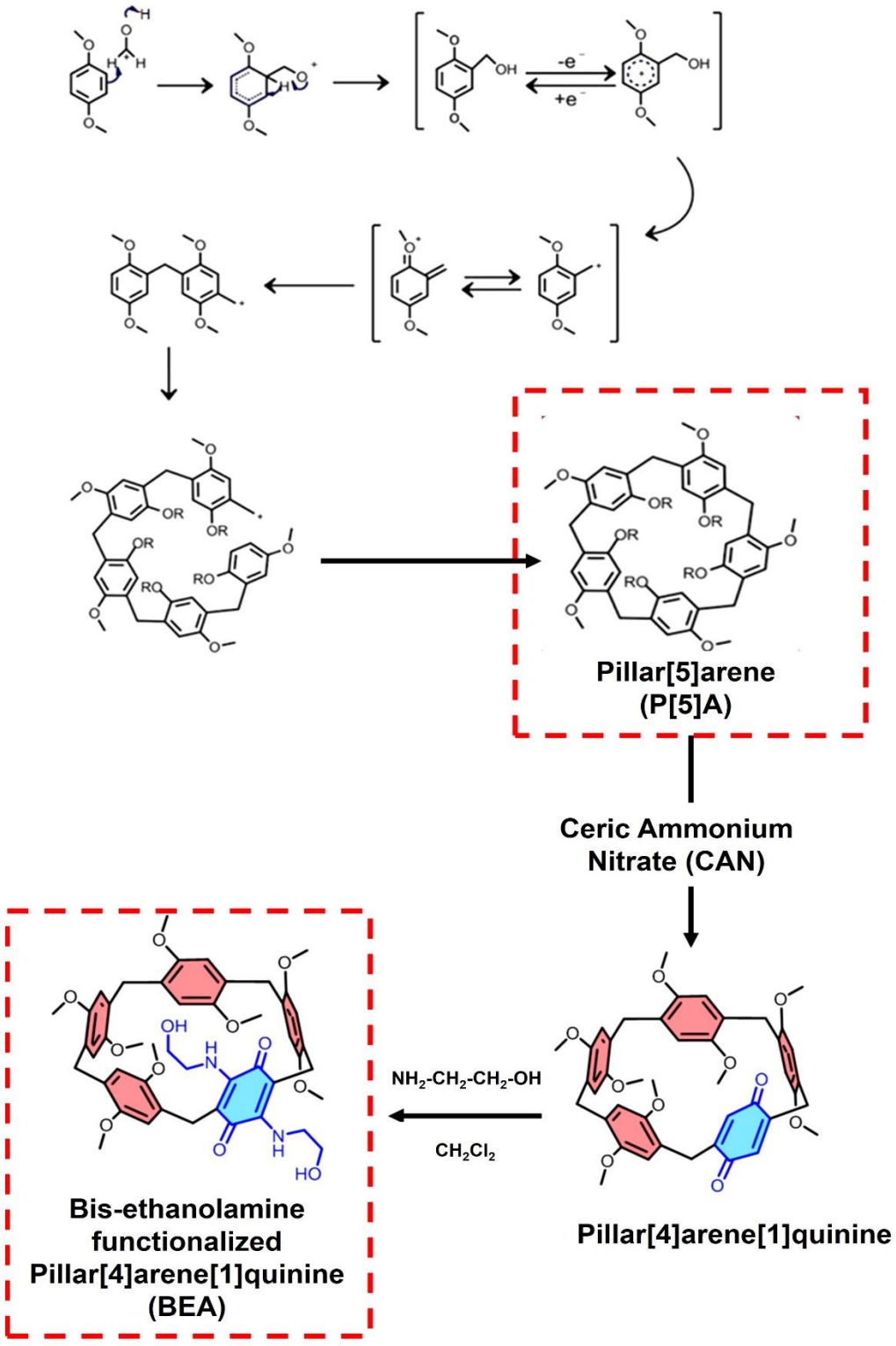


Figure 14: Synthesis of supramolecular host molecules (pillar[5]arene and BEA)

4.1.4. Characterization of synthesized host-guest complexation of pillar[n]arenes and isatin

Host-guest complexation studies in solution involve the investigation of interactions between a host molecule and a guest molecule in solution. Decamethoxypillar[5]arene (P[5]A) and difunctionalized pillar[4]arene[1]quinine derivative (BEA) possess internal cavities that can encapsulate guest molecules such as isatin through non-covalent interactions, such as hydrogen bonding, *van der Waals* forces, and π-π stacking interactions. The characterization of host-guest complexes in solution often involves spectroscopic techniques such as nuclear magnetic resonance (NMR) spectroscopy and UV-visible spectroscopic analysis.

4.1.4.1. Determination of binding affinity of synthesized pillar[5]arene-isatin inclusion complexes by ^{1}H NMR

The binding affinity between pillar[5]arene (P[5]A) and isatin was comprehensively studied using ^{1}H NMR titration experiments, offering valuable insights into their molecular interactions. An equimolar mixture of P[5]A and isatin was prepared in a solvent system of $CDCl_3$-acetone-d_6, and significant changes in the chemical shifts of both components were observed upon mixing, as illustrated in Figure 15. These shifts are indicative of the formation of a stable host-guest complex between P[5]A and isatin.

For isatin, the aryl–CH protons exhibited a pronounced inclusion-induced deshielding effect, a hallmark of complex formation. Notably, the chemical shifts of its protons, Ha, Hb, Hc, and Hd, shifted from their initial positions at 9.95, 7.02, 7.60, and 7.10 ppm to new positions at 9.78, 6.99, 7.57, and 7.09 ppm, respectively. These changes suggest significant interactions between isatin and the P[5]A cavity. Similarly, the protons of P[5]A also showed discernible shifts upon complexation. The aryl–C–H protons (H_1) shifted slightly from 6.87 ppm to 6.86 ppm, and the methoxy protons (H_3) shifted from 3.72 ppm to 3.74 ppm. These changes, while subtle, confirm that the host-guest interaction influences the P[5]A structure as well.

These observations strongly support the conclusion that isatin is effectively encapsulated within the cavity of P[5]A, leading to significant perturbations in their electronic environments. The shifts in chemical shifts for both the host and the guest further affirm the formation of a robust host-guest complex.

These observed changes in the NMR spectra serve as compelling evidence for the occurrence of complexation between P[5]A and isatin, highlighting the intricate interplay of molecular forces governing their interaction. The identified changes in chemical shift values provide valuable information regarding the structural perturbations induced upon complexation, shedding light on the binding mode and affinity between P[5]A and isatin.

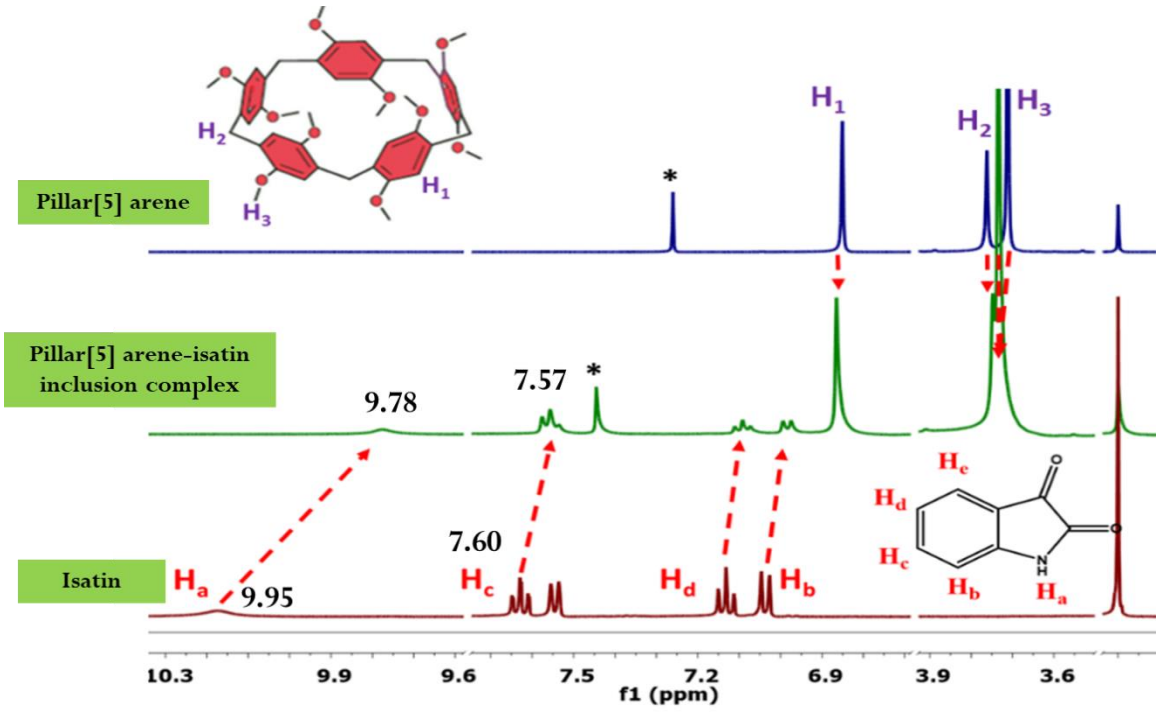


Figure 15: Characterization of synthesized pillar[5]arene-isatin inclusion complexes by ^{1}H NMR

4.1.4.2. Binding constant and host-guest stoichiometry of synthesized pillar[5]arene-isatin inclusion complexes

In an endeavor to comprehensively characterize the binding behavior between P[5]A and isatin, ^{1}H NMR titration experiments were undertaken to determine binding constants and elucidate host-guest binding stoichiometry (Figure 16). However, the dissimilar solubility characteristics of isatin and P[5]A in commonly employed solvents posed a challenge during experimental execution. Attempts to conduct ^{1}H NMR experiments in a mixed solvent system were impeded by sample precipitation, rendering the endeavor unfeasible. To circumvent this obstacle, titration experiments were conducted using a strategic approach. Specifically, aliquots of isatin dissolved in acetone-d6 were incrementally added to a constant concentration of $CDCl_3$ solution containing P[5]A within the NMR tube. This experimental setup facilitated the gradual introduction of isatin to the P[5]A solution, allowing for the systematic exploration of their binding interactions while mitigating issues associated with sample precipitation.

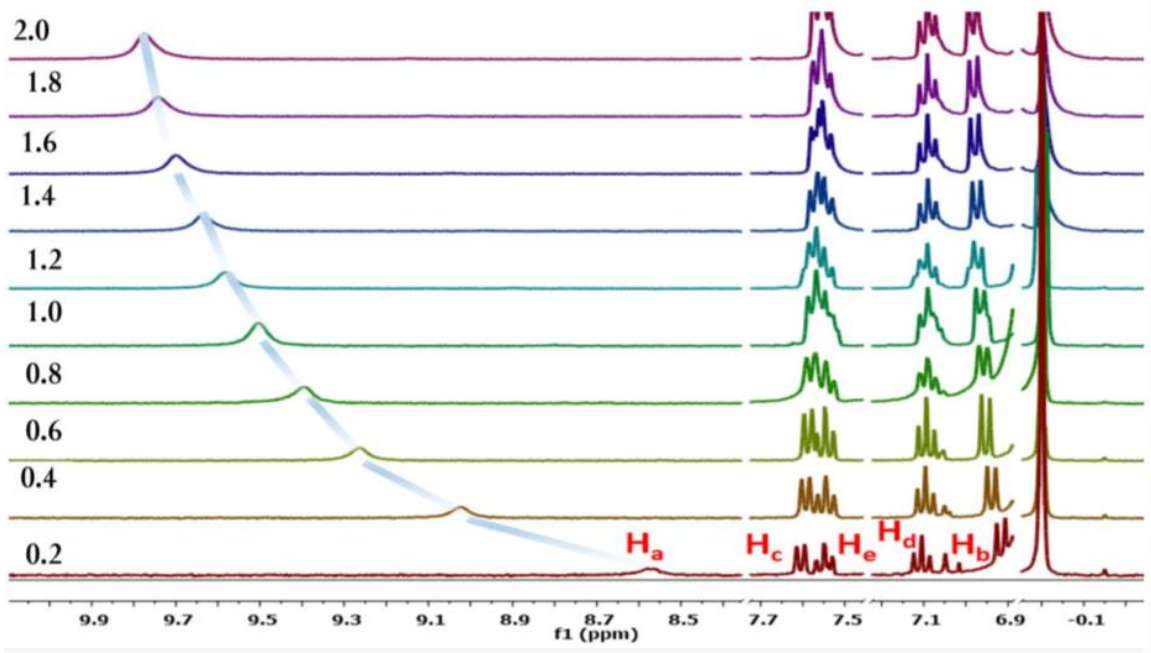


Figure 16: Binding constant and host-guest stoichiometry of synthesized pillar[5]arene-isatin inclusion complexes

4.1.4.3. Job's plot analysis of ^{1}H NMR titration of synthesized pillar[5]arene-isatin inclusion complexes

The comprehensive analysis of the titration data using Job's plot methodology has yielded significant insights into the host-guest binding stoichiometry between P[5]A and isatin (Figure 17). The data revealed a 1:1 binding stoichiometry, indicating the formation of an inclusion complex between P[5]A and isatin. This observation is crucial as it not only corroborates the experimental findings but also reinforces the understanding of the molecular interactions governing their complexation. The establishment of a 1:1 binding stoichiometry provides a clear picture of the structural arrangement within the inclusion complex, enhancing our understanding of the specific molecular recognition events occurring between P[5]A and isatin.

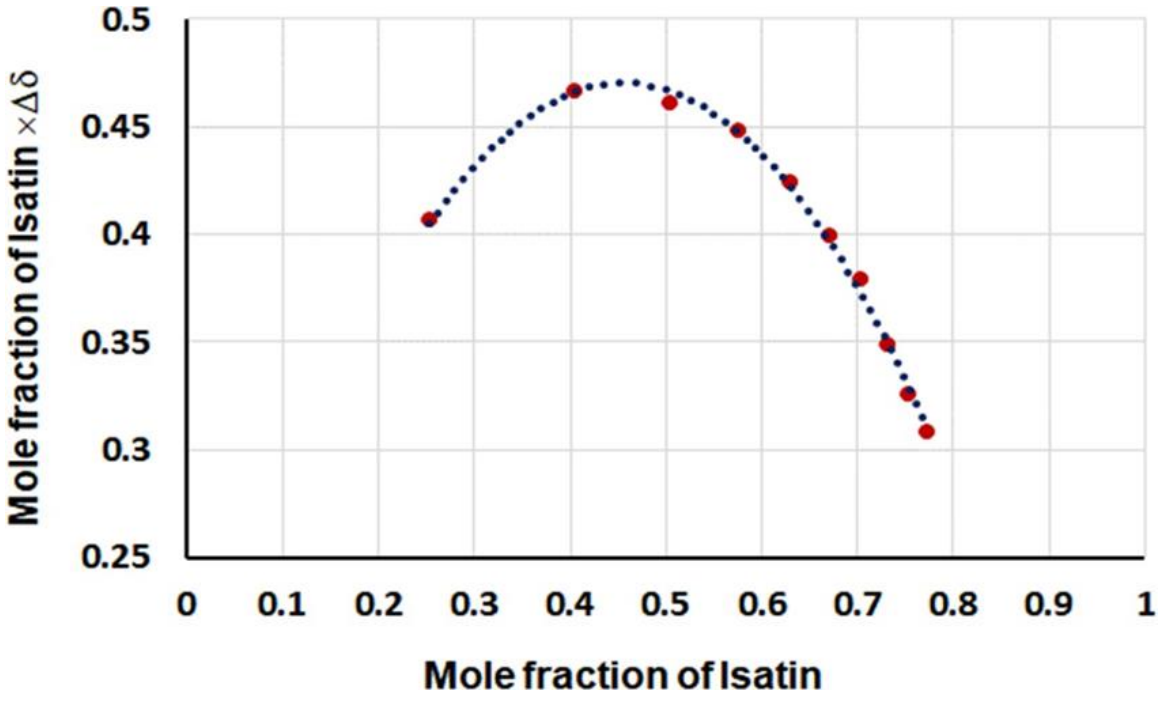


Figure 17: Job's plot of host– guest complexes by ^{1}H NMR

Moreover, the obtained ^{1}H NMR titration data underwent rigorous analysis using WINEQNMR2, a computational tool specifically designed for such analyses (Figure 18). This computational analysis allowed a detailed investigation of the 1:1 host-guest stoichiometry between P[5]A and isatin. As a result, the association

constant log K, which serves as a quantitative measure of the binding strength between the two molecules, was estimated to be 3.3 ± 0.4. This numerical value provides valuable quantitative insights into the affinity of P[5]A for isatin, offering a comprehensive understanding of the thermodynamic aspects of their binding interaction. The precise determination of the association constant further elucidates the energetics driving the formation of the inclusion complex and highlights the stability of the pillar[5]arene-isatin inclusion complexes.

The plots depicting mole fraction against equivalents of isatin (Figure 19) provide valuable insights into the extent of complexation between P[5]A and isatin in solution. Remarkably, the data illustrate that the equimolar mixture of P[5]A and isatin led to the formation of approximately 80% complexation in solution. This high degree of complexation underscores the strong affinity between P[5]A and isatin, highlighting the efficacy of the host- guest interaction under the experimental conditions employed. One factor that could influence the observed binding affinity is the variation in the ratios of $CDCl_3$ and acetone-d_6 used in the ^1H NMR titration experiments. The use of different solvent compositions introduces variability in the polarity of the solvent system, which in turn can impact the binding affinity between P[5]A and isatin. Solvent polarity plays a significant role in molecular interactions, particularly in host-guest complexation processes. Changes in solvent polarity can influence the stability of the inclusion complex by altering the balance of intermolecular forces involved, such as hydrogen bonding, hydrophobic interactions, and van der Waals forces. As a result, variations in solvent composition during the titration experiments may lead to fluctuations in the observed binding constant, reflecting the sensitivity of the host-guest interaction to changes in solvent environment.

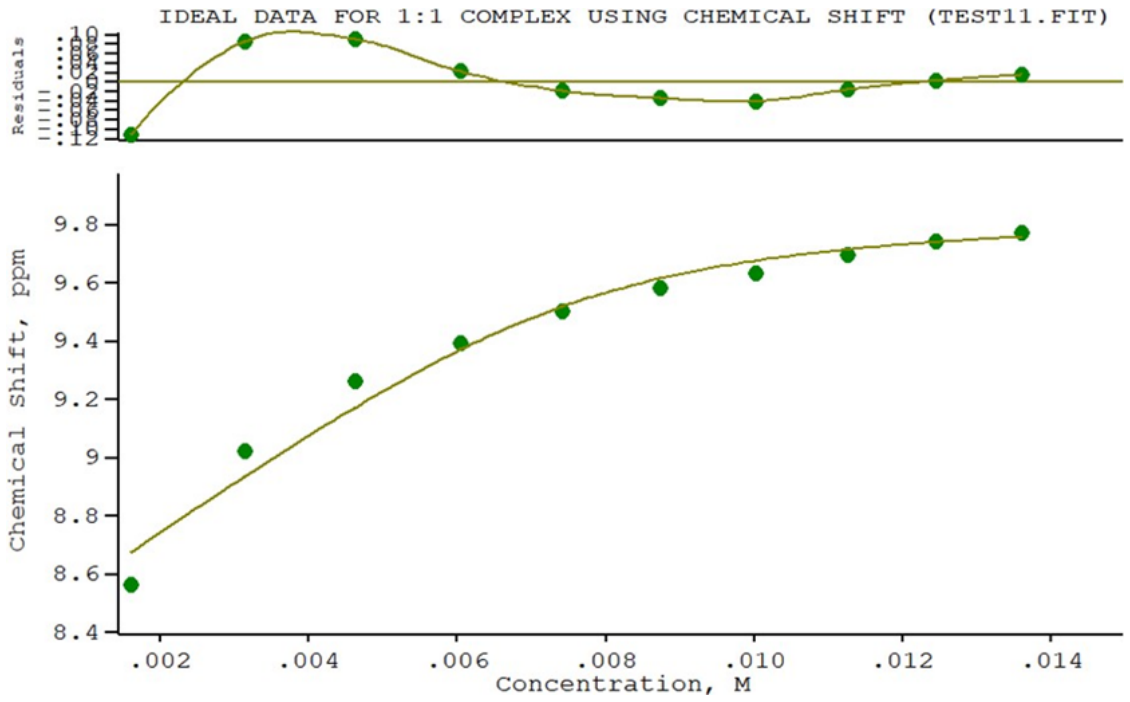


Figure 18: ^{1}H NMR titration curve of pillar[5]arene-isatin inclusion complexes by WINEQNMR2 program

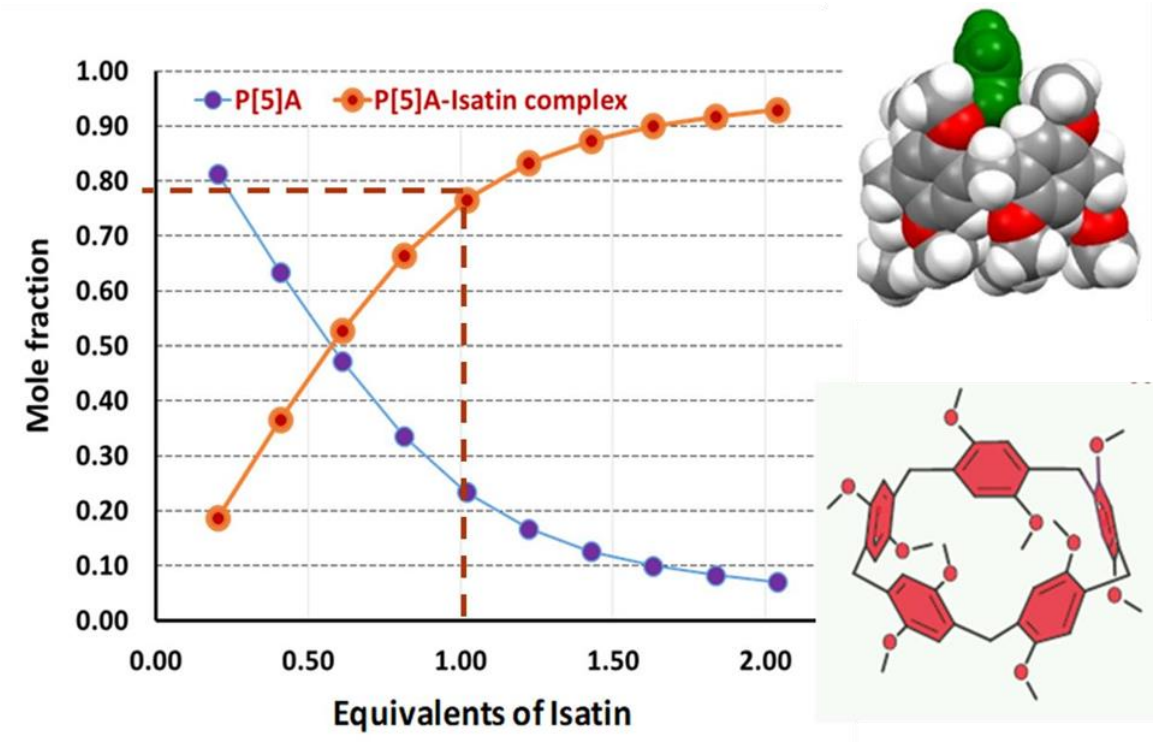


Figure 19: Plots of mole fractions of pillar[5]arene and pillar[5]arene-isatin inclusion complexes versus equivalents of isatin

4.1.4.4. Dynamics of host-guest chemistry in the synthesized pillar[5]arene-isatin inclusion complexes from UV-visible spectra

UV-visible spectroscopic experiments play a pivotal role in elucidating the dynamics of host-guest chemistry. These experiments typically involve the spectral analysis of absorption or emission patterns resulting from electronic transitions within the molecules involved. In host-guest systems, changes in these spectra can indicate the formation of complexes or alterations in the electronic environment of the molecules, offering direct evidence of binding events. UV-visible spectroscopy offers several advantages in studying host-guest chemistry. It is sensitive to changes in molecular environments, allowing for the detection of subtle structural modifications. Additionally, UV-visible spectroscopy is relatively simple to implement and offers rapid data acquisition, making it suitable for both qualitative and quantitative analyses. In host- guest systems, UV-visible spectroscopy can be used to determine binding constants, stoichiometries, and thermodynamic parameters of the complexation process (Figure 20).

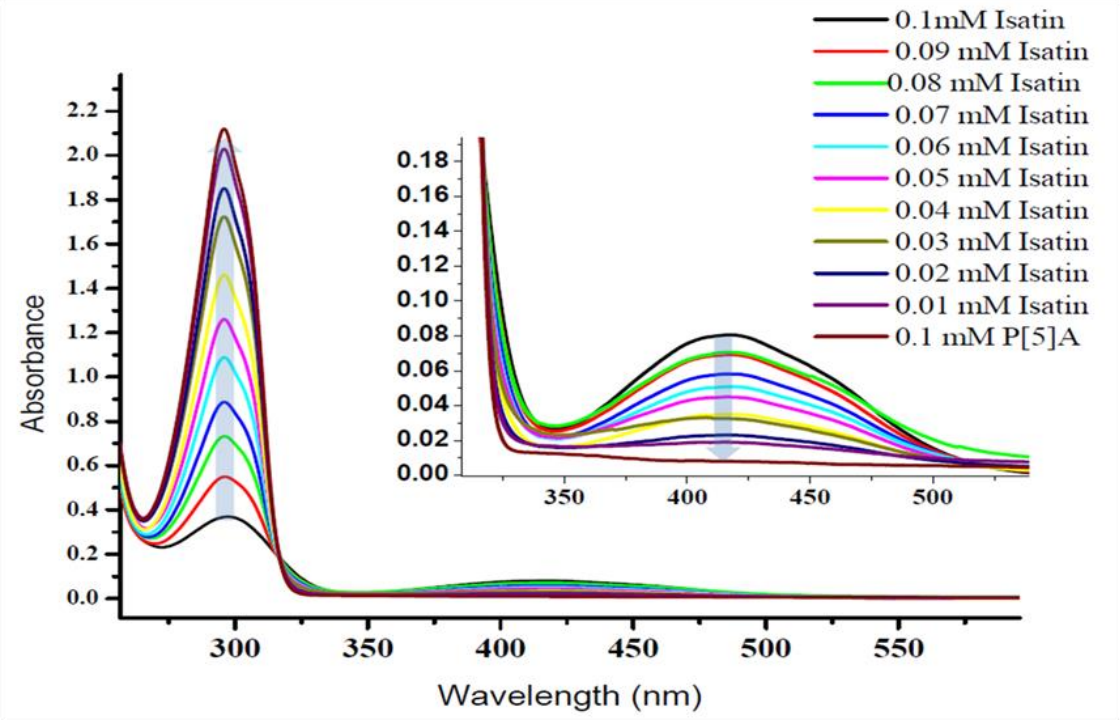


Figure 20: UV-visible spectra of the pillar[5]arene-isatin inclusion complexes

UV-visible spectroscopic analysis was conducted to gain deeper understanding of the host- guest complexation between pillar[5]arene (P[5]A) and isatin. Isatin's UV-visible spectrum in a 1:1 chloroform-acetone (v/v) solvent mixture exhibited distinctive absorption features, notably a strong absorption band with peak intensity at 296 nm and a broad band centered at 417 nm. These absorption bands are characteristic of isatin's electronic transitions, providing valuable information about its molecular structure and electronic properties.

4.1.4.5. Job's plot analysis of UV-visible titration of the synthesized pillar[5]arene-isatin inclusion complexes

To establish the stoichiometry of the host-guest interaction, Job's plot analysis was employed using the UV-visible titration data. Job's plot is a common method utilized in host-guest chemistry to determine the optimal binding ratio between the host and guest molecules. The analysis of the UV-visible titration data using Job's plot confirmed a 1:1 binding stoichiometry between pillar[5]arene and isatin (Figure 21).

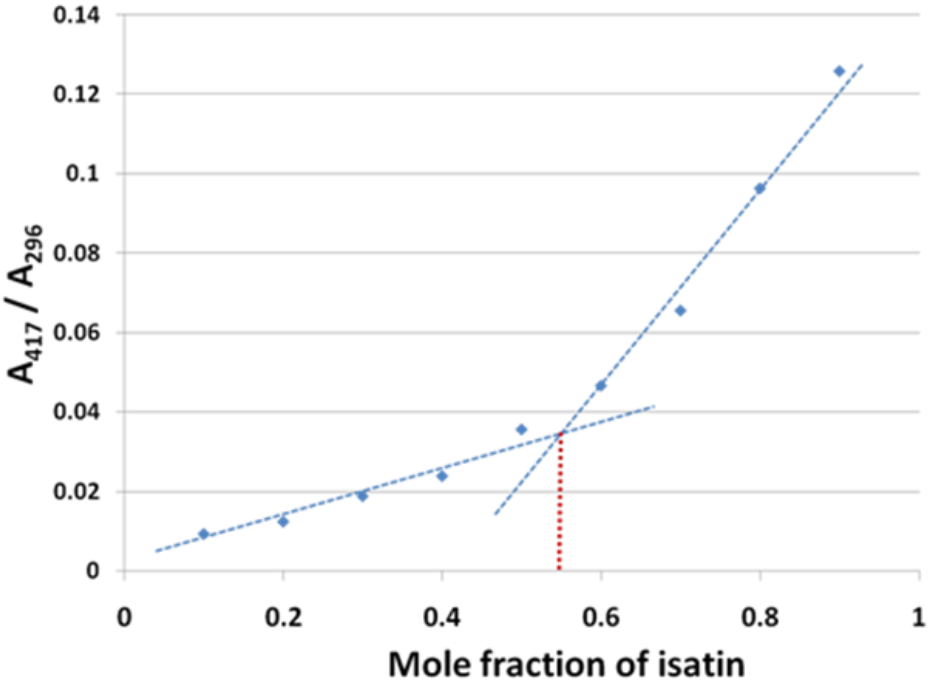


Figure 21: Job's plot of binding stoichiometry of synthesized pillar[5]arene-isatin inclusion complexes by UV-visible spectroscopy

This result corroborates the formation of a 1:1 host-guest complex between P[5]A and isatin, indicating that each pillar[5]arene molecule interacts with one molecule of isatin in the studied system. UV-visible spectroscopic analysis was performed to get further insight on the host-guest complexation between P[5]A and isatin. UV-visible spectrum of isatin in 1:1 $CHCl_3$-acetone (v/v) medium displayed a characteristic strong band with maxima at 296 nm and a broad band at 417 nm. Job's plot analysis of the UV-visible titration data confirmed 1:1 host-guest binding stoichiometry between P[5]A and isatin.

4.1.4.6. Determination of binding affinity of synthesized BEA-isatin inclusion complexes by proton NMR

Host-guest complexation studies were performed using ^1H NMR titration experiments to investigate the interaction between BEA and isatin (Figure 22). The study revealed a striking contrast in binding affinities compared to previously studied systems, particularly with pillar[5]arene (P[5]A), which exhibited strong host-guest interactions. In the case of BEA and isatin, the binding affinity was found to be significantly weaker.

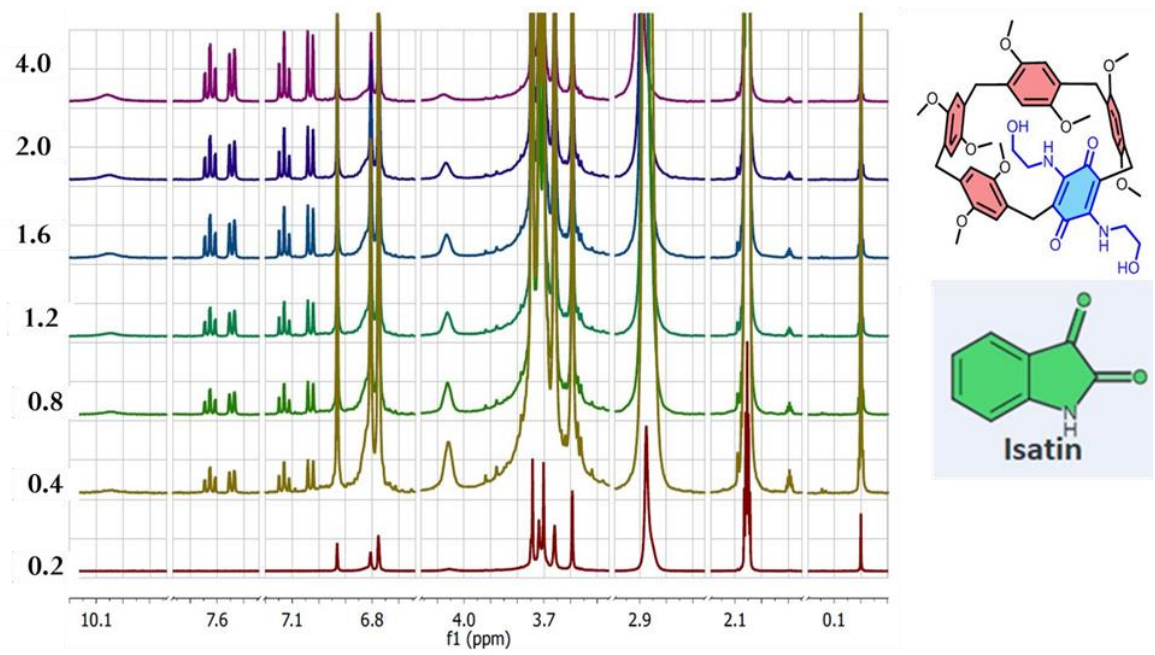


Figure 22: Binding constant and host-guest stoichiometry of synthesized BEA-isatin inclusion complexes

This was evidenced by minimal chemical shift changes in the ^{1}H NMR spectra, even after the addition of four molar equivalents of isatin to BEA. The limited chemical shift perturbations suggested a lack of substantial interaction between the two molecules.

4.1.4.7. Binding constant and host-guest stoichiometry between synthesized BEA-isatin inclusion complexes

The binding affinity between BEA and isatin was further quantified using binding constant calculations. The binding constant, determined from ^{1}H NMR titration data and modeled with a 1:1 stoichiometry using the WINEQNMR2 program, was found to be relatively low (log K = 2.0 ± 0.6). This result reinforces the conclusion of weak host-guest interactions in this system. These findings highlight the importance of structural and electronic compatibility in host-guest chemistry and suggest that BEA does not provide a suitable cavity or complementary interactions for effective binding with isatin.

Additionally, the ^{1}H NMR titration data were rigorously analyzed using the WINEQNMR2 software to elucidate the interaction between BEA and isatin (Figure 23). This analysis confirmed a 1:1 host-guest stoichiometry, consistent with the nature of their interaction. The association constant (log K) derived from this analysis was calculated to be 2.0 ± 0.6, providing a quantitative measure of the binding affinity between BEA and isatin. This binding constant underscores the notably weak interaction in comparison to the interaction of isatin with pillar[5]arene (P[5]A). The binding affinity of BEA for isatin is approximately 20 times lower than that of P[5]A. Such a pronounced disparity in binding strengths reflects the critical role of host-guest compatibility, with P[5]A offering a more complementary cavity and stronger interaction framework for isatin than BEA. This finding highlights the structural and chemical inadequacies of BEA in forming robust complexes with isatin.

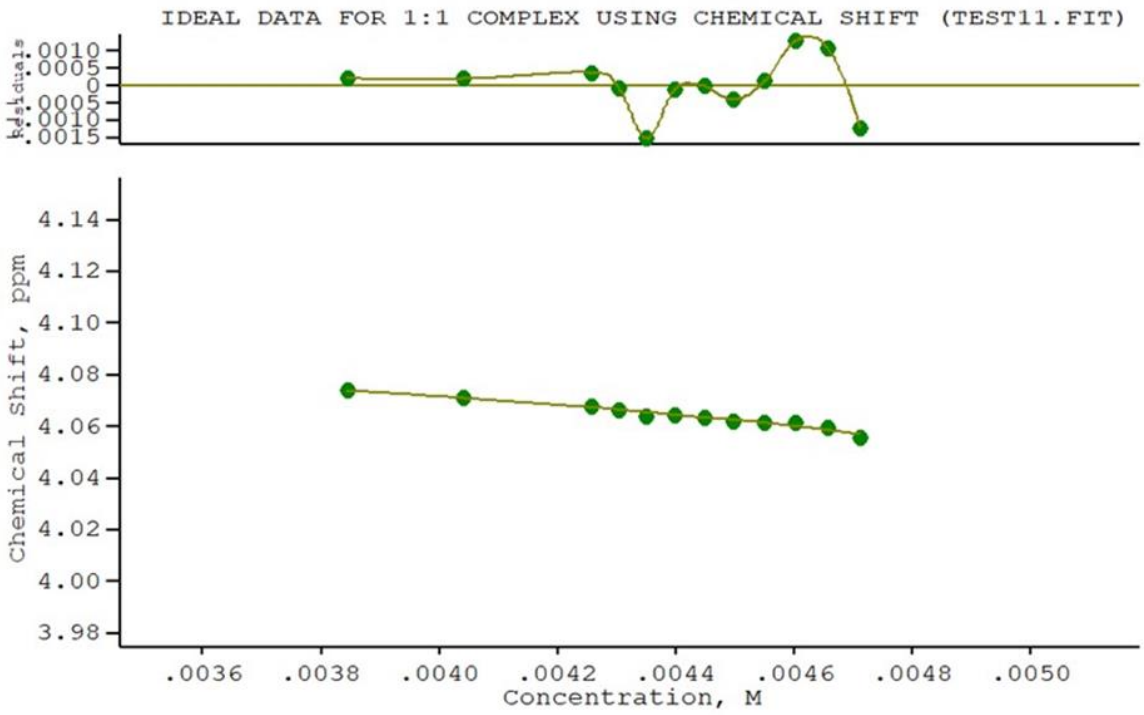


Figure 23: ^{1}H NMR titration curve of BEA-isatin inclusion complexes by WINEQNMR2 program

4.1.4.8. Dynamics of host-guest chemistry in the synthesized BEA-isatin inclusion complexes from UV-visible spectra

The host-guest interaction between BEA and isatin was further explored using UV-visible spectroscopic analysis, a technique highly sensitive to changes in the electronic environment induced by molecular interactions (Figure 24). In acetone, BEA displayed characteristic absorbance peaks at 294 nm and 353 nm, reflective of its intrinsic molecular properties. Upon gradual addition of isatin aliquots to a 10 μM solution of BEA in acetone, significant changes were observed in the UV-visible absorption spectrum.

Notably, there was a reduction in absorbance intensity at 353 nm, coupled with a pronounced increase in intensity at 294 nm and 450 nm. These spectral changes indicate a substantial perturbation in the electronic environment of BEA molecules, attributable to their interaction with isatin. Furthermore, the appearance of well-defined isobestic points at 328 nm and 304 nm provided compelling evidence of the formation of a distinct molecular complex between BEA and isatin.

These isobestic points signify a clear equilibrium between free and bound forms of BEA, affirming the presence of host-guest complexation in the solution. This spectroscopic analysis complements the findings from ^{1}H NMR, further underscoring the interaction, albeit weak, between BEA and isatin.

The presence of isobestic points, characteristic of a transition involving only two species, highlights the specificity and stability of the complex formed between BEA and isatin. This observation reinforces the conclusion that the interaction follows a well-defined binding mechanism.

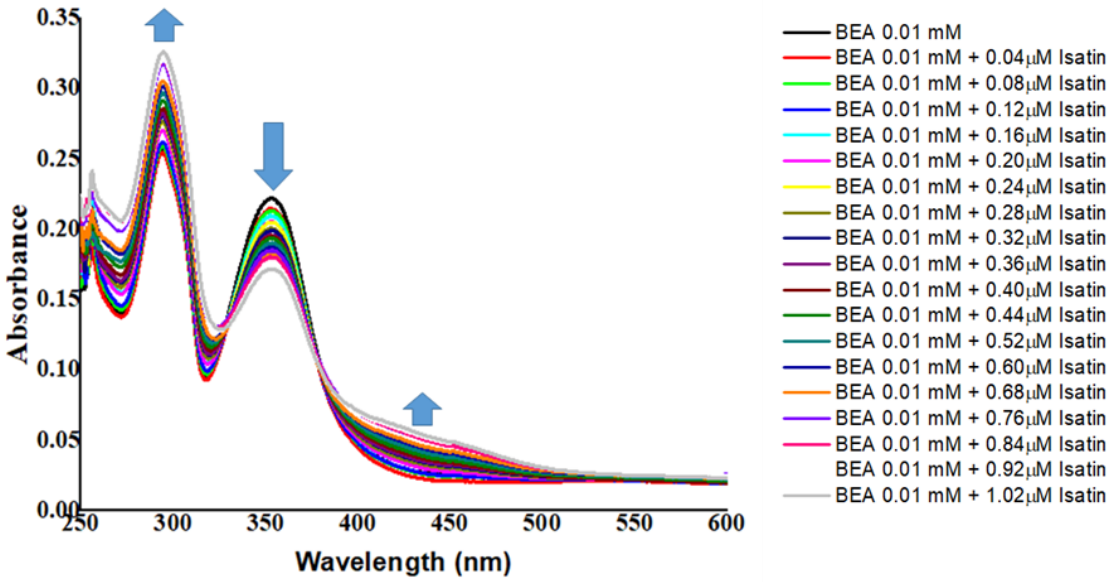


Figure 24: UV-visible spectra of the BEA-isatin inclusion complexes

4.1.4.9. Job's plot analysis of UV-visible titration of the synthesized BEA-isatin inclusion complexes

To further validate the stoichiometry of this host-guest interaction, Job's plot analysis was conducted for isatin and BEA (Figure 25). The UV-visible titration data provided clear evidence of a 1:1 binding stoichiometry between BEA and isatin in solution. This finding is significant, as it provides deeper insight into the molecular recognition events underpinning the interaction between BEA and isatin.

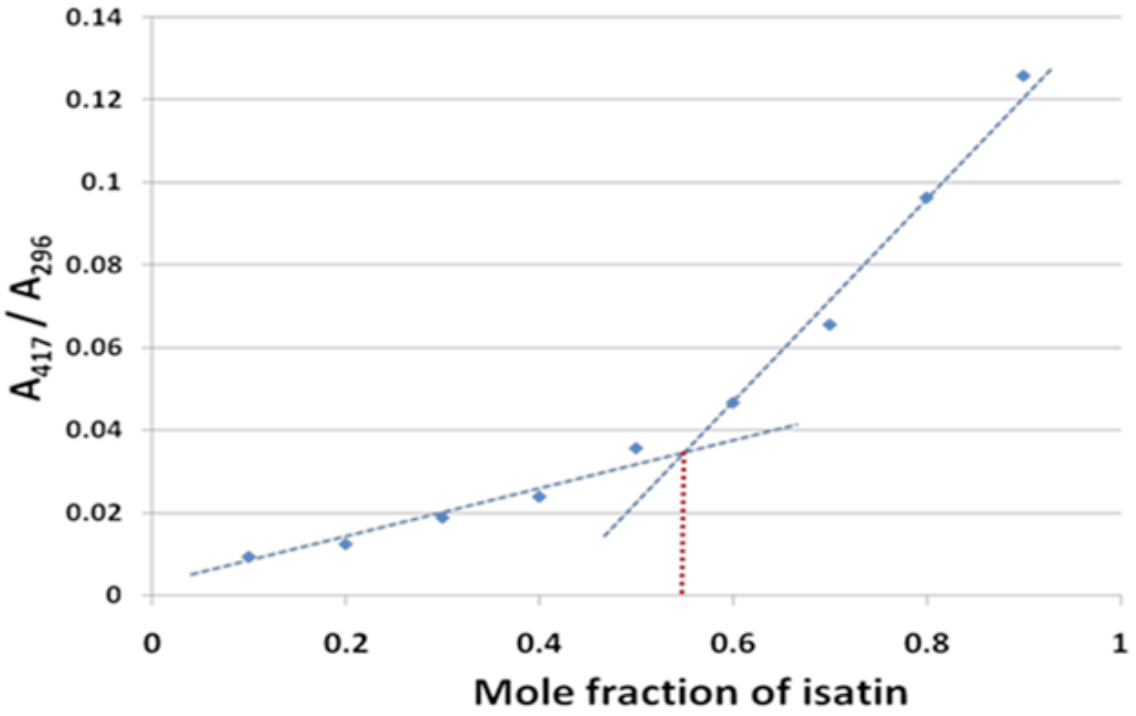


Figure 25: Job's plot of binding stoichiometry of synthesized BEA-isatin inclusion complexes by UV-visible spectroscopy

The Phase I study has successfully identified and synthesized host molecules tailored for drug encapsulation, focusing on interactions between pillar[5]arene (P[5]A) and isatin, as well as BEA and isatin. These host-guest systems were systematically characterized through advanced analytical techniques, including proton nuclear magnetic resonance (NMR) titration experiments and UV-visible spectroscopy, to elucidate the binding properties and structural interactions. Proton NMR titration experiments were pivotal in investigating the inclusion complexation of isatin with the host molecules. For P[5]A, the experiments revealed the formation of a stable inclusion complex, indicating a robust interaction between the host (P[5]A) and the guest (isatin). The binding stoichiometry of this interaction was determined to be 1:1, demonstrating the specificity of the host-guest pairing. In contrast, BEA showed weaker binding affinity for isatin, also adhering to a 1:1 stoichiometry but with less pronounced interactions. These findings suggest a hierarchy in host-guest interaction strengths, with P[5]A being more effective in complexing isatin.

Further insights were provided by UV-visible spectroscopy, which reinforced the NMR findings by illustrating differences in the spectroscopic signatures of the complexes. The analysis confirmed that P[5]A formed a stronger and more stable complex with isatin compared to BEA. This superior binding affinity of P[5]A can be attributed to its structural and chemical properties, which likely facilitate stronger non-covalent interactions, such as π-π stacking with isatin.

Moreover, a study by Chandra *et al*. (2020) has demonstrated the synthesis of serotonin inclusion complexes of cucurbit[7]uril. The NMR experiments revealed the inclusion complexes have been formed at the ratio of 1:1 and elucidated the geometry of the synthesized complexes.

In a similar study, the synthesis of cucurbituril-based supramolecular host–guest complexes with four-armed p-xylene derivative as a guest molecule formed an inclusion complexes at 1:2 ratio and the proton NMR studies revealed that there was no changes in the chemical shift which iterated their stability up to 90 ºC (Wang *et al*., 2024b).

In summary, the Phase I findings established that P[5]A exhibited a superior capacity to encapsulate isatin, making it a promising candidate for drug delivery applications. The combination of NMR and UV-visible spectroscopy provided a comprehensive understanding of the molecular interactions, enabling a clearer distinction between the binding capabilities of P[5]A and BEA.

PHASE II

4.2. Antibacterial Efficacy of Synthesized Pillar[5]arene-Isatin Inclusion Complexes against Clinical Pathogens

4.2.1. Antibacterial efficacy of pillar[n]arenes-isatin inclusion complexes

The antibacterial potential of the selected compounds such as isatin, pillar[n]arenes and their inclusion complexes were assessed against various Gram-positive and Gram-negative pathogenic bacteria namely, *Bacillus subtilis*, *Staphylococcus aureus Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella paratyphi* A, and *Escherichia coli*. Based on the results of stoichiometric binding studies in Phase I, the equimolar concentrations of pillar[5]arene-isatin inclusion complexes was taken for the analysis. The eqimolar concentrations of pillar[5]arene-isatin inclusion complexes corresponds to 15 mg/ml of pillar[5]arene and 3 mg/ml of isatin. In the case of BEA-isatin inclusion complexes, the equimolar concentration corresponds to 17 mg/ml of BEA and 3 mg/ml of isatin. Since chloramphenicol has a wide spectrum of antibacterial efficacy against both pathogenic Gram-positive and Gram-negative bacteria, it was used as antibiotic control throughout the research work. The results of antibacterial efficacy are depicted in Figure 26 and Plate 3.

From the results, all the selected compounds were noted with a broad range of antibacterial activities against the selected bacterial pathogens. The drug, isatin alone was found to have a broader antimicrobial activity against the selected bacterial pathogens. Maximum antibacterial activity of isatin was recorded against *Staphylococcus aureus* (7.7±0.6 mm) and *Pseudomonas aeruginosa* (8±0.6 mm). Compared to BEA, pillar[5]arene has exhibited potential inhibitory activity against all the selected pathogens. BEA has demonstrated less inhibitory activities against the most of the selected bacterial pathogens.

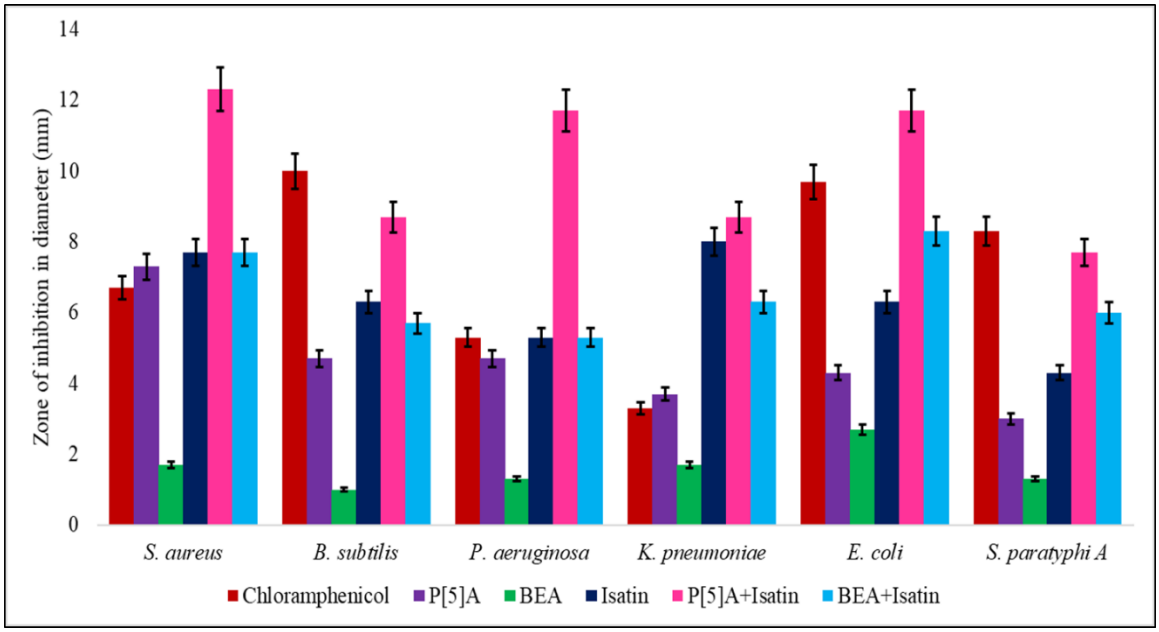


Figure 26: Antibacterial efficacy of pillar[5]arene-isatin inclusion complexes against clinical pathogens

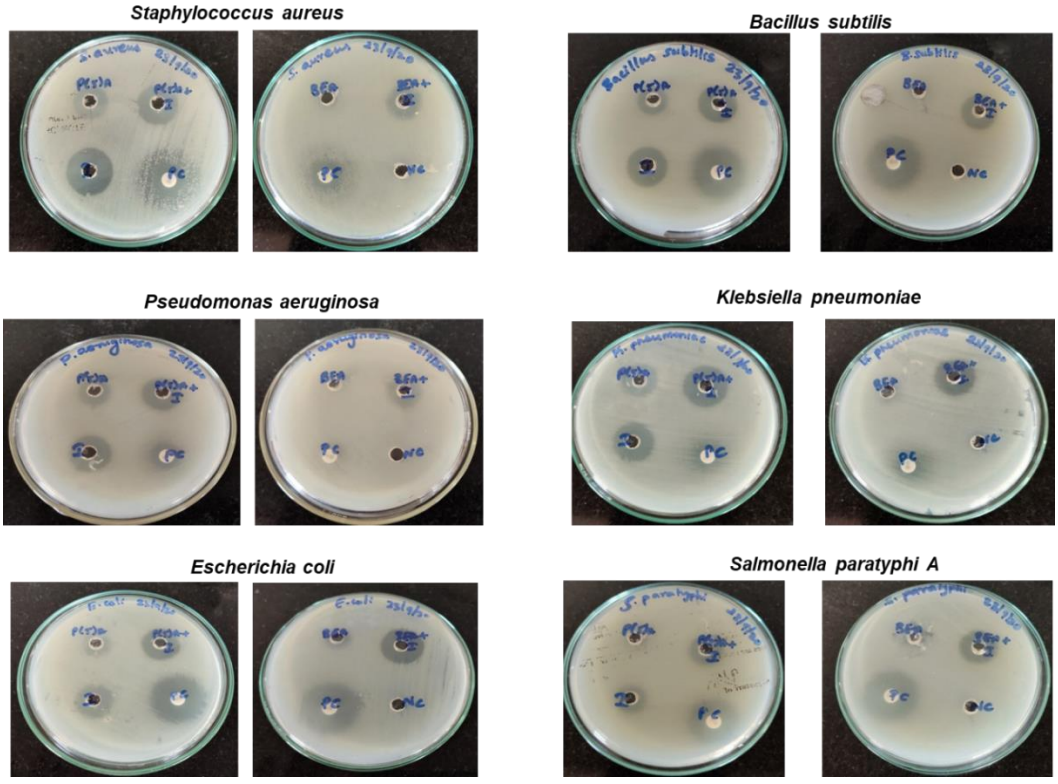


Plate 3: Antibacterial efficacy of pillar[5]arene-isatin inclusion complexes against clinical pathogens

On the other hand, pillar[5]arene-isatin inclusion complexes exhibited greater antibacterial activities against tested bacterial pathogens. Among them, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were found to be more susceptible to the pillar[5]arene-isatin inclusion complexes and the zone of inhibition was found to be 11.7±1.2 mm and 12.3±0.6 mm, respectively. Paradoxically, BEA-isatin inclusion complexes demonstrated less inhibitory activity against all the tested bacterial pathogens. DMSO acted as the negative control or solvent control and was not detected with inhibitory zones against the bacterial pathogens. Chloramphenicol (antibiotic control) was noted for its significant antibacterial activities against the selected bacterial pathogens.

Several scientific studies support the antibacterial activities of natural compounds with supramolecular hosts against several pathogenic fungi.

The host-guest complexation of fluoroquinone, danofloxacin with the macrocyclic host, cucurbit[7]uril (CB7) has positively inhibited the growth of four major pathogens namely, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella paratyphi* (El-Sheshtawy *et al*., 2018).

The host-guest complexation between the β-cyclodextrin units embedded hyaluronic acid chain and azobenzene moiety linked with two enoxacin units have demonstrated their enhanced antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Liu *et al*., 2022).

Another versatile supramolecular structure composed of streptavidin-eosin and biotinylated immunoglobulin G (IgG) has been found to inhibit effectively the growth of *Staphylococcus aureus* (Mussini *et al*., 2022).

Xie *et al*. (2021) had also reported the enhanced antibacterial activity of host-guest driven self-assembly between a branched cyclodextrin and cationic linear peptides affixed with azobenzene side chains against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis*.

The antibacterial activities of pillar[5]arene derivatives against the prominent bacterial pathogens namely, *Staphylococcus aureus*, *Salmonella typhimurium* and *Escherichia coli* were recorded and this emphasized the antibacterial nature of

pillar[5]arene and could be exploited as a potential choice to reduce antimicrobial resistance (Atacan *et al*., 2023).

Therefore, the synthesized supramolecular host-guest complex, pillar[5]arene-isatin inclusion complexes was found to have superior antibacterial activities against the selected bacterial pathogens. Among the selected bacterial pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were recorded as the most susceptible pathogens to the tested compounds such as isatin, pillar[5]arene and their inclusion complexes. In addition, these two pathogens have been noted as the most prominent pathogens involved in wound infections which may further worsen and delay the wound healing processes. Hence, further studies were carried out to elucidate the biotherapeutic potentials of isatin (drug), pillar[5]arene (drug carrier) and pillar[5]arene-isatin inclusion complexes against the two most susceptible pathogens at the wound sites namely, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

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

Minimum inhibitory concentration (MIC) is referred to as the concentration of selected compounds that have the ability to inhibit the growth of bacteria. On the other hand, minimum bactericidal concentration (MBC) is the concentration of selected antimicrobial compounds where no growth of bacteria is observed upon the action of antimicrobial compounds. Hence, the selected compounds such as pillar[5]arene, isatin and pillar[5]arene-isatin inclusion complexes were analyzed to determine the MIC and MBC against the prominent bacterial pathogens in wound infections namely, *Staphylococcus aureus* and *Pseudomonas aeruginosa* using resazurin based microbroth dilution method. The MIC, 1/2 MIC and MBC of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa* are provided in Table 6.

Table 6: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of pillar[5]arene-isatin inclusion complexes

S. No	Compounds	*Staphylococcus aureus*			*Pseudomonas aeruginosa*		
		1/2 MIC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	1/2 MIC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
1.	Isatin	0.375	0.75	1.5	0.75	1.5	3.0
2.	Pillar[5]arene	1.625	3.25	6.5	3.75	7.5	15.0
3.	Pillar[5]arene-isatin inclusion complexes	0.14	0.28	0.56	0.28	0.56	1.125
4.	Chloramphenicol	0.125	0.25	0.5	0.250	0.5	1.0

The results revealed that 0.75 mg/ml of isatin was required to inhibit the growth of *Staphylococcus aureus* and the bacteria was able to continuously grow when the concentration was below 0.75 mg/ml. Hence, the minimum inhibitory concentration of isatin against *Staphylococcus aureus* was considered as 0.75 mg/ml. Parallel to this, the inhibition of *Pseudomonas aeruginosa* upon the action of isatin was observed at 1.5 mg/ml and was noted as the MIC of isatin against *Pseudomonas aeruginosa*. Meanwhile, pillar[5]arene, the supramolecular host was found to have a MIC of 3.25 mg/ml and 7.5 mg/ml against the pathogenic bacteria of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. In comparison with the drug (isatin) and drug carriers (pillar[5]arene) alone, the supramolecular inclusion system (pillar[5]arene-isatin inclusion complexes) was found to effectively inhibit the growth of *Staphylococcus aureus* at 0.28 mg/ml (0.3 mM) and 0.56 mg/ml (0.6 mM). The viable bacterial endurance was observed in wells with the negative control (DMSO) alone. *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth was inhibited at the concentration of 0.25 mg/ml and 0.50 mg/ml of chloramphenicol. Further, the minimum bactericidal concentration (MBC) of the selected compounds was assessed.

The minimum bactericidal concentration (MBC) of the selected compounds was evaluated by plating on Luria-Bertani agar plates. The plates with no growth after 24 hours of incubation were observed as MBC. Isatin exhibited its complete killing action on *Staphylococcus aureus* at the concentration of 1.5 mg/ml and was recorded as the minimum bactericidal concentration. Similarly, *Pseudomonas aeruginosa* was completely killed at 3.0 mg/ml of isatin. Correspondingly, pillar[5]arene was found to show their bactericidal activity against *Staphylococcus aureus* (MBC; 6.5 mg/ml) and *Pseudomonas aeruginosa* (MBC; 15.0 mg/ml). However, the synthesized pillar[5]arene-isatin inclusion complexes exhibited their bactericidal potential at their minimum concentration compared with the isatin and pillar[5]arene at the individual actions. The MBC of the pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was noted at 0.56 mg/ml (0.6 mM) and 1.125 mg/ml (1.2 mM), respectively. The viable bacterial population was observed in the plates with DMSO inoculated wells. Paradoxically, chloramphenicol exhibited its bactericidal potential against the selected pathogens at concentrations of 0.5 mg/ml (*Staphylococcus aureus*) and 1.0 mg/ml (*Pseudomonas aeruginosa*).

On par with our results, fluoroquinolone inclusion complexes of sulfonato-C-methylresorcin[4]arene have showed their bactericidal activity at 12 µg/ml against *Staphylococcus aureus* and 4 µg/ml against *Pseudomonas aeruginosa* (Panigrahi *et al*., 2024).

Interestingly, the minimum bactericidal concentration of vancomycin against *Escherichia coli* was ~15 to 40 fold increased after forming inclusion complexes with guanidinium-functionalized helical polymers, iterating the importance of host-guest chemistry (Liu *et al*., 2022).

Similarly, the ambroxol hydrochloride inclusion complexes of cucurbit[7]uril exhibited a superior bacteriostatic and bactericidal potential against *Pseudomonas aeruginosa* and *Escherichia coli* (Li *et al*., 2020).

Subakaeva *et al*. (2023) have emphasized the antibacterial activity of strptocide inclusion complexes of pillar[5]arene functionalized with sulphonamide

fragments against various pathogenic bacteria with the minimum inhibitory concentration ranges of 182.49 to 364.98 µg/ml.

Similarly, metal based (Zinc and Copper) inclusion complexes of pillar[5]arene with repeated quinolone units have demonstrated their potential inhibitory activities against *Staphylococcus aureus*, *Bacillus cereus*, *Candida albicans* and *Pseudomonas aeruginosa* with the MIC values of 23.43 µg/ml, 46.87 µg/ml, 93.75 µg/ml and 187 µg/ml, respectively (Tosun *et al*., 2024).

All these findings have supported our findings in a way that the pillar[5]arene-isatin inclusion complexes was found to exhibit its bacteriostatic and bactericidal activities against the selected prominent pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, compared with isatin alone. Hence, the supramolecular based host-guest chemistry potentially influencing the antibacterial activities of the small compounds. In this context, the synergistic potential of the host and guest molecule in inhibiting the bacterial growth was further performed.

4.2.3. Synergistic activities of drug (isatin) and drug carrier (pillar[5]arene) against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

The checkerboard method was generally applied to determine the synergistic (positive combined effects) or antagonistic (negative combined effects) activity of two drugs. The synergistic potential of the isatin and pillar[5]arene was assessed against *Staphylococcus aureus* and *Pseudomonas aeruginosa* by checkerboard method. In general, for a synergistic activity, the MIC of one drug will be decreased in the presence of another drug. In line with this, the reduction of MIC of isatin in the presence of pillar[5]arene was determined by analyzing 77 combinations at different concentrations of the two compounds in the medium. FICI (fractional inhibitory concentration index) is generally employed to determine the potential synergistic or antagonistic activity of the selected compounds against the pathogenic bacteria. It was calculated using the interpretations followed by Te Dorsthorst *et al*., 2002. It indicated that FICI<1 shows synergistic activity, while FICI =1 shows partial synergistic or additive activity and FICI>1 shows indifferent

or antagonistic activity. The results of FICI were calculated as 0.75 with isatin and pillar[5]arene against *Staphylococcus aureus* and it has indicated that isatin and pillar[5]arene were found to have a synergistic activity to target bacterial endurance and persistence. The FICI was found to be 1.0 with isatin and pillar[5]arene against *Pseudomonas aeruginosa*. It has exhibited a partial synergism or additive function of isatin and pillar[5]arene towards the inhibition of *Pseudomonas aeruginosa*.

In line with our study, various combinations of drug molecules have demonstrated their synergistic activities.

Usually individual drugs may easily prone to drug resistance. In this context, the combination of glutamine inhibitor and doxorubicin showed a synergistic effect with the FICI values less than 1 (Duan *et al*., 2022).

A similar study by Truszkowska *et al*. (2024) have determined the enhanced antimicrobial activity of daptomycin in complexes with the drug delivery system of ethyl lauroyl arginate against various pathogenic bacteria and fungi.

The combined action of nisin with the drug delivery polymers such as glycol chitosan and/or ε-polylysine has enhanced the antimicrobial activity of nisin up to 6 fold against *Staphylococcus aureus* (Flynn *et al*., 2022).

Nanocomplex carriers loaded with tobramycin antibiotics have indicated their synergistic activity in inhibiting the growth and biofilms of *Pseudomonas aeruginosa* (Finbloom *et al*., 2023).

All these investigations further reinforce the fact that the isatin and pillar[5]arene have enormously inhibited the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* based on the concept of synergism and additive functions. Auxiliary, the mechanistic action of pillar[5]arene-isatin inclusion complexes was investigated.

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

The mechanistic action of the selected drug (isatin), drug carrier (pillar[5]arene) and pillar[5]arene-isatin inclusion complexes on *Staphylococcus*

aureus and *Pseudomonas aeruginosa* were analyzed by time kill kinetics, assessing membrane integrity and permeability damages.

4.2.4.1. Bacterial time-kill kinetics profile of pillar[5]arene-isatin inclusion complexes

The efficacy of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes against the bacterial pathogens, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were evaluated by bacterial time-kill kinetics. The bacterial pathogens were treated with the MIC of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes (0.75 mg/ml, 3.25 mg/ml, 0.28 mg/ml and 1.5 mg/ml, 7.5 mg/ml, 0.56 mg/ml). The activity of selected compounds against the bacterial pathogens were compared with the standard drug, chloramphenicol. The bacteria grown in Luria-Bertani (LB) broth with and without the selected compounds were plated on LB agar plates to identify their viability at various time intervals including 0, 60, 120 and 180 minutes. The results of bacterial time kill kinetics of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes against *Pseudomonas aeruginosa* and *Staphylococcus aureus* are depicted in Figure 27.

The log CFU/ml value was calculated to determine the bacterial time-kill kinetics of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes. The bacterial-time kill kinetics of the selected compounds on the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were analyzed for about 180 minutes of treatment. Isatin was found to decrease the number of *Staphylococcus aureus* colonies at 120 minutes of treatment compared with the control. Similarly, pillar[5]arene also exhibited moderate bacterial time-killing properties against *Staphylococcus aureus* in comparison with isatin. The log CFU/ml values of pillar[5]arene-isatin inclusion complexes treated *Staphylococcus aureus* were found to be 4.2±0.02 at 60 minutes, 3.99±0.02 at 120 minutes and 3.9±0.008 at 180 minutes. It revealed that pillar[5]arene-isatin inclusion complexes had a wide spectrum of antimicrobial activity against *Staphylococcus aureus* by inhibiting their growth and endurance from 60 minutes and higher reduction of *Staphylococcus aureus* number in 120 minutes followed by 180 minutes. Similarly, the antibiotic treated *Staphylococcus aureus* colonies also gradually declined over

time and the log CFU/ml was 4.2±0.01 at 60 minutes, 4.01±0.02 at 120 minutes and 3.8±0.05 at 180 minutes. *Staphylococcus aureus* continuously flourished in control (without compounds) and negative control (with DMSO) and the log CFU/ml at 180 minutes were found to be 4.66±0.001 and 4.65±0.02, respectively.

Likewise, in control (medium with bacteria alone) and negative control (medium with DMSO), the growth of *Pseudomonas aeruginosa* was found be gradual and the log CFU/ml was remarkably increased over the time. It indicated the substantial growth of *Pseudomonas aeruginosa* and their growth was not disturbed with the addition of the solvent, DMSO. Isatin has the ability to kill the bacteria with the time and the great number of reduction in colonies of *Pseudomonas aeruginosa* was observed within 60 minutes of treatment. The reduction of *Pseudomonas aeruginosa* colonies at 120 minutes of treatment with pillar[5]arene was found to be 4.17 log CFU/ml. This exhibited a moderate bacterial-time killing potential against *Pseudomonas aeruginosa*.

Paradoxically, the synthesized pillar[5]arene-isatin inclusion complexes was observed to drastically reduce the number of colonies of *Pseudomonas aeruginosa* within 60 minutes and the log CFU/ml was found to be 4.04±0.03. The control, without the addition of selected compounds, was noted with no or fewer changes in the colony reduction and the number of colonies was found to be 4.3±008 log CFU/ml at 60 minutes. Chloramphenicol was also noted with maximum reduction of number of colonies and the logCFU/ml at 60 minutes of treatment interval was found to be 3.9±0.03. Isatin also exhibited its potential antimicrobial activity after 120 minutes of treatment and the logCFU/ml was found to be 4.06±0.03.

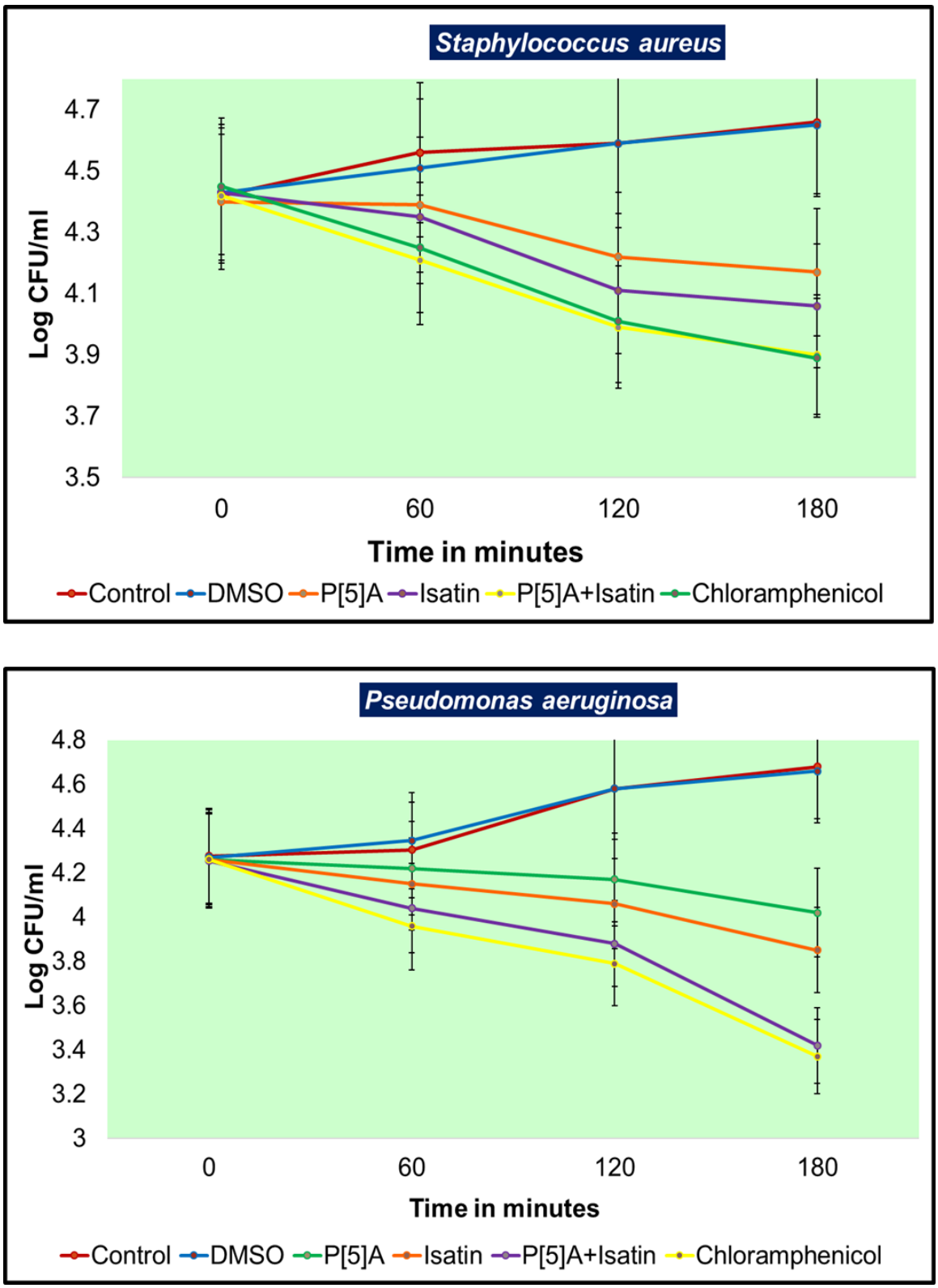


Figure 27: Bacterial time kill kinetics of pillar[5]arene-isatin inclusion complexes treated bacterial pathogens

Various studies have provided scientific evidences on the interaction of synthesized supramolecular host-guest compounds with pathogenic bacteria that affect the growth of bacterial pathogens in a time-dependent manner.

A study by Majumdar *et al*. (2023) has synthesized metals (Hg/Cu/Pb) in complexes with M-SCN (thiocyanate) supramolecular structures. Among them, Hg-Complex has demonstrated a complete inhibition of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Bacillus subtilis* at 4-8 hours of incubation time.

The synthesized daptomycin with ethyl lauroyl arginate with the interaction of ion pairing has demonstrated their greater time-kill kinetic profiles against *Staphylococcus epidemidis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* (Truszkowska *et al*., 2024).

An identical study by Liu *et al*. (2023) has elucidated the extreme reduction in the log CFU values from 6.4 to 4.5 within one hour of treatment with lipoic acid inclusion complexes of β–cyclodextrin in *Escherichia coli*.

A ciprofloxacin loaded self-emulsifying drug delivery systems have dropped the cell viability of *Pseudomonas aeruginosa* from 1.9×10^5 to below 10^2 within 4 hours of treatment which indicated their potential bactericidal activities of the compounds in the drug delivery systems (Asghar *et al*., 2022).

Henceforth, the results of the bacterial time-kill kinetics envisaged the potential antimicrobial efficacy of pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The selected pathogenic bacteria were susceptible to the minimum inhibitory concentration of pillar[5]arene-isatin inclusion complexes.

4.2.4.2. Cellular leakage of protein and glucose in pillar[5]arene-isatin inclusion complexes treated bacterial pathogens

Cell wall components are important in maintaining cell integrity and permeability. The bacterial cell membrane is mainly composed of macromolecules namely, protein and glucose. The bacterial cell membrane is a barrier of selective

permeability that organizes exchanges between the intra and extracellular environment by providing an intracellular environment suitable for the various vital processes. The mechanism of action of the selected compounds on the bacterial cell wall and cell membrane permeability can be assessed by evaluating the release of protein and glucose in the extracellular medium after a treatment period of about 18 hours. Hence, the study focuses on determining the activity of selected compounds in damaging the cell walls of the bacterial pathogens. The results of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes on the cell wall of bacterial pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are provided in Figure 28.

From the results, it was revealed that the selected compounds namely, isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes have acted on the cell membrane which was clearly indicated by the release of proteins in the extracellular environment. The medium with bacteria alone and solvent control have demonstrated that there was no membrane damage against the selected pathogens. The content of protein from the cell walls of isatin treated *Staphylococcus aureus* and *Pseudomonas aeruginosa* was found to be 56.14 µg/ml and 64.33 µg/ml, respectively. It revealed that the isatin was potentially targeting the bacterial cell membrane and was evident by the released content of protein from *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Pillar[5]arene has exhibited a moderate activity on damaging the cell membrane on *Staphylococcus aureus* (35.67 µg/ml) and *Pseudomonas aeruginosa* (31.58 µg/ml).

Conversely, the released content of protein from the cell wall to the extracellular medium and the content was observed to be 76.61 µg/ml (*Staphylococcus aureus*) and 97.08 µg/ml (*Pseudomonas aeruginosa*) after the incubation period of 18 hours. The results have iterated pillar[5]arene-isatin inclusion complexes were found to be remarkably damaging the cell membrane of the selected bacterial pathogens. The results were well correlated with the positive control, chloramphenicol with the drastic release of cellular components from treated bacterial cell wall.

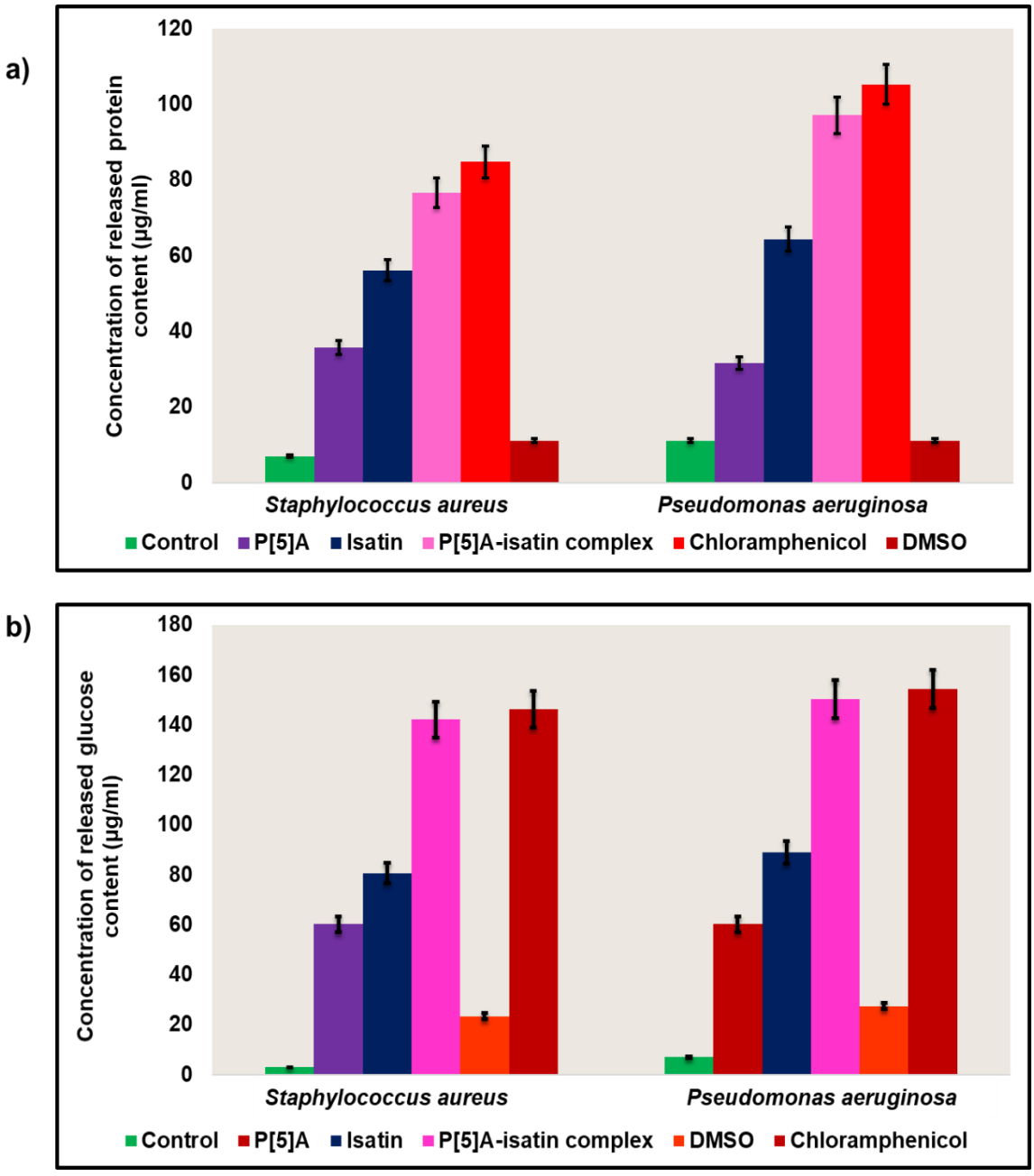


Figure 28: Cellular leakage of protein and glucose in pillar[5]arene-isatin inclusion complexes treated bacterial pathogens.

(a) Leakage of protein (b) Leakage of glucose

The results have envisaged that the significant effect of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes was observed on the cell wall component (glucose) of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In control, only a trace amount of glucose was released after the incubation period of 18 hours and it was in line with the results obtained from the medium of bacteria containing negative control as DMSO. Isatin effectively acted on the cell wall membrane and 80.7 µg/ml and 88.89 µg/ml of glucose content were released from the cell wall of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. Contrarily, the pillar[5]arene treated bacterial pathogens was observed with the glucose release of 60.23 µg/ml which iterated their moderate action on the cell membrane damaging potentials against the selected bacterial pathogens. Inversely, the glucose content from the pillar[5]arene-isatin inclusion complexes treated *Staphylococcus aureus* and *Pseudomonas aeruginosa* was found to be 142.1µg/ml and 150.29 µg/ml, correspondingly. It envisaged that the pillar[5]arene-isatin inclusion complexes was noted with a significant effect on cell wall of the selected bacterial pathogens. Seemingly, chloramphenicol was also observed with their targeted activity on bacterial cell membrane.

Hence, the synthesized pillar[5]arene-isatin inclusion complexes had noticeably decreased the content of protein and glucose from the cell walls of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, thus ensuring the greater impact on the membrane integrity of the selected bacterial pathogens by targeting the cell membrane components.

4.2.4.3. Morphological characterization of pillar[5]arene-isatin inclusion complexes treated cell membrane of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

The cell membrane damaging potential of the synthesized pillar[5]arene-isatin inclusion complexes treated *Staphylococcus aureus* and *Pseudomonas aeruginosa* cell membrane was observed using scanning electron microscopy. The scanning electron microscopic images of cell membrane damage are depicted in Figure 29.

The changes in the morphological view of the bacterial cell membrane of the treated and untreated pathogens were perspicuously demonstrated. In control (untreated), the bacterial surface was found to be very smooth and intact with typical characteristics of cell membranes. Incongruously, the surface of *Staphylococcus aureus* was extremely damaged upon the action of pillar[5]arene-isatin inclusion complexes. The cocci-shaped *Staphylococcus aureus* cells were observed as irregular in shape with aggravated damage. In addition, the incredible leakage of cellular components was noticed by the large pits and holes on the surface of *Staphylococcus aureus* cells. Besides, parallel results were observed with the pillar[5]arene-isatin inclusion complexes treated *Pseudomonas aeruginosa* cells. The rod-shaped *Pseudomonas aeruginosa* surface was found to be clustered and regular in shape (control). Meanwhile, extortionate damages on the cells of inclusion complexes treated *Pseudomonas aeruginosa* cells were observed and the leakage of cellular components was confirmed by the perforated cellular structures.

Various scientific investigations have confirmed the cytoplasmic release of bacterial cell components upon the action of antimicrobial compounds, which would greatly influence the endurance of antimicrobial resistance.

One such study by Yang *et al*. (2023) has revealed the physical disruption of pathogenic bacterial membranes and subsequent leakage of cytoplasmic components in pillar[5]arene with pyridinium groups treated *Staphylococcus aureus* and *Escherichia coli* and it highlighted the superior microbicidal potential of the supramolecular structures.

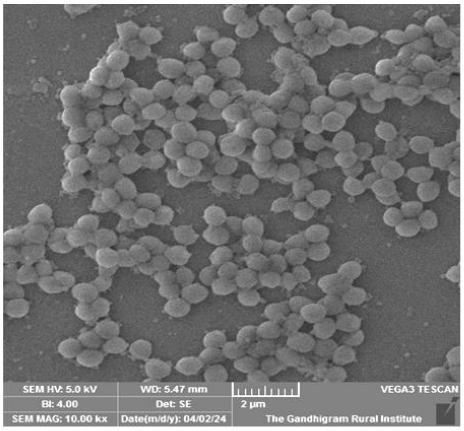
Novel quinazolonthiazoles derivatives were found to disrupt the bacterial membrane permeability to induce bacterial death in *Pseudomonas aeruginosa* (Wang *et al*., 2021).

Moreover, a study by Sun *et al*. (2021) has explained the antibacterial activity of synthesized berberine-derived azolyl ethanols by means of their ability to disintegrate the bacterial membranes of *Escherichia coli*.

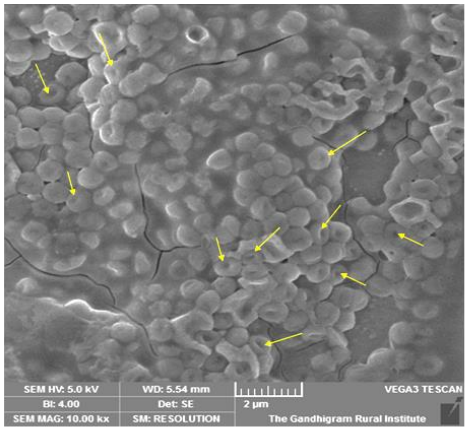
The lipoic acid inclusion complexes of β–cyclodextrin exhibited a severe membrane damage and substantial leakage of cellular components in *Escherichia*

coli compared to mechanistic action of lipoic acid alone on the bacterial cell membrane (Liu *et al*., 2023).

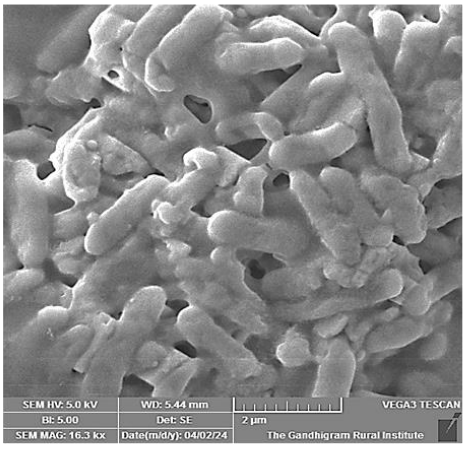
Benzimidazolium inclusion complexes of ionic liquids−β-cyclodextrin supramolecular systems have been found to disrupt the *Escherichia coli* and *Bacillus subtilis* cell wall which leads to the leakage of cellular components in the extracellular environment (Sarkar *et al*., 2021).



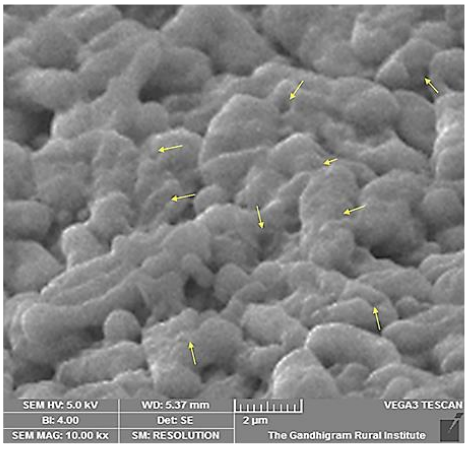
Untreated *Staphylococcus aureus*



Pillar[5]arene-isatin inclusion complexes treated *Staphylococcus aureus*



Untreated *Pseudomonas aeruginosa*



Pillar[5]arene-isatin inclusion complexes treated *Pseudomonas aeruginosa*

Figure 29: Morphological characterization of pillar[5]arene-isatin inclusion complexes treated pathogenic bacteria

Physical damage on the bacterial cell membrane was found to be irreversible, hence the bacterial pathogens could not be able to form their inner membrane cells which ultimately leads to the death of the pathogenic bacteria. In this, the pillar[5]arene-isatin inclusion complexes exhibited their complete killing action on *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

The results of phase II revealed that the drug (isatin), drug carriers (pillar[5]arene, BEA) and the pillar[5]arene-isatin inclusion complexes demonstrated potential antibacterial activities against several Gram-positive and Gram-negative bacterial pathogens. A broad range of antibacterial activities was observed in isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes compared to BEA and BEA-isatin inclusion complexes against the selected bacterial pathogens. The minimum inhibitory and minimum bactericidal concentration of the synthesized pillar[5]arene-isatin inclusion complexes showed a superior bacteriostatic and bactericidal potential against *Staphylococcus aureus* and *Pseudomonas aeruginosa* compared with the action of isatin and pillar[5]arene separately. The checkerboard method of analysis revealed that the selected drug (isatin) and drug carrier (pillar[5]arene) were found to have synergistic activity against *Staphylococcus aureus* and partial synergistic activity against *Pseudomonas aeruginosa*. The mechanism of antibacterial action of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes have strong membrane damaging potential against the selected bacterial pathogens. It was further evident by scanning electron microscopy where the perforated and unorganized cell structures were observed in pillar[5]arene-isatin inclusion complexes treated *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Therefore, the synthesized pillar[5]arene-isatin inclusion complexes has the ability to possess bacteriostatic and bactericidal potentials against the selected bacterial pathogens to completely eradicate them at the target sites.

PHASE III

4.3. Antibiofilm Potential of pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

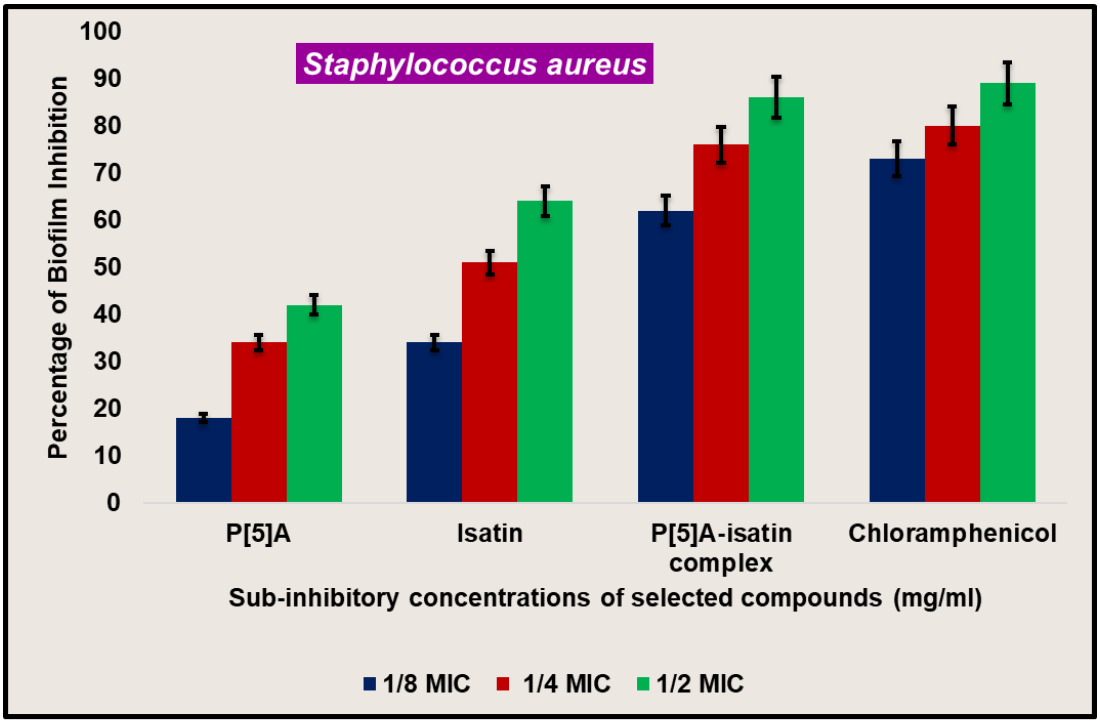
4.3.1. Biofilm inhibitory potential of pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

A self-secreted extracellular polymeric matrix envelops three-dimensional complex microbial communities known as biofilms, which are found all over nature. Multiple factors such as relative impermeability, sub/co-populations and physiological status of strains have been known to contribute to biofilm persistence which renders the commercial antibiotics ineffective and promotes multidrug resistance. Biofilms are protective structures formed by bacterial communities and are notoriously difficult to disrupt and serve as a major barrier to effective antimicrobial treatment. Recent studies have also iterated that, the existing antibiotic therapy may eliminate planktonic forms of biofilms but it does not have a potential effect in eradicating sessile forms of bacterial cells. The unique and deleterious effects of biofilms formed by pathogenic bacteria make them a putative target to disrupt biofilms and prevent their persistence. In this context, efforts have been taken to identify the potential lead compounds that do not harm the host tissue but effectively fight against bacterial biofilms. One such blooming strategy to target bacterial biofilm in this current research world is the supramolecular host-guest system. Macrocyclic host-guest systems have been noticed as potential suppressors of bacterial biofilm systems (Luotonen *et al*., 2024; Lahiri and Basu, 2024). The inhibition and eradication of biofilm on the biotic and abiotic cell surfaces are very difficult to overcome with the existing strategies. Hence, the selected compounds such as pillar[5]arene, isatin and their inclusion complexes were analysed for their antibiofilm potential against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The antibiofilm potential of pillar[5]arene, isatin and pillar[5]arene-isatin inclusion complexes, antibiotic control were studied at their 1/2 MIC, 1/4 MIC and 1/8 MIC (Table 7). The results are shown in Figure 30.

Table 7: Concentrations of drug and drug carriers used for antibiofilm assays

S. No	Compounds	*Staphylococcus aureus*			*Pseudomonas aeruginosa*		
		1/2 MIC (mg/ml)	1/4 MIC (mg/ml)	1/8 MIC (mg/ml)	1/2 MIC (mg/ml)	1/4 MIC (mg/ml)	1/8 MIC (mg/ml)
1.	Isatin	0.375	0.187	0.093	0.75	0.375	0.187
2.	Pillar[5]arene	1.625	0.8125	0.406	3.75	1.875	0.9375
3.	Pillar[5]arene-isatin inclusion complexes	0.14	0.07	0.035	0.28	0.14	0.07
4.	Chloramphenicol	0.125	0.0625	0.031	0.250	0.125	0.0625

From the results, the formation of biofilm was significantly inhibited by 1/2 MIC of pillar[5]arene-isatin inclusion complexes against both *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The biofilm inhibitory percentage of chloramphenicol was found to be 89% against *Staphylococcus aureus* and 86% against *Pseudomonas aeruginosa*. Isatin (1/2 MIC) alone exhibited a notable antibiofilm activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with the biofilm inhibitory percentage of 67% and 64%. The percentage of biofilm inhibition at 1/2 MIC of pillar[5]arene was found to be 42% and 48% against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, correspondingly. The results indicated the moderate biofilm inhibitory efficacy of pillar[5]arene host system. Specifically, in the case of *Staphylococcus aureus*, 1/2 MIC of the pillar[5]arene-isatin inclusion complexes achieved an impressive inhibition of biofilm formation by *Staphylococcus aureus* (84%) and *Pseudomonas aeruginosa* (86%). The 1/8 MIC of pillar[5]arene-isatin inclusion complexes was observed with moderate biofilm inhibition potential against the selected pathogens with <50% inhibitory profiles.



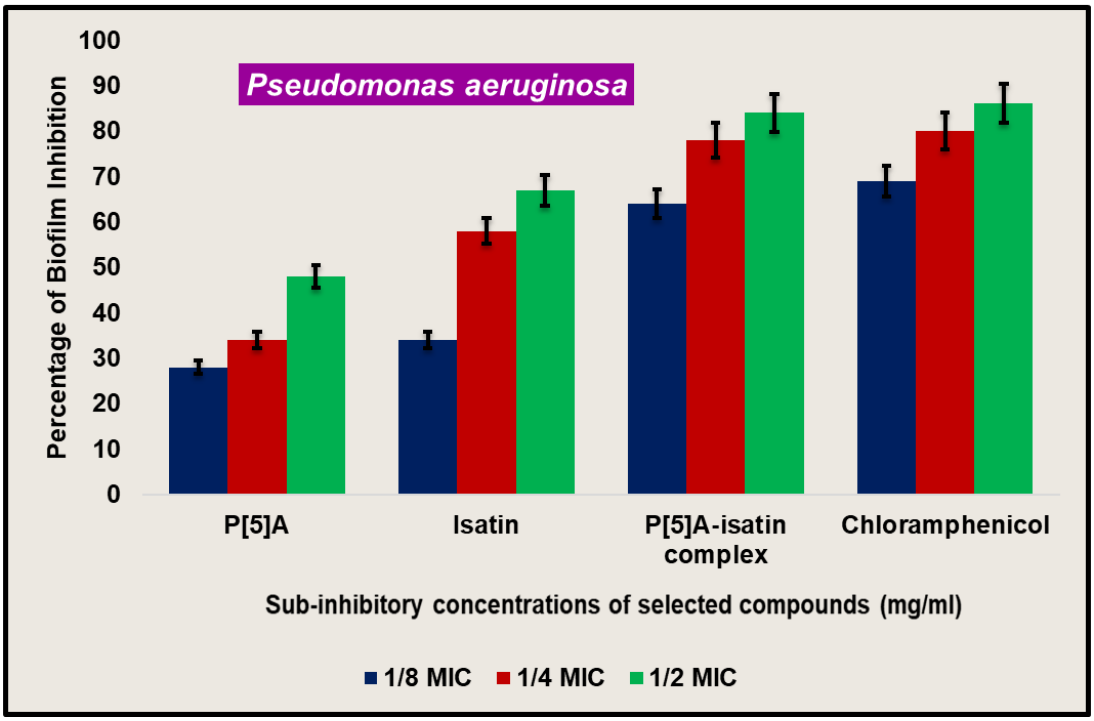


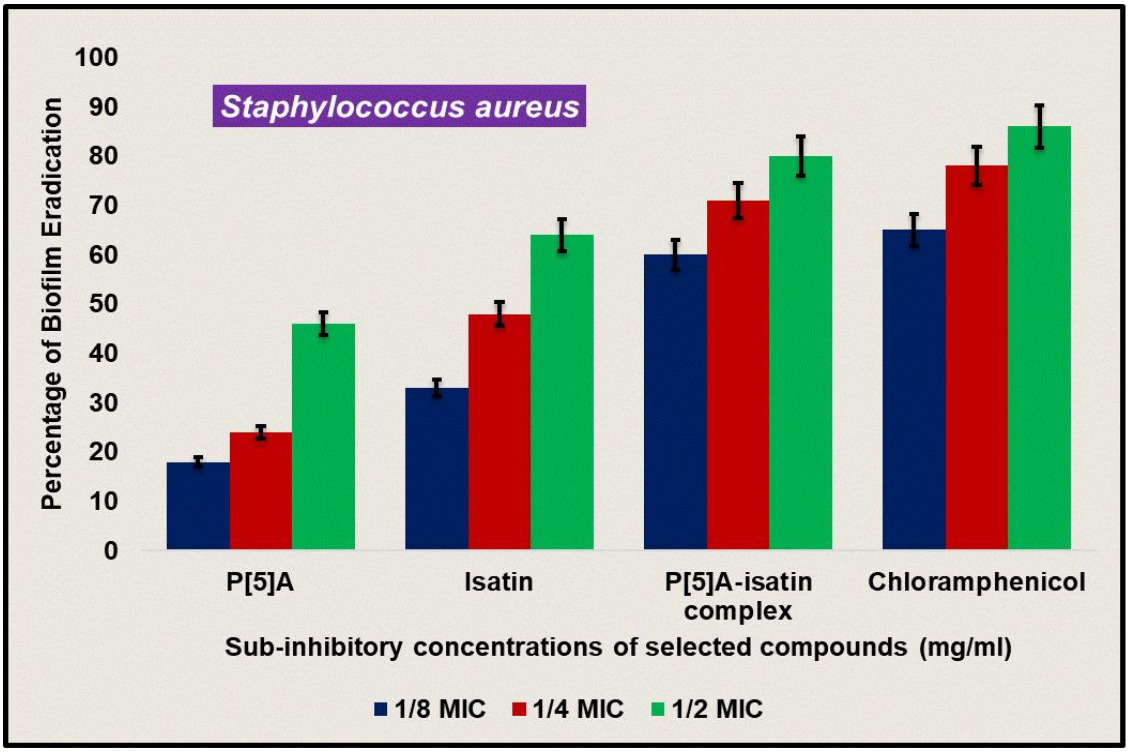
Figure 30: Effect of pillar[5]arene-isatin inclusion complexes on the formation of biofilm by *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Therefore, the biofilm inhibition results have highlighted the superior efficacy of the pillar[5]arene-isatin inclusion complexes compared to the individual components, namely P[5]A and isatin. The enhanced biofilm inhibition emphasized the synergistic interaction between isatin and pillar[5]arene within the synthesized inclusion complexes, which likely improves the bioavailability, stability, and activity of the active compound. The substantial reduction in biofilm formation by the inclusion complex suggests its potential as a powerful strategy to prevent bacterial colonization and persistence, particularly in *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

4.3.2. Biofilm eradication potential of pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Preformed biofilms are difficult to eradicate by conventional antibiotic therapy and they pose a severe threat in clinical and hospital settings. Hence, the biofilm eradication potential of the various concentrations of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes was evaluated on the preformed biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The results of biofilm eradication in selected drug, drug carriers and their inclusion complexes treated *Staphylococcus aureus* and *Pseudomonas aeruginosa* preformed biofilms are shown in Figure 31.

The results of biofilm eradication profiles of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes against the selected prominent bacterial pathogens revealed their significant potential in reducing the preformed biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* . 1/2 MIC of isatin has exhibited 64% biofilm eradication properties against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Only ~33% and 50% of biofilms were eradicated in 1/8 and 1/4 MIC of isatin-treated selected bacterial pathogens. The results revealed that the isatin was exponentially targeting the preformed biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* and the inhibitory activity was concentration-dependent.



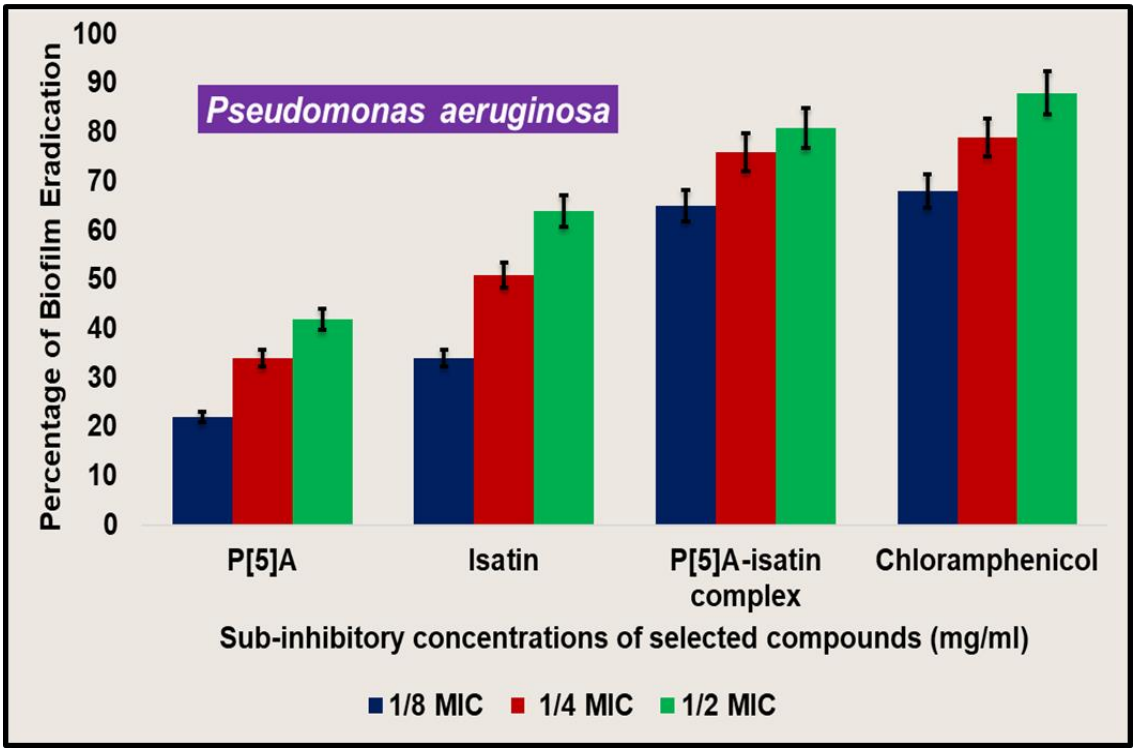


Figure 31: Effect of pillar[5]arene-isatin inclusion complexes on the eradication of preformed biofilms by *Staphylococcus aureus* and *Pseudomonas aeruginosa*

The moderate biofilm eradication potential of pillar[5]arene at 1/2 MIC was observed in both pathogens with a percentage biofilm eradication of 46% (*Staphylococcus aureus*) and 42% (*Pseudomonas aeruginosa*). Inconsistently, the biofilm eradication percentage of the synthesized pillar[5]arene-isatin inclusion complexes (1/2 MIC) was found to be 80% in *Staphylococcus aureus* and 81% in *Pseudomonas aeruginosa*. Even 1/8 MIC of the pillar[5]arene-isatin inclusion complexes have exhibited>65% of eradication percentage.

These observations are supported by Du *et al*. (2023), who deciphered the antibiofilm efficacy of biphen[4,5]arenes in the perspective of inhibiting the assembly of biofilms and eradicating the intractable preformed biofilms formed by *Staphylococcus aureus* and *Escherichia coli*.

β-cyclodextrin/Stilbene-Integrated supramolecular host-guest complexes have been possessing superior biofilm eradication potential at lower concentrations against *Xanthomonas oryzae* pv. *Oryzae*. The results mirrored in eradicating the planktonic biofilm cells and biofilm-enclosed pathogens (Zhou *et al*., 2024).

Luotonen *et al*. (2024) have explored the interference of pillar[5]arene-homoserine lactones inclusion complexes in QS-mediated virulence profiles of *Pseudomonas aeruginosa*. It revealed that the impaired formation of biofilms and the production of pyocyanin was observed in inclusion complexes treated with *Pseudomonas aeruginosa*.

Another investigation revealed the biofilm inhibitory activities of pillar[n]arene derivatives against various Gram-positive pathogens namely, methicillin-resistant *Staphylococcus aureus* and *Enterococcus faecalis*. Among them, pillar[5,6]arene have demonstrated potential biofilm inhibition activities against the selected bacterial pathogens (Kaizerman-Kane *et al*., 2021).

Guo *et al*. (2021) have demonstrated the prospective biofilm inhibitory activities of cefazolin sodium inclusion complexes of guanidinium-functionalized pillar[5]arene against *Staphylococcus aureus* and *Escherichia coli*.

As a result, the synthesized pillar[5]arene-isatin inclusion complexes demonstrated their superior activity in inhibiting the bacterial biofilms and

eradicating the preformed biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

4.3.3. Effect of sub-inhibitory concentrations of pillar[5]arene-isatin inclusion complexes on the swimming and swarming motility behavior of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

Biofilm formation is a complex, multi-step process that begins with bacterial adhesion to a surface or substratum, followed by colonization and subsequent development into a structured community. This initial attachment is primarily facilitated by flagellar motility, which allows bacteria to move towards and adhere to favorable surfaces. Once adhesion is established, the bacteria utilize twitching motility facilitated by the presence of type IV pili, which was used to spread across the surface and form microcolonies. These motility mechanisms are the critical steps in the early phases of biofilm development, as they enable the bacteria to explore, settle, and expand their presence on the surface. Flagella-driven motility, including swimming (individual movement in liquid environments) and swarming (coordinated movement across solid or semi-solid surfaces), plays a pivotal role in bacterial surface attachment. These motilities not only allow bacteria to overcome physical barriers and reach nutrient-rich environments but also ensure the initial distribution of cells across the surface. This spatial spread is essential for the formation of a robust biofilm, as it lays the groundwork for the subsequent secretion of extracellular polymeric substances (EPS) that stabilize the biofilm structure. Disrupting these motilities can significantly impair the ability of bacteria to form biofilms, highlighting their importance in bacterial survival and persistence, particularly in environments where biofilm formation provides protection from external threats such as antimicrobial agents and host immune responses.

In this context, the action of pillar[5]arene-isatin inclusion complexes on the motility behaviors of selected bacterial pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* was elucidated.

4.3.3.1. Effect of pillar[5]arene-isatin inclusion complexes on the swimming motility behavior of *Pseudomonas aeruginosa*

Unicellular behavior such as swimming motility necessitates a functioning polar flagellum with its motor-stator complex. The effect of isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes on the swimming motility behavior of *Pseudomonas aeruginosa* was evaluated on a soft agar medium (Table 8 and Plate 4).

Table 8: Swimming motility behavior of *Pseudomonas aeruginosa* treated with sub-inhibitory concentrations of pillar[5]arene-isatin inclusion complexes

S. No	Treatment groups	Diameter of swimming zone (mm)
1.	Bacterial Control	31.6±0.577
2.	Solvent Control (DMSO)	33.3±0.577
3.	Chloramphenicol	2.5±0.5
4.	Isatin	14.1±0.76
5.	Pillar[5]arene	24.1±0.28
6.	Pillar[5]arene-isatin inclusion complexes	5.3±0.577

The results have elucidated the potential influence of pillar[5]arene-isatin inclusion complexes on the swimming motility behavior of *Pseudomonas aeruginosa*. Untreated *Pseudomonas aeruginosa* (control) utilized their swimming motility action without hindrance in the soft agar medium and the zone of motility was 31.6±0.577 mm. Likewise, the bacteria with the solvent control were observed to freely spread on the media by exploiting its swimming motility patterns. Isatin alone has exerted its maximum activity and reduced the motility behavior of *Pseudomonas aeruginosa* with a diameter of 14.1±0.76 mm. On the other hand, a moderate potential of pillar[5]arene was documented on the motility patterns of *Pseudomonas aeruginosa* (24.1±0.28 mm). Conversely, pillar[5]arene-isatin inclusion complexes significantly reduced the motility behavior of *Pseudomonas*

aeruginosa. It was restricted to move on the soft agar medium pillar[5]arene-isatin inclusion complexes and was noted with 5.3±0.577 mm swimming motility behavior. The results were well correlated with the positive control, chloramphenicol.

4.3.3.2. Effect of pillar[5]arene-isatin inclusion complexes on the swarming motility behavior of *Pseudomonas aeruginosa*

A multicellular phenomenon known as swarming motility occurs when a population of bacteria moves quickly and cooperatively across a semisolid surface. Bacterial cell density, nutritional growth medium, and surface condition moisture content all have a significant influence on the swarming behavior of motility. Swarmer cell differentiation causes significant changes in metabolic bias and gene expression in addition to physical changes like an increase in flagella or cell elongation. These changes suggest that swarming is more than just a form of locomotion, but rather a complex lifestyle adaptation in response to specific medium conditions. Hence, the motility reducing effect of selected compounds such as isatin, pillar[5]arene and synthesized pillar[5]arene-isatin inclusion complexes was determined against *Pseudomonas aeruginosa*. Table 9 and Plate 5 depicts the efficacy of the selected compounds on the swarming motility behavior of *Pseudomonas aeruginosa*.

Table 9: Swarming motility behavior of *Pseudomonas aeruginosa* treated with sub-inhibitory concentrations of pillar[5]arene-isatin inclusion complexes

S. No	Treatment groups	Diameter of swarming zone (mm)
1.	Bacterial Control	48.3±0.57
2.	Solvent Control (DMSO)	33.6±1.5
3.	Chloramphenicol	1.8±0.76
4.	Isatin	16.1±0.76
5.	Pillar[5]arene	22.3±1.5
6.	Pillar[5]arene-isatin inclusion complexes	3.3±0.57

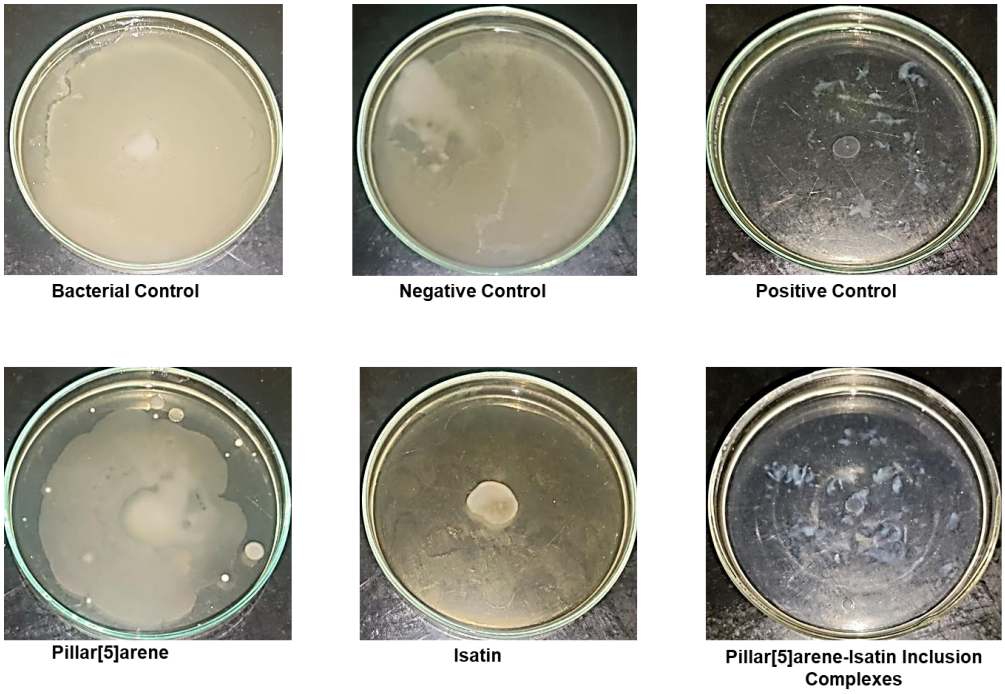


Plate 4: Influence of pillar[5]arene-isatin inclusion complexes on the swimming motility behavior of *Pseudomonas aeruginosa*

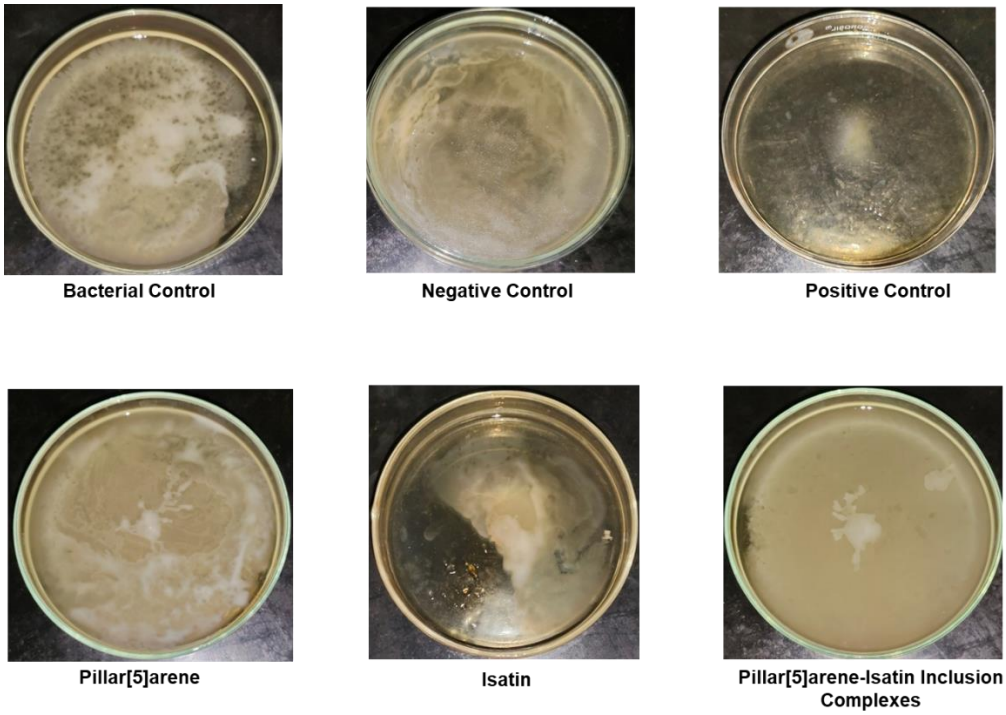


Plate 5: Influence of pillar[5]arene-isatin inclusion complexes on the swarming motility behavior of *Pseudomonas aeruginosa*

From the results, it was evident that the selected compounds efficiently influenced the swarming motility patterns of *Pseudomonas aeruginosa*. Isatin was found to reduce the swarming motility behavior of *Pseudomonas aeruginosa* at its sub-inhibitory concentration with a diameter of 16.1±0.76 mm. The soft agar medium with pillar[5]arene allowed the bacterium to spread for 22.3±1.5 mm and exhibited a moderate activity on swarming behavior of the pathogenic bacteria. The pillar[5]arene-isatin inclusion complexes in the media restricted the spreading of pathogenic bacteria on the medium and 3.3±0.57 mm of swarming motility was recorded. Positively, pillar[5]arene-isatin inclusion complexes had a substantial activity on the swarming motility behavior of *Pseudomonas aeruginosa*. Similarly, chloramphenicol also completely limits the swarming behavior of locomotion of *Pseudomonas aeruginosa* in the soft agar medium. Wholly, the pillar[5]arene-isatin inclusion complexes were noted with a significant reduction in the motility behavior of *Pseudomonas aeruginosa*.

4.3.3.3. Effect of sub-inhibitory concentrations of pillar[5]arene-isatin inclusion complexes on the swarming and hitchhiking motility behavior of *Staphylococcus aureus*

Staphylococcus aureus is a non-motile organism but it may passage on soft agar plates through the process of spreading. The tryptic soy agar (TSA) plates containing 0.4% agarose were prepared to determine the swarming motility patterns of *Staphylococcus aureus*. The inference in the swarming motility behavior of *Staphylococcus aureus* by the selected compounds such as isatin, pillar[5]arene and pillar[5]arene-isatin inclusion complexes was elucidated and the results are provided in Table 10 and Plate 6.

Table 10: Swarming motility behavior of *Staphylococcus aureus* treated with sub-inhibitory concentrations of pillar[5]arene-isatin inclusion complexes

S. No	Treatment groups	Diameter of swarming zone (mm)
1.	Bacterial Control	24.3±0.57
2.	Solvent Control (DMSO)	25.7±0.4
3.	Chloramphenicol	2.7±0.64
4.	Isatin	8.3±0.57
5.	Pillar[5]arene	11.3±0.57
6.	Pillar[5]arene-isatin inclusion complexes	4.7±0.6

Staphylococcus aureus has shown its swarming motility in a medium without containing selected compounds. In the control plate, *Staphylococcus aureus* was independently moving across the medium without any hindrance and the motility zone was found to be 24.3±0.57 mm. Similarly, the medium with DMSO also did not interfere with the swarming locomotion behavior of *Staphylococcus aureus* with a diameter of 25.7±0.4 mm. Chloramphenicol inoculated medium has drastically reduced the motility zone to 2.7±0.64 mm. The swarming motility pattern was decreased in a medium containing 1/2 MIC of isatin and the bacteria was able to move on the media with 8.3±0.57 mm. Moderate swarming motility reduction behavior was observed with pillar[5]arene. pillar[5]arene-isatin inclusion complexes significantly reduced the swarming motility behavior of *Staphylococcus aureus* with the reduction of swarming locomotion of about 4.7±0.6 mm. It iterated the potential activities of synthesized pillar[5]arene-isatin inclusion complexes in reducing the swarming motility behavior of *Staphylococcus aureus*.

Contrariwise, *Staphylococcus aureus* may use the opportunity to interact with neighbouring microbes, especially *Pseudomonas aeruginosa* for rapid spreading and it was observed on TSA plates with soft agar. This kind of motility

is often referred to as hitchhiking motility behavior of *Staphylococcus aureus*. A mid-log phase culture of *Staphylococcus aureus* alone and *Staphylococcus aureus* along with *Pseudomonas aeruginosa* was spotted at the centre of a TSA plate. The plate was subsequently incubated at ambient conditions to allow *Staphylococcus aureus* to swarm along with *Pseudomonas aeruginosa*. The results of hitchhiking motility behavior of *Staphylococcus aureus* along with *Pseudomonas aeruginosa* is given in Figure 32.

The results have demonstrated the potential activities of pillar[5]arene-isatin inclusion complexes in reducing the colonies of *Staphylococcus aureus* on mannitol salt agar. Isatin alone exerted its potential activities in alleviating *Staphylococcus aureus* cells in the media for locomotion and it reduced the cells to $0.97x10^4$ CFU/ml. A moderate reduction in hitchhiking motility pattern was noticed with the pillar[5]arene treated *Staphylococcus aureus*. The viable cells of *Staphylococcus aureus* ($0.74x10^4$ CFU/ml) were observed with the medium containing pillar[5]arene-isatin inclusion complexes. The medium with bacteria alone and along with solvent control have not interfered with the hitchhiking motility behavior of *Staphylococcus aureus* and the viable cells were found to be $3.1x10^4$ CFU/ml in control and $3.2x10^4$ CFU/ml in bacteria with negative control (DMSO). The antibiotic control was also noted with their potential activity in reducing the hitchhiking motility behavior of *Staphylococcus aureus* along with *Pseudomonas aeruginosa*.

Many scientific investigations have been conducted to determine the antibacterial activity of complexes by analyzing their interference with the motility behaviors of pathogenic bacteria.

One such study by Beltran-Torres *et al*. (2022) has determined the drastic reduction of complexes of acyclic bismuth with EDTA-based phenylene on the swimming motility pattern of *Pseudomonas aeruginosa* and *Escherichia coli*. Followed by, it significantly diminished the colony-spreading potential of *Staphylococcus aureus*.

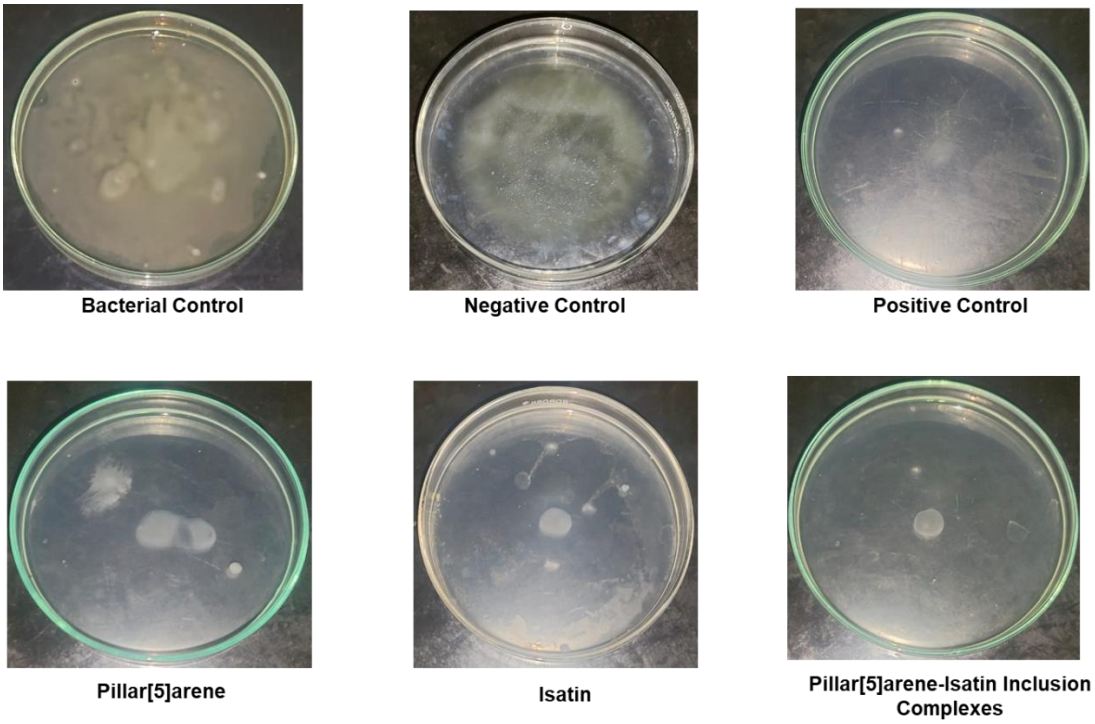


Plate 6: Influence of pillar[5]arene-isatin inclusion complexes on the swarming motility behavior of *Staphylococcus aureus*

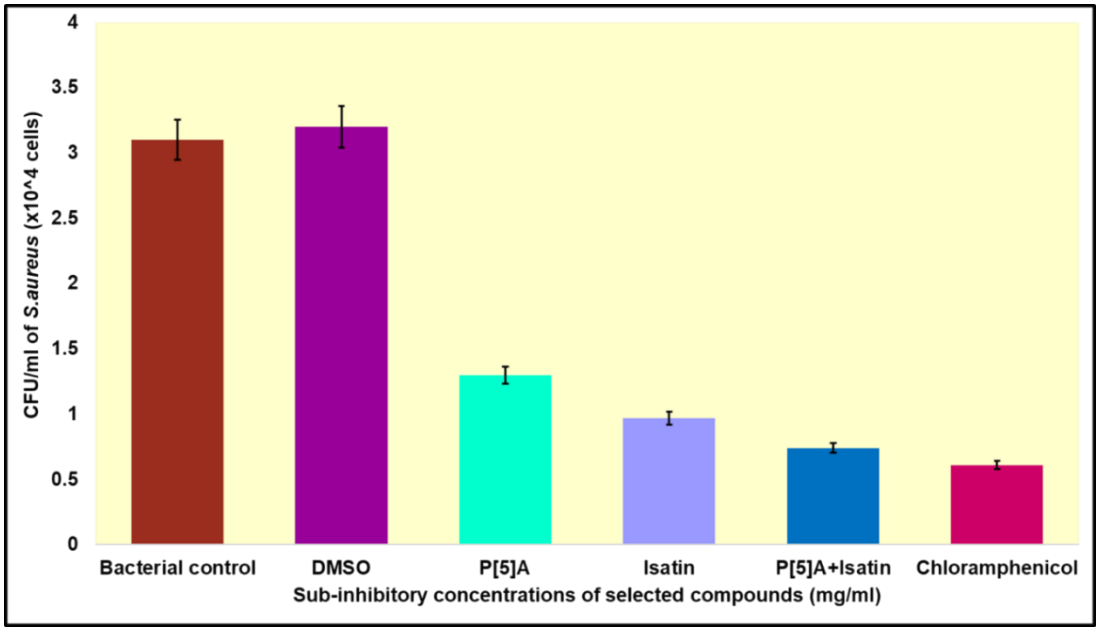


Figure 32: Influence of pillar[5]arene-isatin inclusion complexes on the hitchhiking motility behavior of *Staphylococcus aureus* cultured with *Pseudomonas aeruginosa*

The motility rate of *Staphylococcus aureus* and *Pseudomonas aeruginosa* has been considerably decreased with the action of curd peptide-derived hydrogels (Manna *et al*., 2019).

In response to cyclic-dimeric GMP, the motility behavior including swimming, swarming and twitching patterns of *Pseudomonas aeruginosa* have been greatly affected and in turn, destroyed the formation of biofilms by the bacteria (Cai *et al*., 2020).

Similarly, paeoniflorin (a vital compound) has been observed with the reduction in all types of motility behavior of *Pseudomonas aeruginosa*, envisaging their potential biofilm inhibitory activities (Wang *et al*., 2023a).

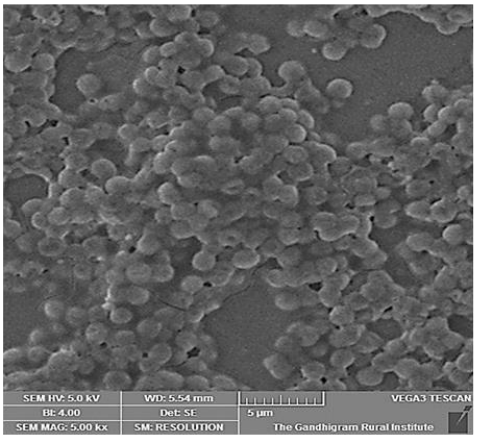
Based on the inference, the motility behaviors of the selected bacterial pathogens were largely affected by the pillar[5]arene-isatin inclusion complexes. Thus, it was evident from enhanced biofilm inhibition and eradication profiles of the synthesized pillar[5]arene-isatin inclusion complexes.

4.3.4. Morphological characterization of bacterial biofilms treated with pillar[5]arene-isatin inclusion complexes

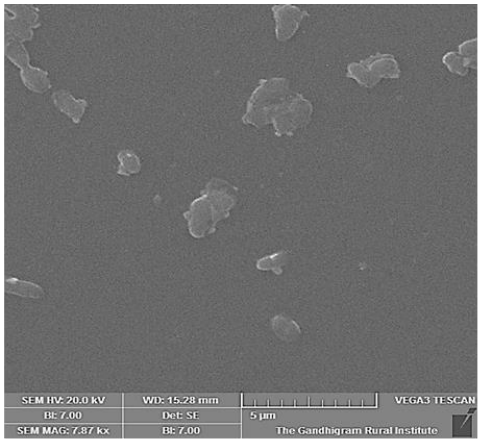
Scanning electron microscopy was used to determine the morphological and structural changes in the biofilm formation of bacterial pathogens. The antibiofilm potential of the synthesized pillar[5]arene-isatin inclusion complexes were observed by visualizing the biofilms of treated and untreated bacterial pathogens under an electron microscope (Figure 33).

Based on the SEM data, the control containing only pathogenic bacteria, the cells were found to be tightly packed, adhered and formed a dense biofilm on the surfaces. In addition, *Staphylococcus aureus* and *Pseudomonas aeruginosa* cells were found to be normal in size and appearance with a multi-layered surface consisting of an outer membrane. Meanwhile, deformed, rumpled and completely distorted morphology of pillar[5]arene-isatin inclusion complexes treated *Staphylococcus aureus* and *Pseudomonas aeruginosa* cells were recorded. Moreover, the formed biofilms on the slides became sparse and only a few adhered

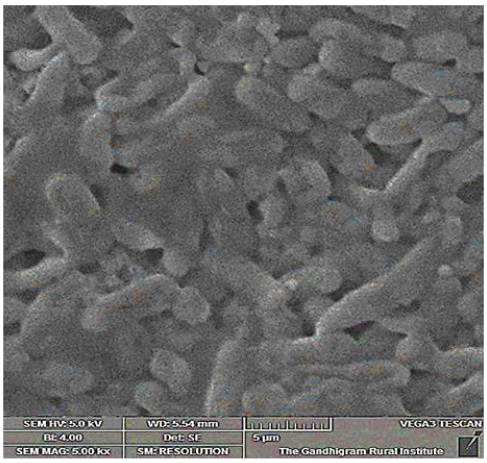
Staphylococcus aureus and *Pseudomonas aeruginosa* were observed. The changes in the biofilm morphology can be modified by a number of key factors including damaging the cytoplasmic cell membrane and interfering with the mechanisms that control bacterial respiration processes. Understanding the fact that there were no changes in the untreated bacterial cells, has provided concrete evidence for the effective antibiofilm potential of synthesized pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.



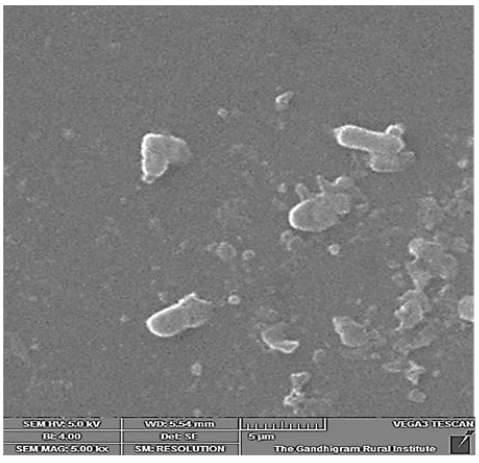
Untreated *Staphylococcus aureus*



Pillar[5]arene-isatin inclusion complexes treated *Staphylococcus aureus*



Untreated *Pseudomonas aeruginosa*



Pillar[5]arene-isatin inclusion complexes treated *Pseudomonas aeruginosa*

Figure 33: Scanning electron microscopic images of bacterial biofilms treated with pillar[5]arene-isatin inclusion complexes

Similarly, the pillar[5]arene films with moxifloxacin have been found to drastically reduce the biofilm mass of *Staphylococcus aureus* and *Klebsiella pneumoniae* on the adhesive sites due to the controlled release of moxifloxacin from the pillar[5]arene films (Shurpik *et al*., 2022).

The host-guest complexes of pillar[5]arene with homoserine lactones signaling molecules have been found to bring out the changes in morphological and structural changes in the biofilms of multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Jonkergouw *et al*., 2023).

Aleksandrova *et al*. (2023) have validated the potential antibiofilm properties of pillar[5]arene in complex with thiaether and tertiary amino groups against *Staphylococcus aureus* which was reflected by the reduced thickness of bacterial biofilms. Pillar[5]arene is a non-toxic DNA binding agent and it could be coupled with antimicrobial compounds to enhance the antibacterial activity to combat bacterial infections in the host.

All these investigations have supported the findings of our study, where the pillar[5]arene-isatin inclusion complexes were found to greatly influence the formation of biofilms on the surfaces. It was confirmed by the detrimental structures of biofilms in the pillar[5]arene-isatin inclusion complexes treated *Staphylococcus aureus* and *Pseudomonas aeruginosa*, compared with the untreated bacterial biofilms.

In sum, the results of phase III demonstrated that the sub-inhibitory concentrations of pillar[5]arene-isatin inclusion complexes were found to effectively inhibit the formation and eradication of biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Further, it was noted to have a prominent activity in reducing the motility behavior of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, thus positively inhibiting the formation and development of biofilms. It was further confirmed by the changes in the morphological characteristics of biofilms treated with the sub-MIC of pillar[5]arene-isatin inclusion complexes in *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Altogether provided a compelling evidence of pillar[5]arene-isatin inclusion complexes was found to be a potent biofilm disruptor against *Staphylococcus aureus* and *Pseudomonas aeruginosa* to promote an infection-free environment in clinical and hospital sectors.

PHASE IV

4.4. Evaluation of Drug Release Kinetics, Formulation and Validation of Pillar[5]Arene-Isatin Inclusion Complexes Based Wound Healing Ointment

4.4.1. *In vitro* drug release potential of isatin from pillar[5]arene-isatin inclusion complexes

The *in vitro* drug release potential of the isatin from the pillar[5]arene-isatin inclusion complexes was evaluated by the direct addition method where phosphate buffered saline was used as dissolution media. The direct addition method was carried out with the dissolution media as phosphate-buffered saline. The absorbance at different time intervals was analysed using UV spectrophotometer. The result of drug release from the inclusion complex is shown in Figure 34a. Nearly 60% of the drug was elevated within 3 hours in the direct addition method.

Further, the release kinetics of isatin from the pillar[5]arene-isatin inclusion complex were confirmed using the HPLC method. The samples from the dissolution medium at different time intervals (0 h, 2 h and 4 h) were collected separately and injected into the HPLC system and analyzed along with the mobile phase for proper detection of released isatin from the pillar[5]arene-isatin inclusion complex. The chromatograms of these samples were compared with the standard chromatogram of pure isatin (Figure 34b). The chromatogram of pure isatin showed a peak at a retention time (RT) of 3.951, corresponding to 100% concentration. In contrast, the chromatograms of the inclusion complex samples collected at different intervals showed peaks at 3.936 RT, 3.938 RT, and 3.926 RT for 0 h, 2 h, and 4 h, respectively. The corresponding concentrations of released isatin were 2.408%, 10.863%, and 33.808% at the respective time points. The gradual increase in isatin concentration over time indicates a controlled release of isatin from the pillar[5]arene–isatin inclusion complex.

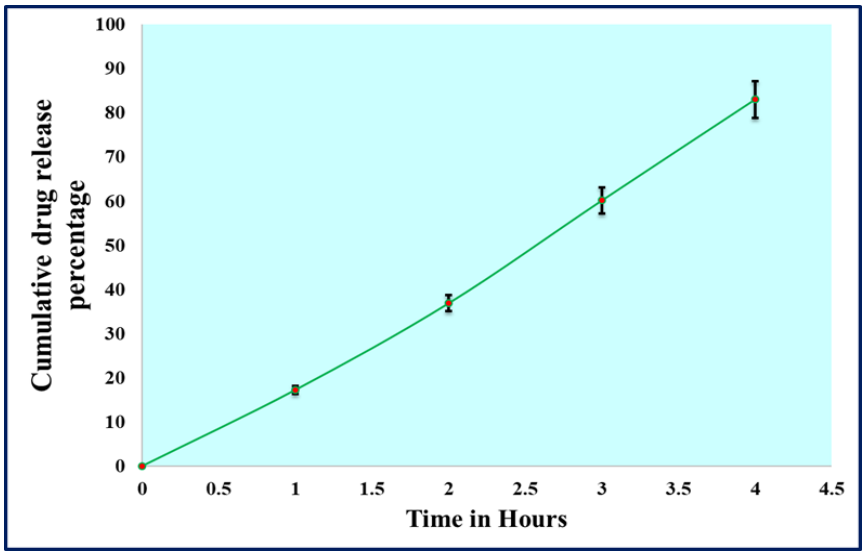


Figure 34a: *In vitro* drug release kinetics of isatin from pillar[5]arene-isatin inclusion complexes derived from UV absorption method

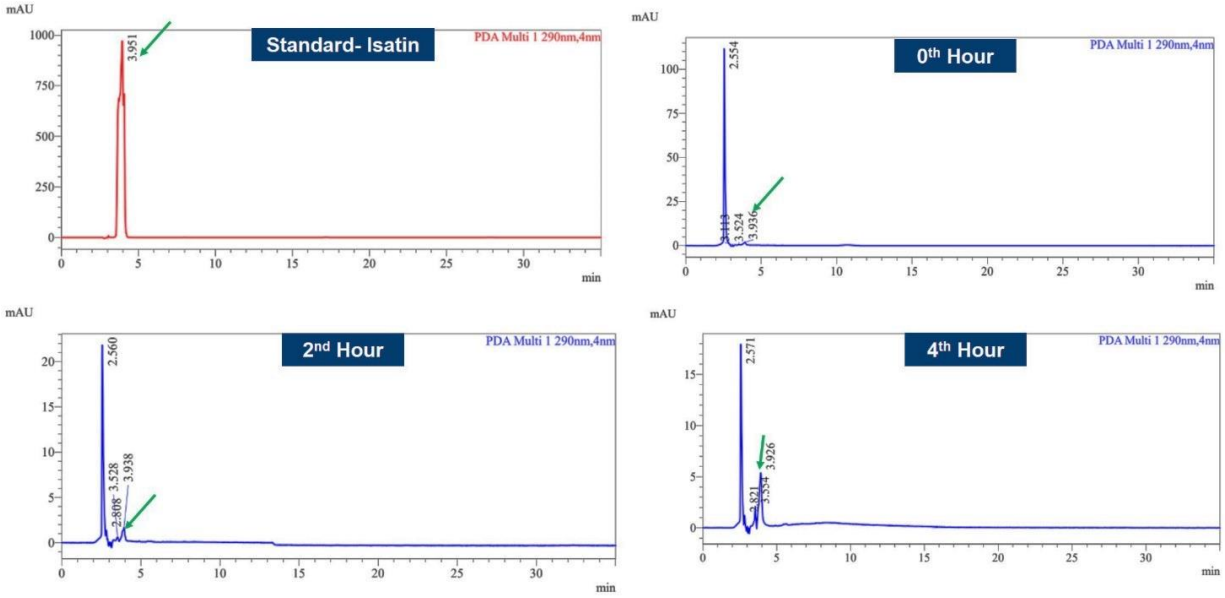


Figure 34b: *In vitro* drug release kinetics of isatin from pillar[5]arene-isatin inclusion complexes by HPLC

In accordance with these findings, the release of alizarin red S from calix[4]arene in a sustainable and controlled manner which recapitulates the potential applications of macrocyclic host as drug delivery systems (Guo et al., 2020).

Shurpik *et al*. (2021) have demonstrated the targeted transport and delivery of therapeutic proteins with enhanced bioavailability through pillar[5]arene based host-guest supramolecular complexes.

Polyphenol-based hydrogel systems have been found to enhance the release of photosensitive drugs to treat bacterial infections by releasing the drugs in a controlled way and maintaining the activity of loaded drugs (Cai *et al*., 2023).

A study by Chandra *et al*. (2020) has established the pH-controlled release of serotonin from the serotonin inclusion complexes of cucurbit[7]uril.

The consistent release of ceftriaxone sodium antibiotic has been observed from the drug delivery material of cross-linking polymeric hydrogels using the phosphate buffered saline as the dissolution media (Nazir *et al*., 2024).

Different types of drugs can be encapsulated with the help of a core-shell fiber mat which releases hydrophilic compounds at a faster rate but hydrophobic compounds have been released in a slow and controlled way to achieve the inhibitory activity against *Staphylococcus aureus* and *Escherichia coli* at the wound sites (Lin *et al*., 2022).

Thus, it was confirmed that the isatin (a hydrophobic compound) was slowly introduced into the external environment from the pillar[5]arene-isatin inclusion complexes in a controlled and sustainable manner. The kinetics of drug delivery from the inclusion complexes were further validated by mathematical models.

4.4.2. Mathematical Models for *in vitro* drug release kinetics of pillar[5]arene-isatin inclusion complexes

Various mathematical models were employed to determine the isatin release from the pillar[5]arene-isatin inclusion complexes and the results are provided in Table 11.

4.4.2.1. Zero-order model

The zero-order model explains the rate of delivery of drugs from the inclusion complex of pillar[5]arene. To evaluate the zero-order model for the drug delivery systems, the graph was plotted between time and cumulative drug release percentage. The zero-order model was employed to evaluate the drug release from the inclusion complex of pillar[5]arene. The R^2 value for the drug release by the direct

addition method was found to be 0.9961. Hence, the isatin release from the pillar[5]arene-isatin inclusion complexes obeys the zero-order kinetics. It implies the drug (isatin) release from the pillar[5]arene-isatin inclusion complexes was at a constant rate. This zero-order kinetics provides an ideal way to improve the therapeutic efficacy of the drug without any side effects.

Table 11: Mathematical models for *in vitro* drug release kinetics of pillar[5]arene-isatin inclusion complexes

Name of the Model	R^2	Equation	Slope	Intercept
Zero-Order Model	0.9961	y = 20.9x - 2.3414	20.9	-2.3414
First-Order Model	0.9129	y = -0.1858x + 2.0813	-0.1858	2.0813
Higuchi Model	0.9514	y = 17.727x - 1.2748	17.727	-1.2748
Korsmeyer-Peppas Model	0.9116	y = 0.79x + 1.9949	0.79	1.9949

4.4.2.2. First-order model

According to First-order drug release kinetics, the release rate of a drug is directly proportional to the concentration of the drug. This indicates that when there is a high concentration of the drug, the release rate also will be high and when the drug concentration decreases, the drug release also decreases. The first-order model was employed to evaluate the drug release from the pillar[5]arene-isatin inclusion complexes. The results exhibited that, the concentration of the isatin has an impact on the release of isatin from the pillar[5]arene-isatin inclusion complexes. This was evident from the correlation coefficient value of 0.9129. Then, the mode of drug delivery by the system was predicted using the Higuchi model.

4.4.2.3. Higuchi Model

Higuchi model explains the drug release from a matrix as a square root of time is based on the process of diffusion. If there is the highest correlation coefficient, then it can be predicted that the drug release is through diffusion. There are some predictions in the Higuchi model and they include: the drug concentration is much higher than the solubility of the matrix at time t_0, sink conditions are

maintained perfectly and constant drug diffusion. To study the Higuchi model of drug release kinetics for various pH, a graph was plotted between the cumulative % drug release and the square root of time. The Higuchi model was plotted for both methods employed to evaluate the drug release from the inclusion complex as a drug delivery system.

The results of the Higuchi model showed that the release of isatin from the pillar[5]arene-isatin inclusion complexes perfectly follows the Higuchi model as indicated by the increased correlation coefficient value. The correlation coefficient (R^2) value for the drug release by the direct addition method was found to be 0.9514. It implies the process of isatin release follows the process of diffusion from the pillar[5]arene-isatin inclusion complexes. To understand the type of diffusion process, the Korsmeyer- Peppas model was constructed.

4.4.2.4. Korsmeyer-Peppas Model

The process of drug release to the target site was identified as diffusion from the Higuchi model. The type of diffusion process was analyzed by constructing the Korsmeyer- Peppas model by plotting the graph between n log cumulative drug release % and log time. The Korsmeyer-Peppas model was plotted to evaluate the drug release from the inclusion complexes-based drug-delivery system.

Based on the value of release exponent (n), the type of drug transport mechanism was identified. The release exponent of the drug release mechanism by the direct addition method was 0.79. The isatin transport mechanism from the pillar[5]arene-isatin inclusion complexes lies in the Non-Fickian transport mechanism with values ranging from $0.45<n<1$. It was indicated that, besides diffusion other transport mechanisms were also involved in the release of drugs at the target place.

Several scientific literature have validated the drug release kinetics by mathematical modeling to predict the *in vivo* dissolution profile of drugs.

In line with our findings, the rosuvastatin drug release from the drug delivery system has been followed by all the proposed models and the best fit model was the first order model. In addition, based on the exponent values in the Korsmeyer-

Peppas model, the drug follows a fickian diffusion pattern for transport of drugs in the host (Ahsan *et al*., 2022).

Different drug data sets with poly(lactic-co-glycolic acid) drug delivery systems have evaluated the drug release kinetics of each drug, which explained that most of the drug molecules do not follow any of the proposed models. All the drugs follow the fickian transport mechanism (Heredia *et al*., 2022).

Another study by Timotius *et al*. (2020) has explained the release of maleic anhydride from the chitosan polymer where the drug release follows all the three proposed models such as first-order model, Higuchi model and Korsmeyer-peppas model.

Similarly, the release of methotrexate drug from the oil-water nanoemulsion drug delivery systems follow all the kinetic models and the best-fit model has been observed as Korsmeyer-peppas model by following non-fickian drug transport mechanisms (Latif *et al*., 2022).

Therefore, the release of isatin from the pillar[5]arene-isatin inclusion complexes follows all the proposed mathematical models for controlled drug release in the *in vivo* conditions.

4.4.3. Development and physiochemical characterization of pillar[5]arene-isatin inclusion complexes based ointment

The pillar[5]arene-isatin inclusion complexes was incorporated into the ointment base and prepared by melting and mixing method. Further, it was proceeded to characterize the physical and chemical characteristics.

4.4.4. Characterization of pillar[5]arene-isatin inclusion complexes based wound healing ointment

Ointments used for commercial applications to treat wounds should possess good quality in terms of odour, texture, colour, and consistency. The physicochemical characteristics of the developed ointment are shown in Table 12.

The developed ointment was found to be semisolid, odourless, pale orange in colour and highly viscous in consistency. Ointment preparations for topical applications should have a maximum pH of 4.5 – 6.8 to match the pH of skin. Therefore, it is considered to be safe and comfortable to use on the skin. If the pH

of the ointment is too acidic, it will cause irritation to the skin, whereas if it is too alkaline the skin will become dry. From the findings of the study, it showed that the pH of the ointment was found to be pH 4.5 to 5.0. It lies within the normal range of the skin pH, it could be considered as safe for topical applications to treat wounds on the skin.

Table 12: Physicochemical characterization of the pillar[5]arene-isatin inclusion complexes based ointment

S.No.	Properties analysed	Observations	
1.	Texture	Semisolid	 Developed ointment
2.	Odour	Odourless	
3.	Color	Pale orange	
4.	Consistency	Thick viscous liquid	
5.	pH	4.5 to 5.0	
6.	Spreadability	5.3 cm/ 50 ml Conc. 4.0 cm/ 10 ml Conc.	

The acidic pH of the ointment could promote the best balance for wound healing and promote faster recovery. Spreadable nature of the developed ointment was analysed for their therapeutic nature. It is used to scrutinize the spreading ability of the ointment when applied on the skin surface. The good spreadable potential of any ointment is 4-7 cm in length, they should spread upon pressure and can be applied on the skin to treat wound infections. The spreadability of the developed ointment on the surfaces was found to be 5.3 cm/ 50 ml concentration and 4.0 cm/ 10 ml concentration.

4.4.5. Cytotoxic effect of developed pillar[5]arene-isatin inclusion complexes based wound healing ointment using human peripheral blood lymphocytes

The cytotoxic effect of developed ointment on human peripheral blood lymphocytes was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and shown in Figure 35. The cells were exposed to different concentrations of ointment for 48 hours. At 1000 µg/ml of the pillar[5]arene-isatin

inclusion complexes based ointment, the peripheral blood lymphocytes (PBLs) were found to be viable with a percentage viability of 87.95% and it iterated their non-cytotoxic nature. Moreover, the sub-inhibitory concentration of pillar[5]arene-isatin inclusion complexes was below 200 µg/ml, and indicating the non-cytotoxic nature of the ointment for topical use. Hence, the developed pillar[5]arene-isatin inclusion complexes based ointment was further validated for its wound healing activities.

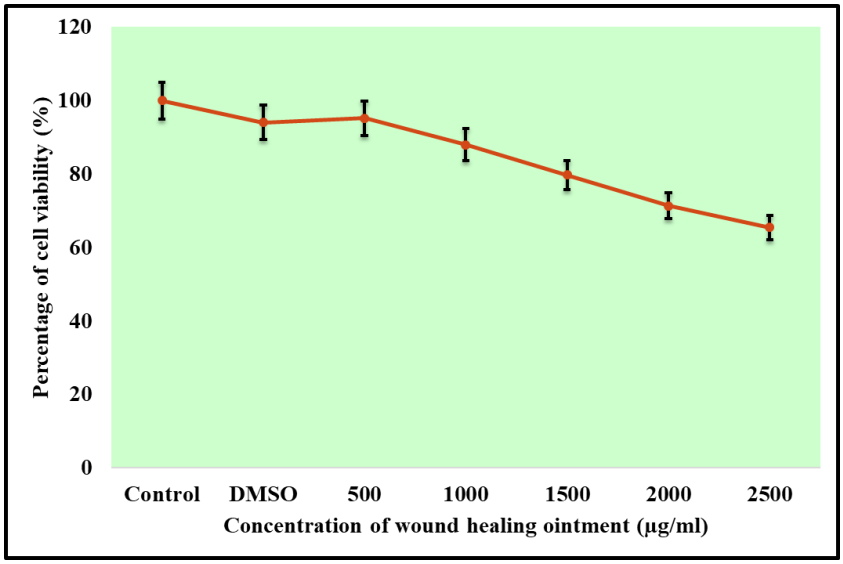


Figure 35: Cytotoxic effect of the developed pillar[5]arene-isatin inclusion complexes based ointment using peripheral blood lymphocyte cells

An identical study has shed light on the safety profile of the N-terminal phenylalanine-containing cytotoxic peptides inclusion complexes of cucurbit[7]uril-based host-guest interactions for the development of therapeutics to treat various illnesses (Wang *et al*., 2020).

The cell viability of the vancomycin-loaded niosomal drug delivery system has been higher than that of free vancomycin which indicates the safer use of synthesized complexes to treat various bacterial infections (Hemmati *et al*., 2024).

The cytotoxicity of chlorhexidine antibiotic has been reduced by forming inclusion complexes with cucurbit[7]uril and also exhibited an enhanced antimicrobial activity (Ruan *et al*., 2023).

The safety profile of the developed pillar[5]arene-isatin inclusion complexes based ointment suggested their applications in treating wound infections and further their antibacterial activities and wound healing potentials were determined.

4.4.6. Antibacterial efficacy of developed pillar[5]arene-isatin inclusion complexes based ointment

The antibacterial efficacy of the developed ointment with a drug-loaded system was analyzed against two important pathogens involved in wound infections namely, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The zone of inhibition was measured and the results are shown in Table 13.

Table 13: Antibacterial efficacy of developed pillar[5]arene-isatin inclusion complexes based ointment

S. No	Samples	Zone of Inhibition in diameter (mm)	
		Staphylococcus aureus	***Pseudomonas aeruginosa***
1.	Positive Control (Cipladine)	12±0.5	13±0.5
2.	Negative Control (DMSO)	-	-
3.	Developed pillar[5]arene-isatin inclusion complexes based ointment	9±0.5	10±0.5

From the results, it showed that the developed pillar[5]arene-isatin inclusion complexes based ointment has good antimicrobial activity against the selected pathogens. The zone of inhibition was found to be 9±0.5mm against *Staphylococcus aureus* and 10±0.5mm against *Pseudomonas aeruginosa*. The negative control did not show any inhibitory profile against the pathogens which indicates that the inhibition against the pathogen was due to the activity of ointment alone.

Various drug loaded drug delivery systems based on topical applications have been elucidated and their broad range of antimicrobial activities against various pathogens have been conducted.

Cephalothin loaded aquasomes have exhibited their antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* with an inhibitory zone of 38 ± 3 mm and 20 ± 5 mm, respectively (Shanmugam and Srinivasan, 2024).

Likewise, many bacterial pathogens including *Streptococcus pneumoniae*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* have demonstrated a higher susceptibility to the developed delafloxacin-loaded Poly(d,l-lactide-co-glycolide), which indicated their potential antibacterial activity (Alshememry *et al*., 2024).

Microsponge drug delivery systems containing clarithromycin have been

found to show an enhanced antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Tomar *et al*., 2022).

Altogether, the developed pillar[5]arene-isatin inclusion complexes based ointment were found to exhibit a wide range of antibacterial activities against the prominent bacterial pathogens at the wound sites.

4.4.7. Wound healing potential of the developed ointment on L929 fibroblast cells

Activation, proliferation and migration of fibroblasts are the primary steps in wound healing, where multiple cell types and other microenvironmental factors are involved. The scratch assay is widely applied as *in vitro* technique for understanding the wound healing capabilities of medicinally important compounds. In our study, L929 cells were treated with 500 µg/ml of developed ointment for up to 48 hours. Cell migration at 0, 24 and 48 hours were captured and wound closure distance was calculated by Image J software. The results indicated that the developed pillar[5]arene-isatin inclusion complexes based ointment closed the gap created by the scratch by 90% in 48 hours. The percentage of wound closure and wound area covered at different time intervals in untreated, developed pillar[5]arene-isatin inclusion complexes based ointment treated and standard control ointment (Cipladine) treated cells are represented in Figures 36 and 37. pillar[5]arene-isatin inclusion complexes based ointment induced the migration of L929 cells resulting in wound closure. In the standard-ointment treated cells, 91% of the gap was closed at 48 h. Figure 38 shows the microscopic images of untreated, cipladine-treated and developed pillar[5]arene-isatin inclusion complexes based ointment treated L929 cells. The inverted microscopic images indicated the increased cell migration in the cipladine and developed ointment treated cells.

Various scientific literatures have supported the enhanced wound healing potential of natural compounds/ drugs encased in the supramolecular structures.

Econazole inclusion complexes of pillar[6]arene with triethylene oxide substituents have enormously accelerated wound healing in *Staphylococcus aureus* infected mice, suggesting their superior topical applications to treat wound infections (Zhang *et al*., 2022).

Similarly, azelaic acid inclusion complexes of cationic pillar[5]arene exhibited a synergistic activity to promote the enhanced wound healing potential by reducing the *Staphylococcus aureus* burden at the wound sites (Ma *et al*., 2022).

Moreover, a study by Wang *et al*. (2023b) has utilized the electron-rich cavity of pillar[5]arene to encapsulate guest molecules which has been noticed to reduce the occurrence of methicillin-resistant strain of *Staphylococcus aureus* in wound affected areas to accelerate wound healing.

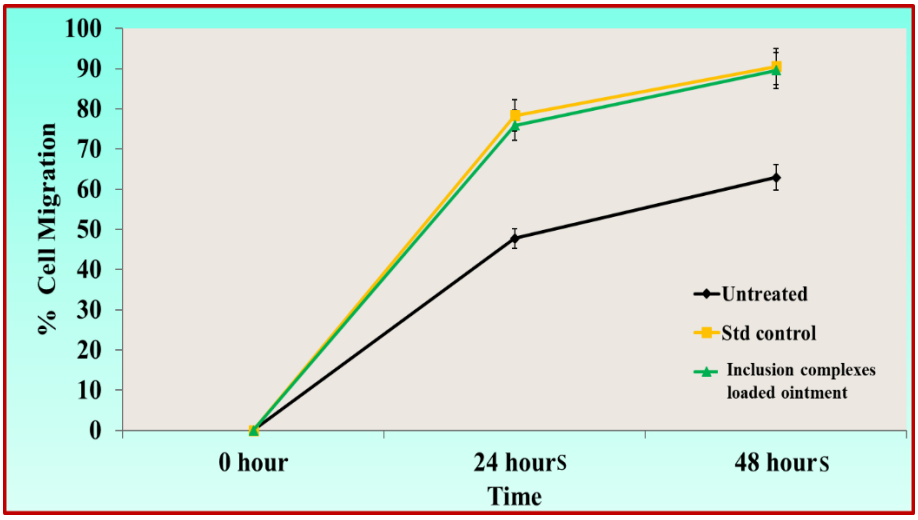


Figure 36: Cell migration rate towards wound area in developed ointment treated L929 fibroblast cells

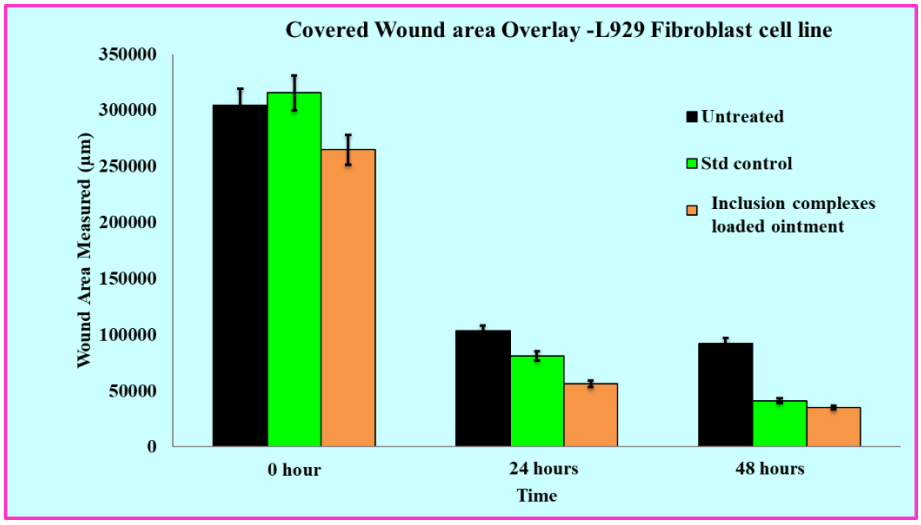


Figure 37: Rate of wound closure by the developed ointment in L929 fibroblast cells

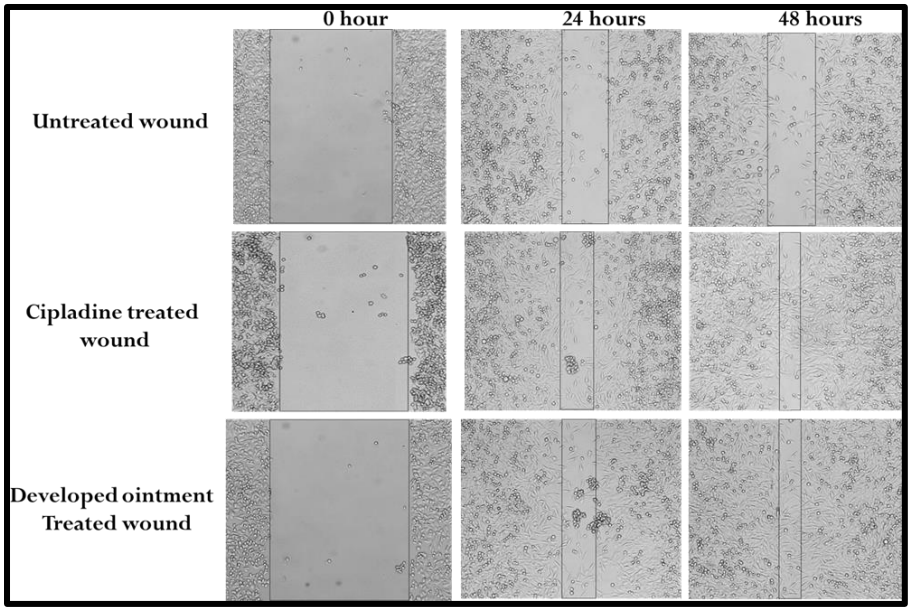


Figure 38: Inverted microscopic images of L929 fibroblast cells treated with the developed wound healing ointment

Phase IV findings revealed that the isatin was found to be released from the pillar[5]arene-isatin inclusion complexes in a controlled manner and it was validated with various mathematical models. The characterization of developed pillar[5]arene-isatin inclusion complexes based ointment showed that it was pale orange in colour, high viscous consistency, odourless, acidic pH and well spread on the surfaces. The *in vitro* wound healing activity using fibroblast L929 cells proved that 90% of the wound was closed by 48 hours of treatment and the developed pillar[5]arene-isatin inclusion complexes based ointment was found to be non-toxic.

From the outcome of the research findings, pillar[5]arene-isatin inclusion complexes found to have a bacteriostatic and bactericidal efficacy against prominent pathogens namely, *Stapylococcus aureus* and *Pseudomonas aeruginosa*, responsible for wound infections and delays wound healing processes. In addition, it has exhibited potential membrane damage and antibiofilm properties against the selected bacterial pathogens. Finally, it envisaged better wound healing potential in fibroblast L929 cell lines.

Hence, the pillar[5]arene-isatin inclusion complexes has proved to be unique for combating wound infections to overcome the antimicrobial resistance of bacterial pathogens at the wound site and promote the wound healing processes.