5.0

SUMMARY AND CONCLUSION

Wound infections play a crucial role in the progression of chronification of wounds thus delaying the wound healing processes. A wound infection is a break in epithelial integrity with a plausible accumulation of microbial load to worsen the wound healing stages. Among the occurrence of various bacterial pathogens at the wound sites, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the two major bacterial pathogens noticed in the wound infection area. These pathogens potentially worsen the wound conditions by forming biofilms on the surfaces to promote microbial colonization. There are various strategies available to manage wound infections to prevent the devastating conditioning of wounds. Plenty of antibiotics are on the frontline to treat wound infections to prevent the delay of wound healing. Since, the endurance of antimicrobial-resistant strains at the wound site, the antibiotics could be difficult to play their action effectively.

Even though there are various strategies including antibiotic therapy, surgery, cleansing, debridement and antiseptics to treat wounds and wound infections, they all are associated with significant disadvantages. 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.

Over the past decades, the pillar[n]arenes have been attracted by many fields by exploiting host-guest interactions for controlled drug release and targeted delivery of drugs without being subjected to premature elimination or degradation. The inclusion complexes-based drug delivery system offers a lot of advantages including improved solubility, enhanced stability, controlled release

of drugs and targeted delivery. Notably, the controlled drug targeted release and efficient therapy to treat infections lies in the utilization of the pillar[n]arene based self-assembly as drug carriers or hosts.

Therefore, the objectives of the study were to synthesize and characterize the pillar[n]arene-isatin inclusion complexes followed by their antibacterial evaluation against clinical pathogens. Next part of the study was to determine the antibiofilm potential of the synthesized pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Finally, the drug release kinetics was investigated and *in vitro* wound healing potential of the pillar[5]arene-isatin inclusion complexes based ointment formulations were performed.

The research work was divided and carried out in four phases. Phase I was involved with the selection of drugs (isatin) and drug carriers (pillar[n]arenes) and the validation of their pharmacokinetic properties to determine their drug-likeliness. Followed by, the synthesis of pillar[n]arenes-isatin inclusion complexes and their characterization was performed to identify the binding stoichiometry between the hosts and guest. Based on the high therapeutic potential of alkaloid fractions of *Couroupita guianensis*. Aubl, isatin (red-orange powdered alkaloids) has been selected as the drug molecule. In order to circumvent the issues of bioavailability, controlled release and premature elimination of isatin in the host, appropriate drug carrier selection is crucial. Due to the burgeoning interest in supramolecular-based drug delivery systems, pillar[5]arene and BEA (difunctionalized pillar[4]arene[1]quinine derivative) 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.

The selected supramolecular hosts (pillar[5]arene and BEA) exhibited good pharmacokinetic profile to become the potential drug carriers for the treatment of various ailments. Further, the synthesis of hosts and host-guest inclusion complexes was performed by two major steps such as condensation

and oxidation. Since these compounds have C-H…π interactions with guest molecules, they act as a driving force for the perfect complexation between pillar[n]arenes and drug molecules. Pillar[5]arene (P[5]A) and BEA were formed as a white solid with a 60% and 73% yield, respectively.

Further, the characterization of host-guest complexes in solution was studied by various spectroscopic techniques such as nuclear magnetic resonance (NMR) spectroscopy and UV-visible spectroscopic analysis, to elucidate the binding properties and structural interactions.^{1}H NMR spectroscopy provided insights into the molecular interactions between pillar[n]arenes-isatin inclusion complexes through chemical shift variations. UV-visible spectroscopy was employed to study changes in the absorption spectra, indicating complex formation.

^{1}H NMR spectroscopy was employed to investigate the binding affinity and molecular interactions between pillar[5]arene (P[5]A) and isatin, providing crucial insights into their complexation behavior. Initially, equimolar mixtures of P[5]A and isatin were prepared in a suitable solvent system, and notable changes in chemical shift values were observed in the NMR spectra of both compounds upon complex formation. Analysis of the titration data using Job's plot methodology revealed a 1:1 binding stoichiometry between P[5]A and isatin, corroborating the formation of an inclusion complex. Moreover, UV-visible spectroscopic analysis complemented the ^{1}H NMR studies, confirming the 1:1 binding stoichiometry between P[5]A and isatin. Isatin's characteristic absorption bands were observed in the UV-visible spectra, and Job's plot analysis confirmed the 1:1 binding ratio between P[5]A and isatin.

The interaction between BEA and isatin was also explored through ^{1}H NMR titration experiments. The results revealed that the interaction between the BEA and isatin showed lesser binding and affinity towards isatin compared to that of P[5]A-isatin inclusion complexes. Minimal changes in chemical shifts were observed even after the introduction of four equivalents of isatin to BEA, indicating lesser complexation and it was confirmed by the calculated binding

constant (K = 100 ± 3). Analysis using WINEQNMR2 confirmed a 1:1 host-guest stoichiometry between BEA and isatin. UV-visible spectroscopic analysis provided further insights into the host-guest interaction between BEA and isatin. Job's plot analysis of UV-visible titration experiments validated the 1:1 binding stoichiometry between BEA and isatin, highlighting the structural and chemical inadequacies of BEA in forming robust complexes with isatin.

The second phase was dealt with the evaluation of antibacterial efficacy of pillar[n]arenes-isatin inclusion complexes and their mechanism of action against the prominent bacterial pathogens responsible for wound infections. The results revealed that 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 namely 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*.

The minimum concentration of pillar [5]arene-isatin inclusion complex that inhibits the growth of the selected bacterial pathogens was identified as 0.28 mg/ml (0.3mM) for *Staphylococcus aureus* and 0.56 mg/ml (0.6 mM) for *Pseudomonas aeruginosa*. The MIC of chloramphenicol against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was found to be 0.25 mg/ml and 0.5 mg/ml, respectively. The MBC was recorded as 0.56 mg/ml (0.6 mM) for *Staphylococcus aureus* and 1.125 mg/ml (1.2mM) for *Pseudomonas aeruginosa*. Isatin was observed to have 1.5 mg/ml of MBC against *Staphylococcus aureus* and 3.0 mg/ml

of MBC against *Pseudomonas aeruginosa*. Henceforth, the pillar[5]arene-isatin inclusion complexes were found to exhibit bacteriostatic and bacteriocidal activities against the selected prominent pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, compared with isatin alone.

Furthermore, the results of the checkerboard method revealed that FICI was 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*.

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. 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.

In addition, 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.

The changes in the morphological view of the bacterial cell membrane of the treated and untreated pathogens were perspicuously demonstrated by scanning electron microscopy (SEM). The results revealed that the physical damage on the bacterial cell membrane by pillar[5]arene-isatin inclusion complexes 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 *Staphylococcus aureus* and *Pseudomonas aeruginosa*. It further iterated that 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 of the work was focused on assessing the antibiofilm potential of selected pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The biofilm inhibition results have highlighted the superior efficacy of the inclusion complexes compared to the individual components, namely P[5]A and isatin. The enhanced biofilm inhibition and eradication have 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 pillar[5]arene-isatin inclusion complexes suggests its potential as a powerful strategy to prevent bacterial colonization and persistence, particularly in *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Further, the action of pillar[5]arene-isatin inclusion complexes on the motility behaviors of selected bacterial pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* was elucidated. The pillar[5]arene-isatin inclusion complexes significantly reduced the swimming motility behavior of *Pseudomonas aeruginosa*. It was restricted to move on the soft agar medium containing pillar[5]arene-isatin inclusion complexes and was noted with 5.3±0.577 mm swimming motility behavior.

In addition, the synthesized pillar[5]arene-isatin inclusion complexes in the media restricted the spreading of *Pseudomonas aeruginosa* 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*. Likewise, 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 by hitchhiking motility. The viable cells of *Staphylococcus aureus* ($0.74x10^4$ CFU/ml) were observed in the medium containing pillar[5]arene-isatin inclusion complexes which emphasized their potential activity in reducing the hitchhiking motility behavior of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. 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.

Further, the synthesized pillar[5]arene-isatin inclusion complexes were found to greatly influence the formation of biofilms on the surfaces at their sub-inhibitory concentrations. It was confirmed by the detrimental structures of bacterial biofilms in the pillar[5]arene-isatin inclusion complexes treated *Staphylococcus aureus* and *Pseudomonas aeruginosa*, compared with the untreated bacterial biofilms. Altogether provided compelling evidence of pillar[5]arene-isatin inclusion complexes were found to be a potent biofilm disruptor against *Staphylococcus aureus* and *Pseudomonas aeruginosa* to promote an infection-free environment in clinical and hospital sectors.

In Phase IV, the drug release kinetics and *in vitro* wound healing potential of formulated ointment loaded with pillar[5]arene-isatin inclusion complexes was assessed using L929 human fibroblast cell lines. The *in vitro* drug release potential of the isatin from the pillar[5]arene-isatin inclusion complexes was evaluated which revealed that 60% of isatin was released from the pillar[5]arene-isatin inclusion complexes within 3 hours of treatment. Thus, it was confirmed by UV and HPLC technique 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.

Besides, the kinetics of drug delivery from the inclusion complexes were further validated by mathematical models. The results envisaged the release of

isatin from the pillar[5]arene-isatin inclusion complexes follows all the proposed mathematical models such as zero-order model, first-order model, Higuchi model and Korsmeyer-Peppas model for controlled drug release in the *in vivo* conditions.

Pillar[5]arene-isatin inclusion complexes based wound healing ointment has been formulated and characterized. The developed ointment was found to be semisolid, odourless, pale orange in colour and highly viscous in consistency. From the findings of the study, it showed that the pH of the ointment was found to bepH 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. The acidic pH of the ointment could promote the best balance for wound healing and promote faster recovery. The spreadable nature of the developed ointment was analyzed for its 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 is4-7 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.

Further, the safety profile of the developed pillar[5]arene-isatin inclusion complexes based ointment using human peripheral blood lymphocytes suggested its applications in treating wound infections. In addition, the developed pillar[5]arene-isatin inclusion complexes based ointment was found to exhibit a wide range of antibacterial activities against the prominent bacterial pathogens (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) at the wound sites.

The *in vitro* wound healing activity of developed pillar[5]arene-isatin inclusion complexes based ointment using fibroblast L929 cells proved that 90% of the wound was closed by 48 hours of treatment, it envisaged their potential bactericidal, biofilm disruption and wound healing potential by targeting the two most deleterious pathogenic bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* at wound sites.

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

Limitations of the study

Although a prominent wound healing potential of synthesized pillar[5]arene-isatin inclusion complexes loaded ointment is proven, detailed *in vivo* studies using animal models can be exploited

Recommendations for future studies

- Gene expression profile of pillar[5]arene-isatin inclusion complexes treated bacterial pathogens can be elucidated
- The efficacy of the compounds can be assessed against prominent fungal pathogens responsible for wound infections
- *In vivo* validation of wound healing ointment can be performed