SPECIMEN FORMAT FOR THESES OF MONTH

Faculty	**:**	School of Biosciences
Department	**:**	Biochemistry, Biotechnology and Bioinformatics
Branch/ Area:	**:**	Biochemistry
Sub Subject Heading:	**:**	Microbiology
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Title of the thesis	**:**	Biological Evaluation of Pillar[5]arene-Isatin Inclusion Complexes to Combat Wound Infections
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Name of Supervisor	**:**	Dr.D.Kavitha
Designation of Supervisor	**:**	Assistant Professor (SS)
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Abstract within 300 words:

Wound infections have become a serious threat at the global level due to biofilm forming bacterial pathogens. There is a need for the development of alternative strategies to prevent complications of drug resistance. Natural compounds have been a prime choice for microbial treatment but have a poor pharmacokinetic profile. To circumvent these challenges, a suitable drug delivery system is of paramount importance, utilizing the host-guest complexation to protect the drug from premature degradation and deactivation. Hence, the current study was designed to explore the host-guest inclusion complexes for the prevention and treatment of wound infections. Isatin, an alkaloid isolated from the *Couroupita guianensis* Aubl. flower, was selected as the drug molecule owing to its traditional use in treating various infections. Likewise, pillar[5]arenes (P[5]A) and bis-ethanolamine functionalized pillar[4]arene[1]quinine (BEA) were selected as supramolecular hosts to capture isatin into their electron-rich cavities to reinforce the controlled release and targeted activity. The pharmacokinetic profiles of the selected hosts revealed their potential druggable nature. The synthesized P[5]A and BEA were characterized by proton NMR and UV-visible spectroscopy which revealed that P[5]A exhibited a superior capacity to encapsulate isatin in comparison with BEA. Job's plot analysis validated the 1:1 binding stoichiometry between P[5]A and isatin, highlighting the specificity and stability of the formed complexes. Further, the synthesized P[5]A-isatin inclusion complexes showed enhanced antibacterial properties and exhibited strong membrane-damaging potential. P[5]A-isatin inclusion complexes have proved their antibiofilm potential against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The isatin was found to be released from the P[5]A-isatin inclusion complexes in a controlled manner as validated by various mathematical models. The formulated P[5]A-isatin inclusion complexes based ointment showed significant wound healing effects *in vitro* with 90% wound closure within 48 hours and was found to be non-toxic. Conclusively, the synthesized pillar[5]arene-isatin inclusion complexes have proved to be unique for combating wound infections and promoting the wound healing processes in an effective manner.

i) Major objectives :

- ✓ To synthesize and characterize the pillar[n]arene-isatin inclusion complexes
- ✓ To assess the antimicrobial action of pillar[n]arene-isatin inclusion complexes against clinical pathogens

- ✓ To determine the antibiofilm potential of pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*
- ✓ To investigate the drug release kinetics and *in vitro* wound healing potential of pillar[5]arene-isatin inclusion complexes based ointment formulations

ii) Hypothesis:

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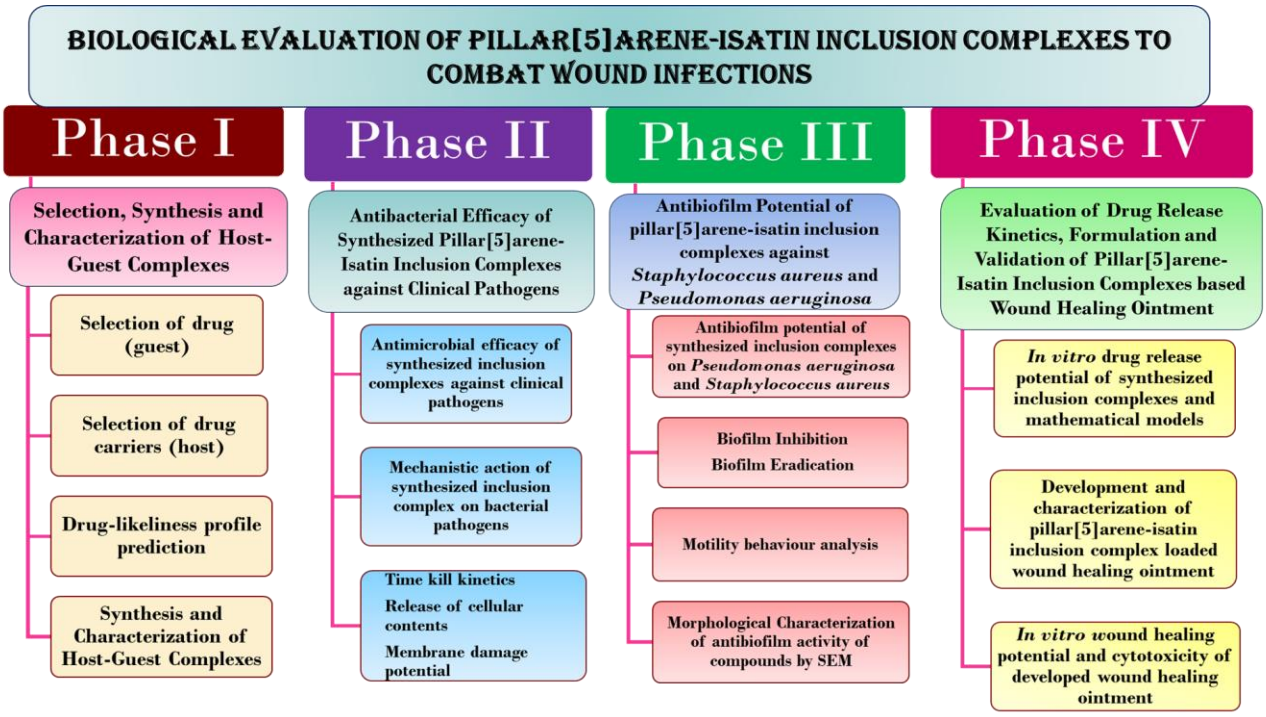
iii) Methodology :

Phase I involved the selection, synthesis and characterization of pillar[5]arene-isatin and BEA-isatin inclusion complexes. Further, the drug-likeliness profile of the selected drug and drug carriers was performed. Phase II was carried out to determine the antibacterial mechanism of synthesized inclusion complexes against several clinical pathogens including, *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli* and *Salmonella paratyphi* A. Phase III dealt with the antibiofilm potential of sub-inhibitory concentrations of synthesized P[5]A-isatin inclusion complexes against prominent pathogens such as *S. aureus* and *P. aeruginosa* in wound affected area. Phase IV comprised of elucidating the drug release kinetics of synthesized P[5]A-isatin inclusion complexes and formulation of inclusion complexes loaded wound healing ointment. Further, the in vitro evaluation of wound healing potential of the developed ointment was assessed using L929 fibroblast cell lines.



iv) Findings:

Phase I - Selection, synthesis and characterization of host-guest complexes

Initially, pillar[n]arene based compounds namely, Decamethoxypillar[5]arene (P[5]A) and bis(ethanolamine) functionalized pillar[4]arene[1]quinone (BEA) were selected as drug carriers. 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. Not only the structural or topological characteristics but the selected pillar[n]arenes are also reported to have various biological functions, including biofilm inhibition. Isatin was selected as a drug molecule, as it is reported with various biological activities including, antimicrobial, anticancer, anticonvulsant and anti-inflammatory. Subsequently, the drug-likeliness properties of the selected compounds, P[5]A, BEA and isatin were analyzed by predicting their ADMET (Absorption, Distribution, Metabolism, Excretion, Toxicity) profiles. The results have indicated that all the compounds have reasonable solubility. It was suggested that P[5]A and BEA had hERG II inhibitory potentials. It is determined that isatin, P[5]A, and BEA were not found to be carcinogenic based on the toxicity data. The maximum tolerated doses of P[5]A, isatin, and BEA suggested that they are safe, and hepatotoxicity projections suggested that isatin and P[5] A are non-toxic.

Based on the data, pillar[n]arene was synthesized by established synthetic protocols and isatin was purchased from a commercial source for the synthesis of host-guest complexation. The inclusion complex was synthesized using predetermined molar equivalents of P[5]A and isatin in a suitable solvent system. The solvent choice was critical to ensure the solubility of both components and facilitate complexation. The formation of the inclusion complex was monitored using ^{1}H NMR spectroscopy and UV-Vis spectroscopy. ^{1}H NMR spectroscopy provided insights into the molecular interactions between P[n]A and isatin 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 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. Chemical shifts of H_a H_b, H_c and H_d of isatin were shifted from 9.95, 7.02, 7.60 and 7.10 to 9.78, 6.99, 7.57 and 7.09 ppm; chemical shifts of aryl – C-H protons of P[5]A labeled as H_1 and methoxy protons labelled as H_3 was shifted from 6.87 and 3.72 to 6.86 and 3.74 ppm, respectively. The aryl–CH protons in isatin exhibited a significant inclusion-induced deshielding effect, while chemical shifts of aryl–C-H protons in P[5]A also experienced discernible shifts. These changes provided compelling evidence for complexation, highlighting the intricate molecular forces governing their interaction.

To comprehensively characterize the binding behavior, ^{1}H NMR titration experiments were conducted to determine binding constants and stoichiometry. 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. Computational analysis of the ^{1}H NMR titration experiments estimated the association constant K to be 1995 ± 3, providing quantitative insights into the binding strength. The plots of mole fraction against equivalents of isatin exhibited the equimolar mixture of P[5]A and Isatin resulted in the formation of almost 80% complexation in solution. Moreover, UV-Vis 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-Vis 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 ($K = 100 \pm 3$). Analysis using WINEQNMR2 confirmed a 1:1 host-guest stoichiometry between BEA and isatin. UV-Vis 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 specificity and stability of the formed complexes.

From phase I, the successful synthesis of P[5]A with isatin showed a binding stoichiometry at a 1:1 ratio for the stable complexation. These synthesized inclusion complexes were taken for phase II experiments to determine their antibacterial mechanisms.

Phase II: Antibacterial efficacy of synthesized pillar[5]arene-isatin inclusion complexes against clinical pathogens

Primarily, the antibacterial activity of the synthesized P[5]A-isatin and BEA-isatin inclusion complexes against various Gram-positive and Gram-negative bacterial pathogens, including *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Salmonella paratyphi* A were evaluated by agar well diffusion method. Since chloramphenicol has a broad spectrum of antibacterial efficacy against both Gram-positive and Gram-negative bacteria, it was used as the antibiotic control throughout the research work. From the agar well diffusion method, the diameter of the zone of inhibition was comparatively low with BEA-isatin inclusion complexes against all the tested bacterial pathogens. The zone of inhibition showed by P[5]A-isatin inclusion complexes against *P. aeruginosa* and *Staphylococcus aureus* was found to be 11.7±1.2 mm and 12.3±0.6 mm, respectively. The antimicrobial efficacy of P[5]A, BEA, isatin and synthesized inclusion complexes have been elucidated against a wide range of clinical pathogens. Further studies were carried out with *Staphylococcus aureus* and *Pseudomonas aeruginosa* since they are the most prominent bacterial pathogens associated with wound infections and delaying wound healing processes. The P[5]A,

isatin and isatin inclusion complex of P[5]A have been observed with potential antibacterial activity than BEA and isatin inclusion complex of BEA against the pathogens. Therefore, P[5]A, isatin and their inclusion complexes have been taken to study their biological activities against selected pathogens.

The minimum inhibitory concentration (MIC) of P[5]A-isatin inclusion complexes against *S. aureus* and *P. aeruginosa* was analyzed by resazurin-based microbroth dilution method. The minimum concentration that inhibited the growth of the selected bacterial pathogens was identified as 0.28 mg/ml (0.3 mM) for *S. aureus* and 0.56 mg/ml (0.6 mM) for *P. aeruginosa*. Followed by, the minimum bactericidal concentration (MBC) was also evaluated by plating the inoculum on Luria-Bertani agar after 24 hours of incubation with the inclusion complexes. The plates with no growth were observed as MBC and it was recorded as 0.56 mg/ml (0.65 mM) for *S. aureus* and 1.125 mg/ml (1.25 mM) for *P. aeruginosa*. The synergistic activity of the P[5]A and isatin was examined by the checkerboard method against *S. aureus* and *P. aeruginosa*. The fractional inhibitory concentration (FIC) index for *S. aureus* was noted as 0.75 and for *P. aeruginosa* was 1.0. It was clearly evident that the FIC values less than 1 indicated synergistic activity and equal to 1 had partial synergy/ additive functions. These findings revealed the synergistic function of P[5]A and isatin against *S. aureus* and partial synergy/ additive functions against *P. aeruginosa*.

The antibacterial mechanism of P[5]A-isatin inclusion complexes was assessed by various parameters including bacterial time kill kinetics, the release of nuclear contents and cellular contents. The membrane permeability damages of treated bacteria were further confirmed by the scanning electron microscopic (SEM) images.

The bacterial time kill kinetics of P[5]A, isatin and P[5]A-isatin inclusion complexes was performed and the logCFU/ml was calculated after 0, 60, 120 and 150 minutes intervals. The results have indicated that the inclusion complexes had a broad range of antimicrobial efficacy against both bacterial pathogens. The inclusion complexes have shown to reduce the number of colonies of *S. aureus* and *P. aeruginosa* within 60 minutes with the logCFU/ml value of 4.2±0.02 and 4.04±0.03. After 180 minutes of treatment, the logCFU/ml was 4.65±0.02 and 4.66±0.08 for *S. aureus* and *P. aeruginosa*, respectively. From the results, it was clearly evident that the solvent

DMSO did not have any inhibitory activity towards *S. aureus* and *P. aeruginosa* and the results were well correlated with the antibiotic control.

Cell integrity and permeability are important phenomena for the bacteria to cause severe infections and for the development of resistance. Hence, the compounds were analyzed for their mechanistic action on damaging the cell wall of bacterial pathogens and release of their cell wall components to prevent their survival and pathogenesis. Protein and glucose are the two major components present in the cell walls of bacterial pathogens to maintain their integrity and permeability. The protein and glucose contents released from the *S. aureus* and *P. aeruginosa* cell walls were monitored after 18 hours of incubation with the inclusion complexes. The leakage of protein and glucose from the cell wall of *S. aureus* was found to be 76.61 µg/ml and 142.1 µg/ml, respectively. Similar results were observed with *P. aeruginosa* treated with P[5]A-isatin inclusion complexes and the released cellular contents were recorded as 97.08 µg/ml of protein and 150.29 µg/ml of glucose. The untreated control was not observed with much leakage of cellular components compared with inclusion complexes treated bacteria. The membrane damage caused by the inclusion complexes was confirmed with the scanning electron microscopic images. The results iterated that the compounds have a targeted action on the bacterial cell wall to make them susceptible to the synthesized P[5]A-isatin inclusion complexes.

Phase III: Antibiofilm potential of pillar[5]arene-isatin inclusion complexes against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

The biofilms play a significant role in the persistence of infections at the wound site and the development of resistance in pathogens by safely encasing their colonies in a protective matrix. Hence, there is a need for the inhibition of biofilms and eradication of the preformed biofilms by the pathogenic microbes. In connection with this, the antibiofilm activities of the synthesized P[5]A-isatin inclusion complexes in terms of biofilm inhibition, eradication and changes in motility behavior of the *P. aeruginosa* and *S. aureus* were evaluated by the standard protocols. 1/2 MIC, 1/4 MIC, 1/8 MIC of all the compounds, including, P[5]A, isatin and inclusion complexes were analyzed for their biofilm inhibitory and eradication profiles against *S. aureus* and *P. aeruginosa*.

Initially, the antibiofilm potential of synthesized inclusion complexes was determined using a standardized crystal violet assay. The percentage of biofilm inhibitory profile of the compounds, P[5]A, isatin and inclusion complexes were determined by measuring their absorbance at 570 nm. The results envisaged that the P[5]A-isatin inclusion complexes have shown the biofilm inhibitory potential against *S. aureus* and *P. aeruginosa* at their sub-MIC level and the inhibitory percentage was found to be 86% and 84%, respectively. The results were well correlated with the 1/2 MIC of antibiotic control and the inhibition of biofilm was 89% and 86%, respectively against *S. aureus* and *P. aeruginosa*.

Similarly, the preformed biofilm eradication profile was also carried out with the same concentrations of all compounds. It showed that the inclusion complexes were observed for a high biofilm eradication profile at their 1/2 MIC and the eradication percentage was 80% for *S. aureus* and 81% for *P. aeruginosa*. Isatin was also noted for its potential biofilm inhibition and eradication against pathogenic bacteria. The percentage of biofilm eradication of isatin at 1/2 MIC, 1/4 MIC and 1/8 MIC for *S. aureus* was 64%, 48% and 33% and for *P. aeruginosa* it was 64%, 51% and 34%. The 1/2 MIC of all the compounds has exhibited good inhibition and eradication profile against the selected bacteria, hence the 1/2 MIC was selected for further experiments.

Indeed, the flagella-type of motility in *P. aeruginosa* was an important phenomenon in biofilm formation and development. Of significance, the role of sub-inhibitory concentrations of P[5]A, Isatin and P[5]A-Isatin inclusion complexes on the motility behavior of *P. aeruginosa* were evaluated by swimming and swarming assay. The compounds, P[5]A, Isatin and P[5]A-Isatin inclusion complexes were able to reduce the swimming motility of *P. aeruginosa* and the diameter was noted as 22.3±1.5 mm, 16.1±0.76 mm and 3.3±0.57 mm, respectively. The swarming motility of *P. aeruginosa* treated with P[5]A, Isatin and P[5]A-Isatin inclusion complexes were also constantly decreased. The diameter of the swarming zone was found to be 14.1±0.28 mm for P[5]A and 10.1±0.76 mm for isatin and 5.3±0.577 mm for inclusion complexes. Colony diameter of the *P. aeruginosa* (control) and the negative control (DMSO) was found to be 31.6±0.57 mm and 33.3±0.577 mm, respectively.

S. aureus is a non-motile organism but it may move on soft agar plates via spreading. It may use the opportunity to interact with neighbouring microbes, especially *P. aeruginosa*. The

tryptic soy agar (TSA) plates with 0.4% agarose were prepared for performing the swarming motility of *S. aureus*. A bacterial culture (2 µl) that contained a mid-log phase culture of *S. aureus* alone and *S. aureus* plus *P. aeruginosa* was spotted at the center of a TSA plate. The swarming motility was decreased in a medium containing 1/2 MIC of P[5]A-isatin inclusion complexes and the zone of motility was recorded as 4.7±0.6 mm. The motility of *S. aureus* along with *P. aeruginosa* was observed due to their hitchhicking motility with neighboring microbes. The CFU/ml of *S. aureus* in soft agar after treatment was enumerated in a specific medium called mannitol salt agar. The results have shown the potential activity of P[5]A-isatin inclusion complexes by reducing the colonies formed on mannitol salt agar and the CFU/ml was found to be $0.74x10^4$ cells. This may be attributed to the activity of isatin, and $0.97x10^4$ cells were noted with isatin treated *S. aureus*. The control medium (only with bacteria) was observed with $3.1x10^4$ CFU/ml. The antibiofilm potential of the inclusion complexes was further confirmed with SEM. The results have supported that the compounds at 1/2 MIC level actively eliminated the biofilm formation and development of *S. aureus* and *P. aeruginosa*. These results have suggested that the P[5]A and isatin inclusion complexes could reduce the number of biofilm cells in *S. aureus* and *P. aeruginosa*, and thus could be used as an effective antibiofilm agent for further studies.

Phase IV: Evaluation of drug release kinetics, formulation and validation of pillar[5]arene-isatin inclusion complexes based wound healing ointment

The controlled drug release potential of P[5]A-isatin inclusion complex was assessed *in vitro* using the direct addition method and the drug release was validated using various mathematical models. The inclusion complexes were directly added to the dissolution media, phosphate buffer saline (pH 7.4). The release of isatin from the inclusion complex was measured at 275 nm using a UV-visible spectrophotometer. Nearly, 60% of the isatin was elevated from the P[5]A inclusion complexes within 3 hours. It iterates the slow release of isatin from the inclusion complexes for their better stability and bioactivity.

In concordance with this, the various mathematical models namely, the zero-order model, first-order model, Higuchi model and Korsmeyer-Peppas model were employed to understand the dissolution mechanism of isatin. The correlation coefficient (R^2) value for the drug release by the direct addition method was found to be 0.9961 and the isatin release from the P[5]A was found to

be at a constant rate by obeying the zero-order kinetics. The release of isatin from the P[5]A perfectly follows the Higuchi model with the increased R^2 value of 0.9514 and it implies the process of isatin release from the P[5]A inclusion complexes follows the process of diffusion. The type of diffusion process was analyzed by constructing the Korsemeyer Peppas model by plotting the graph between n log cumulative drug release % and log time. The release exponent of the drug release mechanism was found to be 0.74. The isatin transport mechanism lies in the Non-Fickian transport mechanism with standard values ranging from $0.45<n<1$. The mathematical model has explained how the compounds were effectively delivered at the target site by various mechanisms.

Based on the outcome of the results, the P[5]A-isatin inclusion complexes were developed as an ointment for the prevention of infections and aid in wound healing. The formulations were prepared using the basic ingredients namely P[5]A-isatin inclusion complexes and ointment base (wool fat, cetostearyl alcohol, hard paraffin and yellow soft paraffin). Subsequently, the physiochemical parameters include texture, odor, colour, transparency, spreadability and pH. The developed inclusions loaded ointment was observed as pale orange coloured semi-solid in nature and has thick viscous consistency without odour. It was well spreading at the surfaces with the value of 4.0 cm/10 ml concentrations of formulations. The pH of the developed ointment also lies in the range of 4.5 to 5.0 and it was well correlated with the skin pH, thus it proved the safer applications at the topical site to prevent wound infections and could promote the wound healing processes. The antimicrobial activities of the ointment against the selected bacterial pathogens, *S. aureus* and *Pseudomonas aeruginosa* were studied using the disk diffusion method. Nearly 10±0.5 mm and 9±0.5 mm zones of inhibition were observed against ointment treated *S. aureus* and *P. aeruginosa*, respectively.

In vitro cell-based scratch assay was performed to determine the wound healing potential of developed ointment formulations against L929 fibroblast cell lines. The L929 fibroblast cells were treated with 1000 µg/ml of ointment formulations for 48 hours. Cell migration at 24 hours and 48 hours were captured and the wound closure distance was calculated using Image J software. Cipladine is the commercial wound healing ointment that was used as the standard. The percentage of wound closure at different time intervals by the developed ointment formulations was recorded as 75.89% at 24 hours and 89.55% at 48 hours. Nearly 90.5% of wounds were closed at 48 hours of treatment with cipladine. The microscopic images also supported the

increased cell migration in developed ointment treated L929 cells compared with untreated control. Almost 56188.16 µm wound area was covered at 24 hours by the action of developed ointment compared with untreated control (wound area covered – 103125.10 µm). The results have suggested that the P[5]A-isatin incorporated ointment has a very good wound healing potential and prevents pathogenic attacks in wounds.

Finally, the cytotoxicity of the developed ointments was assessed using normal peripheral human blood lymphocyte cells using MTT reduction assay. Various concentrations of ointment were studied for their toxicity and viability. The results have proved that the developed ointment did not have any cytotoxicity. Thus, the developed ointment could be used to treat wound infections and also for wound healing without toxicity to the cells.

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