9. Chapter 9: Summary & conclusion

9.1. Summary

Anacardic Acid being a novel molecule, exerts pharmacological effect in several indications, specifically it has proven activity against biofilm and photoaging. However, it's applicability is limited due to certain shorcomings like poor aqueous solubility and physicochemical properties thereby limiting its therapeutic potential. Therefore, our research was hypothesized to improve therapeutic potential of Anacardic Acid, by employing different formulation strategies such as preparation of inclusion complex, encapsulation in solid lipid nanoparticles and development of nanosponge. HP-β-CD inclusion complex was prepared to incorporate $Ana_{C15:3}$ for the enhancement of aqueous solubility and thereby antimicrobial activity. The second study involved the formulation of chitosan and DNase coated SLNs encapsulating $Ana_{C15:3}$ with an aim to improve anti-biofilm potential of $Ana_{C15:3.}$ The last study involved the formulation of HP-β-CD nanosponge encapsulating $Ana_{C15:3,}$ followed by incorporation of the $Ana_{C15:0}$-NS complex in the topical gel with an objective to improve solubility, sustained drug release and finally enhancing the anti-photoaging activity of $Ana_{C15:0.}$

Cashew nut shell liquid (CNSL) was extracted from raw cashew nut shells through soxhlet extraction using light petroleum ether (boiling point range 40-60°C). Anacardic Acid was isolated from the CNSL mixture employing acid-base reaction via formation of calcium anacardate upon treatment of CNSL with calcium hydroxide followed by treatment with hydrochloric acid. Further, Anacardic Acid C15:3 and C15:0 were isolated by using column chromatography using silica C18 as stationary phase and methanol as mobile phase. The isolated compounds were subjected to HPLC, FT-IR and ^{1}H NMR for identification and characterization.

The inclusion complex of $Ana_{C15:3}$ was prepared with HP-β-CD using the co-evaporation technique, and the stability constant of the inclusion complex was determined by performing a phase solubility study. Further, the solid inclusion complex was characterized using different analytical techniques and evaluated for solubility followed by antimicrobial and hence anti-biofilm activity to establish the potency of the developed complex in enhancing bioactivity. The prepared inclusion complex was characterized using FT-IR, DSC, XRD, SEM, and ^{1}H NMR, which provided appropriate evidence to confirm the formation of the inclusion complex. The inclusion complex revealed a distinct FT-IR spectrum with the absence of absorption peaks at 2400–3400 cm^{-1}. The loss of aromatic peaks of $Ana_{C15:3}$ in the inclusion complex suggested the inclusion of $Ana_{C15:3}$ inside the HP-β-CD cavity. The DSC thermogram of $Ana_{C15:3}$ revealed wide diffused peaks around 167°C suggesting crystalline nature whereas the thermogram of inclusion complex indicated the complete absence of melting peaks corresponding to $Ana_{C15:3,}$ however, a broad endothermic peak in the region between 30–132°C suggested possible formation of inclusion complex. XRD study also indicated the transition of $Ana_{C15:3}$ from crystalline to amorphous form whereas substantial evidence was obtained in support of inclusion complex formation from the ^{1}HNMR data. The prepared inclusion complex demonstrated ~2009 times ($Ana_{C15:3}$ solubility of 38.17±0.38 Mm as compared to $Ana_{C15:3}$ solubility of 0.019±0.005 mM) improved aqueous solubility. The developed complex maintained the antimicrobial activity of pure $Ana_{C15:3}$ against *S. aureus*. $Ana_{C15:3}$/HP-β-CD complex exhibited excellent biofilm dispersal potential against mature biofilms as in the case of multiple-dose treatment, the inclusion complex revealed significant biofilm eradication as compared to pure $Ana_{C15:3}$. Confocal laser scanning microscopy (CLSM) demonstrated a remarkable decrease in bacterial biomass and thickness upon treatment with the inclusion complex. The biomass and thickness of $Ana_{C15:3}$ treated biofilms were 22.16±2.1 $\mu m^3/\mu m^2$

and 38.89±3.34 µm while treatment with inclusion complex leads to a reduction in biofilm biomass and thickness to 17.69±1.8 $\mu m^3/\mu m^2$ and 27.6±2.8 µm, respectively.

Chitosan and DNase coated SLNs ($Ana_{C15:3}$ -SLNs-CH-DNase) were prepared in order to target biofilm components and improve the antibiofilm potency of $Ana_{C15:3}$. The study was hypothesized to disassemble biofilm by application of $Ana_{C15:3}$ loaded solid lipid nanoparticles coated with chitosan and DNase I, because SLNs offer better encapsulation of $Ana_{C15:3}$ along with controlled-release delivery leading to a potential tool for targeting biofilms. Though biofilm formation leads to the development of dense colonies of bacteria surrounded by ECM, our study was anticipated to use DNase for disassembling the matrix by neutralizing the e-DNA. The use of chitosan in the formulation can be justified as it adds to the stability and integrity of nanoparticles. Moreover, positively charged nanoparticles are obtained with the use of chitosan which adds the advantage as positively charged nanoparticles would offer better adhesion and binding with the negatively charged biofilm matrix. Thus, the overall aim of the present study was to develop a formulation that can effectively treat the biofilm-mediated infection by simultaneously overcoming the limitations of $Ana_{C15:3}$. Therefore; in this study, we developed $Ana_{C15:3}$ loaded solid lipid nanoparticles (SLNs) which were further coated with chitosan and DNase ($Ana_{C15:3}$-SLNs-CH-DNase). The DNase coating was hypothesized to degrade the e-DNA, while chitosan was coated to yield positively charged SLNs with additional adhesion to biofilms. The SLNs were developed using the homogenization method and further evaluated for particle size, polydispersity index, zeta potential, and entrapment efficiency. Drug excipient compatibility was confirmed by using the FT-IR study, while the encapsulation of $Ana_{C15:3}$ in SLNs was confirmed by X-ray diffraction study. The chitosan and DNase coated SLNs demonstrated a particle size, polydispersity index and zeta potential values 212.8±4.21 nm, 0.285±0.04 and +13.5±1.92 mV, respectively. The SLNs demonstrated a biphasic drug

release pattern (74.86±2.73% in 24h) and Higuchi release kinetics (r^2=0.9724). The developed SLNs were found non-toxic against human immortalized keratinocyte (HaCaT) cells while demonstrated remarkably higher antimicrobial efficacy against *Staphylococcus aureus*. The excellent effect of the developed SLNs on biofilm formation and eradication further confirmed the superiority of the developed formulation strategy. A significant ($p<0.05$) reduction in biofilm thickness and biomass, as confirmed by confocal laser scanning microscopy, was observed in the case of developed SLNs in comparison with pure $Ana_{C15:3}$. Cumulatively, the results suggest the enhanced efficacy of the developed formulation strategy to overcome the biofilm-mediated antimicrobial resistance.

Further, $Ana_{C15:0}$ encapsulated HP-β-CD nanosponge (Ana $_{C15:0}$ -CD-NS) gel was developed to improve the antiphotoaging efficacy of $Ana_{C15:0}$ against UV-B induced skin potoaging. This study was designed to enhance the protective and therapeutic potential of $Ana_{C15:0}$ against UV-B induced skin photoaging. The hypothesis of the objective was to develop $Ana_{C15:0}$ encapsulated HP-β-CD-Nanosponge (CD-NS) based topical gel and thereby to improve its solubility and to ultimately enhance the anti-photoaging potential. The first study under this objective includes development, optimization, and characterization of $Ana_{C15:0}$ encapsulated CD-NS while the second study involves the preparation of topical gel incorporating $Ana_{C15:0}$-CD-NS which were further evaluated for its efficacy against UV-B induced skin photoaging. The nanosponge encapsulating $Ana_{C15:0}$ was prepared and optimized using a 3-level factorial design considering $Ana_{C15:0}$: NS ratio and sonication time as independent variables. Optimized formulation with particle size (197.4±11.6 nm), polydispersity index (0.276±0.028), zeta potential (-22.1±1.9 mV) and entrapment efficiency (74.8±2.9%) was prepared. The solubility study performed to evaluate the solubilization potential of nanosponge revealed a linear relationship of $Ana_{C15:0}$ to NS. A drug excipient compatibility study performed to examine the impact of

excipients on properties of $Ana_{C15:0}$ suggested no interaction and therefore the excipients were found to be compatible with the $Ana_{C15:0}$. Further, the XRD study revealed the transformation of $Ana_{C15:0}$ from crystalline to amorphous nature. *In-vitro* release study suggested diffusion-controlled release following the Higuchi release kinetics (R^2=0.9641). Texture profile analysis (TPA) was performed to assess the gel characteristics and it was found that the data obtained from the study was in the optimal range. The values of Hardness (N), Adhesiveness (N-mm) and Elasticity (mm) obtained from the TPA study were 7.4±1.2, 36.8±3.1, and 4.48±0.5 respectively. The effect of $Ana_{C15:0}$-NS gel was evaluated on photoaged skin which indicated excellent healing potential with a remarkable decrease in the ROS level following the $Ana_{C15:0}$-NS gel treatment. Further, a western blot study was performed to evaluate the expression of MMP-1 and HAT p300 enzymes, which play a key role in the pathogenesis of UV-B induced skin photoaging. The expression of MMP-1 and HAT p300 was significantly reduced which further suggested the excellent healing potential of $Ana_{C15:0}$-NS gel.

9.2. Conclusion

The present research focused on improvement of antimicrobial and antiphotoaging efficacy of Anacardic Acid by applying various formulation approaches. The first strategy included the development of formulations with an aim to improve its anti-biofilm and therefore antimicrobial activity while the second formulation strategy was focused to improve the anti-photoaging potential. We developed an HP-β-CD inclusion complex that not only overcame the solubility issue but also improved the efficacy against the *S. aureus* biofilms. The findings collectively supported the hypothesis to improve the solubility and thus the activity of $Ana_{C15:3}$ following the development of HP-β-CD inclusion complex. The biofilms contain dense colonies surrounded by an extracellular matrix where e-DNA plays a key role in the biofilm progression and develop resistance to antibiotics. Therefore, we

prepared chitosan and DNase coated solid lipid nanoparticles of $Ana_{C15:3.}$The combination of DNase with SLNs was envisaged to improve the antibacterial activity of $Ana_{C15:3}$ against *S. aureus* biofilms whereas chitosan coating provided additional bio adhesiveness as well as the facilitated attachment of SLNs to the biofilms. Thus, the proposed study successfully demonstrated the potential of the developed formulation in eradicating biofilms thereby improving antimicrobial potential.

Chronic skin UV exposure is recognized as a risk factor for the development of skin photoaging and later skin cancer. To overcome skin photoaging, we developed and ptimized $Ana_{C15:0}$ encapsulated HP-β-CD nanosponge which were further incorporated into a topical gel for skin application. The evaluation outcomes of the developed formulation suggested delivery potential of the product which we confirmed after *in-vivo* study. The topical gel applied to photoaged skin indicated excellent healing potential of $Ana_{C15:0}$ which was further supported by the ROS and western blot analysis for the level of different characteristic protens.

Although the data in hand clearly suggest the improved antimicrobial and anti-photoaging efficacy of Anacardic Acid yet, *in-vivo* assessment of anti-biofilm potential of $Ana_{C15:3}$ may further provide a concrete outcome. Being a novel molecule, the understanding of complete pharmacokinetic remains a future challenge for the researchers. The developed forumations may also be employed for enhancement of other pharmacological activities of Anacardic Acid in future. The concept of herbal pesticide is currently under immense deliberation, therefore suitable low cost formulation of Anacardic Acid may also be developed to explore the area.