1. Chapter 1: Introduction

1.1 Background

Cashew nut shell (CNS) is a by-product produced after processing of cashew nuts obtained from the plant *Anacardium occidentale* (family: *Anacardiaceae*). Cashew nut shell contains greenish yellow liquid entrapped inside soft honeycomb like structure of the shell which is popularly known as cashew nut shell liquid (CNSL). CNSL is an extraordinary source of natural polyphenols constituting about 30-35% of the cashew nut shell weight. CNSL primarily contains three major constituents *viz.* Cardol (15-20%), Cardanol (10%) and Anacardic Acid (60-65%) (Hamad and Mubofu 2015). Cardol has been proven for its utility against filarial parasite of cattle setariadigitata while cardanol finds its application in chemical industry in resins, coatings, frictional materials and paints. The third major constituent, Anacardic Acid, has been recognized for its wide range of pharmacological activities *viz.* anticancer, antimicrobial (Kubo, Muroi et al. 1993), and gastro protective activity (Morais, Pinto et al. 2010).

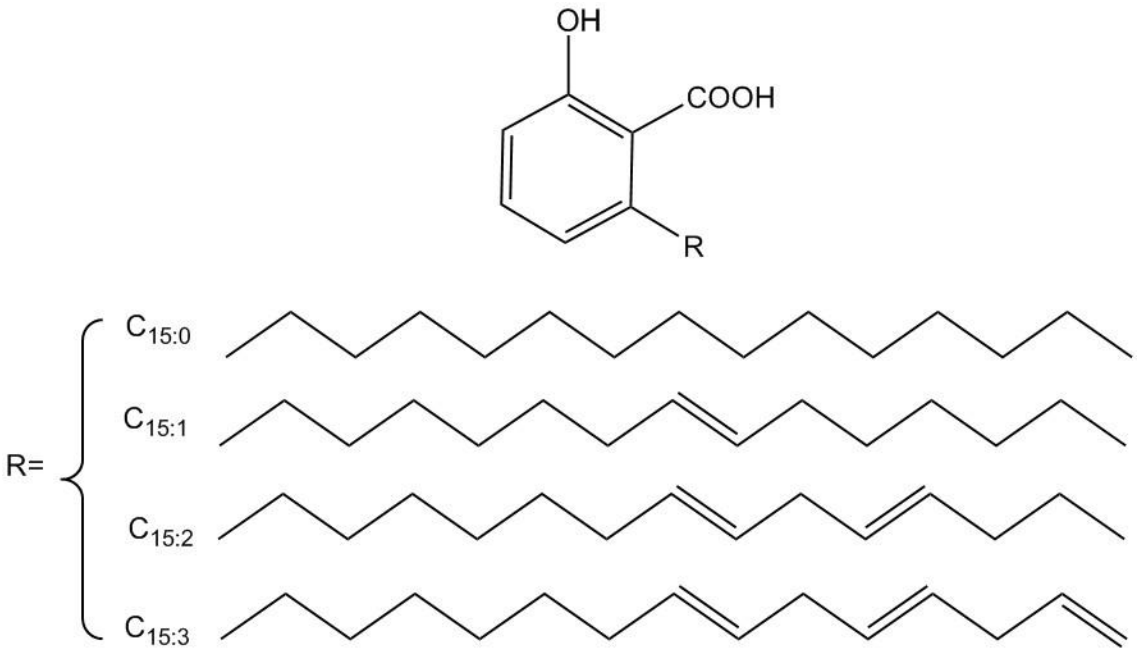

Figure 1.1: Chemical structure of Anacardic Acid and its subtypes

Anacardic Acid has also been reported to inhibit histone acetyl transferase enzyme (HAT) (Kim, Lee et al. 2013), which plays a key role in UV-radiation induced skin photoaging. The long aliphatic side chain of Anacardic Acid is 15 carbons long (Figure 1.1) and found as a mixture of monoene ($C_{15:1}$), diene ($C_{15:2}$), and triene ($C_{15:3}$), with the saturated component ($C_{15:0}$) being present in lesser amount. Although Anacardic Acid is found as mixture of four subcategories, the pharmacological activity of the subtypes varies due to alkyl side chain and unsaturation. The maximum antimicrobial activity is reported for Anacardic Acid triene ($C_{15:3}$) i.e $Ana_{C15:3}$ whereas antiphotoaging activity is reported exclusively for saturated component ($C_{15:0}$) i.e $Ana_{C15:0}$. Anacardic Acid has been found to have diverse range of pharmacological activities but its applicability is very limited due to the physicochemical characteristics like poor aqueous solubility, vesicant nature, short half-life and thereby limited bioavailability (Wasserman and Dawson 1948, Kushwah, Katiyar et al. 2018). This in turn creates absolute hindrance in its therapeutic application and further commercialization.

The earlier researches have been confined to demonstrate only evaluation of different pharmacological activities of Anacardic Acid, therefore a desperate need was observed to develop suitable formulation or delivery system to attain therapeutic and commercial value of this molecule. Further, there is a need to overcome the limitations, improve patient compliance and increase the therapeutic applicability of Anacardic Acid as no formulation of Anacardic Acid involving improvement of antibiofilm and antiphotoaging activities was reported till now.

Microbes/bacteria are capable of developing resistance to antibiotics by several means including biofilm formation. This leads to use of either high dose of antibiotics or its combination, resulting in undesired effects, toxicities and increased mortality (Patel

2005). $Ana_{C15:3}$ is a naturally occuring antibiotic, active against resistant bacteria and therefore it is supposed to be potential molecule to replace the existing antibiotics.

Skin photoaging is mainly caused due to over exposure of UV-B radiation and results in skin related complications like wrinkles, inflammation, psotules, pimples etc. These conditions arise due to damage to extracellular matrix of the skin (Pandel, Poljsak et al. 2013). Most of the available skin photoaging treatments are meant for protective purposes like sunscreens, and very less number of products are available in the market for skin photoaging therapy. Moreover, the available antiphotoaging products cover very high cost and are of synthetic origin. $Ana_{C15:0}$ is one of the subtypes of Anacardic Acid, specifically reported to have therapeutic potential against skin photoaging. $Ana_{C15:0}$ is a part of the naturally abundant cashew nuts and hence can be utilized as replacement for the expensive and synthetic antiphotoaging products available in the market.

Considering the above discussed situation, we propose to develop formulations of $Ana_{C15:3}$ to capitalize on improvement of antimicrobial activity by overcoming limitations like poor solubility and physicochemical characteristics. On the other hand, we hypothesize to improve therapeutic efficacy of $Ana_{C15:0}$ against UV-B induced skin photoaging by developing nanoformulation based topical gel.

1.2 Anacardic Acid activity against Biofilms

Biofilm is described as a sessile community of microbes consisting of complex, differentiated communities embedded in a matrix which is adherent in nature. Adhesion to biotic or abiotic surfaces leads to the secretion of extracellular polymeric substances (EPS) and colonization to develop dense clusters (Donlan 2002). The sessile bacteria exhibit disparate phenotypes in contrast to planktonic ones, as these are capable of communicating with each other through a process called quorum sensing. This in turn

triggers the biofilms to develop immunity against environmental alterations and other lethal threats (Patel 2005). The living microorganism (bacteria) embedded in the biofilm matrix can be over 1000 fold more resistant to antibiotics as compared to planktonic bacteria. It is estimated that over 60% of the bacterial infections affecting humans develop biofilm (Bora, Mazumder et al. 2019). Biofilms exhibit specific characteristics, like surface adherence, formations of clusters stranded in extracellular matrix (ECM), as well as resistance and tolerance to traditional antibiotics. These specific features of biofilms render the invading bacteria very hard to eradicate as they exhibit higher antimicrobial resistance as well as tolerance in comparison with planktonic bacteria. Forming biofilms empowers bacteria to bypass the host immune system. As a result, biofilms may lead to chronic infections, ultimately giving rise to high morbidity and mortality (Abebe 2020). Further, the composition of biofilm matrix includes polysaccharides, phospholipids, proteins, and extracellular DNA (e-DNA). The e-DNA has been reported to have an important role in the stabilization of biofilm structure and safeguard the cells (Montanaro, Poggi et al. 2011). Therefore, the use of deoxyribonuclease I (DNase) additionally with the antimicrobials can result in disassembling of the biofilm matrix and thereby exposing the planktonic cells directly to the antimicrobials. Further, biofilms carry negative charge on its surface and hence the positive charge on the carrier's surface might be an ideal property for targeting the biofilms.

The antimicrobial activity of Anacardic Acid covers wide range of microbes including *Staphylococcus aureus, Staphylococcus mutans* and *Propionibacterium acnes* etc (Hamad and Mubofu 2015). Antimicrobial activity of Anacardic acid is due to the salicyclic acid moiety whereas the alkyl side chain contributes to the variations in the activities of different forms ($C_{15:0}$- $C_{15:3}$). The antibacterial action of Anacardic Acid is

primarily due to the physical disruption of the bacterial cell membrane (Saedtler, Förtig et al. 2020). It has also been reported to act via entering into the cytoplasmic membrane lipid bilayers and thereby disrupting energy converting systems such as electron transport system (ETS) as well as ATPase. It also inhibits bacterial resistance possibly due to its action on the extracytoplasmic region and thereby avoiding cellular pump based resistance mechanisms. The antimicrobial activity of Anacardic acid varies with different forms and depends upon the number of unsaturation in the alkyl side chain. Maximum antimicrobial activity against *Staphylococcus aureus* has been reported for $C_{15:3}$ while the activity is least with $C_{15:0.}$ Anacardic Acid has also been proved to inhibit *Staphylococcus aureus* biofilms by inhibiting quorum sensing (Subhakara Reddy, Kumar et al. 2011, Asfour 2018).

CDs are low molecular weight cyclic oligosaccharides comprising of 6 (α-cyclodextrin), 7 (β-cyclodextrin), 8 (ϒ-cyclodextrin) or more glucopyranose units attached by a-(1, 4) glucosidic linkage which are widely used in pharmaceutical industry for variety of applications (Zhao, Wang et al. 2010). CDs have also found their application in food products, textiles, toilet preparations, cosmetics and different medical preparations. Cyclodextrins offer the ability to form noncovalent associates like "guest–host" inclusion complexes with hydrophobic drug molecules and have a well-designed chemical composition as well as physicochemical stability. β-Cyclodextrin possesses limited aqueous solubility owing to its rigid structure, due to the incurrence of intermolecular hydrogen bonds in the midst of the secondary hydroxyl groups (Yoshida, Shimomura et al. 1999). For further improving the solubility, β-cyclodextrin may be chemically altered to different forms. 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) is produced by substitution of the hydroxyl group with hydroxypropyl substituents in the

positions 2, 3, and 6. HP-β-CD possesses better solubility and lower toxicity than β-cyclodextrin (Garg, Gupta et al. 2010).

Solid lipid nanoparticles (SLNs) are nanosized (50–1000 nm) colloidal lipid nanocarriers comprising lipid matrix for drug encapsulation. The composition of SLNs involves solid lipids and surfactants (stabilizer) (Severino, Silveira et al. 2017). SLNs offer advantages over other nanocarriers in terms of controlled release of encapsulated drugs, increased solubility, stability, and higher payload of drugs. The vital features, like biocompatibility, targeted delivery of a drug, the capability to encapsulate both lipophilic and hydrophilic drugs, and decreased side effects, make this nanocarrier capable of biofilm therapy (Jourghanian, Ghaffari et al. 2016).

As Anacardic Acid is found as a mixture of four sub types and the activity of all the subtypes vary, we propose $Ana_{C15:3}$ formulations for enhancing the antimicrobial potential. Thus the objective of the present study includes development of formulations of $Ana_{C15:3}$ so as to enhance its solubility and improve other physicochemical properties and thereby enhance its antimicrobial potential. Two different formulation approaches were performed under this objective; in the first approach inclusion complex of $Ana_{C15:3}$ with HP-β-CD was developed and evaluated for its antimicrobial efficacy against *Staphylococcus aureus.* The rationale behind the selection of $Ana_{C15:3}$ among different forms is based upon earlier reports in which $Ana_{C15:3}$ has shown the highest antimicrobial activity among all the forms. In this study, the inclusion complex of $Ana_{C15:3}$ was prepared with HP-β-CD, and further stability constant of the inclusion complex was determined by performing a phase solubility study. Furthermore, the solid inclusion complex was characterized using different analytical techniques and evaluated for the solubilisation effect. Finally, the antimicrobial and anti-biofilm activities of $Ana_{C15:3}$ were examined to establish the potency of the developed complex in enhancing

bioactivity. Even though the HP-β-CD inclusion complex yielded substantial antimicrobial activity, further improvement in terms of specific drug targeting and enhanced drug release characteristics were needed.

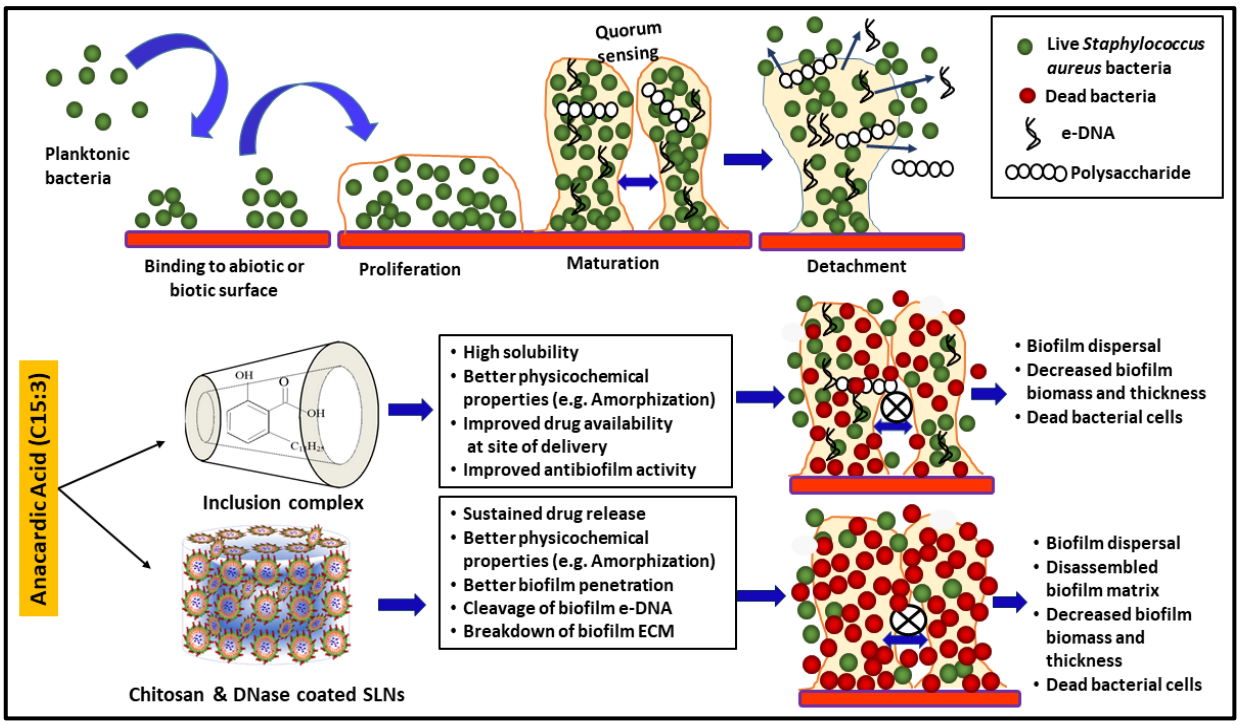

Figure 1.2: Photograph demonstrating various stages of *Staphylococcus aureus* biofilm formation and proposed formulation approaches to combat biofilm.

Since, biofilms comprise of different specific components (e.g., e-DNA, polysaccharides, overall negatively charged matrix) constituting extracellular matrix (ECM), therefore we designed another study to target and disassemble the biofilm matrix and hence to get specific biofilm targeting approach. In the second formulation approach, $Ana_{C15:3}$ loaded solid lipid nanoparticles coated with chitosan and DNase I were developed to have the higher entrapment and controlled release to overcome the biofilm mediated resistance. Though biofilm formation leads to development of dense colonies of bacteria surrounded with extracellular matrix (ECM), our study was anticipated to use DNase for breaking the matrix by disrupting 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 attraction, adhesion, and binding with the negatively charged biofilm matrix. Thus, the overall aim of the present study was to develop formulations which can effectively treat the biofilm-mediated infection by simultaneously overcoming the limitations of $Ana_{C15:3}$ (Figure 1.2).

1.3 Anacardic Acid activity against Photoaging

Chronic exposure to solar radiations especially UV-B (290-320 nm) leads to several skin complications characterized as deep wrinkles, loss of elasticity, dryness, rough texture appearance as well as pigmentation disorders. It is responsible for more than 80% of facial aging. Severity of skin photoaging depends upon type of skin, more eminent in individuals with skin type I & II (fair skin) as compared to less fair skin types (type III) (Bora, Mazumder et al. 2019). The extent of photo-damage also depends on cumulative UV dose accumulated and pigmentation of the skin. The symptoms of photoaged skin are primarily due to damage in the structural components of connective tissue of the dermis. The connective tissue is generated by fibroblasts comprising three major categories of biomolecules: glucosaminoglycans (GAGs), proteoglycans, structural proteins (collagen and elastin) and special macromolecules (fibrillin, fibronectin, laminin and hyaluronan). Collagen contributes maximum to the fibroblasts and forms of scaffolds to provide strength and structure of the skin. The pathological changes due to skin photoaging include formation of erythema, edema as well as inflammatory cellular infiltration (Quan, He et al. 2004). This in turn leads to the skin inflammation and other severe skin complications. Persistent UV-B exposure induces skin inflammatory cytokines like NF- kB mediated interleukin-6 (IL-6) influencing the target gene expression levels such as matrix metalloproteinases (MMPs) (Kim 2016).

This in turn results in formation of wrinkles and acute inflammatory responses. Furthermore, UV-B radiation induces expression histone H-3 acetylation as well as histone acetyltransferase (HAT) p300 ultimately leading to MMP-1 expression (Cho, Bahuguna et al. 2017).

Anacardic Acid ($C_{15:0}$), has been reported to suppress p300 HAT enzyme which plays a key role in the UV radiation mediated skin photoaging (Sung, Pandey et al. 2008). It has also been reported to inhibit MMP-1 gene transcription (Omanakuttan, Nambiar et al. 2012) as well as histone modification human dermal fibroblast. The antioxidant potential of Anacardic Acid may also play a role in fighting with photoaging by reducing the ROS production (Hemshekhar, Sebastin Santhosh et al. 2012).

Nansosponges are hyper cross-linked polymeric colloidal structures composed of sub microscopic particles with cavities ranging in nanometers. Cyclodextrin nanosponges (CD-NS) are novel class of nanosponges characterized as crosslinked, hyperbranched and non-aggregating polymeric nanostructures. These are biocompatible nanosystems prepared by polymerization reaction between CD molecules with cross linkers like carbonyl compounds and organic dianhydrides (Sherje, Dravyakar et al. 2017). Cyclodextrin nanosponges offer enormous opportunities for the development of potential drug delivery system due to its characteristics like ability to modulate physicochemical as well as pharmacokinetic profile of a drug molecule by enhancing its aqueous solubility. Other important features of CD-NS include high drug loading capacity, controlled release, and ability of protecting the drug from physicochemical degradation (Gursalkar, Bajaj et al. 2013).

Anacardic Acid ($Ana_{C15:0}$) also known as 6-pentadecyl salicyclic acid offers antiphotoaging activity exclusively out of the four subtypes of Anacardic Acid, hence we opted $Ana_{C15:0}$ for enhancing the anti-photoaging potential. The objective of this

study includes development of topical gel formulation based on the CD-NS to ultimately enhance the anti-photoaging potential of $Ana_{C15:0}$ (Figure 1.3). The first aim under this objective includes development, optimization and characterization of $Ana_{C15:0}$ encapsulated CD-NS while second approach involves preparation of topical gel incorporating $Ana_{C15:0}$-CD-NS and its efficacy evaluation against UV-B induced skin photoaging.

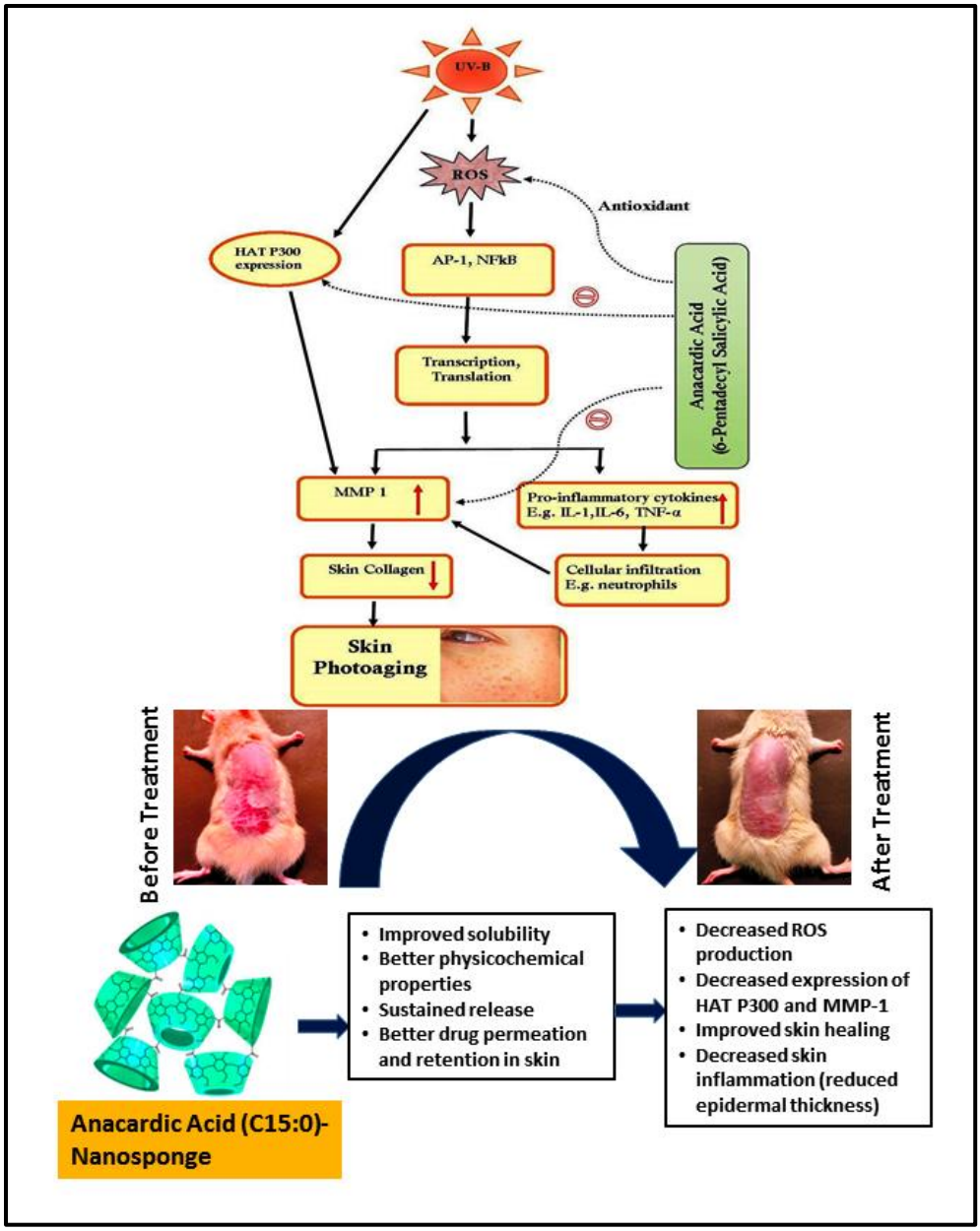

Figure 1.3: Photograph illustrating skin photoaging pathway, Anacardic Acid (C15:0) activity and formulation approach to improve photoaging therapy.