2. Chapter 2: Literature review

2.1. Anacardic Acid

Cashew (*Anacardium occidentale)* is the well-known species of the Anacardiaceae family. India is the largest producer and exporter of cashew kernel, accounting for almost 50% of world's export. As a result of cashew nut processing, the cashew nut shell (CNS) is produced as agricultural by-product (Hemshekhar, Sebastin Santhosh et al. 2012). However, CNS contains soft honeycomb like structure inside, comprising of extravagant greenish-yellow viscous liquid known as cashew nut shell liquid (CNSL). CNSL is an exceptional source of naturally occurring long-chain hydrocarbon phenols constituting approximately 20-25% of the raw cashew weight, and 30%–35% of the CNS weight (Philip, Da Cruz Francisco et al. 2008). Several methods have been reported for the extraction of CNSL from the CNS including hot-oil roasting, solvent extraction and extraction through supercritical carbon dioxide. The composition of CNSL varies and depends upon the method of extraction. Solvent extracted CNSL contains Anacardic Acid (60-65%), Cardol (15-20%) and Cardanol (10-15%) (Figure 2.1) and traces of other phenols as well as less polar molecules. Methods involving high heat result in decarboxylation of Anacardic Acid to form Cardanol and hence the composition changes to Cardanol (60-65%), Cardol (15-20%) and other polymeric materials (about 10%) (Yuliana, Nguyen-Thi et al. 2014). Cardol has proven its utility against filarial parasite of cattle setariadigitata while cardanol finds its application in chemical industry in resins, coatings, frictional materials and paints. Anacardic Acid being major component of the CNSL contains hydrophobic side chains, varying in the number of unsaturation, constituting about 5-8% saturated ($C_{15:0}$), 48-50% monoene ($C_{15:1}$), 16-18% diene ($C_{15:2}$) and 28-30% triene ($C_{15:3}$) (Morais, Silva et al. 2017). Anacardic Acid has attracted remarkable research attention owing to its pharmacological activities like antitumor, anti-

inflammatory, antimicrobial, anti-aging, antioxidant, and gastro-protective etc. moreover, Anacardic Acid has been widely used as synthesis of diversified bioactive compounds with enhanced efficacy (Hamad and Mubofu 2015).

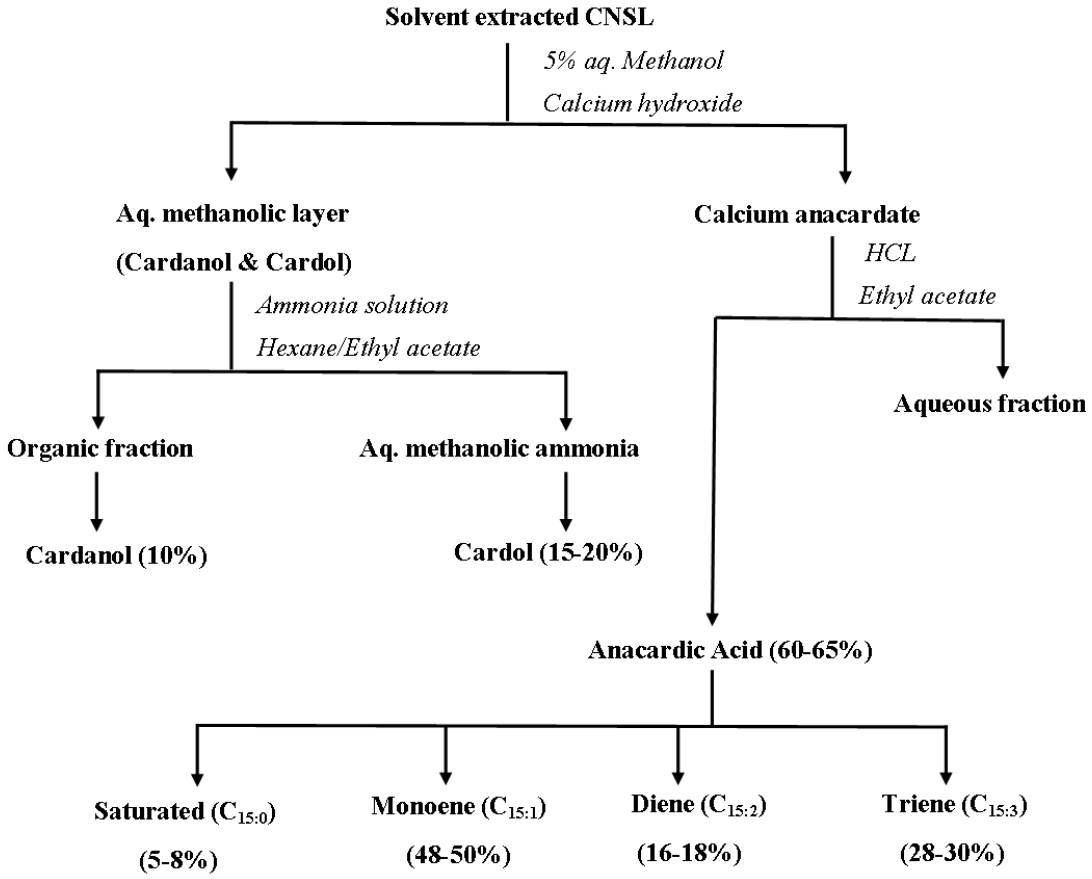

Figure 2.1: Overview of different components mixture of solvent extracted CNSL

2.1.1 Pharmacological Activities of Anacardic Acid

Anacardic Acid being a phytomolecule offers wide range of pharmacological activities *viz.* antimicrobial, anticancer, antiaging etc. Summary of the various reported activities along with its mode of action has been depicted in the Table 2.1.

Table 2.1: Summary of various pharmacological activities of Anacardic Acid.

Activity	Description	Mode of Action
Antimicrobial	Active against following microbes: ▪ *Propionibacterium acnes* ▪ *Corynebacterium xerosis* ▪ *Staphylococcus aureus* ▪ *Staphylococcus mutans* ▪ *Bacillus subtilis* ▪ *Helicobacter pylori*	▪ Inhibits quorum sensing ▪ Physical disruption of the bacterial cell membrane ▪ Disrupts energy converting systems such as electron transport system (ETS) as well as ATPase ▪ Inhibits β-lactamase
Antioxidant	▪ Interacts with free radicals ▪ Decrease oxidative stress by inhibiting reactive oxygen species (ROS) production	▪ Inhibits the propagation of chain reactions by stabilizing the radical species, thus preventing them from causing damage to the body cells
Anticancer	▪ Pituitary adenoma cells ▪ Melanoma cells, HeLa cells ▪ Ovarian cancer	▪ Inhibits histone acetyl transferases (HATs)
Anti-photoaging	▪ Excellent healing potential against UV-B induced skin photoaging	▪ Suppresses levels of different factors (MMP-1, inhibits HAT p300 and decrease ROS) involved in pathogenesis
Anti-inflammatory	▪ Suppresses expression of NF-kappa B ▪ Inhibits lipoxygenases	
Anticavity	▪ Useful in prevention of dental cavities	▪ By inhibiting *Staphylococcus mutans*
Anti-gout	▪ Decreases levels of uric acid	▪ Inhibits xanthine oxidase

2.1.2 Physicochemical properties of Anacardic Acid

Various physicochemical properties of Anacardic Acid (C15:3 and C15:0) is listed in the table (Table 2.2) below;

Table 2.2: Physicochemical properties of Anacardic Acid

Properties	**Anacardic Acid C15:3**	**Anacardic Acid C15:0**
IUPAC name :	2-hydroxy-6-(8Z,11Z)-8,11,14-pentadecatrien-1-yl-benzoic acid	2-Hydroxy-6-pentadecylbenzoic acid
Molecular formula :	$C_{22}H_{30}O_3$	$C_{22}H_{36}O_3$
Molecular weight :	342.5 g/mol	348.5 g/mol
Solubility :	Soluble in organic solvents like ethanol, DMSO, dimethyl formamide, sparingly soluble in aqueous buffer, poorly soluble in water	
logP :	7.69 (ALOGPS)	8.13 (ALOGPS)
pKa (strong acidic):	2.64 (ChemAxon)	2.64 (ChemAxon)
Assay methods :	UV-spectrophotometry and HPLC	UV-spectrophotometry and HPLC

2.1.3 Recent studies on Anacardic Acid

i. Anacardic Acid was isolated from cashew nut shell liquid using preparative high performance liquid chromatography (HPLC), Anacardic acid C15:3, C15:2 and C15:1 was isolated and quantified (Oiram Filho, Zocolo et al. 2019).

ii. Lima et al. 2020 evaluated antimicrobial effect of Anacardic Acid (Ana) loaded-zein nanoparticles on *Streptococcus mutans* biofilm and concluded that both MIC and MBC values for Ana-loaded zein nanoparticles were 0.36 μg/mL. Formulations were very effective in inhibiting *S. mutans* biofilm formation, as no colony-forming units were detected. Further, Ana-based nanoformulations demonstrated very high inhibitory and bactericidal activities against planktonic *S.*

mutans, and the results indicate a strong antiplaque effect (Lima, de Souza et al. 2020).

iii. In a recent study by Rose et al. 2020, reported that Anacardic acid induced IL-33 in the nervous system and resulted in reduced injury in animal models of disease in which loss of myelin was experimentally induced (Ljunggren-Rose, Natarajan et al. 2020).

iv. Yuliana et al. 2014 successfully separated and purified cardol, cardanol and Anacardic acid from cashew (*Anacardium occidentale* L.) nut-shell liquid utilizing a simple two-step column chromatography (Yuliana, Nguyen-Thi et al. 2014).

v. Kim et al. 2017 reported that $Ana_{C15:0}$ amileortates ultraviolet radiarion induced damage to human skin. The topical application of $Ana_{C15:0}$ significantly prevented a UV-induced increase in the erythema index. Moreover, histological findings from skin biopsies demonstrated significant reduction in epidermal thickness in the 0.1% $Ana_{C15:0}$-treated group compared with that in the UV-treated group (Kim, Shin et al. 2017).

vi. Kubo et al. 2006 evaluated antioxidant activity of Ana and concluded that it possess preventive antioxidant activity, also prevents generation of superoxide radicals by inhibiting xanthine oxidase without radical scavenging activity. Further, Ana act as antioxidant in a variety ways, including inhibition of various prooxidant enzymes involved in the production of the reactive oxygen species and chelate divalent metal ions such as Fe^{2+} or Cu^{2+}, but do not quench reactive oxygen species (Kubo, Masuoka et al. 2006).

vii. Huang et al. 2014 reported that Ana induces cell apoptosis associated with induction of ATF4-dependent endoplasmic reticulum stress while induction of

ER stress by Ana was supported by a dose- and time-dependent increase in expression of the ER signalling downstream molecules, like GRP78/BiP, phosphorylated eIF2, ATF4 and CHOP in both HepG2 and U266 cell lines (Huang, Hua et al. 2014).

viii. Kushwah et al. 2018 developed Anacardic Acid modified self-assembled albumin nanoparticles for co-delivery of docetaxel (DTX) and gemcitabine (GEM) for effective breast management of cancer. The developed NPs were reported safe with no marked effect on RBCs, lower hepato and nephro toxicity. Data collectively suggested promising potential of developed NPs in ameliorating the pharmacokinetic and therapeutic profile of combinatorial regimen of DTX and GEM (Kushwah, Katiyar et al. 2018).

ix. In a study by Hollands et al. 2016, reported that Ana from cashew nut shells stimulates neutrophil extracellular trap production and bactericidal activity. Moreover, the molecular modelling and pharmacological inhibitor studies suggested Ana stimulation of neutrophils occurs in a PI3K-dependent manner through activation of surface-expressed G protein-coupled sphingosine-1-phosphate receptors. Neutrophil extracellular traps produced in response to Ana are bactericidal and complement select direct antimicrobial activities of the compound (Hollands, Corriden et al. 2016).

2.2. Microbial infection

The capability of microbes to elicit disease in humans is a global concern and also a leading challenge in all health science sectors. Approximately 15 million out of 57 million annual deaths worldwide are estimated to be associated directly with infectious diseases (Broz and Monack 2011). The commensalism relationship between microorganisms and humans is well known; however, it is stipulated that most

microorganisms have an opportunistic profile, especially in situations where there is a deficit in the host's immune system. These organisms start to adopt an aggressive character and are extremely complex for patient health (Friedman, Newton et al. 2003).

2.3. Microbial resistance to antibiotics

Microbial resistance against antibiotics has become a significant problem, as microorganisms are acquiring the ability to resist drugs, making them a threat to public health. Over the years, antimicrobial resistance has become increasingly widespread, resulting in a significant threat to public health. Some of the commonly known antimicrobials resistance are as follows; sulphonamide, penicillin, methicillin, and vancomycin-resistant *Staphylococcus aureus*, macrolide-resistant *Streptococcus pyogenes,* penicillin-resistant *Streptococcus pneumonia,* etc.(Harriott and Noverr 2009) . Around 40–60% strains of *Staphylococcus aureus* found in hospitals in the United States and United Kingdom are resistant, and most of these are also resistant to multiple antibiotics (Tenover 2006). Bacterial drug resistance has multiple negative effects *viz.* higher doses of drug administration, addition of treatments with higher toxicity, longer hospital stays, and increased mortality (Poole 2005).

2.4. Mechanisms of antibiotic resistance

Development of antibiotic resistance involves several steps including acquisition by microbes of resistance genes, expression of those resistance genes, and selection for microbes expressing those resistance genes. At the beginning, bacteria acquire resistance to single and multiple drugs through horizontal gene transfer by transformation (Redondo-Salvo, Fernandez-Lopez et al. 2020), conjugation, and transduction. Bacteria can also acquire resistance genes by spontaneous mutation of existing genes (Zhang and Heridato 2016). Multiple drug resistance is acquired when a bacterial cell already containing one type of drug resistance gene acquires another type of drug resistance gene

(Van Langeveld, Gagnon et al. 2017). In the second step, in response to exposure to antimicrobial drugs, microbes express the resistance gene. While in the third step, resistance becomes widespread when there is selection for microbes that express resistance genes against the antimicrobial drugs. This selective pressure in favour of resistance occurs whenever microbes are exposed to the drug but not eradicated (either by the bactericidal effects of the drug itself, or by bacteriostatic effects of the drug followed by killing by the host's immune system). Apart from the generalized mode of antimicrobial resistance, there are several specific mechanisms adopted by different bacteria to resist the effect of antibiotics (Nikaido 1989, Sefton 2002). These mechanisms include;

- Decreased uptake and increased efflux of drug from the microbial cell
- Expression of resistance genes that code for an altered version of the substrate to which the antimicrobial agent binds.
- Covalent modification of the antimicrobial drug molecule which inactivates its antimicrobial activity
- Increased production of a competitive inhibitor of antibiotic
- Drug tolerance of metabolically inactive persisters.
- Biofilms

2.5. Biofilm and biofilm mediated antimicrobial resistance

Biofilm is a dense structure comprising clusters of cells encapsulated in a complex extracellular polymeric matrix (EPM). Bacteria in biofilm are more likely to resist to antibiotic treatments, as most drugs do not freely diffuse into the biofilm and thus do not reach optimal therapeutic concentrations (Flemming, Wingender et al. 2016). Biofilm containing bacteria exhibit different physiology as compared to planktonic cells (free

living bacteria) such as diminished metabolic rate, and improved cell to cell communication, which makes antibiotics less effective and increases the chance of developing resistance (Omar, Wright et al. 2017). Bacteria within the biofilm, termed sessile bacteria, exist in a stationary or dormant growth phase and exhibit phenotypes which are distinct from planktonic bacteria (Kumar, Alam et al. 2017). Bacteria in the biofilm demonstrate exceptional resistance to environmental stresses, especially antibiotics making it a major public health problem as 60-80% of human microbial infections are caused by bacteria growing as a biofilm (Crouzet, Le Senechal et al. 2014).

2.5.1 Stages in biofilm formation

Biofilm formation involves total five stages which are as follows;

a. Surface adherence

This process involves early attachment of human host protein (example: proteins from blood or tissues) to the liquid or solid surface (example: human host tissue or an implanted medical device), leading to formation of a layer known as conditioning film. This may also be mediated by non-specific factors like physicochemical factors, and also by specific bacterial protein components. It mainly depends upon the nature of the bacterial cell-wall and superficial proteins in addition to the hydrophobicity of the surface (Satpathy, Sen et al. 2016).

b. Initial attachment

Initial attachment occurs when planktonic bacterial cells attract and attach to the conditioning film via electrostatic, hydrophobic, and london dispersion forces. Further, these bacterial cells divide and recruit other planktonic bacterial cells, thereby increasing the population of bacterial cells (Meliani, Bensoltane et al. 2015).

c. Irreversible attachment

This step is facilitated once the number of initially attached bacterial cells population increases above a certain threshold. These bacterial cells trigger gene expression though quorum sensing leading to synthesis and secretion of a matrix consisting of extracellular polymeric substance (EPS). The EPS matrix mainly composed of polysaccharides, proteins and DNA (e-DNA). The EPS accumulates and eventually surrounds the population of bacterial cells. The EPS matrix is hydrated (contains around 95% water) and also gathers materials from surrounding environment like minerals, blood proteins, and debris (Veerachamy, Yarlagadda et al. 2014). The EPS contains various channels and pores which further facilitates irreversible attachment of cells with each other and the surface on which the cells lie.

d. Maturation

In this step biofilm slowly grows and form the micro colonies embedded inside the EPM. The biomass and EPS production of the biofilm increases, leading to the development of complex 3-dimensional architecture. Specific type of polysaccharides known as poly-*N*-acetyl-glucosamine (PNAG or PIA-Polysaccharide Intercellular Adhesin) is produced when bacteria are embedded within biofilm (mainly from staphylococcal species) (Fey and Olson 2010). The expression of such polysaccharides is specifically regulated by operons and regulons which are especially activated at precise period of biofilm growth. The other macromolecular component of EPS includes proteins, whose role inside the biofilm is to promote cell-cell adhesion, communication and adherence of the cells to the surface (Otto 2013).

e. Detachment or dispersal

Small biofilm segments or single cells are released to promote colonization at other sites. After reaching to the equilibrium, detachment of small lumps from the biofilm happens which results into the release of planktonic bacteria. The released planktonic bacterial

cells travel through growth medium (for example; blood of the human host) to conditioning films on other surfaces leading to the formation of new biofilms (Kostakioti, Hadjifrangiskou et al. 2013). This process is triggered by a plethora of stimuli and precisely regulated with specific gene expression patterns for example; Phenol-Soluble Modulins (PSM) are surfactants produced by *staphylococci* to achieve EPS disruption and subsequent dispersal (Fey and Olson 2010).

2.5.2 Components of biofilm

The extracellular polymeric substance (EPS) matrix of biofilm is mainly composed of extracellular polysaccharides or exopolysaccharides, DNA and proteins. Channels present in the biofilm allow passage of water, air and nutrients to all parts of the EPS matrix (Mann and Wozniak 2012).

a. Exopolysaccharides

Exopolysaccharides are synthesized either extracellularly or intracellularly and look like linear or branched strands, attached to the cell surface which may be stretched to form large networks (Tkhilaishvili, Lombardi et al. 2018). The exopolysaccharides serve as scaffolds for other carbohydrates, proteins, nucleic acids and lipids to adhere. The components, structure and features of the exopolysaccharides differ from each other. The abundant exopolysaccharides in biofilm include mannose, galactose and glucose followed by *N*-acetyl-glucosamine, galacturonic acid, arabinose, fructose, rhamnose and xylose (Jennings, Storek et al. 2015).

b. Extracellular proteins

Extracellular proteins are another major components of EPS matrix; some of the proteins are adhered to cell surface as well as polysaccharides to help in biofilm formation and its stabilization. Key example of extracellular proteins includes glucan binding proteins (Gbps) in *S. mutans* biofilm. Gbps play an important role in biofilm architecture

maintenance by linking bacteria and exopolysaccharides. Biofilms produced by Gbps mutants have lesser height. Another example includes biofilm associated protein (bap) family (Fong and Yildiz 2015) which is comprises of bap protein from *S. aureus* and involved in the biofilm formation and infection processes. Few enzymes are engaged in degradation processes within biofilms and their substrate include polysaccharides, proteins, nucleic acids, cellulose, lipids, other EPS matrix components as well as the objects that are trapped in the EPS matrix (Flemming and Wingender 2010). These enzymes are capable of breaking down biopolymers and provide carbon and energy resources to biofilm cells, especially during starvation. Biofilm detachment and dispersal also need enzymatic activity. Degradation of EPS matrix internally by enzymes releases biofilm cells and initiate a new biofilm lifecycle. For example, DspB protein is responsible for the surface detachment of *Actinobacillus pleuropneumoniae* biofilms (Hathroubi, Loera-Muro et al. 2018).

c. Extracellular DNA (e-DNA)

Extracellular DNAs were earlier believed to be leftovers from lyzed cells until Mattick and coworkers found that DNase I could prevent *P. aeruginosa* biofilm formation (Whitchurch, Tolker-Nielsen et al. 2002). e-DNA is actively secreted from the bacterial cells and plays an important role in biofilm formation, attachment and maturation. The negative charge of e-DNA results in repulsive force in early attachment as the distance between cell and surface ranges within few nanometer. e-DNA interacts with receptors present on the substrate surface to promote adhesion. e-DNA has the potential to chelate metal cations and other positively charged antibiotics owing to its negative charge (Schwartz, Ganesan et al. 2016).

2.5.3 Complications associated with biofilm mediated infections

Biofilms are associated with several human diseases; most of the bacterial infections are biofilm related, like chronic lung, wound and ear infections. Biofilms are also capable of colonization on medical devices like catheters and implants (Lister and Horswill 2014). As per the NIH report, over 80% of the microbial infections are biofilm mediated making very difficult to diagnose and treat (Gries and Kielian 2017). Some of the biofilm related complications are summarised below;

- Results in high resistance to the antibiotic therapy leading to enhancement in minimum inhibitory concentration by 100-1000 times (Høiby, Bjarnsholt et al. 2010).
- Develop chronic infectious disease condition.
- Biofilm has the potential to cause septicaemia at the site of infection (e.g. wounds)
- Very low susceptibility against the host immune system.
- Ability to produce low grade inflammatory response due to host immune mediator like polymeric nucleosides.

2.5.4 Biofilm mediated resistance to antibiotics

Bacteria embedded inside biofilm are highly resistant to antibiotics than planktonic bacteria. Reports estimate that biofilm cells can be up to 10,000 times more resistant to antibiotics than planktonic cells (Mah and O'Toole 2001). Several mechanisms have been proposed for the biofilm associated antibiotic resistance, some are described below;

a. Limited antibiotic penetration

EPS matrix has a key role in antibiotic resistance by limiting antibiotics penetration into the biofilm. Charged polysaccharides and e-DNA has the potential to trap several

antibiotics. This in turn leads to development of tolerance against antibiotics (Suci, Mittelman et al. 1994).

b. Horizontal gene transfer

Many bacteria may acquire antibiotic resistance through random genetic mutations while other harbour antibiotic resistant genes on plasmids. Plasmids can be easily transferred to other cells by horizontal gene transfer. In biofilms, rate of horizontal plasmid transfer is higher than planktonic cells. Study on *S. aureus* biofilms indicated that biofilms promote the spread of plasmid-borne antibiotic resistance genes by conjugation or mobilization (Savage, Chopra et al. 2013).

c. Reduced growth rate

Limited availability of oxygen and nutrients inside biofilms renders biofilm cells with slow metabolic rate, this is even more prominent in deep biofilm layers. In addition to slow metabolism rate, diminished division rate is also observed in biofilms. Such features make biofilm forming bacteria insensitive to antibiotics that target the dividing cells. For instance, the targets of β-lactam antibiotics are dividing cells, as they are used on *E. coli* biofilms, their bacteriolytic activity is decreased (Ashby, Neale et al. 1994).

d. Persister cells

Biofilms contain minute fraction of cells known as persister cells with extremely slow or almost zero growth rate. Most of the currently available marketed antibiotics target the processes involving cell growth or division and hence not effective against persister cells. Therefore, such cells behave like reservoirs which could reactivate to infectious moieties upon removal of antibiotic stress (Lewis 2007).

e. Efflux pumps

Efflux pumps promote bacterial cells to eject out intracellular toxins including antibiotics. The efflux pumps are also expressed by planktonic bacteria however; some efflux pump

genes are expressed in biofilms contributing to antibiotic resistance. *P. aeruginosa* gene A1874–1877 expression in biofilm is much higher than in planktonic cells thereby efflux pump encoded by this gene enhances the resistance to tobramycin, gentamicin and ciprofloxacin (Zhang and Mah 2008).

f. EPS matrix protection

EPS matrix facilitates physical protection to the accumulated biofilm cells. Reports have demonstrated that exopolysaccharide alginate in *P. aeruginosa* biofilms keep biofilm bacteria protected from human leukocyte killing (Leid, Willson et al. 2005).

2.5.5 Diseases associated with biofilm formation

There are several diseases related to biofilm development which leads to complications and mortality. The diseases may be categorised by biofilm formation on biotic or abiotic surfaces. The biofilm formation associated with implants or devices is facilitated by attachment of microbes to abiotic surfaces while attachment to biotic surfaces results in the biofilm formation in tissues (Del Pozo 2018, Jamal, Ahmad et al. 2018). Various biofilm related diseases are listed below;

Diseases involving biofilm formation on abiotic surface	**Diseases involving biofilm formation on biotic surface**
• Cardiac valve infection	• Chronic wound infections
• Urinary catheter related infection	• Chronic otitis media
• Prosthetic joint infection	• Dental plaque
• Contact lenses	• Cystic fibrosis
• Endotracheal tube infections	• Endocarditis
• Breast implants	• Osteomyelitis

2.5.6 Prevention and therapy of biofilm related complications

Several strategies have been reported for management of biofilm associated infection, these cover both preventive as well as therapeutic approaches. These approaches are described below;

2.5.6.1 Prevention of biofilm infections

The preventive approach includes physical removal of the implant or other device to avoid further worsens the infection. Other preventive measures are prevention of bacterial attachment to biotic or abiotic surfaces, antimicrobial coating of implants and devices, application of materials which are anti-adhesive in nature. Vaccination can also be done to avoid chronic biofilm infections.

2.5.6.1.1 Physical removal of the biofilm

Biofilms may be in the form of debridement for wounds or a combination of irrigation and debridement for orthopaedic implants or other devices. Retaining the implants may result in failure to resolve these infections and cause more complications. Therefore, physical removal techniques may be employed to get rid of chronic biofilm related complications (Meyer 2003).

2.5.6.1.2 Preventing attachment of bacteria to surfaces

Biofilm formation is generally associated with different stages like attachment, adherence, proliferation, maturation and dispersal. The attachment of bacteria during biofilm formation may depend upon whether it attaches to biotic or abiotic surface. Attachment to abiotic surfaces like glass, metals and plastics may be nonspecific whereas biotic surface attachment depends on bacterial recognition of host proteins. The problem associated with biofilm related infections has therefore led to the design of anti-infective approaches to prevent initial bacterial colonization. This includes coating the devices to

prevent the attachment of bacteria (anti-adhesion) to and/or growth on (antibacterial) artificial surfaces (Francolini and Donelli 2010).

2.5.6.1.3 Antibacterial coatings

Several approaches have been reported to develop bacteriostatic and bactericidal coatings in addition to surface engineering to prevent attachment of bacteria. For example; titanium, stainless steel and other commonly used implant materials are coated with antibiotics such as vancomycin to prevent growth of *S. aureus* on these surfaces (Jang, Park et al. 2010, Chen, Yu et al. 2013).

2.5.6.1.4 Anti-adhesion surfaces

This approach is aimed to reduce bacterial adhesion to abiotic surfaces and accompanied by development of materials that retard the adhesion and used in combination with co-administration of antibiotics or antimicrobials. Thus, the dual strategy focuses to prevent planktonic bacteria from easily attaching to the implant surface while allowing killing of this antibiotic susceptible population. The anti-adhesion surface can be developed either by changing the surface physical properties (for example; hydrophobicity/hydrophilicity, texture, charge and roughness) in such a way that bacteria would no longer be able to attach easily (Boks, Kaper et al. 2009).

2.5.6.1.5 Vaccination

Research and development of vaccines for prevention of bacterial biofilms especially *S. aureus* has grown remarkably with the rise of antibiotics resistance. Some vaccine approaches have been successful in specifically targeting biofilms. Vaccines against the polysaccharide intercellular adhesin (PIA) component of the biofilm matrix have been developed and tested (Arciola, Campoccia et al. 2015). PIA or poly-N-acetyl-β-(1,6)-glucosamine (PNAG) was one of the first molecules identified in biofilm accumulation

and continues to be a vaccine candidate as antibodies to deacetylated PNAG epitopes behave as better opsonins for humoral protection (Harro, Peters et al. 2010).

2.5.6.2 Therapeutic strategies for biofilm treatment

2.5.6.3 Quorum sensing inhibitors

Bacteria develop the capacity to sense their surviving environments and modify their physiological processes in order to adapt and survive. Quorum sensing (QS) is a process which is used by bacteria to coordinate gene expression as per their density, functioning as a decision making process to regulate the production of virulent factors and create infection (Brackman and Coenye 2015). RNA-III inhibiting peptide has been reported to suppress *staphylococcal* TRAP/agr systems and hence reduce biofilm formation in vivo. The results demonstrated the importance of quorum sensing in biofilm infections in the host. The treatment with RNA-III inhibiting peptide in rats has indicated better prevention of methicillin resistant *S. aureus* graft infections, and suggested that RNA-III-inhibiting peptide can be considered as anti-QS or/and anti-biofilm agent (Giacometti, Cirioni et al. 2003) .

2.5.6.3.1 Bacteriophage therapy

Bacteriophages are kind of viruses that infect and replicate within bacteria which lyse the host bacteria. Bacteriophage therapy for bacterial biofilm infections has been prevalent for decades and the development of antibiotic resistance, especially the multidrug resistant bacteria has attracted significant attention towards the investigation on bacteriophages. Experimental and clinical studies have shown exciting effects in not only wound biofilm infections, but also implants and catheter associated infections. Bacteriophages have the potency to infect and kill both antibiotic sensitive and resistant bacteria (Khalifa, Brosh et al. 2015).

2.5.6.3.2 Matrix degrading enzymes

Extracellular matrix of the biofilm consisting of protein, extracellular DNA (e-DNA) and polysaccharide provides a physical barrier which protects bacteria in the biofilm from host immune defence and antibiotics. Several approaches to disrupt the extracellular matrix are under investigation (Kaplan 2014). These approaches include exogenous addition of enzymes to disrupt the polysaccharide (dispersin B) or the extracellular DNA components of the EPS. Dispersin B, the enzyme produced mainly by the periodontal pathogen, *Actinobacillus actinomycetemcomitans*, disrupts polysaccharide components of staphylococcal biofilms however, susceptibility of *S.aureus* to dispersin B varies between strains. Moreover, DNase-I have been found effective in targeting *S. aureus* biofilm and disrupting the matrix by breaking down the extracellular DNA.

- ***Deoxyribonuclease-I (DNase-I)***

Extracellular DNA (e-DNA) is supposed to be the major structural and functional component of the biofilm matrix in most bacterial species; establishing role of DNase I in biofilm control has been the biggest break though in biofilm research. DNase I not only inhibits biofilm formation but also exhibit antibiofilm acivity in variety of both gram positive and gram negative bacteria (Tetz and Tetz 2010). DNase I has been reported to inhibit biofilm formation by the gram negative species like *P. aeruginosa*, *Bdellovibrio bacteriovorus*, *Comamonas denitrificans*, and *Haemophilus influenzae*, and by the gram positive bacterial species like *S. aureus*, *S. epidermidis* and *Streptococcus intermedius.*

DNase I act by degrading e-DNA which regulates cell to cell adhesion within the biofilm colony. It also restricts cell surface associated nucleic acids thereby inhibit the initial attachment of bacterial cells to the surface (Tetz, Artemenko et al. 2009). DNase-I is a nuclease in nature and cleaves DNA predominantly at phosphodiester linkage near pyrimidine nucleotide producing 5' phosphate-terminated polynucleotides with a free

hydroxyl group on position 3'. It works on single, double stranded DNA, and chromatin. DNase-I has attracted remarkable attention due to its potential in e-DNA degradation and thereby biofilm eradication potential.

- Baelo et al. 2015 utilized DNase-coated nanoparticles with an objective to disassemble the bacterial extracellular matrix and thereby to enhance antibiotic delivery in biofilm infections. Findings of the study indicated three successive dose of DNase I-coated nanoparticles comprising ciprofloxacin was able to reduce biofilm by 95% and then eradicate more than 99.8% of established biofilm (Baelo, Levato et al. 2015).
- Kaplan et al. 2012 investigated anti-biofilm efficacy of recombinant human DNase-I (rhDNase) against *Staphylococcus aureus* and *Staphylococcus epidermidis*. The results indicated that the biofilm formation by *S. aureus* was efficiently inhibited by rhDNase and detached established biofilm significantly whereas in pre-treatment of *S. aureus* biofilms, rhDNase increased their sensitivity to biocide killing by 4–5 log units (Kaplan, LoVetri et al. 2012).
- Patel et al. 2019 reported the DNase-I supplemented solid lipid nanoparticles of silver sulfadiazine (SSD-SLNs) which inhibited around 96.8% of *Pseudomonas aeruginosa* biofilm while in vivo wound healing study demonstrated complete wound healing after 21 days following the SSD-SLNs-DNase-I therapy (Gade, Patel et al. 2019).

2.5.6.4 Cyclodextrins based formulations in biofilm therapy

Cyclodextrins (CDs) are known for their ability to form inclusion complex which increase the aqueous solubility as well as driving force for diffusion across the biological membrane for hydrophobic drugs. However, inclusion complex formation does not alter

molecular structure and permeability characteristics of the drugs. Complexation with cyclodextrins has been widely explored to increase the aqueous solubility and dissolution rate of poor water soluble molecules (Thomsen, Benkovics et al. 2017). Due to hydrophobic central cavities, CDs are able to form stable complex with ideal sized guest molecules. Few chemically modified CDs like hydroxypropyl -β-cyclodextrin (HP-β-CD) have drawn attention, due to their suitable cavity sizes and greater hydrophilicity. There are several reports demonstrating the drug complexed with CDs had remarkable improved antimicrobial as well as antibiofilm activity.

- Aleem et al. 2008 investigated the effect of β-cyclodextrin and hydroxypropyl -β-cyclodextrin complexation on physicochemical properties and antimicrobial activity of cefdinir (CEF) and reported that the antimicrobial activity of CEF increased significantly ($p < 0.001$) against *S. aureus* and *E. coli* following the complexation with β-CD and HP-β-CD. Moreover, inclusion complex developed by using HP-β-CD indicated superior antimicrobial activity than β-CD however β-CD had greater effect on solubility enhancement of CEF (Aleem, Kuchekar et al. 2008).
- Duarte et al. 2015 evaluated antibacterial and antibiofilm activities of resveratrol inclusion complexes and found that apart from improving resveratrol solubility, the inclusion complexes showed anti-*Campylobacter* and anti-*Arcobacter* activity, inhibited biofilm formation and promoted the biofilm dispersion even at sub MIC concentrations for both the microbes. It also indicated the anti-quorum sensing activity of the inclusion complexes via the inhibition of violacein production by *Chromobacterium violaceum* (Duarte, Alves et al. 2015).

- Teodoro et al. 2017 performed a study on Gallic acid/hydroxypropyl-β-cyclodextrin complex (GA/HPβCD) with an aim to improve solubility and antibiofilm activity against *Candida albicans* biofilms. The results demonstrated that GA/HPβCD maintained the antimicrobial activity of the pure GA while GA/HPβCD was effective on *C. albicans* biofilms of 24 and 48h (Teodoro, Gontijo et al. 2017).
- Thomsen et al. 2017 investigated rhodamine lebelled cyclodextrin nanoparticles with different charge moieties, *i.e.*, neutral, anionic, and cationic, and explored their delivery potential against mature *Staphylococcus epidermidis* biofilms, they reported that cationic *β*-CD polymers demonstrated significant higher integration into the biofilms, as compared to neutral and anionic functionalized *β*-CDs (Thomsen, Benkovics et al. 2017).

2.5.6.5 Nanoparticles

Various nanoparticles (NPs) utilize different mechanisms simultaneously to neutralize microbes; these NPs include chitosan-containing nanoparticles (chitosan NPs), metal-containing nanoparticles as well as solid lipid nanoparticles (SLNs). The NPs have distinctive features that make suitable tools for biofilm mediated infections therapy. Size shape, surface and anterior properties of NPs play key role in the control of biofilm mediated infections. The size of NPs is a crucial parameter for penetration into biofilm matrix; should be smaller than the dimensions of water filled channels in biofilms (Qayyum and Khan 2016). The dimensions of channels and pores are in a range of 10 nm to few micrometres. It has been demonstrated that fluorescent NPs with a size of approximately 130 nm could penetrate bacterial biofilm matrix without adsorbing onto the EPS matrix or bacterial cell surfaces. The ideal size range of NPs for biofilm targeting includes from 5 to 200 nm, however NPs size up to 500 nm is promising. Stealth NPs

obtained through surface modification exhibit specialized properties like biocompatible and better penetration to infectious biofilms (Robino and Scavone 2020).

2.5.6.5.1 Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) are nanosized (50-1000 nm) colloidal lipid nanocarriers comprising of solid lipid matrix and surfactants for drug entrapment. SLNs offer advantages over other nanocarriers in terms of controlled release of the entrapped drugs, increased solubility, stability, and higher payload of drugs (Mukherjee, Ray et al. 2009). The vital features like biocompatibility, targeted delivery of drug, the capability to encapsulate both lipophilic and hydrophilic drugs, and decreased side effects make this nanocarrier suitable for biofilm therapy. There are several studies in which SLNs have been successfully utilized to target biofilms and promising results have also been reported, some of them are detailed below;

- Luan et al. 2019 prepared curcumin loaded chinese white wax SLNs for the purpose of inhibiting *Staphylococcus aureus* biofilm formation and concluded that the curcumin loaded SLNs were able to inhibit the growth of *Staphylococcus aureus* and found more effective in reducing the biofilms produced by this bacterium as compared to free curcumin (Luan, Chi et al. 2019).
- Bazzaz et al. 2016 reported time and concentration dependent reduction in biofilm biomass of *Staphylococcus epidermidis* upon treatment with rifampicin-SLNs. Moreover, biofilm count results indicated effective removal of bacteria as compared to free rifampicin (Bazzaz, Khameneh et al. 2016).
- Singh et al. 2014 investigated anti-biofilm efficacy of cefuroxime axetil loaded SLNs (CA-SLNs) against *Staphylococcus aureus* and concluded that CA-SLNs

demonstrated two folds higher anti-biofilm activity *in vitro* than pristine CA against *S. aureus* biofilm (Singh, Vuddanda et al. 2014).

- Taylor et al. 2014 developed a strategy to target *Pseudomonas aeruginosa* biofilm infections by preparing core-shell SLNs made of lauric acid and oleic acid and reported that the SLNs act against bacteria by multiple mechanisms at the nanoscale including disruption of bacteria leading to DNA release in which <1 % adhesion of dead bacteria was observed (Taylor, Kummer et al. 2014).
- Islan et al. 2016 developed SLNs containing levofloxacin and DNase for lung delivery and to combat infections of *Pseudomonas aeruginosa*, particularly in cystic fibrosis (CF) disease. They reported that the developed formulation exhibited antimicrobial activity against *P. aeruginosa* and even against other opportunistic pathogens like *Staphylococcus aureus*. The presence of levofloxacin loaded SLNs reduced the formation of a bacterial biofilm, as after 24 h treatment with the SLNs, all bacteria were red stained due to the antimicrobial activity of levofloxacin loaded nanoparticles (Islan, Tornello et al. 2016).

2.5.6.5.2 Chitosan nanoparticles

Nanoparticles comprising chitosan (chitosan NPs) act through multiple mechanisms to combat microbes, thereby making resistance very unlikely. Chitosan is derived from chitin (a long polymer chain of N-acetyl-glucosamine) being de-acetylated at random monomer residues (Fei Liu, Lin Guan et al. 2001). Therefore, chitosan is simply a long polymer chain consisting of a random number of N-acetyl-glucosamine residues and glucosamine residues arranged in random order. Every de-acetylated amino group of chitosan has pKa value of ~6.5, hence most of these groups on the chitosan molecule become protonated and thereby positively charged at pH lower than 6.5 (as can occur in

epidermal infections). These positive charges exhibit antimicrobial effect by attracting towards the negatively charged cell walls and plasma membranes of microbial cells. This in turn leads to increased permeability of the microbial cell envelope, osmotic damage, and flow of cytoplasmic contents (including ions and proteins) out of the microbial cell (Sudarshan, Hoover et al. 1992). Chitosan also acts by binding to DNA in bacterial and fungal cells, thereby inhibiting transcription of mRNA and hence protein translation. The anti-biofilm activity of chitosan may also be increased by encapsulating chitosan into nanoparticles (DaSilva, Finer et al. 2013). Chitosan nanoparticles increase the surface area to volume ratio, resulting in increased density of positive charge on the surface, stronger and more frequent binding to microbial cell walls and membranes, and increased antimicrobial activity (Endo, Costa et al. 2018).

- Luo et al. 2015 prepared chitosan coated SLNs and concluded that chitosan-coated SLNs were superior as compared to other formulations and found to have promising features for its application as a potential oral drug delivery system for hydrophobic drugs (Luo, Teng et al. 2015).
- Tan et al. 2018 developed functional chitosan nanoparticles to target biofilm cells and biofilm matrix and reported that positively charged chitosan nanoparticles (CSNP) encapsulating oxacillin (Oxa) and Deoxyribonuclease I (CSNP-DNase-Oxa) inhibited biofilm formation in-vitro and eradicated mature biofilm effectively. Further, repeated treatment with CSNP-DNase-Oxa for two consecutive days resulted in 98.4% biofilm reduction. However, CSNP-DNase-Oxa was not only able to affect the biofilm of a standard *S. aureus* strain, but also exhibited highest eradication of biofilms of clinical isolates as compared to control groups (Tan, Ma et al. 2018).

- Paz et al. 2011 evaluated antimicrobial effect of chitosan nanoparticles on *Streptococcus mutans* biofilms and observed that nanocomplexes prepared from high molecular weight chitosans demonstrated low antimicrobial effect (20 to 25% of cells damaged), while those prepared from low molecular weight chitosans showed high antimicrobial effect (>95% of cells damaged) (Chávez de Paz, Resin et al. 2011).
- Cui et al. 2018 investigated antibacterial activity of clove oil/chitosan nanoparticles embedded gelatin nanofibers against *Escherichia coli* O157:H7 biofilms on cucumber and indicated that 9 mg/mL clove oil-loaded chitosan nanoparticles gelatin/CO @CNPs treatment for 24 h, the population of *E. coli* O157:H7 biofilm reduced by about 99.99% in vitro (Cui, Bai et al. 2018).

2.6. UV-B mediated skin photoaging

2.6.1 Aging

Aging is a multifactorial and complex process resulting in functional decline and mortality of the cells and organism. The symptoms of human aging are predominantly visible in skin and characterized by increased wrinkling, sagging, and increased laxity of the skin. Aged skin often has disturbed barrier function, resulting in a dry appearance of the skin and an enhanced risk on skin disorders and is prone to health risk including malignancies (Durai, Thappa et al. 2012). There are mainly two types of aging: intrinsic and extrinsic aging.

2.6.1.1 Intrinsic aging

Intrinsic aging is an uncontrollable process that affects all skin areas. Symptoms include thin, transparent and dry, fine wrinkles and irregular hair growth, unable to sweat

sufficiently, and loss of sub cutaneous fat tissue, leading to hollowed cheeks and eye sockets, insufficient perspiration, and thinning of nail plates. Aged skin only due to intrinsic factors actually does not exist however, individuals living indoor throughout their life may have a close approximation to this type of skin status. Generally, individuals carry skin that reflects various stages of extrinsic aging, superimposed on the level of intrinsic aging (Chung, Yano et al. 2002).

2.6.1.2 Extrinsic aging

Extrinsic aging results due to environmental oxidative factors like solar radiation, cigarette smoke and other pollution factors. These factors add to intrinsic aging. The primary factor responsible for extrinsic aging is mainly UV radiation also known as photoaging (Panich, Sittithumcharee et al. 2016). UV radiation mediated skin degeneration is a cumulative process and rate of degeneration depends on the frequency, duration and intensity of solar exposure and the natural protection by skin pigmentation. Over 80% of the aging cases are due to photoaging and the sysmptoms include deep wrinkling, loss of elasticity, dryness, laxity, rough-textured appearance, teleangiectasies and pigmentation disorders and its appearance is quite distinct from predominantly intrinsically aged skin. Severity of skin photoaging also depends upon the skin type, being more prominent in fair skin individuals (skin types I and II) and less noticeable in people with skin type III or higher. The appearance of photodamaged skin is primarily due to damage of the structural components of the connective tissue of the dermis. The connective tissue is produced by fibroblasts and is composed of three major classes of biomolecules: glucosaminoglycans (GAGs), proteoglycans, structural proteins (collagen and elastin) and special macromolecules (fibrillin, fibronectin, laminin and hyaluronan) (Panich, Sittithumcharee et al. 2016).

2.7. UV radiation and skin photoaging

Solar UV radiation reaching the earth's surface has a wavelength ranging from 290 to 400 nm and has been categorised into three categories according to their wavelength, including UV-A (320–400 nm), UV-B (290–320 nm) and UV-C (200–290 nm) (Figure 2.2) (Wlaschek, Tantcheva-Poór et al. 2001). Solar radiation of wavelengths <290 nm virtually doesn't reach the earth, 290 nm represents a relevant wavelength limit for UV exposure on earth, however it has the potential to form mutagenic DNA lesions and enhance the risk of developing skin cancer. Substantial UV-B radiation reaches to our skin with the sun in overhead position (around noon) and accounts for several types of skin damage. Exposure to UV-B has much more biological impact on the skin than exposure to UV-A, while comparing similar irradiation doses. The radiation intensity (W/m^2) of UVB depends on several factors, including the solar zenith angle, reflection by the soil (albedo) altitude and the presence of clouds or the dust particles in the sky (Wenk, Brenneisen et al. 2001).

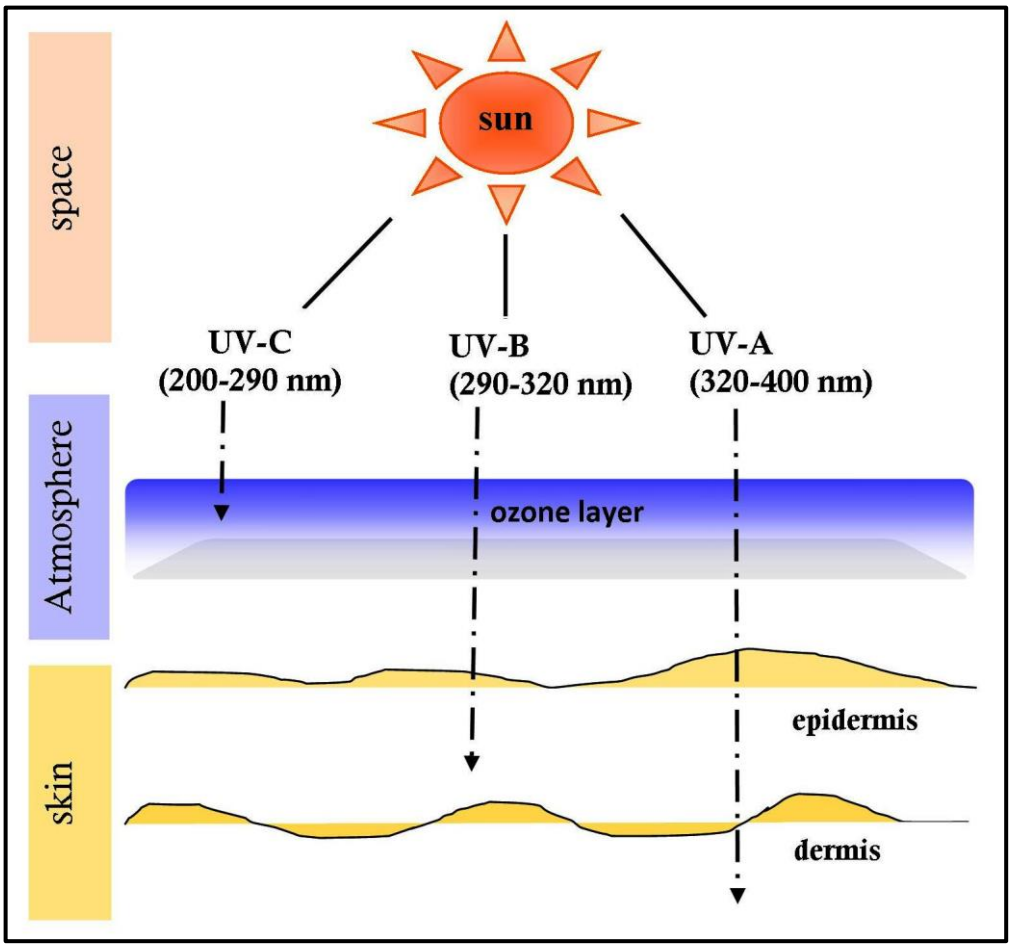

Figure 2.2: Solar UV radiations and its penetration in skin layers

The irradiation dose (J/m^2) is the product of the radiation intensity multiplied by the exposure time, and predominantly determines the UV-induced damage to the skin. Particularly, outdoor workers accumulate more UV-B dose than indoor workers and are therefore more prone to skin damage by UV radiation and the development of skin cancer.

2.8. UV-B radiation and biochemical changes in skin

UV-B radiation is absorbed by chromophores in the skin which lead to various direct or indirect chemical alterations, resulting in direct or indirect damage to biomolecules. Chemical interactions leading to alterations of biomolecules in epidermis and dermis initiate skin photodamage (Sayama, Soushin et al. 2010). UV-B does not penetrate deeply

into the skin, the major portion is absorbed in the epidermis by DNA, aromatic amino acids of proteins, NADH, NADPH, flavins, quinones, porphyrines, carotenoids, urocanic acid, euoro pheomelanin and lipids. UV-B is largely responsible for the development of sunburn. UV-A is not directly associated with sunburn because UV-A is approximately 1000 times less effective than UV-B in creating sunburn (Shah and Rawal Mahajan 2013). UV radiation causes damage to multiple cellular structures directly and indirectly, thereby accelerating the aging process. A substantial part of UV-B is mostly absorbed in the stratum corneum, but attenuated UV-B radiation intensity also reaches the viable epidermal cells causing biological damage. Absorption of UV-B by RNA bases can also have harmful consequences, although more transient, during RNA transcription and translation. Mutations in mRNA can lead to the production of dysfunctional proteins (Wenk, Brenneisen et al. 2001). Reactive oxygen species (ROS) play a crucial role in skin aging. In the skin, approaximately 1.5–5% of the consumed oxygen is converted into ROS by intrinsic processes. ROS are continuously produced as side products in the electron transport chain of the aerobic metabolism in the mitochondria, and are regarded as the main cause of skin aging. Keratinocytes and fibroblasts are the main producers of mitochondrial ROS in the skin. UV-B induced ROS stimulate the synthesis of matrix degenerating metalloproteases (MMPs). MMPs are a family of zinc-dependant endopeptidases. Four members, collagenase (MMP-1), 92 kDa gelatinase (MMP-2), stromelysin (MMP-3) and 72 kDa gelatinase (MMP-9), are in particular responsible for the degradation of ECM (Pittayapruek, Meephansan et al. 2016). MMP1 is the only MMP that can break down intact fibrillar collagen, while the other MMP types can further break down the already degraded collagen fragments. MMPs mainly target connective tissue molecules and basement membrane proteins (Berneburg, Plettenberg et al. 2000). MMPs play a complex role in many physiological and pathological processes, including skin

aging. It has also been reported that histone acetyl transferase (HAT) p300 play a key role in regulation of chromatin remodelling events induced by UV radiation, which in turn activate MMP-1 transcription. HAT p300 senses various stimuli including DNA damage, coordinates and integrates multiple signal-dependent processes at the transcriptional level through its histone acetyl transferase activity.

2.9. Treatment approaches

There are various approaches used now days, to prevent and/or treat the skin photoaging and related indications. The widely used approaches, their benefits, applications and limitations have been discussed in this section.

2.9.1 Preventive approach

Prophylactic measures to avoid the degradation of the dermal extracellular matrix, like collagen and elastin, have yet to reach to the clinic. The prevention strategies are intended for extrinsic aging. These approaches are mainly for precautionary purpose to restrict incidence of photoaging. The effective way to avoid photoaging is the avoidance of direct exposure to sun, sun protective clothing and sunscreens (Pandel, Poljsak et al. 2013). In addition, a person may wear a hat, UV protected sunglasses, white clothes or special UV protected cloths. Regular application of sunscreens (involving UV-B coverage) is an established approach and practised to prevent a subclinical DNA-damaged keratinocyte. Sunscreens are believed to be protective which include dioctyl 4-methoxybenzylidene malonate (DOMBM) and octyl methoxycinnamate (OMC). Moreover, UV filter, paraaminobenzoic acid (PABA) has been found to produce ROS in aqueous solutions, while other sunscreens including benzophenone have been reported as highly photo-allergenic (Berkey, Oguchi et al. 2019).

2.9.2 Topical retinoids

Topical retinoids are considered as effective treatment options for patients with mild to moderate photoaging. Retinoids are a class of naturally occurring or synthetic compounds resembling vitamin A, also known as retinol. Retinol is naturally converted in the body to its most biologically active form, retinoic acid, as well as to its other derivatives, retinaldehyde and retinyl ester. Various natural and synthetic retinoids increase collagen production, induce epidermal hyperplasia, and decrease keratinocyte and melanocyte. Clinically, they reduce the appearance of fine lines, improve skin texture, correct tone and elasticity, and slow the progression of photoaging (Riahi, Bush et al. 2016). Topical retinoids may cause irritant reactions, such as scaling, redness, burning, and dermatitis, limiting patient compliance. Retinoids should be initiated at the lowest effective dose to minimize adverse effects. A minimum of 4 months' use is necessary before benefits are appreciated. Topical retinoids are not recommended during pregnancy or lactation (Pedersen, Voorhees et al. 2019).

Langton et al. 2019 performed a study to examine the histological effects of topical 0.025% all-*trans* retinoic acid (RA) applied to the extensor forearm (under occlusion) in healthy but photoaged white (n = 3; mean age: 70 years) and photoaged black (n = 3; mean age: 67 years) volunteers. The investigation results indicated that RA produced significant epidermal thickening across both cohorts ($P < 0.001$) as compared to untreated baseline control skin; flattening of the dermal-epidermal junction (DEJ) persisted despite application of RA. Further analysis revealed that following RA treatment there was a doubling of keratinocyte cell layers ($P < 0.001$) with each individual keratinocyte having an increased area ($P < 0.01$) (Langton, Chien et al. 2019).

2.9.3 Alpha hydroxy acids

Alpha hydroxy acids are categorised under organic acids traditionally used in clinical practice. The compounds in this category include glycolic acid, lactic acid, and salicylic acid. Earlier, Glycolic acid was used frequently, but recently a new generation of alpha hydroxyl acids known as polyhydroxy acids or polyhydroxy bionic acids have also been reported to exhibit similar effects in photoaging with minimized side effect especially irritation. Alpha hydroxy acids modify cell turnover and alter the extracellular matrix by a number of mechanisms, including increasing collagen synthesis by dermal fibroblasts.

Bernstein et al. 2001 reported that photodamaged skin, upon treatment with topical 20% glycolic acid lotion (pH 4.3), demonstrated increased epidermal thickness, levels of hyaluronic acid within the epidermis and dermis, and collagen gene expression (Bernstein, Lee et al. 2001).

2.9.4 Antioxidants

a. Vitamin C

The skin is subject to great oxidative stressors, such as smoking, pollution, and sunlight, with UV damage being a potent generator of reactive oxygen species (ROS). Given that antioxidants are depleted in the process of protecting skin from oxidative stress, it is reasonable to improve their reservoir in the skin via topical application. Vitamin C, or ascorbic acid, is prevalent in the extracellular matrix of the skin, where it acts as a scavenger of free radicals (Hughes, Williams et al. 2020). The level of ascorbic acid in the epidermis decreases with age. Most important, ascorbic acid is an essential cofactor for the enzymes lysyl and prolyl hydroxylase, which are required for type I and III collagen synthesis. In addition to stimulating transcription of collagen genes, ascorbic acid also decreases the rate of collagen degradation by reducing the production of matrix metalloproteinase enzyme. Topical use of ascorbic acid has been proved to be effective in

decreasing the visible symptoms of photoaging; however, product stability and skin delivery is a key challenge. The products are light sensitive and therefore are generally sold in dark bottles. Vitamin C and its derivatives are also extremely oxygen sensitive and degrade rapidly (Petruk, Del Giudice et al. 2018).

Tarikovich et al. 1999 conducted a randomized, double-blind, vehicle-controlled, split-face study in which 19 patients applied 3% ascorbic acid and vehicle serum to one-half of the face over a 3-month period (level II evidence). Significant improvement was noted in facial wrinkles, roughness, skin laxity, and sallowness as measured by evaluation of standardized photographs and a computer based analysis of skin surface replicas that digitally analysed and quantified surface features (Traikovich 1999).

b. Vitamin E

Vitamin E is an oil soluble antioxidant which is naturally available in eight different forms *viz.* alpha, beta, gamma and delta classes of tocopherol and tocotrienol with alpha tocopherol being the major form in humans (Herrera and Barbas 2001). Vitamin E is delivered to the stratum corneum by sebaceous glands, where it acts by absorbing oxidative environmental stress. Eelier research reports have demonstrated potential photoprotective effects of vitamin E, and other therapeutic benefits of vitamin E including reduction of the cardiovascular risk, immunomodulation and antiallergic effects, neuroprotection properties in models of glutamate excitotoxicity and spino-cerebellar damage, hepatoprotection and prevention of liver toxicity.(Galli, Azzi et al. 2017). Vitamin E and C maintain antioxidant reservoir within the skin due to their synergistic effect, the reservoir is facilitated through a series of redox reactions (Al-Niaimi and Chiang 2017).

Oresazo et al. 2008 performed a study comprising of 10 participants prescribing an antioxidant mixture composed of vitamin C, ferulic acid, and phloretin versus a vehicle

control. The treatments were applied to the human skin followed by the exposure of the subject to the UV irradiation. Results indicated abrogation of photodamage effects, including sunburn cell formation, thymine dimer formation, matrix metalloproteinase expression, and p53 expression (Oresajo, Stephens et al. 2008).

Lin et al. 2003 evaluated effect of a combination of 15% ascorbic acid and 1% alpha tocopherol against vehicle solution applied to pig skin for 4 days and exposed to irradiation. Results indicated 4-fold antioxidant protection as compared to control as well as protection against thymine dimer formation (Lin, Selim et al. 2003).

2.10. Role of Anacardic Acid in management of skin photoaging

Anacardic Acid ($Ana_{C15:0}$) has been reported to suppress p300 HAT activity and also exhibits inhibitory effect on MMP-1 expression (Omanakuttan, Nambiar et al. 2012). $Ana_{C15:0}$ is known to inhibit HAT activity of the transcription co-activators p300 and p300/CREB-binding protein-associated factor (pCAF), NFkB-regulated pathway, and cyclooxygenase (COX) and tyrosinase activity (Sung, Pandey et al. 2008). Antioxidant activity of $Ana_{C15:0}$ and thereby ROS scavenging capability adds to the antiphotoaging potential (Kubo, Masuoka et al. 2006). $Ana_{C15:0}$ has also been reported to have anti-inflammatory activity which may play a key role in mitigating inflammation caused due to skin photodamage. The pathways of skin photoaging and various modes of Anacardic Acid ($Ana_{C15:0}$) activity is depicted in Figure 2.3.

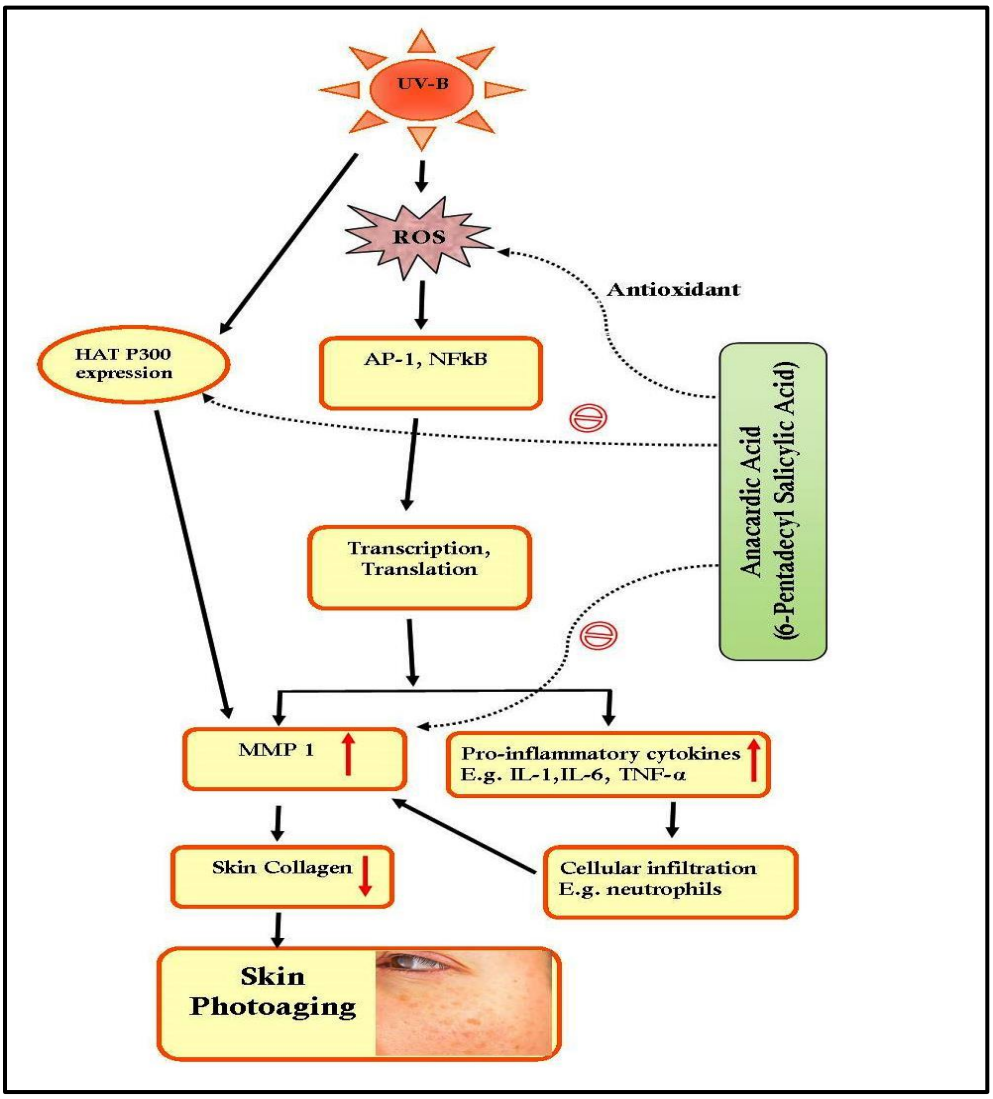

Figure 2.3: Biochemical routes of UV-B mediated skin photoaging and course of Anacardic Acid action

2.11. Cyclodextrin nanosponge for skin delivery

Nanotechnology especially nanomedicine is an outstanding research field, offers range of solutions to several drug delivery and related implications. Several nanocarriers have been reported for improvement of solubility, physicochemical characteristics and pharmacological activity of a drug, including lipid-based nanoparticles, nano-crystals, liposomes and polymer-based delivery systems. However, cyclodextrin based nanosponge (NS) as nanocarrier has vanquished the filed due to its potency in efficiently solubilizing poorly water soluble drugs and offering prolonged release, better bioavailability and

stability. The cyclodextrin based nanosponges (NS) are hyper cross-linked sponge like, polymeric structures, derived from β-cyclodextrins with a high capacity to interact with small molecules in its matrix (Sherje, Dravyakar et al. 2017). The NS can be obtained by crosslinking different types of cyclodextrin using carbonyldiimidazole, pyromellitic dianhydride and diphenylcarbonate cross-linkers (Lembo, Swaminathan et al. 2013). It exhibits high solubilizing efficiency of hydrophobic drug molecules and they are proposed to form inclusion and non-inclusion complexes with different drugs. Drug-loaded NS when dispersed in aqueous vehicle forms colloidal nanosuspension with a tendency to extend drug release (Minelli, Cavalli et al. 2012).

Several applications have been reported in favour of cyclodextrin nanosponge as nanocarriers for different purposes like enhancement of solubility, oral bioavailability, protein delivery, protection of drug from photo degradation. Moreover, NS has also gained remarkable attention as topical delivery carriers and utilized for delivery drugs in therapy of many skin related disorders, some of them are described below;

- Abbas et al. 2019 prepared nanosponge based hydrogel of fluconazole with an objective to improve topical delivery and found adequate drug permeation through rat abdominal skin in ex-vivo permeation experiment. The skin permeation data also demonstrated Higuchi diffusion pattern (Abbas, Parveen et al. 2019).
- Iriventi et al. 2020 developed nanosponge based topical formulation of curcumin and caffeine mixture and reported that the combination of drugs encapsulated in nanosponge gel exhibited significant anti-psoriatic activity upon 10 days treatment while the gel depicted sustained release up to 12 h (Iriventi, Gupta et al. 2020).
- Gangadharappa et al. 2017 carried out in vitro and in vivo evaluation of celecoxib nanosponge hydrogels for topical application and concluded that solubility of

freeze dried nanosponge particles were in the range of 230.49 ± 0.16-490.48 ± 0.88 μg/mL, indicating 30 to 65 fold increases in the solubility as acompared to pure drug. Moreover, in vivo studies revealed that the bioavailability of the plain gel was significantly lesser as compared to the nanosponge formulation and this suggested that prepared topical nanosponge formulation was therapeutically more effective as compared to plain gel (Gangadharappa, Chandra Prasad et al. 2017).

- Argenziano et al. 2019 conducted in vitro skin permeation and retention study of imiquimod (IMQ) loaded β-Cyclodextrin nanosponge hydrogel and revealed that the hydrogel containing IMQ-loaded nanosponges could act as a drug reservoir and provide the sustained release of IMQ through the skin. A greater inhibitory effect on fibroblast proliferation was observed with IMQ loaded nanosponges as compared to the other formulations (Argenziano, Haimhoffer et al. 2019).
- Aggarwal et al. 2016 developed and compared nanosponge and niosome based gel for the topical delivery of Tazarotene and concluded that the nanosponge and niosome gel formulations had lower cumulative amount of drug permeated through the skin with higher skin retention within the skin layers and local accumulation efficiency than plain drug gel and marketed formulation (Aggarwal, Nagpal et al. 2016).