

Table of Contents

List of Figures.....	xii
List of Tables.....	xviii
Abbreviations.....	xix
Preface.....	xxii
1. Chapter 1: Introduction.....	1
9.1 Background	1
9.2 Anacardic Acid activity against Biofilms	3
9.3 Anacardic Acid activity against Photoaging	8
2. Chapter 2: Literature review	11
2.1. Anacardic Acid.....	11
2.1.1 Pharmacological Activities of Anacardic Acid.....	12
2.1.2 Physicochemical properties of Anacardic Acid	14
2.1.3 Recent studies on Anacardic Acid	14
2.2. Microbial infection.....	16
2.3. Microbial resistance to antibiotics.....	17
2.4. Mechanisms of antibiotic resistance	17
2.5. Biofilm and biofilm mediated antimicrobial resistance	18
2.5.1 Stages in biofilm formation	19
2.5.2 Components of biofilm	21
2.5.3 Complications associated with biofilm mediated infections	23
2.5.4 Biofilm mediated resistance to antibiotics.....	23
2.5.5 Diseases associated with biofilm formation	25
2.5.6 Prevention and therapy of biofilm related complications	26
2.6. UV-B mediated skin photoaging.....	36
2.6.1 Aging.....	36
2.7. UV radiation and skin photoaging	38

2.8.	UV-B radiation and biochemical changes in skin	39
2.9.	Treatment approaches.....	41
2.9.1	Preventive approach.....	41
2.9.2	Topical retinoids.....	42
2.9.3	Alpha hydroxy acids.....	43
2.9.4	Antioxidants	43
2.10.	Role of Anacardic Acid in management of skin photoaging	45
2.11.	Cyclodextrin nanosponge for skin delivery	46
3.	Chapter 3: Objectives.....	49
4.	Chapter 4: Plan of study	50
5.	Chapter 5: Extraction and isolation of Anacardic Acid.....	53
5.1	Extraction of Cashew Nut Shell Liquid (CNSL), and group isolation of Anacardic Acid.....	53
5.1.1	Extraction of CNSL.....	53
5.1.2	Isolation of Anacardic Acid from CNSL	53
5.1.3	Isolation of C15:3 and C15:0 among the Anacardic Acid subtypes	54
5.2	HPLC analysis	54
5.3	Identification of the compounds using FT-IR and ¹ H NMR	55
4.4.1	FT-IR.....	55
5.3.1	¹ H NMR.....	55
5.4	Results and Discussion.....	55
5.4.1	HPLC analysis.....	56
5.4.2	FT-IR.....	57
5.4.3	¹ H NMR.....	57
6.	Chapter 6: Preparation and evaluation of Ana_{C15:3}/HP-β-CD inclusion complex	59
6.1	Materials	59
6.2	Methods	59

6.2.1	Analytical method.....	59
6.2.2	Preparation of Ana _{C15:3} /HP-β-CD inclusion complex.....	60
6.2.3	Evaluation of Ana _{C15:3} /HP-β-CD inclusion complex.....	60
6.2.4	Antimicrobial activity.....	63
6.3	Results and Discussion.....	65
6.3.1	Analytical method.....	65
6.3.2	UV-Visible spectroscopy.....	66
6.3.3	Preparation of Anacardic Acid/HP-β-CD inclusion complex.....	67
6.3.4	Phase solubility study.....	68
6.3.5	Fourier transform infrared spectroscopy (FT-IR).....	70
6.3.6	X-ray diffraction (XRD) study.....	72
6.3.7	Differential scanning calorimetry (DSC).....	73
6.3.8	Scanning Electron Microscopy.....	74
6.3.9	¹ H NMR (Proton) Spectroscopy.....	75
6.3.10	Antimicrobial Activity.....	77
7.	Chapter 7: Development of Chitosan and DNase coated solid lipid nanoparticles encapsulating Anacardic Acid (Ana_{C15:3}).....	82
7.1	Materials.....	82
7.2	Methods.....	82
7.2.1	Preparation of Anacardic Acid loaded solid lipid nanoparticles.....	82
7.2.2	Chitosan and DNase coated SLNs.....	83
7.2.3	Characterization of Ana-loaded SLNs.....	83
7.2.4	Antimicrobial study.....	86
7.2.5	Statistical analysis.....	99
7.3	Results and Discussion.....	89
7.3.1	Characterization of Ana _{C15:3} -SLNs.....	89
7.3.2	Antimicrobial study.....	96

8.	Chapter 8: Preparation and evaluation Ana_{C15:0} nanosponge based gel	103
8.1	Materials	103
8.2	Methods	103
8.2.1	Analytical method	103
8.2.2	Synthesis of β -Cyclodextrin Nanosponge	104
8.2.3	Preparation of Ana _{C15:0} loaded Nanosponge complex	104
8.2.4	Optimization of Ana _{C15:0} -NS formulation using 3-level factorial design	105
8.2.5	Evaluation of Ana _{C15:0} -Nanosponge	105
8.2.6	Preparation of Ana _{C15:0} -NS enriched topical gel	107
8.2.7	Evaluation of Ana _{C15:0} -NS topical gel	108
8.2.8	Animal Study	109
8.3	Results and Discussion	111
8.3.1	Analytical method	112
8.3.2	Formulation optimization	113
8.3.3	Solubility study	116
8.3.4	Fourier transform infrared spectroscopy	117
8.3.5	X-ray diffraction (XRD) study	118
8.3.6	Morphology evaluation of Ana _{C15:0} encapsulated nanosponge	120
8.3.7	<i>In-vitro</i> release study	120
8.3.8	Evaluation of Ana _{C15:0} -NS topical gel	121
8.3.9	Physical evaluation of skin photoaging	123
8.3.10	Histopathology study	124
8.3.11	Estimation of biochemical parameters	125
9.	Chapter 9: Summary & conclusion	129
9.1	Summary	129
9.2	Conclusion	133
11.	References	135

List of Figures

Figure No	Description	Page No
Figure 1.1:	Chemical structure of Anacardic Acid and its subtypes	1
Figure 1.2:	Photograph demonstrating various stages of <i>Staphylococcus aureus</i> biofilm formation and proposed formulation approaches to combat biofilm.	7
Figure 1.3:	Photograph illustrating skin photoaging pathway, Anacardic Acid (C _{15.0}) activity and formulation approach to improve photoaging therapy.	10
Figure 2.1:	Overview of different components mixture of solvent extracted CNSL	12
Figure 2.2:	Solar UV radiations and its penetration in skin layers	39
Figure 2.3:	Biochemical routes of UV-B mediated skin photoaging and course of Anacardic Acid action	46
Figure 5.1:	Representative HPLC profiles of Anacardic Acid (a), Ana _{C15.3} (b), and Ana _{C15.0} (c).	56
Figure 5.2:	Comparative FT-IR spectra of Ana _{C15.3} (a) and Ana _{C15.0} (b).	57
Figure 5.3:	¹ H NMR spectra of Ana _{C15.3} (a) and Ana _{C15.0} (b).	58
Figure 6.1:	Calibration curve of Ana _{C15.3} in ethanol (A) and phosphate buffer pH 6.8 (B) for UV spectrophotometry.	66
Figure 6.2:	UV absorption spectra Ana _{C15.3} in phosphate buffer pH 6.8 at different HP-β-CD concentrations: (1) 0.00 mM, (2) 10.00 mM, (3) 20.00 mM and (4) 25.00 mM	67
Figure 6.3:	Phase solubility diagram of Ana _{C15.3} as a function of HP-β-CD concentrations in phosphate buffer pH 6.8 at different temperatures (A) 25°C, (B) 35°C and (C) 45°C.	69
Figure 6.4:	The FT-IR spectra of HP β-CD (A), Ana _{C15.3} (B), and inclusion complex (C).	71
Figure 6.5:	X-ray diffraction patterns of HP-β-CD (A), Ana _{C15.3} (B),	72

- and inclusion complex (C).
- Figure 6.6:** DSC thermograms of HP- β -CD (A), Ana_{C15.3} (B), and the inclusion complex (C). 74
- Figure 6.7:** Scanning electron microscopy image of HP- β -CD (A) and Inclusion complex (B). 75
- Figure 6.8:** Comparative ¹H NMR spectrum of HP- β -CD (A), Ana_{C15.3} (B), and inclusion complex (C). 76
- Figure 6.9:** Disruption effect of free Ana_{C15.3} and Ana_{C15.3}-IC on mature biofilm grown in 96 well plates. The graph depicts the percentage biofilm residue in response to different concentration of Ana_{C15.3} and Ana_{C15.3}-IC. The study demonstrates a reduction of 48 h grown *S. aureus* biofilm on a single dose (Figure 6.9-A) and repeated dose for three recurrent days (Figure 6.9-B) of different Ana_{C15.3} and Ana_{C15.3}-IC concentrations. The data represent a mean \pm standard deviation (n=3). *ap* < 0.005 as compared to Ana_{C15.3} (Two way ANOVA followed by Bonferroni test). *ns* indicates the results are not significant as compared between the two groups. The viable count for the control experiment without Ana_{C15.3} were $2.39 \times 10^9 \pm 1.19 \times 10^8$ CFU/mL. 79
- Figure 6.10:** CLSM image of *S. aureus* biofilm formed on the coverslip surface with media supplementation. The images demonstrate dense biofilms formed without treatment (A) or with Ana_{C15.3} (B) and Ana_{C15.3}-IC (C) treatment. The live cells are indicated by colored fluorescence while the dead cells appear as red colored cells. The control group demonstrates more green colored cells indicating live bacterial cells while the Ana_{C15.3}-IC treated group exhibited maximum red colored cells indicating dead cells. 80
- Figure 7.1:** TEM image of Ana_{C15.3}-SLNs-CH-DNase 91

Figure 7.2:	FT-IR spectra of Ana _{C153} (A), Ana _{C153} -SLNs (B), and Blank SLNs (SLNs without Ana _{C153} (C) and Ana _{C153} -SLNs-CH-DNase (D)	92
Figure 7.3:	XRD plots of Ana _{C153} (A), Blank SLNs (SLNs without Ana _{C153}) (B) and Ana _{C153} -SLNs (C) and Ana _{C153} -SLNs-CH-DNase (D)	93
Figure 7.4:	Plot indicates the cumulative amount of Ana _{C153} release on Y axis with respect to time on X axis from the Ana _{C153} and Ana _{C153} -SLNs-CH-DNase in phosphate buffer (PBS, pH 6.8) as release media containing 0.1% SLS to maintain the sink conditions.	94
Figure 7.5:	Bar diagrams representing the results of the in vitro toxicity study (MTT assay). Data are represented as mean±SD (n=3). No significant differences were observed among the different treatments (One-way ANOVA followed by Newman keul's post hoc test).	95
Figure 7.6:	Inhibition of <i>S. aureus</i> biofilm formation following the different treatments. Graph represents the percentage biofilm residue following the treatment with different concentration of Ana _{C153} . The results are presented as the mean ± standard deviation of three replicates from three independent experiments. Two-way ANOVA was performed (where a represents p <0.05 in comparison with biofilms not receiving any treatment i.e. concentration of Ana _{C153} = 0 µg/mL). The viable cell count in the control experiments without Ana was 2.41×10 ⁹ ±1.09×10 ⁸ CFU/mL.	97
Figure 7.7:	Disassembling of mature biofilm following the treatment with a single dose (A) and repeated dose (B). The data represents the percentage of biofilm residue that remained following the treatments. The values are expressed as mean	100

± standard deviation of three replicates from three independent experiments. Two-way ANOVA was performed (where a represents $p < 0.05$ as compared with Ana_{C15.3}-SLNs-CH-DNase). The viable cell count in the control experiments without Ana_{C15.3} was $2.91 \times 10^9 \pm 1.13 \times 10^8$ CFU/mL.

- Figure 7.8:** CLSM image showing *S. aureus* biofilm formation without any treatment (A) and following the treatment with Ana_{C15.3} (B), Ana_{C15.3}-SLNs (C), Ana_{C15.3}-SLNs-CH (D), Ana_{C15.3}+DNase (E), and Ana_{C15.3}-SLNs-CH-DNase treatment. The cells of the biofilm were stained using the Live/Dead Backlight™ bacterial viability kit. CLSM images demonstrate the staining pattern of live cells (SYTO-9, green) and dead cells (propidium iodide, red). 102
- Figure 8.1:** Calibration curve of Ana_{C15.0} in ethanol (A), and phosphate buffer pH 6.8 (B) by using UV spectrophotometry. The graph indicates the absorbance at Y axis in response to the different concentrations on X axis. 112
- Figure 8.2:** 3D plots indicating the effect of different formulation variables on PS (A) and EE% (B). 115
- Figure 8.3:** Comparative solubility profiles of Ana_{C15.0} and different ratios of Ana_{C15.0}:NS. All experiments were performed in triplicate and data represented as mean±S.D (n=3). a represents significant difference as compared to Ana_{C15.0}, b represents significant difference compared to Ana_{C15.0}:NS (1:2). $P < 0.005$, one-way ANOVA followed by Newman-Keuls multiple-comparison test. 117
- Figure 8.4:** FT-IR spectra of HP-β-CD (A), Ana_{C15.0} (B), and Ana_{C15.0}-Nanosponge (C) 118
- Figure 8.5:** XRD spectra of HP-β-CD (A), Ana_{C15.0} (B) and Ana_{C15.0}-Nanosponge (C). 119

Figure 8.6:	Photograph showing a TEM image of Ana _{C15.0} -NS	120
Figure 8.7:	In-vitro drug release profiles of Ana _{C15.0} and Ana _{C15.0} -NS. Graph represents the cumulative amount of Ana _{C15.0} with respect to time	121
Figure 8.8:	(A) Cumulative amount of drug permeated through skin with respect to time from Ana _{C15.0} -gel and Ana _{C15.0} -NS-gel. (B) Percentage drug retained in different compartments from Ana _{C15.0} -gel and Ana _{C15.0} -NS-gel. All data are presented as mean±SD (n=3) *represents significant difference compared to Ana _{C15.0} -gel, P<0.001 two-way ANOVA followed by Bonferroni posttest.	123
Figure 8.9:	Photographs indicating the effect of UV-B radiation on the animals in different treatment groups. There was no visible lesion or redness in the control group (I), severe photoaging could be observed in the UV-B irradiated group (II), whereas the severity decreases on treatment with Ana _{C15.0} -gel (IV) and Ana _{C15.0} -NS gel (v)	124
Figure 8.10:	Histological examination of the H&E stained dorsal skin samples taken from different groups; control (a), UVB-irradiated (b), Blank gel treated (c), 0.1% Ana _{C15.0} -gel treated, and 0.1% Ana _{C15.0} -NS gel treated group.	125
Figure 8.11:	Effect of different treatment groups on ROS production. All data are presented as mean±SD (n=3) a represents significant difference as compared to UV-B irradiated group b represents significant difference compared to control c represents significant difference compared to Blank gel d represents significant difference compared to Ana _{C15.0} -gel. P<0.005 one-way ANOVA followed by Newman-Keuls multiple-comparison test.	126
Figure 8.12:	Effect of different treatment groups on the expression of MMP-1 & HAT p300 enzymes (A). relative level of HAT	128

P300 among various groups (B), and relative MMP-1 level in different groups under study. All data are presented as mean \pm SD (n=6) a represents significant difference as compared to UV-B irradiated group b represents significant difference as compared to control, c represents significant difference as compared to Blank gel d represents significant difference as compared to Ana_{C15:0}-gel P<0.005 one-way ANOVA followed by Newman-Keuls multiple-comparison test.

List of Tables

Table No:	Description	Page No:
Table 2.1:	Physicochemical properties of Anacardic Acid	13
Table 2.2:	Summary of various pharmacological activities of Anacardic Acid.	14
Table 6.1:	List of chemicals used in the preparation of inclusion complex	59
Table 6.2:	Various parameters for calculation of stability constant	70
Table 6.3:	Variation of the ¹ H NMR chemical shifts (δ /ppm) of Ana _{C15.3} and HP-β-CD in inclusion complex formation.	77
Table 6.4:	Biofilm parameters of S. aureus biofilms without treatment (control), Ana _{C15.3} , and Ana _{C15.3} -IC treatment.	81
Table 7.1:	List of chemicals used in the study	82
Table 7.2:	Data representing particle size, PDI, and zeta potential of different formulations.	90
Table 7.3:	Illustration of variation in particle size and encapsulation efficiency following the storage of SLNs at different temperature conditions.	95
Table 7.4:	Data displaying biomass and thickness of biofilm upon treatment with different formulations.	101
Table 8.1:	List of materials used in the study	103
Table 8.2:	Experimental design demonstrating PS and EE% of the different formulation batches	113
Table 8.3:	Identified independent variables with their levels used in 3-level factorial experimental design and the optimized levels of the variables	115
Table 8.4:	Predicted values of PS and EE% based on the optimized level and the experimental response obtained	116
Table 8.5:	Changes in mechanical properties of hydrogel upon the incorporation of Ana _{C15.0} -NS	122

List of Abbreviations and Symbols

Anacardic Acid	: Ana
Anacardic Acid C15:0	: Anac _{15:0}
Anacardic Acid C15:3	: Anac _{15:3}
Analysis of Variance	: ANOVA
Carbonyldiimidazole	: CDI
Chitosan	: CH
Centimeter	: cm
Colony Forming Unit	: CFU
Confocal Laser Scanning Microscopy	: CLSM
Cyclodextrin	: CD
β -Cyclodextrin	: β -CD
Deoxyribonuclease-I	: DNase
Differential Scanning Calorimetry	: DSC
Dimethyl Formamide	: DMF
Entrapment Efficiency	: EE
Extracellular DNA	: e-DNA
Extra Polymeric Substances	: EPS
Extra Polymeric Matrix	: EPM
Fourier Transform infrared	: FT-IR
Gram	: g
Heanotoxylin and Eosin	: H&E
Hour	: h
High Performance Liquid Chromatography	: HPLC
Histone Acetyl Transferase	: HAT
Hydroxy Propyl- β -Cyclodextrin	: HP- β -CD
Hydroxy Propyl Methyl Cellulose	: HPMC
Kilo Dalton	: KDa
Luria Bertani	: LB

Microgram	: μg
Micrometer	: μm
Milligram	: mg
Minute	: min
Milliliter	: ml
Millimole	: mM
Minimum Inhibitory Concentration	: MIC
Molecular Weight	: MW
Millivolt	: mV
MMP	: Matrix Metallo P _n
Nanometer	: nm
Nanoparticles	: NPs
Nanosponge	: NS
Newton	: N
Poly Dispersity Index	: PDI
Particle Size	: PS
Phosphate Buffer Saline	: PBS
Response Surface Methodology	: RSM
Reactive Oxygen Species	: ROS
Correlation Coefficient	: R^2
Scanning Electron Microscopy	: SEM
Solid Lipid Nanoparticles	: SLNs
Sodium Lauryl Sulfate	: SLS
Standard Deviation	: SD
Texture Profile Analysis	: TPA
Transmission Electron Microscopy	: TEM
Ultra Violet	: UV
X-Ray Diffraction	: XRD
Water in Oil	: W/O
Percentage	: %

Degree Celsius	: °C
Wavelength Maxima	: λ_{\max}
Weight/Weight	: w/w
Weight/Volume	: w/v
Volume/Volume	: v/v
Zeta Potential	: ZP