

1.4.1.4 Scar treatment products available in the markets	10
1.4.2 Natural peptides	10
1.4.2.1 Sources of natural peptides	10
1.4.2.2 Applications of natural peptides	14
1.4.2.3 Protease enzymes	16
A Classification of the protease enzymes	16
B Sources of protease enzymes	17
C Scar treatment by protease enzymes	17
1.4.3 Papain	19
1.4.3.1 Protease enzymes from papaya	19
1.4.3.2 Methods of extraction and purification	20
A. Salt precipitation	20
B. Aqueous/solvent extraction	21
C. Ion exchange chromatography	21
1.4.3.3 Qualitative and quantitative analysis of papain	22
A. Electrophoretic method	22
B. High performance liquid chromatography	23
C. Immunoassay technique	24
1.4.3.4 Papain in pharmaceutical and cosmeceutical applications	25
1.4.3.5 Papain in scar treatment	25
1.4.4 Bromelain	27
1.4.4.1 Protease enzymes from pineapple	28

1.4.4.2	Methods of extraction and purification	29
A.	Precipitation method	29
B.	Aqueous two phase extraction	30
C.	Ion exchange chromatography	30
1.4.4.3	Qualitative and quantitative analysis of bromelain	31
A.	Electrophoretic method	31
B.	High performance liquid chromatography	31
C.	Infrared (IR) spectroscopy	32
D.	Differential scanning calorimetry	33
E.	Lowry and Bradford method	34
1.4.4.4	Bromelain in pharmaceutical and cosmeceutical applications	35
1.4.4.5	Bromelain in scar treatment	36
1.4.5	Problems of protease enzymes in pharmaceutical and cosmeceutical applications	37
1.4.5.1	Chemical stability	37
1.4.5.2	Skin irritation	37
1.4.6	Transdermal delivery systems	38
1.4.6.1	Skin structure and routes of skin penetration	39
1.4.6.2	Advantages of transdermal delivery systems	41
1.4.6.3	Skin penetration enhancement of peptides	42
1.4.6.4	<i>In vitro</i> skin transdermal absorption by Franz diffusion cells	44

1.4.7 Nanocarrier delivery systems	46
1.4.7.1 Nanovesicles	46
A. Classification of nanovesicles	46
B. Methods of preparation	48
C. Physical characteristics of nanovesicles	48
D. Elastic nanovesicles	52
E. Application of elastic nanovesicles in topical pharmaceuticals and cosmeceuticals	56
1.4.7.2 Nanoparticles	58
A. Classification of nanoparticles	59
B. Methods of preparation	60
C. PLGA nanospheres	62
E. Applications of nanoparticulate delivery systems	64
1.4.8 Biological assay of scar treatment	65
1.4.8.1 <i>In vitro</i> biological assays to evaluate scar treatment	65
A. Antioxidative activity	65
B. Cytotoxicity on human skin fibroblast	68
C. Gelatinolytic activity on MMP-2 stimulation (zymography)	70
E. Collagenolytic activity	73
1.4.8.2 <i>In vivo</i> scar treatment evaluation assay	75
A. Rabbit skin testing for irritation	75

B. Rabbit ear model for hypertrophic scar and keloids determination	77
C. Performance test of scar reduction in human volunteers	79
CHAPTER 2 MATERIALS AND METHODS	81
2.1 Materials and equipments	81
2.1.1 Chemicals	81
2.1.2 Cell lines	84
2.1.3 Animals	84
2.1.4 Equipments	84
2.2 Methods	87
Part 1: Development of the modified proper elastic niosomal formulations loaded with the model drug (calcitonin)	88
1.1 Effects of niosomal concentrations and various dispersants on physical characteristics of elastic niosomes loaded with calcitonin	88
1.1.1 Preparation of blank niosomes and niosomes loaded with calcitonin	88
1.1.2 Physical characteristics of niosomes	88
1.1.3 Measurement of deformability index (DI)	88
1.2 Effects of dispersants on the maximum loading and entrapment efficiency of calcitonin in elastic niosomes	90
1.3 Effects of phosphate buffer concentrations on physical characteristics of elastic niosomes	91

1.4 Comparison of physical characteristics and cytotoxicity of elastic niosomes	91
1.4.1 Preparation of elastic niosomes	91
1.4.2 Morphology of elastic niosomes	92
1.4.3 Entrapment efficiency of calcitonin loaded in elastic niosomes	92
1.4.4 Cytotoxicity of elastic niosomes	93
Part 2: Chemical stability and transdermal absorption of elastic niosomes loaded with the model drug (calcitonin)	94
2.1 Physico-chemical stability of calcitonin loaded in niosomes	94
2.2 Transdermal absorption by vertical Franz diffusion cells	95
2.2.1 Preparation of the rat skin	95
2.2.2 Sample preparation	96
2.2.3 Transdermal absorption experiment	96
2.2.4 Extraction of calcitonin from the treated samples	96
2.2.5 Data calculation and statistical analysis	97
Part 3: Preparation and biological activities of the extracted protease enzymes	97
3.1 Preparation of the crude extracts containing protease enzymes	97
3.2 Partial purification of the protease enzymes by precipitation	98
3.3 Qualitative and quantitative analysis of the protease enzymes	99
3.3.1 High performance liquid chromatography (HPLC) analysis	99
3.3.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis	99
3.4 Biological activities	100
3.4.1 Free radical scavenging assay	100

3.4.2 Lipid peroxidation inhibition activity	101
3.4.3 Human skin fibroblast cytotoxicity by the SRB assay	101
3.4.4 Gelatinolytic activity (zymography) on MMP-2	102
Part 4: Development of the elastic niosomes loaded with the extracted papain and bromelain	103
4.1 Preparation of blank elastic niosomes and NaC elastic niosomes loaded with the extracted papain and bromelain	103
4.2 Physical characteristics of niosomes	103
4.3 Measurement of deformability index (DI)	104
4.4 The maximum loading and entrapment efficiency of the extracted papain and bromelain loaded in NaC elastic niosomes	104
4.5 Cytotoxicity on human skin fibroblasts of the NaC elastic niosomes loaded with the extracted enzymes	105
4.6 Gelatinolytic activity (zymography) on MMP-2 stimulation	105
4.7 Physico-chemical stability of the extracted protease enzymes loaded in NaC elastic niosomes	105
Part 5: Development of the PLGA nanospheres loaded with the standard papain	106
5.1 Preparation of the standard papain loaded in PLGA nanospheres	106
5.1.1 Emulsion solvent diffusion method in water	106
5.1.2 Water-oil-water emulsion solvent evaporation method	107
5.2 Physicochemical properties of the standard papain loaded in PLGA nanospheres	108
5.2.1 Particle size and zeta potential determination	108
5.2.2 Morphology investigation	108

5.2.3 Encapsulation efficiency determination	109
5.3 The release profile of the standard papain from the PLGA nanospheres	109
5.4 Human skin fibroblast cytotoxicity by the SRB assay	110
5.5 Physico-chemical stability of the standard papain loaded in PLGA nanospheres	110
Part 6: Development of gel containing papain loaded in nanovesicles and nanoparticles preparation for scar treatment	111
6.1 Preparation of gel containing papain loaded in nanovesicles and nanoparticles	111
6.2 <i>In vitro</i> rat skin transdermal absorption by vertical Franz diffusion cells	111
6.3 Rabbit skin irritation test by the closed patch test	112
6.4 The physico-chemical stability of gel containing papain loaded in nanovesicles	113
6.5 Hypertrophic scar model in the rabbit ears	114
CHAPTER 3 RESULTS AND DISCUSSION	116
Part 1: Development of the proper elastic niosomal formulations loaded with the model drug (calcitonin)	116
1.1 Effects of niosomal concentrations and various dispersants on physical characteristics of elastic niosomes loaded with calcitonin	116
1.2 Effects of dispersants on the maximum loading and entrapment efficiency of calcitonin in elastic niosomes	119

1.3 Effects of phosphate buffer concentrations on physical characteristics of elastic niosomes	121
1.4 Comparison of physical characteristics and cytotoxicity of elastic niosomes containing ethanol or the edge activators (NaC and NaDC)	123
1.4.1 Physical characteristics of elastic niosomes	123
1.4.2 Deformability index of elastic niosomes	128
1.4.3 Entrapment efficiency of calcitonin in elastic niosomes	129
1.4.4 Cytotoxicity of elastic niosomes loaded with calcitonin	130
Part 2: Physical and chemical stability and transdermal absorption of elastic niosomes loaded with calcitonin	133
2.1 Characteristics of non-elastic and elastic niosomes loaded with calcitonin	133
2.2 Physico-chemical stability at various storage temperatures of calcitonin loaded in elastic niosomes	134
2.3 Transdermal absorption of calcitonin loaded in elastic niosomes	138
Part 3: Preparation and biological activities of the extracted protease enzymes	144
3.1 Characteristics of the enzymes	144
3.2 Biological activities of the extracted protease enzymes	147
3.2.1 DPPH radical scavenging activity	147
3.2.2 Lipid peroxidation inhibition activity	150
3.2.3 Cytotoxicity of the extracted protease enzymes	151
3.2.4 Gelatinolytic activity (zymography) on MMP-2	153
Part 4: Development of the elastic niosomes loaded with the extracted papain and bromelain	154

4.1 Physical characteristics of the blank and the non-elastic and NaC elastic niosomes loaded with the enzymes	154
4.2 Deformability index (DI) of the blank and the non-elastic and NaC elastic niosomes loaded with the extracted enzymes	159
4.3 The maximum loading and entrapment efficiency of the extracted enzymes in NaC elastic niosomes	160
4.4 Cytotoxicity of NaC elastic niosomes loaded with the extracted enzymes on human skin fibroblast	162
4.5 Gelatinolytic activity (zymography) on MMP-2 of the extracted protease enzymes	165
4.6 Physical stability of elastic niosomes loaded with the papain and chemical stability of the papain loaded in elastic niosomes	168
Part 5: Development of the PLGA nanospheres loaded with the standard papain	172
5.1 Characteristics of the standard papain loaded PLGA nanospheres	172
5.1.1 Particle size and zeta potential values	173
5.1.2 Morphology of the standard papain loaded in PLGA nanospheres	175
5.1.3 Encapsulation efficiency of the standard papain loaded in PLGA nanospheres	176
5.2 The release profile of the standard papain from the PLGA nanospheres	177
5.3 <i>In vitro</i> cytotoxicity of the standard papain loaded in PLGA nanospheres	181

5.4 Physicochemical stability at various storage temperatures of papain loaded in PLGA nanospheres	184
Part 6: Development of gel containing papain loaded in nanovesicles and nanoparticles for scar treatment	187
6.1 Physical characteristics of gel containing papain loaded in nanovesicles and nanoparticles	187
6.2 Transdermal absorption through rat skin	189
6.3 Rabbit skin irritation	195
6.4 The physico-chemical stability of gel containing papain loaded in nanovesicles	197
6.5 Hypertrophic scar model in the rabbit ears	201
CHAPTER 4 CONCLUSION	204
REFERENCES	212
APPENDICES	250
APPENDIX A	251
APPENDIX B	255
APPENDIX C	257
APPENDIX D	259
CURRICULUM VITAE	263

LIST OF TABLES

Table		Page
1	Various studies on the treatment approaches and their proposed mechanisms of wound healing and scar formation	11
2	The examples of the scar reduction products available in the markets	13
3	Effect of Moricrase-containing ointment application on keloid scars	18
4	Characteristics of protease enzymes from pineapple	28
5	Examples of the delivery methods to enhance protein/peptide across the skin	43
6	Preparation methods of nanovesicles	49
7	The application of elastic nanovesicles for transdermal drug delivery through animal and human skin	57
8	The advantages and drawbacks of the preparation methods	61
9	Studies on various nanoparticle to enhance oral bioavailability of the therapeutic peptides and proteins	66
10	Types of MMPs	71
11	Draize evaluation of dermal reactions	76
12	The Vancouver scar scale	79
13	Descriptions of various niosomal systems for physical characteristic investigation	89
14	Effects of various niosomal concentrations on vesicular deformability index	117

15	Effects of dispersant types on vesicular deformability index	117
16	Entrapment efficiency of calcitonin loaded in ethanol elastic and non-elastic niosomes	121
17	Effects of various concentrations of phosphate buffer on vesicular deformability index	122
18	Physical characteristics and deformability index (DI) of various blank non-elastic and elastic niosomal formulations	124
19	Physical characteristics and deformability index (DI) of the selected non-elastic and elastic niosomes loaded with calcitonin in comparing to their corresponding blank niosomes	126
20	Vesicular sizes (nm) and zeta potential (mv) of blank non-elastic and elastic niosomes after stored at various temperatures for 12 weeks	135
21	Vesicular sizes (nm) and zeta potential (mv) of non-elastic and elastic niosomes loaded with calcitonin (2mg/ml) after stored at various temperatures for 4 weeks	135
22	The cumulative amounts (mg/cm ²) and fluxes (mg/cm ² /h) of calcitonin from various systems in whole skin and receiver compartment solution following transdermal absorption across excised rat skin at 6 h by vertical Franz diffusion cells	140
23	Antioxidant activities of the extracted enzymes in comparing to the standards	148
24	The percentages of cell viability on human skin fibroblast by the SRB assay of the extracted protease enzymes	152

25	Vesicular sizes, zeta potential values and deformability index (DI) of the non-elastic and elastic niosomes loaded with the extracted papain and bromelain	157
26	Comparison biological activities of standard and extracted protease enzymes (papain and bromelain)	169
27	The comparison of particle sizes, zeta potential values and encapsulation efficiency (%EE) of PLGA nanospheres loaded with the standard papain prepared by the ESD and ESE methods	173
28	The release kinetics of the standard papain from the PLGA nanospheres prepared by the the ESD and ESE method in 0.2 M phosphate buffer (pH 7.0) solution at 27 ± 2 °C for 48 hours	180
29	Physical characteristics of gel containing papain loaded in nanovesicles (non-elastic and elastic niosomes) and nanoparticles (PLGA nanospheres)	188
30	Primary irritation index (PII) and category of irritation based on PII of various gel formulations	196
31	Vesicular size, zeta potential values, viscosity of various gel formulations when kept at 4 ± 2 , 27 ± 2 and 45 ± 2 °C for 3 months	199
B.1	Amounts of the composition in the prepared nanovesicles	256

LIST OF FIGURES

Figure		Page
1	The phase of wound healing	7
2	Papaya tree and fruit (<i>Carica papaya</i> L)	19
3	Ion-exchange chromatography of the papaya enzymes on the SP-Sepharose Fast Flow	22
4	The PAG electrophoresis patterns of (a) proteinase obtained by affinity chromatography on the immobilized cystatin, (b) reference papain (Serva), and (c) <i>Carica papaya</i> latex sample: (1) papain; (2) chymopapain; (3) peptidase B; (4) peptidase A	23
5	Rp-HPLC profiles of papain	24
6	Pineapple plant and fruit	27
7	SDS-PAGE electrophoresis of isolated bromelain.	32
8	FTIR spectra of freeze-dried bromelain powder	33
9	DSC thermogram of bromelain powder	34
10	Micrographes from light microscope (A) and transmission electron (B) microscope of human epidermis after 24 h papain treatment	38
11	Skin structure of human skin	39
12	Three main pathways of skin penetration including transappendageal, intercellular and transcellular pathway	40
13	Strategies for optimisation of protein and peptide drug permeation across the skin	42

14	The Franz diffusion cell apparatus	44
15	The fluxes ($\mu\text{g}/\text{cm}^2/\text{h}$) of Gdm from various systems in stratum corneum (SC) and viable epidermis and dermis (VED), at 6 h by vertical Franz diffusion cells	45
16	Mechanism to penetrate the skin of ethosomes	53
17	Chemical structures of sodium cholate (NaC), sodium deoxycholate (NaDC), Tween 80 and Span 80	55
18	Encapsulation mechanism models: drug entrapped in, dissolved or dispersed within, and adsorbed on: a) nanocapsules and b) nanospheres	59
19	Procedure of emulsion solvent diffusion (ESD) method	61
20	Procedure of emulsion solvent evaporation (ESE) method	62
21	Chemical structure of polylactic polyglycolic acid co-polymer	63
22	SEM and TEM images of PLGA nanospheres	64
23	Reaction of the DPPH radical in the presence of the antioxidant during the DPPH assay	67
24	(A) DPPH radical scavenging activities of various hydrolysates from the defatted rice endosperm protein (REP) and (B) Inhibition of linoleic acid autoxidation by the Neutrase hydrolysate from rice endosperm protein (NHREP)	68
25	Representative photographs of cells (original magnification x100). A=Control L929 cells at 24 h; B=L929 exposed to Dentsply gutta-percha for 24 h; C=Control RPC-C2A cells at 48 h; D= RPC-C2A exposed to Resilon for 48 h	70
26	Gelatin zymography	72

27	Profile of plasmin-dependent and independent collagenolytic activity of murine skin fibroblasts	74
28	Hypertrophic scar in the rabbit ear model and the scar elevation index (SEI) evaluation	78
29	Hypertrophic median sternotomy scar at week 0 (A) and week 32 (B)	80
30	Scheme of the scope of the study	87
31	The procedure for the preparation of PLGA nanospheres by the emulsion solvent diffusion method in aqueous PVA solution (ESD)	107
32	The procedure for the preparation of PLGA nanospheres by the water-oil-water (w/o/w) emulsion solvent evaporation method (ESE)	108
33	The maximum loading and entrapment efficiencies of calcitonin in niosomes by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)	120
34	Negative-staining TEM images of the niosomal formulations (20000X) composed of Tween 61/cholesterol (1:1) dispersed in 5 mM phosphate buffer pH 7.0	121
35	The percentages of human skin fibroblast viability by SRB assay of the blank niosomes (A) and calcitonin loaded in niosomes (B)	132
36	The percentages of calcitonin remaining in various systems when stored at various temperatures for 4 weeks	136
37	Cumulative amounts (mg/cm^2) of calcitonin from various systems in whole skin (A) and receiver compartment solution (B) at 1, 3 and 6 h by vertical Franz diffusion cells	141

38	The fluxes ($\text{mg}/\text{cm}^2/\text{h}$) of calcitonin from various systems in whole skin (A) and receiver compartment solution (B) at 1, 3 and 6 h by vertical Franz diffusion cells	143
39	Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the extracted papain (a), extracted bromelain (b) in comparing to the molecular weight marker	145
40	The HPLC chromatograms of the standard papain (A) extracted papain (B) standard bromelain (C) and extracted bromelain (D)	146
41	The amino acid sequences of papain (A) and bromelain (B)	150
42	Gelatinolytic activity of the extracted protease enzymes in comparing to the control and concanavalin A. (a) zymograms and (b) MMP-2 stimulation relative to the control	154
43	Negative-staining TEM images of the protease enzymes loaded in niosomes (20000X)	158
44	Cytotoxicity comparison of both the free standard and extracted enzymes (papain and bromelain) and the enzymes loaded in NaC elastic niosomes on human skin fibroblasts by the SRB assay	164
45	Zymograms of MMP-2 stimulation of both free standard and extracted enzymes (papain and bromelain) and the enzymes loaded in elastic niosomes in comparing to the untreated cells, negative control (vitamin C) and positive control (concanavalin A)	166

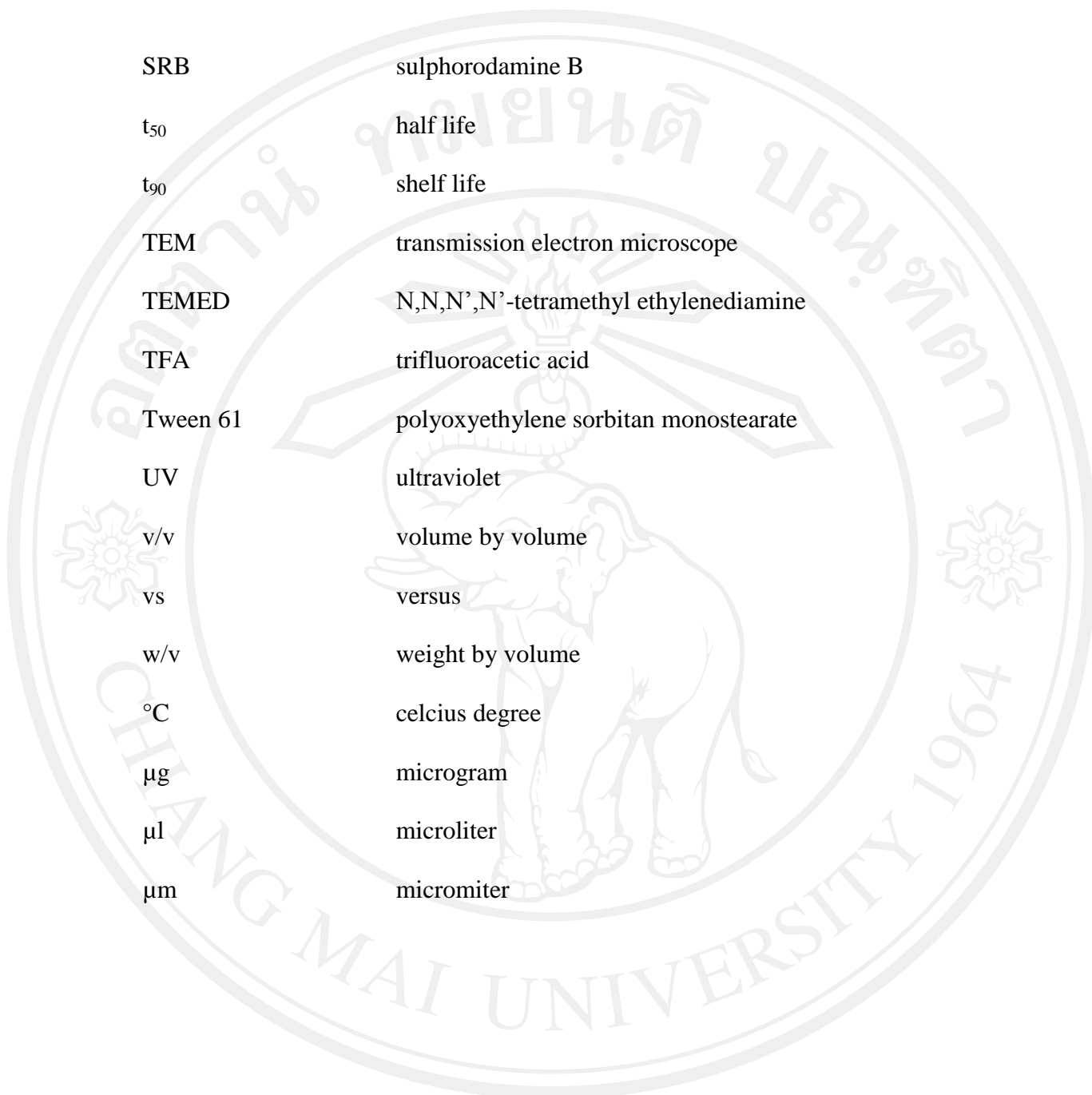
- 46 The relative pro MMP-2 and active MMP-2 stimulation of both free standard and extracted enzymes (papain and bromelain) and the enzymes loaded in non-elastic and NaC elastic niosomes in comparing to the control 167
- 47 The percentages of remaining both free standard and extracted papain and the papain loaded in non-elastic and NaC elastic niosomes stored at different temperatures (4 ± 2 , 27 ± 2 and 45 ± 2 °C) for 12 weeks 171
- 48 The scanning electron (A) and transmission electron (B) microphotographs of the PLGA nanospheres (composed of 100 mg PLGA and 10% w/v PVA403) loaded with 43 µg papain/mg PLGA nanospheres prepared by the ESE method 176
- 49 Comparison of the release profile of the standard papain from the PLGA nanospheres prepared by the ESD and ESE method in 0.2 M phosphate buffer (pH 7.0) solution at 27 ± 2 °C 178
- 50 The percentages of human skin fibroblast viability by the SRB assay of (A) the blank nanospheres and (B) the PLGA nanospheres loaded with papain prepared by the ESD and the ESE method) 182
- 51 The percentages remaining of the free papain and papain loaded in PLGA nanospheres prepared by the ESE method stored at different temperatures (25 ± 2 , 4 ± 2 and 45 ± 2 °C) for 6 weeks 186
- 52 Cumulative amounts (mg/cm^2) of papain from various formulations in whole skin (A) and receiver solution (B) at 1, 3 and 6 h by vertical Franz diffusion cells 190

53	The fluxes (mg/cm^2) of papain from various formulations in whole skin (A) and receiver solution (B) at 1, 3 and 6 h by vertical Franz diffusion cells	191
54	The percentages remaining of papain in various gel formulations when stored at different temperatures for 3 months	200
55	The percentage of hypertrophic scar reduction of various gel formulations after 7, 14, 21 and 28 days of application	202
56	The hematoxylin-eosin-stained cross-section of the induced scars of the rabbits' ear skin treated with various gel formulations after 28 days of application	203
A.1	Chemical structure of Tween 61	251
A.2	Chemical structure of cholesterol	252
A.3	Chemical structure of sodium cholate	253
A.4	Chemical structure of sodium deoxycholate	254
A.5	Structure of concanavalin A	254

ABBREVIATIONS AND SYMBOLS

CC ₅₀	chelating concentration at 50% activity
cm	centimeter
cm ²	square centimeter
DI	deformability index
DLS	dynamic light scattering
D-MEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DPPH	1, 1-Diphenyl-2-picrylhydracyl
EDTA	ethylenediaminetetraacetic acid
ESD	emulsion solvent diffusion method
ESE	emulsion solvent evaporation method
FBS	fetal bovine serum
g	gram
h	hour
HPLC	high performance liquid chromatography
IPC ₅₀	inhibition peroxidation concentration at 50% activity
kDa	kilodalton
kg	kilogram
M	molar
mg	milligram
ml	milliliter

mm	millimeter
mM	millimolar
MMP	matrix metalloproteinase
mV	millivolt
mV	millivolt
MW	molecular weight
NaC	sodium cholate
NaDC	sodium deoxycholate
nm	nanometer
OECD	Organisation for Economic Co-operation and Development
PBS	phosphate-buffered saline
PII	primary irritation index
PLGA	poly (lactide-co-glycolide)
PVA	polyvinyl alcohol
rpm	revolutions per minute
SC	stratum corneum
SC	stratum corneum
SC ₅₀	scavenging concentration at 50% activity
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEI	scar elevation index
SEM	scanning electron microscope
SLS	sodium lauryl sulfate

The background of the page features a large, light gray watermark of the Chiang Mai University logo. The logo is circular and contains a central figure of an elephant standing on a pedestal, with a crown above its head. The text 'CHIANG MAI UNIVERSITY 1964' is written around the bottom inner edge of the circle. Thai script is also present within the logo's border.

SRB	sulphorodamine B
t_{50}	half life
t_{90}	shelf life
TEM	transmission electron microscope
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TFA	trifluoroacetic acid
Tween 61	polyoxyethylene sorbitan monostearate
UV	ultraviolet
v/v	volume by volume
vs	versus
w/v	weight by volume
°C	celcius degree
μg	microgram
μl	microliter
μm	micromiter