LABISIA PUMILA LOADED FLEXIBLE LIPOSOMES FOR ENHANCEMENT OF COLLAGEN SYNTHESIS IN RECONSTRUCTED HUMAN SKIN AND ITS PENETRATION PATHWAY

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Specially dedicated to my husband, Mohd Adli bin Mohd Idris, my children, Anas and Abbas, my mother, Mariam binti Sulaiman, my late father, Roslan bin Mohd Saleh and also my family members.

With all my love and gratitude.

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ABSTRACT

Labisia pumila (LP) contains high antioxidants and has been shown to enhance collagen synthesis in the skin. Thus, LP is suitable to be incorporated in skin care formulations. Flexible liposomes (FL) can be utilized to enhance active ingredients permeation through the skin as they can squeeze through the pores or intercellular junctions of the stratum corneum. This study focused on the development of FL loaded with LP extract for controlled and localized delivery through transdermal route. The formulation of FL-LP was developed using quality by design concept to obtain a comprehensive understanding of the product and process parameters in order to achieve consistent product performance. FL-LP was prepared using thin-film hydration method followed by ultrasonication and the following optimum formulation and process parameter obtained were; 10.43 mg/ml of L- α – phosphatidylcholine, 2.03 mg/ml of β-sitosterol, 0.16 % v/v of Tween 80 and 0.19 % v/v of Span 80. The optimum time of sonication was three minutes at 30 % amplitude. Stability study indicated that the formulation was stable at 4 °C and no significant changes in physical characterization were observed throughout three months of storage. The release kinetics of the FL-LP was analysed *in vitro* and it was found to follow the Higuchi model. Transport properties study showed that the permeability coefficient of FL-LP through rat skin and reconstructed human skin were 1.73 ± 0.4 cm/h and 2.53 ± 0.07 cm/h, respectively. The transdermal penetration pathway of FL-LP was further characterized using Confocal Laser Scanning Microscopy. The pathway of penetration of flexible liposomes was mostly through the hair follicles. The correlation of kinetic release study and permeability study was statistically significant, with R^2 of 0.9024. The amounts of effective transdermal dosage for collagen synthesis assay of non – encapsulated LP extract and FL- LP were estimated based on safe dosage from IC_{50} result. Collagen synthesis analysis was conducted using RHS. FL-LP showed a 2000 fold increase in collagen content, compared to non - encapsulated LP, which resulted in only a 100 fold increase in collagen content in the RHS. The ability of the delivery system to be delivered to the dermis layer was implied in this research work as collagen synthesis by the fibroblasts occurred at the dermal layer. The outcomes in this study suggested that FL-LP was found to be effective in enhancing collagen synthesis in the skin.

ABSTRAK

Labisia pumila (LP) mengandungi kandungan antioksida yang tinggi dan telah menunjukkan boleh meningkatkan sintesis kolagen di dalam kulit. Oleh itu, LP sesuai untuk dimasukkan di dalam formulasi penjagaan kulit. Liposom Fleksibel (FL) boleh digunakan untuk meningkatkan ketelapan bahan aktif menembusi kulit menerusi liang atau simpangan antara sel pada *stratum corneum*. Kajian ini memberi tumpuan kepada pembangunan muatan FL dengan ekstrak LP untuk kawalan dan penghantaran setempat melalui laluan transdermal. Formulasi FL-LP telah dibangunkan menggunakan konsep Kualiti dengan Rekabentuk untuk mendapatkan kefahaman yang menyeluruh ke atas hasil dan parameter proses mengikut tertib bagi mencapai prestasi produk yang konsisten. FL-LP telah disediakan menggunakan kaedah penghidratan filem-nipis diikuti oleh ultrasonikasi dan formulasi optimum serta parameter proses telah diperoleh ialah, 10.43 mg/ml L- α- fosfatidilkolin; 2.03 mg/ml β-sitosterol; 0.16 % v/v Tween 80 dan 0.19 % v/v Span 80. Masa optimum untuk sonikasi ialah tiga minit pada 30 % amplitud. Pengujian kestabilan formulasi menunjukkan paling stabil pada suhu 4 °C dan tiada perubahan ketara terhadap pencirian fizikal yang telah dilihat sepanjang tiga bulan penyimpanan. Kinetik penghantaran FL-LP telah dianalisis secara *in vitro* dan ia didapati mengikut model Higuchi. Kajian sifat pengangkutan menunjukkan pemalar kebolehtelapan FL-LP menerusi kulit tikus dan kulit manusia bina semula (RHS) masing-masing adalah 1.73 ± 0.4 cm/j dan 2.53 ± 0.07 cm/j. Laluan penembusan transdermal oleh FL-LP diperincikan selanjutnya menggunakan mikroskop pengimbasan laser konfokal; laluan penembusan kebanyakannya adalah melalui folikel rambut. Korelasi antara kinetik penghantaran dan kajian ketelapan adalah signifikan dengan R^2 ialah 0.9024. Jumlah dos berkesan transdermal bagi asai sintesis kolagen bagi ekstrak LP yang tidak dikapsulkan dan FL - LP dianggarkan berasaskan keputusan dos selamat IC_{50}. Analisa sintesis kolagen telah dijalankan menggunakan RHS. FL-LP menunjukkan peningkatan 2000 kali ganda kandungan kolagen berbanding ekstrak LP yang tidak dikapsulkan, yang mana ekstrak LP yang tidak dikapsulkan menunjukkan hanya 100 kali ganda peningkatan kandungan kolagen di dalam RHS. Keupayaan sistem pengangkutan ini untuk diangkut ke lapisan dermis diimplikasikan di dalam kajian ini yang mana sintesis kolagen oleh fibroblast berlaku di lapisan dermal. Hasil keputusan kajian ini mencadangkan bahawa FL-LP berkesan bagi meningkatkan sintesis kolagen di dalam kulit.

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LIST OF ABBREVIATIONS

µg/ml	-	microgram per millilitre
ANOVA	-	Analysis of Variance
CLSM	-	Confocal Laser Scanning Microscopy
CO_2	-	Carbon Dioxide
CQA	-	Critical Quality Attribute
CPP	-	Critical Process Parameter
DS	-	Design Space
DOE	-	Design of Experiment
DMEM	-	Dulbecco's Modified Eagle Media
DMSO	-	Dimethyl sulfoxide
DSC	-	Different Scanning Calorimetry
EE	-	Encapsulation Efficiency
FBS	-	Fibroblast cells
FDA	-	Food and Drug Administration
FL	-	Flexible Liposome
FTIR	-	Fourier Transform Infra – Red
GRAS	-	Generally Recognized as Safe
IBD	-	Institute of Bioproduct Development
IC_{50}	-	Inhibition Concentration at 50%
ICH	-	International Conference of Harmonised
kD	-	kilo Dalton

LP	-	*Labisia pumila*
LLP	-	Liposomes loaded with *Labisia pumila*
MMP-1	-	Matrix Metalloproteinases -1
MTT	-	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWCO	-	Molecular Weight Cut Off
NaOH	-	Sodium Hydroxide
NLC	-	Nano-structured Lipid Carrier
OECD	-	Organisation for Economic Co-operation and Development
PBS	-	Phosphate Buffer Saline
PDI	-	Polydispersity Index
PTA	-	Phosphotungstic acid
QbD	-	Quality by Design
QTTP	-	Quality Target Product Profile
RHS	-	Reconstructed Human Skin
RIPA	-	Radioimmunoprecipitation
ROS	-	Reactive Oxygen Species
SC	-	Stratum Corneum
SD	-	Standard Deviation
SLN	-	Solid Lipid Nanoparticles
TDDS	-	Transdermal Drug Delivery System
TEM	-	Transmission Electron Microscopy
TEC	-	Tissue Engineering Centre
TGF-β	-	Transforming Growth Factor – β
TPC	-	Total Phenolic Content

TPP	-	Target Product Profile
U. K	-	United Kingdom
UKM	-	Universiti Kebangsaan Malaysia
UTM	-	Universiti Teknologi Malaysia
UV	-	Ultraviolet
UVB	-	Ultraviolet B
vs.	-	Versus
ZP	-	Zeta Potential

LIST OF MATHEMATICAL SYMBOLS

α	-	alpha
β	-	beta
ζ	-	zeta
∞	-	infinity
√	-	square root
½	-	one – half
°C	-	degree Celcius
%	-	percent
µm	-	micrometre
µg/ml	-	microgram per millilitre
cm	-	centimetre
cm^{-1}	-	per centimetre
cm^{-2}	-	centimetre per square area
cm^{-3}	-	cubic centimetre (volume)
d.nm	-	diameter. nanometer
h	-	hour
h^{-1}	-	per hour
mg/ml	-	milligram per mililitre
ml	-	millimetre
ml/min	-	millimetre per minute
mm	-	millimetre
D	-	Diffusion coefficient

P	-	Permeability coefficient
pH	-	Hydrogen ion concentration in a solution
Q	-	Cumulative amount
rpm	-	revolutions per minute
K	-	Partition coefficient

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Labisia pumila (LP) is from Myrcinaceae family also known as Kacip Fatimah, is a well – known herbal medicine among Malaysian and communities around South-East Asia. It is usually consumed as a traditional medicine to maintain female reproductive function (Fathilah et al., 2012. Chua et al., (2011) reported that LP has high antioxidant concentration such as phenolic acids and flavonoids. This herbal medicine is getting high demands as a food supplement; however, there is an insufficient study on its skin applicaton. The plant also contains estrogen-like constituent that can enhance collagen synthesis in the skin and protect cells from damage due to UVB irradiation (Choi et al., 2010).

Ageing is a common process where the development and deepening of wrinkles, the appearance of age spot and the loosening of the skin will occur with time. This change occurs in all layer of the skin due to the natural ageing process or factor from the environment. The major factor of the formation of wrinkles and loss of skin firmness is the destruction of elastin fibres alongside with the downregulation in collagen synthesis.

Collagen is one of the main building blocks in human skin. It is synthesized from procollagen, which is derived from dermal fibroblast. Collagen is an essential protein that is needed to provide elasticity to the skin. Collagen synthesis is promoted by a cytokine namely the transforming growth factor - beta (TGF-β). Depletion of collagen synthesis in the skin is due to the increase in Matrix Metalloproteinases (MMP-1), a collagenase that

is involved in skin collagen degradation, which is upregulated in aged skin. Excessive exposure to UV radiation from sunlight will trigger the production of reactive oxygen species (ROS) resulting in oxidative damage to cellular components of the skin and induction of pre-mature ageing (Mukherjee et al., 2011). Choi and his co-workers (2010) discovered that a dose-dependent LP extract induced the restoration of collagen in UVB irradiated human fibroblast cells and decreased MMP-1 expression.

Owing to its strong antioxidant contents, it is very useful and important to have methods of protecting the extract to preserve the quality and its biology activity when employed as active ingredients for skin care product. In pharmaceutical and cosmeceutical industries, nanoparticle drug delivery system has been widely used to facilitate the delivery of drug and herbal extract. Current drug delivery technologies commonly used lipid-based carriers such as liposomes, ethosomes, Transfersomes ™, Solid Lipid Nanoparticles (SLN) and Nano-structured Lipid Carrier (NLC) to deliver a therapeutic amount of extract and drug through a topical or transdermal administration of the skin. Interest in the physicochemical aspect of nanoparticle delivery system in any pharmaceutical product has improved the delivery of a free drug or herbal extract to the targeted sites.

Flexible liposomes (FL) is an advanced formulation of liposomes. It is more elastic, more hydrophilic and can penetrate the stratum corneum through intercellular or transcellular route compared to conventional liposomes (Pawar *et al*., 2016). The elastic bilayer of FL resulted from a combination of surfactant and phospholipid. Biocompatible surfactants such as Tween 80, Span 80 and sodium deoxycholate are the types of edge activator frequently used to enhance the flexibility of the liposomes. Stable lamellar structures of the flexible liposomes are achieved by mixing two types of lipids such as phosphatidylcholine and cholesterol or beta-sitosterol. Animal cells contain cholesterol whereas plant cells contain sterol. Plant sterol or phytosterol may also alter the functional and structural properties of the lipid bilayer (Mora *et al*., 1999). Beta-sitosterol, the main component in soybean sterol, was found to be more effective in rearranging the acyl chains

of soybean lecithin bilayers than cholesterol (Farkas *et al*., 2004). It can help maintain membrane integrity to produce a stable core of lipid bilayer (Watson & Preedy, 2004).

In this research work, flexible liposomes were used to encapsulate the LP extract for transdermal delivery administration. Physical and chemical stability of lipid nanocarrier is important and stability depends on the final composition of the carrier. The formulation may have a short shelf – life due to chemical and physical degradation. A systematic approach by implementing the Quality by Design (QbD) is recommended by International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) (ICH 2005, ICH 2009), regulatory agencies for the development of a stable pharmaceutical product. The identification of Target Product Profile (TPP), Quality Target Product Profile (QTPP), Critical Quality Attributes (CQAs), risk assessment and Design Space (DS) were applied in this project for a better understanding of the preparation and formulation process of LP loaded FL. The framework is important to understand the formulation and manufacturing process that influence product quality with an effective control strategy. Design of Experiment (DOE) was used as a tool to minimize the number of experiments in order to obtain the desired product quality and to study the combined effects of the processing factors statistically.

The physical state of the lipid carrier can affect the drug release and the permeation of the nanoparticles through an intact skin barrier**.** The structure and biochemical features of human skin contribute to the barrier function of the skin for molecular transport. Anatomically, the skin is divided into two layers, epidermis and dermis layer. The Stratum Corneum (SC) comprises 15 – 20 layers of keratin-filled corneocytes anchored in a lipophilic matrix. SC is the uppermost layer of the epidermis and is the primary barrier between the body and the environment. There are three possible ways of penetration which drug molecules can cross the intact SC: via appendageal, intercellular or transcellular route. The rationale of using lipid-based delivery system is to overcome the skin barrier including sustaining the release of therapeutic drug molecules. It is believed that FL loaded with LP would return to its original shape once passed the skin barrier due to the function of its edge activator. Improving the formulation of the lipid-based delivery

system could alter the lipid partitioning of the skin barrier and increase the diffusion coefficient of the active molecules to the targeted sites.

1.2 Problem Statement

Reduction of collagen in the skin is due to natural ageing or premature ageing that occur due to an external factor such as excessive exposure to direct sunlight. *Labisia pumila* aqueous extract was found to be effective in inducing collagen synthesis in human skin by reducing the expression of Matrix Metalloproteinase-1 (MMP-1) (Choi et al., 2010). MMP is a gene encoded a member of peptidase. Proteins in MMPs family, involved in the breakdown of extracellular matrix in normal physiological processes such as reproduction and tissue remodelling. A matured protease secreted from preproprotein can break down the interstitial collagen including collagen type I, II and III (Sonoki et al, 2018). Anti-ageing potential of this herb makes it suitable to be incorporated in skin care formulations. The hydrophilic behaviour of this extract makes it difficult to penetrate the skin barrier. The lipophilic nature of the SC limits the permeation of hydrophilic substances. Lipid-based delivery system such as FL can be utilized to deliver a safe and therapeutic dose of LP extract.

The herb used in this study was *Labisia pumila var. alata* procured from Institute of Bioproduct Development, Universiti Teknologi Malaysia. Flexible liposome (FL) was used to encapsulate LP in this research work. The original flexible liposomes coined is a novel formulation Transfersome™, comprises containing a phospholipid, cholesterol and a surfactant to form the lipid bilayers. Elasticity behaviour of the Transfersome™ is due to surfactant such as sodium cholate, Tween 80 or Span 80, which act as an edge activator. The elasticity of Transfersome™ allows it to permeate through the skin barrier area by osmotic force in the skin and then deform back to its original shape. In this work, a flexible

liposomes was made of L-α-phosphatidylcholine, β-sitosterol, and combination of Span 80 and Tween 80.

Transdermal pathways of nanoparticles delivery system have been discussed; however, no specific mechanisms were concluded as different active ingredients might create a different pathway of penetration. The main hypotheses in this study are FL entrapped LP will change the structure of the skin pores and squeeze through the skin layer before returning back to its original shape, owing to its elastic performance. The interaction of the FL-LP with the skin barrier will enhance the delivery and allow the actives to penetrate through the skin. The possible pathway of transdermal delivery would be explained from the results of a series of rat skin and reconstructed human skin (RHS) permeation experiments. RHS was used as an alternative to animal study as using animal is not permitted in cosmeceutical study. Confocal Laser Scanning Microscopy (CLSM) would be used to determine the localization of the penetrant through the skin. The delivery of LP to the dermal layer of the skin would induce collagen synthesis that can be measured through collagen synthesis assay. The findings of this work would contribute to the understanding of the pathway of transdermal delivery of LP loaded FL to enhance collagen synthesis, particularly for cosmeceutical application.

1.3 Research Objectives

The objectives of this study were:

1. To determine the optimum factors in the formulation of *Labisia pumila* loaded flexible liposomes formulation to result in the induction of collagen synthesis in reconstructed human skin (RHS).
2. To study the pathway of flexible liposomes loaded with *Labisia pumila* through the skin.

1.4 Scopes of the Study

Labisia pumila var. alata from the Myrcinaceae family was procured from Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM). This study was done in Cosmeceutical Laboratory, IBD, UTM, Kuala Lumpur. This work focused on the development of an optimum formulation of LP loaded flexible liposomes (FL) to induce collagen synthesis in reconstructed human skin (RHS). A Quality by Design (QbD) approach was used to identify the critical factors involved in the process development. The formulation of FL was developed using Design of Experiment (DOE) by employing 2^k Full Factorial Design. Five factors were selected, namely the amount of L-α-phosphatidylcholine, β-sitosterol, Tween 80, Span 80 and time of sonication. Dependent variables of the design were particle size, polydispersity index (PDI), zeta potential, elasticity and encapsulation efficiency. An optimized formulation from the DOE was validated using ANOVA and was further analysed for physical characterization and storage stability. Cytotoxicity of the formulation was analysed to determine the IC_{50}. It was to ensure a safe formulation and dosage was used throughout the study. The characterization of LP loaded FL was characterized physically using Transmission Electron Microscope (TEM) to determine the morphology of the FL-LP and Nano Zetasizer to measure the particle size, PDI and zeta potential. Gallic acid was used as a marker for LP extract to determine the encapsulation efficiency of FL-LP. Gallic acid concentration was measured using the Total Phenolic Content (TPC) assay. Storage stability of the formulation was conducted in three consecutive months in three batches (triplicate) and leakage rate was measured during the experiment. The interaction of functional groups in FL-LP composition was analysed using Fourier Transform-Infra Red (FTIR) and compared with free LP extract (non-encapsulated) and unloaded FL. The same type of samples was analysed using Differential Scanning Calorimetry (DSC) to measure thermal changes in the formulation.

To understand FL-LP behaviour's as a delivery system, *in vitro* kinetic release study was conducted to identify the kinetic model. Next, *ex vivo* permeability study was carried out using rat skin and full thickness 3-Dimensional RHS to analyse the transport

properties using Franz diffusion unit. The experimental data of *in vitro* and *ex vivo* studies were compared with non-encapsulated LP. The permeability study was also compared with conventional liposomes as a benchmark of delivery efficiency and experimental work.

To study the pathway of permeation, Confocal Laser Scanning Microscopy (CLSM) was used to detect the positioning of the FL-LP in the rat skin. In order to identify the localization of FL-LP during penetration through the rat skin, the FL-LP was dyed with Nile red as a lipid probe. The efficacy study was evaluated using a collagen synthesis assay by measuring collagen concentration in full-thickness RHS. The enhancement of collagen concentration in RHS was compared with non-encapsulated LP and untreated RHS was used as a positive control. The findings of this work will contribute to a new insight into FL's function as a carrier of LP extract to enhance collagen synthesis in the skin.

1.4 Structure of the Thesis

This thesis is structured into five chapters. Chapter 1 is the introduction of the thesis, which covered the problem statement, objectives and scopes of the study, research questions and the significance of the study. Chapter 2 is the literature review on the current progress of therapeutic herbal medicine in cosmeceutical research, types of nanoparticles used to encapsulate the herbal extracts, the influences of physical and chemical characterization to the nanoparticle's stability, transport properties and mode of transdermal delivery of the nanoparticles through the skin. Chapter 3 comprises the experimental procedure and the analysis done in this study. Then, Chapter 4 discusses the results obtained from both the prediction and experimental studies of the nanoparticle's formulation design, characterization of the *Labisia pumila* encapsulated flexible liposomes, kinetic release study of the flexible liposomes and the mode of its transdermal delivery through rat skin and Reconstructed Human Skin. Finally, Chapter 5 concludes

the findings and contribution of this thesis as well as proposes the recommendations or improvements of this study.

1.5 The Significance of the Study

The important finding of this study is the employment of FL as a carrier to deliver LP and enhance collagen synthesis. Since the references on the employment of the delivery system in encapsulating herbal extract is still scarce, this study has pushed the boundary of knowledge by discussing its efficacy in transdermal delivery. The fundamental study on the pathway of *LP* loaded FL for transdermal delivery will help researchers from pharmaceutical and cosmeceutical industry to apply it in a large scale and at the same time provides a wide application of other herbal extract in skin care formulation.

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