OPTIMIZATION OF *ORTHOSIPHON STAMINEUS*-LOADED NANOSTRUCTURED LIPID CARRIER USING D-OPTIMAL MIXTURE DESIGN FOR IMPROVED LIPOLYSIS ACTIVITY

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This thesis is dedicated to my parents and family

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ABSTRACT

Orthosiphon stamineus (OS) is a Malaysian medicinal herb that was reported to have weight reduction effect and normally prepared as herbal infusion. Effective topical utilization of OS requires a good drug delivery system in order to overcome the stratum corneum to reach the targeted area. Nanostructured lipid carrier (NLC) is a promising carrier for topical drug delivery. The goal of the present study was to optimize the formulation of OS-loaded nanostructured lipid carrier (OS-NLC) for improved lipolysis activity. OS-NLC was prepared using melt emulsification homogenization technique with different types of lipid to obtain good homogeneity and miscibility of the formulation. The result revealed the preferred selection of glyceryl monostearate as solid lipid and triglyceride as liquid lipid since they showed good homogeneity. The formulation of OS-NLC was optimized using D-optimal mixture design which consisted of an amount of active ingredients, solid lipid, and liquid lipid as independent variables while particle size, polydispersity index (PDI) and encapsulation efficiency as dependent variables. From the study, it was found that the optimum formulation of OS-NLC was made of 4% OS, 1% glyceryl monostearate, and 5% triglyceride with 88.57 ± 1.187 nm particle size, 0.135 ± 0.007 PDI and 98.10 ± 1.101 % encapsulation efficiency. Coefficient of determination (R^2) indicated a good fit between predicted values and the experimental data points for particle size, PDI and encapsulation efficiency, which were found to be 0.9404, 0.9138 and 0.8754, respectively. Transmission electron microscopy images exhibited the spherical shape of OS-NLC. Fourier transform infrared spectroscopy analysis demonstrated an interaction between OS extract and NLC system. The zeta potential of OS-NLC was -16.7 ± 0.5033 mV. Storage stability of OS-NLC was conducted at cold and room temperature for one month by measuring the particle size, PDI and zeta potential. The results revealed that there were no significant changes in particle size, PDI and zeta potential within one month. The *in vitro* penetration study using Franz diffusion cell showed that the penetration flux of OS-NLC (2188.74 μg $cm^{-1}h^{-1}$) was significantly higher than OS (1614.20 μg $cm^{-1}h^{-1}$). The result proved that NLC encapsulate OS had better delivery system compared to OS extract. In lipolysis study, OS-NLC was found to stimulate the release of glycerol in 3T3-L1 adipocytes cells. Taken together, the optimal OS-NLC formulation had efficiently enhanced penetration into skin and improved the lipolysis activity in adipocyte cell.

ABSTRAK

Orthosiphon stamineus (OS) adalah herba perubatan Malaysia yang dilaporkan mempunyai kesan penurunan berat badan dan biasanya digunakan sebagai infusi herba. Penggunaan berkesan OS memerlukan sistem penghantaran ubat yang baik untuk mengatasi lapisan *stratum corneum*. Pembawa lipid berstruktur nano (NLC) adalah pembawa yang berkebolehan untuk penghantaran ubat topikal. Matlamat kajian ini adalah untuk mengoptimumkan formulasi OS dimuatkan ke dalam pembawa lipid berstruktur nano (OS-NLC) bagi meningkatkan meningkatkan aktiviti lipolisis dalam sel lemak. OS-NLC dihasilkan daripada teknik penyeragaman pengemulsian lebur dengan menggunakan pelbagai jenis lipid untuk mendapatkan keseragaman dan kebolehcampuran formulasi yang baik. Hasil kajian mencadangkan pilihan gliseril monostearat sebagai lipid pepejal dan trigliserida sebagai lipid cecair lebih diutamakan kerana mereka menunjukkan keseragaman yang baik. Formulasi OS-NLC dioptimumkan dengan menggunakan reka bentuk campuran D-optimum yang terdiri daripada bahan aktif, lipid pepejal dan lipid cecair sebagai pemboleh ubah tak bersandar manakala pemboleh ubah bersandar terdiri daripada saiz partikel, indeks kepoliserakan (PDI) dan kecekapan pengkapsulan. Berdasarkan keputusan kajian, formulasi optimum OS-NLC terdiri daripada 4% OS, 1% gliseril monostearat, dan 5% trigliserida dengan saiz partikel 88.57 ± 1.187 nm, 0.135 ± 0.007 PDI dan 98.10 ± 1.101% kecekapan enkapsulasi. Pekali penentuan (R^2) menunjukkan kesesuaian antara nilai ramalan dan data eksperimen untuk saiz partikel, PDI dan kecekapan enkapsulasi masing-masing adalah 0.9404, 0.9138 dan 0.8754. Imej mikroskop elektron transmisi menunjukkan OS-NLC berbentuk sfera. Spektroskopi inframerah transformasi Fourier menunjukkan interaksi antara ekstrak OS dan sistem NLC. Potensi zeta bagi OS-NLC ialah -16.7 ± 0.5033 mV. Kestabilan penyimpanan OS-NLC telah dijalankan pada suhu sejuk dan bilik selama satu bulan dengan mengukur saiz partikel, PDI dan potensi zeta. Hasil kajian mendapati tiada perubahan signifikan dalam saiz partikel, PDI dan potentsi zeta dalam masa satu bulan. Kajian penembusan transdermal *in vitro* menggunakan sel penyebaran Franz menunjukkan bahawa fluks penembusan OS-NLC (2188.74 μg $cm^{-1}h^{-1}$) adalah lebih tinggi daripada OS (1614.20 μg $cm^{-1}h^{-1}$). Hasilnya membuktikan bahawa NLC merangkumi OS menunjukkan sistem penghantaran yang lebih baik berbanding dengan ekstrak OS. Dalam kajian lipolisis, OS-NLC didapati boleh melepaskan gliserol dalam sel adiposit 3T3-L1. Secara keseluruhannya, formulasi OS-NLC yang optimum adalah cekap dalam meningkatkan penembusan ke dalam kulit dan meningkatkan aktiviti lipolysis dalam sel adiposit.

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LIST OF ABBREVATIONS

ANOVA	-	Analysis of variance
ATL	-	Adipose triglyceride lipase
DEX	-	Dexamethasone
DMEM	-	Dulbelcco's modified Eagle's medium
DSC	-	Differential scanning calorimetry
EE	-	Encapsulation efficiency
EUP	-	Eupatorin
FBS	-	Fetal bovine serum
FTIR	-	Fourier transform infrared spectroscopy
GMS	-	Glyceryl monostearate
HPLC	-	High performance liquid chromatography
HSL	-	Hormone sensitive lipase
IBMX	-	3-isobutyl-1-methylxanthine
MAG	-	Monoacylglycerol lipase
MTT	-	3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
NLC	-	Nanostructured lipid carrier
OS	-	*Orthosiphon stamineus*
PBS	-	Phosphate buffered saline
PDI	-	Polydispersity index
PKA	-	Protein kinase A
PKG	-	Protein kinase G
RA	-	Rosmarinic acid
SLN	-	Solid lipid nanoparticles
SN	-	Sinensetin

TMF	-	3’-hydroxy-5,6,7,4’-tetramethoxyflavone
ZP	-	Zeta potential

LIST OF SYMBOLS

cm	-	Centimeter
µg/ml	-	Microgram per mililitre
%	-	Percent
^{o}C	-	Degree celcius
mg/ml	-	Miligram per mililitre
µL	-	Microlitre
nm	-	Nanometer
mM	-	Milimolar
µM	-	Micromolar
nmole	-	Nanomole
mm	-	Milimetre
ml	-	Mililitre
rpm	-	Revolutions per minute
g	-	Gram

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

The largest organ in the human body is the skin. The function of skin is to protect the body from any unwanted influences from the environment. The skin consists of the epidermis, dermis and hypodermis. Stratum corneum is the outermost layer of the human skin. It is known that stratum corneum acts as a skin barrier function. A successful transdermal drug delivery must prevail over the stratum corneum. Thus, many approaches have been done to overcome the barrier function and enhance drug transport into the skin (Ghafourian *et al*., 2004)

Nanotechnology or nanoparticles used as drug delivery vehicle generally have particle size less than 100nm. Nanoparticles are effective transport and delivery system since it improve the bioavaibility of drugs and provide the necessary protection of drugs molecule. Lipid nanoparticles such liposome, nanoemulsion, microemulsion, solid lipid nanoparticles, and nanostructured lipid carrier are gaining a lot of interest as a vehicle for controlled released of active substances and targeting to skin layer. In the beginning of 1990s, solid lipid nanoparticles (SLN) were developed as a carrier

system to substitute emulsion, liposomes and polymeric nanoparticles (Muller *et al*., 2002). SLN is produced by solid lipid only. However, SLN produced a perfect crystal lattice which provides limited space to hold the active, hence causes expulsion of active from the lipid matrix during storage (Bunjes and Koch, 1996).

Nanostructured lipid carrier (NLC) has been introduced to overcome the limitation of solid lipid nanoparticles. Components of NLC are solid lipid, liquid lipid, surfactants and water. NLC is produced by blending both solid and liquid lipids. The combination of solid and liquid lipids alters the formation of perfect crystal thus the matrix is in imperfect form and provides space to hold the active in the lipid matrix. In view of topical administration, NLC has occlusive properties and can reduce transepidermal water loss (TEWL) thus enhance penetration of active ingredient through the stratum corneum by increased hydration (Uprit *et al.,* 2013). NLC offers potential advantages such as protection of active ingredients against degradation, controlled and sustained release of active drug can be achieved, high drug loading capacity, and improve skin permeation (Patel *et al.,* 2013).

Having an excess body fat will lead to several health risk such as being overweight. Adipocytes acts as cells that stored energy as fat. Three different types of adipocytes are white adipocytes, brown adipocytes and beige adipocytes. White adipocytes are responsible to store energy and brown adipocytes to dissipate energy in thermogenesis. While the function of beige adipocytes is still not clear (Stephens, 2012).

Lipolysis is the process of breakdown of triglyceride to free fatty acid and glycerol. This process takes place in white adipose tissue. Enzymes are involved in lipolysis process are adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MAG) (Zechner *et al.,* 2012). In the first step of lipolysis ATGL is responsible to catalyze the conversion of triglyceride into diacylglycerol whereas HSL will hydrolyze diacylglycerol to monoacylglycerol and MAG hydrolyze monoacylglycerol to glycerol. The main pathway for lipolysis process

is through the activation of cAMP dependent protein kinase A (PKA) (Kim *et al.,* 2014).

Orthosiphon stamineus (OS) or commonly known as *Misai kucing* in malay community is a Lamiacae family. OS has been used to treat diabetes, hypertension, menstrual disoder, epilepsy and rheumatic arthritis. Three phytochemical compounds found in different extract of OS are polymethoxylated flavonoids, phenylpropanoids and terpenoids. Major component of aqueous extract in OS is rosmarinic acid which exhibits antioxidant, immunomodulatory and anti-cancer activity (Scheckel *et al.,* 2008; Yam *et al.,* 2009; Ameer *et al.,* 2012). Combination of OS powder with green tea has been utilized for weight reduction effect (Ameer *et al.,* 2012). In addition, previous study by Son *et al.,* (2011) found OS can reduced visceral fat mass and food intake in Sprague-Dawley rats. Study conducted by Sayedan *et al.* (2016) found that the ethanolic extract of OS leaves significantly reduced a gain in body weight which give weight reduction effect in obese mice induced by a high fat diet. This conclude that OS can be a medicinal food for body weight control.

However, most weight reduction drug has been hardly approved by the Food and Drug Administration (FDA) authority, as compared with transdermal patches and beauty creams. In order to loss fat at specific areas, lipolytic agent such as aminophylline and caffeine can be applied to the skin. Aminophylline and caffeine were found to liberate subcutaneous fat, thinning the fat layer and also reducing cellulite (Caruso *et al.,* 2007). Previous study revealed that external medicinal such as topical cream composed of aminophylline possess weight reduction effect (Petrofsky, *et al.,* 2014). Therefore, it was highly potential to use OS as a topical drug to induce lipolysis in adipocyte which located in subcutaneous layer as well as to achieve systemic weight reduction.

Formulation of NLC to encapsulate OS is a challenging task due to the trial and error which can be time consuming and required high cost. Optimization provide significant understanding of develop formulation and their properties can be gained. Experimental design plays bigger role in cosmetic formulation because it provides better understanding about the effect of different product formulation on product properties (Rajin *et al.,* 2007). The desired formulation can be achieved as fast as possible with experimental design because it can reduce number of experiments. Several statistical designs available such as response surface methodology (RSM), factorial design, combined and mixture design. For experiments that have various mixed ingredients, mixture design is employ in order to identify ingredients that give effect on the dependent variables and also to determine the optimal mixing ratio that maximizes or minimizes the dependent variables (Choi, 1998). The D optimal design is suitable for highly constraint design and widely used in drug delivery devices (El-Malah *et al.,* 2006).

The present work was aim to produce cost effective of OS-NLC by optimizing the formulation using D-optimal mixture design in order to improve skin penetration and induce lipolysis activity.

1.2 Problem Statement

Topical drug delivery (TDD) can be described as the application of a drug containing formulation directly to the skin. Once topical formulation was applied to the skin, they must interact with the skin condition which can control the rate of release of the compound. TDD is favoured due to the ease of delivery and most importantly TDD have no intervention with gastric and intestinal fluid (Sharma *et al*., 2013). However, TDD poses a challenge due to the skin barrier. Besides, the protection of active ingredient is a crucial factor in TDD to protect the active ingredient from degradation and successfully delivered the active ingredient to the targeted area.

Nanostructured lipid carriers (NLC) is a lipid based nanoparticles which normally have a particle size less than 100 nm. NLC is considered as a novel drug delivery system to deliver active ingredients with high solubility, stability, effective skin penetration and low irritation. Many studies have found that NLC can increase the encapsulation efficiency due to the involvement of two type of lipids in NLC which gives the imperfection in their structure thus provide more space to accommodate the active ingredient. The addition of liquid lipid is the main factor that contributes higher encapsulation efficiency because of the higher solubility of drugs in liquid lipid in comparison with solid lipid (Muchow *et al.,* 2008). NLC has the ability to incorporate both hydrophilic and lipophilic drugs. Moreover, the main advantage of NLC over other delivery system is the protection of active ingredient from degradation.

Generally OS has been used as diuretic, to treat rheumatism, kidney and bladder inflammation. Most of the compounds found on OS such as terpenoids, polyphenols, orthosiphols were found to have weight reduction effect (Jayaprakasam et al., 2006). Study by Son *et al.,* (2011) found that the OS can reduce appetite and fat deposition, food intake and visceral fat mass in sprague dawley rats which conclude that OS can act as medicinal food application for body weight control. Collectively, it is postulated that OS may play an important role in the treatment of obesity such as numerous in-vivo study were conducted by using Sprague dawley rats. However, the ability of OS

to act as anti-obesity medicinal by inducing lipolysis through direct action on adipose tissue is still not clear

The most effective way to reduce weight is by exercise and healthy diet. However, with dieting and exercise it is hard to target where fat loss occurs. Fat loss can occur in any area of the body. Therefore, if fat loss is to be targeted at specific areas, lipolytic agent can be applied to the skin. In addition, due to the topical administration of drug are much safer compared with oral medication, an external topical formulation of OS seem to have high potential to be used as a weight reduction treatment. However, the poor skin permeation of OS was a challenge to develop OS as transdermal delivery system.

Therefore, in this study, NLC is chosen as a carrier to deliver the OS to the targeted area due to the ability of NLC to encapsulate and protect OS from degradation and to overcome the stratum corneum. In addition, this study emphasized the production of OS-NLC for cosmeceutical application and reveal that NLC is an effective delivery system that enhanced penetration over stratum corneum.

1.3 Objective

The objective of this research is to optimize the formulation of *Orthosiphon stamineus* loaded nanostructured lipid carrier to enhance penetration through the skin and induced lipolysis in adipocyte

1.4 Scope of the Research

In order to achieve the objective of this study, the following scopes have been identified as below:

1. Extraction of OS using maceration method.
2. Formulation of OS-NLC using melt emulsification homogenization technique.
3. Optimization of OS-NLC using D-optimal mixture design by design expert software.
4. Characterization of optimized formulation of OS-NLC using Transmission Electron Microscopy (TEM), Differential Scanning Calorimetry (DSC), Fourier Transform Infrared Spectroscopy (FTIR)
5. In vitro penetration study by Franz type diffusion cell
6. In vitro cytotoxicity study of OS-NLC on 3T3-L1 preadipocyte using MTT assay
7. Investigation on lipolysis induction of OS-NLC on 3T3-L1 adipocytes

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