ENZYMATIC SYNTHESIS OF DILAURYLAZELATE ESTER FOR NANOCOSMECEUTICAL APPLICATION

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FS 2018 110

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By

NURSHAFIRA BINTI KHAIRUDIN

Thesis Submitted to the School Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

March 2018

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March 2018

Chairman : Professor Mahiran Basri, PhD
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Azelaic acid (AzA) and its derivatives have been known to be effective in the treatment of acne and various cutaneous hyperpigmentary disorders for cosmeceutical application. Currently, azelaic acid is available in the market as a 20% azelaic acid cream for the treatment of acne, and has been approved for use by the Food and Drug Administration (FDA). However, high concentration (20%) of azelaic acid is needed to guarantee the drug availability in the skin, which caused an increase the incidence of side effects such as irritation, dryness, and redness of the skin associated with exposure to high levels of undissolved dispersed azelaic acid having an inherent low pH. A derivative of azelaic acid with even better characteristics than the original starting material may be produced through the modification to azelaic acid, especially the efficacy of a relatively low dosage of AzA derivatives in the treatment of acne.

Dilaurylazelate ester was produced through the esterification of azelaic acid with lauryl alcohol using immobilized lipase B from *Candida antarctica* (Novozym 435). The chemical and physical characterization of dilaurylazelate ester was analyzed in order to be effectively applied in cosmeceutical application. The chemical characterization was determined by Fourier-Transform Infrared spectroscopy (FT-IR), Gas Chromatography Mass Spectroscopy (GCMS), and Nuclear Magnetic Resonance (NMR) in order to verify and elucidate the structure of product. The normal fibroblasts cell line (3T3) was used to assess the cytotoxicity of dilaurylazelate ester and the antibacterial activity against the pathogen bacteria *Propionibacterium acnes* ATCC 11827 was studied.

Response surface methodology (RSM) and artificial neural network (ANN) were used to optimize and predict various performance parameters of the enzymatic reaction conditions, namely enzyme amount, reaction time, reaction temperature and molar ratio of substrates that could affect on the degree of percentage conversion of dilaurylazelate ester. The optimization and prediction capabilities of RSM and ANN were then compared. The results obtained showed, ANN model predicted is more accurately

compared to RSM since ANN model has lower percentage of residual square error (0.35%). The higher prediction accuracy of ANN model was due to generation of the model by multiple iterative calculations whereas single step calculation in the case of RSM.

Nanoemulsion has been chosen to be a carrier in encapsulation of dilaurylazelate ester for the treatment of acne. In this work, a D-optimal mixture design was used to determine the optimal composition of nanoemulsion-based system loaded with dilaurylazelate ester. The ultimate goal of the present work was to determine the optimum level of five independent variables (linoleic acid, T80:PF68, glycerol, dilaurylazelate ester, and water) in the nanoemulsion composition with minimum average droplet size. Under the optimal conditions, the predicted average droplet size was obtained 161.90nm, which possessed a good stability over 3 months' storage at different temperatures. In addition, physicochemical characterizations of the optimal nanoemulsion showed its suitability for topical application due to its stability against phase separation.

The permeation study revealed that when nanoemulsion was used as carrier vehicle, permeability of dilaurylazelate ester was significantly improved. Besides, cytotoxicity result further supported the safety and suitability of formulation to be used as a topical product in cosmeceutical fields with IC_{50} higher than 100μg/ml is considered non-toxic and also was classified as dermally non-irritant with a human irritancy equivalent. In vivo skin analysis towards healthy volunteers showed a significant improvement in the stratum corneum in skin hydration, which would be useful in treating the dry skin of acne patients.

Abstrak tesis yang dikemukan kepada Senat Universiti Putra Malaysia bagi memenuhi keperluan untuk ijazah Doktor Falsafah

SINTESIS BERENZIM BAGI ESTER DI-LAURILAZELAT UNTUK APLIKASI NANOKOSMESEUTIKAL

Oleh

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Asid Azelaik (AzA) dan derivatifnya telah diketahui keberkesanannya dalam rawatan jerawat dan pelbagai penyakit kulit bagi aplikasi kosmeseutikal. Pada masa ini, asid azelaik telah berada di pasaran sebagai 20% krim asid azelaik bagi rawatan jerawat, dan kegunaannya telah mendapat kelulusan oleh Pentadbiran Makanan dan Ubat-ubatan (FDA). Walau bagaimanapun, Asid azelaik memerlukan kepekatan yang tinggi iaitu 20% untuk menjamin ketersediaannya di dalam kulit, yang mana telah menyebabkan peningkatan kesan sampingan seperti iritasi pada kulit, kulit kering, dan kulit kemerahan yang dikaitkan dengan pendedahan kepada kandungan asid azelaik yang tinggi dan tidak larut disebabkan oleh pH yang rendah. Derivatif asid azelaik dengan ciri-ciri yang lebih baik boleh dihasilkan melalui pengubahsuaian asid azelaik, terutamanya keberkesanan penggunaan asid azelaik derivatif bagi rawatan jerawat.

Ester di-laurilazelat dihasilkan melalui pengesteran asid azelaik dengan lauril alkohol dengan menggunakan lipase B dari *Candida antarctica* (Novozym 435). Pencirian kimia dan fizikal bagi ketulenan ester di-laurilazelat telah dilakukan untuk digunakan sebagai bahan kosmetik. Pencirian kimia telah ditentukan dengan menggunakan spektroskopi Inframerah (FT-IR), Spektroskopi Jisim Kromatografi Gas (GCMS), dan Resonans Magnet Nukleus (NMR) untuk mengenalpasti produk yang telah diperolehi. Normal sel fibrolast (3T3) telah digunakan untuk mengkaji kesitotoksikan ester di-laurilazelat dan aktiviti antibakteria bagi ester di-laurilazelat terhadap bakteria *Propionibacterium acnes* ATCC 11827 juga telah dikaji keberkesanannya.

Response Surface Methodology (RSM) dan *Artificial Neural Network* (ANN) telah digunakan untuk mengoptimumkan dan meramalkan prestasi parameter iaitu, jumlah enzim, masa tindak balas, suhu tindak balas, dan nisbah molar substrat yang boleh memberi kesan kepada tahap peratusan penukaran ester di-laurilazelat. Seterusnya, ketepatan ramalan pengoptimuman oleh RSM dan ANN dibandingkan. Keputusan yang diperoleh menunjukkan, model yang diramalkan oleh ANN lebih tepat berbanding

RSM, ini adalah kerana model ANN mempunyai peratusan yang lebih rendah ralat piawai residu (RSE) iaitu 0.35%. Ketepatan ramalan yang lebih tinggi dari model ANN adalah disebabkan oleh generasi model dengan pengiraan berulang yang pelbagai manakala pengiraan langkah tunggal dalam kes RSM.

Nanoemulsi telah dipilih untuk menjadi pembawa bagi ester di-laurilazelat yang telah dienkapsulasikan untuk rawatan jerawat. Dalam kajian ini, *D-optimal Mixture Design* (MED) telah digunakan untuk menentukan pengoptimuman sistem komposisi berasaskan nanoemulsi yang mengandungi ester di-laurilazelat. Matlamat utama kajian ini adalah untuk menentukan tahap optimum lima pembolehubah bebas (asid linoleik, T80: PF68, gliserol, ester di-laurilazelat, dan air) dalam komposisi nanoemulsi dengan purata saiz zarah yang minimum. Dalam keadaan yang optimum, diramalkan purata saiz zarah yang diperolehi ialah 161.90 nm, yang mana mempunyai kestabilan yang baik selama 3 bulan penyimpanan pada suhu yang berbeza. Di samping itu, pencirian fizikokimia optimum nanoemulsi menunjukkan kesesuaiannya bagi aplikasi topikal kerana kestabilannya terhadap fasa pemisahan.

Kajian penelapan mendedahkan bahawa apabila nanoemulsi digunakan sebagai pembawa, kebolehtelapan ester di-laurilazelat meningkat dengan ketara. Selain itu, keputusan kesitotoksikan menyokong keselamatan dan kesesuaian formulasi untuk digunakan sebagai produk topikal dalam bidang kosmeseutikal dengan IC_{50} yang lebih daripada 100 μg/ml yang dianggap tidak toksik dan optimum formulasi juga diklasifikasikan sebagai dermal tidak merengsa dengan skor persamaan iritasi manusia. In vivo analisis kulit ke atas sukarelawan yang sihat telah menunjukkan peningkatan yang signifikasi dalam stratum korneum terhadap penghidratan kulit, yang akan berguna dalam merawat kulit kering bagi pesakit yg mempunyai jerawat.

ACKNOWLEDGEMENTS

In the name of Allah, the Most Loving and the Most Merciful. All praises goes to Allah for the strength, health and passion. He has given to me to complete this thesis. I would like to take this opportunity to express my appreciation to those who have contributed directly or indirectly in this thesis.

My deepest gratitude and sincere appreciation is owed to my supervisor late Prof. Dr. Mahiran Basri for her invaluable guidance, continuous support, patience and encouragement from the beginning till the end of this study. My appreciation also goes to members of my supervisory committee, Dr. Siti Efliza Ashari and Dr. Norazlinaliza Salim for their guidance and support.

A special thanks to late Dr. Hamid Reza Fard Masoumi, for his invaluable guidance and insightful comments that insist me throughout my research and publications. My appreciation also goes to all my colleagues in the Research Laboratory of Chemical Synthesis and Nanomolecular (Lab 401), especially Wan Sarah Samiun, Shazwani Samson, and Nur Syafiqah Rizalman. Thank you for making my stay during this research a bearable one with many sweet memories.

Last but not least, love and thanks to my beloved parents, Khairudin bin Mohd, and Rohana binti Ismail whose unwearied support, help, doa and encouragement me throughout my hard time. To my beloved husband, Mohammad Maziz Hafriz, my precious daughter Nur Raissa Kayyisa, and also to all my, brothers and sisters, I would like to express my deepest affection for their never ending love and support. I believed this journey is not only about me, but also about us. Alhamdulillah.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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water content. Notes: before and after 3 hours of optimized nanoemulsion application in 15 healthy volunteers. mean ± standard deviation (n=3)

LIST OF ABBREVIATIONS

AAD	Average absolute deviation
ANN	Artificial neural network
AV	Acne vulgaris
AzA	Azelaic acid
BBP	Batch back propagation
CaLB	*Candida antarctica* lipase B
CCRD	Central composite rotatable design
DCC	Dimethylcyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
FCS	Fetus calf serum
FDA	Food and drug administration
FFA	Free fatty acid
FTIR	Fourier transform infrared
GA	Genetic algorithm
GC	Gas chromatography
GCMS	Gas chromatography mass spectrometry
GUI	Graphical user interface
HIE	Human irritancy equivalent
HLB	Hydrophilic lipophilic balance
HPLC	High performance liquid chromatography
IBP	Incremental back propagation
LM	Levenberg–marguardt algorithm
MED	D-optimal mixture design
MLP	Multilayer perceptron
MTT	3-[4,5-dimethyllthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
OD	Optical density
O/W	Oil-in-water
PBS	Phosphate buffered saline
PDI	Polydispersity index
PEG	Propylene glycol
PF68	Pluronic
QP	Quick propagation
RCM	Reinforced clostridial medium
RMSE	Root mean square error
RSE	Residual standard error
RSM	Response surface methodology
TEWL	Transepidermal water loss
TLC	Thin layer chromatography
W/O	Water-in-oil

CHAPTER 1

INTRODUCTION

1.1 Background of Study

So long as the desire to seek for an ultimate beauty, revitalize skin wellness and eternalise youthful appearance are instilled in oneself, demands for new effective and innovative skincare products in the market will continue to soar. The term "cosmeceutical" is often used in the cosmetic industry which has been classified as cosmetic products which yield therapeutic or medicinal benefits (Choi and Berson, 2006). Similarly to cosmetics, cosmeceuticals are applied topically but vary in which they contain bioactive compounds that can influence the biological function of skin and deliver nutrient to enhance skin health (Brandt *et al.*, 2011).

Azelaic acid (AzA; HOOC-$(CH_2)_7$-COOH) also known as 1,7-heptanedicarboxylic acid is a naturally occurring saturated C9-dicarboxylic acid which is best defined as a cosmeceutical ingredient which it shows both cosmetic and pharmaceutical properties (Gao *et al.*, 2008). The performance of azelaic acid exhibits superior performance for the treatment of acne vulgaris, malasma, and rosacea, as well as shows high performance for hair regrowth and skin lightening by means of enzyme inhibition. Azelaic acid is detrimental for particular organism such as *Propionibacterium acnes* (Thiboutot, 2008).

The European Union considers azelaic acid as a cosmetic ingredient that can be applied without concentration restrictions. A variety of products containing 10-20% azelaic acid are commercially available. However, azelaic acid usage is limited in cosmeceutical and pharmaceuticals application because of poor solubility properties, high melting point and requires high amount of dosage. The latter increases the incidence of side effects such as local irritation, stinging, burning, and redness of the skin which associated with exposure to high levels of undissolved dispersed azelaic acid having an inherent low pH (Al-Marabeh *et al.*, 2016).

Derivative of azelaic acid with even better characteristics than the original starting material may be produced through modification of azelaic acid, especially the efficacy of a relatively low dosage of azelaic acid derivatives in the treatment of acne vulgaris and various cutaneous hyperpigmentary disorders. Modification of azelaic acid by attachment through covalent ester linkages and converting at least one of the carboxylic acid groups into ester group has enhanced not only the technical property of azelaic acid, but also the functional performance of the resulting modified azelaic acid (Tamarkin, 2004).

Derivative of azelaic acid can be synthesized through chemical or enzymatic catalysis. The conventional chemical methods usually involve high temperature, corrosive

catalysts, complex and expensive reaction setup and large amounts of raw materials due to the non-selectiveness of the process (Abdul Rahman *et al.*, 2009). In recent years, enzymatic catalyzed reaction has been widely understood (Castillo *et al.*, 2003). There are many benefits in environmental, economical and product performance by using the expanding arsenal of enzyme in non-traditional ways in synthesis. This will be a direct outcome of milder reaction conditions, increased reaction efficiencies, process that requires less discrete step and the avoidance of toxic and corrosive catalysts (Castro *et al.*, 2001).

As a matter of fact, efficacy of cosmeceutical product largely depends on the bioavailability of bioactive compounds used in formulations. Prausnitz and Langer, (2008) reported that esterification of azelaic acid imparts new pharmacological properties, including increased lipophilicity. In order to improve the bioavailability on human skin, increase in penetration of bioactive compounds into human skin is undoubted necessary (Prausnitz and Langer, 2008). Manufacturers of cosmetic products have recently shown increasing preference for multifunctional products in which different active agent can be incorporated to improve bioavailability and decrease side effect or toxicity due to lower active agent content (Golubovic-Liakopoulos *et al.*, 2011).

A new generation hybrid of nanotechnology and emulsion technology is able to manipulate the delivery of bioactive compounds in achieving the best outcome of the treatment. Nanoemulsions are able to ensure these terms. Nanoemulsions refer to nano-sized emulsions with particle sizes ranging from 20 to 200 nm (Solans *et al.*, 2005). Nanoemulsion have become attractive for application in pharmaceutical and cosmeceutical industry specifically for topical application, has been proven to be better compared to other delivery carriers, such as suspension and gels due to several excellent characteristics possessed by this type of delivery system (Kumar *et al.*, 2011b). These characteristics include high kinetic stability, skin delivery efficiency of active substance or drug besides having excellent sensorial and aesthetic aspects (Rocha-Filho *et al.*, 2014).

The permeability of a substance through the skin is inversely related to its size under certain conditions. Nanoemulsion appeared to be one of the attractive and competitive delivery system in order to permeate through the skin (Kong *et al.*, 2011) because of their nanoscale size. Various bioactive compounds incorporated in nanoemulsions for application in medical research for inflammatory disorder (Peng *et al.*, 2010), have shown enhanced penetration of drugs. Nanoemulsions have also been developed for water insoluble active ingredients such as kojic acid dipalmitate (Al-Edresi and Baie, 2010) as cosmetic delivery system. Eventually, this comes down to the competency of the bioactive compounds and their purported biological activity.

1.2 Research Problems

As in literature, azelaic acid proved to be very beneficial in cosmeceutical skin care. It is an anti-inflammatory, antioxidant, anti-keratinising and bacteriostatic agent, thus it is a very good option for the treatment of acne (Draelos and Kayne, 2008). However, azelaic acid usage is limited in cosmetics and pharmaceuticals because of poor solubility properties, high melting point and requires high amount of dosage. Currently, azelaic acid is available in the market as a 20% azelaic acid cream for the treatment of acne vulgaris (AV), and has been approved for use by the Food and Drug Administration (FDA). High concentration (20%) of azelaic acid is needed to guarantee the drug availability in the skin. The latter increases the incidence of side effects such as local irritation, dryness, scaling, pruritis, erythema, burning, and rarely, hypo-pigmentation of the skin associated with exposure to high levels of undissolved dispersed azelaic acid having an inherent low pH (Al-Marabeh *et al.*, 2016). The side-effects that occurred most, were mild transient erythema and cutaneous irritation. The incorporation of azelaic acid into product formulations has been difficult due to its insolubility, high melting point (103°C) and its crystallinity.

Emulsion stability is a primary concern for formulation design. In nanoemulsion development system, the utmost challenge is to maintain the particle size in the nanometer range while remain physically stable for a period of time. Therefore, the composition of formulation developed is very important in order to obtain stable nanoemulsions with characteristics that are suitable for topical use. This process has been time consuming as the factor affecting interaction between each component had to be individually determined. Thus, well designed data collection process would allow the experiments to achieve high success rates.

1.3 Research Objectives

The main objective of this research was to synthesize dilaurylazelate ester by using Novozym 435 as a biocatalyst and develop a nanoemulsion formulation containing dilaurylazelate ester for nanocosmeceutical application. Therefore, the following specific objectives were pursued:

1) To synthesize dilaurylazelate ester using Novozym 435 and optimize it by using Response Surface Methodology (RSM) and Artificial Neural Network (ANN).
2) To characterize the physico-chemical and biological activities of dilaurylazelate ester.
3) To develop a nanoemulsion formulation containing dilaurylazelate ester as an active component and optimize it by D-optimal Mixture Design (MED) for nanocosmeceutical application.
4) To characterize the physico-chemical properties of the nanoemulsions systems and evaluate the stability of the formulations.
5) To study the safety and efficacy of nanoemulsion formulation containing dilaurylazelate ester.

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