UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF NANOEMULSION CONTAINING KOJIC ACID ESTER FOR COSMECEUTICAL APPLICATION

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FS 2019 6

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

November 2018

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Kojic acid (KA) and its derivatives such as kojic acid ester (KAE) are well-known tyrosinase inhibitor that widely used in food and cosmetic industries. The anti-tyrosinase properties of these compounds known to be effective in the treatment of overproduction of melanin such as hyperpigmentation for cosmeceutical applications. Kojic acid ester was used as the active ingredient and encapsulated in the nanoemulsion system. To design excellent formulation, the nanoemulsion containing kojic acid ester (KAE) was screened with different level of variables by using D-optimal experimental mixture design (MED), solubility determination in various oils and finally the preparation system was further developed. D-Optimal Mixture Experimental Design (MED) was used for optimizing the composition of nanoemulsions suitable for topical delivery system. The optimized nanoemulsion containing kojic acid ester with desirable criteria was 10.00 % w/w of KAE, 3.19 % w/w of T80, 3.74 % w/w of castor oil: lemon essential oil (ratio 9: 1), 0.70 % w/w of xanthan gum and 81.68 % w/w of deionized water.

This optimum nanoemulsion containing KAE showed suitable agreement between the actual droplet size (110.01 nm) and the predicted droplet size (111.73 nm). The residual standard error (RSE) value of nanoemulsion containing KAE was less than 2.0%. The optimized nanoemulsion containing KAE with pH value of 6.28 showed high conductivity value (1492.00 $\mu Scm^{-}1$) indicated that oil-in-water nanoemulsion was obtained. The nanoemulsion remained stable (no phase separation was observed) under accelerated stability during storage at 4°C, 25°C and 45°C within 90 days, centrifugal force as well as freeze-thaw cycles. Rheology measurement justified that the optimized nanoemulsion containing KAE was more elastic (shear thinning and

pseudoplastic properties) rather than viscous characteristics. The permeation study showed that the permeability of KAE was significantly improved and the release increased from 4.94% at 1 h to 59.64% at 8 h of application. The permeation rate of nanoemulsion containing KAE at 8 h was 4659.50 $\mu g.cm^{-2}. h^{-1}$ (initial concentration, C_o = 2000 µg/mL) with permeability coefficient (K_p) value of 0.48 $cm.h^{-1}$. Antimicrobial activity of nanoemulsion containing KAE was studied against the skin pathogen bacteria called *Staphylococcus aureus* ATCC 43300. The results indicated that the inhibition zone size of the optimized nanoemulsion containing KAE (8.00 mm) was slightly bigger than KAE oil (6.5 mm).

In vitro cytotoxicity of the optimized nanoemulsion containing KAE and KAE were tested using fibroblast cell line (3T3). The IC_{50} (50% inhibition of cell viability) of nanoemulsion containing KAE was more than 100 µg/mL. The survival rate of 3T3 cell on nanoemulsion containing KAE (54.76 %) was found to be higher compared to KAE (53.37 %) without any toxicity sign. The in vivo toxicity effect on zebrafish embryos (*Danio rerio*) was also investigated. The calculated LC_{50} (50% lethal concentration) values of nanoemulsion containing KAE showed no toxicity effect with more than 500 µg/mL. Nanoemulsion containing KAE proved to be less toxic and can be applied for cosmeceutical applications. This study has revealed that kojic acid ester could be developed as a new active ingredient with nanoemulsion based system and have a potential to be used for further cosmeceutical applications.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PEMBANGUNAN BAGI NANOEMULSI YANG MENGANDUNGI ESTER ASID KOJIC UNTUK KEGUNAAN KOSMESEUTIKAL

Oleh

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Asid kojik dan terbitannya seperti ester asid kojik terkenal dengan sifat perencat tirosina yang berkembang luas dalam penggunaan industri pemakanan dan kosmetik. Sifat anti-tirosina sebatian- sebatian tersebut diketahui dengan keberkesanannya dalam rawatan lebihan pengeluaran melanin seperti hiperpigmentasi untuk aplikasi kosmeseutikal. Ester asid kojik telah direkabentuk untuk dijadikan ramuan aktif dalam system nanoemulsi. Bagi merekabentuk formulasi yang unggul, pertama, nanoemulsi yang mengandungi ester asid kojik telah diimbas dengan pelbagai saringan julat pembolehubah-pembolehubah, ujikaji tahap kelarutan ester asid kojik dalam minyak berlainan dan akhirnya penyediaan sistem nanoemulsi. Ujian kestabilan di bawah daya empar menunjukkan minyak kastor serta pati minyak lemon merupakan kombinasi terbaik yang sesuai digunakan sebagai campuran minyak dalam kegunaan formulasi sistem nanoemulsi.

'D-Optimal Mixture Experimental Design' telah digunapakai sebagai asas untuk pengoptimuman komposisi nanoemulsi sesuai untuk aplikasi topikal. Komposisi optimum nanoemulsi yang mengandungi ester asid kojik yang telah dicadangkan dengan kriteria yang sesuai adalah 10.0% w/w bagi ester asid kojik , 3.19% w/w bagi T80, 3.74% w/w bagi minyak kastor: pati minyak lemon (nisbah 9: 1), 0.70% w/w bagi xanthan gum dan 81.68% w/w bagi air. Nanoemulsi optimum yang mngandungi ester asid kojik telah menunjukkan persetujuan yang bagus terhadap nilai sebenar saiz partikel (110.01 nm) dan nilai jangkaan saiz partikel (111.73 nm) dengan 'residual standard error' (RSE) kurang dari 2.0 %. Nanoemulsi optimum yang mengandungi ester asid kojik dengan nilai pH (6.28) menunjukkan nilai konduktiviti yang tinggi (1492.00 μScm^{-1}) dan dapat membuktikan bahawa nanoemulsi ini adalah minyak dalam air. Nanoemulsi kekal stabil di bawah ujian kestabilan apabila disimpan pada suhu 4 °C, 25 °C

dan 45 °C selama 90 hari, di bawah ujian sentrifugasi dan kitaran beku. Ukuran reologi telah mempamerkan bahawa nanoemulsi yang mengandungi ester asid kojik tersebut mempunyai ciri elastik (sifat ricih penipisan dan pseudoplastik) dan bukannya ciri kekenyalan. Kajian penyerapan mendedahkan bahawa kebolehtelapan ester asid kojik meningkat dengan ketara dan pembebasan meningkat dari 4.94% bagi 1 jam pertama kepada 59.64% selepas 8 jam. Kadar penyerapan nanoemulsi yang mengandungi ester asid kojik selepas 8 jam adalah 4659.50 $\mu g.cm^{-2}.h^{-1}$ (kepekatan permulaan Co = 2000 µg/mL) dengan nilai pekali kebolehtelapan (K_p) adalah 0.48 $cm.h^{-1}$. Aktiviti antimikrob bagi nanoemulsi yang mengandungi ester asid kojik dikaji terhadap bakteria kulit seperti *Staphylococcus aureus* ATCC 43300. Keputusan menunjukkan zon saiz perencat mikrob bagi optimum nanoemulsi yang mengandungi ester asid kojik adalah lebih besar dari ester asid kojik.

Ujian kesitotoksikan bagi nanoemulsi ester asid kojik yang optimum dan ester asid kojik telah diuji menggunakan sel fibroblast (3T3). Keputusan menunjukkan bahawa nilai IC_{50} bagi nanoemulsi ester asid kojik adalah lebih dari 100 µg/mL.Tahap kehidupan sel 3T3 keatas formulasi ester asid kojik (54.76%) adalah lebih tinggi berbanding minyak ester asid kojik (53.37%) membuktikan tiada penujuk ketotoksikan. Dengan ini, membuktikan bahawa formulasi ester asid kojik adalah tidak toksik dan boleh diaplikasikan untuk kegunaan kosmeseutikal. Kajian *in vivo* ketoksikan keatas embrio zebrafish (*Danio rerio*) telah dijalankan. Keputusan nilai LC_{50} bagi nanoemulsi ester asid kojik menunjukkan kesan ketoksikan adalah lebih 500 µg/mL. Kajian menunjukkan bahawa nanoemulsi ester asid kojik adalah kurang toksik dan boleh diaplikasikan untuk kegunaan kosmeseutikal. Ini membuktikan bahawa ester asid kojic boleh dibangunkan sebagai bahan aktif baru dengan menggunakan sistem nanoemulsi untuk aplikasi kosmeseutikal.

ACKNOWLEDGEMENTS

Alhamdulillah. In the name of Allah the Most Gracious and Most Merciful. All praises and Gratitude are due to Him for giving me strength, health, knowledge and passion in completion of this study and thesis.

Here I would like to take this opportunity to express my deepest gratitude and my sincere appreciation to my beloved supervisor, Dr. Siti Efliza Ashari for her invaluable guidance, constantly encouragement and support as well as endless advice along the study. I am also indebted to all my supportive co-supervisors, Dr. Norazlinaliza Salim and Dr. Syahida Ahmad for their help in giving me useful guidance and suggestions to improve my research quality. My appreciation also goes to all my colleagues in Integrated Chemical Biophysics Centre, UPM members especially Dr. Nur Shafira, Wan Sarah, Nadiatul Atiqah, Fazrieyana, Fatin Izzati and lastly my dear friends from ITMA, UPM, Isshadiba Faikah and Alif Syafiq. Thank you for making my master's life lively with unforgettable sweet memories.

Last but not least, I would like to express my love and thanks to my beloved parents, Syed Azhar Syed Sulaiman and Maziah Yunus for their support, help, duas and encouragement throughout my journey. Not to forget to my beloved sisters, brother and in laws, I would like to express my deepest affection for their never ending love and support. Finally, I would like to express my appreciation to all those unnamed who have contributed directly or indirectly during my research.

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

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LIST OF ABBREVIATIONS

CO	Castor Oil
DMSO	Dimethyl Sulfoxide
FDA	Food and Drug Administration
G'	Storage Modulus
G''	Loss Modulus
HLB	Hydrophilic Lipophilic Balance
Hpf	Hour Post Fertilization
IC_{50}	Inhibition Concentration of 50%
K	Consistency Index
KA	Kojic Acid
KAE	Kojic Acid Ester
L	Litre
LC_{50}	Concentration that cause 50% mortality
LO	Lemon Essential Oil
LVR	Linear Viscoelastic Region
MED	Mixture Experimental Design
Mg	Milligram
mg/mL	Miligram Over Mililiter
mL	Milliliter
MTT	3-[4,5-dimethyl1thiazol-2yl]-2,5-diphenyltetrazolium bromide
N	Flow Behavior Index
η	Viscosity
NEs	Nanoemulsions
O/W	Oil-in-Water
PBS	Phosphate Buffer Saline
PDI	Polydispersity Index
PKOE	Palm Kernel Oil Ester
RSE	Residual Standard Error
RSM	Response Surface Methodology
Sbl	Short Body Length
SO	Soybean Oil
SSO	Safflower Seed Oil
T80	Tween 80
TEM	Transmission Electron Microscopy
TTC	Threshold of Toxicological Concern
UV-Vis	Ultraviolet Visible
VCO	Virgin Coconut Oil
W/O	Water-in-Oil
w/w	Weight Over Weight
µL	Microliter

CHAPTER 1

INTRODUCTION

1.1 Background Study

Cosmeceuticals contain biologically active ingredients that have medical or drug like benefits and supply barrier against degenerative skin conditions. Albert M. Kligman in the late 1970s was the man who popularized the word "cosmeceutical" in the medical field. Basically, cosmeceutical products contain cosmetic actives with therapeutic, disease fighting or healing properties, serving as a bridge between personal care products and pharmaceuticals. Similar to cosmetics, cosmeceuticals are topically applied and the biological active ingredients may enhance the skin tone, texture and radiance as well as wrinkle (Mukul *et al*., 2011). The number of melanin produced in the skin affects the skin colour.

Melanin is biosynthesized in the melanosome of melanocyte and requires enzyme tyrosinase throughout the process. This melanin shields the skin from photocarcinogenesis by absorbing UV sunlight and removes reactive oxygen species (Gupta *et al*., 2006, Sapkota *et al*., 2011, Kim and Uyama, 2005). Furthermore, the overproduction of melanin pigmentation would lead to various dermatological disorders including hyperpigmentation skin for example, lentigo, melasma, post inflammatory melanoderma, freckles, ephelide and age spots (Briganti *et al*., 2003). Thus, the concern of tyrosinase inhibition has become increasingly vital for scientists in many branches of life science research especially in the aesthetic problems. A well-known tyrosinase inhibitor compound called kojic acid may hinder the overproduction of melanin content in the skin.

This acid is common among cosmetic and food industries where it can be found in many class of natural and synthetic compounds (Asadzadeh *et al*., 2015). Kojic acid, 5-hydroxy-2- hydroxymethyl-4H-pyran-4-one is an antibiotic produced by many species of *Aspergillus, Acetobacter* and *Penicillium* in an aerobic process utilizing a wide range of carbon sources (Uher *et al*., 2008). Kojic acid is commonly used as an active ingredient in cosmetic creams for its skin lightening effect by blocking the pigment on the skin (Masse *et al*., 2001). For the reason of its slow and effective reversible competitive inhibition of human malanocyte tyrosinase, kojic acid blocks the melanin production. Therefore, this compound is plays an essential role in the formation of cellular melanin (Kang *et al*., 2009, Raku and Tokiwa, 2003).

Today, cosmetics industries are likely to products such as skin protective lotion to prevent the over exposure of sunlight which then may cause skin cancer if not prevented. It is normally used in the combination with *alpha*-hydroxy acid in the formulation of skin whiteners to control lightened freckles and age spots (Emami *et al*., 2007). Besides that, since hydroquinone has been banned for cosmetic usage in Asia, kojic acid is has been used as an antioxidant and alternative for skin lightening agent by many cosmetic industries (Gupta *et al*., 2006). However, kojic acid itself is unstable towards sun or air and perform less effectiveness as a skin care product. Studies show that the derivatives of kojic acid were more stable at least 15 times more than the source material. This would increase the storage stability, compatibility and oil-solubility of the derivatives thus, more desirable for cosmetics applications (Kim *et al*., 2004).

Recently, methods for the synthesis of various kojic acid derivatives such as kojic acid ester, kojic acid laureate and kojic acid palmitate have been claimed in many research (Ashari *et al*., 2009). These derivatives have been found to improve both the stability and solubility of kojic acid in oily cosmetic products (Mohamad *et al*., 2010). Research also reported that kojic acid esters were safe and nontoxic depigmenting agents as determined in *B16F1* melanoma cells and showed excellent inhibitory results on tyrosinase activity. Therefore, these compounds have potential to be used in the cosmetic formulation (Lajis *et al*., 2012). Besides, kojic acid derivatives have higher tyrosinase inhibitory activities than that of kojic acid (Kim *et al*., 2004). Further evidence by Yuling Li *et al*., (2013) reported that kojic acid derivatives are main class of some natural and synthetic compounds that own high activity profile since they have many biological activities.

Applying the new generation of nanotechnology in the development of cosmeceuticals offers numerous advantages for targeting the active therapeutic component to the desired site to achieve greater skin retention, improvement in the stability of cosmetic ingredients and sustained release of active drug for a long-lasting effect. Some of the nanotechnology-based novel carriers of cosmetics include nanoemulsion, nanocapsule, liposome, niosome, nanocrystal, solid lipid nanoparticle, carbon nanotube, fullerene and dendrimers (Duarah *et al*., 2016). Nanoemulsions (NEs) are considered to be the most advanced nanoparticulate system for cosmetics due to its nanosize range varied from 20 to 200 nm.

Besides, NEs perform as a delivery system in transporting various functional lipophilic compounds in nutraceuticals, drugs, antioxidants, flavors and antimicrobial agents and therefore enhance the bioavailability of the product. These NEs are highly stable towards particle aggregation and gravitational separations shows the improved and efficient activity especially in antimicrobial activity. NEs are a thermodynamically stable system of two phases consisting of at least two immiscible liquids with droplet size in nano range (Dasguptaa *et al*., 2015).

The main advantage of NEs over micro- and macro- counterparts is their high surface area allowing effective transport properties (the smaller the size of the emulsion, the higher the stability and better capability to carry active ingredients). In addition, they do not have inherent creaming like macroemulsions making longer shelf life of the products. In terms of delivering lipophilic compounds, NEs are superior to liposomes due to their lipophilic interior (Sharma *et al*., 2012). Factor affecting interaction between each component of the nanoemulsion has been individually determined thus, time consuming, and the approach of well-designed data collection process would benefits the experiments to achieve high desirable results is considerable in formulation process. The conventional method, focusing on one factor at a time is used where one of the parameter is varied while other parameters are kept constant in order to determine the response.

However, this approach seems to be unreliable and inefficient in terms of time, energy and cost for the determination of optimum formulation and conditions (Gonzalez-Diaz *et al*., 2014, Cafaggi *et al*., 2003). Therefore, optimization using multivariate statistical approach such as mixture experimental design (MED) helps in maximize the amount of acquired information and minimize the number of experiments to be carried out. Besides, the design allow characterization and identification of synergistic and antagonistic interaction effects between different components in the mixture (Ngan *et al*., 2014, Kamairudin *et al*. 2014, Woo *et al*., 2015). D-optimal design has been widely utilized for product formulation in food, pharmaceutical and cosmeceutical industries (Borhan *et al*., 2014). Safety and efficacy of products are another part of assessment that should be consider before providing to consumers. The demand of using natural plant-based in products have raised significantly with new safety issues that need novel approaches for their safety evaluation.

To date, the use of promising tool called Threshold of Toxicological Concern (TTC) able to qualitatively assess the safety of substances present at trace levels as well as minor ingredients of plant-derived substances. Recent finding claims that because of the absence of metabolism in cadaver skin or misclassification of skin residues that, *in vivo*, remain in the stratum corneum or hair follicle openings, the *in vitro* test may overestimate human systemic exposure to products ingredients (Nohynerk *et al*., 2010). Hence, critical and broad test should be taken account for today's safety and efficacy assessment of products and their ingredients not only based on science but on their regulatory status and issues like ethics of animal testing.

1.2 Problems Statement

The development of kojic acid ester as the active ingredient in nanoemulsion system for cosmeceutical application is the subject of considerable research. Kojic acid ester act as tyrosinase inhibitor which can be the alternative for hyperpigmentation treatment with natural based ingredient and non-toxic compare to other harmful tyrosinase inhibitors such as hydroquinone and mercury. Besides, kojic acid ester is more stable, safer and more desirable for cosmeceutical application (Kim *et al*., 2004). However, this kojic acid ester is not very suitable to be applied directly to the skin due to its greasy feeling and its larger droplet size to penetrate deep into skin. Therefore, formulation containing the biological active ingredient must be stable and suitable for consumers use.

The challenging part of topical delivery is to overcome the strong barrier function of the skin to deliver the active ingredient to the target site with sufficient concentration. Hence, using the new generation of nanotechnology system, nanoemulsion system is chosen to encapsulated the active ingredient because the submicron sized of emulsions will give effective delivery for skin absorption with nanosize range between 20-200 nm and the stability of the ingredients can be well maintained (Dasguptaa *et al*., 2015). Nevertheless, nanoemulsion are thermodynamically unstable and may have physical instabilities such as aggregation, flocculation, coalescence and Ostwlad ripening. Therefore, there may be some challenges in maintaining the required droplet size and stability of the nanoemulsion.

In addition, factor affecting interaction between each component of the nanoemulsion has been individually determined thus, time consuming, and the approach of well-designed data collection process would benefits the experiments to achieve high desirable results. To date, there are no report on optimization of kojic acid ester nanoemulsion using statistical method approach of D-optimal mixture experimental design. This approach is suitable to be used in cosmeceutical formulation where it enable to predict more accurate value to the actual response plus the number of experimental run can be reduce.

1.3 Research Objectives

The purpose of this research was to design and develop of nanoemulsion containing kojic acid ester. Therefore, the following specific objectives were pursued:

1) To optimize nanoemulsion containing kojic acid ester using statistical approach of D-optimal mixture experimental design.

2) To characterize the physicochemical properties of the developed nanoemulsion containing kojic acid ester and evaluate the stability properties.

3) To study the efficacy and safety of nanoemulsion containing kojic acid ester for further cosmeceutical application.

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LIST OF PUBLICATIONS

Research Papers

Fatin Amirah Ahmad Norddin, **Sharifah Nurfadhlin Afifah Syed Azhar**, and Siti Efliza Ashari. Evaluation of Direct Esterification of Fatty Acid Derivative of Kojic Acid in Co-solvent System: A Statistical Approach. (Published: Journal of Chemical Engineering and Process Technology, 2017).

Sharifah Nurfadhlin Afifah Syed Azhar, Siti Efliza Ashari and Norazlinaliza Salim. Development of a kojic monooleate-enriched oil-in-water nanoemulsion as a potential carrier for hyperpigmentation treatment (Published: International Journal of Nanomedicine, 2018).

Sharifah Nurfadhlin Afifah Syed Azhar, Siti Efliza Ashari, Syahida Ahmad and Norazlinaliza Salim. Nanotoxicity Studies using Zebrafish Embryo Assay on Nanoemulsion containing Kojic Monooleate (Submitted: Journal of Toxicity and Pharmacology, 2019)

Conferences and Exhibitions

Siti Efliza Ashari,Khairul Azhar Jumbri, Mohd Fakhruddin Al-Hanif Rozy, Rosfarizan Mohamad, Mahiran Basri, Hamid Reza Fard Masoumi and **Sharifah Nurfadhlin Afifah Syed Azhar.** Optimisation and Characterisation of Lipase-catalysed Synthesis of a Plam-based Kojic Acid Monooleate in a solvent-free system by RSM. Exhibition of Invention, Research and Innovation (PRPI) 2016, UPM Serdang, Malaysia, 15-16 November 2016. (Gold medal)

Siti Efliza Ashari, **Sharifah Nurfadhlin Afifah Syed Azhar**, Rosfarizan Mohamad and Norazlinaliza Salim. Anti-tyrosinase riche Nanocosmeceutical Formulation from Palm-based Kojic Acid Ester. Persidangan dan Ekspo Ciptaan Institusi Pengajian Tinggi Antarabangsa 2017 (PECIPTA). 7 -9 Oktober 2017 (Bronze medal)

Sharifah Nurfadhlin Afifah Syed Azhar, Siti Efliza Ashari and Norazlinaliza Salim. An approach optimisation of nanocosmeceutical formulation containing kojic acid ester using D-optimal mixture design. Fundamental Science Centre (FSC) 2017.21-22 November 2017. (Poster presentation)

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