UNIVERSITI PUTRA MALAYSIA

SYNTHESIS OF VIRGIN COCONUT OIL ESTERS AND FORMATION OF NANOEMULSIONS CONTAINING COPPER PEPTIDE FOR COSMECEUTICAL USE

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By

SHAZWANI BINTI SAMSON

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

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Chairperson: Professor Mahiran Basri, PhD
Faculty : Science

Virgin coconut oil esters (VCOE) were synthesized enzymatically through alcoholysis reaction of virgin coconut oil (VCO) and oleyl alcohol. The properties of the esters showed their suitability to be used as cosmetic's ingredient and exhibited non-irritant effect with a Human Irritancy Equivalent (HIE) score below 0.9. Factors (enzyme amount, reaction time, substrate molar ratio) that influenced the percentage yield (%) of esters were investigated using artificial neural network (ANN) and the optimum percentage yield obtained was 96.05%. Pseudo-ternary phase diagrams for VCO/surfactant/water and VCOE/surfactant/water systems were constructed to serve as platform in building the emulsion systems. Surfactants used were mixture of Tween 80 (T80) and Pluronic F68 (PF68) at ratio of 40:1. Based on the results, multiphase regions dominated the two pseudo-ternary phase diagrams. Therefore, several compositions with low surfactants contents from the multiphase regions of the pseudo-ternary phase diagrams were selected for modification, by incorporating xanthan gum as stabilizer into the system. Xanthan gum was found to be able to stabilize the emulsions systems where smaller particle size was obtained at high concentration of surfactant and low concentration of oil and xanthan gum.

The effect of composition on nanoemulsion; VCO, T80:PF68 and xanthan gum on variation of particle size was investigated using D-optimal Mixture Design and ANN. The particle size of nanoemulsions estimated by D-optimal was 120.88 nm while ANN was 124.16 nm. The most suitable method for optimizing the nanoemulsions systems was found out to be ANN due to the lower percentage of residual standard error (RSE). The optimum compositions obtained for VCO nanoemulsion were VCO (10.00%, w/w), T80:PF68 (15.00%, w/w), xanthan gum (0.87%, w/w) and water (74.13%, w/w). The same composition was used for the preparation of VCOE nanoemulsions since there was no significant difference observed in term of particle size. Incorporation of copper peptide in the optimum formulations also showed insignificant effect on the particle size.

Physicochemical characterization and stability evaluation were conducted for the optimum VCO and VCOE nanoemulsions without active (Opt-VCO and Opt-VCOE, respectively) and with the addition of active (VCOCP and VCOECP, respectively). Final formulations were found out to be in 'nano' sized with good stability and were proven to be the oil-in-water type of nanoemulsions with pH suitable for human skin. The micrographs obtained using Transmission Electron Microscopy (TEM) showed that the particle size was in agreement with the measured size. The final formulations exhibited pseudoplastic properties, shear thinning behavior and elastic behavior where wide linear viscoelastic region was observed which correlated well to the high rigidity of the system. In the stability study, the final nanoemulsions showed excellent physical stability against centrifugation test, freeze-thaw cycle test and storage at temperature 25 °C and 45 °C for 90 days. *In-vitro* permeation study indicated the ability of the nanoemulsions to improve the permeability of copper peptide as compared to control. The percentage amount of copper peptide that permeates through skin barrier was observed to be higher for VCOE nanoemulsion as compared to VCO nanoemulsion. In the cytotoxicity test, nanoemulsions showed no cytotoxicity effect on fibroblast cells (3T3) with IC_{50} value higher that 1000µg/ml.

The short-term moisturizing effect on 10 subjects showed increment in skin hydration and reduction of TEWL after application of formulations Opt-VCO and Opt-VCOE. Opt-VCOE exhibited higher skin hydration value after application of the cream to subject's skin. Due to the higher moisturizing capacity of Opt-VCOE as compared to Opt-VCO, VCOE-based nanoemulsion containing copper peptide (VCOECP) was selected for the anti-aging efficacy study. In the irritancy test, VCOECP was found out to be non-irritant with HIE scores lower than 0.9. Anti-aging efficacy measurements showed increment in skin hydration and reduction of the transepidermal water loss (TEWL) after application of VCOECP and placebo. Higher skin hydration value and lower TEWL value was observed for VCOECP as compared to placebo. The mean collagen score was increased significantly from 24.92 ± 4.32 to 41.67 ± 5.57 after application of copper peptide nanoemulsion as compared to at day 0. Placebo did not show any possible therapeutic effect on collagen since there was no significant increment in collagen score from day 0 to day 28. No visible skin reactions were experienced by the subjects during treatment. This work concluded that stable VCO and VCOE nanoemulsions having moisturizing properties and fit for cosmetic use were successfully developed. Copper peptide loaded nanoemulsion showed collagen regeneration in human skin and thus exhibited potential use in the cosmeceutical industry.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

SINTESIS ESTER MINYAK KELAPA DARA DAN PEMBENTUKAN NANOEMULSI MENGANDUNGI KUPRUM PEPTIDA UNTUK KEGUNAAN KOSMESEUTIKAL

Oleh

SHAZWANI BINTI SAMSON

Januari 2017

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Ester minyak kelapa dara (VCOE) telah disintesis secara enzimatik melalui tindak balas alkoholisis minyak kelapa dara (VCO) dan oleyl alkohol. Sifat-sifat ester menunjukkan kesesuaiannya untuk digunakan sebagai bahan kosmetik dan menunjukkan kesan tidak merengsa dengan skor '*Human Irritancy Equivalent*' (HIE) di bawah 0.9. Faktor-faktor (jumlah enzim, masa tindak balas, nisbah molar substrat) yang mempengaruhi hasil peratusan (%) ester telah dikaji dengan menggunakan Rangkaian Neural Tiruan (ANN) dan hasil peratusan optimum yang diperolehi adalah 96.05%. Gambar rajah fasa pseudo-pertigaan bagi sistem VCO/surfaktan /air dan sistem VCOE/surfaktan/air telah dibina sebagai platfom dalam pembinaan sistem emulsi. Surfaktan yang digunakan adalah campuran Tween 80 (T80) dan Pluronic F68 (PF68) pada nisbah 40:1. Berdasarkan hasil keputusan, kawasan multifasa mendominasi kedua-dua gambar rajah fasa pseudo-pertigaan. Oleh itu, beberapa komposisi dengan kandungan surfaktan yang rendah daripada kawasan multifasa pada gambar rajah fasa pseudo-pertigaan telah dipilih untuk pengubahsuaian, dengan menambahkan gam xantan sebagai penstabil ke dalam sistem. Gam xantan didapati berupaya untuk menstabilkan sistem emulsi di mana saiz zarah yang lebih kecil telah diperolehi pada kepekatan surfaktan yang tinggi dan kepekatan minyak dan gam xantan yang rendah.

Kesan komposisi terhadap nanoemulsi; VCO, T80:PF68 dan gam xantan atas perubahan saiz zarah dikaji menggunakan '*D-Optimal Mixture Design*' dan ANN. Saiz zarah nanoemulsi yang dianggarkan oleh D-Optimal adalah 120.88 nm manakala ANN adalah 124.16 nm. Kaedah yang paling sesuai untuk mengoptimumkan sistem nanoemulsi adalah ANN disebabkan peratusan piawai baki (RSE) yang lebih rendah. Komposisi optimum yang diperolehi bagi nanoemulsi VCO adalah VCO (10.00%, w/w), T80: PF68 (15.00%, w/w), gam xantan (0.87%, w/w) dan air (74.13%, w/w). Komposisi yang sama telah digunakan untuk penyediaan nanoemulsi VCOE kerana tidak ada perbezaan yang ketara diperhatikan dari segi saiz zarah. Penambahan kuprum

peptida dalam komposisi optimum juga menunjukkan kesan yang tidak ketara pada saiz zarah.

Pencirian fizikokimia dan penilaian kestabilan telah dijalankan untuk formulasi nanoemulsi VCO dan VCOE optimum tanpa aktif (Opt-VCO dan Opt-VCOE) dan dengan penambahan aktif (VCOCP dan VCOECP). Formulasi akhir didapati bersaiz 'nano' dengan kestabilan yang baik dan dibuktikan sebagai jenis nanoemulsi minyak-dalam-air dengan pH yang sesuai untuk kulit manusia. Mikrograf yang diperolehi menggunakan mikroskop elektron transmisi (TEM) menunjukkan bahawa saiz zarah adalah bersesuaian dengan saiz yang diukur. Formulasi akhir mempamerkan ciri-ciri pseudokenyal, sifat penipisan ricih dan ciri-ciri elastik di mana rantau lurus viskoelastik yang luas telah diperhatikan yang berkait rapat dengan ketegaran sistem yang tinggi. Dalam kajian kestabilan, nanoemulsi akhir menunjukkan kestabilan fizikal yang sangat baik terhadap ujian daya empar, ujian beku-cair dan penyimpanan pada suhu 25 ° C dan 45 ° C selama 90 hari. Kajian penyerapan *in-vitro* menunjukkan keupayaan nanoemulsi untuk meningkatkan kebolehtelapan kuprum peptida berbanding dengan kawalan. Jumlah peratusan kuprum peptida yang meresap melalui penghadang kulit diperhatikan lebih tinggi untuk nanoemulsi VCOE berbanding nanoemulsi VCO. Dalam ujian sitotoksiti, nanoemulsi tidak menunjukkan kesan sitotoksik terhadap sel fibroblast (3T3) dengan nilai IC_{50} yang lebih tinggi daripada 1000μg / ml.

Kesan kelembapan jangka pendek ke atas 10 subjek menunjukkan peningkatan dalam penghidratan kulit dan pengurangan kehilangan air transepidermal (TEWL) selepas penggunaan formulasi Opt-VCO dan Opt-VCOE. Opt-VCOE mempamerkan nilai penghidratan kulit yang lebih tinggi selepas penggunaan krim ke atas kulit subjek. Disebabkan kapasiti pelembapan Opt-VCOE yang lebih tinggi berbanding Opt-VCO, nanoemulsi berasaskan VCOE yang mengandugi kuprum peptida (VCOECP) telah dipilih untuk kajian keberkesanan anti-penuaan. Dalam ujian kerengsaan, VCOECP telah didapati tidak merengsa dengan skor HIE lebih rendah daripada 0.9. Ukuran keberkesanan anti-penuaan menunjukkan peningkatan dalam penghidratan kulit dan pengurangan kehilangan air transepidermal selepas penggunaan VCOECP dan plasebo. Nilai penghidratan kulit yang tinggi dan nilai TEWL lebih rendah diperhatikan untuk VCOECP berbanding dengan plasebo. Skor purata kolagen telah meningkat dengan ketara daripada 24.92 ± 4.32 kepada 41.67 ± 5.57 selepas penggunaan nanoemulsi kuprum peptida berbanding pada hari 0. Plasebo tidak menunjukkan apa-apa kesan mungkin terapeutik terhadap kolagen kerana tidak ada kenaikan ketara dalam skor kolagen dari hari 0 ke hari 28. Sepanjang tempoh rawatan, tidak ada kesan tindak balas pada kulit yang boleh diperhatikan telah dialami oleh subjek. Kajian ini merumuskan bahawa nanoemulsi VCO dan VCOE stabil yang mempunyai ciri-ciri pelembapan dan sesuai untuk kosmetik telah berjaya dibangunkan. Nanoemulsi mengandungi kuprum peptida menunjukkan pertumbuhan semula kolagen dalam kulit manusia dan sekaligus mempunyai potensi penggunaan dalam industri kosmeseutikal.

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I certify that a Thesis Examination Committee has met on (11st January 2017) to conduct the final examination of Shazwani binti Samson on her thesis entitled "Synthesis of Virgin Coconut Oil Esters and Formation of Nanoemulsions Containing Copper Peptide for Cosmeceutical Use" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

ANN	Artificial Neural Network
ANOVA	Analysis of variance
BBP	Batch backpropagation
DAG	Diacylglycerol
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FFA	Free fatty acid
GA	Genetic algorithm
GC	Gas Chromatography
GC-MS	Gas chromatograph-mass spectrometry
GHK	Tripeptide glycyl-L-histidyl-L-lysine
GHK-Cu	Copper-Tripeptide glycyl-L-histidyl-L-lysine
HIE	Human Irritancy Equivalent
HLB	Hydrophilic-lipophilic balance
IBP	Incremental backpropagation
KGF	Keratinocyte growth factor
KKTS	Lysine-theronine-theronine-lysine-serine
LM	Levenberg-Marquardt
LVR	Linear viscoelastic region
MAG	Monoacylglycerol
MSE	Mean squared error
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
O/W	Oil-in-water

PDGF	Platelet-derived growth factor
PF68	Pluronic F68
QP	Quick propagation
RMSE	Root mean squared error
ROS	Reactive oxygen species
RSE	Residual standard error
RSM	Response surface methodology
T80	Tween 80
TAG	Triacylglycerol
TEWL	Transepidermal water loss
TEM	Transmission electron microscopy
TGF	Transforming growth factor
TLC	Thin layer chromatography
UV	Ultraviolet
UVB	Ultraviolet-B
VCO	Virgin coconut oil
VCOE	Virgin coconut oil esters
W/O	Water-in-oil
w/w	Weight per weight
XG	Xanthan gum

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Virgin coconut oil (VCO) is obtained from the fresh and mature kernel of the coconut (*Cocos nucifera* L.) through mechanical and natural means (Dayrit *et al.*, 2011), either with the use of heat or otherwise, provided that it does not lead to alteration or transformation of the oil. It is saturated fat made of medium chain fatty acids, rich in lauric acid content. In terms of cosmetic usage, virgin coconut oil has been reported to have skin moisturizing properties and was found to be useful for skin applications (Mansor *et al.*, 2012) with additional antibacterial and antioxidant properties. The fatty acid composition of VCO is similar to palm kernel oil and the major fatty acid is lauric acid. Vegetable oil esters were reported to be able to increase skin hydration with their moisturizing properties (Keng *et al.*, 2008). VCO esters obtained by enzymatic alcoholysis of triglycerides and oleyl alcohol were used in this study for their unique property of exhibiting excellent wetting behaviour without the oily feeling thus were good candidates as moisturizers. Moisturizers are useful in cosmeceuticals application especially for the treatment of dry skin. Moisturizing agents are able to improve skin hydration, prevent water loss and maintain skin's flexibility.

Cosmeceuticals are applied topically like cosmetic; the difference is it contains biologically active ingredients which can influence the biological function of the skin. Skin appearances can be improved by using cosmeceuticals because it helps in delivering the essential nutrients to the skin. Pharmaceutical active compounds are used in formulating cosmeceuticals and the resulting products are demonstrated to achieve multiple cell protective effects for the purpose of rebuilding healthy skin on cellular level. The future of the cosmeceuticals industry lies in the development of new products which can decrease the amount of time and tedium of a consumer's skin care routine but still able to yield appreciable results. Use of nanotechnology and advanced engineering proves to be effective in enabling innovative formulations and product solutions (Golubovic-Liakopoulos *et al.*, 2011).

Hybrid of nanotechnology and emulsion technology is able to influence the delivery of active compounds in achieving the best outcome of the treatments. Nanoemulsion, emulsion with droplet size in the range of 20-200 nm is suitable to be used as delivery system in cosmeceuticals because it is independence of molecular size of the hydrophilic solute, the nature of aqueous phase and independence of the animal skin characteristics such as the stratum corneum thickness and follicle type (Ng *et al.*, 2013). Nanoemulsion has been reported for cosmeceutical applications such as delivery of moisturizing agent (Ribeiro *et al.*, 2015) and anti-wrinkle agents (Leelapornpisid *et al.*, 2014). In addition, nanoemulsion has been successfully used for efficient delivery of hydrophilic active (Tsai *et al.*, 2014).

Collagen is responsible for the firmness and elasticity of the skin where it confers strength and support to human skin. Degradation of collagen bundles in the dermis through intrinsic and extrinsic aging will reduce the elastic properties of skin causing wrinkles formation and saggy skin. Supplying peptide to skin can regulate the production of fibroblast and therefore facilitates the formation of collagen. Cosmeceutical peptides are important in many natural processes involving skin care. Lupo, (2005) stated that the peptide chain is capable of regulating the production of extracellular matrix's' component such as fibroblast. One example of such compound is copper peptide (copper complex of human tripeptide, glycyl-l-histidyl-l-lysine-Cu) which possesses reparative and antioxidant properties (Pickart and Margolina, 2012, 2010).

Copper peptide (GHK-Cu) is one of the compounds having anti-aging activity which can be found in human plasma and saliva. Copper peptide or copper binding tri-peptide (glycyl-L-histidyl-Lysine) possesses both anti-oxidant and rejuvenating properties. As antioxidant, it acts by quenching the toxic by-products produced from fatty acids lipid peroxidation. On the other hand, the rejuvenating property of copper peptide relies on its ability to stimulate collagen synthesis, maintaining epidermal stem cells, restoring viability of damaged skin cells and many others reparative actions (Pickart and Margolina, 2012, 2010).

1.2 Research Problems

Ageing is characterized by accumulation of molecular damage as well as progressive failure of maintenance and repair of skin which leads to several signs of aging such as wrinkles, sagging, pigmented spots and dryness. Dry skin is a common problem among population and is one of the visible signs of skin ageing. If water is loss more rapidly than it is received, the skin will be dehydrated and loses its flexibility. In addition, decrease in the water-holding capacity of stratum corneum causes dryness which will eventually leads to the appearances of wrinkles. It was reported that water is the only material that is able to plasticize the outer dead layers of epidermis giving the skin its softness and smoothness appearances. Therefore, applying moisturizing agents (occlusive or humectant) to skin will helps to overcome such problems.

On the other hand, excess exposure to UV radiation from the sun rays could leads to premature aging (photoaging) while naturally occurring biological aging processes cause the skin to age intrinsically. Both processes can cause damage to the collagen in the dermis and reduce its productions. Supplying copper peptide to the skin could stimulate collagen synthesis, maintains epidermal stem cells and restoring viability of damaged skin cells. However, oral delivery of peptide limits the amount of peptides that can be delivered into skin due to the physiological processes related to active's absorption and stability. In this case, it would be preferable to administer copper peptide topically. In addition, the barrier property of lipophilic stratum corneum is one of the greatest obstacles for delivery of peptides due to their hydrophilic property and low diffusivity in skin (Gorouhi and Maibach, 2009). Therefore, suitable delivery system which can enhance the delivery of copper peptide is required. Nanoemulsions could be used as the delivery vehicle for copper peptide since they have been reported

to be able to enhance the transportation of drugs or actives through skin (Tsai *et al.*, 2014).

Like any other colloidal systems, nanoemulsion is subjected to various types of physical instability. In fact, one of the main problems with nanoemulsions is stablilization of the develop system and maintaining the physical stability for a period of time without affecting much on the particle size. The procedure for producing nanoemulsions comprised of nano-size droplets emulsion is difficult. The right compositions of each component would be crucial to obtain such sophisticated nanoemulsion system. Finding the best formulation by considering the effect of each component individually involve a tedious and time consuming procedure.

1.3 Research Objectives

The specific objectives of this work are:

1. To synthesize virgin coconut oil esters from virgin coconut oil and optimize the esters' preparation.
2. To develop biocompatible nanoemulsions systems incorporated with copper peptide for topical applications.
3. To conduct optimization of the nanoemulsions composition.
4. To characterize the physicochemical, rheological properties, stability and delivery potential of the newly developed nanoemulsions.
5. To compare the moisturizing efficacy of virgin coconut oil-based nanoemulsions and virgin coconut oil esters-based nanoemulsions.
6. To evaluate the safety of copper peptide-loaded nanoemulsions and their biophysical attributes of human skin.

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