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DESIGN AND OPTIMIZATION OF NANOEMULSION FORMULATION CONTAINING Clinacanthus nutans LINDAU LEAF EXTRACT FOR COSMECEUTICAL APPLICATION

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By

INTAN SORAYA BINTI CHE SULAIMAN

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June 2017

Chairman: Professor Mahiran Basri, PhD
Faculty: Science

Plant-based cosmetics have gained more attention compared to chemical-based product in the cosmetic industry due to its safety and efficacy. However, the primary challenges are incorporation of plant based extract in the formulation owing to its hydrophobicity, instability against oxidation and also difficulties in formulating a stable carrier system. Palm kernel oil esters (PKOEs) with great skin-penetrating abilities were selected to be blended with non-ionic surfactants in the formulation process. The structure of this nanoemulsion is defined as dispersions of self-assembled PKOEs and surfactants molecules in water resulting from solubilization of surfactant micelles. A newly developed nanoemulsion system was designed to incorporate of Clinacanthus nutans (C. nutans) leaves extract for transdermal application. C. nutans or also known as Belalai Gajah is a well-known medicinal plant in tropical Asian countries that has recently attracted attention for its therapeutic characteristics. Numerous reports have documented the biological activity of C. nutans, including its anti-inflammatory, anti-viral, antioxidant and anti-cancer properties. Extract of C. nutans have been well explored for their potential as pharmaceutical agents. However, work on its use in cosmeceutical application is unknown.

Formulating nanoemulsion containing C. nutans extract has much promise as an effective delivery system and leads to the potential use of this plant extract in cosmeceutical application. Preparation of C. nutans leaves extract were obtained via sequential extraction and the optimization of the extraction process were carried out using Response Surface Methodology (RSM). The optimal condition suggested by the RSM model were extraction temperature of 60 °C, at 120 min with solvent ratio (water: ethanol) of 90: 10 v/v% which yielded 23.51% of extract. However, the insolubility of this extract in the methanol testing system has limited their accessibility to antioxidant assay. Thus, in this study extracts from sequential extraction with better solubility in testing system were chosen for further used. The properties of the extract showed their suitability to be used...
as source of antioxidants and exhibited non-toxicity against fibroblasts cells (3T3) which established their safe properties.

Design of nanoemulsions system containing *C. nutans* extract involved several steps including screening of the level of variables, determination of the extract solubility and finally preparation of the nanoemulsions system. Under centrifugal force, all mixture of PKOEs: guava seed oil (GSO) in ratios of 9:1, 8:2 and 7:3 containing the extract did not show any precipitation at the bottom of the test tube. The ratio of 9:1 (PKOEs: GSO) was chosen as the suitable amount of oil mixture to be used in the formulation. The effect of composition on nanoemulsions; oil and surfactant on variation of particle size was investigated using Mixture Experimental Design (MED) and Artificial Neural Network (ANN). The nanoemulsion compositions predicted by different optimization methods were different. MED (labelled as CN1) suggested an optimal formulation containing 8.13% surfactant, 5.00% oil, 1.00% xanthan gum, 0.10% bioactive extract, 0.80% preservative, and 84.97% water to produce particles with a size of 97.38 nm. Meanwhile, ANN (labelled as CN2) suggested an optimal formulation containing 10.32% surfactant, 8.00% oil, 1.00% xanthan gum, 0.10% bioactive extract, 0.80% preservative, and 79.78% water to produce particles with a size of 125.40 nm. Although the particle size obtained by MED optimization was smaller than ANN, the residual standard error (RSE) for ANN was lower than MED which were 1.17% and 2.61%, respectively. This suggested that predicted and actual values of ANN had good correlation, implying that in this work ANN approach was an effective quantitative tool to be used in optimizing formulation design.

Physicochemical characterization and stability evaluation were conducted for both formulations. Based on the results, both formulations showed their suitability for transdermal applications. These formulations were found out to be in nano sized with good stability against phase separation. The formulations were also stable under storage temperature of 25 °C and 45 °C for 90 days, freeze thaw cycles and centrifugal force tests. No distinct changes were observed in the particle size of the ANN formulation, indicating that the nanoemulsion prepared was stable at the chosen optimum composition. However, the particle size of the MED formulation was slightly increased in size over the storage period, nevertheless it was still in the nano-sized range (less than 200 nm).

Transmission Electron Microscopy (TEM) images for both formulations showed the spherical shape of the oil droplets in the colloidal system and the encapsulation of the bioactive extract in the oil droplets. The optimal nanoemulsion had a shear-thinning behavior in both rheological experiments (steady state and oscillatory). This direct interaction fitted the Power Law Model indicating the pseudoplastic behavior in the system and gel structure existence which was one of the criteria in the formulation of transdermally applied cosmetics. Both formulations had pH values within the human skin range which was good for transdermal use. Taking into account the physicochemical characterization of both optimal nanoemulsions, ANN formulation labeled as CN2 was chosen to be the optimal composition in designing the *C. nutans* nanoemulsion. CN2 exhibited non-irritant property with a Human Irritancy Equivalent score of 0.13. In vivo ultrasound attributes of the skin study for CN2 showed that the collagen content increased significantly with the application of the *C.nutans* nanoemulsion among all 21
volunteers during the 21 d of the treatment period. The biophysical attributes of skin studies demonstrated that skin hydration increased without any increment in transepidermal water loss. Thus, a stable *C. mutans* nanoemulsion was successfully developed which had the ability to promote collagen production in human skin and improved the skin barrier function and hence could be potentially used as a system for the delivery of natural antioxidant in cosmetic products.
Abstrak tesis dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

REKABENTUK DAN PENGOPTIMUMAN FORMULASI NANOEMULSI YANG MENGANDUNG EKSTRAK DAUN Clinacanthus nutans LINDAU BAGI KEGUNAAN KOSMESEUTIKAL

Oleh

INTAN SORAYA BINTI CHE SULAIMAN

Jun 2017

Pengerusi: Profesor Mahiran Basri, PhD
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Kosmetik berasaskan tumbuhan mendapat lebih perhatian berbanding produk berasaskan bahan kimia di dalam industri kosmetik. Walau bagaimanapun, cabaran utama adalah menggabungkan ekstrak tumbuhan ke dalam formulasi kerana kehidrofobikannya, mudah teroksida dan kesukaran untuk memformulasikan sebuah sistem pembawa yang stabil. Ester minyak isirong sawit (PKOEs) yang mempunyai kemampuan hebat menembusi kulit telah dipilih untuk digabungkan bersama surfaktan bukan ion di dalam proses formulasi. Struktur nanoemulsi ini ditakrifkan bilamana penyebaran pengumpulan PKOEs dan molekul surfaktan di dalam air hasil daripada kelarutan misel surfaktan. Sebuah sistem nanoemulsi baru telah dicipta dengan menggabungkan ekstrak daun Clinacanthus nutans (C. nutans) untuk aplikasi transdermal. C. nutans atau lebih dikenali sebagai Belalai Gajah adalah tumbuhan ubatan yang popular di negara Asia tropika yang sudah mula mendapat perhatian ramai kerana sifat-sifat terapeutiknya. Terdapat banyak kajian tentang aktiviti biologikal tumbuhan ini termasuk anti keradangan, antiviral, antioksidan dan anti kanser. Ekstrak C. nutans telah dikaji secara mendalam untuk kegunaan sebagai agen farmaseutikal. Walau bagaimanapun, kajian tentang kegunaannya dalam kosmeseutikal masih belum dikenali.

Formulasi ekstrak C. nutans dengan menggunakan sistem pembawa nanoemulsi yang efektif akan menjamin potensi aplikasi ekstrak tumbuhan ini untuk kegunaan kosmeseutikal. Penyediaan ekstrak daun C. nutans dilakukan melalui pengstrakkan berperingkat dan proses pengoptimuman ekstrak dijalankan menggunakan kaedah gerak balas permukaan (RSM). Kondisi optimum yang dicadangkan oleh model RSM adalah pengekstrakkan pada suhu 60 °C, selama 120 minit dengan nisbah pelarut (air: etanol) 90: 10 v/v% untuk memberikan hasil ekstrak sebanyak 23.51%. Walau bagaimanapun, ketidaklarutan ekstrak ini di dalam sistem pelarut methanol telah menghadkan penilaian aktiviti antioksidan ekstrak. Maka, di dalam kajian ini ekstrak
daripada pengestrakkan berperingkat yang mempunyai keterlarutan yang lebih baik di dalam sistem pelarut methanol telah dipilih untuk digunakan dalam formulasi. Sifat-sifat ekstrak menunjukkan kesesuaianya sebagai bahan antioksidan yang selamat untuk digunakan di mana ia mempermerikan kesan tidak toksik ke atas sel fibroblast (3T3).

Rekabentuk nanoemulsi yang mengandungi ekstrak *C. nutans* melibatkan beberapa langkah termasuk saringan jual pemboleh-pemboleh, mengenalpasti kelarutan ekstrak dan akhir sekali penyediaan sistem nanoemulsi. Di bawah daya empar, kesemua campuran PKOEs: minyak biji jambu batu (GSO) di dalam nisbah 9:1, 8:2 dan 7:3 yang mengandungi ekstrak tidak menunjukkan sebarang mendakan di dasar tabung uji. Nisbah 9:1 (PKOEs:GSO) telah dipilih sebagai aman sesuai campuran minyak yang akan digunakan dalam formulasi. Keses komposisi nanoemulsi; minyak dan surfaktan ke atas perubahan saiz zarah di kaji menggunakan rekabentuk campuran eksperimen (MED) dan rangkaian neural tiruan (ANN). Komposisi nanoemulsi yang diramal menggunakan kaedah pengoptimuman berbeza memberikan ramalan yang berbeza. Kaedah MED (dilabelkan sebagai CN1) meramalkan formulasi optimum mengandungi 8.13% surfaktan, 5.00% minyak, 1.00% gam xantan, 0.10% ekstrak bioaktif, 0.80% pengawet, dan 84.97% air untuk menghasilkan zarah bersaiz 97.38 nm. Manakala, ANN (dilabelkan sebagai CN2) meramalkan formulasi optimum mengandungi 10.32% surfaktan, 8.00% minyak, 1.00% gam xantan, 0.10% ekstrak bioaktif, 0.80% pengawet, dan 79.78% air untuk menghasilkan zarah bersaiz 125.40 nm. Walaupun saiz zarah yang dihasilkan dengan kaedah pengoptimuman MED lebih kecil daripada ANN, ralat piawai residu (RSE) menggunakan ANN lebih rendah berbanding MED dengan nilai 1.17% dan 2.61% masing-masing. Ini menunjukkan anggaran nilai eksperimen dan nilai sebenar menggunakan ANN mempunyai hubungkait yang tepat, membuktikan dalam kajian ini kaedah ANN adalah kaedah kuantitatif yang efektif untuk pengoptimuman rekabentuk formulasi.


Imej Mikroskop Elektron Transmisi (TEM) untuk kedua-dua formulasi menunjukkan titisan minyak yang berbentuk sfera di dalam sistem koloid dan pengkapsulan ekstrak bioaktif di dalam titisan minyak. Nanoemulsi optimal mempunyai sifat ricin penipisan di dalam kedua-dua eksperimen reologi (keadaan stabil dan ayunan). Perkadaran terus ini mematuhi model Hukum Power menandakan sistem bersifat pseudoplastik dengan pembentukan struktur gel yang merupakan kriteria untuk kegunaan transdermal. Kedua-dua formulasi juga mempunyai nilai pH di dalam julat kulit manusia yang sesuai untuk kegunaan transdermal. Dengan mengambil kira pencirian fizikokimia ke
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I certify that a Thesis Examination Committee has met on 15 June 2017 to conduct the final examination of Intan Soraya binti Che Sulaiman on her thesis entitled "Design and Optimization of Nanoemulsion Formulation Containing Clinacanthus nutans Lindau Leaf Extract for Cosmeceutical Application" 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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<tr>
<td>AAD</td>
<td>Minimum average absolute deviation</td>
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<tr>
<td>AAPH</td>
<td>2,2’-Azobis (2-methyl propionamidine) dihydrochloride</td>
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<tr>
<td>ANN</td>
<td>Artificial neural network</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BBP</td>
<td>Batch back propagation</td>
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<tr>
<td>BCB</td>
<td>β-Carotene bleaching assay</td>
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<tr>
<td>C. nutans</td>
<td><em>Clinacanthus nutans</em></td>
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<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>DPPH</td>
<td>1,1-Diphenyl-2-picrylhydrazyl</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EFA</td>
<td>Essential fatty acids</td>
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<td>EPP</td>
<td>Entry point project</td>
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<td>ETP</td>
<td>Economic transformation programme</td>
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<td>FCS</td>
<td>Fetus calf serum</td>
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<tr>
<td>FDA</td>
<td>Food and drug administration</td>
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<td>GA</td>
<td>Genetic algorithm</td>
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<tr>
<td>GAE</td>
<td>Gallic acid equivalent</td>
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<td>GSO</td>
<td>Guava seed oil</td>
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<td>HIE</td>
<td>Human irritancy equivalent</td>
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<td>HLB</td>
<td>Hydrophilic lipophilic balance</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IBP</td>
<td>Incremental back propagation</td>
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<tr>
<td>IC₅₀</td>
<td>Concentration of sample that caused inhibition of 50% cell growth</td>
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<tr>
<td>IPL</td>
<td>Intense pulsed light</td>
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<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
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<tr>
<td>LC-MS</td>
<td>Liquid chromatography-mass spectrometry</td>
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<tr>
<td>LM</td>
<td>Levenberg-marquardt</td>
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<td>MD</td>
<td>Molecular dynamics</td>
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<td>MED</td>
<td>Mixture experimental design</td>
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<td>MLP</td>
<td>Multilayer perceptron</td>
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<td>MMPs</td>
<td>Matrix metalloproteinases</td>
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<td>MTT</td>
<td>3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide</td>
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<td>NKEAs</td>
<td>National key economic areas</td>
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<tr>
<td>O/W</td>
<td>Oil-in-water</td>
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<td>OD</td>
<td>Optical density</td>
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<td>ORAC</td>
<td>Oxygen radical absorbance capacity</td>
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<td>OTC</td>
<td>On-the-counter</td>
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<td>PKOEs</td>
<td>Palm kernel oil esters</td>
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<td>PVPP</td>
<td>Polyvinylpolypyrrolidone</td>
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<td>QP</td>
<td>Quick propagation</td>
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<tr>
<td>R&amp;D</td>
<td>Research and development</td>
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<td>RE</td>
<td>Rutin equivalent</td>
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<td>RF</td>
<td>Radiofrequency</td>
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<tr>
<td>RMSE</td>
<td>Root mean squared error</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RSE</td>
<td>Residual standard error</td>
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<td>S80</td>
<td>Sorbitan monooleate</td>
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SB  Stratum basal
SC  Stratum corneum
SG  Stratum granulosum
SS  Stratum spinosum
T20  Polyoxyethylene (20) sorbitan monolaurate
T80  Polyoxyethylene (20) sorbitan monooleate
TE  Trolox equivalent
Teq  Alpha tocopherol equivalent
TEWL  Transepidermal water loss
TFC  Total flavonoid content
TMR  Transparency market research
TPC  Total phenolic content
Trolox  6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
TTC  Total tannin content
U.S.  United States
UV  Ultraviolet
UVB  Ultraviolet B
W/O  Water-in-oil
CHAPTER 1

INTRODUCTION

1.1 Background

By definition, a cosmeceutical is a hybrid category of products lying on the spectrum between drugs and cosmetics (Kligman, 2005). A cosmeceutical product does not merely satisfy the need for beauty but is also enhanced with medicinal or druglike benefits through its biologically active ingredients (Gao et al., 2008). The unique ability of cosmeceutical products is their ability to enhance and beautify the skin appearance from inside out. These functioning ingredients able to penetrate and alter the skin structure by improving skin functionality or texture, protect the skin from harmful effect, maintain the skin hydration and remove minor scar and pigmentation (Bissett, 2009). Cosmeceutical actives are potent substances that may provide a biologic effect to skin. These substances may source from synthetic or plant-based elements. Cosmeceutical products also have been reported to have side issues such as skin irritation and toxicity (Draelos, 2009; Gao et al., 2008). Efforts have been focused on preparing green formulation which is safer than artificial sources where the source of the active ingredients is mainly from the plant kingdom. The advantage of using plant extract instead of pure compounds is one single plants extract may have a diversity of phytochemical properties such as anti-inflammatory, antioxidant, antibacterial, and whitening agent (Bissett, 2009). However, the uses of many active plant-based ingredients have been restricted to poor water solubility, low bioavailability and instability against oxidation and degradation by light.

Consumers are now well educated and demand sophistication in the products that they purchase. This requirement has led to the design of multifunctional cosmetic products which could provide a visible improvement in skin appearances instead of just scent and color to the skin (Draelos, 2009, 2011). In order to formulate a functioning cosmeceutical product mainly for transdermal use, the focus has to be on the ability of the active ingredients to deliver their biologically active forms adequately to the skin. Therefore, an effective carrier vehicle should be addressed to assume the applied product to reach the target site and works efficiently (Draelos, 2011).

One of the most popular use for cosmeceutical products is in improving the appearance of aged skin. Hectic lifestyles and environmental pollution are some of the reasons for cutaneous aging and this is mainly due to the oxidation of skin structure from the generated highly reactive oxygen molecules (Brandt et al., 2011). Cosmeceutical antioxidants of plant origin having the advantage of possessing non-irritating ingredients are indeed the best alternative to protect the skin from radiation and oxidation damage and at the same time, are able to improve the appearance of the skin. Malaysia’s rainforests are well known for their wealth of plant biodiversity. About 12,000 species of flowering plants have been reported in Malaysian forests, and up to 1,300 species have been identified as having therapeutic potential (Jamal et al., 2010). Owing to their beneficial attributes, wide acceptance, and growing awareness of
healthcare, green formulations have been of increasing interest among stakeholders, including wide-spread interest in developing cosmeceutical products containing plant-derived sources. Moreover, there has been rising concern on aging and photo damage leading to skin diseases in the population worldwide. The combination of these factors has caused one of the fastest growth of cosmeceutical products in the personal care industry, which mainly involved products designed to diminish visible signs of aging (Brandt et al., 2011).

_Clinacanthus nutans_ (C. nutans) belonging to the Acanthaceae family, is an herbal plant widely used in folk medicine. This plant is commonly known in Malaysia as Sabah Snake Grass or Belalai Gajah and is widely distributed in tropical Asian countries such as Malaysia, Indonesia and Thailand. Various reports have documented the biological activity of _C. nutans_, including its antioxidant (Yuann et al., 2012), anti-inflammatory (Wanikiat et al., 2008), anti-viral (Direkbusarakom et al., 1998; Kunsoorn et al., 2013; Yoosook et al., 1999) and anti-cancer properties (Yong et al., 2013).

Previous investigation has established the presence of numerous polyphenols such as isomollupentin-7-O-beta-glucopyranoside, vitexin, isovitexin, shaftoside, orientin, isoorientin, 4-vinylphenol, 7-hydroxyflavone and 2,6-dimethoxyphenol from the leaves extract of _C. nutans_ (Mustapa et al., 2015; Teshima et al., 1997). Furthermore, many plants are rich in endogenous antioxidants as they must survive in an environment that is constantly exposed to ultraviolet radiation (Draelos, 2009) and thus have potential to be used as an alternative to synthetic antioxidants.

In line with the Economic Transformation Programme (ETP) as announced in the 10th Malaysia Plan, towards transforming Malaysia into a high income country by 2020; ETP was embodied by the 12 National Key Economic Areas (NKEAs). Agriculture was one of the selected industries chosen due to its potential economic growth and development. Their entry point project (EPP) consisted of capitalizing on the potential of Malaysia to become a supplier of world-class, high quality nutraceutical and cosmeceutical products by leveraging the country’s natural biodiversity. Efforts have therefore been focused on improving product quality including research and development (R&D) and clinical trials using Malaysia’s native plants. _C. nutans_ or also known as Belalai Gajah which was included in the ten leading herbs identified as potential plants for R&D (PEMANDU, 2013).

1.2 Problem Statement

Formulating a product with the beneficial attributes of the bioactive ingredients has been challenging due to various reasons including poor solubility and low bioavailability which may hinder effective transdermal delivery. Moreover, antioxidant bioactive substances are naturally unstable, and easy to oxidise and degrade when exposed to the light (Khatri et al., 2010). An effective transdermal application requires the product to pass through the skin’s inner layers successfully. However, as the biggest organ of the body, the skin’s barrier presents a great challenge for the efficient delivery of therapeutics to the skin dermis. The stratum corneum located on the outer
layer of the skin, prevents the loss of internal body components, particularly water to the external environment (Escobar-chávez et al., 2012). Thus, an effective delivery system was crucial in order to increase the therapeutic effects in transporting agents to target locations by considering the side effects.

Emulsion stability is a primary concern for formulation design. In nanoemulsion development systems, the utmost challenge has been to maintain the particle size in the nanometer range while remaining physically stable for a period of time. Therefore, the composition of formulations developed is very important in order to obtain stable nanoemulsions with characteristics suitable for transdermal use. This process has been time consuming as the factors affecting interactions between each component had to be individually determined. Thus, well-designed data collection processes through optimization using multivariate statistical technique would allow the experiments to achieve high success rates.

1.3 Scope of Study

This study focused on the design and optimization of nanoemulsion systems containing extract of C. nutans leaves for cosmeceutical application. The aim was to capitalize on the antioxidant biological activity of C. nutans leaves extract, in order to improve human skin texture. The work involved two stages of optimization processes. The earlier stage was optimization of the extraction process using Response Surface Methodology, to obtain optimal extract with highest of antioxidant activity. The work was then further carried out to find the optimal composition for formulation by using two optimization methods (Mixture Experimental Design and Artificial Neural Network). The developed formulation was evaluated for its physicochemical properties and efficacy on skin.

This study was limited to the amount of C. nutans extract of 1g/kg of formulation (0.1% w/w). The acute toxicity study of ethanol extract of C. nutans leaves at concentrations as high as 1.3 g/kg of body weight did not exhibit toxicity in the mice model (Chavalittumrong et al., 1995). The use of 1.3 g/kg of C. nutans ethanol extract for mice was reported to be equivalent to 5.44 g/kg of body weight for humans (Kamarudin et al., 2017). Moreover, sub-chronic oral administration (90 days) of C. nutans demonstrated that 1 g/kg body weight in rats showed no any abnormalities of their internal organs (equivalent to 4.18 g/kg of body weight in humans) (Chavalittumrong et al., 1995). In sub-acute toxic effect, after 14 days of oral administration with methanol extract of C. nutans leaves (0.3 g/kg, 0.6 g/kg and 0.9 g/kg) did not cause any adverse effects and organ damages in Sprague Dawley rats (P’ng et al., 2013). Furthermore, the acute oral toxicity study of C. nutans extract demonstrated that acute exposure of 1.8 g/kg of C. nutans was safe in mice without causing any adverse effects or mortality. The oral LD₅₀ of methanol leaves extract of C. nutans was suggested to be greater than 1.8 g/kg body weight in male mice (P’ng et al., 2012).
1.4 Objectives

The specific objectives of this work are:
1) To optimize the extraction process of C. nutans leaves.
2) To determine the antioxidant activity of the extract.
3) To conduct optimization studies of the formulation compositions of nanoemulsion systems containing extract of C. nutans leaves.
4) To characterize the physicochemical properties of the newly developed nanoemulsions.
5) To evaluate the safety of the nanoemulsions and their biophysical attributes to human skin for transdermal use.
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