DEVELOPMENT AND CHARACTERIZATION OF EUGENOL IN CINNAMON LEAF OIL NANOCREAM FOR WOUND HEALING

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DEVELOPMENT AND CHARACTERIZATION OF EUGENOL IN CINNAMON LEAF OIL NANOCREAM FOR WOUND HEALING

by

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APPENDICES

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Nor Azah binti Zainol

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LIST OF ABBREVIATIONS

e.g	example
%	Percent
cm	centimeter
ºF	Degree Fahrenheit
Tween 80	Polysorbate 80
Span 65	Sorbitan tristearate
-OH	Alcohol group
cm^2	Square centimeter
m^2	Square meter
Pa	Pascal
sec	Second
η	Viscosity
F	Shear stress
G	Shear rate
nm	Nanometer
O/W	Oil in water
W/O	Water in oil
º	Degree
mm	Milimeter
gm	Gram
ºC	Degree Celcius
±	Plus minus
N	Slope of Log (S-F) against log G

F	Yield value
r.p.m	Revolution per minute
UK	United Kingdom
USA	United States of America
PTFE	Polytetrafluoroethylene
RH	Relative humidity
mL	Mililiter
&	And
et.,al	And others
S_{mix}	Surfactant mixtures
Pa.s	Pascal second
M	Molar
mV	Milivolt
p.p.m	Part per million
FIDs	Flame-ionization detectors
DSC	Differential scanning calorimetry
LC	Liquid Chromatography
HPLC	High Performance Liquid Chromatography
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
µm	Micrometer
UV-VIS	Ultraviolet visible spectrophotometry
C18	Carbon 18
µg	Microgram
mL/min	Mililiter per minute

mg/mL	Miligram per milliliter
ng/mL	Nanogram per milliliter
mg	milligram
SD	Standard deviation
LOD	Limit of detection
LOQ	Limit of quantification
S/N	Signal to noise
UV	Ultraviolet spectrophotometry
ATCC	American Type Culture Collection
MICs	Minimum inhibitory concentration
NAWD	Nathan's agar well diffusion
L	Liter
min	Minute
OD	Optical density
hrs	Hours
µL	microliter
CFU	Colony-forming unit
kg	Kilogram
Log P	Partition coefficient
H-bonding	Hydrogen bonding
$T_{50\%}$	Time at 50% of drug release
IP	injected intraperitoneal
SPSS	Statistical procedures for social science
ANOVA	Analysis of variance
K Count	number of photons detected in Kilocount persecond

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PEMBANGUNAN DAN PENCIRIAN NANOKRIM EUGENOL DALAM MINYAK DAUN KAYU MANIS UNTUK PENYEMBUHAN LUKA

ABSTRAK

Minyak daun kayu manis (*Cinnamomum zeylanicum)* mengandungi peratusan eugenol yang tinggi dan mempunyai aktiviti antimikrob, anti-oksida dan anti-radang. Walau bagaimanapun, minyak yang tidak dicairkan boleh menyebabkan kerengsaan pada kulit. Oleh itu, untuk mengelakkan kerengsaan minyak daun kayu manis telah dirumuskan menjadi nanokrim. Asas nanokrim telah disediakan dengan menggunakan minyak kelapa sawit, penampan pH 5.5 dan surfaktan (Tween, Span dan Carbitol) pada nilai HLB yang berbeza daripada 13.71, 11.13, 9.84 untuk Tween 80 dan Span 65 manakala 13.92, 12.84 dan 10.68 untuk Tween 80 dan Carbitol. Lima belas rumusan asas nanokrim telah dipilih dari kawasan nanokrim dalam tiga rajah fasa pseudo-ternari dan diuji untuk kajian kestabilan dipercepatkan. Hanya empat rumusan lulus ujian, dan rumusan ini telah diubahsuai dengan menambah 2% alkohol cetostearyl sebagai ejen pemekatan, untuk meningkatkan kestabilan rumusan tersebut. Rumusan yang diubahsuai juga tertakluk kepada ujian kestabilan dipercepatkan dan tiga rumusan lulus ujian. Rumusan asas nanokrim dicirikan dari segi saiz titisan, potensi zeta, kelikatan ketara, dan sifat-sifat reologi. Di dapati bahawa asas nanokrim B2(2) adalah formulasi yang terbaik dan 2% minyak daun kayu manis telah dimasukkan kedalam formulasi ini. Formulasi minyak daun kayu manis yang telah dioptimumkan terdiri daripada penampan pH 5.5, minyak (96% minyak kelapa sawit , 2% minyak daun kayu manis dan 2% cetostearyl alkohol) dan surfaktan (70% Tween 80 dan 30% Span 65 dengan nilai HLB 11.13) pada nisbah

47.4:15.8:36.8. Saiz titisan, potensi zeta, kebolehan sebaran, kelikatan ketara dan nilai "yield" pada nanokrim minyak daun kayu manis yang telah dioptimumkan masing-masing adalah 286.4nm, -29,6 mV, 472,96 g/saat, 10473.14 Pa.s dan 300 Pa,. Di samping itu, formulasi yang telah dioptimumkan mempunyai aliran plastik dengan sifat-sifat penipisan ricih dan imej dari mikroskop elektron transmisi mengesahkan bahawa saiz titisan adalah dalam lingkungan nano. Kajian pembebasan *in vitro* menggunakan sel pembauran Franz menunjukkan bahawa 81% daripada eugenol dibebaskan daripada formulasi dalam masa 24 jam. Ujian mikrobiologi menunjukkan bahawa formulasi nanokrim menghalang pertumbuhan bakteria gram positif dan gram negative kecuali *P. aeruginosa*. Ujian kerengsaan kulit primer menggunakan arnab putih New Zealand menunjukkan bahawa formulasi nanokrim tidak merengsa kulit arnab. Selain itu, kajian penyembuhan luka menggunakan tikus Sprague Dawley menunjukkan bahawa formulasi mempunyai sifat penyembuhan yang sama dengan produk antiseptic krim Dettol yang terdapat dipasaran. Formulasi nanokrim didapati stabil selama 6 bulan pada 25°C±2°C/61-91%RH dalam kajian kestabilan. Hasil kajian ini menunjukkan bahawa 2% nanokrim minyak daun kayu manis mempunyai potensi yang besar untuk digunakan bagi penyembuhan luka.

DEVELOPMENT AND CHARACTERIZATION OF EUGENOL IN CINNAMON LEAF OIL NANOCREAM FOR WOUND HEALING

ABSTRACT

Cinnamon (*Cinnamomum zeylanicum*) leaf oil contains high percentage of eugenol and has antimicrobial, antioxidant and anti-inflammatory activities. However, the undiluted oil can cause irritation to the skin. Therefore, to avoid irritation cinnamon leaf oils was formulated into nanocream. The nanocream base was prepared using palm oil, buffer pH 5.5 and surfactants (Tween, Span and Carbitol) at different HLB values of 13.71, 11.13, 9.84 for Tween 80 and Span 65 while 13.92, 12.84 and 10.68 for Tween 80 and Carbitol. Fifteen nanocream base formulations were selected from nanocream areas in three pseudo-ternary phase diagrams, and tested for accelerated stability study. Only four formulations passed the test, and these formulations were modified by adding 2 % cetostearyl alcohol as thickening agent, to improve their stability. The modified formulations were also subjected to accelerated stability test and three formulations passed the test. The nanocream base formulations were characterized in term of droplet size, zeta potential, apparent viscosity, and rheological properties. It was found that the nanocream base B2(2) was the best formulation and 2% cinnamon leaf oil was incorporated to this formulation. The optimized cinnamon leaf oil formulation consists of buffer pH 5.5, oil (96% palm oil, 2% cinnamon leaf oil and 2% cetostearyl alcohol) and surfactants (70% Tween 80 and 30% Span 65 with HLB value 11.13) at the ratio of 47.4:15.8:36.8. The droplet size, zeta potential, spreadability, apparent viscosity and yield value of the optimized cinnamon leaf oil nanocream were 286.4nm, -29.6 mV, 472.96 g/sec, 10473.14 Pa.s and 300 Pa, respectively. In addition, the optimized formulation had plastic flow with

shear thinning behavior and image from transmission electron microscope confirmed that the droplets size was in nano range. The *in vitro* release study using a Franz diffusion cell showed that 81% of eugenol was released from the formulation in 24 hours. The microbiological test revealed that the nanocream formulation inhibited the growth of gram positive and gram negative bacteria except *P. aeruginosa*. Primary skin irritation test using New Zealand White rabbit showed that the nanocream formulation was non irritant to the rabbit's skin. Moreover, wound healing study using Sprague dawley rats indicated that the formulation had healing properties similar to the marketed product antiseptic Dettol cream. The nanocream formulation was found stable for 6 months at 25°C±2°C/61-91%RH in the stability study. The results of the present study suggested that the 2% cinnamon leaf oil nanocream has great potential to be used for wound healing.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Dermatological formulations are among the most frequently used products because of their wide range of potential uses. In modern-day pharmaceutical practice, semisolids are the preferred vehicle for dermatological therapy because they deliver drugs over the extended time periods. The dermatological formulations are normally found in the form of solution, suspensions and gels, ointment, emulsions, lotion and creams (Aulton,2007).

Regardless of the formulation, all the dermatological formulations are applied to the skin in order to treat skin disease or restore a good skin condition. Skin is the largest organ in the human body that protects the internal body structure of harmful organisms existed in the environment. Skin can easily damage, whether by mechanically (e.g. cuts, bruises, burns, bites and stings), chemically (e.g. Detergent), biologically (e.g microorganism) or radiation (e.g plants deliver contact allergens) (Aulton,2007).

1.2 Anatomy of skin

Skin consists of three layers. The outer layer is called as epidermis, the middle layer is the dermis and the innermost layer is hypodermis or subcutaneous tissue (Figure 1.1)

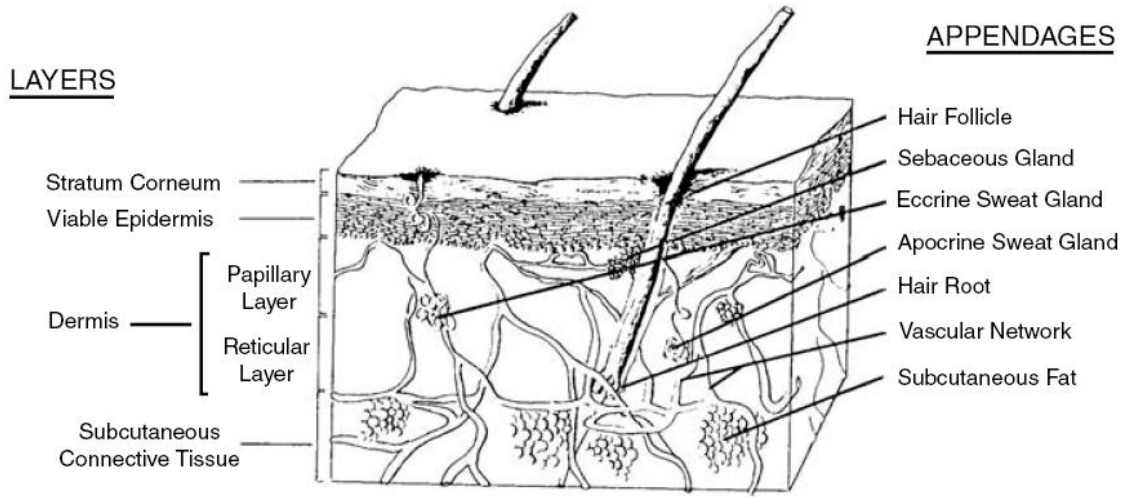

Figure 1.1: Structure of skin (Baheti et al.,2011)

1.2.1 Epidermis

Epidermis consists of five layers in which the stratum corneum is the outermost layer. The other layers of the epidermis from top to bottom are stratum lucidum (only in palms of hands and bottoms of feet), stratum granulosum, stratum spinosum and stratum basale. The epidermis has different thickness in various body sites. The thinnest layer is on the eyelids which is about 0.006mm and the thickest layer is about 0.8mm on the palms and soles of the feet. There are no blood vessels found in this layer (Aulton,2007).

Stratum corneum consists of 10-25 cell layers of keratinocytes which are dead cells and do not contain nucleus. The thickness of this layer is only 10μm when it is dry but it swells several-fold in the water. It acts as a natural barrier to external agents or chemicals. Stratum corneum also controls trans epidermal water loss. The lowest of the stratum corneum layers is surrounded by wet layers below the skin and the

uppermost layer is exposed to various environmental conditions. The life cycle of this cell continues up to 28 days.

Stratum basale is the lower layer of epidermis. It contains three types of cells which are Melanocytes, Langerhans cells and Merkel cells. These cells have different roles in the skin. Melanocytes are responsible for the synthesis of the melanin pigment which provides colour to the skin. Langerhans cells are immune responder cells. They have an ability to bind antigen and transfer them to the lymphocytes in the lymph nodes. Merkel cells are present in the stroke sensitive locations of the body and accounts for sensation (Aulton,2007)

1.2.2 Dermis

Dermis is the non-descriptive lying area in between of the epidermis and the subcutaneous fat regions. It is composed of an elastic network of collagen fibrils embedded in a muccopolysacharide gel with the layer thickness of 3-5mm (William,2003; Aulton,2007). The elasticity of our skin is caused by this network or gel that constructs the cells. The dermis contains hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic nodes and blood vessels. The dermis requires an efficient blood supply to deliver nutrients, remove waste products, regulate pressure and temperature and also contribute to skin colour. This layer also synthesizes collagen and elastin.

1.2.3 Subcutaneous Layer

The subcutaneous layer is the innermost first layer of the skin that covers the body part. These layers are covered by the dermis and composed of fat and adipose tissue.

It is a thick layer in most part of the body, but absent in some part of the body such as the eyelids. It provides high energy molecules to the body, takes part in the thermoregulation procedure and acts as a mechanical quantity of the external shock (Williams,2003).

1.2.4 Appendages

The appendages include 3 kinds of structures that are oriented from the dermis. Sweat glands in the dermis are associated with sweat ducts. There are two types of the sweat glands which are eccrine glands and apocrine glands. Eccrine glands are found in most parts of the body (2-5 million) and they produce sweat (pH 4.0-pH.8) and may also secrete drugs, proteins, antibodies and antigens. Their functions are to aid heat control and provoke sweating when there is emotional stress. On the other hands, the apocrine glands are found in certain locations in the body. These glands provide characteristic of adult distribution in the armpit (axilla), the breast areola and the perianal region. They secrete milky, oily and may be coloured liquid that contains proteins, lipids, lipoproteins, and saccharides. External bacteria metabolize this odourless fluid to produce the body smell (William,2003).

Hair follicles are developed over all skin except the red part of the lips, the palm and sex organs. These follicles are related to the secretory glands that are recognized as the sebaceous glands. They are the most numerous and largest on the face and forehead, in the ear, on the midline of the back and anogenital surfaces. Sebaceous glands secrete fatty lubricant materials that aid to lubricate the skin and keep the skin at pH of 4-6. This fatty secretion is recognized as sebum.

1.3 Chemical penetration enhancers used in dermatological formulation.

Chemical enhancers are the agents that interact with skin constituent that promotes drug flux. It is the most widely method studied to approach the dermatological enhancement. However, a chemical penetration enhancer should be pharmacologically inert, nontoxic, nonirritating, nonallergic, odourless, tasteless, colourless, inexpensive, and have good solvent properties. Chemical penetration enhancer can be classified into: Solvent (water, alcohols, alkyl methyl sulfoxide, dimethyl sulfoxide, dimethyl acetamide and dimethyl formamide), Azone, Terpenes, Terpenoids, Essential oil, Pyrrolidones, Fatty acids and esters, Surfactants, Bile salts and Miscellaneous Chemicals (Jadhav & Sreenivas,2012).

Alcohol which acts as a solvent can enhance the penetration of drug by various mechanisms such as extraction of lipids and proteins, swelling of the stratum corneum or improving drug partitioning into the skin or increasing solubility of the drug in the formulation. However, surfactants enhance drug delivery by solubilizing lipid within the stratum corneum. Surfactant can be divided into anionic, cationic and non-ionic. Anionic and nonionic surfactants are more widely used. Anionic (example: sodium lauryl sulfate) is a strong penetration enhancer but high toxicity and may cause skin irritation. Nonionic surfactants (example: Tween 80) are preferable in industrial application because it is low toxicity and less irritant.

1.4 Wound

A wound can be defined as a defect or a break in the skin, resulting from either mechanical or thermal damage or as a result of the presence of an underlying medical or physiological condition. Wounds are described as normal (acute) if they heal

rapidly with minimum scarring and called as chronic if they take longer than 8-12 weeks to heal (Aulton,2007). The primary causes of acute wounds include mechanical injuries due to external factors such as abrasions which are caused by frictional contact between the skin and hard surfaces, penetrating wounds caused by knives and gun shots and surgical wounds caused by surgical incisions for example removing tumors. While chronic wound is caused by diabetes and malignancies, persistent infections, poor primary treatment and other patient related factors. Skin wounds can also be classified according to the number of skin layers that are affected. Superficial wounds are the damage to the epidermis alone. Partial thickness wounds are the damage to the epidermis and deeper layers include blood vessels, hair follicles and sweat glands. While full thickness wounds are the damage to fat or deeper tissue as well.

1.4.1 Wound healing

Wound healing may be considered as a dynamic process in which cellular and matrix components perform together to reestablish the integrity of damaged tissue and replace lost tissue. These overlapping can be classified in four stages (Aulton,2007).

- Inflammation
- Migration
- Proliferation
- Maturation

Inflammation: this stage starts almost at the same time as homeostasis. It occurs between few minutes to up to 24 minutes after injury. In this stage histamine and serotonin are released into the wound area and activate phagocytes to enter the wound area and engulf dead cells (Aulton,2007).

Migration: in this stage the reestablishment of wound begins. The epithelial cells, fibroblast and keratinocytes move into the injured area to replace damaged and lost tissue (Aulton,2007).

Proliferation: It involves the development of new tissue and occurs simultaneously or just after the migration phase. This stage has three characteristics. First, the granulation tissue is formed by growth of capillaries. Second, lymphatic vessels enter into the wound and the third one, synthesis of collagen starts providing form and strength to the injured tissue (S. Boateng et al.,2008).

Maturation: Final phase of wound healing is also called as remodeling phase. It involves an enlargement of collagen fibres which increase the tensile strength of the repair wound. The timescale for wound repair is from 3 weeks to 2 years (Aulton,2007).

1.4.2 Wound dressing

Dressing can be classified based on their function in wound(antibacterial, absorbent), type of material employed in producing the dressing (collagen, hydrocolloid), physical form of the dressing (ointment, film and gel), traditional and modern dressings. Traditional wound dressing can be divided into topical pharmaceutical formulations and traditional dressing (S. Boateng et al.,2008). However, topical pharmaceutical formulation such as lotion, cream or ointment can be used in the initial stages of wound healing as antibacterial (S. Boateng et al,2008). While traditional dressing such as cotton wool and natural or synthetic gauze are used more in wounds such as burn wounds because liquid and semi liquid dressings do not remain on the wound over optimal time.

Modern wound dressings are often classified as hydrocolloid dressings, alginate dressings, hydrogel dressings, dressings in the form of gels, foams and films, etc.

The modern dressings do not enhance the reepithelialization, but stimulate collagen synthesis and promote the angiogenesis. They can provide a pain relief feeling to patients. The modern dressings can inhibit bacterial growth by maintaining a barrier against external contamination and some of them by decreasing the pH at the wound surface. They provide a moist environment for the wound bed to enhance the healing process (S. Boateng et al.,2008).

1.5 Nanocream for dermatological application

Nanocream or semisolid emulsion is one of the pharmaceutical topical formulations that apply to external uses only. The size of the droplet in nano is 100-600nm (Sharma et al.,2011). Nanocream can be prepared by using high energy methods such as high shear stirring, high pressure homogenizers or ultrasound generators (Fernandez et al.,2004). Generally, nanocream is very useful in personal care and cosmetics because the small size of the droplets allow them to deposit uniformly into the skin and enhance the efficiency of active ingredients delivery through the skin. Basically, the cream contains various drugs for different remedial properties in an appropriate semi solid base either hydrophobic or hydrophilic in character (Aulton,2007). Both o/w or w/o type of cream is available.

1.5.1 O/W and W/O cream

Pharmaceutical cream consists of oil and water. Water in oil or oil in water is depending on whether the continuous phase is water or oil. Oil droplets dispersed in water is called oil in water. Suitable surfactant should be selected in the production of oil in water or water in oil as the cream type depends on HLB number. However, production of oil in water creams facing no problem as they are miscible in water and

easily diluted. Oil in water cream is nice to apply on our skin surface as it has no oily feel. Thus, it is easily washed from the skin. Oil in water cream will dissolve lipophilic drug while water in oil cream will dissolve hydrophilic drug (Azeem et al.,2009). Normally water in oil cream has greasy texture and is not always accepted in cosmetic field. It is less efficient as cleanser but usually, suitable as moisturizing cream. It prevents moisture loss from the skin and inhibits drying of the stratum corneum. There are several tests that are normally used for identification of cream types. If the cream soluble in water, it is oil in water type. In contrast, if the cream soluble in oil, it is classified as water in oil cream. Another analysis that can identify the cream types is by using conductivity measurement. Oil in water cream will conduct electricity while water in oil cream does not conduct electricity. Besides, staining test using methylene blue is always used in the cream type detection. Oil in water cream can result in more intense blue than water in oil cream. The water phase will be in blue color while oil phase is colourless.

1.6 Surfactants as emulsifying agent

Surfactants or surface active agents are termed as amphiphiles and are used in cream preparation as an emulsifying agent. It has the features of two different areas of the hydrophilic and hydrophobic. Hydrophobic part is usually saturated or unsaturated hydrocarbon chain, or aromatic heterocyclic ring system. The hydrophilic part can be anionic, cationic or nonionic. Surfactants are classified according to the nature of the hydrophilic groups that are anionic surfactant, cationic surfactant and non-ionic surfactant.

Anionic and cationic surfactants are toxic. However, anionic surfactant has been always a perfect choice in industrial application because of their cheapness. Unlike

the cationic surfactant, they tend to be used only for the formulation of antiseptic cream due to the toxicity. In addition, this compound naturally has antiseptic properties.

Nonionic surfactants are normally used because it is low toxicity and less irritation to the skin. It also has a higher level of compatibility with other materials compared to anionic and cationic surfactants. Each surfactant has its own HLB number. Polysorbate 80 (Tween 80), Sorbitan tristearate (Span 65) and Carbitol have HLB number of 15, 2.1 and 4.2, respectively. Tween 80 is more soluble in water so it tends to form oil in water cream. However, Span 65 is soluble in oil. So, it tends to form water in oil cream. Generally, nonionic surfactant is safely used in the pharmaceutical, cosmetic and food products. They are uncharged thus the products based on this surfactant are not affected by pH changes (Mahdi et al.,2011).

1.6.1 Hydrophile-lipophile balance (HLB)

HLB is primarily requested for nonionic surfactant. Every single surfactant is endowed an HLB number instead of the comparison the lipophilic and hydrophilic molecules. High numbers of HLB surfactants have hydrophilic or polar properties while low numbers of HLB exhibit lipophilic or non-polar characteristic (Aulton,2007).

In hydrophile – lipophile balanced (HLB) systems, the more hydrophilic interfacial barrier favours more oil in water cream while the more non-polar barrier favours more water in oil cream. Type of cream is dependent on the material goods of the emulsifying agent.

Oil in water cream is formed when HLB cream is in a range of 8 to 16 and water in oil cream is formed when HLB is about 3 to 6. High HLB emulsifier such as a mixture of Tween 80 and Span 65 will form oil in water cream. However, the Span 65 itself with HLB 2.1 will form water in oil cream.

Table 1.1: HLB scale of surfactant function (Aulton,2007):

HLB	Functions
2-3	Antifoaming agents
3-6	Water in oil emulsifying agents
7-9	Wetting and spreading agents
8-16	Oil in water emulsifying agents
13-15	Detergent
15-18	Solubilizing agents

1.6.2 Co-surfactant

The second amphiphile or surface active agent such as carbitol is also known as co - surfactant. A combination of both surfactant and co-surfactant gives very low interfacial tension. It is necessary to enhance stability of the products. A mixture of surfactant and co-surfactant produces a good emulsifying agent and is more stable than the surfactant alone (Abdulkarim et al,2011).

1.7 Selection of Palm Oil or Palm Olein as the oil phase in topical cream

Malaysia and Indonesia are the largest producers of palm oil, accounting for 84% of the world production (Basiron et al.,2004). Palm oil is derived from the fruit of the palm tree *Elaesis guineenis*. Palm oil is a combination of triglyceride compounds

(Abdulkarim et al., 2010). It is the main natural source of tocotrienol. It is also high in vitamin K and dietary magnesium. The palm oil is derived of saturated and unsaturated fatty acids. According to Mukherjee and Mitra(2009), the composition of saturated fatty acid in palm oil is Palmitic (C16) 44.3%, Stearic (C18) 4.6% and Myristic (C14) 1.0% while unsaturated fatty acids are Oleic acid (C18) 38.7%, Linoleic acid (C18) 10.5% and other/unknown 0.9 %.

90% of the palm oil production is used as food and only 10% in non-food industry. The major uses in the foods are as cooking oil, shortening, margarine and spread and substitutes of dairy product. Non-food application of palm oil is mainly for manufacturing of creams, soaps, softeners and etc. Recently, there is a higher demand for palm oil in non-food industries due to lower price and easier to obtain compared to other vegetable oil. It is also renewable, nontoxic, environmentally friendly and not harmful to the environment and humans (Hsu & Nacu,2003).

Palm oil has a characteristic of an emollient. In cream, palm oil functions as a carrying agent for the active ingredient. It is used to substitute liquid paraffin that is widely used before. Consumption of palm oil in cream preparation is safer and lack of toxicity. Besides that, palm oil in a cream formulation affects the viscosity of the final product and the transport of the drug into the skin. Compare to coconut oil, consumption of palm oil in cream preparation generates no rancidity.

1.8 Cetostearyl alcohol in cream preparation

Cetostearyl alcohol is a white flake and melts to a clear colour when it is heated. It is insoluble in water and soluble in oil (Liebert,1988). Cetostearyl alcohol may be used

as an emollient, emulsifying agent or as a thickening agent in cream formulations. Generally, it has been used to increase the viscosity of topical pharmaceutical formulations to both w/o and o/w creams. Furthermore, cetostearyl alcohol stabilizes creams and can act as a co-emulsifier. It is stable when stored in a well-closed container in a cool and dry place. It is also non-toxic to the skin.

1.9 Literature review of Cinnamon leaf oil (*Cinnamomum zeylanicum)*

Cinnamon leaf oil is extracted from the leaves part of *Cinnamomum zeylanicum* species. This brownish yellow to dark brown oil is volatile and slightly soluble in water. It has a warm and spicy musky smell and generally used as aromatherapy to reduce stress. It contains a variety of chemicals that work in medicine. Eugenol and cinnamaldehyde is the major compounds in cinnamon leaf oil (Ayala – Zavala et al.,2008). According to Trajano et al.,(2010), other components of the essential oil obtained from the leaves are eugenol acetate, cinnamic aldehyde, benzyl benzoate, linalool, trans- β-cariophyllene, α-humullene, α-pinene and p-cymene.

Cinnamon leaf oil is recognized for their flavour and aroma as well as antimicrobial medical applications. In the whole world, the aroma of leaves and bark cinnamon are used as spices in food industries, flavouring in perfumes, ice cream and chewing gum. From the previous reports, cinnamon leaf oil compounds such as eugenol and cinnamaldehyde have the characteristics of strong and astringent properties of anti-infectious, anti-bacterial, anti-parasitic, anti-spasmodic and anti-diarrohea (Jakhetia et al.,2010). Thus, these herbs have been used in healing a number of diseases, such as cardiovascular, respiratory, digestive, immune, urinary, lymphatic, reproductive,

nervous system complaints and several other disorders. In addition, cinnamon leaf oil also shows a very effective mosquito repellent (Cheng et al.,2011).

Eugenol in cinnamon leaf oil provides anti-inflammatory, antioxidant, anaesthetic, vascular and muscle relaxant properties (Javdani & Nikousefat,2012). All the properties of cinnamon leaf oil help in reducing pain and wound healing. Farahpour & M. Habibi(2012) reported that Eugenol shows similar anti-inflammatory effects of COX antagonist that helps to accelerate wound healing. It is known that different mechanisms may be involved in the inflammatory process.

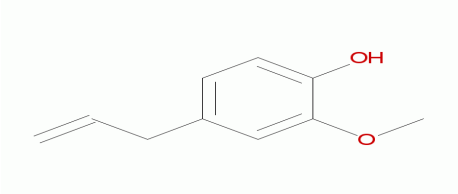

Figure 1.2: Chemical structure of eugenol

Meanwhile, cinnamaldehyde is a compound that is found in cinnamon bark oil and cinnamon leaf oil. Cinnamaldehyde is easily obtained from the steam distillation of the oil of cinnamon bark. It is a yellowish liquid with a strong pleasant aroma. Trans-cinnamaldehyde has strong antimicrobial effects against animal and plant pathogens, food poisoning and spoilage bacteria and mold (Schmidt et al.,2006).

Due to its antibacterial, antifungal, antiviral, antiparasitic and antiseptic properties of cinnamon leaf oil it is effective in fighting vaginal yeast infections, oral yeast infections and stomach ulcers and head lice, as reported by Vangalapati et al.,(2012). Besides, it is used externally as an antiseptic skin to treat minor bacterial and fungal infections of the skin and promote a bright face that enhances the beauty of skin.

1.10 Problem statement

Cinnamon leaf oil has anti-inflammatory, antioxidant and antimicrobial activities. However, this oil can cause irritation and allergic reaction if undiluted oil is applied directly to the skin. Therefore, in overcoming the shortcomings the present study develops cinnamon leaf oil nanocream formulation. In addition, nanocream formulation has droplets in nanosize range which increases surface area for drug absorption. Hence, it will improve penetration of drug through the skin.

1.11 Objectives of the study

The objective of the study was to develop cinnamon leaf oil nanocream. This study was sectioned into various stages with the following objectives:

i. To validate HPLC method for determination of eugenol in nanocream preparation.
ii. To evaluate the nanocream formulation based on droplet size and rheological properties.
iii. To evaluate the *in vitro* release of eugenol from selected cinnamon leaf oil nanocream formulation.
iv. To examine the *in vitro* antimicrobial activity of cinnamon leaf oil nanocream.
v. To evaluate the *in vivo* performance of the optimized formulation of cinnamon leaf oil nanocream on wound healing in rats.
vi. To conduct stability studies of selected cinnamon leaf oil nanocream at 25°C±2°/61-91%RH temperature and 40°C/75%RH for 6 months.

CHAPTER TWO

FORMULATION AND CHARACTERIZATION OF NANO-CREAM PREPARATION

2.1 Introduction

Cream or semisolid emulsion is one of the pharmaceutical topical formulations derived from the combination of oil and water. It is applied for external used. Commonly this semi-solid base that is either hydrophobic or hydrophilic in character is added with effective drug that has remedial value (Aulton,2007). The creams can be categorized as water-in-oil or oil in water. If there is difficulty in joining immiscible compounds, it can be done by mechanical agitation or heating process(Kumar et al.,2011). An area of cream or Emulgel structure is usually understood by constructing a pseudoternary phase diagram. This diagram can be used to show the effect of changes in volume fraction of different phases on the phase behaviour of the system. The selected formulation from pseudoternary phase diagrams must have features for droplet size, zeta potential and rheological properties.

2.2 Rheological Properties

Materials can be categorized as solid or fluid depending on their flowability. Rheology can be defined as the study of the flowability of materials especially in the liquid state when force is applied. However, in response to the applied force, 'soft solids' or solids will react in plastic flow rather than deforming elastically. Generally, the shear stress is force per unit area is required to produce flow. The unit of stress

measurement is *dyne/cm*^{2} or *newton/m*^{2} which is equal to 1 Pa. The flow of fluids is called viscous flow. When very low force such as gravity force is applied, the flow and shape of the fluid will change.

The viscosity of the fluid is described as its resistance to flow or movement and the unit of viscosity measurement is poise and Pascal second. Viscosity or flow of the fluid can be determined or analysed using different kinds of instruments such as capillary viscometer and rotational viscometer. The relation between viscosity (η), shear stress (F) and the shear rate (G) is summarized by Newtonian equation (Abdulkarim et al.,2010):

$$\eta = F/G \qquad \text{(Eq.2.1)}$$

According to this relationship between shear stress and shear rate, the flow of fluids can be divided into Newtonian and non- Newtonian. Fluids that follow Newtonian equation are called as Newtonian fluids. Newtonian fluid can be described by a steady viscosity that is not affected by shear rate or shear stress value. The rheogram of Newtonian fluids pass through the origin and the rate of shear are directly proportional to the shear stress. Meanwhile, Non-Newtonian flow can be subdivided into plastic, pseudoplastic and dilatants based on rheogram. Rheogram is the curve obtained by plotting rate of shear on the ordinate and the corresponding shear stress on the abscissa.

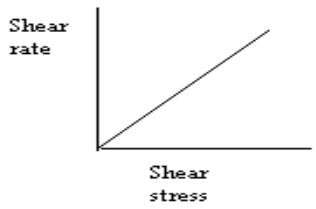

Figure 2.1: Rheogram of Newtonian flow

Plastic or Bingham flow is due to the rheogram that does not pass through the origin, but intersect with the shear stress axis at the point normally has yield value. Plastic substance displays elastic behaviour at the low shear stress with no obvious movement. At shear stress higher than the elastic forces, it shows a nonlinear increase of shear rate. The slope of the curve is slowly decreased with the increasing rate of shear, therefore the viscosity decreased as the shear rate is increased. The system is called shear thinning or thixotrophy.

Pseudoplastic flow is characterized by a rheogram passing through the origin and has no yield value. The material will flow as soon as a shear stress is applied and the slopes of the curve slowly decrease with increasing of shear rate. The viscosity is derived from the slope and therefore it is decreased as the shear rate is increased. Materials exhibiting this behavior have no single value of viscosity.

Characteristic of dilatant is opposite to the pseudoplastic whereas the viscosity increases alongside increases in shear rate. Thus, this system is called as shear thickening.

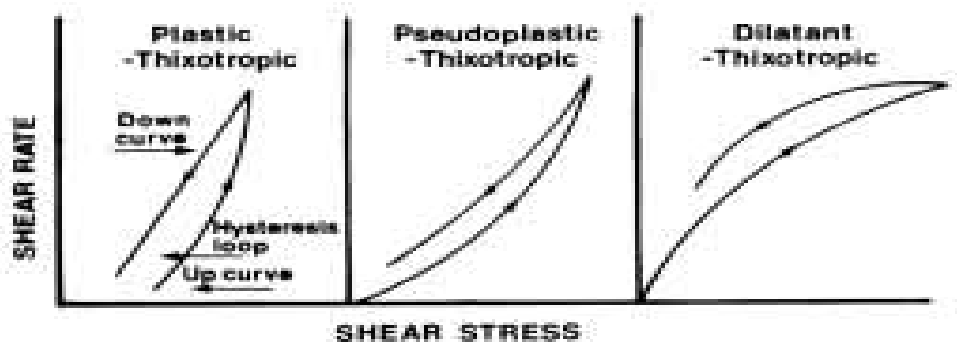

Figure 2.2: Rheogram of different types non- Newtonian flow properties (Korhonen,2004).

2.3 Droplet Size and Zeta Potential

Normally, nano-emulsions are called as submicron emulsion, mini-emulsion or ultrafine emulsion with the size range around 100-600nm (Sharma et al.,2011). In a stability study of cream and emulsion, dispersity of the droplets is very important. If the distribution is more uniform, it can produce a stable emulsion with a long lifetime.

Zeta potential is an acronym for electrokinetic potential in colloidal systems. Zeta potential indicates the number of repulsion between closest, similarly charged particles in dispersion. When potential is too low, attraction exceeds repulsion and the dispersion will flocculate and form aggregates. So, colloids with high zeta potential are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate.

2.4 Accelerated stability tests

Accelerated stability tests are conducted to assess the physical stability of the cream using macroscopic examination. It consists of two methods which are storage at temperature cycles (heating and cooling) and centrifugation. Temperature cycles consisting of storage for several hours at about 40°C are followed by refrigerator or freezing until instability becomes evident have been successfully. This method of accelerated stability testing is particularly useful for assessment of crystal growth in cream. It is also important to ensure that the cream is deflocculated. Meanwhile, centrifugation is the suitable method for artificially increasing the rate of sedimentation of the cream. The process of centrifugation may destroy the structure

of the flocculated system and give a useful indication of the relative stabilities of the trial products (Aulton,2007).

2.5 Objectives of the study

The objectives of this study are:

i. To select the most suitable ratio of palm oil, surfactant mixtures at a particular HLB value and water, which yields a large cream area within the pseudoternary phase diagram

ii. To characterize the selected formulations in term of droplet size, zeta potential and rheological properties.

2.6 Materials and Methods

2.6.1 Materials

Palm oil (Seri Murni) was purchased at Tecso Sg Nibong, Penang, Polysorbate 80 (Tween 80), cetostearyl alcohol and Cinnamon leaf oil were purchased from Euro Chemo-Pharma Sdn Bhd (Penang), Sorbitan tristearate (Span 65) was purchased from Fluka (Malaysia), propyl paraben, methyl paraben and Carbitol (Diethylene glycol monoethyl ether) were purchased from Sigma-Aldrich (Malaysia), Sodium citrate and citric acid were purchased from R & M Chemicals (UK).

2.6.2 Methods

2.6.2 (a) Pseudo Ternary Phase Diagram Construction

The study began with constructing various pseudoternary phase diagrams by using different ratios of palm oil, surfactant of different HLB values and water. Phase

diagrams of a mixture containing palm oil, surfactants of different HLB values and water were constructed using the water titration method modified from Abdulkarim et al.,(2011). From the diagrams, several suitable formulas in different HLB values will be selected to produce nanocream containing cinnamon leaf oil.

Oil and surfactant mixtures were prepared at ratios of 9.0: 1.0, 8.0:2.0, 7.0:3.0, 6.0:4.0, 5.0:5.0, 4.0:6.0, 3.0:7.0, 2.0:8.0, and 1.0:9.0 in a separated universal bottle which was enough to make the end weight of 20gm. Distilled water was added 1ml every fifteen minutes and the changes in the mixtures were recorded. Generally, gel or cream phase can be identified if the formulations do not flow when the container is tilted at 90°. All the mixtures forming O/W, W/O and gels were marked and plotted in the ternary phase diagrams. Six pseudo-ternary phase diagrams were constructed using two different surfactants in the ratios of 90:10, 70:30 and 60:40. Mixtures of Tween 80 and Span 65 which had the HLB value of 13.71, 11.17 and 9.84 were investigated. In addition, mixtures of Tween 80 and Carbitol producing HLB value of 13.92, 12.84 and 10.68 were also studied. The mixtures were kept for 24hours at room temperature to achieve equilibrium. Then, the final visual observation was recorded according to the categories shown in the Table 2.1 (Abdulkarim et al.,2011). The conductivity of resulting mixtures was measured using electrical conductometer to classify them as an O/W emulsion or W/O emulsion. The results were plotted in the pseudoternary phase diagram using software Chemix School 3.

Table 2.1: Categories of visual observation

Category	Description
Microemulsion	Transparent or translucent and can flow easily
Liquid crystal	Transparent or translucent nonflowable when inverted 90°
Emulsion	Milky or cloudy and can flow easily
Emollient gel or cream	Milky or cloudy nonflowable when inverted 90°
Bicontinuous phase	More than one type of dispersion exists in the mixture, as indicated by the presence of more than one abbreviation of dispersions

2.6.2 (b) Method of preparation primary nanocream

The method was modified from Baie and Sheikh K.A(2000). The oil and water phase were heated in the water bath separately in two different beakers at 55°C with continuous stirring at 350 r.p.m for 30 minutes using a magnetic stirrer. The oil phase consists of palm oil, propyl paraben (0.05%), and Span 65 while the water phase containing Tween 80, buffer pH 5.5 and methyl paraben (0.1%). Then, the oil phase was dispersed in the water phase. Nanoemulsion systems were continuously mixed using a magnetic stirrer at 350 r.p.m with the aid of spatula to overcome the liquid crystalline phase. After a while, the mixture was stirred at 1500 r.p.m for 30 minutes. Finally, the mixture was homogenized using T25 Ultra-Turrax (IKA, USA) at 19,100 r.p.m for 2 minutes.

2.6.2 (c) Method of preparation of formulation

The rheological properties of the primary nanocream were modified using cetostrearyl alcohol. The percentage of cetostearyl alcohol as rheology modifiers were derived from the palm oil percentage in the primary nanocream. The oil phase: cetostearyl alcohol, cinnamon leaf oil, palm oil, Span 65 and propyl paraben were heated in the water bath at temperatures 55°C while the water phase: Tween 80, buffer pH 5.5 and methyl paraben were heated separately at the same temperature for 30 minutes. The mixtures were stirred separately at 350 r.p.m using a magnetic stirrer. Then, the oil phase was dispersed in the water phase and mixed using a magnetic stirrer at 350 r.p.m with the aid of spatula to overcome the liquid crystalline phase. After a while, the mixture was stirred at 1500 r.p.m for 30 minutes. Finally, the mixture was homogenized using T25 Ultra-Turrax (IKA, USA) at 19,100 r.p.m for 2 minutes or 4 cycles (1 cycle = 30 second homogenization and 30 second rest) and the preparation was left to cool to room temperature before further characterization.

The buffer pH 5.5 (0.1M) was prepared by dissolving 0.73gm of citric acid in 38.2mL distilled water and 0.35gm sodium citrate in 11.8mL distilled water and the solutions were mixed together.

2.6.2 (d) Accelerated stability study

Two methods were used in the accelerated stability study, centrifugation and heating cooling cycle. In centrifugation method, 6mL cream formulation was placed in the 10mL graduated tubes and centrifuged at 4000 r.p.m for 30 minutes (Eppendorf centrifuge 5702 R) (Alam et al.,2012). Heating cooling cycle method was carried out in two different temperatures. Firstly, 6mL of the nanocream formulation was placed

in a 10mL graduated tube and freezed at temperature -8°C for 24 hours followed by storing at 45°C for 24 hours to complete 1 cycle. The experiment was repeated for 6 cycles to determine the stability of the nanocream by observing separation and coagulation in the nanocream (Baie and Sheikh K.A,2000).

2.6.2 (e) Droplet size measurement

The droplet sizes of formulation were measured by using Zeta Sizer 1000 HS_A, (Malvern Instrument,UK) which is based on the basic principle of photon correlation spectroscopy. The sample was diluted with the buffer to get the K count in between 50-200 as required by machine consistency before reading the droplet size (Abdulkarim et al.,2010).

2.6.2 (f) Zeta potential Measurement of Nanocream

Zeta potential of the formulation was measured using Zetasizer Nano ZS (Malvern,UK). Zeta potential of the formulated nanocream was determined to ensure that they are within the limit of ±30 because within this value the droplets do not coalesce. The formulations were diluted with the same buffer solution used as the external phase in the formula to fix the ionic strength and reduce the droplet count. The bubbles were eliminated from the samples before measurement to prevent change in the mobility of the droplets in the samples (Mahdi et al.,2011)

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2.6.2 (g) Rheological and apparent viscosity measurements

Rheological study was conducted on several formulations which contain no cinnamon leaf oil was modified from Abdulkarim et al.,(2010). The rheological measurements were carried out by using rheometer (rheological instrument AB,