DEVELOPMENT AND CHARACTERIZATION OF TOPICAL PHOSPHOLIPID FORMULATIONS CONTAINING *CHROMOLAENA ODORATA* EXTRACT TO AMELIORATE SKIN AGEING

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by

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LIST OF ABBREVIATIONS

AP-1	Activator protein
CH	Cholesterol
CLSM	Confocal Laser Scanning Microscopy
CO	*Chromolaena odorata*
DCFDA	2',7'-dichlorofluorescein diacetate
DPPH	2,2- diphenyl-1-picrylhydrazyl
ECM	Extracellular matrix
EE	Entrapment efficiency
EGCG	pigallocatechin-3-gallate
Etho	Ethosomes
HDFa	Normal adult human primary dermal fibroblasts
HPLC	High performance liquid chromatography
ICH	International Conference on Harmonization
IPP	Isopropyl palmitate
Lipo	Liposomes
LO	Lecithin organogels
LOD	Limit of detection
LOQ	Limit of quantification
MAPK	Mitogen-activated protein kinase
MMPs	Matrix metalloproteinases
mV	Millivolt
M.Pa	Megapascal
NMR	Nuclear Magnetic Resonance
Nrf2	Nuclear factor-erythroid 2 Related Factor 2
o/w	Oil by Water
PBS	Phosphate buffer saline
PDI	Polydispersity index
PEO	Polyethylene oxide
PI	Isoelectric point
ppm	Parts per million

PPO	Polypropylene oxide
PS	Phosphatidylserine
PTFE	Polytetrafluoroethylene
Qu	Quercetin
r	Correlation coefficient
RH	Relative humidity
R	Retention
ROS	Reactive oxygen species
rpm	Rotation per minute
RSD	Relative standard deviation
S	Slope
SC	Stratum corneum
SD	Standard deviation
SDC	Sodium deoxycholate
SFE	Supercritical fluid extraction
TEM	Transmission Electron Microscopy
Trans	Transfersomes
TGF-β	Transforming growth factor beta
UV	Ultraviolet
v/v	Volume per volume
w/o	Water in oil

PEMBANGUNAN DAN PERINCIAN FORMULASI FOSFOLIPID TOPIKAL MENGANDUNGI EKSTRAK *CHROMOLAENA ODORATA* UNTUK MENGURANGKAN PENUAAN KULIT

ABSTRAK

Penuaan kulit dipercepatkan oleh faktor luaran seperti sinaran UV dan pencemaran persekitaran. Ketidakstabilan antioksidan semulajadi yang telah digunakan untuk mengurangkan kesan yang merosakkan, bersama dengan keperluan untuk menembusi kulit untuk sampai pada lapisan yang terkesan, adalah cabaran besar. Kajian ini melibatkan penyediaan empat formulasi topikal berasaskan fosfolipid yang mengandungi ekstrak *Chromolaena odorata* (CO) untuk memperbaiki kesan penuaan kulit. Liposom, transfersom, ethosom dan organogel lesitin (LOs) telah dipilih kerana dijangka dapat menyampaikan dan mengekalkan ekstrak tersebut di lapisan dermis dengan mencukupi untuk menghasilkan kesan anti-penuaan. Kajian pencirian menunjukkan saiz purata partikel yang terkecil iaitu 281.77 ± 6.33 nm, telah dibentuk oleh liposom bermuatan CO. Keupayaan zeta formulasi didapati bernilai dalam julat -88.7 ± 8.70 hingga -27.07 ± 0.86 mV, yang mencadangkan bahawa formulasi akan mempunyai kestabilan elektrostatik jangka panjang. Nilai EE yang paling tinggi, iaitu 77.12% ± 2.10, telah ditunjukkan oleh liposom bermuatan CO. Dapatan TEM menunjukkan bahawa semua vesikel adalah berbentuk sfera manakala ^{1}H NMR mengesahkan pemerangkapan molekul ekstrak dalam dwilapisan fosfolipid. pH formulasi yang didapati adalah dalam julat 6.8 hingga 7.4. Kajian kestabilan yang

telah dijalankan untuk menentukan kesan keadaan penyimpanan yang berbeza, mendedahkan bahawa ethosom bermuatan CO mempunyai kestabilan yang paling lama antara semua formulasi, pada 4 °C ± 2 °C dan 30 °C ± 2 °C sehingga satu tahun. Kajian resapan kulit dan enapan kulit *in vitro* yang telah dijalankan menggunakan kulit telinga khinzir dalam sel bauran Franz, menunjukkan bahawa kesemua formulasi bermuatan ekstrak telah menyebabkan enapan ekstrak yang lebih banyak dan lagi kurang resapan merentasi kulit berbanding dengan ekstrak sahaja. Penembusan formulasi yang mengandungi ekstrak ke lapisan kulit yang lebih dalam dengan berkesan, telah diperhatikan dengan menggunakan mikroskopi imbasan laser konfokal (CLSM). Kesan ekstrak CO dan formulasinya terhadap kerosakan yang disebabkan oleh sinaran ultraungu B (UVB) dan H_2O_2 pada fibroblas dermis manusia dewasa (HDFa) telah diselidik. Rawatan dengan ekstrak CO dalam kultur HDFa yang terdedah pada UVB dan H_2O_2 mengekalkan amaun kolagen, dan mengurangkan paras ROS dan MMP-1. Pra-rawatan dengan transfersom bermuatan CO dan ethosom bermuatan CO sebelum pendedahan kepada UVB, menaikkan daya hidup sel, mengekalkan amaun kolagen dan mengurangkan paras ROS dan MMP-1. Walaupun kedua-dua formulasi tidak menunjukkan peningkatan paras kolagen dalam sel HDFa yang ditegas dengan H_2O_2, sel tersebut menunjukkan peningkatan kadar kelangsungan hidup, dan penurunan paras ROS dan MMP-1. Secara menyeluruh, hasil kajian telah mendedahkan potensi ethosom bermuatan CO sebagai formulasi yang dapat menyasar kulit untuk memperbaiki kesan penuaan kulit.

DEVELOPMENT AND CHARACTERIZATION OF TOPICAL PHOSPHOLIPID FORMULATIONS CONTAINING *CHROMOLAENA ODORATA* EXTRACT TO AMELIORATE SKIN AGEING

ABSTRACT

Skin ageing is accelerated by external factors such as UV radiation and environmental pollutants. The instability of natural antioxidants that have been used to counteract the damaging effects, along with the need to penetrate the skin to reach the affected layer, are great challenges. This study involved the preparation of four phospholipid-based topical formulations containing *Chromolaena odorata* (CO) extract to ameliorate the effects of skin ageing. Liposomes, transfersomes, ethosomes and lecithin organogels (LOs) were selected as they were deemed to be able to deliver the extract to the dermal layer and retain it adequately to exert anti-ageing effects. Characterization studies revealed that the smallest mean particle size of 281.77 ± 6.33 nm, was formed by the CO-loaded liposomes. The zeta potential of the formulations was in the range of -88.7 ± 8.70 to -27.07 ± 0.86 mV. The pH of the formulations was in the range of 6.8 to 7.4. Entrapment efficiency (EE) of CO-loaded liposomes and CO-loaded ethosomes was 77.12% ± 2.10 and 69.40% ± 2.29, respectively. Transmission Electron Microscopy (TEM) results showed that all vesicles were spherical in shape whilst ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) confirmed the entrapment of extract molecules in the phospholipid bilayers. Stability studies revealed that CO-loaded ethosomes showed the longest stability, among all the formulations, at both 4 °C

± 2 °C and 30 °C ± 2 °C up to one year. The *in vitro* release study outcomes revealed that the highest percentage of the extract release was observed from CO-loaded ethosomes (68%). The *in vitro* skin permeation and skin deposition studies demonstrated that all the extract-loaded formulations led to more extract deposition in the skin and less permeation across the skin, compared to the extract alone. The effective penetration of the extract-loaded formulations into the skin was observed using Confocal Laser Scanning Microscopy (CLSM). The effects of CO extract and the formulations against ultraviolet B (UVB) rays and H_2O_2-induced damage in human dermal fibroblasts (HDFa) were investigated. Pre-treatment with CO-loaded transfersomes and CO-loaded ethosomes before UVB exposure improved cell viability, maintained collagen amount and reduced ROS and MMP-1 levels. Although both formulations did not show an increase in collagen level in HDFa cells stressed with H_2O_2, the cells displayed an increased survival rate, and reduced ROS and MMP-1 levels. Overall, the results demonstrated that CO-loaded ethosomes has the potential to be used in a skin targeting formulation to ameliorate the effects of skin ageing.

CHAPTER 1

INTRODUCTION

1.1 Skin

Skin is the largest and most vital organ that covers the whole human body and characterized by its significant protective functions (Kanitakis, 2002). Anatomically, it consists of three distinct layers; epidermis, dermis and hypodermis, also known as subcutaneous tissue. The superficial layer is called the epidermis, which is the topmost layer of the skin; dermis is the second and thicker layer; and the hypodermis layer is the bottom layer of the skin (Figure 1.1) (Vanić, 2015). The skin surface area of an average human adult exceeds 2 m^2 (Kolarsick *et al.,* 2011). Skin thickness varies according to the function, in which skin over the eyelids is 0.1 mm thick and over feet soles is up to 2 mm in thickness (Powell and Soon, 2002).

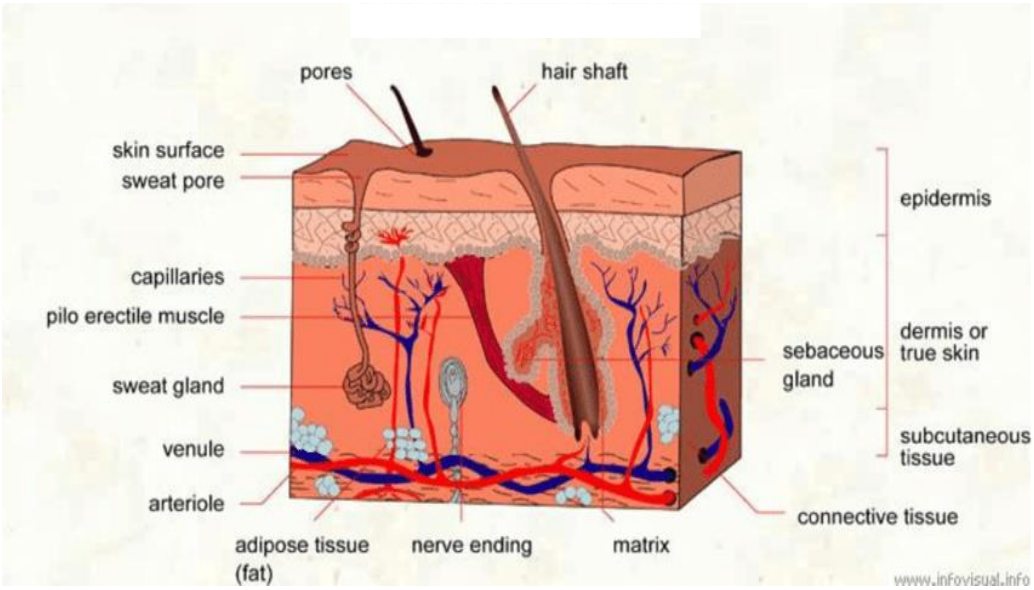

Figure 1.1 Cross section of the human skin (Raju *et al.*, 2019)

The epidermis is the external layer of the skin. Keratinocytes is the principle cell type in this skin layer, comprising 80% of the total epidermis cells. The epidermis also contains the melanocytes, Merkel cells and Langerhans' cell (Gaboriau and Murakami, 2001). The epidermis is divided into four layers depending on the various stages of differentiation of keratinocytes including the top layer called stratum corneum (SC), stratum spinosum, stratum granulosum and the germinative layer (McGrath *et al.*, 2004). At birth, the epidermis is thin and becomes thicker at youth and during early adulthood, then get thin again in the fifth to sixth decades of life (Gaboriau and Murakami, 2001). Keratin is the major fibrous protein of the epidermis which is synthesised by keratinocytes. The epidermal keratin filaments first appear in small, wavy and lateral aggregates. These filament bundles grow in size and length as they travel through the cells, forming complex intracellular networks that bind to desmosomal junctions on the cell membrane (Powell and Soon, 2002). Melanocytes are another cell type found in the basal layer of epidermis. These cells produce melanin, a pigment that acts as a protector for the nucleus of the keratinocytes from ultraviolet (UV) radiation (Gaboriau and Murakami, 2001).

The dermis is the inner layer of the skin, that is found in between epidermis and the hypodermis layers. The dermis is attached to the epidermis by the dermoepidermal junction, that provides mechanical support to the epidermis layer, as well as acts as a barrier to cells and chemicals (Gaboriau and Murakami, 2001; Kolarsick *et al.*, 2011). Dermal fibroblasts are the most common cell type in the dermis layer. The primary role of fibroblasts cells in the dermis layer is to synthesise extracellular matrix (ECM) proteins including collagen and elastin (Halper, 2018). Collagen fibres, which are tough bundles of collagen and a major

component of the ECM that supports most tissues, play an important role in skin mechanical strength and extensibility. Collagen makes up 75% of the fat-free dry weight of the skin (Joodaki and Panzer, 2018). Therefore, the dermis plays an important role in maintaining the elasticity and firmness of the skin as dermal fibroblasts secretes two major components, collagen and elastin fibres, providing the structural skeleton to the skin (Bravo *et al.,* 2017). Finally, the hypodermis which is the deepest layer of skin and contains adipose lobules, sensory neurons and blood vessels (Kanitakis, 2002).

1.1.1 Skin ageing

There are several problems that the skin encounters, one of these is skin ageing which is an unavoidable aspect of human life and defined as the amassing of skin damage overtime (Tobin, 2017). Skin ageing is a complex multifactorial mechanism involving physical and biological changes in the skin. It can be indicated by certain signs for instance wrinkles, lines, spots, falling hair, acne and pimples, loss of skin elasticity and strength as well as development of pigmentation. Skin ageing is also characterised by thinning of the epidermis due to a reduction in vascularity and hydration, with no change in the number of epidermal layers (Greenwald *et al.,* 2016; Bravo *et al.,* 2017). When skin ages, the amount of skin cells is reduced and consequently disturbs the homeostasis in the skin. The loss of collagen and elastin fibres increases the rate of depletion in ECM. Besides, the production of serum and the ability to hold moisture on the skin is reduced. Thus, aged skin tends to have thinner epidermis and dermis, formation of wrinkles and irregular pigments, poor wound healing and laxity (Swalwell *et al.,* 2012). These contribute to an old appearance in skin. Many factors can lead to premature skin

ageing like environmental pollutants, poor care and exposure to UV radiation (Waller and Maibach, 2005; Gaur *et al.*, 2017).

Generally, skin ageing can be caused by two biologically distinct mechanisms, intrinsic and extrinsic ageing. Intrinsic ageing is unpredictable and unavoidable, which will happen in all the organs in the body due to the physiological changes of hormones and steroid production. Meanwhile, extrinsic ageing involves external and environmental factors, such as pollution, radiation, repeated facial expressions, gravity, sleeping positions, smoking, life style and exposure to sun light (Fisher *et al.,* 2002; Makrantonaki *et al.,* 2006; Maity *et al.,* 2011). Photo ageing is a term used to describe extrinsic ageing caused by prolonged exposure to sunlight, especially UV light. Age lines, rough and leathery skin, spider veins on the forehead, fine wrinkles that vanish when stretched, solar elastosis, actinic keratosis, loose skin, blotchy complexion and skin cancer are all signs of photo ageing (Wulf *et al.,* 2004).

Collagen, a natural protein found in the dermis, gives the skin elasticity and strength. Collagen synthesis decreases with age, resulting in a loss of elasticity and the appearance of wrinkles (Moon *et al.,* 2010). According to previous reports, changes in collagen precursors can play a role in the wrinkled appearance of human skin (Talwar *et al.,* 1995; Lubin *et al.,* 2004). Therefore, in aged skin, higher levels of collagen degradation and a reduction in collagen synthesis occur (Papakonstantinou *et al.,* 2012).

Matrix metalloproteinases (MMPs) are zinc-containing endopeptides which mediate the degradation of different components in ECM (Kim *et al.,* 2011). It is secreted by keratinocytes and dermal fibroblasts due to exposure of multiple

stimuli such as oxidative stress and UV radiation. There are about 28 types of MMPs, which can be grouped into collagenases, gelatinases, stremelysins, matrylysins, membrane-type and others (Pittayapruek *et al.,* 2016). Collagenase acts as a starter, which degrades the collagen into fragments, while other MMPs such as gelatinase do the rest of the degradation such as hydrolyse the fragments and cause the deterioration of the ECM (Kim *et al.,* 2011). Three types of collagenase, namely MMP-1, MMP-8 and MMP-13, have similar structures with small differences in binding sites; however, MMP-1 takes the most important role in degradation of collagen specifically collagen type I and III (Sbardella *et al.,* 2012). Collagen type I is the most abundant type of collagen found in the ECM of skin, while collagen type III is present in a small amount. Meanwhile, UV has been reported to have limited effect on the up regulation of MMP-8, indicating that it has minimal influence on UV-induced collagen loss in skin (Brennan *et al.,* 2003). On the other hand, MMP-13 does degrade collagen type I and III, but it is less potent compared to MMP-1 (Sbardella *et al.,* 2012). Thus, MMP-1 contributes the most to the degradation of collagen. Furthermore, fibroblasts cells play a key role in ageing as they can synthesise collagen and degrade collagen through secretion of MMPs (Fisher *et al.,* 2009).

While intrinsic and extrinsic skin ageing are two separate processes, their molecular mechanisms are similar. Reactive oxygen species (ROS) and free radical produced by cell metabolism, for example, play an important role in both processes (Papakonstantinou *et al.,* 2012). Furthermore, photo ageing is thought to occur as a result of the development of ROS, the subsequent activation of activator protein-1 (AP-1), the subsequent induction of MMPs, reduced collagen synthesis and inflammation (Fisher *et al.,* 1997). Past research showed that the

antioxidant enzymes and antioxidants are dominant factors which affect the process of photo ageing. Four antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, along with antioxidants, namely α-tocopherol, ascorbic acid, uric acid and glutathione were tested *in vivo* after exposure of UV. Results showed that catalase activity and antioxidant level became significantly lower in dermis after exposure to UV (Rhie *et al.*, 2001). This indicates that UV is lowering the antioxidant capacity in skin which leads to oxidative-induced cell damage in later stage. Besides, excessive ROS also activates the local inflammatory response. Overexposure of these inflammatory cytokines promotes further damage to the cell, which leads to apoptosis and cell death (Abdel-Daim *et al.*, 2010). Moreover, the high amount of ROS also affects the activity of protein-tyrosine phosphate-K, which is important in maintaining the activity of cell surface receptors (Sahu *et al.*, 2013). Suppression of this enzyme induces the activity of mitogen-activated protein kinase (MAPK). Subsequently, the MAPK pathway produces AP-1 transcription factor complex, which modulates the expression of MMPs, transforming growth factor beta (TGF-β) signalling and the production of procollagen type 1 (Schwartz *et al.*, 1993; Hwang *et al.*, 2014; Chen *et al.*, 2015; Sun *et al.*, 2015). Invisible solar scars result despite the subsequent repair procedure, which includes tissue inhibitors of MMPs. A lifetime of acute exposures with the accumulation of invisible solar scars will gradually result in visible solar scars, causing photo damaged skin to wrinkle (Kang *et al.*, 2001). Furthermore, the imbalance of removal and formation of ROS and free radical causes oxidative damage to cell such as mitochondrial dysfunction, apoptosis and activation of inflammatory response in skin, which leads to cell death (Limtrakul *et al.*, 2016; Mesa-Arango *et al.*, 2017). Moreover, the

superoxide radicals and hydrogen peroxide (H_2O_2) formed by the mitochondria in the course of normal metabolism, causes damage to nucleic acid, lipids and proteins including collagen. This cumulative collagen damage, disrupts the structural integrity of skin and contributes to the formation of wrinkles (Yang and Li, 2015; Tong *et al.*, 2019). Therefore, the most important factor in skin ageing is oxidative damage, which contributes to the loss of ECM through gene/protein pathways while also inhibiting fibroblasts proliferation.

1.1.2 Protection against skin ageing

There exists an intrinsic defence mechanism against UV radiation because the human skin is continuously exposed to the elements. After UV exposure, the epidermal thickness increases, which helps protect against more UV damage. By absorbing and scattering harmful UV rays, the distribution of melanin pigments is thought to provide protection against sunburn, photo ageing and carcinogenesis (Kaidbey *et al.*, 1979; Rabe *et al.*, 2006). These natural defence mechanisms, however, are considered inadequate in the search to look younger and more attractive. As a result, using cosmeceuticals and other formulations has become a daily habit. There are three photo ageing treatment techniques to protect human skin (Rabe *et al.*, 2006). The elimination of risk factors prior to the incidence of a disease or illness is referred to as primary prevention. The primary prevention is classified as photo protection/sun protection (Glaser, 2004). Primary photo security measures include clothing, hats and sunglasses, among others (Rai and Srinivas, 2007). Antioxidants, retinoids, growth factors, oestrogen and cytokines are examples of secondary prevention, which delays or diminishes the condition (Isnard *et al.*, 2004). Tertiary prevention is the treatment of an existing symptomatic disease in order to reduce the signs and manifestation of the ageing

process. Chemical peels, laser systems, resurfacing procedures, botulinum toxins, radiofrequency technology and soft tissue augmentation are examples of tertiary preventions (Tanzi *et al.,* 2003; Ghersetich *et al.,* 2004).

Although it is impossible to reverse ageing, cosmetics and pharmaceuticals may help to mitigate and postpone the effects. Topical applications of biocompatible and biodegradable lipid carriers for transporting skin care compounds have been considered of great interest in minimising skin ageing (Shehzad *et al.,* 2016).

1.2 Topical delivery systems

Topical delivery system is a type of pharmaceutical dosage form that can be used in a variety of ways, and encompasses dosage types such as gels, ointments and creams. They have a number of advantages over other dosage forms (Gibson *et al.,* 2002). First, topical dosage formulations circumvent the liver first pass effect, which is a possible barrier or restricting factor for most orally administered drugs. Second, patients prefer topically applied systems because of the ease with which they can be applied; in other words, topical dosage forms are applicable to a relatively broad body area and can be easily terminated as required. Since they work topically, they avoid the complications and inconveniences of intravenous injections. Furthermore, topical drug delivery can bypass the absorptive conditions of the gastrointestinal tract, such as pH differences from mouth to colon, enzyme activity and gastric emptying time. Finally, topical dosage forms allow for the treatment of various skin disorders by targeting the skin (Lee *et al.,* 2000; Torin Huzil *et al.,* 2011; Joshi *et al.,* 2014; Singh Malik *et al.,* 2016). Topical dosage formulations have several drawbacks in addition to their many advantages. To

begin with, skin irritations and allergies are fairly common problems that are typically caused by medications and/or excipients, leading to skin dermatitis in some cases. Second, due to the complexities of skin structure in general, and SC, the outermost layer in particular, skin permeation of certain drugs is sometimes low (Kute and Saudagar, 2013; Sultana *et al.*, 2014). The absorption of drugs may be impaired if the dosage type contains large droplets (Sandri *et al.*, 2014). Furthermore, enzymes found primarily in the epidermis of the skin have the ability to denature the applied preparation. Finally, these dosage forms are more successful locally than systemically, meaning that they are applied to a particular area of skin (Cypess *et al.*, 2009; Sharadha *et al.*, 2020). There is currently no optimal drug delivery system that can produce all of the desired outcomes. However, genuine attempts can be made to achieve them by novel drug delivery strategies. Liposomes, niosomes, transfersomes and ethosomes are phospholipid vesicular drug delivery systems that have been developed for topical and transdermal drug delivery (Opatha *et al.*, 2020; Sguizzato *et al.*, 2021; Witika *et al.*, 2021). These novel drug delivery systems have been identified for a variety of routes of administration in order to achieve controlled local and systemic drug delivery.

1.3 Permeation of drugs through skin

The skin, which is made up of various layers, is the key barrier that prevents drugs applied topically from passing through. Because of its structure, especially the SC, the skin serves as a primary barrier, preventing the passage of both foreign and endogenous molecules. In fact, the SC is considered to be the most significant barrier to drug penetration through the skin (Trommer and Neubert, 2006). As a result, the major challenge for topical formulations is to

achieve a sufficient increase in drug penetration into the skin without creating permanent changes to the barrier feature (Wen *et al.,* 2021).

Permeation of drugs through the skin occurs primarily through three pathways. These are intercellular route (restricted to lipid matrix), transcellular route (through both corneocytes and lipid matrix) and transappendageal route (Larin *et al.,* 2011). Lipophilic drug molecules permeate primarily through intercellular route from in between the cells in the lipid matrix. While, through transcellular route, the movement of drugs across the alternate layers of hydrophilic corneocytes and lipophilic lipid bilayers requires partitioning in both (Parhi and Mandru, 2020). Moreover, passive diffusion is considered as the primary mechanism for drug transport through the SC via both intercellular or transcellular pathway (N'Da, 2014). Therefore, permeation of compound along such pathways is thus influenced by the affinity of the compound with lipid environment, with the internal environment of the corneocyte and ability of molecule to permeate through corneocyte cell wall (Jepps *et al.,* 2013). The transappendageal route refers to the delivery of drugs through hair follicles and sweat ducts (Verma *et al.,* 2016). Hair follicles are an invagination of the epidermis that reaches deep into the dermis, providing a larger surface area for drug absorption (Patzelt and Lademann, 2015). Micro particulate systems have been used for follicular drug delivery because hair follicles have a lot of capacity for drug delivery into viable skin layers (Kajimoto *et al.,* 2011).

Physicochemical properties of the drug and dosage form, skin structure and position, and skin physiological conditions all influence the rate and degree of drug absorption through the skin, which most likely affect drug transportation to the systemic circulation (Lee *et al.,* 2000). Furthermore, the assembling of SC matrix

and the hydration level of the skin are main criteria for drug penetration, and they are influenced by a variety of factors such as human race, sex, age, skin type, anatomical location and environmental humidity (Darlenski and Fluhr, 2012). The SC is made up of insoluble keratin bundles encased in a cell envelope and held together by cross-linked proteins and covalently bound lipids (Eckert *et al.,* 2005). To overcome skin barrier properties, a number of advanced technologies have been developed. Physical methods such as iontophoresis, sonophoresis, and microneedles are used in some technologies (Cross and Roberts, 2004; Nanda *et al.,* 2006). There are also chemical means which can induce irritation, cause damage, and reduce skin barrier function (Kanikkannan and Singh, 2002). On the other hand, in the past decades, phospholipid vesicular systems for topical delivery of drugs such as liposomal formulations have gain considerable interest in achieving the desired skin penetration and targeting delivery of drugs (Shim *et al.,* 2003; Thong *et al.,* 2007).

1.4 Herbal medicine

There are many different definitions of herbal medicine, simply it is the science of using plants to treat medical conditions (Pal and Shukla, 2003). Herbal medicines have been used by many people to directly treat a medical problem or relieve their symptoms, such as using aloe vera to soothe a mild skin wound irritation (Rajeswari *et al.,* 2012). Some herbals can also be used to help treat disease, such as cinnamon in the treatment of urinary tract infections (Fazly Bazzaz *et al.,* 2021). Hippocrates, an ancient Greek physician known as the "Father of Medicine," described over 500 different plants that could be used medicinally (Pollio *et al.,* 2008). Before the introduction of modern medicine, this was one of the resources available to people. People apparently had to rely on other items to

help cure their medical ailments before we had antibiotics and medical lifesaving equipment (Pan *et al.,* 2014). As a result, the importance of these plants to the survival of cultures all over the world was critical. Even with rapidly evolving medical technology, herbals are still important today (Colombo *et al.,* 2020). In fact, herbals are still being used widely in some developing countries due to economic reasons or a lack of pharmaceuticals (David *et al.,* 2015). Furthermore, several pharmaceuticals that have been investigated in research laboratories have been extracted from medicinal plants (Gurib-Fakim, 2006).

Pharmaceuticals, in general, have a higher potency due to the single concentrated active ingredients, and thus have a greater therapeutic effect. As a result, there is a higher chance of further side effects. Moreover, the single concentrated ingredient in pharmaceuticals also means it basically exerts a single action. Herbals, on the other hand, may have numerous effects due to the large variety of active constituents found in a single plant (Schmidt *et al.,* 2008). These active ingredients also function synergistically, which means that they complement each other and support one another (Patwardhan and Gautam, 2005). Furthermore, several factors such as the plant component, season, environment, growth phase, harvesting conditions, extraction solvent, extraction process and plant material to solvent ratio influence the biological activity of medicinal plants (Maji *et al.,* 2010; Kothari *et al.,* 2012). On the other hand, these factors are not considered variables that will affect the characteristics of a synthetic drug compound commonly used in conventional pharmaceuticals.

1.4.1 Herbal medicine for treating skin ageing

For centuries, several herbs have been used in medicine and cosmetics. Their ability to treat a variety of skin diseases as well as to improve the appearance of the skin is well-known (Delfan *et al.*, 2014). Since UV radiation can cause sunburns, premature ageing, wrinkles, lowered immunity to infections and cancer, it is important to protect the skin at all times (Ralf Paus *et al.*, 2008; Yamada and Prow, 2020). Herbs and herbal preparations have a great deal of promise due to their components activity. Recently, natural bioactive components such as phenolic acids, flavonoids and high molecular weight polyphenols have been unveiled for their photo protective activity and capability in dealing with ageing, either through topical application or oral administration. In fact, antioxidants such as vitamins, flavonoids and phenolic acids are important in suppressing free radical species, which are the root cause of a variety of undesirable skin changes (Korać and Khambholja, 2011; Wojdyło *et al.*, 2018; Siddeeg *et al.*, 2021).

Involvement in up-regulating oxidative stress related genes, by several compounds available in different plant extracts, plays an important role in enhancing the survival rate and flexibility of skin cells towards stress. Nuclear factor-erythroid 2 related factor 2 (Nrf2), a transcription factor that regulates cellular redox homeostasis, plays an important part in combating oxidative stress in cells (Schmidlin *et al.*, 2019). In the presence of free radicals, the active Nrf2 will bind to Adenylate uridylate-rich element (ARE) and initiate the transcription of genes that are involved in oxidative stress, such as heme oxygenease-1 (HO-1), superoxide dismutase (SOD) and catalase (CAT) (Itoh *et al.*, 1997; Kensler *et al.*, 2007; Buendia *et al.*, 2016). Besides, previous research showed that Nrf2 was downregulated in older fibroblasts and more sensitive towards the effect of

oxidative stress (Kapeta *et al.,* 2010). Therefore, extract and bioactive compounds which can up-regulate Nrf2 transcription can potentially reduce oxidative stress. In fact, compounds from plants have been shown to protect against oxidative stress through the activation of the transcription of oxidative genes such as Nrf2 and HO-1 (Liu *et al.,* 2019) SOD and CAT (Hahn *et al.,* 2017). Several mechanisms could be involved to explain the protection of fibroblasts from UVB radiation and H_2O_2-induced stress. For example, an unloading effect on the expression of AP-1 and Nuclear factor kappa B (NF-κB) (Mohamed *et al.,* 2014), stimulation of TGF-β (Hwang *et al.,* 2013), genetic upregulation of the genes COL1a1 and COL3a1 (Permatasari *et al.,* 2014), or reduced intracellular ROS production, triggering cell signalling pathways, leading to the cleavage and affect synthesis of collagen (Bravo *et al.,* 2017). Previous report stated that oral feeding of UVB-radiated mice with anthocyanin enriched purple sweet potato extract showed improvement in collagen deposition and moisture content, through increasing antioxidant activities and suppressing the inflammatory activity (Zhi *et al.,* 2020). Similarly, UVB-radiated mouse showed reduction in the level of protein oxidation and MMPs after drinking the infusion of green tea polyphenols, a mixture of major catechin with water (Vayalil *et al.,* 2004). Topical application of red raspberry extract, which is mainly cyanidin and ellagic acid, on the UV-irradiated nude mouse skin prevented the oxidative damage through activation of oxidative signalling pathway, ROS scavenging activity and inhibition of the apoptosis and inflammatory pathways (Wang *et al.,* 2019b). *In vitro* studies of green tea extract, *Litchi chinensis* leaf extract and *Morella parvifolia* extract demonstrated that these extracts protected the skin cells from UV injured through their ability to absorb certain intensity of UVA and UVB and their antioxidant activity (Kaur and Saraf, 2011;

Ebrahimzadeh *et al.*, 2014; Thiesen *et al.*, 2017; Puertas-Mejía *et al.*, 2018). Furthermore, polysaccharides from aloe showed protective effects on UVB-irradiated skin nerve cells through lowering the oxidative stress caused by UV (Yuan *et al.*, 2020). These evidences showed that plant extracts and its bioactive components act through several signalling pathways, which is suitable in multifactorial disease such as skin ageing. Furthermore, the majority of biologically active plant constituents are polar or water soluble molecules. However, water soluble phyto-constituents like tannins, flavonoids, glycosides are poorly absorbed either because of their poor lipid solubility or due to their large molecular size that cannot be absorbed by passive diffusion, strictly limiting their ability to pass across the biological lipid membrane of the skin and lack of specific sites targeting, leading to poor bioavailability. As a result, it must be formulated in order to produce the desired therapeutic effects in the targeted area (Monica and Naik, 2014).

1.5 *Chromolaena odorata* (CO)

According to King and Robinson (1970), *Chromolaena odorata* (CO) belongs to the Eupatorieae family. The Eupatorieae belongs to the Asteroideae subfamily and is well-defined taxonomically. It is native to Florida and Texas, as well as Mexico and the Caribbean. It is an aggressive introduced species that forms dense stands in tropical Asia, West Africa, and parts of Australia, preventing the establishment of other plant species (Gautier, 1992; Zachariades *et al.*, 2009; Vaisakh and Pandey, 2012; Omokhua *et al.*, 2016).

Many parts of CO, obtained from various locations, were used to determine the chemical composition (Apoxi *et al.*, 2000; Akinmoladun *et al.*, 2007; Owolabi

et al., 2010; Prabhu *et al.,* 2011; Félicien *et al.,* 2012; Usunomena and Efosa, 2016). Sinensetin, salvigenin, lupeol, rhamnetin, tamarixetin, ombuin, isosakuranetin, and luteolin are only a few of the compounds that have been isolated from CO (Barua *et al.,* 1978; Valant-Vetschera and Wollenweber, 1995; Suksamrarn *et al.,* 2004). Numerous flavonoids like isosakuranetin (Bose et al., 1973), quercetagetin (Wollenweber and Roitman, 1996), salvigenin (Talapatra et al., 1974), scutellarein tetramethyl ether and sinensetin (Barua et al., 1978) can be obtained from the leaves of this plant. Coumarins, tannins, steroids, saponins, terpenoids, terpenes, flavonoids and cardiac glycosides are also contained in the leaf extract (Thophon *et al.,* 2016). CO has historically been used to speed wound healing in the Malay culture by crushing the leaves before adding to the wound (Ebrahimi *et al.,* 2020). CO was one of eleven medicinal plants used commonly in Ghana (Agyare *et al.,* 2009). In Thailand, the juice of the leaves is used as a haemostatic on wounds and as an anti-inflammatory treatment (Intekhab and Aslam, 2009). Despite the fact that multiple compounds have been extracted from this plant extract using various methods, the majority of published studies over the years have not used a single constituent of CO extract. Instead, the majority of researchers used either the whole CO extract or fractions of it. In a study, Suksamrarn *et al.* (2004) discovered that in traditional medicine, CO leaves decoction is used as a cough remedy and as an ingredient in malaria treatment with lemongrass and guava leaves. Flavonoids derived from CO flowers have had moderate to poor anti-mycobacterial activity, moderate cytotoxicity activity against human small cell lung cancer and mild cytotoxicity activity against breast cancer cells (Suksamrarn *et al.,* 2004). Other bioactive properties reported to be shown by CO are antiprotozoal, astringent, antibacterial, hepatotropic, antitrypanosomal, diuretic,

antihypertensive, analgesic, anthelimintic, antispasmodic, anti-inflammatory and antipyretic activities (Taiwo *et al.,* 2000; Owoyele *et al.,* 2008; Patel *et al.,* 2010). Furthermore, over many years, interest in CO extract has been noticed because of its numerous activities in treating skin disorders including wound healing properties (Phan *et al.*, 2001), in addition to its ability to accelerate blood clotting (Triratana *et al.,* 1991). In general, plant extracts containing flavonoids have long been used in dermatology and cosmetics (Arct and Pytkowska, 2008). CO has been stated to have a variety of biological activities due to its high flavonoid and phenolic content. It has been found that CO extracts have antioxidant properties in general, protecting fibroblasts and keratinocytes from induced damage (Phan *et al.*, 2001). Moreover, CO methanolic leaf extracts display potentially high antioxidant activity, according to a previous report (Melinda *et al.,* 2010). As a result, it would be a good candidate for formulation into a cosmeceutical for skin health purposes.

In this study, CO was extracted using supercritical fluid extraction (SFE) method, which is carbon dioxide-based fluid that is fully evaporate. When a fluid is heated and compressed past its critical stage, it becomes supercritical. The properties of supercritical fluids are similar to those of both liquid and gas phases. Supercritical fluids have a lower viscosity than liquids and are comparable to gases in terms of diffusivity. Carbon dioxide is a relatively inexpensive, non-flammable, safe and mild solvent that can be used in both experimental and industrial applications. It is a non-polar molecule with some polarity due to its quadrupole moment (Perrut, 2000; Reverchon *et al.,* 2007; Gönen *et al.,* 2009; Sapkale *et al.*, 2010).

In general, SFE has gotten a lot of attention in recent years as a result of rising environmental and health concerns about conventional organic solvent

extraction (Zougagh *et al.*, 2004). In contrast to ethanol extraction, the advantages of SFE using carbon dioxide are that it is relatively fast and environmentally friendly, with low operating temperature (thus no thermal degradation of most active compounds), high selectivity in the extraction compounds and no solvent residue (Tan and Lee, 2011). The disadvantage of using SFE-CO_2 is the high cost of supplementary equipment and its low polarity, which makes polar part extraction difficult. However, small quantities of polar modifiers, such as methanol or ethanol, can improve the efficiency of extraction (Karale Chandrakant *et al.*, 2011). On the other hand, loss of volatile compounds, long extraction times, toxic solvent residues and heat-induced degradation of active compounds are drawbacks of solvent extraction method (Liang *et al.*, 2008; Yang *et al.*, 2012).

1.6 Phospholipids

Phospholipids belong to a class of lipids that is a main constituent of cell membranes (Chang *et al.*, 2019). In general, the phospholipid molecule has a tail that consists of two hydrophobic fatty acids and a head that consists of a hydrophilic phosphate group and a glycerol molecule connects these two components (Tan *et al.*, 2017). The long fatty acid tail chains are uncharged and do not dissolve in water, while the phosphate head group is very negatively charged and can quickly dissolve in water by forming hydrogen bonds. In aqueous solution, hydrophilic heads and hydrophobic tails of phospholipids interact to produce a phospholipids membrane or phospholipids bilayer that is two molecules thick, the head groups are directed outwards where they interact with the surrounding water and the tails are packed together in the interior of the bilayer. Poor hydrophobic interactions hold the bilayer together, preventing several materials from passing through (Anamourlis, 2020). Membrane fluidity and

versatility are due to the ability of individual phospholipid molecule to travel inside the bilayer. This fluidity allows membranes to spontaneously split and reform (endocytosis and exocytosis) (Rasch *et al.,* 2010). In biological systems, phospholipids are found in a bilayer cell membrane with other molecules such as proteins, glycolipids and sterols. Since lipids act as a solvent for all the substances and proteins within the membrane, proteins and lipid molecules are free to spread through the lipid matrix and travel through it (Ramanathan *et al.,* 2013). Therefore, phospholipids have a unique amphiphilicity and an eminent biocompatibility characteristic which make them suitable agent or excipient in drug delivery systems for the purpose of therapeutic applications (Li *et al.,* 2015). Phospholipids have been used in drug delivery systems to increase the bioavailability of drugs with low aqueous solubility or low membrane penetration, improve drug uptake and release profiles, protect sensitive active agents from degradation in the gastrointestinal tract, minimise side effects and mask bitter taste (Date and Nagarsenker, 2008).

Phospholipids are classified into two categories: natural phospholipids and synthetic phospholipids (Li *et al.,* 2015). Phospholipids are found in both animals and plants, with soybean, sunflower, corn, cotton seed, and rapeseed oil being the most common sources in plants. Egg yolk and bovine brain are considered essential sources of phospholipids in animal tissues. Egg yolk and soybean are the most important sources of natural phospholipids in terms of output (Lordan *et al.,* 2017). Meanwhile, since chromatographic purification techniques have yet to isolate a single constituent of naturally occurring phospholipids, researchers have turned their attention to chemical synthesis, which can produce a specific component with a specified structure and configuration. Phospholipids synthesis

is divided into two mechanisms, semi-synthesis and total synthesis. Naturally isolated phospholipids are always less expensive compare to that obtained by synthetic or semi-synthetic methods (Li *et al.,* 2015).

Vegetable phospholipids, such as soya lecithin, are commonly used for topical applications in cosmetics and dermatology because they contain a high concentration of unsaturated fatty acids, especially linoleic acid, which is thought to improve drug permeation through the skin (Ahmad and Ahsan, 2020). The packaging nature of unsaturated fatty acids altered the fluidity of SC lipid structure, allowing bioactive molecules to pass through more easily (Valenta *et al.,* 2000). Furthermore, the properties and integrity of the skin permeability barrier are affected by topically applied lipid vesicles. When they bind to the keratin filament, they can extract lipid from the skin or disrupt the order within and between the corneocytes (Gupta *et al.,* 2005). In general, transdermal biomolecule transmission can be facilitated by two forms of interactions between the skin and vesicles: (1) Adsorption and fusion of drug loaded vesicles onto the surface of skin lead to high thermodynamic activity gradient of the drug-SC surface. (2) The effect of vesicle on SC may change the bioactive permeation kinetics due to an impaired barrier function of the SC (Touitou *et al.,* 1994; Fang *et al.,* 2001a).

1.6.1 Lecithin

Lecithin is a minor name for 1, 2-diacyl-sn-3-phosphocholine that belongs to the phospholipids class. They contribute to the formation of the lipid matrix of biological membranes as well as playing a key role in the cellular metabolism (Shchipunov, 2001). Lecithin structural formula is shown in Figure 1.2. Lecithin is a mixture of many components of glycerophospholipids including phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine and phosphatidic acid. Soybeans, eggs, milk, marine organisms, rape seeds, cotton seeds and sunflower seeds are all popular sources (Alhajj *et al.*, 2020). In general, water solubility of lecithin is limited. However, it is an excellent emulsifier and lubricant (van Nieuwenhuyzen and Tomás, 2008). Its phospholipids can be converted into liposomes, bilayer sheets, micelles or lamellar structures in aqueous solution, in particular upon the control of the level of hydration and temperature (Martiel *et al.*, 2014). In the pharmaceutical industry, lecithin is used as a stabilizing agent and as a carrier that assists in encapsulation and emulsification processes, and is a good dispersing agent as well (van Hoogevest and Fahr, 2019). Therefore, it can be used in the manufacturing of many pharmaceutical preparations such as topical dosage forms and intravenous fat infusions (Li *et al.*, 2015).

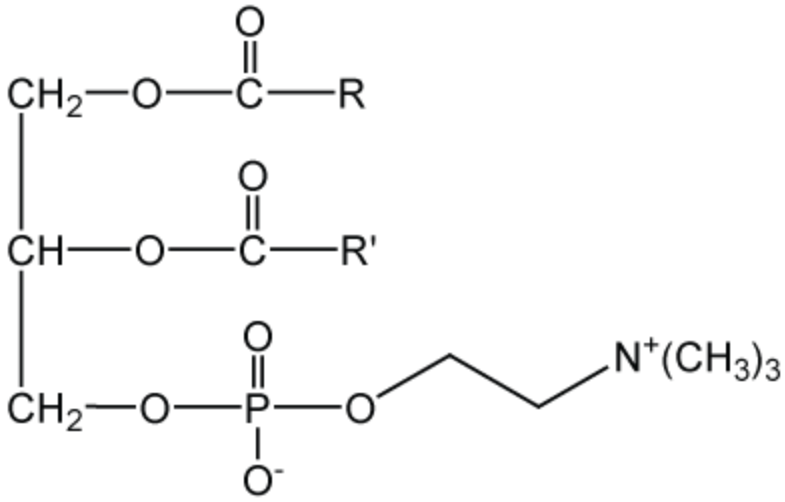

Figure 1.2 Lecithin chemical structure (The Merck Index, 2010)

1.7 Phospholipid formulations

1.7.1 Lecithin organogels (LOs)

LOs are a clear, viscoelastic, thermodynamically stable and biocompatible jelly-like matters (Raut *et al.*, 2012). In general, it is made up of hydrated phospholipids and a suitable organic liquid (Kumar and Katare, 2005). Because of their functional and structural properties, these structures are of great interest to pharmaceutical scientists. Moreover, LOs have gotten much attention lately for topical application of medications (Shaikh *et al.*, 2009). Many therapeutic agents have been formulated as LOs due to their efficiency of carriage via topical routes including dermal and transdermal. The biphasic drug solubility is the main reason for the improvement of topical drug delivery using organogels, as well as the desired drug partitioning and the modification of skin barrier function (Raut *et al.*, 2012). In addition, LOs possess prolonged shelf life as they are prepared by spontaneous emulsification and therefore considered thermodynamically stable (Kumar and Katare, 2005).

LOs were first described by Scartazzini and Luici in 1988. They discovered that adding trace amount of water to non-aqueous lecithin solutions induced a rapid increase in viscosity. The initial non-viscous solution transforms into a jelly-like state as a result of the rapid rise in viscosity (Scartazzini and Luisi, 1988; Luisi *et al.*, 1990; Shchipunov, 2001). As water is added to a nonpolar organic solution, the mainly spherical reverse micelles produced by lecithin molecules become cylindrical. The one-dimensional growth of micelles is caused by the formation of hydrogen bonds between water molecules and the phosphate groups of lecithin molecules, in which two modifying lecithin molecules are bound together by one water molecule. Water molecules could interact in a simultaneous manner with phosphate groups of neighbouring lipid molecules by hydrogen bonding, which acts as a bridge between them. In this case, the solvent molecules and the lecithin phosphate groups can be arranged to form a hydrogen-bonding network (Cypess *et al.*, 2009). Long tubular and flexible micelles can develop as the volume of water increases. These polymer-like, worm-like, or spaghetti-like micelles can entangle, which is why they can form a transient three-dimensional network that controls the viscoelastic properties of LOs. As the water concentration reaches a critical level, the network shrinks and phase separation occurs. Also at higher water concentrations, a transformation to a solid, non-transparent precipitate can be seen. The rod-like micelles in this diluted solution have insufficient lengths to overlap and form a three-dimensional network (Murdan *et al.*, 1999; Nasseri, 2002).

Pluronic® F-127 is hydrophilic non-ionic surfactant, utilized in the preparation of LOs, and it is more soluble in cold water than in hot water owing to the increase in the solvation and hydrogen linkages at low temperature (Escobar-Chávez *et al.*, 2006). The aqueous solutions of Pluronic® F-127 reversibly turn into

gels at a certain temperature, that is they become more liquid at lower temperature (4-5 ºC) and turn into gels at body temperature and this transformation is reversible, thus solutions return to a liquid state at low temperature (Lee *et al.,* 2004; Escobar-Chávez *et al.,* 2006; Feng *et al.,* 2011). As the temperature of a Pluronic® F-127 aqueous solution rises, the polypropylene oxide (PPO) block dehydrates, forming a core surrounded by hydrated polyethylene oxide (PEO) chains that aggregate into spherical micelles. In an aqueous environment, the micellar structure of this copolymer can be used to incorporate hydrophilic and hydrophobic drugs, and it prolongs drug release (Hosseinzadeh *et al.,* 2012)**.**

1.7.2 Liposomes

Topical formulations based on liposomes have been shown to be extremely promising for improvement in drug penetration, diminished side effects, improved pharmacological effects, controlled drug release and drug photo protection (Padamwar and Pokharkar, 2006). Liposomes was first described in 1964 (Bangham and Horne, 1964). Since then, liposomes have been more commonly used as drug carriers to mitigate drug toxicity or transport drugs to their target sites of action (Gregoriadis, 1991). The resemblance of the phospholipid bilayer membrane of the vesicles to the normal membrane of the skin cells is the basis for the use of liposomes in skin care (Betz *et al.,* 2005). This involves the ability of lipid vesicles to alter cell membrane fluidity and fuse with cells, allowing the drug to be delivered to the desired location (Chen *et al.,* 2013).

Liposome vesicles are made up of two components: phospholipids and cholesterol, which self-assemble into a bilayers structure. The polar group in the hydrophilic head comprises choline, glycerol, and phosphate, while the central fatty acid chain is made up of the two hydrophobic tails (Kulkarni *et al.,* 2011).