A NON-INVASIVE MELANOGENESIS INHIBITION METHOD AND DEVICE

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A NON-INVASIVE MELANOGENESIS INHIBITION METHOD AND DEVICE

ABSTRACT

Skin whitening research is an important field of cosmetic science research in Asia as bright and fair skin is preferred by Asian women. L-ascorbic acid is used in many topical cosmetic preparations since it has many favourable effects in skin, the most important being antioxidant action. L-ascorbic acid is known to both inhibiting melanin formation and reducing oxidized melanin and thus, can cause skin whitening. The percutaneous absorption of L-ascorbic acid is however limited by its impermeability and instability and the skin whitening function of L-ascorbic acid in topical cosmetic preparations is thus limited. In this present study, a transdermal iontophoresis device is fabricated to promote percutaneous absorption of L-ascorbic acid in human skin for *in vivo* melanogenesis inhibition and the efficacy of the transdermal iontophoresis device has been evaluated in the present study. The results indicated that the *in vivo* melanogenesis inhibition effect from the topical application of L-ascorbic acid is limited while the effect of topical application of L-ascorbic acid combined with the use of the invented transdermal iontophoresis device is obvious. In conclusion, the invented transdermal iontophoresis device is a safe, effective, non-invasive and cost effective portable device to promote percutaneous absorption of L-ascorbic acid in human skin for *in vivo* melanogenesis inhibition.

Keywords: skin whitening, L-ascorbic acid, transdermal iontophoresis device

KAEDAH DAN ALAT TIDAK INVASIF UNTUK MENGHALANG PEMBENTUKAN MELANIN

ABSTRAK

Penyelidikan untuk mencerahkan kulit merupakan satu bidang yang penting dalam penyelidikan sains kosmetik di Asia memandangkan kulit cerah lebih digemari oleh wanita Asia. Asid L-askorbik telah digunakan secara meluas dalam penyediaan kosmetik topikal kerana ia memberi pelbagai kesan yang baik terhadap kulit, terutamanya, kesan antioksidan. Asid L-askorbik telah diketahui dapat menghalang pembentukan melanin dan mengurangkan melanin teroksida dan dengan sebab itu, boleh menyebabkan kulit menjadi cerah. Walaubagaimanapun, kesan penyerapan asid L-askorbik adalah terhad disebabkan oleh sifatnya yang tidak telap dan tidak stabil dan ini telah menyebabkan fungsi pencerahan asid L-askorbik di dalam penyediaan kosmetik topikal menjadi terhad. Di dalam kajian ini, satu peralatan yang baru iaitu alat transdermal iontophoresis telah direka untuk menggalakkan kesan penyerapan asid L-askorbik di dalam kulit manusia bagi menghalang pembentukan melanin secara *in vivo* dan keberkesanan alat transdermal iontophoresis telah dinilai melalui kajian ini. Keputusan kajian menunjukkan bahawa kesan untuk menghalang pembentukan melanin secara *in vivo* daripada aplikasi asid L-askorbik adalah sangat terhad. Sementara, aplikasi asid L-askorbik digabungkan dengan penggunaan alat transdermal iontophoresis menunjukkan kesan yang sangat nyata. Kesimpulannya, alat transdermal iontophoresis yang mudah alih, selamat, berkesan, tidak invasif dengan kos yang lebih efektif berupaya menggalakkan kesan penyerapan asid L-askorbik di dalam kulit manusia untuk menghalang pembentukan melanin secara *in vivo.*

Kata kunci: mencerahkan kulit, asid L-askorbik, alat transdermal iontophoresis

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LIST OF SYMBOLS AND ABBREVIATIONS

The following symbols and abbreviations have been used in this thesis.

A	Estimated daily exposure values
Å	Angstrom
AC	Alternating current
ADI	Animal defenders international
C	Concentration of the substance
C/N	Carbon-to-nitrogen
C/S	Carbon-to-sulfur
C-4	Carbon atom number 4
C-5	Carbon atom number 5
CAS	Chemical abstracts service
C-C	Carbon-carbon bonds
C_D	Concentration of the solute
cm	Centimeter
CMC	Carboxymethyl cellulose
CMT	Critical micellization temperature
cps	Centipoise
Dap	Dermal absorption
DC	Direct current
DHI	Dihydroxyindole
DHICA	Dihydroxyindole carboxylic acid
DOPA	Dihydroxyphenylalanine
DP	Degree of polymerization
DS	Degree of substitution
E	Total potential difference
e.g.	exempli gratia

EC number	Enzyme commission number
Er	Standard cell potential
et al.,	et alia
etc	et cetera
EU	European Union
F	Faraday's constant
FDA	The Food and Drug Administration
g	Gram
g/cm^3	Gram per cube centimeter
g/m^2	Grams per square meter
g/mL	Gram per mililitre
g/mol.	Gram per mol
GRAS	Generally recognized as safe
GSH	Glutathione
GSSG	Glutathione disulfide
$[H^+]$	Hydrogen ion concentration
i.e.	id est/that is (to say)
ID	Identity document
INCI	International nomenclature of cosmetic ingredients
IPL	Intense pulse light
IUPAC	International Union of Pure and Applied Chemistry
J_D	Flux
J_D^{EO}	Drug flux due to the electroosmotic mechanism
KDF-Skin model	Keratinocytes, dendritics and fibroblasts-skin model
kg	Kilogram
LD50	Lethal dose 50%

L-DOPA	L-3,4-dihydroxyphenylalanine
m^2	Metre square
MC1R	Melanocortin 1 receptor
MD	Doctor of Medicine
µA	Microampere
mF	Millifarad
mg	Miligram
mg/cm^2	Miligram per square centimetre
mg/kg	Miligram per kilogram
MITF	Microphthalmia-associated transcription factor gene
mm	Millimeter
MoS	Margin of Safety
MSDS	Material safety data sheet
MSH	Melanin stimulating hormone
n.d.	Not determine
NDDS	Novel drug delivery systems
nm	Nanometer
NOAEL	No-observed-adverse-effect level
NSAIDs	Nonsteroidal antiinflammatory drugs
^oC	Degree celsius
Pa	Pascal
Pa·s	Pascal second
PETA	People for the Ethical Treatment of Animals
PhD	Doctor of Philosophy
p*K*a	Acid dissociation constant
PMNs	Polymorphonuclear Leukocytes
p-value	Probability value

R	Gas constant
RPE	Retinal pigment epithelial
γ	Shear rate
τ	Shear stress
SED	Systemic exposure dosage
SEM	Standard error of the mean
T	Absolute temperature
t_d	Transport number
TDDS	Transdermal drug delivery systems
TEWL	Trans epidermal water loss
TYRP1	Tyrosinase-related protein 1
TYRP2	Tyrosinase-related protein 2
UK	United Kingdom
UV	Ultra violet
η	Viscosity
V	Volt
$\mathcal{U}$	Solvent volume flow
w/v	Weight over volume
wt %	Weight percent
z_d	Valence of the drug

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Skin Whitening Products and Skin Whitening Agent

Skin whitening products are commercially available for cosmetic purposes for whiter or lighter skin complexion. Clinically, they are also used to treat hyperpigmentary disorders such as melasma, solar lentigo, postinflammatory hyperpigmentation and etc. Skin is prone to damage due to environmental agents, physiological changes and psychological factors regardless whatever the skin colour. Skin whitening products become the world centre of attraction as they give promising outcomes of flawless skin, age spots, blemishes and scars free. The desire to eliminate localized hyperpigmentation as well as to lighten the general skin tone contribute to the increment of demand for skin lightening products (Prakash & Majeed, 2009).

There are many reasons culturally why people use skin whitening products. In Western countries, skin whitening products are used to eliminate or inhibit the development of irregular pigmentation including melasma (chloasma or localized discoloration), age spots (Lentigo senilis) or liver spots (associated with sun damage or aging sometimes appearing as raised spots or Seborrheic keratoses) and freckles (Lentigo aestiva). In Asia, a whiter or lighter skin colour is associated with beauty and aristocracy. Thus people in Asia use skin whitening products mostly to lighten and brighten their skin tone (Prakash & Majeed, 2009).

Skin whitening research is an important field of cosmetic science research in Asia as bright and fair skin is preferred by Asian. Many Asians associate bright or fair skin with higher social status and wealth as they believe that women of higher class do not have to work outdoor and therefore are not subject to the sun UV rays radiation.

Although such beliefs are no longer accurate, modern Asian women are still of the opinion that skin fairness will directly affect the appearance and they always prefer to have bright and fair skin (Pan, 2013).

Human skin tone results from the process of melanogenesis. During melanogenesis, pigment cells or melanocytes produce melanin in the melanosomes. These melanosomes are then transferred and distributed within the keratinocytes (Ando et al., 2012; Park et al., 2010). In more detail, melanin is formed, first, by the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) using the enzyme tyrosinase (Slominski et al., 2012; Espin et al., 2000). The DOPA is subsequently oxidized by tyrosinase and/or peroxidase to DOPAquinone which leads to the formation of eumelanin and pheomelanin (D'Mello et al., 2016). Once melanin is formed in the melanosomes, it migrates into the melanocyte's dendrite tips through the myosin V filaments and dynein "motor" (Wu & Hammer, 2014; Byers et al., 2003). The melanosomes are then transferred to the neighboring keratinocytes or into the dermis. Each melanocytes actively transfers its melanosomes to about 40 basal keratinocytes (Seiberg et al., 2000). Ultimately, skin reflectance is determined by the amount and distribution of melanosomes within keratinocytes rather than melanocytes. In general, melanosomes of dark skin are larger and dispersed widely into the keratinocytes than in light skin (Minwalla et al., 2001).

Skin whitening products are widely used in cosmetics to help lighten or increase the fairness of human skin. It contains skin whitening or lightening agents that cause depigmenting activity in human skin. These skin whitening agents act at various levels of melanogenisis process in the melanocytes such as inhibiting the formation of melanin or preventing the transfer of melanosomes from melanocytes to surrounding keratinocytes. Currently, there are many skin whitening agents used in the skin

whitening products. Amongst the most common skin whitening agents used are hydroquinone, arbutin, kojic acid, niacinamide and L-ascorbic acid.

Hydroquinone has been used as topical skin whitening agent for more than 50 years to treat many forms of epidermal hyperpigmentation, such as melasma, freckles and postinflammatory hyperpigmentation, etc (Hua et al., 2009; Levitt, 2007). It is potentially carcinogenic and known to be skin and respiratory irritant. As such, it has been banned in some countries due to fear of cancer risk (Osman et al., 2015). The World Health Organization (WHO) also reported that 1% hydroquinone aqueous solution or a 5% hydroquinone cream can cause dermal irritation in humans (WHO, 1996).

Arbutin is a glycosylated form of hydroquinone (O'Donoghue, 2006) and is reported to be able to inhibit tyrosinase and further prevent the formation of melanin (Zhu & Gao, 2008). Arbutin exist naturally in bearberry plant and the leaves of Bergenia crassifolia (Pop et al., 2009). Application of arbutin onto cultured human melanoma cells and three-dimensional human skin model showed it to not affect cell viability and has good efficacy in the reduction of melanin synthesis. Arbutin is thus thought to be safe and effective skin whitening agent to be used in cosmetic product (Sugimoto et al., 2004).

Kojic acid is white crystalline hydrophilic antioxidant powder derived from fungus and is very effective in reducing skin whitening. It works by inhibiting the tyrosinase by a copper chelating action (Bentley, 2006; Lajis et al., 2012). Kojic acid has long been used in Japan as natural alternative to hydroquinone. Despite the fact that kojic acid is a good skin whitening agent, topical skin whitening product formulated

with more than 1% kojic acid cause risk to the consumer's health. In addition, kojic acid has the potential to induce skin sensitisation (SCCS, 2012).

Niacinamide is a form of vitamin B3 found in food and use as dietary supplement and medication. Niacinamide has been shown to reduce the production of melanin (pigment) and acts as an effective skin-lightening agent when added to skin creams (Lee et al., 2016). Unlike other skin whitening agents, niacinamide does not act as tyrosinase inhibitor or inhibiting the formation of melanin in the melanocytes. Rather, it inhibits the transfer of melanosome from melanocytes to keratinocytes in cell culture (Hakozaki et al., 2002).

L-ascorbic acid is a form of vitamin C and is a natural occurring organic compound which is recognized for its antioxidant (Darr et al., 1992; Darr et al., 1996; Eeman et al., 2014). It has been reported to show skin whitening properties (Maeda at al., 1996) in skin care products.

1.2 Why L-Ascorbic Acid is Used in This Study

In this study, L-ascorbic acid has been chosen as the skin whitening agent to be investigated further since L-ascorbic acid is the safest compound when compared to other common skin whitening agents. Besides the skin whitening effect, L-ascorbic acid consists of various favourable effects to the skin, such as antioxidant, UV photoprotection, improve morphogenesis of epidermal and promote collagen synthesis.

From years 2009 to 2014, L-ascorbic acid have been used in over 27% of new skin care products that claim skin whitening or lightening effect (Eeman et al., 2014). L-ascorbic acid is also an important antioxidant that protects the skin by scavenging and

destroying free radicals and reactive oxygen-derived species (Darr et al., 1992) and is reported to be able to improve the morphogenesis of dermal epidermal junction in addition to its skin whitening properties (Pinell & Madey, 1998; Walter 1998). As a UV photoprotection agent, it works synergistic when used in conjunction with vitamin E, a lipophilic vitamin (Lin et al., 2003). L-ascorbic acid is also used topically because of its ability to reduce wrinkles by promoting collagen synthesis (Smaoui et al., 2013).

L-ascorbic acid is a co-factor for the enzymatic activity of prolyl hydroxylase, an enzyme that hydroxylates prolyl residues in procollagen, elastin and other proteins with collagenous domains prior to triplex helix formation. Deficiency of L-ascorbic acid leads to impaired collagen production, resulting in scurvy (Algahtania et al., 2010). In addition, L-ascorbic acid is able to inhibit melanin production by reducing oxidized dopaquinone, interrupting DHICA oxidation (Ros et al., 1993) and interacting with copper ions at the active site of tyrosinase (Ebanks et al., 2009; Briganti et al., 2003).

1.3 Limitation of L-Ascorbic Acid in Skin Whitening Products

The effectiveness of topical formulations incorporating L-ascorbic acid is challenged by its intrinsic lack of stability and its poor penetration or impermeability into the skin (Kameyama et al., 1996).

L-ascorbic acid is highly susceptible to oxidation, especially in water-based systems and when exposed to air (Eeman, 2014). Under aerobic conditions, it is reversibly oxidized to dehydroascorbic acid, which can be irreversibly degraded to oxalic acid (Kleszczewska, 2000; Smaoui et al., 2013). The physico-chemical degradation of L-ascorbic acid in water is 11% after 7 days and 50% after 28 days (OECD, 1994). In addition, it is difficult to design a cosmetic product with L-ascorbic

acid that can remain stable for a long period of time since most of cosmetic products contain relatively high percentage of water.

A number of different strategies can be followed to improve stability of L-ascorbic acid in a cosmetics, such as exclusion of oxygen during production of the cosmetics, use of oxygen impermeable packaging to pack the cosmetics, encapsulation of L-ascorbic acid before mixing into the cosmetics, designing low pH and minimization of water content in the cosmetics, and inclusion of other antioxidants into the cosmetics (Sheraz et al., 2011). Besides, a promising approach for increasing L-ascorbic acid stability is to synthesize its less reactive derivatives such as L-ascorbyl-2-phosphate, sodium L-ascorbyl-2-phosphate and L-ascorbyl palmitate. These derivatives have been employed in a variety of cosmetic and pharmaceutical formulations, primarily as antioxidants.

As mentioned earlier, the effectiveness of topical formulations incorporating L-ascorbic acid is mainly challenged by its intrinsic lack of stability and its poor penetration into the skin. Deriving various derivaties of L-ascorbic acids is only able to resolve the issue of its intrinsic lack of stability but not its impermeability. Although some derivatives provided some stability, but, at the same time, it reduces the efficacy of L-ascorbic acid as these derivatives are not able to convert back to L-ascorbic acid after absorption or penetration into skin (Telang, 2013).

In order to overcome the impermeability of L-ascorbic acid, scientists have utilized different delivery systems. L-ascorbic acid has been successfully formulated in multiple emulsions (Farahmand et al., 2006) and in microemulsions (Pakpayat et al., 2009) showing improved stability. Encapsulation in chitosan microspheres (Desai et al., 2006) and nanoparticles (Jang & Hyeon, 2008) was also successfully attempted. Ascorbate derivatives have also been incorporated into colloidal carriers. For example,

ascorbyl palmitate has been incorporated into solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) with increased stability (Uner et al., 2005). The effectiveness of the lipid particles loaded with ascorbyl palmitate and sodium ascorbyl phosphate against free radical formation has been demonstrated in UV-A irradiated pig skin (Kristl et al., 2005) and was shown to depend significantly on the carrier system (i.e. the type of microemulsion and its concentration), rather than on the time of application (Jurkovic et al., 2004). Although the impermeability of L-ascorbic acid is enhanced through different delivery systems, the activity of the L-ascorbic acid is reduced as the L-ascorbic acid is firmly bound with delivery systems and the L-ascorbic acid is thus difficult to release from the delivery systems and utilized by the skin (Moribe et al., 2011).

1.4 Used of Transdermal Iontophoresis Approach in This Study

Nowadays, skin has increasingly become a route for the delivery of drugs. The passive delivery of most compounds across the skin is limited due to the barrier properties of the epidermis. Transdermal iontophoresis through electromotive force is an effective technique to facilitate the transport of permeants across the skin. The underlying principles of transdermal iontophoresis involve placing two oppositely charged electrodes at appropriate sides of the skin. The drug, in its ionic form, is placed under the electrode bearing the same charge as the drug and the voltage source most often supplies a constant electric current that is converted to an ionic current by oxidation-reduction reactions at the electrodes. As the ions carry this current through the skin barrier, charged ions are repelled from the active electrode into the skin and into the systemic circulation. This technique has been extensively explored as a potential mean for delivery of hydrophilic, large and charged molecules (Wang et al., 2005).

In view of this, there emerge a new approach that is able to overcome the instability and impermeability of the L-ascorbic acid and to deliver the L-ascorbic acid into the skin with optimal efficacy. This study has addressed to this issue by developing an electrically assisted, safe, intensive, effective and non-invasive method to promote percutaneous absorption of L-ascorbic acid into human skin. Besides, this study aims to develope a transdermal iontophoresis device to promote percutaneous absorption of L-ascorbic acid in human skin. With the application of the study, it manages to reduce or avoid any difficulties faced by the formulators in developing and formulating an effective L-ascorbic acid skin lightening products.

1.5 Objectives of the Study

The objectives of this study are:

1. To formulate a topical viscous hydrocolloids solution that suitable to be used as the delivery carrier for the L-ascorbic acid and which is suitable to be used in transdermal iontophoresis process;
2. To fabricate a transdermal iontophoresis device for promoting percutaneous absorption of L-ascorbic acid in human skin;
3. To evaluate the skin whitening efficacy of the formulated L-ascorbic acid topical hydrocolloid solution with the use of the fabricated transdermal iontophoresis device;
4. To detemine the optimum concentration of L-ascorbic acid in topical viscous hydrocolloids solution for optimum skin whitening efficacy when treated with the fabricated transdermal iontophoresis device; and
5. To detemine the optimum current densities of the fabricated transdermal iontophoresis device.

1.6 Economic of Scale

Consumers frequently use cosmetics to protect and care for their skin as well as to improve the health and/or physical appearance of their skin. Skin roughness and hyperpigmentations (such as age spots, freckles and brown patches associated with sunlight exposure, skin aging or environmental damage to the human skin) are areas where consumers typically seek to treat using cosmetics. Skin whitening or brightening is of particular interest in Asian populations. Thus, cosmetics containing skin whitening agents have been reported to be the best selling skin care products amongst Asian women (Tokiwa et al., 2007). The numbers of whitening cosmetics in the market are continuously increasing and the commercial potential in this area is high due to the high demand.

In the past decades, there have been a large number of scientific reports on *in vitro* testing to indicate L-ascorbic acid to be an effective substance for melanogenesis inhibition. Due to this reason, L-ascorbic acid has been added to many whitening cosmetics. However, the melanogenesis inhibition and/or reduction effect of L-ascorbic acid via external cosmetic application is very limited due to its impermeability and instability. In this study, the transdermal method and device have been developed to enhance percutaneous absorption of L-ascorbic acid into the human skin. The results obtained in this study observed that the formulated L-ascorbic acid topical viscous hydrocolloid solution together with the use of the fabricated transdermal iontophoresis device is a safe, intensive, effective and non-invasive method to promote percutaneous absorption of L-ascorbic acid in human skin for instant skin whitening.

In the current cosmeceutical treatment practice, the popular devices for skin whitening are skin laser and intense pulse light (IPL). Both devices are expensive. A

good skin laser device may cost up to RM100,000.00 while a good IPL device may cost up to RM50,000.00. In addition, operating the skin laser and IPL are complicated and requires well-trained profesional to handle. Inappropriate handling of these devices may cause skin burns or injuries. Thus, this study aims to develop an effective skin whitening device that is cost efficient and easier to operate.

1.7 Outline of the Study

There are 6 main chapters in this thesis. The first chapter includes the introduction of this study and objectives to be achieved. Chapter 2 comprehensively describes about the knowledge of skin melanogenesis process, skin whitening mechanism, the safety and efficacy of L-ascorbic acid when used on human skin, hydrocolloids solution and transdermal drug delivery. Chapter 3 presents the methods and approaches used in this study. Chapter 4 presents the results obtained in this study. Chapter 5 discusses the results obtained in this study. Chapter 6 summarizes the important results obtained in this study and provides a conclusion to the entire study.

CHAPTER 2: LITERATURE REVIEW

2.1 Skin Whitening

The term skin whitening (also called skin lightening) covers a variety of cosmetic methods used in an attempt to whiten the skin (Arbab & Eltahir, 2010). Skin whitening products are increasing in demand especially in Asia. According to Ollagnier et al. (2011), Asia currently makes up approximately 90 percent of the market in skin whiteners by value. On the other hand, Datamonitor Consumer Survey by Chipalkatti (2014) had reported that the Asian show highest percentage (50%) in response to consider skin whitening benefits to be key feature of skincare products as compared to North American (24%) and Western European (22%).

The increasing demand for skin whitening products among Asian are most probably due to the perception of "white skin" which traditionally been associated with higher social status and wealth in Asia (Pan, 2013). The skin lightness does not only affect perceptions of a woman's beauty, it also affects her marital prospects, job prospects, social status and earning potential (Li et al., 2008). Each country has different perception of skin complexion. For example, in China, pure white skin is a beauty symbol and in India, a lighter complexion stands for high caste (Li et al, 2008). According to the study of Li et al (2008), Indian culture portray "black" as underprivileged people and is a symbol of "dark," "dirty," "wrong," "hell," and "unfairness" and is opposite to "good," "bright," and "well-being". White skin is always associated with positive messages in Indian and Hindu cultures. It is taken as a sign of "beauty," "purity," "cleanliness," and "happiness," and is a symbol of power and privilege (Arif, 2004). In Hindu religion, *Kali*, a dark-skin goddess, is a symbol of

ugliness, cruelty, and destruction and manifests the negative association of dark skinned women in Indian society (Arif, 2004; Leeming, 2001).

Based on the perception of white skin potrayed by the Asian, it can be seen that "whiteness" is an important sign in presenting and constructing beauty in many non-white cultures. Desires for "whiteness," under this chain of associations, is pursued for mixed reasons by women in everyday life. In the social context, white face and white skin can be identified as a form of performance, which presents and re-represents the beauty and virtue of an individual within the community. Therefore, skin whitening cosmetics are highly preferable with high demand among the Asian.

As the Asian women prefer to have white skin, they used to obsessively protect themselves from the sun, by using umbrellas on sunny day and wearing gloves to maintain pale skin color on their hands, face and other parts of their body. Sunblock and skin-whitening creams are also widely used and applied to avoid skin from being tanned. Over 80% of the skin care products available in any grocery store or pharmacy aim to lighten skin tone and protect skin from sun damage (Pan, 2013).

Normally, the primary target of these skin whitening products is women (women of color in Pacific Asia and White women in North America and Europe). However, the cosmetic industry is increasingly targeting men (especially Asian men). There was a media outcry after Unilever, the maker of a new Vaseline skin whitening brand for men, posted an advertisement for this brand on the Facebook social networking site. The advertisement sought to promote skin whitening to dark-skinned Indian men by promising them a few shades of lighter skin tone with the use of the skin whitening creams (Mire, 2012).

Research found that men with lighter skin are viewed to be more intelligent and men with dark skin are seen as lacking intelligence (Glenn, 2008; Rambaran, 2013). Thus, the demand of skin whitening products will be continuosly increased as the skin whitening products are not only targeting women consumers but also men consumers as they also desire to have lighter skin.

The demands of skin whitening cosmetics make no limits and extend across race, sex and all age groups in communities (Rambaran, 2013). According to Chipalkatti, 2014 in her report for Datamonitor consumer of trends to watch in skin whitening, she reported that around 1 in 5 consumers in Asia say they use skin whitening products daily or almost every day. The Datamonitor consumer also reported that skin whitening cosmetics are so popular because it can boost up the consumers' confident as the cultural perceptions denotes the white and fair skin to higher social class and youthful look (Chipalkatti, 2014).

Nevertheless, there are also demands for skin whitening cosmetics from European countries. Lighter skin complexion owned by the European tend to accumulate patchy or irregular pigmentations such as freckles, melasma, age spots or liver spots when exposed to the sun rays. Thus, skin whitening or skin fairness cosmetics are also needed by European in order for them to get even skin tone and eliminate irregular pigmentations (Prakash & Majeed, 2009). Besides, there are also other disorders of pigmentation that can cause uncomfortable feelings and distractions such as postinflammatory hyperpigmentation, "tanning-bed" lentigines (may lead to skin cancer), solar lentigos and under-eye circles which need skin whitening cosmetics to reduce or treat the affected skin area (Baumann, 2009).

From the consumers' perceptions and the medical skin pigmentations disorders that had been described above, it can be seen that the reasons of using skin whitening cosmetics are varied. Although Asian are dominating the usage of skin whitening cosmetics, there are still considerable demands from European for the usage of skin whitening cosmetics to reduce and treat the uneven skin tone and pigmentation disorders experienced by those with lighter skin complexion. Therefore, it can be concluded that the demand for skin whitening cosmetics or products are not likely to be decreased, in fact, the skin whitening cosmetics or products are predicted to bloom across the regions with more stable and effective ingredients or wtih a device to promote maximum whitening effects towards the consumers' skin.

2.2 Skin

Skin is the outer covering of human body. Skin is the largest organ of the human body as it covers about 15% of the total of adult body weight, with a surface area of 1.8 m^2 and it is continuous, with the mucous membrane lining the body's surface (Kanitakis, 2002; Kolarsick et al., 2011). Skin is essential for protections against external physical, chemical and biological assailants. Besides, skin is also important to maintain the normal body temperature by preventing excess water loss from the body. Skin is a dynamic organ in a constant state of change, as cells of the outer layers are continuously shed and replaced by inner cells moving up to the surface. Although structurally consistent throughout the body, skin varies in thickness according to anatomical site and age of the individual.

2.2.1 Skin Anatomy

Skin is made up by three different layers which are epidermis, dermis, and subcutaneous tissue. Hair, nails, sebaceous, sweat and apocrine glands are regarded as derivatives of skin. Epidermis is the outermost layer serving as the physical and chemical barrier between the interior body and exterior environment. Epidermis consists of layers of keratinocytes with a protective role, also containing melanocytes, Langerhans cells and Merkel cells.

Dermis, the middle layer provides structural support of the skin, is fundamentally made up of the fibrillar structural protein known as collagen. It is an area of supportive connective tissue between the epidermis and the underlying subcutis which contains sweat glands, hair follicles, nervous cells and fibres, blood and lymph vessels. The subcutaneous tissue or subcutis is layer of loose connective tissue and fat beneath the dermis. Figure 2.1 is a human skin antomy drawing showed the three layers of skin and associated glands and vessels e.g. epidermis, dermis, subcutaneous tissue, blood vessels, follicle, sebaceous gland and sweat gland.

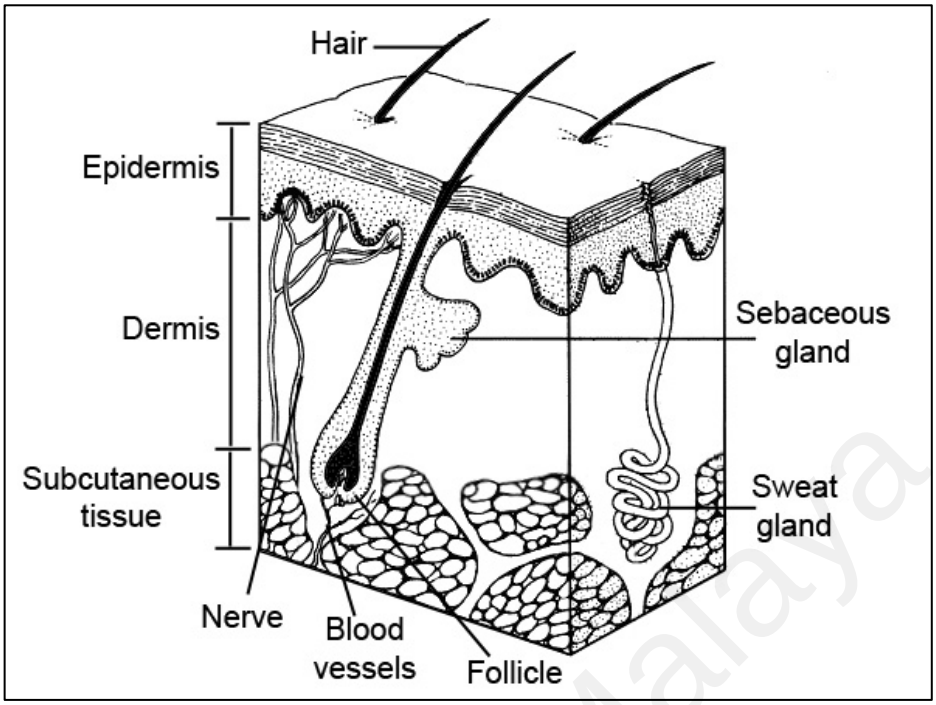

Figure 2.1: Human skin anatomy

2.2.1.1 Epidermis

Epidermis consists of a specific constellation of cells known as keratinocytes, which functions to synthesize keratin, a long, threadlike protein. Keratinocytes are connected by protein bridges known as desmosomes which are in a constant state of transition from the deeper layers to the superficial. The thickness of these layers varies considerably, depending on the geographic location on the anatomy of the body. For example, the eyelid has the thinnest layer of the epidermis, measuring less than 0.1 mm, whereas the palms and soles of the feet have the thickest epidermal layer, measuring approximately 1.5 mm (James et al., 2006; Kolarsick et al., 2011).

There are five separate layers of the epidermis which are formed by the different stages of keratin maturation. Moving from the lower layers upwards to the surface, the five layers of the epidermis are stratum basale (basal or germinativum cell layer),

stratum spinosum (spinous or prickle cell layer), stratum granulosum (granular cell layer), stratum lucidum and stratum corneum (horny layer) (Kolarsick et al., 2011). Figure 2.2 showed the layers of epidermis.

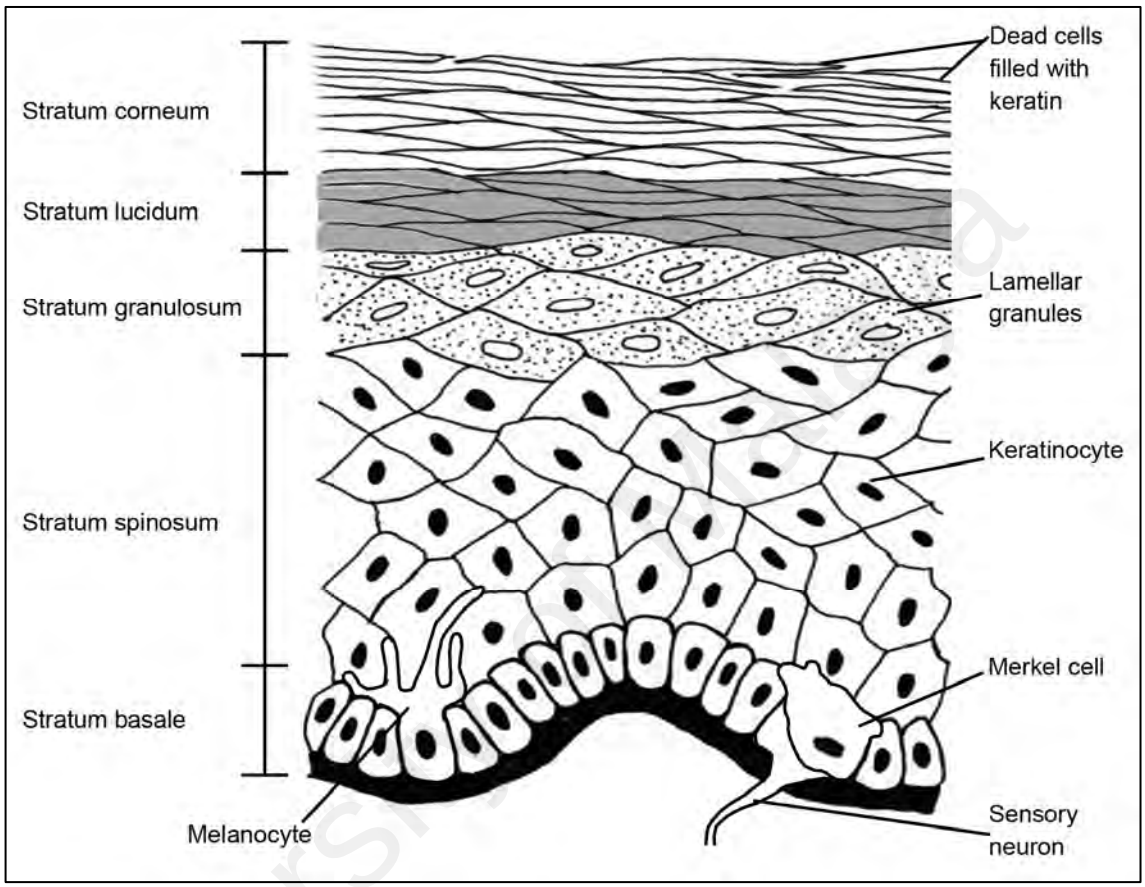

Figure 2.2: The layers of epidermis

As mentioned before, epidermis contains 3 other cells which are melanocytes, Langerhans cells and Merkel cells. Melanocytes are produced in stratum basale layer which is the innermost layer of the epidermis. Stratum basale layer lies adjacent to the dermis comprises mainly dividing and non-dividing keratinocytes, which are attached to the basement membrane by hemidesmosomes. As keratinocytes divide and differentiate, they move from this deeper layer to the surface. Making up a small proportion of the basal cell population is the pigment (melanin) producing melanocytes. These cells are

characterised by dendritric processes, which stretch between relatively large numbers of neighbouring keratinocytes (Ro & Dawson, 2005).

Melanin accumulates in melanosomes that are transferred to the adjacent keratinocytes where they remain as granules. Melanin pigment provides protection against ultraviolet (UV) radiation; chronic exposure to light increases the ratio of melanocytes to keratinocytes, so more are found in facial skin compared to the lower back and a greater number on the outer arm compared to the inner arm. The number of melanocytes is the same in equivalent body sites in white and black skin but the distribution and rate of production of melanin is different. Intrinsic ageing diminishes the melanocyte population. Merkel cells are also found in the basal layer with large numbers in touch sensitive sites such as the fingertips and lips. They are closely associated with cutaneous nerves and seem to be involved in light touch sensation. Langerhans cells are dendritic, immunologically active cells derived from the bone marrow and are found on all epidermal surfaces but are mainly located in the middle of stratum spinosum layer. They play a significant role in immune reactions of the skin, acting as antigen-presenting cells (Ro & Dawson, 2005).

2.2.1.2 Dermis

Dermis contributes for about 90% of the weight of skin (Quan & Fisher, 2015), and forms the foundation of this organ system. It lies between the epidermis and the subcutaneous fat. There are two distinct zones that can be seen in the dermis. The uppermost zone of dermis layer which lies beneath the epidermis layer is known as papillary dermis, and the lower zone is known as the reticular dermis (Baumann, 2009).

Fibroblast are the primary cell type in the dermis which produce collagen, elastin, other matrix proteins, and enzymes such as collagenase and stromelysin. Immune cells such as mast cells, polymorphonuclear leukocytes (PMNs), lymphocytes, and macrophages are also present in the dermis. However, dermis is mostly compose of collagen which responsible for the thickness of the skin and plays a key role in cosmetic appearance. The thickness and moisture of dermis layer are decreased with aging (Baumann, 2009).

2.2.1.3 Subcutaneous Tissue or Hypodermis

The subcutaneous tissue or subcutis also named as hypodermis is located beneath the dermis. The subcutaneous tissue is separated from dermis above and the muscle tissue below by layers of connective tissue, called fascias. The subcutaneous fat layer is mainly composed of two components; adipose tissue and interstitial tissue, shown in Figure 2.3. The interstitial tissue is placed between the adipocytes and consists mainly of a fibre framework made of collagen (Agache & Humbert 2004). The subcutaneous tissue mostly consists of fat which is an essential energy source for the body. Besides, it also contains collagen types I, III, and V. As humans age, some of the subcutaneous fat is lost or redistributed into undesired areas. This phenomenon contributes to the aged appearance.

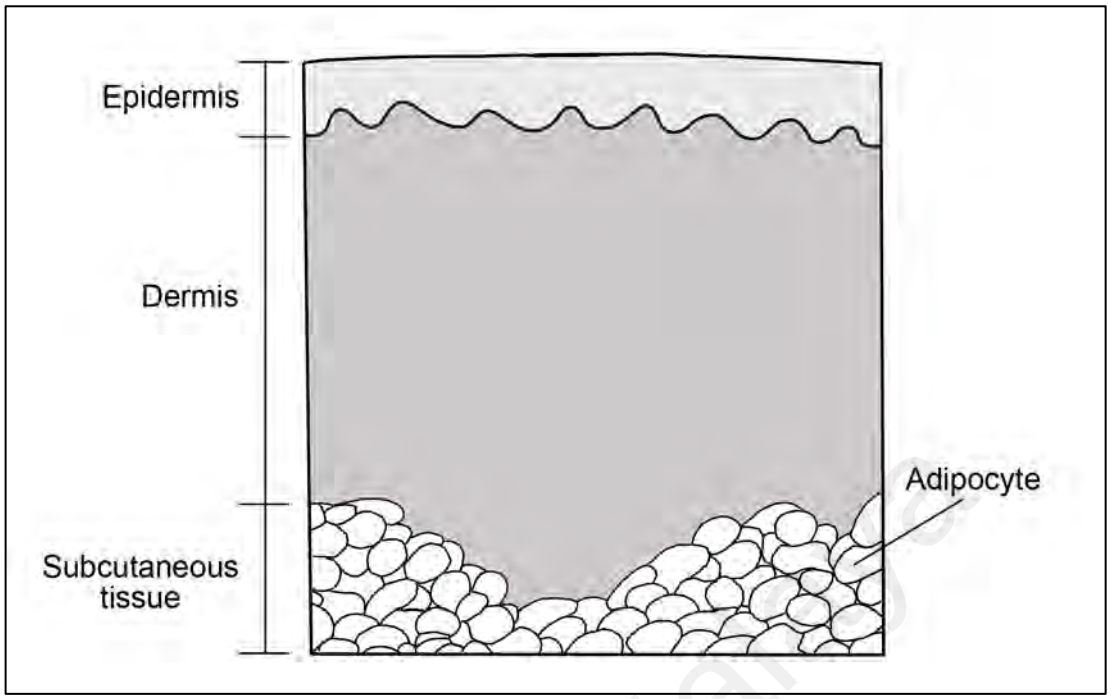

Figure 2.3: The layer of subcutaneous tissue is below the dermis layer

2.3 Melanocytes

Melanocytes are specialized melanin producing cells which are responsible for skin, hair, and eye pigmentation in human. Melanocytes synthesize pigment melanin within a special organelle recognized as melanosome. There are several specific enzymes and structure proteins assembled to synthesize melanin from tyrosine or phenylalanine (Slominski et al., 2004; Osawa, 2009). Visible pigmentation of the skin, hair, and eyes depends on the function of melanocytes in those tissues and can be influenced by a wide variety of factors that work at different levels. Melanocytes in the skin are found in two distinct populations, those residing at the dermal-epidermal junction, which give rise to skin color, and those residing in hair follicles, which give rise to hair color (Yamaguchi & Hearing, 2006). Figure 2.4 showed the melanocyte located in the stratum basale layer of epidermis of the human skin.

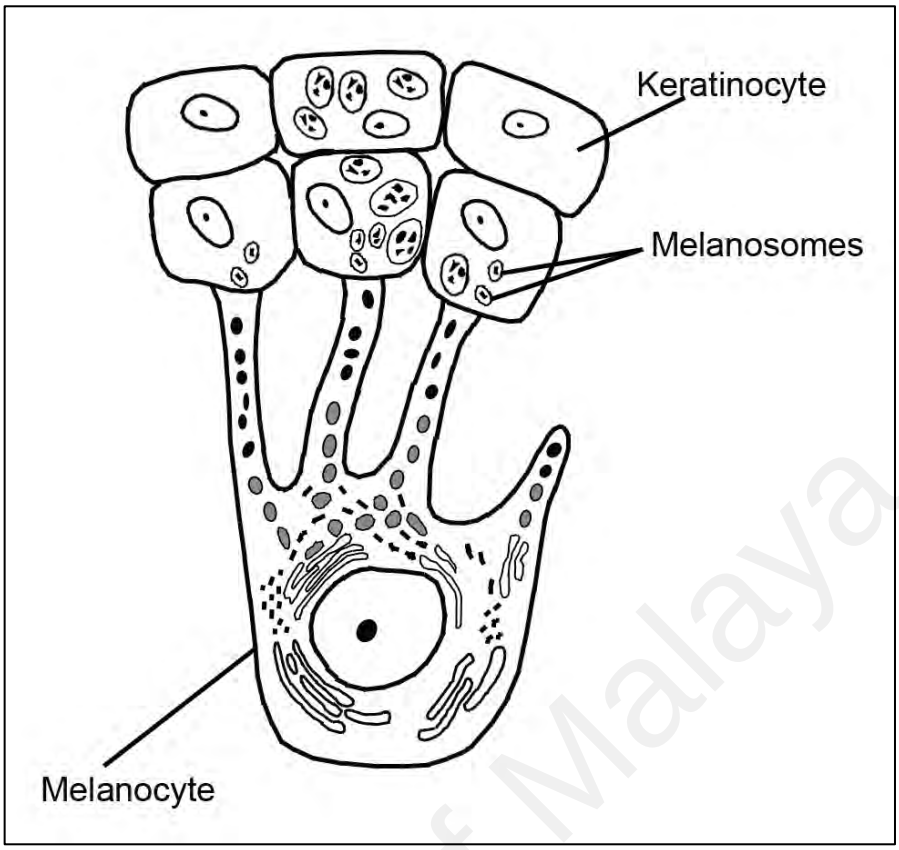

Figure 2.4: Melanocyte in stratum basale layer

2.3.1 Melanin (Eumelanin & Pheomelanin)

Melanin is a complex mixture of biopolymers derived from tyrosine, produced in melanocytes and passed to surrounding keratinocytes (Hennessy et al., 2005). It plays an essential role in defending the body against harmful UV rays and other environmental challenges (Costin & Hearing, 2007).

Melanin can be classified broadly into two types which are eumelanin and pheomelanin. The eumelanin is prevalent in individuals with black and brown hair and the pheomelanin (yellow-reddish) is responsible for red hair and freckles. Both melanins have a common organization of repeating units linked by carbon bonds; however, the pigments differ from each other in terms of chemical, structural and physical properties (Nasti & Timares, 2015).

2.3.2 Melanosome

Referring to Figure 2.4, melanosomes, the pigment granules that provide tissues with colour and photoprotection, are the cellular site of synthesis, storage and transport of melanin pigments. They are synthesised in mammalian skin melanocytes, in choroidal melanocytes and retinal pigment epithelial (RPE) cells in the eye, and in melanophores (a class of pigment-containing cells) in lower vertebrates. Melanosomes are large organelles (~500 nm in diameter) and, because of their dark pigment, are easily visible by brightfield microscopy (Wasmeier et al., 2008).

2.3.3 Melanogenesis

Melanogenesis has been defined as the entire process leading to the formation of dark macromolecular pigments, that is, melanin, which are formed by a combination of enzymatically catalyzed and chemical reactions (Chang, 2012). Melanogenesis is initiated in melanosomes, the special organelles of melanocytes, with the first step of L-tyrosine oxidation to L-DOPA (L-3,4-dihydroxyphenylalanine) and then to DOPAquinone, which is catalyzed by tyrosinase. This is a rate-limiting step in melanin synthesis because the remainder of the reaction sequence can proceed spontaneously at a physiological pH value (Ding et al., 2011). The biosynthetic pathway of melanogenesis has been elucidated, where two types of melanin are synthesized within melanosomes: eumelanin and pheomelanin (Chang. 2012).

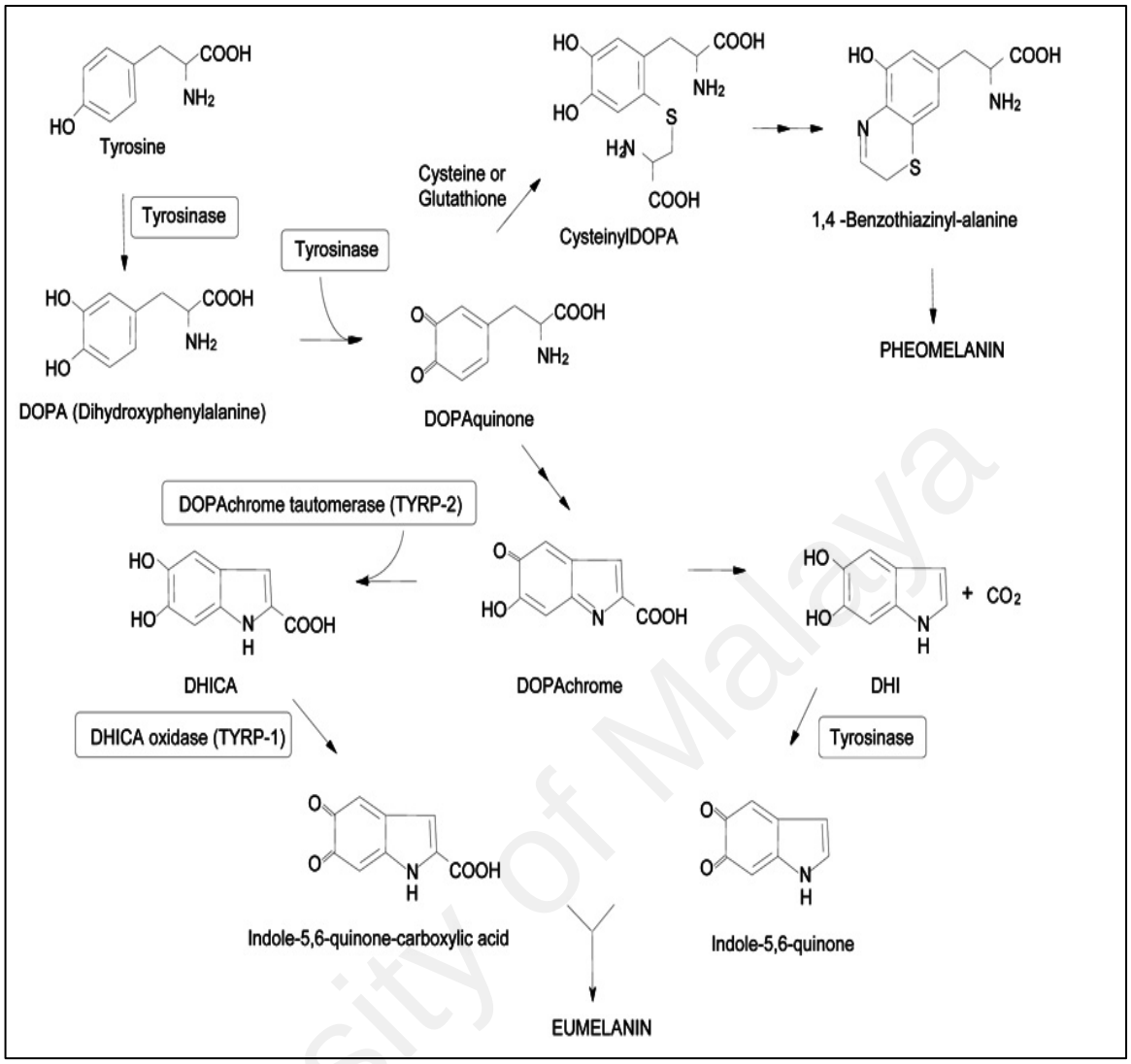

Figure 2.5: Process of melanogenesis within epidermal melanosomes (Ebanks et al., 2009)

2.3.4 Skin Color

Melanin is a heterogeneous biopolymer produced only by melanocytes, which synthesize and deposit the melanin pigment in specialized membrane-bound organelles known as melanosomes. It regulates pigmentation of the skin, eyes, and hair.

Melanins, the end-products of complex multistep transformations of L-tyrosine, are polymorphous and multifunctional biopolymers, represented by three basic types:

eumelanin, pheomelanin and neuromelanin. The melanin which associate with skin colour are eumelanin, which is prevalent in individuals with black and brown hair and pheomelanin (yellow-reddish) which is responsible for red hair and freckles (Nasti and Timares, 2015).

Melanin biosynthesis can be initiated from either the hydroxylation of L-phenylalanine to L-tyrosine (nonobligatory step, operative *in vivo*) or directly from L-tyrosine, which is then hydroxylated to L-3,4-DIHYDROXYPHENYLALANINE (L-DOPA) (obligatory step both *in vitro* and *in vivo*). L-DOPA serves as a precursor to both melanins and catecholamines, acting along separate pathways (Slominski et al., 2004).

The next step, oxidation of L-DOPA to DOPAquinone, is common to both eu- and pheomelanogenic pathways (Prota, 1992; Prota, 1995). Eumelanogenesis involves the further transformation of DOPAquinone to leukodopachrome, followed by a series of oxidoreduction reactions with production of the intermediates dihydroxyindole (DHI) and DHI carboxylic acid (DHICA), that undergo polymerization to form eumelanin. Pheomelanogenesis also starts with DOPAquinone; this is conjugated to cysteine or glutathione to yield cysteinyldopa and glutathionyldopa, for further transformation into pheomelanin (Slominski et al., 2004; Ito, 2003; Prota, 1992; Prota, 1995).

Generally, the arrangement of melanin pigments (eumelanins and pheomelanin) are about the same, whereby several units linked by carbon-carbon bonds (C-C). However, the melanin pigments are differ from each other in chemical composition, as well as structural and physical properties. For example, the eumelanins are polymorphous nitrogenous biopolymers (predominantly copolymers of DHI and DHICA), black to brown in color, insoluble in most solvents and tightly associated with

proteins through covalent bonds while the pheomelanin has a backbone of benzothiazine units and exhibits a yellow to reddish-brown color and is alkali soluble (Slominski et al., 2004; Ito, 2003; Prota, 1992; Prota, 1995). Pheomelanin is also tightly bound to proteins, indicating that *in vivo* it occurs as a chromoprotein , with high variability in nitrogen and sulfur content (C/N and C/S ratios). Pheomelanin can also act as a binding agent for drugs and chemicals. Other than that, pheomelanins are photolabile, and its photolysis products include superoxide, hydroxyl radicals, and hydrogen peroxide (Boyan et al., 2002; Mars et al., 1999).

Melanogenesis produces mixtures of eumelanin and pheomelanin at different mixed ratios. The ratio is determined by tyrosinase activity and the substrate concentrations of tyrosine and cysteine (Simon et al., 2009). As the melanosome mature, they are transported toward the tip of the dendrites of the melanocyte (Nasti & Timares, 2015).

Melanogenesis is also regulated at the cellular level via the controlling formation of melanosomes, which can be produced in varying sizes, numbers, and densities depending on melanin content (Chang, 2012). Pigment-loaded melanosomes translocate from their site of origin in the perinuclear cytoplasm towards the melanocyte dendrite tips using microtubule-based and actin-based motor proteins (Lambert et al., 1999; Wu & Hammer, 2000). They are then transferred into the recipient keratinocytes, where they are dispersed into the cytoplasm (Seiberg, 2001).

After transfering to the keratinocyte, the melanosomes are assembled predominantly to the apical pole of the nucleus, facilitated again by microtubule transport. This supranuclear melanin cap effectively absorbs and scatters incident UV

light, providing protection against mutagenic damage (Boissy, 2003; Yamashita et al., 2005; Van Den Bossche et al., 2006; Droste et al., 2006).

Human skin can be classified in certain ranges according to the Fitzpatrick Skin Type Classification system developed by Harvard Medical School dermatologist, Thomas Fitzpatrick, MD, PhD. The system ranges from (Type I) very fair skin to (Type VI) very dark skin. The test is based on the reaction to the sun exposure and genetic disposition. Figure 2.6 showed the skin colour classifiction according to the Fitzpatrick Scale.

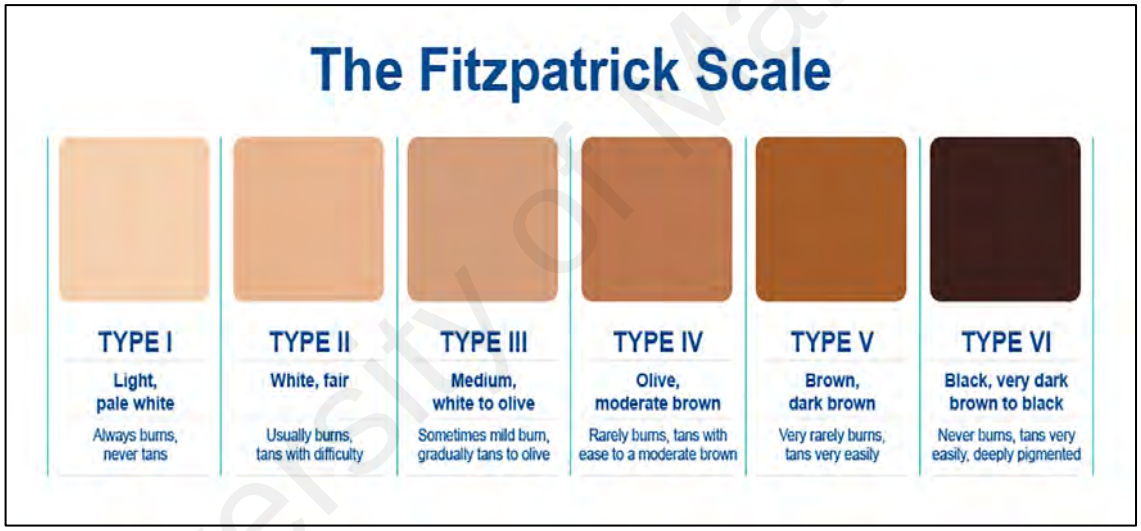

Figure 2.6: The Fitzpatrick Scale which indicates skin types classification based on skin colour and reaction to the sun exposure (Perfect Image, 2016)

The structural organisation of melanosomes within the keratinocytes varies according to skin type. In dark skin of Fitzpatrick's skin phototypes V and VI, melanosomes measure approximately 100 – 250 nm lengthwise and up to 100 nm across, and are maintained as individual organelles throughout the cytoplasm. Lighter skin types usually exhibit smaller melanosomes (50 – 150 nm along, 50 – 80 nm across), the oval shape being more pronounced than in dark skin (Droste et al., 2006).

A characteristic feature of light skin types (Fitzpatrick's skin types I and II), is the aggregation of melanosomes into clusters of 2-10 organelles. Skin of phototypes III and IV, and sometimes type V, present a combination of both distribution patterns, i.e. individual and clustered melanosomes (Thong, 2003; Droste et al., 2006). In all skin types, the majority of melanosomes are observed in the basal keratinocytes, and melanosome number decrease toward the surface of the skin (Figure 2.4). While in skin phototypes I to III, i.e. Caucasian skin, the melanosomes disappear until the mid stratum spinosum, they are still present in the stratum granulosum of skin types IV and V, i.e. Asian skin. In dark skin types, predominantly phototype VI, i.e. dark Asian and Negroid skin, melanosomes are observed throughout the entire epidermis and remain visible in the corneocytes. The amount of clustered melanosomes decreases toward the upper layers of the epidermis, and only individual melanosomes are observed from the stratum granulosum upward (Lu et al., 1996; Alaluf et al., 2002; Thong et al., 2003; Droste et al., 2006).

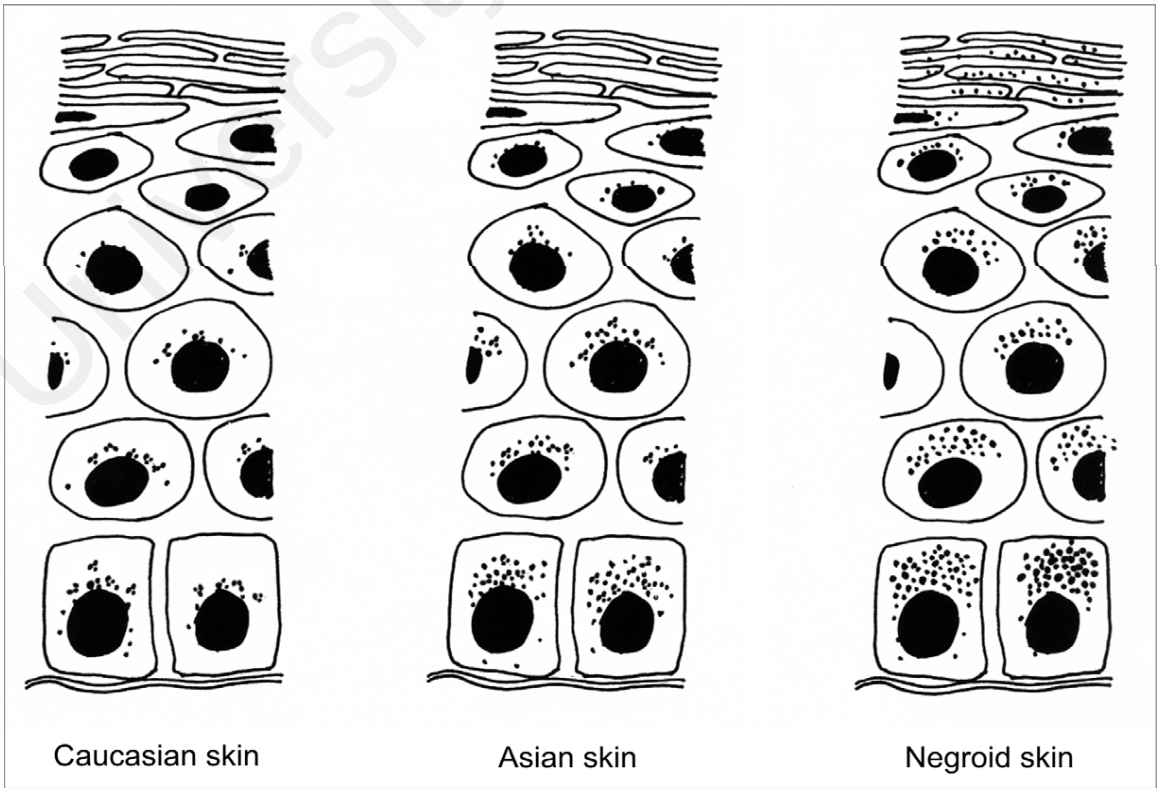

Figure 2.7: Melanosome distribution patterns in different ethnic skin types

Figure 2.7 showed the melanosome distribution patterns in different ethnic skin types. Independently of the skin type, the largest amount of melanosomes is observed in the stratum basale, and number decrease toward the skin surface. In skin phototypes I to III, i.e. Caucasian skin, melanosomes are predominantly aggregated into clusters, and disappear until the mid stratum spinosum. In skin types IV and V, i.e. Asian skin, melanosomes are visible until the stratum granulosum, while they are even present in the surface corneocytes in very dark skin (Negroid skin) (Droste et al., 2006).

A survey was conducted in Malaysia to determine the skin color types for the three largest ethnic group in Malaysia i.e. Malay, Chinese and Indian. For Malay ethnic, the skin type range was from type I to type VI, for Chinese, it was from type I to type IV and Indian, from type IV to type VI. Overall, the predominate skin color type for Malaysian ranges from type III to type IV (Isa et al., 2016).

2.3.5 Mechanism of Inhibition of Melanogenesis for Skin Whitening

Skin whitening products help to brighten up human skin or increase the fairness of skin. It contains skin whitening or lightening agents that cause depigmenting activity in human skin and they have been widely used in pharmaceutical topical whitening and cosmetic products. The formation of pigmentation in the skin involved several types of enzymes and genes, therefore, some mechanism of action to inhibit the involved enzymes and genes to stop or reduce skin pigmentation have been applied in order to produce lighter skin. The mechanism of action to inhibit the pigmentation activities in skin including tyrosinase inhibition, microphthalmia-associated transcription factor gene (MITF) expression inhibition, down regulation of melanocortin 1 receptor (MC1R) activity, interference with melanosome maturation and transfer, melanocyte dendricity

inhibition, melanin stimulating hormone (MSH) inhibition and serine protease inhibitors to inhibit melanosome transfer (Kamakshi, 2011).

Knowledge of the type of inhibition may be important in order to achieve better skin whitening effects since combined treatments may result in synergistic effects (Smit et al., 2009). The most common target for melanogenesis inhibitors is direct inhibition of tyrosinase catalytic activity. Among the clinical applications in cosmetic and pharmaceutical products, tyrosinase inhibitors are the most popular and wildly-used hypopigmenting agents. The main interest of inhibiting tyrosinase is because of tyrosinase produces only by melanocytic cells (Setty et al., 2008), hence, tyrosinase inhibitors have highly specific targeting to melanogenesis in the cells without other side effects. In contrast, melanogenesis inhibitors targeting to the tyrosinase gene expressions or protein degradations are rarely used as clinical hypopigmenting agents, due to their non-specific and global effects via intracellular signaling pathways. Hence, searching for new melanogenesis inhibitors based on direct inhibition of tyrosinase catalytic activity seems to still be the major field of interest for further study (Chang, 2012).

According to Smit et al. (2009), the tyrosinase inhibitors can be classified as competitive, uncompetitive, mixed type and non-competitive inhibitors (Smit et al., 2009). The nature of tyrosinase inhibition can be disclosed by measuring enzyme inhibition kinetics using Lineweaver-Burk plots with varying concentrations of L-DOPA as the substrate. This has been shown in case of the competitive tyrosinase inhibitor, arbutin and the non-competitive inhibitor, aloesin (Parvez et al., 2007; Jin et al., 1999). A 2009 paper by Chang stated that a large majority of tyrosinase inhibitors show reversible inhibition (Chang, 2009). In irreversible inhibition, covalent binding with the enzyme may cause its inactivation by altering the active site of the enzyme

and/or by conformational changes to the protein molecule. Irreversible inhibition may also occur via the so-called suicide inhibition mechanism as described in the model by Land et al. (Land et al., 2008). Other than that, the tyrosinase activity can also be inhibited by interacting with copper at the active site.

There are also other several examples of inhibitions to reduce pigmentation, resulting lighter skin complexion. For example, reducing agents causing chemical reduction of DOPAquinone such as ascorbic acid, which is used as a melanogenesis inhibitor because of its capacity to reduce DOPAquinone to DOPA, thus avoiding dopachrome and melanin formations. Another example of mechanism for pigmentation inhibition is DOPAquinone scavenger such as most thio-containing compounds, which are well-known melanogenesis inhibitors and react with DOPAquinone to form colorless products. The melanogenetic process is therefore slowed until all the scavenger is consumed, and then it goes at its original rate (Chang, 2009).

Other than that, alternative enzyme substrates such as some phenolic compounds, whose quinoid reaction products absorb in a spectral range different from that of dopachrome also manage to inhibit the pigmentation activity. When these phenolics show a good affinity for the enzyme, dopachrome formation is prevented, and they could be mistakenly classified as inhibitors. Besides, non-specific enzyme inactivators such as acids or bases could also act as inhibitor for pigmentation activity by non-specifically denature the enzyme, thus inhibiting its activity. Specific tyrosinase inactivators such as mechanism-based inhibitors, which are also called suicide substrates is also one of the activities which could inhibit pigmentation. These inhibitors can be catalyzed by tyrosinase and form covalent bond with the enzyme, thus, irreversibly inactivating the enzyme during catalytic reaction. They inhibit tyrosinase activity by inducing the enzyme catalyzing “suicide reaction (Chang, 2009).

2.4 L-Ascorbic Acid

Ascorbic acid (International Union of Pure and Applied Chemistry (IUPAC) name (5*R*)-[(1*S*)-1,2-Dihydroxyethyl]-3,4-dihydroxyfuran-2(5*H*)-one; synonym: L-ascorbic acid, Vitamin C) is a natural occurring organic compound and rich with antioxidant properties. It occurs as a white crystal solid or sometimes as a yellowish powder and almost odorless. L-ascorbic acid is one form of vitamin C. The molecular structure of L-ascorbic acid is shown in Figure 2.8.

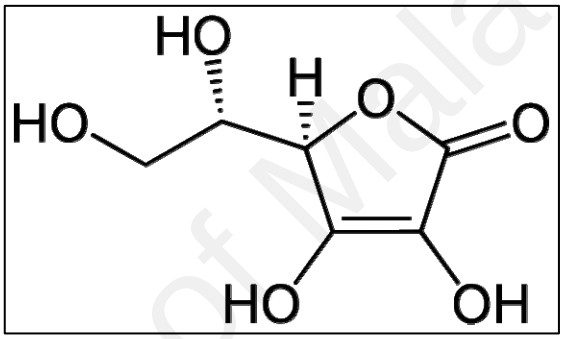

Figure 2.8: Molecular structure of L-ascorbic acid

The molecular formula of L-ascorbic acid is $C_6H_8O_6$ with molecular weight of 176.12 g/mol. It has a melting point of 191-192 °C and has a bulk density of approximately 1.65 g/cm^3. The solubility of L-ascorbic acid in g/mL is 0.33 in water, 0.033 in 95 wt% ethanol, and 0.05 in propylene glycol, while the pH of a 5% (w/v) solution in water is 2.2 to 2.5. It also has two p*K*a value; 4.10 and 11.60.

2.4.1 Stability of L-Ascorbic Acid

L-ascorbic acid is a dibasic with an enediol group built into 5 membered heterocylic lactone rings. The chemical and physical properties of L-ascorbic acid are related to its structure. Figure 2.9 shows the structure of L-dehydroascorbic acid, the

first oxidation product of L-ascorbic acid that has been analyzed by x-ray crystallography to be a dimer.

Electrochemical studies have indicated that L-ascorbic acid and L-dehydroascorbic acid for a reversible redox couple.

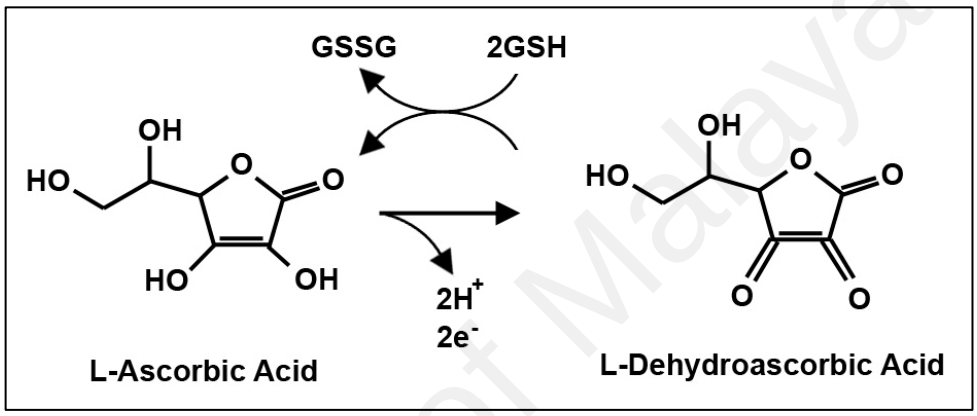

Figure 2.9: L-ascorbic acid and L-dehydroascorbic acid

Refer to Figure 2.9, the oxidized form, L-dehydroascorbic acid can be reduced back to L-ascorbic acid by glutathione (Aguirre & May, 2008). The L-ascorbic acid molecule consists of two asymmetric carbon atoms, C-4 and C-5.

L-ascorbic acid is stable when dry, but solutions readily oxidize, especially in the presence of trace amount of copper, iron and alkali. The main limitation of L-ascorbic acid is lack of stability. Even though ascorbic acid is stable when dry, but according to Smaoui and his team (Smaoui et al., 2013), L-ascorbic acid can be easily reversibly oxidized to L-dehydro ascorbic acid under aerobic condition, then, can be irreversibly degraded to oxalic acid. L-ascorbic acid is highly susceptible to oxidation (Kleszczewska, 2000), especially in water-based systems and when exposed to air

(Eeman, 2014). The physico-chemical degradation of L-ascorbic acid in water is 11% after 7 days and 50% after 28 days (OECD, 1994). The rate of L-ascorbic acid oxidation also influenced by other factors such as concentration, temperatures, light and pH (Davey et al., 2000).

2.4.2 Safety and Toxicology Profile of L-Ascorbic Acid

L-ascorbic acid is generally recognized as safe substances to be used as chemical preservatives in cosmetic product, food and as a nutrient or dietary supplement. The Food and Drug Administration (FDA) lists L-ascorbic acid as Generally Recognized As Safe (GRAS) substance.

According to a study by Elmore (Elmore, 2005) on different percentage from 5-10% L-ascorbic acid in cosmetic product shows no dermal sensitization to human skin (Ascorbic acid MSDS, 2005; Ascorbic acid solution, 5% MSDS, 2012). In addition, dermal application of L-ascorbic acid to patients with radiation dermatitis and burn victims had no adverse effects.

L-ascorbic acid as nutrient in food or dietary supplement appears to be generally low oral toxicity to humans. However, high dosage of L-ascorbic acid can cause diarrhea, acidification of the urine (low urinary pH) which may cause stones in the urinary tract and renal failure (Ascorbic acid. PubChem Open Chemistry Database, 2015). Oral test on pregnancy rat and mice showed intake of L-ascorbic acid up to 1 g/kg body weight per day had no detrimental effect, teratogenic, or fetotoxic effect (Elmore, 2005). The toxicological information of ascorbic acid: Oral (LD50): Acute: 11900 mg/kg [Rat] and oral (LD50): 3367 mg/kg [Mouse] (Ascorbic acid MSDS, 2005; L(+)-Ascorbic acid MSDS, 2008).

2.4.3 Benefits of L-Ascorbic Acid on Human – Skin and Health

L-ascorbic acid has a variety of biological, pharmaceutical and dermatological functions; it promotes collagen biosynthesis, provides photoprotection, causes melanin reduction, scavenges free radical, and enhances the immunity (anti-virus effect) and others (Yang, et al., 2003). L-ascorbic acid is an essential element to human. Human needs L-ascorbic acid as dietary supplement because human bodies do not produce this vitamin. L-ascorbic acid would be mostly depleted after 3 weeks if without ingestion. The minimum daily requirement for L-ascorbic acid is 200 mg.

L-ascorbic acid is a very well known substance that has many benefits to human health and skin related with its antioxidant properties. L-ascorbic acid has been used in pharmaceutical and cosmetic products because of its favorable effects. It has a role to influence production of collagen. According to an article by Traikovich (Traikovich, 1999), ascorbate influence quantities of collagen synthesis in addition to stimulating quantitative changes to the collagen molecule. It is necessary for the formation of propyl hydroxylase, an enzyme essential for producing a stable collagen molecule. Besides, it is also used to form lysyl hydroxylase, an enzyme necessary for cross linking of one collagen molecule to another that will give effect to tissue strength.

In health aspect, L-ascorbic acid is able to repair various tissues in the body including bone, cartilage, ligament and tendons, blood vessel walls and teeth. Lack of this substance can lead to scurvy, which resulting easy bruising, bleeding, joint and muscle pains.

L-ascorbic acid is also famous with its capability in developing lighter skin complexion. Act as skin whitening agents, L-ascorbic acid undergoes chemical reduction to inhibit melanogenesis by reducing back DOPAquinone to DOPA, thus, avoiding dopachrome and melanin formations (Chang, 2009). In addition, L-ascorbic acid is able to inhibit melanin production by interrupting DHICA oxidation (Ros et al., 1993) and interacting with copper ions at the active site of tyrosinase (Ebanks et al, 2009; Briganti et al, 2003).

L-ascorbic acid also owns antioxidants characteristics against reactive oxygen and free radical from UV light. Since skin relies on antioxidants for protection against reactive oxygen species, and since skin predominantly receives and must deal with the free radical assault resulting from UV light, increasing the antioxidant defense of skin becomes an attractive strategy for increased photoprotection (Al-Niaimi & Chiang, 2017; Pinnel et al, 2001). If antioxidants could be delivered in high concentration through the stratum corneum barrier into the human skin, then, the antioxidant protective reservoir could be increased and photoprotection might be enhanced (Pinnel et al, 2001).

2.5 Skin Whitening Efficacy Test

The improvement of living standard globally increases the concern about well-being and beauty, thus a lot of new cosmetics are produced with various functions to improve the skin condition. Many functional products for moisturizing, whitening, reducing acne, wrinkle, cellulite, anti-ageing, and improving atopic skin have been launched. However, cosmetics for skin whitening are observed to be the most demanding products among the consumers. Ideal skin whitening compounds should have a potent, rapid and selective bleaching effect on hyper-activated melanocytes,

carry no short- or long-term side effects, and lead to a permanent removal of undesired pigment, acting on one or more steps of the pigmentation process. In the course of screening such functional substances, various *in vitro* and/or *in vivo* studies to determine the efficacy and safety must be conducted (Son & Heo, 2013).

2.5.1 The Limitation of *In Vitro* Test

Methods for evaluation of skin whitening agents efficacy include *in vitro* tests and *in vivo* studies. *In vitro* tests focus on the screening of skin whitening agents through the investigations of mechanisms for the activation or inhibition of signal transfer factors or enzymes involved in the formation of melanin. Tyrosinase is known to be a key enzyme for melanin biosynthesis, the ability of inhibiting tyrosinase activity is the best indication to evaluate the effectiveness of skin whitening agents. Thus, cellular tyrosinase assay is the important *in vitro* test to determine whether the substance owns any anti-tyrosinase activity that further inhibits the formation of melanin.

Besides, measuring the inhibition of L-DOPA auto-oxidation (Jani & Setty, 2016), melanin content assay conducted on a cultured cell and measuring the inhibition of melanin production in melanocytes (Kao et al, 2013) are also widely used to indicate the level of effectiveness of skin whitening agents. However, these *in vitro* methods are not suitable to measure the skin whitening efficacy of a finished cosmetic product.

Finished cosmetic product is formulated with skin whitening active ingredients and excipients that help to stabilise the entire formulation. The excipients may consist of the solvent, humectant, emollients, emulsifying agent, preservative and the like. These excipients subtances may be cytotoxic to the cell culture that use for assays and further affect the inhibition assays result.

Besides, the inhibition assays results obtained from the *in vitro* methods do not reflect the actual skin whitening results of the finished cosmetic product. The finished cosmetic product is to be applied onto the surface of the skin epidermal layer. The skin epidermal layer serves as a barrier to protect the body against microbial pathogens, oxidant stress (UV light) and chemical compounds, thus, most of the ingredients in finished cosmetic product are prevented from penetrating into the skin.

2.5.2 Ban on Animal Test for *In Vivo* Cosmetic Testing

In vivo testing for cosmetic purposes is carried out on mice, rats, guinea pigs, rabbit, and fish. The use of animal during finished cosmetic product testing is ethically unacceptable in view of the dosing levels, effects of mixtures, repeated-dose toxicity, reproductive toxicity and toxicokinetics testing which can lead to the extended and chronic suffering of the animal (PETA Deutschland e.V, 2011; Regulation (EC) No 1223/2009 on cosmetic products (OJL342, 22.12.2009).

Thus, considering the animal welfare, Animal Defenders International (ADI) has fought (including through the National Anti-Vivisection Society) for a ban on the use of animals in cosmetic product testing since the 1990s. As a result of the sustained public campaign, cosmetic testing has been banned in the UK since 1998, which then led to the EU-wide ban on animal testing for cosmetic products implemented in 2009 and the sale of cosmetic products containing ingredients tested on animals outside the European Union from 2013 (ADI; Elena Ares, 2011, Cosmetic Testing on animals: EU ban). Likewise, the Japanese cosmetics company, Shiseido recently cited the 2013 sales ban as a reason for stopping animal testing and develop new alternative methods.

Since implementation of the animal testing and marketing bans, alternative test methods and its validation have been developed and conducted to replace animal testing. One of the alternatives is use of a device to measure the whitening effect of a skin whitening product applied on the human skin. This *in vivo* test method is a method of testing that uses human volunteer to study the effect of skin whitening product. As the test is performed directly to the human skin, a device called Mexameter is used to prove and measure the whitening effect of the product. Mexameter measures two components that are responsible for skin colour i.e. melanin and erythema content by reflectance.

2.5.3 Mexameter MX 18

Human beings are unique because they come in a spectrum of different skin colour. The colour of human skin is made up of many different components including melanin (protective tanning pigment) and redness (erythema) and will vary depending on time of year and environmental influences. Human eye is very sensitive for the distinction of colours viewed side by side. But, as soon as they are separated by space or time, differences are frequently undetected. Furthermore, the impression the eye receives from colours depends on the ambient lighting. This is where objective measurement of colour can be so valuable.

Mexameter MX 18 is a spectrometer measurement technique based on light absorption and reflection. It is a very easy, quick and economical tool to measure the two components, mainly responsible for the colour of the skin: melanin (pigmentation) and haemoglobin (erythema) by reflectance. Mexameter MX 18 is worldwide established and used in many scientific studies for *in vivo* melanogenesis inhibition evaluation (Chalermchai & Rummaneethorn, 2018; Hayashi et al., 2018; Spencer et al., 2018).

2.6 Safety Assessment of Cosmetic Product

According to European Commission Cosmetics Directive (76/768/EEC) and ASEAN Cosmetic Directive, the cosmetic product should not cause damage to human health when applied under normal or reasonably foreseeable conditions of use. Thus, safety assessment must be conducted on a cosmetic product before it is used on human skin.

The safety assessment takes account of:-

i. The general toxicological profile of each ingredients used;

ii. The chemical structure of each ingredient;

iii. The level of exposure of each ingredient;

iv. The specific exposure characteristics of the areas where the product will be applied;

v. The population intended to use the product.

The European Commission and ASEAN Cosmetic Directives have listed all the banned and restricted cosmetic ingredients in the Annex of II to VII of the both Cosmetics Directive. Besides, the new chemical substance that is not listed in the cosmetic ingredient database of European Commission will be treated as non-permissible ingredients for use in cosmetic product.

Thus, a cosmetic product formulated according to the requirements and guidelines of European Commission and ASEAN Cosmetic Directives is confirmed safe and no damage is caused to human health when applied under normal or reasonably foreseeable conditions of use.

2.7 Transdermal Drug Delivery System

Transdermal drug delivery systems (TDDS) are dosage forms designed to deliver a therapeutically effective amount of drug across a patient's skin. In details, the TDDS are dosage forms involves drug transport to viable epidermal and/or dermal tissues of the skin for local therapeutic effect while a very major fraction of drug is transported into the systemic blood circulation. The importance of the TDDS is to provide an alternative way or solution to improve the effectiveness of oral drug administration as about 74% of drugs taken orally are found not to be as effective as desired (Kumar et al., 2010).

Other than that, the TDDS provide a painless drug delivery into the bloodstream because the patient does not need to inject himself, there are no bulky delivery devices to manage or dangerous needles to dispose of, and there are few or no gastrointestinal effects from the drug itself. In addition, transdermal delivery is useful for those drugs that have a high first pass effect through the liver, have poor oral uptake, need frequent administration, or that interact with stomach acid. During the application of the TDDS, drugs absorbed through the skin, and then, enter the general circulation directly avoiding the liver, with less total drug absorption occurring. Thus, using TDDS is a better way to deliver substances that are brokendown by the stomach acids, not well-absorbed from the gut, or extensively degraded by the liver. Conclusion, the TDDS has fewer side effects than oral medications or supplements, easier to use and remember, provide an alternative to people who cannot, or prefer not to take medications or supplements orally and cost-effective (Patel et al., 2012).

Through time, the application of the TDDS are seen to be improvised to increase the efficacy and provide maximum effect of the drugs towards the patients. Newer dosage forms and drug delivery systems providing excellent improvement in drug therapy are termed novel drug delivery systems (NDDS). These are termed 'novel' because of recent development with satisfactory results in the field of drug delivery. The primary objective of NDDS is to ensure safety and to improve efficacy of drugs as well as patient's compliance. Some of these novel advanced transdermal technologies include iontophoresis (Dhote et al., 2011).

2.7.1 Iontophoresis

Iontophoresis is a minimally invasive technique that enhances the transport of charged and highly polar molecules across the skin by the application of a small electrical current. Iontophoresis passes a few milliamperes of current to a few square centimeters of skin through the electrode placed in contact with the formulation, which facilitates drug delivery across the barrier (Patel et al., 2012). The two main mechanisms of transport of this electrically enhanced method are electromigration and/or electroosmosis (Kalia et al., 2004; Delgado-Charro & Guy, 2001).

Electromigration originates from the direct interaction of the electrical field and the ions present in the formulation and the skin and will therefore enhance the transport of cationic drugs from the anode and, inversely, negatively charged drugs from the cathode. Conservation of charge requires that the sum of the electrical current carried by each ion equals the total electrical current supplied by the power source. Practically, as far as drug delivery is concerned, this means that the drug competes with all the other ions present in the system (Kalia et al., 2004; Delgado-Charro & Guy, 2001). The efficiency of transport, i.e., the fraction of the total charge transported by a given drug

(its transport number, t_d), can be determined experimentally by measuring its flux (J_D) and applying the relation:

$$J_D = \frac{I \cdot t_d}{F \cdot z_d} \qquad \text{------ Equation (1)}$$

where I is the total current passed, F is the Faraday's constant and z_d is the valence of the drug .

Electroosmosis has its origin in the fact that the skin is a negatively charged membrane at physiological pH. When an electrical potential is applied across a membrane containing fixed charge, a bulk volume flow of solution occurs in the direction of the counterion movement (Pikal, 2001). This means that for the negatively charged skin, the electoosmotic flow is in the anode-to-cathode direction. This assists the transport of cations and retards that of anions. This flow of solvent carries through the skin any dissolved solute and is therefore the mechanism enhancing the transdermal delivery of neutral, polar, molecules. The electroosmotic flow is the dominant mechanism of transport for the delivery of larger molecules (Delgado-Charro & Guy, 2001). The drug flux due to the electroosmotic mechanism (J_D^{EO}) is proportional to the concentration (C_D) of the solute:

$$J_D^{EO} = U \cdot C_D \qquad \text{------ Equation (2)}$$

where U is the solvent volume flow (Pikal and Shah, 1990). The pH and the ionic strength are the main parameters of the formulation that can be used to modulate electroosmosis by, respectively, modifying and screening the skin's charge (Santi & Guy, 1996).

2.8 Hydrocolloids

A hydrocolloid is defined as a colloidal system, in which the colloid particles are dispersed in water. A hydrocolloid particles spread throughout the water and depending on the quantity of water available, that system can be a gel or sol (liquid). Some hydrocolloids can exist in both a gel and a sol state, and can alternate between the states with the addition or elimination of heat.

Hydrocolloids which also called gums are colloidal substances that are generally non-crystalline in nature. They are water-soluble or water-dispersible hydrophilic compounds which are insoluble in alcohol and organic solvents. They are high molecular weight polysaccharides which are formed from sugars and uronic acid units (Ofori-Kwakye et al., 2010).

Hydrocolloids can be classified into 3 classes which are natural gums, semi-synthetic gum and synthetic gums. Natural gums are usually obtained from living organism such as plants, animals and microorganism and they are formed by natural processes. Semi-synthetic (modified gums) is produced by chemical modification of natural gums. The cellulose derivatives can be made by etherification, esterification, cross-linking or graft copolymerization, while synthetic gums are chemically produced gums (Ofori-Kwakye et al., 2010).

Hydrocolloids are polymeric substances that are widely used because of their hydrophilicity, rheological properties, and with mild to moderate surface activity (Munoz et al., 2007). In industrial application, hydrocolloids are extensively utilized in food making and processing, pharmaceutical, cosmetic, textiles, and etc. They serve for various purposes such as suspending, emulsifying, wetting, foaming, solubilizing, and

bioadhesive agents as well as permeation enhancers (Olorunsola & Adedokun, 2014). They are also widely use because of their low cost, non-toxicity, high biodegradability and high compatibility with drugs (Ofori-Kwakye et al., 2010).

2.8.1 Carboxymethyl Cellulose - Plant Based Hydrocolloid

Carboxymethyl cellulose (CMC) $[C_6H_7O(OH)_{3\_x}(OCH_2COOH)_x]_n$ or cellulose gum is a derivative of cellulose with carboxymethyl groups ($-CH_2-COOH$) bound to some of the hydroxyl groups of the glucopyranose monomers that make up the cellulose backbone. It is generally used as its sodium salt which is sodium carboxymethyl cellulose. The molecular structural of sodium carboxymethyl cellulose is shown in Figure 2.10.

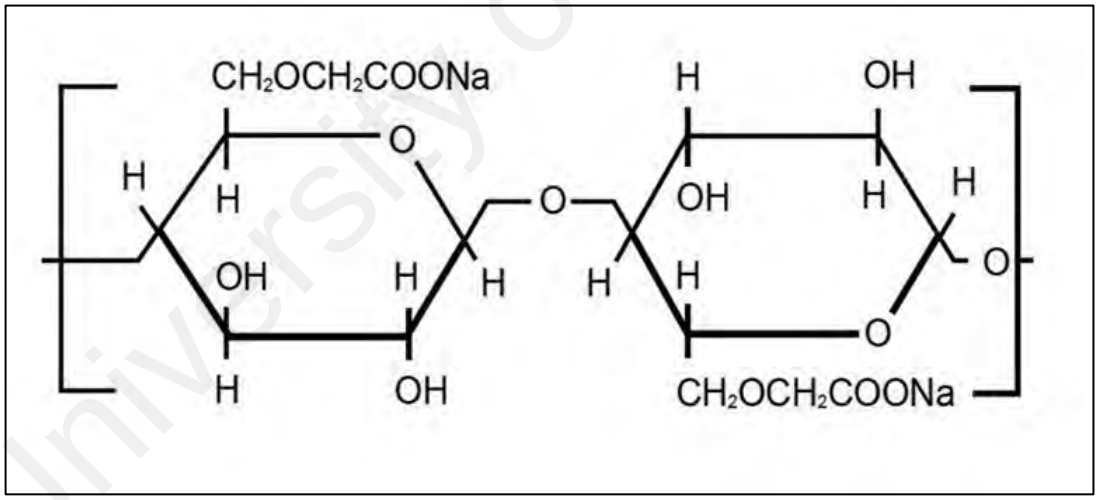

Figure 2.10 Molecular structure of sodium carboxymethyl cellulose

CMC is one of the plant based hydrocolloid and it is obtained by the chemical modification of natural plant derived cellulose. It is prepared by first treating cellulose with alkali (alkali cellulose), and then treating with monochloroacetatic acid or its sodium salt in an organic medium (rigidly controlled conditions). The degree of substitution (DS) with the carboxyl groups is generally between 0.6 to 0.95 per

monomeric unit (maximum DS is 3), and occurs at *O*-2 and *O*-6, and occasionally at *O*-3 positions (Boruvkova & Wiener, 2011; Milani & Maleki, 2012; Chaplin, 2015).

The CMC structure is based on the β-(1 ⟶ 4)-D-glucopyranose polymer of cellulose. CMC molecules are somewhat shorter, on average, than native cellulose with uneven derivatization giving areas of high and low substitution. This substitution is mostly 2-O- and 6-O-linked, followed in order of importance by 2,6-di-O- then 3-O-, 3,6-di-O-, 2,3-di-O- lastly 2,3,6-tri-O-.linked (Chaplin, 2015).

As CMC is derived from cellulose, it makes this compound water-soluble by a chemical reaction. The water-solubility is achieved by introducing carboxymethyl groups along the cellulose chain, which makes hydration of the molecule possible (Kelco, 2006).

CMC is also a long-chain polymer. Its solution characteristics and functional properties depend on the average chain length or degree of polymerization (DP) as well as the degree of substitution of the cellulose structure. For example, it depends on how many hydroxyl group taken part in the substitution reaction. The degree of substitution will determine molecular weight of the polymer. As molecular weight increases, the viscosity of CMC solutions increases rapidly. Besides, average chain length of the cellulose bone structure and the degree of clustering of the carboxymethyl substituents also affect the functional properties of CMC (Aqualon, 1999). The degree of neutralization of carboxymethyl groups also impacts viscosity. In solution, the degree of neutralization is controlled by the pH.

The substituents are irreversibly linked to the cellulose backbone with ether bridges, and thus, CMC belongs to the group of substances called cellulose ethers. It is important to note that the carboxymethyl group has an acid function meaning that CMC is an anionic polyelectrolyte (Grządka, 2012).

As mentioned, CMC is an anionic polyelectrolyte and water-soluble polymer which capable of forming very viscous solutions. As it is an anionic water soluble polymer derived from cellulose, it has many beneficial properties and functions for use in a broad range of applications in food, pharmaceutical, cosmetic, paper, and other industries. It can act as a thickener, binder, stabilizer, protective colloid, suspending agent, and rheology, or flow control agent. It also can form films that are resistant to oils, greases, and organic solvents, and maintain adequate flow properties at high temperature and pressure. Moreover, CMC also dissolves rapidly in cold or hot water and suitable for use in food systems. Besides, it is physiologically inert and can act as anionic polyelectrolyte. (Aqualon, 1999; Benchabane & Bekkour, 2008; Milani & Maleki, 2012).

The excellent properties and functions of CMC makes this compound widely used in many industries. The representative listing as given below shows the application of CMC based on its properties and functions. The most common grades of CMC used are highly purified grade and standard grade (Aqualon, 1999; Benchabane & Bekkour, 2008).

Table 2.1: Application of purified CMC in cosmetics, foods and pharmaceuticals industries

Application for Purified CMC		
Types of Uses	**Specific Applications**	**Properties Utilized**
Cosmetics	Toothpaste	Thickener; Flavour Stabilizer; Suspending Aid; Binder
	Shampoos; Foamed Products	Suspending Aid; Thickener; Foam Stabilizer; High Water-Binding Capacity
	Creams; Lotions	Emulsion Stabilizer; Film-Former; Thickener
Foods	Frozen Desserts; Soft-Serve	Controls Ice Crystal Growth; Improves Mouth Feel, Body, and Texture
	Baked Goods	Batter Viscosifier; Improves Moisture Retention and Texture
	Beverages	Suspending Aid; Rapid Viscosifier; Improves Mouthfeel And Body; Protein Stabilizer in Acidified Drinks
	Desserts; Icings; Toppings	Odorless And Tasteless; Thickens; Controls Sugar Crystal Size; Improves Texture; Inhibits Syneresis
	Protein Foods	Retains Water; Improves Mouth Feel
Pharmaceuticals	Ointments; Creams; Lotions Tablet Binder; Granulation Aid Syrups Suspensions	Stabilizer; Thickener; Film-Former High-Strength Binder Thickener Thickener; Suspending Aid

Table 2.2 : Application of standard grade CMC in others industries

Applications For Standard Grade of CMC		
Types of Uses	**Specific Applications**	**Properties Utilized**
Adhesives	Wallpaper Paste	Water-Binding Aid; Adhesion; Good Open Time; Non Staining
	Latex Adhesives	Thickener; Water-Binding Aid
Ceramics	Glazes Porcelain Slips Vitreous Enamels Refractory Mortars	Binder for Green Strength; Thickener; Suspending Aid
Coatings	Latex Paints; Paper Coatings	Rheology Control; Suspending Aid; Protective Colloid
Detergents	Laundry	Whiteness Retention Through Soil Suspension
Paper	Internal Addition	High-Strength Binder; Improves Dry Strength of Paper Products
Textiles	Latex Adhesives; Backing Compound Printing Pastes And Dyes	Rheology Control; Thickener; Water Binding and Holdout Compounds
Tobacco	Cigar and Cigarette Adhesive	Good Wet Tack; High Film Strength

2.8.2 Safety and Toxicology Profile of CMC

CMC is generally recognized as safe substances to be used in cosmetic product, food and health supplement industries. The Food and Drug Administration (FDA) lists CMC as Generally Recognized as Safe (GRAS) substance when used in dry food packaging, while its sodium salt is listed as GRAS as a miscellaneous or general purpose food additive (Carboxymethyl cellulose (packaging) and Sodium carboxymethyl cellulose, 2015).

Cellulose gum is another term of CMC used in cosmetic product. CMC shows a slightly acute symptom which may cause slight skin irritation and eye irritation when contact with eye or skin but, no information on chronic effect to human (CMC MSDS, 2013). The laboratory test on animals (rabbit and rat) also shows an occurrence of irritation effect of the skin of the animal (CMC MSDS, 2009).

In food industry, sodium CMC is a permitted food additive in which is used for the purpose of viscosity modifier or thickener, and also as stabilizer for emulsion in various products. This compound also has been used in dietary supplements as disintegrant in sweetener tablet. According to report by European Cosmission, the sodium CMC shows a low toxicity and long history of safe use. However, it is poorly absorbed in man and laboratory animal and shows little degradation in the gastrointestinal tract. When sodium CMC is modified into cross linked sodium CMC, the cross linking makes it less soluble in water and in simulated gastric and intestinal environments than sodium CMC. The acute oral toxicity studies in rats and mice (13 weeks oral feeding) shows no significant toxic effects, only reduced on body weight and reduced efficiency of food utilization (European Commission, 1998). The acute toxicological information of sodium CMC: Dermal (LD50):>2,000 mg/kg [Rabbit] and oral (LD50): 27,000 mg/kg [Rat].

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Carboxymethyl Cellulose (CMC) was purchased from Euro Chemo-Pharma Sdn. Bhd., Malaysia. L-ascorbic acid was purchased from Roche, Swaziland. 12 V Step-down transformer (12 V/0.25 A Teletron Power Transformer) was purchased from Justron Technology Sdn. Bhd., Malaysia. 13A/250V power plug, alternating current (AC) mains power switch; 0.2 A fuse, rectifiers; 3300 mF capacitor, voltage step down adjuster, direct current (DC) volt meter, 100 μA meter, voltage output terminals and coupling devices were purchased from Tonsin Component, Malaysia. Hand straps were modified from antistatic wrist straps that purchased from Wanfy (M) Sdn. Bhd., Malaysia. Disposable cotton mask sheets were purchased from Guardian Health and Beauty Sdn. Bhd., Malaysia.

3.2 Preparation of Topical Viscous Hydrocolloid Solution

3.2.1 Preparation of Base Topical Viscous Hydrocolloid Solution

In order to obtain the most suitable base topical viscous hydrocolloid solution, 3 different types of base topical viscous hydrocolloid solutions with 3 different viscosity were prepared in this study. These 3 different types of base topical viscous hydrocolloid solutions were 1% carboxymethyl cellulose topical hydrocolloid solution, 2% carboxymethyl cellulose topical hydrocolloid solution and 3% carboxymethyl cellulose topical hydrocolloid solution. These 3 different viscosity were prepared by respectively dissolving 1 g, 2 g and 3 g of CMC into 99 g, 98 g and 97 g of distilled water respectively at the temperature of 25 °C with agitation in a 100 mL beaker until a clear, almost colourless base topical viscous hydrocolloid solution was obtained. Table 3.1,

Table 3.2 and Table 3.3 listed the quantitative composition of these 3 types of carboxymethyl cellulose topical hydrocolloid solutions.

Table 3.1: Quantitative composition of 1% carboxymethyl cellulose topical hydrocolloid solution

	Ingredients	**Concentration in %**
1	Distilled Water	99%
2	Carboxymethyl Cellulose	1%
	Total	100%

Table 3.2: Quantitative composition of 2% carboxymethyl cellulose topical hydrocolloid solution

	Ingredients	**Concentration in %**
1	Distilled Water	98%
2	Carboxymethyl Cellulose	2%
	Total	100%

Table 3.3: Quantitative composition of 3% carboxymethyl cellulose topical hydrocolloid solution

	Ingredients	**Concentration in %**
1	Distilled Water	97%
2	Carboxymethyl Cellulose	3%
	Total	100%

3.2.2 Preparation of L-Ascorbic Acid Topical Viscous Hydrocolloid Solution

The clear and almost colorless L-ascorbic acid topical viscous hydrocolloid solution with different concentrations of L-ascorbic acid (10%, 15%, 20% and 25% respectively) was prepared by respectively mixing 1 g, 1.5 g, 2.0 g and 2.5 g of L-ascorbic acid into 9 g, 8.5 g, 8.0 g and 7.5 g of the 2% carboxymethyl cellulose topical hydrocolloid solution with agitation in a 100 mL beaker. In this study, as the viscosity of 2% carboxymethyl cellulose topical hydrocolloid solution is the most suitable viscosity for application on human skin and it could statically maintain on the human skin, thus, it was chosen as the base topical hydrocolloid solution. The 2% carboxymethyl cellulose topical hydrocolloid solution will be hereinafter referred to as base topical viscous hydrocolloid solution.

L-ascorbic acid topical viscous hydrocolloid solutions with the concentrations of 10% and 15% are very easy to prepare as these amounts of L-ascorbic acid are very easy to dissolve into the topical viscous hydrocolloid solution. The time required to dissolve these amounts of L-ascorbic acid is less than 5 minutes. However, 20% L-ascorbic acid required more time to dissolve in the viscous hydrocolloid solution and for 25% L-ascorbic acid, increase of the temperature of viscous hydrocolloid solution is required before L-ascorbic acid can be dissolved fully.

Table 3.4: The Summary of L-ascorbic acid topical viscous hydrocolloid solution preparation

Types of Topical Viscous Hydrocolloid Solutions	Preparation
10% L-ascorbic acid topical viscous hydrocolloid solution	1 g of L-ascorbic acid mixing into 9 g of 2% carboxymethyl cellulose topical viscous hydrocolloid solution
15% L-ascorbic acid topical viscous hydrocolloid solution	1.5 g of L-ascorbic acid mixing into 8.5 g of 2% carboxymethyl cellulose topical viscous hydrocolloid solution
20% L-ascorbic acid topical viscous hydrocolloid solution	2 g of L-ascorbic acid mixing into 8 g of 2% carboxymethyl cellulose topical viscous hydrocolloid solution
25% L-ascorbic acid topical viscous hydrocolloid solution	2.5 g of L-ascorbic acid mixing into 7.5 g of 2% carboxymethyl cellulose topical viscous hydrocolloid solution

3.3 Principle of pH Measurement of the Topical Viscous Hydrocolloid Solution

The pH of a solution measures the degree of acidity or alkalinity relative to the ionization of water. It involves comparison of the potential of solutions with unknown $[H^+]$ to a known reference potential. pH meter converts the voltage ratio between a reference half-cell and a sensing half-cell to pH values. The changes in potential and detection of $[H^+]$ were represented by Nernst equation (Equation 3),

$$E = Er + \left(\frac{2.303RT}{zF}\right) log\left(\frac{[H^+]_u}{[H^+]_s}\right) \quad \text{------ Equation (3)}$$

where E is the total potential difference (V), Er is the standard cell potential (V), R is the gas constant of 8.3145 J K^{-1} mol^{-1}, T is the absolute temperature (K), z is the valence or charge of the ion, F is the Faraday's constant of 9.65×10^4 C mol^{-1}, $[H^+]_u$ is

the hydrogen ion concentration of measured sample (mol m^{-3}), $[H^+]_i$ is the hydrogen ion concentration of internal reference (mol m^{-3}).

The pH of prepared base topical viscous hydrocolloid solution and 4 types of L-ascorbic acid topical viscous hydrocolloid solutions were measured using the EUTECH pH 510 pH Meter (Mettler Toledo, Switzerland). The measurement was carried out at the temperature of 25 ºC in three replicates.

Figure 3.1: EUTECH pH 510 pH Meter that used for pH measurement of the topical viscous hydrocolloid solution

3.4 Principle of Viscosity Measurement of the Topical Viscous Hydrocolloid Solution

The viscosity measures the flowability of a material moving through surfaces. Fluids resist relative motion of layers with differing velocities within them, opposing changes in the initial formation, hence generating internal resistance to flow. A fluid showing high resistance to opposing flow is said to be thick or viscous while those having small resistance

to flow are thin or runny. The viscosity of a sample is defined by shear stress over shear rate as Equation 4 below,

$$\eta = \frac{\tau}{\gamma} \qquad \text{------ Equation (4)}$$

where η is the viscosity of the sample (Pa·s), τ denotes the shear stress where amount of force applied to sample per unit area (Pa), and γ signifies the shear rate where velocity per unit height (s^{-1}).

Newtonian fluid is independent on the shear rate whereby the shear stress is proportional to shear strain rate. For non-Newtonian fluid, the viscosity is affected by the shear rate which causes the sample to be shear thickening or shear thinning.

The viscosity of the prepared base topical viscous hydrocolloid solution and 4 types of L-ascorbic acid topical viscous hydrocolloid solutions were determined using a Physica MCR 301 Rheometer (Anton Paar, Austria). The measurements were performed at a controlled temperature of 25 ± 0.01 ˚C in a shear range of 1 - 100 s^{-1}. The apparent viscosity of samples were recorded at a shear rate of 47.9 s^{-1}.

Figure 3.2: Physica MCR 301 Rheometer that used for viscosity measurement of the topical viscous hydrocolloid solution

3.5 Transdermal Iontophoresis Device Design and Fabrication

Transdermal iontophoresis device was fabricated and used to promote percutaneous absorption of L-ascorbic acid into human skin. The fabricated transdermal iontophoresis device is a lightweight portable device which is able to produce a constant electrical direct current in the range of 1 V to 12 V. The internal structure of the transdermal iontophoresis device was fabricated in a transparent casing so that it is easier to been seen. Besides, in order to make the transdermal iontophoresis device portable and handy, it was fabricated in small size. The actual size of the transdermal iontophoresis device is 14 cm (length) x 10 cm (width) x 10 cm (height) and its weight is less than 700 g. Figure 3.3 shows the photo of the actual transdermal iontophoresis device fabricated in this study.

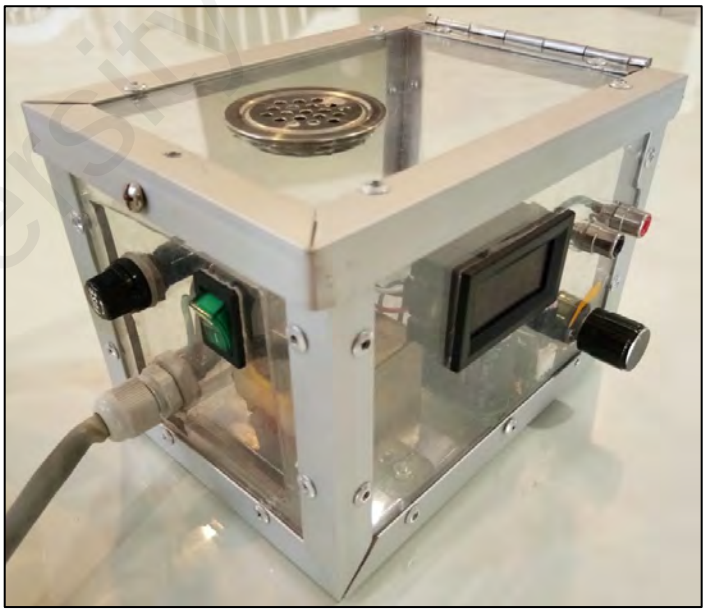

Figure 3.3: The photo of the actual transdermal iontophoresis device fabricated in this study

Couple sets of coupling devices which connected to the negative terminal of the voltage output terminal were fabricated as the working electrode and a pair of hand straps which connected to the positive terminal of the voltage output terminal was fabricated as the counter electrode. Figure 3.4 shows the couple sets of coupling devices that used as the working electrode and a pair of hand straps that used as the counter electrode.

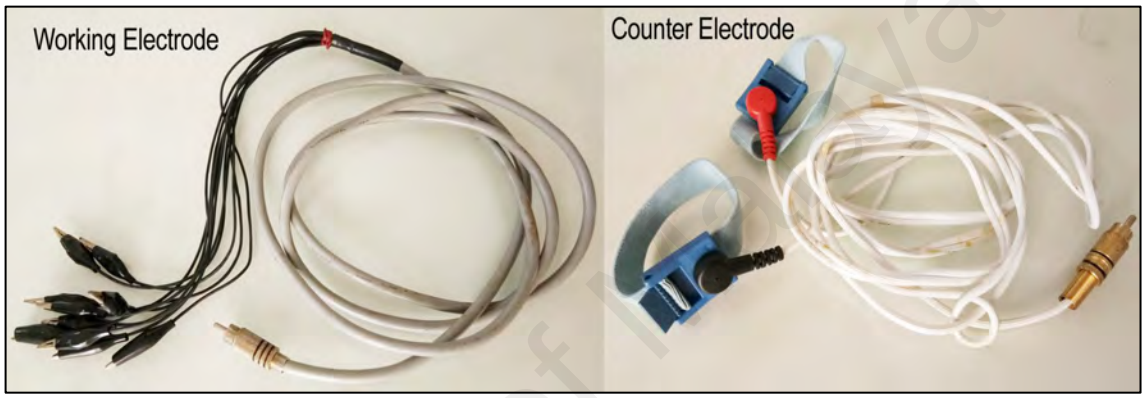

Figure 3.4: The couple sets of coupling devices that used as the working electrode and a pair of hand straps that used as the counter electrode

In order to distribute the direct current evenly onto the face of the subject, a disposable cotton mask sheet was used as medium to connect the working electrode and the human skin. Figure 3.5 shows the disposable cotton mask sheet that used to connect the coupling devices of the working electrode and the human skin.

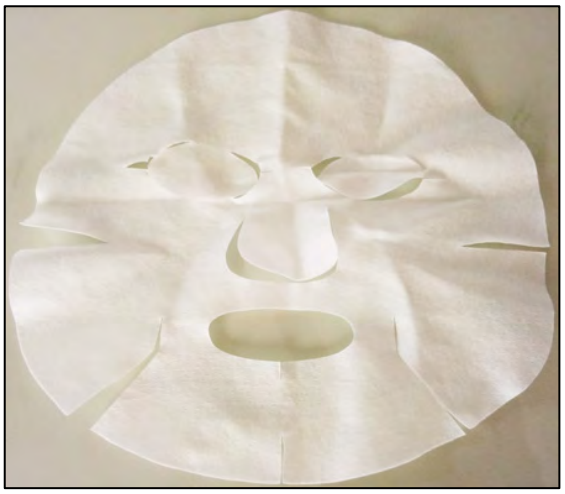

Figure 3.5: The disposable cotton mask sheet that used to connect the coupling devices of the working electrode and the human skin

To provide better understanding, the entire fabricated transdermal iontophoresis device together with the 2 electrodes and the disposable cotton mask sheet were presented in a schematic diagram and Figure 3.6 showed the schematic diagram of the fabricated transdermal iontophoresis device according to this study. The fabricated transdermal iontophoresis device consists of: a 13A/250V power plug; an alternating current (AC) mains power switch; a 0.2 A fuse; a transformer; a set of 4 rectifier diodes; a 3300 mF capacitor; a voltage step down adjusters; a DC volt meter; a 100 μA meter; a pair of voltage output terminals; a pair of hand straps; couple sets of coupling devices and the disposable cotton mask sheet.

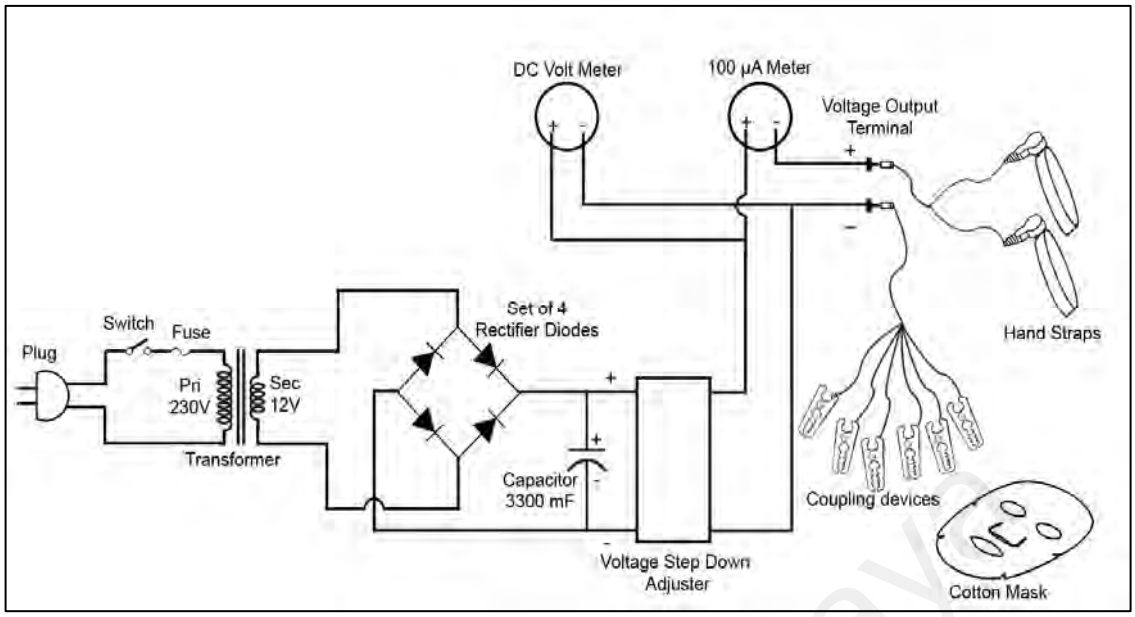

Figure 3.6: The schematic diagram of a transdermal iontophoresis device

Referring to Figure 3.6, a 13A/250V power plug was used for connecting the device to the alternating current (AC) power source. An alternating current (AC) mains power switch was used to turn the device on or off. 0.2 A fuse was used to connect with the AC mains power switch as a safety component to cut off the AC power source supply if the current exceeds a safe level.

A 12 V/0.25A step down transformer was used to convert the 230 V AC input power to 12 V/0.25 A AC output power. A set of 4 rectifier diodes was used to convert the 12 V AC power to a 12V direct current (DC) power. A 3300 mF capacitor was used to smooth down the distortion and make the DC output voltage as flat as possible. A voltage step down adjuster was used in the device to allow the stepping down of the 12 V DV power to a specific voltage. A DC volt meter was fabricated into the device to determine and monitor the voltage applied onto the subject's body and a 100 µA meter was also fabricated into the device to determine and monitor the quantity of the electricity applyied onto the subject's body. A pair of positive and negative voltage output terminals was fabricated to connect the working electrode and the counter

electrode. A pair of hand straps that used as the counter electrode was connected to the positive terminal of the voltage output terminal for the subject to wear on her arm and couple sets of coupling devices used as the working electrode connected to the negative terminal of the voltage output terminal and the disposable cotton mask sheet.

3.6 Safety Assessment of L-Ascorbic Acid Topical Viscous Hydrocolloid Solution

3.6.1 Safety Assessment

The safety of a cosmetic product is based on the safety of the ingredients used in the product. Accoding to European Commission Cosmetics Directive and ASEAN Cosmetic Directive, safety assessment must be conducted on a cosmetic product prior to the use.

To comply with the requirements of European Commission Cosmetics Directive and ASEAN Cosmetic Directive, the safety assessment of the L-ascorbic acid topical viscous hydrocolloid solutions used in this study were conducted based on the quantitative composition of the L-ascorbic acid topical hydrocolloid solution listed in Table 3.5 to Table 3.8 and this safety assessment was conducted in accordance with:-

i) the ASEAN Cosmetic Directive Annex I, Part 6: “Guidelines for the safety assessment of a cosmetic product”; and

ii) the European Commission Scientific Committee on Consumer Safety (SCCS) notes of Guidance for The Testing of Cosmetic Ingredients and Their Safety Evaluation 9th revision, 2016.

3.6.2 Quantitative Composition of the L-Ascorbic Acid Topical Viscous Hydrocolloid Solution

The quantitative composition of all ingredients used in the L-ascorbic acid Topical Viscous Hydrocolloid Solution are listed in Table 3.5 to Table 3.8 declared in a percentage format.

Table 3.5: Quantitative composition of 10% L-ascorbic acid topical viscous hydrocolloid solution

	Ingredients	**Concentration in %**
1	Distilled Water	88.2%
2	Carboxymethyl Cellulose	1.8%
3	L-Ascorbic Acid	10%
	Total	100%

Table 3.6: Quantitative composition of 15% L-ascorbic acid topical viscous hydrocolloid solution

	Ingredients	**Concentration in %**
1	Distilled Water	83.3%
2	Carboxymethyl Cellulose	1.7%
3	L-Ascorbic Acid	15%
	Total	100%

Table 3.7: Quantitative composition of 20% L-ascorbic acid topical viscous hydrocolloid solution

	Ingredients	Concentration in %
1	Distilled Water	78.4%
2	Carboxymethyl Cellulose	1.6%
3	L-Ascorbic Acid	20%
	TOTAL	100%

Table 3.8: Quantitative composition of 25% L-ascorbic acid topical viscous hydrocolloid solution

	Ingredients	Concentration in %
1	Distilled Water	73.5%
2	Carboxymethyl Cellulose	1.5%
3	L-Ascorbic Acid	25%
	Total	100%

3.6.3 No Observed Adverse Effect Level (NOAEL) Value

The no observed adverse effect level (NOAEL) value of the each ingredient used in the L-ascorbic acid topical hydrocolloid solution was obtained from the literature review and listed in Table 3.9. According to the SCCS notes of Guidance for The Testing of Cosmetic Ingredients and Their Safety Evaluation 9th revision, 2016, the NOAEL is defined as the highest dose or exposure level where no adverse treatment-related findings are observed.

Table 3.9: The NOAEL value of the each ingredient used in the L-ascorbic acid topical hydrocolloid solution

Ingredients	NOAEL mg/kg bw/day	NOAEL Value Reference
Distilled water	Safe	-
Carboxymethyl cellulose	6000	Retrieved on 15 May 2013, from http://www.inchem.org/documents/jecfa/jecmono/v042je10.htm
L-ascorbic acid	2000	SIDS Initial Assessment Report for SIAM 2

3.6.4 Calculation of the Systemic Exposure Dosage (SED) of a Cosmetic Ingredient

The Systemic Exposure Dosage (SED) values listed below were calculated based on dermal absorption reported as a percentage of the amount of substance applied according to the suggestion of SCCS notes of Guidance for The Testing of Cosmetic ingredients and Their Safety Evaluation 9th revision, 2016.

The calculation of the SED will be as follows:

SED = **A** (mg/kg bw/day) x **C** (%)/100 x **Dap** (%)/100 ------ Equation (5)

With: SED (mg/kg bw/day) = Systemic Exposure Dose

A (mg/kg bw/day) = Estimated Daily Exposure to a Cosmetic Product Per Kg Body Weight, Based Upon the Amount Applied and the Frequency of Application

C (%) = Concentration of the Substance Under Study in the Finished Cosmetic Product on the Application Site

Dap (%) = Dermal Absorption Expressed As a Percentage of the Test Dose Assumed to be Applied in Real-Life Conditions

The estimated daily exposure values (**A**) to a face cosmetic product per kg body weight, based upon the amount applied and the frequency of application (mg/kg bw/day) adopted in the above calculation is assumed to be 24.14 mg/kg bw/day (The adult's body weight was accepted 60 kg.) based on the suggestion of SCCS notes of Guidance for The Testing of Cosmetic ingredients and Their Safety Evaluation 9th revision, 2016.

Besides, the maximum value of 100% is used for the dermal absorption (**Dap**) expressed as a percentage of the test dose assumed to be applied in real-life conditions.

3.6.5 Calculation of the Margin of Safety (MoS) of a Cosmetic Ingredient

The last step in the Safety assessment of a cosmetic ingredient is the calculation of the Margin of Safety (MoS), which is the ratio between a NOAEL and an estimate of the exposure.

$$MoS = NOAEL / SED \quad \text{------ Equation (6)}$$

The MoS value is compared with a reference MoS, which is comparable to the uncertainty/assessment factor used in general to extrapolate from a group of test animals to an average human being, and subsequently from average humans to sensitive subpopulations (see Figure 3.7). A default value of 100 (10×10) is generally accepted and a MoS of at least 100 therefore indicates that a cosmetic ingredient is considered safe for use.

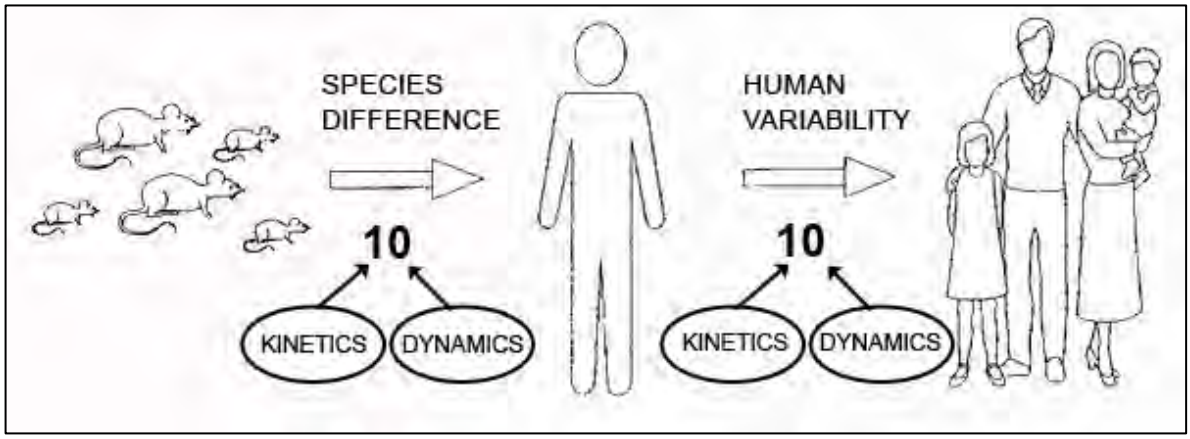

Figure 3.7 Schematic representation of the extrapolation from animal to man (SCCS notes of Guidance for The Testing of Cosmetic ingredients and Their Safety Evaluation 9th revision, 2016)

As shown in Figure 3.7, the default value of 100 consists of a factor of 10 for the extrapolation from test animals to an average human being and another factor of 10 taking into account the intra-species (inter-individual) variations within the human population.

3.7 Principle of Mexameter MX 18 for *In Vivo* Melanogenesis Inhibition

In this study, Mexameter MX 18 was used to evaluate the *in vivo* melanogenesis inhibition. The most important part in Mexameter MX18 is a probe. A probe is used to measure the status of melanin and erythema (redness) by reflection. The modern and high quality electronics probe allows very quick measurement because it is very sensitive to the slightest skin colour changes. A spring in the measuring head provides constant pressure on the skin. Moreover, its low weight ensures easy handling during operation. The new technology of the probe does not require frequent, complicated and time consuming recalibration. The accuracy of the probe can be checked at anytime with a special cap.

The measuring principle of the Mexameter is illustrated in Figure 3.8. The probe emits 3 specific light wavelengths which are 568, 660 and 880 nm. These wavelengths are respectively corresponding to green, red and infrared light (Baquie & Kasraee, 2013). A receiver measures the light reflected by the skin. The positions of emitter and receiver guarantee that only diffuse and scattered light is measured. As the quantity of emitted light is defined, the quantity of light absorbed by the skin can be calculated.

The skin melanin content (i.e. melanin value with arbitrary units) is measured by specific wavelengths chosen to correspond to different absorption rates by the pigments. The measurement is based on analysis of reflected light from wavelengths in the red and near-infrared. For the cutaneous hemoglobin content (i.e. erythema value with arbitrary units), the measurement is based on photodetection of the reflected light after emissions in the green and red wavelengths. The results for both parameters are shown within 1 second as index numbers (0-999) (Mexameter MX 18 – Assessing melanin content and eryhtma level, n.d; Baquie & Kasraee, 2013).

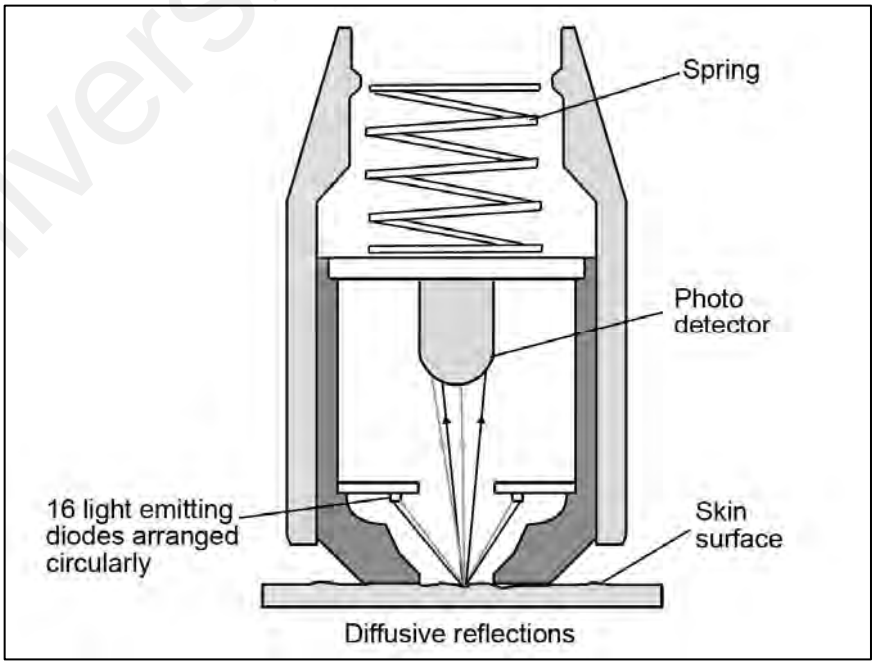

Figure 3.8 The measuring principles of the Mexameter MX 18

There are many fields of applications where changes in the skin colour are of interest. Many international scientific studies demonstrate its benefits in important skin related and cosmetological application fields.

In dermatology, Mexameter is used for objective clinical diagnosis, allergy and patch testing and measurement of melanoma and scar colour. It is indispensable in efficacy testing and claim support for cosmetic and pharmaceutical products (especially sunscreen and skin whitening products). It is also used in an assessment of skin soothing products effectiveness. Besides, in occupational health, skin irritation (erythema) is of special interest to educate the necessity of protection.

3.8 Study Subjects

Asian females with Fitzpatrick skin types III and IV aged from 18 to 50 years old were enrolled as the subjects in this study. This study only enrolled females with Fitzpatrick skin types III and IV because the predominate skin color type for Malaysian ranges from type III to type IV (Isa et al., 2016). Besides, subjects with closer skin color will provide smaller value of standard error of the mean when performing the melanin index measuring using the Mexameter MX 18. During the study, the subjects were refrained from excessive exposure to direct sunlight.

Besides, to reduce the interference of the melanogenesis inhibition activity evaluation, subjects were excluded if they had any of the following conditions:

i) smokers;

ii) alcoholics;

iii) drug abusers;

iv) pregnant;

v) had used the following substances (topical steroids, hormones, antibiotics, NSAIDs, antihistamines, or medicated cosmetics containing alpha hydroxy acids, retinoids, isotretinoin, azelaic acid, kojic acid, hydroquinone, chemical peels or other substances) which might induce or inhibit melanogenesis activity of the skin during the entire period of study;

vi) had known allergy or sensitivity to L-ascorbic acid or had any disease that might interfere with the evaluation of melanogenesis inhibition activity.

3.9 Melanogenesis Inhibition Evaluation of L-Ascorbic Acid Topical Viscous Hydrocolloid Solution – Treatment Time Optimization Study

3.9.1 Study Design

The evaluation were conducted on 20 subjects. The subjects were arranged in a room with air conditional controlled at 25 ºC for 30 minutes. After 30 minutes, the face of each subject was cleaned and the melanin index value of both sides of the face was measured by using Mexameter MX18.

After the melanin index value were taken, 5 g of 10% L-ascorbic acid topical viscous hydrocolloid solution were applied to one side of the face of 20 subjects while 5 g of base topical viscous hydrocolloid solution as placebo was applied to the other side of the face as a control. Both sides of the face were treated with the invented transdermal iontophoresis device (8 V) for 15 minutes, 30 minutes, 45 minutes and 60 minutes respectively.

After 15 minutes, 30 minutes, 45 minutes and 60 minutes treatment time respectively, the L-ascorbic acid topical viscous hydrocolloid solution and base topical viscous hydrocolloid solution were removed by cleansing the face and the melanin index value of both sides of the face were measured by using Mexameter MX18.

The quantitative evaluation of *in vivo* melanogenesis inhibition activity or skin whitening results were conducted by using Mexameter MX18 before treatment and after the treatment.

3.9.2 Study Statistical Analysis

All data of melanin index of the 20 subjects were expressed as the mean ± standard error of the mean (SEM). The differences between treated group and control group were analyzed for statistical significance by Student's one-tailed and paired t-test using a standard software package. The criterion for statistical significance was expressed as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.10 Melanogenesis Inhibition Evaluation of L-Ascorbic Acid Topical Viscous Hydrocolloid Solution – L-Ascorbic Acid Concentration Optimization Study

3.10.1 Study Design

The evaluation were conducted on 20 subjects. Subjects were arranged in a room with air conditional controlled at 25 ºC for 30 minutes. After 30 minutes, the face of each subject was cleaned and the melanin index value of both sides of the face were measured by using Mexameter MX18.

After the melanin index value were taken, 5 g of 10%, 15%, 20% and 25% of L-ascorbic acid topical viscous hydrocolloid solution were applied respectively to one side of the face of 20 subjects while 5 g of base topical viscous hydrocolloid solution as placebo was applied to the other side of the face as a control. Both sides of the face were treated with the invented transdermal iontophoresis device (8 V) for 45 minutes.

After 45 minutes, the L-ascorbic acid topical viscous hydrocolloid solution and base topical viscous hydrocolloid solution were removed by cleansing the face and the melanin index value of both sides of the face were measured by using Mexameter MX18.

The quantitative evaluation of *in vivo* melanogenesis inhibition activity or skin whitening result was conducted by using Mexameter MX18 before treatment and after 45 minutes of treatment.

3.10.2 Study Statistical Analysis

All data of melanin index of the 20 subjects were expressed as the mean ± standard error of the mean (SEM). The differences between treated group and control group were analyzed for statistical significance by Student's one-tailed and paired t-test using a standard software package. The criterion for statistical significance was expressed as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.11 Melanogenesis Inhibition Evaluation (4 Weeks) of 15% L-Ascorbic Acid Topical Viscous Hydrocolloid Solution

The evaluation were conducted on 40 subjects. The subjects were divided into 2 groups with each group consists of 20 subjects. The first group was conducted with the the evaluation of 4 Weeks *In-Vivo* melanogenesis inhibition evaluation of 15% L-ascorbic acid topical viscous hydrocolloid solution without using transdermal iontophoresis device. The second group was conducted with a 4 weeks *in vivo* melanogenesis inhibition evaluation of 15% L-ascorbic acid topical viscous hydrocolloid solution by using the invented transdermal iontophoresis device fabricated according to the method disclosed herein before.

3.11.1 Study Design of 4 Weeks *In-Vivo* Melanogenesis Inhibition Evaluation of 15% L-Ascorbic Acid Topical Viscous Hydrocolloid Solution without Transdermal Iontophoresis Device

The evaluation was conducted on the first group of 20 subjects. The 15% L-ascorbic acid topical viscous hydrocolloid solution was applied twice daily (in the morning and before bed after cleansing the face) for a continuous period of 4 weeks in a dose of 5 mg/cm^2 to one side of the face of the 20 subjects, while a base topical viscous hydrocolloid solution as placebo was applied to the other side as a control. Both 15% L-ascorbic acid topical viscous hydrocolloid solution and base topical viscous hydrocolloid solution were kept in a fridge (4 °C) before and after use.

The quantitative evaluation of *in vivo* melanogenesis inhibition activity was conducted by using Mexameter MX18 before treatment (as a control) and 1 day after the 4 weeks treatment.

3.11.2 Study Design of 4 Weeks *In-Vivo* Melanogenesis Inhibition Evaluation of 15% L-Ascorbic Acid Topical Viscous Hydrocolloid Solution and Treated with the Invented Transdermal Iontophoresis Device

The evaluation was conducted on the second group of 20 subjects. The evaluation process was repeated once daily for the period of 4 weeks. 5 g of 15% L-ascorbic acid topical viscous hydrocolloid solution was applied to one side of the face of 20 subjects while 5 g of base topical viscous hydrocolloid solution as placebo was applied to the other side as a control. Both sides of the face were treated with the invented transdermal iontophoresis device with the current densities of 70 μA (Output electrical voltage the device is selected from the range of 6V to 8 V from) for 45 minutes. After 45 minutes, the L-ascorbic acid topical viscous hydrocolloid solution and base topical viscous hydrocolloid solution were removed by cleansing the face.

The quantitative evaluation of *in vivo* melanogenesis inhibition activity were conducted by using Mexameter MX18 before treatment (as a control) and 1 day after the 4 weeks treatment.

3.11.3 Statistical Analysis

All data of melanin index of the 20 subjects in the first group and the 20 subjects in the second group were expressed as the mean ± standard error of the mean (SEM). The differences between treated group and control group were analyzed for statistical significance by Student's one-tailed and paired t-test using a standard software package. The criterion for statistical significance was expressed as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

CHAPTER 4: RESULT

4.1 pH Measurement of the Topical Viscous Hydrocolloid Solution

One of the factors that might affect the stability and viscosity of the viscous hydrocolloid solution is its pH. In this study, the pH of the topical viscous hydrocolloid solution preparations were measured at the temperature of 25 ºC in three replicates and the pH values were displayed in Tables 4.1 and 4.2. Table 4.1 showed the pH value of all topical viscous hydrocolloid solution preparations measured in three replicates, while, Table 4.2 showed the summary of the pH value of all topical viscous hydrocolloid solution preparations presented as mean ± standard deviation.

Table 4.1: The pH mesurement of all topical viscous hydrocolloid solution preparations

	pH at 25 ºC				
	Base	10%	15%	20%	25%
Measurement 1	6.42	3.08	2.87	2.75	2.68
Measurement 2	6.49	3.07	2.85	2.73	2.66
Measurement 3	6.50	3.06	2.86	2.72	2.65
Mean	6.47	3.07	2.86	2.73	2.67
Standard deviation (SD)	0.04	0.01	0.01	0.02	0.012

Base represented base topical viscous hydrocolloid solution; 10% represented 10% L-ascorbic acid topical viscous hydrocolloid solution; 15% represented 15% L-ascorbic acid topical viscous hydrocolloid solution; 20% represented 20% L-ascorbic acid topical viscous hydrocolloid solution; and 25% represented 25% L-ascorbic acid topical viscous hydrocolloid solution.

Table 4.2: The pH value of all types of topical viscous hydrocolloid solution preparations presented as mean ± standard deviation

Types of Topical Viscous Hydrocolloid Solutions	pH at 25 ºC (Mean ± SD)
Base	6.47 ± 0.04
10%	3.07 ± 0.01
15%	2.86 ± 0.01
20%	2.73 ± 0.02
25%	2.67 ± 0.02

Base represented base topical viscous hydrocolloid solution; 10% represented 10% L-ascorbic acid topical viscous hydrocolloid solution; 15% represented 15% L-ascorbic acid topical viscous hydrocolloid solution; 20% represented 20% L-ascorbic acid topical viscous hydrocolloid solution; and 25% represented 25% L-ascorbic acid topical viscous hydrocolloid solution.

The changes of the pH of all topical viscous hydrocolloid solution preparations were plotted in a graph (Figure 4.1) to provide clearer visual image for exploring the trend of the pH change.

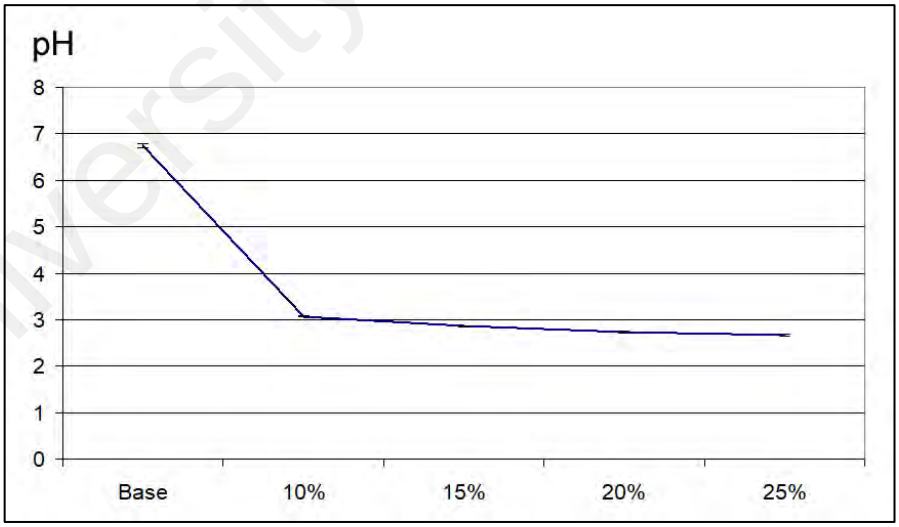

Figure 4.1: The trend of the pH changes by adding the 10%, 15%, 20% and 25% L-ascorbic acid into base topical viscous hydrocolloid solution

As referimg to Figure 4.1, it was observed that the pH of the base topical viscous hydrocolloid solution was much affected by adding 10% of L-ascorbic acid as the pH value of the base topical viscous hydrocolloid solution was initially from 6.47 ± 0.04

decreases to 3.07 ± 0.01 after adding 10% L-ascorbic acid into it. Nevertheless, the pH value of the topical viscous hydrocolloid solution was not much decreased when the amount of L-ascorbic acid was increased from 10% to 25%.

4.2 Viscosity Measurement of the Topical Viscous Hydrocolloid Solution

Viscosity is important property of the topical viscous hydrocolloid solution as more viscous solution is capable to maintain the topical viscous hydrocolloid solution on the skin surface of the subject longer. Thus, in this study, the viscosity of the topical viscous hydrocolloid solution preparations were measured at the temperature of 25 ºC in three replicates and the viscosity values were displayed in Tables 4.3 and 4.4. Table 4.3 showed the viscosity value of the 3 base topical viscous hydrocolloid solutions prepared by dissolving CMC (1 g, 2 g and 3 g respectively) into distilled water (99 g, 98 g and 97 g respectively) at the temperature of 25 °C with agitation in a 100 mL beaker until a clear, almost colourless base topical viscous hydrocolloid solution was obtained. The viscosity value were measured in three replicates and presented as mean ± standard deviation. The results of the viscosity value were showed in Table 4.3 and Figure 4.2.

Table 4.3: The viscosity value of base topical viscous hydrocolloid solution prepared with 1%, 2% and 3% CMC. The viscosity value were presented as mean ± standard deviation

Shear Rate (1/s)	Viscosity (Pa·s)		
	1% CMC	2% CMC	3% CMC
1	1.433 ± 0.186	10.667 ± 0.379	39.260 ± 0.314
6.21	0.897 ± 0.085	5.630 ± 0.171	16.660 ± 0.242
11.4	0.740 ± 0.025	4.407 ± 0.165	11.167 ± 0.231
16.6	0.655 ± 0.023	3.747 ± 0.153	9.023 ± 0.115
21.8	0.587 ± 0.006	3.280 ± 0.089	7.553 ± 0.098
27.1	0.512 ± 0.010	2.853 ± 0.061	6.183 ± 0.029
32.3	0.478 ± 0.006	2.490 ± 0.030	5.483 ± 0.029
37.5	0.448 ± 0.015	2.337 ± 0.025	5.027 ± 0.029
42.7	0.425 ± 0.006	2.130 ± 0.030	4.643 ± 0.038
47.9	0.408 ± 0.006	2.013 ± 0.025	4.367 ± 0.021
53.1	0.388 ± 0.007	1.853 ± 0.025	4.030 ± 0.026
58.3	0.378 ± 0.001	1.793 ± 0.025	3.853 ± 0.025
63.5	0.368 ± 0.003	1.667 ± 0.015	3.607 ± 0.006
68.8	0.345 ± 0.005	1.580 ± 0.010	3.443 ± 0.006
74	0.339 ± 0.006	1.527 ± 0.006	3.207 ± 0.006
79.2	0.328 ± 0.001	1.470 ± 0.010	3.090 ± 0.010
84.4	0.323 ± 0.001	1.413 ± 0.006	2.983 ± 0.006
89.6	0.316 ± 0.004	1.357 ± 0.006	2.813 ± 0.006
94.8	0.304 ± 0.003	1.310 ± 0.000	2.700 ± 0.010
100	0.295 ± 0.002	1.267 ± 0.006	2.597 ± 0.006

To provide clearer visual image, the viscosity value of the above 3 types of base topical viscous hydrocolloid solutions were plotted in a graph and showed in Figure 4.2.

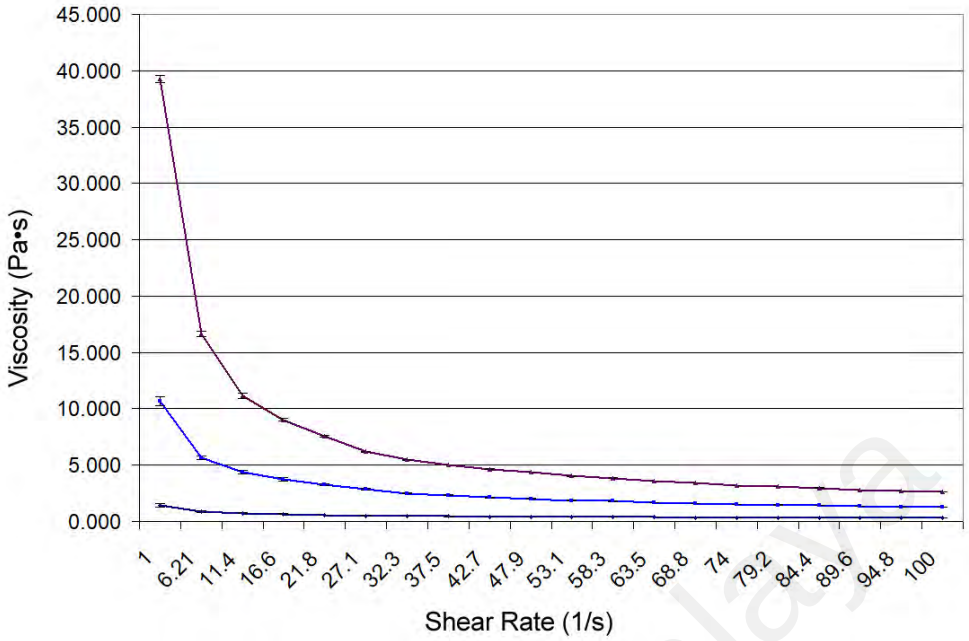

Figure 4.2: The viscosity value of the base topical viscous hydrocolloid solutions prepared by 1%, 2% and 3% CMC

The lowest line (dark blue line) represented the viscosity value of the 1% CMC hydrocolloid solution; the medium line (blue line) represented the viscosity value of the 2% CMC hydrocolloid solution; the top line (purple line) represented the viscosity value of the 3% CMC hydrocolloid solution.

The viscosity of 2% carboxymethyl cellulose topical hydrocolloid solution is the most suitable viscosity for application on human skin and it could statically maintain on the human skin, it was thus chosen as the base topical hydrocolloid solution. The 2% carboxymethyl cellulose topical hydrocolloid solution will be used as base topical viscous hydrocolloid solution to mix with 10%, 15%, 20% and 25% L-ascorbic acid.

L-ascorbic acid topical viscous hydrocolloid solutions with different concentrations of L-ascorbic acid (10%, 15%, 20% and 25% respectively) were prepared by respectively mixing 1 g, 1.5 g, 2.0 g and 2.5 g of L-ascorbic acid into 9 g, 8.5 g, 8.0 g and 7.5 g of the 2% carboxymethyl cellulose topical hydrocolloid solutions. Table 4.4 showed the viscosity of base topical viscous hydrocolloid solution (with 2% CMC) preparation and 10%, 15%, 20% and 25% L-ascorbic acid topical viscous

hydrocolloid solutions. The viscosity value were also measured in three replicates and presented as mean ± standard deviation.

Table 4.4: The viscosity value of base topical hydrocolloid solution and 10%, 15%, 20% and 25% L-ascorbic acid topical viscous hydrocolloid solutions were presented as mean ± standard deviation

Shear Rate (1/s)	Viscosity (Pa·s)				
	Base	10%	15%	20%	25%
1	10.667 ± 0.379	9.953 ± 1.725	7.940 ± 0.185	6.937 ± 0.125	6.280 ± 0.185
6.21	5.630 ± 0.171	3.880 ± 0.167	4.333 ± 0.126	3.983 ± 0.075	3.767 ± 0.100
11.4	4.407 ± 0.165	3.037 ± 0.057	3.897 ± 0.031	3.063 ± 0.058	2.867 ± 0.081
16.6	3.747 ± 0.153	2.637 ± 0.068	3.160 ± 0.085	2.590 ± 0.052	2.370 ± 0.050
21.8	3.280 ± 0.089	2.260 ± 0.070	2.433 ± 0.050	2.283 ± 0.055	2.040 ± 0.044
27.1	2.853 ± 0.061	1.910 ± 0.044	2.127 ± 0.031	2.023 ± 0.021	1.837 ± 0.040
32.3	2.490 ± 0.030	1.737 ± 0.015	1.857 ± 0.035	1.807 ± 0.021	1.657 ± 0.035
37.5	2.337 ± 0.025	1.613 ± 0.015	1.760 ± 0.030	1.613 ± 0.015	1.533 ± 0.015
42.7	2.130 ± 0.030	1.467 ± 0.025	1.627 ± 0.029	1.563 ± 0.015	1.470 ± 0.010
47.9	2.013 ± 0.025	1.420 ± 0.017	1.533 ± 0.015	1.417 ± 0.012	1.350 ± 0.010
53.1	1.853 ± 0.025	1.303 ± 0.012	1.413 ± 0.015	1.363 ± 0.006	1.300 ± 0.017
58.3	1.793 ± 0.025	1.243 ± 0.012	1.370 ± 0.010	1.257 ± 0.006	1.213 ± 0.015
63.5	1.667 ± 0.015	1.203 ± 0.012	1.270 ± 0.010	1.193 ± 0.006	1.153 ± 0.006
68.8	1.580 ± 0.010	1.127 ± 0.006	1.207 ± 0.006	1.163 ± 0.006	1.113 ± 0.006
74	1.527 ± 0.006	1.067 ± 0.006	1.173 ± 0.006	1.107 ± 0.006	1.053 ± 0.006
79.2	1.470 ± 0.010	1.037 ± 0.006	1.133 ± 0.006	1.053 ± 0.006	0.997 ± 0.002
84.4	1.413 ± 0.006	1.007 ± 0.006	1.087 ± 0.006	0.997 ± 0.002	0.960 ± 0.002
89.6	1.357 ± 0.006	0.974 ± 0.006	1.053 ± 0.006	0.959 ± 0.001	0.926 ± 0.001
94.8	1.310 ± 0.000	0.940 ± 0.005	1.013 ± 0.006	0.927 ± 0.001	0.896 ± 0.001
100	1.267 ± 0.006	0.906 ± 0.004	0.977 ± 0.001	0.901 ± 0.001	0.866 ± 0.001

Base represented base topical viscous hydrocolloid solution; 10% represented 10% L-ascorbic acid topical viscous hydrocolloid solution; 15% represented 15% L-ascorbic acid topical viscous hydrocolloid solution; 20% represented 20% L-ascorbic acid topical viscous hydrocolloid solution; and 25% represented 25% L-ascorbic acid topical viscous hydrocolloid solution.

To provide clearer visual image, the viscosity value of the base topical viscous hydrocolloid solution and all types of L-ascorbic acid topical viscous hydrocolloid solutions were ploted in a graph and showed in Figure 4.3.

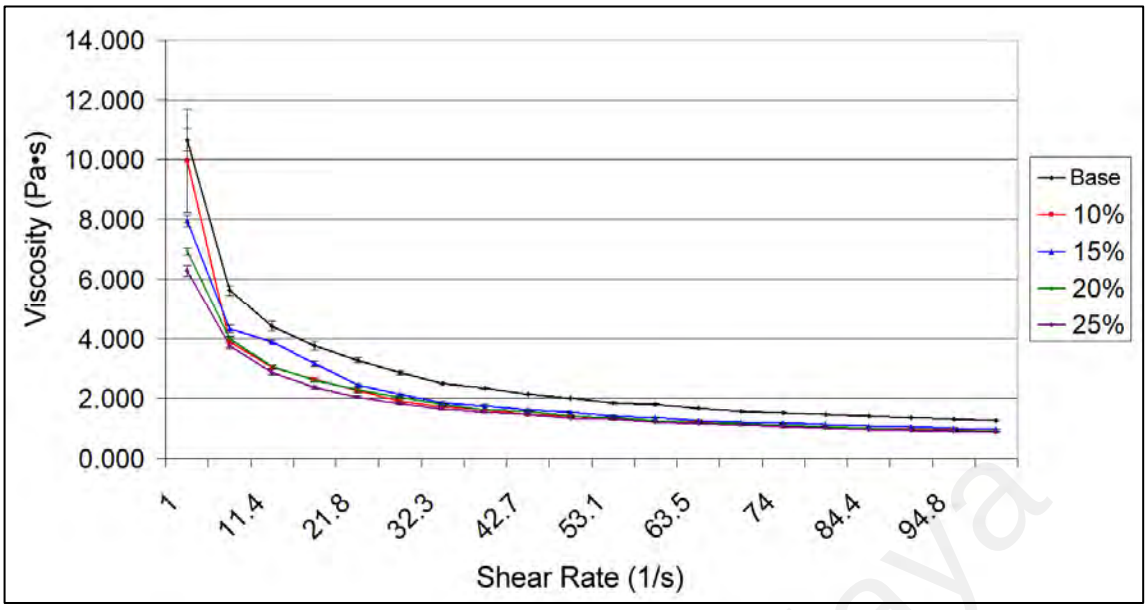

Figure 4.3: The viscosity value of the base topical viscous hydrocolloid solution and all types of L-ascorbic acid topical viscous hydrocolloid solutions

The topest line represented the viscosity value of the base topical viscous hydrocolloid solution, followed by 10%, 15%, 20% and 25% L-ascorbic acid topical viscous hydrocolloid solutions.

As referring to Figure 4.3, it was observed that the viscosity of the base topical viscous hydrocolloid solution was not affected by adding 10% of L-ascorbic acid as the viscosity of the base topical viscous hydrocolloid solution only slightly decreased after adding 10% of L-ascorbic acid into it. Besides, the viscosity of the topical viscous hydrocolloid solution is almost similar when the amount of L-ascorbic acid was increased from 10% to 25%.

4.3 Safety Assessment of L-Ascorbic Acid Topical Viscous Hydrocolloid Solution

In order to determine the safety of the L-ascorbic acid topical viscous hydrocolloid solutions used in this study, the safety assessment of the L-ascorbic acid topical viscous hydrocolloid solutions used in this study were conducted based on the calculation of the Systemic Exposure Dosage (SED) listed in Equation (5) and the Margin of Safety (MoS) listed in Equation (6) of the cosmetic ingredients used in the L-ascorbic acid topical viscous hydrocolloid solutions.

4.3.1 Safety Assessment of 10% L-Ascorbic Acid Topical Viscous Hydrocolloid Solution

4.3.1.1 Calculation of the Systemic Exposure Dosage (SED) and Margin of Safety (MoS) for 1.8% of CMC

SED = **A** (mg/kg bw/day) x **C** (%)/100 x **Dap** (%)/100

The L-ascorbic acid topical viscous hydrocolloid solution is intended to be used on adult's face. Thus, based on the SCC's Notes of Guidance for The Testing of Cosmetic Ingredients and Their Safety Evaluation 9th Revision, 2016, where,

i) **A** is 24.14 mg/kg bw/day for leave on face cream, lotion, gel or liquid. The adult's body weight was accepted as 60 kg;

ii) **C** is 1.8 as the concentration of CMC is 1.8%; and

iii) **Dap** is **100 as** the maximum value of 100% is used for the dermal absorption (**Dap**) expressed as a percentage of the test dose assumed to be applied in real-life conditions.

Thus,

SED = 24.14 (mg/kg bw/day) x 1.8 (%)/100 x 100 (%)/100

= 0.434 mg/kg bw/day

MoS = NOAEL / SED

= 6000 / 0.434

= 13,824.885

4.3.1.2 Calculation of the Systemic Exposure Dosage (SED) and Margin of Safety (MoS) for 10% L-Ascorbic Acid

SED = **A** (mg/kg bw/day) x **C** (%)/100 x **Dap** (%)/100

The L-ascorbic acid topical viscous hydrocolloid solution is intended to be used on adult's face, thus, based on The SCC's Notes of Guidance for The Testing of Cosmetic Ingredients and Their Safety Evaluation 9th Revision, 2016, where,

i) **A** is 24.14 mg/kg bw/day for leave on face cream, lotion, gel or liquid. The adult's body weight was accepted as 60 kg;

ii) **C** is 10 as the concentration of L-ascorbic acid is 10%; and

iii) **Dap** is **100 as** the maximum value of 100% is used for the dermal absorption (**Dap**) expressed as a percentage of the test dose assumed to be applied in real-life conditions.

Thus,

SED = 24.14 (mg/kg bw/day) x 10 (%)/100 x 100 (%)/100

= 2.414 mg/kg bw/day

MoS = NOAEL / SED

= 2000 / 2.414

= 828.500

The MoS values of 1.8% of CMC and 10% of L-ascorbic acid are greater than 100 (MoS >100). Thus, it can be concluded that the 10% L-ascorbic acid topical viscous hydrocolloid solution used in this study is not likely to be hazardous when use on human face.

4.3.2 Safety Assessment of 15%, 20% and 25% L-Ascorbic Acid Topical Viscous Hydrocolloid Solutions

The SED and MoS values of 1.7%, 1.6% and 1.5% of CMC; and 15%, 20% and 25% L-ascorbic acid were calculated based on the Equation (5) and Equation (6). The actual method of calculation of SED and MoS value are similar with the calculation listed in Section 4.3.1.1 and 4.3.1.2. The results of these SED and MoS value were summarized and listed in Table 4.5 and Table 4.6.

Table 4.5: The SED and MoS values of 1.7%, 1.6% and 1.5% CMC

1.7%		1.6%		1.5%	
SED	MoS	SED	MoS	SED	MoS
0.410	14,634.146	0.386	15,544.041	0.362	16,574.586

Table 4.6: The SED and MoS value of 15%, 20% and 25% L-ascorbic acid

15%		20%		25%	
SED	MoS	SED	MoS	SED	MoS
3.621	552.333	4.828	414.250	6.035	658.978

The MoS values of 1.7%, 1.6% and 1.5% CMC; and 15%, 20% and 25% L-ascorbic acid were greater than 100 (MoS >100). Thus, it can also be concluded that the 15%, 20% and 25% L-ascorbic acid topical viscous hydrocolloid solutions used in this study are not likely to be hazardous when use on human face.

4.4 Melanogenesis Inhibition Evaluation of L-Ascorbic Acid Topical Viscous Hydrocolloid Solution – Treatment Time Optimization Study

5 g of 10% L-ascorbic acid topical viscous hydrocolloid solution was applied to one side of the face of each subject while 5 g of base topical viscous hydrocolloid solution as placebo was applied to the other side of the face as a control. Both sides of the face were treated with the invented transdermal iontophoresis device (8 V) for 15 minutes, 30 minutes, 45 minutes and 60 minutes respectively. The melanin index value of both sides of the face were measured by using Mexameter MX18 before and after the treatment. The melanin index value of the 20 subjects before and after the treatment are summarized and listed in Table 4.7 to Table 4.10.

Table 4.7: Melanin index value (15 minutes treatment)

	Treated Side		Control Side	
Subject	Before	After	Before	After
1	286	273	287	285
2	298	283	304	302
3	227	217	228	225
4	327	320	325	323
5	286	277	306	303
6	285	277	298	297
7	230	219	222	221
8	231	222	233	230
9	325	319	320	321
10	329	320	317	314
11	197	188	201	200
12	175	169	178	176
13	180	169	182	181
14	251	244	241	240
15	177	165	178	176
16	183	173	186	183
17	179	171	182	179
18	176	169	179	178
19	251	246	248	246
20	238	232	236	235
Mean	241.55	232.65	242.55	240.75
SEM	12.31	12.33	12.21	12.23

Table 4.7 showed the melanin index value for both treated side (treated with 10% L-ascorbic acid topical viscous hydrocolloid solution) and control side (treated with base topical viscous hydrocolloid solution) of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 15 minutes. In Table 4.7, the mean of melanin index value and the standard error of the mean (SEM) were calculated and listed on the last two rows of the table.

Table 4.8: Melanin index value (30 minutes treatment)

	Treated Side		Control Side	
Subject	Before	After	Before	After
1	286	258	288	286
2	304	271	308	306
3	230	205	231	228
4	327	304	328	326
5	295	264	301	299
6	289	262	298	299
7	226	194	229	226
8	231	207	233	230
9	325	305	321	319
10	329	319	319	316
11	199	180	201	200
12	176	162	178	176
13	184	148	184	181
14	246	238	243	242
15	177	144	178	176
16	183	148	183	183
17	179	165	182	181
18	176	161	180	179
19	251	244	248	247
20	237	229	234	233
Mean	242.5	220.4	243.35	241.65
SEM	12.43	12.72	12.27	12.25

Table 4.8 showed the melanin index value for both treated side (treated with 10% L-ascorbic acid topical viscous hydrocolloid solution) and control side (treated with base topical viscous hydrocolloid solution) of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 30 minutes. In Table 4.8, the mean of

melanin index value and the standard error of the mean (SEM) were calculated and listed on the last two rows of the table.

Table 4.9: Melanin index value (45 minutes treatment)

	Treated Side		Control Side	
Subject	Before	After	Before	After
1	271	241	286	282
2	258	222	304	301
3	227	199	228	225
4	327	302	325	323
5	256	222	307	299
6	263	233	296	298
7	233	199	222	218
8	228	201	233	230
9	325	302	320	324
10	329	318	317	314
11	197	176	200	200
12	175	159	178	176
13	180	142	182	181
14	251	242	240	244
15	168	130	180	176
16	180	140	186	180
17	178	162	180	175
18	173	156	179	178
19	251	243	245	245
20	250	241	235	235
Mean	236	211.5	242.15	240.2
SEM	11.69	12.42	12.19	12.31

Table 4.9 showed the melanin index value for both treated side (treated with 10% L-ascorbic acid topical viscous hydrocolloid solution) and control side (treated with base topical viscous hydrocolloid solution) of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 45 minutes. In Table 4.9, the mean of melanin index value and the standard error of the mean (SEM) were calculated and listed on the last two rows of the table.

Table 4.10: Melanin index value (60 minutes treatment)

	Treated Side		Control Side	
Subject	Before	After	Before	After
1	271	238	287	284
2	298	256	304	301
3	227	196	228	225
4	327	299	327	325
5	256	219	298	297
6	273	233	286	284
7	233	199	230	224
8	235	201	232	230
9	325	302	325	324
10	329	317	317	314
11	203	175	202	200
12	174	154	178	176
13	180	141	182	181
14	249	238	243	244
15	178	130	182	179
16	180	139	183	180
17	178	161	176	175
18	176	156	179	178
19	254	240	246	245
20	238	225	236	235
Mean	239.2	210.95	242.05	240.05
SEM	11.88	12.51	12.13	12.12

Table 4.10 showed the melanin index value for both treated side (treated with 10% L-ascorbic acid topical viscous hydrocolloid solution) and control side (treated with base topical viscous hydrocolloid solution) of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 60 minutes. In Table 4.9, the mean of melanin index value for and the standard error of the mean (SEM) were calculated and listed on the last two rows of the table.

For data analysis purpose, the variation and the percentage of variation (% variation) for both treated side and control side were calculated and the results were tabulated in Table 4.11 to Table 4.14. Besides, the differences between treated group and control group were analyzed for statistical significance by Student's one-tailed and

paired t-test using a standard software package. The criterion for statistical significance was expressed as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Table 4.11 showed the melanin index variation and % variation for both treated side and control side of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 15 minutes.

Table 4.11: The variation and % variation of 15 minutes treatment

	Melanin Index of Treated Side		Melanin Index of Control Side	
	Before	After	Before	After
Mean ± SEM	241.55 ± 12.31	232.65 ± 12.33	242.55 ± 12.21	240.75 ± 12.23
Variation (After – Before)	- 8.9		- 1.8	
% Variation (Control After - Treated After /Treated After) (%)	3.48%			
Statistical Significance (/Control)	$p<0.001$			

Table 4.12 showed the melanin index variation and % variation for both treated side and control side of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 30 minutes.

Table 4.12: The variation and % variation of 30 minutes treatment

	Melanin Index of Treated Side		Melanin Index of Control Side	
	Before	After	Before	After
Mean ± SEM	242.50 ± 12.43	220.40 ± 12.72	243.35 ± 12.27	241.65 ± 12.25
Variation (After – Before)	- 22.1		- 1.7	
% Variation (Control After - Treated After /Treated After) (%)	9.64%			
Statistical Significance (/Control)	$p<0.001$			

Table 4.13 showed the melanin index variation and % variation for both treated side and control side of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 45 minutes.

Table 4.13: The variation and % variation of 45 minutes treatment

	Melanin Index of Treated Side		Melanin Index of Control Side	
	Before	After	Before	After
Mean ± SEM	236.00 ± 11.69	211.50 ± 12.42	242.15 ± 12.19	240.20 ± 12.31
Variation (After – Before)	- 24.5		- 1.95	
% Variation (Control After - Treated After /Treated After) (%)	13.57%			
Statistical Significance (/Control)	$p<0.001$			

Table 4.14 showed the melanin index variation and % variation for both treated side and control side of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 60 minutes.

Table 4.14: The variation and % variation of 60 minutes treatment

	Melanin Index of Treated Side		Melanin Index of Control Side	
	Before	After	Before	After
Mean ± SEM	239.20 ± 11.88	210.95 ± 12.51	242.05 ± 12.13	240.05 ± 12.12
Variation (After – Before)	- 28.25		- 2.0	
% Variation (Control After - Treated After /Treated After) (%)	13.79%			
Statistical Significance (/Control)	$p<0.001$			

To provide clearer visual image, the % variation for 15 minutes to 60 minutes treatment run time were plotted in a graph and showed in Figure 4.4.

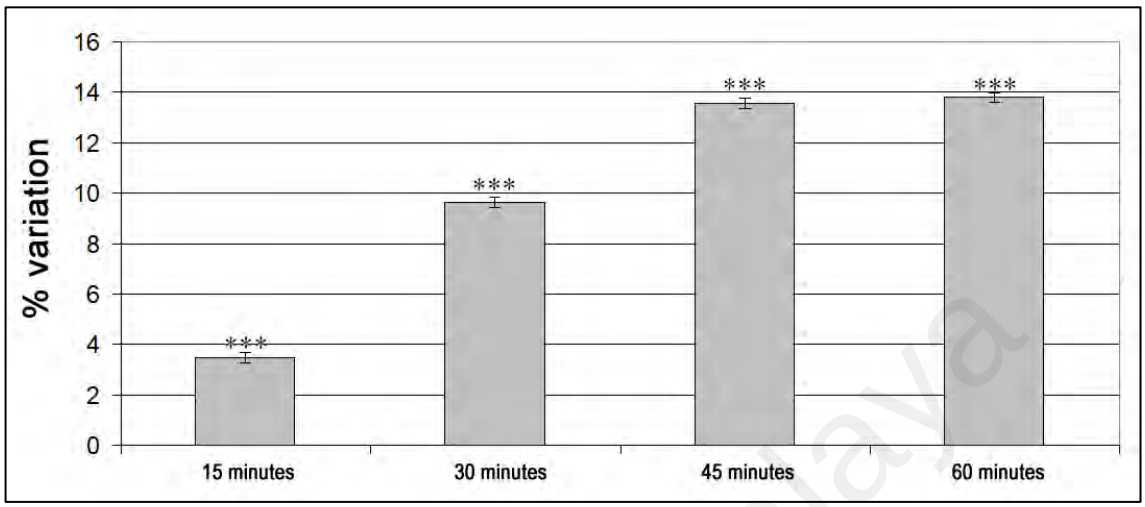

Figure 4.4: The % variation between 15 minutes, 30 minutes, 45 minutes and 60 minutes transdermal iontophoresis treatment run time

*p<0.05, **p<0.01, ***p<0.001: statistically significant compared with control group

From the data indicated in Table 4.11 to Table 4.14 and Figure 4.4, it can be concluded that subjects treated with 10% L-ascorbic acid topical viscous hydrocolloid solution with 15 minutes, 30 minutes, 45 minutes and 60 minutes treatment run time were able to inhibit the melanogenesis activity in the human epidermis (*in vivo* inhibition) effectively. The p value between the treated group and control group for 15 minutes, 30 minutes, 45 minutes and 60 minutes treatment run time were smaller than 0.001. This indicated that the melanogenesis inhibition activity between the treated group and control group were highly statistically significant. In other words, the melanogenesis inhibition activity results between the treated group and control group were obvious and not likely to occur randomly or by chance, but, instead was likely to be attributable to a specific cause.

However, from the % variation showed in Figure 4.4, 45 minutes transdermal iontophoresis treatment run time was the most suitable treatment run time as after 45 minutes transdermal iontophoresis treatment run time no significant melanogenesis inhibition activity was observed. Besides, extension of the treatment run time will increase the discomfort of the subjects and the chance of adverse skin reactions. Thus, 45 minutes of treatment run time was chosen as the optimal treatment run time for conducting the melanogenesis inhibition evaluation in this study.

4.5 Melanogenesis Inhibition Evaluation of L-Ascorbic Acid Topical Viscous Hydrocolloid Solution – L-Ascorbic Acid Concentration Optimization Study

5 g of 10%, 15%, 20% and 25% L-ascorbic acid topical viscous hydrocolloid solutions were respectively applied onto one side of the face of each subject while 5 g of base topical viscous hydrocolloid solution as placebo was applied onto the other side as a control. Then, both sides of the face were treated with the invented transdermal iontophoresis (8V) for 45 minutes. The melanin index value of both sides of the face were measured by using Mexameter MX18 before and after the treatment. The melanin index value of the 20 subjects before and after the treatment were summarized and listed in Table 4.15 to Table 4.18.

Table 4.15 showed the melanin index value for both treated side (treated with 10% L-ascorbic acid topical viscous hydrocolloid solution) and control side (treated with base topical viscous hydrocolloid solution) of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 45 minutes. In Table 4.15, the mean of melanin index value and the standard error of the mean (SEM) were calculated and listed on the last two rows of the table.

Table 4.15: Melanin index value of 45 minutes treatment of 10% L-ascorbic acid topical viscous hydrocolloid solution and base topical viscous hydrocolloid solution

	Treated Side		Control Side	
Subject	Before	After	Before	After
1	271	241	286	282
2	258	222	304	301
3	227	199	228	225
4	327	302	325	323
5	256	222	307	299
6	263	233	296	298
7	233	199	222	218
8	228	201	233	230
9	325	302	320	324
10	329	318	317	314
11	197	176	200	200
12	175	159	178	176
13	180	142	182	181
14	251	242	240	244
15	168	130	180	176
16	180	140	186	180
17	178	162	180	175
18	173	156	179	178
19	251	243	245	245
20	250	241	235	235
Mean	236	211.5	242.15	240.2
SEM	11.69	12.42	12.19	12.31

Table 4.16 showed the melanin index value for both treated side (treated with 15% L-ascorbic acid topical viscous hydrocolloid solution) and control side (treated with base topical viscous hydrocolloid solution) of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 45 minutes. In Table 4.16, the mean of melanin index value and the standard error of the mean (SEM) were calculated and listed on the last two rows of the table.

Table 4.16: Melanin index value of 45 minutes treatment of 15% L-ascorbic acid topical viscous hydrocolloid solution and base topical viscous hydrocolloid solution

	Treated Side		Control Side	
Subject	Before	After	Before	After
1	280	241	274	277
2	265	211	290	286
3	233	189	221	226
4	343	324	311	320
5	254	206	287	282
6	274	210	285	282
7	232	185	218	217
8	237	193	221	219
9	340	324	315	315
10	342	326	310	315
11	197	170	200	200
12	171	150	178	176
13	170	121	182	181
14	251	240	240	244
15	169	120	180	176
16	166	121	186	180
17	169	148	180	175
18	171	152	179	178
19	241	232	245	245
20	254	241	235	235
Mean	237.95	205.2	236.85	236.45
SEM	13.26	14.51	11.14	11.54

Table 4.17 showed the melanin index value for both treated side (treated with 20% L-ascorbic acid topical viscous hydrocolloid solution) and control side (treated with base topical viscous hydrocolloid solution) of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 45 minutes. In Table 4.17, the mean of melanin index value and the standard error of the mean (SEM) were calculated and listed on the last two rows of the table.

Table 4.17: Melanin index value of 45 minutes treatment of 20% L-ascorbic acid topical viscous hydrocolloid solution and base topical viscous hydrocolloid solution

	Treated Side		Control Side	
Subject	Before	After	Before	After
1	277	241	276	260
2	271	243	283	264
3	223	169	232	203
4	337	311	312	313
5	273	243	280	264
6	271	245	282	269
7	235	175	221	205
8	216	169	229	207
9	338	301	312	309
10	338	307	313	315
11	217	195	216	218
12	190	182	190	197
13	187	158	215	210
14	273	245	242	247
15	192	180	191	191
16	194	184	193	200
17	181	155	213	209
18	189	159	217	210
19	280	251	239	237
20	265	242	242	255
Mean	247.35	217.75	244.9	239.15
SEM	11.75	11.53	9.15	9.05

Table 4.18 showed the melanin index value for both treated side (treated with 20% L-ascorbic acid topical viscous hydrocolloid solution) and control side (treated with base topical viscous hydrocolloid solution) of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 45 minutes. In Table 4.18, the mean of melanin index value and the standard error of the mean (SEM) were calculated and listed on the last two rows of the table.

Table 4.18: Melanin index value of 45 minutes treatment of 25% L-ascorbic acid topical viscous hydrocolloid solution and base topical viscous hydrocolloid solution

	Treated Side		Control Side	
Subject	Before	After	Before	After
1	268	242	253	252
2	260	234	280	269
3	219	178	193	196
4	325	313	286	291
5	257	227	271	262
6	261	238	289	269
7	220	174	194	193
8	220	175	198	206
9	322	312	286	276
10	327	304	293	293
11	237	214	228	227
12	232	212	224	222
13	199	164	205	205
14	280	266	254	253
15	228	209	227	221
16	232	213	224	223
17	202	165	203	199
18	196	161	206	209
19	278	266	251	246
20	279	255	258	260
Mean	252.1	226.1	241.15	238.6
SEM	9.13	10.97	7.86	7.24

For data analysis purpose, the variation and % variation for both treated side and control side were calculated and the results were tabulated in Table 4.19 to Table 4.22. Besides, the differences between treated group and control group were analyzed for statistical significance by Student's one-tailed and paired t-test using a standard software package. The criterion for statistical significance was expressed as * $p<0.05$, ** $p<0.01$ and ***$p<0.001$.

Table 4.19 showed the melanin index variation and % variation for both treated side (10% L-ascorbic acid) and control side of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 45 minutes.

Table 4.19: The melanin index variation and % variation of 10% L-ascorbic acid

	Melanin Index of Treated Side		Melanin Index of Control Side	
	Before	After	Before	After
Mean ± SEM	236.00 ± 11.69	211.50 ± 12.42	242.15 ± 12.19	240.20 ± 12.31
Variation (After – Before)	- 24.5		- 1.95	
% Variation (Control After - Treated After /Treated After) (%)	13.57%			
Statistical Significance (/Control)	$p<0.001$			

Table 4.20 showed the melanin index variation and % variation for both treated side (15% L-ascorbic acid) and control side of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 45 minutes.

Table 4.20: The melanin index variation and % variation of 15% L-ascorbic acid

	Melanin Index of Treated Side		Melanin Index of Control Side	
	Before	After	Before	After
Mean ± SEM	237.95 ± 13.26	205.20 ± 14.51	236.85 ± 11.14	236.45 ± 11.54
Variation (After – Before)	- 32.75		- 0.4	
% Variation (Control After - Treated After /Treated After) (%)	15.22%			
Statistical Significance (/Control)	$p<0.001$			

Table 4.21 showed the melanin index variation and % variation for both treated side (20% L-ascorbic acid) and control side of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 45 minutes.

Table 4.21: The melanin index variation and % variation of 20% L-ascorbic acid

	Melanin Index of Treated Side		Melanin Index of Control Side	
	Before	After	Before	After
Mean ± SEM	247.35 ± 11.75	217.75 ± 11.53	244.9 ± 11.14	239.15 ± 9.05
Variation (After – Before)	- 29.6		- 5.75	
% Variation (Control After - Treated After /Treated After) (%)	9.83%			
Statistical Significance (/Control)	$p<0.001$			

Table 4.22 showed the melanin index variation and % variation for both treated side (25% L-ascorbic acid) and control side of the 20 subjects before and after conducting the transdermal iontophoresis (8V) for 45 minutes.

Table 4.22: The melanin index variation and % variation of 25% L-ascorbic acid

	Melanin Index of Treated Side		Melanin Index of Control Side	
	Before	After	Before	After
Mean ± SEM	252.10 ± 9.13	226.10 ± 10.97	241.15 ± 7.86	238.6 ± 7.24
Variation (After – Before)	- 26.0		- 2.55	
% Variation (Control After - Treated After /Treated After) (%)	5.53%			
Statistical Significance (/Control)	$p<0.001$			

To provide clearer visual image, the % variation for 10%, 15%, 20% and 25% L-ascorbic acid treated with the invented transdermal iontophoresis were plotted in a graph and shown in Figure 4.5.

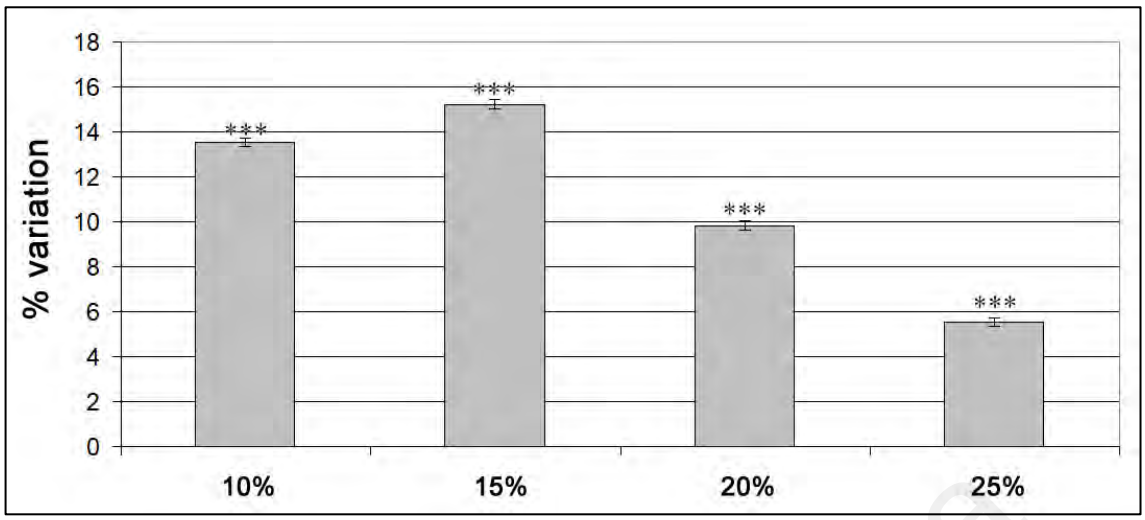

Figure 4.5: The % variation between 10%, 15%, 20% and 25% L-ascorbic acid with transdermal iontophoresis

*p<0.05, **p<0.01, ***p<0.001: statistically significant compared with control group

From the data indicated in Table 4.19 to Table 4.22 and Figure 4.5, it can be concluded that 10%, 15%, 20% and 25% L-ascorbic acid with transdermal iontophoresis were able to inhibit the melanogenesis activity in the human epidermis (*in vivo* inhibition) effectively. The *p* value between the treated group and control group for 10%, 15%, 20% and 25% L-ascorbic acid transdermal iontophoresis were smaller than 0.001. This indicated that the melanogenesis inhibition activity between the treated group and control group were highly statistically significant.

In other words, the melanogenesis inhibition activity results between the treated group and control group were obvious and not likely to occur randomly or by chance.

However, from the % variation showed in Figure 4.5, 15% L-ascorbic acid with transdermal iontophoresis seems to be the most effective method to inhibit the melanogenesis activity in human epidermis whilst 25% L-ascorbic acid with

transdermal iontophoresis seems to be the least effective method to inhibit the melanogenesis activity in human epidermis.

4.6 Effect of the 4 Weeks Topical Application of 15% L-Ascorbic Acid Topical Viscous Hydrocolloid Solution

15% L-ascorbic acid topical viscous hydrocolloid solution was applied twice daily for a continuous period of 4 weeks in a dose of 5 mg/cm^2 to one side of the face of each subject, while a base topical viscous hydrocolloid solution as placebo was applied to the other side as a control. The quantitative evaluation of *in vivo* melanogenesis inhibition activity was conducted by using Mexameter MX18 before treatment and 4 weeks after treatment. The results were tabulated in Table 4.23.

Table 4.23 showed the melanin index for both treated side (with 15% L-ascorbic acid) and control side of the 20 subjects before and after applying the 15% L-ascorbic acid topical viscous hydrocolloid solution and a base topical viscous hydrocolloid solution for the period of 4 weeks. In Table 4.23, the mean of melanin index value for and the standard error of the mean (SEM) were calculated and listed on the last two rows of the table.

Table 4.23: Melanin index value of 4 weeks topical application of 15% L-ascorbic acid topical viscous hydrocolloid solution

	Treated Side		Control Side	
Subject	Before	After	Before	After
1	274	277	275	277
2	290	286	292	289
3	221	226	221	226
4	301	310	301	310
5	287	278	287	286
6	245	241	245	245
7	218	215	220	214
8	221	214	221	218
9	315	313	315	313
10	300	305	299	305
11	220	215	233	223
12	178	176	197	188
13	182	181	182	181
14	240	244	240	244
15	180	176	188	176
16	210	202	210	208
17	254	249	254	249
18	179	175	178	183
19	233	228	233	228
20	225	219	245	246
Mean	238.65	236.5	241.8	240.45
SEM	9.77	10.16	9.32	9.85

For data analysis purpose, the variation and % variation for both treated side and control side were calculated and the results were tabulated in Table 4.24. Besides, the differences between treated group and control group were analyzed for statistical significance by student's one-tailed and paired t-test using a standard software package. The criterion for statistical significance was expressed as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Table 4.24 showed the melanin index variation and % variation for both treated side (with 15% L-ascorbic acid) and control side of the 20 subjects before and after applying the 15% L-ascorbic acid topical viscous hydrocolloid solution and a base topical viscous hydrocolloid solution for the period of 4 weeks.

Table 4.24: The melanin index variation and % variation of 4 weeks topical application of 15% L-ascorbic acid topical viscous hydrocolloid solution

	Melanin Index of Treated Side		Melanin Index of Control Side	
	Before	After	Before	After
Mean ± SEM	238.65 ± 9.77	236.50 ± 10.16	241.80 ± 9.32	240.45 ± 9.85
Variation (After – Before)	- 2.15		- 1.35	
% Variation (Control After - Treated After /Treated After) (%)	1.67%			
Statistical Significance (/Control)	*P*>0.05			

From the data indicated in Table 4.24, it can be concluded that applying the 15% L-ascorbic acid topical viscous hydrocolloid solution topically for the period of 4 weeks was not able to inhibit the melanogenesis activity in the human epidermis (*in vivo* inhibition) effectively.

The *p* value between the treated group and control group were greater than 0.05. This indicated that the melanogenesis inhibition activity between the treated group and control group were not statistically significant. In other words, the melanogenesis inhibition activity results between the treated group and control group were not obvious and likely to occur randomly or by chance.

4.7 Effect of the 4 Weeks Topical Application of 15% L-Ascorbic Acid Topical Viscous Hydrocolloid Solution with Transdermal Iontophoresis Device

5 g of 15% L-ascorbic acid topical viscous hydrocolloid solution was applied topically onto one side of the face of each subject, while 5 g of base topical viscous hydrocolloid solution, as placebo, was applied to the other side as a control. Both sides were treated with the invented transdermal iontophoresis device with the current densities of 70 μA for 45 minutes. The quantitative evaluation of *in vivo* melanogenesis

inhibition activity were conducted by using Mexameter MX18 before treatment and 1 day after the 4 weeks treatment. The results were tabulated in Table 4.25.

Table 4.25 showed the melanin index for both treated side (15% L-ascorbic acid) and control side of the 20 subjects before and after applying the 15% L-ascorbic acid topical viscous hydrocolloid solution and the base topical viscous hydrocolloid solution and treated with the invented transdermal iontophoresis (current densities of 70 μA) for 45 minutes for the period of 4 weeks. In Table 4.25, the mean of melanin index value and the standard error of the mean (SEM) were calculated and listed on the last two rows of the table.

Table 4.25: Melanin index value of 4 weeks 15% L-ascorbic acid topical viscous hydrocolloid solution treated with invented transdermal iontophoresis device

	Treated Side		Control Side	
Subject	Before	After	Before	After
1	275	241	275	269
2	264	231	292	289
3	231	199	221	226
4	343	312	301	310
5	254	216	287	286
6	272	234	245	245
7	232	194	220	214
8	237	203	221	218
9	338	309	315	313
10	335	315	299	305
11	197	176	233	225
12	171	150	197	188
13	170	160	182	181
14	251	235	240	244
15	176	149	188	179
16	164	149	210	208
17	169	152	180	175
18	168	152	179	178
19	241	230	245	238
20	254	236	235	233
Mean	237.1	212.15	238.25	236.2
SEM	12.99	12.21	9.77	10.28

For data analysis purpose, the variation and % variation for both treated side and control side were calculated and the results were tabulated in Table 4.26. Besides, the differences between treated group and control group were analyzed for statistical significance by Student's one-tailed and paired t-test using a standard software package. The criterion for statistical significance was expressed as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Table 4.26 showed the melanin index variation and % variation for both treated side (15% L-ascorbic acid topical viscous hydrocolloid solution treated with invented transdermal iontophoresis device) and control side of the 20 subjects before and after applying the 15% L-ascorbic acid topical viscous hydrocolloid solution and a base topical viscous hydrocolloid solution for the period of 4 weeks.

Table 4.26: The melanin index variation and % variation of 4 weeks 15% L-ascorbic acid topical viscous hydrocolloid solution treated with invented transdermal iontophoresis device

	Melanin Index of Treated Side		Melanin Index of Control Side	
	Before	After	Before	After
Mean ± SEM	237.10 ± 12.99	212.15 ± 12.21	238.25 ± 9.77	236.20 ± 10.28
Variation (After – Before)	- 24.95		- 2.05	
% Variation (Control After - Treated After /Treated After) (%)	11.33%			
Statistical Significance (/Control)	$P<0.001$			

From the data indicated in Table 4.26, it can be concluded that 15% L-ascorbic acid and treated with the invented transdermal iontophoresis were able to inhibit the melanogenesis activity in the human epidermis (*in vivo* inhibition) effectively. The *p* value between the treated group and control group for 15% L-ascorbic acid transdermal iontophoresis were smaller than 0.001. This indicated that the melanogenesis inhibition activities between the treated group and control group were highly statistically

significant. In other words, the melanogenesis inhibition activity results between the treated group and control group were obvious and not likely to occur randomly or by chance, but instead was likely to be attributable to a specific cause.

CHAPTER 5: DISCUSSION

5.1 The preparation of Base Topical Viscous Hydrocolloid Solution and L-Ascorbic Acid Topical Viscous Hydrocolloid Solution

A hydrocolloid solution is a colloid system wherein the colloid particles are hydrophilic polymers dispersed in water. The hydrophilic polymers in the hydrocolloid solution are generally contained many hydroxyl groups and may be polyelectrolytes. Besides, the two important properties of the hydrocolloid solution are viscosity (including thickening and gelling) and water binding. A viscous hydrocolloid solution is important in this study as viscous solution can form a thin layer and maintain the negatively charged L-ascorbic acid ion (anionic form) longer on the surface of the skin. The polyelectrolytes properties of the hydrocolloid solution also help to transfer the electrical current supply from the transdermal iontophoresis device to the inner surface of the skin. This ensures the successful of transdermal iontophoresis process.

In the study, CMC was chosen as the viscosity enhancer or gelling agent as it has been deemed safe and is used widely in food industry as food viscosity modifier. CMC is one of the plant based hydrocolloid and it can dissolve in water to produce viscous hydrocolloid solution. The easy way in producing viscous hydrocolloid solution is to add the dry CMC powder carefully into the water so that it is well dispersed (well-wetted). Adding the dry CMC powder in portions may be necessary. Adding the dry CMC powder into water must be followed with gently stirring to well disperse the dry CMC powder. Adding water to the dry CMC powder will produce a "clump" of solid that is very difficult to dissolve and required more time to dissolve completely. The time frame for preparing 2% CMC Topical Hydrocolloid Solution (completely dissolve 2 g of CMC into 98 g of distilled water) may require up to 40 minutes.

2% carboxymethyl cellulose topical hydrocolloid solution was chosen as the base topical viscous hydrocolloid solution and used to prepare the 10%, 15% L-ascorbic acid topical viscous hydrocolloid solution, this is due to the viscosity of this 2% carboxymethyl cellulose topical hydrocolloid solution is the most suitable viscosity (with the apparent viscosity of 2.013 ± 0.025 Pa·s) for application on human skin and it could statically maintain on the human skin and form a thin layer of viscous hydrocolloid solution. As mentioned earlier, maintaining the thin layer of viscous hydrocolloid solution onto human skin surface is very important during the entire transdermal iontophoresis process as it acts as conductive medium to facilitate the transdermal iontophoresis process and the reservoir to continually supply the L-ascorbic acid into skin.

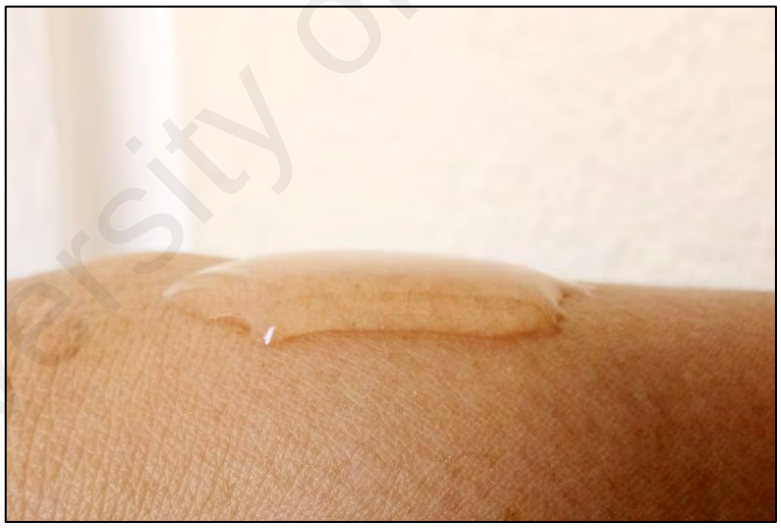

Figure 5.1: The actual photo showed the 2% CMC topical viscous hydrocolloid solution can be statically maintained on the human skin and form a thin layer of viscous hydrocolloid solution

The pH of 2% carboxymethyl cellulose topical hydrocolloid solution (the base topical viscous hydrocolloid solution) was 6.47 ± 0.04, after adding 10%, 15%, 20% and 25% of L-ascorbic acid, the pH decreased to 3.07 ± 0.01, 2.86 ± 0.01, 2.73 ± 0.02 and 2.67 ± 0.02 respectively. The adding of L-ascorbic acid and the decrease of pH

were not affect much on the viscosity of the solution. The viscosity of 2% carboxymethyl cellulose topical hydrocolloid solution was stable even after dissolving 25% L-ascorbic acid at a low pH (2.67 ± 0.02). Thus, it can be concluded that 2% carboxymethyl cellulose topical hydrocolloid solution was suitable to be used as the base topical viscous hydrocolloid solution to produce L-ascorbic acid topical viscous hydrocolloid solution up to 25% L-ascorbic acid concentration.

A viscous solution preparation is important in this study as viscous solution is required to maintain the L-ascorbic acid longer on the surface of the skin. In this study, L-ascorbic acid must be maintained on the surface of skin for at least 45 minutes during the transdermal iontophoresis treatment. The viscous topical hydrocolloid solution produced by 2% CMC is suitable for cosmetic use as it is non-sticky, comfortable and flakeless when it is dry.

L-ascorbic acid topical viscous hydrocolloid solutions with the concentrations of 10% and 15% L-ascorbic acid are very easy to prepare as these amounts of L-ascorbic acid are very easy to dissolve into the base topical viscous hydrocolloid solutions. The time required to dissolve these amounts of L-ascorbic acid is less than 5 minutes. However, 20% L-ascorbic acid required more time to dissolve into the base topical viscous hydrocolloid solution. 25% L-ascorbic acid is difficult to dissolve into the base topical viscous hydrocolloid solution and it required increase of the temperature of the base topical viscous hydrocolloid solution before the L-ascorbic acid can be completely dissolved into the base topical viscous hydrocolloid solution. The added temperature or heat increases the kinetic energy of the base topical viscous hydrocolloid solution molecules, allowing them to react and dissolve the L-ascorbic acid more quickly.

5.2 The Design and Fabrication of Transdermal Iontophoresis Device

In order to overcome chemical impermeability of L-ascorbic acid, a transdermal iontophoresis device was developed to promote percutaneous absorption of L-ascorbic acid into human skin for *in-vivo* melanogenesis inhibition. This device is an electrically assisted, safe, intensive, effective and non-invasive method which promotes percutaneous absorption of L-ascorbic acid into human skin. The device allows direct penetration of L-ascorbic acid into the human skin to promote ultimate absorption of L-ascorbic acid into the skin. Thus, maximum absorption of L-ascorbic acid could be performed and sufficient amount of L-ascorbic acid could be supplied to produce radiant and lighter skin complexion to the user.

Transdermal iontophoresis through electromotive force is an effective technique to facilitate the transport of permeants across the skin. The underlying principles of transdermal iontophoresis involve placing two oppositely charged electrodes at the appropriate sides on the skin. The active compound in its ionic form is placed under the electrode bearing the same charge as the active compound. The voltage source most often supplies a constant electric current that is converted to an ionic current at the electrodes. As the ions carry this current through the skin barrier, charged molecule is repelled from the working electrode into the skin.

An actual transdermal iontophoresis device fabricated according to this study is shown in Figure 5.2.

Figure 5.2: The transdermal iontophoresis device

Referring to Figure 5.2, all the electronic components are designed and built in a small transparent outer casing, the dimension of the outer casing is 14 cm (length) x 10 cm (width) x 10 cm (height). The pair of hand straps functioned as a counter electrode and the couple sets of coupling devices that connected to the disposable cotton mask sheet functioned as a working electrode. The total weight of the actual transdermal iontophoresis device is less than 700 g, thus, it can classify as a light weight portable device.

In the study, the ionic form of the dissolved L-ascorbic is negatively charged ion (anionic form). Figure 5.3 showed how the invented transdermal iontophoresis device is used. As shown in Figure 5.3, one end of the plurality set of coupling devices are

connected to the negative terminal of the voltage output terminal and another end is connected to the disposable cotton mask sheet. These plurality set of coupling devices are worked as the working electrode to deliver the negatively charged L-ascorbic acid ion into the skin. The two hand straps that work as counter electrode are connected to the positive terminal of the voltage output terminal.

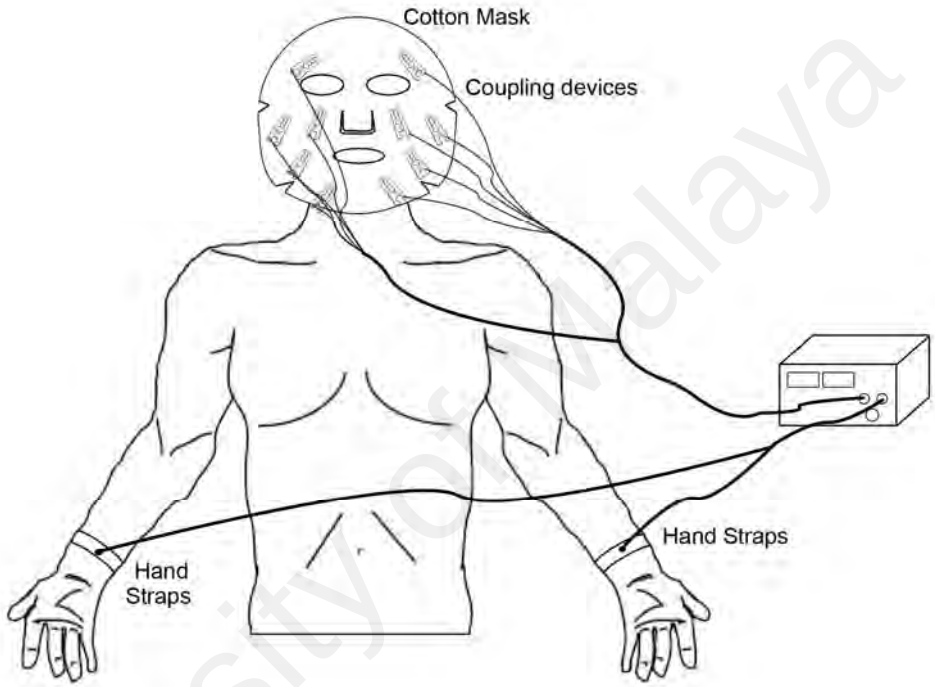

Figure 5.3: Use of the invented transdermal iontophoresis device

As illustrated in Figure 5.3, the disposal cotton mask sheet connected to coupling devices was placed onto the face of the subject. Before placing the mask, the face of the subject was cleansed and L-ascorbic acid topical viscous hydrocolloid solution was topically applied onto one side of the face of the subject while a base topical viscous hydrocolloid solution as placebo was applied onto the other side as a control. Both hand straps were put on both of the forearms of the subject. During the transdermal iontophoresis treatment, the electrical current that transferred by the coupling devices onto the subject's face was measured by the electrical multimeter and confirmed that the cotton mask sheet was able to successfully transfer the electrical current to the face of

the subject evenly. The design was comfortable for the subject during the 45 minutes transdermal iontophoresis treatment.

5.3 Safety Assessment of L-Ascorbic Acid Topical Viscous Hydrocolloid Solution

The MoS value is compared with a reference MoS, which is comparable to the uncertainty/assessment factor used in general to extrapolate from a group of test animals to an average human being, and subsequently from average humans to sensitive subpopulations. A default value of 100 (10×10) is generally accepted and a MoS of at least 100 therefore indicates that a cosmetic ingredient is considered safe for use.

The MoS value of CMC and L-ascorbic acid calculated in Sections 4.3 concluded that CMC and L-ascorbic acid are safe to use as leave on face gel at all the four concentrations because MoS >100. Thus, it can be concluded that the L-ascorbic acid topical viscous hydrocolloid solutions used in this study are not likely to be hazardous when use on human face.

5.4 Study Subjects

This study is aimed to develop a novel non-invasive melanogenesis inhibition method for Asian females. Therefore only Asian females are chosen as subjects in this study. During the study, the subjects are asked to refrained from excessive exposure to direct sunlight as excessive direct sunlight will directly increase the melanogenesis inhibition activity and affect the results of this study.

Besides, in order to ensure the accuracy of this study, subjects are excluded if they are smokers, alcoholics, drug abusers, and had used any substance which might induce or inhibit melanogenesis activity of the skin during the entire period of study.

Lastly, for safety reason, subjects are also excluded if they are pregnant and had known allergy or sensitivity to L-ascorbic acid.

5.5 Optimal Transdermal Iontophoresis Voltage Study

Besides the viscosity of the topical viscous hydrocolloid solution, other factors affecting transdermal iontophoresis of L-ascorbic acid include electrical voltages used during the transdermal iontophoresis and the run time of transdermal iontophoresis process.

Before conducting the study, the optimal voltage to conduct *in vivo* transdermal iontophoresis was determined. The transdermal iontophoresis device invented in this study consists of a voltage step down adjuster that allows the user to adjust the voltage output up to maximun 12 V. In this study, prior to the transdermal iontophoresis, a maximum voltage output is determined by appling the voltage output from the lowest of 1 V to 12 V. 8 V of the voltage output was observed as the maximum voltage output. Pain, bruises and inflammation on the part of the face treated may occur if the voltage is increased beyond 8 V. Thus, 8 V of the voltage output was adopted in this study for the optimal melanogenesis inhibition activity. When treated with 8V of electrical current, the current densities for transdermal iontophoresis are between 60 μA to 80 μA depending upon the skin moisture content of the subject.

5.6 Treatment Time Optimization Study

From this study, it was observed that the melanogenesis inhibition activity increased progressively with the increase of time until 45 minutes. However, the melanogenesis inhibition activity did not show significant increase beyond the 45 minutes. Thus, 45 minutes transdermal iontophoresis run time was adopted in this study for the optimal melanogenesis inhibition activity.

5.7 Effect of 10%, 15%, 20% and 25% L-Ascorbic Acid with Transdermal Iontophoresis

It was demonstrated that L-ascorbic acid combined with transdermal iontophoresis provided good melanogenesis inhibition activity, or in other words, provided good skin whitening effect. The increase of concentration of L-ascorbic acid will cause the total amount of L-ascorbic anion increase which then increase the possibility of L-ascorbic anion penetration into the human epidermis. Thus, the melanogenesis inhibition activity was observed to increase when concentrations increased from 10% to 15%.

However, it was also observed that the melanogensis inhibition activity decreased with the concentration of L-ascorbic acid at 20%. Presumably, the total amount of L-ascorbic acid anion at the boundary layer between the solution and epidermis has reached its saturated condition at this concentration. Thus, percutaneous absorption of L-ascorbic acid into human epidermis is no longer dependent on the increase of L-ascorbic acid concentration in the topical viscous hydrocolloid solution. At the saturated condition, a drug reservoir phenomenon occurred in the epidermis, resulting in the formation of a negative charge barrier layer. This causes an increase in skin resistant for percutaneous absorption of L-ascorbic acid. In addition, during the 45 minutes

transdermal iontophoresis process, the water content of the topical viscous solution would decrease causing L-ascorbic acid to crystallise out. All these directly contributed to the decrease in melanogensis inhibition efficacy of the L-ascorbic acid.

The topical viscous solution with 25% L-ascorbic acid concentration was observed to be the least effective concentration for inhibition of the melanogenesis activity in human epidermis. This is due to the low solubility at 25% concentration L-ascorbic acid that dissolved in the viscous solution. To enhance solubility, heating of the viscous solution is required. Heating would destroy the activity of the L-ascorbic acid and in addition, there are several limitations such as saturated condition, drug reservoir phenomena and crystallization of the L-ascorbic acid that occurred as discussed earlier. Thus, 25% L-ascorbic acid is the least effective concentration for inhibition of melanogenesis activity in human epidermis.

It can therefore be concluded that 15% L-ascorbic acid with iontophoresis 8V for 45 minutes is the optimal parameter for *in vivo* melanogenesis inhibition. Besides, this parameter is safe with minimum skin sensitivity.

5.8 Effect of the 4 Weeks Topical Application of 15% L-Ascorbic Acid

A large number of scientific reports (Maeda at al., 1996; Eeman et al., 2014) have indicated L-ascorbic acid to be an effective substance for melanogenesis inhibition via *in vitro* testing such as extracellular tyrosinase inhibition activity assay, intracellular tyrosinase inhibition activity and melanin content spectroscopic measurement. However, melanogenesis inhibition and/or reduction effect of L-ascorbic acid via external cosmetic application is almost ineffective due to its impermeability. In this study, 15% L-ascorbic acid topical viscous hydrocolloid solution was applied twice daily on the

subject's face for a continuous period of 4 weeks. After 4 weeks, it was observed that no significant decrease of the melanogensis inhibition activity on the subject's epidermis. It can therefore be concluded that although L-ascorbic acid is the effective substance for melanogenesis inhibition but external application of L-ascorbic preparation will not provide any melanogenesis inhibition activity. This result is corresponding to the results published in Pinnell et al., (2001) and Ebihara et al., (2003).

5.9 Effect of the 4 Weeks 15% L-Ascorbic Acid with Transdermal Iontophoresis

It was demonstrated that applying 15% L-ascorbic topical viscous hydrocolloid solution together with the treatment of transdermal iontophoresis using the transdermal iontophoresis device invented in the study provide good and stable melanogenesis inhibition activity after 4 weeks of treatment.

Before conducting the study, the optimal transdermal iontophoresis current density and treatment run time to conduct the *in vivo* transdermal iontophoresis were determined. The melanogenesis inhibition activity increased progressively with increasing application time until it reaches its optimal time at 45 minutes. Melanogenesis inhibition activity did not show significant increase beyond 45 minutes. The melanogenesis inhibition activity is also increased progressively with increase of current densities.

It was discussed in Section 5.5 and 5.6 that the transdermal iontophoresis with 8 V and run time of 45 minutes were adopted for the optimal melanogenesis inhibition activity. However, the current density used at 8V is different from the range of 60 μA to 80 μA. Thus, in order to provide an optimal effect with minimum skin sensitivity, it is recommended that a fixed current densities of 70 μA for all the subjects during the

transdermal iontophoresis treatment since this level is safe to be used on human. In addition, no detection of pain, bruises and inflammation was observed at this current density when applied onto the skin for 45 minutes.

CHAPTER 6: CONCLUSION

The results obtained in this study indicated, from the cosmetic product safety assessment conducted and the actual use on human skin, that 15% L-ascorbic acid topical viscous hydrocolloid solution is safe to be used on human skin. However, the melanogenesis inhibition and skin whitening effect of L-ascorbic acid via external cosmetic application is almost ineffective due to its impermeability.

Combination of transdermal iontophoresis and 15% L-ascorbic acid topical viscous hydrocolloid solution provide promising, optimal and non-invasive treatment for effective *in vivo* melanogenesis inhibition and skin whitening. The results obtained in the study also showed that the transdermal iontophoresis device invented in this study is able to promote the permeation of L-ascorbic acid through human skin, effectively inhibite melanogenesis activity and provide consistent skin whitening effect.

The transdermal iontophoresis device was designed consisting of multiple sets of coupling devices and the two hand straps that evenly distribute the current densities on the entire face of the subject. This design is able to provide balance melanogenesis inhibition activity on the face and minimise skin sensitivity towards the electrical current. In addition, the transdermal iontophoresis device can be directly and very easily applied to the subject's face without causing undesirable irritation on the skin especially skin burns and rubefaction.

As conclusion, this study has sucessfully formulated a topical viscous hydrocolloids solution that is suitable to be used as the delivery carrier for the L-ascorbic acid and also suitable to be used in transdermal iontophoresis process. In addition, this study has also

sucessufully invented a transdermal iontophoresis device that is able to promote percutaneous absorption of L-ascorbic acid in human skin for skin whitening effect.

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