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DEVELOPMENT OF A CREAM CONTAINING PROTOCATECHUIC ACID-RICH FRACTION FROM *Clinacanthus nutans* LEAVES

BY

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A thesis submitted in fulfilment of the requirement for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Technology)

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MAY 2019

ABSTRACT

Premature aging is an aesthetic problem that degrades youthful appearance and leads to the lack of one's self-confidence. This may be the reason behind the rocketed sales of the anti-aging and whitening skin care in most of the world markets. One of the common anti-aging bioactives in cosmetics is phytochemicals that possessed antioxidant capacity such as protocatechuic acid, which may be capable of protecting the skin from premature aging due to ultraviolet radiation and free radicals. Therefore, this research was aimed to extract PCA-rich fraction from *C. nutans* leaves and screened its antioxidant and anti-tyrosinase capacities, to develop the cream containing PCA-rich fraction, and to characterise the developed cream. The extraction processes started with the sequential maceration of dried pulverised leaves of *C. nutans* with the *n*-hexane as the first in sequence followed by dichloromethane and lastly methanol. Screening of the crude extract for the protocatechuic was done by developing the TLC profiles and compared them to TLC pattern of PCA standard. Since TLC profile of methanol crude extract showed compound with R_f similar to the PCA standard, it had been chosen for further fractionation by vacuum liquid chromatography which yielded 2 g of PCA-rich fraction. PCA-rich fraction had found to have antioxidant and anti-tyrosinase activities as compared to other extracts. As for the development of the cream containing PCA-rich fraction, the development was started with the fabrication of four base cream. Based on the preliminary physical evaluation consisted of odour, colour, texture, homogeneity and spreadability, F4 base cream (placebo) was selected to be incorporated with 1% of PCA-rich fraction to produce PCA cream. Then, characterisation and stability studies were performed for both F4 base cream and PCA cream in accelerated and real-time storage condition for weekly sampling point for one month. The result for the characterisation and stability study showed some of the parameters which were droplet size, zeta potential and pH changed within the period of storage, however, the changes were acceptable since the results were within the acceptance limit. Physical properties and rheological behaviour of the creams were unchanged throughout the period of storage. Microbial limit test also showed negative result in which there were no growth observed in total aerobic microbial count (TAMC), total yeast and mold count (TYMC) and specific microbial tests for *Pseudomonas aeruginosa* and *Staphylococcus aureus.* For PCA content in cream analysis, the results showed only a slight insignificant reduction of PCA content in the cream for both accelerated and real-time storage condition. Last part of the research was about the *in vitro* release study of PCA from the cream by using Franz diffusion cell and silicone membrane as barrier between cream sample and receptor medium. The HPLC analyses showed there was no PCA permeated into the receptor medium. However, PCA had been recovered on the surface of the silicone membrane as well as deposited within the silicone membrane. As a conclusion, protocatechuic acid-rich fraction had been successfully obtained and a cream containing the PCA-rich fraction was successfully developed.

خلاصة البحث

الشيخوخة المبكرة هي مشكلة جمالية تشوه المظهر الشبابي وتؤدي إلى انعدام الثقة بالنفس، وقد يكون ذلك السبب وراء المبيعات المتزايدة لمستحضرات العناية بالبشرة المضادة للشيخوخة والمبيضة للبشرة في معظم الأسواق العالمية. أحد المواد النشطة حيويا المضادة للشيخوخة والشائعة في مستحضرات التجميل هي المواد الكيميائية النباتية التي تمتلك نشاطا مضادا للأكسدة، مثل حمض البروتوكاتيك، والقادرة على حماية البشرة من الشيخوخة المبكرة بسبب الأشعة فوق البنفسجية والجذور الحرة. لذلك كان الهدف من هذا البحث هو استخراج جزء غني بحمض البروتوكاتيك من مستخلصات أوراق كليناكانتوس نوتانس وفحص قدراته المضادة للأكسدة والمضادة للتيروزيناز، ولتطوير مرهم محتو على جزء غني بحمض البروتوكاتيك، وتوصيف المرهم المطور. بدأت عمليات الاستخلاص بالنقع المتسلسل لأوراق كليناكانتوس نوتانس المجففة باستخدام هيكسان–n كالمذيب الأول في التسلسل، كلورو الميثان، الميثانول. تم فحص وجود البروتوكاتيك في المستخلص الخام من خلال تطوير بروفايلات كرموتوغرافيا الفصل بالطبقة الرقيقة (TLC) ومقارنتها بنمط الTLC لحمض البروتوكاتيك المعياري. نظرًا لأن بروفايل الTLC الخاص بمستخلص الميثانول الخام قد أظهر مركبًا بمعامل احتجاز (Rf) مشابه لحمض البروتوكاتيك المعياري، فقد تم اختياره لمزيد من التجزئة بواسطة الفصل الكروماتوجرافي السائل المفرغ والذي نتج عنه 2 جم من الجزء الغني بحمض البروتوكاتيك. أما بالنسبة لتطوير مرهم محتو على جزء غني بحمض البروتوكاتيك، فقد بدأ التطوير بتصنيع مرهم بأربعة مكونات أساسية. استنادًا إلى التقييم الفيزيائي الأولي المكون من الرائحة واللون والملمس والتجانس والانتشار، تم اختيار كريم الأساس F4 (الدواء الوهمي) لدمجه مع 1٪ من الجزء الغني بحمض البروتوكاتيك لإنتاج مرهم حمض البروتوكاتيك. بعد ذلك أجريت دراسات التوصيف والاستقرار لكل من مرهم الأساس F4 ومرهم حمض البروتوكاتيك في حالتي تخزين متسارعة وفورية لنقاط أخذ عينات أسبوعية لمدة شهر واحد. أظهرت نتائج دراسات التوصيف والاستقرار تغير بعض المعايير وهي حجم القطرة، وكمون زيتا، ودرجة الحموضة خلال فترة التخزين، ومع ذلك فإن التغيرات كانت مقبولة لأن النتائج كانت في حدود القبول. لم تتغير الخصائص الفيزيائية والسلوك الريولوجي للمراهم طوال فترة التخزين. أظهر اختبار الحد الميكروبي أيضًا نتائج سلبية حيث لم يلاحَظ أي نمو في إجمالي عدد الميكروبات الهوائية (TAMC) ، والتعداد الكلي للخمائر والعفن (TYMC)، وأيضا في الاختبارات الجرثومية المحددة لكل من الزائفة الزنجارية والمكورة العنقودية الذهبية. أما بالنسبة لمحتوى حمض البروتوكاتيك في تحليل المراهم، أظهرت النتائج انخفاضًا ضئيلا بسيطا في محتوى حمض البروتوكاتيك في المرهم لكل من حالة التخزين المتسارع والفوري. كان الجزء الأخير من البحث متعلقا بدراسة إطلاق حمض البروتوكاتيك من المرهم خارج الجسم الحي باستخدام خلية النشر لفرانز وحاجز غشاء السيليكون بين عينة المرهم والوسيط المستقبل. أظهرت تحاليل الكروماتوغرافيا السائلة عالية الأداء عدم تخلخل حمض البروتوكاتيك في الوسيط المستقبل. ومع ذلك تم استرداد حمض البروتوكاتيك على سطح غشاء السيليكون وكذلك الممتص داخل غشاء السيليكون. ختاما تم بنجاح الحصول على جزء غني بحمض البروتوكاتيك من المستخلصات وتم بنجاح تطوير مرهم محتو على الجزء الغني بحمض البروتوكاتيك.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Pharmaceutical Sciences (Pharmaceutical Technology)

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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Signature Date

ACKNOWLEDGEMENTS

Firstly, I wish to express my gratefulness to The Creator for His Blessings and granting me strength to complete my study.

It is my greatest pleasure to dedicate this work to my beloved parents: Zamery Baba and Tengku Fatimah, my family and my wife, who granted me the gift of their untiring trust in my ability to achieve this goal: thank you for your support and patience.

I wish to express my appreciation and thanks to my research mates and friends who provided their time, effort and support for this project: Nadzira, Akilah, Suganya, Ghazali, Hakimah, Shakirah, Nazrin and Faiz. I would rather not imagine my research life without your presences.

A special appreciation dedicated to all members of Pharmaceutical Technology Department who have spent their time, energy and support for this research especially to Sr Haryanti, Sr Maznera, Sr Zaililah, Sr Ain and Br Dzadil. Without their assistances, my research progression would not be so smooth.

Finally, a special thanks to my dedicated, enthusiastic and energetic supervisor, Associate Professor Dr Hazrina Ab Hadi and my supportive co-supervisor, Assistant Professor Dr Norazian Hassan for their continuous support, encouragement and leadership, and for that, I will be forever grateful.

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

1.1.1 Skin Physiology

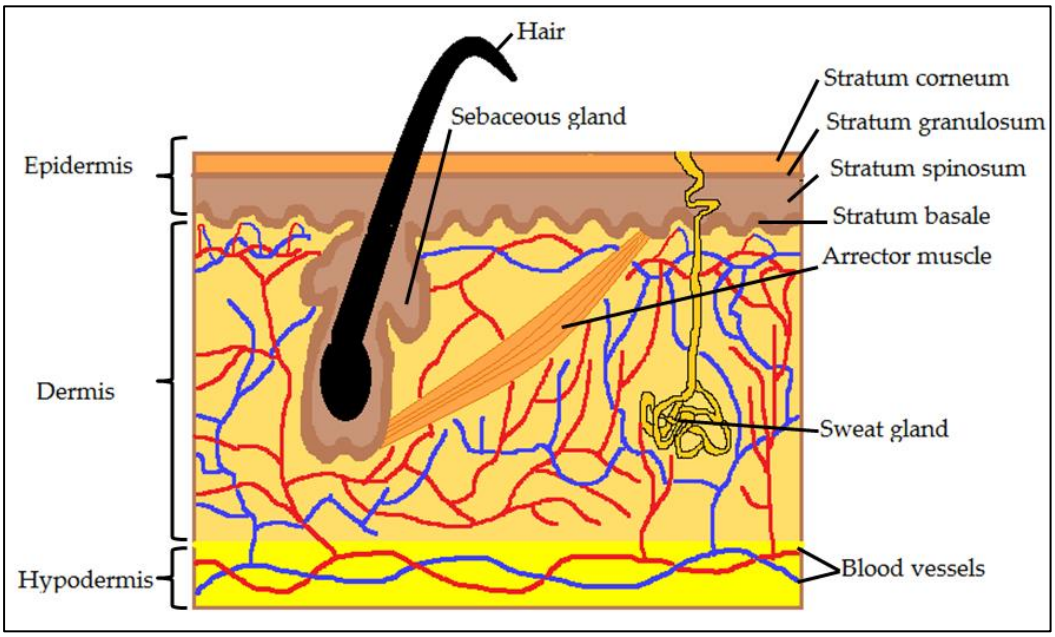

Figure 1.1: Layer of the skin comprised of three which are epidermis, dermis and hypodermis. Adapted from Lai-Cheong et al. (2013).

Besides being the outermost, heaviest and largest multifunctional organ of human body, skin bears important roles in maintaining the numerous critical physiological functions which are fortification against UV irradiation, inhibiting microorganism invasion and chemical penetration, and regulating water as well as electrolyte passages (Firooz et al., 2012; Quan, 2016). Major roles of skin apart from providing protection are sensory,

absorption, excretion, thermoregulation as well as metabolism (Atif, Shoaib, & Naveed, 2016; Darlenski, Kazandjieva, & Tsankov, 2011). According to Figure 1.1, human skin comprised of three different layers which are the outmost epidermis, the thick, intermediate dermis and the innermost hypodermis (Quan, 2016). The skin defensive function aims to preserve skin moisture by preserving homeostasis and avoiding the uncontrolled loss of water, ions and serum proteins into the environment. This skin defensive function is realized by the existence of protective skin barrier (Atif et al., 2016; Darlenski et al., 2011).

1.1.1.1 Epidermis: Primary Protective Skin Barrier

Over 90% of protective skin barrier comprised from epidermis and predominantly belongs to its outermost stratum corneum (Baroni et al., 2012). Figure 1.2 exhibits a schematic diagram that illustrates stratum corneum. Stratum corneum is developed from the differentiation of keratinocytes which are the predominant cell type of epidermis. The differentiation ceases with the complex modifications in their organization which leading to a alteration into flat and non-nucleated squamous cell corneocytes of stratum corneum (Baroni et al., 2012). The main components of stratum corneum which are the corneocytes, epidermal protein (cornified envelope) and the intercellular epidermal lipid, are considered the core structures that influence the speed of the transcutaneous exchange of substances and mechanical resistance (Casetti et al., 2011; Darlenski et al., 2011; Rinnerthaler et al., 2013). Particularly, lipids in the intercellular space of stratum corneum functions in counteracting the excessive loss of water and salt from the skin (Baroni et al., 2012; Darlenski et al., 2011).

Several epidermal proteins that make up the cornified envelope where corneocyte is embedded and tight junction (corneodesmosome) also act as components

participating in the mechanical resistance of epidermal barrier such as keratins, loricrin, involucrin, corneodesmosin and filaggrin. For maintaining moisture of skin, filaggrin is an important protein since it will be degraded into naturally moisturizing factors (NMF) which are free amino acids, uronic acid, inorganic salts, sugars, lactic acid and urea (Kim et al., 2012; Wan et al., 2014). Apart from stratum corneum, sebum also plays a role in skin barrier by forming water-lipid mantle on the skin surface (Schneider et al., 2016). Sebum is produced by sebaceous gland and composed of fatty acids, wax esters, ceramides, squalene, cholesterol, triglycerides and cholesterol esters (Darlenski et al., 2011; Guo et al., 2015). As sebum contains fatty acids, it also contributes to the acidity of the skin surface which is a vital defensive mechanism against pathogenic bacteria and preservation of barrier homeostasis. Changes in sebum secretion also had been found to be part of the pathogenesis of dry skin condition (Darlenski et al., 2011).

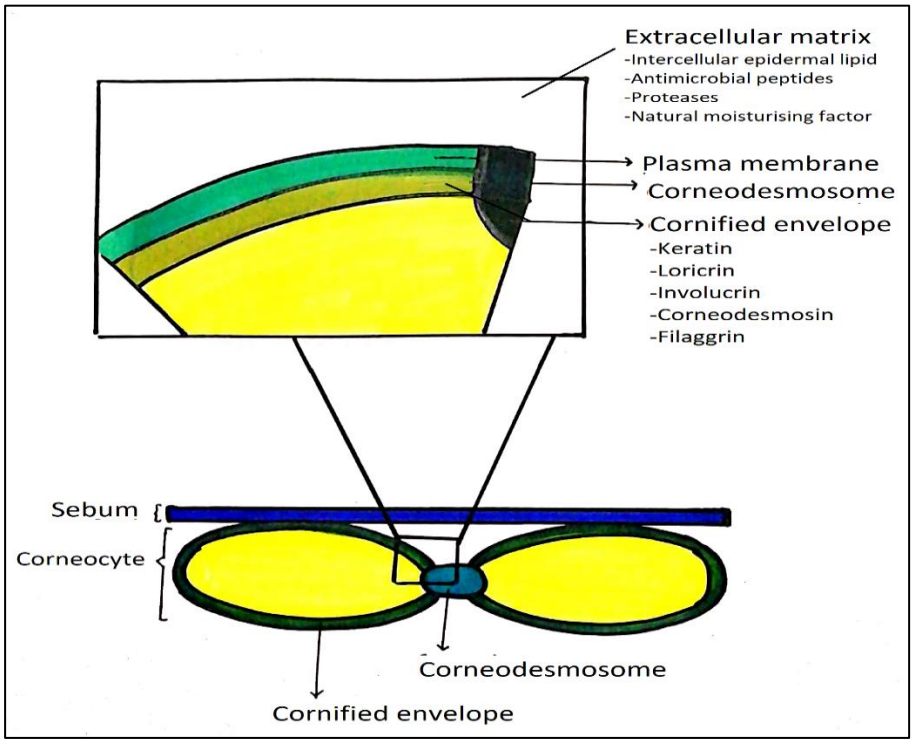

Figure 1.2: Stratum corneum with schematic structure of epidermal barrier (Ananthapadmanabhan, Mukherjee, & Chandar, 2013).

Next, the other component of epidermis which is important for skin protection is melanin. Skin colour is mainly determined by the pigment named melanin (eumelanin and pheomelanin) which is synthesized by neural crest-derived melanocytes that are found in various sites of body including skin, eye, ear, hair and central nervous system (Farage, Miller, & Maibach, 2010). Melanin also has another functions besides giving the skin its colour such as protection against UV radiation (photoprotection), contributes in hearing function and central nervous system (Farage et al., 2010). Melanin synthesis (Figure 1.3) or also known as melanogenesis begin with a substrate called tyrosine in which it will be hydrolyzed to dihydroxyphenylalanine (DOPA) by tyrosinase catalysis and will further proceed to form DOPAquinone followed by eumelanin and pheomelanin via two different pathways (Farage et al., 2010; Videira et al., 2013). Tyrosinase is a rate-limiting and key enzyme of melanogenesis since it controls the rate-limiting stage in melanin synthesis (catalysis of conversion from tyrosine to DOPA (Videira et al., 2013).

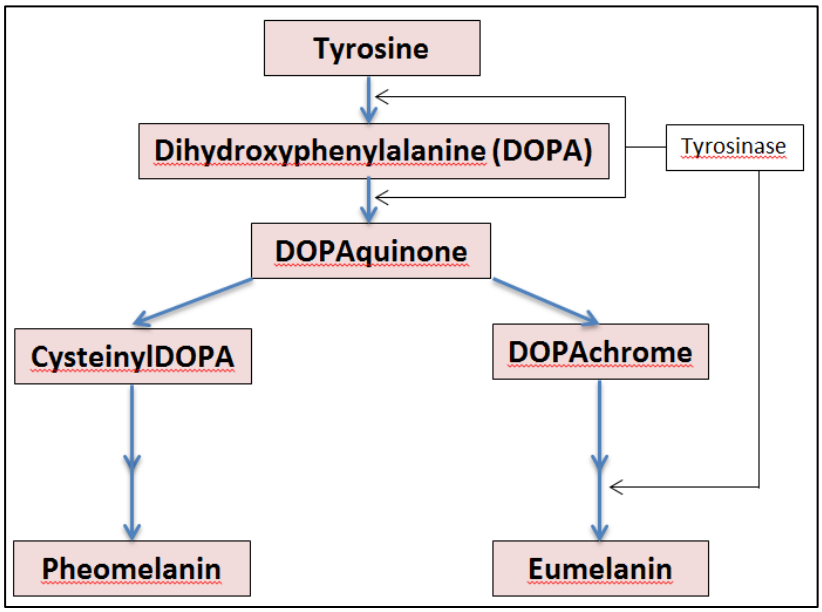

Figure 1.3: Melanin biosynthesis (melanogenesis) pathway for the production of eumelanin and pheomelanin.

1.1.1.2 Dermis: Contributions in Human Skin Integrity

The dermis of skin is comprised of a dense collagen-rich connective tissue and also dermal fibroblast, a type of stromal cells. This middle skin layer intermingles and interacts with epidermis and hypodermis to provide structural and mechanical integrity of the skin. The integrity of human skin is also contributed by extracellular matrix (ECM) protein which composed of collagen, elastin, fibronectin and proteoglycan in which dermal collagen plays the major role (Quan, 2016). Dermal fibroblasts synthesize, organize and maintain collagen-rich ECM by secreting the precursors of all components of the ECM proteins in skin. Therefore, dermal fibroblasts is important in controlling skin's homeostasis and the alteration of dermal fibroblasts will lead to substantial impact on collagen homeostasis (Quan et al., 2015; Quan, 2016). Collagen comprised of several collagen type in which type I collagen is the most abundant and common type of collagen and it is the major component of ECM and human skin (Dutov et al., 2016). Each structural unit of type I collagen will accumulate and associated with other collagen types as well as ECM proteins to form collagen fibrils. Collagen fibril is the one that provide mechanical support and serve as scaffold for cellular attachment (Dutov et al., 2016; Quan, 2016).

1.1.2 Cosmetics

According to Article 2 of the EU Cosmetics Regulation (Regulation (EC) No. 1223/2009), cosmetic product refers to any substance or mixture intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance and/or correcting body odours and/or protecting them or

keeping them in good condition (European Commission, 2009). Federal Food, Drug and Cosmetic Act includes the following products as the cosmetic products which are skin moisturizers, perfumes, lipsticks, fingernail polishes, eye and facial make-up preparations, cleansing shampoos, permanent waves, hair colors, and deodorants, as well as any substance intended for use as a component of a cosmetic product (FDA, 2012).

Cosmetic products had been generally known to enhance and beautify the appearance of the skin. However, in fact, the cosmetic products also had been developed to protect and keep the external parts of human body such as skin to be in their good condition. The condition of the skin intensely influences the appearance of human individual as well as perception of other people towards the individual. For instance, the facial condition or appearance can be correlated with the age of the individual in which aging can be characterized by patchy skin tone and wrinkles (Quan, 2016). The example of skin appearance that causes cascade of skin problems concerned with beauty (aesthetic) is premature skin aging which will lead to other skin problems which are dry skin, pigmentation and wrinkles.

1.1.2.1 Categories of cosmetics

Different countries possessed their own classifications of cosmetics outlined by their own regulatory bodies. For example, United State Food and Drug Administration (USFDA) had outlined thirteen major categories of cosmetics such as baby products, bath preparations, eye makeup preparations, fragrance preparations and hair preparations while in Japan, there were five major categories of cosmetics in their regulation on manufacturing and sales of cosmetics includes hair care products, skincare products, makeup products, perfumes and cologne, and others (bath preparations, nail

cosmetics, body powder). In addition, according to the Guideline for Control of Cosmetic Products in Malaysia by National Pharmaceutical Regulatory Agency (NPRA), there were twenty categories of cosmetic products had been listed as follows:-

1. Creams, emulsions, lotions, gels and oils for the skin.
2. Face masks (with the exception of chemical peeling products)
3. Tinted bases (liquids, pastes, powders)
4. Make-up powders, after-bath powders, hygienic powders, etc.
5. Toilet soaps, deodorant soaps, etc.
6. Perfumes, toilet waters and eau de cologne
7. Bath and shower preparations (salts, foams, oils, gels, etc.)
8. Depilatories
9. Deodorants and anti-perspirants
10. Hair care products
11. Shaving products (creams, foams, lotions, etc.)
12. Products for making-up and removing make-up from the face and the eyes
13. Products intended for application to the lips
14. Products for care of the teeth and the mouth
15. Products for nail care and make-up
16. Products for external intimate hygiene
17. Sunbathing products
18. Product for tanning without sun
19. Skin-whitening products
20. Anti-wrinkle/anti-aging products

1.1.2.2 Common materials used in cosmetics

In cosmetic products development, there were several common materials used such as aqua (water), oils, surfactant, polymers, organic solvent, acid and alkali salt, colourant (pigment), proteins, plant extracts, vitamins, antioxidants and aromatic essential oils (Hiroshi & Shimada, 2013). Basically, each of the common materials used have their own functional contribution to the product form either to produce the intended cosmetic product form, to give enough stability to the product physically and chemically, to exert the beneficial effect to the intended application site and to have effects on senses of end-users (Gabriella & Kenneth S., 2015; Hiroshi & Shimada, 2013).

There are ingredients that composed of the primary part of the cosmetic products and these ingredients provide them with good aesthetic quality, appropriate texture of cosmetic product and define their product form. The ingredients typically consisted of waters, oils, surfactants, silicone, polyhydric alcohols, polymers and powders (Hiroshi & Shimada, 2013). Waters, polyhydric alcohols (glycerols, glycols) and oils (waxes such as beeswax, oils such as squalene, jojoba oil and almond oil, fats, fatty acids) are solvents which play crucial parts in most cosmetics. They are utilized to dissolve solid materials, blend liquid materials, act as a vehicle for the cosmetic formulation and give the texture of the products (Gabriella & Kenneth S., 2015). Other than acting as a solvent, polyhydric alcohols for example glycerol are able to contribute as moisturizing agent, solubilizer and stabilizer to the products (Hiroshi & Shimada, 2013).

In addition, the other part of materials which also play an important role in cosmetic product formulation are preservatives (antimicrobial agents), pH buffer, antioxidant and chelating agents. These materials are vital ingredients since they provide adequate stability and protection against deterioration of cosmetic products

(Hiroshi & Shimada, 2013). Table 1.1 showed the role of the mentioned materials with examples (Gabriella & Kenneth S., 2015).

Table 1.1 Stabilizing materials, their roles and examples

Stabilizing materials	**Role**	**Example**
Preservatives	Prevent undesirable growth of molds, yeast and bacteria in cosmetic product.	Parabens, benzalkonium chloride, phenoxyethanol.
pH Buffers	Adjustment of pH in stabilizing cosmetic product since certain ingredients are stable at specific pH values.	Citric acid, lactic acid, sodium hydroxide and triethanolamine.
Antioxidants	Provide protection against oxidative reactions and prevent undesirable chemical changes within cosmetics.	Propyl gallate, vitamins, natural extracts, isoflavone and polyphenols
Chelating Agents	Molecules that able to complex with metal ions and deactivate them to prevent clarity reduction, fragrance integrity disturbance and rancidity.	Ethylenediaminetetraacetic acid (EDTA), citric acid and phosphoric acid derivatives.

1.1.3 Photoaging and Cosmetics' Roles Against It

Skin aging is a dermatologic transformation that develops as one ages or is exposed to ultraviolet radiations (UVR) with no treatment is adopted (Jadoon et al., 2015). Aging skin is influenced by combinations of two key processes which are intrinsic aging (cellular metabolism, metabolic processes and genetics) and extrinsic aging (chemical, toxin, chronic light exposure and pollution) (Cevenini et al., 2008; Nikolakis,