การประเมินฤทธิ์ชะลอวัยของสารสกัดว่านเพชรหึงเพื่อพัฒนาเป็นซีรัมสำหรับต้านริ้วรอย

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ANTI-AGING ACTIVITY EVALUATION OF *GRAMMATOPHYLLUM SPECIOSUM* EXTRACT FOR DEVELOPMENT INTO AN ANTI-WRINKLE SERUM

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmaceutics
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Thesis Title	ANTI-AGING ACTIVITY EVALUATION OF *GRAMMATOPHYLLUM SPECIOSUM* EXTRACT FOR DEVELOPMENT INTO AN ANTI-WRINKLE SERUM
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วีริสา เช้าเจริญ : การประเมินฤทธิ์ชะลอวัยของสารสกัดว่านเพชรหึงเพื่อพัฒนาเป็นซีรัมสำหรับต้านริ้วรอย (ANTI-AGING ACTIVITY EVALUATION OF *GRAMMATOPHYLLUM SPECIOSUM* EXTRACT FOR DEVELOPMENT INTO AN ANTI-WRINKLE SERUM) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภก. ดร. ภาคภูมิ เต็งอำนวย, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ภญ. ดร. อุบลทิพย์ นิมมานนิตย์, รศ. ภก. ดร. ปิติ จันทร์วรโชติ, หน้า.

ว่านเพชรหึง *Grammatophyllum speciosum* Blume พืชวงศ์ Orchidaceae เป็นกล้วยไม้ขนาดใหญ่ที่สุดที่พบในภาคทวีปเอเชียตะวันออกเฉียงใต้ การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ชะลอวัยของสารสกัดว่านเพชรหึง ทั้งในหลอดทดลองและในร่างกาย โดยการศึกษาฤทธิ์ชะลอวัยในหลอดทดลอง เป็นการศึกษาที่ไม่ใช้เซลล์และการศึกษาในเซลล์ ผลการศึกษาพบว่าสารสกัดจากลำต้นเทียมของว่านเพชรหึงจากแอลกอฮอล์มีสารสำคัญ คือ gastrodin และมี total phenolic content เท่ากับ 48.19 ± 0.39 มิลลิกรัมของสาร epigallocatechin gallate (EGCG) equivalent ต่อกรัมของสารสกัด สารสกัดจากลำต้นเทียมของว่านเพชรหึงด้วยแอลกอฮอล์ มีฤทธิ์การต้านการทำงานของเอนไซม์คอลลาจีเนส แต่น้อยกว่า EGCG (IC_{50} 1179.47 และ 0.056 ไมโครกรัมต่อมิลลิลิตรตามลำดับ) แต่มีฤทธิ์ในการต้านการทำงานของเอนไซม์อีลาสเตสมากกว่า EGCG (IC_{50} 71.10 และ 92.52 ไมโครกรัมต่อมิลลิลิตรตามลำดับ) อันเป็นสาเหตุของการเกิดริ้วรอยที่ผิวหนัง นอกจากนี้สารสกัดว่านเพชรหึงมีศักยภาพในการเพิ่มคุณสมบัติของเซลล์ต้นกำเนิดคีราติโนไซต์ และสามารถป้องกันการตายของเซลล์คีราติโนไซต์ที่เกิดจากสารประกอบที่มีออกซิเจนในโมเลกุลได้ที่ความเข้มข้น 20 ไมโครกรัมต่อมิลลิลิตร ส่วนการศึกษาทางคลินิก ศึกษาการระคายเคืองของสารสกัดโดยใช้วิธี prophetic patch test ในอาสาสมัครคนไทย และทำการศึกษาประสิทธิศักย์ของสารสกัด โดยใช้วิธี randomized double-blind, placebo-controlled trial เป็นเวลา 56 วัน และประเมินโดยใช้เครื่องมือ Visioface และ Cutometer ซีรัมสารสกัดจากลำต้นเทียมของว่านเพชรหึง (0.5% โดยน้ำหนัก) ยังเพิ่มความยืดหยุ่น (distensibiltity) ของผิวหนังในอาสาสมัคร สมบัติหยุ่นหนืด (viscoelasticity) และริ้วรอยลดลงอย่างมีนัยสำคัญทางสถิติ

โดยสรุป สารสกัดจากลำต้นเทียมของว่านเพชรหึง มีฤทธิ์ชะลอวัย (anti-aging) ในหลอดทดลองและในอาสาสมัคร ดังนั้น จึงควรมีการพัฒนาสารสกัดจากลำต้นเทียมของว่านเพชรหึง เพื่อชะลอวัยและลดการเหี่ยวย่น และให้ได้สารสกัดที่มรมาตรฐาน

ภาควิชา	วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม	ลายมือชื่อนิสิต	.....
		ลายมือชื่อ อ.ที่ปรึกษาหลัก	.....
สาขาวิชา	เภสัชกรรม	ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....
ปีการศึกษา	2560	ลายมือชื่อ อ.ที่ปรึกษาร่วม	.....

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KEYWORDS: GRAMMATOPHYLLUM SPECIOSUM / ANTI-AGING / ANTI-COLLAGENASE / ANTI-ELASTASE / CLINICAL TRIAL

VERISA CHOWJAREAN: ANTI-AGING ACTIVITY EVALUATION OF *GRAMMATOPHYLLUM SPECIOSUM* EXTRACT FOR DEVELOPMENT INTO AN ANTI-WRINKLE SERUM. ADVISOR: ASSOC. PROF. PARKPOOM TENGAMNUAY, Ph.D., CO-ADVISOR: ASSOC. PROF. UBONTHIP NIMMANNIT, Ph.D., ASSOC. PROF. PITHI CHANVORACHOTE, Ph.D., pp.

Grammatophyllum speciosum Blume (Orchidaceae) is a large-sized orchid in Southeast Asia. The aim of this study was to evaluate *G. speciosum* pseudobulb extract for anti-aging activities, both *in vitro* and *in vivo*. *In vitro* anti-aging activity was performed using non-cell and cell-based assays. *In vitro* study, *G. speciosum* pseudobulb extract exhibited a high content of gastrodin, which was used as an active analytical marker. The total content of the antioxidant polyphenols in *G. speciosum* ethanolic extract was 48.19 ± 0.39 mg epigallocatechin gallate (EGCG) equivalent / g extract. The anti-collagenase enzyme activity of the ethanolic extract of the pseudobulb of *G. speciosum* was lower than EGCG (IC_{50} 1179.47 and 0.056 µg/mL, respectively). The clinical test for skin irritation using prophetic patch test method was conducted in healthy Thai volunteers. The randomized double-blind, placebo-controlled trial was further conducted for efficacy assessment during 56 days of serum application, as observed by Visioface© and Cutometer©. However, the *G. speciosum* extract possessed higher anti-elastase activity than that of EGCG (IC_{50} 71.10 and 92.52 µg/mL, respectively), that was to confirm the activity of anti-wrinkle on the skin. Moreover, *G. speciosum* extract showed a potential to enhance stem cell behaviors and markers of human keratinocyte cells (HaCaTs). This ethanolic extract was also able to protect cells against superoxide anion-induced cell death at concentration of 20 µg/mL in HaCaTs. The *G. speciosum* pseudobulb extract was subsequently formulated into a viscous serum at 0.5% w/w for further safety and efficacy evaluation. The serum was found to be non-irritating and safely increase the skin distensibility (R0) level in healthy volunteers. The skin viscoelasticity (R6) and wrinkle volume were also significantly reduced, indicating the extract's efficacy as a novel anti-aging agent.

In summary, *G. speciosum* pseudobulb extract is beneficial for skin aging treatments, as indicated through both the *in vitro* tests and *in vivo* skin evaluation in human Thai volunteers. *G. speciosum* pseudobulb extract thus has a potential for development into a novel active standardized extract for anti-aging and/or treatment of age-related skin disorders.

Department:	Pharmaceutics and Industrial Pharmacy	Student's Signature	.....
		Advisor's Signature	.....
Field of Study:	Pharmaceutics	Co-Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....

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CHAPTER I
INTRODUCTION

1. Background and significance of the study

Thai cosmetic products nowadays have interesting adaptive strategies. As seen in 2016 market capitalization, there is 2.8 trillion baht and growth rate is increasing every year, especially cosmetics and beauty products made from natural ingredients. As naturalism is gaining supportive factor, Thailand becomes relatively high advantageous with trading due to the country's diverse and abundant natural resources. Anti-wrinkles cosmetics and beauty markets are specially growing and highly competitive. Today, most of the natural cosmetics and beauty products sold in the mainstream market have problems with consistency and efficiency. Consumers' greater confidence in these products and the aforementioned problems could be resolved if there is rigorous product quality assurance and evaluation.

This study researched the capability of herbs with anti-wrinkles properties because free radicals have a major effect towards causing skin fine lines and wrinkles. Preliminary screening study has been undertaken to select herbs with antioxidant activity property by DPPH radical scavenging assay from 10 interesting herbal extracts (Table 1). Herbs selections were considered from ancient Thai traditional medicine recipe, usage history, herbal properties and literature reviews (Table 1). The results showed that the *Grammatophyllum speciosum* (*G. speciosum*) extract had the smallest 50 percent inhibitory

concentration (IC_{50}). This demonstrated that the extracts have the highest antioxidant activity property by DPPH radical scavenging activity on the selected 10 herbal extracts.

Table 1: IC_{50} of the extracts with antioxidant activity by DPPH radical scavenging assay.

Plant species	Part used	Family	Activities	IC_{50} (mg/mL)	Ref
Pueraria mirifica	root	Leguminosae	Anti-elastase and Estrogen-like effect	1.1609	(Chattuwatthana, and Okello, 2015; Sakong et al., 2011)
Kaempferia parviflora	rhizomes	Zingiberaceae	Anti-inflammatory and Anti-aging	0.2375	(Narayanaswamy, and Ismail, 2015; Sutthanut et al., 2009)
Coffea Arabica	coffee grounds	Rubiaceae	Stimulate the synthesis of collagen and elastin, Improving wrinkles and MMP inhibition	0.2204	(Binic et al., 2013; Del Carmen Velazquez Pereda et al., 2009)
Nelumbo nucifera Gaertn	lotus pollen	Nelumbonaceae	Antioxidant activity and treatment of tissue inflammation	0.3162	(Rai et al., 2006)
Piper retrofractum Vahl	fruit	Piperaceae	Antioxidant activity	0.5977	(Jadid et al., 2017)
Carthamus tinctorius L.	flower	Compositae	Antioxidant activity and Melanin synthesis inhibition	0.4593	(Roh et al., 2004)
Citrus hystrix DC.	peel	Rutaceae	Ttyrosinase inhibition and antioxidant activity	0.3432	(Manosroi, and Manosroi, 2003; Wungsintaweekul et al., 2010)
Piper nigrum L.	dried fruit	Piperaceae	Anti-inflammatory activity	0.2693	(Tasleem et al., 2014)
Garcinia atroviridis Griff.	fruit	Guttiferae	Antioxidant and anti-inflammatory activity	1.0107	(Taher et al., 2017)
Grammatophyllum speciosum	pseudobulb	Orchidaceae	Anti-inflammatory activity	0.1056	(Uawonggul et al., 2006)

Grammatophyllum speciosum Blume (Thai name: Waan-Phet-Cha-hueng; Orchidaceae ว่านเพชรหึง) is a gigantic-sized orchid, which widely spreads throughout the tropical rainforests in Southeast Asia such as Thailand, Malaysia, Indonesia, Laos, Burma and the Philippines (Nontachaiyapoom, Sasirat, and Manoch, 2011; Sahakitpichan et al., 2013; Teoh, 2016). The most gigantic orchid of 12 species in *Grammatophyllum* genus, *G. speciosum*, is the only species originates in Thailand (Sopalun, Thammasiri, and Ishikawa, 2010) and can be found in tropical regions of Asia and south pacific islands. In Thailand alone, this species has been found in natural habitats of Phitsanulok, Chachoengsao, Loei, Chonburi, Chumphon, Suratthani, Trang and Narathiwat provinces.

Thai traditional medicine has reported the essential properties of *G. speciosum* for being an "elixir" by dissecting its trunk and leaf stalk, washing and putting them in a vessel full of alcohol for drinking. The trunk can cure lymph nodes-involved dermatitis by masking the skin with the trunk mixture in rain and rice water to treat skin abscess (108 Panmaithai, 2560: Online). It can also cure lymph node tuberculosis and sore throat (Folk Doctor Foundation, 2552: Online). The extract of *G. speciosum* root was found to have resistance effect of 23% against *Heterometrus laoticus* at 0.706 mg/ml concentration (Uawonggul et al., 2006). Phytochemicals of its pseudobulbs methanol extracts were found to have novel compounds which are derivatives of glucosyloxybenzyl of (R)-2-benzylmalic acid and (R)-eucomic acid, grammatophyllosides A – D (Sahakitpichan et al., 2013). Moreover, other phytochemicals, namely, gastrodin, orcinol glucoside and isovitexin are also found. With the mentioned preliminary study result and elixir property in Thai traditional medicine, *G. speciosum* was

selected to test for its properties and uses. Additionally, dermatological research on *G. speciosum* lacks sufficient information, especially a cosmetic assessment of its effects on anti-skin aging. Therefore, this study researched the *G. speciosum* capability to reduce or decelerate skin fine lines and wrinkles using both the *in vitro* and *in vivo* clinical studies.

Skin aging can occur naturally within human skin by many factors. The aging of skin appears clearly as wrinkles and fine lines mainly as a result of long-term free radical accumulation. Functionality of the skin cells is reduced and so the balance between producing new and disposing aged skin cells is lost, therefore causing skin aging or damages. For those reasons, skin aging is corresponding with old age and wrinkles on skin. Thus, the skin can serve as a direct organ to study for its bodily aging.

There are many obvious reasons for skin aging such as keratinocyte stem cell functional decline, reactive oxygen species (ROS) accumulation from aging organ, ultraviolet (UV) radiation and increased activity of collagenase and elastase enzymes (Alonso, and Fuchs, 2003; Krutmann et al., 2017; Mukherjee et al., 2011).

So, this study was examined *in vitro* without cell culture (non cell-based assay) to investigate the extracts of *G. speciosum* for their ability to inhibit collagenase and elastase enzymes activity. In addition, an *in vitro* activity assay with cell culture (cell-based assay) was performed to examine whether *G. speciosum* has an ability to improve stem cell functions of keratinocytes by observing changes in stem cell marker proteins, namely, CD133, beta-catenin (*β*-catenin) and aldehyde dehydrogenase 1A1 (ALDH1A1) using SDS-PAGE and

western blot analysis. Moreover, prevention of keratinocyte cell death induced by molecular oxygenated compounds was evaluated with 2 types of ROS, namely, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) and hydrogen peroxide (H_2O_2), which are a superoxide anion ($O2^{\bullet-}$) generator, and a non-radical ROS, respectively.

After research on *G. speciosum* capability to reduce or decelerate wrinkles has been done, the extract of *G. speciosum* was subsequently studied for physicochemical properties to further develop into a serum formulation. The serum formula was tested for consistency as well as skin irritation and efficacy in healthy Thai volunteers. Knowledge acquired from this study could benefit implementing the extracts of *G. speciosum* for inclusion as an anti-aging agent in skin products in order to promote and develop Thai herb to the international standards. At the same time, the herb could be promoted as a new economic crop which would add values to local Thai herb products according to Government policy.

2. Objectives of the study

2.1. To investigate the cell cytotoxicity, cell protective, cell stemness, anti-elastase and anti-collagenase activities of *G. speciosum* extract.

2.2. To formulate a stable and non-irritating serum containing *G. speciosum* extract with promising anti-wrinkle activities in human volunteers.

3. Expected outcomes

3.1 *G. speciosum* may have a potential to increase stem cell function of keratinocytes.

3.2 *G. speciosum* extract possesses anti-elastase and anti-collagenase properties.

3.3 The serum formulation containing *G. speciosum* extract can deliver the active constituents without skin irritation and with promising anti-wrinkle activities in human volunteers.

CHAPTER II
LITERATURE REVIEW

1. *Grammatophyllum speciosum* Blume

Grammatophyllum speciosum Blume (Orchidaceae; Thai name: Waan-Phet-Cha-hueng: ว่านเพชรหึง; Figure 1) is a large-sized orchid which spreads widely in the tropical rainforests of Southeast Asia such as Thailand, Malaysia, Indonesia, and the Philippines (Nontachaiyapoom, Sasirat, and Manoch, 2011; Sopalun, Thammasiri, and Ishikawa, 2010). In Thai folk medicine, the decoction made from *G. speciosum* is used to treat sore throats and bronchitis. Additionally, the pseudobulb extract is applied externally to relieve pain from the venom of scorpion (*Heterometrus laoticus*) (Uawonggul et al., 2006). The picture of *G. speciosum* is shown in Figure 1.

Figure 1: The picture of *Grammatophyllum speciosum* Blume

Phytochemical compounds that have been found in *G. speciosum* pseudobulb include glucosyloxybenzyl derivatives of (R)-2-benzylmalic acid and of

(R)-eucomic acid, grammatophyllosides A - D, cronupapine, vandateroside II, gastrodin, vanilloloside, orcinol glucoside, and isovitexin (Sahakitpichan et al., 2013).

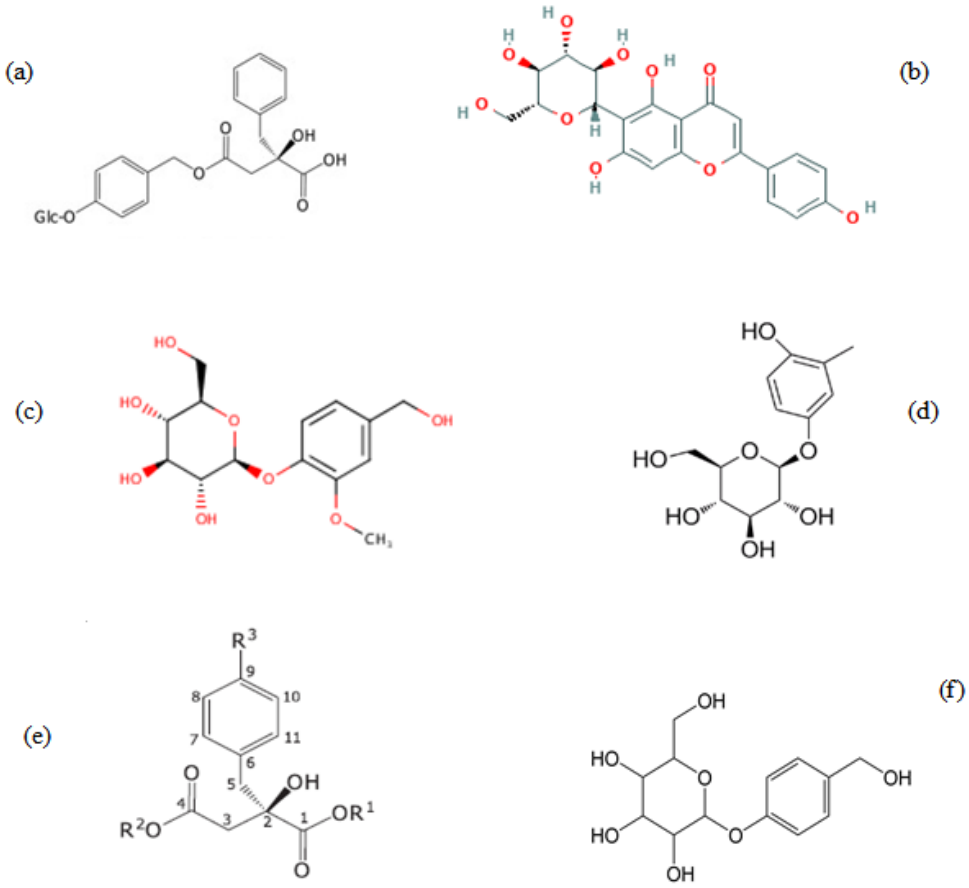


Figure 2**:** The molecular structure of phytochemical compounds in *G. speciosum* pseudobulb (a) Grammatophyllosides A (b) Isovitexin (c) Vanilloloside (d) Orcinol glucoside (e) Vanilloloside II (f) Gastrodin

Gastrodin (Figure 2 (f)), a phenolic glycoside [p-hydroxymethylphenyl-β-D-glucopyranoside] found as a major active component in *G. speciosum*, is a potent antioxidant. Gastrodin was also found to function as anti-apoptotic activities in brain areas (Kumar et al., 2013), a memory enhancer (Hsieh, Wu, and Chen, 1997; Wu et al.,

1996), as well as having anti-epileptics (W. Wang et al., 2013), anti-cell death (H.-J. Kim et al., 2003), anti-Alzheimer's disease (Hu, Li, and Shen, 2014), anti-Parkinson's disease activities (X.-L. Wang et al., 2014), as well as protective effects against osteoporosis linking to a reduction in ROS (Huang et al., 2015). This study selected gastrodin as an active marker to control the quality of *G. speciosum* ethanolic extract due to its presence in significant amount, thereby facilitating detection and analysis. It was also used as an analytical marker for quantitative evaluation of *Rhizoma gastrodiae* (Tianma) extracts using HPLC (W.-C. Chen et al., 2015; Lee et al., 2015).

In addition, this plant contains various other phytochemicals that may benefit medicinal usage including the effects on skin. Up to the presence, the effects of *G. speciosum* extract on the anti-aging of human skin have never been evaluated.

2. Skin aging

2.1 Factors affecting skin aging

Skin aging can occur naturally with human skin by many factors. The aging of skin appears clearly as wrinkles for having been accumulating free radicals or ROS a long time. Functionality of the skin cells is reduced and so the balance between producing new and disposing aged skin cells is lost, therefore causing skin aging or damages. For these reasons, skin aging is related to old age and wrinkles on skin (Escoffier et al., 1989). Skin is thus an organ that could be taken for study of its bodily aging (Guinot et al., 2002). Table 2 and Figure 3 are shown of cellular and molecular features of young and aged cells.

Table 2: Function of skin cellular component and Changes that occur with age (Stojiljković, Pavlović, and Arsić, 2014).

Cell type/component	Function	Change with age
Keratinocytes	Barrier function, mechanical protection, cytokine production	↓barrier function, ↓proliferation and differentiation
Melanocytes	Synthesize pigment for protection from UV radiation	↓melanocyte number, ↓life span
Fibroblasts	Synthesis and degradation of ECM	↓in number
Collagen	ECM component	↓biosynthesis,↑stability and resistance to enzymatic degradation
Elastin	ECM component	↓ microfibril content, porous, indistinct, and fragmented

ECM (extracellular matrix), UV (ultraviolet)

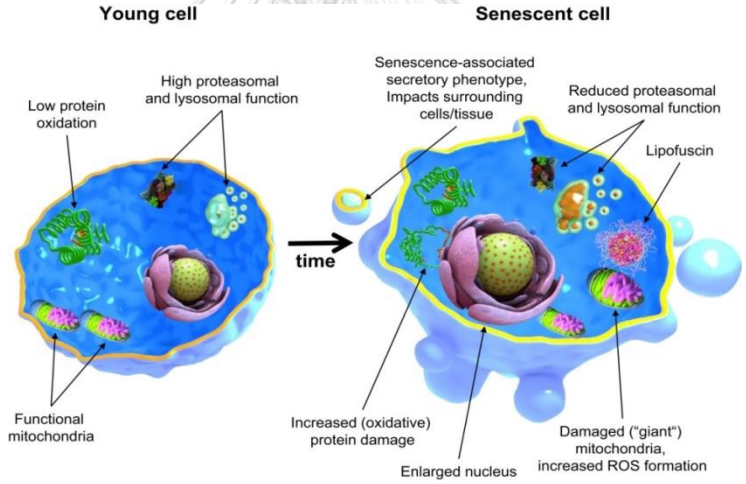


Figure 3**:** Cellular and molecular features of young skin cell compared to aged skin cells (Höhn et al., 2017)

Aging occurs via 2 factors: 1) Internal factors, which are influenced by cells, internal system or intrinsic aging and 2) External factors or extrinsic aging such as ultraviolet (UV) radiation (Figure 4 - Figure 6). These intrinsic and extrinsic factors thin out the dermis layer of the skin by breaking down collagen elastin and

glycosaminoglycans, causing the skin to lose its elasticity (Zouboulis, and Makrantonaki, 2011).

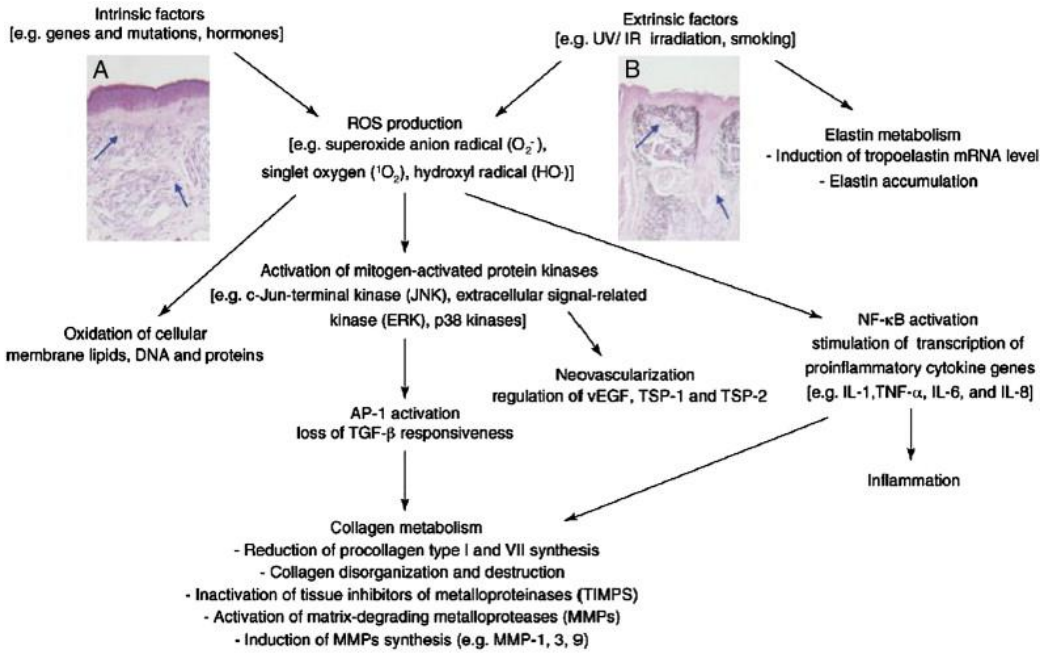


Figure 4**:** A schematic diagram describing biochemical signaling pathways underlying intrinsic (A) and extrinsic (B) skin aging mechanism. Sample (A) was obtained from inner side of the upper arm of an 83-year-old subject representing sun-protected area of the skin affecting only from intrinsic factors. Sample (B) was from the facial skin of a 75-year-old female subject showing sun-exposed skin influenced by extrinsic factors. It was clearly seen that sample (B) had more unorganized structure of elastin tissue as illustrated with blue arrow. (Zouboulis, and Makrantonaki, 2011)

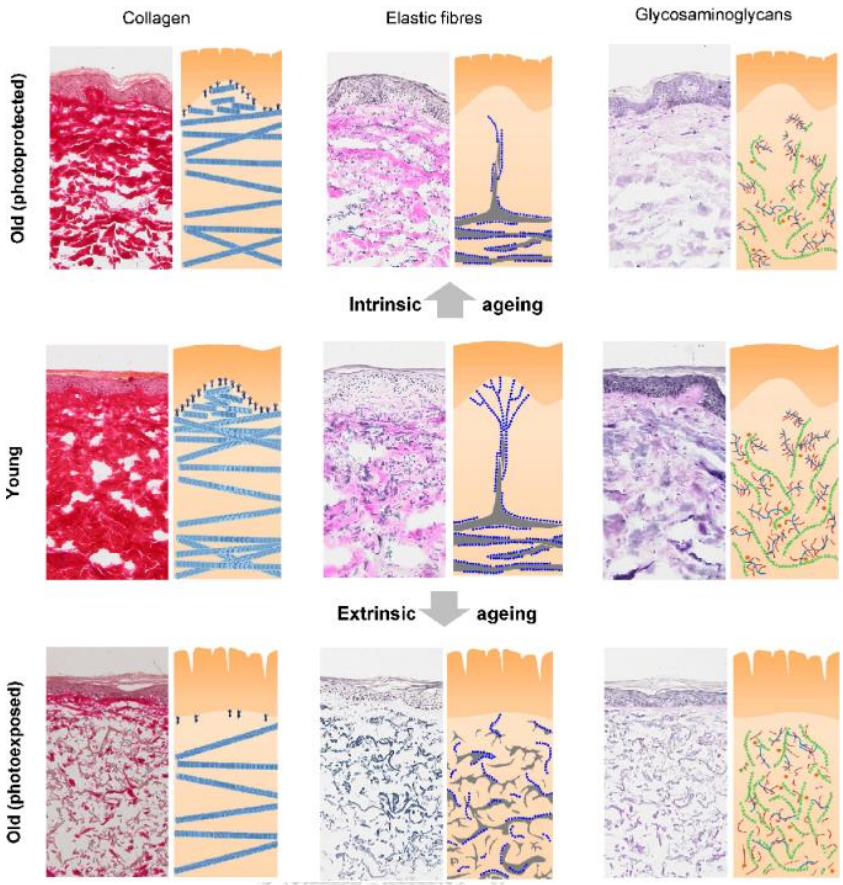


Figure 5**:** The ECM remodeling influenced by intrinsic and extrinsic factors are illustrated. Skin aging significantly diverse between photo protected area and photo exposed area. The aged photo protected area was represented by the buttock skin of a 75-year-old subject while the aged photo exposed area was obtained from the forearm of the same subject. The skin of the upper inner arm of a 23-year-old was used to reflect the young skin. Collagen was stained with picrosirruis red, elastin was marked by Miller's substrate and glycosaminoglycan was shown with periodic acid. The unexposed aged skin showed an atrophy of the reticular collagens, elastin and glycosaminoglycans whereas sunlight exposure severely caused fibrillar collagen destruction specifically collagen type VII and disorder of the elastin fiber, together with glycosaminoglycans pattern in the extracellular matrix (Naylor, Watson, and Sherratt, 2011).

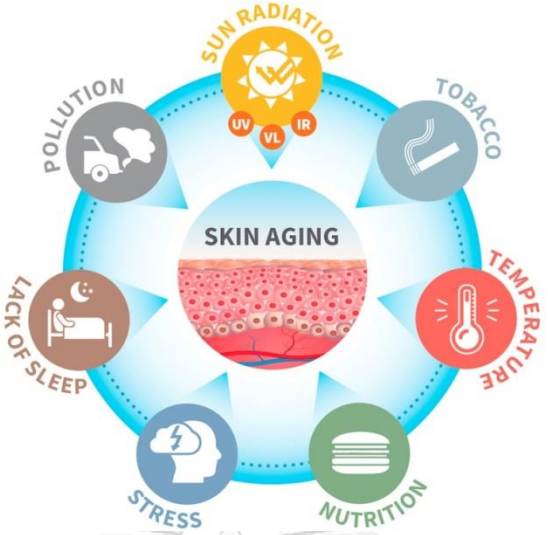


Figure 6: The factors influenced skin aging is called exposome. UV radiation, tobacco and pollution exposure are the primary cause of skin structure damage. Even though other factors. (Krutmann et al., 2017)

Intrinsic and extrinsic aging

The two types of skin aging have clear sources: the intrinsic and extrinsic; however, their results become synergistic, leading to the aged look of the skin.

Intrinsic aging is an unavoidable factor occurring as a natural process of the body. Different ethnicities present different level of melanin. The high level of melanin protects the skin from photoaging. It was evidenced that Asian skins are less prone to sun light and develop wrinkle at a slower rate compared to Caucasian skins with less amount of melanin. Anatomic variation is another factor impacting skin aging internally. Signs of skin aging are more likely to appear in the area with thinner skin such as lips and eye area as compared to palm of the hand or sole of the foot. Moreover, hormonal changes can also affect the changes of the skin. The present of estrogens activate fibroblast to produce collagen leading to generation of hyaluronic

acid that increases the water retention in the extracellular matrix. A sharp decline in estrogens has an essential effect on skin hydration. Therefore, women during menopause are likely to have thin and sagged skin which can be restored by administering estrogen hormones back into the body.

On the other hand, *extrinsic aging* can be controlled and avoided. For example, high temperature causes water to evaporate at faster rate compared to low temperature. Thus, skin in high temperature trend to lose more water and become dehydrated faster than within lower temperature. Also, it was reported that some peptides and lipids required specific temperature to be properly structured. In addition, some drugs contain side effect that can be observe on skin. According to xeroderma and desquamation are the undesirable side effects of hypocholesterolemic drugs. Furthermore, Smoking has long been established as an enemy of young skin even more potent that sun exposure. It was claimed that smoking is directly related to the intensity of skin aging appearance. Smoking disturbs skin arrangement in the cellular level by inducing elastosis, telangiectasias, and decrease angiogenesis depriving the dermal tissue from essential nutrients and oxygen Also, smoking emphasis on the accumulation of free radicals which contribute to skin roughness and hyperpigmentation. Patients with long time exposure to smoke lost their ability to response upon cutaneous hormonal therapy. Photo-aging is also one of the most significant factors inducing skin disorder which responsible for more than 90% of the observable skin aging. Sun light exposure causing changes in the skin down to cellular level, however the destructions are generally visible after age 50. Table below indicates the changes in skin after exposing to sun light (Ramos-e-Silva et al., 2013).

Rabe et al., (2006) categorized strategies to treat skin aging according to the severity as shown in Table 3 (Rabe et al., 2006). The most non-invasive approach is to prevent photo-induced aging by using sunscreen protection. Both physical and chemical sun filer should be applied on the skin before exposing to the sun. The next strategy is to use topical products to delay or reduce the appearance of skin aging. By using retinoic acid, antioxidants, or hormonal therapy can improve skin physiology and return skin cells back to the homeostasis. The tertiary approach is the most invasive strategy which involves triggering of the skin regenerating mechanism. Chemical peelings and microdermabrasion strip off an outer layer of the skin containing the aging signs expecting the new, undisturbed layer of skin to be restored. Botulinum toxin and filler involve injecting foreign substrate into the cells replacing the natural skin environment.

Table 3**:** Skin therapy categorized by treatment intensity (Rabe et al., 2006)

Primary	Secondary	Tertiary
Photoprotection	Photoprotection	Chemical peelings
	Retinoic acid	Microdermabrasion/microcoblation
	Antioxidants	Laser
	Estrogens	Botulinum toxin
	Growth factors/cytokines	Fillers

2.2 Anatomy of the skin barrier

Skin consists of three layers, epidermis, dermis and hypodermis (Figure 7). The dermis and epidermis of human skin are the source of wrinkles (Zouboulis et al., 2008). Dermis is a connective tissue under the outer skin layer (epidermis) which is composed

of various fibrous proteins such as collagen (80% of skin's dry weight) resulting in skin tensile strength, elastin (2 - 4%) which scatters connectively and embedded in a ground tissue resulting in skin elasticity which can regain shape after stretching or shrinking, as well as GAGs, which function to make the skin moist. The cell that produces collagen, elastin and GAGs is fibroblast. Skin aging caused by internal factors affects the dermis to degenerate and become thinner. Also, an area between the epidermal and dermal layers would have flattening or become flatter as skin cell production is reduced. The enzymes responsible for collagen degradation are collectively called collagenases. As the skin ages, collagenase activity would not become balanced but tend to increase (Thring, Hili, and Naughton, 2009).

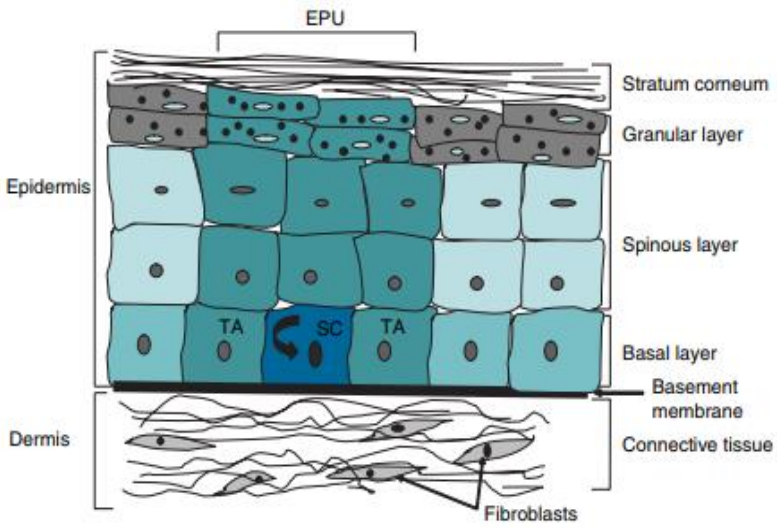


Figure 7: Diagrammatic representation of skin histology (Dayan, 2008).

2.3 Molecular mechanisms of skin aging

Collagenases are metalloproteinases (MMPs) enzymes which break down a structure of fibrous proteins of the connective tissue under the skin such as collagen, elastin and GAGs (Figure 8). Moreover, old age influences a decrease in the fibroblast

amount of the dermis, thereby decreasing collagen, elastin and GAGs and affecting a change in the skin structure, resulting in the loss of elasticity of the dermal layer. This can lead to dermal atrophy. Decreasing elastin amount is due to the fewer expression of elastin gene after the age of 40 - 50 years, and thus reducing the skin elasticity (Jenkins, 2002). Elastin structure and other fibrous proteins are also degraded by elastase of serine protease (Figure 9). So, inhibition of collagenase and elastase activity could help prevent skin elasticity loss which leads to wrinkles (Pillai, Oresajo, and Hayward, 2005; Thring, Hili, and Naughton, 2009).

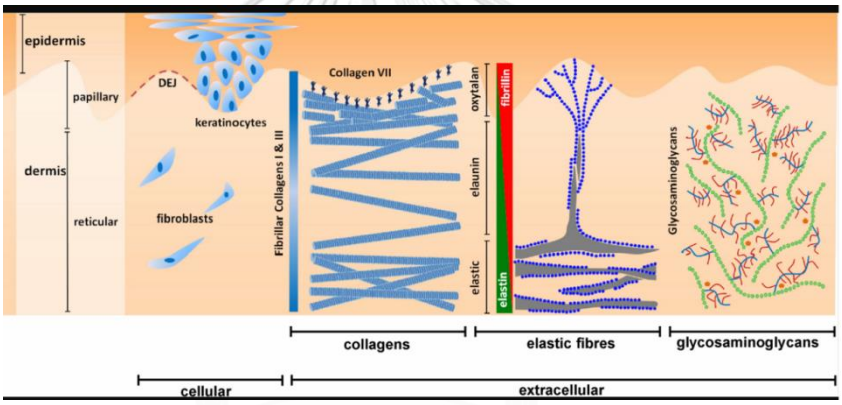


Figure 8: The illustration of composition of young skin is indicated. Collagen, elastin, and glycosaminoglycan are the main structure providing strength and integrity in the dermis layer. (Naylor, Watson, and Sherratt, 2011)

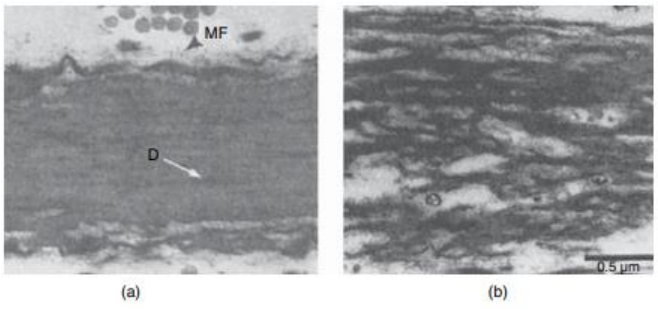


Figure 9**:** The figure shows dermis elastin disorder in aged skin. Figure (a) represent skin of 30-70-year-old human showing dense microfibrillar elastin fibers marked as (D) and harmonized microfibrils marked as MF. Figure (b) represent skin of 50-70-year-old human having discrete layer of elastin fiber. (Dayan, 2008)

Collagen

One of the most fundamental proteins in mammals is collagens which account for more than 30% of the total body protein mass. Collagen is a general term for triple helix polypeptide chain. These supramolecular structures are the main character of Collagen family abundantly found in extracellular matrix with various size, function and tissue contribution (Figure 10). Collagen family proteins can be categorized according to their structure and organization into fibril-forming collagen which account for more than 90% of all collagen in the body. Fibril-forming collagen is further divided into type I which is the main substrate of bone, dermis, tendons, ligament and cornea that often found together with type V. The cartilage and vitreous body are consisted of type II and type XI whereas type III is found in the reticular fibres. Basement membrane collagens provide anchoring ground for dermis layer of the skin which consists of type IV collagen. Microfibrillar collagens provide strength and structure to the skin. Moreover, Anchoring fibrils, Hexagonal network-forming collagens, fibril-associate collagens (FACIT), transmembrane collagens, and

multiplexins are also the subcategory of collagens as shown in Table 5. So far, 28 genetically distinct collagen types have been described (Figure 11). Different collagen types have different structures and complexities leading to different functions (Gelse, Pöschl, and Aigner, 2003).

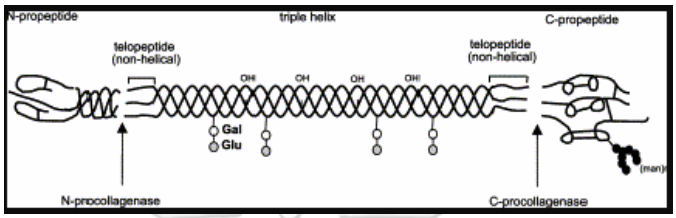


Figure 10: Fibrillar Collagens type I are arranged in triple helix strands with subdomains including cleavage sites for N-procollagens and C-procollagens. (Gelse, Pöschl, and Aigner, 2003)

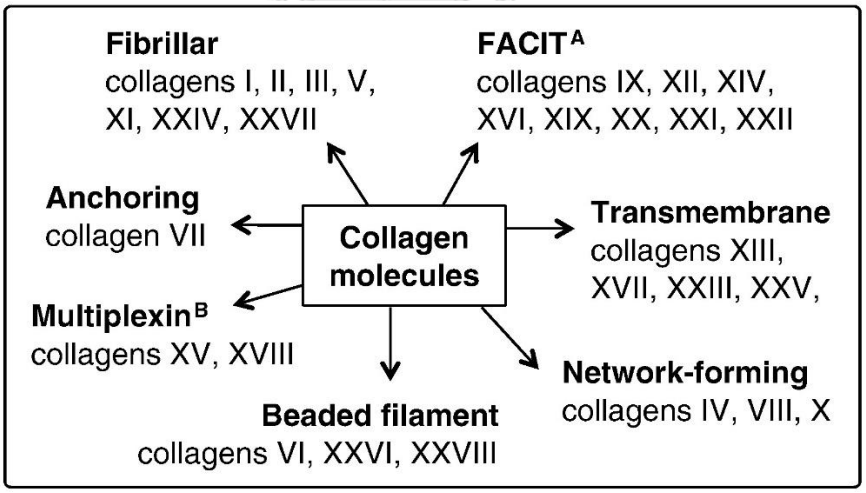


Figure 11: Collagen family proteins are highly diverse. The supramolecular structures of collagens are grouped and listed in the diagram above. ([A] Fibril-associated collagen with interrupted triple helices and [B] Multiple triple helix domains and interruptions). (Muiznieks, and Keeley, 2013).

Based on their structure and supramolecular organization, they can be grouped into fibril-forming collagens, fibril-associated collagens (FACIT), network-forming collagens, anchoring fibrils, transmembrane collagens, basement membrane collagens and others with unique functions (Table 4). The different collagen types are characterized by considerable complexity and diversity in their structure, their splice variants, the presence of additional, non-helical domains, their assembly and their function. The most abundant and widespread family of collagens with about 90% of the total collagen is represented by the fibril-forming collagens (Figure 12). Types I and V collagen fibrils contribute to the structural backbone of the bone and types II and XI collagens predominantly contribute to the fibrillar matrix of articular cartilage. Their torsional stability and tensile strength lead to the stability and integrity of these tissues (Muiznieks, and Keeley, 2013).

Table 4: Table illustration the variety of collagen types as they belong to the main collagen groups. (Muiznieks and Keeley, 2013)

Type	Molecular composition	Genes (genomic localization)	Tissue distribution
Fibril-forming collagens			
I	$[\alpha1(I)]_2\alpha2(I)$	COL1A1 (17q21.31–q22) COL1A2 (7q22.1)	bone, dermis, tendon, ligaments, cornea
II	$[\alpha1(II)]_3$	COL2A1 (12q13.11–q13.2)	cartilage, vitreous body, nucleus pulposus
III	$[\alpha1(III)]_3$	COL3A1 (2q31)	skin, vessel wall, reticular fibres of most tissues (lungs, liver, spleen, etc.)
V	α1(V),α2(V),α3(V)	COL5A1 (9q34.2–q34.3)	lung, cornea, bone, fetal membranes; together with

Type	Molecular composition	Genes (genomic localization)	Tissue distribution
		COL5A2 (2q31)	type I collagen
		COL5A3 (19p13.2)	
		COL11A1 (1p21)	
XI	α1(XI)α2(XI)α3(XI)	COL11A2 (6p21.3)	cartilage, vitreous body
		COL11A3=COL2A1	
Basement membrane collagens			
		COL4A1 (13q34)	
		COL4A2 (13q34)	
IV	$[\alpha1(IV)]_2\alpha2(IV)$; α1–α6	COL4A3 (2q36–q37)	basement membranes
		COL4A4 (2q36–q37)	
		COL4A5 (Xq22.3)	
		COL4A6 (Xp22.3)	
Microfibrillar collagen			
		COL6A1 (21q22.3)	widespread: dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
VI	α1(VI),α2(VI),α3(VI)	COL6A2 (21q22.3)	
		COL6A3 (2q37)	
Anchoring fibrils			
VII	$[\alpha1(VII)]_3$	COL7A1 (3p21.3)	skin, dermal–epidermal junctions; oral mucosa, cervix,
Hexagonal network-forming collagens			
		COL8A1 (3q12–q13.1)	endothelial cells, Descemet's membrane
VIII	$[\alpha1(VIII)]_2\alpha2(VIII)$	COL8A2 (1p34.3–p32.3)	
X	$[\alpha3(X)]_3$	COL10A1 (6q21–q22.3)	hypertrophic cartilage
FACIT collagens			
IX	α1(IX)α2(IX)α3(IX)	COL9A1 (6q13)	cartilage, vitreous humor,

Type	Molecular composition	Genes (genomic localization)	Tissue distribution
		COL9A2 (1p33–p32.2)	cornea
XII	$[\alpha1(XII)]_3$	COL12A1 (6q12–q13)	perichondrium, ligaments, tendon
XIV	$[\alpha1(XIV)]_3$	COL9A1 (8q23)	dermis, tendon, vessel wall, placenta, lungs, liver
XIX	$[\alpha1(XIX)]_3$	COL19A1 (6q12–q14)	human rhabdomyosarcoma
XX	$[\alpha1(XX)]_3$		corneal epithelium, embryonic skin, sternal cartilage, tendon
XXI	$[\alpha1(XXI)]_3$	COL21A1 (6p12.3–11.2)	blood vessel wall
Transmembrane collagens			
XIII	$[\alpha1(XIII)]_3$	COL13A1 (10q22)	epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver
XVII	$[\alpha1(XVII)]_3$	COL17A1 (10q24.3)	dermal–epidermal junctions
Multiplexins			
XV	$[\alpha1(XV)]_3$	COL15A1 (9q21–q22)	fibroblasts, smooth muscle cells, kidney, pancreas,
XVI	$[\alpha1(XVI)]_3$	COL16A1 (1p34)	fibroblasts, amnion, keratinocytes
XVIII	$[\alpha1(XVIII)]_3$	COL18A1 (21q22.3)	lungs, liver

Given are the molecular compositions, the genomic localization of the different chains as well as the basic tissue distribution.

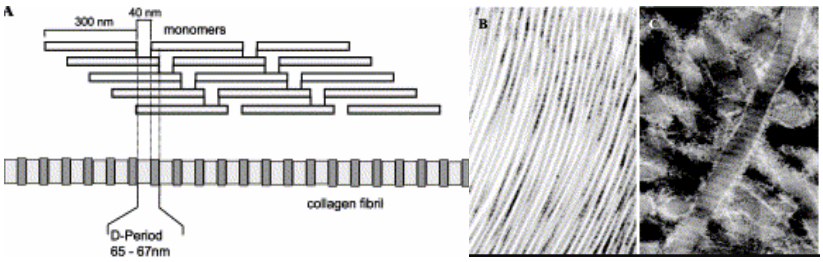


Figure 12: (A) Schematic representation of the supramolecular assembly of the collagen fibrils in the characteristic quarter-staggered form. The monomers are 300-nm long and 40-nm gaps separate consecutive monomers causing the characteristic appearance of the collagen type I fibrils on the ultrastructural level. (B+C) Collagen type I (B) and II (C) fibrils as they are arranged in normal tendon (B) and articular cartilage (C). Whereas they are arranged in tendon in a parallel manner, they show a rather network-like supramolecular arrangement in articular cartilage (Gelse, Pöschl, and Aigner, 2003).

Elastin

The fundamental function of elastin is to hold the integrity of the tissue enabling the tissue to be extended and bounce back to its formal shape. The tropoelastin (Figure 13) secreted from fibroblast and smooth muscle allows elastin assembly forming into elastin fibers. Even though both collagen and elastin are consisted of glycine and proline amino acids, they are arranged in different structure leading to different roles in supporting the extracellular space. Collagen and elastin are also cross-linked to form into fibrous extracellular network to perform even more variety of functions though out the body. The body response to the external mechanical force is truly rely on the extracellular matrix networks of collagens and elastin (Karsdal, 2016; Muiznieks, and Keeley, 2013).

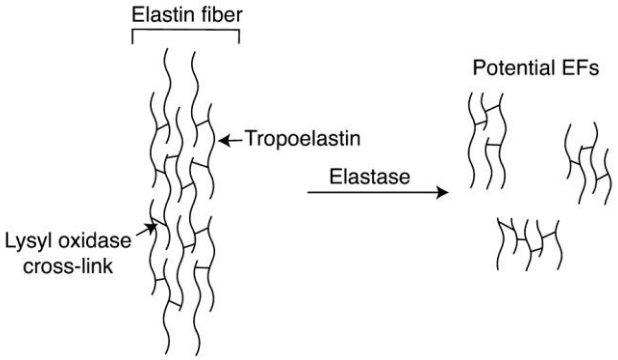


Figure 13: Depiction of an elastin fiber. The schematic depicts an elastin fiber that consists of multiple tropoelastin monomers cross-linked together by lysyl oxidase. Potential degradation products and their cross-linked nature are shown (Houghton et al., 2006).

Matrix metalloproteinases (MMPs)

MMPs comprise a family of extracellular matrix degrading enzymes (Table 4 and Table 5) that are believed to play pivotal roles in embryonic development and growth as well as in tissue remodeling and repair. Excessive or inappropriate expression of MMPs may contribute to the pathogenesis of many tissue destructive processes, including highly prevalent diseases (Shapiro, 1998).

Table 5: Matrix metalloproteinase substrates (Shapiro, 1998)

Matrix metalloproteinase substrates*.

MMP	Interstitial collagens	Basement membrane	Elastin	Non-matrix proteins
Collagenases				
MMP-1	III > I >(+/− II); VII, X	+/− FN, LN, EN, PG	−	L-selectin is a substrate for collagenases
MMP-8	I > III > II; (? VII, X)	+/− FN, LN, EN, PG	−	
MMP-13	II > I, III; GL (? VII, X) and telopeptidase	+/− FN, LN, EN, PG	−	
Stromelysins				
MMP-3	−	FN, LN, EN, PG, +/− PS col IV	+/−	EGF-like growth factor and plasminogen are substrates for stromelysins
MMP-10†	−	FN, LN, EN, PG, +/− PS col IV	+/−	
Stromelysin-like				
MMP-7	−	FN, LN, EN, PG	+	Stromelysin-like enzymes are most potent at converting plasminogen to angiostatin and degrading α_1 AT
MMP-12	−	FN, LN, EN, PG, PS col IV	++	
Gelatinases				
MMP-2	GL, I, VII, X, XI	col IV/V, FN, LN, EN, PG, PS	++	
MMP-9	GL	col IV/V, FN, LN, EN, PG, PS	++	
Furin-recognition sites				
MMP-11‡	−	−	−	
Membrane type				
MMP-14§	+/− I > III, II	FN, LN, EN, PG		
MMP-15		FN, LN, EN, PG		
MMP-16	?			
MMP-17	?			
Newly described				
Enamolsin	GL (amelogenin)	?	?	?

*Note this list is inherently incomplete, representing only selected substrates tested to date. These substrates guide potential biological functions, but the actual *in vivo* substrates are unknown. †MMP-10 has the same substrate specificities as MMP-3 but is less potent. ‡Human enzyme not catalytically active to known ECM components. §Soluble recombinant protein was tested. α_1 AT, α_1 antitrypsin; EN, entactin; FN, fibronectin; GL, gelatin; colIV/V, types IV and V collagen); LN, laminin; PG, proteoglycan; PS col IV, pepsinized type IV collagen.

In addition, UV irradiation disrupts the skin collagen matrix by two interdependent pathways: stimulating collagen degradation and inhibiting procollagen production (Fisher et al., 1996). UV irradiation activates MAP kinase signaling, which in turn induces transcription factor AP-1, which stimulates expression of matrix metalloproteinases (MMP) including MMP-1 and MMP-3 (Brenneisen, Sies, and Scharffetter and Kochanek, 2002; Pittayapruek et al., 2016).

Table 6: Classification of matrix metalloproteinase enzymes (Verma, and Hansch, 2007)

No.	MMP No.	Class	Enzyme
1	MMP-1	Collagenases	Collagenase-1
2	MMP-8		Neutrophil collagenase
3	MMP-13		Collagenase-3
4	MMP-18		Collagenase-4
5	MMP-2	Gelatinases	Gelatinase-A
6	MMP-9		Gelatinases-B
7	MMP-3	Stromelysins	Stromelysin-1
8	MMP-10		Stromelysin-2
9	MMP-11		Stromelysin-3
10	MMP-27		Homology to stromelysin-2 (51.6%)
11	MMP-7	Matrilysins	Matrilysin (PUMP)
12	MMP-26		Matrilysin-2
13	MMP-14	MT-MMP (membrane type)	MT1-MMP
14	MMP-15		MT2-MMP
15	MMP-16		MT3-MMP
16	MMP-17		MT4-MMP
17	MMP-24		MT5-MMP
18	MMP-25		MT6-MMP
19	MMP-12	Other enzymes	Macrophage metalloelastase
20	MMP-19		RASI 1
21	MMP-20		Enamelysin
22	MMP-21		MMP identified on chromosome 1
23	MMP-22		MMP identified on chromosome 1
24	MMP-23		From human ovary cDNA
25	MMP-28		Epilysin
26	MMP-29		Unnamed

Apart from dermis of the human skin whereas wrinkles are often seen, the epidermis is another factor. Epidermis can also be determined to have 4 major layers as follows.

1. Stratum basale is the deepest epidermal layer close to dermis and laid on basement membrane. A cell is a cylinder-shaped or cuboid-shaped cell and constantly going through mitosis. Therefore, the newly produced cells are pushed superficially away to the next layer. Keratinocyte stem cells settle in this layer.

2. Stratum spinosum is the upper layer. Shape of a cell is flatter and spiny with joints to attach between the cells.

3. Stratum granulosum is fusiform-shaped and its nucleus is not clearly visible.

4. Stratum corneum is the top epidermal layer and also the thickest layer. This layer has a significant role of barrier layer for all transporting substances. Cells in this layer are aligned in rows. They are dead and prokaryotic (corneocytes) cells which are shed periodically. This stratum corneum is sometimes also known as cornified, horny layer.

Epidermis functions as a fort to prevent foreign substances from outside and to store water within the cell surface. This layer of cells has capability to renew and rejuvenate itself in response to the damage (Baroni et al., 2012; Darlenski, Kazandjieva, and Tsankov, 2011). The cell that undertakes homeostasis and restores epidermal skin are keratinocyte stem cell which divides and develops itself into keratinocyte, the major cells in epidermis constituting 90 - 95% of all epidermal layers while keratinocyte stem cell (Figure 14**)** is found in the stratum basale layer. When a keratinocyte stem cell has divided itself, those new cells either inherit all

biochemical properties and characteristics in order to be another source for stem cells or can differentiate in biochemical properties and characteristics in order to have a capability to further divide and proliferate into TA cell (transit amplifying cell). TA cells are capable to rapidly amplify into keratinocytes cell in other layer (post-mitotic differentiating cell). TA cells are found mainly in the stratum basale. (Koster, and Roop, 2007; Zouboulis et al., 2008).

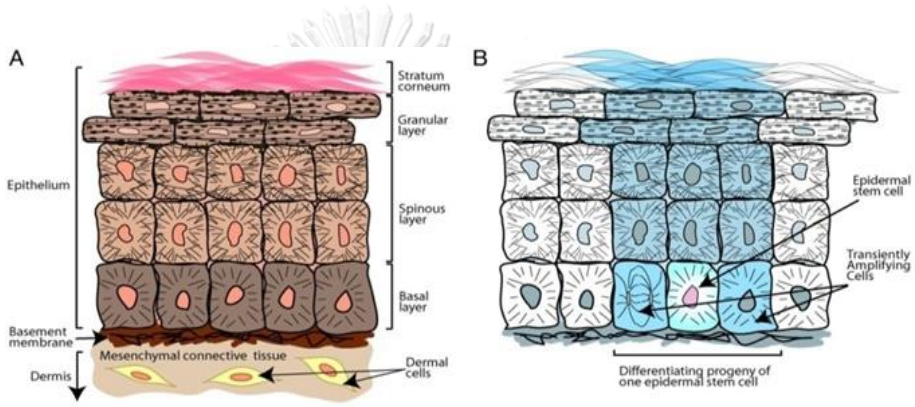


Figure 14**:** A: Structure of human epidermal skin and B: Position and scattering of keratinocyte stem cell and TA cells in stratum basale (Alonso, and Fuchs, 2003)

2.4 Basic knowledge of stem cell: Stem cell types based on cell potency

Stem cells can be classified according to the cells' potency to develop into mature cell or the differentiation potential (Figure 15).

2.4.1. Totipotent (Omnipotent) stem cells

Totipotent stem cells are produced from the fertilization of egg and sperm into blastomere which have the potential to differentiate into any linages of organism. This type is the most potent type of stem cells.

2.4.2. Pluripotent stem cells

Pluripotent stem cells are able to develop into more than 1 cell linages.

2.4.3. Multipotent stem cells

Multipotent stem cells or progenitor cells give rise into any adult cells within a cell linage. For example, hematopoietic stem cell can give rise to red blood cells, white blood cells, and lymphoid cells.

2.4.4. Unipotent stem cells

Unipotent stem cells can only differentiate into only 1 cell type. For instance, hepatoblasts can only develop into hepatocytes or liver cells (Jaenisch, and Young, 2008; Knoepffler, Schipanski, and Sorgner, 2007)

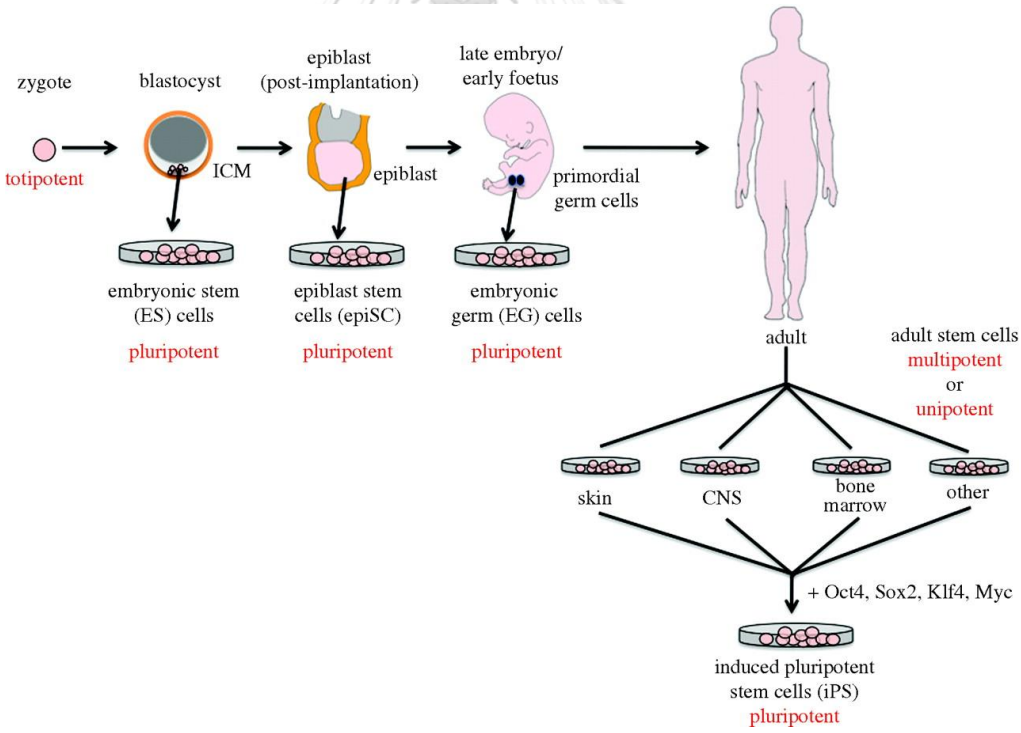


Figure 15: Stem cell types based on cell potency(Fiona M. Watt, and Driskell, 2009)

2.5 Epidermal Stem cell (Morris, 2012)

2.5.1. Proliferative hierarchy in epithelium

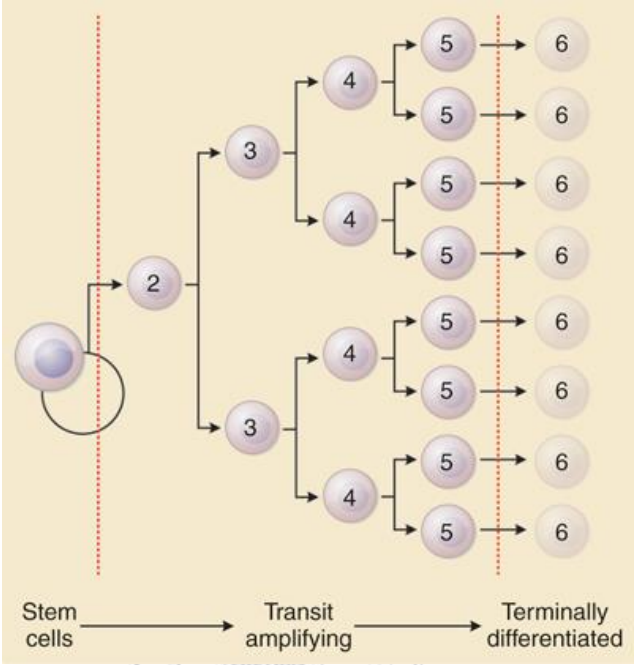


Figure 16: Proliferative hierarchy in epithelium (Morris, 2012)

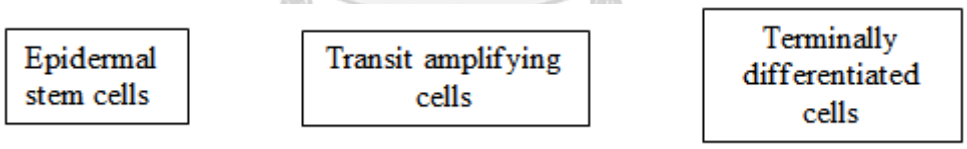


Figure 17: Diagram of proliferative hierarchy in epithelium

The diagram described the hierarchy of epidermal stem cell differentiation to product epithelial cell (Figure 16 and 17).

- At the top of the hierarchy presents epidermal stem cells which are unipotent stem cells. Epidermal stem cell has the undifferentiated phenotype with a long-lived cycle. There are only a few number of progenitor stem cells in the stem cell niche.

- Transit amplifying cells or TAC are progenitor cells with fast cycling period and able to give rise to fully differentiated daughter cells

- The epithelium cells are the fully differentiated version of dermal skin cells maintaining the mass of epithelial tissue.

2.5.2. Stem cells within the adult skin epithelium

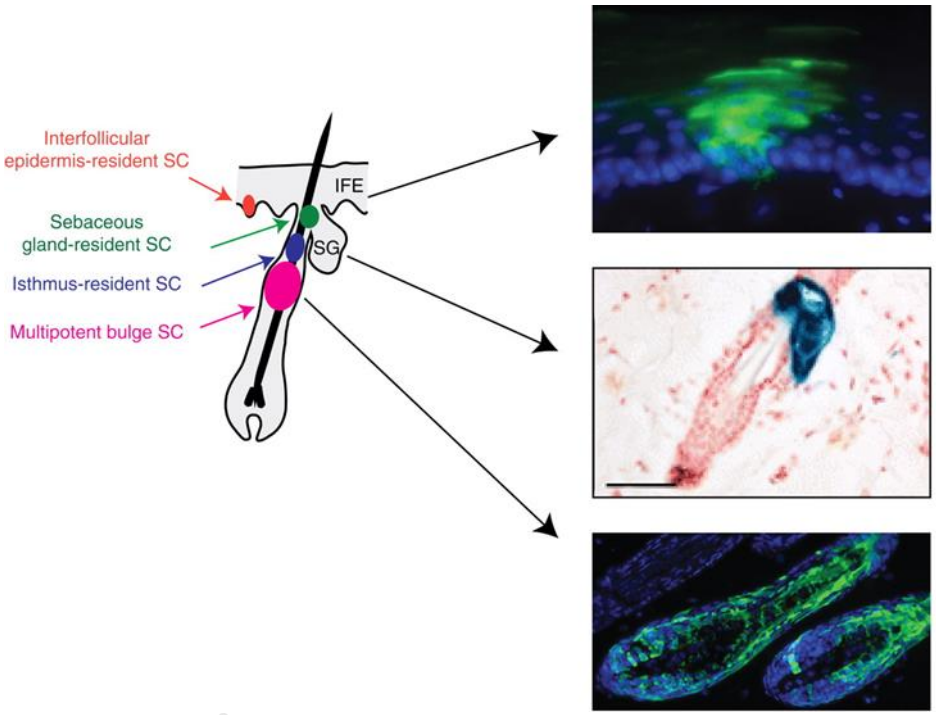


Figure 18: Different types of stem cells regulate epidermal homeostasis (Blanpain, and Fuchs, 2009)

In the adult skin epithelium, there are combinations of dermal building block. Each of the blocks includes hair follicles or pilosebaceous unit, sebaceous unit and the surrounding intrafollicular epidermis or IFM. Each of the unit has its' own progenitor cells to reproduce themselves. Moreover, hair follicles possess multipotent stem cell which would be activated for repairing process at the beginning of the new hair cycle or when any damage occurs (Figure 18) (C. Blanpain, and E. Fuchs, 2006).

Many studies have been done to support that the progenitor cells in the epidermis are responsible for skin repair and skin renewal. The research on mouse epidermis found a group of hexagonal cells presenting along the basal dermis. It was theorized that these cells are epidermal stem cell niche containing a proliferative unit or EPU and a unipotent stem cell which are responsible for reproducing dermal tissue (Mackenzie, 1970; C. Potten, 1981; C. S. Potten, 1974).

2.5.3. The epidermal proliferative unit (EPU) concept: organization and stem cells location

Epidermal stem cells present within the basal layer (Hsu, Li, and Fuchs, 2014) among the epidermal proliferative unit or EPU which is a three-dimensional organized microenvironment surrounding a single-stem cell (Figure 19).

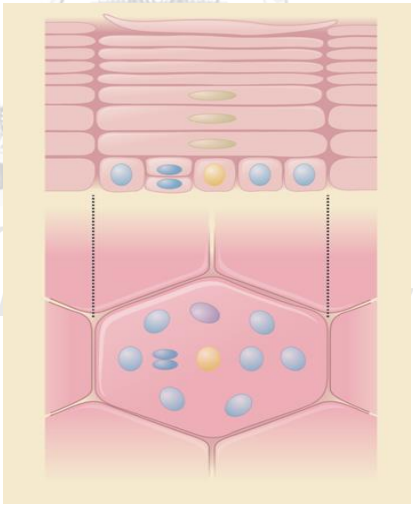


Figure 19: Epidermal Proliferative Unit (Morris, 2012)

Organization of the epidermal proliferative unit (Morris, 2012)

Each EPU contains approximately 10 basal cells which consists of an epidermal stem cell (yellow), immediate transit-amplifying progenitor cells (blue) and early-differentiating cells (purple)

From EPU, the epidermal stem cell proliferates to produce daughter cells and differentiate into differentiated keratinocytes (green) and mature squamous cells which enucleated.

2.5.4. Relationship between compartment of epidermal stem cells

There is more and more evidence supporting the presence of stem cells and progenitor cell niche within the cutaneous dermis. During the normal uninterrupted stage, the stem cell niche has no interaction of the surrounding tissue (Ghazizadeh, and Taichman, 2001). However, when the damage occurs, the proliferative unit has the potential to assist with the skin repair process (Morris, 2012).

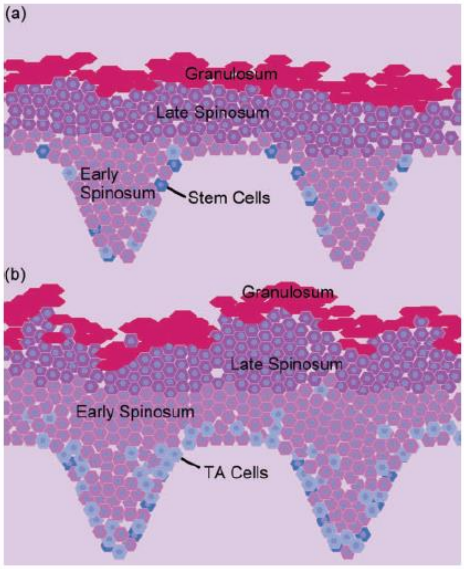


Figure 20: Diagram to demonstrate location and distribution of epidermal stem cells (N. Grabe, and K. Neuber, 2007)

2.5.5. Epidermal keratinocytes morphogenesis sequence

In order to maintain the tissue self-renewal property, the keratinocytes slowly undergo morphogenesis (Niels Grabe, and Karsten Neuber, 2007). The keratinocytes constantly proliferate from the dermal basal layer towards the suprabasal layer to compensate the shredded squamous cells. The differentiation of epidermis is tightly regulated through gene expression (Niels Grabe, and Karsten Neuber, 2007).

Morphology of keratinocytes in each layer of epidermis is their unique characteristics. Morphology of a keratinocyte could help us to define its stage in differentiation (Figure 20 and 21).

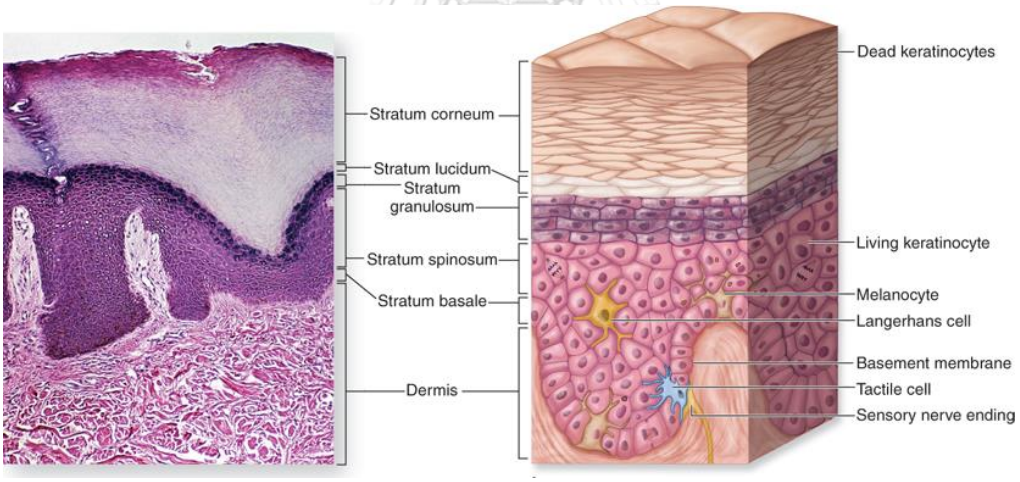


Figure 21: Epidermal layer (Left) Micrograph of thick skin (X100; H&E)

(Right) Illustrated diagram (Mescher, 2013)

2.5.6. Skin Diseases Arising from Proliferative Dysfunction (Morris, 2012)

Psoriasis is one of the diseases caused by irregular proliferation of the progenitor cells. The increase number of β-1 integrin dim cells in the suprabasal layers marked the onset of the disease together with Ki67 and C-myc.(N. Grabe, and K. Neuber, 2007)

2.6 Inhibitory Effect of Age-associated Inflammation on Epidermal Stem Cell Function

The age-associated skin inflammation is an unavoidable dermal disease. The inflammation causes a severe decrease in progenitor cell proliferation activity. As evidence indicating that the stem cells not only act as a new cell reservoir for regeneration, but they also produce several cytokines and growth factors supporting skin functions. Thus, the decrease of stem cells function and population from age-associated inflammation skin would deteriorate skin function.

2.6.1. Alterations in stem cells during skin aging (Doles et al., 2012a; Doles et al., 2012b)

- Increased numbers
- Decreased function
- An inability to tolerate stress

2.6.2 Phenotypical & functional changes (Doles et al., 2012b)

- Decreased hair cycling
- Epidermal thinning
- Diminished sebaceous gland function
- An impaired wound response

The key players in maintaining the epidermal homeostasis and repair machinery are keratinocyte stem cells (Senoo, 2013). Regarding stem cell biology, evidence has indicated that several proteins play important roles in maintaining stem cell phenotypes, including CD133, β-catenin, and ALDH1A1 (aldehyde dehydrogenase 1A1).

CD133 has been shown to control cell growth and development as well as cellular functions associated with stem cell maintenance and activity (Kiratipaiboon, Tengamnuay, and Chanvorachote, 2015, 2016; Li, 2013). β-catenin, a co-trancription factor of T-cell factor/lymphoid enhancing factor (TCF/LEF), was reported to induce the expression of transcription factors of stem cells (Fukumoto et al., 2001). Also, ALDH1A1 has been found in stem cells functioning as a detoxifying enzyme (Levi et al., 2009). As stem cells have been shown to be an important key player of physiological regulation, the augmentation of stem cell functions is possible to be a potential way to improve tissue homeostasis and protective mechanisms (Kaur et al., 2004; Racila, and Bickenbach, 2009; Senoo, 2013).

Oxidative stress has been thought to play an important role in the pathogenesis of diseases such as cancer, cardiovascular diseases, atherosclerosis, diabetes mellitus, and neurodegenerative disorders (Pham-Huy, He, and Pham-Huy, 2008; Uttara et al., 2009). In terms of skin biology, reactive oxygen species (ROS) are strongly shown to mediate skin aging and attenuate barrier function (Pillai, Oresajo, and Hayward, 2005; Racila, and Bickenbach, 2009). In keratinocyte, exposure to the UV radiation leads to increased level of ROS (Figure 22), which alters gene and protein structure resulting in cell damage (Helfrich, Sachs, and Voorhees, 2008; Oh, Lee, and Wagers, 2014). Previously, it has been shown that UVB irradiation induced keratinocyte damage via

superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical generation (Dhumrongvaraporn, and Chanvorachote, 2012). As the stem cell component of the epidermis has previously been shown to possess a high level of superoxide dismutase (SOD), an important anti-oxidant enzyme detoxifying $O_2^{\bullet-}$ (Stern, and Bickenbach, 2007), the increase in the stem cell viability or stemness should protect these cells against oxidative damage.

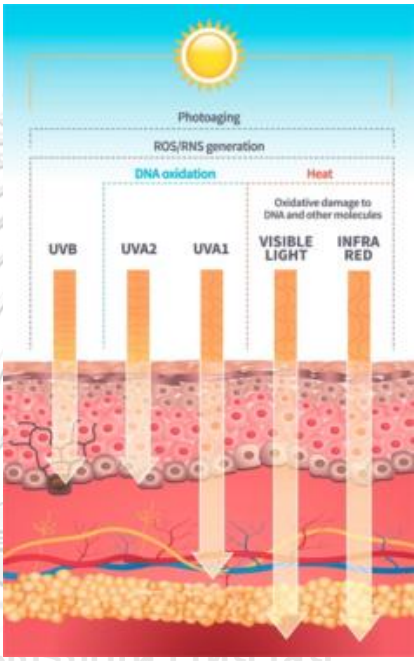


Figure 22: The solar spectrum is composed of various wavelengths, which penetrate into skin at different levels. The longer the wavelength the deeper the rays penetrate the skin. Each wavelength has both different and overlapping effects. UV = Ultraviolet radiation, ROS = Reactive oxygen species, RNS = reactive nitrogen species. (Krutmann et al., 2017)

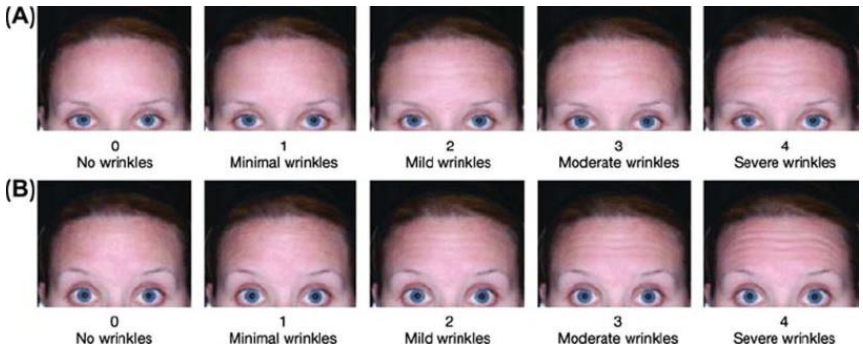


Figure 23**:** Reference images for the five-point Forehead Lines Grading Scale: (A) static grading scale; (B) dynamic grading scale. (A. Carruthers, and Carruthers, 2010)

Skin dryness, rough skin, darker skin, wrinkle, groove and loss of elasticity are the results of skin aging (Figure 23). It is enhanced by the presence of ROS that cause inflammation and disorder of proteins in the skin matrix. In the short state of oxidative stress, it would have minor effect to the body (Nimse, and Pal, 2015). In contrast, a longer state of oxidative stress would risk tissues such as cell membrane or DNA to be destroyed and cause skin elasticity loss, ultimately leading to wrinkles (Figure 24 and 25). To overcome a skin aging, most cosmetic company has been working on the development of anti-aging products, especially ones containing herbal products as they are attractive trends for most cosmetic companies (Mukherjee et al., 2011).

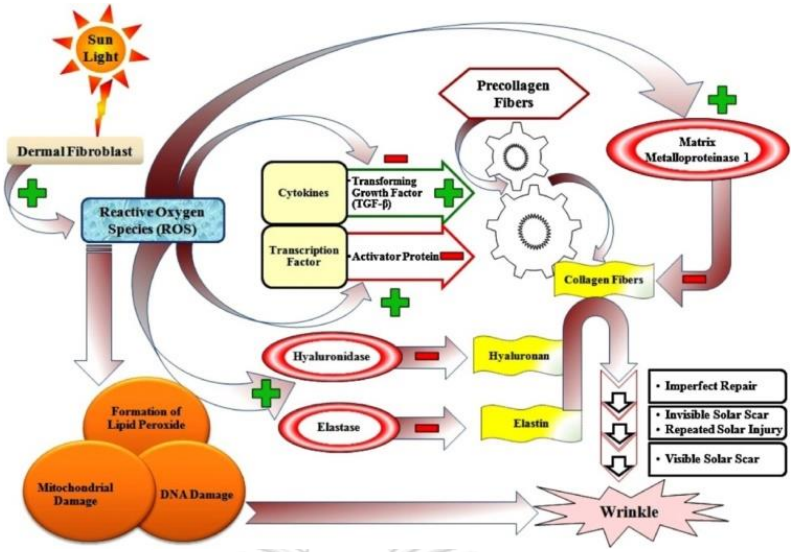


Figure 24: Pathway of premature skin aging [+, induction; −, inhibition] (Mukherjee et al., 2011).

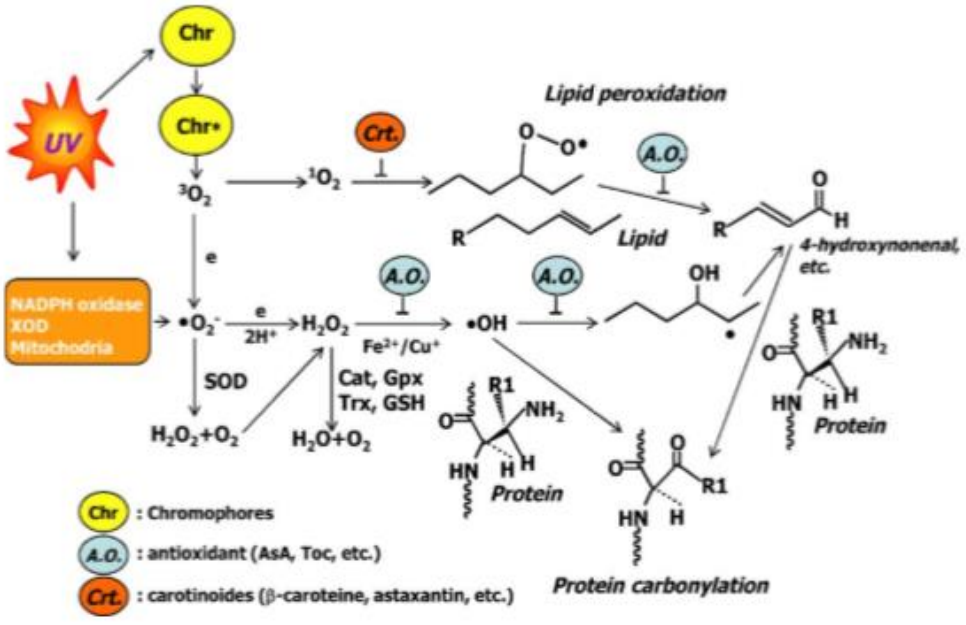


Figure 25**:** ROS-initiated oxidative chain reactions and scavengers (Masaki, 2010)

HaCaT cell lines

HaCaT is an immortalized human epithelial cell line which was developed from an adult skin by Boukamp et al. in 1988. Boukamp et al. created HaCaT from epithelial cells from the periphery of a melanoma located on the upper half of the back of a 62-yr-old male patient who were not extensively sun-exposed. Then these cells were infected by Simian virus 40 (SV40). Regardless of the ability to reproduce itself endlessly, HaCaT is almost identical to normal keratinocytes. HaCaT when mature, also present K1, K10, involucrin and filagrin on the cell surface. These proteins are markers for a fully differentiated epidermis. Moreover, it was proven that the DNA fingerprint pattern of HaCaT is not affected by long-term cultivation.Therefore, it is acceptable to use HaCaT to represent human keratinocytes in the *in vitro* experiment (Boukamp et al., 1988).

2.7. Skin anti-aging prevention and therapy

The skin anti-aging strategies attempted to reverse the dermal and epidermal signs of intrinsic and extrinsic aging can be grouped under the following approaches in Table 6.

Cosmetological care Cosmeceutical refers to products exerting both therapeutic and cosmetic effects. Several cosmeceuticals contain active biological ingredients, and have generally been tested for their safety, but the clinical effects are often unsubstantiated (Kligman, 2005). They have only been initiated to address the quality control, industry standards and regulations (Dureja et al., 2005). However, studying the effect of a cosmeceutical product on the skin can be challenging, since

there are no skin non-effective placebos. Cosmeceutical agents are shown in Table 7 and the targets for Cosmeceutical delivery are shown in Table 8.

Table 7**:** Skin anti-aging approaches (Ganceviciene et al., 2012)

Cosmetological care	Daily skin care Correct sun protection Aesthetic non-invasive procedures
Topical medical agents or topical agents	Antioxidants Cell regulators
Invasive procedures	Chemical peelings Visible light devices Intense pulsed light (IPL) Ablative and nonablative laser photo–rejuvenation Radiofrequency (RF) Injectable skin biostimulation and rejuvenation Prevention of dynamic wrinkles Correction of static, anatomical wrinkles Restoration(redistribution) of fat and volume loss, skin augmentation and contouring
Systemic agents	Hormone replacement therapy Antioxidants
Avoiding of exogenous factors of aging, correction of life style and habits	Smoking Pollution Solar UV irradiation Stress Nutrition, diet restriction and alimentary supplementation Physical activity Control of general health

Table 8: Cosmeceutical Agents (Dahiya, and Romano, 2006)

Cosmeceutical Agents	
α-Hydroxy acids	Citric acid, glycolic acid, lactic acid, salicylic acid, tartaric acid
Retinoids, retinol	Adapalene, retinol, tazarotene, tretinoin
Vitamins	Vitamins A, B3 (niacinamide), C, E
Antioxidants	α-Lipoic acid, green tea, kinetin, ubiquinone (coenzyme Q10)
Growth factors	Epidermal growth factor, platelet-derived growth factor, transforming growth factor-b, vascular endothelial growth factor
Peptides	Argireline®, glycyl-L-histidyl-L-lysine, valine-glycine-valine-alanineproline-glycine
Lightening agents	Aloesin, arbutin, azelaic acid, glabridin, hydroquinone, kojic acid, melatonin, mequinol, niacinamide, paper mulberry extract, soy

Table 9: Targets of Cosmeceuticals (Dahiya and Romano, 2006)

Targets of Cosmeceuticals	
Antiaging Target	**Cosmeceutical Agent**
Collagen turnover	AHAs, growth factors, peptides, retinol, vitamin B3 (niacinamide)
Antioxidant effect	a-Lipoic acid, green tea, kinetin, ubiquinone (coenzyme Q10), vitamins B3 (niacinamide), C, and E
Pigmentation	AHAs, aloesin, arbutin, azelaic acid, glabridin, hydroquinone, kojic acid, melatonin, mequinol, paper mulberry extract, retinol, soy, vitamin B3 (niacinamide)

Furthermore, the term "cosmeceutical" does not universally refer to the same products. For instance, sunscreens are considered to be OTC medications in North America, while they are categorized as cosmeceuticals in Europe. Future

classifications and regulations may ultimately depend on how product claims are presented.

- Photoprotection

Photoprotection is the measures that can prevent human skin from sun light or solar radiation which causes skin damage. The measures include applying sunscreens, limiting sun exposure, and wearing sun-protective clothes.

Topical Pharmacological Agents with Anti-Aging Properties

- Antioxidants

Antioxidants are widely-used ingredients added in some skin anti-aging products. Antioxidants include vitamins C and E, which are demonstrated to prevent the photodamage by inhibiting the free radicals produced by UV radiation. As previously discussed, UV rays promote the production of these toxic electron-missing molecules, thus its name "free radicals" (Goukassian, and Gilchrest, 2004; Muller et al., 2007). These damaged cells seek for electron donation from healthy cells, which, in turn, damages the latter cells. Antioxidants readily "offer" electrons to free radicals, thus protecting cells from damage. Vitamin C is not only an antioxidant, but also exerts the collagen repairing and its production stimulating effects. The most effective formulations are those that contain exceeding 10% L-ascorbic.

Ferulic acid is generally found in the cell walls of plants. It has been used as a combination with vitamins A and E to provide a more effective antioxidant activity. Ferulic acid has strong UV light absorption, therefore

protecting skin from UV radiation. A recent study reported that ferulic acid can promote vitamins C and E to reach doubled photoprotecting effect (Lin et al., 2005).

Alpha-lipoic acid has anti-inflammatory effects which have been demonstrated to inhibit the inflammatory cytokines production (Beitner, 2003).

Coenzyme Q10 (CoQ10) is a nutrient on cell membrane playing an important role in ATP transport chain in mitochondria (Shindo et al., 1994).

Idebenone is thought to be a more potent and more efficient antioxidant compared to other substances. Idebenone is not only a powerful radical scavenger, but also acts as an effective electron carrier in hypoxic conditions (Imada et al., 1989).

McDaniel et al. (2005) evaluated the antioxidant effects of several popular substances. Idebenone, CoQ10, kinetin, vitamin C, and alpha-lipoic acid, were investigated for their inhibiting effects against UV-B. The investigators found that idebenone had the highest antioxidant property, followed by vitamin E, kinetin, CoQ10, alpha-lipoic acid, and vitamin C, respectively. Hence, clinical research proves the aging preventing effect of idebenone (McDaniel et al., 2005).

Kinetin is commonly found in yeast, and functions as a growth regulator. Kinetin has been proved to prevent some aging-associated alteration in cell cultures. By adding kinetin to fibroblasts, it retarded cell shape alterations, cell size, growth rates, and molecular synthesis. According to some investigations, the mechanism behind the age decelerating action may relate to

the genes influencing aging. Some patients were reported having skin texture improvement and mottled pigmentation reduction (Quan et al., 2006; Rattan, and Clark, 1994).

Vitamins C, B3, and E are the most important antioxidants because of their ability to penetrate the skin through their small molecular weight (Bissett et al. 2004). The water-soluble, heat-labile local L-ascorbic acid (vitamin C) in concentrations between 5 and 15% was proven to have a skin anti-aging effect by inducing the production of Collagens I and III, as well as enzymes important for the production of collagen, and inhibitors of MMP1 or collagenase 1 (Haftek et al., 2008; Nusgens et al., 2001).

Vitamin E (α-tocopherol) used as a component of skin products has anti-inflammatory and antiproliferative effects in concentrations between 2 and 20%. It acts by smoothing the skin and increasing the ability of the stratum corneum to maintain its humidity, to accelerate the epithelialization, and contribute to photoprotection of the skin. The effects are not as strong as with vitamins C and B3 (Zhai et al., 2005).

- Botanical Compounds

Botanicals are the largest category of cosmeceutical additives, which their use is often unregulated without clinical data supports. Some botanicals believed to nourish the skin include green tea extract, ferulic acid, and grape seed extract.

Green tea polyphenols inhibit collagenase activity and increase collagen biosynthesis in human fibroblasts. It can also inhibit tyrosinase

activity. Evidence has shown that, after 40 kGy of gamma ray irradiation, the mentioned effects were all unregulated (An et al., 2005).

Ferulic acid is derived from plants and is deemed to be a strong antioxidant that has photoprotecting effect to the skin (D. H. Kim et al., 2011). When combined with vitamins C and E, this compound has provided more substantial UV protection for human skin. In addition, ferulic acid could be a supplement to enhance the sun protection provided by sunscreens (Saija et al., 2000).

Grape seed extract is an antioxidant and exerts wound healing acceleration. The grape seed extract topical application has also been demonstrated to enhance SPF (Murray et al., 2008).

Genistein, an isoflavone isolated from soy, is another useful antioxidant. Topical genistein inhibited UVB-induced skin tumors in mice and blocked UVB-induced acute skin burns and cutaneous wrinkling. Topical genistein also can inhibit UVB-induced erythema in human skin (Wei et al., 2003).

- Cell regulators

Cell regulators, such as vitamin A derivatives, polypeptides and botanicals, act directly on the collagen metabolism and stimulate the production of collagen and elastic fibers. Vitamin A (retinol) and its derivatives (retinaldehyde and tretinoin) are a group of agents that also have antioxidant effects. They can induce the biosynthesis of collagen and reduce the expression of MMP 1 (collagenase 1). Retinol is, at the moment, the substance that is most often used as an anti-aging compound and, compared

with tretinoin, causes less skin irritation (Kafi et al., 2007; Varani et al., 2000). Polypeptides or oligopeptides are composed of amino acids and can imitate a peptide sequence of molecules such as collagen or elastin. Through topical application, polypeptides have the ability to stimulate collagen synthesis and activate dermal metabolism (Lupo, and Cole, 2007).

Invasive Procedures

- Chemical Peels

Chemical peels are categorized as superficial, medium, and deep, regarding to the depth and the duration that agents remain on the skin. Superficial peels [α-β-, lipo-hydroxy acids (HA), trichloroacetic acid (TCA) 10–30%] exfoliate epidermal layers without going beyond the basal layer; medium-depth peels (TCA above 30 to 50%) reach the upper reticular dermis; deep peels (TCA > 50%, phenol) penetrate the lower reticular dermis (Fischer et al., 2010).

- Visible Light Devices: IPL, Lasers, RF for the Skin Rejuvenation, Resurfacing and Tightening

Nonablative skin rejuvenation or “subsurfacing” comes as a low risk and short downtime technology which can improve aging structural changes without disruption of cutaneous integrity (Sadick, 2003). The mechanism of action is supposed to be a selective, heat induced denaturalization of dermal collagen that leads to subsequent reactive synthesis. Nonablative skin rejuvenation is not a precise term since rejuvenation is a controlled form of skin wounding aimed at achieving a more youthful appearance after the

wound heals. The epidermis and superficial dermis can be selectively damaged by two basic mechanisms: (a) by targeting discrete chromophores in the dermis or at the dermal-epidermal junction or (b) by utilizing mid infrared (IR) lasers (Hardaway, and Ross, 2002).

Unfortunately, there is a lack of long-term studies of efficacy and analysis of side effects for the skin using this method of skin rejuvenation. It is obvious that different treatment modalities using visible light devices have resulted in varying clinical effects and allow to select individual treatment parameters for different indications (Raulin, Greve, and Grema, 2003).

- Injectable Skin Rejuvenation and Dermal Fillers

The goal of skin biorejuvenation is to increase the biosynthetic capacity of fibroblasts, inducing the reconstruction of an optimal physiologic environment, the enhancement of cell activity, hydration, and the synthesis of collagen, elastin, and HA (hyalorunic acid). The desired effect could be achieved by the microinjections in the superficial dermis of products containing only one active ingredient or cocktails of different compounds which are perfectly biocompatible and totally absorbable: HA, vitamins, minerals, nutrients, hormones, growth factor (GF), amino acids, autologous cultured fibroblasts, homeopathic products, etc. These may be grouped into temporary, semipermanent (lasting between 1–2 years), or permanent materials (lasting longer than 2 years) (Lacarrubba et al., 2008).

- Autologous Platelet-Rich Plasma (PRP)

Autologous Platelet-rich Plasma (PRP) has attracted attention for skin rejuvenation. PRP is derived from fresh whole blood, which contains a high

concentration of platelets (Zimmermann et al., 2001). It has been shown that PRP may induce the synthesis of collagen and other matrix components by stimulating the activation of fibroblasts, thus, rejuvenating the skin. However, the molecular mechanisms underlying PRP-inducing wound healing processes are still largely unknown and experimental studies confirming the effects of PRP on aged fibroblasts are very limited (D. H. Kim et al., 2011).

- Botulinum Toxin Type A

Botulinum neurotoxin type A (Botox), is a natural exotoxin produced by *Clostridium botulinum*, whose effect is blocking local neuromuscular transmission. In 1980, Scott first reported its effect in strabismus and later blepharospasm treatments (Scott, 1980). Botulinum toxin-A can inhibit muscle contraction and reduce the severity of glabellar lines (J. A. Carruthers et al., 2002). In spite of not directly curing photodamage, it provides rejuvenating appearance by relaxing the underlying musculature. The structure of botulinum toxin-A is a dichain linked with a disulfide bond. The light chain, cleaves SNAP-25, a protein that responses for exocytocis of acetylcholine, resulting in musculature weakness or paralysis. The effect of botulinum toxin-A lasts approximately 3–4 months, or a bit longer, depending on patients and injection sites (J. Carruthers, Fagien, and Matarasso, 2004).

Systemic agents

- Hormone replacement therapy (HRT)

It is well known that there is a progressive decrease of hormone synthesis with age. Levels of growth hormone (GH) and insulin-like growth

factor-1 (IGF-1), melatonin (nocturnal), TSH, thyroid hormones (T3), dehydroepiandrosterone (DHEA), estrogens and testosterone are progressively decreasing (Hertoghe, 2005). On the contrary, HRT has been accused to have a higher cardiovascular risk and increase of the risk of breast cancer. However, it has clear, positive preventive effects on osteoporosis, and an early, low-dose estrogen monotherapy can be considered to have advantages (Ganceviciene et al., 2012).

So, this study was first examined *in vitro* without cell culture (non-cell-based assay) to investigate the extracts of *G. speciosum* for inhibiting collagenase and elastase enzyme activities. In addition, an *in vitro* activity assay with cell culture (cell-based assay) was performed to examine whether *G. speciosum* may have a potential to increase stem cell function of keratinocytes. Also, the augmentation of stemness was evaluated whether it could protect these keratinocytes against ROS-induced cell death. *G. speciosum* extract was subsequently prepared and developed into a topical serum. The stability testing was evaluated in accordance with the ASEAN Guideline on Stability of Drug Product. Anti-aging activities of *G. speciosum* serum were investigated by clinical study. The clinical efficacy was evaluated in terms of dermatological irritation, elasticity, and wrinkle, the dermatological indications of cutaneous aging. Knowledge gained form this study may be beneficial to the development and further investigation of novel active compounds for anti-aging or treatment of skin disorders.

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 Raw material

1. Fresh *G. speciosum* pseudobulbs (Khao Hin Sorn Royal Development Study Center's Area, Chachoengsao, Thailand)

1.2 Equipment and instruments

1. Rotary evaporator (RE 120) (Bŭchi, USA)

2. Inertsil® ODS-3 C18 column (4.6 x 150 mm, 5 µm) (GL Sciences B.V., USA)

3. HPLC Thermo separation systems; Spectra Systems P400 pump, Sprecta System As 3000 Autosampler, Sprecta System UV6000LP detector, SN4000 Software (Thermo Fisher Scientific, USA)

4. Vortex mixer (Scientific industries, USA)

5. Micropipettes (50-200 µL), (100-1000 µL), (1-5 mL) (BIOHIT, Finland)

6. Micropipette tips (BIOHIT, Finland)

7. Membrane filters (13 mm, 0.45 µm Nylon, LUBITECH, China)

8. Microplate reader (Anthos, North Carolina, USA)

9. Analyical balance (PB3002, Mettler-Toledo Ltd., Switzerland)

10. Analytical balance (ML303, Mettler-Toledo Ltd., Switzerland)

11. Analytical balance (XP205, Mettler-Toledo Ltd., Switzerland)

12. Karl Fischer titrator (Metrohm 785 DMP, Metrohm Ltd., Switzerland)

13. Microplate reader (Spectramax M5, Molecular Devices, LLC., USA)

14. 96-well microplates (SPL Life Science, Ltd., Korea)

15. Sonicator (Elmasonic S70H, Elma Electric Inc., USA)

16. Incubator (MEMMERT, Germany)

17. Cooler (HETOFRIG, UK)

18. pH meter (PB20, SARTORIUS, USA)

19. Water bath (BecThai, Thailand)

20. Mobile phase filtration kit (KONTES, USA)

21. 0.22 µm syringe filters (Membrane solution, USA)

22. Centrifuge tubes, capacity 50 mL (Corning Incorporated, USA)

23. Centrifuge tubes, capacity 15 mL (Corning Incorporated, USA)

24. Micro Centrifuge tube (Eppendorf tube), capacity 1.5 mL (USA Scientific, Inc., USA)

25. Disposable syringes 1 mL without needle (NIPRO, USA)

26. 24-Well ultra-low attachment multiple-well plates (Corning Incorporated, USA)

27. Phase-contrast microscope (Olympus 1X51 with DP70)

28. 0.45 µm Nitrocellulose membranes (Bio-Rad, Hercules, CA).

29. SDS-polyacrylamide electrophoresis (Bio-Rad, Hercules, CA)

30. Analyst/PC densitometry software (Bio-Rad, Hercules, CA).

31. Cutometer® MPA 580, (Courage and Khazaka, Germany)

32. Corneometer® CM 825 (Courage and Khazaka, Germany)

33. Visioface® RD (Courage and Khazaka, Germany)

34. Brookfield viscometer model DV-II (Brookfield engineering laboratories, USA)

1.3 Materials

1. Absolute ethanol, AR grade (Lab Scan Co., Ltd., Thailand)

2. Water (HPLC grade, RCI Labscan, Thailand)

3. Acetronitle (HPLC grade, RCI Labscan, Thailand)

4. Methanol (HPLC grade, RCI Labscan, Thailand)

5. Gastrodin standard (% purity > 98%) (Chengdu Biopurify Phytochemicals, Sichuan, China).

6. (−)-Epigallocatechin gallate (EGCG) (Sigma-Aldrich, MO, USA)

7. Folin-Ciocalteu reagent (Merck Ltd., Germany)

8. 0.2 mM Tris-HCL (Sigma-Aldrich Co., MO, USA)

9. N-Succinyl-Ala-Ala-Ala-p-nitroanilide (AAAPVN) (Sigma-Aldrich Co., MO, USA)

10. Porcine pancreatic enzyme or PE (Enzyme Commission Number 3.4.21.36) (Sigma-Aldrich, MO, USA)

11. 50 mM Tricine (Sigma-Aldrich Co., MO, USA)

12. NaCl (Sigma-Aldrich Co., MO, USA)

13. CaCl2 (Sigma-Aldrich Co., MO, USA)

14. Clostridium histolyticum or ChC (Enzyme Commission Number. 3.4.23.3) (Sigma-Aldrich Co., MO, USA)

15. N-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA) (Sigma-Aldrich Co., MO, USA)

16. Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA).

17. Fetal bovine serum, FBS (Gibco, Grand Island, NY, USA).

18. L-glutamine (Gibco, Grand Island, NY, USA).

19. Penicillin/Streptomycin (Gibco, Grand Island, NY, USA).

20. 0.25% Trypsin (Gibco, Grand Island, NY, USA).

21. EDTA (Gibco, Grand Island, NY, USA).

22. Phosphate-buffered saline (PBS) (Gibco, Grand Island, NY, USA).

23. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen, Carlsbad, CA, USA)

24. Dimethylsulfoxide (DMSO) (Sigma-Aldrich Co., MO, USA)

25. Triton X (Roche Molecular Biochemicals, Indianapolis, IN, USA)

26. Glycerol (Roche Molecular Biochemicals, Indianapolis, IN, USA)

27. Sodium orthovanadate (Roche Molecular Biochemicals, Indianapolis, IN, USA)

28. Sodium fluoride (Roche Molecular Biochemicals, Indianapolis, IN, USA)

29. Phenylmethylsulfonyl fluoride (Roche Molecular Biochemicals, Indianapolis, IN, USA)

30. Commercial protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA)

31. BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

32. Non-fat milk (Bio-Rad, Hercules, CA, USA)

33. CD133 (Cell Applications, San Diego, CA, USA)

34. Aldehyde dehydrogenase 1A1 (Santa Cruz Biotechnology, Dallas, TX, USA)

35. *β*-actin (Cell Signaling, Danvers, MA, USA)

36. *β*-catenin (Cell Signaling, Danvers, MA, USA)

37. Super Signal West Pico Chemiluminescence substrate (Pierce Biotechnology, Rockford, IL, USA)

38. 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) (Sigma-Aldrich Co., LLC, MO, USA)

39. Hydrogen peroxide (H2O2) (Sigma-Aldrich, MO, USA)

40. Horseradish peroxidase-coupled secondary antibodies (Cell Signaling, Danvers, MA, USA)

41. Tryphan Blue (Gibco, USA)

42. Polyethylene glycol 400 (Merck KGaA, Darmstadt, Germany)

43. Ammonium acryloyldimethyltaurate/VP Copolymer (Clariant International Ltd., USA)

44. Phenoxyethanol, chlorphenesin and glycerin (Thor Personal Care, France)

45. Non-woven fabric (3M, Thailand)

46. 2′,7′-Dichlorodihydrofluorescein diacetate (Sigma-Aldrich Co., MO, USA)

1.4 Cell line

1. Human keratinocyte (HaCaT) cells, catalog number: 300493 (Cell Lines Service, Heidelberg, Germany)

2. Methods

2.1. Preparation of *G. speciosum* extract

The fresh pseudobulbs of *G. speciosum* Blume were collected from the Khao Hin Sorn Royal Development Study Center's Area, Chachoengsao province, and were prepared by the maceration technique. The dry pseudobulbs of *G. speciosum* were ground and macerated for 3 days in 95% ethanol at 25ºC (1:9 w/v crude powders to ethanol ratio). The process was repeated to produce 3 batches. Each of the three ethanolic extracts was filtered and evaporated under vacuum at the temperature below 40ºC. The percent yield of each extract was calculated by the following equation:

Equation 1: $$\% \text{ yield} = \frac{(\text{mass of vacuum-dried } \textit{G. speciosum} \text{ extract})}{(\text{initial mass of ground } \textit{G. speciosum})} \times 100$$

2.2. Quality control of *G. speciosum* extract

Gastrodin, the major compound in the *G. speciosum* extract, was characterized by high performance liquid chromatography (HPLC) technique as follows:

2.2.1 Analytical method development for the determination of gastrodin in G. speciosum extract.

HPLC technique was developed to analyze the quantity of gastrodin in *G. speciosum* extract. The chromatographic condition was modified from (W.-C. Chen et al., 2015; Lee et al., 2015). HPLC condition was performed under gradient condition. Acetronitrile and water were used as moblie phase solvents (Table 9). The flow rate was set at 1.0 mL/min and the column used was Inertsil® ODS-3 C18 (4.6 x 150 mm, 5 µm). DAD signal was set at 190-400 nm with the monitoring wavelength at 220 nm. The method was developed and validated according to the Association of

Analytical Communities (AOAC) Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanical (2002)(Horwitz, 2002). Solvent A and solvent B were filtered through 0.22 µm membrane filter and then degassed by sonication for 30 minutes prior to use.

Table 10**:** The gradient HPLC system

Time (min)	% Solvent A (water)	% Solvent B (Acetonitrile)
0	100	0
24	99	1
30	80	20
35	20	80
40		100
50		100

Analytical method validation

HPLC system was validated using standard addition method. Selectivity, linearity, accuracy and precision were performed in accordance with the AOAC Guidelines for Dietary Supplements and Botanicals, 2002.

- *System suitability*

The system suitability was performed using HPLC-DAD by injecting six replicates of *G. speciosum* extract in comparison with the gastrodin standard solution to obtain HPLC peak responses.

- *Selectivity*

Selectivity was examined by separately injecting gastrodin standard solution, *G. speciosum* extract, and *G. speciosum* extract plus gastrodin standard mixture using HPLC-DAD to obtain HPLC peak responses.

- *Linearity*

Linearity of the system was examined using the standard addition method. Six different concentrations of gastrodin standard solution were used to construct the calibration curve. G. speciosum extract of about 57.69 mg was accurately weighed into a series of 25 mL volumetric flasks. 10.0 mL of ethanol was added to each flask and sonicated for 5 min until dissolved. From the preliminary study, 2.31 mg/mL of G. speciosum extract was found to contain gastrodin of about 0.347 mg/mL. 220, 250, 280, 334, 400 and 500 µL of gastrodin standard solutions at 0.231, 0.260, 0.291, 0.347, 0.416 and 0.520 mg/mL was added to each G. speciosum extract solution and adjusted to 25 mL with ethanol to obtain final concentration (gastrodin in extract plus gastrodin standard) concentrations of about 0.578, 0.607, 0.638, 0.694, 0.763 and 0.867 mg/mL of gastrodin. The solution was filtered through a 0.22 µm syringe filter prior to use. Each concentration was analyzed in triplicate. A calibration curve was constructed by plotting the peak area against the concentration. The slope, y-intercept and correlation coefficient (r) were calculated using the Least Squares Regression method.

- *Accuracy and Precision*

Accuracy and precision of the system were evaluated using the standard addition method. Three different concentrations were prepared by accurately weighing 57.69 mg *G. speciosum* extract into a 25-mL volumetric flask, and 10.0 mL of ethanol was added and sonicated for 5 min until dissolved. A gastrodin standard solution at 80%, 100% and 120% concentration of marker in *G. speciosum* extract was added to each *G. speciosum* extract sample, adjusted to 25 mL with ethanol, to obtain final concentrations of 0.555, 0.694 and 0.833 mg/mL. The solution was

filtered through a 0.22 μm syringe filter prior to use. Each concentration was analyzed in triplicates. Inter-day precision and accuracy were performed on three different days. Intra-day precision was obtained after injection of 6 replicates of each concentration within one day of analysis. Average % recovery and % relative standard deviation (%RSD) were calculated to determine the accuracy and precision of the system using the following equations:

Equation 2: % Recovery = (amount found/ amount added) x 100

Equation 3: % RSD (Inter-day or Intra-day precision) = (SD / mean) x 100

- *Detection and Quantitation Limits (LOD and LOQ)*

The signal-to-noise method was used to determine LOD and LOQ. The signal-to-noise ratio of the analyte was measured, and subsequently, the concentration of the analyte that would yield a signal equal to certain value of noise-to-signal ratio is estimated. The noise magnitude was calculated by an auto integrator of the instrument. A signal-to-noise ratio (S/N) of three is generally accepted for estimating LOD and signal-to-noise ratio of ten is used for estimating LOQ. This method is commonly applied to analytical methods that exhibit baseline noise (Shrivastava, and Gupta, 2011).

2.2.2. Chemical analysis of G. speciosum extract

- Standard preparation

Standard gastrodin was individually dissolved in ethanol to prepare a stock solution. The stock solution was used to prepare standard solutions of gastrodin by diluting with ethanol to final concentrations of about 0.231, 0.260, 0.291, 0.347, 0.416

and 0.520 mg/mL, respectively. The solutions were filtered through a 0.22 µm syringe filter prior to use.

- Sample preparation

57.69 mg of *G. speciosum* extract was accurately weighed and placed in a 25 mL-volumetric flask. About 10 mL of ethanol was added into the container and sonicated for 5 min until dissolved. The solution was further diluted with ethanol to 25.0 mL and filtered through a 0.22 µm syringe filter prior to use. Calculated the quantity of marker according to the following equation:

Equation 4:

$$\text{Quantity of gastrodin in extract (mg)} = (A_{GSE} / A_{std}) \times C_{std}\,(\mu g/mL) \times \text{purity of std.} \times \text{dilution factor} /1000$$

A_{GSE} is the peak area response of marker *G. speciosum* extract.

A_{std} is the peak area response of gastrodin standard.

C_{std} is the concentration of gastrodin standard (µg/mL).

2.3 Biological activity and properties of *G. speciosum* extracts.

2.3.1. In vitro testing

2.3.1.1. Non cell-based assay

2.3.1.1.1. Total phenolic content (TPC)

The TPC of *G. speciosum* ethanolic psuedobulb extracts was determined using Folin-Ciocalteu method (Singleton, and Rossi, 1965). In this study, EGCG was used as standard and water was used as blank. *G. speciosum* extracts (1 mL) were thoroughly mixed with 5% (v/v) Folin-Ciocalteu reagent (1 mL). Subsequently, 10%

(w/v) sodium carbonate solution (400 µl) was added and incubated in the dark at 25°C for 60 min. Transferred 100 µL of this solution in 96-well plate to measure the UV absorbance in a microplate spectrophotometer at 765 nm. The TPC was calculated in comparison with standard EGCG at the concentration of 62.50 - 500.00 mg/mL. The results were expressed in mg of EGCG equivalents per gram of *G. speciosum* extract. Experiments were performed in triplicate.

2.3.1.1.2. Anti-elastase effect

To evaluate the elastase inhibition activity, the method was performed according to (Thring, Hili, and Naughton, 2009). The solvent used in this assay was 0.2 mM Tris-HCL buffer at pH 8.0. The prepared buffer was used to dissolve the synthetic substrate N-Succinyl-Ala-Ala-Ala-p-nitroanilide (AAAPVN) at 1.6 mM. Porcine pancreatic enzyme or PE (Enzyme Commission Number 3.4.21.36) was used to represent elastase at 3.33 mg/mL stock solution. *G. speciosum* extracts in 1 % ethanol were treated with the enzyme at 0.02 - 0.14 mg/mL concentrations for 15 min. The substrate was subsequently added to the extract and incubated for 20 min. EGCG at concentrations of 0.04 - 0.16 mg/mL in water were used as positive control and sterile water was used as a negative control. The final sample solution contained 0.8 mM of AAAPVN, 1 µg/mL of PE and 25 µg/mL of the tested extract. The test mixtures were analyzed using microplate spectrophotometer at the monitoring wavelength of 410 nm. The % elastase inhibition at each concentration of the extract and EGCG was calculated by the following equation:

Equation 5:

$$\% \text{ Enzyme inhibition} = [(A410 \text{ control} - A410 \text{ sample}) / A410 \text{ control}] \times 100$$

The anti-elastase activities of all samples were finally expressed as an IC_{50} value.

2.3.1.1.3. Anti-collagenase effect

The method to determine anti-collagenase activities were modified from Thring et al, (2009). The 50 mM Tricine buffer pH 7.5 was initially prepared using the combination of 400 mM NaCl and 10 mM $CaCl_2$. The buffer was then used for dissolving collagenase (Type I) from *Clostridium histolyticum* or ChC (Enzyme Commission Number 3.4.23.3) at the concentration of 2 units/mL. In addition, the N-[3-(2-furyl) acryloyl]-Leu-Gly-Pro-Ala (FALGPA) was also dissolved in the previously prepared buffer to make 2 mM solution. *G. speciosum* extracts at various concentrations of 1.38 - 1.32 mg/mL in DMSO were transferred to 96-well plates and later treated with the enzyme buffer for 15 min prior to the addition of substrate. The final treated solution including the collagenase ChC, the substrate FALGPA and *G. speciosum* extracts at various concentrations was dissolved in Tricine buffer and was analyzed using microplate spectrophotometer. EGCG at concentrations of 0.03 - 0.09 mg/mL in water were used as a positive control while sterile water was used as a negative control. The absorbance of the samples at each concentration was measured at 340 nm at 0 min and 20 min after adding the substrate. The % collagenase inhibition was calculated by the following equation:

Equation 6:

% Enzyme inhibition = (A340 control - A340 sample) x 100 / (A340 control)

The anti-collagenase activities of all samples were finally expressed in terms of IC_{50} value.

2.3.1.2. Cell-based assay in skin keratinocyte cell line

Human keratinocyte (HaCaT) cells were obtained from the Cell Lines Service catalog number was 300493. HaCaT cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and 100 U/ml penicillin and 100 µg/ml streptomycin. Cell cultures were maintained in a 37°C humidified incubator with 5% CO_2. Cells were routinely passaged at a pre-confluent density using 0.25% trypsin solution with 0.53 mM EDTA.

2.3.1.2.1. Cell Viability Assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cell viability. Cells (passage 58-60) were seeded at a density of 1 x 10^4 cells/well and cultivated for 12 h in 96-well plate. Afterward, the cells were incubated with various concentrations of *G. speciosum* extract (0–100 µg/mL) in complete DMEM for 24 h. After indicated treatments, the cells were incubated with 5mg/mL of MTT for 4 h at 37°C. Then, the supernatant was removed and replaced with 100 µl of dimethylsulfoxide (DMSO) to dissolve the formazan crystal. The intensity of MTT product was measured at 570 nm using a microplate reader. Cell viability was calculated by the following formula and presented as a percentage to non-treated control value.

Equation 7:

$$\text{Cell viability (\%)} = \frac{\text{A570 of treatment}}{\text{A570 of control}} \times 100$$

2.3.1.2.2. Spheroid Formation Assay

Spheroids culture assay was slightly modified from Yongsanguanchai et al. (2014) and Bhummaphan et al. (2015). Cells (passage 67-69) were treated with *G. speciosum* extract (5–20 µg/mL) in DMEM for 3 days. When the *G. speciosum* extract treatment was completed, cells were detached into single cells using 1 mM EDTA and 2.5×10^3 cells per well were grown in 24-well ultralow attach plate. Primary spheroids were collected at day 7 of culture (37°C, 5% CO_2). These primary spheroids were disaggregated into single cells by using 1mM EDTA and resuspended with serum free-DMEM at 2.5×10^3 cells/well in 24-well ultralow attach plate. Phase-contrast images of spheroids contained at least 2 cells were captured at day 14 using a phase-contrast microscope. Spheroid number and area were counted and measured, respectively, using ImageJ software. The relative spheroid number and area were determined by dividing the values of the treated cells by those of the control cells.

2.3.1.2.3. Western Blot Analysis

Cells were seeded at a density of 5×10^4 cells/well onto 6-well plate for 12 h and cultured in the presence of various concentrations of extract (5-20 µg/mL) in complete DMEM for 3 days. After washing cells with PBS, cell lysates were prepared by incubating cells in ice-cold lysis buffer containing 20 mM Tris-HCl (pH7.5), 0.5% Triton X, 150 mM sodium chloride, 10% glycerol, 1mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and commercial protease inhibitor cocktail for 45 min on ice. Protein content was then analyzed using BCA protein assay kit from Pierce Biotechnology. Equal amounts of proteins (40 µg) were boiled in LaemmLi loading buffer for 5 min at 95°C. Proteins

were subsequently loaded on 10% SDS-polyacrylamide gel electrophoresis. After separation, proteins were transferred onto 0.45 µm nitrocellulose membranes. Subsequently, the membranes were blocked with 5% non-fat milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, and 0.05% Tween-20) for 1 h at room temperature, membranes were incubated with specific primary antibodies against CD133, aldehyde dehydrogenase 1A1, *β*-catenin and *β*-actin at 4°C overnight. Membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase-coupled secondary antibodies for 1 h at room temperature. The immune complexes were detected by Super Signal West Pico Chemiluminescence substrate and quantified using analyst/PC densitometry software.

2.3.1.2.4. Cell protective effect of G. speciosum extract against ROS-induced cell death

Cells were seeded at a density of 8 x 10^3 cells/well onto 96-well plate for 12 h and cultured in the presence of various concentrations of *G. speciosum* extract (5-20 µg/mL) in DMEM for 3 days and at various times (1-6 days) for the 20 µg/mL extract. Afterward, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) or hydrogen peroxide (H_2O_2) was added at the concentration of 25 µM for 24 hr. The protective effect of *G. speciosum* extract against DMNQ (superoxide anion generator)- or hydrogen peroxide (H_2O_2)-induced cytotoxicity in HaCaTs was determined by MTT assay.

2.3.1.2.5. Determination of intracellular ROS level

After penetrating into cells, DCFH-DA is converted to fluorescent dichlorofluorescein (DCF) by intracellular ROS. Cells were seeded at a density of 5 x 10^3 cells/well onto 96-well plate for 12 h and cultured in the presence

of *G. speciosum* extract 20 µg/mL in complete DMEM for 3 days. At indicate time point, the cells were then incubated with $DCFH_2$-DA (10 µM) in the dark, at 4°C for 30 min. After fluorescence staining, the cells were treated with DMNQ (25 µM) for 3 h or H_2O_2 (25 µM) for 30 min. The fluorescence intensity was analyzed by a fluorescence microplate reader. Intracellular ROS of the cells were visualized under a fluorescence microscope for comparison with untreated control (no extract).

2.4. Stability and physicochemical characteristic of the *G. speciosum* extract

The physicochemical properties of *G. speciosum* extract were evaluated by the determination of color, solubility, and pH. The chemical and physical stability of *G. speciosum* extract were investigated including the heating-cooling cycle test where the *G. speciosum* extract was kept at 4 ± 2 °C for 48 h and alternated to 45 ± 2 °C for 48 h. After 6 cycles, the physicochemical properties and the amount of gastrodin were re-evaluated (Kanlayavattanakul, Lourith, and Chaikul, 2016).

2.5. Formulation and stability of serum containing *G. speciosum* extract

In this topic, Solubility and stability testing were performed. Stability testing consists of both physical and chemical stability testing. In this study, water was chosen as solvent and 4 different solvents which are polyethylene glycol 400, glycerin, propylene glycol and butylene glycol were tested for solubility to use as a co-solvent. These chosen solvents are non-toxic and were widely used in cosmetic product. Base serum and Active serum with various concentrations of *G. speciosum* extract were formulated using cosmetic grade ingredients. Ingredients of the final formulation of *G. speciosum* serum are shown in Table 10. The serums contains PEG 400 as co-solvent, aristoflex AVC as thickener, microcare PHC as preservative, water as solvent. Both formulations were evaluated for the physicochemical properties

including appearance, color, odor, pH, viscosity and gastrodin content. The accelerated stability of the *G. speciosum* serum was studied at 40 ± 2 °C and 75 ± 5 % relative humidity (RH) for 3 months according to ASEAN guideline condition (Asean Guideline on Stability Study of Drug Product)(Countries, 2005). The stability was evaluated at the interval of 0, 1, 2 and 3 months.

Table 11**:** Ingredients of base and *G. speciosum* serum.

Ingredients	% (w/w)	
	Base	*G. speciosum* Serum
G. speciosum extract	-	0.5
PEG 400	12	12
aristoflex AVC®	0.5	0.5
microcare PHC®	1	1
DI water	100	100

Preparation of samples and standards

Serum containing *G. speciosum* ethanolic extract (0.5%) was dissolved in an appropriate volume of polyethylene glycol (PEG) 400 prior to assay. Standard curve was constructed from peak areas of gastrodin standard solution in 20% ethyl alcohol in a concentration range of 231 - 520 µg/mL. A typical standard curve showed linearity with $R^2 > 0.99$. Concentration of gastrodin in the formulation was calculated from the corresponding standard curve.

2.6. Clinical trial testing

2.6.1. Research Ethics Committee of Bhumibol Adulyadej Hospital

The method for clinical study was conducted according to the declaration of Helsinki and Tokyo for medical research involving human subjects. Prior to the clinical trial studies, the study protocol was filed and approved by the ethical board of Bhumibol Adulyadej Hospital, Royal Thai Air Force (See Appendix A).

2.6.2. Volunteer recruitment

Thai volunteers with age between of 25 and 55 years (both male and female) were selected. Volunteers with skin diseases or hypersensitive skin were excluded in this research. None of the recruited volunteers were pregnant or lactating. All the selected volunteers were clearly informed about the procedure of the study prior to signing an informed consent form.

2.6.3. Skin irritation patch test of serum containing G. speciosum extract

Irritation test was conducted regarding to the method reported by Internatinal Contact Dermatitis Research Group (ICDRG) (Ivens, Serup, and O'goshi, 2007). Briefly, 32 volunteers (2 males and 30 females) aged 25 to 55 years were enrolled in the test. Individuals were excluded if they had any history of skin diseases or conditions that may interfere with the evaluation of skin reaction consent. About 0.2 gram of test formulations (serum containing *G. speciosum* crude extract, serum base, and water) was applied on one square inch of an individual back. Each volunteer received 3 patches each containing water, serum base and 0.5% (w/w) *G. speciosum* serum labeled as 1, 2, and 3, respectively (Figure 26). An adhesive tape was placed on the applied area. In this study, water used as negative control. The patches were left in place for 24 hours. After 24 hours, the patches were removed. The applied areas were cleaned with soaked cotton prior to immediately evaluate for any irritation by a dermatologist. The applied areas were re-evaluated at 48 and 72 hours according to International Contact Dermatitis Research Group (ICDRG) scale (Table 4). The procedure was repeated after 10 days to assure washout period.

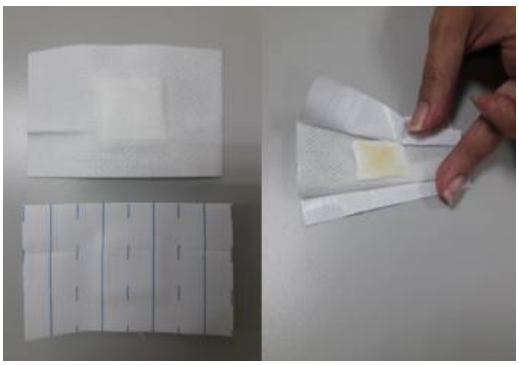


Figure 26**:** This is the picture of the 1 inch square patch and 0.2 g of serum was transferred into the patch

Table 12: International Contact Dermatitis Research Group (ICDRG) irritation scale

Grade	Description
0	No observable reaction occurred
+	Slight reaction occurred (small rednees of skin attached by the patch is observed)
++	Moderate reaction occurred (redness of skin attached by the patch is clearly observed)
+++	Severe reaction occurred (redness and swellness of skin attached by the patch is clearly observed)

2.6.4. Clinical evaluation

To determine the clinical effects of the extract serum on human skin, randomized double-blind, placebo-controlled experimentation was conducted. Two male and twenty-two female subjects from the previous patch test who had not been exposed to any steroids for 4 weeks and had no allergic reactions for the past week were selected for the *in vivo* study. The subjects were randomized into two groups (n=12 per group). The first group used 0.5% (w/w) *G. speciosum* extract serum (test serum) on the right face and the base serum (control serum) on the left face. The second group used 0.5% (w/w) *G. speciosum* extract serum (test serum) on the left

face and the base serum (control serum) on the right face. The subjects were instructed to apply the provided system twice a day in the morning and at the bed time.

The treated skin was investigated by Cutometer® MPA 580, Corneometer® CM 825, and Visioface® RD (Courage and Khazaka, Germany) for skin elasticity, hydration and wrinkle depth, respectively, at base line (D0) and after application at 28, 42 and 56 days of the application. The topically tested area was protected from daylight, UV exposure and any skin insults during the treatment period. In addition, the subjects were also prevented from smoking and alcohol drinking. The experimental area was adjusted to 25 °C and 40 - 60 % RH (Futrakul, Kanlayavattanakul, and Krisdaphong, 2010).

2.7. Sensory assessment

After the clinical trial study, the 24 volunteers were also subjected to sensory evaluation. The volunteers were allowed to score their preferences from 1 – 5 (disfavor – most favor) according to questionnaires specifically designed for the experiment.

2.8. Statistical analysis

Data are expressed as the mean ± standard deviation (SD) (triplicate analysis). The results from the human study are shown as the mean ± standard error of measurement (SEM). The parameters from the *in vitro* studies were evaluated using one-way ANOVA followed by post-hoc tests, whereas the statistical analysis for the clinical study was repeated-measure ANOVA and paired t-test. Values of $p < 0.05$ were considered as statistically significant using the SPSS program version 21.

CHAPTER IV
RESULTS AND DISCUSSION

1. Plant material preparation of *G. speciosum*

The crude extract of *G. speciosum* Blume pseudobulbs was 194.6 g accounted as 6.49 % w/w yield compared to the initial dry weight. Crude herb of *G. speciosum* were ground and extracted according to the extraction process in section 1, chapter 3, to obtain herb-to-extract ratio (HER) of 1:9. The *G. speciosum* extract obtained was highly viscous with a dark-brown color. The appearance of *G. speciosum* extract is shown in Figure 27.

Figure 27**:** The extract from *G. speciosum* pseudobulbs

2. Quality control of *G. speciosum* extract

G. speciosum extract was analyzed by high performance liquid chromatography (HPLC) (Agilent Technologies, CA, USA). Gastrodin, a major compound of *G. speciosum* extract, was selected as a marker. Method validation was developed in accordance with AOAC international guidelines for validation of botanical

identification methods (2013) (AOAC, and Group, 2012). The mobile phase consisted of solvent A (water) and solvent B (acetonitrile), the gradient started with 100% solvent A, was adjusted to 99% solvent A for 24 min, to 80% solvent A for 6 min, to 20% solvent A for 5 min, and finally to 100% solvent A for 15 min (Table 13). The flow rate of the system was 1.0 mL/min with the column temperature controlled at 25°C. The injection volume was set at 20 µl. The photodiode array detector (DAD) was adjusted at 200-380 nm with a monitoring wavelength of 220 nm. The HPLC column used was Inertsil® ODS-3 C18 (4.6 x 150 mm, 5 µm) column. The results were calculated using ChemStation© software. Standard and sample solutions were prepared using ethanol as solvent and filtered through Whatman 0.22 µm nylon syringe filter prior to the injection. Gastrodin was identified by comparing the retention time and the spectrum of *G. speciosum* extract with gastrodin standard (%purity > 98%). Gastrodin content was determined by comparing the peak areas of gastrodin in *G. speciosum* extract and the gastrodin standard (corrected for 98%purity). Solvent A and solvent B were filtered through 0.22 µm membrane filter and then degassed by sonication for 30 minutes prior to use.

Table 13**:** The gradient HPLC system showing Time (min), % Solvent A and % Solvent B

Time (min)	% Solvent A (water)	% Solvent B (acetonitrile)
0	100	0
24	99	1
30	80	20
35	20	80
40		100
50		100

3. Analytical method validation

The analytical method validation was evaluated by using standard addition. In this study, a standard solution was added into the analyte to compensate for the matrix effect of the sample. Selectivity, linearity, accuracy and precision were performed in accordance with the AOAC Guidelines for Dietary Supplements and Botanicals, 2013.

System suitability

System suitability tests were an integral part of HPLC methods. It was used to verify that the chromatographic system is adequate for the intended analysis. The system suitability test was performed by collecting data from six successive replicates injections of standard (347 µg/mL) The %RSD of peak areas, tailing factor and theoretical plates complied with Validation of Chromatographic Methods, Center for Drug Evaluation and Research (1994) which stated that the %RSD of peak areas should be not more than 1.0%, tailing factor should be not more than 2 and theoretical plates should be not less than 2000 (Table 14).

Table 14**:** System suitability of the HPLC system

Analytical marker	% RSD (n=6)	Tailing factor	Number of theoretical plates (N)
Gastrodin	0.961	0.818	3753.5

Selectivity

Peak responses of the marker in standard solutions were clearly separated from each other, with the retention time similar to gastrodin standard. The spectra of gastrodin were then confirmed for its characteristic by diode array detection (DAD) at range 200 - 380 nm as shown in Figure 28. The retention time of *G. speciosum* extract samples and gastrodin standard solutions were found to elute at 24.287 and 25.355 min, respectively (Figure 29). The results showed that the spectra of gastrodin in sample and standard were identical to each other.

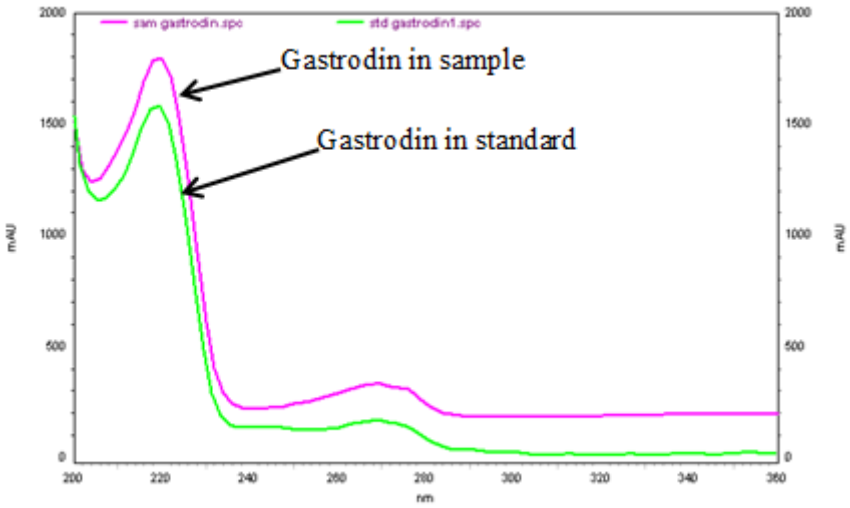


Figure 28**:** LC-UV selectivity spectra of gastrodin in the *G. speciosum* Pseudobulb ethanolic extract (upper line) in comparison with gastrodin standard (lower line)

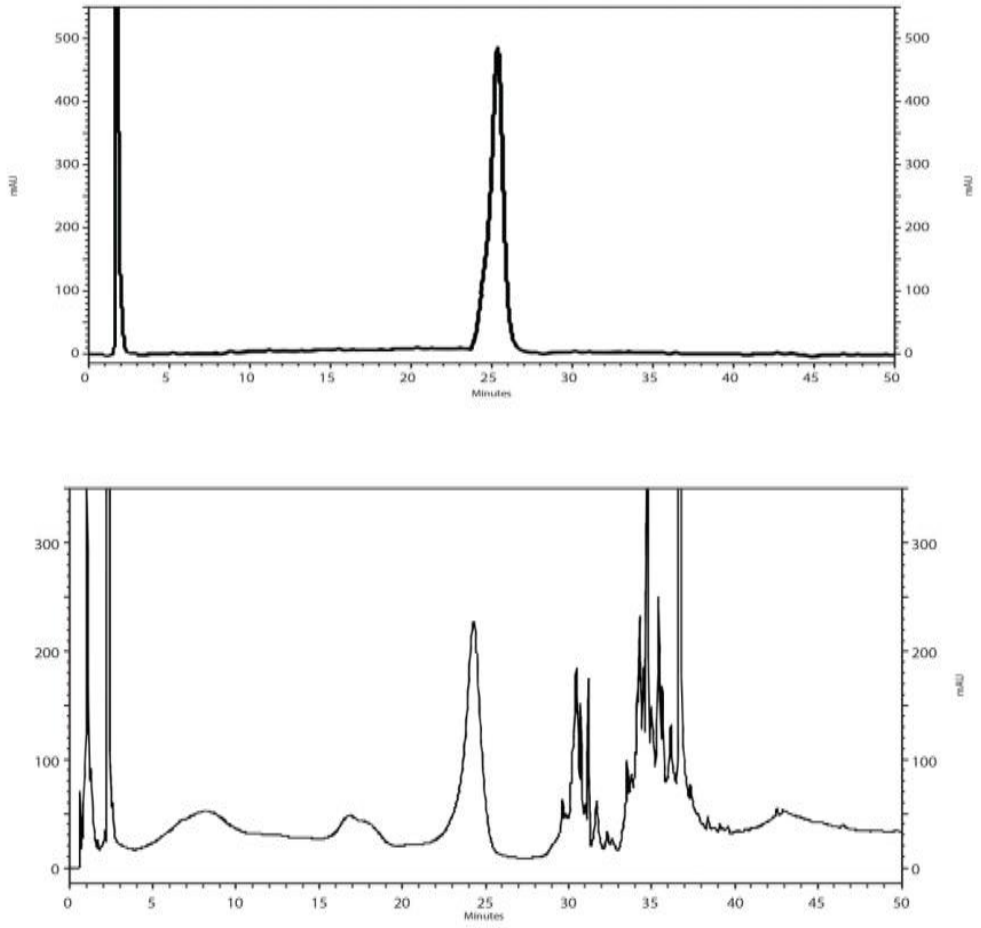


Figure 29**:** Typical HPLC chromatogram of the gastrodin in the standard (top) and the *G. speciosum* ethanolic extract (bottom). The retention times of *G. speciosum* extract sample and gastrodin standard solution were found to elute at 24.287 and 25.355 min, respectively.

Linearity

Linearity parameters of the HPLC system are shown in Table 10. The results exhibited good linearity with correlation coefficient (r) of 0.9994. These results complied with the AOAC Guidelines for Dietary Supplements and Botanicals, 2013,

which stated that the correlation coefficient (r) should be > 0.99 for acceptable linearity (Figure 30). The regression equation was y = 61486199.8x + 2329106.7, where y is the peak area and x is the corresponding concentration of gastrodin standard. Calibration curves were plotted using 6 independent sets of gastrodin dilutions to get a linearity range of concentration between 231 - 520 μg/mL (Table 15).

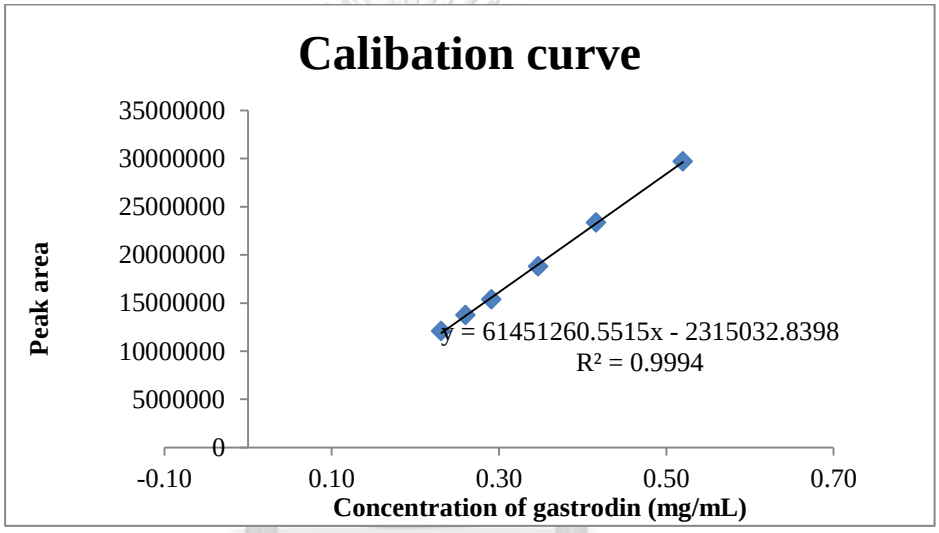


Figure 30**:** The calibration curve of gastrodin standards analyzed by HPLC method

Table 15**:** The calibration data of gastrodin standards analyzed by HPLC method.

	Conc. mg/mL	Inj. 1	Inj. 2	Inj. 3	average	SD	%RSD
1	0.231	12064475	12047473	12079550	12063833	16048	0.13
2	0.260	13764580	13708080	13724923	13732528	29008	0.21
3	0.291	15392911	15352426	15404311	15383216	27267	0.18
4	0.347	18698752	18881437	18760716	18780302	92904	0.49
5	0.416	23318711	23338060	23386763	23347845	35065	0.15
6	0.520	29622047	29688558	29786205	29698937	82570	0.28

Accuracy and Precision

Precision of the system, which reflects the closeness of the analytical results following analyses of replicate samples, is shown as a %RSD values in Table 16 and Table 17. The intra-day and inter-day precisions were 1.78 and 1.78 %, respectively. The precision results for gastrodin showed %RSD values of less than 2%, which complied with the AOAC Guidelines. Accuracy of the system, which reflects the interference of matrix to gastrodin content in *G. speciosum* ethanolic extract, is shown as an average %recovery calculated from following equation;

% Recovery = (total gastrodin - gastrodin in sample) x100 / gastrodin standard

In this study, the accuracy of the system is shown in Table 18. The average % recovery was found to be 100.02, 100.20 and 100.68 % for 80 % (0.278 mg/mL), 100 % (0.347 mg/mL) and 120 % (0.416 mg/mL) concentrations of gastrodin, respectively. The average % recovery of gastrodin complied with the AOAC Guidelines, which stated that this value should be between 98-102% (% RSD for both precisions must be less than 2% and percent analytical recovery must be between 98-102%). These results indicate that the developed HPLC method was accurate and precise in the determination of gastrodin content in the ethanolic extracts of *G. speciosum.*

Table 16**:** Intra-day precision of the HPLC system determined.

Number of replicates	Concentration of *G. speciosum* extract (mg/ml)	Gastrodin content (mg/ml)
1	8.475	0.366
2	8.385	0.349
3	8.510	0.366
4	8.515	0.362
5	8.475	0.357
6	8.525	0.360
Mean		0.360
SD		0.006
%RSD		1.778

Table 17**:** Inter-day precision of the HPLC system determined.

Number of days	Concentration of *G. speciosum* extract (mg/ml)	Gastrodin content (mg/ml)
1	8.585	0.359
2	8.630	0.367
3	8.525	0.368
4	8.515	0.362
5	8.450	0.350
6	8.500	0.362

Mean	0.361
SD	0.006
%RSD	1.781

Table 18**:** Accuracy (% analytical recovery) of the HPLC system.

Concentration (mg/ml)	% recovery					
	1	2	3	Mean	SD	%RSD
0.278 (80%)	99.452	100.079	100.531	100.021	0.542	0.542
0.347 (100%)	100.492	100.667	99.448	100.202	0.659	0.658
0.416 (120%)	100.500	100.937	100.616	100.684	0.226	0.225

All validation data parameters of gastrodin, the major constituent analyzed by the above HPLC method, are summarized in Table 19. The LOQ (Figure 31) and LOD (Figure 32) of gastrodin were 0.0128 and 0.0071 mg/mL, respectively.

Table 19**:** Validation data of gastrodin, the major constituent analyzed by HPLC method.

Parameters	Gastrodin
Linearity	
Range (µg/mL)	231-520
Coefficient of determination (r^2)	0.9994
Equation	
Intercept	2329106.7
Slope	61486199.8
Precision (%RSD)	
Intra-day	1.778
Inter-day	1.781
Accuracy	
Recovery (%)	99.452-100.937
Limit of quantitation (µg/mL)	12.8
Limit of detection (µg/mL)	7.1

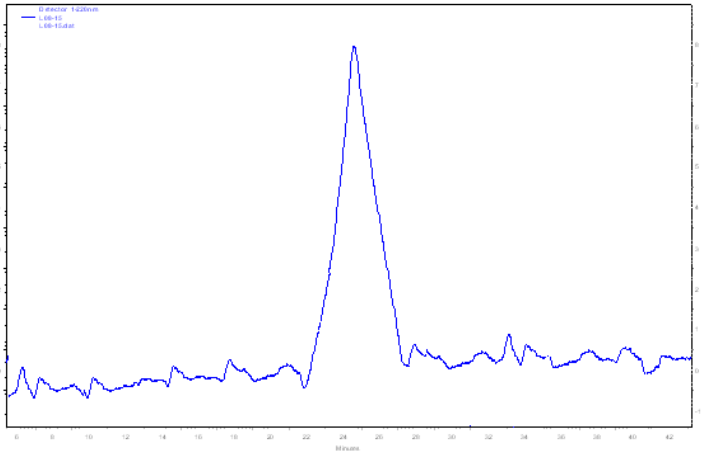


Figure 31**:** HPLC chromatogram of gastrodin standard at quantitation limit of 0.0071 mg / mL (see Gastrodin Peak; t_R = 25.227 min).

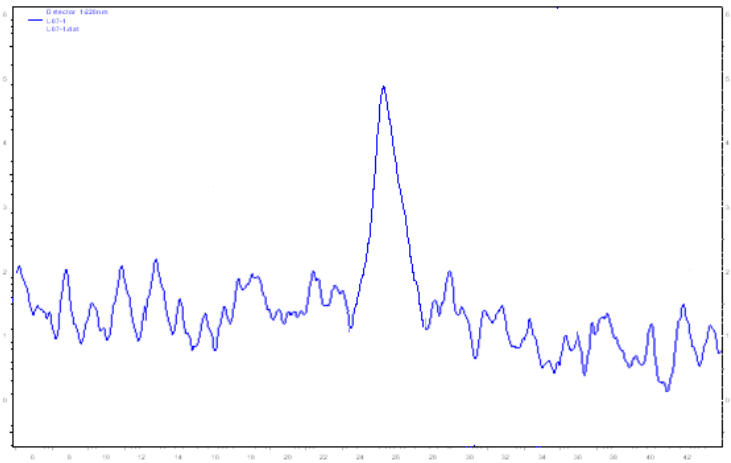


Figure 32**:** HPLC chromatogram of gastrodin standard at detection limit of 0.0128 mg / mL (see Gastrodin Peak; t_R = 25.227 min).

The amount of gastrodin in 3 different batches of G. speciosum ethanolic extract was analyzed using the above validated method. The results showed that the amount of gastrodin from batch 1, batch 2, and batch 3 contained 58.12±4.6, 57.24±2.5, and 55.50±1.4 mg/g, respectively (Table 20), which was considered to be not significantly different from one another ($p > 0.05$).

Table 20**:** Gastrodin contents in *G. speciosum* extracts of 3 different batches (values = mean ± S.D., n = 3 replicates).

Batch	1	2	3
Gastrodin (mg/g)	58.12±4.6	57.24±2.5	55.50±1.4

4. *In vitro* anti-aging efficacy

The presence of phenolic compounds in plants was claimed to provide an anti-aging effect such as the scavenging of oxidants, especially reactive oxygen species (ROS), preventing some degenerative disorders.

In this study, the total content of polyphenols in *G. speciosum* ethanolic extract was 48.19±0.39 mg EGCG equivalent / g extract. The antioxidant activity of *G. speciosum* extract possessed therapeutic activities for skin disorders (Elsner, and Maibach, 2000; Thring, Hili, and Naughton, 2009). In this study, the anti-elastase and anti-collagenase activities were evaluated *in vitro* and the data are demonstrated in Table 21 - Table 28 in comparison with the known antioxidant polyphenol EGCG**.** The anti-collagenase enzyme activity was lower than EGCG. However, the *G. speciosum* extract possessed higher anti-elastase

activity (IC_{50} 71.10 ± 1.04 µg/mL) than that of EGCG (IC_{50} 92.52 ± 0.62 µg/mL) ($p \leq 0.05$) (Thring, Hili, and Naughton, 2009).

Table 21**:** The percent elastase activity inhibition of *G. speciosum* ethanolic extract**.**

Concentration *G. speciosum* extract (µg/mL)	**%Inhibition Elastase**			**Mean ± S.D.**
	run1	**run2**	**run3**	
20	25.3267	24.8603	25.4495	25.21 ± 0.31
40	36.5588	35.0714	34.8109	35.48 ± 0.94
60	45.0840	44.1962	43.1804	44.15 ± 0.95
80	54.9782	54.2520	53.6268	54.29 ± 0.68
100	64.7791	63.7803	63.9492	64.17 ± 0.53
120	74.5177	73.5258	73.6206	73.89 ± 0.55
140	82.4829	82.0298	83.7880	82.77 ± 0.91

Table 22**:** IC_{50} of elastase enzyme inhibition of *G. speciosum* ethanolic extract**.**

run	**IC_{50} (µg/mL) anti-elastase activity**
1	69.90
2	71.73
3	71.68
MEAN	71.10
SD	1.04
%RSD	1.47

Table 23**:** The percent elastase activity inhibition of EGCG standard.

EGCG Concentration (µg/mL)	**%Inhibition Elastase**			**Mean ± S.D.**
	run1	**run2**	**run3**	
40	24.2499	23.4820	25.5886	24.44 ± 1.07
60	34.3025	35.0991	36.5242	35.31 ± 1.13
80	45.4067	44.3928	45.8798	45.23 ± 0.76
100	54.8407	53.3147	53.9033	54.02 ± 0.77
120	62.2023	63.0731	62.0818	62.45 ± 0.54
140	71.4507	72.9244	70.7249	71.70 ± 1.12
160	81.0393	81.4746	81.0409	82.18 ± 0.25

Table 24**:** IC_{50} of elastase enzyme inhibition of EGCG standard**.**

run	IC_{50} (µg/mL) anti-elastase activity of EGCG
1	91.80
2	92.92
3	92.83
MEAN	92.52
SD	0.62
%RSD	0.67

Table 25**:** The percent collagenase activity inhibition of *G. Speciosum* ethanolic extract

Concentration (µg/mL)	%Inhibition Collagenase			Mean ± S.D.
	run1	run2	run3	
1,080	25.3267	24.8603	25.4495	25.29 ± 0.14
1,120	36.5588	35.0714	34.8109	35.51 ± 0.22
1,160	45.0840	44.1962	43.1804	45.43 ± 0.43
1,200	54.9782	54.2520	53.6268	55.45 ± 0.21
1,240	64.7791	63.7803	63.9492	64.59 ± 0.23
1,280	74.5177	73.5258	73.6206	73.74 ± 0.58
1,320	82.4829	82.0298	83.7880	81.41 ± 1.38

Table 26**:** IC_{50} of collagenase enzyme inhibition of *G. speciosum* ethanolic extract

run	IC_{50} (µg/mL) anti-collagenase activity
1	1,180
2	1,179
3	1,179
MEAN	1,179
SD	0.001
%RSD	1.466

Table 27**:** The percent collagenase activity inhibition of EGCG standard.

EGCG Concentration (µg/mL)	%Inhibition Collagenase			Mean ± S.D.
	run1	run2	run3	
30	21.7241	24.2634	24.5675	23.52 ± 1.56
40	32.4138	33.6222	34.7751	33.60 ± 1.18
50	42.9310	44.1941	44.8097	43.978 ± 0.96
60	53.4483	53.0329	55.1903	53.89 ± 1.14
70	63.6207	61.5251	65.0519	63.40 ± 1.77
80	75.1724	71.5771	73.5294	73.43 ± 1.80
90	82.5862	83.3622	82.6990	82.88 ± 0.42

Table 28**:** The IC_{50} of collagenase enzyme inhibition of EGCG standard**.**

Run	IC_{50} (µg/mL) anti-collagenase activity of EGCG
1	55.50
2	56.97
3	56.81
MEAN	56.43
SD	0.81
%RSD	1.43

Elastase is a member of the chymotrypsin family of proteases. It is responsible for cleave of elastin. Elastin is an important protein in extracellular matrix (ECM), which gives elasticity to skin, arteries, lungs and ligaments. In normal conditions, elastase is necessary to enable tissue repair after wounding. It is responsible to degrade unnecessary proteins in the ECM during phagocytosis. In terms of anti-aging, elastase enzymes inhibitors might be useful to prevent skin elasticity losing. As the loss of elastin in the skin is the important sign of aging, both wrinkles and sagging (Y.-J. Kim, Uyama, and Kobayashi, 2004; Thring, Hili, and Naughton, 2009; Tu, and Tawata, 2015).

The *in vitro* study (non-cell base assay) utilized EGCG as a standard compound (positive control) because EGCG showed potent anti-collagenase and anti-elastase activities and it was used as a positive control in various studies (Tansirikongkol, 2016; Thring, Hili, and Naughton, 2009).

Gastrodin was preliminary studied for anti-elastase and anti-collagenase activity in *in vitro* non-cell-based assay. Gastrodin showed anti-elastase and anti-collagenase activity at very high concentration (data not shown). The activity of gastrodin, a compound in phenolic glycoside group, was low because the activity of phenol group might be hidden by glycoside group in the structure. Therefore, gastrodin was not be used as active marker in further study.

In this study, *G. speciosum* extract possess anti-elastase activity, therefore, *G. speciosum* extract could be the candidate for treatment of skin aging disorder, particularly the skin elasticity improvement.

Regarding the stability of the *G. speciosum* extract before and after the heating-cooling cycle tests, the results are shown in Table 29. The result demonstrated that Gastrodin content, color and pH of *G. Speciosum* extract did not change significantly throughout the process. It may conclude that the *G. speciosum* extract was stable at these temperatures, subjected to further longer term study.

Table 29**:** Stability of the *G. speciosum* extracts before and after the heating-cooling cycle tests.

Parameter	Standard EGCG	*G. speciosum* extract	
		Before HC	After HC
TPC (mg EGCG/g extract)		48.187± 0.393	nd
Anti-elastase (IC_{50}:µg/mL)	92.52 ± 0.62	71.10 ± 1.04	nd
Anti-collagenase (IC_{50}:µg/mL)	56.43 ± 0.81	1,179 ± 0.01	nd
Gastrodin content (µg/g)	-	27.48 ± 0.12	28.33 ± 0.16
Color	-	Brownish yellow	Brownish yellow
pH	-	5.2	5.2

* HC; heating-cooling cycle, ** EGCG; epigallocatechin gallate, *** nd; not determined

5. Cell-based assay in skin keratinocyte cell line.

5.1. Effect of *G. speciosum* extract on the viability of HaCaT cells.

To evaluate the effect of *G. speciosum* extract on stemness, the cytotoxicity of the extract at 0-100 µg/mL was first carried out using the human keratinocyte HaCaT cells as the model. The non-cytotoxic concentrations of extract were determined and subsequently utilized to evaluate the preventive effects of *G. speciosum* extract in human keratinocyte HaCaT cells. Cell viability was determined after 24 h treatment with the extract by MTT assay. Results indicated that the extract at the concentrations of 0-20 µg/mL caused no significant change in the cell viability compared with the non-treated control (Figure 33 (a) and Table 30). This information may help to clarify that the following effects of *G. speciosum* extract on HaCaTs were not a consequence of cytotoxic effect or cell stress.

This information may help to clarify that the following effects of *G. speciosum* extract on HaCaTs were not a consequence of cytotoxic effect or cell stress. On the other hand, the cytotoxicity observed at higher concentrations of *G.speciosum* extract might be due to a pro-oxidant effect, leading to a decrease in cell viability. As high concentration of vanillin (1 mM), one of the compounds found in *G.speciosum* extract, has been previously reported to have a pro-oxidant effect on Fe(III)-superoxide-induced damage to deoxyribose and some amino acids. Higher concentration of vanillin (10 mM) also exhibited the DNA damage property (Jiankang, and Akitane, 1993). This could be a partial reason that contributed to the cytotoxicity of *G. speciosum* at higher concentrations.

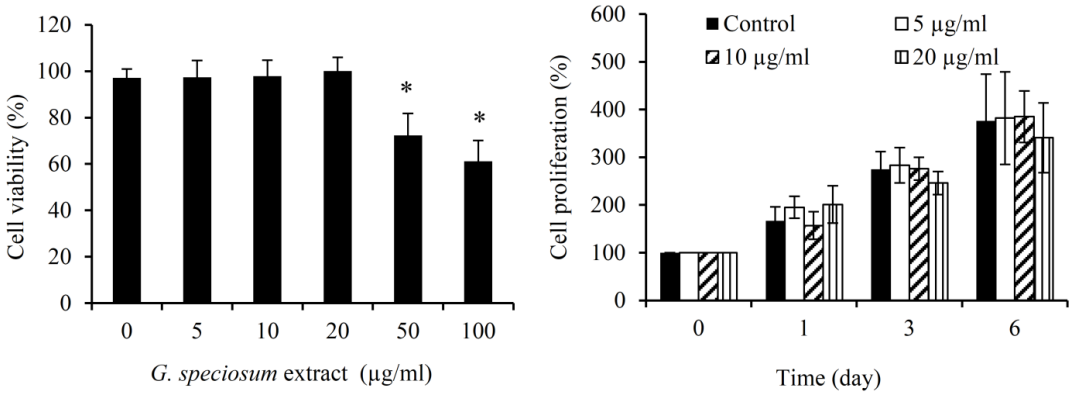


Figure 33: Effect of *G. speciosum* extract on viability of HaCaT cells by MTT assay. (a) Cell viability of HaCaTs were determined after treated with *G. speciosum* extract (0-100 µg/ml) for 24 h. (b) Effect of *G. speciosum* extract on HaCaTs proliferation. Cells were treated with *G. speciosum* extract (0-20 µg/ml) at 37°C for 0, 1, 3 and 6 days. The data represent the means of four independent experiments ± SD. $^{*}p < 0.05$ versus non-treated control.

Table 30**:** Effect of *G. speciosum* extract on viability of HaCaT cells by MTT assay.

G. speciosum extract (µg/mL)	% Cell viability 1	2	3	4	Mean ± SD
	93.18	91.29	94.12	93.18	
	102.35	93.18	103.29	100.47	
	99.76	96.71	93.65	96.00	
0	100.00	100.00	100.24	96.71	97.13 ± 3.77
	100.00	108.00	93.06	100.94	
	89.41	91.06	105.88	96.71	
	102.35	93.18	103.06	93.65	
5	97.88	84.24	100.47	102.59	97.65 ± 6.45
	93.18	104.24	100.00	101.88	
	101.41	91.76	108.00	102.82	
	90.35	102.12	92.00	102.35	
10	92.47	84.47	91.53	107.06	97.85 ± 6.94
	103.29	99.29	98.59	111.53	
	101.88	92.94	105.65	93.18	
	100.00	100.47	103.29	97.65	
20	107.76	92.47	90.59	103.29	100.12 ± 5.84
	91.76	71.76	73.41	70.82	
	65.18	85.65	74.12	63.06	
	89.18	74.12	66.82	60.00	
50	61.41	65.88	74.12	69.41	72.29 ± 9.45
	69.65	67.53	49.41	62.59	
	50.82	65.41	53.41	71.53	
	67.29	69.41	60.94	52.94	
100	65.41	65.65	40.00	65.88	61.12 ± 9.04

5.2. Effect of *G. speciosum* extract on HaCaT cells proliferation

The effect of *G. speciosum* extract on the proliferation of HaCaT cells is shown in Figure 33 (b). Cells were exposed to 0-20 μg/mL *G. speciosum* extract for up to 6 days in normal culturing condition. MTT results indicated that all concentrations of *G. speciosum* extract did not affect the proliferative activity of the cells as compared to the non-treated control ($p > 0.05$). There was no significant difference in the percentage of cell proliferation among cells treated with 0-20 μg/mL. This range of concentration was used in further study to inform whether the increment of stemness was not from the proliferation of the cells.

5.3. Effect of *G. speciosum* extract on stem cell behavior in HaCaT keratinocyte cells.

The effect of *G. speciosum* extract on the stem cell behaviors and its functionality was carried out. Stemness-inducing effect of *G. speciosum* extract was then tested in HaCaT cells. As the ability of the stem cells to grow and form spheroids in anchorage-independent condition has been widely accepted as a hallmark of stem cell clonogenicity (Bhummaphan, and Chanvorachote, 2015; Yongsanguanchai et al., 2014), cells were pretreated with the non-cytotoxic concentrations of *G. speciosum* extract for 3 days, and analyzed for spheroid formation in ultralow attached plates. The treated cells were seeded at low density in DMEM serum-free medium. Treatment of the cells with *G. speciosum* extract significantly increased cell ability to form spheroid colonies (Figure 34(a)). Moreover, spheroid numbers were counted and expressed as relative values over non-treated control levels. *G. speciosum* extract treatment at the concentration of 5, 10 and 20 μg/mL significantly increased the size

of spheroids (Figure 34 (c). At 10 and 20 µg/mL of *G. speciosum* extract significantly increased the spheroid number (Figure 34 (b)). This effect was not found at low concentration (5 µg/mL), at this concentration might be not strong enough to increase spheroid number.

Spheroid forming is the conventional method to evaluate stem cell properties of the cells. Suspension cells with restricted nutrients (serum free media without growth factors) were used in this study. Cells with the ability to increase and form into spheroid were possibly distinguished as stem cells. The result of stemness-inducing suggested that *G. speciosum* extract significantly increased cell ability to form spheroids which was first detected at day 21 (Figure 34). At day 21, spheroid numbers were counted and expressed as relative values over non-treated control levels. *G. speciosum* extract treatment significantly increased in the spheroid number in dose-dependent manner (Figure 34). These results demonstrated the role of *G. speciosum* extract as an inducer of stem cell-like behaviors in human keratinocyte cells regards from the clonogenicity of stem cells.

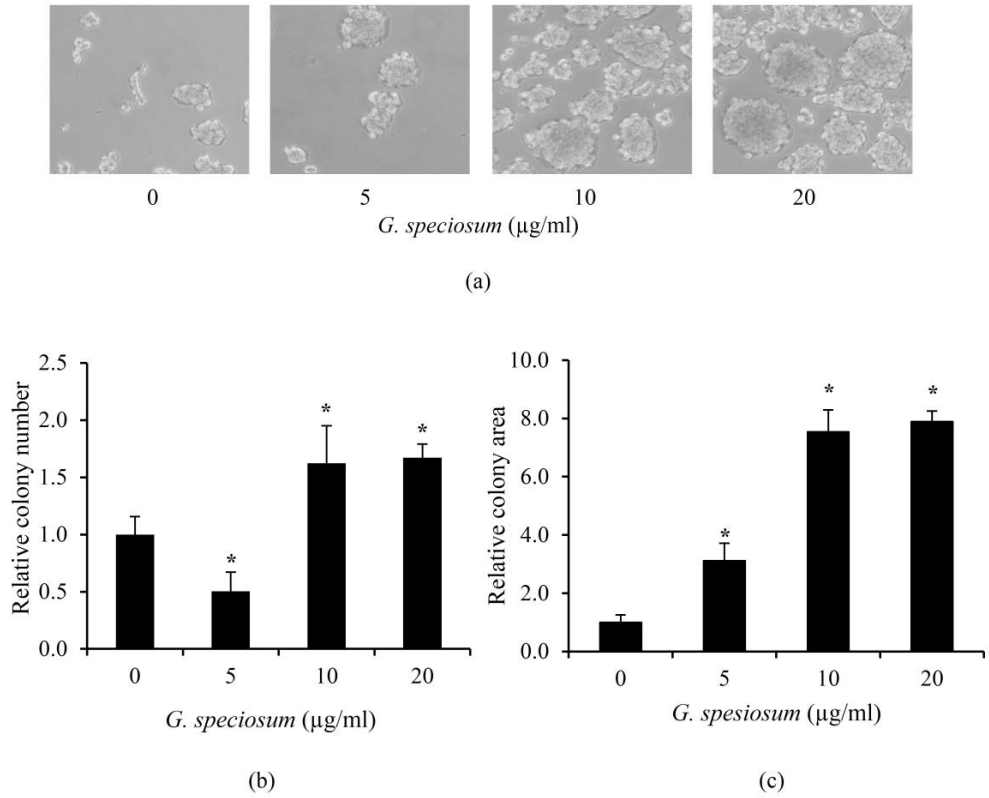


Figure 34: Effect of *G. speciosum* extract on spheroid formation. After treat with *G. speciosum* extract 0-20 µg/ml for 3 days, HaCaT cells were subjected to spheroid formation assay as a single cell suspension. (a), 10 x phase-contrast images of spheroids at day 14 were captured. Effect of *G. speciosum* extract on (b) spheroid colony number and (c) spheroid colony area, as determined by spheroid-formation assay. Relative spheroid number and area were determined by dividing the values of the treated cells by those of the control cells. Triplicate samples were averaged for each group. Error bars show SD of triplicate experiments (n = 9). *$p < 0.05$ versus non-treated cells.

5.4. *G. speciosum* extract increased expression level of stem cell markers in HaCaTs

To further confirm the stemness-inducing effect, the expression of stem cell key molecular markers was investigated. The CD133, *β*-catenin and ALDH1A1 expressions have been recognized as the indicators for stemness of cells (Cédric Blanpain, and Elaine Fuchs, 2006; Kiratipaiboon, Tengamnuay, and Chanvorachote, 2015, 2016; Nordvig, Owens, and Morris, 2012; Zhang et al., 2012). Cells were treated with *G. speciosum* extract in dose- and time-dependent manners and the expression level of CD133, *β*-catenin and ALDH1A1 was determined by Western blot analysis. Results indicated that treatment with *G. speciosum* extract at the concentration of 20 µg/mL significantly increased expression of CD133 and ALDH1A1 as early as day 1 and all markers significantly increased at day 3 (Figure 28(a)). Also, *G. speciosum* extract increased *β*-catenin and ALDH1A1 in a concentration-dependent manner after 3 days of treatment compared with the non-treated control (Figure 35(b)). There are some minor differences between relative protein level of CD133, *β*-catenin and ALDH1A1 of HaCaT cells after treating with *G. speciosum* extract at the concentration of 20 µg/mL for days, as shown in Fig. 35 (a) and (b). The difference of cell passages could affect the consistency of the result. The increment of stem cell markers might be from the increase in stemness of keratinocyte stem cells, as at this concentration of *G. speciosum* extract did not affect the cell proliferation. Consequently, *G.speciosum* may delay keratinocyte stem cell death, leading to augmentation of skin hydration.

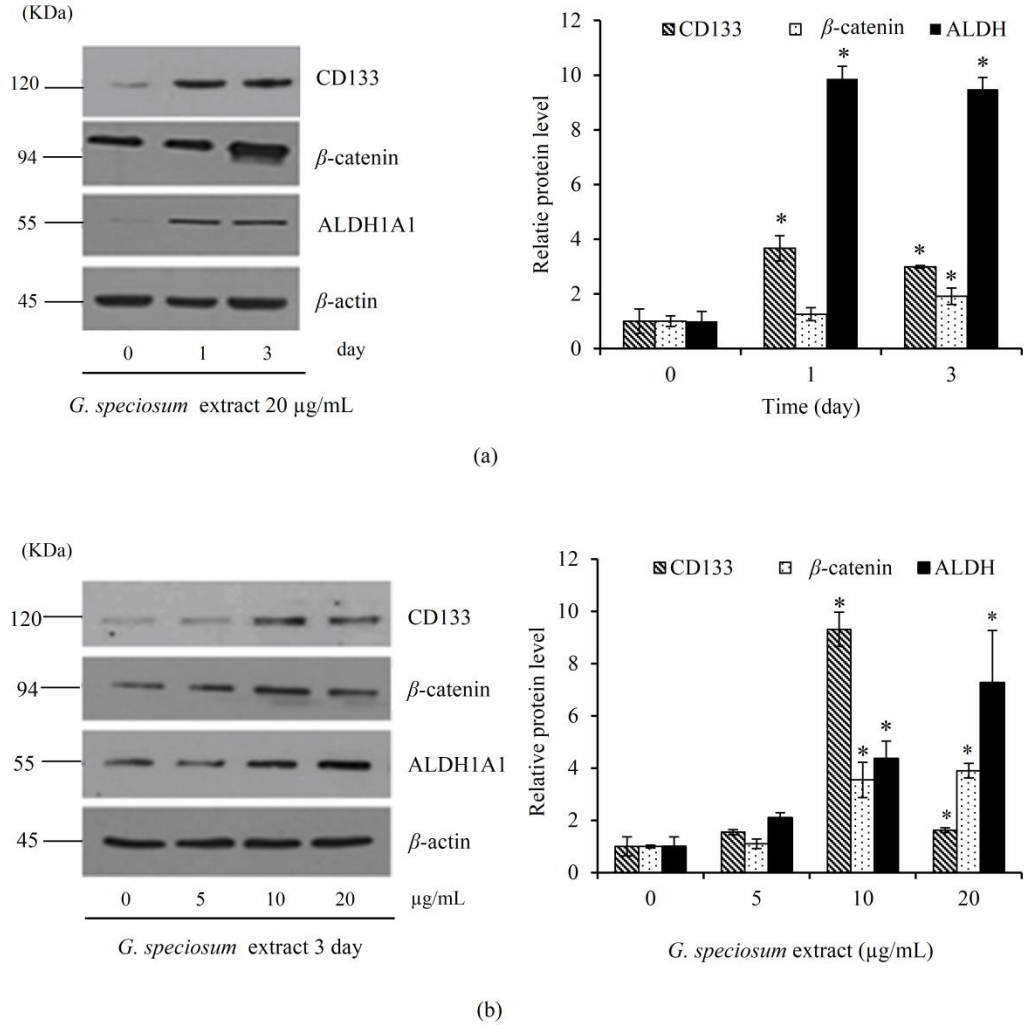


Figure 35: Effect of *G. speciosum* extract on stem cell markers. The levels of CD133, *β*- catenin and ALDH1A1 were analyzed by Western blotting. Blots were re-probed with *β*-actin to confirm equal loading. The Western blot signals were quantified by densitometry and the mean data from independent experiments were normalized to the non-treated control. (a) Time-dependent effect of *G. speciosum* extract treatment on the expression of stem cell markers. Cells were cultured in the presence or absence of *G. speciosum* extract (20 µg/mL) for 0–3 days. (b) Dose-dependent effect of *G. speciosum* extract on the expression of stem cell markers was determined. Cells were treated with *G. speciosum* extract (5– 20 µg/mL) for 3 days.

The data represent the means of three independent experiments ± SD. $^{*}p < 0.05$ versus non-treated control.

We have revealed for the first time that *G. speciosum* extract positively regulates the stemness of keratinocyte cells. The *G. speciosum* extract increased both the spheroid and the colony formation, along with up-regulation of the known stem cells markers.

Epidermal stem cells are found in the basal layer of the skin among the epidermal proliferative unit (EPU) which is a 3-dimensional structure of cells surrounding a single stem cell creating the microenvironment. The epidermal stem cells possess a self-renewal potential which is responsible for maintaining epidermal homeostasis throughout human lifespan, indicating the delay in cell aging (Doles et al., 2012a). In this study, we discovered an effective compound that can enhance the stem cell characteristics in human keratinocytes.

Stem cells are known to express their specific surface markers, such as aldehyde dehydrogenase (ALDH), ATP-Binding Cassette (ABC), CD133, CD44, and some other proteins (Peitzsch et al., 2013). ALDH has a role during the differentiation process of normal stem cells (Russo, and Hilton, 1988). ABC is one of the transporter superfamily protein which is activated in response to environmental factors and functions in controlling chemical homeostasis (Bleau et al., 2009). CD133 is associated with stem cell phenotype maintenance and it is generally reported with an increased phosphor-Akt level. Additionally, it is evidenced that cell transition ability from epithelial to mesenchymal (EMT) is required for stem cells to undergo metastasis. Thus, the induction of EMT has been reported to increase the stem cell-like phenotype.

Having shown that *G. speciosum* extract enhanced stem cells phenotypes in keratinocytes, we next confirmed such observation by using well-known epidermal stem cell markers (14). Cells were treated with *G. speciosum* extract in the dose-and time-dependent manner experiments and the expression level of CD133 and ALDH1A1 were determined by Western blot assay. Also, the assays that mimic the stem cells aggressive behaviors including spheroid and colony formation (Cédric Blanpain, and Elaine Fuchs, 2006; Hsu, Li, and Fuchs, 2014) were performed in this study.

In stem cell research, co-transcription factors, for example, *β*-catenin, have been accepted as a dominant stemness mediator, and were widely used as biomarkers for human stem cells detection (Mescher, 2013). The rising of the cellular levels of *β*-catenin in HaCaT keratinocytes could indicating the augmenting effect of the compound on keratinocyte stem cell phenotype. Hence, to sustain self-renewal, stem cells must reside within the niche anchoring via adhesion molecules (S. Chen, Lewallen, and Xie, 2013).

As previously reported in human dermal papilla cells, the improvement of stemness in this cells was found after treatment with ciprofloxacin. The results showed that ciprofloxacin increased stemness through ATP-dependent tyrosine kinase/glycogen synthase kinase3*β*-dependent mechanism, which in turn upregulated *β*-catenin similar to our results (Kiratipaiboon, Tengamnuay, and Chanvorachote, 2015). Clonogenicity, another behaviour characteristic of stem cells, was also detected in ciprofloxacin treatment group (Kiratipaiboon, Tengamnuay, and Chanvorachote, 2015).

5.5. Protective effect of *G. speciosum* extract on ROS-induced cell death in HaCaTs

The protective activity of *G. speciosum* extract against ROS-induced cell damage was evaluated. As the stem cells have been shown to have enhanced cellular detoxifying mechanisms as well as defenses against oxidative damages (Kobayashi, and Suda, 2012), protective activity of *G. speciosum* extract against ROS-induced cell damage were evaluated. HaCaTs were pretreated with *G. speciosum* extract for various times (1-6 day) at 20 µg/mL or various concentrations (5-20 µg/mL) for 3 days. Then, the cells were exposed to 2,3-dimethoxy-1,4-naphthoquinone (DMNQ (superoxide anion ($O_2^{\bullet-}$) generator)) or H_2O_2 at the concentrations of 25 µM, and cell viability was measured by MTT assay. Results showed that pre-treatment of the cells with *G. speciosum* extract significantly protected against $O_2^{\bullet-}$-induced cell death mediated by DMNQ (Figure 36 (a) and (b)), but not H_2O_2 (Figure 36 (c) and (d)). Also, the protective effect of the extract against $O_2^{\bullet-}$-induced cell death was in a dose-dependent manner (Figure 36 (b)). We found that the protective effect of the extract against $O_2^{\bullet-}$ was maximum at day 3 after pre-treatment. In order to clarify the underlying mechanism of *G. speciosum* extract in reduction of ROS-induced cell death, we investigated the change in cellular ROS. Cellular ROS levels were determined with the $DCFH_2$-DA fluorescence dyes. Results indicated that pre-treatment of the cells with 20 µg/mL *G. speciosum* extract significantly decreased overall intracellular ROS-induced by DMNQ (Figure 37 (a)), but not H_2O_2 (Figure 37 (b)). Immunocytochemistry was examined to confirm the ROS reduction effect of *G. speciosum* extract in ROS inducer-treated cells. Consistently, the increased ROS signal induced by DMNQ, but not H_2O_2, was suppressed by the addition of *G.*

speciosum extract (Figure 37 (c)). Taken together, these results pointed out that *G. speciosum* extract prevented $O_2^{\bullet-}$-induced cell death in HaCaTs through ROS reduction, in particular the superoxide anion, which was parallel with the stemness augmentation.

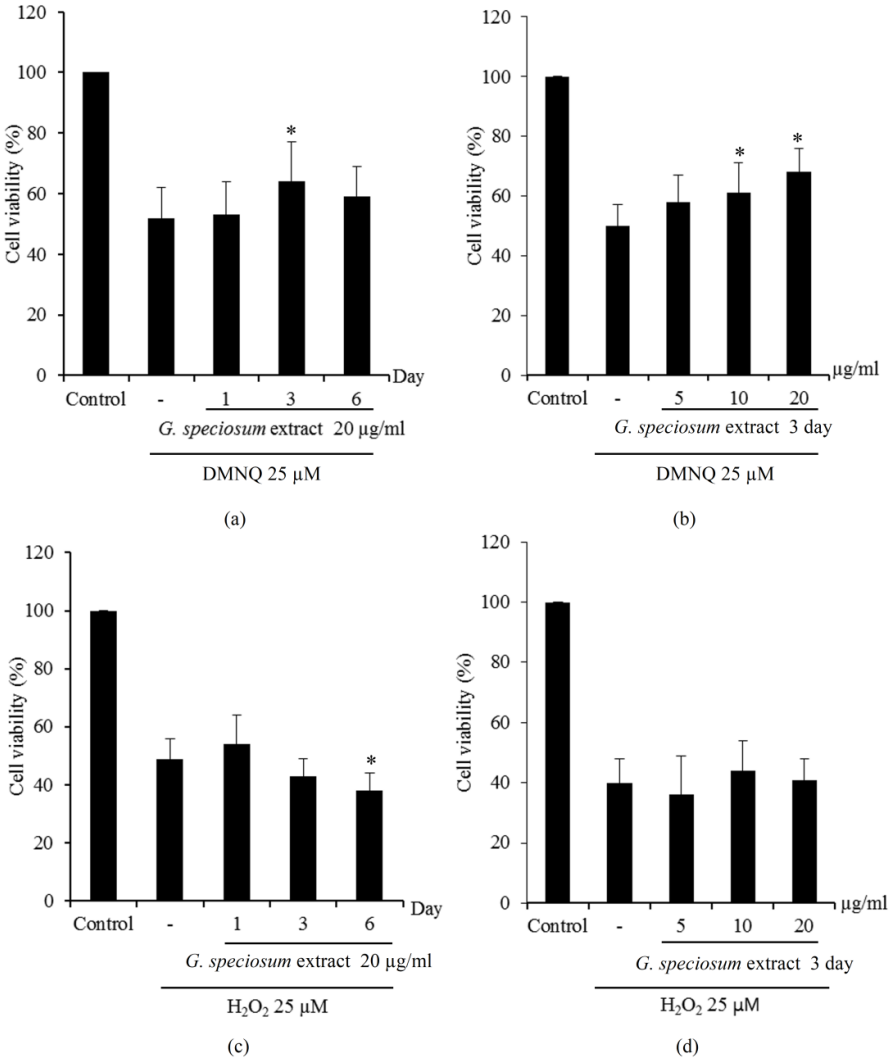


Figure 36: Effect of *G. speciosum* extract on ROS-induced cell death. (a) Cells were pretreated with 20 µg/ml *G. speciosum* extract for various times (1-6 days) and followed by treatment with 25 µM DMNQ. (b) Cells were pretreated with *G. speciosum* extract at various concentrations (0-20 µg/ml) for times 3 days and followed by treatment with by 25 µM DMNQ. (c) Cells were pretreated with 20

µg/ml *G. speciosum* extract for various times (1-6 days) and induced cell death by 25 µM H_2O_2. (d) Cells were pretreated with *G. speciosum* extract at various concentrations (0-20 µg/ml) for 3 days and treated with 25 µM H_2O_2. The data represent the means of four independent experiments ± SD. $^*p < 0.05$ versus non-treated control.

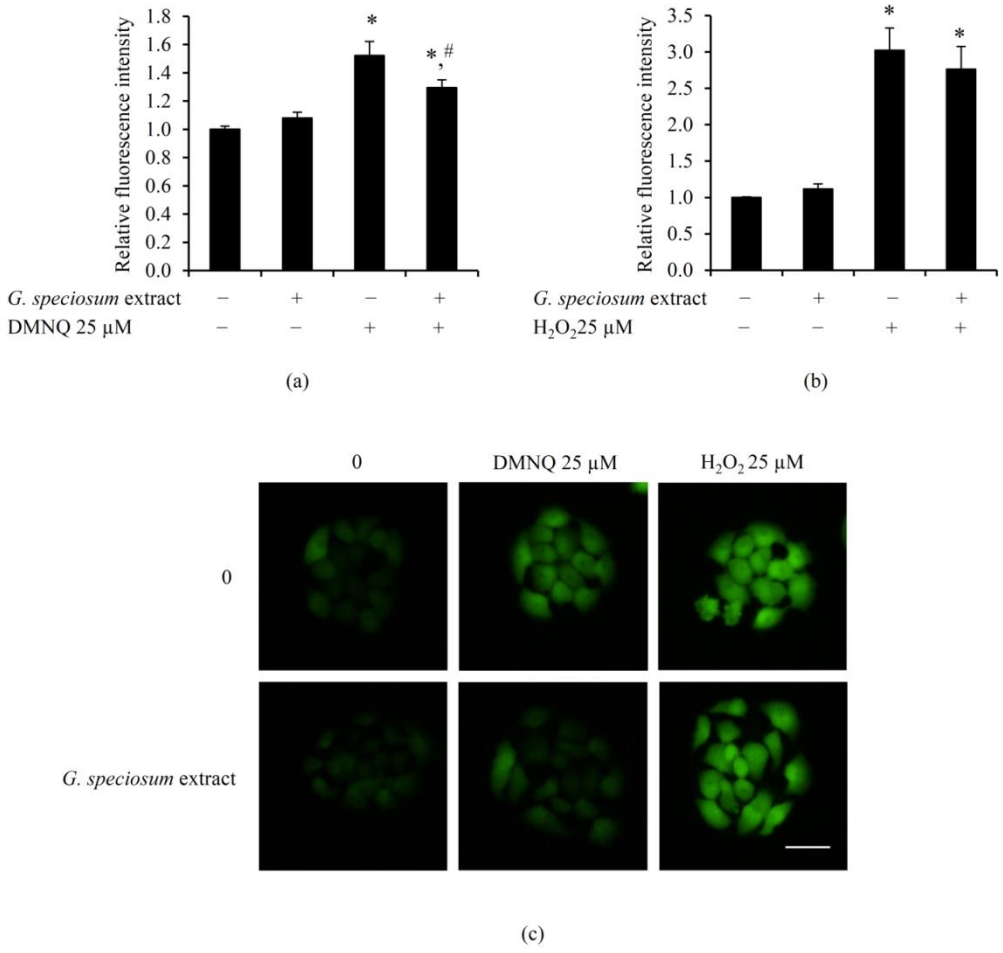


Figure 37: Cellular ROS level of *G. speciosum* extract on stress induced by ROS-inducer in HaCaTs cells. Subconfluent (70-80%) monolayers of HaCaTs cells were treated with 20 µg/ml of *G. speciosum* extract for 3 days. The cells were then incubated with ROS detection probe, $DCFH_2$-DA, at 4°C for 30 min prior to the treatment with (a) DMNQ (25 µM) for 3 h or (b) H_2O_2 (25 µM) for 30 min. The

fluorescence intensity was analyzed by a microplate reader. Mean intensity was normalized to non-treated control cells and represented as relative ROS levels. Values are means of independent triplicate experiments ± SD. *p<0.05 versus non-treated control. $^{\#}p$<0.05 versus ROS inducer treated alone. (c) Intracellular ROS of the cells stained with $DCFH_2$-DA were visualized under a fluorescence microscope; scale bar = 50 μm.

Epidermis as a defensive barrier of the body is maintained and repaired by the activities of stem cells within. For maintaining the epidermal homeostasis and repair machinery, the key players are keratinocyte stem cells (Senoo, 2013). There are evidences indicating that keratinocyte stem cells are enriched in the skin of newborns, but the number of such cells declines during aging (Barrandon, 1993; Barrandon, and Green, 1987). Keratinocyte stem cells are located at basal layer of the epidermis with the responsibility of producing cells for the upper epidermis. The entire unit of keratinocyte stem cells and the differentiated cells are termed an epidermal proliferative unit (EPU) and evidence supported that stem cells hold the function to generate and maintain such a column structure (Webb, Li, and Kaur, 2004).

Regarding stem cell research, CD133, a transmembrane glycoprotein, has been widely used as a standard biomarker of human stem cells (Li, 2013). The β-catenin functions as a co-trancription factor of T-cell factor/lymphoid enhancing factor (TCF/LEF) and consequently increases the expression of proteins facilitating stem cell functions (Fukumoto et al., 2001; Fiona M Watt, 2002). Together with the clonogenicity assay, our data indicated that *G. speciosum* extract increased the stemness of keratinocyte cells. The induction of these stem cell markers along with

enhanced stem cell phenotypes may support the conclusion that *G. speciosum* extract potentiates stemness of human keratinocytes.

Increasing of stem cell markers indicated the improvement in stemness of human keratinocytes. Keratinocyte stem cells that are located at the basal layer of the epidermis are responsible of producing cells for the upper epidermis. Hence, when the stemness of the basal layer was enhanced, the released markers will promote the production of new upper epidermal layers, resulting in the new and younger looking outer skin layer (Webb, Li, and Kaur, 2004). The results of anti-aging and anti-wrinkle effects of *G. speciosum* extract serum are reported in the clinical study part.

Ultraviolet (UV) radiation is a potent initiator of ROS generation in the skin (Ichihashi et al., 2003; Mukherjee et al., 2011). The type(s) of ROS generated, however, depends on the UV wavelength. UVB mainly stimulates the production of $O_2^{\bullet-}$ through the activation of NADPH oxidase and respiratory chain reactions (Lushchak, 2014). The increase in cellular $O_2^{\bullet-}$ may cause direct cellular damage or $O_2^{\bullet-}$ can be an initial generator of other highly reactive oxygen species such as peroxynitrite and hydroxyl radical. In terms of keratinocytes, we have revealed that the exposure to UVB can generate $O_2^{\bullet-}$ and subsequently cause cell damage (Heck et al., 2003). As evidence indicated that the epidermal stem cells possess high level of SOD1 (Racila, and Bickenbach, 2009; Stern, and Bickenbach, 2007), the increase in stem cell features may not only improves the barrier function of epidermis (Kaur et al., 2004; Racila, and Bickenbach, 2009; Senoo, 2013) but also may increase cellular defense against UVB-mediated cell injury.

According to critical role of $O_2^{\bullet-}$ in regulating epidermal stem cells (Racila, and Bickenbach, 2009), gastrodin, the phenolic glycoside [p-(hydroxymethyl) phenyl-

b-D-glucopyranoside], found in *G. speciosum* extract might play a critical role in potentiating stemness in human keratinocytes. The studies indicate that gastrodin is an effective antioxidant via augmentation of SOD level (Huang et al., 2015; Zhao et al., 2012). These data were consistent with our present results that the increase in keratinocyte stemness protect the cells against DMNQ, an $O_2^{\bullet-}$ generator, but not H_2O_2. Therefore, we did not observe the protective effect of *G. speciosum* from H_2O_2 -induced cell death in our current study.

Moreover, the ALDH enzyme represents an important class of enzymes that significantly attributes to the management of oxidative stress and lipid peroxidation in mammal (Singh et al., 2013). Reduction of ROS in keratinocyte cells might be a consequence from ALDH augmentation by *G. speciosum* extract.

There are at least two possibilities that the *G. speciosum* extract can reduce the superoxide anion inside the cell: 1) by direct scavenging of the ROS (Kumar et al., 2013) and 2) by stimulating the production of SOD (Kumar et al., 2013; Zhao et al., 2012)

The *in vitro* anti-aging activities (non-cell-based assay) were performed using both anti elastase and collagenase enzyme. These enzymes presented throughout the extracellular matrix in dermis layer. Therefore, this study represented the dermis layer, however, it lacks of the information in fibroblast cells. The *in vitro* anti-aging activities (cell-based assay) were performed using keratinocyte cell which presented in epidermis layer.

6. Development of the serum product containing *G. speciosum* extract

After evaluation of physicochemical properties and *in vitro* biological activities of the *G. speciosum* extract, it was further formulated into to a topical serum for human study in order to prove its clinical anti-aging/anti-wrinkle efficacy and safety.

The *G. speciosum* extract was completely dissolved in propylene glycol 400 at 0.5% (w/w) giving a solution. Thus, in the formulation of *G. speciosum* extract serum, propylene glycol 400 was used as a co-solvent. Other ingredients used in the topical serum are listed in the Table 31. The physiochemical properties including appearance, pH, and viscosity of *G. speciosum* extract solution are reported in Table 31. All formulations were stable according to ASEAN guideline at condition of 40 °C and 75 % RH for 3 months as shown in Table 32.

Table 31**:** Ingredients of base and *G. speciosum* serums.

Ingredients	% (w/w)	
	Base Serum	*G. speciosum* Serum
G. speciosum extract	-	0.5
PEG 400	12	12
Aristoflex AVC® *	0.5	0.5
Microcare PHC® **	1	1
DI water q.s.	100	100

*Aristoflex AVC; Ammonium Acryloyldimethyltaurate/VP Copolymer ,
** Microcare PHC; Phenoxyethanol (and) Chlorphenesin (and) Glycerin

Table 32**:** Stability of the *G. speciosum* extracts before and after the accelerated tests.

Parameter	*G. speciosum* extract	
	Before HC*	After HC*
Gastrodin content (μg/g)	27.48 ± 0.12	28.33 ± 0.16
Color	Brownish yellow	Brownish yellow
pH	5.2	5.2

* HC; heating-cooling cycle (n = 3, value = mean ± S.D.)

According to the previous stability evaluation, the solutions with various concentrations of *G. speciosum* extract resulted in homogenous formulations with slightly different physicochemical properties. The lower concentration of the extract in the formulation resulted in a less viscous and lower pH product, particularly with the 0.5% *G. speciosum* as shown in Table 33. However, these changes were not statistically significant ($p > 0.05$) and were still in an acceptable range for topical serums. *G. speciosum* serum formulation at 0.5% w/w extract was therefore selected for further skin irritation assessment in human volunteers due to its good physicochemical stability.

Table 33**:** Stability of the 0.5% *G. speciosum* serum formulation.

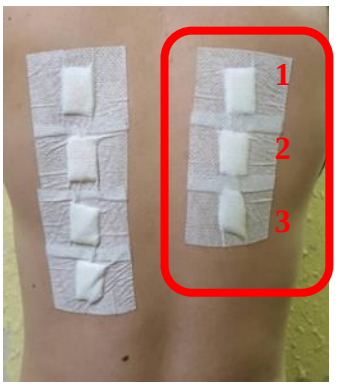
Condition 40 °C 75 % RH	0.5% *G. speciosum* Serum	
	Before 3 month (D0)	After 3 month (D90)
Gastrodin content (µg/g)	28.62 ± 1.26	27.28±0.24
pH	5.16	5.13
Viscosity* (cps)	2240 ± 2.21	2185 ± 3.08

*Spindle no. 95, 100 rpm and % torque > 50 (n = 3, value = mean ± S.D.)

7. Preliminary skin irritation assessment in human volunteers.

Skin irritation evaluation was carried out in 32 volunteers using the closed patch skin irritation test. There was no significant difference on skin irritation observed from both the control water-based serum and the *G. speciosum* serum.

The serums, with and without *G. speciosum* ethanolic extract, were separately applied to the right side of each volunteer's back, respectively, for 24 hours (Figure 38). The irritancy was observed after 24, 48, and 72 hours according to International Contact Dermatitis Research Group (ICDRG) scale. There was no significant difference on skin irritation observed from both the control water-based serum and the *G. speciosum* serum (Figure 39, Figure 40 and Figure 41). This formulation of serum was subsequently further studied for anti-aging efficacy in human volunteers.

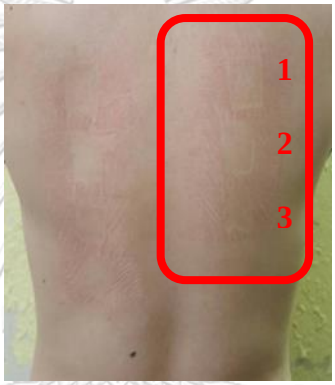


1: Base serum

2: Control (water)

3: *G. speciosum* serum

Figure 38**:** Patch test of serum with, without *G. speciosum* crude extract and control (water)

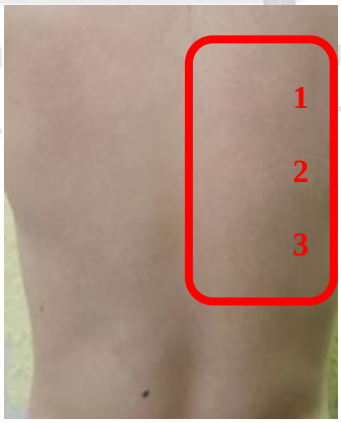


1: Base serum

2: Control (water)

3: *G. speciosum* serum

Figure 39**:** Volunteer's back after left the patch for 24 hours

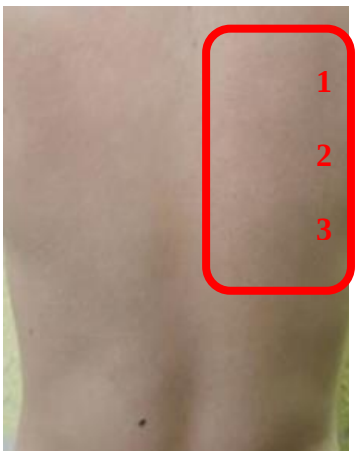


1: Base serum

2: Control (water)

3: *G. speciosum* serum

Figure 40**:** Volunteer's back after left the patch for 48 hours



1: Base serum

2: Control (water)

3: *G. speciosum* serum

Figure 41**:** Volunteer's back after left the patch for 72 hours

8. *In vivo* efficacy assessment in human volunteers

The Thai volunteers (2 males and 22 females) average age of 45.75 ± 0.89 years were assigned to a randomized, double-blind placebo-controlled experiments using the 0.5% (w/w) *G. speciosum* serum and serum base applied on either left or right side of their face (total 2 serums for each volunteer: each facial side for each formulation). The subjects in the both groups (n = 12 per group), the first group were managed to apply *G. speciosum* extracted serum (test serum) on the right side of the face and the base serum on the left side of the face meanwhile the second group proceeded the same procedure at the opposite side of the face (randomized). Subjects were instructed to apply 0.2 g of each provided serum twice a day for 56 days in the morning and at bed time.

Non-invasive measurements were performed to track the anti-aging efficacy of the pre-treatment at the base line serums and 28, 46, and 56 days after treatment (Kanlayavattanakul et al., 2015) (Figure 42). There were no adverse effects reported on any volunteers over the entire study period.

Figure 42**:** Study on volunteer skin elasticity measurement by Cutometer©

The cutometer© generated a graph depicting immediate deformation or skin extensibility (Ue), delayed distension (Uv), final deformation (Uf), immediate retraction (Ur), total recovery (Ua), and residual deformation at the end of measuring cycle (R) (Figure 43) (Tran et al., 2015).

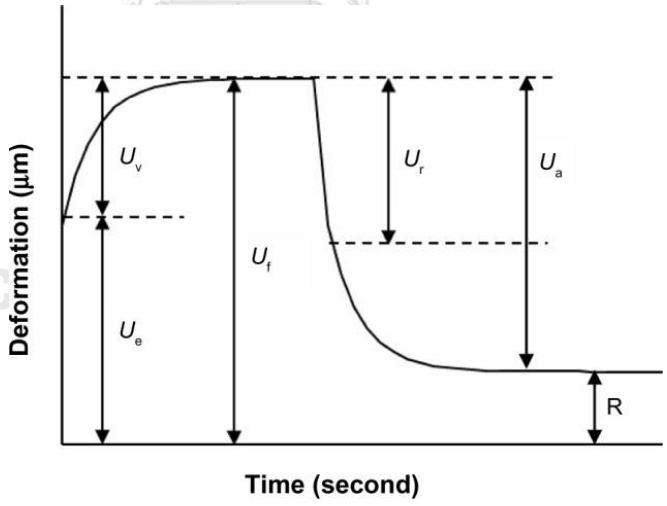


Figure 43**:** Skin deformation curve obtained with a cutometer© depicting the immediate deformation or skin extensibility (Ue), delayed distension (U_v), final deformation (U_f), immediate retraction (U_r), total recovery (U_a), and residual deformation at the end of the measuring cycle (R). (Berardesca, Maibach, and Wilhelm, 2013; Tran et al., 2015)

8.1 skin viscoelasticity efficacy

R6 indicates the portion of the viscoelasticity on the elastic part of the curve, and is represented by the ratio of "viscoelastic" to "elastic distension", R6= Uv/Ue. Since this parameter measures the stretch capacity of the skin, negative values reflect improved skin condition. In this study, % efficacy of skin elasticity was calculated by the following:

Equation 8:

% efficacy of skin viscoelasticity = [R6 (d0) - R6 (dx)] * 100 / R6 (d0)

Elasticity improvement of skin after using serum base and *G. speciosum* serum at different time were compared and shown in Table 34. Skin applied with *G. speciosum* serum showed higher percent improvement than skin applied with serum base in every time point (D28, 42 and 56). Statistical analysis of almost every time point, with except at D42, indicated that *G. speciosum* serum significantly improve skin elasticity higher than serum base ($p < 0.05$).

Table 34**:** Clinical efficacies of the 0.5*% G. speciosum* serum.

Efficacy (%)	Time interval	Serum	
		Base	0.5% *G. speciosum*
Skin viscoelasticity	D0-D28	38.45±3.97	51.55±2.95*
R6	D0-D42	38.54±3.63	45.58±3.56
	D0-D56	38.86±3.67	48.63±2.70*
Skin distensibility	D0-D28	40.90±6.39	54.63±5.67
R0	D0-D42	31.79±3.98	50.76±5.56*
	D0-D56	36.83±4.21	50.42±5.88*

* $p<0.05$ = compare between Base and 0.5% *G. speciosum* serum

R6 represents the relative contribution of skin viscoelastic and viscous deformation to elastic deformation. This parameter indicates a decrease in the viscosity of interstitial fluid, leading to increasing of water content. When water is accumulated in dermis, the friction between fibers and interstitial fluid is decreased (Akhtar et al., 2010; Berardesca, Maibach, and Wilhelm, 2013). In this study, *G. speciosum* serum improves the stretch capacity of the skin.

The decreasing Uv and increasing Ue represent the improvement of skin viscoelasticity. Also water accumulation in the dermis can decrease Uv (Dobrev, 2014). In this study, *G .speciosum* serum improves the stretch capacity/viscoelasticity of the skin (R6) by decreasing Uv and/or increasing Ue.

8.2. Skin distensibility efficacy.

R0 is the first maximum amplitude or the highest point of the first curve, i.e., Uf. It is known as final distension or skin dispensability (Akhtar et al., 2010). This has an implication for the firmness of skin. It represents passive behavior of the skin to force. In this study, % efficacy of skin distensibility was calculated by the following:

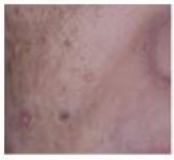
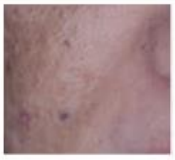
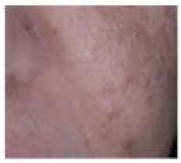
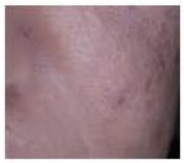
Equation 9: % efficacy of skin distensibility = [R0 (dx) -R0 (d0)] * 100 / R0 (d0)

Following application of *G. speciosum* serum, % skin distensibility increased to 54.63, 50.76 and 50.42% at day 28, 42 and 56, respectively. For base serum, R0 values showed a variable tendency to increase and decrease up to the end of the study period of 8 weeks. Percent skin distensibility increased to 40.90 at day 28 then decrease to 31.79 and 36.83 at day 56 and 84, respectively. Changes in R0 values throughout the study period produced by the *G. speciosum* serum over the serum base

were significant during a period of 8-week study ($p < 0.05$). Since R0, or skin distensibility, indicates skin firmness and the skin's passive resistance after negative pressure is applied. R0 is dependent on skin thickness, the positive change is representative of an increase in skin firming and an improvement in stratum corneum thickness (Berardesca, Maibach, and Wilhelm, 2013). Therefore, it could be summarized that *G. speciosum* serum improves skin firming and increase in stratum corneum thickness.

8.3. Skin wrinkle reduction efficacy

At day 0 of treatment, the wrinkle volume was similar between *G. speciosum* serum treatment and base serum treatment groups. The *G. speciosum* serum was shown to significantly reduce the wrinkle volume than base serum ($p \leq 0.001$) at day 56 of treatment, as shown in Figure 44 and Figure 45. At day 56 of *G. speciosum* serum application, the skin was smoother and the wrinkle was smaller than the base line.

Time	D0	D56	Different Wrinkle Volume (px^3)	*p* values
Base serum			- 647 ± 1222	
G. speciosum serum			18199 ± 2210	< 0.001

[a] *p<0.05 = compare between base and 0.5% G. speciosum serum*

Figure 44**:** Skin surfaces at the base line and following the treatment for 56 days.

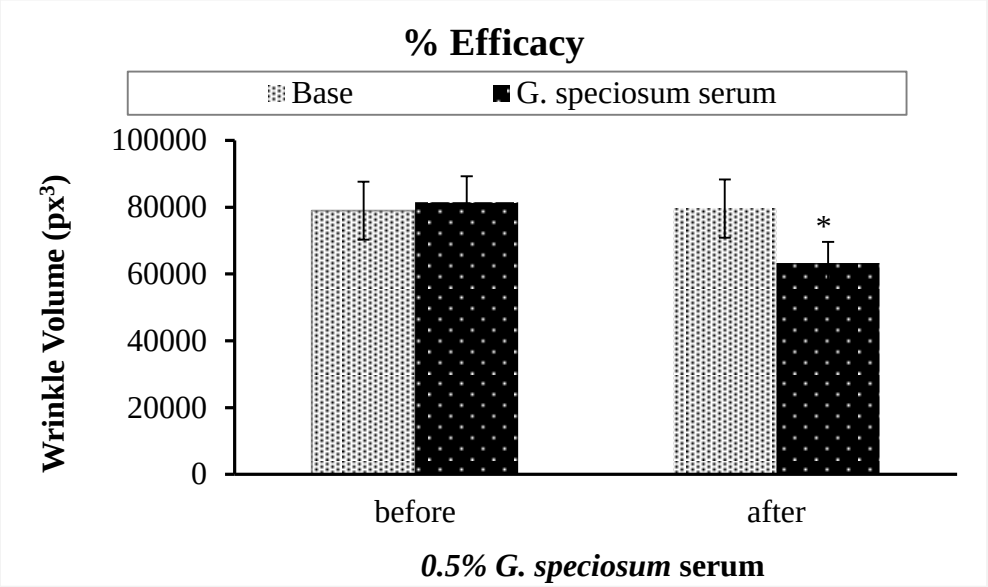


Figure 45**:** Wrinkle volume of base serum and *G. speciosum* serum at the base line (D0) and following the treatment for 56 days (D56). *$p < 0.05$ versus D0 (before treatment)

9. Consumer self-evaluation of the 0.5% (w/w) *G. speciosum* serum

The opinions of 24 volunteers aged 25-55 years on the *G. speciosum* serum were collected by means of a questionnaire. According to the records, most volunteers clearly (>80%) preferred the 0.5 % (w/w) *G. speciosum* serum over the serum base in terms of stickiness, color, spreadability, skin adsorption and greasiness (Table 35). Most volunteers preferred overall efficacy of *G. speciosum* serum (86.45%), both whitening effect (85.81%) and anti-wrinkle effect (84.52%). For self-evaluation of side effect, most volunteers preferred of no side effect (86.45%). They received the positive feedback from people (87.10%) and preferred to continuing use

this serum more than 2 months (86.45%). Base on the volunteer self-evaluation, the *G. speciosum* serum has the potential to be developed for using as cosmeceutical product in the future.

Table 35: Summary consumer self-evaluation by questionnaire (n=24)

Questionnaires	**% Preferred**
Overall efficacy	86.45
Whitening effect	85.81
Anti-wrinkle effect	84.52
Positive feedback from people	87.10
No side effect occurred	86.45
Plan to use more than 2 months	86.45

CHAPTER V
CONCLUSION

This study examined the anti-aging property of *G. speciosum* pseudobulb ethanolic extract both *in vitro* (using non-cell based and cell-based assays) and *in vivo* (clinical study). The compounds in *G. speciosum* pseudobulb extract were determined in this study. In non-cell based assay it was found that *G. speciosum* ethanolic extract contained polyphenols with the major active constituent being gastrodin. Previous studies reported that gastrodin exhibited antioxidant activity and possessed therapeutic activities for many disorders such as memory enhancer (Hsieh, Wu, and Chen, 1997; Wu et al., 1996), anti-Alzheimer (Hu, Li, and Shen, 2014), anti-Parkinson (Wang et al., 2014), as well as having protective effects against osteoporosis, the mechanism of which was linked to a reduction in reactive oxygen species or ROS (Huang et al., 2015). Moreover, the result from this study showed that *G. speciosum* extract possessed higher anti-elastase activity than that of EGCG at the similar concentration. However, the anti-collagenase enzyme activity was lower than EGCG. The results of this study indicated that the ethanolic extract of the pseudobulb of *G. speciosum* exhibited potent elastase inhibitory activity.

In cell-based assay, the protective effect of *G. speciosum* extract against superoxide anion-induced cell death was assessed in HaCaT cells which represented the human keratinocytes. The enhancement of stemness by *G. speciosum* extract was also investigated in the same cells. The results demonstrated that *G. speciosum* extract has a potential to increase stem cell phenotypes of human keratinocyte cells. As the stemness of keratinocytes is an important factor determining the function of epidermis

as well as it regeneration processes, *G. speciosum* extract may be a potential candidate for the enhancement of the skin protective and repair functions. Beside the ability to increase in stem cell markers like *β*-catenin, ALDH1A1, and CD133, the extract was able to protect the cells against $O2^{\bullet-}$ -induced cell death. Hence, this study provides for the first time of the interesting effects of *G. speciosum* extract on cell stemness enhancement that warrants further investigation and may provide useful information for future therapeutic and/or cosmeceutical applications.

Subsequently, topical serum containing the *G. speciosum* extract was formulated at 0.5% w/w. A skin irritation test was conducted in 32 healthy Thai volunteers. No irritation was found in volunteers who were assigned to treatment group, which was similar to the control. For efficacy evaluation, the randomized double-blind, placebo-controlled trial was conducted in 24 subjects during 56 days. It was shown to effectively increase firmness and smoothness as well as having therapeutic benefits to improve skin wrinkle.

This study has proven that the traditional remedies of *G. speciosum* can be scientifically and systematically studied to obtain a pseudobulb extract formulation for skin disorder treatment. These results highlight the potential feasibility of *G. speciosum* extract as a candidate for treatment of skin age-related problems and for cosmetic applications.

Further studies should be done in fibroblast cells which are within the dermis layer to fulfill a better understanding of anti-aging mechanisms of this herbal extract.

Regarding the previous research, the individual phytochemical compounds found in *G. speciosum* include vandateroside II, gastrodin, vanilloloside, orcinol glucoside, and isovitexin. A further study should be done to identify other possible

principal compounds in the *G. speciosum* extract that may be accounted for the anti-elastase and wrinkle-reducing effects an order to fulfill the understanding of anti-aging mechanism of this herbal extract.

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APPENDIX

เอกสารรับรองโครงการวิจัย

โดย

คณะกรรมการจริยธรรมการวิจัย โรงพยาบาลภูมิพลอดุลยเดช

กรมแพทย์ทหารอากาศ

ขอรับรองว่า

โครงการ การทดสอบการระคายเคือง และประสิทธิศักย์ของผลิตภัณฑ์ด้านริ้วรอยที่มีส่วนผสมสารสกัดสมุนไพรว่านเพชรหึงจากพื้นที่โครงการพระราชดำริเขาหินซ้อนจากพื้นที่โครงการพระราชดำริเขาหินซ้อน

โดย พลอากาศตรีสันติ ศรีเสริมโภค

สังกัด โรงพยาบาลภูมิพลอดุลยเดช กรมแพทย์ทหารอากาศ

เอกสารที่พิจารณา:
๑. โครงร่างงานวิจัย
๒. เอกสารข้อมูลสำหรับผู้ป่วย และ เอกสารแสดงความยินยอมเข้าร่วมการวิจัย ฉบับภาษาไทย

คณะกรรมการจริยธรรม โรงพยาบาลภูมิพลอดุลยเดช กรมแพทย์ทหารอากาศ ได้พิจารณารายละเอียดโครงร่างงานวิจัย เอกสารข้อมูลสำหรับผู้ป่วย เอกสารแสดงความยินยอมเข้าร่วมการวิจัยภาษาไทยแล้วเห็นว่าไม่ขัดต่อสวัสดิภาพหรือก่อให้เกิดภยันตรายแก่ผู้ถูกวิจัยแต่ประการใด

จึงเห็นสมควรให้ดำเนินการวิจัยในขอบข่ายของโครงการที่เสนอได้ ณ วันที่ ๗ มิถุนายน ๒๕๖๐

นาวาอากาศเอก

(ทวีพงษ์ ปาจรีย์)

ประธานคณะกรรมการจริยธรรมการวิจัย

พลอากาศตรี

(สันติ ศรีเสริมโภค)

ผู้อำนวยการโรงพยาบาลภูมิพลอดุลยเดช

กรมแพทย์ทหารอากาศ

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