THE EFFECT OF GLYCYRRHIZIC ACID AND AQUEOUS LICORICE EXTRACT ON HAIR GROWTH RETARDATION: *IN VITRO* MECHANISTIC AND EFFICACY STUDIES IN HUMAN

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Pharmaceutical Technology
Department of Pharmaceutics and Industrial Pharmacy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2016

ผลของกรดกลีเซอไรซิกและสารสกัดด้วยน้ำจากชะเอมเทศต่อการชะลอการงอกของเส้นขน : การศึกษากลไกการออกฤทธิ์แบบนอกกายและประสิทธิศักย์ในมนุษย์

นางสาวชญานิน กีรติไพบูลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีเภสัชกรรม ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

Thesis Title	THE EFFECT OF GLYCYRRHIZIC ACID AND AQUEOUS LICORICE EXTRACT ON HAIR GROWTH RETARDATION: *IN VITRO* MECHANISTIC AND EFFICACY STUDIES IN HUMAN
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ชญานิน กีรติไพบูลย์ : ผลของกรดกลีเซอไรซิกและสารสกัดด้วยน้ำจากชะเอมเทศต่อการชะลอการงอกของเส้นขน : การศึกษากลไกการออกฤทธิ์แบบนอกกายและประสิทธิศักย์ในมนุษย์ (THE EFFECT OF GLYCYRRHIZIC ACID AND AQUEOUS LICORICE EXTRACT ON HAIR GROWTH RETARDATION: *IN VITRO* MECHANISTIC AND EFFICACY STUDIES IN HUMAN) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภก. ดร.ปิติ จันทร์วรโชติ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ภก. ดร.ภาคภูมิ เต็งอำนวย, 111 หน้า.

การเจริญของเส้นขนเกี่ยวข้องโดยตรงกับการทำงานของเซลล์ dermal papilla ซึ่งเป็นเซลล์ต้นกำเนิดภายในต่อมรากขน งานวิจัยนี้เป็นงานวิจัยแรกที่แสดงให้เห็นว่ากรดกลีเซอไรซิก (GA) ซึ่งเป็นสารสำคัญที่อยู่ในชะเอมเทศ หรือ *Glycyrrhiza glabra* L.และสารสกัดด้วยน้ำจากชะเอมเทศ (GE) สามารถลดความเป็นเซลล์ต้นกำเนิดของเซลล์ dermal papilla ได้ โดยเซลล์ dermal papilla ที่ได้รับ GA และ GE มีลักษณะของเซลล์ต้นกำเนิดที่ลดลง ได้แก่ พฤติกรรมการรวมกลุ่มที่ลดลงและการแสดงออกของโปรตีนที่บ่งชี้ความเป็นเซลล์ต้นกำเนิดลดลงลดลง จากการศึกษากลไกการออกฤทธิ์พบว่าเซลล์ dermal papilla ที่ได้รับ GA และ GE สามารถลดการทำงานของ ATP-dependent tyrosine kinase/glycogen synthase kinase3 beta-dependent (Akt/GSK3 beta) ทำให้ beta-catenin มีการแสดงออกของโปรตีนลดลงจึงส่งผลให้ transcription factor ซึ่งมีหน้าที่คงความเป็นเซลล์ต้นกำเนิด ได้แก่ Oct-4, Nanog และ Sox2 ร่วมกับ transcription factor ที่กระตุ้น epithelial-mesenchymal transition (EMT) ได้แก่ ZEB1, Snail and Slug มีการแสดงออกของโปรตีนลดลง นอกจากนี้พบว่าลักษณะความเป็นเซลล์ต้นกำเนิดของเซลล์ dermal papilla ที่แยกโดยตรงจากมนุษย์ลดลงเมื่อได้รับ GA และ GE ในการศึกษาก่อนตั้งตำรับเมื่อวิเคราะห์ปริมาณสารสำคัญ GA ด้วยวิธี TLC-densitometric พบว่าตัวอย่างของ GA และ GE ที่ใช้ในงานวิจัยมีปริมาณ GA อยู่ 80.49% and 11.42% ตามลำดับ สูตรตำรับที่ใช้ทดสอบในอาสาสมัครของเจลที่มีส่วนผสมของ GA เข้มข้น 15% w/w เตรียมโดยใช้ propylene glycol และ carbomer 940 เข้มข้น 2% w/w ส่วนสูตรตำรับเจลที่มีส่วนผสมของ GE เข้มข้น 15% w/w เตรียมโดยใช้น้ำ : propylene glycol (1:1 w/w) และ carbomer 940 เข้มข้น 1% w/w เป็นตัวทำละลายและสารก่อเจลตามลำดับ ผลการทดสอบความคงตัวแสดงให้เห็นว่าความใส สี ค่าความเป็นกรด-เบสและความหนืดของเจลไม่เปลี่ยนแปลงเมื่อทดสอบด้วยวิธี Heating - cooling cycle ที่อุณหภูมิ 45 °C และ 4 °C จำนวน 6 รอบ และยังพบว่าปริมาณสารสำคัญ GA ภายในเจล ไม่เปลี่ยนแปลงภายในเวลา 30 วัน เมื่อเก็บที่อุณหภูมิ 30 ° C การทดสอบอาการระคายเคืองแสดงให้เห็นว่าสูตรตำรับเจลที่มีส่วนผสมของ GA และ GE เข้มข้น 15% w/w นั้นไม่ก่อให้เกิดการระคายเคืองในอาสาสมัครจำนวน 22 คน จากนั้นให้อาสาสมัครทาเจลที่มีส่วนผสมของ GA หรือ GE เข้มข้น 15% w/w ที่รักแร้บริเวณที่ถูกโกนขน วันละ 2 ครั้งเช้าและเย็น โดยทาต่อเนื่องเป็นเวลา 28 วัน ผลการศึกษาพบว่าเจลที่มีส่วนผสมของ GA เข้มข้น 15% w/w และ GE เข้มข้น 15% w/w สามารถลดพื้นที่ผิว ความหนา ความยาว และจำนวนเส้นขนที่รักแร้ได้อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับยาพื้นเจลหลังจากทาเจลเป็นเวลา 14, 21 และ 28 วัน และพบว่าเจลที่มีส่วนผสมของ GA เข้มข้น 15% w/w มีประสิทธิภาพชะลอการเจริญของเส้นขนเหนือกว่าเจลที่มีส่วนผสมของ GE เข้มข้น 15% w/w อย่างมีนัยสำคัญทางสถิติ นอกจากนี้เพื่อเป็นการลดต้นทุนในการผลิตผู้วิจัยจึงเตรียมเจลที่มีส่วนผสมของ GA เข้มข้น 7.5% w/w และเจลที่มีส่วนผสมของ GE เข้มข้น 10% w/w และนำมาใช้ในการศึกษานี้ด้วย ผลการศึกษาพบว่าเจลที่มีส่วนผสมของ GA เข้มข้น 7.5% w/w และเจลที่มีส่วนผสมของ GE เข้มข้น 10% w/w ไม่ก่อให้เกิดอาการระคายเคืองในอาสาสมัครจำนวน 22 คน และพบว่าเจลที่มีส่วนผสมของ GA เข้มข้น 7.5% w/w และเจลที่มีส่วนผสมของ GE เข้มข้น 10% w/w สามารถลดพื้นที่ผิว ความหนา ความยาว และจำนวนเส้นขนที่รักแร้ได้อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับยาพื้นเจล หลังจากทาเจลเป็นเวลา 14, 21 และ 28 วัน โดยผลการศึกษาแสดงให้เห็นว่าเจลที่มีส่วนผสมของ GA เข้มข้น 7.5% w/w และ GE เข้มข้น 10% w/w มีประสิทธิภาพในการชะลอการเจริญของเส้นขนใกล้เคียงกับเจลที่มีส่วนผสมของ GA เข้มข้น 15% w/w และ GE เข้มข้น 15% w/w ตามลำดับ ดังนั้นการศึกษานี้จึงแสดงให้เห็นถึงกลไกที่ GA และ GE ควบคุมการทำงานของเซลล์ dermal papilla และให้ข้อมูลทางวิทยาศาสตร์ที่สนับสนุนการใช้ GA และ GE เพื่อชะลอการงอกของเส้นขน

ภาควิชา	วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม	ลายมือชื่อนิสิต .....
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5576557733 : MAJOR PHARMACEUTICAL TECHNOLOGY

KEYWORDS: GLYCYRRHIZIC ACID / DERMAL PAPILLA CELL / EMT / STEMNESS / LICORICE

CHAYANIN KIRATIPAIBOON: THE EFFECT OF GLYCYRRHIZIC ACID AND AQUEOUS LICORICE EXTRACT ON HAIR GROWTH RETARDATION: *IN VITRO* MECHANISTIC AND EFFICACY STUDIES IN HUMAN. ADVISOR: ASSOC. PROF. PITHI CHANVORACHOTE, Ph.D., CO-ADVISOR: ASSOC. PROF. PARKPOOM TENGAMNUAY, Ph.D., 111 pp.

The growth rate of hair is tightly associated with the functions of dermal papilla cells (DPCs), stem cells component of hair follicle. The present study has revealed for the first time that glycyrrhizic acid (GA), the primary active ingredient of licorice or *Glycyrrhiza glabra* L., and aqueous *G. glabra* L. extract (GE) significantly reduced the stem cell-related pathways of DPCs. Stem cell features including clonogenic growth and stem cell markers in DPCs were found to be dramatically decreased in response to GA and GE treatments. Regarding molecular mechanism, GA and GE were shown to suppress ATP-dependent tyrosine kinase/glycogen synthase kinase3 beta-dependent (Akt/GSK3 beta) leading to b-catenin down-regulation. Besides, the down-stream stem cell transcription factors of Wnt/beta-catenin pathway, namely, Oct-4, Nanog and Sox2 were significantly down-regulated in the GA-treated and GE-treated cells. In addition, GA and GE were shown to depress epithelial-mesenchymal transition (EMT) in DPCs as the transcription factors ZEB1, Snail and Slug were markly decreased. The effect of GA and GE on the decline of stem cell features were also exhibited in the primary DPCs directly isolated from human hair follicles. TLC-densitometric method demonstrated GA content of GA and GE samples with value of 80.49% and 11.42%, respectively. The final test formulation for *in vivo* studies of 15% w/w GA gel was prepared by using propylene glycol and 2% w/w carbomer 940 whereas 15% w/w GE gel was prepared utilizing water : propylene glycol (1:1 w/w) and 1% w/w carbomer 940 as solvent and gelling agent, respectively. Stability testing showed no changes of clarity, color, pH value and viscosity after subjected the gels to 6 cycles of heating-cooling cycle and there was no significant change of GA content in the gels from time zero to 30 days at 30 °C. Furthermore, the gels showed no short term and long term irritation effect on 22 volunteers. After treatment with 15% w/w GA and 15% w/w GE gels, the results indicated that 15% w/w GA and 15% w/w GE gels significantly decreased underarm hair growth in respect of area, thickness, length and number after 14, 21 and 28 days compared with its gel base. Besides, 15% w/w GA gel showed superior hair growth retardation effect than 15% w/w GE gel after 28 days. In order to reduce cost of production, 7.5% w/w GA and 10% w/w GE gel were prepared and tested in the present study. Likewise, the results demonstrated that 7.5% w/w GA and 10% w/w GE gels showed no short term and long term irritation effects (n=22) and significantly decreased underarm hair growth in respect of area, thickness, length and number after 14, 21 and 28 days compared with its gel base. Moreover, hair growth retardation effect of 7.5% w/w GA and 10% w/w GE gels were found to be comparable with 15% w/w GA and 15% w/w GE gel, respectively. Therefore, these findings unveiled a novel molecular mechanism regulating DPCs' function of GA and GE as well as provided the scientific information supporting the use of these compounds for suppressing the growth of unwanted hair.

Department:	Pharmaceutics and Industrial Pharmacy	Student's Signature	.....
Field of Study:	Pharmaceutical Technology	Advisor's Signature	.....
Academic Year:	2016	Co-Advisor's Signature	.....

ACKNOWLEDGEMENTS

This dissertation could have not been possible without my thesis advisors, Associate Professor Dr. Pithi Chanvorachote, who not only carefully planned out and intensively advised me through my Ph.D. years, but also gave me the priceless opportunity to study and developed critical thinking of my knowledge. Also, I would like to express my sincere gratitude to co-advisor, Associate Professor Dr. Parkpoom Tengamnuay who kindly gave me invaluable intellectual advices and encouragement throughout the course.

I would like to acknowledge Associate Professor Dr. Boonchoo Sritularak, Associate Professor Dr. Wanchai De- Eknamkul, Assistant Professor Dr. Varisa Pongrakhananon and Dr. Voraphol Vejjabhinanta for their generous and invaluable help to complete my manuscript and dissertation. Furthermore, I would like to express warmest thanks to Professor Dr. Yon Rojanasakul who offered me the great research experience in West Virginia University.

Besides, I would like to also thank my thesis committee for their instructive and insightful comments to help improving my dissertation. Special thanks to my laboratory members for their assistance and making the tough working hours seemed easy. I would like to thank their beautiful friendships and I will keep our precious memories forever. My deepest gratitude goes to my dear family for love and support not only during my degree seeking, but throughout my life. Without their encouragement and understanding, I would not have finished this course. This dissertation is dedicated to my parents.

Finally, this research was supported by the Thailand Research Fund through a Research and Researchers for industries program (PHD56I0002) and Greater Pharma Company Limited.

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LIST OF ABBREVIATIONS

DPC	=	dermal papilla cell
GA	=	glycyrrhizic acid
GE	=	*G. glabra* L. extract
IRS	=	inner root sheath
HS	=	hair shaft
ORS	=	outer root sheath
M	=	melanocyte
HM	=	hair matrix
EMT	=	epithelial-mesenchymal transition
GSK	=	glycogen synthase kinase
Akt	=	ATP-dependent tyrosine kinase
TCF/LEF	=	T-cell factor/lymphoid enhancing factor
ALDH1A1	=	aldehyde dehydrogenase 1A1
DMSO	=	dimethylsulfoxide
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PI	=	propidium iodide
TUNEL	=	Terminal deoxynucleotidyl transferase dUTP nick end labeling
SCMC	=	sodium carboxymethylcellulose
HPMC	=	hydroxypropyl methylcellulose
HEC	=	hydroxyethyl cellulose

TLC	=	thin layer chromatography
LOD	=	limit of detection
LOQ	=	limit of quantification

CHAPTER I

INTRODUCTION

Unwanted hair can cause the embarrassment and be a source of distress leading to a significant emotional burden (Dawber, 2005). Nowadays, many hair removal products have been claimed to decrease the rate of hair growth; however, the scientific evidence of their effect as well as the mechanism on hair growth retardation remain unclear.

The dermal papilla cells (DPCs), the specialized mesenchymal cells located at the base of hair follicle, have been recognized as the key players in hair follicle formation and hair growth (Stenn and Paus, 2001). Various cytokines secreted from DPCs were shown to induce keratinocytes proliferation resulting in hair shaft outgrowth. Interestingly, substantial evidence indicated that cytokine level and the amount of DPCs lying underneath hair follicles determine the rate as well as the duration of hair growth (Elliott et al., 1999; Paus and Cotsarelis, 1999; Paus and Foitzik, 2004). Therefore, DPCs are indicated as the signaling center in hair follicle cycling. Furthermore, DPCs are multipotent stem cells, their function in regulation of the hair follicle as described were shown to link with their stemness (Ito et al., 2007; Driskell et al., 2009; Driskell et al., 2012; Clavel et al., 2012). This concept was supported by the fact that knockout of Sox2, one of the major transcription factors that maintain stemness of stem cells, in DPCs led to the impairment of the hair shaft outgrowth in transgenic animals (Clavel et al., 2012). Moreover, Wnt pathway and fibroblast growth factor genes that maintain capability in inducing hair growth of DPCs were more prominent in the Sox2-positive DPCs (Driskell et al., 2009). Hence, suppression of stemness in DPC may lead to hair growth retardation.

Importantly, the Wnt/β-catenin signaling appears to lend strong support to maintain the stemness in human stem cell as well as hair follicle morphogenesis and regeneration (Enshell-Seijffers et al., 2010; Kishimoto et al., 2000; Shimizu and Morgan,

2004). A research in mouse model demonstrated that knockout of β-catenin in DPCs resulted in the decrease of fibroblast growth factors and insulin-like growth factors expressions which are the crucial signaling pathway in hair follicle formation as well as growth cycling (Enshell-Seijffers et al., 2010). Furthermore, in the same study also reported the inhibition of hair follicle formation was observed after β-catenin in DPCs had been genetically inhibited (Enshell-Seijffers et al., 2010). Moreover, the transcription factors that maintain stemness of stem cells, including Oct-4, Nanog and Sox2, are the downstream target of Wnt/β-catenin signaling (Van Raay et al., 2005; Pereira et al., 2006; Cole et al., 2008). Therefore, the interruption of this pathway may account for the molecular mechanism of the impairment of the hair shaft outgrowth through reducing stemness in DPCs.

Glycyrrhiza glabra L. (Fabaceae), colloquially known as licorice, is a medicinal plant of ancient origin and distributed in subtropical climate of Europe, Middle East and Western Asia. Recent study demonstrated that treatment of idiopathic hirsutism patients with 755 nm alexandrite laser plus topical *G. glabra* L. extract (GE) was more effective in decreasing number of facial hairs than alexandrite laser alone (Faghihi et al., 2015). Interestingly, this ability is likely to be associated with glycyrrhizic acid (GA), one of the active constituents of *G. glabra* L.. GA has demonstrated a promising effect on reduction of hair growth as well as the number of hairs in rat without any signs of skin irritation even after long-term treatment (Petrou, 2010; Ivosevic-Zaper et al., 2014). Nevertheless, the effects of GA as well as GE on stemness of DPCs have not yet been investigated. Furthermore, the effect of GA and GE on hair growth in human still remains largely unclear. Therefore, this study aimed to investigate the molecular basis of GA and GE on the cellular signals regulating stem cell phenotypes in DPCs and evaluate the effect of these compounds on hair growth in human.

Knowledge gained from the present study would unveil the molecular mechanism regulating stem cell features in DPCs, which facilitate the development of novel strategies to minimize the unwanted hair growth. In addition, it may provide some scientific evidence supporting the use of these relatively safe compounds for suppressing the growth of unwanted hair.

CHAPTER II

LITERATURE REVIEW

The presence of excessive hair growth is not dangerous; nevertheless it can become a suffering in silence, especially in women. Several studies clearly showed that the affected women suffered from anxiety and depression leading to a reduced quality of life (Loo and Lanigan, 2002; Guyatt et al., 2004). Hence, unwanted hair growth should be viewed as health issue and should not be included as a purely cosmetic concern.

1. Hair follicle structure

Hair follicle structure (Figure 1) undergoes cyclical changes during each stage of hair growth cycle. In anagen, hair structure are comprised of 2 distinct compartments i.e. hair follicle and hair shaft. Hair follicles reach into the deep dermis and their bulbs are embedded in subcutaneous fat layer. The pluripotent keratinocytes of hair matrix are contained within hair follicle bulb. Matrix cells at the lower part of hair bulb are rapidly proliferating cells, while the matrix cells in the upper part of hair bulb have a slow mitosis rate and can differentiate into the middle and innermost layers of hair follicle, which are the inner root sheath (IRS) and hair shaft. The outermost layer of hair follicle, outer root sheath (ORS), separates hair follicle structure from dermis and be a source of epithelial stem cell at its bulge region. Pigmentation of hair shaft results from melanocytes, which locate within the hair matrix of hair follicle. The melanin granule produced by melanocytes was transferred to keratinocytes of the growing hair shaft leading to pigmentation of hair shaft. Importantly, a cluster of mesenchymal DPCs also reside at the base of hair follicle and contain capillary loops that provide nutrient from the blood. Several lines of evidence indicated that DPC number is correlated with the size of hair follicle and hair shaft, therefore, DPC function as a regulator of hair growth (Stenn and Paus, 2001; Paus and Foitzik, 2004).

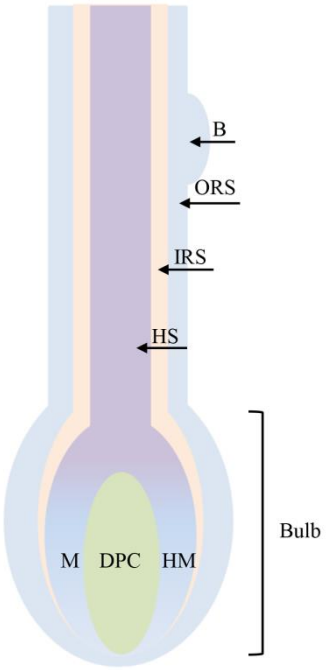


Figure 1 Diagram of hair follicle structure. Abbreviation: B, bulge; ORS, outer root sheath; IRS, inner root sheath; HS, hair shaft; M, melanocyte; DPC, dermal papilla cell; HM, Hair matrix.

2. Hair growth cycle

Each hair follicle grows in repeated cycles of 3 distinct phases (Figure 2) which are anagen, catagen and telogen phases (Paus and Cotsarelis, 1999; Stenn and Paus, 2001; Schneider et al., 2009). In the anagen or active growth phase, daughter cells of pluripotent keratinocytes move upwards and fully differentiate into distinct epithelial hair lineages of IRS and hair shaft. The fully differentiated keratinocytes giving rise to hair shaft extrude their organelles and tightly pack to build cysteine-rich keratin. The growing hair shaft moves toward the skin surface and remain in its canal through interlock of IRS and their cuticle structures. The length of hair shaft in different sites of the body is proportional to the duration of anagen. After that, the hair follicle enters the catagen or regression phase. During catagen phase, hair follicle rapidly regresses by apoptosis of matrix keratinocyte, IRS and ORS resulting in the formation of epithelial strands, while pluripotent keratinocytes in the bulge region and DPCs escape apoptosis.

Thus, hair shaft stops growing and form the club hair, which move up until it reaches the bulge region. The DPCs become inactive and move upward to maintain contact with the bulge region. Subsequently, the hair enters the telogen or resting phase. In this phase, DPCs still attach to the bulge region containing hair follicle stem cells this allows the interaction between DPCs and hair follicle stem cells to re-enter the anagen phase of hair follicle by stimulating the hair follicle stem cells (Schneider et al., 2009). At the end of this stage, hair shaft can be shed passively by mechanical forces.

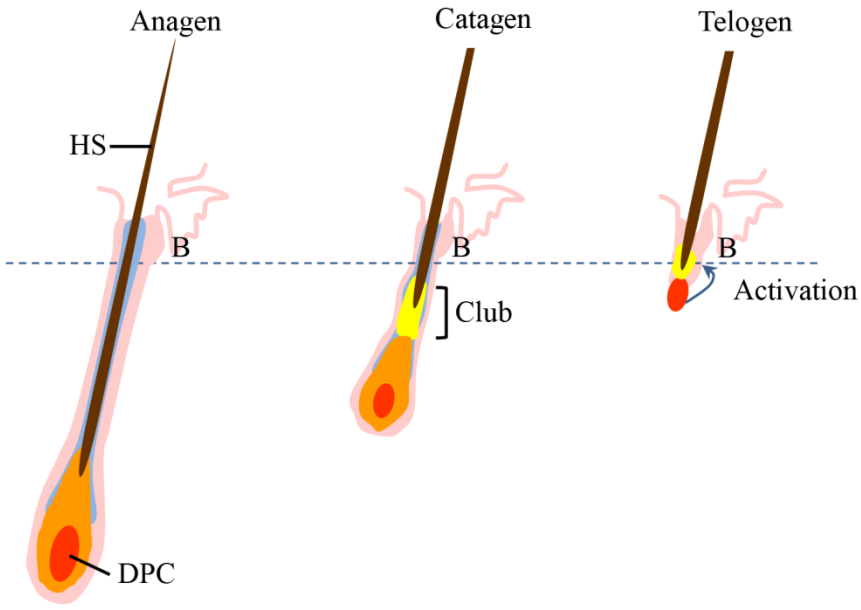


Figure 2 Hair growth cycle. Hair follicle produces new hair shaft during anagen. As the fully differentiated keratinocytes are tightly packed to form cysteine- rich keratin throughout anagen, hair shaft continuously grows and reaches the skin surface. During catagen, the lower two third of epithelial compartments of hair follicle are underwent apoptosis and club structure is developed where hair shaft is retained. Subsequently, the hair follicle enters telogen until DPCs activate hair follicle stem cell within bulge in order to form a new hair shaft of the next growth cycle. Abbreviations: B, bulge; DPC, dermal papilla cell; HS, hair shaft.

3. Stemness of DPCs and hair growth regulation

Hair follicle comprises of epithelial and mesenchymal compartments. DPCs, the major cell population existing in the mesenchymal compartment at the base of hair follicle, function as the most important driver to instruct the hair follicle cycling (Paus and Cotsarelis, 1999; Stenn and Paus, 2001). These cells send the specific cues through different cytokines to the epithelial stem cells and matrix cells within hair follicle bulb to migrate and rapidly proliferate in order to differentiate into distinct cell lineages that make up of IRS and hair shaft resulting in the growing hair shaft. Therefore, the number of DPCs has been reported to determine the duration as well as the rate of hair growth (Elliott et al., 1999; Paus and Cotsarelis, 1999; Paus and Foitzik, 2004).

Recent findings indicated that DPCs are multipotent stem cell since it can form sphere with capacity to differentiate into adipogenic, osteogenic and hematopoietic lineages by using specific inducers (Driskell et al., 2011); however, the DPCs spontaneously differentiate into fibroblast in culture (Richardson et al., 2005). Surprisingly, several lines of evidence has suggested that stemness of DPCs is important for regulating hair follicle as described above (Driskell et al., 2009; Driskell et al., 2012; Clavel et al., 2012). Ito et al. (2007) demonstrated that DPCs expressed CD133, a putative marker of stem cells, during early anagen phase. They found that transplantation of CD133-positive DPCs isolated from adult skin of mice and embryonic mouse epithelial cells could induce new hair follicle formation in nude mice while CD133-negative DPCs could not. Furthermore, expression of Sox2, one of the major transcription factors that maintain stemness of stem cells, in DPCs was reported to regulate migration rate of hair shaft progenitors through BMP pathway. Sox2 null DPCs showed increased *Bmp6* and decreased BMP inhibitor *Sostdc1*, Sox2 transcriptional target, led to the reduction of progenitor cells migration and impairment of hair shaft out growth in animals (Clavel et al., 2012). Recently, (Driskell et al., 2009) demonstrated that Wnt pathway and fibroblast growth factor genes that maintain hair inductive ability of DPCs were more prominent in the Sox2-positive DPCs. Therefore, the loss of stem cell-like phenotypes or the suppression of stemness-related signals in DPCs may

shorten the anagen phase as well as reduce the rate of hair growth and benefit the treatment of hirsutism or managing unwanted hair.

4. Wnt/β-catenin signaling in regulation of hair growth and DPCs'stemness

DPCs' stemness was shown to be associated with its ability to produce growth factors for stimulating hair growth since many studies demonstrated that Wnt/β-catenin signaling in DPCs was strongly involved in the hair follicle morphogenesis and regeneration as well as stemness maintenance. Transcription factors in maintaining stemness including stemness related transcription factors, Oct-4, Nanog and Sox2 and epithelial-mesenchymal transition (EMT) activating transcription factors, ZEB1, Snail and Slug, are target genes of Wnt/β-catenin signaling. Also, growth factor inducing hair growth, FGF7, FGF10 and noggin were found to be target genes of Wnt/β-catenin signaling (Cole et al., 2008; Pereira et al., 2006; ten Berge et al., 2008; Enshell-Seijffers et al., 2010). This can be suggested that reduced activation of this signaling cascade may cause a decrease in stemness as well as suppressing hair growth through reduced production of growth factor-inducing hair growth.

Regarding Wnt/β-catenin signaling on hair growth, Kishimoto et al. (2000) reported that exposure of Wnt3 or Wnt7a on mice' DPC by co-culturing with feeder cells resulted in the expression of gene encoding in the Wnt signaling pathway including frizzled7, disheveled 2, GSK3, β-catenin and Lef1 in comparison of control DPC. Moreover, Wnt3a or Wnt7a treated-DPCs were combined with primary keratinocytes and grafted onto nude mice, the result showed that Wnt treated-DPCs significantly induced hair growth compared to control DPC. This can be suggested that Wnt signaling functions in maintenance of the hair inducing ability in DPCs.

Likewise, Shimizu and Morgan (2004) performed the experiment in order to clarify whether Wnt acts on the epithelial compartments of hair follicle to initiate secondary signals that act on DPC or Wnts directly act on PDC. They demonstrated that forced expression of Wnt3a in mice' DPC could maintain hair inducing ability compared with that of control DPCs after grafting on nude mice with keratinocytes, suggesting direct

activity of Wnt on DPC and maintenance of ability to induce hair growth of DPC by Wnt signaling cascade.

Recently, Enshell-Seijffers et al. (2010) illustrated that deletion of β-catenin allele of DPC in mice caused dramatically shorter and thinner of awl, guard, zigzag and auchene hairs in mutant mice compared with that of control animals suggesting the reduction of proliferation rate of pluripotent keratinocytes of hair matrix and shortening of the anagen phase. They also found the decrease BrdU-positive cells of the hair matrix in mutant mice confirming the crucial role of β-catenin in reducing proliferation rate of pluripotent keratinocytes of hair matrix and their progeny. Furthermore, β-catenin deleted DPCs and control DPCs of the animal were purified and evaluated for changes in gene expression by using real-time PCR. Consistent with the decreased proliferation of pluripotent keratinocytes of hair matrix and their progeny, it was found that transcript levels of signaling molecule stimulating proliferation of pluripotent keratinocyte, namely, FGF7, FGF10 and noggin were significantly decreased in β-catenin deleted DPCs indicating the regulation of gene expression by β-catenin in DPCs. As the length of anagen phase is proportional to the hair length, tissue sections of mutant and control animals were collected and analysed for phase of hair follicle. The mutant mice were found to have a premature induction of catagen phase in comparison to control animals, suggesting the role of β-catenin in DPC for anagen maintenance and normal growth of hair follicle.

With extensive insight of stem cell biology, the evidence suggested that Wnt/β-catenin signal played an important role in maintenance of stemness in human stem cells (Miki et al., 2011; Merrill, 2012). In this regard, the expression of stemness and epithelial-mesenchymal transition (EMT) activating transcription factors, which are the downstream targets of Wnt/β-catenin signaling pathway, were found to account for maintenance of stemness in stem cells (Mani et al., 2008; Shenghui et al., 2009). Stemness related transcription factors were found to maintain stemness by suppressing differentiation inducing genes expression(Shenghui et al., 2009). Furthermore, forced expression of EMT activating transcription factors was shown to enhance stem cell

characteristic meanwhile how these transcription factors maintain stemness remains unanswered (Mani et al., 2008).

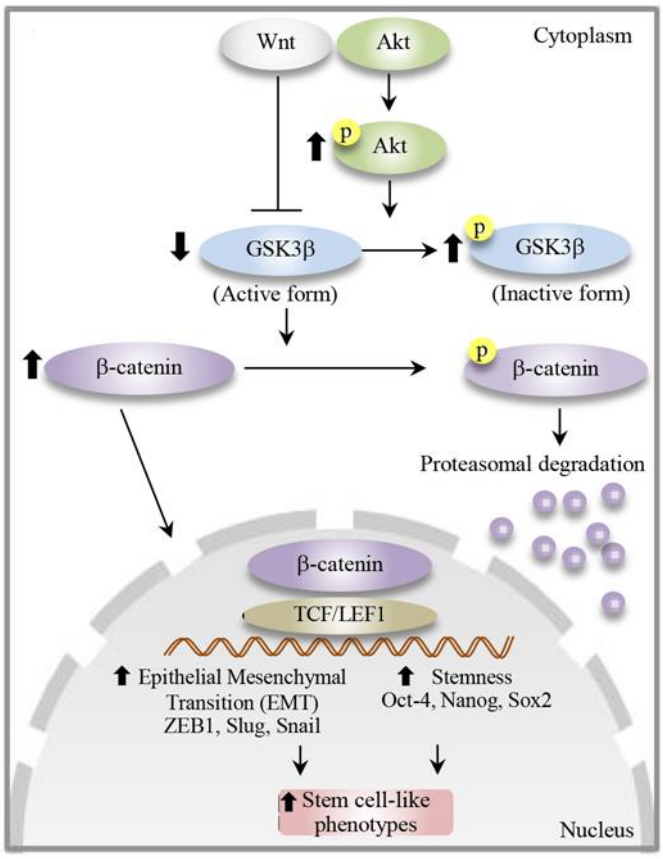


Figure 3 The mechanism of Wnt/β-catenin signaling in maintenance of stemness. (a) Upon activation of Wnt, GSK3β is inactivated. Also, the phosphorylation at GSK3β through the function of active Akt leads to the inhibition of such enzyme. GSK3β negatively regulates β-catenin by the phosphorylation leading to the degradation of phosphorylated β-catenin. Hence, in the presence Wnt and activated Akt cause the accumulation of cellular β-catenin and in turn trigger transcription of genes-activating EMT and stemness.

Molecular mechanism of Wnt/β-catenin signaling in maintaining stem cell features is summarized as shown in Figure 3. The presence of Wnt leads to the inactivation of glycogen synthase kinase (GSK) 3β. The GSK3β is also inactivated by the phosphorylation through the function of activated ATP-dependent tyrosine kinase

(Akt). The function of GSK3β is to phosphorylate β-catenin leading to β-catenin degradation. Therefore, the presence of Wnt and activated Akt, β-catenin accumulates in the cells and functions as a co-trancription factor of T-cell factor/lymphoid enhancing factor (TCF/LEF) and consequently regulates expression of proteins facilitating stem cell functions, including stemness and EMT activating transcription factors (Fukumoto et al., 2001; Miki et al., 2011; Merrill, 2012). Therefore, it is highly possible that attenuating stemness in DPCs through decreased activation of Wnt/β-catenin signaling may be the key mechanism for hair growth reduction (Figure 4).

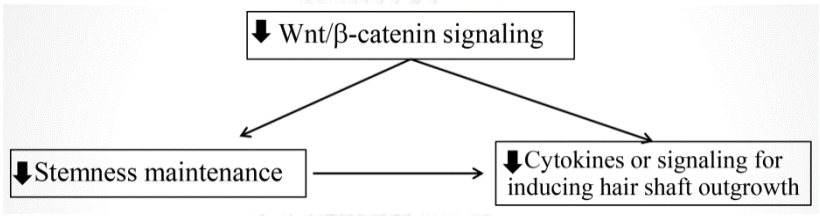


Figure 4 A proposed concept on the interplay between stemness maintenance and ability to induce hair growth of DPCs

5. Growth factor inducing hair growth in DPCs regulated by Wnt/β-catenin signaling

FGF7 and FGF10 are also known as keratinocyte growth factor and keratinocyte growth factor-2, respectively. RNA in situ hybridization analysis revealed its expression in DPCs during anagen phase of hair growth cycle however they were found to down regulate in the late of anagen IV stage. These growth factor functions on stimulating proliferation, migration and differentiation of the pluripotent keratinocytes in bulge and outer root sheath through FGF receptors 1 and 2 on the keratinocyte stem cells' surface (Rosenquist and Martin, 1996; Yang et al., 2010).

Noggin is expressed in DPCs. It was found that genetic inhibition of noggin in mice demonstrated significant retardation of hair follicle induction. Noggin counteracts

with BMP-4, telogen inducer, resulting in the increase expression of Lef-1 transcription factor and the cell-adhesion molecule NCAM, as well as through BMP4-independent downregulation of neurotrophin receptor leading to stimulation of pluripotent keratinocyte proliferation (Botchkarev et al., 1999; Botchkarev et al., 2001)

5. *Glycyrrhiza glabra* L.

Family Fabaceae

Common names

Lacrisse (German), Licorice, Liquorice**,** Sweet wood (English), Reglisse (French), Regolizia (Italian) (Lakshmi and Geetha, 2011; Parvaiz et al., 2014).

Description

Licorice belongs to the pea family and grows in subtropical climates to a height of 4 or 5 feet. The plant is an herbaceous perennial, up to 2 m high. It has oval with odd pinnate leaflets. The individual flower is 1 to 1.5 cm long white to purplish. The taproot is 15 cm long and subdivided into 3 to 5 subsidiary roots. The taproot of licorice has a bright yellow which is important part for medicinal use (Lakshmi and Geetha, 2011; Parvaiz et al., 2014)

Phytochemistry

G. glabra L. root contains glycyrrhizic acid (GA; Figure 5), triterpenoid saponins, , 10-25% of *G. glabra* L. extract (GE) and it is counted as the main active ingredient. GA accounts for sweet taste of *G. glabra* L. root as it is 50 times as sweet as sugar. Currently, it is used as a food flavoring agent. Other active components of *G. glabra* L. were listed as follows.

Saponin: liquiritic acid, glycyrretol, isoglaborlide and licorice acid

Flavonoids and chalcones: liquiritin, rhamniliquiritin, neoliquiritin, liquiritigenin, chalcones, isoliquiritin, iso liquiritigenin, neoisoliquiritin, licuraside, glabrolide, licoflavonol, 5, 8- dihydroxy- 8- methoxyl- flavone- 7- O- beta- D- glucuronide and glychionide B

Isoflavones: glabridin, galbrene, glabrone, shinpterocarpin, licoisoflavones A and B, formononetin, glyzain, kumatakenin, hispaglabridin A, hispaglabridin B, 4′-O-methylglabridin, 3′-hydroxy-4′-O-methylglabridin, glabroiso flavanone A, glabroiso flavanone B

Other components are polysaccharides, pectin, sugar, amino acids, mineral salts, asparagines, bitters, essential oil, fat, female hormone estrogen, gums, mucilage, protein, resins, starches, sterol, volatile oils, tannins and glycosides (Lakshmi and Geetha, 2011).

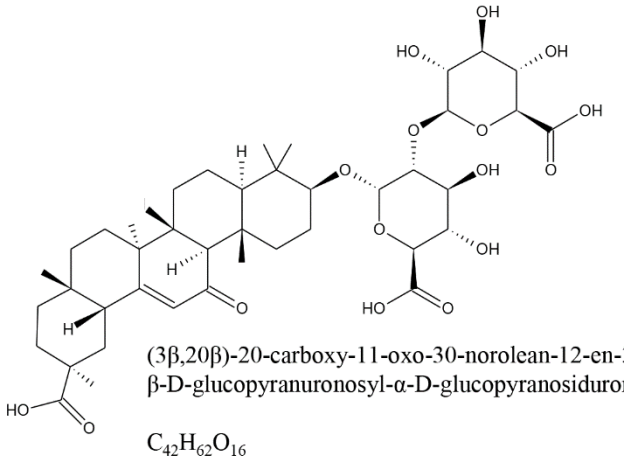


Figure 5 Structure of glycyrrhizic acid (GA)

6. GA and *G. glabra* L. extract (GE) on hair growth retardation

GA was assessed for the effect on hair growth utilizing animal model. The neck area of rats was twice daily treated with 3×500 µl of 15% w/w GA solution. The effect was observed after 3 and 6 days of treatment. The results revealed that there was a visible loss of hair of 20-30% after treatment for 3 days in GA-treated animals compared with that of control animals. The long-term effect of GA treatment was observed by repeated treatment of 15% w/w GA solution for 2 weeks and left untreated for 4 weeks. The results indicated that hair growth was impaired by 10-20% with no skin irritation in the GA-treated animals after 5 and 7 treatment cycles; however, the cuticle of regrowing hair shafts showed smaller and smoother than the control animals (Ivosevic-Zaper et al., 2014). Moreover, the treatment cycles of GA in the animals were

continuously conducted for 2 years by the same group of researchers. Histologic features of hair follicle were then determined using scanning electron microscopy and *in situ* TUNEL assay. The histology of hair follicle of the GA-treated animals obtained from scanning electron microscopy demonstrated destruction of anchoring structures of the hair cuticle and a detachment of the hair shaft from the IRS. Besides, the *in situ* TUNEL assay displayed apoptotic cells in the bulge region after treatment with GA. They also found that the regrowing hair shaft become thinner and the hair density were decreased in the GA-treated animals after each treatment cycle and there was no abnormal skin surface or dysplastic changes at the GA-treated area (Petrou, 2010).

GE had been used for hair removal in Turkmenistan for a long time ago (Ivosevic-Zaper et al., 2014); however, there is no scientific evidence proof of this activity. Recently, the effect of GE on hair growth was investigated in idiopathic hirsutism patients. Each patient was provided with 15% w/w GE gel or blank gel and was instructed to apply twice daily at the dose of half tip finger unit for 24 week on the hirsute areas. All patients were also received 5 treatment sessions at 6-week intervals of alexandrite laser. Subsequently, the changes in terminal hair density after treatment of alexandrite laser plus 15% w/w GE gel and alexandrite laser were determined. The result showed that the treatment of alexandrite laser plus 15% w/w GE gel treatment significantly decreased the mean number of terminal hairs in comparison to alexandrite laser treatment alone and there was no dermatitis or hypersensitivity reaction throughout the follow-up period (Faghihi et al., 2015). Thus, it is likely that GA and GE may suppress the growth of unwanted hair in human. Nevertheless, their effect as well as the molecular mechanism on hair growth retardation still remains unclarified.

CHAPTER III

MATERIALS AND METHODS

Part1. Evaluation of the effect of GA and GE on stemness of DPCs

1. Cell culture

Immortalized dermal papilla cells (DPCs) were obtained from Applied Biological Materials Inc (Richmond, BC). The cells were cultured in Prigrow III medium (Applied Biological Materials Inc, Richmond, BC) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml of penicillin/streptomycin (Life technologies, MD, USA). For primary human DPCs, they were purchased from PromoCell (Heidelberg, Germany). The cells were cultivated in medium containing bovine pituitary extract 4 µl/ml, fetal calf serum 0.05 ml/ml, basic fibroblast growth factor 1 ng/ml, recombinant human insulin 5 µg/ml and phenol red 0.62 ng/ml from PromoCell (Heidelberg, Germany), and 100 units/ml of penicillin/streptomycin. All cells were maintained at 37°C in a humidified incubator with a 5% CO_2 atmosphere and passaged at near confluent with trypsin-EDTA.

2. Plant material

Glycyrrhizic acid (GA) has 99.5% purity was bought from Sigma-Aldrich (Cat. no. 91443; St.Louis, MO). GA was prepared in dimethylsulfoxide (DMSO; Lab-Scan, Dublin, Ireland) and stock sample was further diluted in PBS resulting in working concentrations, with final concentration of 0.1% DMSO. The final concentration of DMSO used in all experiments was 0.1%.

G. glabra L. extract (GE) was kindly given from Greater Pharma Company Limited. GE was prepared by dissolving in PBS resulting in working concentrations.

3. Cell viability assay

Cell viability was evaluated utilizing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Cells were seeded in 96-well plates at 1×10^4 cells per well

and incubated overnight for cell attachment. After exposure to various doses of GA and GE for indicated time, the serum was replaced with 100 µl of MTT solution. Then, the plates were incubated at 37°C for 4 h. After removing supernatant, 100 µl of DMSO was added into each well to dissolve the formazan crystal. The absorbance at the wavelength of 570 nm was measured by microplate reader. Cell viability was calculated by dividing the absorbance of the treated cells by the control cells and presented as a percentage to untreated control value.

4. Nuclear staining assay

To examine apoptotic and necrosis cell death, Hoechst 33342 and propidium iodide (PI) co-staining was carried out. Cells were seeded in 96-well plates at 1×10^4 cells per well and incubated overnight for cell attachment. After exposure to various doses of GA and GE for indicated time, the cells were stained with 10 µM of Hoechst and 5 µg/mL of PI (Molecular Probes, Eugene, OR) for 30 min at 37°C, visualized and imaged by a fluorescence microscope (Olympus IX 51 with DP70; Olympus America Inc., Center valley, PA).

5. Cell cycle analysis

Cells were seeded in 6-well plates at 3×10^4 cells per well and incubated overnight for cell attachment. After exposure to GA and GE for indicated time, the cells were serum-deprived for 24 h. Then, the serum-deprived cells were incubated with complete media for 12 h, trypsinized and fixed with 70% absolute ethanol at 20°C overnight. Afterward, the cells were collected and incubated in PI solution containing 0.1% Triton-X, 1 µg/ml RNase, and 1 mg/ml propidium iodide at 37°C for 30 min. The early passage cells (passages 2-3) without serum-deprivation were used as a non-treated control at 0 h. DNA in whole cells were stained with PI, and cell cycle profile was evaluated using flow cytometry (FACSort, Becton Dickinson, Rutherford, NJ, USA).

6. Cell morphology and aggregation behavior evaluation

Cells were seeded in 24-well plate at 6×10^3 cells per well and incubated overnight for cell attachment. After exposure to various doses of GA and GE for indicated time, morphology and aggregation behavior of cells were observed and

photographed by a phase-contrast microscope (Olympus IX51 with DP70; Olympus America Inc., Center valley, PA).

7. Immunofluorescence

Cells were seeded onto each coverslip in 6-well plate at 3×10^4 cells/well and incubated overnight for cell attachment. After exposure to GA and GE for indicated time, the early passage cells (passages 2-3) were used as a non-treated control at 0 h. The coverslips were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton-X for 10 min and incubated with 3% BSA for 30 min at room temperature to block unspecific binding. The coverslips were then washed and incubated with CD133 rabbit monoclonal antibody (Cell Applications, San Diego, CA) overnight at 4°C. After incubation with primary antibody, the coverslips were washed with PBS and subsequently incubated with Alexa Fluor 488 conjugated secondary antibody (Invitrogen, Carlsbad, CA) for 1 h at room temperature. The expression of CD133 of all samples was examined using Confocal Laser Scanning Microscopy (Zeiss LSM 510).

8. Western blot analysis

To detect protein expression in response to treatment with GA and GE, the cells were washed twice before being incubated in lysis buffer containing 20 mM Tris·HCl (pH 7.5), 0.5% Triton X, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadade, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride and commercial protease inhibitor cocktail (Roche Molecular Biochemicals) for 30 min on ice. Cell lysates were then separated and measured for protein concentration using BCA protein assay kit (Bio-Rad, Hercules, CA). Equal amount of protein samples (40 µg) were denatured by heating in Laemmli loading buffer at 95°C for 5 min and subsequently loaded on 10% SDS-polyacrylamide electrophoresis before being transferred to 0.45-µm nitrocellulose membranes (Bio-Rad, Hercules, CA). The transferred membranes were blocked with 5% non-fat milk in TBST [25 mM Tris·HCl (pH 7.5), 125 mM NaCl, 0.05% Tween-20] at room temperature for 1 h and incubated with specific primary antibodies against CD133 (Cell Applications, San Diego, CA), aldehyde dehydrogenase 1A1 (ALDH1A1; Santa Cruz Biotechnology, Dallas, Texas),

integrinβ1, phosphorylated ATP-dependent tyrosine kinase (Akt; Ser 473), Akt, phosphorylated glycogen synthase kinase3β (GSK3β; Ser 9), GSK3β, ZEB1, Oct-4, Nanog, Sox2, Slug, Snail and β-actin (Cell Signaling, Danvers, MA) at 4°C overnight. Afterward, membranes were washed twice with TBST for 10 min and incubated with horseradish peroxidase-coupled secondary antibodies for 1 h at room temperature. The bands were visualized using film exposure with chemiluminescence detection system (super signal West Pico; Pierrce, Rockford, IL) and quantified using analyst/PC densitometry software (Bio-Rad, Hercules, California).

Part 2. Preformulation study in preparation of GA and GE gels

9. Determination of GA content in GA and GE samples by thin layer chromatography (TLC)-densitometric

9.1. Instruments and chromatographic conditions

Estimation of GA content using TLC-densitometric was done according to the method of Khera et al. (2011) and Alam et al. (2014) with some modifications. The solution samples were applied as bands of width 6 mm with a CAMAG 100 µl syringe on a 20×10 cm precoated silica gel aluminium plate 60 F254 (EMD Millipore, Corp Billerica, MA) using CAMAG Linomat 5 automatic sample applicator (Muttenz, Switzerland). The application rate was set at 150 nl/s. The TLC plate was developed in a previously saturated CAMAG glass twin-through chamber (20×20 cm) at room temperature using ethyl acetate: glacial acetic acid: methanol: water (8:3:2:1 v/v; EMD Millipore, Corp Billerica, MA) as mobile phase. The plate was then dried with the help of an air dryer. Subsequently, the developed TLC plates were scanned using CAMAG TLC scanner 3 in the absorbance mode at 254 nm and integrated winCATS software version 1.4.4 was used for the analysis. Each sample was prepared and analysed in triplicate.

9.2. Method validation

The validation of TLC densitometric method was performed in terms of linearity, precision, accuracy, specificity, limit of detection (LOD) and limit of quantification (LOQ), according to International Conference on Harmonization (ICH) guidelines (ICH, 2005).

9.2.1. Linearity

A stock solution of standard GA (cat. no. 91443; Sigma-Aldrich, St. Louis, MO) was prepared at the concentration of 1000 µg/ml in methanol : water (80 : 20 v/v). The stock solution was further diluted with methanol : water (80 : 20 v/v) to acquire the working concentration of 250 µg/ml. Aliquots of standard GA solution 0.5, 1, 2, 4, 6, 8 and 10 µl were spotted on TLC plate to furnish amount in the range of 0.125, 0.25, 0.5, 1, 1.5, 2 and 2.5 µg/spot. The peak areas were plotted against the corresponding concentrations to obtain the calibration curve. A linear calibration curve was generated using least-squares linear regression analysis.

9.2.2. Precision

The precision of the method was assessed in terms of intra- and inter-day variations by spotting different concentrations of standard GA 0.125, 0.25 and 0.5 µg/spot onto TLC plate on three different times within the same day for intra-day variation. Inter-day variations were determined by analyzing same concentrations of standard GA as described above in three different days over a period of week. The peak areas were recorded and standard GA content was analysed. The precision was expressed as the standard deviation (SD) and percent relative standard deviation (RSD).

9.2.3. Accuracy

To ensure the accuracy of the method, the recovery studies were performed by overspotting the pre-analysed samples of GE with extra 0.125, 0.25 and 0.5 µg/spot of standard GA on TLC plate. The GA content was then analysed and calculated as percentage recovery using the following formula

$$\text{Recovery (\%)} = \frac{\text{Experimental content}}{\text{Theoretical content}} \times 100$$

9.2.4 Specificity

The specificity of the method was verified by analyzing the standard GA, GA and GE samples. The GA and GE samples containing GA was confirmed by comparing R_f values and UV spectra of standard. The peak purity of GA was assessed by comparing the overlay spectra of standard GA, GA and GE samples at three different positions, peak start, peak apex, and peak end of the spot detected at 254 nm.

9.2.5 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were calculated based on standard deviation (SD) of the response and the slope (S) of the calibration curve (y =4669.5x+84.144) according to the formula: LOD = 3.3(SD/S) and LOQ = 10(SD/S), respectively (ICH, 2005). The standard deviation of the response was determined based on the standard deviation of y-intercepts of regression lines.

9.3 Measurement of GA content in GA and GE samples

Aliquots of standard GA solution (250 µg/ml) were spotted on TLC plate in the range of 0.125, 0.25, 0.5, 1, 1.5, 2 and 2.5 µg/spot.

GE samples were prepared by dissolving 5 mg of GE in 1 ml of methanol : water (80 : 20 v/v). The extract solution was then centrifuged at 8,000 rpm for 2 min and the supernatant solution was used for GA content analysis.

GA samples (cat. no. G2137; Sigma-Aldrich, St.Louis, MO) were prepared by dissolving 5 mg of GA in 5 ml of methanol : water (80 : 20 v/v). The GA solution was further diluted in methanol : water (80 : 20 v/v) to obtain the final concentration at 0.5 mg/ml.

An aliquot of 2 µl of each sample was applied on TLC plate followed by development and scanning as described in the section 9.1. All samples were freshly prepared and analysed in triplicate. The content of GA was assessed utilizing the calibration curve.

10. Selection of solvent system

In order to select solvent system, GA and GE solutions at the concentration of 15% w/w were prepared in various solvents including, water, ethanol, polyethylene glycol 400, propylene glycol and their combinations (Table 1 and 2). These compounds were mixed with vortex mixer at 25 °C for 10 min and allowed to equilibrate at room temperature for 24 h. Each solvent was then determined for its color, clarity and sedimentation.

Table 1 The amount of each solvent used in preparation of 15% w/w GA solution

No.	GA (g)	Solvent			
		Water	Ethanol	Polyethylene glycol 400	Propylene glycol
1	0.37	1.64	-	-	-
2	0.37	-	1.64	-	-
3	0.37	-	-	1.64	
4	0.37	-	-	-	1.64
5	0.37	0.82	0.82	-	-
6	0.37	0.82	-	0.82	-
7	0.37	0.82	-	-	0.82

Table 2 The amount of each solvent used in preparation of 15% w/w GE solution

No.	GE (g)	Solvent			
		Water	Ethanol	Polyethylene glycol 400	Propylene glycol
1	0.3	1.7	-	-	-
2	0.3	-	1.7	-	-
3	0.3	-	-	1.7	
4	0.3	-	-	-	1.7
5	0.3	0.85	0.85	-	-
6	0.3	0.85	-	0.85	-
7	0.3	0.85	-	-	0.85

11. Selection of concentration of gelling agents

GA and GE gels at the concentration of 15% w/w were prepared by separate dissolving GA and GE in the chosen solvent system. The solutions of these compounds

were separately added with different gelling agents which are 1) 1-3% w/w sodium carboxymethylcellulose (SCMC), 2) 0.5-2% w/w carbomer 940, 3) 2-4% w/w hydroxypropyl methylcellulose (HPMC) and 4) 1-3% w/w hydroxyethyl cellulose (HEC) in order to select the proper concentration of gelling agent (Tables 3 and 4). Concentration of gelling agents was selected based on viscosity, spreadability, clarity and pH. Preparations of GA and GE gels utilizing different gelling agents were briefly described as follows:

SCMC were prepared by dispersing in glycerin then water was added while gently stirring. GA and GE solutions were separately added into different concentrations of SCMC gel. The mixture was stirred gently until homogeneous gel was formed.

Carbomer 940 was gradually added into water while continuous stirring in order to obtain carbomer solution. GA and GE solutions were then separately added into different concentrations of carbomer solution. Subsequently, triethanolamine was added into the carbomer solution until homogeneous gel was formed.

Prior to addition to water, 1/3 of the required volume of water was heated to approximately 70 °C then HPMC powder was added to the hot water while continuous stirring until the particles were thoroughly wetted and evenly dispersed. The remainder of the water was then added as cold water. GA and GE solutions were separately added to HPMC solution during continuous stirring until homogeneous gel was formed.

HEC was dispersed into room temperature water and heated to approximately 80 °C with continuous stirring until the mixture began to thicken. The mixture was then removed from heat and cooled down. GA and GE solutions were separately added to HEC while continuing to stir until completely hydrated.

Table 3 Formulation of 15% w/w GA gels using different gelling agents

Composition (g, %w/w)	S1	S2	S3	C1	C2	C3	HP1	HP2	HP3	HE1	HE2	HE3
GA	1.86	1.86	1.86	1.86	1.86	1.86	1.86	1.86	1.86	1.86	1.86	1.86
SCMC	0.1	0.2	0.3	–	–	–	–	–	–	–	–	–
Carbomer 940	–	–	–	0.05	0.1	0.2	–	–	–	–	–	–
HPMC	–	–	–	–	–	–	0.2	0.3	0.4			
HEC	–	–	–	–	–	–	–	–	–	0.1	0.2	0.3
Propylene glycol	2.97	2.95	2.93	3	2.97	2.95	2.95	2.93	2.9	2.97	2.95	2.93
Triethanolamine	–	–	–	qs	qs	qs	–	–	–	–	–	–
Distilled water	5.08	4.99	4.92	5.09	5.08	4.99	4.99	4.92	4.84	5.08	4.99	4.92

Abbreviation: S1, 1% w/w SCMC; S2, 2% w/w SCMC; S3, 3%w/w SCMC; C1, 0.5% w/w carbomer 940; C2, 1% w/w carbomer 940; C3, 2% w/w carbomer 940; HP1, 2% w/w HPMC; HP2, 3% w/w HPMC; HP3, 4% w/w HPMC; HE1, 1% w/w HEC; HE2, 2% w/w HEC; HE3, 3% w/w HEC.

Table 4 Formulation of 15% w/w GE gels using different gelling agents

Composition (g, %w/w)	S1	S2	S3	C1	C2	C3	HP1	HP2	HP3	HE1	HE2	HE3
GE	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
SCMC	0.1	0.2	0.3	–	–	–	–	–	–	–	–	–
Carbomer 940	–	–	–	0.05	0.1	0.2	–	–	–	–	–	–
HPMC	–	–	–	–	–	–	0.2	0.3	0.4	–	–	–
HEC	–	–	–	–	–	–	–	–	–	0.1	0.2	0.3
Propylene glycol	1.48	1.45	1.43	1.49	1.48	1.45	1.45	1.43	1.4	1.48	1.45	1.43
Triethanolamine	–	–	–	qs	qs	qs	–	–	–	–	–	–
Distilled water	6.97	6.95	6.76	6.99	6.97	6.95	6.95	6.76	6.7	6.97	6.95	6.76

Abbreviation: S1, 1% w/w SCMC; S2, 2% w/w SCMC; S3, 3%w/w SCMC; C1, 0.5% w/w carbomer 940; C2, 1% w/w carbomer 940; C3, 2% w/w carbomer 940; HP1, 2% w/w HPMC; HP2, 3% w/w HPMC; HP3, 4% w/w HPMC; HE1, 1% w/w HEC; HE2, 2% w/w HEC; HE3, 3% w/w HEC.

12. Formulation of GA and GE in gel base

GA and GE gels were prepared utilizing the selected concentration of the gelling agents. These formulations were separately added with preservative, chelating agent, emulsifier and perfume that were the mixture of caprylyl glycol and ethylhexyl glycerin, sodium EDTA, tween 20 and Floreintal TL 9501 as well as anti-oxidants which are sodium metabisulfite and vitamin E at the fixed concentration to obtain the final test formulation. All formulations for each of GA and GE gels were further studied for physicochemical stability.

13. Stability test

13.1. Physical stability

All formulations for each of GA and GE gels were subjected to the physical stability test by using heating-cooling cycles. Each formulation was kept at 4 °C for 24 h and subsequently kept at 45 °C for 24 h, which is completed for one cycle. These formulations were repeated for 6 cycles (Grimm, 1998). After completion of each heating-cooling cycle, they were observed for physical appearances which are color, clarity, pH and viscosity.

13.2. Chemical stability

Samples of GA and GE gels were kept at 30 °C for 30 days and evaluated for their concentration of GA present in the formulation using TLC-densitometry technique as mentioned in section 9.

14. Selection criteria of appropriate formulation of GA and GE in gel

After 13, the two formulations (one for GA and one for GE gels) having the most stable physicochemical and chemical stabilities were used to formulate GA and GE gels for irritation and efficacy studies in human.

Part 3.Evaluation of safety and efficacy of GA and GE on hair growth retardation

15. Protocol of clinical study (Irritation and efficacy evaluation)

15.1. Selection of the volunteers

22 volunteers which were female and male were recruited for this study by the following criteria.

15.1.1 Inclusion criteria

1) Healthy persons aged between 18-50 years

2) Show clearly visible hairs on their armpits

3) Do not show any signs of irritation to the test formulations (test gel and gel base) after irritation test

4) No history of allergies to cosmetic products or any dermatological compounds

5) Voluntarily enroll in this study.

Subjects were required to read and sign a consent form summarizing the discussion before enrolling in this study and were assured that they can withdraw from the study any time.

15.1.2 Exclusion criteria

1) Had history of hyper allergic reactions.

2) Had history of eczema or psoriasis within 6 months before this study.

3) Had used any topical medicine or cosmetics on their armpits within 7 days before participating in this study.

4) Pregnancy or lactating

15.1.3 Discontinuous criteria

1) Develop allergic reactions from using the test product.

2) Concomitantly use any cosmetic products during the study.

3) Request for withdrawal from the study.

4) Other non-compliance to instruction procedure during study

In addition, the volunteers are given a proper medical care from Dr. Voraphol Vejjabhinanta (dermatologist) in case of any suspected allergic reactions.

16. Irritation test of GA and GE gels

Prior to the study, irritation test were done according to the guideline provided by International Contact Dermatitis Research Group (ICDRG; Table 5) (Curry et al., 1991). Briefly, all volunteers were randomly assigned into 2 treatment groups (11 volunteers per group) according to the 2 formulations (1 GA and 1 GE gels). Individuals were excluded if they had any active or history of skin diseases that might interfere with the evaluation of skin irritation. About 0.5 gram of gel containing the test compound and gel base is applied on one square inch at the ventral side of the right and the left subject's forearms, respectively, for 24 h in an occlusive condition. The tested areas were then cleaned with normal saline-soaked cotton prior to immediately evaluate for skin irritation. The tested area was re-evaluated at 48 and 72 h according to International Contact Dermatitis Research Group (ICDRG). Subjects who show no sign of irritation (Grade 0) were included for further study.

Table 5 ICDRG visual grading scale in skin irritation

Grade	Description
0	No observation reaction occurred
+	Slight reaction occurred (small redness 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 of skin attached by the patch is clearly observed)

17. Efficacy evaluation of GA and GE gels on hair growth retardation

The remaining volunteers who passed the irritation test in each group were provided with the assigned formulation of test gel and its corresponding gel base which were the same formulations used in the irritation test. Approximately 0.5 g of the test gel and the gel base were separately applied on the shaved area with diameter 1 inch of each armpit after skin cleansing in the morning and the night for 28 days. Each side of the armpits was randomly assigned so that about half of the subjects in each group were received test product and blank gel on the left and right armpits, respectively. The other half were received the product in reverse order.

In the present study, the effect of each formulation of GA and GE gels on hair growth in the volunteers was compared to its gel base. This effect was focused on area, thickness, length and number of hair. These parameters were determined by Image Pro Plus at day 0, 7, 14, 21 and 28.

18. The data analysis

18.1. Digital camera image of indicated area on both armpits were used for comparison of hair growth between each formulation of the test gel and its gel base.

18.2. The parameters representing hair growth in terms of area, thickness, length and number of hair of both armpits were calculated as % Hair growth at days 0 (after shaving), 7, 14, 21 and 28 in order to compare the effect on hair growth between each formulation of the test gel and its gel base from the following equations;

$$\%\ \text{Area of hair growth} = \frac{\text{Area of hair}_{\text{day x}} - \text{Area of hair}_{\text{day 0}}}{\text{Area of hair}_{\text{day 0}}} \times 100$$

$$\%\ \text{Thickness of hair growth} = \frac{\text{Thickness of hair}_{\text{day x}} - \text{Thickness of hair}_{\text{day 0}}}{\text{Thickness of hair}_{\text{day 0}}} \times 100$$

$$\%\ \text{Length of hair growth} = \frac{\text{Length of hair}_{\text{day x}} - \text{Length of hair}_{\text{day 0}}}{\text{Length of hair}_{\text{day 0}}} \times 100$$

$$\%\ \text{Number of hair growth} = \frac{\text{Number of hair}_{\text{day x}} - \text{Number of hair}_{\text{day 0}}}{\text{Number of hair}_{\text{day 0}}} \times 100$$

18.3. The difference in % Hair growth (Δ % Hair growth) of area, thickness, length and number of hair between each formulation and its gel base at day 28 was determined in order to compare the efficacy on reducing hair growth between the 2 formulations using the following equations.

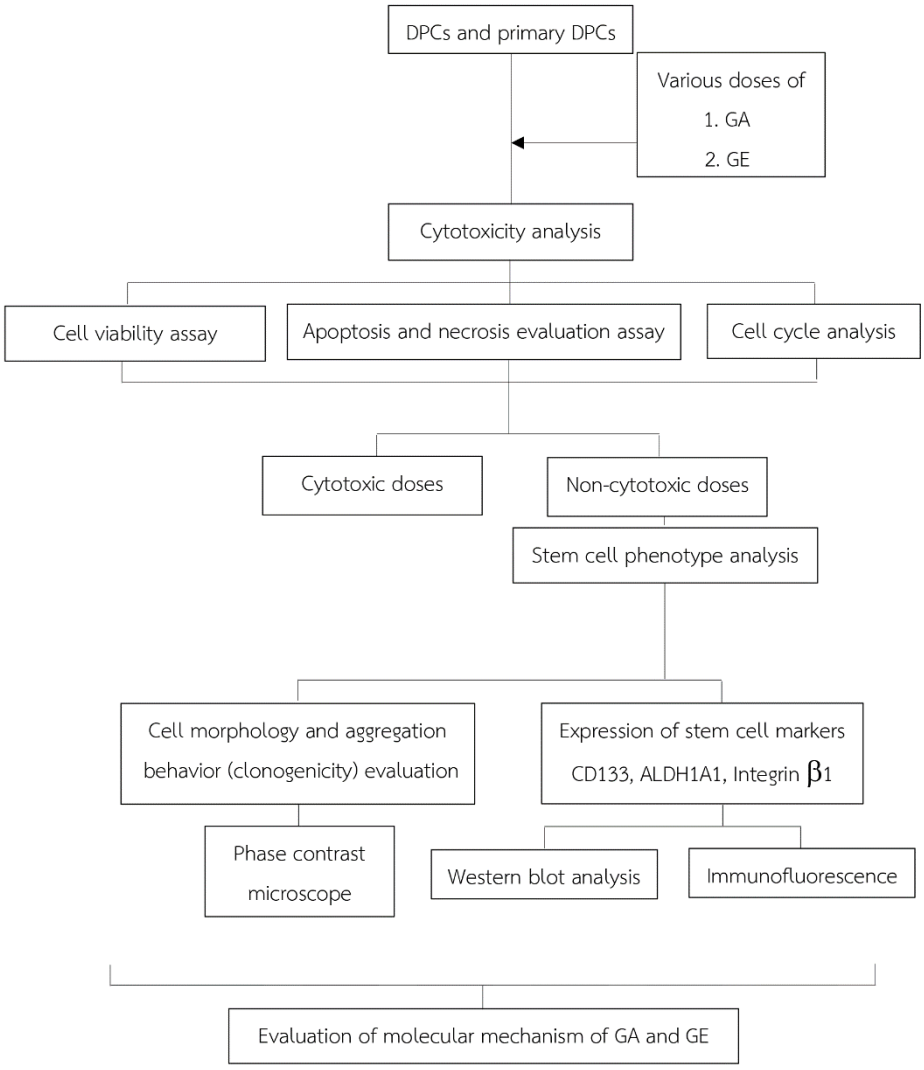
$$\Delta \text{ \% Hair growth} = \text{\% Hair growth}_{\text{gel base}} - \text{\% Hair growth}_{\text{test formulation}}$$

19. Statistical analysis

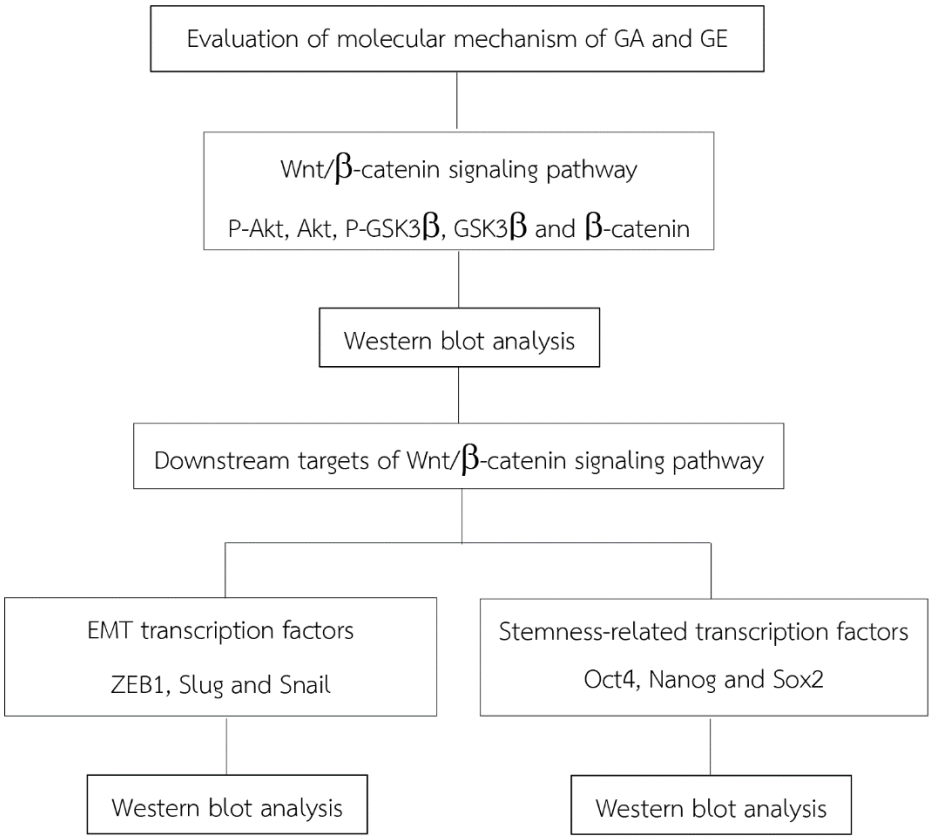
Quantitative data were expressed as means ± standard deviation (SD) from at least three independent experiments. Statistical analysis was performed by student's *t* test and one-way ANOVA followed by multiple comparison test. *P* value less than 0.05 was considered statistical significance.

Research design

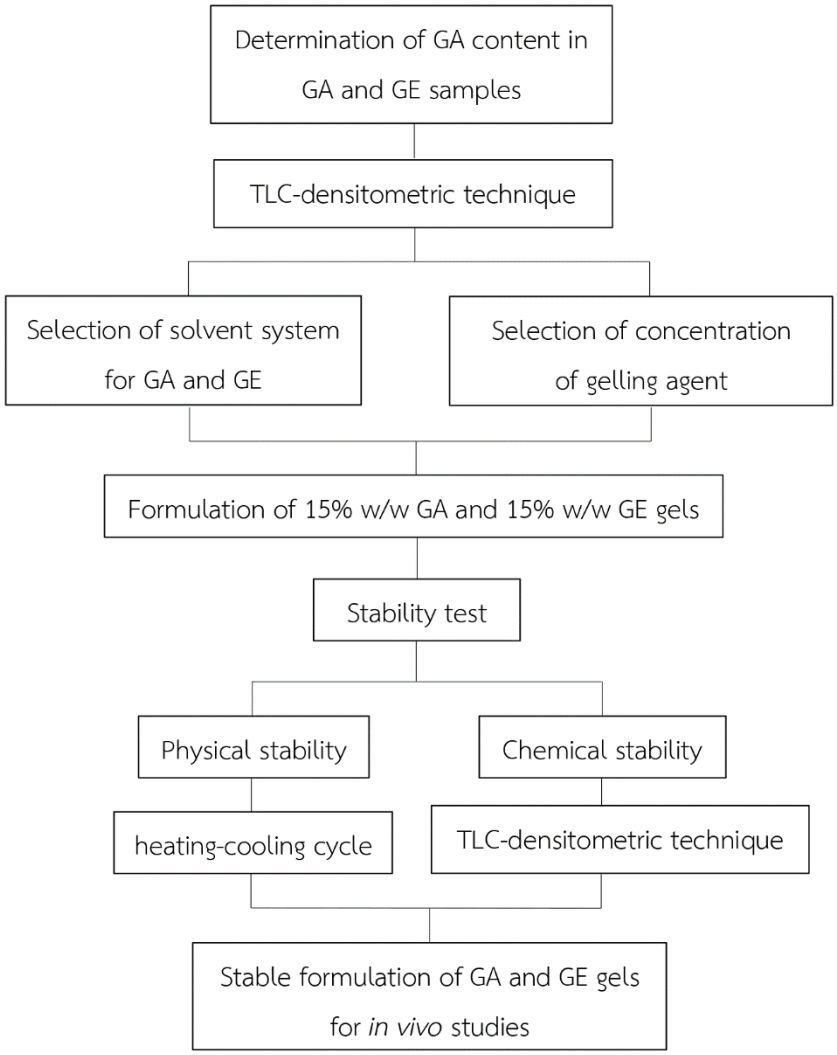
Part 1. Evaluation of the effect of GA and GE on stemness of DPCs



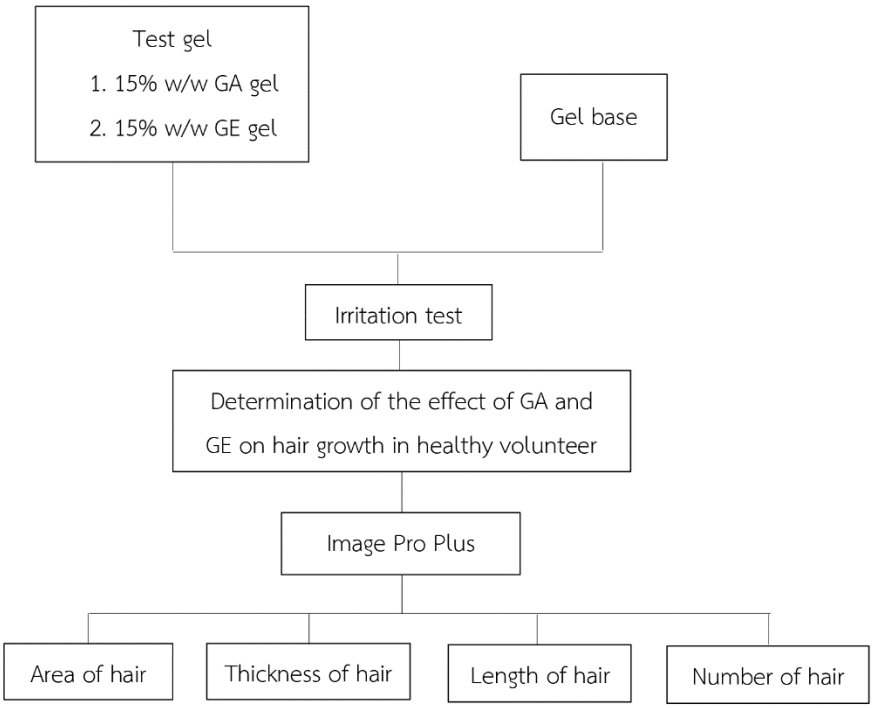
Part1. Evaluation of the effect of GA and GE on stemness of DPCs (cont.)



Part 2. Preformulation study in preparation of GA and GE gels



Part 3. Evaluation of safety and efficacy of GA and GE on hair growth retardation



CHAPTER IV

RESULTS

Part1. Evaluation of the effect of GA and GE on stemness of DPCs

1. Cytotoxicity analysis

1.1 Effect of GA and GE on viability of DPCs.

Prior to determining the effect of GA and GE on stem cell property of DPCs, the non-cytotoxic concentrations of GA and GE were first characterized using cell viability MTT. Treatments of the cells with various concentrations of GA (0-200 μM) and GE (0-200 μg/ml) for 24 h caused no significant change in cell viability compared with the non-treated control (Figure 6). Consistent with apoptosis and necrosis detection by Hoechst 33342/propidium iodide (PI) co-staining showed the absence of apoptotic and necrotic cell death (Figure 7). These data indicated that the treatment of GA and GE at such concentrations caused no cytotoxic effect in DPCs.

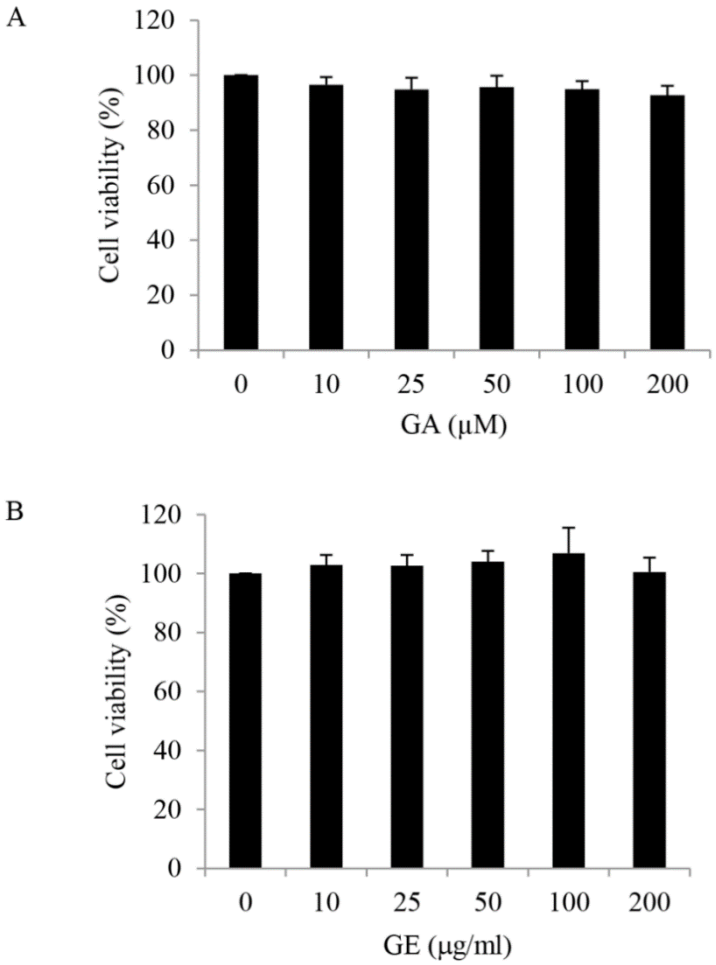


Figure 6 Cytotoxicity of GA and GE on DPCs. DPCs were treated with various concentrations of (A) GA (0-200 μM) and (B) GE (0-200 μg/ml) for 24 h. Cell viability was evaluated by MTT assay. The data is presented as mean ± SD (n=3).

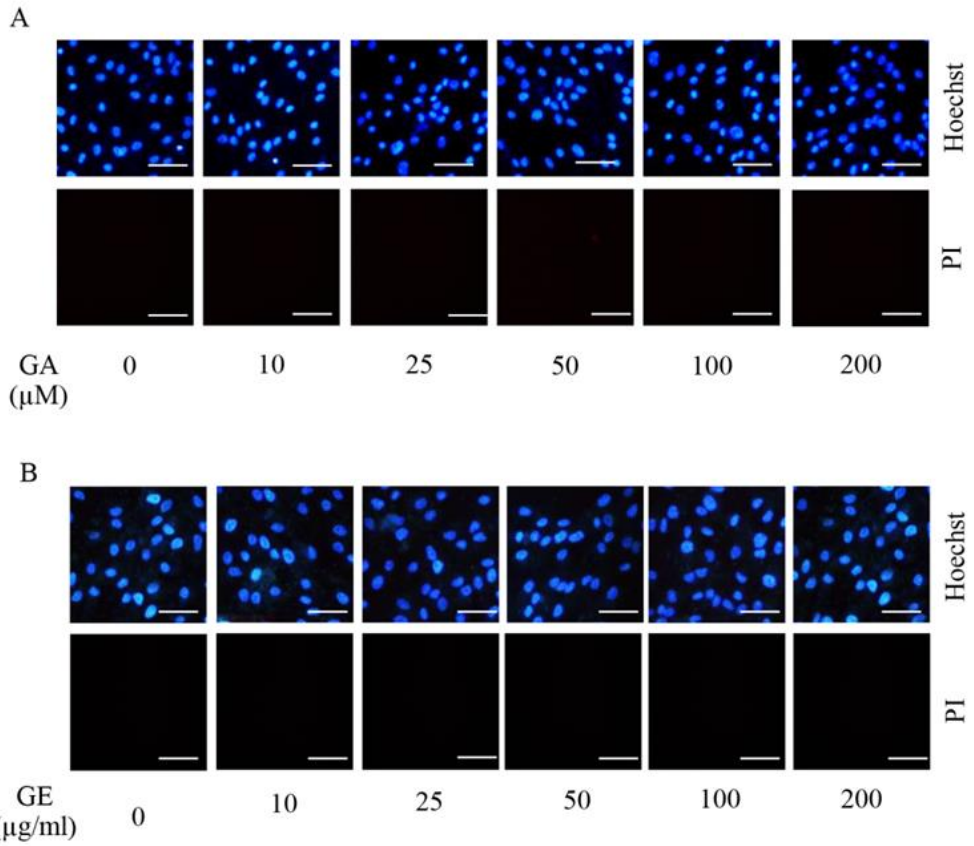


Figure 7 Effect of GA and GE on apoptotic and necrotic cell death. DPCs were treated with various concentrations of (A) GA (0-200 μM) and (B) GE (0-200 μg/ml) for 24 h. Mode of cell death was determined by Hoechst 33342/PI co-staining assay. Scale bar is 100 μm.

1.2 The effect of GA and GE on cell cycle distribution of DPCs

To confirm the above effect of GA and GE on viability of DPCs, the effects of GA and GE on the cell cycle distribution of DPCs were determined. The DPCs were cultivated in the presence or absence of GA (200 μM) and GE (200 μg/ml) for 72 h and subjected to cell cycle evaluation. The cells were serum starved for 24 h. Then, the cells were incubated with complete media for 12 h and the percentage of cells in each phase of cell cycle including G1, S and G2/M was determined by flow cytometer using PI. Furthermore, the early passage DPCs (passages 2-3) without serum-deprivation were used as a control. Figures 8 and 9 illustrate that at 12 h after receiving growth factors, GA-treated and GE-treated cells showed no significant difference in terms of cell proliferation compared with that of the non-treated control cells. These results

indicated that treatment of the cells with GA and GE caused no significant effect on the cell cycle suggesting that the following effects of GA and GE on DPCs were not a consequence of cytotoxic effect or cell stress.

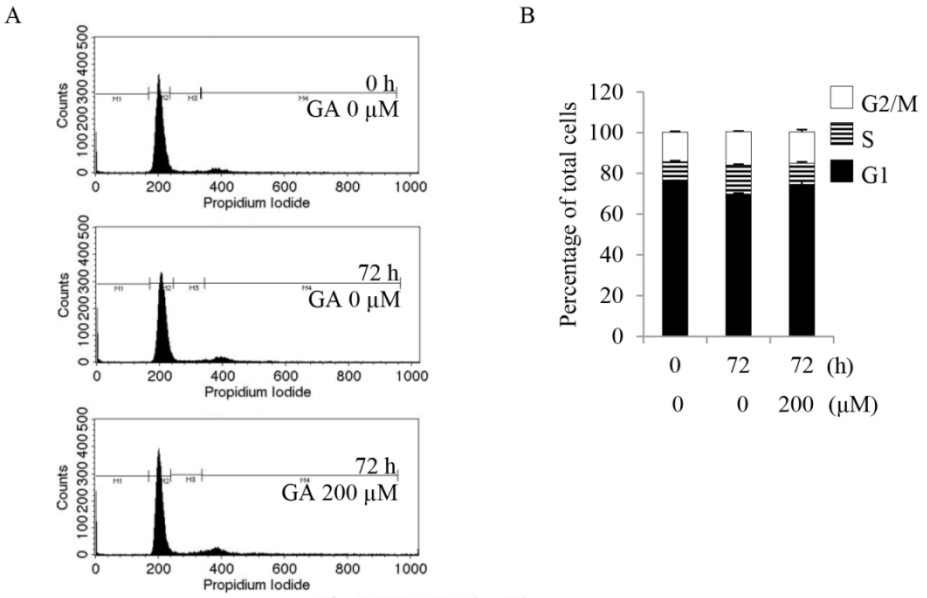


Figure 8 The effect of GA on cell cycle distribution in DPCs. (A-B) Cells were cultured in the presence or absence of GA (200 µM) for 72 h. After indicated treatment, the cells were serum-deprived for 24 h and incubated with complete media for 12 h. The percentage of non-treated and treated cell in G1, S and G2/M phases of cell cycle was assessed by PI staining and flow cytometry. The early passage cells (passages 2-3) without serum-deprivation were used as a non-treated control at 0 h.

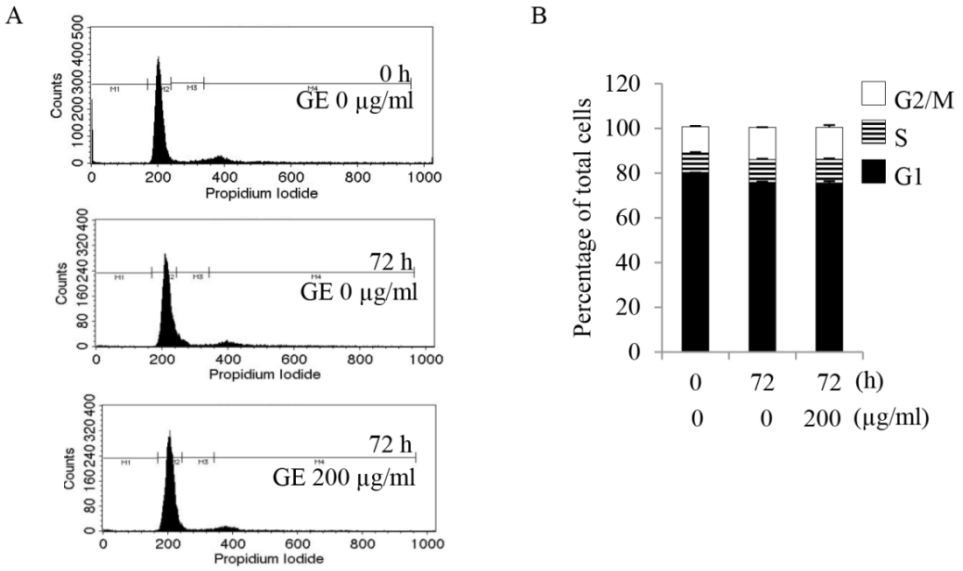


Figure 9 The effect of GE on cell cycle distribution in DPCs. (A-B) Cells were cultured in the presence or absence of GE (200 µg/ml) for 72 h. After indicated treatment, the cells were serum-deprived for 24 h and incubated with complete media for 12 h. The percentage of non-treated and treated cell in G1, S and G2/M phases of cell cycle was assessed by PI staining and flow cytometry. The early passage cells (passages 2-3) without serum-deprivation were used as a non-treated control at 0 h.

2. The effects of GA and GE on the stem cell-like characteristics of DPCs.

DPCs have been reported to function as multipotent stem cells and the stemness of DPCs was tightly associated with their ability to induce hair follicles and control hair growth (Ito et al., 2007; Driskell et al., 2009; Driskell et al., 2012; Clavel et al., 2012). In order to investigate whether GA and GE affect the stem cell-like characteristics in these DPCs, the cells were cultivated in various concentrations of GA (0-200 µM) and GE (0-200 µg/ml) for 0-72 h. Subsequently, morphology of the cells and aggregative pattern were assessed. These results indicated a clearly noticeable change in shape and appearance of most GA-treated and GE-treated cells at the concentrations of 25-200 µM and 50-200 µg/ml, respectively, toward fibroblast-like morphology at 48 and 72 h (Figure 9). Meanwhile, the morphology of most GA-treated and GE-treated cells at the concentration of 10 µM and 10-25 µg/ml, respectively,

remained unaltered (Figure 10). Previous study showed that the hair follicle inductive property of the DPCs was involved in their aggregate behaviors (Osada et al., 2007), therefore, the effect of GA and GE treatments on the aggregative growth pattern of in these cells was investigated. The early passage DPCs (passages 2-3) were cultivated in the presence or absence of GA and GE for 72 h and the aggregate size as well as the number were then evaluated. Figure 11 and 12 demonstrate a significant decrease in size and the number of cell aggregations of GA-treated and GE-treated cells at the concentrations of 100-200 μM and 100-200 μg/ml, respectively, in comparison to those of non-treated control at 72 h.

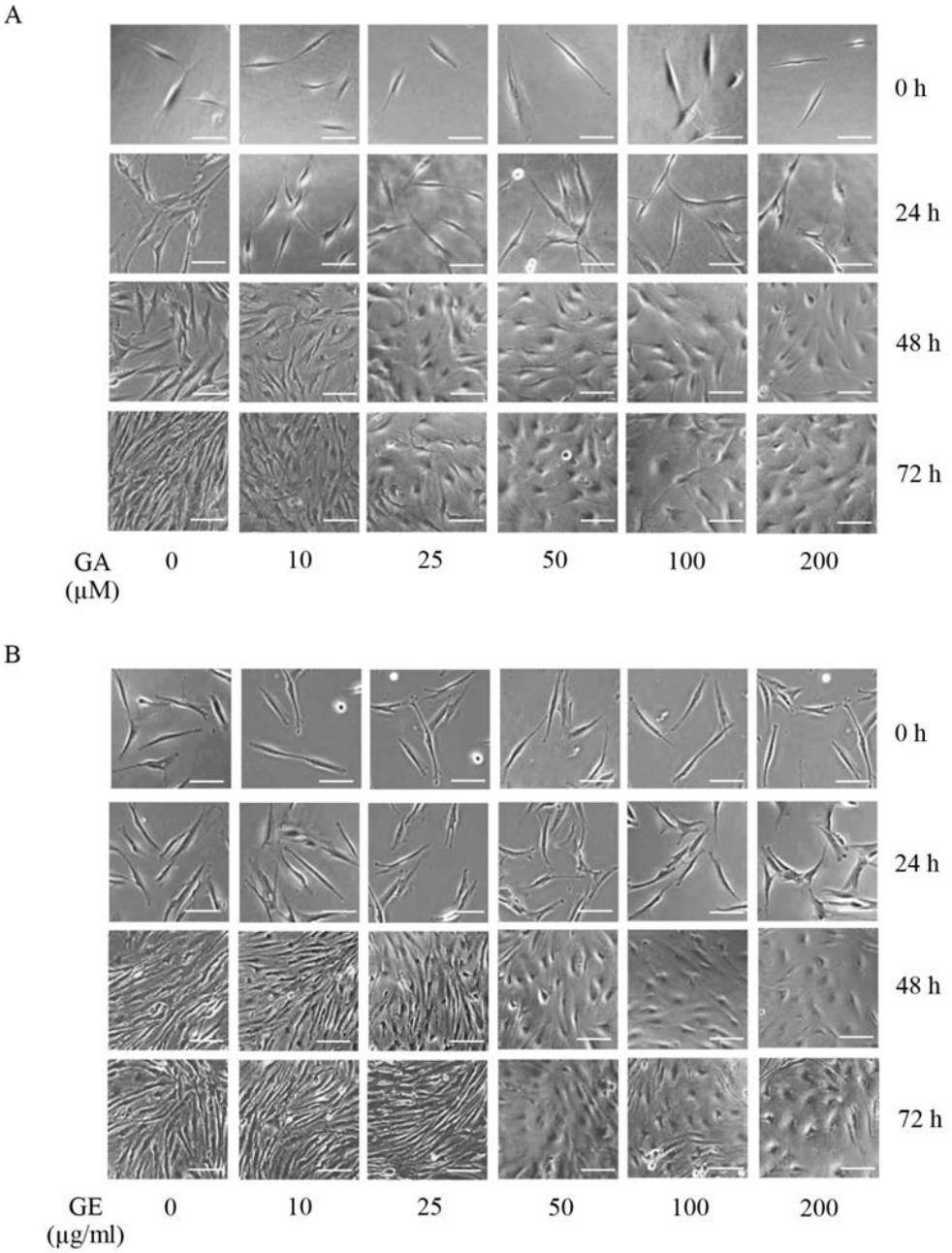


Figure 10 Effect of GA and GE on DPCs' morphology. After cells were treated with (A) GA (0- 200 μM) and (B) GE (0-200 μg/ml) for 72 h, cell morphological changes was continuously examined by phase contrast microscopy (10X) for various times (0-72 h) after treatment. Scale bar is 100 μm.

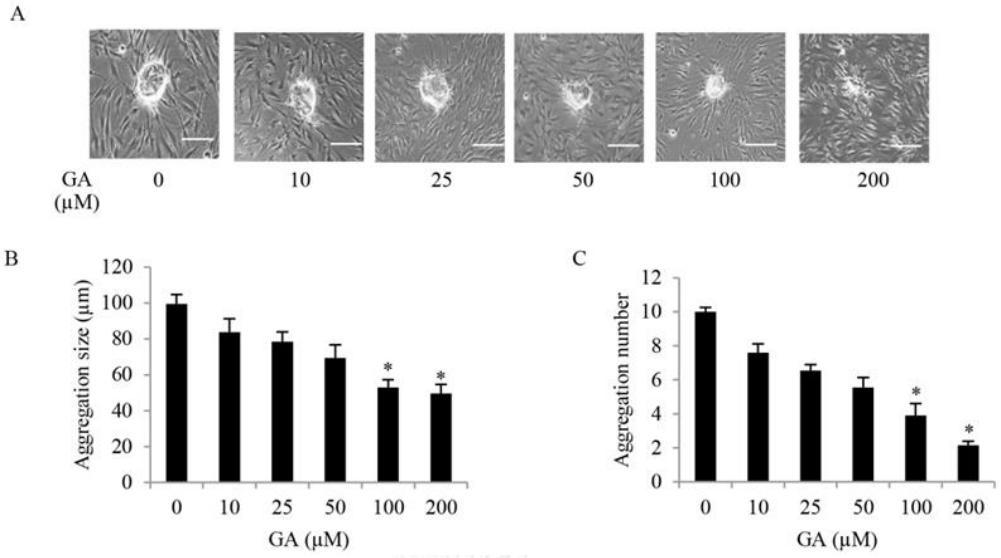


Figure 11 Effect of GA on DPCs' aggregation. (A) After being treated with GA (0-200 μM) for 72 h, phase contrast images of aggregation behavior at 72 h were captured for non-treated and treated cells. Scale bar is 100 μm. (B) Aggregation size and (C) aggregation number were determined by image analyser. The data is presented as mean ± SD (n=3). *P < 0.05 versus non-treated control.

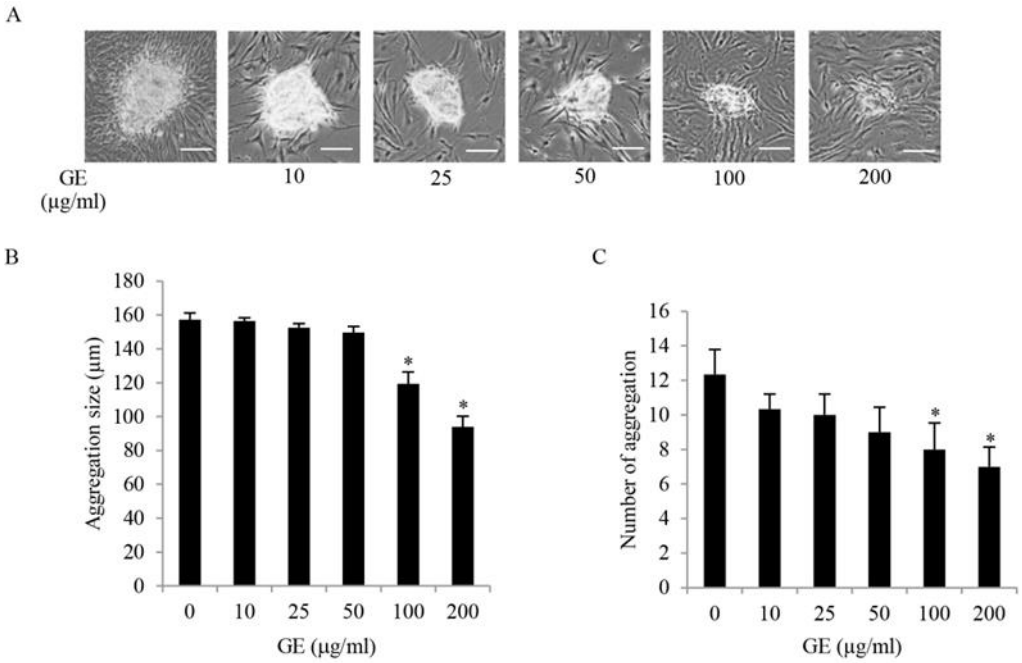


Figure 12 Effect of GE on DPCs' aggregation. (A) After being treated with GE (0-200 μg/ml) for 72 h, phase contrast image of aggregation behavior at 72 h were captured for non-treated and treated cells. Scale bar is 100 μm. (B) Aggregation size and (C) aggregation number were determined by image analyser. The data is presented as mean ± SD (n=3). *$P < 0.05$ versus non-treated control.

3. The effect of GA and GE on stem cell markers in DPCs.

Having shown that GA and GE caused the decline of the stem cell-like phenotypes in DPCs, the presence of the well-known stem cell marker in these cells were then evaluated utilizing immunocytochemistry analysis. Emerging evidence has suggested CD133 as a marker of several stem cells including DPCs (Ito et al., 2007; Li, 2013), the expression of such a protein in GA-treated and GE-treated DPCs was determined at the concentrations of 200 μM and 200 μg/ml, respectively, for 72 h and non-treated control cells. Figure 13 shows that the expression of CD133 was decreased in the cells treated with GA and GE for 72 h in comparison to that of control cells at 0 and 72 h.

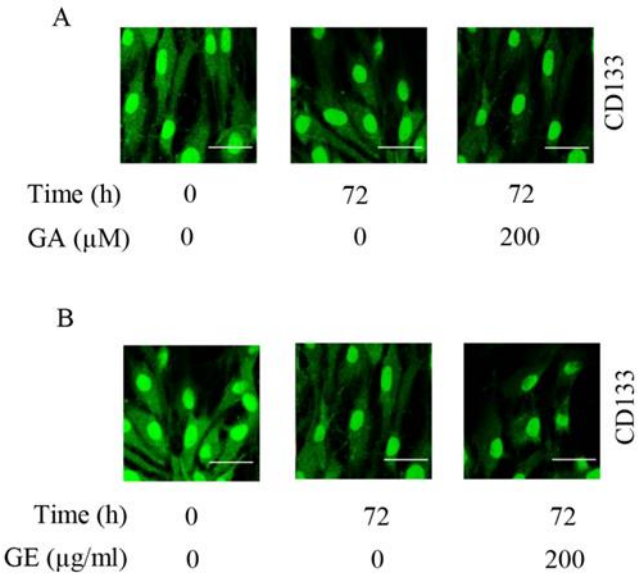


Figure 13 GA and GE alleviated the expression of stem cell markers fluorescence in DPCs. Cells were treated with (A) GA (200 μM) and (B) GE (200 μg/ml) for 72 h. The early passage cells (passages 2-3) were used as a non-treated control at 0 h. The expression of CD133 was analysed using fluorescence microscopy. Scale bar is 50 μm.

Therefore, the information on the effect of GA and GE on DPCs stemness was further investigated. The expression levels of stem cell markers including CD133, integrin β1 and ALDH1A1 in response to GA and GE treatment in time- and concentration-dependent manners were analysed for the proteins by western blot analysis. For time-dependent experiments, the cells were cultured in the presence or absence of GA (200 μM) and GE (200 μg/ml) and protein expression was evaluated at various times (0-72 h). Figures 14 and 15 show that all mesenchymal-related proteins including CD133, Integrin β1 and ALDH1A1 were gradually decreased in the cell-treated with GA and GE in a time-dependent manner compared with a non-treated control. The dose-dependent study was also performed in order to assure the effect of GA and GE on stemness of DPCs. The cells were cultivated in various concentrations of GA (0-200 μM) and GE (0-200 μg/ml) and protein expression was examined at 72 h. The results indicated the suppressive effect of GA and GE on the expression of stem cell markers in DPCs (Figure 16).

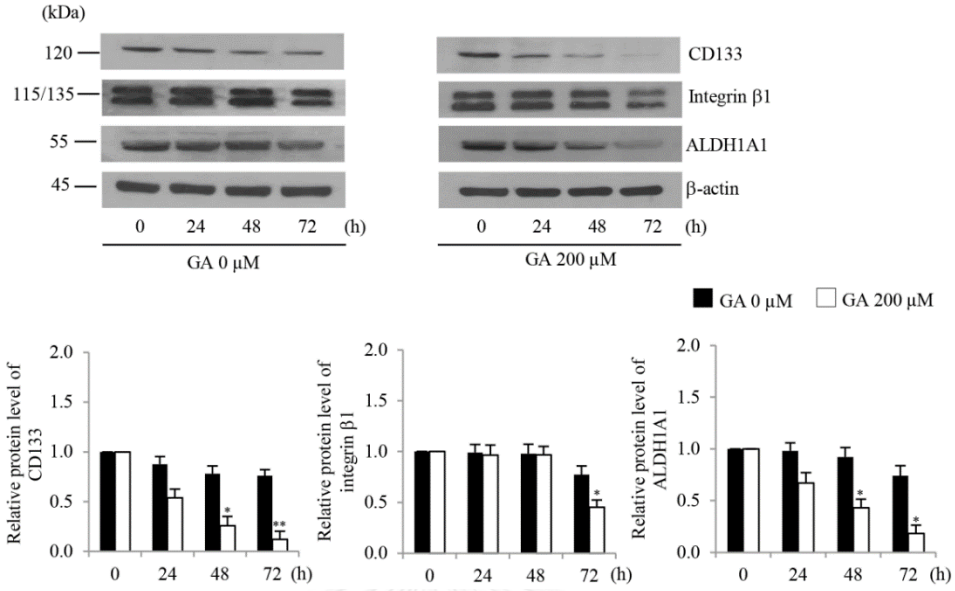


Figure 14 Time course measurement on the expression of stem cell markers in DPCs after being treated with GA. Cells were treated with or without GA (200 μM) for various times (0-72 h) and the level of stem cell markers, CD133, integrin β1 and ALDH1A1 was analysed by western blotting. Blots were reprobed with β-actin to confirm equal loading of samples. The immunoblot signals were quantified by densitometry, and the mean data from independent experiments were normalized and presented. The data is presented as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$ versus non-treated control at 0 h.

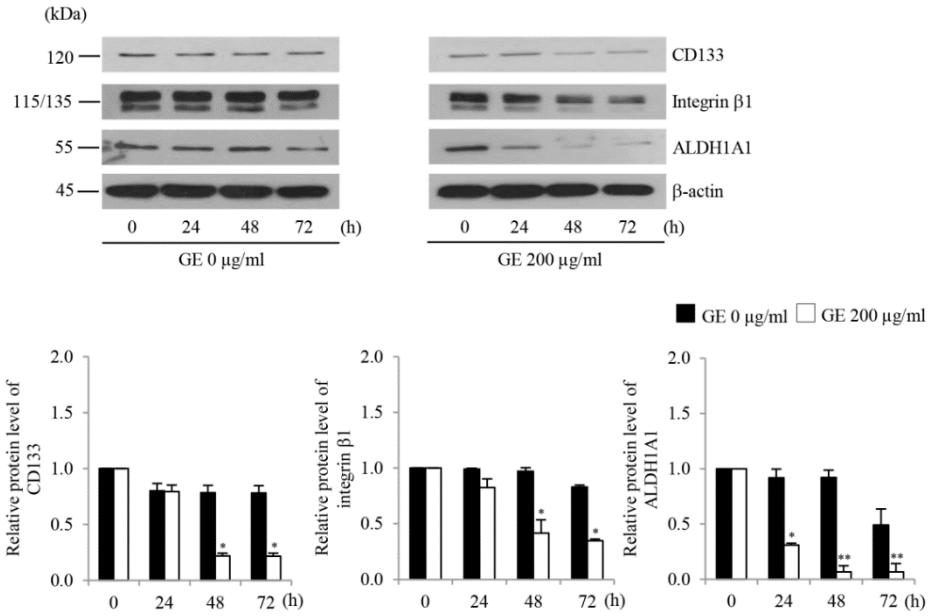


Figure 15 Time course measurement on the expression of stem cell markers in DPCs after being treated with GE. Cells were treated with or without GE (200 μg/ml) for various times (0-72 h) and the level of stem cell markers, CD133, integrin β1 and ALDH1A1 was analysed by western blotting. Blots were reprobed with β-actin to confirm equal loading of samples. The immunoblot signals were quantified by densitometry, and the mean data from independent experiments were normalized and presented. The data is presented as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$ versus non-treated control at 0 h.

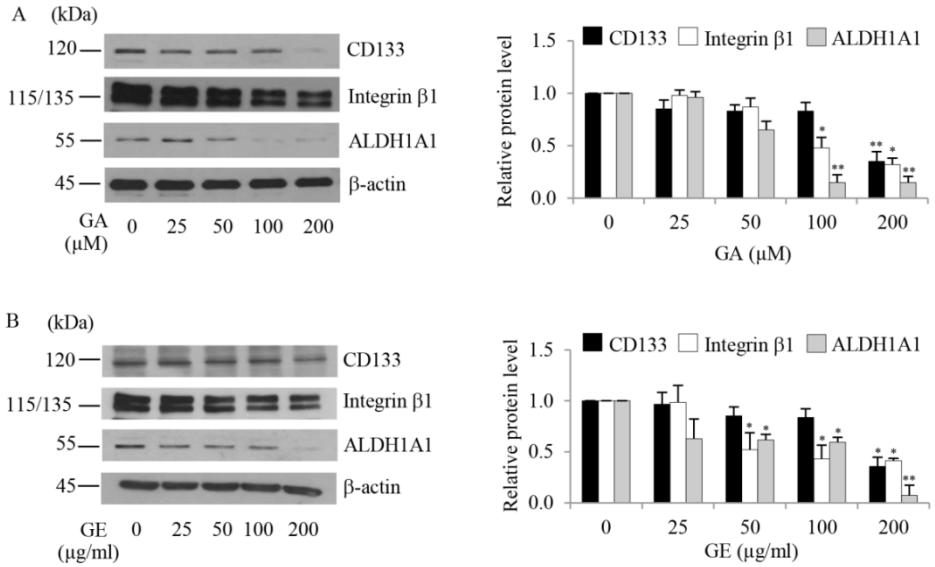


Figure 16 Effect of GA and GE on the expression of stem cell markers in DPCs. After the cells were treated with (A) GA (0-200 μM) and (B) GE (0-200 μg/ml) for 72 h, the level of stem cell markers was determined by western blotting. β-actin was served as the loading control. The immunoblot signals were quantified by densitometry, and the mean data from independent experiments were presented. The data is presented as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$ versus non-treated control

4. The effect of GA and GE on Wnt/β-catenin signaling cascade in DPCs

The Wnt/β-catenin signal has been implicated in the maintenance of stem cell features (Merrill, 2012). In addition, the exposure of Wnt/β-catenin activator could prolong the hair follicle inductive effect of DPCs during culture (Kishimoto et al., 2000; Shimizu and Morgan, 2004). To determine the effect of GA and GE on Wnt/β-catenin pathway, the signaling proteins associated with Wnt/β-catenin including activated Akt (phosphorylated Akt at Ser 473), total Akt, inactivated glycogen synthase kinase3β (phosphorylated GSK3β at Ser 9), parental GSK3β and β-catenin were determined by western blot analysis. The activated Akt inhibits the function of GSK3β by phosphorylating at serine 9 of GSK3β. The cellular level of β-catenin is tightly controlled by GSK3β. The phosphorylation of β-catenin by GSK3β resulted in ubiquitination and proteasomal degradation of β-catenin. As a negative regulator of

β-catenin, such phosphorylation results in the inhibition of GSK3β function, which successively leaves the β-catenin to function as a co-transcription factor. Figure 17 shows that treatment of the cells with GA and GE at the concentrations of 100-200 μM and 100-200 μg/ml, respectively, significantly decreased the level of activated Akt. Furthermore, it was demonstrated the significant decrease of phosphorylated GSK3β in the GA-treated and GE-treated DPCs in accordance with the decrease of activated Akt level (Figure 17). As GSK3β was reported to play the major role in the degradation process of β-catenin, these data showed corresponding results indicated that the decrease of phosphorylated GSK3β leads to a dramatic decrease of cellular β-catenin in GA-treated and GE-treated DPCs (Figure 17). Together, this finding suggested that GA and GE suppressed the stem cell features of DPCs, at least in part, by the reduction of cellular β-catenin through Akt/GSK3β-dependent pathway.

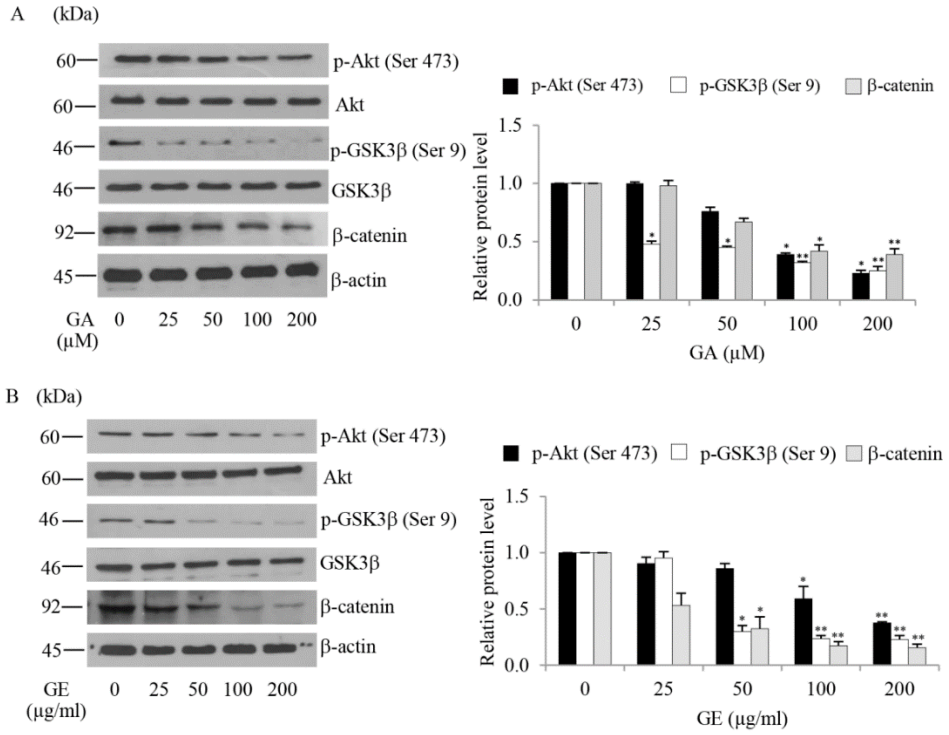


Figure 17 Effect of GA and GE on Wnt/β-catenin signaling in DPCs. After being treated with (A) GA (0-200 μM) and (B) GE (0-200 μg/ml) for 72 h, the cells were collected and the cellular level of Wnt/β-catenin signaling (Akt, p-Akt (Ser 473), GSK3β, p-GSK3β (Ser 9) and β-catenin) were analysed by western blotting. β-actin was used as the

loading control. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized and presented. The data is presented as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$ versus non-treated control.

5. The effect of GA and GE on epithelial-mesenchymal transition (EMT) in DPCs.

Recently, the process of the cell transition from epithelial-mesenchymal phenotypes (EMT) has gained increasing attention in cell biology as it is reported to increase the stem cell-like phenotypes in various cells (Mani et al., 2008; Shimono et al., 2009). Furthermore, the up-regulated transcription factors during EMT like Snail were shown to maintain the stem cell-like phenotypes in many cells (Mani et al., 2008; Shimono et al., 2009). In order to test whether the treatment of cells with GA and GE plays a part in the reduction of stem cell phenotypes through this pathway, the EMT-activating transcription factors including ZEB1, Slug, and Snail were investigated by western blot analysis. Figure 18 illustrates the significant down-regulation of cellular levels of ZEB1 and Snail after incubation with GA for 72 h. However, Slug was slightly decreased in response to GA treatment. Consistently, after incubation with GE for 72 h, the cellular levels of ZEB1, Slug, and Snail were found to significantly down-regulate (Figure 18). Taken together, these results demonstrated that GA and GE suppresses the stem cell-like phenotypes in the DPCs by the negative regulation of β-catenin and EMT.

6. The effect of GA and GE on the expression of self-renewal transcription factors in DPCs.

As the stem cells retain the capacity for renewing themselves (Shenghui et al., 2009), we next investigated whether GA and GE could affect the self-renewal-related proteins. The major transcription factors involving the maintenance of pluripotency and self-renewal in human stem cells (Shenghui et al., 2009), namely, Oct-4, Nanog, and Sox2 were analysed in the GE-treated and non-treated cells. Figure 18 shows Oct-4 was significantly decreased in response to GA and GE treatment at the concentrations of 50-200 μM and 50-200 μg/ml, respectively. As expected, the expression levels of Nanog were found to significantly decrease after treatment with GA and GE at 100-200

μM and 50-200 μg/ml, respectively (Figure 18). Consistently, Sox2 was strongly decreased in response to GA and GE treatment at 100-200 μM and 25-200 μg/ml, respectively. These results suggested that the treatment of GA and GE was capable of decreasing the self-renewal machinery in DPCs by suppressing stemness-related transcription factors.

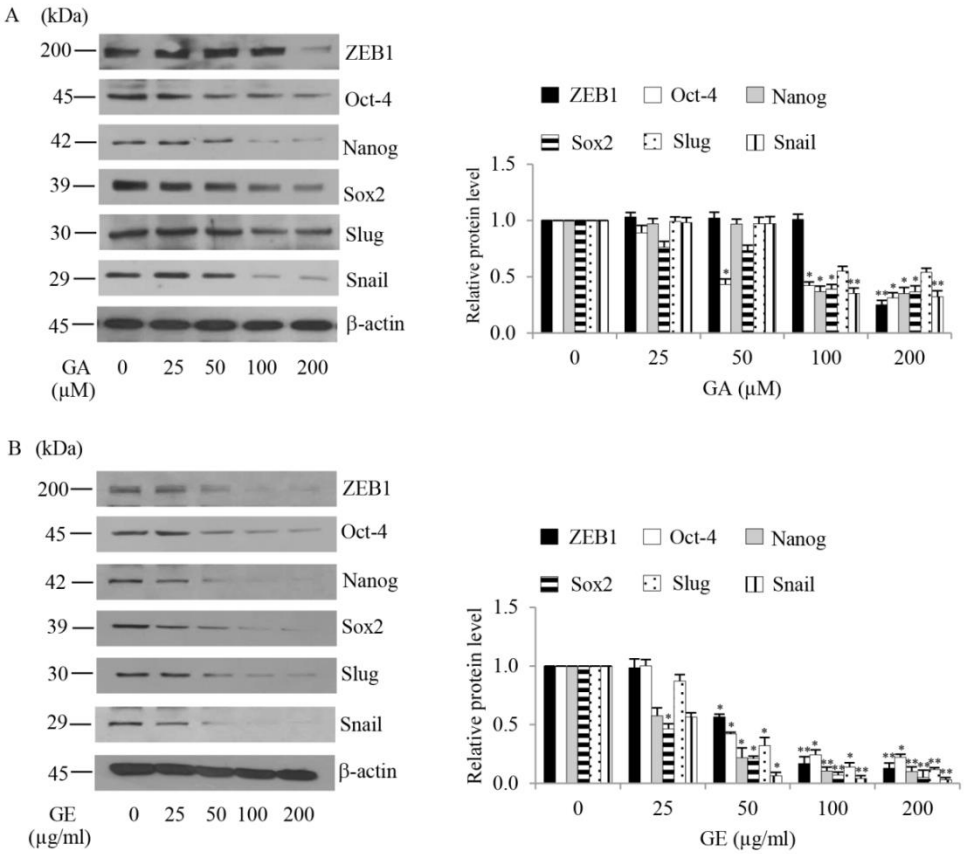


Figure 18 Effect of GA and GE on EMT and self-renewal transcription factors in DPCs. After being treated with (A) GA (0-200 μM) and (B) GE (0-200 μg/ml) for 72 h, the cells were collected and EMT and self-renewal-activating transcription factors (ZEB1, Oct-4, Nanog, Sox2, Slug and Snail) were evaluated by western blotting. β-actin was used as the loading control. The immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized and presented. The data is presented as mean ± SD (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus non-treated control.

7. The effect of GA and GE on the stem cell-like phenotypes in primary human DPCs

To confirm the aforementioned effect of GE on DPCs, the effect of GA and GE was then evaluated in primary human DPCs. The isolated human DPCs were cultivated in primary DPC medium in the presence of various concentrations of GA (0-200 μM) and GE (0-200 μg/ml) and the stem cell-like characteristics were investigated as described.

Figures 19 and 20 show that treatment of the cells with GA (0-200 μM) and GE (0-200 μg/ml) caused no direct cytotoxicity in these cells. Western blot analysis revealed that levels of stem cell markers including CD133, integrin β1 and ALDH1A1 significantly decreased in response to GA and GE treatment for 72 h (Figure 21). Furthermore, treatment of the cells with GA and GE significantly suppressed the expression of proliferation and stemness signals such as active Akt, inactive GSK3β, and β-catenin (Figure 22). Additionally, EMT-activating transcription factors ZEB1 and Snail were found to be down-regulated in GA-treated cell (Figure 22). Also, the ZEB1, Slug and Snail were significantly down-regulated in the GE-treated primary DPCs (Figure 23). Taken together, this data supported the earlier findings that GA and GE attenuates the stemness of DPCs.

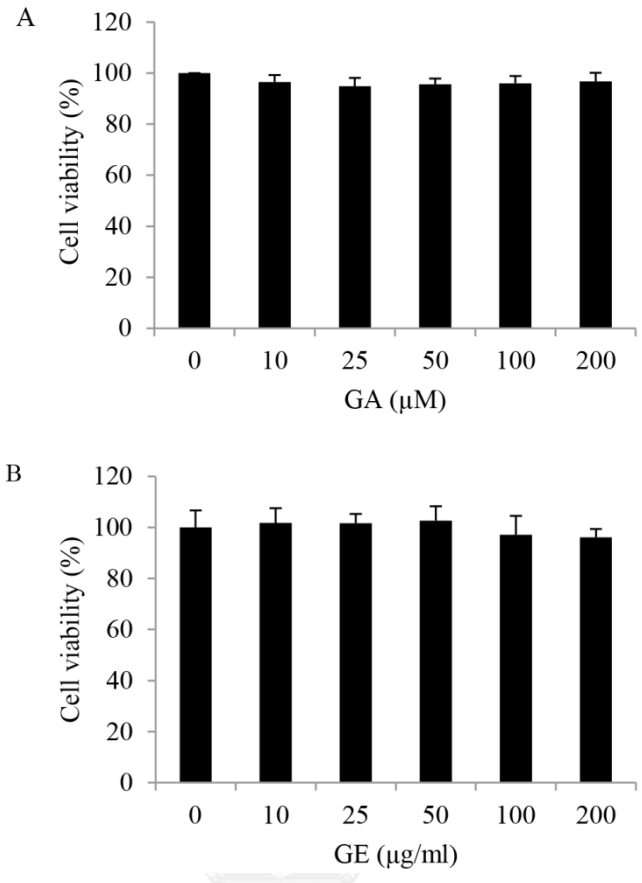


Figure 19 Cytotoxicity of GA and GE on primary human DPCs. Cells were treated with various concentrations of (A) GA (0-200 μM) and (B) GE (0-200 μg/ml) for 24 h. Cell viability was evaluated by MTT assay. The data is presented as mean ± SD (n=3).

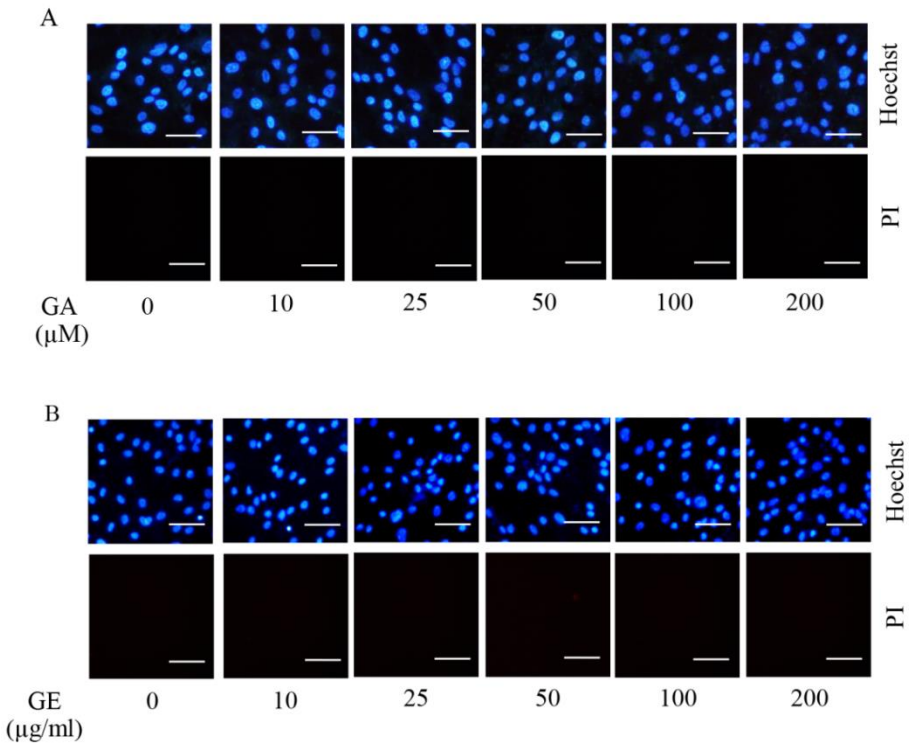


Figure 20 Effect of GA and GE on apoptotic and necrotic death in primary human DPCs. DPCs were treated with various concentrations of (A) GA (0-200 μM) and (B) GE (0-200 μg/ml) for 24 h. Mode of cell death was determined by Hoechst 33342/PI co-staining assay. Scale bar is 100 μm.

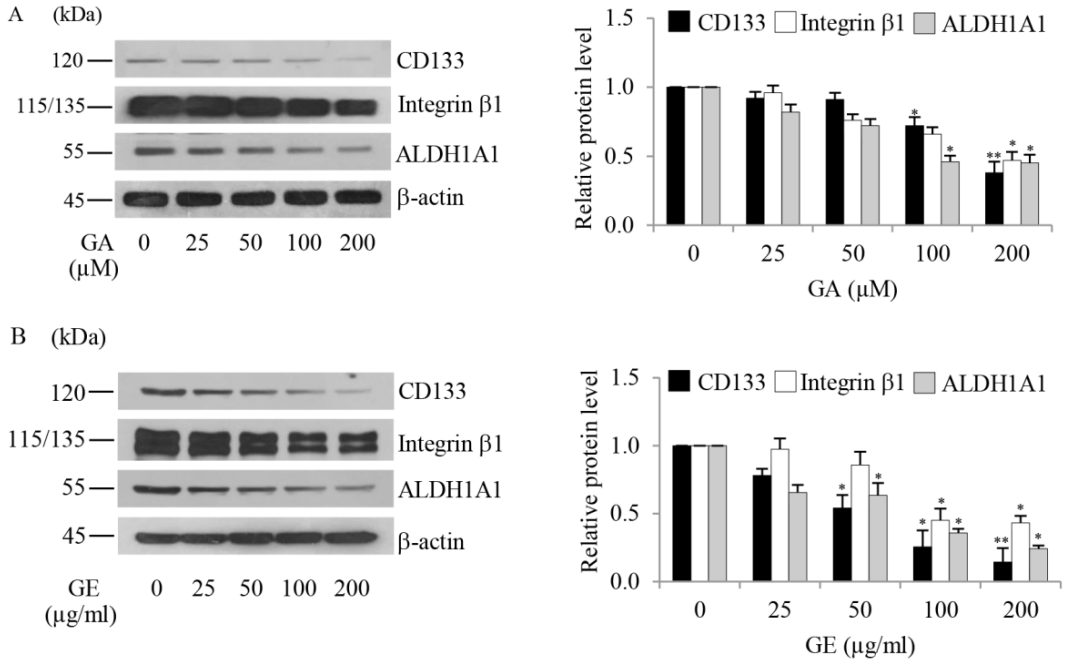


Figure 21 Effect of GA and GE on the expression of stem cell markers in primary human DPCs. After the cells were treated with (A) GA (200 μM) and (B) GE (0-200 μg/ml) for 72 h, the level of stem cell markers was determined by western blotting. μ-actin was served as the loading control. The immunoblot signals were quantified by densitometry, and the mean data from independent experiments were presented. The data is presented as mean ± SD (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus non-treated control.

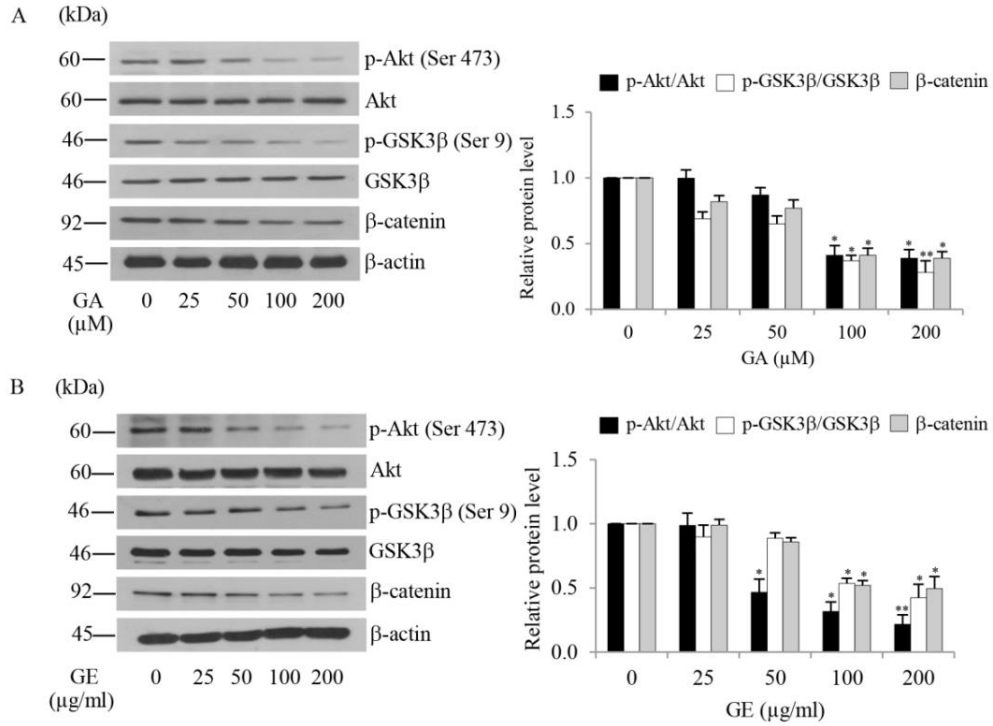


Figure 22 Effect of GA and GE on Wnt/β-catenin signaling in primary human DPCs. (A-B) After being treated with GA (0-200 μM) and (GE (0-200 μg/ml) for 72 h, the cells were collected and the cellular level of Wnt/β-catenin signaling (Akt, p-Akt (Ser 473), GSK3β, p-GSK3β (Ser 9) and β-catenin) were analysed by western blotting. β-actin was used as the loading control. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized and presented. The data is presented as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$ versus non-treated control.

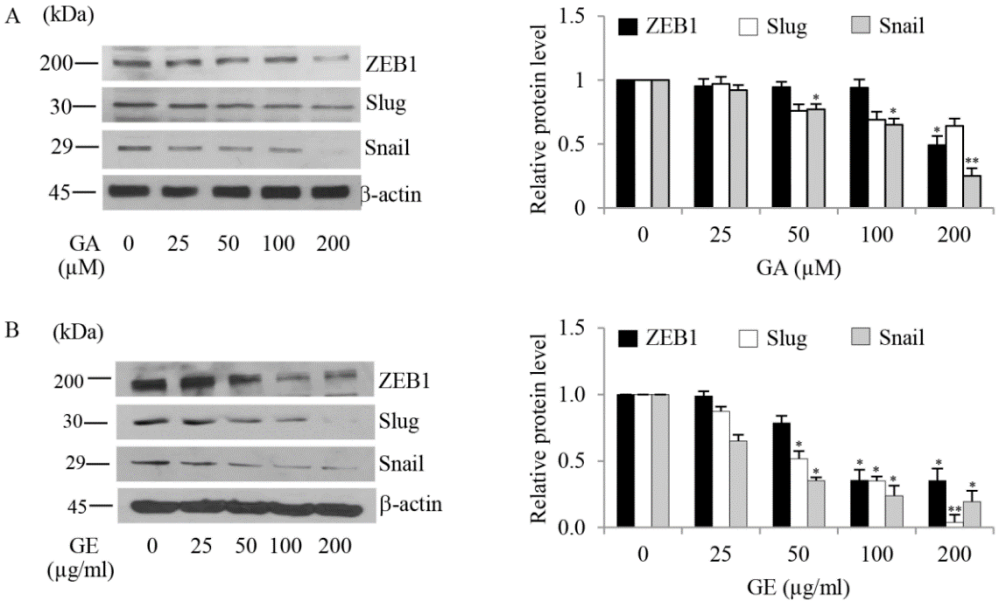


Figure 23 Effect of GA and GE on EMT-activating transcription factors in primary human DPCs. After being treated with (A) GA (0-200 μM) and (B) GE (0-200 μg/ml) for 72 h, the cells were collected and EMT and self-renewal-activating transcription factors (ZEB1, Slug and Snail) were evaluated by western blotting. β-actin was used as the loading control. The immunoblot signals were quantified by densitometry and mean data from independent experiments were normalized and presented. The data is presented as mean ± SD (n=3). *$P < 0.05$, **$P < 0.01$ versus non-treated control.

Part 2. Preformulation study in preparation of GA and GE gels

8. Validation of TLC-densitometric method

Before estimating content of GA in GA and GE samples by TLC-densitometric method, the method was verified for its linearity, LOD, LOQ, accuracy, precision, and specificity.

8.1 Linearity

The linear regression data for the calibration curves showed good linear relationship within the concentration range 0.125-2.5 μg/spot. Linear regression equation was found to be y = 4669.5x+84.144 with a correlation coefficient of 0.9989 (Figure 24).

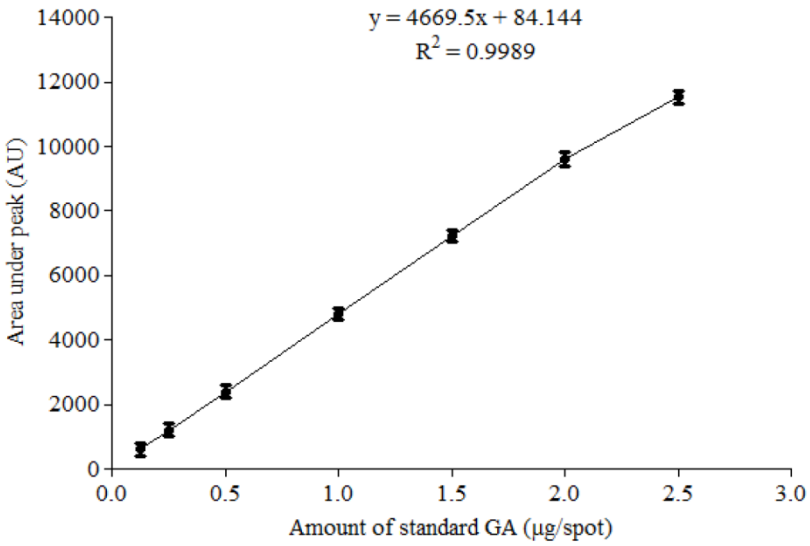


Figure 24 Calibration curve of standard GA by TLC-densitometric method

8.2 Detection and quantification limit

LOD and LOQ were determined by the method as described in Materials and Methods section. LOD and LOQ values of standard GA were 0.109 and 0.329 µg/spot, respectively. Then, standard GA solution was further diluted and spotted on TLC plate at 0.054, 0.082, 0.109 and 0.125 µg/spot. It was found that standard GA at 0.054 and 0.082 µg/spot could not be detected by TLC scanner whereas standard GA at 0.109 µg/spot could be detected but its chromatogram showed inconsistent peak area suggesting a detection limit at 0.1089 µg/spot. Conversely, standard GA at 0.125 µg/spot showed a steady peak area indicating a quantification limit of this method. Therefore, after doing experiment, LOD and LOQ values of standard GA were 0.109 and 0.125 µg/spot indicating adequate sensitivity of the method.

8.3 Accuracy

The accuracy of the method was validated by adding a known quantity of standard at three levels with pre-analysed sample, and the percentage recovery values of 99.65, 99.82 and 99.63 were obtained as listed in Table 6.

Table 6 Accuracy determined for the TLC-densitometric method

Sample no.	GA standard added (µg/spot)	Theoretical content (µg/spot)	Experimental content (µg/spot)	Recovery (%)
1	0.125	0.925	0.92179±0.0015	99.65±0.30
2	0.25	1.05	1.0481±0.0061	99.82±0.55
3	0.5	1.3	1.2951±0.0015	99.63±0.16

The data is presented as mean ± SD (n=3).

8.4 Precision

The intra- and inter-day precisions were expressed in terms of %RSD, the result depicted high precision of the method as presented in Table 7.

Table 7 Intra- and Inter-day precision

Amount of GA standard (µg/spot)	Intra-day precision		Inter-day precision	
	Mean area±SD	%RSD	Mean area±SD	%RSD
0.125	664.73±5.40	0.0081	660.05±11.75	0.0178
0.25	1,250.12±2.84	0.0022	1,249.03±3.97	0.0032
0.5	2,417.34±7.13	0.0029	2,418.89±9.339	0.0038

The data is presented as mean ± SD (n=3).

8.5 Specificity

The densitometric analysis was performed at 254 nm in the reflectance mode. Chromatogram of standard GA, GA and GE samples was developed under chamber saturation condition using ethyl acetate: glacial acetic acid: methanol: water (8:3:2:1 v/v) as mobile phase gave a consistent peak of GA with R_f value of 0.48 (Figure 25). Also, three different levels, peak start, peak apex, and peak end of GA peak in GA and GE samples were consistent with the peak of standard GA (Figure 26).

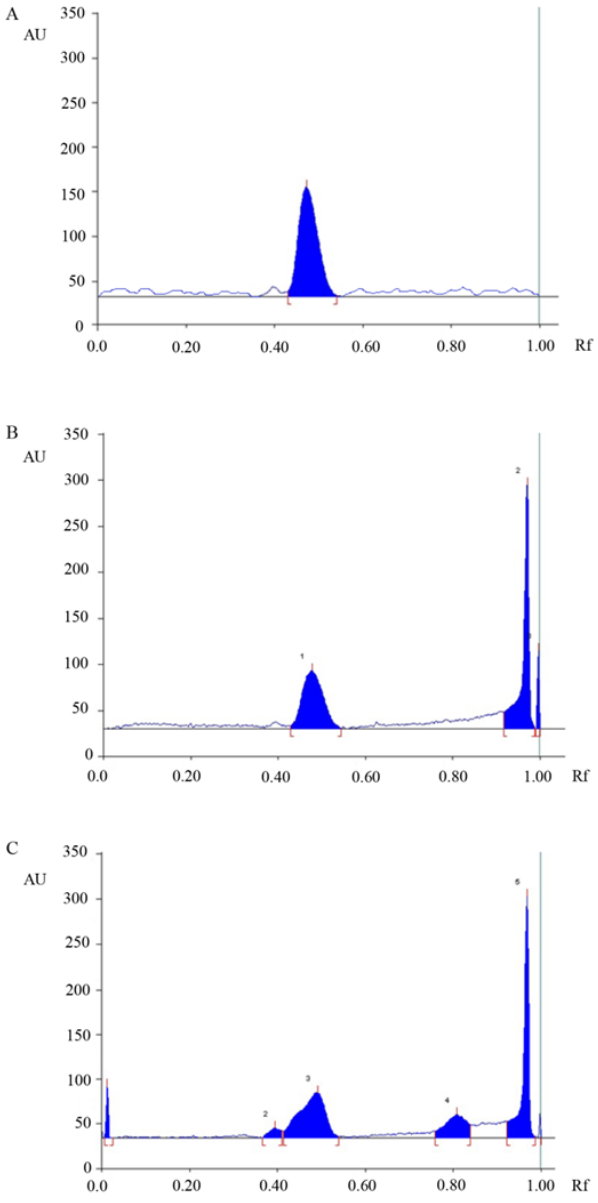


Figure 25 TLC chromatogram of (A) standard GA 1.5 µg/spot, (B) GA sample 1 µg/spot and (C) GE sample 10 µg/spot. Mobile phase is ethyl acetate: glacial acetic acid: methanol: water (8:3:2:1 v/v).

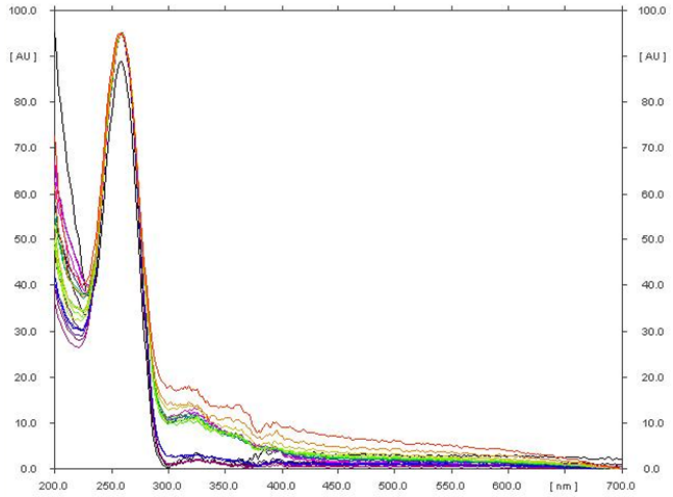


Figure 26 UV spectral comparison of standard GA 1 µg/spot, GA sample 1 µg/spot and GE sample 10 µg/spot detection at 254 nm

8.6 Estimation of GA content in GA and GE samples

According to previous reports *G. glabra L.* contains amount of GA ranging from 10-25% depending on source and it was considered the primary active ingredient (Lakshmi and Geetha, 2011). GA was therefore selected as a marker constituent in this study. The amount of GA was determined by seven-point standard curve of standard GA. The GA contents in GA and GE samples were 80.49% and 11.42% (Table 8).

Table 8 Percentage of GA in GA and GE samples

Sample	Mean area±SD	% of GA
GA	3,861.62±25.29	80.49 ± 0.5391
GE	5,442.04±53.09	11.42 ± 0.1131

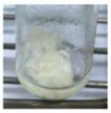
The data is presented as mean ± SD (n=3).

9. Selection of solvent system

The objective of this experiment was to determine the proper solvent for GA and GE at the concentration of 15% w/w. This decision for selecting concentration of the test compounds was based on previous studies (Ivosevic-Zaper et al., 2014; Faghihi et al., 2015). After separate dissolution of GA and GE in various solvent systems, all solutions were agitated with vortex mixer for 10 min and allowed to equilibrate at room temperature for 24 h. The result revealed that GA was poorly soluble in water

and its solution clearly showed sedimentation of GA. Also, it was found to be poorly soluble in ethanol and water : ethanol (1:1 w/w; Table 9) and color of these solutions was turbid white. Furthermore, GA was found to be slightly soluble in polyethylene glycol 400, water : polyethylene glycol 400 (1:1 w/w) and water : propylene glycol (1:1 w/w) and these solutions were nearly colorless with slightly turbid meanwhile it was found to completely dissolve in propylene glycol and the solution was colorless as shown in Table 9. Table 10 demonstrates that GE was found to be poorly soluble in ethanol, polyethylene glycol 400 and propylene glycol and these solutions were markedly turbid. It was found to slightly dissolve in water : ethanol (1:1 w/w), and water : polyethylene glycol 400 (1:1 w/w) and these solutions were yellow with noticeable sedimentation. Whereas it was found to be completely soluble in both water and water : propylene glycol (1:1 w/w) and these solutions were yellow, although the solution in water : propylene glycol (1:1 w/w) showed more clarity as illustrated in Table 10. The proper solvent for each compound should completely dissolve these compounds with an acceptable clarity of solution. Therefore, propylene glycol and water: propylene glycol (1:1 w/w) were selected as a solvent for GA and GE, respectively.

Table 9 Physical properties of 15% w/w GA solution in various solvents

Solvent	Physical appearance	Color	Clarity	Sedimentation	pH
Water		colorless	+++	+++	5.24
Ethanol		white	–	+++	5.0
Polyethylene glycol 400		colorless	–	+++	5.26
Propylene glycol		colorless	+++	–	5.45
Water : ethanol (1:1 w/w)		white	–	+++	5.25
Water : Polyethylene glycol 400 (1:1 w/w)		colorless	+	++	5.25
Water : Propylene glycol (1:1 w/w)		colorless	++	+	5.40

Clarity: – no clarity, + low, ++ medium, +++ high

Sedimentation: –no sedimentation, + low, ++ medium, +++ high

Table 10 Physical properties of 15% w/w GE solution in various solvents

Solvent	Physical appearance	Color	Clarity	Sedimentation	pH
Water		yellow	++	–	5.45
Ethanol		colorless	++	+++	5.0
Polyethylene glycol 400		white	–	+++	5.25
Propylene glycol		white	–	+++	5.20
Water : ethanol (1:1 w/w)		yellow	+++	++	5.19
Water : Polyethylene glycol 400 (1:1 w/w)		yellow	++	++	5.23
Water : Propylene glycol (1:1 w/w)		yellow	+++	–	5.52

Clarity: – no clarity, + low, ++ medium, +++ high

Sedimentation: –no sedimentation, + low, ++ medium, +++ high

10. Selection of concentration of gelling agent

This study was performed in order to screen the proper concentration of gelling agent for GA and GE gel formulation. GA and GE were dissolved in their selected solvent that were propylene glycol and water : propylene glycol (1 : 1 w/w). Afterward, the solution of these compounds was mixed with different concentrations of each gelling agent solution. Tables 11 and 12 indicate that GA and GE gels prepared by using 1% w/w SCMC, 0.5% w/w carbomer 940, 2% w/w HPMC and 1% w/w HEC as a gelling agent were still viscous liquid meanwhile the gels prepared by 2-3% SCMC, 1-2% carbomer 940, 3-4% HPMC and 2-3% HEC could form gel with clear texture and fine spreadability. The pH value of GA and GE gels ranged from 6.52 to 6.72 as shown

in Table11 and 12. Consequently, 2-3% SCMC, 1-2% carbomer 940, 3-4% HPMC and 2-3% HEC were chosen to prepared GA and GE gels for further experiment.

Table 11 Physical properties of 15% w/w GA gel prepared by different gelling agents

Gelling Agent	Concentration (%w/w)	Viscosity	Spreadability	Clarity	pH
SCMC	1%	+	very good	very good	6.55±0.01
	2%	++	very good	very good	6.61±0.01
	3%	+++	very good	very good	6.65±0.01
Carbomer 940	0.5%	-	very good	very good	6.65±0.01
	1%	++	very good	very good	6.69±0.01
	2%	++	very good	very good	6.72±0.01
HPMC	2%	+	very good	very good	6.56±0.01
	3%	++	very good	very good	6.52±0.01
	4%	+++	very good	very good	6.59±0.01
HEC	1%	+	very good	very good	6.63±0.01
	2%	++	very good	very good	6.60±0.01
	3%	+++	very good	very good	6.63±0.01

Vicosity; – liquid-like, + viscous liquid, still flowable, ++ moderately viscous, not too sticky, +++ too viscous, sticky feel

Table 12 Physical properties of 15% w/w GE gel prepared by different gelling agents

Gelling Agent	Concentration (%w/w)	Viscosity	Spreadability	Clarity	pH
SCMC	1%	-	very good	moderate	6.62±0.01
	2%	++	very good	moderate	6.55±0.01
	3%	+++	very good	moderate	6.51±0.01
Carbomer 940	0.5%	+	very good	moderate	6.65±0.02
	1%	++	very good	moderate	6.68±0.01
	2%	++	very good	moderate	6.63±0.01
HPMC	2%	+	very good	moderate	6.62±0.01
	3%	++	very good	moderate	6.59±0.01
	4%	+++	very good	moderate	6.69±0.01
HEC	1%	-	very good	moderate	6.64±0.01
	2%	++	very good	moderate	6.61±0.01
	3%	+++	very good	moderate	6.57±0.01

Vicosity; - liquid-like, + viscous liquid, still flowable, ++ moderately viscous, not too sticky, +++ too viscous, sticky feel

11. Formulation of GA and GE in gel base

Subsequently, GA and GE gels at the concentrations of 15% w/w were prepared by using the chosen concentration of gelling agent according to the content of formulation listed in Tables 13-20. The prepared GA and GE gels were found to be transparent, homogenous appearance and easily spreadable (Figures 27-34). However, GA and GE gel prepared by using SCMC, HPMC and HEC as gelling agent gave more sticky feeling after skin application than carbomer 940. All formulations of GA and GE gels were then evaluated for their stability.

Table 13 Formulation of 15% w/w GA gel using varied concentrations of SCMC as gelling agent

Composition (g, %w/w)	Formulation			
	F1	F2	F3	F4
GA	18.6	18.6	18.6	18.6
SCMC	2	2	3	3
Propylene glycol	29.5	29.5	29.25	29.25
Distilled water	47.75	47.75	47	47
Sodium metabisulfite	0.1	-	0.1	-
Vitamin E	-	0.1	-	0.1
Sodium EDTA	0.05	0.05	0.05	0.05
Floreintal TL 9501	0.001	0.001	0.001	0.001
Tween 20	1	1	1	1
Triethanolamine	-	-	-	-
Caprylyl glycol	0.67	0.67	0.67	0.67
Ethylhexyl glycerin	0.33	0.33	0.33	0.33

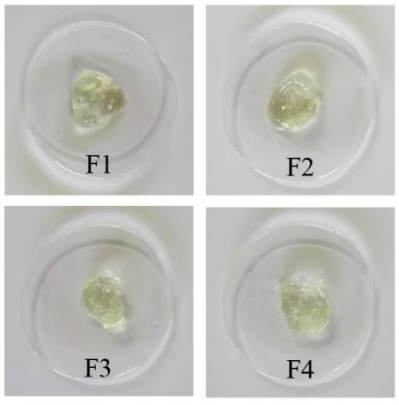


Figure 27 General appearances of 15% w/w GA gel formulations containing varied concentrations of SCMC as gelling agent

Table 14 Formulation of 15% w/w GA gel using varied concentrations of carbomer 940 as gelling agent

Composition (g, %w/w)	Formulation			
	F5	F6	F7	F8
GA	18.6	18.6	18.6	18.6
Carbomer 940	1	1	2	2
Propylene glycol	29.75	29.75	29.5	29.5
Distilled water	48.5	48.5	47.75	47.75
Sodium metabisulfite	0.1	-	0.1	-
Vitamin E	-	0.1	-	0.1
Sodium EDTA	0.05	0.05	0.05	0.05
Floreintal TL 9501	0.001	0.001	0.001	0.001
Tween 20	1	1	1	1
Triethanolamine	qs	qs	qs	qs
Caprylyl glycol	0.67	0.67	0.67	0.67
Ethylhexyl glycerin	0.33	0.33	0.33	0.33

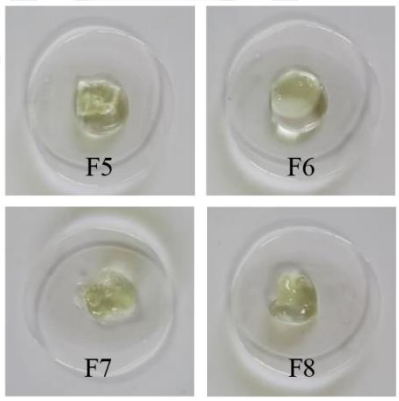


Figure 28 General appearances of 15% w/w GA gel formulations containing varied concentrations of carbomer 940 as gelling agent

Table 15 Formulation of 15% w/w GA gel using varied concentrations of HPMC as gelling agent

Composition (g, %w/w)	Formulation			
	F9	F10	F11	F12
GA	18.6	18.6	18.6	18.6
HPMC	3	3	4	4
Propylene glycol	29.25	29.25	29	29
Distilled water	47	47	46.25	46.25
Sodium metabisulfite	0.1	-	0.1	-
Vitamin E	-	0.1	-	0.1
Sodium EDTA	0.05	0.05	0.05	0.05
Floreintal TL 9501	0.001	0.001	0.001	0.001
Tween 20	1	1	1	1
Triethanolamine	-	-	-	-
Caprylyl glycol	0.67	0.67	0.67	0.67
Ethylhexyl glycerin	0.33	0.33	0.33	0.33

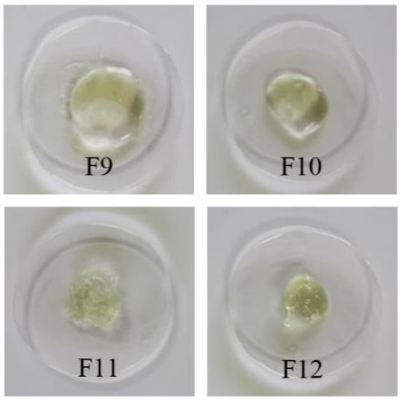


Figure 29 General appearances of 15% w/w GA gel formulations containing varied concentrations of HPMC as gelling agent

Table 16 Formulation of 15% w/w GA gel using varied concentrations of HEC as gelling agent

Composition (g, %w/w)	Formulation			
	F13	F14	F15	F16
GA	18.6	18.6	18.6	18.6
HEC	2	2	3	3
Propylene glycol	29.5	29.5	29.25	29.25
Distilled water	47.75	47.75	47	47
Sodium metabisulfite	0.1	-	0.1	-
Vitamin E	-	0.1	-	0.1
Sodium EDTA	0.05	0.05	0.05	0.05
Floreintal TL 9501	0.001	0.001	0.001	0.001
Tween 20	1	1	1	1
Triethanolamine	-	-	-	-
Caprylyl glycol	0.67	0.67	0.67	0.67
Ethylhexyl glycerin	0.33	0.33	0.33	0.33

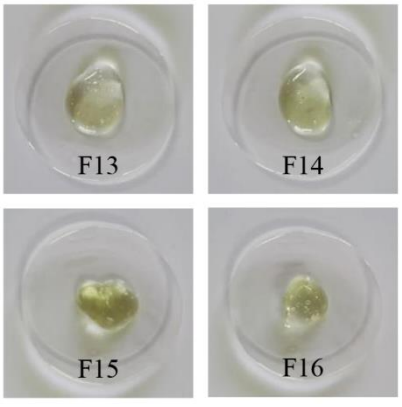


Figure 30 General appearances of 15% w/w GA gel formulations containing varied concentrations of HEC as gelling agent

Table 17 Formulation of 15% w/w GE gel using varied concentrations of SCMC as gelling agent

Composition (g, %w/w)	Formulation			
	F17	F18	F19	F20
GE	15	15	15	15
SCMC	2	2	3	3
Propylene glycol	14.35	14.35	14.25	14.25
Distilled water	66.50	66.50	66.35	66.35
Sodium metabisulfite	0.1	-	0.1	-
Vitamin E	-	0.1	-	0.1
Sodium EDTA	0.05	0.05	0.05	0.05
Floreintal TL 9501	0.001	0.001	0.001	0.001
Tween 20	1	1	1	1
Triethanolamine	-	-	-	-
Caprylyl glycol	0.67	0.67	0.67	0.67
Ethylhexyl glycerin	0.33	0.33	0.33	0.33

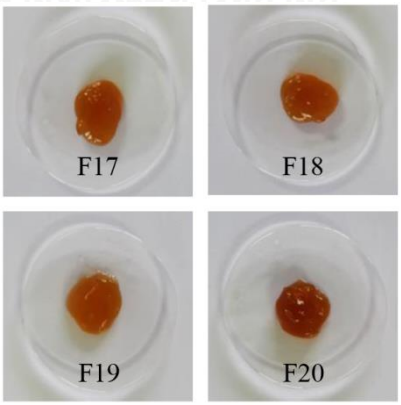


Figure 31 General appearances of 15% w/w GE gel formulations containing varied concentrations of SCMC as gelling agent

Table 18 Formulation of 15% w/w GE gel using varied concentrations of carbomer 940 as gelling agent

Composition (g, %w/w)	Formulation			
	F21	F22	F23	F24
GE	15	15	15	15
Carbomer 940	1	1	2	2
Propylene glycol	14.50	14.50	14.35	14.35
Distilled water	67.35	67.35	66.50	66.50
Sodium metabisulfite	0.1	-	0.1	-
Vitamin E	-	0.1	-	0.1
Sodium EDTA	0.05	0.05	0.05	0.05
Floreintal TL 9501	0.001	0.001	0.001	0.001
Tween 20	1	1	1	1
Triethanolamine	qs	qs	qs	qs
Caprylyl glycol	0.67	0.67	0.67	0.67
Ethylhexyl glycerin	0.33	0.33	0.33	0.33

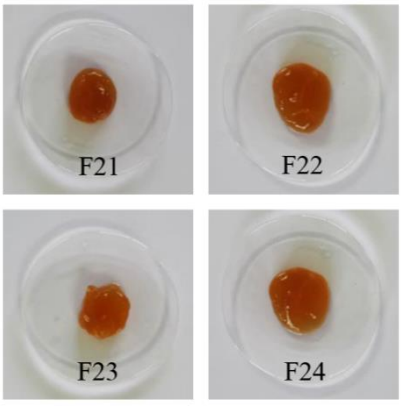


Figure 32 General appearances of 15% w/w GE gel formulations containing varied concentrations of carbomer 940 as gelling agent

Table 19 Formulation of 15% w/w GE gel using varied concentrations of HPMC as gelling agent

Composition (g, %w/w)	Formulation			
	F25	F26	F27	F28
GE	15	15	15	15
HPMC	3	3	4	4
Propylene glycol	14.25	14.25	14	14
Distilled water	66.35	66.35	64.85	64.85
Sodium metabisulfite	0.1	-	0.1	-
Vitamin E	-	0.1	-	0.1
Sodium EDTA	0.05	0.05	0.05	0.05
Floreintal TL 9501	0.001	0.001	0.001	0.001
Tween 20	1	1	1	1
Triethanolamine	-	-	-	-
Caprylyl glycol	0.67	0.67	0.67	0.67
Ethylhexyl glycerin	0.33	0.33	0.33	0.33

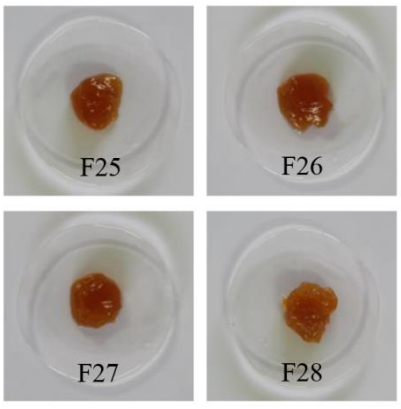


Figure 33 General appearances of 15% w/w GE gel formulations containing varied concentrations of HPMC as gelling agent

Table 20 Formulation of 15% w/w GE gel using varied concentrations of HEC as gelling agent

Composition (g, %w/w)	Formulation			
	F29	F30	F31	F32
GE	15	15	15	15
HEC	2	2	3	3
Propylene glycol	14.35	14.35	14.25	14.25
Distilled water	66.50	66.50	66.35	66.35
Sodium metabisulfite	0.1	-	0.1	-
Vitamin E	-	0.1	-	0.1
Sodium EDTA	0.05	0.05	0.05	0.05
Floreintal TL 9501	0.001	0.001	0.001	0.001
Tween 20	1	1	1	1
Triethanolamine	-	-	-	-
Caprylyl glycol	0.67	0.67	0.67	0.67
Ethylhexyl glycerin	0.33	0.33	0.33	0.33

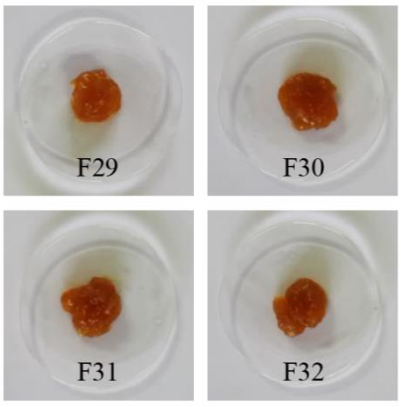


Figure 34 General appearances of 15% w/w GE gel formulations containing varied concentrations of HEC as gelling agent

12. Physical stability

In order to ensure physical stability of the final formulation, all formulations were then subjected to heating-cooling cycle for 6 cycles. The results illustrated that color and clarity of all formulations remained unchanged (Tables 21 and 22). Besides, pH value was quite stable as shown in Tables 23 and 24. These results indicated that viscosity value of GA gel formulation F1-F8 and GE gel formulation F17-F24 was relatively stable (Tables 23 and 24). However, it was found that upon storage at 4 ℃ and 45 ℃ caused a markedly decrease in viscosity of GA gel formulation F9-F16 and GE gel formulation F25-F32. Hence, GA gel formulation F1-F8 and GE gel formulation F17-F24 were subjected to chemical stability evaluation.

Table 21 Color and clarity of different formulations of 15% w/w GA gels after 6 heat-cool cycles

Formulation	Physical properties	Heat-Cool cycle						
		Cycle 0	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
F1	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F2	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F3	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F4	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F5	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F6	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F7	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F8	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F9	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F10	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F11	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F12	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F13	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F14	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F15	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear
F16	Color	–	–	–	–	–	–	–
	Clarity	very clear	very clear	very clear	very clear	very clear	very clear	very clear

Color; – no change (pale yellow)

Table 22 Color and clarity of different formulations of 15% w/w GE gels after 6 heat-cool cycles

Formulation	Physical properties	Heat-Cool cycle						
		Cycle 0	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
F17	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F18	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F19	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F20	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F21	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F22	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F23	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F24	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F25	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F26	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F27	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F28	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F29	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F30	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F31	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear
F32	Color	–	–	–	–	–	–	–
	Clarity	clear	clear	clear	clear	clear	clear	clear

Color; – no change (dark yellow)

Table 23 pH value and viscosity of different formulations of 15% w/w GA gels after 6 heat-cool cycles

Formulation	Physical properties	Heat-Cool cycle						
		Cycle 0	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
F1	pH	6.69±0.02	6.69±0.01	6.69±0.01	6.69±0.01	6.69±0.01	6.69±0.01	6.69±0.01
	Viscosity (cPs)	6,718.63±413.37	6,728.5±541.97	6,723.9±481.852	6,718.5±502.45	6,712.67±491.50	6,709.57±610.70	6,707.67±650.97
F2	pH	6.55±0.02	6.55±0.02	6.55±0.01	6.55±0.01	6.55±0.01	6.55±0.01	6.55±0.01
	Viscosity (cPs)	6,704.33±502.95	6,702.20±644.75	6,700.30±408.70	6,696.90±528.85	6,695.37±641.02	6,692.50±721.10	6,690.33±570.32
F3	pH	6.62±0.01	6.62±0.01	6.62±0.01	6.62±0.01	6.62±0.01	6.62±0.01	6.61±0.01
	Viscosity (cPs)	24,893.33±2,019.27	25,496.67±2,315.04	25,430.00±2,108.17	25,110.00±2,100.37	24,990.00±2,455.68	24,706.67±2,083.27	24,506.67±2,405.77
F4	pH	6.51±0.01	6.51±0.02	6.51±0.01	6.52±0.01	6.51±0.01	6.51±0.01	6.51±0.01
	Viscosity (cPs)	24,356.67±2,035.12	24,370.00±2,126.46	24,146.67±2,020.82	24,070.00±2,149.33	23,800.31±2,140.25	23,653.33±2,237.86	23,436.67±2,135.03
F5	pH	6.51±0.02	6.51±0.01	6.51±0.01	6.51±0.02	6.51±0.01	6.51±0.01	6.51±0.01
	Viscosity (cPs)	4,224.00±207.32	4,371.33±396.65	4,300.00±421.29	4,190.00±249.03	4,126.67±442.52	4,059.67±437.68	4,102.67±405.23
F6	pH	6.59±0.02	6.59±0.02	6.59±0.01	6.59±0.01	6.59±0.01	6.59±0.01	6.59±0.01
	Viscosity (cPs)	4,925.04±407.12	4,881.62±4102.53	4,955.31±427.24	4,969.16±422.92	4,954.00±407.34	4,934.11±387.02	4,926.12±397.21
F7	pH	6.55±0.01	6.55±0.01	6.55±0.01	6.55±0.01	6.55±0.01	6.55±0.01	6.55±0.01
	Viscosity (cPs)	24,296.34±2,431.52	24,640.00±2,046.53	24,986.19±2,027.24	24,996.11±2,912.92	24,733.33±1,526.02	24,580.01±2,206.91	24,373.33±2,382.34
F8	pH	6.61±0.01	6.61±0.02	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01
	Viscosity (cPs)	24,004.00±2,620.11	24,056.18±1,947.42	24,026.18±2,385.46	24,033.33±2,262.14	24,033.38±1,729.82	24,086.10±2,397.18	24,033.56±2,391.42
F9	pH	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01
	Viscosity (cPs)	10,800.00±1,028.28	10,810.93±1,026.46	1,860.00±117.32	1,903.33±115.28	1,866.67±101.55	1,843.33±149.33	1,750.00±134.64
F10	pH	6.48±0.01	6.48±0.02	6.48±0.01	6.48±0.02	6.48±0.01	6.48±0.01	6.47±0.01
	Viscosity (cPs)	10,686.67±1,240.41	10,660.00±1,236.06	1,643.33±149.33	1,546.67±147.26	1,530.00±126.46	1,526.67±140.41	1,526.67±137.86
F11	pH	6.57±0.01	6.57±0.01	6.57±0.01	6.57±0.01	6.57±0.01	6.57±0.01	6.57±0.01
	Viscosity (cPs)	20,280.33±2,104.58	20,280.67±2,013.21	4,284.33±242.08	4,251.67±456.89	4,266.33±415.27	4,207.67±314.93	4,269.67±417.04
F12	pH	6.51±0.01	6.51±0.02	6.51±0.01	6.51±0.01	6.52±0.01	6.51±0.02	6.51±0.01
	Viscosity (cPs)	21,278.33±2,201.58	21,278.67±2,005.35	1,277.67±102.47	1,278.67±114.27	1,263.00±124.02	1,276.67±174.36	1,207.33±156.73
F13	pH	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.6±0.01
	Viscosity (cPs)	12,669.73±1,019.49	12,695.57±1,201.97	2,691.97±262.25	2,684.80±221.28	2,679.76±201.90	2,673.66±251.11	2,669.27±214.60
F14	pH	6.52±0.01	6.52±0.01	6.52±0.01	6.52±0.01	6.52±0.01	6.52±0.01	6.52±0.01
	Viscosity (cPs)	8,665.60±811.25	8,661.70±805.15	1,659.17±101.47	1,607.20±129.80	1,654.07±109.29	1,651.97±119.49	1,540.23±101.55
F15	pH	6.61±0.02	6.61±0.02	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01
	Viscosity (cPs)	20,720.33±1,802.02	21,440.06±2031.15	1,713.33±104.08	1,766.05±158.94	1,923.36±181.61	1,946.63±115.82	1,910.32±155.78
F16	pH	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01
	Viscosity (cPs)	21,990.23±2079.37	21,070.13±2,131.15	2,208.84±195.29	2,206.34±212.29	2,221.67±293.66	2,205.72±102.02	2,218.36±252.69

Table 24 pH value and viscosity of different formulations of 15% w/w GE gels after 6 heat-cool cycles

Formulation	Physical properties	Heat-Cool cycle						
		Cycle 0	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
F17	pH	6.52±0.01	6.52±0.01	6.52±0.01	6.52±0.01	6.52±0.01	6.52±0.01	6.52±0.01
	Viscosity (cPs)	7,651.23±685.05	7,650.77±675.41	7,647.7±741.32	7,645.07±723.18	7,644.4±735.43	7,643.03±725.41	7,640.83±684.21
F18	pH	6.61±0.02	6.61±0.02	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01
	Viscosity (cPs)	7,674.83±701.24	7,675.03±695.23	7,668.63±665.15	7,666.6±801.73	7,660.82±781.04	7,657.61±719.45	7,654.9±721.33
F19	pH	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01	6.54±0.01
	Viscosity (cPs)	20,953.31±2018.36	21,226.18±2103.61	20,703.27±1902.41	20,470±215.94	20,050±1905.19	19,743.33±1104.98	19,383.61±1403.15
F20	pH	6.62±0.01	6.62±0.01	6.62±0.01	6.62±0.01	6.62±0.01	6.62±0.01	6.62±0.01
	Viscosity (cPs)	19,206.12±1,082.19	18,853.41±1,708.19	18,623.62±1,705.63	18,356.71±1,171.39	18,140±1,705.63	17,930±1,712.35	17,800±1,905.34
F21	pH	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.62±0.01	6.61±0.01
	Viscosity (cPs)	4,905.17±380.54	4,932.62±367.04	4,915.94±375.28	4,893.36±389.31	4,863.51±381.25	4,843.48±395.62	4,832±371.05
F22	pH	6.65±0.01	6.65±0.01	6.65±0.01	6.64±0.01	6.65±0.01	6.64±0.01	6.65±0.01
	Viscosity (cPs)	4,818.97±418.06	4,809.61±398.12	4,797.34±416.72	4,781±396.24	4,773.19±414.10	4,764.86±485.12	4,747.67±386.32
F23	pH	6.59±0.01	6.59±0.01	6.59±0.01	6.59±0.01	6.6±0.01	6.59±0.01	6.59±0.01
	Viscosity (cPs)	27,416.66±2,462.88	26,870±2,705.89	27,110±1,905.43	27,196.15±2,866.11	27,183.57±2,102.16	27,270±2,359.18	27,413.61±2,605.11
F24	pH	6.59±0.01	6.59±0.01	6.6±0.01	6.59±0.01	6.61±0.01	6.59±0.01	6.59±0.01
	Viscosity (cPs)	27,453.53±2,230.31	27,393.46±1,638.22	27,363.12±2,550.06	27,343.77±2,601.41	27,300±2,605.12	27,356.6±2,871.34	27,413.41±2,420.56
F25	pH	6.64±0.01	6.64±0.01	6.64±0.02	6.64±0.01	6.64±0.02	6.64±0.01	6.64±0.01
	Viscosity (cPs)	13,100±1,241.04	13,456.81±1,281.14	1,506.62±105.25	1,513.31±95.10	1,490±85.03	1,560.13±100.91	1,376.68±99.41
F26	pH	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.02	6.61±0.01	6.61±0.01	6.61±0.01
	Viscosity (cPs)	13,316.67±1,134.16	13,270±1,206.63	1,353.37±105.75	1,293.48±97.11	1,366.25±107.16	1,330±75.12	1,333.67±95.32
F27	pH	6.57±0.01	6.57±0.01	6.57±0.01	6.57±0.01	6.57±0.01	6.58±0.01	6.57±0.01
	Viscosity (cPs)	30,833.26±3,325.21	31,000.19±2,905.66	1,453.34±895.14	1,433.63±125.09	1,360.41±98.23	1,443.33±95.20	1,334.02±99.40
F28	pH	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01
	Viscosity (cPs)	29,233.31±2,194.30	29,133.04±2,805.03	1,290.17±135.66	1,193.33±145.26	1,303.71±112.34	1,270.89±805.15	1,285.39±115.61
F29	pH	6.57±0.01	6.57±0.01	6.57±0.01	6.57±0.01	6.57±0.01	6.57±0.01	6.57±0.01
	Viscosity (cPs)	9,741.3±881.63	9,736.1±876.74	2,728.66±285.94	2,723.53±205.52	2,719.43±184.62	2,714.07±805.15	2,711.17±214.13
F30	pH	6.61±0.01	6.61±0.01	6.62±0.01	6.61±0.01	6.6±0.01	6.61±0.01	6.61±0.01
	Viscosity (cPs)	9,666.29±927.09	9,670.64±874.12	2,660.47±179.40	2,651.71±246.71	2,661.63±241.23	2,663.8±185.06	2,658.23±182.19
F31	pH	6.61±0.02	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.62±0.01
	Viscosity (cPs)	28,396.62±2,608.29	27,830.14±2,605.45	6,410.35±612.92	6,403.33±503.46	5,803.03±545.78	5,816.76±483.42	5,590.42±515.43
F32	pH	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.01	6.61±0.02
	Viscosity (cPs)	28,290.17±2,702.11	27,893.33±2,705.86	6,906.65±601.35	6,566.07±800.33	6,786.14±725.12	6,771.49±605.11	6,819.56±599.15

13. Chemical stability

The content of GA in GA gel formulation F1-F8 and GE gel formulation F17-F24 after stored at 30 °C for 30 days were calculated as percent GA remaining relative to initial value in order to evaluate the chemical stability of GA in the formulation. Tables 25 and 26 indicate that there was no significant change in the concentration of GA in the formulation over 30 days suggesting that GA gel formulation F1-F8 and GE gel formulation F17-F24 were chemically stable at 30 °C for at least 30 days. The final test formulation was selected based on the best chemical stability, therefore, GA gel formulation F8 and GE gel formulation F22 were selected as a final test formulation for *in vivo* studies.

Table 25 Chemical stability of GA in each formulation of GA gel relative to initial value at room temperature

Formulation	% GA at day 0	% GA at day 30	% GA Remaining relative to day 0
F1	14.9633±0.02	14.9403±0.01	99.85±0.04
F2	14.9733±0.02	14.9512±0.01	99.85±0.04
F3	14.9900±0.01	14.9400±0.01	99.67±0.04
F4	14.9667±0.02	14.9365±0.01	99.80±0.08
F5	14.9767±0.02	14.9461±0.01	99.80±0.04
F6	14.9867±0.01	14.9511±0.01	99.76±0.08
F7	14.9900±0.01	14.9433±0.01	99.69±0.04
F8	14.9933±0.01	14.9689±0.01	99.90±0.04

The data is presented as mean ± SD (n=3).

Table 26 Chemical stability of GA in each formulation of GE gel relative to initial value at room temperature

Formulation	% GA at day 0	% GA at day 30	% GA Remaining relative to day 0
F17	1.7000±0.01	1.6967±0.01	99.80±0.34
F18	1.7066±0.01	1.6967±0.01	99.41±0.68
F19	1.6966±0.01	1.6933±0.01	99.80±0.34
F20	1.7033±0.01	1.6983±0.01	99.71±0.74
F21	1.7066±0.01	1.7000±0.01	99.61±0.59
F22	1.7133±0.01	1.7113±0.01	99.88±0.03
F23	1.7033±0.01	1.6983±0.01	99.71±0.74
F24	1.6966±0.01	1.6933±0.01	99.80±0.34

The data is presented as mean ± SD (n=3).

Part 3. Evaluation of safety and efficacy of GA and GE on hair growth retardation

14. Demographic data

All volunteers were consisted of 2 men and 20 women aged 30-50. There was no pre-existing medical conditions and history of drug or cosmetic allergy in all subjects.

15. Irritation testing of GA and GE gels in human volunteers

Gel containing the test compound and its gel base were subjected to irritation testing in order to ensure safety of these formulations in human volunteers. Twenty-two volunteers in the age range 18-50 were included to the test and randomly assigned into 2 treatment groups according to 2 formulations of 15% w/w GA and 15% w/w GE gels. Gel containing the test compound and its gel base were applied to the ventral side of the right and the left subject's forearms, respectively, for 24 h in an occlusive condition. The irritation was monitored after 24, 48 and 72 h according to International Contact Dermatitis Research Group (ICDRG) scale. The results revealed that there was no short-term irritancy including redness or swelling of skin in 22 volunteers after left the patches for 24 h. Moreover, all volunteers did not have any irritation after 48 and 72 h. It can be suggested that these formulations are relatively safe for human although 6 volunteers denied participating in the evaluation of the effect of GA and GE gels on hair growth. Thus, 16 subjects were subjected to the further experiment.

16. The effect of GA and GE gels on hair growth

Having shown that treatment DPCs with GA and GE resulted in decreased stem cell-like phenotypes. Furthermore, previous studies showed that maintaining hair growth greatly depended on stemness of DPCs (Ito et al., 2007; Driskell et al., 2009; Driskell et al., 2012; Clavel et al., 2012), therefore, the effect of GA and GE treatments on hair growth were investigated. Sixteen subjects who passed the irritation testing were provided with the test gel and its corresponding gel base which were the same formulation of the irritation test. The test gel and its gel base were separately applied on the shaved area of each underarm twice daily in the morning and the night after

skin cleansing for 28 days. Figures 35 and 36 illustrate that 15% w/w GA and 15% w/w GE gels significantly decreased hair growth in respect of area, thickness, length and number of underarm hair after 14, 21 and 28 day compared with its gel base. It can be suggested that GA and GE may decrease the rate of hair growth through suppressing stemness of DPCs. In addition, the effect on hair growth between 15% w/w GA gel and 15% w/w GE gel was compared utilizing the different percent of hair growth between test gel and its gel base. This result revealed that 15% w/w GA gel showed higher hair growth retardation effect over 15% w/w GE gel after 28 day of application as indicated in Figure 37. All subjects (100%) completed this study and there was no irritancy or any skin reaction during the study.

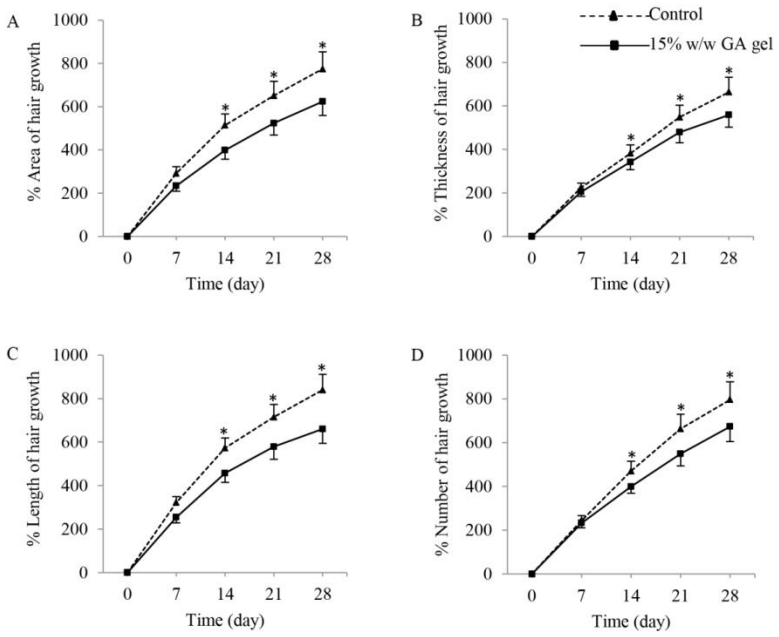


Figure 35 Effect of 15% w/w GA gel on underarm hair growth. The shaved area of each armpit was separately applied with 15% w/w GA gel and gel base after skin cleansing in the morning and the night for 28 days, digital camera images of indicated area were captured. (A-D) Parameters representing hair growth (area, thickness, length and number of hair) were determined by image processing software for various times (0-28 days) and expressed as the percentage of hair growth compared with the baseline on day 0. The data is presented as mean ± SD (n=8). *$P < 0.05$ versus gel base.

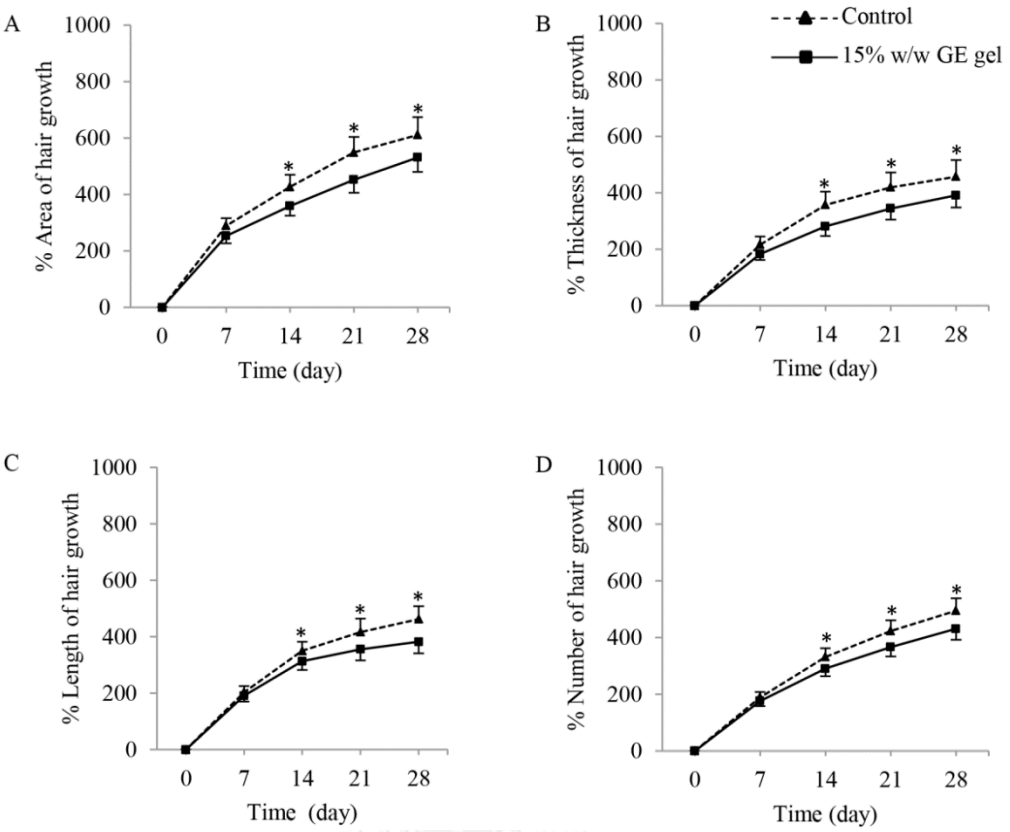


Figure 36 Effect of 15% w/w GE gel on underarm hair growth. The shaved area of each armpit was separately applied with 15% w/w GE gel and gel base after skin cleansing in the morning and the night for 28 days, digital camera images of indicated were captured. (A-D) Parameters representing hair growth (area, thickness, length and number of hair) were determined by image processing software for various times (0-28 days) and expressed as the percentage of hair growth compared with the baseline on day 0. The data is presented as mean ± SD (n=8). *$P < 0.05$ versus gel base.

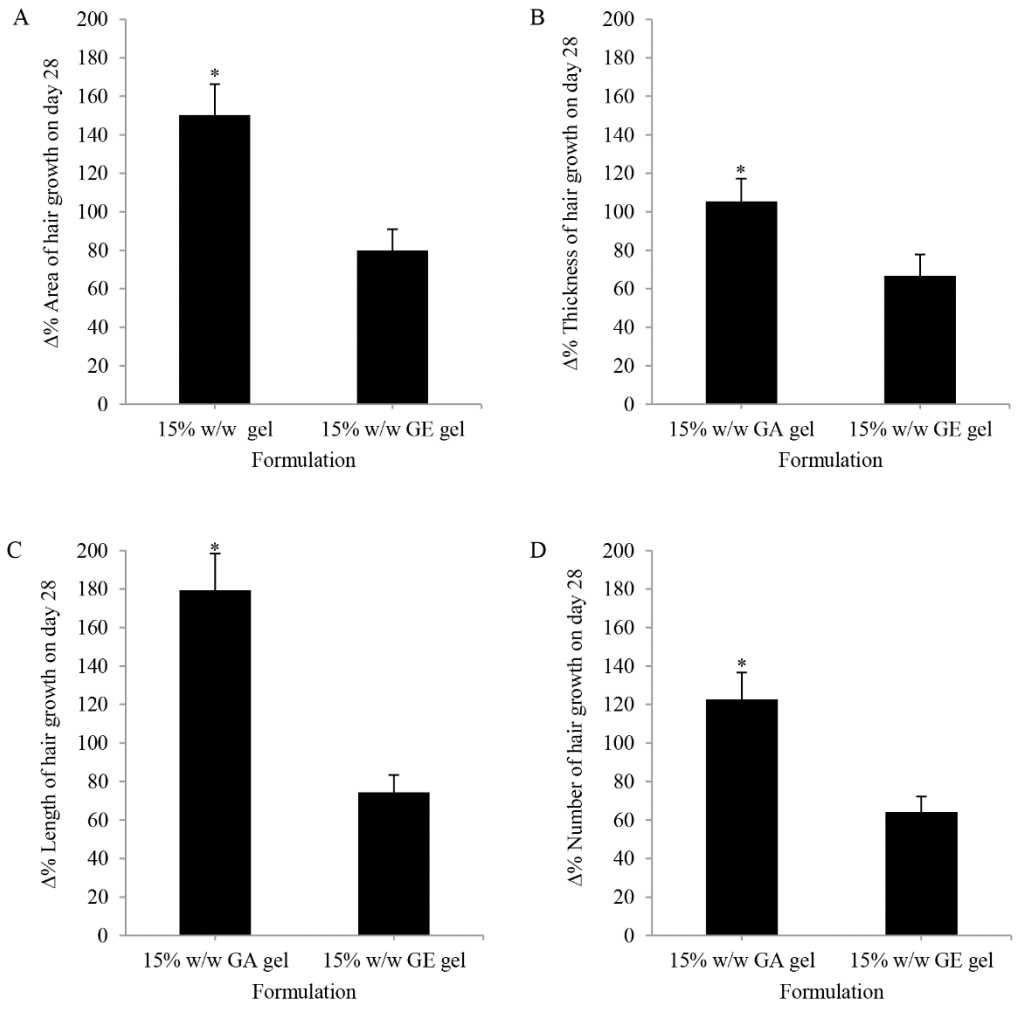


Figure 37 Comparison of the effect on underarm hair growth between 15% w/w GA and 15% w/w GE gel. The shaved area of each armpit was separately applied with the test gel and gel base after skin cleansing in the morning and the night for 28 days, digital camera images of indicated area were captured. (A-D) Parameters representing hair growth (area, thickness, length and number of hair) were determined by image processing software and expressed as the difference of hair growth in percentage between each formulation and its gel base on day 28. The data is presented as mean ±SD (n=8). *$P < 0.05$ versus 15% w/w GE gel.

Furthermore, in order to minimize the cost of production and risk of long term irritation, lower concentrations of the test compounds were utilized. 7.5% w/w GA and 10% w/w GE gels were prepared according to the content of formulation listed in Table 27 and 28, after that the analysis of irritating and their effect on hair growth were

performed. Twenty-two volunteers were therefore recruited for irritation test and then randomly assigned into 2 treatment groups according to 2 formulations of 7.5% w/w GA and 10% w/w GE gels for the efficacy test. All subjects were consisted of 1 man and 22 women aged 24-50. All study subjects have no pre-existing medical conditions and history of drug or cosmetic allergy. The result indicated that there was no short term irritation after testing for 24 h. Besides, all subjects showed no irritation after 48 and 72 h. However, there were only 16 subjects participating in the efficacy study. These subjects were provided with the test gel and its corresponding gel base which were the same formulation of the irritation test. The result of the efficacy study showed that 7.5% w/w GA and 10% w/w GE gel significantly decreased the underarm hair growth in respect of area, thickness, length and number after 14, 21 and 28 days in comparison to its gel base as illustrated in Figures 38 and 39. Moreover, the effect on hair growth between 7.5% w/w GA gel and 15% w/w GA gel was compared utilizing the differences in percent of the hair growth between test gel and its gel base. Interestingly, it was found that 7.5% w/w GA and 15% w/w GA gels exhibited similar effect on the underarm hair growth after 28 day in respect of area, thickness, length and number as shown in Figure 40. Likewise, treatment of 10% w/w GE gel for 28 days demonstrated comparable effect on the underarm hair growth in respect of area, thickness and number in comparison to 15% w/w GE gel whereas length of underarm hair growth was significantly decreased by treatment of 15% w/w GE gel compared to 10% w/w GE gel (Figure 41). Therefore, it can be suggested that the lower concentration of GA and GE at 7.5% w/w and 10% w/w may be suitable for an alternative treatment for suppressing the growth of unwanted hair. There was no irritancy or any skin reaction throughout and all study volunteers completed this study.

Table 27 Formula for preparation of 7.5% w/w GA gel

Composition	Content (% w/w)
GA	9.3
Carbomer 940	2
Propylene glycol	33.4
Distilled water	53.2
Vitamin E	0.1
Sodium EDTA	0.05
Floreintal TL 9501	0.001
Tween 20	1
Triethanolamine	qs
Caprylyl glycol	0.67
Ethylhexyl glycerin	0.33

Table 28 Formula for preparation of 10% w/w GE gel

Composition	Content (% w/w)
GE	10
Carbomer 940	1
Propylene glycol	15.45
Distilled water	71.4
Vitamin E	0.1
Sodium EDTA	0.05
Floreintal TL 9501	0.001
Tween 20	1
Triethanolamine	qs
Caprylyl glycol	0.67
Ethylhexyl glycerin	0.33

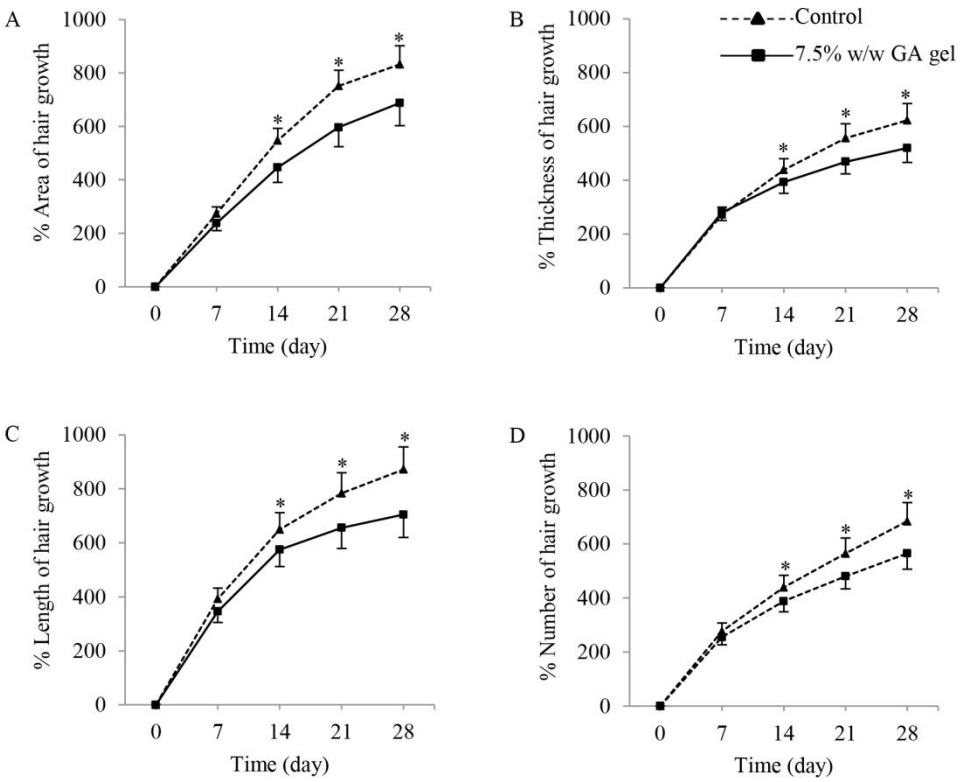


Figure 38 Effect of 7.5% w/w GA gel on underarm hair growth. The shaved area of each armpit was separately applied with 7.5% w/w GA gel and gel base after skin cleansing in the morning and the night for 28 days, digital camera images of indicated area were captured. (A-D) Parameters representing hair growth (area, thickness, length and number of hair) were determined by image processing software for various times (0-28 days) and expressed as the percentage of hair growth compared with the baseline on day 0. The data is presented as mean ± SD (n=8). *$P < 0.05$ versus gel base.

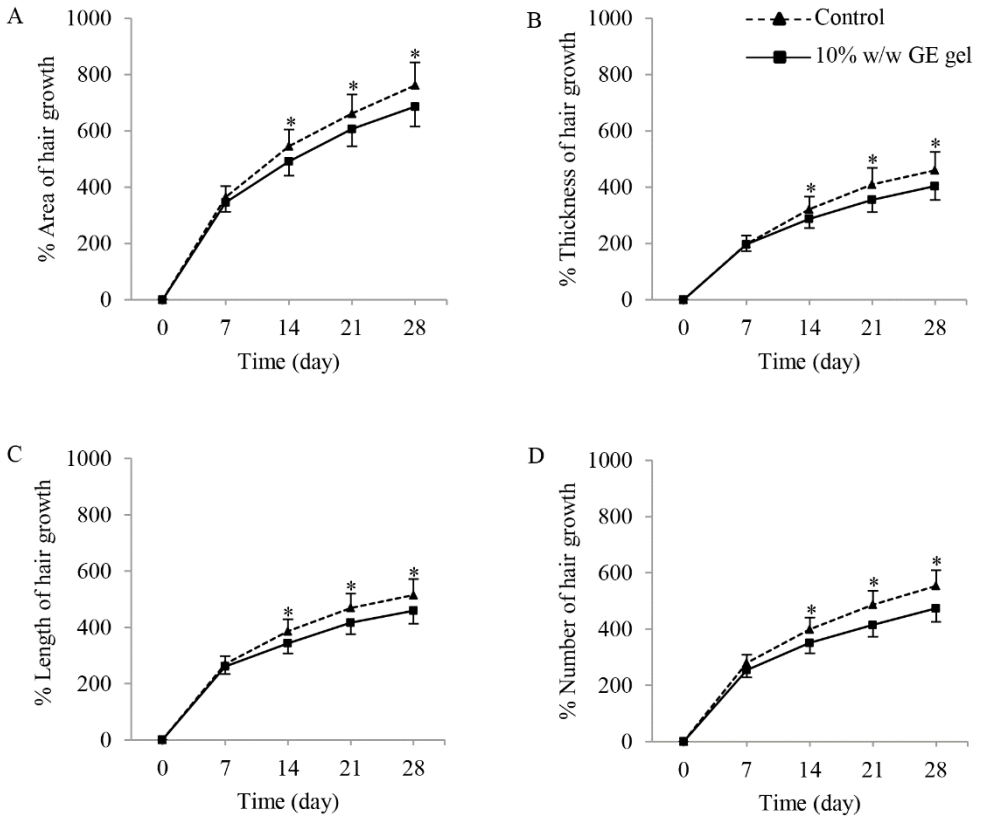


Figure 39 Effect of 10% w/w GE gel on underarm hair growth. The shaved area of each armpit was separately applied with 10% w/w GE gel and gel base after skin cleansing in the morning and the night for 28 days, digital camera images of indicated were captured. (A-D) Parameters representing hair growth (area, thickness, length and number of hair) were determined by image processing software for various times (0-28 days) and expressed as the percentage of hair growth compared with the baseline on day 0. The data is presented as mean ± SD (n=8). *$P < 0.05$ versus gel base.

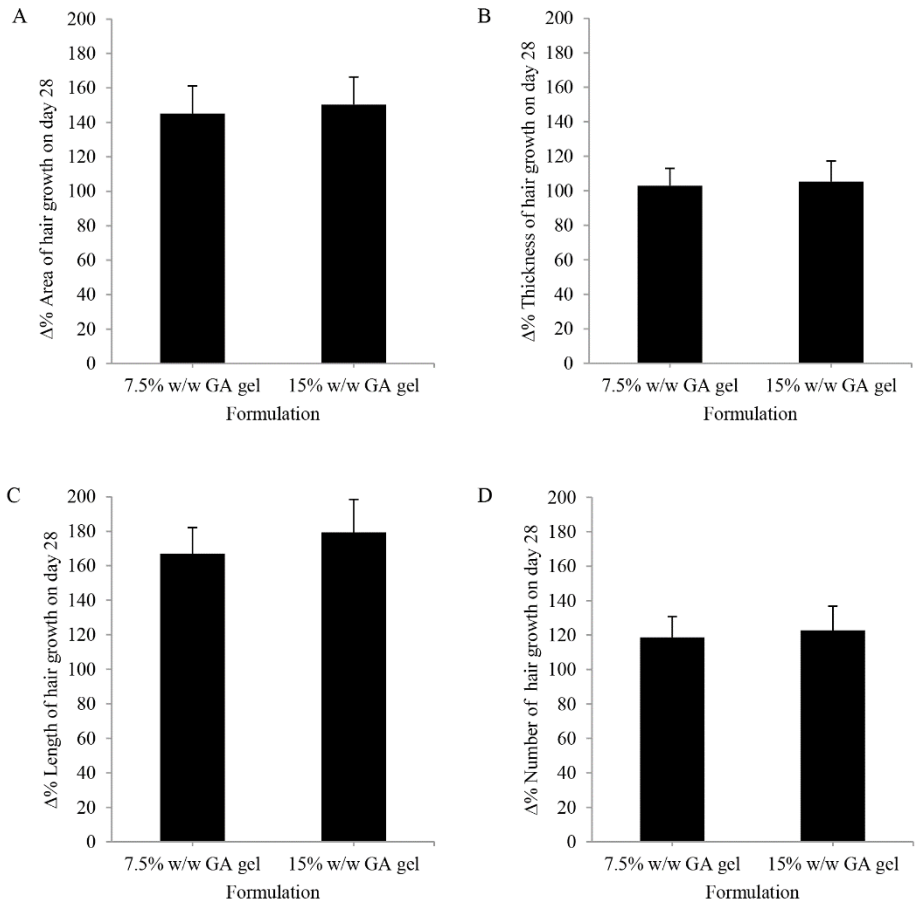


Figure 40 Comparison of the effect on underarm hair growth between 7.5% w/w GA and 15% w/w GA gel. The shaved area of each armpit was separately applied with the test gel and gel base after skin cleansing in the morning and the night for 28 days, digital camera images of indicated area were captured. (A-D) Parameters representing hair growth (area, thickness, length and number of hair) were determined by image processing software and expressed as the difference of hair growth in percentage between each formulation and its gel base on day 28. The data is presented as mean±SD (n=8).

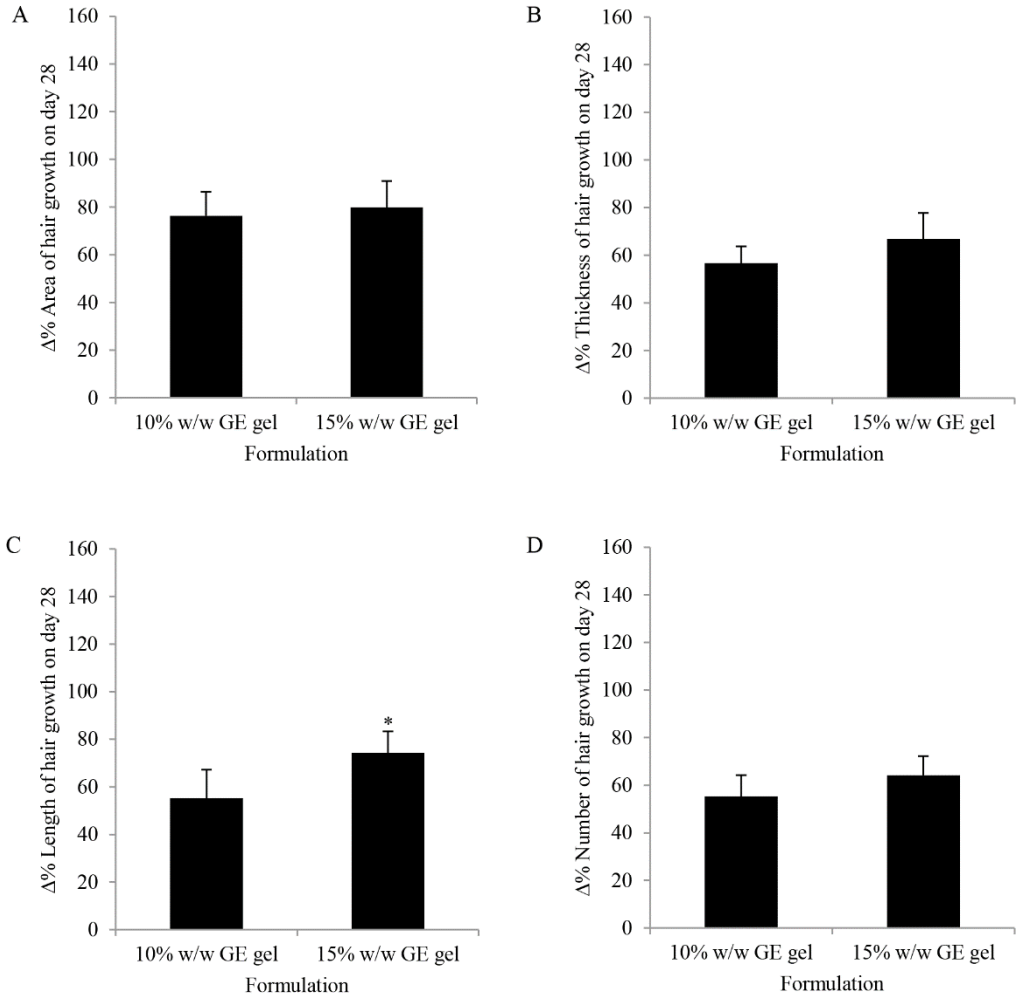


Figure 41 Comparison of the effect on underarm hair growth between 10% w/w GE and 15% w/w GE gel. The shaved area of each armpit was separately applied with the test gel and gel base after skin cleansing in the morning and the night for 28 days, digital camera images of indicated area were captured. (A-D) Parameters representing hair growth (area, thickness, length and number of hair) were determined by image processing software and expressed as the difference of hair growth in percentage between each formulation and its gel base on day 28. The data is presented as mean±SD (n=8).

CHAPTER V

DISCUSSION AND CONCLUSION

Stem cell- like plasticity of DPCs is well recognized by their ability to differentiate into different cell types (Richardson et al., 2005). Interestingly, stemness and multipotency of DPCs have been shown to be important factors potentiating hair follicle formation (Ito et al., 2007; Driskell et al., 2009; Driskell et al., 2012; Clavel et al., 2012). Sufficient studies pointed out that the key functions of active DPCs are to promote hair growth and to maintain or to prolong anagen phase (Elliott et al., 1999; Paus and Foitzik, 2004). Therefore, it is likely that diminishing the stemness in these specialized cells may decrease the rate of hair growth. In an attempt to clarify the possible mechanism of GA, the active ingredient of *G. glabra* L., and GE in negatively regulating hair growth, it has been shown for the first time that GA and GE mediates the reduction of stemness in DPCs. Treatment of the DPCs with non- toxic concentrations of GA and GE caused the spontaneous morphological change toward fibroblast-like cells (Figure 10). Likewise, the results of immune-cytochemistry as well as western blot analysis portrayed that treatment of DPCs with GA and GE resulted in the significant decline of stem cell markers (Figure 13-16).

In relevance to stem cell research, CD133, a transmembrane glycoprotein, has been generally used as a standard biomarker of human stem cells (Li, 2013). These results demonstrated that DPCs at the early passages had a high level of CD133. Treatment of the cells with GA and GE led to the loss of stem cell features in DPCs including CD133, ALDH1A1, and integrin β1 (Figure 13- 16). With regard to molecular mechanism in regulating stemness, tremendous advances have been made to elucidate stem cell signaling pathways during the past decades, and in particular Wnt/β-catenin was found to be a crucial factor for maintaining functions as well as characteristics of stem cells (Merrill, 2012). Previous study reported that the genetic inhibition of β-catenin in DPCs caused the substantial suppression of hair growth and

regeneration (Enshell-Seijffers et al., 2010). The function of β-catenin, a co-trancription factor of T-cell factor/lymphoid enhancing factor (TCF/LEF), was shown to up-regulate of the expression of proteins facilitating stem cell functions (Fukumoto et al., 2001). Indeed, the phosphorylation of β-catenin by its negative regulator, namely, GSK3β contributes to ubiquitin-mediated proteasomal degradation of β-catenin. The active Akt, the very well-known proliferative and survival signals, is shown to inhibit the function of GSK3β by the phosphorylation of GSK3β at serine 9 (Fukumoto et al., 2001). Therefore, the activation of Akt results in the increase of cellular β-catenin and cellular stemness. Herein, it was shown that treatment with GA and GE decreased the level of activated Akt leading to the decrease of β-catenin level (Figure 17). These results revealed that the decline of stemness in GA-treated and GE-treated cells caused by the interruption of Akt/β catenin pathway.

Several studies demonstrated that β-catenin interacted with many signaling pathways associated with pluripotency and EMT (Kim et al., 2002; Miki et al., 2011; Li et al., 2012; Merrill, 2012; Su et al., 2014). The expression of EMT proteins and pluripotent-activating transcription factors were found to up-regulate by the activation of Wnt/β-catenin signaling (Van Raay et al., 2005; Pereira et al., 2006; Cole et al., 2008; ten Berge et al., 2008; Lambertini et al., 2010; Wu et al., 2012). According to the previous studies, the presence of transcription factor Snail and ZEB1 is important for the EMT (Mani et al., 2008; Shimono et al., 2009), our findings displayed the significant reduction of such proteins in the DPCs treated with GA and GE (Figure 18), and the decrease of EMT proteins was found to be consistent with the stem cell-like morphology and aggregative behavior of DPCs. These results also support the concept that Akt/GSK3β-dependent β-catenin activation is required for the DPCs to retain their stem cell phenotypes. Our findings revealed that the down-stream transcription factors of Wnt/β-catenin, namely Oct-4, Nanog and Sox2 were decreased in the GA-treated and GE-treated cells (Figure 18). Essentially, the role of GA and GE treatment on the reduction of stemness in primary human DPCs was systematically evaluated (Figure

22). We have shown that GA and GE treatments inhibit stemness in DPCs through the suppression of Akt/GSK3β-dependent β-catenin regulation (Figure 17 and 22).

In closing, the present study also provided further details of the effect of GA and GE on hair growth in human utilizing the prepared GA and GE gels since increasing evidence pointed out that DPCs' stemness was strongly associated with hair growth (Ito et al., 2007; Driskell et al., 2009; Driskell et al., 2012; Clavel et al., 2012). Our results illustrated the significant reduction of hair growth by GA and GE gel treatments (Figure 35, 36, 38 and 39) and it was found to be consistent with the decrease of stemness in GA treated and GE treated DPCs. Interestingly, our findings were consistent with obtained preliminary data from Terakosolphan et al. (2011) that revealed a significant hair growth retardation effect of GA on arm hairs of study volunteers. Therefore, these results support the concept that stemness of DPCs may be a key player in hair growth regulation and treatment with GA and GE leads to hair growth retardation (Figure 42). Based on our findings, it is likely that GA may be one of the primary active substances of *G. glabra* L. influencing stemness-suppressing the effect in DPCs and hair growth retardation as *G. glabra* L. was reported to contain several classes of substances (Lakshmi and Geetha, 2011; Parvaiz et al., 2014). Importantly, our results demonstrated a significant stemness-suppressing activity in DPCs and hair growth retardation activity in GE sample that were not proportional to its GA content compared with that of GA sample. This suggests that there may be other active substances of *G. glabra* L. that accounts for these activities. Therefore, this data initially provides the information for provoking further investigations that may lead to the use of these compounds for therapeutic as well as cosmeceutical approaches.

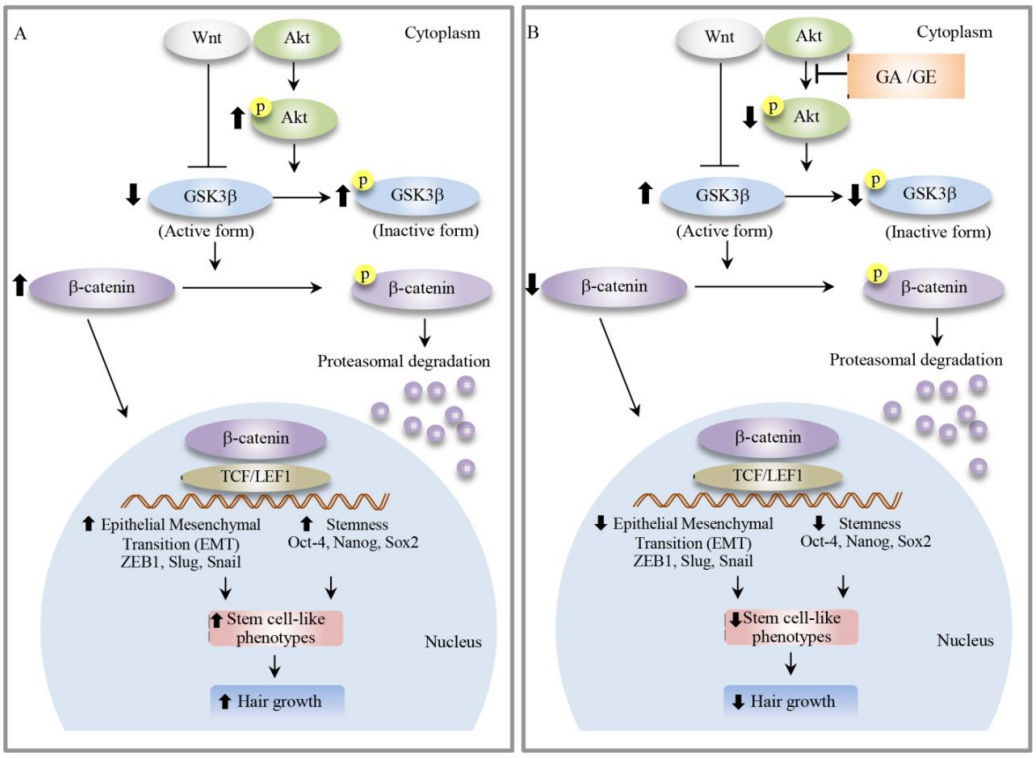


Figure 42 Schematic diagram illustrating the proposed mechanism in reducing hair growth of GA and GE through the down-regulation of stemness in human DPCs. (A) Upon activation of Wnt, GSK3β is inactivated. Also, the phosphorylation at GSK3β through the function of active Akt leads to the inhibition of such enzyme. GSK3β negatively regulates β-catenin by the phosphorylation leading to the degradation of phosphorylated β-catenin. Hence, in the presence Wnt and activated Akt cause the accumulation of cellular β-catenin and in turn trigger transcription of genes-activating EMT and stemness that may result in the increase of hair growth. (B) The addition of GA or GE was responsible for the decrease of activated Akt resulting in the augmented level of active GSK3β and phosphorylated β-catenin. As a consequence, phosphorylated β-catenin undergoes proteasomal degradation and in turn decreases transcription of target genes associated with stem cell phenotypes in human DPCs that may result in the decrease of hair growth.

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APPENDIX

APPENDIX A

TABLES OF EXPERIMENTAL RESULTS

Table 29 Cytotoxicity of GA and GE on DPCs

GA (μM)	% Cell viability	GE (μg/ml)	% Cell viability
0	100±0.00	0	100±0.00
10	96.50±2.76	10	96.12±2.39
25	94.84±3.26	25	99.18±4.79
50	95.58±2.24	50	98.99±2.76
100	95.92±2.97	100	100.33±6.79
200	96.68±3.49	200	97.25±3.07

The data is presented as mean ± SD (n=3).

Table 30 The effect of GA on cell cycle distribution in DPCs

Treatment	Time (h)	Percentage of total cells		
		G1	S	G2/M
GA 0 μM	0	76.37±6.62	9.42±3.28	14.40±4.11
GA 0 μM	0	69.94±5.16	14.10±4.61	16.47±5.20
GA 200 μM	72	74.45±4.53	10.51±4.97	15.18±2.46

The data is presented as mean ± SD (n=3).

Table 31 The effect of GE on cell cycle distribution in DPCs

Treatment	Time (h)	Percentage of total cells		
		G1	S	G2/M
GE 0 μg/ml	0	80.22±5.41	8.76±2.98	11.62±4.29
GE 0 μg/ml	0	75.82±4.10	10.14±4.31	14.36±3.51
GE 200 μg/ml	72	74.65±3.69	11.48±5.72	14.18±2.14

The data is presented as mean ± SD (n=3).

Table 32 Effect of GA on DPCs' aggregation

GA (μM)	Aggregation size (μm)	Aggregation number
0	99.45±5.32	10±0.25
10	83.69±7.59	7.59±0.52
25	78.55±5.31	6.550.34
50	69.32±7.31	5.55±0.59
100	52.95±4.31*	3.91±0.69*
200	49.59±4.99*	2.14±0.25*

The data is presented as mean ± SD (n=3). $^{*}P < 0.05$ versus non-treated control.

Table 33 Effect of GE on DPCs' aggregation

GE (μg/ml)	Aggregation size (μm)	Aggregation number
0	157.27±3.90	12.33±1.45
10	156.40±1.9	10.33±0.88
25	152.47±2.42	10.15±1.20
50	149.60±3.73	9.04±1.58
100	119.40±6.91*	8.12±1.53*
200	93.97±6.28*	7.03±1.15*

The data is presented as mean ± SD (n=3). $^{*}P < 0.05$ versus non-treated control.

Table 34 Time course measurement on the expression of stem cell markers in DPCs after being treated with GA

Time (h)	Stem cell markers	GA 0 μM	GA 200 μM
0	CD133	1.00±0.00	1.00±0.00
	Integrin β1	1.00±0.00	1.00±0.00
	ALDH1A1	1.00±0.00	1.00±0.00
24	CD133	0.87±0.08	0.54±0.09
	Integrin β1	0.99±0.08	0.96±0.09
	ALDH1A1	0.98±0.08	0.67±0.09
48	CD133	0.78±0.08	0.26±0.09*
	Integrinβ1	0.98±0.09	0.97±0.08
	ALDH1A1	0.92±0.09	0.43±0.08*
72	CD133	0.76±0.06	0.12±0.08**
	Integrin β1	0.77±0.09	0.45±0.07*
	ALDH1A1	0.74±0.09	0.18±0.08*

The data is presented as mean ± SD (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus non-treated control.

Table 35 Time course measurement on the expression of stem cell markers in DPCs after being treated with GE

Time (h)	Stem cell markers	GE 0 µg/ml	GE 200 µg/ml
0	CD133	1.00±0.00	1.00±0.00
	Integrin β1	1.00±0.00	1.00±0.00
	ALDH1A1	1.00±0.00	1.00±0.00
24	CD133	0.80±0.50	0.79±0.07
	Integrin β1	0.99±0.09	0.82±0.03
	ALDH1A1	0.92±0.31	0.31±0.02*
48	CD133	0.78±0.51	0.22±0.06*
	Integrinβ1	0.97±0.08	0.41±0.03*
	ALDH1A1	0.92±0.06	0.07±0.05**
72	CD133	0.78±0.49	0.22±0.06*
	Integrin β1	0.83±0.08	0.35±0.02*
	ALDH1A1	0.49±0.07	0.07±0.05**

The data is presented as mean ± SD (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus non-treated control.

Table 36 Effect of GA and GE on the expression of stem cell markers in DPCs

Treatments		CD133	Integrin β1	ALDH1A1
GA (µM)	0	1.00±0.00	1.00±0.00	1.00±0.00
	25	0.85±0.05	0.98±0.06	0.96±0.08
	50	0.83±0.06	0.87±0.08	0.65±0.09
	100	0.83±0.08	0.48±0.09*	0.15±0.06**
	200	0.35±0.08**	0.32±0.07*	0.15±0.06**
GE (µg/ml)	0	1.00±0.00	1.00±0.00	1.00±0.00
	25	0.96±0.11	0.98±0.17	0.63±0.2
	50	0.85±0.09	0.52±0.16*	0.61±0.06*
	100	0.84±0.08	0.43±0.13*	0.59±0.05*
	200	0.36±0.09*	0.31±0.02*	0.07±0.10**

The data is presented as mean ± SD (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus non-treated control.

Table 37 Effect of GA and GE on Wnt/β-catenin signaling in DPCs

Treatments		p-Akt (Ser 473)	p-GSK3β (Ser 9)	β-catenin
GA (μM)	0	1.00±0.00	1.00±0.00	1.00±0.00
	25	0.99±0.01	0.48±0.02*	0.98±0.04
	50	0.76±0.04	0.45±0.01*	0.67±0.03
	100	0.39±0.01*	0.32±0.01**	0.42±0.05*
	200	0.23±0.02*	0.25±0.04**	0.39±0.05**
GE (μg/ml)	0	1.00±0.00	1.00±0.00	1.00±0.00
	25	0.90±0.06	0.95±0.06	0.53±0.11
	50	0.86±0.04	0.30±0.05*	0.32±0.10*
	100	0.59±0.11*	0.24±0.03**	0.17±0.04**
	200	0.38±0.01**	0.23±0.03**	0.16±0.03**

The data is presented as mean ± SD (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus non-treated control.

Table 38 Effect of GA and GE on EMT and self-renewal transcription factors in DPCs

Treatments		ZEB1	Oct-4	Nanog	Sox2	Slug	Snail
GA (μM)	0	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
	25	1.03±0.04	0.89±0.06	0.97±0.04	0.76±0.05	0.99±0.04	0.98±0.04
	50	1.02±0.05	0.65±0.05*	0.97±0.04	0.73±0.05	0.97±0.06	0.97±0.06
	100	1.04±0.04	0.53±0.03*	0.37±0.05*	0.39±0.04*	0.55±0.04	0.35±0.05**
	200	0.25±0.04**	0.31±0.05*	0.35±0.05*	0.36±0.05*	0.54±0.04	0.32±0.05**
GE (μg/ml)	0	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00	1.00±0.00
	25	0.98±0.07	1.00±0.05	0.58±0.07	0.46±0.04*	0.87±0.06	0.56±0.04
	50	0.56±0.02*	0.42±0.01*	0.22±0.08*	0.21±0.02*	0.32±0.07*	0.06±0.03*
	100	0.17±0.06**	0.24±0.04*	0.11±0.03**	0.07±0.03**	0.14±0.03*	0.04±0.02**
	200	0.13±0.04**	0.22±0.03*	0.10±0.04**	0.05±0.01**	0.12±0.02**	0.03±0.02**

The data is presented as mean ± SD (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus non-treated control.

Table 39 Cytotoxicity of GA and GE on primary human DPCs

GA (µM)	% Cell viability	GE (µg/ml)	% Cell viability
0	100±0.00	0	100±0.00
10	96.63±3.48	10	100.03±3.98
25	93.23±3.42	25	98.41±2.52
50	94.16±2.61	50	99.20±3.72
100	95.12±2.98	100	94.65±4.63
200	96.04±3.02	200	93.71±2.24

The data is presented as mean ± SD (n=3).

Table 40 Effect of GA and GE on the expression of stem cell markers in primary human DPCs

Treatments		CD133	Integrin β1	ALDH1A1
GA (µM)	0	1.00±0.00	1.00±0.00	1.00±0.00
	25	0.92±0.04	0.96±0.05	0.82±0.08
	50	0.91±0.05	0.76±0.06	0.72±0.08
	100	0.72±0.04*	0.66±0.05	0.46±0.06*
	200	0.38±0.05**	0.47±0.04*	0.45±0.06*
GE (µM)	0	1.00±0.00	1.00±0.00	1.00±0.00
	25	0.78±0.05	0.97±0.08	0.65±0.06
	50	0.54±0.09*	0.86±0.09	0.63±0.09*
	100	0.25±0.12*	0.45±0.08*	0.36±0.03*
	200	0.14±0.10**	0.43±0.05*	0.24±0.02*

The data is presented as mean ± SD (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus non-treated control.

Table 41 Effect of GA and GE on Wnt/β-catenin signaling in primary human DPCs

Treatments		p-Akt (Ser 473)	p-GSK3β (Ser 9)	β-catenin
GA (μM)	0	1.00±0.00	1.00±0.00	1.00±0.00
	25	0.99±0.06	0.69±0.05	0.82±0.04
	50	0.87±0.06	0.65±0.06	0.77±0.06
	100	0.41±0.07*	0.37±0.04*	0.41±0.05*
	200	0.39±0.06*	0.28±0.09**	0.39±0.05*
GE (μg/ml)	0	1.00±0.00	1.00±0.00	1.00±0.00
	25	0.98±0.09	0.91±0.09	0.93±0.04
	50	0.53±0.10*	0.83±0.04	0.72±0.03
	100	0.27±0.07*	0.68±0.04*	0.50±0.04*
	200	0.16±0.07**	0.46±0.10*	0.39±0.09*

The data is presented as mean ± SD (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus non-treated control.

Table 42 Effect of GA and GE on EMT-activating transcription factors in primary human DPCs

Treatments		ZEB1	Slug	Snail
GA (μM)	0	1.00±0.00	1.00±0.00	1.00±0.00
	25	0.95±0.06	0.97±0.05	0.92±0.04
	50	0.94±0.04	0.76±0.05	0.77±0.04
	100	0.94±0.06	0.69±0.06	0.65±0.05*
	200	0.49±0.07*	0.64±0.06	0.32±0.06**
GE (μg/ml)	0	1.00±0.00	1.00±0.00	1.00±0.00
	25	0.88±0.03	0.82±0.03	0.65±0.05
	50	0.63±0.05	0.56±0.06*	0.32±0.02*
	100	0.26±0.08*	0.40±0.03*	0.24±0.07*
	200	0.24±0.06*	0.03±0.05**	0.19±0.07*

The data is presented as mean ± SD (n=3). $^{*}P < 0.05$, $^{**}P < 0.01$ versus non-treated control.

Table 43 Calibration curve of standard GA by TLC-densitometric method

Amount of standard GA (µg/spot)	Area under peak (AU)
0.125	604±15.65
0.250	1,200±16.36
0.500	2,404±18.48
1.000	4,805±14.95
1.500	7,212±15.66
2.000	9,616±15.41
2.500	11,520±16.32

The data is presented as mean ± SD (n=3).

Table 44 Effect of 15% GA gel on underarm hair growth

Treatment	% Area of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	291.39±31.15	514.09±52.17*	651.19±66.29*	774.23±79.31*
15% w/w GA gel	0.00±0.00	234.05±25.08	398.59±41.17	523.49±55.20	623.94±64.21
Treatment	% Thickness of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	224.37±21.09	381.68±39.14*	547.96±55.18*	663.60±68.25*
15% w/w GA gel	0.00±0.00	205.10±21.08	342.10±35.14	478.95±48.15	558.34±56.15
Treatment	% Length of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	321.99±27.09	572.88±46.17*	714.88±58.25*	839.28±72.24*
15% w/w GA gel	0.00±0.00	254.47±26.08	457.05±42.14	578.66±58.17	659.90±65.25
Treatment	% Number of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	243.59±23.09	469.98±45.16*	663.04±66.23*	795.88±82.23*
15% w/w GA gel	0.00±0.00	232.43±22.08	398.82±30.13	548.95±55.16	673.20±68.25

Data represent mean ± SD (n=8). $^{*}P < 0.05$ versus gel base.

Table 45 Effect of 15% GE gel on underarm hair growth

Treatment	% Area of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	289.25±27.09	426.73±43.13*	548.53±55.13*	611.25±62.20*
15% w/w GE gel	0.00±0.00	253.28±26.08	359.20±34.14	452.31±46.15	531.40±51.14
Treatment	% Thickness of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	216.18±29.09	357.41±46.12*	419.39±52.15*	457.37±58.17*
15% w/w GE gel	0.00±0.00	183.05±21.08	280.78±34.12	344.36±39.14	390.65±43.15
Treatment	% Length of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	203.27±22.09	349.92±32.17*	416.38±47.19*	462.34±45.24*
15% w/w GE gel	0.00±0.00	190.77±21.09	312.52±30.13	355.25±39.15	381.62±40.18
Treatment	% Number of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	189.00±19.09	331.57±30.13*	423.01±37.15*	494.53±43.16*
15% w/w GE gel	0.00±0.00	176.30±18.08	289.95±26.12	366.00±33.14	430.38±38.15

Data represent mean ± SD (n=8). $^{*}P < 0.05$ versus gel base.

Table 46 Comparison of the effect on underarm hair growth between 15% w/w GA and 15% w/w GE gel.

Treatment	Δ% Area of hair growth at day 28
15% w/w GA gel	150.29±16.05*
15% w/w GE gel	79.85±11.02
Treatment	Δ% Thickness of hair growth at day 28
15% w/w GA gel	105.26±12.04*
15% w/w GE gel	66.72±11.04
Treatment	Δ% Length of hair growth at day 28
15% w/w GA gel	179.38±19.05*
15% w/w GE gel	74.34±9.02
Treatment	Δ% Number of hair growth at day 28
15% w/w GA gel	122.68±14.05*
15% w/w GE gel	64.15±8.02

Data represent mean ± SD (n=8). $^{*}P < 0.05$ versus 15% w/w GE gel.

Table 47 Effect of 7.5% GA gel on underarm hair growth

Treatment	% Area of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	274.09±28.09	547.21±56.16*	751.85±72.26*	832.61±85.23*
7.5% w/w GA gel	0.00±0.00	238.17±24.08	446.63±45.16	596.69±58.29	687.56±69.26
Treatment	% Thickness of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	272.51±28.09	438.32±42.17*	556.30±53.17*	622.44±62.14*
7.5% w/w GA gel	0.00±0.00	280.34±30.08	392.80±41.16	468.64±45.13	519.52±53.14
Treatment	% Length of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	392.40±40.09	649.46±62.14*	783.65±76.15*	871.44±84.18*
7.5% w/w GA gel	0.00±0.00	345.79±35.08	574.99±51.13	655.69±64.13	704.41±65.15
Treatment	% Number of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	276.88±31.09	439.24±45.13*	564.74±57.17*	683.79±69.19*
7.5% w/w GA gel	0.00±0.00	254.77±28.08	387.87±39.15	480.30±46.16	565.17±58.16

Data represent mean ± SD (n=8). *$P < 0.05$ versus gel base.

Table 48 Effect of 10% GE gel on underarm hair growth

Treatment	% Area of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	364.44±39.12	545.24±59.12*	661.47±68.17*	761.92±81.19*
10% w/w GE gel	0.00±0.00	345.54±33.09	491.08±50.12	606.62±62.12	685.64±70.16
Treatment	% Thickness of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	198.42±30.11	321.37±45.14*	409.31±59.17*	460.03±65.18*
10% w/w GE gel	0.00±0.00	196.89±24.09	287.27±32.13	354.94±43.13	403.37±48.15
Treatment	% Length of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	269.22±29.13	385.69±42.13*	468.72±51.13*	514.06±57.16*
10% w/w GE gel	0.00±0.00	260.90±27.09	343.01±36.16	416.13±41.14	458.79±46.15
Treatment	% Number of hair growth after shaving at different time				
	Day 0	Day 7	Day 14	Day 21	Day 28
Gel base	0.00±0.00	279.47±29.13	399.07±41.14*	486.68±49.14*	553.37±56.15*
10% w/w GE gel	0.00±0.00	254.35±26.03	350.75±37.13	414.51±42.12	473.42±48.14

Data represent mean ± SD (n=8). *$P < 0.05$ versus gel base.

Table 49 Comparison of the effect on underarm hair growth between 7.5% w/w GA and 15% w/w GA gels

Treatment	Δ% Area of hair growth at day 28
7.5% w/w GA gel	145.05±18.06
15% w/w GA gel	150.29±16.05
Treatment	Δ% Thickness of hair growth at day 28
7.5% w/w GA gel	102.92±10.01
15% w/w GA gel	105.26±12.04
Treatment	Δ% Length of hair growth at day 28
7.5% w/w GA gel	167.03±15.06
15% w/w GA gel	179.38±19.05
Treatment	Δ% Number of hair growth at day 28
7.5% w/w GA gel	118.62±12.05
15% w/w GA gel	122.68±14.05

Data represent mean ± SD (n=8).

Table 50 Comparison of the effect on underarm hair growth between 10% w/w GE and 15% w/w GE gels

Treatment	Δ% Area of hair growth at day 28
10% w/w GA gel	76.28±10.03
15% w/w GE gel	79.85±11.02
Treatment	Δ% Thickness of hair growth at day 28
10% w/w GA gel	56.66±7.04
15% w/w GE gel	66.72±11.04
Treatment	Δ% Length of hair growth at day 28
10% w/w GA gel	55.38±12.01
15% w/w GE gel	74.34±9.02*
Treatment	Δ% Number of hair growth at day 28
10% w/w GA gel	55.27±9.01
15% w/w GE gel	64.15±8.02

Data represent mean ± SD (n=8). $^{*}P < 0.05$ versus gel base.

APPENDIX B

FIGURES OF EXPERIMENTAL RESULTS

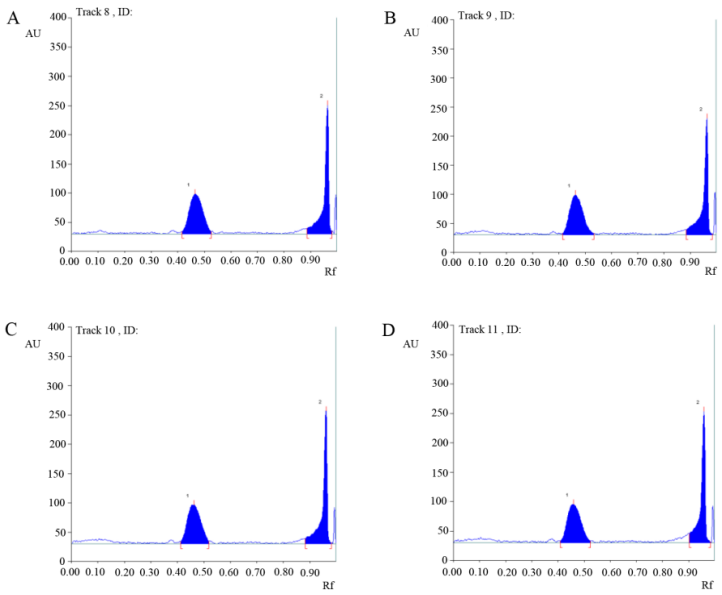

Figure 43 TLC chromatogram of GA in F1 (A), F2 (B), F3 (C), F4 (D) formulations. Mobile phase is ethyl acetate : glacial acetic acid : methanol : water (8:3:2:1, v/v)

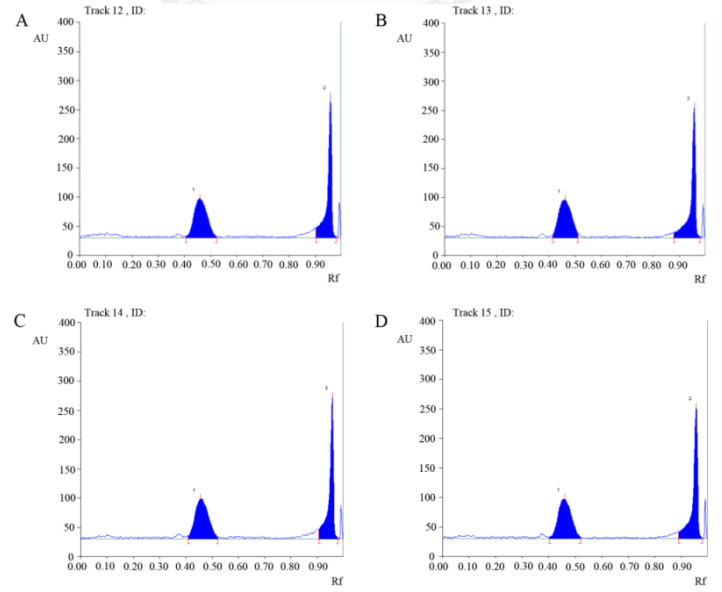

Figure 44 TLC chromatogram of GA in F5 (A), F6 (B), F7 (C), F8 (D) formulations. Mobile phase is ethyl acetate : glacial acetic acid : methanol : water (8:3:2:1, v/v)

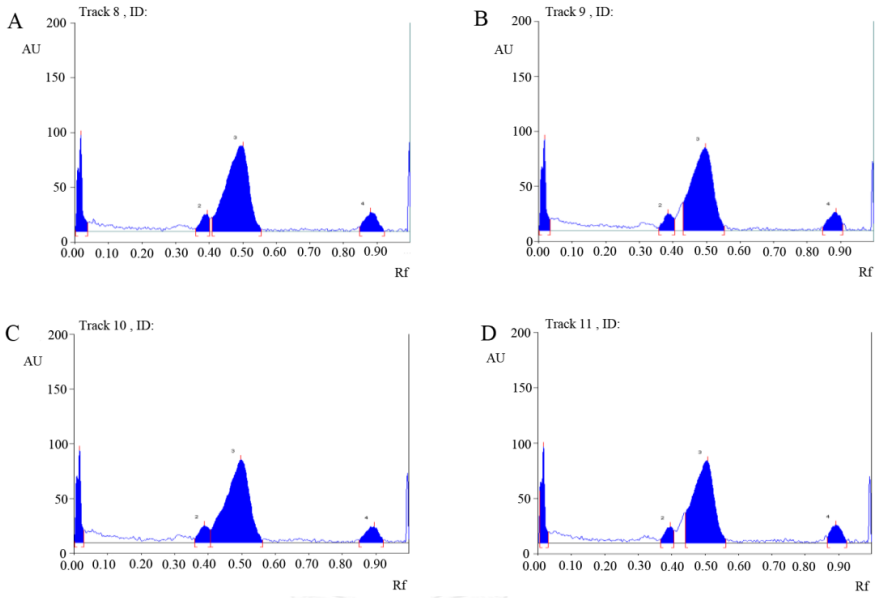

Figure 45 TLC chromatogram of GA in F17 (A), F18 (B), F19 (C), F20 (D) formulations. Mobile phase is ethyl acetate : glacial acetic acid : methanol : water (8:3:2:1, v/v)

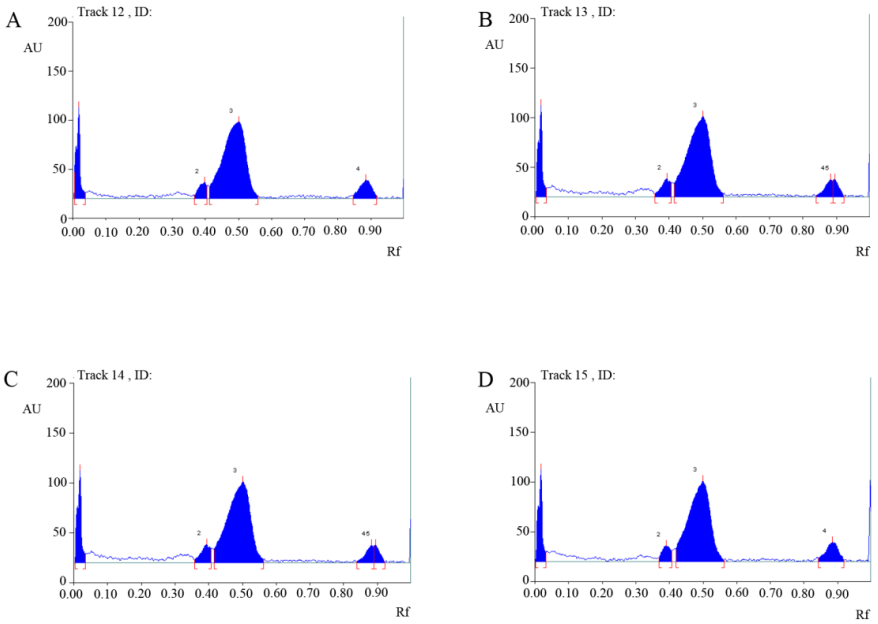

Figure 46 TLC chromatogram of GA in F21 (A), F22 (B), F23 (C), F24 (D) formulations. Mobile phase is ethyl acetate : glacial acetic acid : methanol : water (8:3:2:1, v/v)

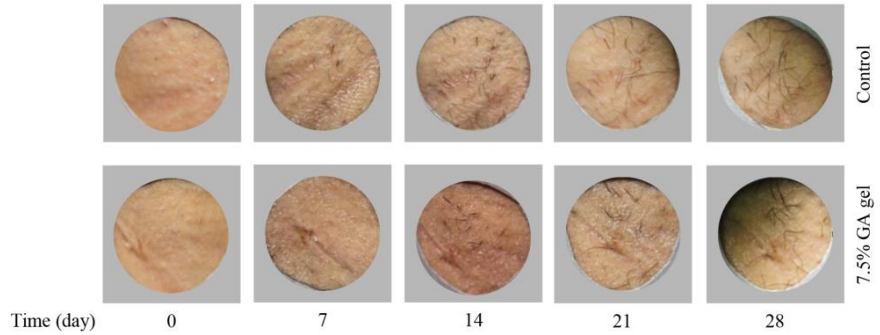

Figure 47 Representative images of underarm hair after treatment with 7.5% w/w GA gel and gel base for various times (0-28 days). The shaved area of each armpit was separately applied with 7.5% w/w GA gel and gel base after skin cleansing in the morning and the night for 28 days, digital camera images of indicated area were captured.

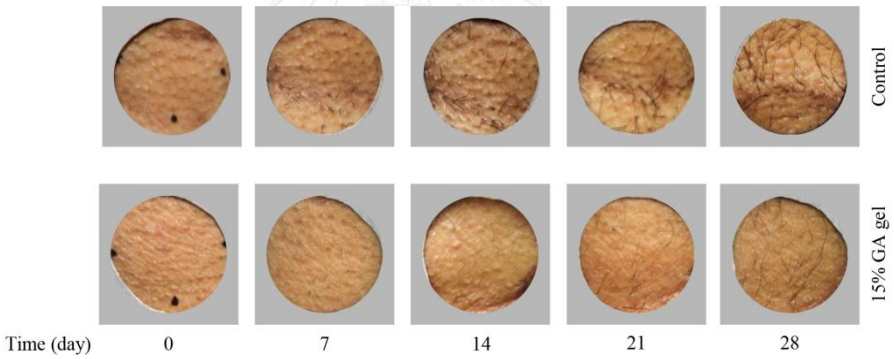

Figure 48 Representative images of underarm hair after treatment with 15% w/w GA gel and gel base for various times (0-28 days). The shaved area of each armpit was separately applied with 15% w/w GA gel and gel base after skin cleansing in the morning and the night for 28 days, digital camera images of indicated area were captured.

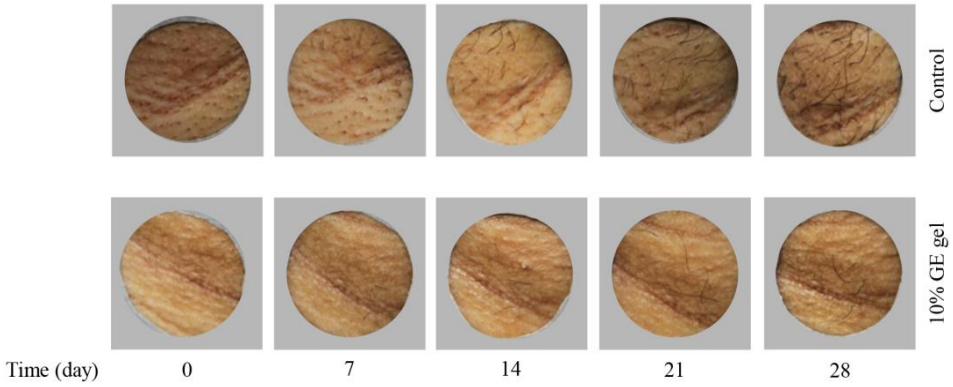

Figure 49 Representative images of underarm hair after treatment with 10% w/w GE gel and gel base for various times (0-28 days). The shaved area of each armpit was separately applied with 10% w/w GE gel and gel base after skin cleansing in the morning and the night for 28 days, digital camera images of indicated area were captured.

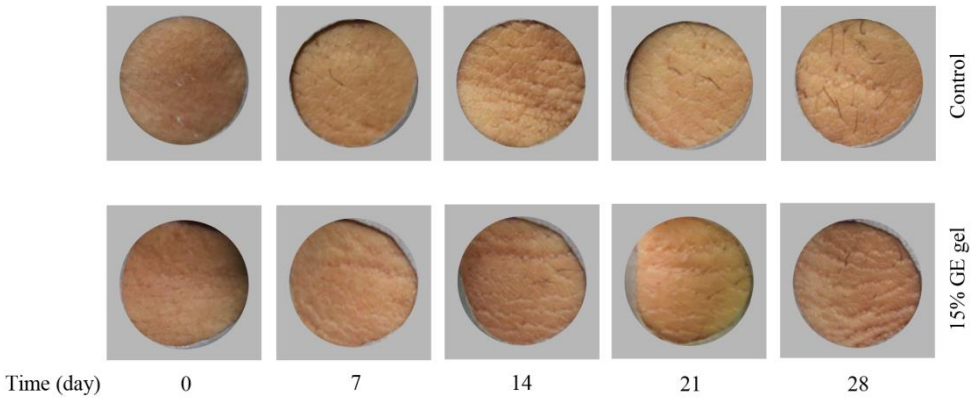

Figure 50 Representative images of underarm hair after treatment with 15% w/w GE gel and gel base for various times (0-28 days). The shaved area of each armpit was separately applied with 15% w/w GE gel and gel base after skin cleansing in the morning and the night for 28 days, digital camera images of indicated area were captured.

APPENDIX C

Study Protocol Approval by The Research Ethics Review Committee for Research Involving Human Research Participants, Health Sciences Group, Chulalongkorn University, Bangkok, Thailand

AF 02-12

The Research Ethics Review Committee for Research Involving Human Research Participants, Health Sciences Group, Chulalongkorn University
Jamjuree 1 Building, 2nd Floor, Phyathai Rd., Patumwan district, Bangkok 10330, Thailand,
Tel/Fax: 0-2218-3202 E-mail: eccu@chula.ac.th

COA No. 005/2016

Certificate of Approval

Study Title No. 218.1/58 : **THE EFFECT OF GLYCYRRHIZIC ACID AND AQUEOUS LICORICE EXTRACT ON HAIR GROWTH RETARDATION: *IN VITRO* MECHANISTIC AND *IN VIVO* EFFICACY STUDIES IN HUMAN**

Principal Investigator : MISS CHAYANIN KIRATIPAIBOON

Place of Proposed Study/Institution : Faculty of Pharmaceutical Sciences, Chulalongkorn University

The Research Ethics Review Committee for Research Involving Human Research Participants, Health Sciences Group, Chulalongkorn University, Thailand, has approved constituted in accordance with the International Conference on Harmonization – Good Clinical Practice (ICH-GCP) and/or Code of Conduct in Animal Use of NRCT version 2000.

Signature: Prida Tasanapradit Signature: Nuntaree Chaichanawongsaroj

(Associate Professor Prida Tasanapradit, M.D.) Chairman

(Assistant Professor Nuntaree Chaichanawongsaroj, Ph.D.) Secretary

Date of Approval : 14 January 2016 Approval Expire date : 13 January 2017

The approval documents including Protocol No. 218.1/58
Date of Approval 14 JAN 2016
Approval Expire Date 13 JAN 2017

1) Research proposal
2) Patient/Participant Information Sheet and Informed Consent Form
3) Researcher

The approved investigator must comply with the following conditions:

1. *The research/project activities must end on the approval expired date of the Research Ethics Review Committee for Research Involving Human Research Participants, Health Sciences Group, Chulalongkorn University (RECCU). In case the research/project is unable to complete within that date, the project extension can be applied one month prior to the RECCU approval expired date.*
2. *Strictly conduct the research/project activities as written in the proposal.*
3. *Using only the documents that bearing the RECCU's seal of approval with the subjects/volunteers (including subject information sheet, consent form, invitation letter for project/research participation (if available).*
4. *Report to the RECCU for any serious adverse events within 5 working days*
5. *Report to the RECCU for any change of the research/project activities prior to conduct the activities.*
6. *Final report (AF 03-12) and abstract is required for a one year (or less) research/project and report within 30 days after the completion of the research/project. For thesis, abstract is required and report within 30 days after the completion of the research/project.*
7. *Annual progress report is needed for a two- year (or more) research/project and submit the progress report before the expire date of certificate. After the completion of the research/project processes as No. 6.*

VITA

Chayanin Kiratipaiboon was born on the 12th of February 1983 in Bangkok, Thailand. She finished her elementary and secondary programs in Anuban Wat Pitula Tirat Rangsarit and Benchmaracharungsarit schools, respectively. In 2007, she recieved her undergraduate degree in pharmaceutical sciences (PharmD) majoring in clinical pharmacy at Srinakharinwirot University, Nakhon Nayok, Thailand. After graduation, she had worked at Bangkok Pattaya Bangkok Hospital Pattaya, Chonburi, Thailand for 2 years. After that she enrolled in the Graduate Program in Pharmacology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for her Master degree. In 2012, she received her Master of Pharmaceutical Sciences Degree and entered in the International Graduate Program in Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for her doctoral degree.