EFFECTS OF *Tacca chantrieri* ANDER, *Centella asiatica* LINN AND *Eleutherine americana* (AUBL.) MERR. ON ANTIOXIDANT ACTIVITIES AND MODULATION ON DERMAL PAPILLA CELLS, *in vitro*

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Thesis entitled "EFFECTS OF *Tacca chantrieri* ANDER, *Centella asiatica* LINN AND *Eleutherine americana* (AUBL.) MERR. ON ANTIOXIDANT ACTIVITIES AND MODULATION ON DERMAL PAPILLA CELLS, *in vitro*"

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has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy in Biochemistry of Naresuan University

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ABSTRACT

Tacca chantrieri, *Centella asiatica*, and *Eluetherine americana* have long been applied in Thai folk medicine as a hair treatment. However, to date there has been inadequate scientific information on these substances allowing a greater understanding of their active properties. The objectives of this study were to add to the body of knowledge by determining the biological activity, cytotoxicity, and cytokine expressitivity of extracts of these plants on human follicle dermal papilla cells (HFDPC), after appropriate preparation. Dried and ground extracts of each medicinal plant were macerated with 70% ethanol, and each extract was then separated by liquid-liquid partition, followed by lyophilization. Five extracts were then derived using ethanol (EtOH), hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and distilled water (H_2O) in the extraction procedure. Anti-oxidation against DPPH and ABTS free radicals was determined by DPPH, and ABTS methods. The total phenolic compound contents were determined by the Folin-Ciocalteu method, and the total flavonoid compounds were determined by the aluminium chloride colorimetric method. The results showed that the EtOAc fraction of those plants had the highest level of anti-oxidants, total phenolic compounds and total flavonoids compounds. In order to test the cell viability, the EtOH extract, EtOAc fraction, and H_2O fraction were incubated with the cells and determined by Presto-blue method. The results showed that the EtOAc fraction harmed the HFDPC but the EtOH extract and H_2O fraction did not. The EtOH extract and H_2O fraction were used to study a cytokine

expression assay by the real time PCR method. The VEGF expressions were induced after incubation with EtOH extract. From our results, we can conclude that the extracts of these plants are active in modulating the HFDPC via VEGF expression. These findings will beneficially inform the further development of cosmetic products with natural ingredients.

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ABBREVIATIONS

µG	=	Microgram
µl	=	Microliter
ABTS	=	2,2'-azino-bis(3-ethylben-zthiazoline-6-sulphonic acid
Al_2Cl_3	=	Aluminium chloride
bp	=	Base pair
°C	=	Degree Celsius
DMSO	=	Dimethyl sulfoxide
cDNA	=	Complementary deoxyribonucleic acid
DNA	=	Deoxyribonucleic acid
dNTPs	=	Deoxyribonucleotide triphosphate mix, nucleotides mix
EDTA	=	Ethylenediaminetetraacti acid
EtOH	=	Ethanol
EtOAc	=	Athylacetate
h (hr.)	=	Hour
mM	=	Millimolar
min	=	Minute
mRNA	=	Messenger ribonucleic acid
NaCl	=	Sodium chloride
Na_2CO_3	=	Sodiumbicarbonate
OD	=	Optical density
pH	=	Power of hydrogen ion concentration
PCR	=	Polymerase chain reaction
RNA	=	Ribonucleic acid
RT-PCR	=	Real time polymerase chain reaction

CHAPTER I

INTRODUCTION

Background and Significance of the Study

Hair is a protein filament that grows from follicles found in the dermis. At the base of the hair follicle, there are dermal papilla cells (DPCs); the tissue was surrounded by the epithelial matrix cells. DPCs provide nutrients and regulating factors for supporting epithelial matrix cells proliferation and differentiation in hair cycle progression (Park, et al., 2012). There are three phases in the hair growth cycle; anagen, catagen and telogen. The anagen phase is a growth phase. It begins in the papilla and can last from two to six years (approximately 1,000 days). During the anagen phase, the cells in the papilla divide to produce new hair fibers, and the follicle buries itself into the dermal layer of the skin to nourish the strand. About 85% of the hairs on the head are in the anagen phase at any given time. The catagen phase or the transitional phase, allows the follicle to renew itself. During this phase, which lasts about 10 days, the hair follicle shrinks and disintegrates and the papilla detaches and "rests," cutting the hair strand off from its nourishing blood supply. The hair is not growing during this phase. The length of the terminal fibers increase when the follicle pushes them upward. In the telogen or resting phase, the follicle remains dormant for up to 100 days. Ten to fifteen percent of the hairs on the head are in this phase, and it can grow at any given time. In this phase the epidermal cells line the follicle channel which continue to grow as normal and may accumulate around the base of the hair. The hair base will break free from the root which shrinks. Within two weeks a new hair shaft will begin to emerge. The anagen phase begins again once the telogen phase is completed. The process results in normal hair loss known as shedding. The hair follicle consists of dermal papilla cells which control the hair growth cycle. Therefore, these cells are important in a study of the hair growth cycle. Previous studies found that the size and amount of dermal papilla cells play a key role in the hair growth cycle in the anagen phase (Hoffmann and Happle, 2000; Trueb, 2002).

Hair loss occurs in the catagen phase. It is a critical problem (Hibino and Nishiyama, 2004). Androgen is the hormone that controls the catagen phase via the thyroid hormone and vitamin D (Hsieh, et al., 2003). Recent studies reveal that there

are many factors involved in the catagen phase such as transforming growth factor-β (TGF-β) that affects hair loss or dermal papilla cells atrophy (Rho, et al., 2005; Zatelli, et al., 1998), fibroblast growth factor-5 (FGF-5) that promotes the hair growth cycle in the anagen phase (Ogihara, et al., 2000), insulin like growth factor (IGF) that affects the hair growth cycle and the remainding dermal papilla cells (Rho, et al., 2005). Previous studies have also shown the promotion of dermal papilla cells growth or the inhibition of dermal papilla cells atrophy using cytokine as a marker to compare with minoxidil or finasteride which are the drugs of choice for hair loss treatment nowadays (Messenger and Rundegren, 2004; Park, et al., 2012). Natural products have been discussed as being important in the development of herbal medicines as a key to global health. Many herbs have been found to have medicinal qualities, and it is suggested that further studies of the chemical makeup of naturally occuring herbs would reveal a substantial natural storehouse of beneficial medicinal herbs. Thailand has a commendable history of such studies and analyses, and the use of natural remedies.

The dermal papilla cells (DPCs) play a major role in the expression of personality relating to health problems. The previous study found that the DPCs death via apoptosis pathway involved with cytokine signaling and free radical. Therefore, DPCs are the role model for studying the relationship between hair growth cycles and pathogenesis of hair loss. Resulting the DPCs play a critical role in hair growth and secret extracellularmatrix (Messenger, et al., 1991).

Natural products have always been significant for mankind. They have been used as, and in, food, drink, cosmetics, and medicines. Several studies have shown that natural products demonstrate numerous medicinal effects including being antioxidants, having anti-cancer effects, and anti-metastasis and anti-inflammatory properties. In Thailand, many kinds of natural products have been traditionally used for disease treatment or prevention, as well being included in beauty products and cosmetics. *Butea superb* Roxb, for example, contain phytoestrogens that are anti-oxidant and encourage stimulation of immune cells via the NF- κB signaling pathway and the induced binding of estrogen or androgen and their receptors that could prolong the hair growth cycle. *Butea superb* Roxb also contains isoflavones (biochanin A, daidzein, formononetin and genistein) which protect against bone degradation and promote blood circulation. (Beck, Rohr, and Jungbauer, 2005). In 2000, Chansakaow, S., et al.

found that isoflavonoids extracted from *Pueraria candollei* Graham ex Benth. varmirifica could stimulate estrogen (Chansakaow, et al., 2000). Evidence from traditional medicine practice shows that *Tacca chantrieri* Ander, *Centella asiatica* Linn and *Eleutherine americana* (Aubl.) Merr have a long history of use as an anti-inflammatory and anti-infection treatment. Interestingly, all of these natural products have been reported to have an antioxidation effect which may inhibit hair loss. However, these natural products have never been scientifically studied in regard to their affect on hair loss. These natural products have only been used in traditional medicines in the northern part of Thailand.

Given the extent of naturally occuring baldness, primarily in men, and also to a lesser extent in women, and further given the often negative impact this may have on the social life, and even career prospects of those suffering baldness, the study of the anti-hair loss effect of naturally occurring medicinal plants, such as *Tacca chantrieri* Ander, *Centella asiatica* Linn and *Eleutherine americana* (Aubl.) Merr was considered to be an important matter. Our study, therefore was to identify and test medicinal plant extracts that can modulate HFDPCs and lead to the development of the extracts effective for the promotion hair growth.

Purposes of the Study

1. To study the antioxidation effect of crude extract from etanol and fractions of water, ethanol, ethyl acetate, dichloromethane, hexane of *Tacca chantrieri* Ander, *Centella asiatica* Linn and *Eleutherine americana* (Aubl.) Merr.

2. To study the effect of the extract from *Tacca chantrieri* Ander, *Centella asiatica* Linn and *Eleutherine americana* (Aubl.) Merr to human follicle dermal papilla cells (HFDPCs) function or modulation of HFDPCs.

Hypotheses of the Research

1. The extracts and some active fractions from *Tacca chantrieri* Ander, *Centella asiatica* Linn and *Eleutherine americana* (Aubl.) Merr contain active compounds which scavenge free radicals.

2. The extracts from *Tacca chantrieri* Ander, *Centella asiatica* Linn and *Eleutherine americana* (Aubl.) Merr. express non toxicity and modulate the gene expression related to HFDPCs proliferation.

Delimitation of the Study

The study was limited to an analysis of the extracts of, and biological activities of, *Tacca chantrieri* Ander, *Centella asiatica* Linn and *Eleutherine americana* (Aubl.) Merr. These natural products were identified by the taxonomist of the Naresuan University. They were extracted by ethanol and fractionation with hexane, dichloromethane, ethyl acetate, and water. These extracts were determined the antioxidant activities and phytochemical screening assay *in vitro*. The highest antioxidant activity and the highest antioxidant compound among the extracts were selected for the next study including the cell viability test in HFDPCs using Presto Blue cell viability assay. To determine the relationship between these extracts and gene that control the expression of growth factor and receptor including, vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor (VEGFR, KDR), insulin-like growth factor-1 (IGF-1), insulin-like growth factor-1 receptor (IGF-1R), transforming growth factor-β (TGF-β) and transforming growth factor-β receptor (TGF-βR) in HFDPCs were done by using real-time PCR. The relationship between these extracts and the expression of cytokine and their receptor was determined in the molecular mechanism.

Keywords

Dermal papila, *Tacca chantrieri* Ander, *Centella asiatica* Linn, *Eleutherine americana* (Aubl.) Merr. growth factor

CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Biology of the hair growth cycle

Hair follicle is a mammalian skin organ that produces hair. There are many hair follicles all over the skin, except on the lips, the palms, and the soles of the feet. The development of hair follicle is special in mammal that produces by itself and the hair growth could be stimulate through many pathways (Kligman, 1959). The hair follicle also plays an important role in survival. The mammal loss of hair leads to the death by cold. Besides, the previous reports found that androgen can stimulate hair growth. Contrastingly, the inhibition of hair follicle formation leads to hair loss (Randall, 1994a). Although, hair loss is not importance in clinical as it has no effect in everyday life, however, it dose importance for people's personality (Jansen and van Baalen, 2006). Hair loss is claimed as a social problem (Girman, et al., 1998).

Hair growth cycle is driven by hormone and ages in an individual. The environment factor is also a major problem that affect hair function, hair structure and hair growth cycle. Therefore, the control of hair growth cycle is a key for decreasing of hair loss.

1. Hair function

The skin of mammal can produce hair, except on the lips, the palms of the hands, and the soles of the feet. However, in some areas of the human skin are shown thin or small hairs. The body has different types of hair, including vellus hair and androgenic hair, each of with has its own type of cellular construction. The different constructions give unique characteristics of hair, and serve specific purposes with regards, mainly warmth and protection. The outer layer of hair is thin and flexible. It is produced by the connective tissue, cells consisting of keratin that differentiated the expression such as; color, length, diameter, and shape. Inside the hair, it consist of hair follicle, melanocyte, dermal papilla that showed in Figure 1 (Philpott, et al., 1994).

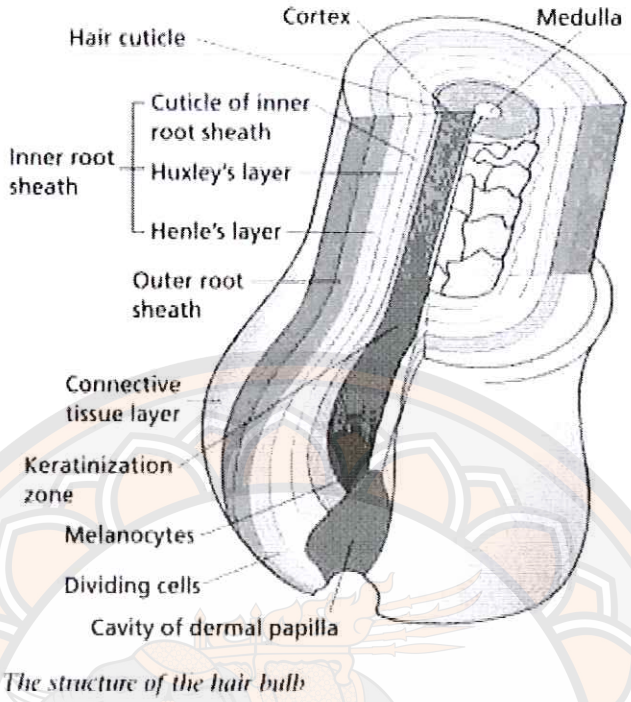

Figure 1 Structure of the hair

Note: http://www.texascollaborative.org/hildasustaita/module%20files/topic1.htm

Many mammals have fur and other hair which serve different functions. The location of hair generally indicates its role. The hair on head protects the scalp against the burning sun or UV and helps to hold in the body heat (Courtois, et al., 1996). Nostril hair helps to regulate the temperature of inhaled air before it enters to the body. Eyelashes and eyebrow hair help to keep foreign matter out of the eyes, and hair in the nostrils and ear canal help to catch dust, debris and even insects from entering the body. Human bodies hairs are connected to touch receptors in the skin that allow us to feel and sense, as well as they collectively serve as a protective warning device. Moreover, hair is important for personality in which the healthy hair is indicated the healthy person while the brittle hair indicated the malnutrition or disease (Bradfield, 1971). Hair plays key role in many cultures such as the removal of hair represents punishment of person. In contrast, persons who have healthy hair represent the strong. Therefore hair loss condition is a social problem that affects

human confidence (Randall, 2001). However the human hair follicle functions in health and disease is not fully understood. This is the reason why many hair disorders are poorly controlled. Androgens have much greater effect with body site of a follicle determining what type of response the follicle will be exhibited. Androgenetic alopecia in men, or male pattern baldness, is increasingly recognized as a physically and psychologically harmful medical condition that can be managed effectively by generalist clinicians. (Stough, et al., 2005)

2. Anatomy of hair follicle

Hair follicle is a complex organ consists of many layer of epithelial cells and mesenchymal cells called dermal papilla cells (DPCs). In a hair growth cycle contains three phase including the anagen (growth phase), the catagen (transitional phase) and the telogen (resting phase). Although, the pathway that control hair growth cycle is not fully understood. But there are many factors affecting hair loss in the catagen phase. Previous study has been reported that human genome contain many data that affect human. (Hibino and Nishiyama, 2004; Ozeki and Tabata, 2003).

The hair follicle can be divided into three regions: the lower segment (bulb and suprabulb), the middle segment (isthmus), and the upper segment (infundibulum).The infundibulum extend from the entrance of the sebaceous gland duct to the follicular orifice. The isthmus is a short section that extends from the insertion of the arrectorpili muscle to the entrance of the sebaceous gland duct. The suprabulb area and hair bulb extends from the base of the follicle to the insertion of the arrectorrpili muscle. In the hair bulb, it consists of keratinocytes cells that always proliferation and melanocyte cells that produce the melanin pigment that locates around the dermal papilla cells. The dermal papilla cells contain fibroblast cell in the extracellular matrix and separate from keratinocyte cells by basement membrane (Nutbrown and Randall, 1995).

Keratinocyte cells are moved and changed to be a hair. Whereas, melanocyte produce the melanin that represent the hair color. The hair follicle is surrounded by the protective root sheath, consisting of the external and internal root sheath. The internal root sheath, in turn, has three layer including, the innermost internal cuticle, the medial Huxley's layer, and the outermost Henle's layer. The internal cuticle is continuous with the outermost layer of the hair fiber. The hair fiber

also has three layers: the cuticle, the intermediate cortex, and the inner medulla (Figure 1).

2.1 Hair

Hair is a protein filament that grows from hair follicles found in the dermis, or skin. Hair is one of the defining characteristics of mammals. Each strand of hair is made up of the medulla, cortex, and cuticle and the part that exhibit hair is called prekeratogenous zone (Langbein and Schweizer, 2005). The medullar is the center of hair that specific express of keratin which controlled by androgen (Jave-Suarez, et al., 2004). Located within the cuticle is the "cortex," which contains melanin. The cortex determines the coloring, flexibility, and the "tensile strength" of the hair strand. The outermost of these sections is the "cuticle," which is comprised of intertwined colorless scales of keratin lending to the elasticity, water-resistance, and the thickness of the hair.

2.2 Internal root sheath of a hair

Inner root sheath of the hair follicle is located between the outer root sheath and the hair shaft The internal root sheath composes of 4 layer including, cuticle, Huxley's layer, Henley's layer and companion layer. Inner root sheath fastens on hair follicle and produces keratin (1/10) and trichohyalin that support the inner root sheath and promote hair growth. Recent study showed that GATA-3 is the transcription factor involved with inner root sheath differentiation and development. Previous study revealed that the mice that knockout of GATA-3 gene could not produce inner root sheath (Kaufman, et al., 2003).

2.3 External root sheath of a hair

External root sheath is relating to with the stratum mucosum (Stratum germinativum and Stratum spinosum) of the epidermis and resembles it in the round form and soft character of its cells; at the bottom of the hair follicle these cells become continuous with those of the hair. The external root sheath consists of difference cells such as keratinocyte that express keratin type 5 and 14, mesenchymal cells and melanocyte (Botchkareva, et al., 2001, Byrne and Fuchs, 1993, Oshima, et al., 2001). Between arrectorpili muscle and sebaceous gland is a source of multipotent cells (Cotsarelis, Sun, and Lavker, 1990). These cells can be divided by biochemistry or detected by immunology for cytokeratins 15 and 19, CD 34 (mice), and CD 200

(human) (Commo and Bernard, 1997, Michel, et al., 1996, Ohyama, et al., 2006). Besides, these cells also divide by rate of proliferation and promotion of cells growth such as keratinocyte, melanocyte and dermal papilla cells (Cotsarelis, 2006). Nestin is a type VI intermediate filament (IF) protein. These intermediate filament proteins are expressed mostly in nerve cells where they are implicated in the radial growth of the axon. The nestin also expressed in hair root. It plays a role in regulation of the assembly and disassembly of intermediate filaments, which is together with other structural proteins, participate in remodeling of cells. The cells that positive to nestin can develop to melanocyte *in vitro* study. Moreover, in vivo study found that mesenchymal cells that express nestin can develop to blood vessel after transplant in Subcutis of nude mice (Hoffman, 2007). The result showed that mesenchymal cells may be a source of multi potent cells that can apply for the hair loss treatment (Hoffman, 2007).

2.4 Dermal papilla

Hair bulb is near the dermal papilla cells consisting of mesenchyme-derived cells, dermal papilla, mucopolysaccharide-rich stroma, nerve fiber and single capillary loop.The dermal papilla contains tiny blood vessels or capillaries, which is brings oxygen through the blood supply as well as nutrients. The hair bulb, which surrounds the papilla, absorbs those nutrients. The dermal papilla cells play importance role in hair growth cycle, specification of size and color of hair. Previous study has been reported that dermal papilla cells which transplant into scalp in hair loss can induce new hair growth (Jahoda, Horne, and Oliver, 1984; Reynolds, et al., 1999). The dermal papilla cells could produce and secrete paracrine factor for hair growth and pigment formation led to the hypothesis that the dermal papilla cells is a key factor to control hormone circulation. The example for molecule that produced by dermal papilla cells involved with hair growth such as noggin. Noggin is a protein which induce hair growth via inhibition signal bone morphogenetic protein (BMP) and induce expression of BMP receptor type IA on the root hair epithelial cells (Botchkarev, et al., 2002). Keratinocyte growth factor (KGF) and keratinocyte growth factor receptor 2 (KGFR2) was produced from dermal papilla cells in anagen phase and mostly found in keratinocyte cells. This study was determined by injection of KGF into nude mice and lead to increasing of hair growth at injection site (Danilenko,

et al., 1995). Besides, the dermal papilla cells express hepatocyte growth factor (HGF) and the study found that after transfect HGF gene can stimulate hair growth (Lindner, et al., 2000). Insulin-like growth factor-I (IGF-I) which found in dermal papilla cells play a key role for morphogenesis of hair root (Rudman, et al., 1997). Moreover, stem cell factor (SCF) that produced by dermal papilla cells involved with melanocyte cells division, differentiation and pigment formation via signaling pathway related with SCF and SCF receptor (c-kit) (Botchkareva, et al., 2001). From the previous report, high alkaline phosphatase level could induce dermal papilla cells that start the new hair growth but when inhibit alkaline phosphatase activity leads to decrease new hair growth. However, there is no clear evidence to support this study (Handjiski, et al., 1994). The recent study reveals that dermal papilla cells could be used as mesenchymal cells for hair growth. Previous study from Jahoda, et al. showed that mouse dermal papilla cells could differentiation to adipocyte and osteocyte (Lako, et al., 2002; Richardson, et al., 2005).

2.5 Hair growth cycle

Human hair growth and shedding is random and not seasonal or cyclical. At any given time, a random number of hairs will be in one of three stages of growth and shedding: anagen, catagen, and telogen (Figure 2). The alterations of hair root in hair growth cycle depend on the interaction between keratinocyte cells and dermal papilla cells. During the hair growth and development are controlled by the related molecules that control cells survival, differentiation of cells and cells apoptosis. At the same time, degradation of hair root is stimulated by signaling pathway related with apoptosis pathway (Botchkarev and Kishimoto, 2003; Botchkareva, Ahluwalia, and Shander, 2006).

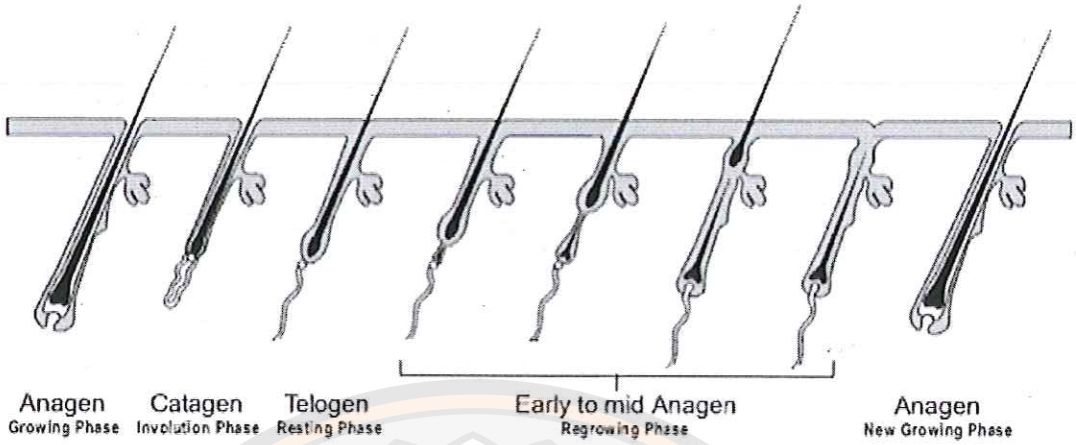

Figure 2 Hair growth cycle

Note: http://www.riolaserhair.com/laser-hair-removers/growth-cycle.html

2.6 Anagen (growth phase)

Anagen is the active phase of the hair. The cells in the root of the hair are dividing rapidly. A new hair is formed and it pushes the club hair (a hair that has stopped growing or is no longer in the anagen phase) up the follicle and eventually out. Some people have difficulty growing their hair beyond a certain length because they have a short active phase of growth. On the other hand, people with very long hair have a long active phase of growth. Anagen can be divided into six phases; the beginning phase is anagen initiation. In this phase, there are cells proliferation, dermal papilla cells growth and development to hair and external root sheath of a hair. In the intermediate phase of anagen, melanocyte cells will remove to the hair medulla and produce the pigment of hair. Moreover, this phase is also producing the new hair growth. In the last phase of anagen, there is the formation of new hair that composes of hair bulb of epithelial cells surrounding with dermal papilla cells that deeply locates in subcutaneous tissue and cuticle that is deep in the skin (Stenn and Paus, 2001). In the anagen phase, the signaling pathway involved with hair bulb of epithelial cells and dermal papilla cells have been changed. The keratinocyte cells in the hair medullar continue proliferation and express receptor on the cell surface for signaling pathway

including, P-catenidef-1, c-kit, c-met, FGFR2 and IGF-IR. Additionally, the dermal papilla cells express molecules including, WntSa, SCF, HGF, FGF7 and IGF-1 (Botchkarev and Kishimoto, 2003; Paus and Cotsarelis, 1999). Besides, alteration of hair root structure and nerve system is also occurred in the anagen phase (Mecklenburg, et al., 2000). The formation of blood vessel to the hair follicle is increased relating to the stimulation of expression molecules for blood vessel formation such as vascular endothelial growth factor (VEGF). Previous study found that mRNA extract from keratinocyte cells in the external root sheath of hair of mice that high expression of VEGF showed the new blood vessel formation (angiogenesis) to the hair follicle leads to increase the hair growth, size of hair follicle and cuticle (Yano, Brown, and Detmar, 2001). In contrast, after the mice were obtained chemical that can inhibit angiogenesis resulted in decrease of hair growth (Telek, et al., 2007). Therefore, angiogenesis is the key factor of hair growth from dermal papilla cells and hair follicle.

2.7 Catagen (transitional phase)

Catagen is the phase that occur after the anagen phase, catagen (Kligman, 1959, and Straile, Chase, and Arsenault, 1961). In the initiation of catagen phase, the proliferation and development of keratinocyte cells is obviously decreased. In this phase, catagen is a highly controlled process of coordinated cell differentiation and apoptosis, involving the cessation of cell growth and pigmentation, release of the papilla from the bulb, loss of the layered differentiation of the lower follicle, substantial extracellular matrix remodeling, and vectorial shrinkage (distally) of the inferior follicle by the process of apoptosis. The catagen stage of the hair cycle has been divided into eight subphases that beginning with late anagen and end in the early telogen (Muller-Rover, et al., 2001). The first ultra-structural sign of regression in the catagen follicle is the withdrawal of papilla cell fibroblast projections from the basement membrane. The papilla shrinks, probably through the loss of extracellular matrix substance. The cessation of bulbar epithelial cell division coincides with massive epithelial cell apoptosis in well-defined regions of the regressing hair follicle. In addition, there is a dramatic change in the cytoskeletal proteins of follicle epithelial cells in that trichohyalin, transglutaminase I, and desmoglein production cease. The catagen is involved with program cell death (apoptosis) that express at hair follicle.

Apoptosis may be activated by loss of growth factors, loss of cell-cell or cell-substrate interactions, changing cytokine interactions, hormone exposure, immune action, viral infection, sub lethal damage (chemotherapy, radiation), or purely genetic initiation factors such as in tissue modeling. The control of apoptosis pathway depend on type of cells in the hair follicle (Botchkareva, et al., 2006). The hair follicle epithelial cells and melanocyte cells are sensitive for apoptosis pathway, whereas dermal papilla cells and keratinocyte cells resist to apoptosis pathway (Lindner, et al., 1997). The relationship between hair follicle physiology and deletion of dermal papilla-derived growth factors in the anagen phase was controlled by signaling pathway via death receptors (Figure 3).

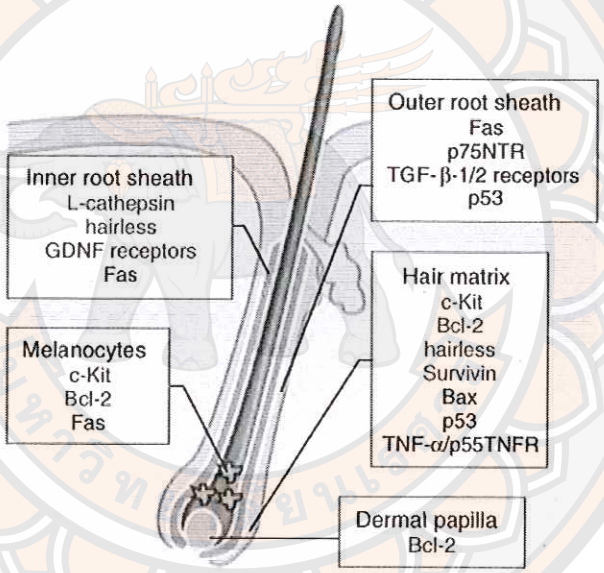

Figure 3 Molecular mechanisms of apoptosis control in the distinct hair follicle compartments (Botchkareva, et al., 2006)

2.8 Telogen (resting phase)

The telogen phase is the resting phase of the hair follicle. When the body is subjected to extremely stress, about 70 percent of hair can prematurely enter a phase of rest, it is called the telogen phase. This phase is the resting period where no new cell division occurs. This hair begins to fall, causing a noticeable loss of hair. This condition is called telogen effluvium. The club hair is the final product of a hair follicle in the telogen stage, and it is dead, fully keratinized hair. The balance of

stimulator and inhibitor of telogen phase was changed to drive the telogen phase to the anagen phase. The previous study has been reported that the stimulation of Sonic hedgehog (Shh) could drive the hair follicle from the telogen to the anagen phase (Sato, Leopold and Crystal, 1999). The sensitivity of hair root to Shh in the telogen phase was confirmed by the previous report that Nonpeptidyl small molecule, a hedgehog (Hh) stimulator, could induce transition from the resting (telogen) to the growth (anagen) stage of the hair cycle (Paladini, et al., 2005). In contrast, the skin in the telogen phase may contain molecules that inhibit hair growth cycle (Paus, Stenn and Link, 1990). Moreover, Bone morphogenetic protein4 (BMP4) could inhibit BMP4 signaling pathway and leads to stimulation of hair growth on the skin by noggin (Botchkareva, et al., 2001). Noggin also enhance expression of Shh mRNA in the hair root epithelial cells but BMP4 showed the contrast result (Botchkareva, et al., 2001). The cell division of dermal papilla cells in telogen phase was stimulated by chemical and physical such as testing of the elimination of hair in telogen phase by pull out the hair lead to new hair growth (Muller-Rover, et al., 2001). Due to, the molecular mechanisms have not been clearly studied. However, the stimulation of anagen phase by pulling out the hair was widely used as a model for study of gene expression during hair growth cycle in animal model. Besides, the *in vivo* study of the driven from telogen to anagen phase, found that the hair follicle was stimulated by immunosuppressant such as cyclosporin A and FK506 (Iwabuchi, et al., 1995). Therefore, these stimulation of excesses hair were occurred in patient who treated with immunosuppressant or treatment of autoimmune disease (Lutz, 1994).

The tumor suppressor gene, p53, is a molecule that involved with apoptosis pathway of keratinocyte cells in the hair medulla. Previous study found that the mice that knock out p53 gene were slow down the catagen phase compare with control (Botchkareva, et al., 2001).The balance between apoptosis pathway and cells proliferation is essential for controlling hair growth cycle. This process can be controlled by an anti-apoptotic protein, surviving (apoptosis inhibitor protein family), that control cells proliferation and inhibit apoptosis pathway (Altieri, 2003). Survivin is widely expressed in keratinocyte cells in the hair medulla and external root sheath of a hair in the anagen phase. The survivin will disappear in catagen phase (Botchkareva, et al., 2007). Before catagen or during catagen phase, keratinocyte cells in the external

root sheath of a hair will secrete molecules that could stimulate catagen including, fibroblast growth factor-5 short isoform, neurotrophins, transforming growth factor-β1/2 (TGF-β1/2), IGF binding protein 3, and thrombospondin-1 (Botchkarev, et al., 2004; Foitzik, et al., 2000; Hebert, et al., 1994; Yano, et al., 2003). These molecules were found to be a control of catagen phase via inhibition of related molecules. The essential gene is fibroblast growth factor-5 (fgfs) that increase 50% of length of hair in animal model compare with the control group. Neurotrophins and TGF-βI could stimulate catagen phase and the study in animal model showed that the highly expression of neurotrophin family (BDNF, NT-3) can be drived the cells to catagen phase and induced apoptosis pathway via neurotrophin receptor on the external root sheath of a hair. The mice were inhibited expression of TGF-β1 lead to the slowdown of catagen phase (Foitzik, et al., 2000). Moreover, the catagen phase is also stimulated by various molecules including endothelin-I, insulin like growth factor binding proteins-3/4/5, interleukin-1, vitamin D receptor, prolactin, endocannabinoids orthrombospondin-1 (Foitzik, et al., 2006; Stenn and Paus, 2001) (Figure 3).

Factors related to hair growth cycle

Hair follicle is under the control of physical effect and biochemical effect.

1. Physical effect

The hair growth rate and hair alternate are related with seasons. For example, in winter, the hair is rapidly growth compared with in summer. The control of hair alternation was involved with the hormone similar to the control of pairing season in animal (Lincoln and Richardson, 1998). The hair growth cycle has been studied in healthy fourteen men (age; between 18 and 39 years) for 18 months at Sheffield, United State of America. It was found that season affect the hair alternation by the people who expose with sunlight lead to increasing of hair follicle more that 90% in the anagen phase during spring (Nixon, et al., 2002). The study in France has also shown the similar data that in summer was resulting in increasing of sperm in men (Reinberg, et al., 1988). This data related with androgen level including, luteinising hormone (LH), testosterone and 17β-oestradiol that highest level in autumn (Lamba, Goswami, and Sundararaj, 1983; Reinberg, et al., 1975). Moreover, the nutrients

are the most factors that affect hair growth due to hair growth cycle contain high metabolism to obtain energy.

There is no evidence to confirm that which factor control a new hair growth but previous report revealed that minerals are importance for new hair growth. Some people who lack of zinc (Zn) would result in weak hair and hair loss. Vitamin B5 or panthenol play an important role in new hair growth by treatment of physical property of hair.

2. Biochemical effect

2.1 Hormone related to hair loss

The hair growth cycle also involve with hormone such as thyroid hormone that can stimulate new hair growth in resting hair follicle. Additional, the hair is sensitive for androgen that found in both women and men. Androgen is the most factors for hair growth cycle and the thickness of hair shaft. The androgen could stimulate body hair growth but inhibit head hair. Estrogens showed the contrast result to androgen and prolong hair growth cycle. In pregnancy women, the level of high estrogen leads to the high rate of hair growth. Therefore, the balance of androgen and estrogen indicates the hair growth in women and men (Hibino and Nishiyama, 2004).

2.2 Cytokine related to hair loss

In the previous reported by Rho S. S., et al. in 2005 found that many signaling molecules can control hair growth cycle including, epidermal growth factor (EGF), keratinocyte growth factor (KGF), insulin like growth factor-1 (IGF-1), interleukin-1 (IL-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF). The molecules that control hair loss are transforming growth factor-α (TGF-α) and TGF-β. Transforming growth factor-α (TGF-α) and transforming growth factor-β (TGF-β) can cause hair loss, aging, and stimulation of caspase protein related to apoptosis pathway (Inoue, et al., 2009; Zatelli, et al., 1998). The present study should be evaluated the effect of natural product to hair growth cycle that related with hair loss.

2.3 Oxidation that related to hair loss

The alternation of hair in human is difficult to investigate because the hair did not loss all of the head. Hair is natural photoprotectant which helps to assure that sunlight is filtered before reaching the scalp. The outer factors; age increased, thin

hair, and the scalp is exposed to more sunlight. They can cause the production of free radicals, which leads to oxidative injury, and contributes to follicular inflammation. The living cells have to use oxygen for living. Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA (Nawar, 1996). The oxidation sometime occurs by stimulation of enzyme (Halliwell, 1995) and found in inflammation pathway (Konstan and Berger, 1993) or extrinsic factor such as medicine, UV or smoking. In generally the body has anti-oxidant enzyme for balancing but when high oxidative stress that cause disease. The importance mechanism is apoptosis pathway. Apoptosis is the process of programmed cell death (PCD) that may occur in multicellular organisms that play key role in the control of cells development. Therefore, the abnormality of apoptosis pathway leads to pathological such as cancer or auto-immune disease.

Androgen signaling pathway in hair root

The androgens function as paracrine hormones is required by the Sertoli cells to support sperm production. It is also required for masculinization of the developing male fetus (including penis and scrotum formation). Androgen can diffuse through cells membrane to activate the target cells by binging with specific receptor. After binding with their receptors, androgen will translocate into the nuclease and complementary binding with the target DNA at hormone response elements (HRE) with adaptive protein to promote a target gene expression (Stanford, et al., 1997). The androgen function is more complicate than the other steroid hormones. Testosterone is a main androgen in the men that react to a target tissue such as skeletal muscle. In prostate, testosterone is metabolites by 5α-reductase to 5α-dihydrotestosterone that has effective function with their receptors to promote target gene expression (Randall, 1994b). The previous study found that dermal papilla cells are expressing 5α-reductase mRNA (Asada, et al., 2001)

After that, the synthesis of transforming growth factor-β2 (TGF-β2) in dermal papilla cells will be occurred. TGF-β2 inhibits epithelial cells proliferation and

production of caspase in apoptosis pathway. TGF-β2 interacts with caspase for inducing epithelial cell death via apoptosis pathway. The chemical or compounds that inhibit TGF-β lead to alteration of catagen phase including physiological changes, and also decrease the dermal papilla cell number. After the hair cycle progress, if the new cells have no growth they cause the hair loss (Hibino and Nishiyama, 2004; Zatelli, et al., 1998). The previous study found that the factor that cause hair loss in human are (1) the change of testosterone to dihydrotestosterone (DHT) by 5α-reductase; (2) the secretion of TGF-β2 from dermal papilla cells; and (3) the stimulation of caspase in apoptosis pathway which lead to epithelial cells death. All of these data support that the reduction time of hair cell cycle leads to hair loss (Foitzik, et al., 2000; Handa, et al., 2008).

Cytokine and receptor related hair growth cycle

Cytokine are broad and loose category of small proteins that are important in cell signaling. Many types of cytokine play an important role in hair growth control or new hair growth, including epidermal growth factor (EGF), keratinocyte growth factor (KGF), insulin like growth factor-1 (IGF-1), interleukin-1 (IL-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF). Whereas the signaling protein related to aging are transforming growth factor-α (TGF-α) and TGF-β. When the dermal papilla was stimulated by these cytokines, it leads to environment change around the cells and affect the hair growth (Danilenko, Ring, and Pierce, 1996; Jindo, et al., 1994). Besides, hormone is important in a hair growth and hair loss cycle. Androgen is the main factor that affects hair loss and this condition related with genetic disorder. Another hormone related with hair loss are testosterone and dihydrotestosterone (DHT). Testosterone was changed to dihydrotestosterone (DHT) using 5-reductase type 1 and 2. Previous study have been reported that cytokines and their receptors involve with apoptosis pathway in hair follicle cells such as IGF-1 that induces cell survival via PI3K/AKT and MEK/ERK (Hayashi, et al., 2013). Furthermore, inflammation cytokine can stimulate apoptosis pathway in the hair root cells via JNK and p53 (Wei, et al., 2012). Reactive oxygen species (ROS) such as hydroxyl radical produced from cisplatin induce dermal papilla cells apoptosis by inhibition of anti-apoptotic protein, Bcl-2 (Luanpitpong, et al., 2011)

similar to fluoride that induces lipid peroxidation and generate ROS cause hair root cells apoptosis but can be inhibited by selenium as an anti-oxidant compound (Wang, et al., 2010). Moreover, the antioxidant compound such as procyanidin can inhibit TGF-β induced cells apoptosis via MEK-1/2 (Kamimura, et al., 2006) (Figure 4).

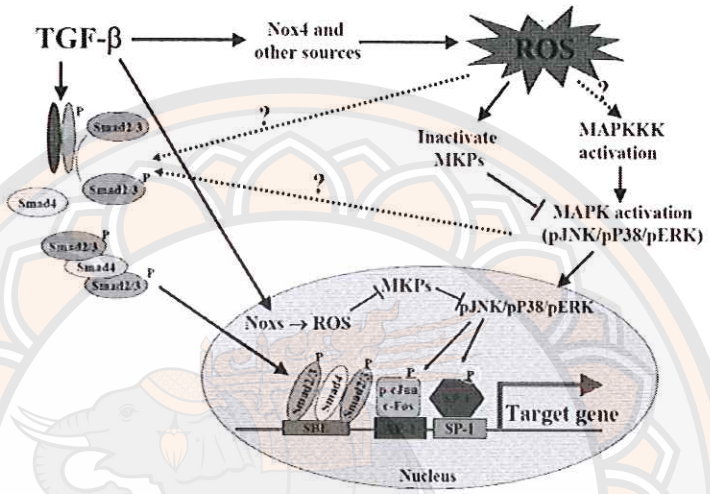

Figure 4 Signaling pathway related apoptosis via MAPK/JNK/p38 (Liu, Gaston and Pravia, 2010)

1. Transforming growth factor-beta (TGF-β)

TGF-β plays importance role in the inhibition of hair follicle proliferation (Philpott, Green and Kealey, 1992). Besides, the expression of hepatocyte growth factor or HGF induced keratinocyte cells proliferation and also inhibited by TGF-β (Shimaoka, Imai and Ogawa, 1994). From previous study, TGF-β2 specific expressed in dermal papilla cells (Inoue, et al., 2009). The secretion of TGF-β produced by dermal papilla cells (Chiu, et al., 1996). Moreover, androgen can induce apoptosis in keratinocyte cells when co-culture with dermal papilla cells. The dermal papilla cells were induced by androgen which increases expression of TGF-β1 mRNA (Inui, et al., 2002). TGF-β was induced apoptosis by open Kir 6.0 channel pore and sulfonylurea receptor (SUR) at inner membrane of mitochondria (Otomo, 2002) and it was induced cell apoptosis via smad2/3 in the anagen and catagen phase (Soma, et al., 2003). Dihydrotestosterone (DHT) produces TGF-β2 in dermal papilla cells which inhibit epithelial cells proliferation and stimulate caspase via apoptosis pathway in catagen

phase (Hibino and Nishiyama, 2004). Due to the dermal papilla cells that expresses the androgen receptor that induces TGF-β expression (Itami, 2004). Moreover, the induction of TGF-β expression by androgen depends on the reactive oxygen species (ROS) in the cells. Anti-oxidant compound can lead to TGF-β1 expression (Shin, et al., 2013).

2. Epidermal growth factor (EGF)

Epidermal growth factor or EGF is a growth factor that stimulates the cell growth, proliferation, and differentiation by binding to its receptor EGFR. Dermal papilla cells and the hair root cells were induced by EGF increase cells proliferation comparing with the hydrocortisone and minoxidil that cannot stimulate cells proliferation (Katsuoka, et al., 1987). Dermal papilla cells secrete EGF for stimulating the surrounding cells that is paracrine and autocrine. Dermal papilla cells and cuticle cells were more response to EGF than hair epithelial cells (Pisansarakit, du Cros, and Moore, 1991). Moreover, the EGF decreased the movement of external root sheath of a hair (Philpott and Kealey, 1994). In addition, the proliferation and development of skin and keratinocyte did not changed but the proliferation of adipocyte in subcutaneous was decreased (Sugawara, et al., 2010). EGF can stimulate leucine uptake in the cells but inhibit hair root cells proliferation (Philpott, et al., 1992). Dermal papilla cells express of EGF-R1 that the receptor on the cell surface in the anagen phase (Green and Couchman, 1985). However, the studies of molecular mechanism of EGF signaling have never been reported.

3. Insulin-like growth factor-1 (IGF-1)

IGF-1 is a hormone similar to the molecular structure of insulin. It plays a key role in childhood's growth and continues to have anabolic effects in adults. Previous study showed that the expression of IGF-1 and IGF binding protein found in hair loss person more than normal hair person. This mechanism is controlled by androgen (Panchaprateep and Asawanonda, 2014) and also stimulated via Wnt/beta-catenin and ERK (Hwang, et al., 2012). IGF-1 was secreted from dermal papilla to control proliferation and differentiation of hair root cells (Weger and Schlake, 2005). Besides, flavone (Zhao, et al., 2011) or anti-oxidant compound can induce expression of IGF-1 via PI3K pathway (Kwack, et al., 2009).

4. Keratinocyte growth factor (KGF)

The Keratinocyte Growth Factor (KGF) is a growth factor presented in the epithelialization-phase of wound healing. In this phase, keratinocytes are covering the wound, and forming the epithelium. KGF is expressed in the dermal papilla cells in hair follicle and the specific receptor which is a fibroblast growth factor receptor 2 or FGFR-2. The signaling pathway through KGF affects dermal papilla cells proliferation and differentiation (Botchkareva, et al., 1999). The inhibition of 5α-reductase type2 leads to increase expression of KGF (Roh, et al., 2002). Moreover, treatment of KGF-2, a recombinant protein (Jang, 2005), or IL-1α could stimulate dermal papilla cells for an increase of KGF secretion (Boivin, et al., 2006). However, the studies of molecular mechanism of EGF signaling have never been reported.

5. Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis. Dermal papilla cells express VEGF that stimulate cells proliferation, epithelial cells differentiation for angiogenesis (Lachgar, et al., 1996). VEGF plays an important role in angiogenesis in hair growth cycle (Kozlowska, et al., 1998). Moreover, VEGF also stimulates collagen synthesis from dermal papilla cells (Lachgar, et al., 1996). Glucocorticoid (Choi, et al., 2013) and androgen can inhibit VEGF expression (Lachgar, et al., 1999). The VEGF receptor (VEGFR-2) is expressed in hair root of keratinocyte cells which stimulated VEGFR-2, Erk1/2, c-Jun and p38 (Wu, et al., 2014). Besides, ginsenosideare, a class of natural product steroid glycosides and triterpene saponins can increase VEGF expression (Shin, et al., 2013).

From these data, it can conclude that the stimulation of dermal papilla cells through cytokines such as EGF, IGF, IL, KGF, and VEGF. But the inhibition of cytokine TGF-β leads to promote hair loss.

Although, androgen induced hair loss in dermal papilla cells via inhibition of nexin-1 expression, it plays key role in inhibition of serine proteases enzyme that involves with cells proliferation and differentiation (Sonoda, et al., 1999). Androgen also induced dermal papilla cells to produce TGF-β and TGF-β2 (Hibino and Nishiyama, 2004; Inui, et al., 2002). TGF-β is a molecule that inhibits activity of keratinocyte cells in hair loss person by inhibiting the cells growth in a catagen phase

for both human and mouse(Soma, Tsuji and Hibino, 2002). A keratinocyte also expresses TGF-β receptor on the cells surface (Wollina, et al., 1996). Besides, the cultured medium from hair loss cells can inhibit dermal papilla cells growth. This led the secretion molecule (TGF-β) that control cells growth, it drives from an anagen to a catagen phase (Soma, et al., 2003). The receptor is mostly expressed in keratinocyte cells (Wollina, et al., 1996) so that the study in dermal papilla cells was determined some molecule related to hair growth such as IGF- 1, nexin- 1 and TGF-β. The alteration of these molecules in molecular mechanism plays an important role for controlling of biochemistry of hair root cells. Therefore, the further study related to these molecules may describe the relationship between hair follicle cells and pathology of hair loss that induced by androgen.

Hair loss treatment

According to the hair is significant for an individual personality and social life so that the hair loss is concerned a big problem and cause stress. There are many ways to control hair loss and stimulate hair growth presently. The treatment of hair loss including; chemical, medicine, vitamin, mineral and natural products has been used. Minoxidil (Regaine) is a one of the choices of drug in nowadays. It is plays an important role in stimulation of hair growth. In the animal study found that minoxidil can reduce the time in the telogen phase and inhibit 5α-reductase (Rendl, Lewis, and Fuchs, 2005) so the hair follicle can rapidly drive to anagen phase. Moreover, minoxidil can prolong the anagen phase and increase size of hair follicle. The previous study revealed that the epidermal cells treated with minoxidil increase cells viability and enhance DNA replication more that non-treated cells. The treatment of high blood pressure with minoxidil cause side effect with stimulation of hair growth so this drug is used in the aspect of inhibits hair loss. From the study of Olsen found that minoxidil can increase 25% of new hair growth and the new hair control drugs were widely used including, Natural benzyl amino peptide (Duvic, et al., 1996; Gibson, et al., 2005). Minoxidil affected in adenosine-triphosphate-sensitive potassium channel (KATP) to increase calcium level in the cells and it produces VEGF (Li, et al., 2001) and prostaglandin (Messenger and Rundegren, 2004). Similarly, Messenger and Rundegren also reported that minoxidil can increase blood circulation. In 1998,

Lachgar, et al. found that minoxidil also increase expression of VEGF mRNA and VEGF in dermal papilla cells *in vitro*. In conclusion, minoxidil enhances dermal papilla cells proliferation, increases blood circulation, and increase expression of VEGF mRNA and VEGF (Lachgar, et al., 1998). Besides, minoxidil also increases the expression of AKT and ERK in cells survival signaling and increases Bcl-2 expression that is an anti-apoptotic protein (Han, et al., 2004). However, the side effect of minoxidil has never been reported.

Finasteride is another widely used drug for hair loss control. It can inhibit 5α-reductase type 2, leads to decreasing of dehydrotestosterone that causes hair loss, but this drug does not affect to dehydrotestosterone type 2 (Dallob, et al., 1994; Tilakaratne and Soory, 2001). However, the side effect of finasteride causes the change of neuroactive steroid levels (Caruso, et al., 2014). Therefore, the use of this drug is considered for permanent abnormality in patient (Irwig and Kolukula, 2011). The best way to treat hair loss in nowadays is the hair transplantation in the hair follicle with no response to androgen and keeps the hair condition. The recent study reveals that many researchers tried to culture hair follicle cells from patient in vitro before transplanting back to the patient (Orentreich and Durr, 1982).

Moreover, the vitamin and mineral such as biotin (vitamin H, B7) that used for stimulating of cells proliferation, fatty acid synthesis, lipid and amino function. Biotin was used for supplementing of nail and hair care. Besides, hair follicle interact with vitamin B 5 derivatives (panthothenic acid; panthenol). In cosmetics and personal-care products, panthenol is a humectant, emollient, and moisturizer. Vitamin A (Retinoic acid) and vitamin E (tocoperol) also increase moisturizer for hair and lead to the growth of hair follicle and prolong survival.

The natural product that stimulates hair growth is γ-linolenic acid, α-linolenic acid, linoleic, and oleic acid. These compounds inhibit both type 1 and 2 of 5α-reductase and also contain anti-inflammatory effect. Azelaic acid purified from family Gramineae including, *Hordeum vulgare* L. and *Triticum* spp, can inhibit 5α-reductase. The study from Japan revealed that procyanidin can stimulate new hair growth by induce hair epidermal function and also increase new hair growth in anagen phase in the volunteer. In 2007, Kwon, O. S., et al. found that all-trans Retinoic acid (tretinoin) promote the hair growth and inhibit caspase enzyme in apoptosis pathway of epithelial

cells via p53 and p21 functions lead to inhibition of apoptosis in dermal papilla cells (Kwon, et al., 2007). The data confirmed that the active compound that most found in plant was a group of hydrophilic compound (phenolic compound and flavonoids) (Basu, et al., 1999 and Packer, et al., 1999).

The treatment of anti-oxidant compound from natural product in hair loss

Triterpenoids purify from *Ganoderma lucidum* in ethanol fraction inhibit androgen, 5α-reductase type 1 and 2. It also inhibits testosterone induced prostate cancer cells proliferation but cannot inhibit dihydrotestosterone induced prostate cancer cells proliferation. Thin layer chromatography was used for separation of active compound including triterpenoids (Liu, et al., 2007). Moreover, in 2005, Fujita R., et al. found that the extract from *Ganoderma lucidum* significantly inhibit 5α-reductase *in vivo* study (Fujita, et al., 2005). Besides, these extracts significantly inhibit testosterone induced prostate cancer cells proliferation *in vitro* (Murkies, 1998). The extract from *Aconiti ciliare* stimulates dermal papilla cells growth *in vitro* and *in vivo* study via Wnt/β-catenin signaling pathway leads to stimulate of new hair growth and prolong the anagen phase (Park, et al., 2012).

***Centella asiatica* Linn**

Previous study found that *Centella asiatica* Linn (*C. asiatica*) contain biology activities including immune stimulant (Punturee, 2005), control of cytochrome P450 and anti-oxidant (Pan, et al., 2010) (Figure 5). The active compound are saponins (asiaticosides, braminoside, brahmoside, brahminoside, isothankuniside, madecassosides, and thankusiside), essential oil, flavones derivatives, sesquiterpenes, triterpenic acid, and triterpenic steroids (Brinkhaus, et al., 2000). Previous study found that active compound in *C. asiatica* is triterpenoids consisting of four compounds including, asiatic acid, madecassic acid, asiaticoside, and madecassoside. The active compound can stimulate fibroblast cells to produce growth factor.(Coldren, et al., 2003). The another compound is kaempferol, a flavonoid, (Vasavi, Arun, and Rekha, 2014), contain anti-oxidant, decrease lipid profile (Zhao, et al., 2014), and decrease blood sugar (Kabir, et al., 2014). Triterpenoid can inhibit coronary disease (Belcaro, et al., 2014). The extracted from the leaf of *C. asiatica* Linn consist of gallic acid and

ferulic acid that high anti-oxidant effect and anti-inflammatory effect (Ramesh, et al., 2014).The extracted from *C. asiatica* Linn also inhibit alpha-synuclein that the cause of Parkinson disease (Berrocal, et al., 2014). Caffeoylquinic acids from water extract of *C. asiatica* Linn can inhibit amyloid beta that the cause of alzheimer disease (Gray, et al., 2014) and induce signaling pathway via AKT and ERK-1/2 (Wanakhachornkrai, et al., 2013).

Figure 5 ***Centella asiatica*** **Linn**

Tacca chantrieri **Ander**

Tacca chantrieri Ander (*T. chantrieri*) was used in traditional medicine that contain active compound including, saponins (compesterol, spirostanol, arginin, etc), flavonoid, poly phenol, glycoside, and phenolic glycoside (Yokosuka and Mimaki, 2007) (Figure 6). The biological activities of these herbal have been widely study including, anti-oxidant, inhibition of lipidperoxidation and decrease blood sugar in diabetes patient (Steinrut, Itharat, and Ruangnoo, 2011), inhibition of virus, parasite, anti-inflammation, and anti-cancer. (Yokosuka, et al., 2005). Benzoquinone-type retro-dihydrochalcone are extracted from root of *T. chantrieri* Ander can inhibit breast cancer, prostate cancer and cervical cancer. (Peng, et al., 2010). Moreover, taccalonolides E and A, a steroid from this plant, can inhibit microtubule rearrangement (Tinley, et al., 2003) and inhibit expression of p-glycoprotein and inhibit MDR cancer cells

proliferation (Risinger, et al., 2008). Additionally, the ethanol fraction from *T. chantrieri* Ander can decrease blood pressure, relieve pain and anti-inflammation in animal model (Tiamjan, et al., 2007) but have no report *in vitro* study. For the study in skin and hair, found that *T. chantrieri* Ander is an ingredient of shampoo for elimination of louse (Watcharawit and Soonwera, 2013). However the molecular mechanism of *T. chantrieri* Ander for inhibition of hair loss has not been study.

Figure 6 ***Tacca chantrieri*** **Ander**

Eleutherine americana **(Aubl.) Merr.**

Eleutherine americana (Aubl.) Merr (*E. americana*) was used in traditional medicine for coronary thrombosis, common cold and study effect of active compound; napthoquoinone (Paramapojn, et al., 2008) (Figure 7). Moreover, there are active compound that inhibit α-glucosidase that suitable to use in diabetes patient (Coman, Rugina, and Socaciu, 2012; Triggiani, et al., 2006). The other active compounds are isoeleutherin and eleutherinol that stimulate and control immune system in human body. The isoeleutherin also decrease expression of interleukin-1β and interferon-βthat is cytokine in inflammatory cascade. The isoeleutherin can inhibit reactive oxygen species (ROS) which induced by lipopolysaccharide via inhibition of iNOS and NF-κB (Song, et al., 2009). Pyranonaphthoquinone, a derivative of eleutherin, β-lapachone, and β-lapachone can inhibits cancer cells proliferation by control DNA topoisomerase II (Krishnan and Bastow, 2000). Moreover, the study of skin and hair

found that naphthoquinone and eleutherin can inhibit skin disease from fungus and inhibit melanin pigment formation in the cells as same as arbutin and less toxicity (Kusuma, et al., 2010).

Figure 7 ***Eleutherine americana*** **(Aubl.) Merr**

CHAPTER III

MATERIALS AND METHODS

Schematic of the Study

To study the effect of *T. chantrieri*, *C. asiatica*, and *E. americana* on antioxidant activities and modulation on dermal papilla cell, *in vitro*, various experiments were performed. Brifely, according to Figure 8, the plants were identification with taxonomist of Naresuan Univeristy. Then, each dried plant was macerated with 70% ethanol and further fractionated with solvent. After that, each extract was study the phytochemical properties and biochemistry effects.

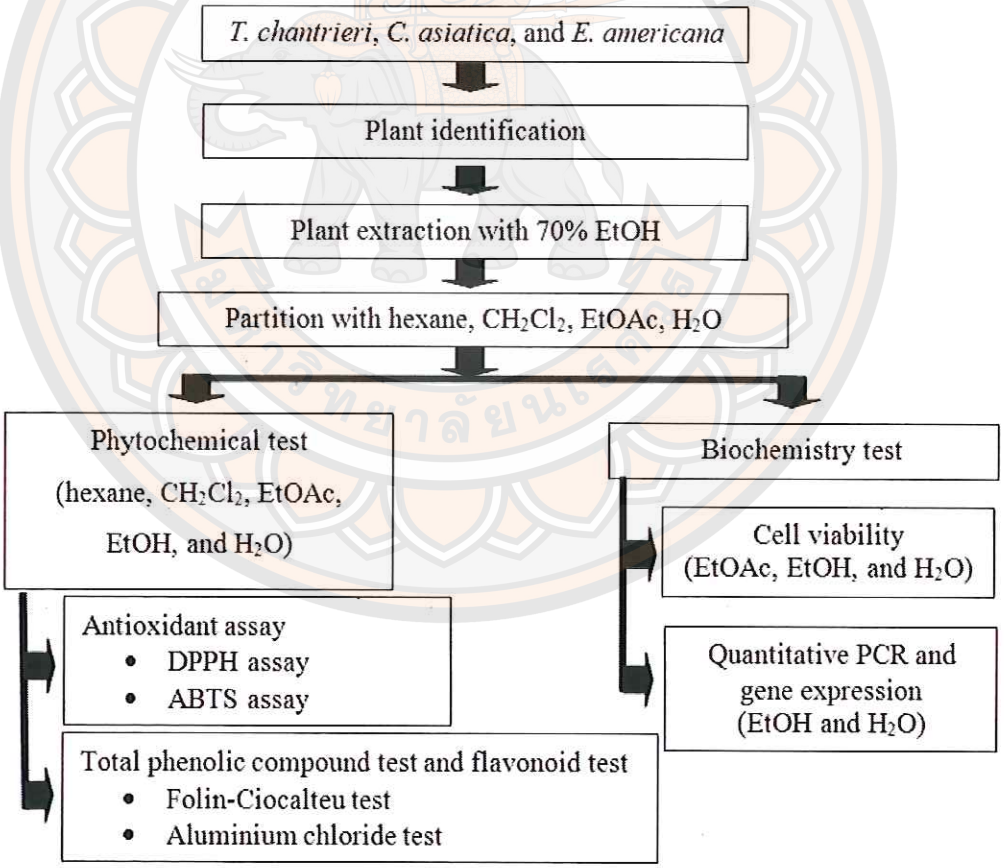

Figure 8 Show the scope of the study

Plant preparation and extraction

1. Plant materials

The *Tacca chantrieri* Ander (*T. chantrieri*), *Centella asiatica* Linn (*C. asiatica*) and *Eleutherine americana* (Aubl.) Merr (*E. americana*) were harvested from Chiang Rai province. Then, the raw material was dried by hot air oven at 30-45 °C. The dried material was grounded by grinder. The sample was identified by the taxonomist of Nareasuan University.

2. Extraction procedure

One kilogram of each dried sample (*T. chantrieri*, *C. asiatica* and *E. americana*) was fermented by maceration (70% ethanol 4L for 24h). The chlorophyll was later separated from the extracted by charcoal. The macerated sample was filtrated by a thin cloth for separation of residue from the natural products. The macerated sample was evaporated by rotary vacuum evaporator to recive 100 ml of the ethanolic extract. The ethanolic extract was filtrated by filter paper (whatman No.1) under the vacuum condition. The filtrated sample was dried using freeze dryer. The sample powder was kelp at -20°C and avoided the light. These extracts will be used in the next experiment.

2.1 Extraction based on Liquid-Liquid partition

The *T. chantrieri*, *C. asiatica*, and *E. eamericana* extracts that derived from 3.2.1 were separated by the partition technique. The crude extract from ethanolic fraction was firstly dissolved in water. Then, the extract was separated by different polarity of solvent begin with hexane, dichloromethane, and ethyl acetate, respectively. After that, each separated fractions were freeze-dried using freeze dryer. The samples were further used in the phytochemical tests and biological activity tests.

The first step of the separation, each of *T. chantrieri*, *C. asiatica* and *E. americana* extract was performed by adding hexane ande water in the ratio 1:1. After that, the hexane fraction was separated and evaporated by rotary vacuum evaporator. The hexane fraction was freeze-dried using freeze dryer to obtain the powder of hexane fraction. This process was performed in three times. In the second step, the remaining of crude extract in water fraction from the first step was mixed with dichloromethane in the ratio 1:1. Then the dichloromethane fraction was separated and evaporated by rotary vacuum evaporator. The dichloromethane fraction

was freeze-dried using freeze dryer to obtain the powder of dichloromethane fraction. This process was performed in three times. The third step was performed by mixing the remaining crude extract in water fraction from the second step with ethyl acetate in the ratio 1:1. Then the ethyl acetate fraction was separated and evaporated by rotary vacuum evaporator. The ethyl acetate fraction was freeze-dried using freeze dryer to obtain the powder of ethyl acetate fraction. This process was performed in three times. Finally, the remaining crude extract in the water fraction was evaporated by rotary vacuum evaporator. The water fraction was freeze-dried using freeze dryer to obtain the powder of water fraction. After the partition process, the four fractions would be obtained (hexane fraction, dichloromethane fraction, ethyl acetate fraction and water fraction) from crude ethanolic extract.

3. Quantification of total phenolic and flavonoid content

3.1 Quantification of total phenolic content in the extracts (Amin I, 2006)

The total phenolic content in the extract was determined using Folin-Ciocalteu assay with some modification. The Folin-Ciocalteu reagent (FCR) is a mixture of phosphotungstate and phosphomolybdate that used for determining the phenolic and polyphenolic antioxidants in the colorimetric assay *in vitro*. The phosphomolybdic-phosphotungstic acid reagents were reduced by phenolic hydroxyl groups in the structure of phenolic compound lead to formation of tungsten and molybdenum blue. The blue color of molybdenum blue was measured at 731 nm using UV-spectrophotometer.

3.2 The calibration curve of gallic acid

The Folin-Ciocalteu reagent also called the gallic acid equivalence method (GAE). The gallic acid was used as the standard for phenolic compound compared with the phenolic content in the extract. The gallic acid (100 ppm) was diluted with ethanol to obtain concentration 0, 20, 40, 60 and 80 ppm. Each concentration of gallic acid (0.2 ml) was added with 0.2 ml of Folin-Ciocalteu reagent and mixed by vortex. The mixtures were added with 2 ml of 7% sodium bicarbonate (Na_2CO_3) and mixed by vortex. Then, the mixture was further incubated in a dark place at a room temperature for 90 minutes. The absorbance of blue complex was evaluated at 731 nm (A_{731}) using UV-visible spectrophotometer. The calibration curve

was plotted between concentration of gallic acid and the absorbance. The concentration of phenolic content in the extract was calculated.

3.3 Quantification of total phenolic content in the extracts

The total phenolic content in the extract was determined by the modified Folin-Ciocalteu assay. Briefly, fifty milligram of each extract was dissolved with 20 ml of ethanol. The extract (0.2 ml) was added in the test tube. The water (2.5 ml) and the Folin-Ciocalteu reagent (0.2 ml) were added in the test tube and mixed by vortex. The mixtures were added with 2 ml of 7 % sodium bicarbonate (Na_2CO_3) and mixed by vortex. Then, the mixture was further incubated in a dark place at a room temperature for 90 minutes. The absorbance of blue complex was evaluated at 731 nm (A_{731}) using UV-visible spectrophotometer comparing with a standard curve prepared with various concentrations of gallic acid (GA) solution. The total phenolic content was shown as milligrams of GA equivalents per gram of extract (mg GAE/g extract). These data represented in mean ± S.D. with three independent experiments.

3.4 Quantification of total flavonoid content in the extract (Jia, et al., 1998)

Total flavonoid content was measured by the aluminium chloride ($AlCl_3$) colorimetric assay with a slightly modification. The principle of aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl. The product of the colorimetric was determined by UV-visible spectrophotometer at 510 nm.

3.5 The calibration curve of catechin

The catechin (CE) was use as the standard for flavonoid compared with the phenolic content in the extract. The catechin (100 ppm) was diluted with methanol to obtain concentration 0, 20, 40, 60 and 80 ppm. Each concentration of catechin (2.5 ml) was added with 10 ml of DI water and mix by vortex. The mixtures were added with 0.75 ml of 5% sodium nitrite ($NaNO_2$) and mixed by vortex for 5 min. After that, 0.125 ml of 10 % $AlCl_3$ was added into the mixture and incubated for 6 minutes. Then, five ml of sodium hydroxide (NaOH) was added and adjusted the volume to 25 ml with DI water. The solution was mixed and the absorbance was measured at 510 nm (A_{510}) using spectrophotometer.

3.6 Quantification of total flavonoid content in the extract

Total flavonoid content was measured by the aluminium chloride ($AlCl_3$) colorimetric assay with a slightly modification. Briefly, each extract was dissolved in methanol. The extract (2.5 ml) was added with 10 ml of DI water and mixed by vortex. The mixtures were added with 0.75 ml of 5% sodium nitrite ($NaNO_2$) and mix by vortex for 5 min. After that, 0.125 ml of 10 % $AlCl_3$ was added into the mixture and incubated for 6 minutes. Then, five ml of sodium hydroxide (NaOH) was added and adjusted the volume to 25 ml with DI water. The solution was mixed and the absorbance was measured at 510 nm (A_{510}) compared with the standard catechin using spectrophotometer. The total flavonoid content was expressed as mg catechin equivalents per gram extract (mg CE/g extract). These data represented in mean±S.D. with three independent experiments.

4. **Determination of antioxidant activity of the extract by DPPH assay (Fenglin, 2004)**

Scavenging of DPPH free radical is the basis of a common antioxidant assay. This method use for determining the antioxidant activity of the extract that can react with DPPH (2,2-diphenyl-1-picrylhydrazyl) (Figure 8–9). Therefore, the reduction of a chemical reaction upon addition of DPPH was used as an indicator of the radical nature of that reaction. Due to a strong absorption band centered at about 517 nm, the DPPH radical has a deep violet color in solution, and it becomes colorless when neutralized. This property allows visual monitoring of the reaction, and the number of initial radicals can be counted from the change in the optical absorption at 517 nm.

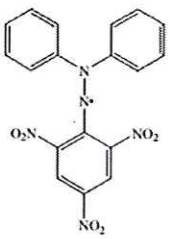

Figure 9 The structure of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH radical) that can react with antioxidant (AH) or radical species (R•)

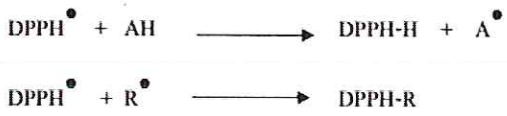

Figure 10 The scavenging reaction between DPPH• and an antioxidant (AH)

The 50% inhibition (IC_{50}) of antioxidant activity was calculated as the concentrations of sample that sacavenge a half of DPPH radical activity.

The extract was prepared with various concentrations between 10-1000 ppm. Then, nine ml of 0.2 mM methanolic DPPH radical was added in each concentration of the extract (1 ml). The mixture was mixed and kept in the dark for 30 minutes. The absorbance of sample was measured at 517 nm using UV-spectrophotometer. These data represented in mean ± S.D. with three independent experiments. The 50% inhibition (IC_{50}) of antioxidant activity of the sample was calculated compared with standard vitamin C. The percent inhibition of antioxidant activity of the sample was calculated from the absorbance of these extracts compared with standard vitamin C using the following formula:

$$\% \text{ inhibition} = \{(A_{blank} - A_{abs}) \times 100\} \div A_{blank}$$

5. Determination of antioxidant activity of the extract by ABTS free radical scavenging assay (Kriengsak, 2006)

The peroxidase substrate 2, 2'-azino-bis (3-ethylben-zthiazoline-6-sulphonic acid) or ABTS, forming a relatively stable radical (ABTS) upon one-electron oxidation, is a popular substrate for determination of total antioxidant activity. ABTS is converted to its radical cation by adding the of sodium persulfate ($Na_2S_2O_8$). This radical cation is in a blue color and absorbs the light at 734 nm using spectrophotometry. During the reaction, the blue ABTS radical cation is converted back to its colorless neutral form (Figure 10). This assay is referred to as the trolox equivalent antioxidant capacity (TEAC) assay. The reactivity of the various antioxidants tested of extract is compared with trolox, which is a water-soluble analog of vitamin E. Another standard reagent

that widely used is ascorbic acid. This advantage of ABTS assay is that soluble in water and organic solvent which rapid reaction in the wide range of pH.

$$ABTS^{\bullet+} + AH\ (antioxidant) \longrightarrow ABTS\ (colorless) + A^{\bullet}$$

Figure 11 The scavenging reaction between ABTS• and an antioxidant (AH)

The antioxidant activity of the extract was measured using ABTS (2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) assay. Briefly, the ABTS and potassium persulfate were dissolved in distilled water to a final concentration of 7 mM and 2.45 mM respectively. Both of solutions were mixed by vortex and the mixture was kept in the dark place at a room temperature for 12-16 h before use. For the study of antioxidant capacity in the extract, the ABTS radical solution was diluted with distilled water to an absorbance value of 0.7-0.9 at 734 nm. These data represented in mean ± S.D. with three independent experiments. The reactivity of the various antioxidants tested of extract was compared with Trolex. The 50% inhibition (IC_{50}) of antioxidant activity of the sample was calculated comparing with the standard trolox. The % inhibition was also calculated with the same equation as 3.2.4.

Biochemistry

1. Cell culture (Inoue, et al., 2009)

Primary human follicle dermal papilla cells (HFDPC) were cultured in follicle dermal papilla cell growth medium supplemented with 4% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, 0.4% bovine pituitary extract, basic fibroblast growth gactor (1 ng/mL), and insulin. The cells were maintained in a 5% CO_2 humidified incubator at 37 °C. The cells were harvested after 70-80% confluence for the further experiment.

2. Cell viability test (Lall, et al., 2013)

Cell viability was determined for cytotoxicity testing of the natural product in the cells. The measurement of metobolic activity is the popular method for cytotoxicity testing. The cells with low metabolic activity mean the decreasing of cell viability. PrestoBlue cell viability assay is a commercial available, ready-to-use,

and water-soluble preparation. The mitochondrial reductase in the viable cells was use as a marker of cell viability. The pestoBlue resazurin-based solution (purple) can transport into the cells and prestoBlue was reduced by mitochondria reductase. Viable cells convert the purple oxidized form of the dye (resazurin) into a resorufin (red-fluorescent) reduced form. The fluorescent intensity was measured at 570 and 600 nm. This system is specific for cell viability as non-viable cells, rapidly lose metabolic capacity to reduce resazurin, and therefore no fluorescent signal is detected. Due to the dermal papilla cells is a primary cells, therefore the number of cell is lower than the cancer cells. Hence, the prestoBlue is suitable for these cells. The prestoBlue is not toxic in the cells and the cells can reuse after detecting by prestoBlue assay. Beside, prestoBlue is high sensitivity to reducing condition inside the cells that can use only 10 min compare with MTT or XTT assay.

Primary human follicle dermal papilla cells (HFDPCs) were cultured in follicle dermal papilla cell growth medium. After the cells reach to 70-80% confluence, the cells were seeded in 96-well culture plate (10^3 cells/ well) and maintained in a 5% CO_2 humidified incubator at 37°C for 18 h. Then, the cells were incubated with 200 µl of each fraction of the solvent extract (ethanol, dichloromethane, hexane, ethyl acetate and water fraction) that gave the best chemistry assay in various concentrations for example; 10 µg/ml to 1,000 µg/ml.

The control cells were incubated with 200 µl of follicle dermal papilla cell growth medium without serum and extracts were further incubated in a 5% CO_2 humidified incubator at 37°C for 24h, 48h, and 72h. After the indicated of time, the prestoBlue was added and incubated in 5% CO_2 incubator at 37°C for 10 minutes. The absorbance was measured using microplate reader at 570 and 600 nm. The cell viability was expressed as a percentage relating to the cells untreated with natural product. The percentage of cell viability was calculated using the following formula:

$$\%\text{cell viability} = \frac{\text{Mean O.D. of test compound} \times 100}{\text{Mean O.D. of control}}$$

The phytochemical test, the EtOAc fraction with *T.chantrieri, C. asiatica,* and *E. americana* extracts were test the cell viability to HFDPC. But the *T.chantrieri, C. asiatica, E. americana* fraction of EtOAc harm to cell. The safer solvents that used in extraction were used (EtOH extract and H_2O fraction).

3. The study of effect of the extract using real time polymerase chain reaction (Foitzik, et al., 2005; Inoue, et al., 2009)

The study of effect of the extracts and to the hair growth cycle related gene expression including, vascular endothelial growth factor (VEGF), vascular endothelial growth factor receptor (VEGFR, or KDR), insulin-like growth factor-1 (IGF-1), insulin-like growth factor-1 receptor (IGF-1R), transforming growth factor-β (TGF-β), and transforming growth factor-β receptor (TGF-βR) was show in Figure 11. By cell viability test, the incubation time at 24h, 48h, and 72h to HFDPC with *T.chantrieri, C. asiatica, E. americana* extracts did not show significant in difference. The incubation time at 24h to HFDPC *T.chantrieri, C. asiatica, E. americana* extracts was used to study gene expression by real-time PCR (qPCR).

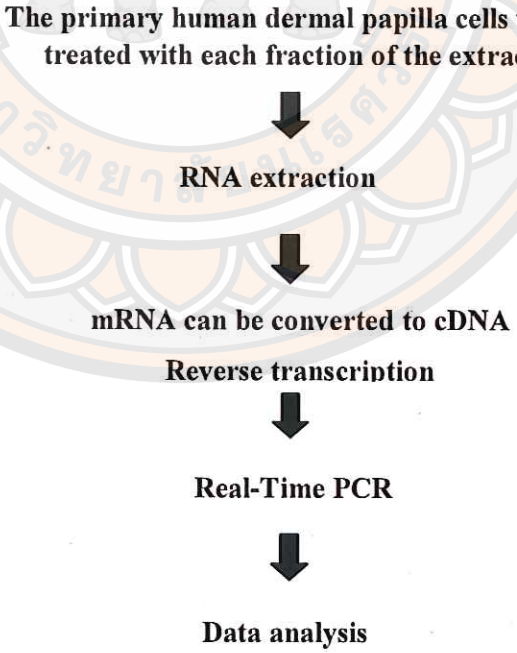

Figure 12 Over view of gene expression study

4. RNA extraction from primary human follicle dermal papilla cells

HFDPCs that treated with or without extracts unds, were extracted to recive the RNA by RNAzol REAGENT kit (Sigma-Aldrich) that contain guanidine thiocyanate and phenol. The samples were mixed and incubated at 15-30°C for 2-3 min. The samples were centrifuged at 4°C, 12,000g for 15 minutes. After centrifugation, the samples were separated into three fractions. The upper phase was RNA, the inter phase was protein or membrane and the lower phase was DNA. Then, the RNA fraction was collected in a Zymo-Spin™ IIC column (collection tube). Then, centrifuged at 12,000g 1 min, 4°C (RNA sample was in the column). After that, RNA sample was transferred into another tube and Direct-zol™ RNA prewash 400 µl was added. The RNA sample was centrifuged at 12,000g 1 min, for 4°C and discarded the flow through (bottom part). The RNA sample was added with 80 µl of DNase I reaction mix, centrifuged 12,000g 1 min, 4°C, discarded the flow through and transferred the RNA free DNA into another tube. The DNase/RNase-free water was added into RNA free DNA (centrifuged 12,000g for 1 min at 4°C). The total RNA was determined by spectrophotometer at 260 nm. The amount of the RNA was measured the ratio of O.D. 260 to O.D. 280 (The value of ratio of O.D. 260 to O.D. 280 more than 1.8 mean the high purity of RNA).

5. Reverse transcription (cDNA synthesis)

The reaction mixtures of reverse transcription were prepared for 20 µl containing of 1 µg of the RNA, 0.5 µg of oligo-dT, 1 mM dNTPs, 20 unit of ribonuclease inhibitor and 100 units of reverse transcriptase and adjust volume by DEPC-treated water. The reaction mixture was used as a template of cDNA synthesis for real-time PCR using thermal cycler (Appendix C). The reverse mRNA to cDNA condition was set as 37°C for 15 minutes, 50°C for 5 minutes and 98°C for 5 minutes, 1 cycle. The cDNA will be obtained from this step.

6. Gene expression by Real-Time PCR

Real-time polymerase chain reaction (qPCR) was the most used technique for determining of alteration of RNA or DNA in the molecular mechanism. The qPCR was the technique used for amplification of DNA and could detect the PCR products at that time. The amplified DNA (PCR product) was detected as the reaction progresses

in "real time" after the end of reaction. Two general methods for detecting of the PCR products in qPCR was: (1) non-specific fluorescent dyes (SYBR Green I) that intercalate with minor groove of double-stranded DNA, and (2) sequence-specific DNA probes that consist of oligonucleotides which were labeled with a fluorescent reporter that permits detection only after hybridization of the probe with its complementary sequence of DNA to quantify messenger RNA (mRNA) and non-coding RNA in cells or tissues. Our study, the qPCR condition and the specific primers including, VEGF, VEGFR (KDR), IGF-1, IGF-1R, TGF- β, or TGF- βR were used and the house keeping gene; beta-actin was used as an internal control (Appendix C). The alteration of hair growth cycle related to gene such as VEGF, VEGFR (KDR), IGF-1, IGF-1R, TGF- β, or TGF- βR was determined by qPCR compare with control.

The data was represented by Ct (cycle threshold) which was defined as the number of cycles required for the fluorescent signal (SYBR green) to cross the threshold. The result was calculated for $2^{-\Delta\Delta Ct}$, as fold change which normalized by reference gene (β-actin). The Ct value of gene was considered which not below than 30 cycles.

$2^{-\Delta\Delta Ct}$ calculation

Fold change = $2^{-\Delta\Delta Ct}$

; $\Delta Ct = Ct_{(target\ gene)} - Ct_{(\beta\text{-}actin)}$

; $\Delta\Delta Ct = \Delta Ct_{(target\ gene)} - \Delta Ct_{(negative\ control)}$

Ct is the threshold cycle for target or reference (β-actin) amplification.

ΔCt is equal to the difference in threshold cycle for target and reference.

Statistical analysis

All datas were represented as the mean ± standard deviation (S.D.) values. Statistical analysis was analyzed by SPSS software using one-way ANOVA. Statistical significance was determined at * $p < 0.05$.

CHAPTER IV

RESULTS

Plant extraction and partition

The *Tacca chantrieri* Ander (*T. chantrieri*), *Centella asiatica* Linn (*C.* asiatica) and *Eleutherine americana* (Aubl.) Merr (*E.* americana) were extracted by ethanol to recive ethanolic extract (EtOH). The liquid-liquid partition chromatography (LLPC) to obtain hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAC), and water (H_2O) fraction were derived from EtOH. The fractions of *T. chantrieri*, *C. asiatica* and *E. americana* were dried to obtain a powder and calculated percent yield (Table 1). Each percent yield of *T. chantrieri* powder in hexane, CH_2Cl_2, EtOAc, and water fractions were 2.20%, 1.40%, 5.53% and 52.73%, respectively. Each percent yield of *C. asiatica* powder in hexane, CH_2Cl_2, EtOAc, and water fractions were 9.00%, 0.80%, 3.20% and 59.20%, respectively. Each percent yield of *E. americana* powder in hexane CH_2Cl_2, EtOAc, and water fractions were 16.80%, 9.20%, 4.80% and 44.00%, respectively.

Table 1 The percent yield of *T. chantrieri*, *C. asiatica* and *E. americana* in each fractions

Sample	Dried (g)	Yield (g) Percent yield to EtOH				
		EtOH	Hexane	CH_2Cl_2	EtOAC	H_2O
T. chantrieri	100	15.00	0.33 2.20%	0.21 1.40%	0.83 5.53%	7.91 52.73%
C. asiatica	100	5.00	0.45 9.00%	0.04 0.80%	0.16 3.20%	2.96 59.20%
E. americana	100	5.00	0.84 16.80%	0.46 9.20%	0.24 4.80%	2.20 44.00%

Determination of antioxidant activity to DPPH radicals

The percent inhibition of *T. chantrieri*, *C. asiatica* and *E. americana* with various concentrations were presented in Figure 13 – 15. The percent inhibition of *T. chantrieri* in EtOH extract and its fractions including hexane, CH_2Cl_2, EtOAc, and H_2O fraction were significantly increased in dose dependent. The EtOAC fraction and EtOH extract were the most effectively anti-oxidant activity among the 5 fractions of *T. chantrieri* extract (Figure 13). The highest antioxidation value of *T. chantrieri* extract with EtOH against DPPH radicals at 200 µg/ml was 97.52 ± 0.12 follow by *T. chantrieri* extract with EtOH against DPPH radicals at 100 µg/ml was 96.47 ± 0.06.

The percent inhibition of *C. asiatica* in EOH extract and its fractions including hexane, CH_2Cl_2, EtOAc, and H_2O fractions were significantly increased in dose dependent. The EtOAC fraction and EtOH extract were the most effectively anti-oxidant activity among the five fractions of *C. asiatica* extract (Figure 14). The highest antioxidation value of *C. asiatica* extract with EtOH against DPPH radicals at 200 µg/ml was 97.01 ± 0.42 followed by *C. asiatica* extract with EtOH against DPPH radicals at 100 µg/ml was 95.78 ± 0.14.

The percent inhibition of *E. americana* in EtOH extract and its fractions including hexane, CH_2Cl_2, EtOAc, and H_2O fractions were significantly increased in dose dependent. The EtOAC fraction and EtOH extract were the most effectively anti-oxidant activity among the five fractions of *E. americana* extract (Figure 15). The highest antioxidation value of *E. americana* extract with EtOAc against DPPH radicals at 200 µg/ml were 99.39 ± 0.01.

The highest antioxidation activity against DPPH radicals of *T. chantrieri* and *C. asiatica* was EtOH extract. But the highest antioxidation of *E.americana* was given by EtOAc fraction. The antioxidant values were relative to dose dependent. Among these plants, *E. americana* gave the best antioxidation aginst DPPH radicals followed by *T. chantrieri* and *C. asiatica*. The IC_{50} of the extracts to DPPH radicals was shown in table 2.

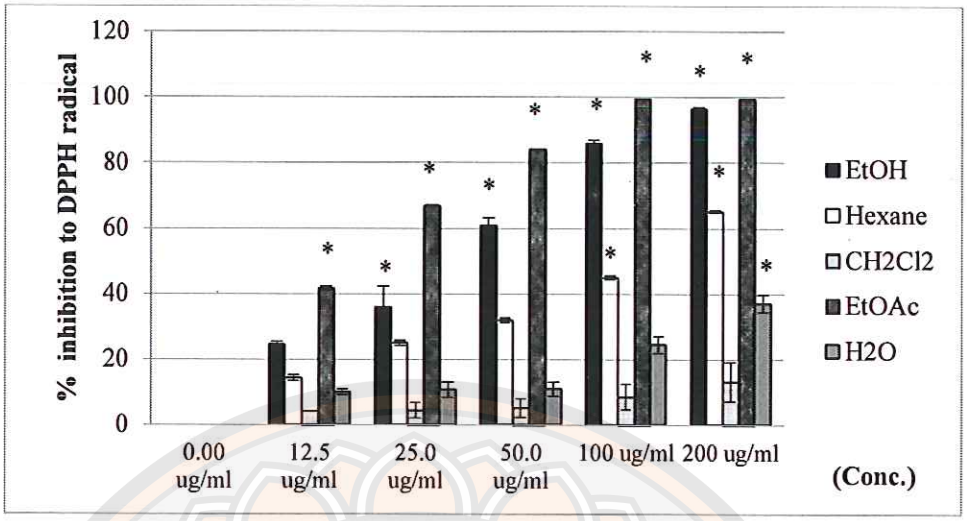

Figure 13 Percent inhibition of *T. chantrieri* to DPPH radicals

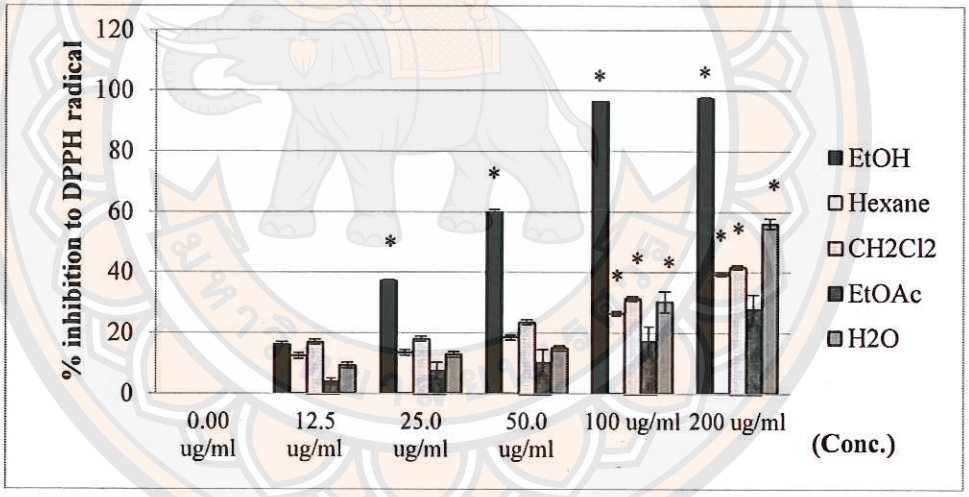

Figure 14 Percent inhibition of *C. asiatica* to DPPH radicals

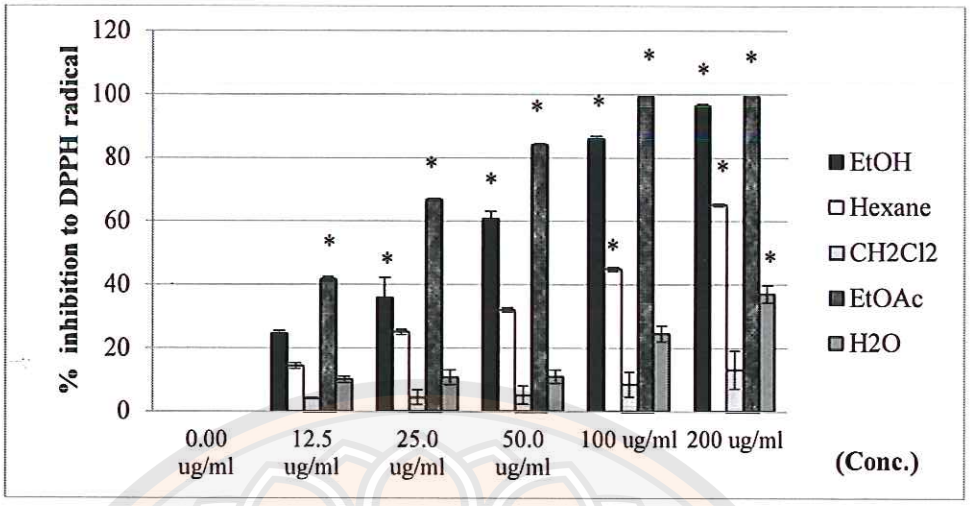

Figure 15 Percent inhibition of *E. americana* to DPPH radical

Table 2 IC_{50} of the extracts to DPPH radicals

Fraction	IC_{50} of Extract to DPPH radicals (μg/ml)		
	T. chantrieri	*C. asiatica*	*E. amercana*
EtOH	36.85 ± 3.38	28.41 ± 1.47	30.48 ±2.30
Hexane	> 200	>200	159.34 ± 10.04
CH_2Cl_2	> 200	>200	>200
EtOAc	> 200	119.05 ± 2.12	22.71 ± 2.43
H_2O	178.57 ± 4.92	>200	>200

Determination of antioxidant activity of the extracts to ABTS radicals

The percent inhibition of *T. chantrieri*, *C. asiatica* and *E. americana* in various concentrations was presented in Figure 16 – 18. The percent inhibition of *T. chantrieri* in EtOH extract and its fractions were significantly increased in dose dependent.

The percent inhibition of EtoAC fraction of *T. chantrieri* was the most effective anti-oxidant activity among the five fractions (Figure 16). The EtOAc fraction of *T. chantrieri* (200 µg/ml) gave the highest antioxidation value against ABTS radicals, it was 88.24 ± 4.37 follow by EtOAc fraction of *T. chantrieri* (100 µg/ml) was 85.81 ± 7.38.

The percent inhibition of *C. asiatica* in EtOH extract and its fractions including hexane, CH_2CL_2, EtOAC, and H_2O fractions were significantly increased in dose dependent. The percent inhibition of EtoAC fraction of *C. asiatica* gave the most effective anti-oxidant activity among the five fractions (Figure 17). The highest antioxidation value of EtOAc fraction of *C. asiatica* (200 µg/ml) against ABTS radicals was 65.33 ± 2.09 follow by EtOAc of *C. asiatica* (100 µg/ml) was 51.56 ± 1.70.

The percent inhibition of *E. americana* in EtOH extract and its fractions including hexane, CH_2CL_2, EtOAC, and H_2O fractions was significantly increased in dose dependent. The percent inhibition of EtoAC fraction of *E. americana* was the most effective anti-oxidant activity among the five fractions (Table 3 and Figure 18). The highest antioxidation value of *E. americana* in EtOAc fraction against ABTS radicals at 200 µg/ml was 75.04 ± 0.69 follow by *E. americana* extract with EtOAc against ABTS radicals at 100 µg/ml was 66.98 ± 4.26.

The highest antioxidation of *T. chantrieri, C. asiatica,* and *E.americana* was given by EtOAc fraction.The antioxidant values were relative to dose dependent. Among these plants, *T. chantrieri* gave the best antioxidation aginst ABTS radicals followed by *E. americana* and *C. asiatica*. The IC_{50} of the extracts to ABTS radicals was shown in Table 3.

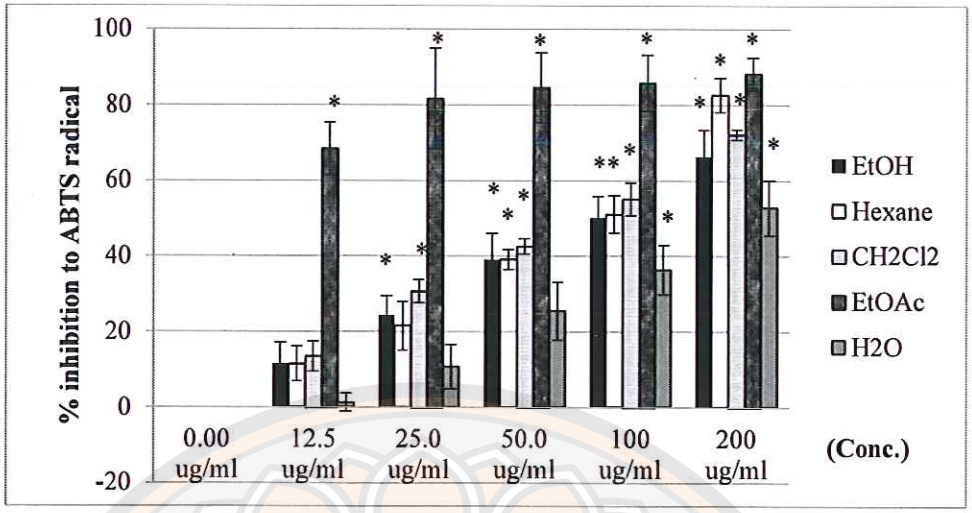

Figure 16 Percent inhibition of *T. chantrieri* to ABTS radicals

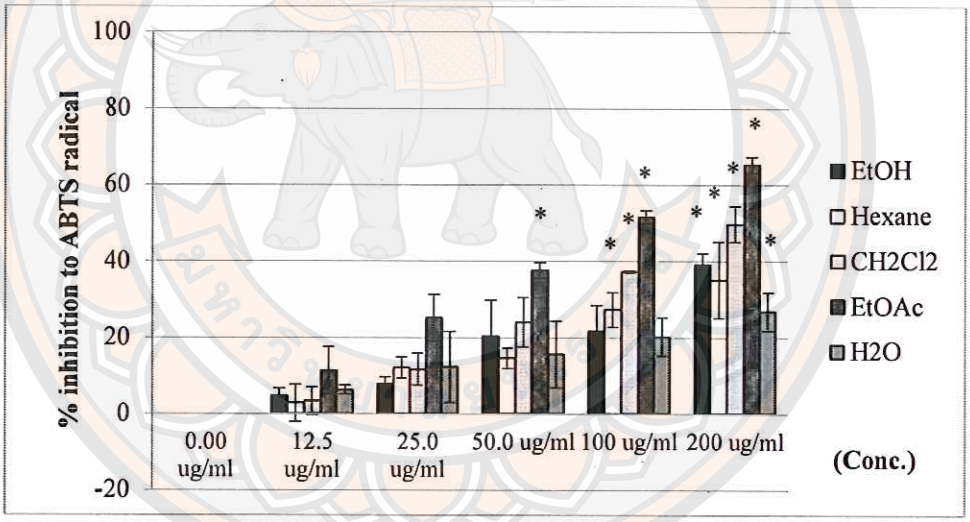

Figure 17 Percent inhibition of *C. asiatica* to ABTS radicals

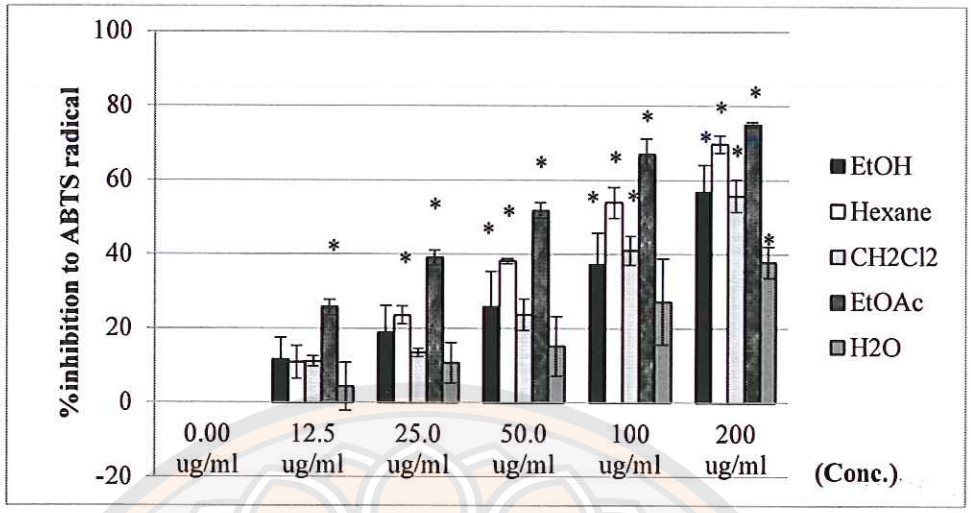

Figure 18 Percent inhibition of *E. americana* to ABTS radicals

Table 3 IC_{50} of the extracts to ABTS radicals

Fraction	IC_{50} of Extract to ABTS radicals (μg/ml)		
	T. chantrieri	*C. asiatica*	*E. amercana*
EtOH	120.37 ± 45.29	>200	164.92 ± 33.98
Hexane	98.44 ± 9.48	>200	114.56 ± 5.21
CH_2Cl_2	164.66 ± 17.23	174.30 ± 4.10	164.66 ± 17.24
EtOAc	9.19 ± 0.98	122.22 ± 7.49	70.37 ± 4.71
H_2O	175.26 ± 2.09	>200	>200

Determination of antioxidant compounds of the extracts by phenolic assay

The phenolic content of *T. chantrieri* in EtOH extract and its fractions was presented in Figure 19 – 21. The phenolic content of the *T. chantrieri* in EtOH and its fractions including hexane, CH_2CL_2, EtOAC, and water fractions was significantly

increased in dose dependent. The phenolic content was found highly in EtOAc fraction compared with the other fractions (Figure 19). The highest amount of phenolic content of *T. chantrieri* fractionated with EtOAc at 100 µg/ml was 196.06 ± 0.03 mg GAE/g extract follow by *T. chantrieri* fractionated with EtOAc at 50 µg/ml was 193.92 ± 0.04 mg GAE/g extract.

The phenolic content of *C. asiatica* in EtOH extract and its fractions including hexane, CH_2CL_2, and EtOAC, fractions was increased in dose dependent but not significant whereas H_2O fraction was could not found phenolic compound. The phenolic content was found highly in EtOAc fraction compared with the other fractions (Figure 20). The highest amount of phenolic content of *C. asiatica* fractionated with EtOAc at 100 µg/ml was 21.70 ± 0.03 mg GAE/g extract follow by *C. asiatica* fractionated with EtOAc at 200 µg/ml was 17.75 ± 0.02 mg GAE/g extract.

The phenolic content of *E. americana* in EtOH extract and its fractions including hexane, CH_2CL_2, EtOAC, and H_2O fractions was significantly increased in dose dependent. The phenolic content was found highly in EtOAc fraction compared with the other fractions (Figure 21). The highest amount of phenolic content of *E. americana* fractionated with EtOAc at 50 µg/ml was 137.51 ± 0.02 mg GAE/g extract follow by *E. americana* fractionated with EtOAc at 200 µg/ml was 136.26 ± 0.00 mg GAE/g extract.

The highest amount of phenolic content of *T. chantrieri, C. asiatica,* and *E.americana* was derived by EtOAc fraction.The amount of phenolic contents were relative to dose dependent. Among these plants, *T. chantrieri* gave the highest amount of phenolic content follow by *E. americana* and *C. asiatica*.

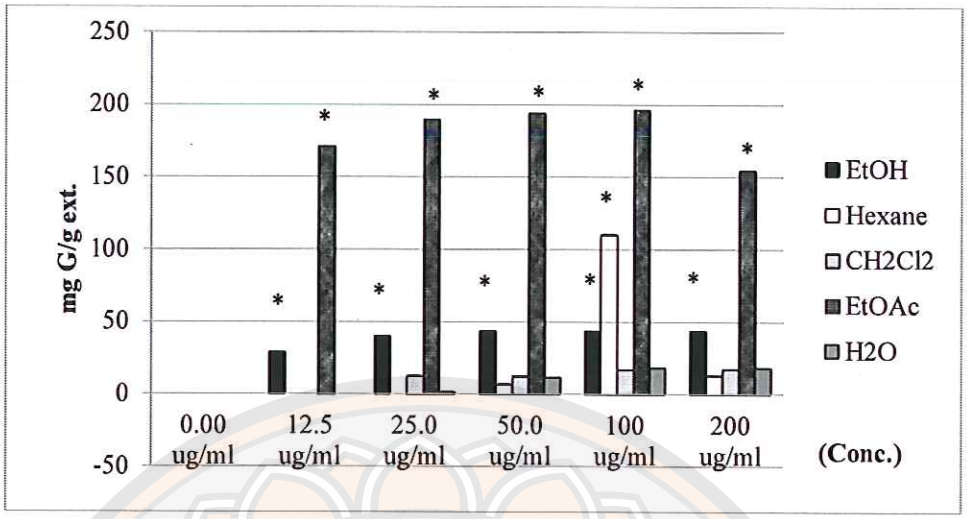

Figure 19 Phenolic content (µg G/g.ext) in fraction of *T. chantrieri*

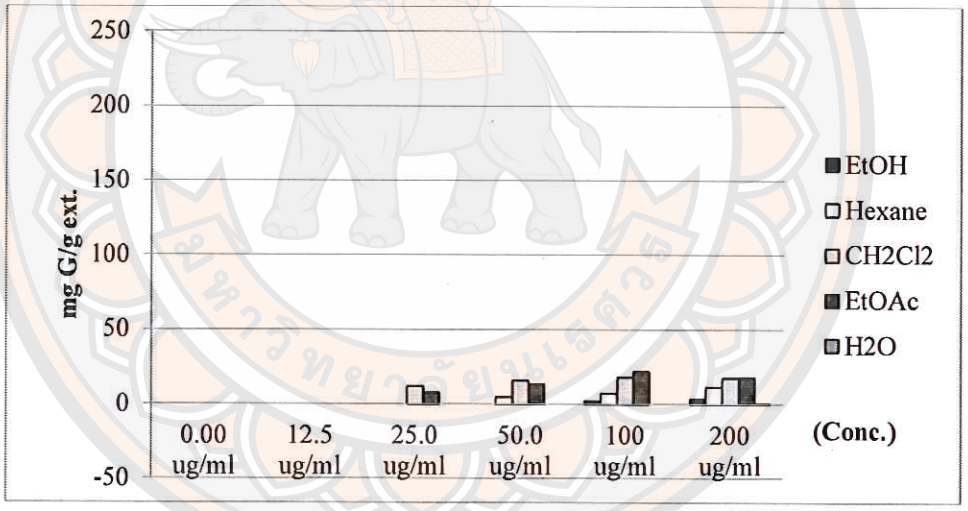

Figure 20 Phenolic content (µg G/g.ext) in fraction of *C. asiatica*

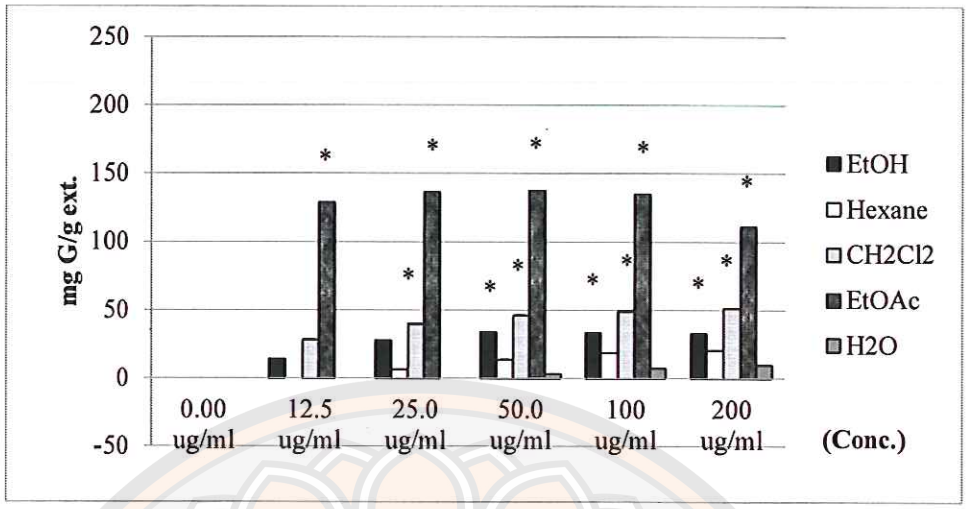

Figure 21 Phenolic content (µg G/g.ext) in fraction of *E. americana*

Determination of antioxidant compound of the extracts by flavonoid assay

The flavonoid content of *T. chantrieri* in EtOH extract and its fractions was presented in Figure 22 – 24. The flavonoid content of *T. chantrieri* in EtOH extract and its fractions including hexane, CH_2CL_2, EtOAC, and H_2O fractions was increased in dose dependent but no significant except EtOH extract (Figure 22). The highest amount of flavonoid content of *T. chantrieri* extract with EtOH at 100 µg/ml was 18.01 ± 0.36 mg CE/g extract follow by *T. chantrieri* extract with EtOH at 200 µg/ml was 9.00 ± 0.00 mg CE/g extract.

The flavonoid content of *C. asiatica* in EtOH extract and its fractions including hexane, CH_2CL_2, and H_2O fractions was not found flavonoid content except in EtOH extract (Figure 23). The highest amount of flavonoid content of *C. asiatica* extract with EtOH at 200 µg/ml was 2.52 ± 0.00 mg CE/g extract follow by *C. asiatica* extract with EtOH at 100 µg/ml was 0.94 ± 0.00 mg CE/g extract.

The flavonoid content of *E. americana* in EtOH extract and its fractions including hexane, CH_2CL_2, and EtOAc fractions was increased in dose dependent but no significant except EtOAc fraction whereas H_2O fraction was could not found flavonoid. The flavonoid content was found highly in EtOH extract compared with the other fractions (Figure 24). The highest amount of flavonoid content of *C. asiatica*

extract with EtOH at 50 µg/ml was 33.58 ± 0.00 mg CE/g extract follow by *C. asiatica* extract with EtOH at 100 µg/ml was 33.26 ± 0.00 mg CE/g extract.

The highest amount of flavonoid content of *T. chantrieri, C. asiatica,* and *E.americana* was derived by EtOH fraction. The amount of flavonoid content was relative to dose dependent. Among these plants, *E. americana* gave the highest amount of flavonoid content follow by *T. chantrieri* and *C. asiatica*.

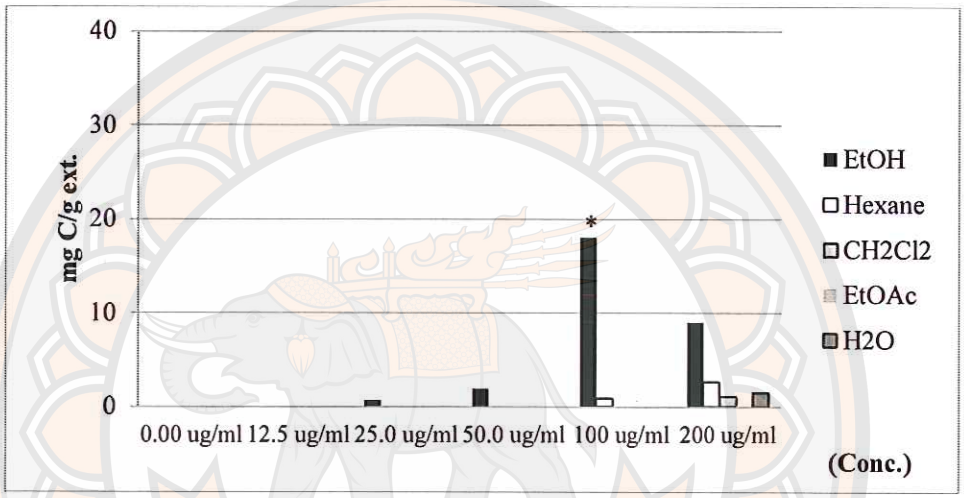

Figure 22 Flavonoids content (mg CE/g.ext) in fraction of *T. chantrieri*

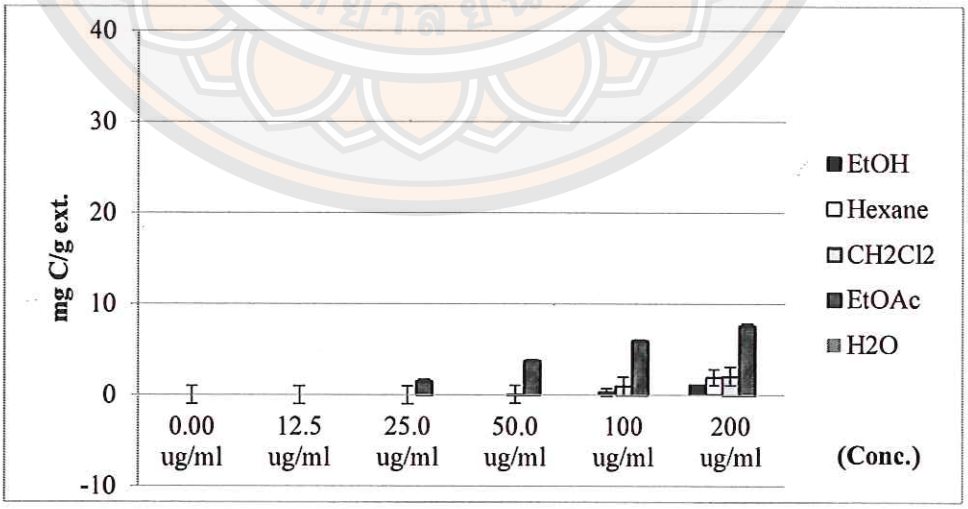

Figure 23 Flavonoids content (mg CE/g.ext) in fraction of *C. asiatica*

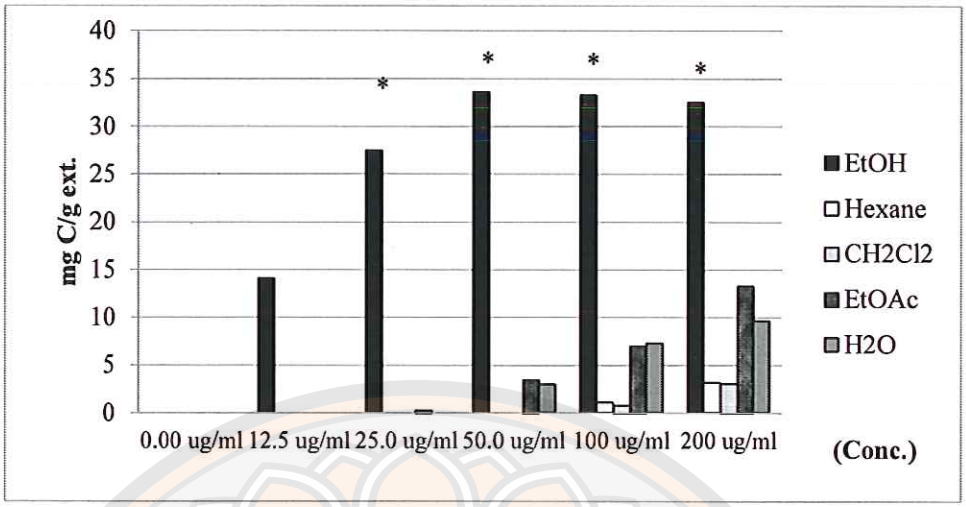

Figure 24 Flavonoids content (mg CE/g.ext) in fraction of *E. americana*

Cell viability testing of extracts in ethyl acetate fraction on Human Folicle Dermal Papilla Cell (HFDPC)

The ethyl acetate (EtoAC) fraction showed the highest anti-oxidant, phenolic and flavonoid contents among all of the three extracts. Therefore, the toxicity testing of *T. chantrieri*, *C. asiatica* and *E. americana* in EtOAC fraction to HFDPC was determined using PrestoBlue cell viability assay. The HFDPC was incubated with various concentrations of EtOAc fraction of the extracts for 24h, 48h and 72h.

The *T. chantrieri* in EtOAc was not enhancing the growth of HFDPC when incubated for 24h, 48h, and 72h. The cells viability was slightly decreased with incubation time (Figure 25). The morphology of the HFDPC changed after incubated with EtOAc fraction of *T. chantrieri* (Figure 26). The HFDPC changed the oval large shape form with nucleus to shrink of cytoplasm and extinct after increasing the concentration of the extracted from 0 µg/ml to 100 µg/ml. This was occure with 24h, 48h, and 72h of incubation time of EtOAc fraction.

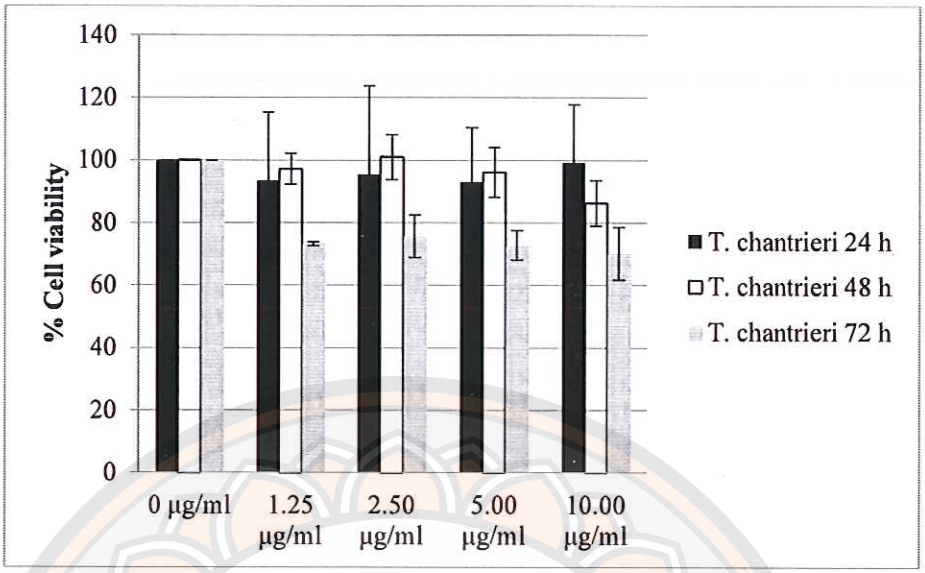

Figure 25 The HFDPC viability when treated with EtOAc fraction of *T. chantrieri* using PrestoBlue cell viability assay for 24h, 48h and 72h.

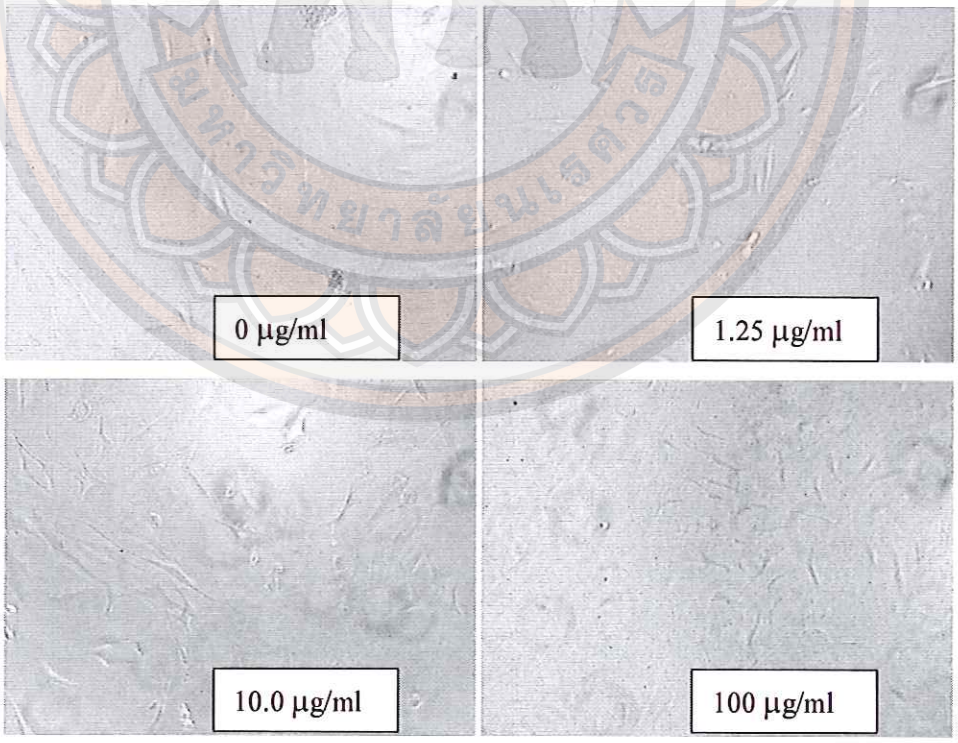

Figure 26 HFDPC morphology (10X) after treated with various concentrations of *T. chantrieri* in EtOAc fraction for 72h.

The EtOAc fraction of *C. asiatica* was not enhancing the growth of HFDPC when incubated for 24h, 48h or 72h (Figure 27). As the result, the morphology of the HFDPC changed after incubated with EtOAc fraction of *C. asiatica* (Figure 28). That it harm to HFDPC.

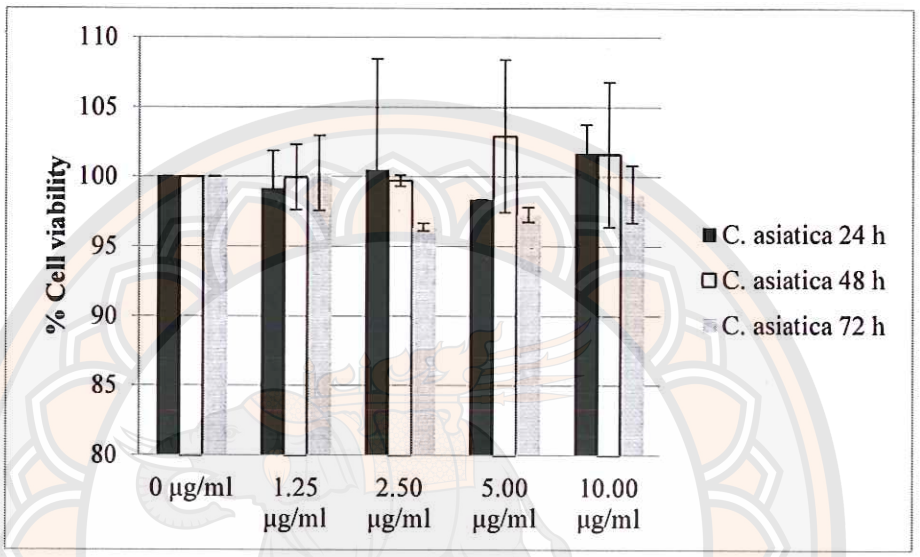

Figure 27 The HFDPC viability when treated with EtOAc fraction of *C. asiatica* using PrestoBlue cell viability assay for 24h, 48h and 72h.

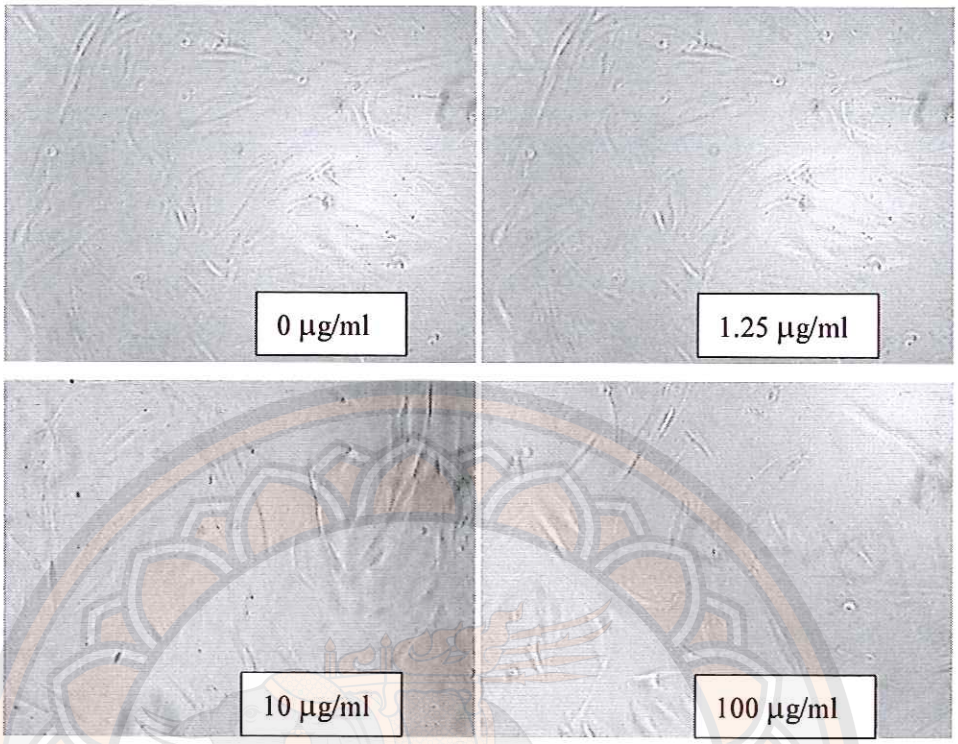

Figure 28 HFDPC morphology (10X) after treated with various concentrations of *C. asiatica* in EtOAc fraction for 72h.

The *E. americana* in EtOAc fraction was inhibit the growth of HFDPC when incubated for 24h, 48h or 72h (Figure 29). The morphology of the HFDPC has changed after incubated with EtOAc fraction (Figure 30). The HFDPC changed oval large shape form with nucleus to shrink of cytoplasm and extinct after increase the concentration of the extracted from 0 µg/ml to 100 µg/ml.

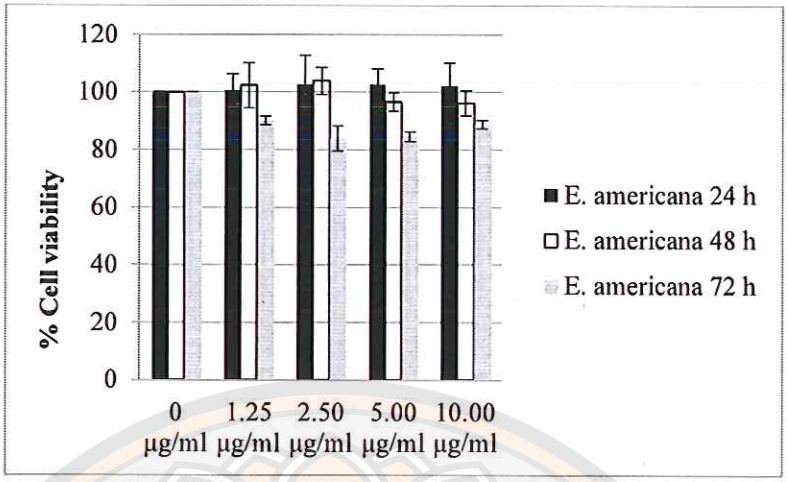

Figure 29 The HFDPC viability when treated with *E. americana* in EtOAc fraction using PrestoBlue cell viability assay for 24, 48 and 72h.

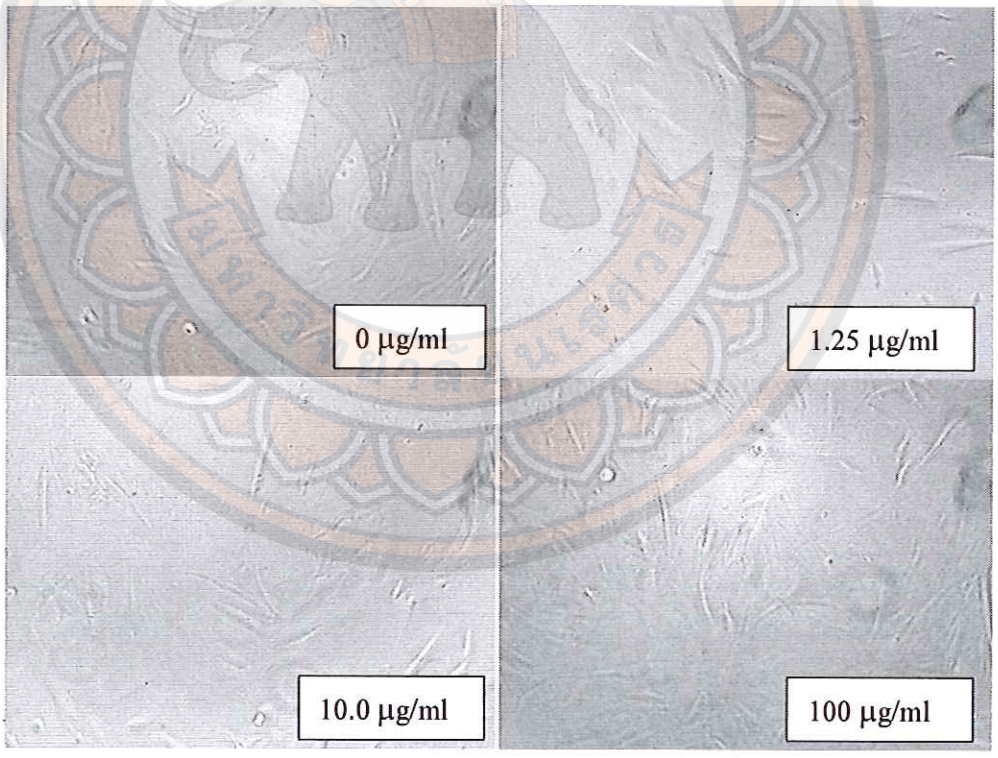

Figure 30 HFDPC morphology (10X) after treated with various concentrations of *E. americana* extract in EtOAc fraction for 72h.

The cytotoxicity datas showed that when treate HFDPC with the *T. chantrieri*, *C. asiatica* and *E. americana* in EtOAc fraction. The EtOAc fractions was decreased % cells survival when treated for 72h using PrestoBlue cell viability assay. Therefore, before drive to next study, the ethanol extract and H_2O fraction, which reported as a non-toxic solvent for use *in vitro* study for 24h and 48h, were determined. Moreover, the EtOH extracts also contains high phenolic and flavonoid contents that related with anti-oxidant activities.

The incubation of the extracts (*T. chantrieri*, *C. asiatica* and *E. americana*) in H_2O fraction for 24h and 48h was not toxic to HFDPC, which showed no effect of cells survival (Figure 31 – 32). The EtOH extracts were significantly increased % cells survival compared with control within 24h (Figure 33), and 48h of incubation times (Figure 32). Therefore, the H_2O fraction and EtOH extract were used for studing the mRNA expression. The qPCR technique was used in this study.

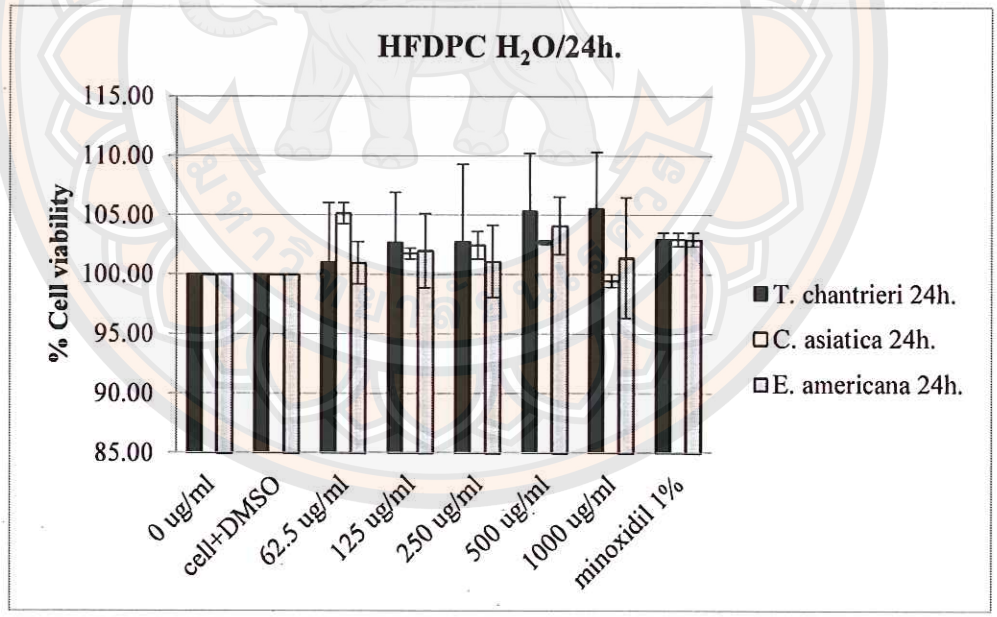

Figure 31 The percent HFDPC viability of extracts in H_2O fraction using PrestoBlue cell viability assay for 24h.

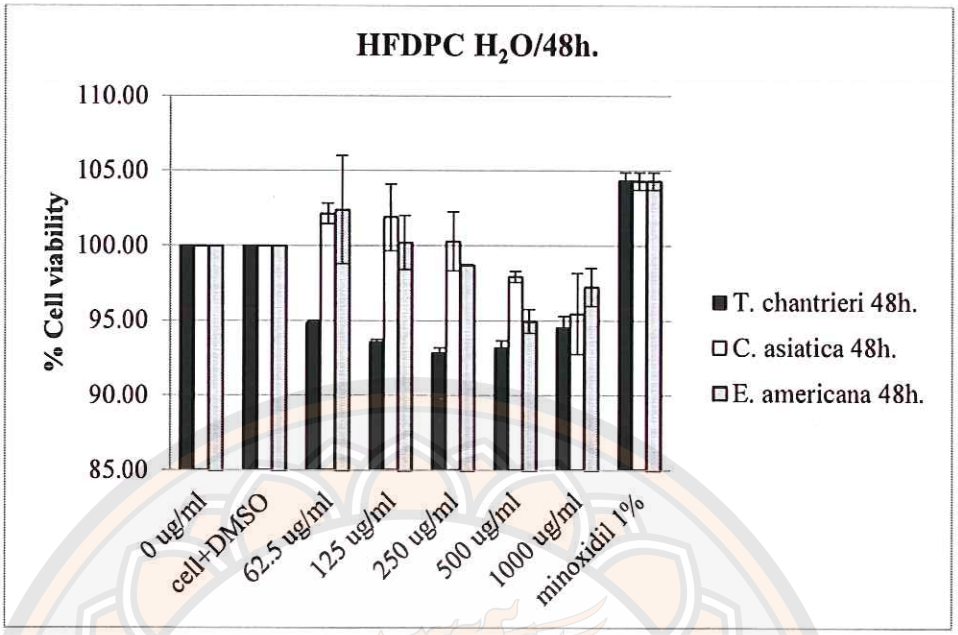

Figure 32 The percent HFDPC survival of extracts in H_2O fraction using PrestoBlue Cell viability assay for 48h.

By cell viability assay, the *T. chantrieri*, *C. asiatica* and *E. americana* extracts in H_2O fraction at 24h and 48h did not show scientific different with time incubation. The concentration of incubation also did not show scientific different when increase the concentration to 1,000 μg/ml.

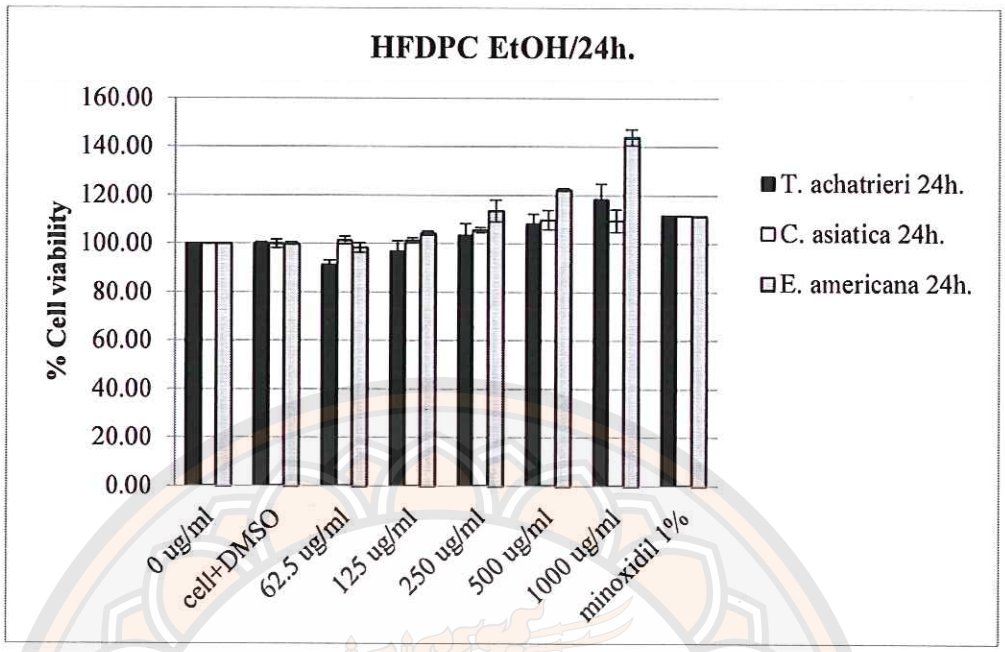

Figure 33 The percent HFDPC survival of EtOH extracts using PrestoBlue cell viability assay for 24h.

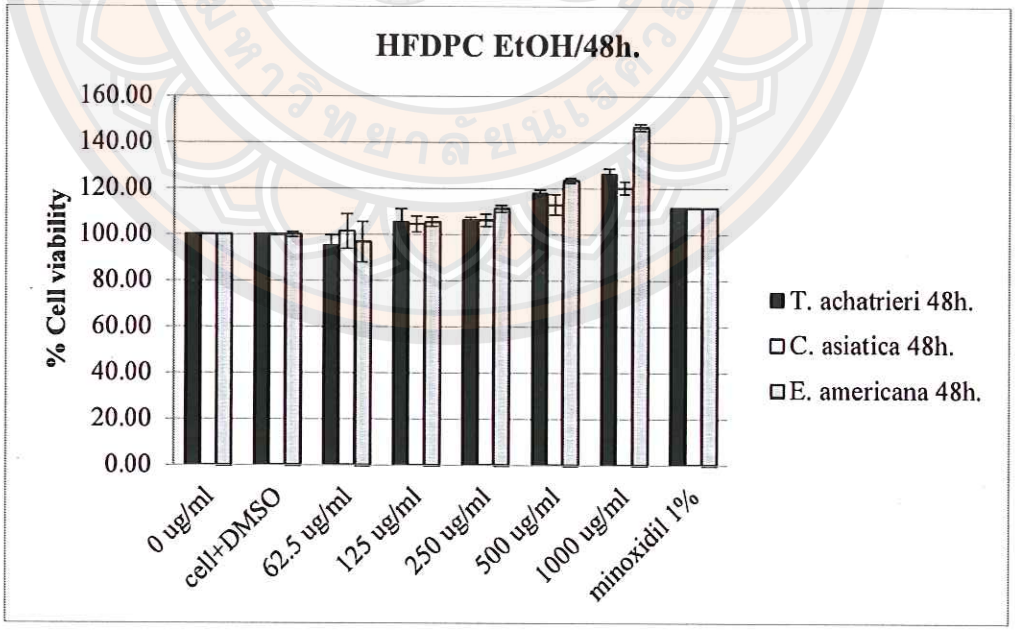

Figure 34 The percent HFDPC survival of EtOH extracts using PrestoBlue cell viability assay for 48h.

The *T. chantrieri*, *C. asiatica* and *E. americana* in EtOH extract did not show scientific different of 24h and 48h incubation times. The EtOH of extracts at 1,000 µg/ml showed significant increased amount of HFDPC.

Alteration of mRNA expression using real time PCR (qPCR)

HFDPCs were treated with or without various concentrations of the *T. chantrieri*, *C. asiatica* and *E. americana* extract (EtOH extract or water fraction). Then, HFDPC extracted RNA by RNAZOL reagent kit. The expression of mRNA level was determined using qPCR. The incubation time of the extracts (EtOH extract and H_2O fraction) to HFDPC was 24h. The result was derived in 2 times of experiment.

The expressions of VEGF, VEGFR (KDR), IGF-1, IGF-1R, TGF-βR and TGF-β mRNA level when HFDPC was incubated with *T. chantrieri* in H_2O fraction were increased. The β-actin was used as a reference gene at 1st (Table 4) and 2nd treatment (Table 5).

Table 4 Gene expression of HFDPC (H_2O fraction of *T. chantrieri* at 1st treatment).

1st treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1%	1.09	0.16	1.70	0.89	1.02	0.52
T. chantrieri 500 µg/ml	ND	ND	ND	ND	ND	ND
T.chantrieri 1000 µg/ml	ND	ND	ND	ND	ND	ND

ND = Not detected

Table 5 Gene expression of HFDPC (H_2O fraction of *T. chantrieri* at 2nd treatment).

2nd treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1%	1.04	0.83	4.02	1.04	1.01	0.70
T. chantrieri 500 µg/ml	ND	ND	ND	ND	ND	ND
T.chantrieri 1000 µg/ml	ND	ND	ND	ND	ND	ND

ND = Not detected

The expressions of VEGF, KDR, IGF-1, IGF-1R, TGF-β and TGF-βR mRNA level were decresed when incubated HFDPC with EtOH extract of *T. chantrieri*. The β-actin was used as an internal control at 1st treatment (Table 6) and 2nd treatment (Table 7)

Table 6 Gene expression of HFDPC when incubated with EtOH extract of *T. chantrieri* at 1st treatment.

1st treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1 µg	0.50	0.62	0.18	0.59	1.15	0.67
T. chantrieri 500 µg/ml	ND	ND	ND	ND	ND	ND
T.chantrieri 1000 µg/ml	ND	ND	ND	ND	ND	ND

ND = Not detected

Table 7 Gene expression of HFDPC when incubated with EtOH extract of *T. chantrieri* at 2nd treatment.

2nd treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1 μg	1.04	1.22	0.16	0.50	0.87	0.64
T. chantrieri 500 μg/ml	ND	ND	ND	ND	ND	ND
T.chantrieri 1000 μg/ml	ND	ND	ND	ND	ND	ND

ND = Not detected

The expression of VEGF, VEGFR, and IGF-1R mRNA level was increased when incubated HFDPC with H_2O fraction of *C. asiatica*. The expression of IGF-1R, TGF-β, and TGF-βR mRNA level was decreased after incubated HFDPC with H_2O fraction of *C. asiatica*. The β-actin was used as an internal control at 1st treatment (Table 8) and 2nd treatment (Table 9).

Table 8 Gene expression of HFDPC when incubated with H_2O fraction of *C. asiatica* at 1st treatment

1st treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1 μg	0.91	0.03	ND	ND	ND	ND
C. asiatica 500 μg/ml	15.63	1.66	ND	ND	1.11	ND
C. asiatica 1000 μg/ml	61	8.03	ND	ND	0.97	ND

ND = Not detected

Table 9 Gene expression of HFDPC when incubated with H_2O fraction of *C. asiatica* at 2nd treatment

2nd treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
Control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1 μg	1.56	5.52	ND	ND	ND	ND
C. asiatica 500 μg/ml	30	3.27	ND	ND	1.30	ND
C. asiatica 1000 μg/ml	160	96	ND	ND	0.96	ND

ND = Not detected

The expression of VEGF, VEGFR, IGF-1, IGF-1R, TGF-β and TGF-βR mRNA level was increased when incubated HFDPC with EtOH extract of *C. asiatica*. The β-actin was used as a reference gene at 1st treatment (Table 10) and 2nd treatment (Table 11).

Table 10 Gene expression of HFDPC when incubated with EtOH extract of *C. asiatica* at 1st treatment

1st treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
Control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1 μg	1.04	1.03	ND	0.63	1.11	ND
C. asiatica 500 μg/ml	4.47	ND	ND	ND	3.19	ND
C. asiatica 1000 μg/ml	21	ND	ND	ND	ND	ND

ND = Not detectd

Table 11 Gene expression of HFDPC when incubated with EtOH extract of *C. asiatica* at 2nd treatment

2nd treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
Control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1 μg	1.28	4.53	ND	1.42	1.14	ND
C. asiatica 500 μg/ml	6.70	ND	ND	ND	ND	ND
C. asiatica 1000 μg/ml	42	ND	ND	ND	ND	ND

ND = Not detected

The expression of VEGF, TGF-β mRNA level was increased when treated HFDPC with H_2O fraction of *E. americana.* But the expression of VEGFR, IGF-1, IGF-1R, and TGF-βR mRNA level was decreased when incubated HFDPC with H_2O fraction of *E. Americana.* The β-actin was used as a reference gene at 1st treatment (Table 12) and 2nd treatment (Table 13).

Table 12 Gene expression of HFDPC when incubated with H_2O fraction of *E. americana* at 1st treatment

1st treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
Control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1 μg	1.11	ND	ND	ND	ND	ND
E. americana 500 μg/ml	2.49	ND	ND	1.27	1.31	ND
E. americana 1000 μg/ml	9.52	ND	ND	2.02	2.13	ND

ND = Not detected

Table 13 Gene expression of HFDPC when incubated with H_2O fraction of *E. americana* at 2nd treatment

2nd treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
Control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1 μg	0.90	ND	ND	ND	ND	ND
E. americana 500 μg/ml	3.81	ND	ND	1.00	1.47	ND
E. americana 1000 μg/ml	9.95	ND	ND	0.96	1.85	ND

ND = Not detected

The expression of VEGF, IGF-1, IGF-1R, TGF-β, and TGF-βR mRNA level was increased when incubated HFDPC with EtOH extract of *E. americana*. But the expression of VEGFR mRNA level was decreased when incubated HFDPC with EtOH extract of *E. americana*. The β-actin was used as a reference gene at 1st treatment (Table 14) and 2nd treatment (Table 15)

Table 14 Gene expression of HFDPC when incubated with EtOH extract of *E. americana* at 1st treatment

1st treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
Control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1 μg	ND	ND	0.85	0.70	0.93	0.83
E. americana 500 μg/ml	5.54	ND	58.32	4.54	2.58	ND
E. americana 1000 μg/ml	2.56	ND	3.22	1.12	1.67	ND

ND = Not detected

Table 15 Gene expression of HFDPC when incubated with EtOH extract of *E. americana* at 2nd treatment

2nd treatment	VEGF	VEGFR	IGF-1	IGF-1R	TGFβ2	TGFβ2R
Control	1.00	1.00	1.00	1.00	1.00	1.00
Minoxidil 1 µg	ND	ND	0.98	0.82	1.24	0.87
E. americana 500 µg/ml	10	ND	113	8.08	4.92	ND
E. americana 1000 µg/ml	3.17	ND	1.42	1.17	1.83	ND

ND = Not detected

CHAPTER V

DISCUSSION AND CONCLUSION

Tacca chantrieri Ander (*T. chantrieri*), *Centella asiatica* Linn (*C. asiatica*) and *Eleutherine americana* (Aubl.) Merr. (*E. americana*) have a long history of use as traditional medicines in Thailand and other countries. The evidence from studies into the medicinal properties of these herbs supports that *T. chantrieri*, *C. asiatica* and *E. americana* have anti-inflammatory and anti-infection properties and uses (Keardrit, Rujjanawate, and Amornlerdpison, 2010, Ifesan, et al., 2009, and Saha, et al., 2013). However, there is no evidence of previous scientifically controlled studies on the use of these natural products in the prevention of hair loss. Our study fills this gap in the body of knowledge on natural remedies and use of medicinal herbs.

Extracts from *T. chantrieri*, *C. asiatica* and *E. americana* were taken using Liquid-Liquid Chromatography to obtain hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAC), and water (H_2O) fractions from the ethanol (EtOH) extract. This method was widely used in many studies. This method is used to separate the substance by like dissolve like. The substances in each plant will separate into categories by the solvent to solvent polarity. The polarity of the substance groups will be increased by the solvent polarity, since the plant has many substances or many active compounds. To classify the biological activity or phytochemical activity of the plant compound, fractionation should be used to separate the fractions. As discussed in (Li, 2016), this is a simple and practical method to use.

***Tacca chantrieri* (*T. chantrieri*)**

The *T. chantrieri* extracted with EtOH is used by Thai hill tribes as a hair treatment (Rujjanawate, et al., 2009). In the oxidation process, peroxides and free radicals are produced. These are damaging to all components of the cell, including proteins, lipids, and DNA (Nawar, 1996). These products are reactive oxygen species (ROS) that can harm normal cells, causing cell aging (Kwon, et al., 2007). Previous reports have indicated that there is a relationship between anti-oxidant activity and dermal papilla death by apoptosis. This mechanism was expressed via the TGF-β

pathway (Soma, et al., 2003 and Hibino and Nishiyama, 2004). Our study included analysing the affect of the antioxidant compounds found in *T. chantrieri* extract on the antioxidation activity, which we measured by DPPH and ABTS assays. These assays showed that the EtOAc fraction of *T. chantrieri* is the highest of all the fractions. This study followed the study of (Wang, et al., 2016) in which the EtOAc fraction of *Polygonum cuspidatum* was found to have an antioxidation affect *in vitro*, by gathering the substances that can dissolve in both polar and non polar polarities. Our experiment showed the antioxidation activity of each extract in both a polar solvent and an organic solvent. The polar solvent was represented by ABTS assay because water was used as the solvent. The organic solvent was represented by DPPH assay because methanol was used as the solvent. The assays investigated both strong radicals (ABTS assay) and not strong radical (DPPH assay). Many studies have suggested that plants have a pharmacodynamic potential as an antioxidant (Lin, et al., 2015). The antioxidant activity of *T. chantrieri* against DPPH radicals and ABTS radicals showed in our study as being dose dependent, and also demonstrated the ability to scavenge both strong radicals and not strong radicals. The 200 μg/ml of the *T. chantrieri* was used in the experiment because it did not dissolve when a higher concentration was used. The *T. chantrieri* plant extract inhibited the caspase, p53, and p21 protein that cause apoptotic cell death. The apoptotic cell death in HFDPC was involved with TGF-β that is related to antioxidant activity (Kamimura, A., et al., 2006). The highest anti-oxidant activity was found in the EtOAc fraction of *T. chantrieri*. Anti-oxidant activity was also found in the EtOH extract. The *T. chantrieri* extracts with the EtOAc fraction and EtOH extract had high phenolic and flavonoid content. Oxidative stress reflects an imbalance between the systemic manifestation of ROS and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting cell damage (Nawar, 1996). The active compound found in plants has anti aging effects, immune modulation, anti hyperlipidemia, hepatoprotective effect, anti cancer, etc with non cytotoxicity effect (Lin, et al., 2015).

Phenolic compound and flavonoid compound are active against many diseases (Chen, et al., 2014). The extract of *T. chantrieri* showed dose dependent phenolic compounds and flavonoid compounds. Interestingly, the phenolic compounds and flavonoid compounds in the EtOAc fraction and the EtOH extract of *T. chantrieri*

gave the highest level of phenolic compounds and flavonoid compounds of all the other fractions. The series of solvents has an effect on the total phenolic compounds and total flavonoid compounds. Our study found the same data as (Wang, et al. 2016), that the moderate polarity of the solvent (EtOAc) showed the ability to gather higher amounts of phenolic compounds and flavonoid compounds than other fractions. The EtOH extract also gave the highest levels of anti-oxidant activity and anti-oxidant compounds because of the EtOAc being fractionated from EtOH. The remaining anti-oxidant activity and anti-oxidant compounds were also found in EtOAc fraction.

Phenolic compounds contain anti-oxidant effects such as flavonoid, phenolic and tannin (Liu and Malta, 2014, Yang, et al., 2014). Phenolic compounds act as an anti-oxidant which scavenges oxidants such as peroxyl (Liu and Malta, 2014). The phenolic compounds are a protective oxidative reaction (Basu, et al., 1999). It can be concluded that the total amount of phenolic compounds and total flavonoid compounds are related to the antioxidant activity. Phenolic compounds from plants have an effect on cytokine expression and an anti-aging effect on hair. Phenolic compounds and flavonoid compounds found in plants induce a strong increase in the proliferation of dermal papilla cells and increased hair-fiber length (Sun, et al., 2013). These are involved in inhibiting oxidative stress, inflammation, and the expression of TGF-β1 (Lin, et al., 2015). Therefore, it can be concluded that the anti-oxidant effect of *T. chantrieri* involves the phenolic and flavonoid compounds, as indicated in those previous studies.

In our study, the EtOAc fraction of *T. chantrieri* gave the highest activity against DPPH radicals and ABTS radicals. This fraction also gave the highest total phenolic compounds and total flavonoid compounds. Therefore, the EtOAc fraction of *T. chantrieri* was selected for determining the cytotoxicity on HFDCP using PrestoBlue cell viability assay for 24, 48 and 72h. The results showed that the EtOAc fraction of *T. chantrieri* was toxic to HFDPC when incubated for 24h, 48h and 72h. Therefore, the cytotoxicity testing in EtOH extract and H_2O fraction, which reported as a non-toxic solvent, used *in vitro* study for 24h and 48h, were evaluated. The EtOH extract and H_2O fraction of *T. chantrieri* were not toxic to HFDPC when incubated for 24h, 48h, but it was toxic to HFDPC when incubated for 72h. The EtOH extract of *T. chantrieri* significantly increased the percent of cells survival compared with the

control group when incubating with *T. chantrieri* for 24h and 48h. This is a similar result to the study of (Sparg, et al., 2004) which also showed that some saponin extracted from *T. chantrieri* did not show cell growth inhibitory activity.

Cytokines are a broad category of small proteins that are important in cell signaling. Many types of cytokine play important roles in hair growth control and in new hair growth, including epidermal growth factor (EGF), keratinocyte growth factor (KGF), insulin like growth factor-1 (IGF-1), interleukin-1 (IL-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) whereas the signaling protein related to aging is the transforming growth factor-α and the transforming growth factor-β (TGF-α and TGF-β). The HFDPC was stimulated by these cytokines which led to environmental change around the cells and affected hair growth (Danilenko, et al., 1996; Jindo, et al., 1994). Therefore, these genes were used to determine the mRNA level in HFDPC after being treated with extracts of *T. chantrieri*.

The *T. chantrieri* in the H_2O fraction and EtOH extract did not increase the expression of VEGF, KDR (VEGFR), IGF-1, IGF-1R, TGF-βR mRNA level except for TGF-β mRNA when β-actin was used as an internal control. These results showed that the *T. chantrieri* in the H_2O fraction and EtOH extract could not stimulate VEGF, KDR (VEGFR), IGF-1, IGF-1R, TGF-β and TGF-βR mRNA. It can be concluded that *T. chantrieri* shows the ability to scavenge free radicals and anti-oxidant components. The H_2O fraction and EtOH extract of *T. chantrieri* can modulate the growth of HFDPC but can not reduce the expression of the genes involves with HFDPC aging, such as TGF-β and TGF-βR mRNA. The H_2O fraction and EtOH extract of *T. chantrieri* could not induce the expression of the genes involved with HFDPC aging, such as VEGF, KDR (VEGFR), IGF-1, and IGF-1R mRNA.

***Centella asiatica* (*C. asiatica*)**

C. asiatica extract showed evidence of anti-inflammation, anti-oxidation, gene expression (Ruszymah, et al, 2012; Punturee, et al., 2005; Ramesh, et al., 2015). The antioxidation effect was found in *C. asiatica* extract. The anti-oxidant activity measured by DPPH and ABTS assay showed that the EtOAc fraction of *C. asiatica* gave the highest level of anti-oxidant activity among the other fractions. This study

gave the same data as Wang, et al., 2016 in which the EtOAc fraction of *Polygonum cuspidatum* was found to have an antioxidation affect *in vitro*, by gathering the substances that can dissolve in both polar and non polar polarities. Our experiment showed the antioxidation activity of each extract in both a polar solvent and an organic solvent. The polar solvent was represented by ABTS assay because water was used as the solvent. The organic solvent was represented by DPPH assay because methanol was used as the solvent. The assays investigated both strong radicals (ABTS assay) and not strong radical (DPPH assay). Many studies have suggested that plants have a pharmacodynamic potential as an antioxidant (Lin, et al., 2015). The antioxidant activity against DPPH radical and ABTS radical of *C. asiatica* showed as being dose dependent. The extract of *C. asiatica* showed the ability to scavenge both strong radicals similarly to not strong radicals. The concentration of *C. asiatica* extract at 200 μg/ml gave the highest level of anti-oxidant activity. The present study showed that in a higher concentration of the extract it will not dissolve in the design condition. Plant extract affected the growth of dermal papilla cells which was related to TGF-β that caused oxidation (Kamimura, A., et al., 2006). The *C. asiatica* extract could induce VEGF protein production (Punturee, et al., 2005). The anti-oxidant activity of EtOAc fraction gave the highest scavenging activity of the DPPH radicals and ABTS radicals. The anti-oxidant activity was also found in the EtOH extract of *C. asiatica*. Our study found that the *C. asiatica* extract gave high phenolic content and flavonoid content in EtOAc fraction and EtOH extract. The study of (Lin, et al., 2015) found that the active compound found in plants has biological effects such as anti aging, and is antioxidant with non cytotoxicity effects. Phenolic compounds and flavonoid compounds that are found in plants are active against many diseases (Chen, et al., 2014). The phenolic and flavonoid content in *C. asiatica* showed as being dose dependent. The EtOAc fraction and EtOH extract of *C. asiatica* gave a lower amount of total phenolic compounds and flavonoid compounds than *T. chantrieri* extract. Interestingly, the phenolic and flavonoid content in EtOAc fraction and EtOH extract of *C. asiatica* were highest among the other fractions. This series of solvents from non polarity up to high polarity has an effect on the total phenolic compounds and total flavonoid compounds. In the present study, the moderate polarity of the solvent showed the ability in gathering amounts of phenolic compounds and flavonoid compounds in the middle of the range

of polarity (Wang, et al. 2016). The EtOH extract was also found to have a higher level of anti-oxidant activities and anti-oxidant compounds than the EtOAc fraction, because the EtOAc was fractionated from EtOH. The remaining anti-oxidant activities and anti-oxidant compounds were also found in the EtOAc fraction as EtOH extract.

The phenolic compounds that show anti-oxidant activities are flavonoid, phenolic and tannin (Liu and Malta, 2014, Yang, et al., 2014). Phenolic compounds act as an anti-oxidant for scavenging oxidants such as peroxyl (Liu and Malta, 2014). The phenolic compounds protect against oxidative reaction (Basu, et.al., 1999). It can be concluded that the phenolic compounds and flavonoid compounds were related to the antioxidant activity. The phenolic compounds of plant have an effect on cytokine expression and the anti aging effect of HFDPC. Phenolic compounds and flavonoid compounds of plants induce the proliferation of HFDPC and increase the hair-fiber length (Sun, et al., 2013). Therefore, the anti-oxidant effect of *C. asiatica* involved with the phenolic compounds and flavonoid compounds was similar to that observed in the previous study.

The EtOAc fraction of *C. asiatica* gave the highest level of activity against DPPH radicals and ABTS radicals. This fraction contains the highest phenolic compounds and flavonoid compounds levels. The EtOAc fraction of *C. asiatica* was selected for determining the cytotoxicity on HFDPC using PrestoBlue cell viability assay for 24, 48 and 72h. The result showed that the EtOAc fraction of *C. asiatica* was toxic to HFDPC when incubated for 24h, 48h and 72h. The cytotoxicity was tested in EtOH extract and H_2O fraction, which are non-toxic solvents, using *in vitro* study for 24h and 48h. The EtOH extract and H_2O fraction of *C. asiatica* were not toxic to HFDPC when incubated for 24h, 48h, but it was toxic to HFDPC after being incubated with *C. asiatica* extracts for 72h. The EtOH extract and H_2O fraction of *C. asiatica* modulated the percent of cells survival when compared with the control group. *C. asiatica* extract has a potential effect on skin re-epithelialization; this extract could be a possible candidate for treating epithelial wounds (Ruszymah, et al., 2012).

Cytokines expressions such as VEGF, VEGFR, IGF-1, IGF-1R, TGF-β2, and TGF-β2R were designed for this study. The HFDPC stimulated by these cytokines led to environmental change around the cells and affected hair growth (Danilenko, et al.,

1996; Roh, et al., 2002; Jindo, et al., 1994). Therefore, these genes were used to determine the mRNA level in HFDPC after treatment with extracts of *C. asiatica*.

The *C. asiatica* in the H_2O fraction increased the expression of VEGF, KDR (VEGFR) mRNA level when β-actin was used as an internal control. The *C. asiatica* in H_2O fraction reduced the expression of TGF-β2 and TGF-β2R mRNA level. The TGF-β2 is especially express in HFDPC (Inoue, et al., 2009) and play importance role in inhibition of HFDPC proliferation (Philpott, et al., 1992). The inhibition of TGF-β2 signaling pathway could induce HFDPC proliferation. This study showed that the *C. asiatica* in H_2O fraction reduced the expression of TGF-β2 and TGF-β2R but increased the expression of VEGF and VEGFR. The result was similar to previous studies that the plant extracts showed ability to scavenge free radicals, had anti-oxidant components and induced the expression of VEGF and VEGFR (Roh, et al., 2002, Rho, et al., 2004). The expressions of VEGF and VEGFR cause angiogenesis in hair growth cycles (Kozlowska, et al., 1998). *C. asiatica* in H_2O fraction showed ability to scavenge free radicals, had anti-oxidant components, induced the expression the gene that involve with HFDPC proliferation, and reduced the expression the gene that involve with HFDPC aging. A drug, minoxidil, was similar to this study that showed the reduction of hair fall. It reduced the oxidative stress (Otomo, S., 2002; Messenger, et al., 2004).

The *C. asiatica* in EtOH extract increased the expression of VEGF, KDR (VEGFR) mRNA level when β-actin was used as an internal control. The *C. asiatica* in EtOH extract showed ability to scavenge free radical and had anti-oxidant component, and induced the expression of VEGF and VEGFR genes (Roh, et al., 2005; Rho, et al., 2004). The expressions of VEGF and VEGFR genes induced angiogenesis in hair growth cycle (Kozlowska, et al., 1998). The *C. asiatica* in EtOH extract showed ability to scavenge free radical, had the anti-oxidant compound, and induced the expression the gene that involve with HFDPC proliferation. The minoxidil effected to the HFDPC proliferation as same as our studied (Otomo, S., 2002; Messenger, et al., 2004). The *C. asiatica* in water fraction and EtOH extract showed the modulation of HFDPC as the minoxidil action.

***Eleutherin americana* (*E. americana*)**

The *E. americana* extract could protect cell against to nitric oxide synthase that involve with anti-oxidant activity (Song, S. H., et al., 2009) and its active compounds were isoeleutherin, eleutherinol that improve immune system (Hong, J. O., et al., 2008). Our study founded that the *E. americana* extract showed anti-oxidation effect. The anti-oxidant activities were measured by DPPH and ABTS methods. The *E. americana* in EtOAc fraction showed the highest anti-oxidant activities among the other fractions. This study was in accordance to the study of Wang, et al., 2016 that was EtOAc fraction of plant extract showed ability to antioxidant *in vitro*. The fraction of EtOAc was gathering the substances that can dissolve in both polar and non polar polarities. Our experiment showed the high anti-oxidant activities of *E. americana* in EtOAc fraction. The methods maintain both strong radical (ABTS method) and not strong radical (DPPH method). Many studies have suggested that plants and their extracted have a pharmacodynamics potential as an antioxidant (Lin, et al., 2015). The antioxidant activity against DPPH radicals and ABTS radicals of *E. americana* showed as being dose dependent. The extract of *E. americana* showed the ability to scavenge both strong radicals similarly to strong radicals. The concentration of *E. americana* extract at 200 μg/ml gave the highest level of anti-oxidant activity. The present study showed that in the concentration of the extract it will not dissolve in the design condition. The information of TGF-β showed the relationship to anti-oxidation activity and dermal papilla cell aging (Kamimura, A., et al., 2006). The *E. americana* in EtOAc fraction gave the highest level of anti-oxidant activities. The anti-oxidant activity also found in EtOH extract. The anti-oxidant activity of *E. americana* extracts were related to the high level of phenolic and flavonoid contents in the EtOAc fraction. An imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to detoxify the reactive oxygen species reflected to cell survival (Nawar, 1996). The activities in anti aging and any biological effects were found in plant compounds (Lin, et al., 2015). The phenolic compounds and flavonoid compounds have an activity in preventing many diseases (Chen, et al., 2014). The phenolic compounds and flavonoid compounds in *E. americana* showed the dose dependent. Interestingly, the *E. americana* in EtOAc fraction and EtOH extract gave higher amount of the phenolic compounds and flavonoid compounds than

the other fractions. This series of solvents from non polarity up to high polarity has an effect on the total phenolic compounds and total flavonoid compounds. In the present study, the moderate polarity of the solvent showed the ability in gathering amounts of phenolic compounds and flavonoid compounds in the middle of the range of polarity (Wang, et al., 2016). The EtOH extract was also found to have a higher level of anti-oxidant activities and anti-oxidant compounds than the EtOAc fraction, because the EtOAc was fractionated from EtOH. The remaining anti-oxidant activities and anti-oxidant compounds were also found in the EtOAc fraction as EtOH extract.

Phenolic compounds contain anti-oxidant effect such as flavonoid, phenolic and tannin (Liu and Malta, 2014, Yang, et al., 2014). Phenolic compound acts as an anti-oxidant for scavenging oxidant such as peroxyl (Liu and Malta, 2014). The anti-oxidant activities of *E. americana* were relate to the phenolic compounds and flavonoid compounds. Our study gave the same data as the previous study. The phenolic compounds protect oxidative reaction (Basu, et al., 1999). It can be concluded that the phenolic compounds and flavonoid compounds were related to the antioxidant activity. The phenolic compounds from plants have an effect to cytokine expression and anti aging of hair. The phenolic compounds and flavonoid compounds induced the proliferation of HFDPC and increased the hair-fiber length (Sun, et al., 2013). This information was involved with the inhibition of oxidative stress, inflammation, and the expression of TGF-β1 (Lin, et al., 2015).

Our study showed that the *E. americana* in EtOAc fraction gave the highest activity against DPPH radicals and ABTS radicals. This fraction gave the highest amount of phenolic compounds and flavonoid compounds. The *E. americana* in EtOAc fraction was selected for determining the cytotoxicity on HFDPC using PrestoBlue cell viability assay for 24, 48 and 72h. The EtOAc fraction of *E. americana* was toxic to HFDPC when incubated for 24h, 48h and 72h. Therefore, the cytotoxicity was tested in EtOH extract and H_2O fraction, which non-toxic solvent. The *E. americana* in EtOH extract and H_2O fraction were not toxic to HFDPC when incubated for 24h, 48h, but there were toxic to HFDPC when incubated for 72h. The *E. americana* in EtOH extract was significant increased percent of cells survival when compared with the control group. The cytotoxicity test of isoeleutherine, which was isolated from the bulb of *E. americana* was not harm to RAW 264.7 cell that gave by

MTT test (Song, et al., 2009). These results related to our study. This study the mRNA expression of HFDPC at 24h of incubation with *E. americana* by real time PCR was determined in EtOH extract and H_2O fraction.

Cytokines are small proteins that are important in cell signaling. Many types of cytokine play important role in a hair growth control or new hair growth including EGF, KGF, IGF-1, IL-1, bFGF, VEGF, and HGF whereas the signaling proteins which are related to aging are TGF-α and TGF-β. The HFDPC was stimulated by these cytokines led to environment change around the cells and affecting hair growth (Danilenko, et al., 1996; Jindo, et al., 1994). Therefore, the expression of these genes was used to determine the mRNA level in HFDPC after treatment with extracted of *E. americana.*

The *E. americana* in H_2O fraction was increase the expression of VEGF, KDR (VEGFR) mRNA level when β-actin was used as an internal control. The *E. americana* in H_2O fraction reduced the expression of TGF-β2R mRNA level. TGF-β2 and TGF-β2 receptor were specific express in HFDPC (Inoue, et al., 2009) and played importance role in inhibition of HFDPC proliferation (Philpott, et al., 1992). The inhibition of TGF-β2 signaling pathway may induce HFDPC proliferation. The results showed that the *E. americana* in H_2O fraction reduced the expression of TGF-β2R and increased the expression of VEGF and VEGFR. The result was similar to previous studies that the plant extracts in Korea showed ability to scavenge free radical, had anti-oxidant compounds, and induced the expression of VEGF and VEGFR (Roh, et al., 2005; Rho, et al., 2004). The expressions of VEGF and VEGFR genes cause angiogenesis in hair growth cycle (Kozlowska, et al., 1998). It can be concluded that *E. americana* in H_2O fraction showed ability to scavenge free radical, anti-oxidant component. The *E. americana* in H_2O fraction induced the expression the gene that involve with HFDPC proliferation and reduced the expression the gene that involve with HFDPC aging.

The *E. americana* in EtOH extract was increase the expression of VEGF and VEGFR mRNA levels but not TGF-β2 and TGF-β2R mRNA levels when β-actin was used as an internal control. The result was similar to previous studies that the plant extracts showed ability to scavenge free radical, had anti-oxidant compounds, and induced the expression of VEGF and VEGFR genes (Roh, et al., 2005; Rho, et al.,

2004). The expressions of VEGF and VEGFR genes caused angiogenesis in hair growth cycle (Kozlowska, et al., 1998). It can be concluded that the *E. americana* in H_2O fraction and the EtOH extract showed ability to scavenge free radical, had anti-oxidant compound, and induced the expression the genes that involve with HFDPC proliferation. The *E. americana* in H_2O fraction and the EtOH extract showed the same ability as minoxidil study, that could promote growth of the HFDPC (Otomo, S., 2002; Messenger, et al., 2004).

The effect of extracts on HFDPC

HFDPCs were isolated from the dermis of the scalp. The HFDPCs stained positive for alkaline phosphatase. They were then embedded in a laminin and collagen IV rich extracellular matrix at the base of the hair follicles. Laminin and collagen IV are essential for the induction and maintenance of hair growth and since HFDPCs have androgen receptors they can be used for *in vitro* screening of androgen blocking reagents.

The HFDPCs had 22.5h or more of doubling time (www.promocell.com). The growth of the HFDPC is caused by β-catenin signaling (Driskell, et al., 2011). β-catenin activity in the HFDPC regulates a number of other signaling pathways, including the phosphorelation of the downstream signaling such as FGF pathway (Driskell, et al., 2011), VEGF pathway (Lachgar, et al., 1999), VEGFR pathway (Li, et al., 2012), IGF pathway and its receptor (Kwack, 1999), and TGF-β pathway and its receptor (Botchkareva, et al., 2006). This information led to our study about the expression of the genes incubating the HFDPC with the extracts of *T. chantrieri*, *C. asiatica* and *E. americana*.

The real time PCR method was used in our study. This method has been validated in many previous scientific studies using a model an *in vivo* animal model and *in vitro* cell cultures which have shown that TGF-β was implicated in signaling in the low levels of the epidermal and hair cycles. The TGF-β increased HFDPC apoptosis of the anagen phase (Lin and Yang, 2013, Niimori, et al. 2012). In the anagen phase, the fold induction value of the VEGF mRNA expression can be increased up to 400 (Roh, et al., 2002). The fold induction value of $VEGF_{165}$ can be increased to 140 (Rho, et al., 2005) and IGF-1 can be increased up to 300 (Roh, et al.,

2002). Also, the fold induction of mRNA expression of FGF can be increased over 100 fold (He, et al., 2016). However, the expression of TGF-β in the anagen phase has not been found (Rho, et al., 2005; Roh, et al., 2002). The expression of paracrine factors in HFDPC by real time PCR occurred within 24h after administering 10 - 400 μg/ml of ginseng extracts (Park, et al., 2015). The real time PCR did not detect any mRNA expressions of HGF, or IGF-1 in HFDPC, but could be found by western blot analysis after being incubated with ginseng extracts. The dominant mRNA expression was found in VEGF or VEGFR genes when treated with some plant extracts (Rho, et al., 2005; Roh, et al., 2002). Both real time PCR and western blot analysis were used and the expression of VEGFR mRNA and proteins were detected in cultured HFDPC (Wu, et al. 2014). This information related to our study which found that the H_2O fractions of *C. asiatica* and *E. americana* showed the expression of VEGF and VEGFR mRNA. The involvement of plant extracts such as RK found in red raspberries increased HFDPC cytokine production and promoted hair growth in animal (*in vivo*) and cell culture experiments (*in vitro*) (Harada, et al. (2008). In the real time PCR study, Minoxidil could only induce the expression of VEGF genes (Rho, et al., 2005; Otamo, S., 2002) and sometimes it could induce the expression of the IGF-1 gene but this was not dominant (Roh, et al., 2002; Messenger, et al., 2004). Minoxidil co-incubated with retinoic acid could inhibit the apoptoic protein and TGF-β protein in dermal papilla cell (Kwon, et al., 2007).

The HFDPC was in a steady state within 24h and 48h when co-incubated with the extracts or alone (Rastegar, 2015). This was related to our study which showed no growth of HFDPC when the extracts were with HFDPCs more than 48h. Some study commented that the study of cell cycle of HFDPC should be normalized by Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) because the GAPDH was increased with metabolism of cell. Besides, the β-actin is a synthesis while cell have had differentiation (Rastegar, 2015). The cell had no differentiation; the cell stopped DNA synthesis and had no expression of β-actin mRNA. The β-actin slowed down the rate of an expression but the other genes still had the same rate of expression of expression of metabolic genes (Rastegar, 2015). In some plant extracts it was founded that the Glychyrrhizic acid (saponin) of plants increased the size of HFDPC and slowed DNA synthesis by Comet assay (Kiratipaiboon, et al., 2015). Adhirajan, et al.,

2003 studied *in vivo* and *in vitro* studied the effect of *Hibiscus rosa-sinensis* flower and its leaf also potential maintain of hair growth. The *Aconiti ciliare* tuber extract induced the size and maintenance stability of the HFDPC (Park, et al., 2012). By these previous studies, it can be concluded that the extracts of *C. asiatica* and *E. americana* showed potential to keep HFDPC in the anagen phase.

Our study discovered by DPPH and ABTS assays that the EtOH extracts of *T. chantrieri*, *C. asiatica* and *E. americana* demonstrated the ability to protect against radicals. Only the *C. asiatica* and *E. americana* in H_2O fractions showed the strong evidence of expression of mRNA genes such as, VEGF and KDR (VEGFR). The extracts showed only a slightly expression of IGF-1 and IGF-1R genes. The *C. asiatica* and *E. americana* in H_2O fractions of also showed ability to reduce the expression of TGF-β2 and TGF-β2R genes. The EtOH extract of *C. asiatica* and *E. americana* showed the ability to express of mRNA genes such as, VEGF and KDR (VEGFR) genes.

Suggestion for further study

The EtOAc fractions of *T. chantrieri*, *C. asiatica* and *E. americana* demonstrated the highest antioxidant activities, total phenolic compound, and total flavonoid compound. Further study should be conducted to determine why the EtOAc fractions of *T. chantrieri*, *C. asiatica* and *E. americana* had cytotoxicity to HFDPC. Also, the protein synthesis after mRNA expression should be observed. The other studies suggested that $VEGF_{165}$ showed specificity to mRNA expression in HFDPC, so $VEGF_{91}$ specificity in HFDPC should be compared. Extracts of *T. chantrieri*, *C. asiatica* and *E. americana* can be studied and applying to production of cosmetics.

Summary

The extracts and fractions of *T. chantrieri*, *C. asiatica*, and *E. americana* demonstrated the ability to scavenge DPPH radicals and ABTS radicals. The most effective extracts of those plants were mostly found in EtOAc fraction. The antioxidant compounds were also found in the EtOH extract and other fractions of extracts. The antioxidant compounds were found in EtOH extract and EtOAc fraction.

The extracts could modulate HFDPC growth and gene expression. The H_2O fraction and EtOH extract of the plants modulated the growth of HFDPC in 24h. The H_2O fractions and EtOH extracts incubated with HFDPC showed the ability to enhance gene expression of HFDPC in 24h.

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APPENDIX

APPENDIX A List of chemical and materials used in this study

Name of chemicals	Company
Absolute ethanol	E.Merck, Germany
Acetic acid	Sigma-Aldrich, USA
Acetone	E.Merck, Germany
Aluminium chloride	Sigma-Aldrich, USA
2,2'-azino-bis(3-ethylben-zthiazoline-6-sulphonic acid	Sigma-Aldrich, USA
β-actin	Sigma-Aldrich, USA
Calcium chloride	Sigma-Aldrich, USA
Cateachin	Sigma-Aldrich, USA
Chloroform	E.Merck, Germany
Dibasic sodium phosphate	Sigma-Aldrich, USA
Dichloromethane	Northern chemical, Thailand
Diethyl ether	RCI Labscan, Bangkok, Thailand
Dimethyl sulfoxide (DMSO)	E.Merck, Germany
2,2-diphenyl-1-picrylhydrazyl	Sigma-Aldrich, USA
Ethanol	E.Merck, Germany
Ehtylacetate	Northern chemical, Thailand
EDTA	Sigma-Aldrich, USA
Follicle Dermal Papilla Cell Growth Medium	Promocell, Thailand
Fetal bovine serum	Hyclone, USA
Hexane	Northern chemical, Thailand
Human Follicle Dermal Papilla	BioMed Diagnostic, Thailand
Hydrochloric acid	E.Merck, Germany
Gallic acid	Sigma-Aldrich, USA
l-Glutamine	E.Merck, Germany

Methanol	E.Merck, Germany
N,N,N,N-tetramethyl ethylene diamine	Sigma-Aldrich, USA
Penicillin-streptomycin	GIBCO, USA
Phosphotungstic acid	Sigma-Aldrich, USA
Potassium chloride	Sigma-Aldrich, USA
Potassium dihydrogen phosphate	Sigma-Aldrich, USA
Potassium persulfate	Sigma-Aldrich, USA
Potassium phosphate	Sigma-Aldrich, USA
Revertra Aca qPCR Master Mix	TOYOBO, Japan
RNase Wiper	Pacific Science, Thailand
Sodium bicarbonate	Sigma-Aldrich, USA
Sodium chloride	Sigma-Aldrich, USA
Sodium hydroxide	Sigma-Aldrich, USA
Sodium nitrite	Sigma-Aldrich, USA
Trichloroacetic acid (TCA)	Sigma-Aldrich, USA
Tris-base	Sigma-Aldrich, USA
Triton X-100	Sigma-Aldrich, USA

APPENDIX B List of instrument used in this study

Instrument	Company
ABI 7500 Real-Time PCR system	Applied Biosystems, USA 4
Analytical balance	METTLER TOLEDO ME5
Autoclave	Tomy autoclave SS-240
Automatic pipette	GIBCO, Gilson
Bio-Max MR film	Eastman, Kodak, USA
Carbondioxide incubator	Forma Scientific
Centrifugation	Eppendorf 5702R, Germany
Deionized water machine	Barnstead
Distilled water machine	Hamilton
Fluorescence microscope	Hollywood international Ltd., Bangkok, Thailand
Freezer (-80 °C)	Forma scientific
Freezer (-20 °C)	Sanyo
Glassware	Pyrex
Hot air oven	Haraeus
Laminar flow biological cabinet	AIR2000 Fembrook Lane Plymouth, MN55447
Light microscope	Olympia Tokyo
Liquid nitrogen tank	Taylor-wharton
Lyophilizer	Christ Alpha1-4
Magnetic stirrer	Sybron / Thermolyne
Mastercycler® PCR machine	EppendorfMastercycler gradient, Tokyo, Japan
Microcentrifuge, bench-topped	Clay
Microplate reader	MicroplateAutoreader EL311s

Instrument	Company
Pasture pipette	Pyrex
pH meter	Hanna Instruments 8417
Refrigerator	Sanyo, Hitachi
Shaker bath	Unitronic 320 OR
Sonicator	Sci Med
25 or 75 cm^3 T-flask	Nunc
6 or 24 or 24 well plate	Nunc
UV-visible spectrophotometer	UV-1700 pharmaspec SHIMADZU
Vortex	Scientific industries
Water bath	GFL 1083

APPENDIX C Preparation of some reagents and buffers

Phytochemical assay

1. Total flavonoid assay

1.1 Standard Catechin (Standard solution)

1.1.1 Catechin 1.00 mg dissolved in MeOH 1 ml (concentration 1.00 mg/ml)

1.1.2 for Standard Catechin Curve concentration 80, 40, 20 ,10, 5, 2.5 µg/ml (@ final concentration)

1.2 5% NaNO2 solution

$NaNO_2$ 5.0 gram dissolved in distilled water, adjust the volume to 100 ml with distilled water

1.3 10% AlCl3 .6H2O solution

AlCl3 .6H2O 10.0 gram dissolved in distilled water, adjust the volume to 100 ml with distilled water

1.4 1 M NaOH solution

NaOH 4.0 gram dissolved in distilled water, adjust the volume to 100 ml with distilled water

2. Total Phenolic assay

2.1 Standard Catechin (Standard solution)

2.1.1 Gallic acid 1.00 mg dissolved in EtOH 1 ml (concentration 1.00 mg/ml)

2.1.2 For Standard Catechin Curve (concentration 80, 40, 20 ,10, 5, 2.5 µg/ml) (@ final concentration)

2.2 7 % sodium bicarbonate (Na2CO3) solution

$NaNO_2$ 7.0 gram dissolved in distilled water, adjust the volume to 100 ml with distilled water

3. DPPH assay

0.2 mM of DPPH

0.0078 gram of DPPH dissolved in MeOH 1 ml

4. ABTS assay

4.1 7 mM of ABTS

0.0192 gram of ABTS dissolved in distilled water, adjust the volume to 1000 ml with distilled water

4.2 2.45 mM of potassium persulfate

0.0331 gram of potassium persulfate dissolved in distilled water, adjust the volume to 1000 ml with distilled water

Cell culture

1. Completed culture media

Follicle Dermal Papilla Cell Growth Medium	445	ml
Fetal bovine serum	50	ml
Penicillin-streptomycin	5	ml

Stored at 4°C

2. Freezing solution

Incomplete Follicle Dermal Papilla Cell Growth Medium	7	ml
Fetal bovine serum	2	ml
DMSO	1	ml

Stored at 4°C

3. Tris-EDTA (pH 7.4)

NaCl	8.6	g
KCl	0.38	g
Na2HPO4	3.7	g
Tris-base	3.02	g
EDTA	0.038	g
Deionize distilled water	800	mL

Adjust pH to 7.4 then add volume with deionized water to 1,000 ml and sterile by autoclave and suction filter (membrane pore size 0.2 μM)

4. 0.25% Trypsin

2.5% Trypsin	10	mL
Tris-EDTA	90	mL

Reverse Transcription mixture

Reagent	Final Concentration	
5X RT master mix	2.00	µl
RNA Template	1.00	µg
Nuclease-free water	x.00	µl
Total volume	**10.0**	**µl**

Real Time PCR

1. Reaction mixture

Reagent	Final Concentration	
SensiFAST™ SYBR® Lo-ROX master mix	10	µl
Forward Primer 10 µM	0.4	µl
Reverse Primer 10 µM	0.4	µl
Nuclease-free water	7.2	µl
cDNA dilution 1:5	2.0	µl
Total volume	**20**	**µl**

2. Real time PCR condition

The reaction was run by ABI 7500 Real-Time PCR systems with 2-step cycling the condition;

Pre-denaturation	95°C	2	min.	
Denaturation	95°C	15	sec.	
Annealing and extension	60°C	1	min.	for 40 cycles

3. VEGF primer

F (5′ to 3′) ATGACGAGGGCCTGGAGTGTG

R (5′ to 3′) CCTATGTGCTGGCCTTGGTGAG

4. KDR (VEGF receptor) primer

F (5′ to 3′) CTT CCA AGT GGC TAA GGG CA

R (5′ to 3′) GGC GAG CAT CTC CTT TTC TG

5. IGF-1 primer

F (5′ to 3′) CAG CAG TCT TCC AAC CCA AT

R (5′ to 3′) CAT GCA CTC CCT CTA CTT GC

6. IGF-1 receptor primer

F (5′ to 3′) AAT GAG TGC CAC CCC GA

R (5′ to 3′) ACA CAG CGC CAG CCC TCA AA

7. TGF-β primer

F (5′ to 3′) GTG GAA ACC CAC AAC GAA AT

R (5′ to 3′) CTA CCT CCA CCA TGC CAA GT

8. TGF- β receptor primer

F (5′ to 3′) AGC ATC ACG GCC ATC TGT G

R (5′ to 3′) TGG CAA ACC GTC TCC AGA GT

9. β–actin primer

F (5′ to 3′) CTTCCAGCCTTCCTTCCTGG

R (5′ to 3′) TTCTGCATCCTGTCGGCAAT

APPENDIX D Absorbance and figure of absorbance of phytochemical assay

1. Absorbance of DPPH assay and standard vitamin C

Table 16 The absorbance of *T. chantrieri*, *C. asiatica* and *E. americana* in each fraction

ABS 517	Hexane	CH_2Cl_2	EtOAc	EtOH	H_2O
T. chantrieri 0.00 µg/ml	0.736 ± 0.008	0.736 ± 0.008	0.736 ± 0.008	0.736 ± 0.008	0.736 ± 0.008
T. chantrieri 12.5 µg/ml	0.644 ± 0.000	0.611 ± 0.000	0.707 ± 0.000	0.617 ± 0.000	0.667 ± 0.000
T. chantrieri 25 µg/ml	0.636 ± 0.000	0.602 ± 0.000	0.680 ± 0.013	0.462 ± 0.007	0.640 ± 0.000
T. chantrieri 50 µg/ml	0.599 ± 0.000	0.562 ± 0.000	0.659 ± 0.024	0.296 ± 0.004	0.626 ± 0.000
T. chantrieri 100 µg/ml	0.541 ± 0.000	0.505 ± 0.000	0.608 ± 0.029	0.026 ± 0.001	0.513 ± 0.020
T. chantrieri 200 µg/ml	0.446 ± 0.000	0.428 ± 0.000	0.531 ± 0.031	0.018 ± 0.001	0.323 ± 0.009
ABS 517	**Hexane**	**CH_2Cl_2**	**EtOAc**	**EtOH**	**H_2O**
C. asiatica 0.00 µg/ml	0.736 ± 0.008	0.736 ± 0.008	0.736 ± 0.008	0.736 ± 0.008	0.736 ± 0.008
C. asiatica 12.5 µg/ml	0.623 ± 0.000	0.612 ± 0.000	0.704 ± 0.000	0.608 ± 0.000	0.743 ± 0.000
C. asiatica 25 µg/ml	0.628 ± 0.000	0.602 ± 0.000	0.638 ± 0.018	0.409 ± 0.012	0.726 ± 0.005
C. asiatica 50 µg/ml	0.611 ± 0.000	0.574 ± 0.000	0.567 ± 0.027	0.239 ± 0.030	0.704 ± 0.005
C. asiatica 100 µg/ml	0.586 ± 0.000	0.525 ± 0.000	0.425 ± 0.037	0.031 ± 0.001	0.684 ± 0.016
C. asiatica 200 µg/ml	0.536 ± 0.000	0.452 ± 0.000	0.221 ± 0.024	0.022 ± 0.003	0.655 ± 0.023

Table 16 (cont.)

ABS 517	Hexane	CH_2Cl_2	EtOAc	EtOH	H_2O
E. americana 0.00 µg/ml	0.736 ± 0.008	0.736 ± 0.008	0.736 ± 0.008	0.736 ± 0.008	0.736 ± 0.008
E. americana 12.5 µg/ml	0.630 ± 0.000	0.429 ± 0.000	0.414 ± 0.000	0.555 ± 0.000	0.662 ± 0.000
E. americana 25 µg/ml	0.551 ± 0.000	0.244 ± 0.000	0.703 ± 0.010	0.472 ± 0.051	0.656 ± 0.010
E. americana 50 µg/ml	0.501 ± 0.000	0.117 ± 0.000	0.698 ± 0.013	0.288 ± 0.014	0.655 ± 0.009
E. americana 100 µg/ml	0.405 ± 0.000	0.005 ± 0.000	0.673 ± 0.022	0.104 ± 0.006	0.555 ± 0.013
E. americana 200 µg/ml	0.257 ± 0.000	0.005 ± 0.000	0.638 ± 0.037	0.026 ± 0.004	0.463 ± 0.015

Table 17 The absorbance and percent inhibition of ascorbic acid (vitamin C) in various concentrations

Ascorbic acid	sample blank	A_{517}	sample-blank	% inhibition
0 ug/ml	0.047	**0.752**	0.705	
1.25 ug/ml	0.045	**0.683**	0.638	10
2.5 ug/ml	0.048	**0.632**	0.584	17
5 ug/ml	0.045	**0.502**	0.457	35
10 ug/ml	0.052	**0.198**	0.146	79
20 ug/ml	0.054	**0.066**	0.012	98

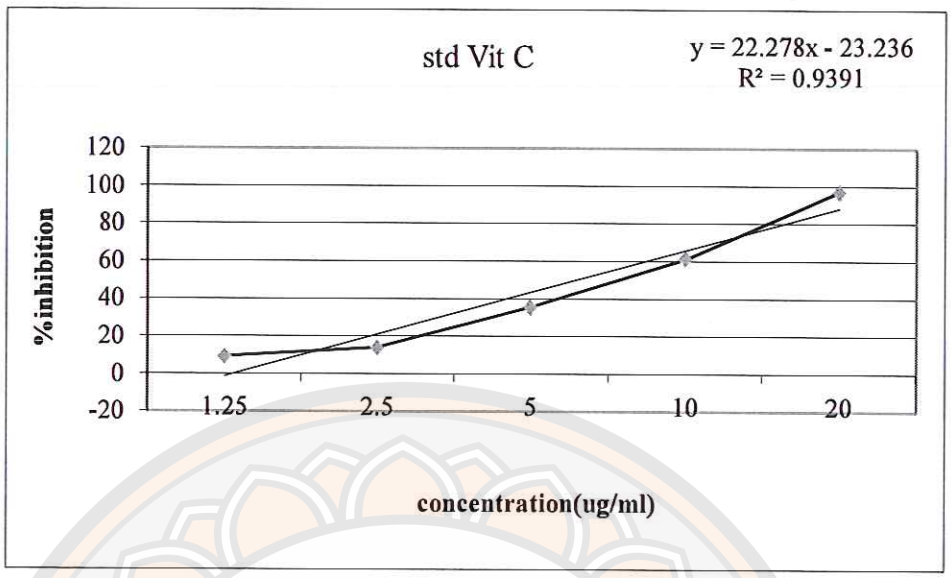

Figure 35 The standard curve of various concentrations of ascorbic acid and % inhibition

2. Absorbance of ABTS assay and standard

Table 18 The absorbance (mean ± SD) of *T. chantrieri*, *C. asiatica* and *E. americana* in each fraction using ABTS assay

ABS 735	Hexane	CH_2Cl_2	EtOAc	EtOH	H_2O
T. chantrieri 0.00 µg/ml	0.680 ± 0.056	0.680 ± 0.056	0.680 ± 0.056	0.680 ± 0.056	0.680 ± 0.056
T. chantrieri 12.5 µg/ml	0.657 ± 0.008	0.642 ± 0.008	0.231 ± 0.045	0.650 ± 0.016	0.749 ± 0.014
T. chantrieri 25 µg/ml	0.582 ± 0.024	0.515 ± 0.010	0.135 ± 0.098	0.556 ± 0.012	0.655 ± 0.012
T. chantrieri 50 µg/ml	0.452 ± 0.000	0.427 ± 0.028	0.115 ± 0.068	0.447 ± 0.029	0.547 ± 0.029
T. chantrieri 100 µg/ml	0.363 ± 0.035	0.333 ± 0.022	0.104 ± 0.052	0.366 ± 0.027	0.466 ± 0.027
T. chantrieri 200 µg/ml	0.128 ± 0.028	0.207 ± 0.003	0.086 ± 0.033	0.247 ± 0.044	0.346 ± 0.042

Table 18 (cont.)

ABS 735	**Hexane**	**CH2Cl2**	**EtOAc**	**EtOH**	**H2O**
C. asiatica 0.00 µg/ml	0.680 ± 0.056	0.680 ± 0.056	0.680 ± 0.056	0.680 ± 0.056	0.680 ± 0.056
C. asiatica 12.5 µg/ml	0.714 ± 0.006	0.713 ± 0.017	0.640 ± 0.035	0.708 ± 0.090	0.674 ± 0.011
C. asiatica 25 µg/ml	0.649 ± 0.015	0.652 ± 0.029	0.539 ± 0.037	0.664 ± 0.007	0.633 ± 0.055
C. asiatica 50 µg/ml	0.629 ± 0.012	0.560 ± 0.022	0.449 ± 0.018	0.574 ± 0.063	0.609 ± 0.052
C. asiatica 100 µg/ml	0.534 ± 0.018	0.461 ± 0.021	0.349 ± 0.007	0.566 ± 0.048	0.576 ± 0.031
C. asiatica 200 µg/ml	0.476 ± 0.062	0.370 ± 0.023	0.249 ± 0.020	0.439 ± 0.019	0.529 ± 0.037
ABS 735	**Hexane**	**CH2Cl2**	**EtOAc**	**EtOH**	**H2O**
E. americana 0.00 µg/ml	0.680 ± 0.056	0.680 ± 0.056	0.680 ± 0.056	0.680 ± 0.056	0.680 ± 0.056
E. americana 12.5 µg/ml	0.646 ± 0.038	0.643 ± 0.008	0.521 ± 0.013	0.620 ± 0.042	0.669 ± 0.045
E. americana 25 µg/ml	0.554 ± 0.016	0.626 ± 0.014	0.427 ± 0.014	0.570 ± 0.052	0.626 ± 0.039
E. americana 50 µg/ml	0.448 ± 0.011	0.553 ± 0.040	0.338 ± 0.017	0.520 ± 0.067	0.594 ± 0.058
E. americana 100 µg/ml	0.334 ± 0.027	0.426 ± 0.020	0.231 ± 0.031	0.441 ± 0.061	0.511 ± 0.082
E. americana 200 µg/*ml*	0.220 ± 0.021	0.319 ± 0.026	0.175 ± 0.005	0.303 ± 0.052	0.436 ± 0.031

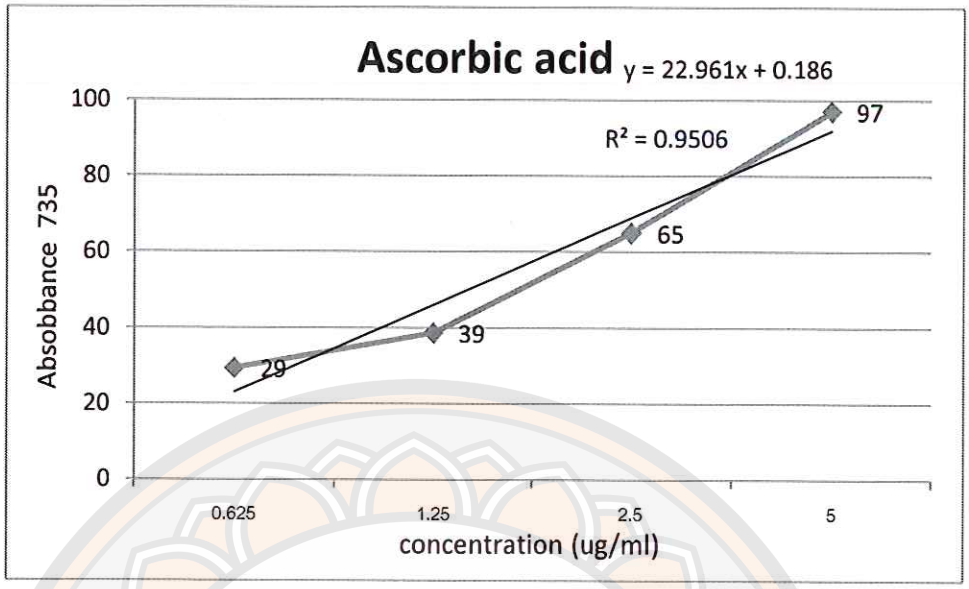

Figure 36 The standard curve of various concentrations of ascorbic acid and absorbance at 735 nm

3. Absorbance of phenolic assay and standard gallic acid

Table 19 Phenolic content in the *T. chantrieri* , *C. asiatica* and *E.americana* in each fraction measured at 731 nm (mean ± SD).

ABS 731	Hexane	CH_2Cl_2	EtOAc	EtOH	H_2O
T. chantrieri 0.00 µg/ml	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T. chantrieri 12.5 µg/ml	0.00 ± 0.00	0.02 ± 0.01	0.12 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
T. chantrieri 25 µg/ml	0.02 ± 0.02	0.04 ± 0.02	0.23 ± 0.00	0.06 ± 0.00	0.02 ± 0.00
T. chantrieri 50 µg/ml	0.04 ± 0.00	0.05 ± 0.00	0.45 ± 0.04	0.11 ± 0.00	0.05 ± 0.00
T. chantrieri 100 µg/ml	0.51 ± 0.38	0.10 ± 0.01	0.89 ± 0.03	0.21 ± 0.00	0.10 ± 0.00
T. chantrieri 200 µg/ml	0.14 ± 0.00	0.18 ± 0.02	1.39 ± 0.07	0.40 ± 0.02	0.18 ± 0.01
ABS 731	**Hexane**	**CH_2Cl_2**	**EtOAc**	**EtOH**	**H_2O**
C. asiatica 0.00 µg/ml	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. asiatica 12.5 µg/ml	0.01 ± 0.00	0.02 ± 0.02	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. asiatica 25 µg/ml	0.01 ± 0.00	0.04 ± 0.02	0.03 ± 0.01	0.01 ± 0.00	0.00 ± 0.00
C. asiatica 50 µg/ml	0.03 ± 0.01	0.06 ± 0.01	0.05 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C. asiatica 100 µg/ml	0.06 ± 0.00	0.10 ± 0.01	0.12 ± 0.03	0.03 ± 0.00	0.01 ± 0.00
C. asiatica 200 µg/ml	0.13 ± 0.01	0.18 ± 0.01	0.18 ± 0.02	0.04 ± 0.00	0.03 ± 0.00

Table 19 (cont.)

ABS 731	Hexane	CH_2Cl_2	EtOAc	EtOH	H_2O
E. americana 0.00 µg/ml	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
E. americana 12.5 µg/ml	0.02 ± 0.00	0.04 ± 0.01	0.09 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
E. americana 25 µg/ml	0.03 ± 0.00	0.07 ± 0.01	0.17 ± 0.00	0.05 ± 0.00	0.01 ± 0.00
E. americana 50 µg/ml	0.05 ± 0.00	0.13 ± 0.01	0.33 ± 0.02	0.09 ± 0.00	0.03 ± 0.00
E. americana 100 µg/ml	0.11 ± 0.01	0.24 ± 0.01	0.62 ± 0.04	0.16 ± 0.00	0.06 ± 0.00
E. americana 200 µg/ml	0.20 ± 0.01	0.48 ± 0.02	1.00 ± 0.01	0.30 ± 0.01	0.11 ± 0.01

Table 20 The absorbance (A_{731}) of various concentrations of gallic acid

Sample	OD 731	OD 731	OD 731	Average
blank water	0.038	0.043	0.040	0.040
gallic acid 2.5 µg/ml	0.155	0.145	0.151	0.150
gallic acid 5 µg/ml	0.26	0.249	0.254	0.254
gallic acid 10 µg/ml	0.446	0.451	0.453	0.450
gallic acid 20 µg/ml	0.853	0.851	0.846	0.850
gallic acid 40 µg/ml	1.598	1.592	1.589	1.593

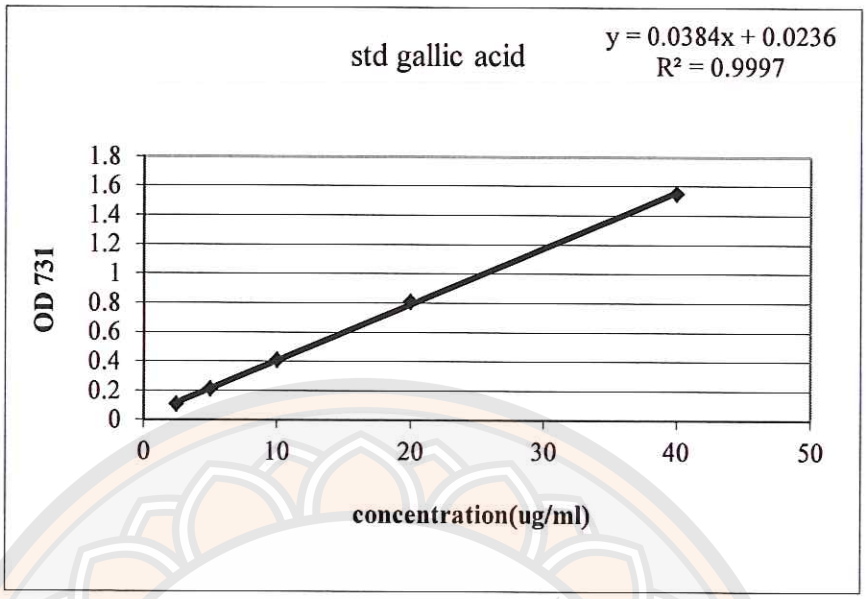

Figure 37 Standard curve of various concentrations of gallic acid and absorbance at 731 nm

4. Absorbance of total flavonoid assay and standard catechin

Table 21 A_{510} of Flavonoids content (mean ±SD) in *Tacca chantrieri* Ander in each fraction

A510	Hexane	CH_2Cl_2	EtOAc	EtOH	H_2O
T. chantrieri 0.00 µg/ml	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T. chantrieri 12.5 µg/ml	0.01 ± 0.00	0.00 ± 0.00	0.05 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
T. chantrieri 25 µg/ml	0.01 ± 0.00	0.01 ± 0.00	0.09 ± 0.01	0.02 ± 0.00	0.03 ± 0.03
T. chantrieri 50 µg/ml	0.02 ± 0.00	0.01 ± 0.00	0.18 ± 0.01	0.04 ± 0.00	0.02 ± 0.00
T. chantrieri 100 µg/ml	0.04 ± 0.00	0.01 ± 0.04	0.35 ± 0.01	0.28 ± 0.06	0.03 ± 0.00
T. chantrieri 200 µg/ml	0.07 ± 0.00	0.05 ± 0.00	0.70 ± 0.01	0.14 ± 0.00	0.06 ± 0.00

Table 21 (cont.)

A510	Hexane	CH_2Cl_2	EtOAc	EtOH	H_2O
C. asiatica 0.00 µg/ml	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. asiatica 12.5 µg/ml	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C. asiatica 25 µg/ml	0.03 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
C. asiatica 50 µg/ml	0.05 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
C. asiatica 100 µg/ml	0.07 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.00 ± 0.00
C. asiatica 200 µg/ml	0.08 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.05 ± 0.00	0.00 ± 0.00
A510	**Hexane**	**CH_2Cl_2**	**EtOAc**	**EtOH**	**H_2O**
E. americana 0.00 µg/ml	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
E. americana 12.5 µg/ml	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
E. americana 25 µg/ml	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
E. americana 50 µg/ml	0.02 ± 0.00	0.02 ± 0.00	0.09 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
E. americana 100 µg/ml	0.05 ± 0.00	0.04 ± 0.00	0.15 ± 0.03	0.03 ± 0.00	0.01 ± 0.00
E. americana 200 µg/ml	0.08 ± 0.00	0.08± 0.01	0.26 ± 0.01	0.05 ± 0.00	0.05 ± 0.07

Table 22 The absorbance (A_{510}) of various concentrations of catechin

Sample	OD 510	OD 510	OD 510	Average
blank	0.041	0.044	0.045	0.043
catechin 5 ug/ml	0.152	0.156	0.145	0.151
catechin 10 ug/ml	0.238	0.247	0.239	0.241
catechin 20 ug/ml	0.421	0.419	0.427	0.422
catechin 40 ug/ml	0.734	0.755	0.740	0.743
catechin 80 ug/ml	1.436	1.434	1.495	1.455

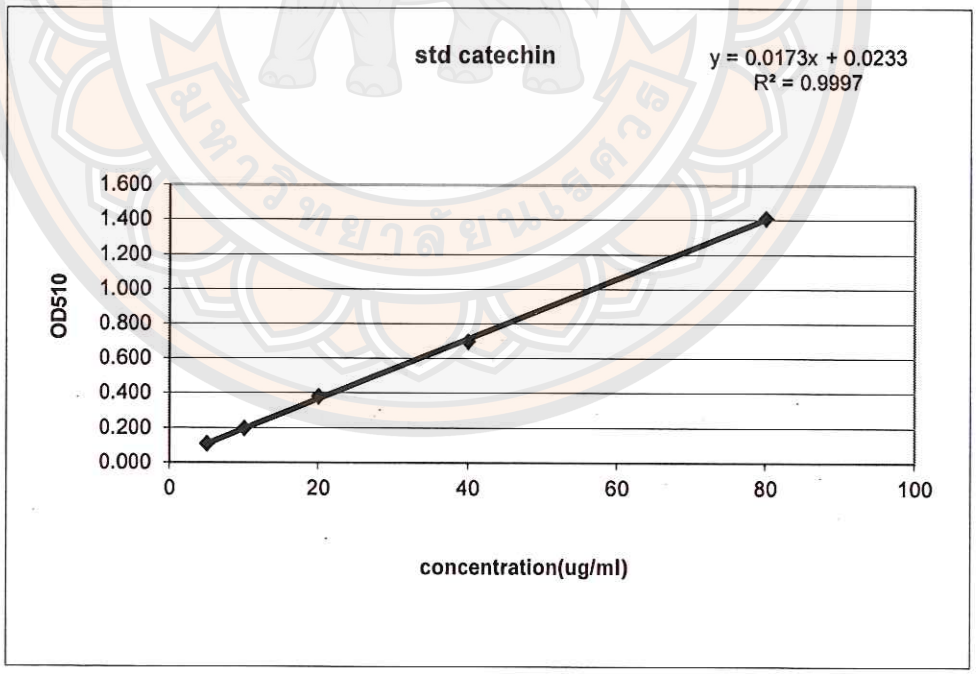

Figure 38 Standard curve of various concentration of catechin and absorbance at 510 nm

5. Cell viability testing by PrestoBlue assay

Table 23 The absorbance of *T. chantrieri* extract in EtOAc fraction on HFDPC using PrestoBlue cell viability assay for 24, 48 and 72h

Concentrations	24h	48h	72h
0 µg/ml	0.151±0.012	0.160±0.023	0.184±0.000
1.25 µg/ml	0.152±0.044	0.162±0.015	0.166±0.001
2.50 µg/ml	0.155±0.054	0.165±0.012	0.154±0.012
5.00 µg/ml	0.155±0.037	0.154±0.010	0.156±0.009
10.00 µg/ml	0.154±0.040	0.153±0.008	0.164±0.016

Table 24 The absorbance of *C. asiatica* extract in EtOAc fraction on HFDPC using PrestoBlue cell viability assay for 24, 48 and 72h

Concentration	24h	48h	72h
0 µg/ml	0.151±0.012	0.160±0.023	0.184±0.000
1.25 µg/ml	0.149±0.0160	0.159±0.020	0.184±0.049
2.50 µg/ml	0.152±0.024	0.159±0.023	0.177±0.000
5.00 µg/ml	0.148±0.012	0.164±0.015	0.179±0.001
10.00 µg/ml	0.153±0.015	0.161±0.015	0.1817±0.004

Table 25 The absorbance of *E. americana* extract in EtOAc fraction on HFDPC using PrestoBlue cell viability assay for 24, 48 and 72h

Concentrations	24h	48h	72h
0 µg/ml	0.151±0.012	0.160±0.023	0.184±0.000
1.25 µg/ml	0.152±0.021	0.162±0.011	0.1658±0.003
2.50 µg/ml	0.155±0.028	0.165±0.017	0.154±0.008
5.00 µg/ml	0.155±0.025	0.154±0.017	0.156±0.003
10.00 µg/ml	0.154±0.024	0.153±0.016	0.164±0.003

Table 26 The absorbance of *T. chantrieri*, *C. asiatica* and *E. americana* extract in H_2O fraction on HFDPC using PrestoBlue cell viability assay for 24h.

concentration ug/ml	*T. chantrieri* (24 h)	*C. asiatica* (24 h)	*E. Americana* (24h)
0	0.812 ± 0.012	0.812 ± 0.012	0.812 ± 0.012
62.5	0.820 ± 0.028	0.854 ± 0.025	0.820 ± 0.020
125	0.834 ± 0.017	0.826 ± 0.006	0.828 ± 0.015
250	0.834 ± 0.019	0.832 ± 0.031	0.821 ± 0.010
500	0.855 ± 0.004	0.834 ± 0.026	0.845 ± 0.024
1000	0.857 ± 0.030	0.808 ± 0.093	0.823 ± 0.028
std minoxidil 1 µg	0.575 ± 0.029	0.575 ± 0.029	0.575 ± 0.029

Table 27 The absorbance of *T. chantrieri*, *C. asiatica* and *E. americana* in H_2O fraction on HFDPC using PrestoBlue cell viability assay for 48h.

concentration ug/ml	*T. chantrieri* (48 h)	*C. asiatica* (48 h)	*E. americana* (48h)
0	0.917 ± 0.029	0.917 ± 0.029	0.917 ± 0.029
62.5	0.870 ± 0.014	0.937 ± 0.035	0.939 ± 0.036
125	0.858 ± 0.005	0.934 ± 0.015	0.919 ± 0.006
250	0.851 ± 0.003	0.920 ± 0.037	0.905 ± 0.026
500	0.854 ± 0.007	0.898 ± 0.038	0.871 ± 0.013
1000	0.866 ± 0.049	0.875 ± 0.037	0.891 ± 0.032
std minoxidil 1 µg	0.575 ± 0.029	0.575 ± 0.029	0.575 ± 0.029

Table 28 The absorbance of *T. chantrieri*, *C. asiatica* and *E. americana* extract in EtOH extract on HFDPC using PrestoBlue cell viability assay for 24h.

concentration ug/ml	*T. chantrieri* (24 h)	*C. asiatica* (24 h)	*E. Americana* (24h)
0	0.603 ± 0.012	0.603 ± 0.012	0.603 ± 0.012
62.5	0.550 ± 0.019	0.611 ± 0.002	0.594 ± 0.019
125	0.583 ± 0.019	0.612 ± 0.000	0.629 ± 0.012
250	0.624 ± 0.021	0.637 ± 0.014	0.685 ± 0.019
500	0.652 ± 0.017	0.663 ± 0.017	0.738 ± 0.005
1000	0.714 ± 0.030	0.660 ± 0.020	0.868 ± 0.010
std minoxidil 1 µg	0.575 ± 0.029	0.575 ± 0.029	0.575 ± 0.029

Table 29 The absorbance of *T. chantrieri*, *C. asiatica* and *E. americana* extract in EtOH extract on HFDPC using PrestoBlue cell viability assay for 48h.

concentration ug/ml	*T. chantrieri* (48 h)	*C. asiatica* (48 h)	*E. Americana* (48h)
0	0.602 ± 0.003	0.602 ± 0.003	0.602 ± 0.003
62.5	0.583 ± 0.024	0.610 ± 0.043	0.583 ± 0.050
125	0.635 ± 0.030	0.630 ± 0.018	0.635 ± 0.008
250	0.669 ± 0.004	0.640 ± 0.012	0.669 ± 0.006
500	0.743 ± 0.011	0.680 ±0.023	0.743 ± 0.010
1000	0.882 ± 0.012	0.724 ± 0.020	0.882 ± 0.005
std minoxidil 1 µg	0.575 ± 0.029	0.575 ± 0.029	0.575 ± 0.029

6. Gene expression by qPCR

6.1 Gene expression of *T. chantrieri* in H_2O fraction by qPCR

Table 30 Effect of *T. chantrieri* in H_2O fraction on VEGF expression using qPCR

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	VEGF	β-actin			
control	29.58	19.48	10.10	0.00	1.00
Minoxidil 1 µg	28.84	18.87	9.98	-0.13	1.09
500 µg/ml	29.08	36.52	-7.45	-17.55	ND
1000 µg/ml	30.10	37.78	-7.68	-17.78	ND

ND = Not detected

Table 31 Effect of *T. chantrieri* in H_2O fraction on VEGFR (KDR) expression using qPCR

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	KDR	β-actin			
control	31.02	19.48	11.54	0.00	1.00
Minoxidil 1 µg	33.05	18.87	14.18	2.65	ND
500 µg/ml	31.49	36.52	-5.03	-16.57	ND
1000 µg/ml	30.50	37.78	-7.28	-18.82	ND

ND = Not detected

Table 32 Effect of *T. chantrieri* in H_2O fraction on IGF-1 expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1	β-actin			
control	34.56	19.48	15.07	0.00	1.00
Minoxidil 1 µg	33.18	18.87	14.31	-0.77	1.70
500 µg/ml	38.16	36.52	1.64	-13.43	ND
(1000 µg/ml	31.20	37.78	-6.57	-21.65	ND

ND = Not detected

Table 33 Effect of *T. chantrieri* in H_2O fraction on IGF-1R expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1R	β-actin			
control	29.65	19.48	10.17	0.00	1.00
Minoxidil 1 μg	29.21	18.87	10.34	0.17	ND
500 μg/ml	37.60	36.52	1.08	9.09	ND
1000 μg/ml	36.56	37.78	-1.22	11.39	ND

ND = Not detected

Table 34 Effect of *T. chantrieri* in H_2O fraction on TGF-β expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β	β-actin			
control	24.70	19.48	5.22	0.00	1.00
Minoxidil 1 μg	24.05	18.87	5.19	-0.03	1.02
500 μg/ml	38.11	36.52	1.59	3.64	ND
1000 μg/ml	39.15	37.78	1.37	3.85	ND

ND = Not detected

Table 35 Effect of *T. chantrieri* in H_2O fraction on TGF-β2R expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β2R	β-actin			
control	32.06	19.48	12.57	0.00	1.00
Minoxidil 1 µg	32.38	18.87	13.52	0.94	ND
500 µg/ml	34.67	36.52	-1.85	14.42	ND
1000 µg/ml	37.15	37.78	-0.63	13.20	ND

ND = Not detected

6.2 Gene expression of *T. chantrieri* in EtOH extract by qPCR

Table 36 Effect of *T. chantrieri* in EtOH extract on VEGF expression using qPCR

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	VEGF	β-actin			
control	26.68	16.44	10.24	0.00	1.00
Minoxidil 1 µg	27.78	16.56	11.21	0.99	ND
500 µg/ml	32.40	29.96	2.44	-7.80	ND
1000 µg/ml	32.20	27.31	4.90	-5.34	ND

ND = Not detected

Table 37 Effect of *T. chantrieri* in EtOH extract on KDR expression using qPCR

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	KDR	β-actin			
control	31.02	16.44	14.58	0.00	1.00
Minoxidil 1 µg	31.84	16.56	15.28	0.70	ND
500 µg/ml	32.10	29.96	2.14	-12.45	ND
1000 µg/ml	31.61	27.31	4.30	-10.28	ND

ND = Not detected

Table 38 Effect of *T. chantrieri* in EtOH extract on IGF-1 expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1	β-actin			
control	26.05	16.44	9.61	0.00	1.00
Minoxidil 1 µg	28.67	16.56	12.11	2.50	ND
500 µg/ml	32.46	29.96	2.50	-7.11	ND
1000 µg/ml	31.33	27.31	4.02	-5.58	ND

ND = Not detected

Table 39 Effect of *T. chantrieri* in EtOH extract on IGF-1R expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1R	β-actin			
control	25.97	16.44	9.53	0.00	1.00
Minoxidil 1 µg	26.84	16.56	10.29	0.76	ND
500 µg/ml	31.27	29.96	1.31	-8.21	ND
1000 µg/ml	30.28	27.31	2.97	-6.55	ND

ND = Not detected

Table 40 Effect of *T. chantrieri* in EtOH extract on TGF-β expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β	β-actin			
control	22.30	16.44	5.86	0.00	1.00
Minoxidil 1 µg	22.22	16.56	5.66	-0.20	1.15
500 µg/ml	33.17	29.96	3.20	-2.65	ND
1000 µg/ml	31.19	27.31	3.88	-2.00	ND

ND = Not detected

Table 41 Effect of *T. chantrieri* in EtOH extract on TGF-β2R expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β2R	β-actin			
control	29.88	16.44	13.44	0.00	1.00
Minoxidil 1 µg	30.57	16.56	14.01	0.57	ND
500 µg/ml	33.32	29.96	3.37	-10.07	ND
1000 µg/ml	32.53	27.31	5.22	-8.22	ND

ND = Not detected

6.3 Gene expression of *C. asiatica* in H_2O fraction by qPCR

Table 42 Effect of *C. asiatica* in H_2O fraction on VEGF expression using qPCR

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	VEGF	β-actin			
control	28.90	19.18	9.72	0.00	1.00
Minoxidil 1 µg	28.25	18.39	9.86	0.14	ND
500 µg/ml	26.35	16.63	9.72	0.00	1.00
1000 µg/ml	25.29	15.85	9.45	0.27	ND

ND = Not detected

Table 43 Effect of *C. asiatica* in H_2O fraction on KDR expression using qPCR

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	KDR	β-actin			
control	31.11	19.20	11.93	0.00	1.00
Minoxidil 1 µg	35.18	18.39	16.79	4.86	ND
500 µg/ml	31.93	16.63	15.30	3.37	ND
1000 µg/ml	31.72	15.85	15.87	3.94	ND

ND = Not detected

Table 44 Effect of *C. asiatica* in H_2O fraction on IGF-1 expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1	β-actin			
Control	32.96	19.18	13.78	0.00	1.00
Minoxidil 1 µg	32.48	18.39	14.08	0.30	ND
500 µg/ml	30.82	16.63	14.19	0.41	ND
1000 µg/ml	30.76	15.85	14.92	1.13	ND

ND = Not detected

Table 45 Effect of *C. asiatica* in H_2O fraction on IGF-1R expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1R	β-actin			
control	29.58	19.18	10.40	0.00	1.00
Minoxidil 1 μg	29.00	18.39	10.62	0.22	ND
500 μg/ml	27.48	16.63	10.85	0.45	ND
1000 μg/ml	26.49	15.85	10.64	0.24	ND

ND = Not detected

Table 46 Effect of *C. asiatica* in H_2O fraction on TGF-β expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β	β-actin			
control	24.50	19.18	5.32	0.00	1.00
Minoxidil 1 μg	23.98	18.39	5.59	0.27	ND
500 μg/ml	21.80	16.63	5.17	-0.15	1.11
1000 μg/ml	21.21	15.85	5.36	-0.04	0.97

ND = Not detected

Table 47 Effect of *C. asiatica* in H_2O fraction on TGF-β2R expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β2R	β-actin			
control	32.39	19.18	13.21	0.00	1.00
Minoxidil 1 µg	31.62	18.39	13.23	0.02	ND
500 µg/ml	30.76	16.63	14.13	0.92	ND
1000 µg/ml	30.43	15.85	14.58	1.37	ND

6.4 Gene expression of *C. asiatica* in EtOHextract by qPCR

Table 48 Effect of *C. asiatica* in EtOH extract on VEGF expression using qPCR

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	VEGF	β-actin			
control	27.22	16.16	11.05	0.00	1.00
Minoxidil 1 µg	27.04	16.035	11.00	-0.05	1.04
500 µg/ml	32.74	28.32	4.42	-6.64	ND
1000 µg/ml	25.87	19.27	6.60	-4.45	ND

ND = Not detected

Table 49 Effect of *C. asiatica* in EtOH extract onKDR expression using qPCR

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	KDR	β-actin			
control	29.47	16.16	13.31	0.00	1.00
Minoxidil 1 μg	29.30	16.03	13.27	-0.04	1.03
500 μg/ml	36.83	28.32	8.51	-4.80	ND
1000 μg/ml	31.36	19.27	12.09	-1.22	ND

ND = Not detected

Table 50 Effect of *C. asiatica* in EtOH extract on IGF-1 expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1	β-actin			
control	26.07	16.16	9.91	0.00	1.00
Minoxidil 1 μg	30.25	16.03	14.22	4.31	ND
500 μg/ml	37.11	28.32	8.79	-1.12	ND
1000 μg/ml	29.55	19.27	10.28	0.37	ND

ND = Not detected

Table 51 Effect of *C. asiatica* in EtOH extract on IGF-1R expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1R	β-actin			
control	25.93	16.16	9.77	0.00	1.00
Minoxidil 1 μg	26.48	16.04	10.45	0.68	ND
500 μg/ml	37.58	19.27	9.26	-0.52	ND
1000 μg/ml	28.99	28.32	9.72	-0.05	1.04

ND = Not detected

Table 52 Effect of *C. asiatica* in EtOH extract on TGF-β expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β	β-actin			
control	22.12	16.16	5.96	0.00	1.00
Minoxidil 1 μg	21.85	16.04	5.81	-0.15	1.11
500 μg/ml	23.56	19.27	4.29	-1.67	3.19
1000 μg/ml	33.65	28.32	5.33	-0.63	ND

ND = Not detected

Table 53 Effect of *C. asiatica* in EtOH extract on TGF-β2R expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β2R	β-actin			
control	29.61	16.16	13.45	0.00	1.00
Minoxidil 1 µg	30.07	16.03	14.03	0.58	ND
500 µg/ml	33.84	19.27	14.57	1.12	ND
1000 µg/ml	33.67	28.32	5.35	-8.10	ND

ND = Not detected

6.5 Gene expression of *E. americana* in H_2O fraction by qPCR

Table 54 Effect of *E. americana* in H_2O fraction on VEGF expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	VEGF	β-actin			
control	29.42	19.14	10.28	0.00	1.00
Minoxidil 1 µg	28.59	18.31	10.27	-0.01	1.00
500 µg/ml	25.67	17.02	8.65	-1.62	3.08
1000 µg/ml	24.44	17.45	6.99	-3.28	9.73

ND = Not detected

Table 55 Effect of *E. americana* in H_2O fraction on KDR expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	KDR	β-actin			
control	31.30	19.14	12.16	0.00	1.00
Minoxidil 1 µg	32.42	18.31	14.10	1.94	ND
500 µg/ml	31.49	17.02	14.47	2.31	ND
1000 µg/ml	33.00	17.45	15.56	3.39	ND

ND = Not detected

Table 56 Effect of *E. americana* in H_2O fraction on IGF-1 expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1	β-actin			
control	29.36	19.14	10.22	0.00	1.00
Minoxidil 1 µg	32.55	18.31	14.24	4.01	ND
500 µg/ml	31.66	17.02	14.64	4.41	ND
1000 µg/ml	31.82	17.45	14.37	4.14	ND

ND = Not detected

Table 57 Effect of *E. americana* in H_2O fraction on IGF-1R expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1R	β-actin			
control	29.30	19.14	10.16	0.00	1.00
Minoxidil 1 μg	28.65	18.31	10.34	0.18	ND
500 μg/ml	26.83	17.02	9.81	-0.34	1.27
1000 μg/ml	26.59	17.45	9.14	-1.02	2.02

ND = Not detected

Table 58 Effect of *E. americana* in H_2O fraction on TGF-β expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β	β-actin			
control	24.19	19.14	5.06	0.00	1.00
Minoxidil 1 μg	23.54	18.31	5.23	0.17	ND
500 μg/ml	21.68	17.02	4.66	-0.39	1.31
1000 μg/ml	21.41	17.45	3.96	-1.09	2.13

ND = Not detected

Table 59 Effect of *E. americana* in H_2O fraction on TGF-β2R expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β2R	β-actin			
control	30.17	19.14	11.03	0.00	1.00
Minoxidil 1 µg	31.35	18.31	13.04	2.01	ND
500 µg/ml	30.36	17.02	13.34	2.31	ND
1000 µg/ml	29.95	17.45	12.50	1.47	ND

ND = Not detected

6.6 Gene expression of *E. americana* in EtOH extract by qPCR

Table 60 Effect of *E. americana* in EtOH extract on VEGF expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	VEGF	β-actin			
control	27.46	16.06	11.40	0.00	1.00
Minoxidil 1 µg	27.72	16.40	11.32	-0.08	1.06
500 µg/ml	30.77	21.83	8.93	-2.47	5.54
1000 µg/ml	30.25	20.21	10.04	-1.36	2.56

ND = Not detected

Table 61 Effect of *E. americana* in EtOH extract on KDR expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	KDR	β-actin			
Control	30.16	16.06	14.40	0.00	1.00
Minoxidil 1 µg	32.73	16.40	16.33	2.24	ND
500 µg/ml	31.20	21.84	9.36	-4.74	ND
1000 µg/ml	34.34	20.21	14.13	-0.27	ND

ND = Not detected

Table 62 Effect of *E. americana* in EtOH extract on IGF-1 expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1	β-actin			
control	30.39	16.06	14.32	0.00	1.00
Minoxidil 1 µg	30.96	16.40	14.56	0.23	ND
500 µg/ml	30.30	21.84	8.46	-5.87	58.32
1000 µg/ml	32.85	20.21	12.64	-1.69	3.22

ND = Not detected

Table 63 Effect of *E. americana* in EtOH extract on IGF-1R expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	IGF-1R	β-actin			
control	25.80	16.06	9.74	0.00	1.00
Minoxidil 1 µg	26.66	16.40	10.26	0.52	ND
500 µg/ml	29.39	21.84	7.55	-2.18	4.54
1000 µg/ml	29.78	20.21	9.57	-0.17	1.12

ND = Not detected

Table 64 Effect of *E. americana* in EtOH extract on TGF-β expression using qPCR.

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β	β-actin			
control	21.86	16.06	5.80	0.00	1.00
Minoxidil 1 µg	22.10	16.40	5.70	-0.11	1.08
500 µg/ml	26.28	21.84	4.44	-1.36	2.58
1000 µg/ml	25.28	20.21	5.07	-0.74	1.67

ND = Not detected

Table 65 Effect of *E. americana* in EtOH extract on TGF-β2R expression using qPCR.3

Treatment	CT		ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
	TGF-β2R	β-actin			
control	29.20	16.06	13.14	0.00	1.00
Minoxidil 1 µg	29.77	16.40	13.37	0.23	ND
500 µg/ml	31.03	21.84	9.19	-3.94	15.41
1000 µg/ml	31.57	20.21	11.36	-1.78	3.43

ND = Not detected

GLOSSORY

1 The anagen phase is a growth phase of hair. It begins in the papilla and can last from two to six years (approximately 1,000 days).

2 The catagen phase or the transitional phase of hair, allows the follicle to renew itself. During this phase, this lasts about 10 days.

3 The telogen or resting phase of hair, the follicle remains dormant anywhere from 100 days.

4 Vascular endothelial growth factor (VEGF) is a cytokine that involved with hair growth.

5 Vascular endothelial growth factor receptor (VEGFR) or kinase insert domain receptor (KDR) is a VEGF receptor that involved with hair growth.

6 Insulin-like growth factor-1 (IGF-1) is a cytokine that involved with hair growth.

7 Insulin-like growth factor-1 receptor (IGF-1R) is a IGF-1 receptor that involved with hair growth.

8 Transforming growth factor-β (TGF-β) is a cytokine that involved with hair reduction or transitioning phase of hair growth.

9 Transforming growth factor-β receptor (TGF-βR) is a TGF-β receptor that involved with hair reduction or transitioning phase of hair growth.

10 Androgenetic is a genetic which involved with androgen hormone. It causes alopecia.

11 Apoptosis is a program cell death.

12 Melanocyte is a cell which contain melanin or pigment

13 Basic fibroblast growth factor (bFGF) is a cytokine that involved with hair growth.

14 Hepatocyte growth factor (HGF) is a cytokine that involved with hair growth.

15 5α-reductase is an enzyme that convert testosterone hormone to dihydrotertosterone