Enhanced VEGF Expression in Hair Follicle Dermal Papilla Cells by *Centella asiatica* Linn.

Pahol Saansoomchai[1], Apinun Limmongkon[1], Damratsamon Surangkul[1], Teera Chewonarin[2] and Metawee Srikummool[1*]

[1]*Department of Biochemistry, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand*
[2]*Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*

**Corresponding author. E-mail: metaweesr@nu.ac.th*

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ABSTRACT

Centella asiatica Linn. (C. asiatica) extract has been shown to possess high antioxidant activity due to its phenols and flavonoids. This study tested the efficacy of 70%-ethanol (EtOH) crude extracts of C. asiatica and its fractions (H_2O, EtOAc, CH_2Cl_2, and hexane) to modulate human follicle dermal papilla cells. In addition, we analyzed the extracts for major phytochemicals as well as free radical scavenging activity. Our results from ABTS and DPPH assays showed that the amounts of phenolic and flavonoid compounds in the extracts were both related to its free radical scavenging activity. While the EtOAc fraction of C. asiatica demonstrated the highest free radical scavenging activity, it was toxic to human follicle dermal papilla cells. The cell viability test was positive when cells were treated with EtOH crude extract and H_2O fraction. VEGF gene expression, quantified by real-time PCR analysis of the EtOH crude extract, showed a significant level of induction, indicating that the growth promotion effect in human follicle dermal papilla cells was related to VEGF gene expression, which has a positive hair growth stimulating effect. The EtOH crude extract of C. asiatica may offer potential in hair growth promoting products.

Keywords: Antioxidant activities, *Centella asiatica*, Phytochemical screening, Real-time PCR, Gene expression

INTRODUCTION

Hair protects the scalp from the environment, including heat, cold, and UV radiation, and serves as a measure of beauty. As its loss can result in distress and psychological problems, prevention or treatment strategies need to be investigated. So far, only two drugs, minoxidil and finasteride, have been approved for the treatment of hair loss in men by the US Food and Drug Administration (Park et al., 2012).

Hair follicles of any hair type have a unique life cycle comprised of three main stages – anagen, catagen, and telogen, each of which leads to the destruction and regeneration of hair follicles over a lifetime. The regulation of the hair cycle is complicated and involves several

factors (Hibino and Nishiyama, 2004) that are not well understood. Genetic factors, cytokine imbalance, and oxidative stress can cause abnormal hair follicle cycling and subsequent hair loss (Rho et al., 2005; Aron et al., 2013). Many cytokines and receptors are involved in the cell cycle of human follicle dermal papilla cells, including the vascular endothelial cell growth factor (VEGF) (Shin et al., 2014), vascular endothelial cell growth factor receptor (VEGFR) (Li et al., 2012), fibroblast growth factor (FGF) (Rho et al., 2005), insulin-like growth factor (IGF) (Panchaprateep and Asawanonda, 2014), epidermal growth factor (EGF) (Bressan et al., 2014), keratinocyte growth factor (KGF) (Gopu et al., 2015), and transforming growth factor (TGF) (Kang et al., 2013; Shin et al., 2014). Some hair regeneration has been achieved by molecular effect and growth factors (Danilenko et al., 1996) and follicle dermal stem cells (Rahmani et al., 2014). Some evidence has suggested that VEGF and VEGFR could induce the proliferation of human follicle dermal papilla cells through ERK activation (Li et al., 2012). TGF has been related to human follicle dermal papilla cell death involving free radicals (Soma et al., 2003; Rho et al., 2005).

Medicinal plants, including *Centella asiatica* Linn. (*C. asiatica*), are natural sources of bioactive compounds that possess health-promoting effects. *C. asiatica* has been used to treat a range of ailments, including the common cold (Roy et al., 2013). In Thailand, its fresh leaves have been used to treat wounds and burns and its extract has been used to reduce swelling and infection. *C. asiatica* extract is widely available in Thailand and cost effective (Taemchuay et al., 2009). Many scientific studies have researched traditional applications of *C. asiatica* extract (Bylka et al., 2014; Hashim, 2014). *C. asiatica* extract contains several bioactive compounds, including saponins, essential oils, flavone derivatives, sesquiterpenes, triterpenic acid, and triterpenic steroids (Roy et al., 2013). It also has been reported to contain bioactive compounds, such as terpenes, flavonoids, and polyphenols, that are related to its potent antioxidative activities (Hashim et al., 2011; Nurlaily et al., 2012; Orhan et al., 2013). Extracts from *C. asiatica* leaves consist of gallic acid and ferulic acid, which have antioxidant and anti-inflammatory effects (Ramesh et al., 2014). Another study has shown that *C. asiatica* extracts exhibited antioxidant activity and UV protection effects (Hashim et al., 2011). Many beauty products are currently available that incorporate *C. asiatica* extracts, such as cosmetic creams, hand and body lotions, eye gel, and face mask products (Bylka et al., 2014). A previous study found that *C. asiatica* extract enlarged hair follicles (Jain and Dass, 2015) and inhibited the activity of 5α-reductase that causes hair loss (Jain et al., 2016). However, few hair care or restoration products contain the extract, as its effect on the hair root remains unclear; the molecular mechanisms involved in plant extracts modulating gene expression are not well understood.

The *in vitro* treatment of human follicle dermal papilla cells could, possibly, provide a gateway to hair regeneration and sustainably protect against hair loss. The objective of this study was to search for any potential effect, especially involving antioxidant activity, of *C. asiatica* extract on growth and molecular regulation in human follicle dermal papilla cells. Positive findings would indicate the potential for developing accessible and affordable value added hair growth promoting products using ingredients extracted from natural sources rather than synthetic drugs.

MATERIALS AND METHODS

Plant material

A *C. asiatica* plant was collected from Chiang Rai, Thailand and positively identified by the taxonomist of Nareasuan University. The leaves were cleaned and dried in an oven at 40°C, then stored at -20°C until use.

Preparing the ethanol crude extract

One kilogram of dried samples were ground into powder and macerated in 4 L of 70% (v/v) ethanol (EtOH) for 24 h at room temperature. The extraction was performed twice under the same conditions. Chlorophyll was removed using the charcoal absorption method with some modification (Limtrakul et al., 2004). Briefly, each extract was bleached with 160 g of activated charcoal. The chlorophyll-free extract was then filtered through Whatman's No.1 filter paper and the solvent was removed using a vacuum rotary evaporator (Buchi, Switzerland) at room temperature. The concentrated aqueous portion was lyophilized (Christ Alpha1-4 LD, UK) into a powder and further partitioned using four different solvents: hexane, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and water (H_2O). The EtOH crude extract and four fractions with the highest antioxidant activity were then used in subsequent experiments.

Evaluating the free radical scavenging activity of the crude extract

Two methods measured the free radical scavenging activity of *C. asiatica* crude extract: 1) a DPPH inhibition assay following the method of Padmanabhan and Jangle (2012) and 2) an ABTS inhibition assay as described by Gorjanović et al. (2012). With treatments of various concentrations of the extract, the decrease in absorbance was measured at 517 nm for the DPPH assay and 735 nm for the ABTS assay; the % inhibition and IC_{50} value were also reported.

Measuring total phenolic and flavonoid contents of the crude extract

Total phenolic content was determined using the Folin-Ciocalteu method. Quantification was expressed as milligrams of gallic acid equivalent per gram of extract (mg GE/g of ext) (Saikia et al., 2012). The total flavonoid content (TF) was measured by aluminium chloride colorimetric assay and expressed in milligrams of catechin equivalent per gram of extract (mg CE/g of ext) (Saikia et al., 2012).

Human follicle dermal papilla cell cultures and cell viability testing

Human follicle dermal papilla cells cultures were obtained from PromoCell, Germany. The cells were cultured and maintained in Follicle Dermal Papilla Cell Growth Medium (PromoCell, Germany) at 37°C in 5% (v/v) CO_2. Cytotoxicity of the extract of the human follicle dermal papilla cells was performed using the Presto-blue (Invitrogen, USA) assay according to the PrestoBlue™ cell viability reagent protocol. Briefly, 2×10^3 of the human follicle dermal papilla cells were seeded into a 96-well, flat-bottomed, microliter plate and cultured for 24 h. A 100-µl sample of *C. asiatica* extract at different concentrations was

added to each well and the cells were further cultured for 24 h for one group of cells, and 48 h for another. Then, 20 µl of Presto-blue solution was added to each well and the cells were incubated for 20 min. The *C. asiatica* extracts were compared to 1% standard minoxidil (Sigma-Aldrich, USA) as the control. The absorbance was measured at 570 nm. The effective time of incubation to human follicle dermal papilla cells was used for studying the mRNA expression.

Detecting VEGF mRNA expression in *C. asiatica*–treated human follicle dermal papilla cells

The human follicle dermal papilla cells were treated with the indicated concentrations of *C. asiatica* extract for 24 h. Total RNA was isolated using RNAZol® RT (Molecular Research Center Inc., USA) according to the manufacturer's protocol. RNA quality was assessed by RNA/Protein sample PCR amplification of cDNA performed in a RevertraAce®qPCR RT Master Mix (Toyobo, Japan). cDNA was obtained from 2 µg/ml of RNA by one cycle of reverse transcription. Gene expression was quantified using real-time PCR (RT-PCR). Targeted genes and the details are presented in Table 1. The PCR cycle steps consisted of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and a final extension step at 72°C for 1 min within 40 cycles. Each gene expression was calculated according to the threshold cycle (C^T) value, normalized using the value of sample with the lowest level for each product, and the data were corrected according to the level of β-actin.

Table 1. Sequences of gene specific primers used in RT-PCR.

Genes	Sequences	Size	Reference
VEGF	forward 5'- ATGACGAGGGCCTGGAGTGTG -3' reverse 5'- CCTATGTGCTGGCCTTGGTGAG -3'	91	Soulitzis et al., 2006
β-actin	forward, 5'- CTTCCAGCCTTCCTTCCTGG -3' reverse, 5'- TTCTGCATCCTGTCGGCAAT -3'	162	Soulitzis et al., 2006

To verify the RT-PCR, PCR products were analyzed by electrophoresis in 2% agarose gels. They were then stained with ethidium bromide and photographed on a UV light transilluminator. PCR product length for VEGF growth factor was analyzed, as well as β-actin.

Statistical analysis

Each experiment was performed in triplicate. All values were presented as a mean value (Mean ± SD). The statistically significant differences between the means of the samples were calculated by one-way ANOVA and the differences were considered significant at a level of $p<0.05$ (*).

RESULTS

Antioxidant activity of *C. asiatica* extracts

The antioxidant activity of the *C. asiatica* extract was measured by its ability to scavenge DPPH and ABTS radicals.

Figure 1 shows the DPPH (A) and ABTS (B) free radical scavenging assays. The free radical scavenging activity of *C. asiatica* extract showed the highest activity in the EtOH crude extract for DPPH assay and the highest activity in the EtOAc fraction for ABTS assay.

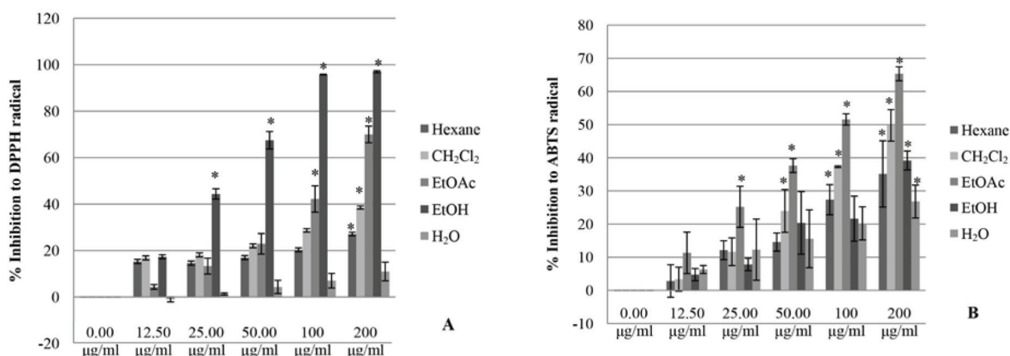

Figure 1. The free radical scavenging activities of *C. asiatica* extracts.

Note: *The differences were considered significant at $p<0.05$.

Table 2. IC_{50} of the *C. asiatica* extracts against DPPH and ABTS radicals.

Fraction	IC_{50} of *C. asiatica* extract (µg/ml)	
	DPPH assay	ABTS assay
Hexane	>200	>200
CH_2Cl_2	>200	173.20 ± 3.47
EtOAc	134.76 ± 12.09	122.22 ± 7.49
EtOH	34.63 ± 0.76	>200
H_2O	>200	>200
Ascorbic acid	13.95 ± 0.01	-
Trolox	-	6.41 ± 0.03

Note: The presence of IC_{50} of *C. asiatica* and standards against DPPH and ABTS radicals are presented as mean ± SD.

As shown in Figure 1 and Table 2, the free radical scavenging activity of *C. asiatica* extract at 0-200 µg/ml was found to scavenge the DPPH radicals and ABTS radicals in a dose dependent manner when compared with the positive control, ascorbic acid and Trolox.

The EtOH crude extract of *C. asiatic*a at a concentration of 200 µg/ml displayed the highest inhibitory effect; it inhibited DPPH radicals at 97.01 ± 0.42% of the ascorbic acid and also inhibited IC_{50} at 34.63 ± 0.76 µg/ml. CH_2Cl_2, hexane, and H_2O fractions also displayed inhibitory effects at the same concentration levels of IC_{50} (>200 ug/ml).

C. asiatica extract in the EtOAc fraction displayed the highest inhibitory effect on ABTS radicals at a concentration of 200 µg/ml, inhibiting 65.33 ± 2.09% of Trolox with IC_{50} at 122.22 ± 7.49 µg/ml. The CH_2Cl_2 fraction inhibited at IC_{50} of 173.20± 3.47 µg/ml. EtOH crude extract, hexane fraction, and H_2O fraction had the same concentration levels (>200 ug/ml).

The two active fractions, the EtOH crude extract and EtOAc fraction, have been linked to solvent polarity that can extract different fractions of polar/nonpolar constituents from the plant. This finding agreed well with the total phenolic and flavonoid contents of each fraction, as shown in Table 3.

Total phenolic and flavonoid content in *C. asiatica* extracts

The amount of phenols and flavonoids contained in *C. asiatica* extracts are shown in Table 3. The EtOAc fraction contained the most phenolic compounds, at 19.72 ± 0.02 mg GE/g of extract, closely followed by the CH_2Cl_2 fraction. The EtOAc fraction contained the most flavonoids, at 6.79 ± 0.12 mg CE/g of extract, with all other fractions containing only small or trace amounts.

Table 3. Total phenolic (TP) and flavonoid (TF) content of *C. asiatica* extracts.

C. asiatica	Hexane	CH_2Cl_2	EtOAc	EtOH	H_2O
TP (mg GE/g of ext)	7.71 ± 0.01	17.04 ± 0.01	19.72 ± 0.02	2.68 ± 0.00	0.13 ± 0.00
TF (mg CE/g of ext)	1.10 ± 0.64	1.51 ± 0.36	6.79 ± 0.12	0.54 ± 0.14	0.00 ± 0.00

Note: The present chemical components, including TP and TF, are presented as mean ± SD.

Cytotoxicity of the *C. asiatica* extracts

The cytotoxicity of the *C. asiatica* extract from EtOH crude extract and each fraction were further examined by treating human follicle dermal papilla cells with the extract at different concentrations. The EtOH crude extract and H_2O fraction were evaluated for cytotoxic effects on human follicle dermal papilla cells at different doses up to 1,000 µg/ml extract for 24 h and 48 h (Figure 2). The cell viability was 100% or more compared with the non-treated cells (0 µg/ml extract). The *C. asiatica* extract at concentrations of 500 µg/ml and 1,000 µg/ml slightly induced cell proliferation. From this, it can be concluded that the EtOH crude extract and H_2O fraction of *C. asiatica* did not show any toxicity to the human follicle dermal papilla cells, and in this regard were as effective as 1 µg of minoxidil (control). No statistical differences were found between the viability of the cells treated with minoxidil,

and the viability of the cells in the control. Unfortunately, the screening data revealed that the EtOAc fraction, which possessed the greatest antioxidant activity, was toxic to human follicle dermal papilla cells (data not show). Therefore, we did not use this fraction for further study. We then focused on the extract from the safer solvents – EtOH and H_2O.

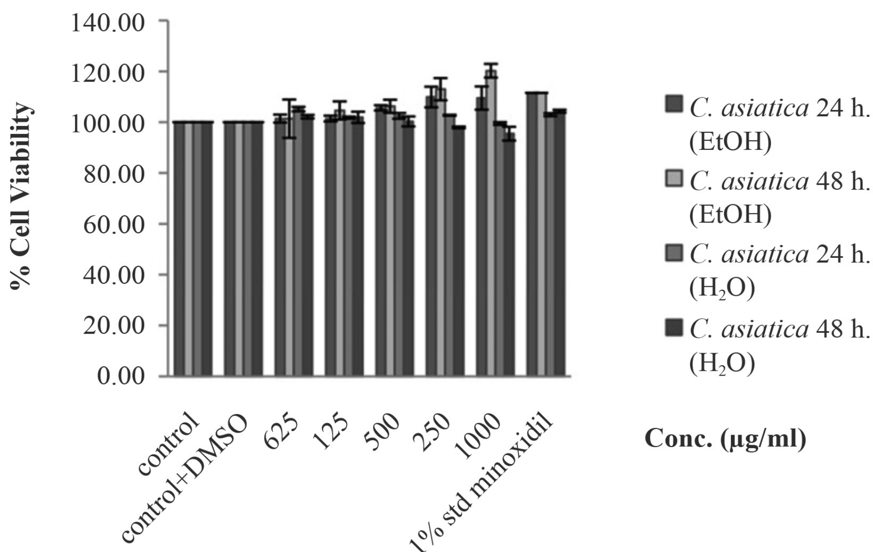

Figure 2. Cell viability of EtOH crude extract and H_2O fraction of *C. asiatica* extract at 24 h and 48 h. The EtOH crude extract and H_2O fraction did not display cytotoxicity to human follicle dermal papilla cells.

Note: *The differences were considered significant at $p<0.05$.

VEGF gene expression

The EtOH crude extract and H_2O fraction (the safer solvents) of *C. asiatica* at concentrations of 500 µg/ml and 1,000 µg/ml were used for a gene expressivity assay by RT-PCR. As shown in Figure 3, both concentrations of EtOH crude extracts of C. asiatica induced VEGF gene expression ($p<0.05$), with 500 µg/ml the most at 37.30 ± 9.47. This far exceeded the slightly induced VEGF gene expression of minoxidil (1.99 ± 0.07); the H_2O fraction did not induce gene expression (data not shown).

The size of the PCR products corresponded to the data shown in Table 1. The band of VEGF growth factor was presented after incubating the *C. asiatica* extract with human follicle dermal papilla cells. *C. asiatica* extract at the concentration of 500 µg/ml showed a more intense band than the *C. asiatica* extract at the concentration 1,000 µg/ml. Similarly to the β-actin, these concentrations showed the same result as VEGF growth factor (data not show). The PCR products coresponded to RT-PCR.

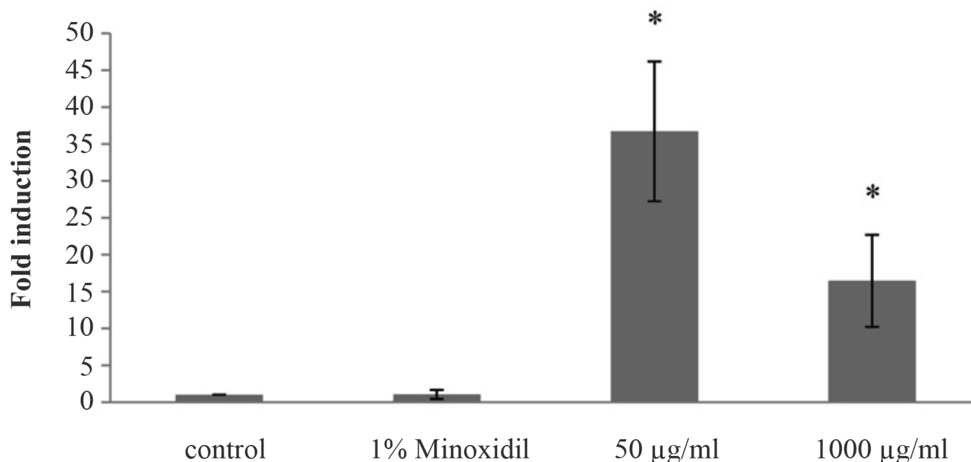

Figure 3. VEGF expression of human follicle dermal papilla cells by the induction of EtOH crude extract of *C. asiatica*.

Note: *The differences were considered significant at $p<0.05$.

DISCUSSION

ABTS and DPPH assays determined the anti-oxidative activity of the 70%-EtOH extract of *C. asiatica* and its partition fractions (hexane, CH_2Cl_2, EtOAc, and H_2O fractions). The different fractions inhibited the ABTS and DPPH radicals differently. The EtOH crude extract showed the strongest inhibitory effect on DPPH radicals, while the EtOAc fraction had the strongest inhibitory effect on the ABTS radicals. These results indicated that the free radical scavenging activities correlated to the phenolic and flavonoid content in the extract. Moreover, the inhibitory effect of both the EtOH crude extract and the EtOAc fraction depended on not only their phytochemical ingredients, but also the solvents used to generate the radicals. Water was used as the solvent in the ABTS assay, representing the polar solvent borne radicals (Gorjanović et al., 2012), while methanol used as the solvent in the DPPH assay, representing the organic solvent borne radicals (Padmanabhan and Jangle, 2012). Rahman et al. (2013) and Shalaby and Shanab (2013) found that the free radical scavenging activity of an extract related to the polarity of the solvent, which occurs because the antioxidant molecules engage in strong interactions with free radicals.

The results showed that the free radical scavenging activity of the *C. asiatica* extracts related to the variety of chemicals in the phenols and flavonoids, which included many lipophilic phytochemicals or hydrophilic phytochemicals. Many studies have reported levels and activities of phenols and flavonoids using a chlorophyll-free extraction method (Limtrakul et al., 2004; Paula et al., 2012). In our study, the *C. asiatica* extracts and fractions showed total phenols ranging from 0.13-19.72 mg GE/g of extract. This result agreed with Frederico et al. (2009) for different parts of *C. asiatica*. The flavonoids levels in the *C. asiatica* extracts were

high, up to 6.79 mg CE/g extract. The EtOAc fraction showed the highest amount of phenols and flavonoids. This was consistent with previous reports that indicated that the moderate polarity of a solvent, such as EtOAc, yields more phenols and flavonoids than other solvents (Wang et al., 2016) and that the polarity of a solvent affects the amount of each (Rahman et al., 2013).

The cytotoxicity tests showed that the EtOAc fraction harmed human follicle dermal papilla cells. Natural glycosides were extracted from the plant by the low polarity of the solvent, causing the harm. Podolak et al. (2010) also described the haemolytic activity and cytotoxicity to cells of these natural glycosides.

RT-PCR analysis tested the stimulating effect of treating cells with EtOH crude extract of *C. asiatica*, observed as the expression of VEGF mRNA. The EtOH crude extract of *C. asiatica* induced VEGF expression in human follicle dermal papilla cells, possibly leading to cell proliferation. Our results agreed with other studies of plant extracts (such as *Asiasari radix* and *Panax ginseng*) in enhancing the expression of VEGF (Rho et al., 2005; Shin et al., 2014). Other reports showed that the proliferation of human follicle dermal papilla cells was involved with cytokines signaling (Soma et al., 2003; Rho et al., 2005). β-catenin causes the signaling of human follicle dermal papilla cell proliferation (Driskell et al., 2011). The β-catenin activity in the human follicle dermal papilla cells regulates a number of other signaling pathways, including the phosphorelation of downstream signalling, such as the VEGF pathway, that stimulates cell proliferation (Lachgar et al., 1999; Driskell et al., 2011). VEGF also plays an important role in angiogenesis in follicle dermal papilla cells (Yano et al., 2001).

Previously, minoxidil was reported to have a concentration-dependent, biphasic effect on proliferation and differentiation, as well as on growth stimulation in low doses, and to be an anti-proliferative through the expression of cytokines (Kwon et al., 2007). In solution at less than 5%, Minoxidil can safely be applied to the human scalp; this equals a concentration of 1 mM (Han et al., 2004). Our results showed that minoxidil in solution stimulated the expression of VEGF, corresponding to Lachgar et al. (1998) and Li et al. (2001). The higher VEGF expression after treatment with the EtOH crude extract of *C. asiatica* more efficiently promoted human follicle dermal papilla cells than minoxidil.

CONCLUSION

While many reports have studied *C. asiatica* extracts, few have looked at its effects on human follicle dermal papilla cells and the molecular mechanisms that promote the proliferation of the cells. This study focused on gene expression after incubating the cells with the *C. asiatica* extract. The EtOH crude extract of *C. asiatica* induced the expression of VEGF mRNA in human follicle dermal papilla cells. Moreover, the phenols and flavonoids found in the *C. asiatica* extracts demonstrated antioxidant activity that could maintain the growth of human follicle dermal papilla cells. This study has clearly indicated that the EtOH crude extract of *C. asiatica* will be of benefit in the development of hair care products and hair loss therapy.

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