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Effect of *Orthosiphon stamineus* plant extract on *in vitro* dermal papilla cell proliferation and *ex vivo* hair growth

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Background : *The study of Orthosiphon stamineus (OSE) on hair growth is still limited. The potential use of OSE as hair growth promotion in both human dermal papilla cells and androgenic alopecia (AGA) patient hair follicle model may be used to develop a novel hair promoting products derived from Thai natural resources.*

Objective : *The objective of this study was to investigate the effect of Orthosiphon stamineus plant extract on in vitro dermal papilla cell proliferation and ex vivo hair growth.*

Methods : *Dermal papilla cell lines were cultured with medium and OSE at concentration 25 - 250 μg/mL for 24 hr for acute cytotoxicity evaluation and 5 days to detect proliferation. In ex vivo hair follicle culture, 45 hair samples from three AGA patients were cultured for 14 days in medium and OSE at 125, 250,500 μg/mL. Hair length was measured every two days and the results were compared with vehicle treated control and positive control.*

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Results : *OSE at concentration 25 - 250 μg/mL showed no acute cytotoxicity to cell after 24 hours culture. Furthermore, OSE at all concentrations could stimulate dermal papilla cell proliferation when cultured for 5 days, (P <0.05). Also, OSE at 500 μg/mL could enhance hair follicles of androgenic alopecia patients in vitro by 12% after 14 days of culturing which were more than vehicle (6.46%) (P <0.05). And based on area under the curve from day 0 – day 14, OSE at 500 μg/mL could enhance total hair growth significantly at 217% (P <0.05), compared to vehicle but OSE at lower concentration and positive control were not significantly different from vehicle.*

Conclusion : *OSE could significantly stimulate growth of human hair follicle of AGA patients ex vivo and promoted proliferation of the dermal papilla cells compared to vehicle treated control.*

Keywords : *Hair growth, Orthosiphon stamineus extract, Java tea, dermal papilla cell.*

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เหตุผลของการทำวิจัย : *การศึกษาผลของสารสกัดหญ้าหนวดแมวต่อการเจริญของเซลล์รากผมและเส้นผมในหลอดทดลอง อาจนำไปใช้ในการพัฒนาผลิตภัณฑ์บำรุงเส้นผมที่ได้จากสมุนไพรไทยทดแทนการนำเข้า และลดผลข้างเคียงจากการใช้ยาหรือสารเคมี*

วัตถุประสงค์ : *เพื่อศึกษาผลของสารสกัดหญ้าหนวดแมวต่อการเพิ่มจำนวนของเซลล์รากผมในหลอดทดลอง และการเจริญของเส้นผมแบบนอกกาย*

วิธีการวิจัย : *การทดสอบความเป็นพิษต่อเซลล์โดยการเลี้ยงเซลล์รากผมเพาะเลี้ยงในอาหารเลี้ยงเซลล์ และสารสกัดหญ้าหนวดแมวที่ระดับความเข้มข้น 25 - 250 μg/mL เป็นเวลา 24 ชั่วโมง และทำการเพาะเลี้ยงเป็นเวลา 5 วัน เพื่อศึกษาการเพิ่มจำนวนของเซลล์รากผมเพาะเลี้ยง หลังจากนั้นได้ทำการทดลองเลี้ยงปุ่มรากผมจำนวน 45 เส้น จากอาสาสมัคร 3 รายที่ได้รับการวินิจฉัยว่าเป็นโรคผมร่วงจากพันธุกรรม โดยทำการเพาะเลี้ยงในน้ำยาเลี้ยงเซลล์และสารสกัดหญ้าหนวดแมวที่ความเข้มข้น 125, 250, 500 μg/mL เป็นเวลา 14 วัน โดยวัดความยาวของเส้นผมทุก 2 วัน นำมาวิเคราะห์ความยาวและรูปร่างเปรียบเทียบกับกลุ่มควบคุมโดยใช้ ANOVA และ Dunnett's post hoc test*

ผลการวิจัย : *สารสกัดหญ้าหนวดแมวที่ค่าความเข้มข้น 25 - 250 μg/mL ไม่มีพิษต่อเซลล์รากผมเพาะเลี้ยง ภายหลังการเพาะเลี้ยงที่ 24 ชั่วโมง แต่มีฤทธิ์กระตุ้นการเพิ่มจำนวนของเซลล์รากผมได้อย่างมีนัยสำคัญได้ทุกความเข้มข้น (ร้อยละ 124 -139%) เมื่อเทียบกับตัวอย่างควบคุมภายหลังการเพาะเลี้ยงเป็นเวลา 5 วัน ($P < 0.05$) ส่วนการทดลองเลี้ยงปุ่มรากผมที่ได้จากอาสาสมัคร พบว่าสารสกัดหญ้าหนวดแมวที่ค่าความเข้มข้น 500 μg/mL สามารถเพิ่มความยาวของเส้นผมได้ร้อยละ 12 หลังจากการเพาะเลี้ยง 14 วัน ซึ่งมากกว่ากลุ่มควบคุมซึ่งมีความยาวเพิ่มขึ้นเพียงร้อยละ 6.5 ($P < 0.05$) และเมื่อเปรียบเทียบร้อยละของการเจริญของเส้นและปุ่มรากผมตลอดระยะเวลา 14 วันเทียบกับกลุ่มควบคุม โดยวิเคราะห์พื้นที่ใต้กราฟพบว่าสารสกัดหญ้าหนวดแมวที่ความเข้มข้น 500 μg/mL สามารถเพิ่มการเจริญของผม ได้ดีที่สุดอย่างมีนัยสำคัญ (ร้อยละ 217) เมื่อเทียบกับกลุ่มควบคุม ($P < 0.05$) ขณะที่สารสกัดในความเข้มข้นที่ต่ำกว่านี้ และ EGCG ที่ใช้เป็นสารมาตรฐานเปรียบเทียบไม่พบความแตกต่างจากกลุ่มควบคุม ($P > 0.05$)*

สรุป : *สารสกัดหญ้าหนวดแมวสามารถกระตุ้นการเจริญของเซลล์รากผมในหลอดทดลอง และเพิ่มความยาวของเส้นผมจากอาสาสมัครที่เป็นโรคผมร่วงจากพันธุกรรมได้อย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มตัวอย่างควบคุม*

คำสำคัญ : *การเจริญของเส้นผม, หญ้าหนวดแมว, เซลล์รากผม.*

Orthosiphon stamineus, commonly called Java tea (Thai: Ya Nuad Maew), is a widely distributed plant in Southeast Asia. Traditionally, it is used in folk medicine by preparing tea from the leaves as beverage to improve health and for treatment of kidney, bladder inflammation, gout and diabetes. *O.stamineus* contains several chemically active constituents including polyphenols such as sinensetin, eupatorin and rosmarinic acid. The extract using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) in vitro model system exhibited significant radical scavenging activity at 60 - 90% compared to quercetin and BHA at 0.05mg/ml. [1] There are several interests in *O.stamineus* health-related benefits but there is limited research in cosmetic applications.

Androgenic alopecia (AGA) is a major type of hair loss commonly found among males aged over 50 and females after menopause period. [2] In AGA patient, there is an over production of dihydrotestosterone (DHT) which results in the premature entry of the hair follicle into the catagen phase, thus shortens the hair growth cycle. The key 5α-reductase enzymes catalyze the conversion of testosterone to DHT. 5α-reductase enzyme type 1 is mainly present in the dermal papilla cell [2], which is a major regulator of hair growth cycle as it plays essential role in induction of new hair follicles and maintaining hair growth.

O. stamineus extract (OSE) was shown to inhibit 5α-reductase enzymes type 1 by 64% and possessed capabilities to reduce the oily appearance of facial skin. [3] However, there has been no research on the effect of *O. stamineus* on human hair growth. This study is aimed to investigate the effect of OSE on the proliferation of human dermal papilla cells and the elongation of cultured hair follicles obtained from AGA patients.

Materials and Methods

Materials

O. staminues leaf extract (OSE) was obtained from SNP Specialty Co., Ltd., Bangkok, Thailand, (500 mg% polyphenol). Epigallocatechin gallate (EGCG) was obtained from Merck (Thailand), Bangkok. All other reagents were purchased from Gibthai Co., Ltd., Bangkok, Thailand and used as received.

Cell proliferation assay

Culture of immortalized human hair dermal papilla cell line (Applied Biological Materials Inc., Richmond, BC, Canada) was a gift from Dr. Pithi Chanvorachote. The cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS) and 100 units/mL of penicillin/streptomycin (Life Technology, MD, USA) at 37°C in a 5% CO2 atmosphere. [4] The cells were seeded at a density of 1 $\times$ 105 cells per well in a 96-well plate and cultivated for 24 hours. [5] The cells were then treated with 100 μL of OSE (25, 50, 125 and 250 μg/mL dissolved in the medium with 1% DMSO) and with 1% DMSO as a blank control. The cells were cultured for 24 hours for viability (acute cytotoxicity) evaluation and 5 days to detect proliferation. [6] The tests were performed in triplicates. Cell viability was measured at 24 hours using PrestoBlue® reagent (Life Technologies) in RPMI medium. In the presence of viable cells, PrestoBlue® changes from a non-fluorescent blue color to a fluorescent purplish-pink color, which was detected by fluorospectrophotometer with the

excitation/emission of 535/615 nm (Beckman Coulter DTX880 Multimode Detector, Indianapolis, USA). Results were expressed as percentage of cell viability relative to untreated control. [4]

Hair follicle organ culture

Human hair follicle samples were donated by three male volunteers who were diagnosed as AGA by a dermatologist. Samples were taken from donor area at the back of the scalp using a special instrument, Follicular Unit Extraction (FUE). Therefore, single hair follicles were obtained with less tissue and unwanted fat. Then the samples were washed with PBS and cultured in a 24-well plate for 14 days in Williams E medium (Gibco BRL, Gaithersburg, USA), containing 10 ng/ml hydrocortisone, 10 mg/ml insulin, 2mM L-glutamine, 100 mg/ml streptomycin and 100 U/ml penicillin at 37°C in a 5% CO2 atmosphere. [7] OSE was added to culture media at 125, 250 and 500 μg/mL and EGCG at 2.3 μg/mL (5 μM) was used as a positive control. [6] Thus 5 treatments were compared in this study (3 concentrations of OSE plus EGCG and a non-treated control). The culture media and treatments were changed every other day. The total of 45 anagen-phase hair follicles from 3 different AGA male volunteers were cultured (15 follicles taken from each subject and then separated into 3 follicles for each of the 5 treatments). Each treatment was thus performed in triplicate for each subject (n = 3 follicles per treatment). The hair length was measured under invert light microscope supported with digital camera (US300, VezuTech), calibrated with micrometer. The results were first averaged for each subject and the values were then statistically analyzed among the three donors (n = 3 subjects). Statistics were performed using ANOVA followed by post hoc Dunnett test at 5% significance level.

Results

Cell proliferation assay

To study the effect of OSE on the growth of dermal papilla cells, we first characterized cell viability in response to OSE treatment after 24 hours - incubation using PrestoBlue®. The result confirmed that OSE at 25 – 250 μg/mL was not cytotoxic to the cells (Figure 1. White colored bar). Then the test was repeated but cultured for a longer period of 5 days. We found that human dermal papilla cells treated with OSE at all concentrations significantly proliferated over the untreated control (133 - 139% vs 100%) ($P < 0.05$, one-way ANOVA and Dunnett's Post hoc test) (Figure 1. Gray colored bar).

Hair follicle elongation study

The results of hair follicle elongation study are shown in Figure 2. OSE increased the AGA hair follicle elongation in a time-dependent manner. The hair follicles treated with OSE at 500 μg/mL grow significantly by 12% after 14 days of culturing which is more than vehicle (6.46%) ($P <0.05$, two-way repeated measure ANOVA and Dunnett's post hoc test).

To compare the overall effect of OSE versus control, we calculated the area under the curve (AUC) of % hair elongation as a function of time from day 0 to day 14 as shown in Figure 3. The graph shows that the hair follicle treated with OSE at 500 μg/mL gave the highest relative growth over 14 days (217%) which was significantly greater than the non-treated control ($P < 0.05$, one-way ANOVA and Dunnett test) whereas other treatments including EGCG did not show significant effect.

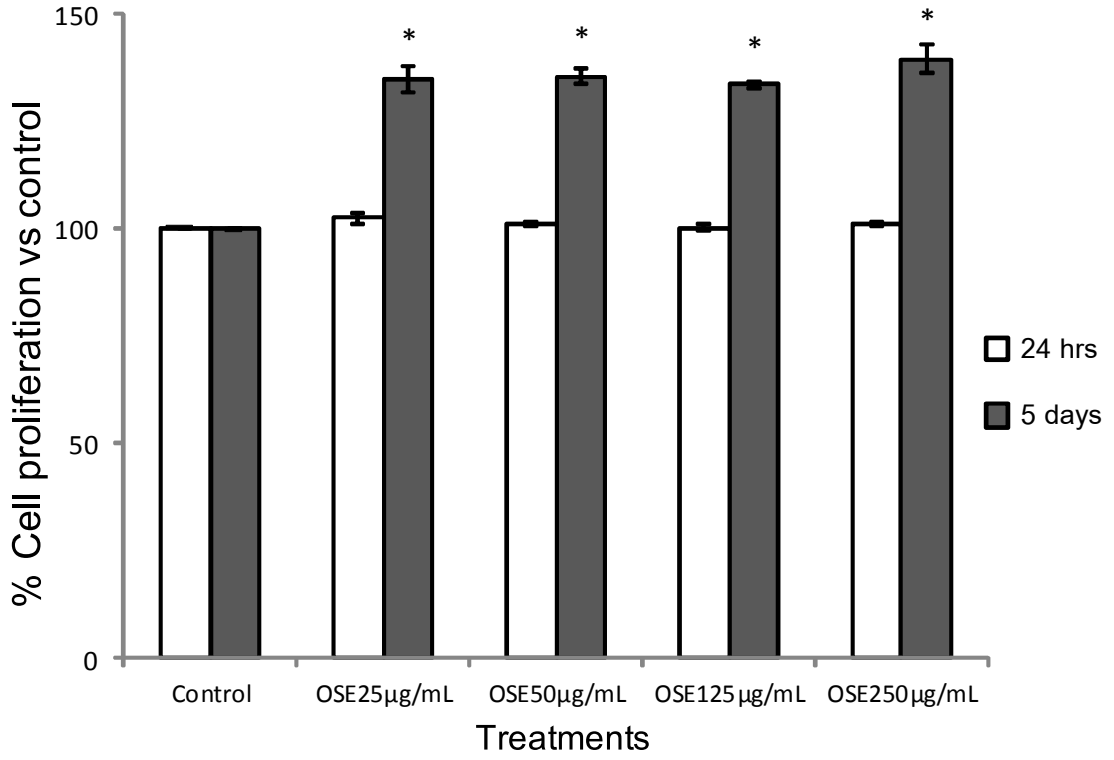

Figure 1. *Orthosiphon stamineus* extract after 24 hours incubation (White-colored bar) showed no cytotoxicity to human dermal papilla cells at all concentrations. OSE at 250 µg/mL, however, showed slight but significant cell proliferation compared to the non-treated control ($P < 0.05$). After repeating the test and incubating for 5 days, OSE at all concentrations enhanced the proliferation of human dermal papilla cells compared with the non-treated control (green-colored bar). Values are means ± SD (n = 3 subjects, $P < 0.05$).

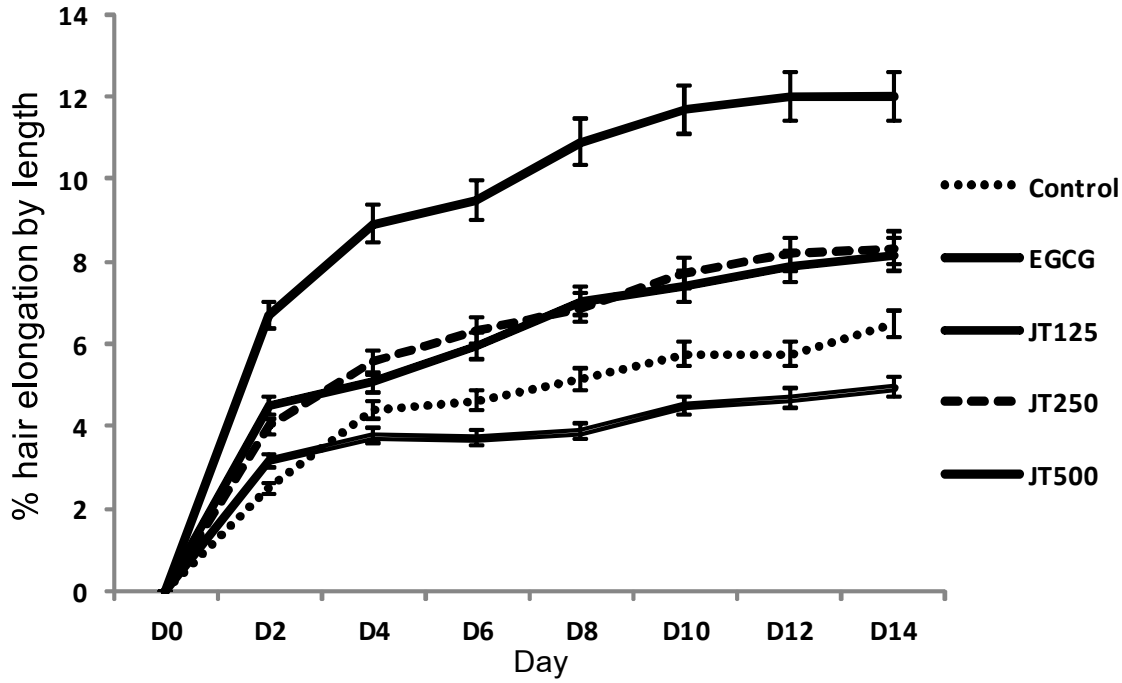

Figure 2. Effect of different treatments on AGA hair follicles in culture for 14 days. Graph showed elongation by length as %cumulative compared to day 0. Results are expressed as mean ± SD of 3 volunteers.

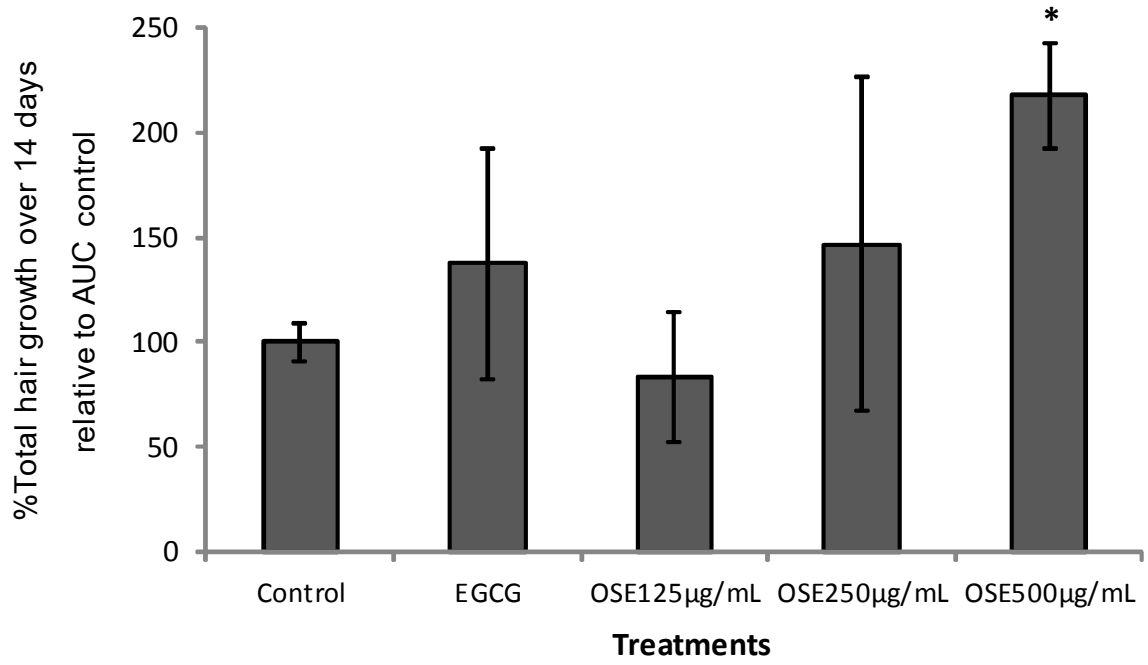

Figure 3. Effect of various concentrations of OSE on cumulative human hair follicle growth ex vivo over 14 day culture period. Only OSE at 500 µg/mL shows highest and significant growth compared to non-treated control). Results are expressed as mean ± SD of 3 volunteers. *$P < 0.05$

Morphology evaluation

The maintaining effect of OSE on the gross morphology of the human hair follicles cultured up to 14 days is shown in Figure 4. We observed that the whole hair follicles treated with OSE at 500 µg/mL (Figure 4A.) could retain their normal morphology until day 8 before thinning of the pre-cortex and starting to lose the matrix cells surrounding the dermal papilla. As seen in Figure 4B, the non-treated control hair's pre-cortex started to thin and lose the bulb volume as early as day 2. The follicles then quickly lose matrix cells as observed from a retraction of the hair fiber and losing volume and pigmentation. At day 14, the control-treated hair shaft almost became detached from the bulb.

Discussion

This study explored the potential of OSE on the human hair dermal papilla cell proliferation as well as the hair growth promotion efficacy in AGA patients' hair follicles. We found that OSE at concentration 25 - 250 µg/mL showed no toxicity to cells after 24 hours incubation. However, we could not further increase the concentration of OSE due to limited solubility of OSE in 1% DMSO. To further study the effect of OSE on dermal papilla cell proliferation, we repeated the cell viability test but extended cell culture for 5 days. The results showed that OSE treatment at concentration 25 - 250 µg/mL can enhance dermal papilla cell proliferation but mechanism was not yet identified. *O.stamineus* is known to have antioxidant properties[1] that might contribute to the growth of dermal papilla cells. To further prove that the extract could have beneficial effect on the whole hair follicles, an *ex vivo* hair follicle elongation study was performed. OSE 500 µg/mL could enhance hair follicle elongation

(A) OSE (B) Vehicle

Figure 4. Gross morphology of hair follicles in culture over 14 days. (A) OSE at 500 μg/mL could prolong the anagen phase of the hair follicles by maintaining hair morphology from day 0 – day 8. (B) Hair follicles treated with the medium alone (control) entered the catagen phase as early as day 2.

compared to vehicle treated control while positive control EGCG was not significantly different from the control. The much more economical OSE showed a better result on AGA patient hair follicles compared to EGCG may be due to its ability to inhibit 5α-reductase type 1. [3] Furthermore, OSE was able to maintain normal morphology of hair follicle and delay entering the catagen phase. The continuation of hair matrix growth by OSE may be due to ability to enhance dermal papilla cell proliferation and anagen prolongation. [8]

Conclusion

In conclusion, our results revealed that OSE could significantly stimulate growth of human hair follicle of AGA patients ex vivo by prolonging hair in the anagen phase from prematurely entering the catagen phase in a dose-dependent manner, with the highest effect observed at 500 μg/mL. At 250 μg/mL, OSE still exerted the hair promoting effect which was similar to the reference compound EGCG (5 μM). The effect of OSE may be due to its ability to promote proliferation of the dermal papilla cell which is the key inducer of new hair follicles and regulator of hair growth. [9] The effects of OSE on maintaining the hair follicle anagen phase and promoting dermal papilla cell proliferation observed in this study encourage further study on its mechanism of action and may have potential for the development of a novel hair promoting products derived from natural resources.

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