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Research Article

Effects of alcoholic extract from *Clitoria ternatea* flowers on the proliferation of human dermal papilla cells and hair growth in C57BL/6Mlac mice

Narongchai Chaksupa[1], Nongluck Sookvanichsilp[1*], Noppamas Soonthornchareonnon[1], Primchanien Moongkarndi[1], Orapin Gerdprasert[2]

[1] Faculty of Pharmacy, Mahidol University, Bangkok, Thailand
[2] Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand

ABSTRACT

Clitoria ternatea is a vine native to tropical and equatorial Asia. Previous review articles have mentioned about different biological activities of extracts from flowers and other parts of the plant, but none being related to hair growth-promoting activity. Scientific reports dealing with hair growth-promoting activity of this plant are scarce. In the present study, the effect of alcoholic extract from its flowers on the proliferation of dermal papilla cells (DPCs) from isolated hair papillae of normal human scalp hair follicles was performed in comparison with minoxidil. Moreover, its effect on hair growth was also tested in C57BL/6Mlac mice of both sexes in comparison with minoxidil and latanoprost. The results have indicated that the extract could increase human DPC proliferation and stimulate the initial hair growth of C57BL/6Mlac mice, but it has no ability to increase the number of hair follicles or to prolong the anagen hair follicles. The effects of the *C. ternatea* alcoholic extract were similar to those of minoxidil.

Keywords:
Clitoria ternatea flower extract, Human dermal papilla cells, Hair growth in mice

1. INTRODUCTION

Although hair loss is not a disease or life-threatening condition, it can cause psychosocial distress and quality of life impairment in both sexes. Of these, androgenetic alopecia is the most common form of hair loss on scalp in genetically susceptible men and women. Drugs such as topical minoxidil and oral finasteride are approved in some countries to treat this condition. Unfortunately, both drugs have exhibited the limited success rate in improvement of hair loss[1-3].

Clitoria ternatea L. is a vine native to tropical and equatorial Asia. It grows well in moist neutral soil and requires little care. It belongs to Fabaceae (Leguminosae) family with various common names such as Asian Pigeon Wings, Butterfly Pea, Blue Pea and Anchan (Thai name)[4-5]. The most important substance in *C. ternatea* flowers (Figure 1) is anthocyanin, which is also found in other plants, flowers and fruits with blue, red or purple pigments[6-7]. Previous review articles have mentioned about different biological activities of extracts from flowers and other parts of *C. ternatea* such as antioxidant, antidiabetic, antimicrobial, antihelminthic, hepatoprotective, immunomodulatory, antiasthmatic and memory enhancing activities[7-10]. but none being related to hair growth-promoting activity. Although this plant has little scientific evidence to show hair growth-promoting activity, Thai people have used the flowers to grow hairs and eyebrows for infants, to reduce hair loss and to dye gray hair since the former days[11]. Different commercial shampoos containing *C. ternatea* flowers are available in Thailand. In an effort to elucidate the hair growth-promoting effects of *C. ternatea*, both *in vitro* and *in vivo* experiments were therefore performed.

***Corresponding author:**
*Nongluck Sookvanichsilp Email: nongluck.soo@mahidol.ac.th

Figure 1. *Clitoria ternatea* fresh and dried flowers.

2. MATERIALS AND METHODS

2.1. Preparation of the extract

Dried flowers of *C. ternatea* were purchased from Wechaphong-osoth herb shop in Bangkok, Thailand. Two-hundred grams of the dried flowers were further dried in a hot air oven at 50°C for 45 minutes, then ground into powder by a blender. The dried powder was macerated with 50% ethanol (1,200 mL) for 6 days, and filtered through cotton wool and Whatman no.1 filter paper. The same volume of solvent was added to the marc and the extraction process was repeated. Two portions of the filtrates were combined and concentrated under reduced pressure at 43-45°C. The crude extract was further evaporated on a water bath for 2 hours. The dried extract was kept in refrigerator until use.

2.2. Phytochemical detection of the extract

With pH-dependent characteristics, anthocyanins in the plant extract are able to change color from red to blue when ammonium hydroxide is added, and from blue to red when hydrochloric acid is added. Other tests for alkaloids, flavonoids, tannins and polyphenolic compounds, and saponins were also performed with the extract.

To determine the presence of quercetin, TLC chromatograms of the extract on precoated silica gel aluminium plates 60 F254 were performed simultaneously with quercetin, the reference standard, by using 2 suitable developing systems, ethyl acetate: hexane: formic acid: water = 9 : 2 : 0.5 : 0.5 and ethyl acetate: dichloromethane: acetic acid: formic acid: water = 10 : 5 : 0.5 : 0.5 : 0.5. After development, the TLC plates were examined under a UV-short wave (254 nm) and long wave (366 nm). To visualize the zones, the plate sprayed with 0.5% anisaldehyde/sulfuric acid was heated at 110°C for 10 minutes in a hot air oven, whereas the plate sprayed with natural product/polyethylene glycol (NP/PEG) reagent was detected under UV light at 366 nm.

2.3. Effects of the extract on the proliferation of human DPCs

2.3.1. Isolation of dermal papillae from human scalp skin

This part of study was approved by Ethical Review, Mahidol University Institutional Review Board. Normal human female scalp skins were obtained from persons aged 40-60 years, who underwent to full frontal face-lift surgery at Yanhee Hospital, Bangkok, Thailand. Dissected scalp tissues were kept in cooled 0.9% normal saline and dermal papillae were isolated within 12 hours after dissection. In brief, all hair shafts were trimmed and the following aseptic procedure was performed. The scalp tissues were soaked in povidone iodine solution for 30 minutes and washed 4-5 times with sterile normal saline. The epidermal layer was separated from dermal layer with blade and hair follicles were pulled by forceps from dermal layer under a dissecting microscope. Anagen hair follicles were selected, placed into a 35-mm culture dish containing sterile 2% collagenase D in Hanks' balanced salt solutions (HBSS) plus 1% penicillin-streptomycin-amphotericin B, and incubated at 37°C for 25-30 minutes. Dermal papillae were released from stalks by gentle pipetting and the supernatant was drawn into a 15-mL tube, centrifuged at 2,000 rpm, 5 minutes for 3 times,[12] and the supernatant was discarded. A portion of complete medium (see 2.3.2.) was added into the tube, centrifuged at 500 rpm for 1 minute thrice, and the supernatant was discarded. Complete medium at the volume of 5 mL was added and the dermal papillae were transferred into a 25-cm^2 culture flask.

2.3.2. Culture and subculture of human DPCs

Approximately 10 dermal papillae were cultured in complete medium which is a minimum essential medium (MEM) containing penicillin (100 units/mL), streptomycin (100 µg/mL), amphotericin B (0.25 µg/mL) and supplemented with 10% fetal bovine serum (FBS) in a 25-cm^2 culture flask in humidified atmosphere of 5% CO_2 and 95% air at 37°C for 5 days. After this period, the DPC migration became apparent and the medium was refreshed every other day. When human DPCs reached 90-95% confluence, cells were subcultured with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) solution.

2.3.3. Effects of the extract on the proliferation of human DPCs

Cell viability was determined by 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in 96-well plates. With this method, the survival cells after exposure with test compounds could convert the yellow MTT tetrazolium salt to insoluble purple formazan crystals by the mitochondrial enzyme succinate dehydrogenase. Survival cells were directly proportional to the intensity of purple formazan[13]. In brief, human DPCs in passage 6 (1×10^4 cells per well in 100 µL of medium) were transferred into 96-well plates. After 24 hour of incubation, the medium was removed and cells were cultured with 100 µL of FBS-free MEM with or without various concentrations of the extract (0.001-0.1 mg/mL) or minoxidil (0.01-1.0 µM) for either 24 (1 day) and 120 hours (5 days). The medium was then removed and 100 µL of fresh medium plus 25 µL of MTT (5 mg/mL) in HBSS were added. At the end of 4-hour incubation at 37°C, 100 µL of lysis buffer were added into each well, and further incubated overnight. Absorbance was determined spectrophotometrically at 570 nm using a microplate reader. All determinations were repeated three times in triplicate samples.

2.4. Effects of the extract on hair growth

2.4.1. Animals and treatments

The study was performed in six-week-old male and female C57BL/6Mlac mice. This part of study was approved by Mahidol University Animal Care and Use Committee. Mice were purchased from the National Laboratory Animal Center, Mahidol University at Salaya campus, Nakorn Pathom province, Thailand. Mice were housed at 25±2°C in individual cages under 12 hours light periods, and fed with water and mouse chow *ad libitum*.

After one-week acclimatization, under general anesthesia, the dorsal area of all mice was carefully depilated by scissors and disposable razor blade sets for anagen induction. Twenty-four hours later, male and female mice which had pink dorsal skin were divided into 4 treatment groups of 6 males and 6 females in each group to receive different treatments as follows: group 1–10% *C. ternatea* extract in 60% ethanol, group 2–5% minoxidil solution (Regaine®, topical preparation for treatment of androgenetic alopecia in males), group 3–0.005% latanoprost eye drops (Xalatan®, ophthalmic preparation for treatment of glaucoma and it can cause increased thickness and length of eyelashes and hypertrichosis), and group 4–60% ethanol solution (as negative control). A volume of 100 µL of each treatment agent was applied daily on denuded area by using a micropipette and a straw with flat end for 7 days. From day 8 till day 21, the volume of each agent was increased to 200 µL (hair growth starting after 1 week, an increased volume then required to cover the whole depilated area) and applied in the same manner.

2.4.2. Assessment of the hair growth-promoting effects

During 21-day experiment period, hair growth was observed daily. Area of hair growth was analyzed from photographs taken from each mouse at days 9, 10, 12, 14, 16, 18 and 21 postdepilation. At the end of study, all mice were sacrificed by an overdose of volatile diethyl ether. Twenty newly grown hairs from selected mice in each group were randomly plucked by forceps in 5 areas (upper left and right, lower left and right, and central regions of depilated skin) for determining the hair length by using a Vernier caliper. The depilated skin from each animal was then harvested and formalin-fixed paraffin-embedded skin tissue was performed. A 10-µm-thick section was stained with hematoxylin and eosin, and observed histologically and a number of hair follicles per millimeter of skin were determined[14-15].

3. RESULTS AND DISCUSSION

3.1. Phytochemical detection of the extract

Color conversion between red and blue color after adding acid or alkali showed the unique pH characteristic of anthocyanins and with the Shinoda test, a positive red color indicated the presence of flavonoids in this extract. From TLC chromatograms showed the presence of quercetin. This phytochemical detection of the extract provided the evidence to support the presence of anthocyanins, flavonoids and quercetin in *C. ternatea* flowers from previous findings[16-18].

3.2. Effects of the extract on the proliferation of human DPCs

Human DPCs have been widely used as the *in vitro*

screening model for hair growth[19-21]. In the present study, human DPCs began to migrate onto the plastic substrate 5 or 6 days after the harvesting. The cells grew slowly in culture and became confluent after 2-3 weeks. The extract at various concentrations (0.001-0.1 mg/mL) as well as minoxidil, an agent applied topically to treat hair loss in androgenetic alopecia[22], at different concentrations (0.01-1.0 μM) could increase DPC proliferation either on day 1 (24 hours) or day 5 of treatment with significant differences from each control at some concentrations since day 1 of treatment and at all concentrations on day 5 (Figure 2). Neither the extract nor minoxidil at the highest concentration could inhibit the DPC proliferation. This could probably support the benefit and safety of "Anchan" flowers in traditional use for hair and the eyebrow in Thailand[11]. Although many biological activities of the extracts from *C. ternatea* (flowers or other parts of the plant) such as antioxidant, antidiabetic, antimicrobial, antihelminthic, hepatoprotective, anti-allergy, anti-tussive and memory enhancing activities have been mentioned[7-10,23], to our knowledge, scientific reports dealing with hair growth-promoting activity of this plant are scarce. The result of human DPC growth-promoting activity from the present study can be added.

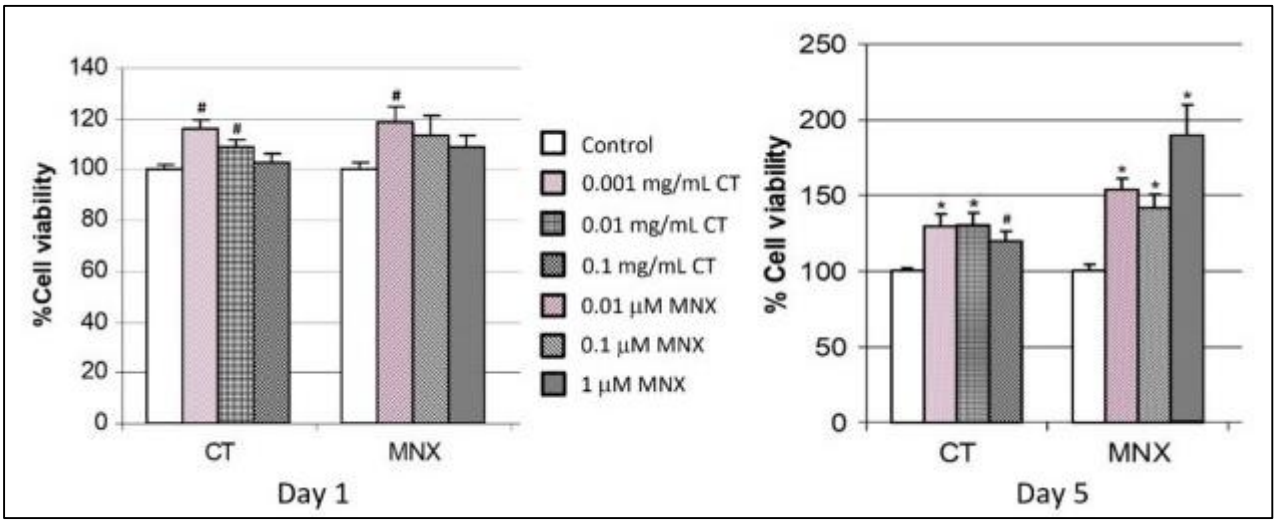

Figure 2. Effect of *C. ternatea* flower extract (CT) and minoxidil (MNX) on cell viability of human dermal papilla cells at day 1 and day 5 of treatment. Data are expressed as means±S.E.M. of 3 independent experiments (each one in triplicate). $^{\#}p<0.05$ and $^{*}p<0.01$, compared with the corresponding control.

3.3. Effects of the extract on hair growth in mice

3.3.1. Hair growth rate

Gender, hair diameter and health status are significant factors influencing hair growth rates and there is a slightly faster mean growth rate in females as compared to males[24]. In the present study, gender difference in the speed of hair growth was also found in C57BL/6Mlac mice (with a slightly faster in females) as evaluated in the control groups either by the initial hair growth (the first hair growth being visualized) as well as complete hair growth (the whole depilated area being occupied with hair) in Table 1, or percent area of hair growth in Table 2.

Time required for the initial hair growth (as well as complete hair growth) on the dorsal skin area of C57BL/6Mlac mice was found shorter than that of the control in mice treated with the extract and minoxidil (but not latanoprost) although significant differences could be detected only in some groups (Table 1). Moreover, the extract and minoxidil (but not latanoprost) could significantly stimulate and prolong hair growth throughout the study period (as evaluated by percent area of hair growth starting from day 9) when compared with control particularly in male mice (Table 2). A previous study of the *C. ternatea* flower extract in C57BL/6Mlac male mice found that the extract could promote the hair growth (as evaluated by % growth on the depilated area) during the first 14 days of the experiment while during the last 14 days the hair growth rate was constant[25]. This could be due to the depilated area was almost completely occupied by new hairs as it was found in the present study that percent area of hair growth in the extract group had been approaching 100% since day 10.

From these results indicate that *C. ternatea* extract, similar to minoxidil, could stimulate the initial hair growth throughout the study particularly in male mice which could probably reflect a higher benefit of these preparations for those with delayed hair growth rate.

Minoxidil is a vasodilator, but its mechanisms of action as a hair growth stimulator are not well-established. Different mechanisms have been suggested such as shortening the telogen phase, causing the quiescent hair follicles to enter prematurely into the anagen phase, acting on the potassium channels of vascular smooth muscles and hair follicles−which may induce the stimulation of the microcirculation near the hair follicles, inducing vascular endothelial growth factor (VEGF)

expression to increase vascularization around the hair follicles, inhibiting of androgen effects on the androgen-sensitive hair follicles, delaying their aging, and etc[3,26]. It is recommended that further studies on the effects of *C. ternatea* flower extract be undertaken in those areas to find the possibility of its mechanisms of action.

Table 1. Time (days after depilation) required for the initial and complete hair growth in C57BL/6Mlac mice.

Treatment	Initial hair growth (day)		Complete hair growth (day)	
	Male	Female	Male	Female
10% *C. ternatea* extract	9.17 ± 0.31*	9.17 ± 0.17	N/A	16.33 ± 1.28$^{\#\#}$
5% Minoxidil	9.17 ± 0.17**	9.17 ± 0.17	13.67 ± 1.08	11.33 ± 1.20**
0.005% Latanoprost	11.17 ± 0.17$^{\#\#}$	11.17 ± 0.70$^{\#}$	N/A	18.67 ± 0.80$^{\#\#}$
60% Ethanol	10.33 ± 0.21	9.83 ± 0.31	N/A	16.83 ± 0.98

Values are means±S.E.M (n=6 in each group).
* p<0.05 and ** p<0.01, compared with 60% ethanol (control group).
$^{\#}$ p<0.05 and $^{\#\#}$ p<0.01, compared with minoxidil.
N/A, data not available due to none could achieve a complete hair growth at the end of study (21 days).

Table 2. Percent area of hair growth in C57BL/6Mlac mice.

Days after depilation	Male				Female			
	10% CT	5% MNX	0.005% LTN	60% Ethanol	10% CT	5% MNX	0.005% LTN	60% Ethanol
Day 9	59.24 ±18.86*	79.72 ±16.25**	0.00 ±0.00	0.00 ± 0.00	62.82 ±13.02	77.07 ±15.50*	0.00 ± 0.00	0.00 ± 0.00
Day 10	92.32 ± 2.00*	99.65 ± 0.35*	0.00 ±0.00*	48.04 ±15.46	85.97 ± 4.68	98.69 ± 0.91*	0.00 ± 0.00*	63.74 ±13.52
Day 12	94.13 ± 1.90**	99.57 ± 0.43**	14.32 ±3.12**	74.30 ± 2.18†	93.22 ± 3.33	99.27 ± 0.73*	47.54 ±14.10*	87.47 ± 4.29
Day 14	95.61 ± 1.45**	99.74 ± 0.26**	15.30 ± 3.00**,††	79.98 ± 1.73††	95.28 ± 2.56	99.39 ± 0.61*	66.03 ±10.59	91.86 ± 2.87
Day 16	96.60 ± 1.31**	99.87 ± 0.13**	27.52 ± 7.94**,††	82.93 ± 1.89††	97.43 ± 1.49	99.73 ± 0.27	86.72 ± 4.92	97.12 ± 2.08
Day 18	98.10 ± 1.10**	100.00 ± 0.00**	69.50 ± 5.05*,††	84.63 ± 1.94††	99.42 ± 0.58	100.00 ± 0.00	96.46 ± 2.26	98.93 ± 1.07
Day 21	99.35 ± 0.65**	100.00 ± 0.00**	79.26 ± 3.33*,††	89.14 ± 2.27††	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00

Values are means±S.E.M (n=6 in each group).
CT=*C. ternatea*, MNX=minoxidil, LTN=latanoprost
$^{*}p$<0.05 and $^{**}p$<0.01, compared with 60% ethanol (control) group.
$^{\dagger}p$<0.05 and $^{\dagger\dagger}p$<0.01, compared with female mice in the same treatment group.

3.3.2. Hair length and number of hair follicles per millimeter of skin

Significant differences from control in hair length and number of hair follicles were found only in mice treated with latanoprost (but not the extract and minoxidil) (Table 3). Moreover, histological sections of tissues from dorsal skin at day 21 postdepilation showing more anagen hair follicles (follicles in growth phase) and higher skin thickness than control were also found only in mice treated with latanoprost (Figure 3).

Latanoprost, an ophthalmic preparation for treatment of open-angle glaucoma, can cause increased thickness and length of eyelashes and hypertrichosis[27-28]. The hypertrichotic effect of latanoprost has been used therapeutically to treat loss of eyelashes[29]. Although it did not stimulate the initial hair growth in C57BL/6Mlac mice as the extract and minoxidil could do, it increased hair length as well as the number of anagen hair follicles while those two compounds could not. The present results could support the previous finding that latanoprost can increase the length of lashes in consistency with the ability to prolong the anagen phase of the hair cycle[30].

4. CONCLUSION

In conclusion, the alcoholic extract of *C. ternatea* flowers could increase human DPC proliferation and stimulate the initial hair growth in male and female C57BL/6Mlac mice, similar to minoxidil. However, it has no ability to increase the number of hair follicles or to prolong the anagen hair follicles in both male and female

Table 3. Effects on hair length and number of hair follicles per millimeter of skin in C57BL/6Mlac mice.

Treatment	Hair length (millimeter)[a]		Number of hair follicles per millimeter skin[b]	
	Male	Female	Male	Female
10% *C. ternatea* extract	5.93 ± 0.12	6.27 ± 0.10	85.33 ± 2.12	86.33 ± 0.90
5% Minoxidil	6.09 ± 0.08††	6.59 ± 0.08	86.00 ± 0.98	85.67 ± 1.24
0.005% Latanoprost	6.18 ± 0.10††	6.78 ± 0.14*	107.00 ± 0.66*	106.67 ± 0.44*
60% Ethanol	5.78 ± 0.15††	6.41 ± 0.08	85.67 ± 1.15	85.33 ± 0.78

Values are means ± S.E.M.
[a] n = 20 hairs from each group and [b] count and average the number of hair follicles per millimeter of skin in 3 fields (100x magnification) of 3 skin sections of 6 mice.
$^{*}p$<0.05, compared with 60% ethanol (control) group.
$^{\dagger\dagger}p$<0.01, compared with female mice in the same treatment group.

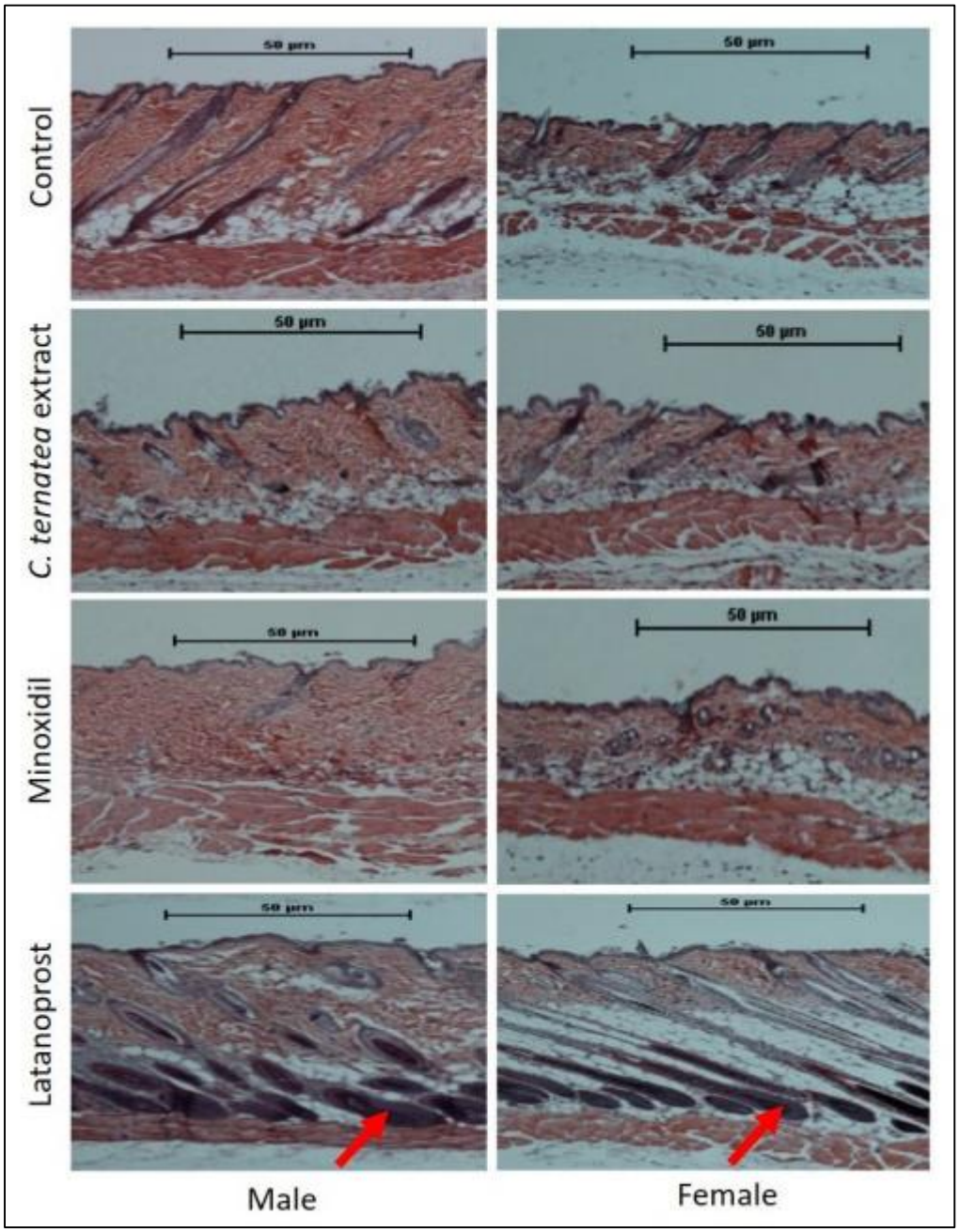

Figure 3. Histological sections of the representative tissues from dorsal skin of male and female C57BL/6Mlac mice treated topically with different compounds (10% *C. ternatea* extract, 5% minoxidil and 0.005% latanoprost) at day 21 postdepilation (100x magnification). More anagen hair follicles (follicles in growth phase, with proximal part located deeply in subcutis as indicated by arrows) and higher skin thickness than control were found only in mice (both males and females) treated with latanoprost.

mice which is similar to topical minoxidil, but different from topical latanoprost.

5. ACKNOWLEDGEMENT

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Conflict of interest

There is no conflict of interest.

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Ethics approval

1. Mahidol University Animal Care and Use Committee (No.PYT009/2553).
2. Ethical Review, Mahidol University Institutional Review Board (COE.No.MU-IRB 2010/012.0804).

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