Author's response to reviews

Title: Hair promoting activity of a hot water extract of Thuja orientalis

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Version: 5 **Date:** 10 October 2012

Author's response to reviews: see over

Title: Hair promoting activity of Thuja orientalis

Version: 1 **Date:** 2 July 2012

Dear Dr. Sandhya S

We greatly appreciate the efforts of the Editorial Board and reviewers for giving us the valuable comments on our manuscript for publication *BMC Complementary and Alternative Medicine.*

As you requested, we have enclosed a revised version of manuscript in response to the extensive and insightful reviewer comments. Included below is a point-by-point description of our responses to the reviewer's comments. The revision made in the manuscript was written in blue. We hope that you and the reviewers find this revised manuscript acceptable for publication in *BMC Complementary and Alternative Medicine.*

Thank you.

Yours sincerely,

Hye-Jin Park, Ph.D.

Major revisions in the manuscript;

1) The nature of extract used was described in the revised manuscript, including abstract.

2) The extraction method for the leaves of *Thuja orientalis* was described in the methods section of the revised manuscript.

Reviewer's report:

Major compulsory revisions

1. In the year 2004, Young Jun Kim et al, had proved the hair growth promoting activity of *Thuja orientalis* in DBA1 J mice model; then what made you repeat it in different model?

Response: As alopecia is one of the most common side effects of chemotherapy, Kim et al, investigated the effects of *Thuja orientalis* to treat chemotherapy-induced alopecia using DBA1J mice. Some of the toxic effects of chemotherapy drugs are caused by the increased production of inflammatory mediators [1]. Kim's group described that anti-inflammatory property of *Thuja orientalis* contributes to its anti-hair loss activity on chemotherapy-induced alopecia.

In our study, we investigated the hair promoting activity of a hot water extract of *Thuja orientalis*, using C57BL/6N mice to characterize its role in anagen induction of hair follicles. The skin color of this mice in telogen phase is pink and becomes dark along with anagen initiation [2]. Since the active growth of hair follicles and black pigmentation occur in C57BL/6N mice during anagen phase [3], we used C57BL/6N mice to characterize the role of a hot water extract of *Thuja orientalis* in anagen induction of hair follicles. As a result, we found that a hot water extract of *Thuja orientalis* promoted hair growth through inducing anagen phase in telogenic C57BL/6N mice. Histomorphometry analysis data indicated that topical application of a hot water extract of *Thuja orientalis* could induce the earlier anagen phase and prolong the mature anagen phase compared to either control or 1% minoxidil treated group. In a hot water extract of *Thuja orientalis* treated group, we observed an

increase in the number and the size of hair follicles. Immunohistochemical analysis revealed that earlier induction of β-catenin and Shh protein in the hair follicles of a hot water extract of *Thuja orientalis* treated group was observed than that in control group or 1% minoxidil treated group.

References

1. Mills PJ, Ancoli-Israel S, Parker B, Natarajan L, Hong S, Jain S, Sadler GR, von Kanel R: **Predictors of inflammation in response to anthracycline-based chemotherapy for breast cancer.** *Brain Behav Immun* 2008, **22(1):**98-104.
2. Paus R, Foitzik K: **In search of the "hair cycle clock": a guided tour.** *Differentiation* 2004, **72(9-10):**489-511.
3. Peters EM, Botchkarev VA, Muller-Rover S, Moll I, Rice FL, Paus R: **Developmental timing of hair follicle and dorsal skin innervation in mice.** *The Journal of Comparative Neurology* 2002, **448(1):**28-52.

Minor Essential Revisions

1. 1) Please mention the nature of extract used (aqueous, alcoholic etc) in the abstract.

Response: As the reviewer suggested, we mentioned the nature of extract used in the abstract.

<Before revision>

~~The present study sought to investigate hair growth promoting activity of *Thuja orientalis* and its mechanism of action.~~

<After revision> page 1, line 15-16

The present study sought to investigate hair growth promoting activity of a hot water extract of *Thuja orientalis* and its mechanism of action.

2) What do you want to convey through this sentence mentioned in the background of the study: *Thuja orientalis* has been traditionally used to prevent or promote hair growth in the oriental medicine.

Response: *Thuja orientalis* has been traditionally used to prevent or promote hair growth in the oriental medicine. However, the mechanism responsible for its hair promoting effect remains unknown. Therefore, the hair promoting activity of a hot water extract of *Thuja orientalis* was investigated using telogenic C57BL/6N mice.

<Before revision>

~~However, it has never been investigated the hair promoting effect of *Thuja orientalis* extract and its cellular mechanism.~~

<After revision> page 3, line 8-9

However, the mechanism responsible for its hair promoting effect remains unknown. Therefore, the hair promoting activity of a hot water extract of *Thuja orientalis* was investigated using telogenic C57BL/6N mice.

2. What was method of extraction adopted for extracting the active principles from the leaves of the plant?

Response: An authenticated voucher specimen of the leaves of *Thuja orientalis* (Kucari 1108) was deposited in the Herbarium at the College of Bioscience and Biotechnology, Konkuk

University (Seoul, Korea). The leaves of *Thuja orientalis* were ground to a fine powder with a grinder. The powder was extracted four times with hot water extract for 4 hours. The hot-water extract was chilled, filtrated through Advantech No.2 filter paper (Osaka, Japan) and evaporated to dryness. The residue was extracted with hot water at room temperature and filtered again. The extract was dried by a rotary evaporator under vacuum at 40°C and stored at −20°C until use. A hot water extract of *Thuja orientalis* was dissolved in water and used for the animal experiments [4].

<Before revision>

~~The dried and powdered leaves of *Thuja orientalis* were provided by Cell Activation Research Institute (CARI, Seoul, Korea). Authenticated voucher specimen of *Thuja orientalis* (Kucari 1002) is deposited in the Herbarium at College of Bioscience and Biotechnology, Konkuk University (Seoul, Korea). In brief, water extracts of *Thuja orientalis* were prepared in 850 ml autoclaved deionized water for 5 min. The hot-water extract was chilled, filtrated through Advantech No.2 filter paper (Osaka, Japan) and evaporated to dryness.~~

<After revision> page 3, line 24-27

Page 4, line 1-3

An authenticated voucher specimen of the leaves of *Thuja orientalis* (Kucari 1108) was deposited in the Herbarium at the College of Bioscience and Biotechnology, Konkuk University (Seoul, Korea). The leaves of *Thuja orientalis* were ground to a fine powder with a grinder. The powder was extracted four times with hot water extract for 4 hours. The hot-water extract was chilled, filtrated through Advantech No.2 filter paper (Osaka, Japan) and evaporated to dryness. The residue was extracted with hot water at room temperature and filtered again. The extract was dried by a rotary evaporator under vacuum at 40°C and stored at −20°C until use. *Thuja orientalis* extracts were dissolved in water and used for the animal

experiments.

References

4. Park HJ, Han ES, Park DK, Lee C, Lee KW: **An extract of Phellinus linteus grown on germinated brown rice inhibits inflammation markers in RAW264.7 macrophages by suppressing inflammatory cytokines, chemokines, and mediators and up-regulating antioxidant activity**. *J Med Food* 2010, **13**(6):1468-1477.

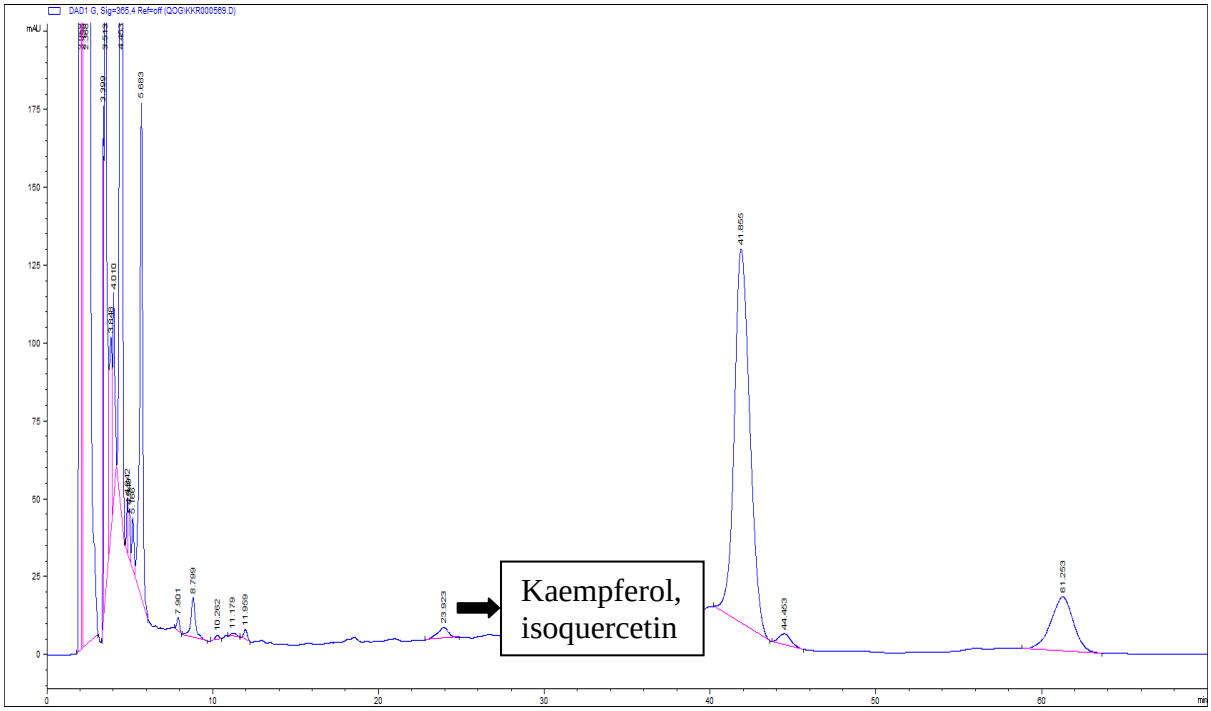
3. Did you perform any preliminary chemical screening for the identification of chemical nature of the bioactive components in your plant extract?

Response: In this study, the whole hot water extract of *Thuja orientalis*, not individual components, is used to prove its biological activity and mechanism against pathogenic alopecia.

We agree to reviewer's comments that it would be better to identify the bioactive components in hot water extract of *Thuja orientalis*. Other studies show that the leaves of *Thuja orientalis* contain large amounts of the flavonoid constituents, including quercetin, rutin [5], isoquercetin [6], myricitrin, hypoletin-7-O-β-D-xylopyranoside, quercitrin, kaempferin, kaempferol and amentoflavone [7].

The obtained High performance liquid chromatography (HPLC) chromatogram profiles are shown below.

A)



B)

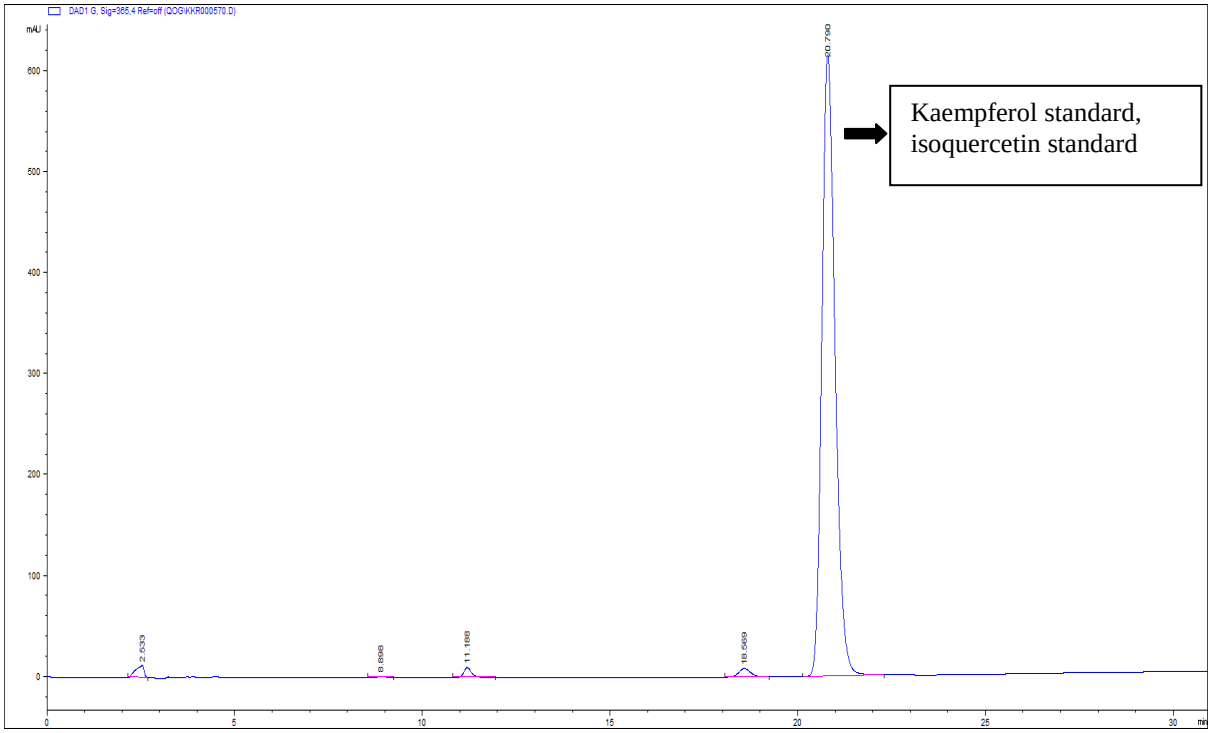


Figure. **High performance liquid chromatography (HPLC) chromatogram of A) a hot**

water extract of *Thuja orientalis* and B) kaempferol and isoquercetin standard.

HPLC chromatogram indicated that kaempferol and isoquercetin were found in hot water extract of *Thuja orientalis* leaves. It has been reported that kaempferol or isoquercetin, a polyphenolic flavonoid, possesses antioxidants [8-10], anti-inflammatory [11] and inhibitory activity in cellular events, which associated with initiation, promotion and progression of carcinogenesis [12, 13]. These activities of two components might be contributed to hair promoting activity of a hot water extract of *Thuja orientalis*. However, we cannot rule out the possibility that other components in a hot water extract of *Thuja orientalis* exert hair promoting activity. Further chemical screening analysis for the other bioactive components in a hot water extract of *Thuja orientalis* leaves will help to understand the detailed mechanism of its hair promoting activity.

References

5. Zhu JX, Wang Y, Kong LD, Yang C, Zhang X: **Effects of Biota orientalis extract and its flavonoid constituents, quercetin and rutin on serum uric acid levels in oxonate-induced mice and xanthine dehydrogenase and xanthine oxidase activities in mouse liver**. *J Ethnopharmacol* 2004, **93**(1):133-140.
6. Jung SH, Kim BJ, Lee EH, Osborne NN: **Isoquercitrin is the most effective antioxidant in the plant Thuja orientalis and able to counteract oxidative-induced damage to a transformed cell line (RGC-5 cells)**. *Neurochemistry International* 2010, **57**(7):713-721.
7. Lee EH, Song DG, Lee JY, Pan CH, Um BH, Jung SH: **Flavonoids from the Leaves of Thuja orientalis Inhibit the Aldose Reductase and the Formation of Advanced**

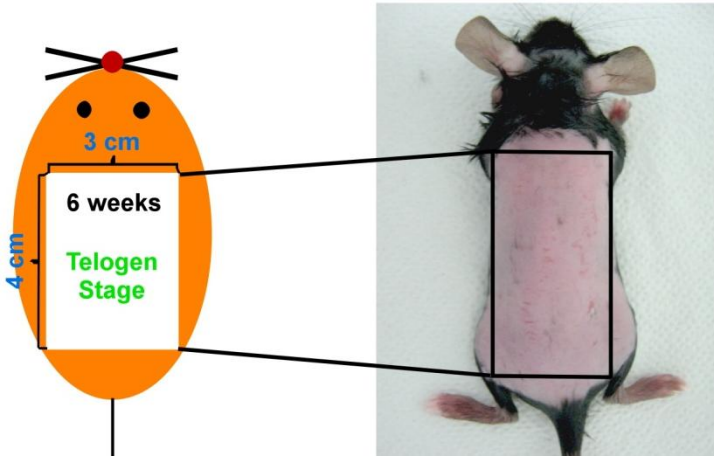
Glycation Endproducts. *J Korean Soc Appl Bi* 2009, **52**(5):448-455.

8. Rice-Evans CA, Miller NJ, Paganga G: **Structure-antioxidant activity relationships of flavonoids and phenolic acids**. *Free Radic Biol Med* 1996, **20**(7):933-956.
9. Hou L, Zhou B, Yang L, Liu ZL: **Inhibition of human low density lipoprotein oxidation by flavonols and their glycosides**. *Chem Phys Lipids* 2004, **129**(2):209-219.
10. Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF, Lin JK: **Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages**. *Carcinogenesis* 1999, **20**(10):1945-1952.
11. Hamalainen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E: **Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages**. *Mediators Inflamm* 2007, **2007**:45673.
12. Nguyen TT, Tran E, Ong CK, Lee SK, Do PT, Huynh TT, Nguyen TH, Lee JJ, Tan Y, Ong CS *et al*: **Kaempferol-induced growth inhibition and apoptosis in A549 lung cancer cells is mediated by activation of MEK-MAPK**. *J Cell Physiol* 2003, **197**(1):110-121.
13. Yang CS, Landau JM, Huang MT, Newmark HL: **Inhibition of carcinogenesis by dietary polyphenolic compounds**. *Annu Rev Nutr* 2001, **21**:381-406.

4. On what basis have you calculated the dose as 5.05mg/cm^2/day?

Response: In this study, a 12 cm^2 (Horizontal length = 3 cm, longitudinal length = 4 cm) area

(Shown as below) of dorsal portion of 6 week-old C57BL/6N mice was shaved with animal clipper at which of the hair follicles of mouse were synchronized in the telogen stage.



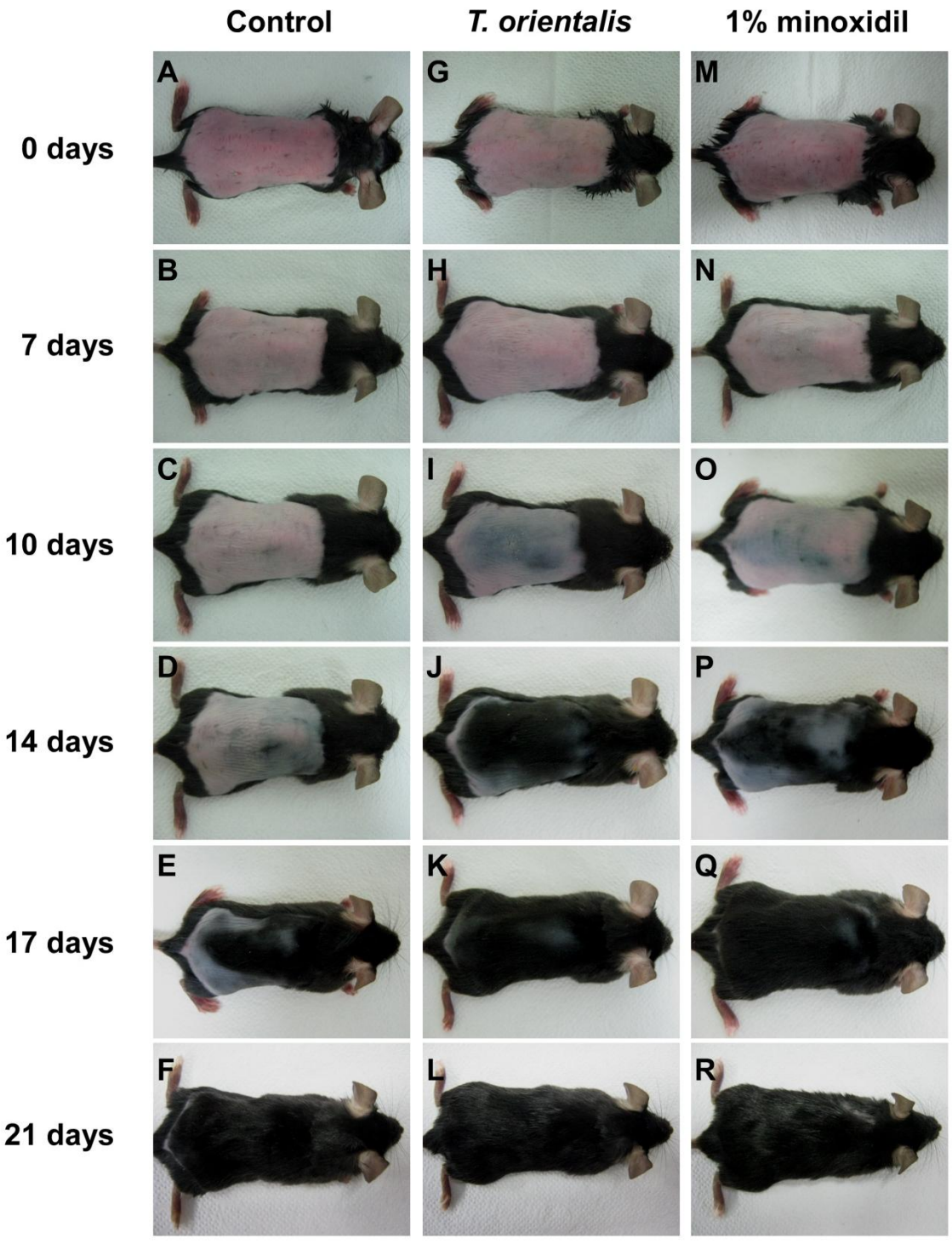
The hot water extract or the vehicle was applied topically on dorsal back with a total volume of 400 μl for 21 days. A hot water extract of *Thuja orientlis* (200 μl) with equal volume (propylene glycol 96.5% (v/v) and DMSO 3.5% (v/v)) were topically applied to the shaved area of C57BL/6N mice. A hot water extract of *Thuja orientalis* (200 μl) was lyophilized by freezer drying process then its weight was measured with microbalance. The dose of *Thuja orientlais* hot water extract applied to the shaved dorsal skin was calculated as below:

5.05 mg/cm^2/day = 60.6 mg (a hot water extract of *Thuja orientalis*)/12 cm^2 (horizontal length = 3 cm, longitudinal length = 4 cm)/day

Daily amount that topically applied (60.6 mg/12 cm^2)	**A hot water extract of *Thuja orientalis* concentration**	**Area**
	60.6 mg	**12 cm^2**

5. Why did you measure the hair only on 14th and 21st days?

Response: Re-grown hairs of shaved dorsal skin were visible from 10th day. In this study, mice from each group were sacrificed to obtain skin specimen at 0, 7, 14 and 21 days. Therefore, re-grown hairs were plucked from representative area of shaved dorsal skin from the sacrificed mice and measured the average hair length on 14 and 21 days. Visible hair growth was recorded at 0, 7, 10, 14, 17 and 21 days, respectively, as shown below:

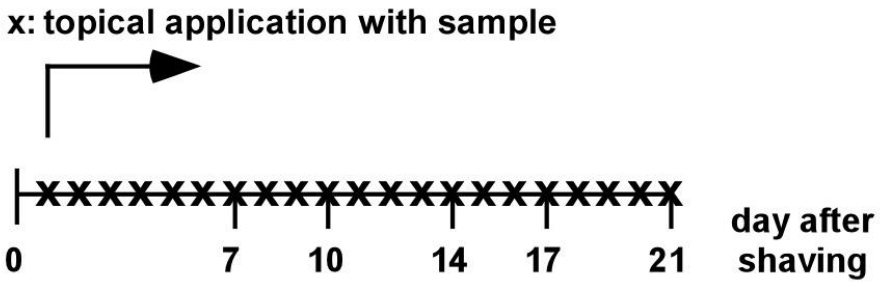


6. Was there any change in hair length, when you consider this parameter before and after the experiment?

Response: As reviewer suggested, we measured the average hair length before and after the experiment. In this study, the experiment was performed with 6 week-old male C57BL/6N mice and the hot water extract or the vehicle was applied topically on dorsal back for 21 days. The hair length data of before and after experiment are shown as below:

	Before experiment (6 week-old C57BL/6N mice)			**After experiment (9 week-old C57BL/6N mice)**		
Group	**Control**	***T. orientalis***	**1% minoxidil**	**Control**	***T. orientalis***	**1% minoxidil**
Hair length	**4.5 ± 0.5 mm**	**4.5 ± 0.5 mm**	**4.5 ± 0.5 mm**	**3.3 ± 0.7 mm**	**5.2 ± 0.8 mm**	**5 ± 0.6 mm**

The hair length data of 6 week-old male C57BL/6N mice is provided by Orient Bio Inc.



As the data shown, the average hair length of 6 week-old male C57BL/6N mice is 4.5 ± 0.5 mm in before experiment. After 3 week of treatment, the average hair length of control group, a hot water extract of *Thuja orientalis* treat group and 1% minoxidil group are 3.3 ± 0.7, 5.2 ± 0.8 and 5 ± 0.6 mm, respectively. X indicates the day(s) of topical application of a hot water extract of *Thuja orientals* or the vehicle application.

7. Typographical errors should be taken care.

Response: As the reviewer suggested, we checked the typographical errors and corrected it.

1) <Before revision>

~~5α-Reductase is an enzyme which converts testosterone to dihydrotestosterone (DHT) that triggers androgenetic alopecia in individuals who are genetically susceptible.~~

<After revision> Page 3, line 4-6

5α-reductase is an enzyme which converts testosterone to dihydrotestosterone (DHT) that triggers androgenetic alopecia in individuals who are genetically susceptible.

2) <Before revision>

~~The experimental data are expressed as mean ± SD.~~

<After revision> Page 6, line 7

The experimental data are expressed as mean ± standard deviation (S.D.).

8. In the sub heading: Background-correct the sentence "A number of patients suffering from hair loss or alopecia are dramatically increasing [3] and [4]".

Response: we corrected it.

<Before revision>

~~A number of patients suffering from hair loss or alopecia are dramatically increasing [3] and [4].~~

<After revision> Page 2, line 13-14

The number of patients suffering from hair loss or alopecia is dramatically increasing [3] and [4].

9. Why didn't you perform the T.S of Immunohistochemistry analysis?

Response: We performed image analysis to do the T.S of Immunohistochemistry analysis. This quantitative analysis of digitized IHC-stained tissue sections is widely used in research studies and clinical practice [14, 15]. Image analysis is used to quantify the staining intensity of β-catenin or Shh proteins. The percentage of protein expression was calculated with the following formula [16, 17].

$$\text{percentage of DAB stained area} = \frac{\text{DAB stained area}}{\text{Total stained area}}\%$$

DAB stained area	The stained area of tissue for β-catenin or Sonic hedgehog (brown staining)
Total stained area	The area of tissue (brown + non-brown staining)

Data are shown as below:

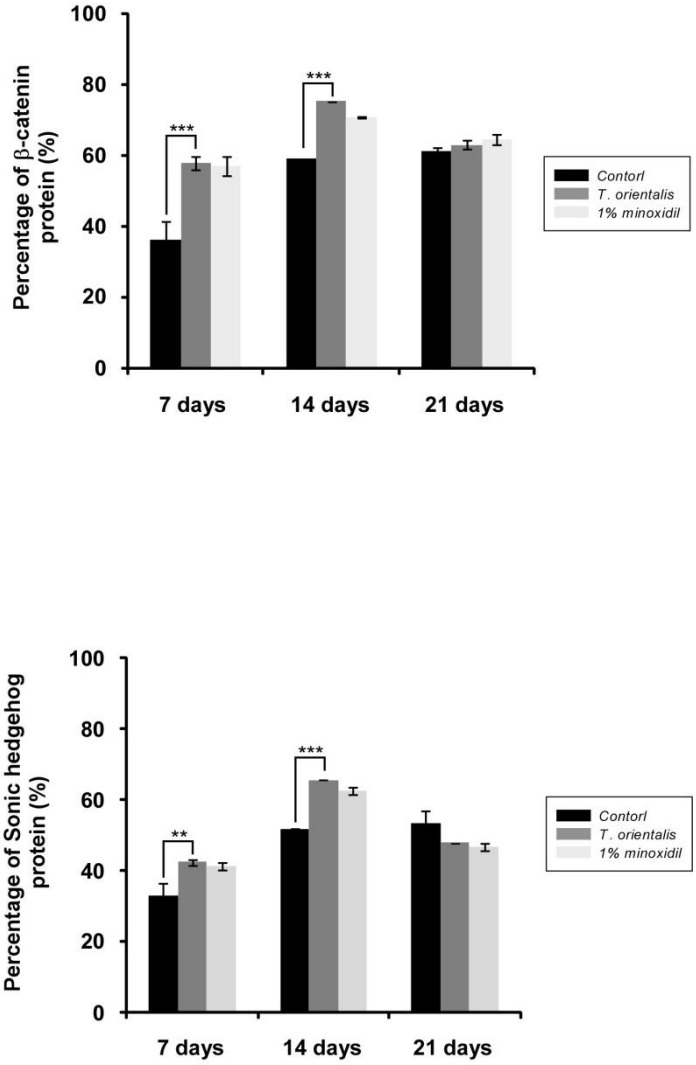


Figure legend

Area (%) of β-catenin and Sonic hedgehog (Shh) proteins in the tissue was investigated using Image analysis. Image analysis data indicated that levels of β-catenin and Shh proteins in a hot water extract of *Thuja orientalis* group or 1% minoxidil group were higher than that those in control group. Further, the level of β-catenin and Shh protein gradually reduced in a hot water extract of *Thuja orientalis* group or 1% minoxidil group at 21 days. Data shown represent means ± standard deviation (S.D.) of three independent experiments ($^{**}p<0.01$, $^{***}P<0.001$ vs. control).

Methods

Software

For image analysis the freeware ImageJ v1.33 as well as the Colour Histogram plug-in, both downloaded from the NIH website (http://rsb.info.nih.gov/ij) were used.

Automated quantitative analysis

The IhcJ algorithm first divides the acquired image of the IHC stained specimen in RGB colour space into separate colour channels by a colour deconvolution method. The ImageJ plugin for colour deconvolution has a built in vector for separating methyl green and diaminobenzidine (DAB) stainings. After colour deconvolution, methyl green and DAB images are processed separately. By using five random test samples stained for β-catenin or Sonic hedgehog, suitable threshold levels for methyl green and DAB were determined. These thresholds were used on both methyl green and DAB images, respectively, and kept constant for the analysis of the main image dataset. Thresholding creates binary masks of methyl green and DAB positive areas and the two areas may overlap. Binary masks were merged into a single result image. In the result image, the area of methyl green-positive and DAB-negative pixels is pseudocoloured with red colour. The area of DAB-positive pixels regardless of methyl green-status is pseudocoloured with black colour. The background, where both values are negative, is indicated with white colour.

The extent of staining is calculated as the total number of DAB-positive pixels divided by the union of the total number of methyl green-positive pixels and the total number of DAB-positive pixels. The intensity of staining is calculated from DAB-positive area, as a mean pixel value of original DAB image. The mean intensity value is scaled to range from 0 to 100 percent.

References

14. ten Berge O, van Velsen SG, Giovannone B, Bruijnzeel-Koomen CA, Knol EF, Guikers K, van Weelden H: **Assessment of cyclobutane pyrimidine dimers by digital photography in human skin**. *J Immunol Methods* 2011, **373**(1-2):240-246.
15. Zehntner SP, Chakravarty MM, Bolovan RJ, Chan C, Bedell BJ: **Synergistic tissue counterstaining and image segmentation techniques for accurate, quantitative immunohistochemistry**. *J Histochem Cytochem* 2008, **56**(10):873-880.
16. Brey EM, King TW, Johnston C, McIntire LV, Reece GP, Patrick CW, Jr.: **A technique for quantitative three-dimensional analysis of microvascular structure**. *Microvasc Res* 2002, **63**(3):279-294.
17. Brey EM, Lalani Z, Johnston C, Wong M, McIntire LV, Duke PJ, Patrick CW, Jr.: **Automated selection of DAB-labeled tissue for immunohistochemical quantification**. *J Histochem Cytochem* 2003, **51**(5):575-584.

Discretionary Revisions

1. Did the animals treated with 1% minoxidil show any change in β-catenin and Shh proteins level?

Response: Immunohistochemistry data showed that the levels of β-catenin and Shh protein in 1% minoxidil treated-group were significantly increased compared to those in control group at the day of 7th and 14th. Also the levels of β-catenin and Shh protein in 1% minoxidil treated-group at the day of 14th were significantly increased compared to those at the day of 7th. Immunohistochemistry data are shown as below:

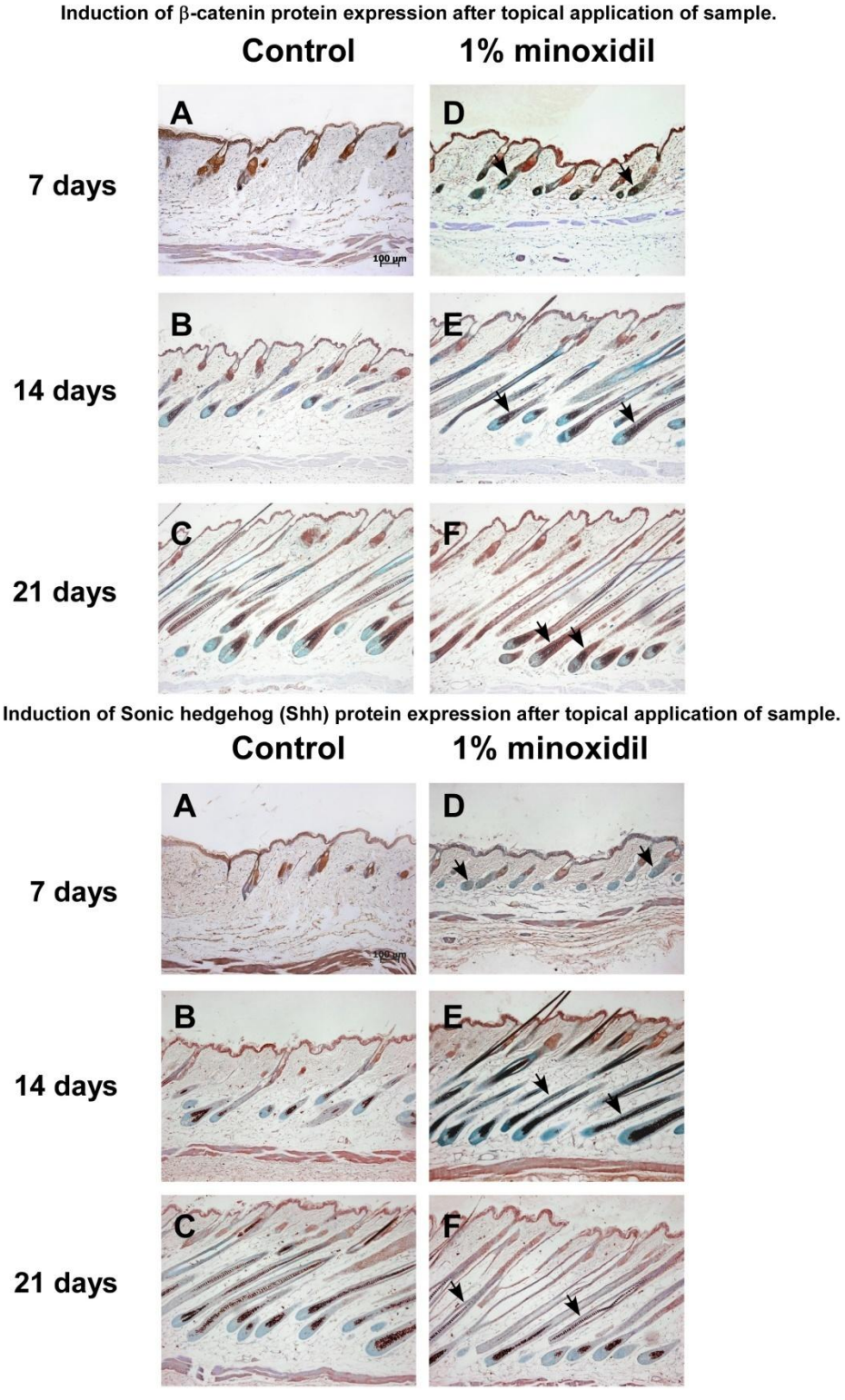


Figure legend

Induction of β-catenin and Sonic hedgehog (Shh) proteins expression after topical application of sample. Immunohistochemistry analysis monitors β-catenin and Sonic

hedgehog (Shh) proteins (arrows) expression in the longitudinal sections of the dorsal skins (7, 14 and 21 days) from control group and 1% minoxidil group. Bars: 100 μm. Control (A-C); 1% minoxidil (D-F).

Level of interest: An article of importance in its field

Quality of written English: Needs some language corrections before being published

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.