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Original Article

Moringa oleifera seed oil promotes hair growth in mice and modulates the genetic expressions of factors affecting hair growth cycle in skin cell culture

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Abstract

Moringa oleifera Lam. seed oil (MO) has traditionally been used for hair loss and several products are commercially available with limited scientific evidence. This study aimed to investigate the hair growth-promoting effect of MO using cell-based and animal models. MO samples were selected to test for the effects on the genetic expressions of four factors related to hair growth cycle (VEGF, TGF-β1, 5α-reductase I and II) by RT-PCR method. MO up-regulated the expression of VEGF gene and down-regulated the expressions of the other three genes. MO showed hair growth-promoting effects via hair growth-related gene expression on both keratinocytes and derma papilla cells and comparable to the effects of the minoxidil. After application on C57BL/6 mice, MO increased the hair growth of the mice. Histological examination of the mouse skin showed that MO increased skin thickness, number of hair follicles and anagen-to-telogen ratio. The obtained results suggest the hair growth-promoting effect of moringa oils.

Keywords: moringa oil, hair growth, skin thickness, genetic expressions

1. Introduction

Hair loss is a common dermatological disease, which significantly influences patient psychology and quality of life. There are several factors that cause hair loss such as environmental pollution, drug side effects and social-environmental stress. Each hair grows through a cycle of the anagen, catagen, and telogen phases, which are the growing, regressing, and resting phases, respectively (Stenn & Paus, 2001). Various factors are involved in the regulation of the hair growth such as vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF), and epidermal growth factor (EGF) (Ozeki & Tabata, 2003). The VEGF plays important roles in angiogenesis, which brings the oxygen supply to tissues (Yano, Brown, & Detmar, 2001). Likewise, the important factor that causes hair loss is 5α-reductase. 5α-reductase stimulates the testosterone to be dihydrotestosterone (DHT) which triggers hair loss by stimulation of the expression of the transforming growth factor-β1 (TGF-β1) (Rho *et al*., 2005). To date, 2 drugs including finasteride and minoxidil have been approved for the treatment of hair loss in men by the Food and Drug Administration (FDA) of the USA. Minoxidil promotes hair growth through increasing the duration of anagen and finasteride is a competitive inhibitor of type2 5α-reductase (Sawaya & Shapiro, 2000). However, these drugs have limited therapeutic uses due to their unsatisfactory cure rates (Park *et al*., 2012). The efficacy of minoxidil is variable and temporary, and finasteride may cause malformation of the external genitalia of male fetuses (Sinclair, 2004). To complement these drugs, some researchers have focused their attentions on medicinal herbs, such as the hair growth-

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promoting herbs *Carthanus tinctorius* floret, *Platycladus orientalis* leaves, *Asiasari radix* rhizome and *Zizyphus jujube* essential oil (Junlatat & Sripanidkulchai, 2014; Rho *et al*., 2005; Yoon, Al-Rezs, & Kang, 2010; Zhang *et al*., 2016).

Moringa oleifera Lam. (miracle tree or drumstick tree) is a native plant of the Himalayan region of India that is now widely cultivated in several subtropical and tropical countries including China, India, Philippines and Thailand. The plant belongs to the family of Moringaceae and it is a multipurpose tree as being a part of culinary and Ayurveda medicine (Razis, Ibrahim, & Kntayya, 2014). Every part of *M. oleifera* can be utilized for sources of food products including leaf, seed, flower, pod, root and bark (Falowo *et al*., 2018). The seed oil of *M. oleifera* is called moringa oil or also ben oil, resembles olive oil and is generally applied in lighting, cooking, perfume and cosmetics industries (Leone *et al*., 2016). Major bioactive agents of moringa oil were reported to include the abundance of the unsaturated fatty acid, especially oleic acid, B-sitosterol and stigmasterol (Özcan, Ghafoor, Al Juhaimi, Ahmed, & Babiker, 2019). Several pharmacological activities of these bioactive compounds were shown such as antioxidant, anti-inflammatory, anticancer and neuroprotective effects (Leone *et al*., 2016). Recently, the antioxidant and anti-inflammatory activity of moringa oil were demonstrated to prevent chemical-induced toxicity in several organs of rats, to abrogate cerebral neurotoxicity induced by antineoplastic agents, and to modulate sleeping behavior in mice (Liu, Wu, Shang, Zhao, & Huang, 2020). All data support the health benefits of moringa oil and its use in functional food and pharmaceuticals. Even though moringa oil has been traditionally utilized for hair care and loss and many products are commercially available, the studies on its action and mechanism of action on hair growth are limited. Taking into consideration of the lack of information, this study aimed to investigate the cell-based effects of moringa oil on the expressions of selected hair growth-related genes including VEGF and TGF-β1 that are involved in the hair growth cycle and 5α-reductase that is involved in the conversion of testosterone to be dihydrotestosterone which affects hair loss. The effect of moringa oil on hair growth in mice was also elucidated. The obtained data would support the use of moringa oil for hair care products and can strengthen the industrial demand of *M. oleifera* seed oil.

2. Materials and Methods

2.1 Sources of *Moringa oleifera* oil

M. oleifera seed oil (moringa oil) samples used in this study were from cold press preparation. The commercially available samples labelled with moringa oil (MO-1) and with organic moringa oil (MO-2) which claims to use raw moringa seeds grown using organic methods were purchased from the local distributors. The third sample (MO-3) was given by Assistant Professor Pradit Sukontavarin, Faculty of Medicine, Khon Kaen University, Thailand.

2.2 Animals

Five-week-old female C57BL/6 mice (18-20 g) were purchased from the National Laboratory Animal Center (Mahidol University, Thailand). The mice were maintained on a standard laboratory diet with water provided *ad libitum*. They were housed in an automatically air-conditioned room (25±3°C and 65±5% humidity) with a 12:12 h light and dark cycle for 7 days prior to the experiments. The experimental protocol was approved by the Animal Ethics Committee of Khon Kaen University (record number AEKKU 11/2554 and reference number 0514.1.12.2/92).

2.3 Cell culture

Human keratinocytes (HaCaT) were purchased from CLS-cell lines service (Germany) and carefully maintained in a complete media containing DMEM, 10% FBS, 2mM L-glutamine, 4.5 g/L glucose, 100 IU penicillin and 100µg streptomycin. Human follicle dermal papilla cells (DPCs) were obtained from PromoCell (Germany) and cultured in a Basal medium supplemented with SupplementMix which contains 0.04 ml/ml fetal calf serum, 0.004 ml/ml bovine pituitary extract, 1 ng/ml basic fibroblast growth factor and 5 µg/ml insulin. The cells were cultured at 37°C in a humidified atmosphere with 5%CO_2. The DPCs passage number 5 were used in this study.

2.4 Cell-based experiments

2.4.1 Cytotoxicity test

The cells were treated with various concentrations of moringa oil and then incubated at 37ºC in the humidified atmosphere with 5%CO_2 for 24h. Cell viability was analyzed by using MTT assay (Mosmann, 1983) and the absorbance measured at 570 nm. The results were calculated for the percentage of cell viability.

2.4.2 Hair growth-related gene expression

Semi-quantitative-reverse transcription-polymerase chain reaction (RT-PCR) was used and modified from previous studies (Iehle *et al*., 1999; Rho *et al*., 2005). The cells were seeded at a density of 10^6 cells / well in a 12-well tissue culture plate and incubated overnight. The cells were treated with samples and incubated at 37°C in a humidified atmosphere with 5%CO_2 for 24h. Then, the cells were harvested by trypsinization. Total RNA was isolated using an RNA isolation kit according to the manufacturer's instructions. Specific oligonucleotides were based on the published sequences as shown in Table 1. cDNA was synthesized from 40 ng of total RNA with a two-step RT-PCR kit using a thermal cycler (Biometra, Germany). The conditions were denaturation at 94ºC for 1 min (all other genes); annealing at 60ºC, 1 min (β-actin), 58ºC, 1 min (VEGF, TGF-β1), 57ºC, 1 min (5αRI, 5αRII), and primer extension at 72ºC for 1 min (all other genes). The amplification was completed for 30 cycles and the PCR products were then analyzed on 2% agarose gel, visualized by Novel Juice staining and RT-PCR product density was measured by Gel documentation and a system analysis machine (Gel Documentation InGenius L, Bio-rad Lab, Hercules, CA, USA). Expression of hair growth related genes was determined as the relative mRNA expression level with β-actin from the same template.

Table 1. Nucleotide sequences of the primers of the studied genes

Gene	Nucleotide sequence	Size (bp)
β -actin	Forward: 5´-GGCACCACACCTTCTACAATGAG-3´ Backward: 5´-CGTCATACTCCTGCTTGCTGATC-3´	661
VEGF	Forward: 5´-CTACCTCCACCATGCCAAGT-3´ Backward: 5´-GCGAGTCTGTGTTTTTGCAG-3´	536
TGF-β1	Forward: 5´-GTGGAAACCCACAACGA-3´ Backward: 5´-GAGGTCCTTGCGGAAGT-3´	579
5αRI	Forward: 5´-GGTTTTGGCTTGTGGTTA-3´ Backward: 5´-CAAAATAGTTGGCTGCAG-3´	142
5αRII	Forward: 5´-TACACAGACATACGGTTT-3´ Backward: 5´-CTTGTGGAATCCTGTAGC-3´	127

2.5 Animal study

The C57BL/6 mice were divided into 6 groups, each with 5 animals (untreated, minoxidil, vehicle and 3 concentrations of moringa oil (MO-2). The hair (2x3 cm^2) of the dorsal portion of the animals was shaved off with a razor blade. All the shaved animals were synchronized in the telogen stage (Rho *et al*., 2005). Then, 100 µl of the tested solution were applied daily to the shaved area. This treatment was continued for 20 days. Promotion of hair growth was evaluated by observing the skin color, which is indicative of the telogen-to-anagen conversion. The mice were killed at day 20. The 10 mm skin pieces were cut and further processed by using paraffin technique, and stained with haematoxylin and eosin. From the histological characterization of the sections, the number of hair follicles per square millimeter of the skin and the percentage ratio of hair follicle in anagen and telogen phases were determined using a microscope fitted with an ocular micrometer facility. Follicles were counted manually in the dermis and subcutis layers by a blinded observer at a fixed size. The anagen phase follicles are in the deep subcutis while the follicles in telogen phase lie only in the dermis (Datta *et al*., 2009; Junlatat & Sripanidkulchai, 2014). Skin thickness from epidermis to panniculus carnosus also was measured.

2.6 Determination of fatty acid contents

2.6.1 Sample preparation

Moringa oil samples were prepared at a concentration of 8 mg / ml using 0.5 M NaOH in MeOH as a solvent. Then, 1 ml of 2000 ppm internal standard (C17: 0) was added and mixed. This mixture was then refluxed in the water bath and left at room temperature for 5 minutes. After that, 14% BF3 in Methanol was added and refluxed at 100’C for 15 minutes and left at room temperature for 5 minutes. 10 ml saturated NaCl solution and 4 ml hexane were added, mixed and left to separate the layers between hexane and water. The samples in the hexane layer were injected into the gas chromatography.

2.6.2 Gas chromatography condition

The samples were analyzed using CHRMPACK (CP9001) gas chromatography (GC). GC system was comprised of 280°C flame ionized detection (FID), WCOT FUSED SILICA 50 M x 0.25 MM ID COATING CP-SIL 88 TAILOR MADE FAME CP7488 columns. The injection temp was 270°C and the oven temp was 140°C (5 min) - 200°C (15 min). The injection volume was 1 µl and carrier gas was 100 kPa He.

3. Results

3.1 Cytotoxicity test

Three sources of moringa oils up to 5% concentration were tested for the cytotoxicity in keratinocytes (HaCaT) and dermal papilla cells (DPCs). As shown in Figure 1, these moringa oils gave a similar pattern of effects in both HaCaT and DPCs. At low concentration, moringa oils stimulated the cell proliferation, whereas at high concentration they were cytotoxic to the cells. The proliferative stimulating and cytotoxic effects of these three sources of moringa oils on HaCaT were not different (Figure 1A). In contrast, at concentrations up to 0.625%, these three moringa oils showed

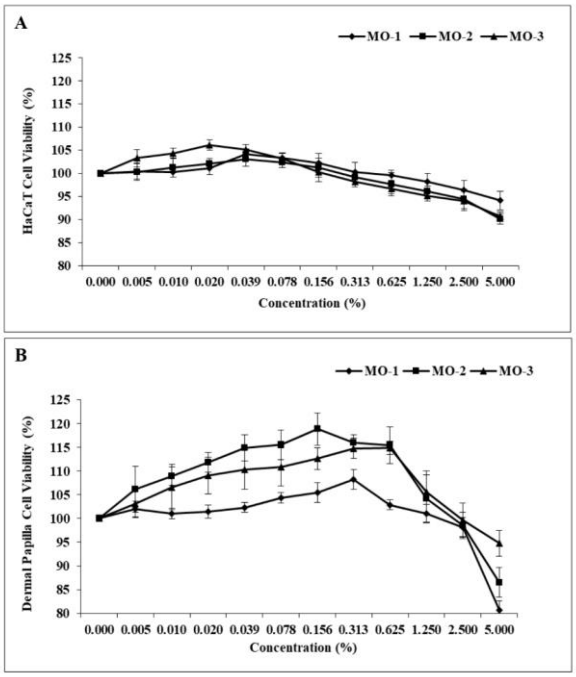

Figure 1. Effects of moringa oils on the viability of HaCaT (A) and DPCs (B)

different proliferative stimulating effects on DPCs with the relative order of higher to lower of MO-2>MO-3>MO-1 (Figure 1B). Taken from these results further experiments were conducted with the highest concentration at 5% of these three moringa oils.

3.2 Efficacy study

To investigate the efficacy of moringa oils, the cell-based experiments on the hair-growth related gene expressions in both HaCaT and DPCs, and the *in vivo* hair growth-promoting effect in mice were performed. By semi-quantitative-RT-PCR method, the moringa oils up-regulated VEGF gene and down-regulated TGF-β1, 5α-reductase type I and type II in both HaCaT and DPCs with dose-dependent manners (Figure 2, 3). However, the modulatory effects on these hair-growth related genes were different among these three moringa oils. Considering from the effective concentration at 20 % (EC_{20}) (Table 2), MO-2

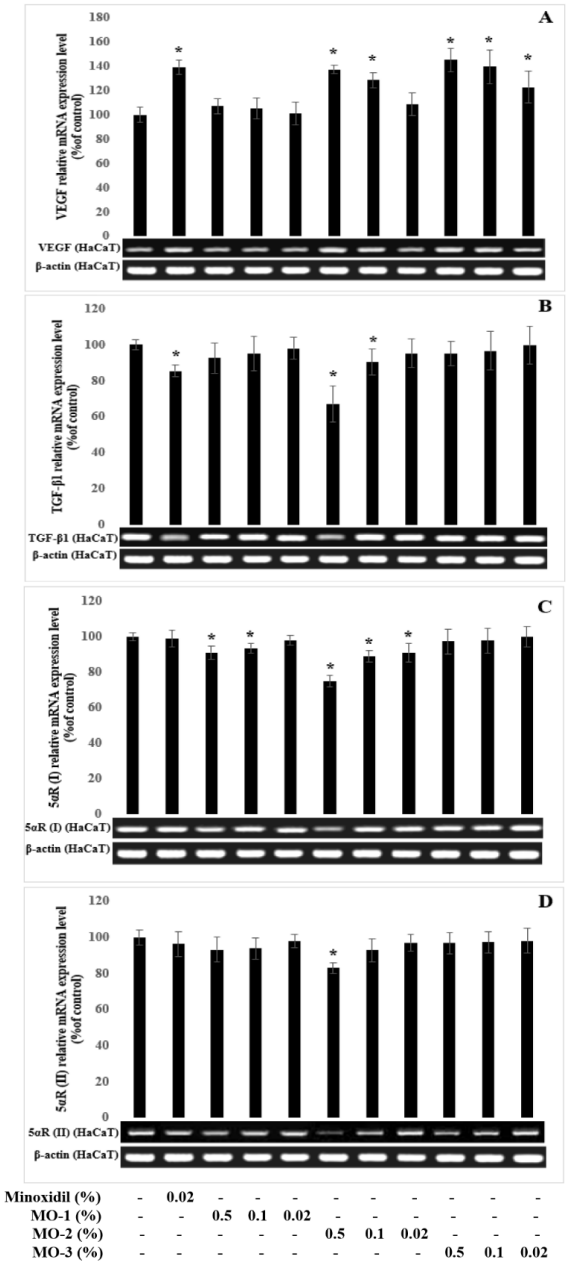

Figure 2. Effects of moringa oils on the genetic expressions of VEGF (A), TGF-β1 (B), 5αR (I) (C) and 5αR (II) (D) in HaCaT cells, * Significant difference from untreated group at $p < 0.05$

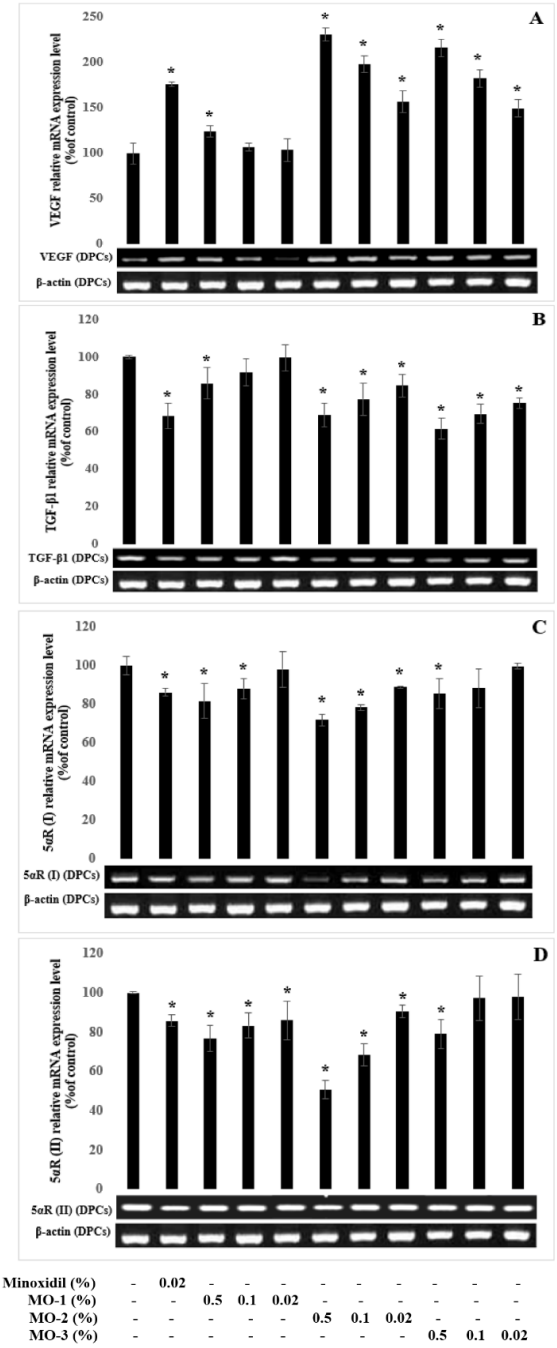

Figure 3. Effects of moringa oils on the genetic expressions of VEGF (A), TGF-β1 (B), 5αR (I) (C) and 5αR (II) (D) in dermal papilla cells, * Significant difference from the untreated group at $p < 0.05$

Table 2. Effective concentration at 20% (EC_{20}) of moringa oils on the expressions of the hair growth related genes in keratinocytes and dermal papilla cells

Genes	EC_{20} (%)*			
	MO-1	MO-2	MO-3	Minoxidil
up - regulation				
VEGF				
HaCaT	>0.50	0.10±0.01	<0.02	<0.02
DPCs	0.40±0.09	<0.02	<0.02	<0.02
down-regulation				
TGF-β1				
HaCaT	>0.50	0.28±0.05	>0.50	0.03±0.00
DPCs	>0.50	0.11±0.01	<0.02	<0.02
5αRI				
HaCaT	>0.50	0.35±0.02	>0.50	0.38±0.02
DPCs	>0.50	0.19±0.01	>0.50	0.03±0.00
5αRII				
HaCaT	>0.50	>0.50	>0.50	0.12±0.01
DPCs	0.31±0.03	0.06±0.00	0.48±0.06	0.03±0.00

*Expressed as percentage of mean ± SD of the effective concentration at 20%

and MO-3 showed similar effects on the up-regulation of VEGF gene in both HaCaT and DPCs, whereas MO-1 did not stimulate the expression of VEGF in HaCaT and slightly increased VEGF expression in DPCs. The differentiated effects on the down regulation of TGF-β1 gene was also observed. Only MO-2 decreased the genetic expression of TGF-β1 in HaCaT, whereas in DPCs all moringa oils inhibited the expression with the relative order of higher to lower effects of MO-3>MO-2>MO-1. The differentiated effects of moringa oils on the down-regulation of the 5α-reductase enzymes were also found. MO-2 showed the highest effect on the expressions of 5α-reductase type I and type II in both HaCaT and DPCs with the relative effects as MO 2>MO-1>MO-3. These results implied the differential and selective effects of these moringa oils on the hair growth related genes. Moreover, these effects were comparable with the effect of the standard drug minoxidil. Based on the finding that MO-2 possessed the highest activity in the cell-based experiment, it was further studied for the hair growth effect in the animal model. By applying MO-2 on the dorsal part of the hair-shaved mice for 20 days, the hair growth promotion was observed as a dose-dependent manner and similar to the effect of minoxidil (Figure 4A). Histological examination of the skin section also demonstrated the promoting effect of MO-2, as observed that the skin layer was thicker with the higher anagen to telogen ratio and number of hair follicles (Figure 4B and Table 3). The hair growth-promoting effect of MO-2 was comparable with the effect of minoxidil.

3.3 Fatty acid analysis

In general, the physical characteristics of the three moringa oils were similar with a slightly viscous consistency and yellow color. The fatty acid contents were determined and unsaturated oleic acid was found to be the highest amount in these moringa oils. The contents of other fatty acids were slightly different, in which the unsaturated fatty acids included palmitoleic, elaidic, linoleic and eicosenoic acids and the saturated fatty acids included palmitic, stearic, arachdic and behenic acids (Table 4). The GC-chromatogram of 3 sources moringa oils is shown in Figure 5.

4. Discussion

The problem of hair loss has a great impact on personality and quality of life. The searching for novel products from herbs with high efficacy and safety is an important goal of industries. With the multipurpose usage of moringa oil for foods, cosmetics and medicines, the development of its products requires the supportive scientific data. Currently, several hair growths-promoting products containing moringa oil are commercially available. Our study has provided the evidence that moringa oil promotes the hair growth by modulating the genetic expressions of factors affecting the hair growth cycle. The cold pressed preparation of the three moringa oil products used in this study showed a similar pattern of fatty acid content as previously reported (Leone *et al*., 2016), which is a very high amount of unsaturated oleic acid. The differences of each fatty acid content in the tested samples reflect the different source of the raw material seeds and to provide the high quality and reliable products, the consistency of fatty acid content should be more of a concern. There are several studies support fatty acid as a hair growth enhancer. The main compound in moringa oil is a fatty acid group, therefore the hair growth enhancing effect possibly comes from these compounds (Shimizu *et al*., 2000; Ryu *et al*., 2021). In cell-based study, dermal papilla cells and keratinocytes were used as models in this study because they are important in the growth of hair. Both cells are major components of hair, and produce a number of growth factors and the major protein of hair (Junlatat & Sripanidkulchai, 2014). Our results from the cell-based study suggest the modulatory effect of moringa oil on the hair growth cycle. The up-regulation of VEGF gene was found in both keratinocytes and dermal papilla cells, which suggests the role in angiogenesis of the moringa oil, to facilitate the oxygen supply to the cells and promote the hair growth (Yano *et al*., 2001). There were several herbal hair growth supplements that

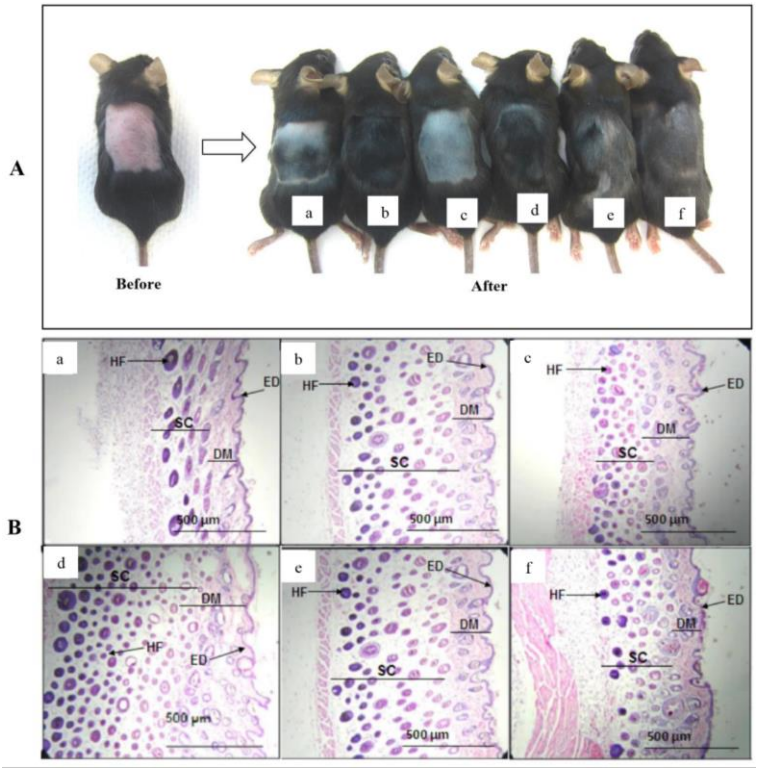

Figure 4. Hair growth-promoting effect of MO-2 in mice (A) and the representative transverse sections of mouse skin (B). a=untreated, b=2% minoxidil, c=vehicle, d=2%MO-2, e=1%MO-2 and f=0.5%MO-2 *(HF=hair follicle, SC=subcutis, DM=dermis, ED=epidermis)*

Table 3. Effect of MO-2 on the skin thickness, number and growth phase of hair follicles in mice (n=5)

Group	Skin thickness (µm) [a]	Hair follicle number (% of control) [b]	Anagen/telogen ratio (%) [c]
Untreated	402.13 ± 31.91	100.0	70.4 / 29.6
Vehicle	548.94 ± 34.04*	149.6 ± 11.25*	71.4 / 28.6
2% Minoxidil	980.85 ± 39.15*	317.3 ± 25.36*	87.9 / 12.1*
0.5%MO-2	563.83 ± 48.94*	184.1 ± 13.24*	74.9 / 25.1*
1%MO-2	576.60 ± 44.68*	193.5 ± 15.23*	79.5 / 20.5*
2%MO-2	651.06 ± 46.81*	217.8 ± 15.69*	80.2 / 19.8*

[a] Measured from epidermis to subcutis layer, expressed as mean±SD
[b] Hair follicle number/mm^2, expressed as % of control
[c] Hair follicle number in subcutis layer / hair follicle number in dermis layer, expressed as %average
*P < 0.05 vs. untreated

Table 4. The fatty acid contents in moringa oils

Fatty acid content (%)	Moringa oils		
	MO-1	MO-2	MO-3
Saturated fatty acid			
Palmitic acid	13.08	11.30	9.32
Stearic acid	4.97	7.26	5.71
Arachidic acid	2.17	3.10	3.39
Behenic acid	2.42	2.91	6.51
Unsaturated fatty acid			
Palmitoleic acid	0.00	1.60	1.03
Elaidic acid	1.43	2.41	1.32
Oleic acid	59.52	66.16	69.45
Linoleic acid	14.95	3.57	0.54
Eicosenoic aicd	1.46	1.69	2.73

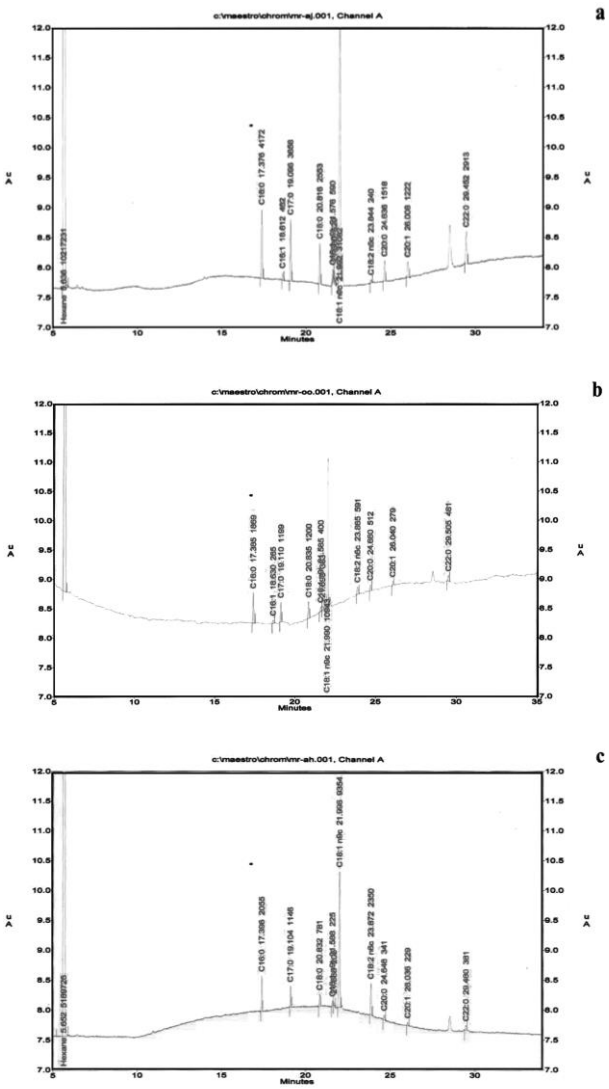

Figure 5. GC-chromatograms of MO-1 (a), MO-2 (b) and MO-3 (c)

have an effect on the expression of VEGF such as *Carthamus tinctorius* (Junlatat & Sripanidkulchai, 2014) and *Asiasari radix* (Rho *et al*., 2005). In contrast, the down-regulations of TGF-β1, 5α-reductase type I and II genes were also observed in both keratinocytes and dermal papilla cells. Inhibition of TGF-β1 and 5α-reductase of other herbs have previously been reported, such as *Rosmarinus officinalis* (Murata *et al*., 2013) and *Forsythia supensa* (Shin *et al*., 2015). These findings confirm the promoting effect of moringa oil on the decrease of factors interfering with hair growth cycle. As previously reported, 5α-reductase stimulates the conversion of testosterone to be dihydrotestosterone which could trigger hair loss by the action of TGF-β1 (Rho *et al*., 2005). Taken together, moringa oil affects the hair growth cycle via dual actions, one is in a similar fashion as minoxidil by increasing the duration of anagen phase and another one is in a similar action of finasteride to inhibit 5α-reductase (Sawaya & Shapiro, 2000; Sudduth & Koronkowski, 1993). In addition, moringa oil can inhibit both expressions of 5α-reductase type I and II. Both types play an important role in hair loss. 5α-reductase type II has a relatively clear report of its role in hair loss. A type I 5α-reductase, which also metabolizes testosterone to DHT, is distinguished from the type II enzyme and is the principal isoenzyme in sebaceous and sweat glands. Currently, there is an effective hair loss treatment that can inhibit both types I and II, dutasteride (Olsen *et al*., 2006). This study was conducted in keratinocytes (HaCaT) and dermal papilla cells (DPCs) because they are important in the growth of hair. Keratinocytes are part of the hair follicle and hair shaft, and produce the major protein of hair, keratin. Besides, the DPCs are major components of hair and produce a number of growth factors (Rushan, Fei, Zhirong, & Yu-zhang, 2007). With these dual effects, promoting hair growth and inhibiting hair loss factors, and without irritation or redness, moringa oil should be a potential candidate for the therapy of hair loss. Our results of the animal study obviously support the cell-based data. The hair growth-promoting effect of moringa oil in mice can be observed after 20 days of topical administration of moringa oil (Figure 4). Moreover, the histological analysis confirmed the promoting effect, in which the increase in dermis thickness and number of hair follicles of the moringa oil-treated mice is a dose-response and comparable to the effect of minoxidil. The increase in the ratio of anagen to telogen of hair follicles also indicated that moringa oil prolonged the anagen phase and shortened the telogen phase. Moringa oils from three different sources showed a similar pattern on the gene expressions but with different levels of action. Since MO-2 was the most potent sample and for the ethical concerns on minimal animal use, the animal study was conducted only with MO-2. However, the differentiated and selective effects of moringa oil on each gene expression may require further studies on the actual effective dose for clinical uses of this products. Furthermore, the constituents of moringa oil that give the effectiveness are required for further elucidation.

5. Conclusions

In conclusion, this study is the first report on the effect of moringa oil on the level of genetic expressions of genes related to hair growth promotion. Moringa oil promoted the hair growth via stimulation of VEGF and suppression of TGF-β1, 5α-reductase type I and II in both keratinocytes and dermal papilla cells. Moringa oil also increased hair growth in mice, similar to the effects of minoxidil. The findings confirm the traditional use of moringa oil as a hair growth-promoting product.

Acknowledgements

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