

SUPPLEMENTARY MATERIAL

Multiple-physiological benefits of bird's nest fern (*Asplenium australasicum*) frond extract for dermatological applications

Wen-Wen Zeng^a and Lih-Shiuh Lai^{a*}

^a*Department of Food Science and Biotechnology, National Chung Hsing University, Taichung, Taiwan.*

*Corresponding author. E-mail: ls Lai@ dragon.nchu.edu.tw

Abstract

p-coumaric acid and fucose-rich polysaccharide have been studied for many bio-functions in skin including cutaneous protection from oxidative damage and anti-aging, respectively, as well as wound healing. The physiological activities of various bird's nest fern extracts (BNFE), containing considerable fucose-rich mucilage and *p*-coumaric acid, on fibroblast and human skin were first investigated. BNFE with higher polysaccharide content generally contributed to a better moisture holding capability. Furthermore, BNFE showed pronouncedly enhancing effect on collagen production and growth of fibroblast (NIH-3T3), clinical trial results revealed that the emulsions with 1% BNFE showed good moisturizing effect and improved the elasticity of human skins effectively. The potential of BNFE for cosmetics and medical applications such as natural moisturizer, anti-aging and wound repairing was possibly related to the fucose-rich mucilage and various phenolic compounds including *p*-coumaric acid in BNFE.

Keywords: bird's nest fern, fibroblast, skin aging, collagen, fucose-rich mucilage, *p*-coumaric acid

Experimental

Materials

The edible fronds of *Asplenium australasicum* (J.Sm.) Hook, harvested in September 2011 and purchased from a contracted farmer in Hualien, Taiwan (24.03' N, 121°59' E), with midrib strongly and acutely keeled on lower surface which is consistent with features of voucher specimen (No. 248383) identified by Assoc. Prof. Chen-Meng Kuo (Institute of Ecology and Evolutionary Biology, National Taiwan University, Taiwan) and deposited at Herbarium of National Taiwan

University, Taiwan. The bird's nest fern fronds were freeze-dried with a freeze dryer (Atlas Freeze Drying Batch Plant RAYTM125, Denmark) and then ground, sieved (40 meshes) for further analysis (Zeng and Lai 2016).

Preparation of bird's nest fern extracts (BNFE) and mucilage (WM)

The lyophilized powders (15 g) of bird's nest fern were mixed with 750 ml of deionized water, 25% and 40% aqueous ethanol, respectively. The extraction was performed by using an ultrasonicator (Elma Schmidbauer GmbH, Elmasonic P 120H, Germany) under a condition of 80 Hz, 100% Power (330 W), 40°C for 30 min. The extract was then centrifuged (6000 g, 4°C, 15 min) and filtered through a Whatman No.1 paper to remove the solid debris. The filtrate was concentrated to around 90 ml by using a rotary evaporator at 40°C. The concentrate was lyophilized (Eyela FDU-506, Tokyo, Japan) to obtain the water, 25% and 40% aqueous ethanol extracts, which were expressed as WE, AE25 and AE40, respectively. The mucilage of the bird's nest fern was isolated by adding three volumes of 95% (v/v) ethanol to the filtrates as above described and standing at 4°C overnight in order to precipitate the polymers. After centrifugation (6000 g, 20 min), the precipitate was then lyophilized to obtain water-extracted mucilage (WM).

Chemical components in BNFE

Protein, polysaccharide, total phenolics and flavonoid content

The total protein content of bird's nest fern extracts was quantified by using a Bradford protein assay kit according to Bradford method (Bradford 1976). The polysaccharide content was determined by using phenol-sulfuric acid method (Dubois et al. 1956).

The total phenolics content was quantified by performing a Folin-Ciocalteu assay according to (Magalhães et al. 2010) with slight modification. 200 µL of bird's nest fern extracts (2 mg/ml) were mixed with 200 µL of Folin-Ciocalteu reagent (diluted with water for 1/5 times), followed by the addition of 400 µL of sodium hydroxide solution (0.35M). The absorbance of the blue complex formed was measured at 750 nm after 3 min. The flavonoid content was determined according to the method of (Quettier-Deleu et al. 2000) with slight modification. 400 µL of bird's nest fern extracts (4 mg/ml) was mixed with 400 µL of 2% aluminium chloride aqueous solution, the absorbance of yellow complex formed was measured at 430 nm after 3 min.

Monosaccharide composition of WM

Monosaccharide composition of WM was analyzed according to the method of Zeng and Lai (2016). Briefly, after bird's nest fern mucilage was hydrolyzed and derived with

p-aminobenzoic ethyl ester (ABEE) derivatization reagent, the ABEE-labeled monosaccharide was separated by using an HPLC system equipped with a Gemini-NX C18 column (46×250 mm, phenomenex, USA). Elution was conducted under an isocratic condition (90% of 0.04 M potassium buffer (pH8.9) and 10% of acetonitrile) at a flow rate of 0.8 ml/min. The optical density at 308 nm was recorded by a UV detector (HITACHI, L-2400, Japan). Quantification was performed by using standard curves constructed with various concentrations of standard mixtures containing GalA, GlcA, Gal, Man, Glc, Ara, Xyl, Fuc and Rha.

Phenolic acid analysis of BNFE

The phenolic acids of BNFE were analyzed according to the method of Mattila and Kumpulainen (2002) with slight modification. Briefly, 0.2 g of sample and 10 mL of the mixture of 0.2% BHA in methanol and 10% acetic acid (85:15) were added into a 50-mL tube, and then ultrasonicated for 30 min by using an ultrasonicator (Elma Schmidbauer GmbH, Elmasonic P 120H, Germany). Distilled water and 10 M NaOH were added into the mixture, followed by rotating overnight at room temperature (about 16 h) using a mixer (Intelli Mixer, SLRM-2M, Mylab, Korea). The solution was adjusted to pH 2, and the liberated phenolic acids were extracted three times with 15 mL of a mixture of cold diethyl ether (DE) and ethyl acetate (EA) (1:1) and centrifuged (4020 g, 4 °C, 10 min). DE/EA layers were combined, evaporated (40 °C) by using a rotary vacuum evaporator (EYELA, SN series, Tokyo Rikakikai Co., Ltd., Japan) to dryness, and dissolved into 1.5 mL of methanol (80%). The above sample was injected into an HPLC system equipped with a Synchronis C18 column (250 × 4.6 mm, 5 μm, Thermo scientific™, USA). Gradient elution was employed with a mobile phase consisting of 0.1% formic acid (solution A) and methanol (solution B) as follows: 90% A/10% B at 0 min; 80% A/20% B at 10-20 min; 60% A/40% B at 40 min; 50% A/50% B at 60-80 min at a flow rate of 1.0 ml/min. The absorbance at 280 nm was recorded by a UV detector. Quantification was performed by using standard curves constructed with various concentrations of standards including gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, vanilic acid, syringic acid, sinapic acid, p-coumaric acid, ferulic acid, o-coumaric acid, m-coumaric acid, cinnamic acid, and salicylic acid.

Physical moisture holding capability against harsh environments

The moisture holding capability of BNFE was determined according to the method of (Zeng and Lai 2016). Ten grams of 0.5% *A. australasicum* mucilage sample solution or deionized water were poured into a 6-cm dish. The dishes containing sample (W_1) were placed in a controlled temperature–humidity chamber (GTH-072S, Giant Force Instrument Enterprise Co.,

Ltd., Taipei, Taiwan) at 45 °C and 40% relative humidity for 5 hours and weighed (W_2). The weight loss (g) of moisture (W_1-W_2) for deionized water and mucilage was calculated respectively. The moisture holding capability (%) of extracts was calculated according to the following equation:

$$\text{MHC (\%)} = \frac{\text{water loss (H}_2\text{O)} - \text{water loss (sample)}}{\text{water loss (H}_2\text{O)}} \times 100 \quad (1)$$

The effect of bird's nest fern extracts on the viability of fibroblast NIH-3T3

The murine fibroblast NIH-3T3 (BCRC 60008, ATCC CRL-1658) were purchased from the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan). The NIH-3T3 cells were cultured in DMEM media plus 10% calf serum and 1% penicillin and then maintained in a humidified incubator (Forma[®] Direct Heat CO₂ incubator, Bioway corporation, USA) with 5% CO₂ at 37 °C. For the cell viability assays, cells were seeded in a 24-well plate at a density of 2×10^4 cells/well for 24-hour incubation. Culture media (as control, A_C), BNFE, WM and *p*-coumaric acid (as sample, A_S) dissolved in culture media and filtered through a 0.2- μm -filter were added to each well of the plate, and the plate was incubated for an additional 24 and 72 h. Cell viability was determined via an MTT assay (Ciapetti et al. 1993). The formazan dissolved in DMSO was detected at 540 nm by a microplate reader (FLUOstar[®] Omega, BMG LABTECH, Germany). Blank (A_B) was carried out by an incubation without cell seeding but only culture media. Cell viability (%) was calculated by $(A_S - A_B) / (A_C - A_B) \times 100$.

Collagen accumulation in fibroblast NIH-3T3 cultures

Collagen content was determined with a Sircol Collagen kit through hydroxyproline estimation (Lareu et al. 2010). For collagen production secreted from fibroblasts NIH-3T3, the incubated media were collected after 72 h as described above (section 4.6). The absorbance of collagen-dye complex was determined at 555 nm. 0.5 M acetic acid and various concentrations of Type I rat collagen standard (10-90 $\mu\text{g/ml}$) were used as blank and standards, respectively. The percentage of collagen production relative to control was calculated by:

$$\text{Relative collagen production (\%)} = \frac{C_S}{C_C} \times 100 \quad (2)$$

where C_S is the collagen content of fibroblast cultured with sample, and C_C is the collagen content of fibroblast cultured with control.

Preparation of model emulsions with WE and AE40

Model emulsions with 1.0% WE, AE and control were prepared for the human experiment according to (Yih et al. 2014) with some modifications. The control o/w model emulsion was prepared for a vehicle tested as control, which was produced via the same procedure but excluding 1.0% WE or 1.0% AE. The emulsion formula included 3.0 % jojoba oil, 3.0 % capric triglyceride, 2 % span 80 (HLB=4.3), 1.4 % stearic acid, 0.5 % complex preservatives (containing 90% phenoxyethanol and 10% ethylhexylglycerin), 8 % glycerol, 2 % tween 20 (HLB=16.7), 0.4 % sodium alginate, 0.184% CaCl₂, deionized water and 1% WE or AE40.

Study protocol of human clinical experiment

All healthy female subjects (18 - 40 years old) volunteer went through an allergic test via smearing the tested formula emulsions at their inner skin of the forearms (5 x 5 cm²) for five hours. The subjects without allergic reaction to tested emulsions were divided into two groups (23 persons per group) for emulsion -WE/control and emulsion-AE40/control. The formula emulsions, with 1.0% WE or 1.0% AE40, were used on a constant arm skin while the control emulsion (vehicle) was for the other arm, and were applied on subjects' forearm (inner and outer) twice daily by self-control for four weeks according to the protocols of (Chang et al. 2008). The formula emulsions -WE and -AE40 were randomly given between subjects. All subjects were allowed to wash their skin normally, but were instructed not to apply any other skincare products on their forearms skin during the four-week testing period. After cleaning their forearm with water and detergent and drying with tissue gently, subjects were asked to stay in the room where the skin properties measurement would be conducted for at least 30 min for full skin adapt to the temperature and humidity of the room, prior to the skin properties measurements. The forearm skin of subjects was then measured at the 0 day (baseline) and the day after applying tested formula emulsions for one to four weeks.

Moisture content in stratum corneum

Moisture content in superficial epidermal layers (stratum corneum) of subjects was measured to evaluate the skin hydration by using Multi Skin Test Center[®] MC 1000 (Courage-Khazaka electronic GmbH, Germany) connected with a Corneometer[®] probe and a camera (Visioscope[®] PC35) to image the fine skin structure of subjects. The moisture content of stratum corneum, based on a capacitive method (Wu et al. 2003), is expressed as an arbitrary unit (A.U.) on a scale of 0-99. The change of moisture content was determined by taking the

measured value of before-using minus that of after-using for each subject every week during the testing period.

Elasticity of human skin

The elasticity of skin was measured with Multi Skin Test Center[®] MC 1000 connected with an elasticity measurement probe. The principle of skin elasticity measurement is to assess how the skin can resist the negative pressure of 400 mbar (*a*) in relation to its ability to return to the original position evaluated (*b*) by a suction method. Elasticity % is calculated by $(a-b)/a \times 100$ in a range of 0-99. The increases of elasticity was determined by taking the measured value of before-using minus that of after-using for each subject every week during the testing period.

Pigmentation of human skin

The melanin pigment of skin was measured with Multi Skin Test Center[®] MC 1000 connected with a melanin measurement probe with a foam ring for avoiding the interference of ambient light. The melanin content in a range of 0-99, based on the absorption/reflection principle, was calculated through the quantity of light with defined wavelength (660 and 880 nm, that correspond to pigments absorption (Soica et al. 2014)) absorbed by the tested skin. The inhibition of melaninization was determined by taking the measured value of before-using minus that of after-using for each subject every week during the testing period.

Statistical analysis

All data was reported as the mean of experiments conducted in triplicate. The statistical analysis was performed using IBM[®] SPSS[®] software version 19 (SPSS Inc., USA). One-way analysis of variance (ANOVA) was used for comparisons between groups. Student's *t*-test was used for comparisons between control and treatments. The intergroup of sample analysis was performed by using a Duncan's multiple-comparison test. Statistical differences between means were determined under a confidence level of 95% ($p < 0.05$).

Ethical approval

Forty six female volunteers with healthy skin were examined in the present study. Before the clinical experiment, each subject was informed about the details of this study and signed a letter of consent, which was approved by an Institutional Review Board (IRB) of National Cheng-Kung University Hospital, Taiwan. All procedures performed in studies involving

human participants were in accordance with the ethical standards of the institutional or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Tables

Table S1. Protein, carbohydrate, total phenolics, flavonoid contents, moisture holding capability (MHC) and inhibitory effect on tyrosinase activity of bird's nest fern extracts (BNFE)

	Protein^a	Carbohydrate^b	MHC	Total phenolics^c	Flavonoid^d	<i>p</i>-coumaric acid
	(SAE %)	(GE %)	(%)	(GAE mg/g)	(QE mg/100 g)	(mg/g)
WE	3.02 ±0.27 a	24.73 ±0.32 a	14.08 ±1.91 a	9.85 ±0.35 b	53.75 ±3.01 c	3.25 ±0.04 a
AE25	2.67 ±0.07 b	19.80 ±0.16 b	10.97 ±0.67 b	10.09 ±0.41 b	85.65 ±1.18 b	4.24 ±0.22 b
AE40	1.74 ±0.09 c	13.98 ±0.51 c	10.93 ±0.54 b	11.55 ±0.62 a	93.63 ±5.48 a	7.52 ±0.04 c

^a Protein content was expressed as serum albumin equivalent (SAE).

^b Total carbohydrate content was expressed as glucose equivalent (GE).

^c Total phenolics content was expressed as gallic acid equivalent (GAE).

^d Flavonoid content was expressed as quercetin equivalent (QE).

Means with different letters within the same column differ significantly ($p < 0.05$).

Table S2. Effect of bird's nest fern mucilage (WM) and *p*-coumaric acid on the viability of NIH-3T3 mouse embryonic fibroblasts cultivated in DMEM plus CS medium

		Concentration of aqueous extracts (µg/ml)				
days		10	25	50	75	100
WM	1 d	105.8 ±1.1 *	115.1 ±5.2 *	127.3 ±5.0 *	112.0 ±9.3	111.5 ±1.7 **
	3 d	106.4 ±0.9 **	103.0 ±1.3	103.0 ±1.3	97.8 ±3.9	96.8 ±1.4
		Concentration of aqueous extracts (µg/ml)				
days		0.5	1	2	5	10
<i>p</i>-Coumaric acid	1 d	107.7 ±1.4 *	110.4 ±2.7 *	112.9 ±1.6 **	113.7 ±0.9 **	112.2 ±2.1 **
	3 d	107.6 ±0.7 **	112.4 ±2.8 *	112.0 ±0.6 ***	112.5 ±1.2 **	108.6 ±0.4 ***

Each data was expressed as the mean of three replications ± standard deviation. Means with *, ** and *** differ significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively) with control (viability=100).

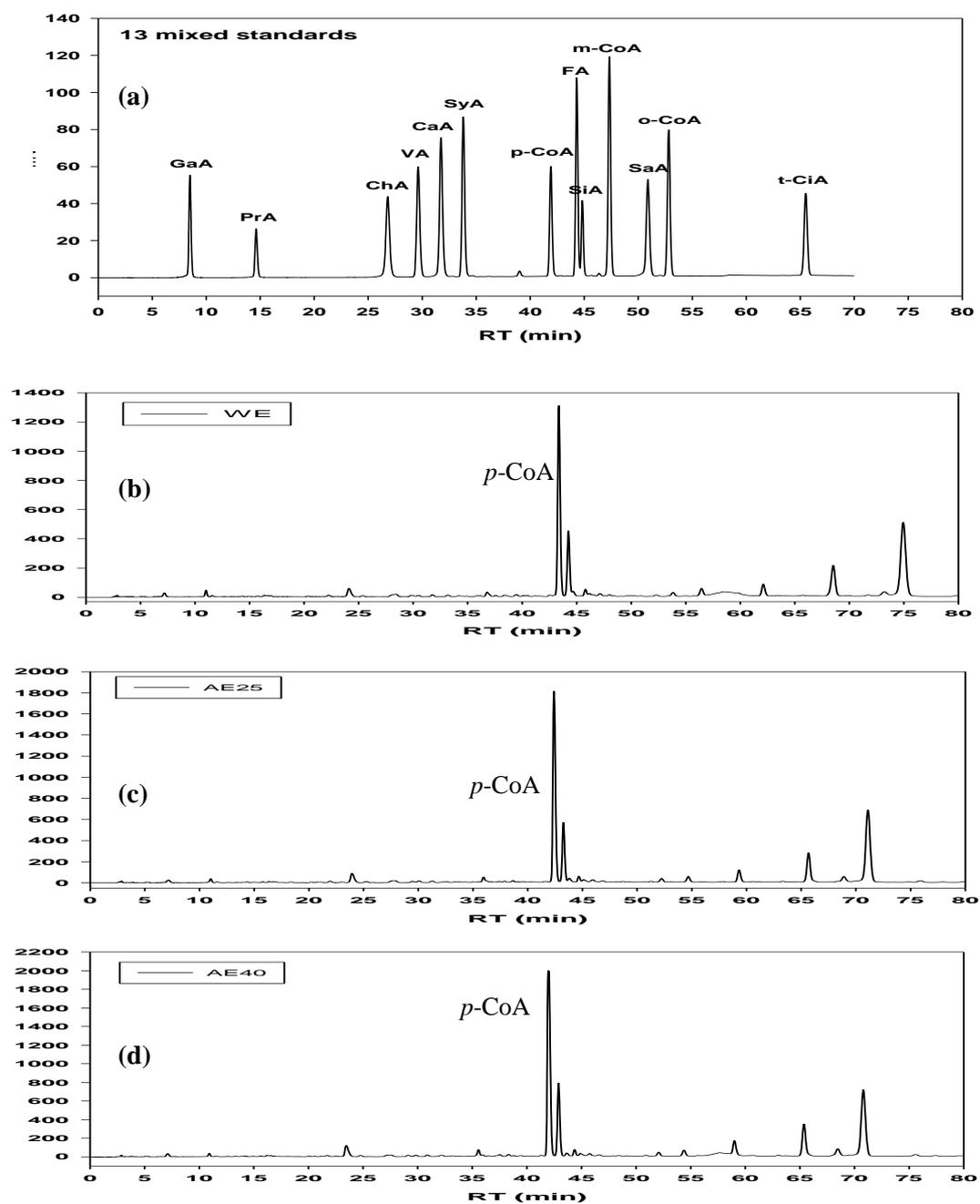


Figure S1. HPLC chromatogram of 13 mixed standards (a) and bird's nest fern extracts (b-d); GaA: gallic acid, PrA: protocatechuic acid, ChA: chlorogenic acid, CA: caffeic acid, VA: vanilic acid, SyA: syringic acid, SiA: sinapic acid, *p*-CoA: *p*-coumaric acid, FA: ferulic acid, *o*-CoA: *o*-coumaric acid, *m*-CoA: *m*-coumaric acid, *t*-CiA: cinnamic acid, and SaA: salicylic acid.

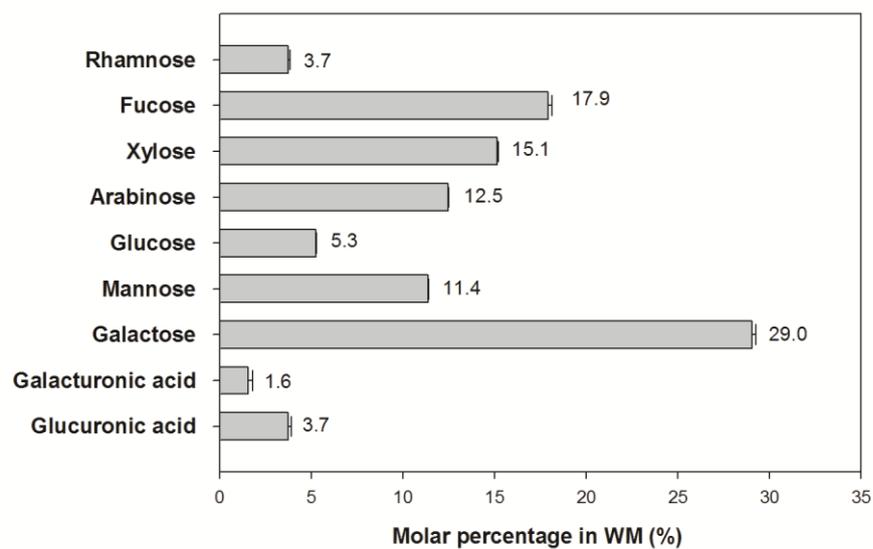


Figure S2. Monosaccharide composition of water-extracted bird's nest fern mucilage (WM).

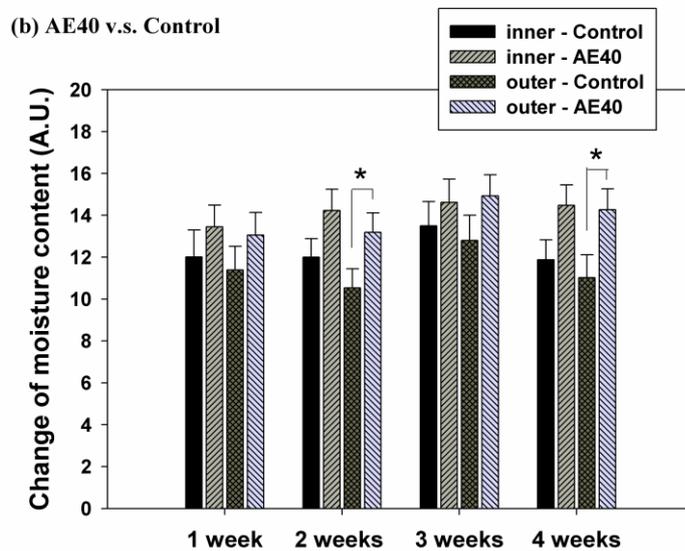
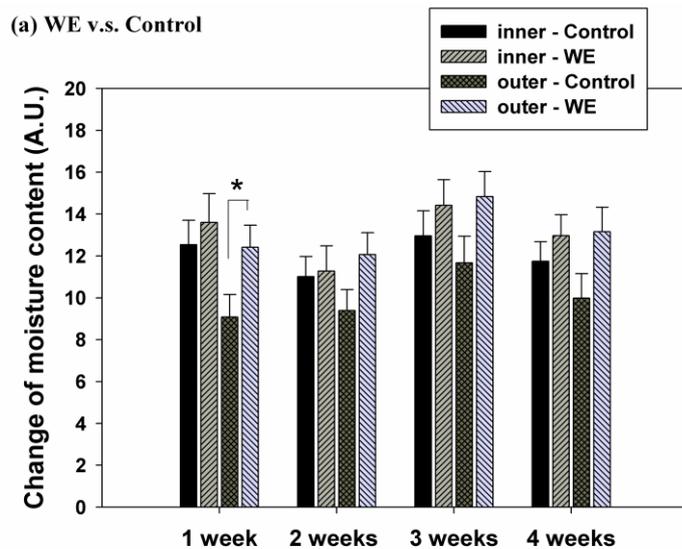


Figure S3. Change of moisture content on the forearms of female volunteers during 4-week period of daily application of emulsions with (a) WE and (b) AE40. Values are mean±S.E. of twenty three individuals. * denoted the mean was significantly greater than that of control ($p < 0.05$ analyzed by independent- t test) at the same week.

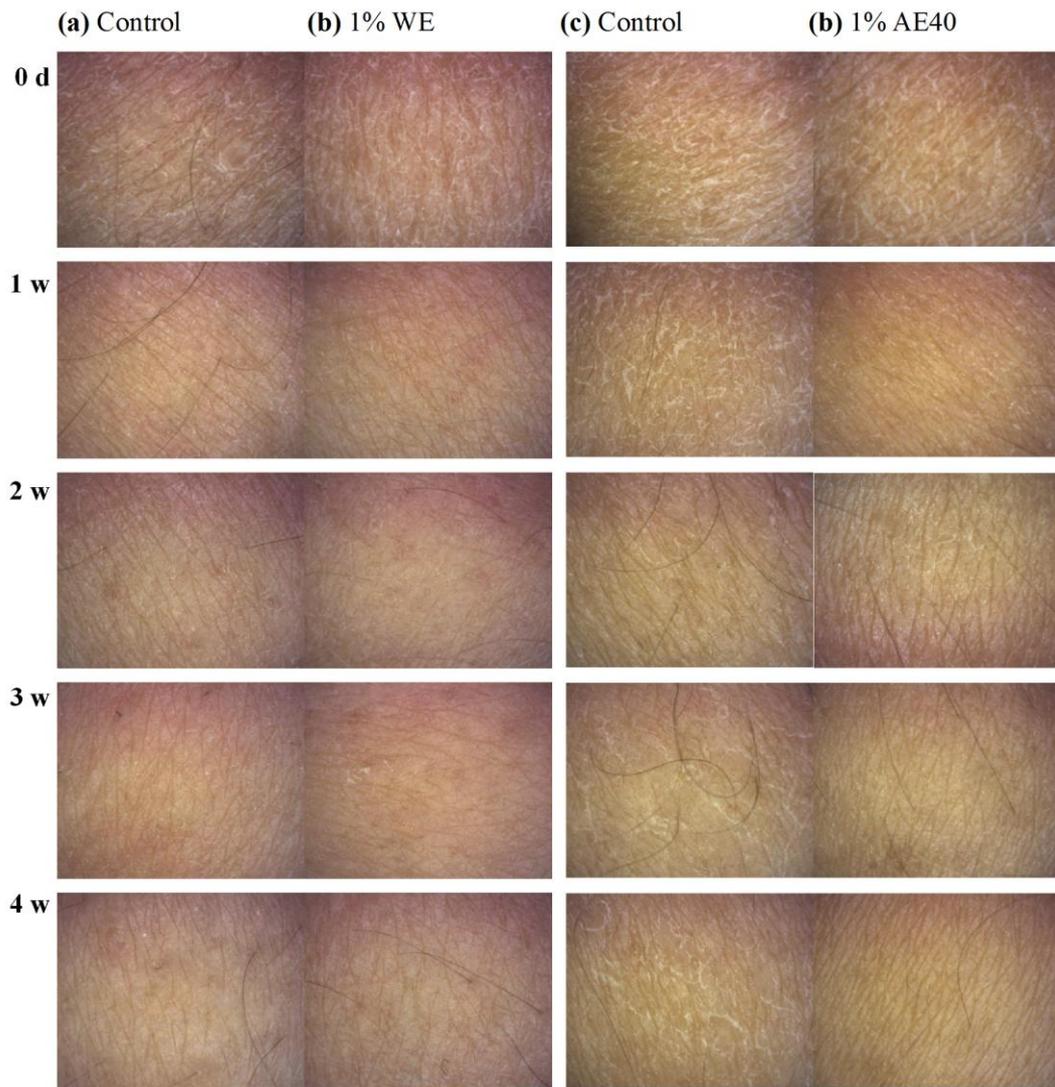
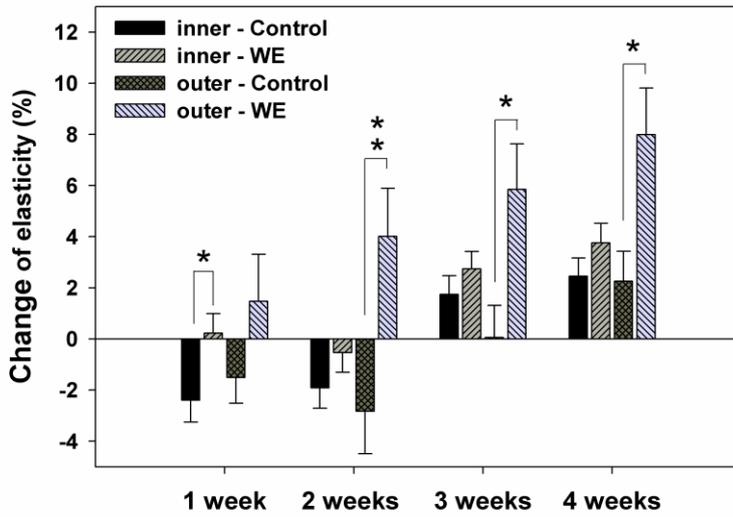


Figure S4. Typical images of the outside of forearm skin of subjects before (0 day) and after 1, 2 3 and 4 weeks of usage of emulsions with 1% WE (b) and 1% AE40 (d) as comparing to the control emulsion (a) and (c), respectively.

(a) WE v.s. Control



(b) AE40 v.s. Control

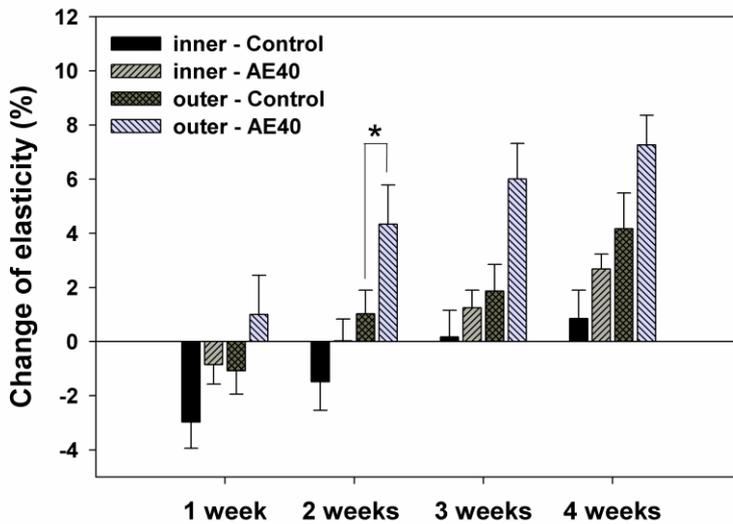


Figure S5. Change of elasticity on the forearms of female volunteers during 4-week period of daily application of emulsions with (a) WE and (b) AE40. Values are mean \pm S.E. of twenty three individuals. * and ** denoted the mean was significantly greater than that of control ($p < 0.05$ and $p < 0.01$, respectively, analyzed by independent- t test) at the same week.