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The protective effect of alfalfa (*Medicago sativa* L.) seed extract containing polysaccharides on human keratinocytes and fibroblasts

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Abstract: To protect the body against environmental threats, cosmetics can play important antiinflammatory and anti-aging roles. Many researchers have developed cosmetic ingredients using natural plant extracts or the active compounds of such extracts. In this study, we assessed the ability of extracts from the seeds of alfalfa (*Medicago sativa* L.) to affect skin cells. In a keratinocyte-derived cell line (HaCaT cells), alfalfa polysaccharide extract (APE) treatment triggered cornified envelope formation and increased the gene expression levels of filaggrin and involucrin, suggesting that APE increased epidermal cell differentiation. In addition, APE dramatically increased the mRNA and protein levels of claudin1, suggesting that it enhances tight junction formation. Finally, APE exerted antiinflammatory effects in keratinocytes by decreasing the LPS-induced production of proinflammatory cytokines. In primary normal human dermal fibroblasts (NHDF), APE increased procollagen synthesis and decreased elastase activity and metalloproteinase-1 (MMP) secretion. APE also showed superoxide dismutase (SOD)-like activity in a cell-free system, resulting in antioxidant effects. Finally, we found that the polysaccharides from alfalfa seeds used in our experiments consist mainly of galactose and mannose. Based on our findings, we conclude that APE may have potent skin-protective and skin-improving effects and could be developed as an important ingredient in cosmetics and medicines.

Keywords: alfalfa, fibroblasts, keratinocytes, *Medicago sativa* L., polysaccharides

INTRODUCTION

The skin embodies a number of critical functions necessary for animal survival, such as protection from water loss, radiation, trauma and infection, and animal perception of the environment [1]. Human skin shows numerous visible signs of aging, and many people invest in cosmetics and pharmaceutical products that help them prevent or partially reverse skin aging [2]. The physical barrier function of skin mainly resides in the stratum corneum (SC). Barrier dysfunction often correlates with downregulation of barrier-related molecules such as filaggrin (FLG), loricrin (LOR) and involucrin (IVL) [3]. The nucleated cells of the epidermis contribute to the barrier function by forming tight, gap and adherent junctions. The tight junction barrier is located in the granular layer of the epidermis [4]. Claudins are representative tight junction membrane proteins expressed in epithelial and endothelial cells that form paracellular barriers and pores that determine tight junction permeability [5].

Skin inflammation is observed after skin is exposed to various external stimuli, such as ultraviolet (UV) radiation, allergen uptake, microbial challenge, or contact with irritants [6]. Inflammaging has recently emerged to link aging and age-related diseases to inflammation. As the largest organ of the body and home to a significant portion of the human microbiome, the skin may play a unique role in inflammaging. Therefore, many researchers have tried to reduce the inflammation of the skin to achieve anti-aging effects [7]. UV irradiation

is an important factor in skin aging and inflammation because it triggers the formation of intracellular reactive oxygen species (ROS). UV and ROS enhance the expression of matrix metalloproteinase-1 (MMP-1) to increase collagen degradation in fibroblasts. Impaired collagen turnover and increased collagen degradation lead to skin aging and loss of firmness and elasticity [8]. Therefore, inhibiting the effects of UV radiation and/or the production of ROS could be a crucial anti-aging strategy for the skin.

Medicago sativa L., commonly known as alfalfa, belongs to the Fabaceae family and has a long history of dietary and medicinal use in traditional herbal medicine in Asian countries [9,10]. The high antioxidant capacity of *M. sativa* L. extracts suggests that these extracts could be valuable ingredients for the cosmetic and pharmaceutical industries [11,12]. *M. sativa L.* is widely used as animal feed due to its high content of fiber, protein, minerals, vitamins, chlorophyll and carotenoids [13]. In a previous study, soluble carbohydrates were identified and quantified during the development, maturation and desiccation of somatic embryos of *M. sativa* L. and compared with soluble carbohydrates in leaflets and mature seeds, with the aim of relating changes in soluble carbohydrates to maturation events [14]. We aimed to find out determine how alfalfa seed extract-containing polysaccharides can affect the physiology of epidermal HaCaT keratinocytes and normal human dermal fibroblasts *in vitro*. We also examined the protective efficacy of alfalfa seed extracts against cultured skin cells stimulated by external environmental factors, such as lipopolysaccharide (LPS) or UV.

MATERIALS AND METHODS

Preparation of alfalfa polysaccharide extract (APE)

Alfalfa seeds were grown in Australia and purchased from Danong Co. Ltd (Korea). The alfalfa seeds were ground and extraction was performed with purified water at a ratio of 1:10, at 65°C for 3 h. To remove impurities after extraction, 2% of the extract and celite was mixed for 1 h and then passed through a 1.0-μm filter. The filtered extract was slowly added to ethanol with stirring. The precipitated polysaccharide was collected, washed with ethanol, dried at 90°C for 48 h and ground using a mortar.

Cell culture

The human adult low-calcium high-temperature (HaCaT) keratinocytes were cultured in Dulbecco's modified Eagle's medium (DMEM; Welgene, Gyeongsan-si, Gyeongsangbuk-do, Korea) supplemented with 5% fetal bovine serum (FBS; Welgene) and 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. Normal human dermal fibroblasts (NHDF) were purchased from Lonza Ltd. (Basel, Switzerland) and grown in Fibroblast Basal Medium (FBM; Lonza Ltd., Basel, Switzerland), supplemented with a Bullet kit (FGM-2 SingleQuot Supplement & Growth Factors kit; Lonza Ltd., Basel, Switzerland).

Cell viability (WST-1 assay)

Cell viability was determined using a WST-1 assay kit (EZ-Cytox; DoGenBio, Seoul, Korea) according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates, cultured for 24 h, and then treated with different concentrations of the test samples. The cells were incubated for 24 h, 100 μL of medium containing 10 μL of water-soluble tetrazolium salt (WST) solution was added to each well, and the plates were incubated for 1 h at 37°C. The absorbance of each well at 450 nm was measured using an Absorbance Microplate Reader (Multiskan GO; Thermo Scientific, Waltham, MA, USA). Cell viability was determined by the equation:

$$\text{Cell viability (\%)} = \text{sample treatment group absorbance/control group absorbance} \times 100.$$

Cornified envelope formation assay

To induce HaCaT cells to form the cornified envelope, which contributes to the skin barrier, the calcium concentration was altered. HaCaT cells were seeded in 6-well plates (Falcon, Glendale, AZ, USA) containing 15×104 cells/well, cultured for 24 hours, then switched to calcium-free medium and cultured for another day. The cells were treated with APE and cultured for 96 h in a calcium-free medium and then collected. The collected cells were lysed in 0.1 M Tris-HCl containing 2% sodium dodecyl sulfate (SDS) and 20 mM dithiothreitol (DTT), all from Sigma-Aldrich, USA. The samples were boiled at 95°C for 2 min, and absorbance at 340 nm was measured to confirm cornified envelope formation.

Real-time qPCR

For quantitative real-time qRT-PCR analysis, total RNA was extracted using TRIzol solution (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual. First-strand cDNA was synthesized from 1 μg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). The cDNA samples were analyzed for the mRNAs encoding filaggrin (qHsaCED0036604), involucrin (qHsaCED0046054) and claudin1 (qHsaCID0006097). The expression levels of the target mRNAs were normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, qHsaCID0015464). A high-performance real-time PCR instrument (LightCycler® 96 System; Roche, Basel, Switzerland) was used to determine the gene expression levels. Primers were purchased from Bio-Rad (Hercules, CA, USA). The cycling conditions were as follows: 95°C for 10 min followed by 45 cycles at 95°C for 10 s, at 60°C for 10 s, and at 75°C for 10 s. Quantitation of gene expression was carried out by the comparative Ct method. All data were obtained from three or more independent experiments carried out in triplicate.

Sandwich enzyme-linked immunosorbent assay (ELISA) for IL-6 and IL-8

HaCaT cells (1.5×10^4 cells/well) were seeded in 96-well plates. After 24 h, the cells were treated with APE in the presence of 1 μg/mL LPS (Sigma-Aldrich, MO, USA). After incubation for 24 h, the culture media were collected and the concentrations of IL-6 and IL-8 were determined using ELISA Kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The results were measured using an absorbance microplate reader (Multiskan GO; Thermo Fisher Scientific, USA).

ELISA for MMP-1 and procollagen

To stimulate MMP-1 production by primary normal human dermal fibroblasts (NHDF), HaCaT cells were irradiated with 20 mJ/cm2 UVB as the major cause of sunburn and incubated for 24 h. The culture medium was used as a conditioned medium (CM). NHDF were seeded with 0.6×10^4 cells/well in a 96-well plate (Falcon) and cultured for 24 h and then switched to FBM without additives and cultured for another day. APEs were diluted in CM by concentration and applied to the NHDF. The cells were cultured for 24 h, and the supernatant was obtained and examined by ELISA for MMP-1 (R&D Systems, MN, USA) and procollagen ELISA (Takara Bio, Kusatsu, Shiga, Japan) according to the manufacturer's recommendations. Absorbance was measured at 450 nm.

Elastase activity assay

NHDF cell lysates were used to assess the effect of APE on elastase activity in fibroblasts. NHDF were seeded at 45×10^4 cells/flask in T75 flasks (SPL, Pocheon-si, Gyunggi-do, Korea), grown to confluency, washed with Dulbecco's phosphate buffered saline (DPBS) (Welgene, Korea), and collected. The obtained cells were lysed with 0.2 M Tris-HCl containing 0.1% Triton X-100 (both from Sigma-Aldrich) and then centrifuged to obtain the supernatant. A DC protein assay kit (Bio-Rad, Hercules, CA, USA) was used to quantify the protein contents. Equal amounts of protein were mixed with the substrate, STANA (Sigma-Aldrich), APE, or the positive control, phosphoramidon (Sigma-Aldrich) and allowed to react at 37°C for 90 min; absorbance was measured at 405 nm using a microplate spectrophotometer (Multiskan GO; Thermo Scientific).

SOD-like activity assay

APE and ascorbic acid, a positive control, were diluted with distilled water (DW) and SOD activity was measured using an EZ-SOD assay kit (DoGenBio, Korea) according to the manufacturer's recommendations. Absorbance was measured at 450 nm and SOD-like activity was determined by the equation:

$$\text{SOD-like activity (\%)} = (\text{absorbance of control} - \text{absorbance of sample})/\text{absorbance of control} \times 100.$$

Immunocytochemistry

HaCaT cells were seeded onto coverslips in 6-well plates (50×10^4 cells/well) and incubated for 24 h. After washing with 1× phosphate-buffered saline (PBS), the cells were serum-starved overnight and then treated with 100 or 200 μg/mL APE for 24 h. For immunocytochemistry, the cells were fixed for 10 min in 4%

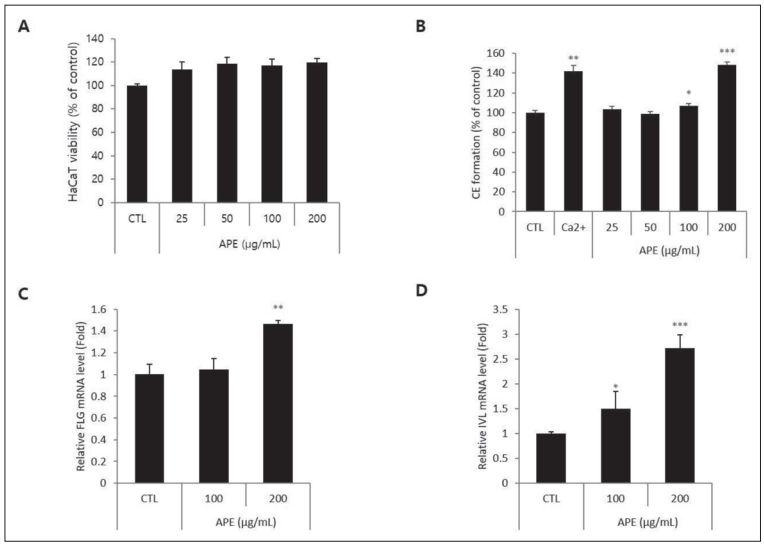


Fig. 1. Effects of APE on HaCaT cell viability and differentiation markers. **A** – Effect of APE on HaCaT cell viability. Cells were incubated 25-400 μg/mL APE for 24 h, and cell viability was assessed using the WST-1 assay. **B** – Effect of APE on cornified envelope (CE) formation. Cells were treated with 25-200 μg/mL APE or 1.2 mM calcium (Ca^{2+}, positive control) for 24 h. CE formation was determined using an absorbance microplate reader. **C**, **D** – Effects of APE on the mRNA levels of filaggrin (FLG) and involucrin (IVL) in HaCaT cells. Cells were treated with 100 and 200 μg/mL APE for 24 h. mRNA levels were determined by real-time PCR and normalized relative to that of GAPDH. Data are expressed as the mean±SD of at least three independent measurements. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control (CTL).

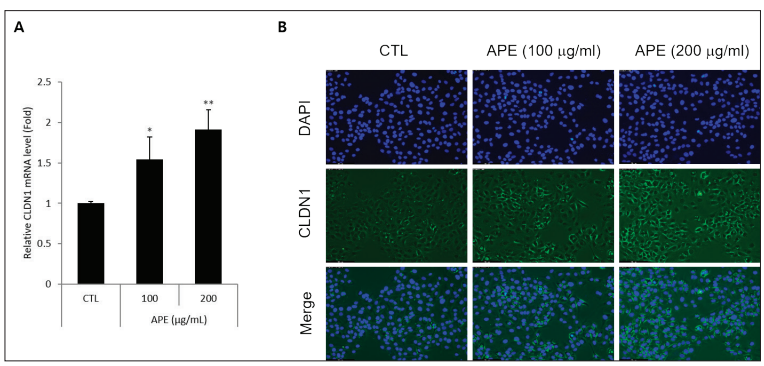


Fig. 2. Effect of APE on mRNA and protein levels of claudin1. **A –** Effect of APE on claudin1 (CLDN1) mRNA levels in HaCaT cells. Cells were treated with 100 and 200 μg/mL APE for 24 h. mRNA levels were determined by real-time PCR and normalized relative to that of GAPDH. **B** – Representative immunofluorescence staining of CLDN1 in HaCaT cells. The green shows CLDN1 and the blue shows DAPI. Data are expressed as the mean±SD of at least three independent measurements. $^{*}P<0.05$, $^{**}P<0.01$ versus control (CTL).

paraformaldehyde at 4°C, permeabilized with 0.1% Triton X-100 for 20 min and blocked with 10% bovine serum albumin (BSA) for 30 min at room temperature. The primary antibody against claudin1 (Invitrogen, Fisher Scientific, MA, USA) was diluted in 3% BSA and incubated with cells overnight at 4°C. Alexa Fluor 488-conjugated anti-rabbit IgG (Cell Signaling Technology, MA, USA) in 3% BSA was used as the secondary antibody at room temperature. The nuclei were stained with the fluorescent stain 4',6-diamidino-2-phenylindole (DAPI, Sigma Aldrich), and the slides were visualized using an inverted digital microscope (DMI8, Leica, Germany).

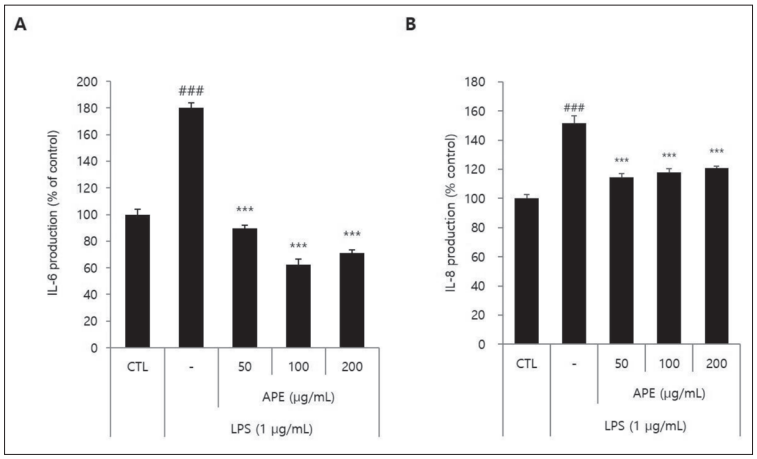
Statistical analysis

The results are expressed as the mean±standard deviation (SD). The data were analyzed using the Student's t-test, and a two-tailed value of $P<0.05$ was considered statistically significant.

RESULTS

Effects of APE on HaCaT cell viability and differentiation markers

Prior to examining the effects of APE on skin cells, we purified and identified its component polysaccharides. Our results showed that APE contained primarily galactose, followed by mannose, and these two components accounted for 99.69% of the total polysaccharide composition (Supplementary Fig. S1). To examine the effect of APE on epidermal keratinocytes, we treated HaCaT cells with increasing concentrations of APE, ranging from 25 to 400 μg/mL. Ape did not exhibit cytotoxicity against HaCaT cells (Fig. 1A). Treatment of HaCaT cells with 100 or 200 μg/mL yielded a significant formation of CE by 96 h (Fig. 1B) and significantly increased the mRNA expression levels of filaggrin and involucrin (Fig. 1C and D). These data suggest that APE induces CE formation and upregulates filaggrin and involucrin in epidermal keratinocytes, and thus could increase skin barrier function.



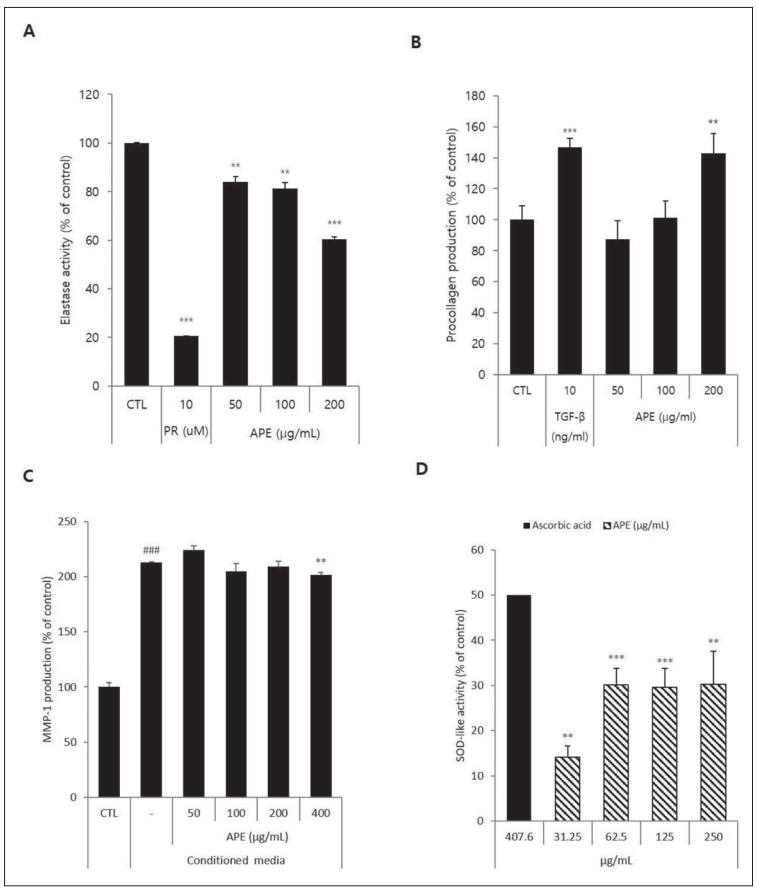


Fig. 4. Anti-aging and antioxidant effects of APE. **A** – Effect of APE on elastase activity in NHDF. Elastase solutions prepared using NHDF lysates were mixed with 50, 100, and 200 μg/mL APE or 10 μM phosphoramidon (PR, positive control) for 90 min. **B** – Effect of APE on procollagen production in NHDF. Cells were treated with 50, 100, and 200 μg/mL APE for 24 h and procollagen levels were determined by ELISA. **C** – Effect of APE on MMP-1 production in NHDF. NHDF were treated with 50, 100, 200 and 400 μg/mL APE in conditioned media (CM; 20 mJ/cm^2 UVB-irradiated HaCaT cultured media), and the amount of MMP-1 production was determined by ELISA. **D** – Anti-oxidant effect assessed by measuring the SOD-like activity of APE. Data are presented as the mean±SD. **P<0.01, ***P<0.001 versus control (CTL; A, B and D). $^{###}$P<0.001 compared with control (CTL); **P<0.01 versus conditioned media (C).

Fig. 3. Effect of APE on inflammatory cytokines IL-6 and IL-8 in HaCaT cells. **A**, **B** – Effect of APE on the LPS-stimulated productions of IL-6 and IL-8 in HaCaT cells. Cells were treated with 50, 100 and 200 μg/mL APE for 24 h and then stimulated with 1 μg/mL lipopolysaccharide (LPS). Protein levels were determined by ELISA. Data are presented as the mean±SD of at least three independent measurements. $^{###}$P<0.001 versus control (CTL); ***P<0.001 versus the LPS-only group.

Effect of APE on the expression levels of claudin1 and inflammatory cytokines IL-6 and IL-8 in HaCaT cells

To assess whether APE could alter tight junctions, we monitored the expression of claudin1, which is a representative tight junction protein. APE significantly up-regulated claudin1 transcripts, as shown by real-time qPCR (Fig. 2A), and increased the level of claudin1 proteins at the cell membrane, as shown by immunocytochemistry (Fig. 2B).

We next examined whether APE could exert antiinflammatory effects to protect keratinocytes exposed to inflammatory stimuli. We briefly stimulated HaCaT cells with LPS. After 24 h, ELISA was used to analyze the culture media for secreted proinflammatory cytokines IL-6 and IL-8. APE in a concentration of 50 to 400 μg/ml strongly inhibited the secretion of IL -6 and IL -8 from LPS-stimulated HaCaT cells (Fig. 3A and B). These data indicate that APE has a potent antiinflammatory effect on LPS-stimulated keratinocytes.

Anti-aging and antioxidant effects of APE

Finally, to assess the ability of APE to protect dermal skin cells, we used primary normal human dermal fibroblasts. First, we observed elastase activity and procollagen production in APE-treated NHDF, as elastin and collagen are important in the maintenance of dermal strength. As shown in Fig. 4A, APE significantly and dose-dependently

inhibited elastase activity and increased procollagen production in NHDF. These results suggest that APE may protect the dermis by inhibiting elastin degradation and inducing collagen synthesis. We also assessed the expression of MMP-1, which degrades collagen fibers. To this end, we collected conditioned medium from UVB-irradiated HaCaT cells and applied it to NHDF in the presence or absence of APE. As shown in Fig. 4D, the conditioned media induced MMP-1 secretion by NHDF, and this was slightly but significantly inhibited by treatment with 400 μg/mL APE. Finally, we evaluated whether APE possesses antioxidative efficacy, because oxidative stress has a negative effect on normal skin physiology. In a cell-free system, APE showed SOD-like activity, indicating that APE could act as an antioxidant (Fig. 4D).

DISCUSSION

The outer epidermis is a primary barrier that protects the body from extrinsic factors such as UV radiation, chemicals, and bacteria, and prevents water loss [15,16]. The physical barrier of the skin consists mainly of the stratum corneum and the tight junctions [17]. The complete epithelialization of keratinocytes in the epidermis is essential for enhancing the skin's barrier function. Keratinocytes form a protein envelope called the cornified envelope through the cross-linking of specialized structural proteins, including involucrin and loricrin [15]. Tight junctions, which seal neighboring cells of the granular layer and control paracellular pathways, are formed in the human epidermis through the critical actions of occludin and claudin [17]. Therefore, normal skin differentiation and keratinization, as well as strengthening of tight junctions, are considered critical to the skin's ability to maintain homeostasis in the face of external stimuli. To support these protective functions, many researchers are seeking to develop natural extracts or synthetic compounds for use in cosmetics and/or medicines.

In the current study, we demonstrate for the first time that alfalfa seed extract possessing polysaccharides (APE) has protective effects on skin cells. Our results suggest that APE regulates the formation of the cornified envelope and the expression of proteins related to epidermal differentiation and tight junctions. In addition, APE shows an antiinflammatory effect in LPS-treated keratinocytes. These data suggest that APE can regulate physiological conditions and suppress external stimulus-induced inflammation in cells of the epidermis. We also found that APE inhibits elastase and collagenase and promotes procollagen production and SOD-like activity in dermal fibroblasts, indicating that APE exerts anti-aging activities in cells of the dermis. In a previous report, alfalfa extracts were found to have high antioxidant activity, suggesting that they have the potential to be a valuable ingredient for the cosmetic and pharmaceutical industries [12]. In the present study, we identified a much broader range of alfalfa extracts as potentially skin-enhancing agents. Several mechanisms have been proposed to explain the effects of alfalfa extracts on cells, and conflicting results have been reported. Notably, contrasting results have been reported for MAPK and NF-κB signaling [18-21]. These discrepancies are thought to reflect differences in the stage/type of extracted alfalfa material, the variety and amounts of active components and the utilized cells. In future studies, we plan to closely examine the molecular mechanism(s) by which APE acts on skin cells.

In considering the skin-protective effects of APE, we have focused here on the polysaccharides of APE as potent active components. Several previous studies found that galactose and/or mannose exhibited skin-protective, antioxidative, and antityrosinase activities. These polysaccharides have also been shown to ameliorate skin inflammation, such as those seen in atopic dermatitis and UVB irradiation-induced injury [22-25]. Another report found that alfalfa polysaccharides extracted from full flowers exhibited growth-promoting and immune-enhancing functions; in this work, an iron chromatography-based saccharide composition analysis revealed that the alfalfa polysaccharides mostly consisted of galacturonic acid, glucose and glucuronic acid [20]. In the present study, our polysaccharide analysis of APE revealed that most of the polysaccharides consisted of galactose and mannose, which were found in a ratio of about 2:1. This suggests that galactose and mannose may play major roles in the skin-protective effects of APE. Our study is the first to show that polysaccharides extracted from the seeds of alfalfa differ from those extracted from its flowers or stems and have remarkable effects on skin cells. Further research is needed to determine which polysaccharides or combinations thereof or which components other than polysaccharides are the main active ingredient in APE.

In summary, we report here preliminary evidence that alfalfa seed extract containing polysaccharides may regulate skin physiology and protect the skin from environmental irritants, and thus may prove useful in cosmetics and/or pharmaceuticals.

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Conflict of interest disclosure: The authors have no conflicts of interest to declare.

Data availability: The data underlying the findings presented in this study are available in the Supplementary Material.

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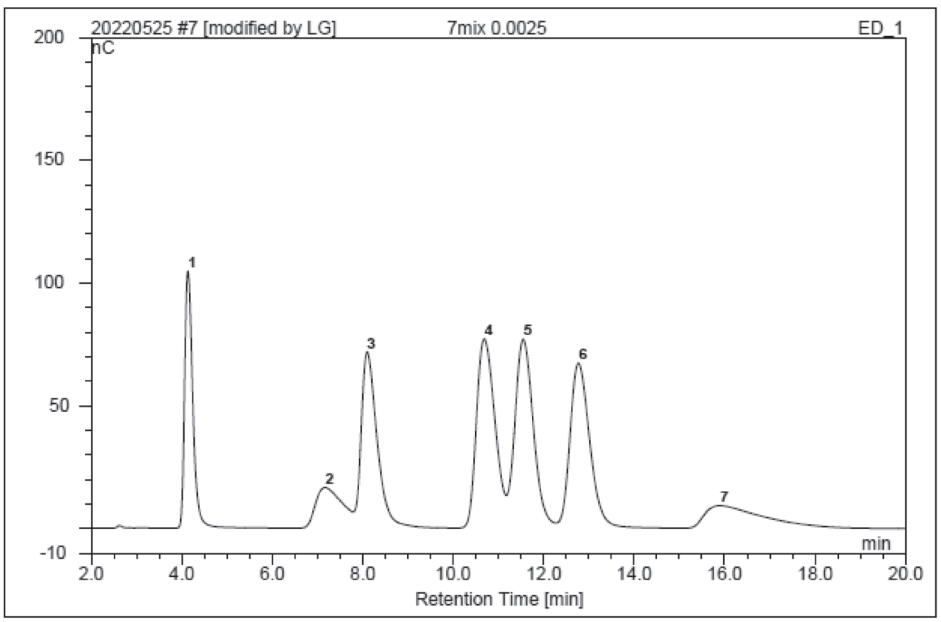
SUPPLEMENTARY MATERIAL

The data presented in this study are available as a raw data set that can be accessed via the following links:
https://www.serbiosoc.org.rs/NewUploads/Uploads/Kim%20et%20al_8609-Data%20Set-xlsx.xlsx
https://www.serbiosoc.org.rs/NewUploads/Uploads/Kim%20et%20al_8609-Data%20Set.1.pdf
https://www.serbiosoc.org.rs/NewUploads/Uploads/Kim%20et%20al_8609-Data%20Set.2.pdf



No.	Ret.Time min	Peak Name	Height nC	Area nC*min	Rel.Area %
1	4.14	Fucose	104.618	21.791	11.99
2	7.17	Rhamnose	16.489	11.316	6.23
3	8.10	Arabinose	71.650	28.979	15.94
4	10.69	Galactose	77.032	36.512	20.09
5	11.55	Glucose	76.938	36.709	20.20
6	12.77	Xylose	67.180	33.989	18.70
7	15.90	Fructose	9.008	12.447	6.85
Total:			422.915	181.744	100.0

Supplementary Fig. S1. Identification of polysaccharides in APE. The polysaccharides in APE were identified using high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD).