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글리세릴 베이스의 신규베지클 이용 캡슐화를 통한 항산화성 아스타잔틴의 성질 강화

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The Cosmeceutical Property of Antioxidant Astaxanthin is Enhanced by Encapsulation Using Glyceryl Based New Vesicle

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요 약: 새로운 베지클인 glyceryl citrate/ lactate/ linoleate/ oleate를 이용한 수중유형 형태의 아스타잔틴 나노에멀젼에 대해 항산화 효과, 세포 생존력, 단백질과 관련한 효소의 영향, 피부 침투도 그리고 피부에 대한 보습 및 탄력 등의 약용화장품적인 측면에서의 전반적 연구를 실시하였다. 항산화력 및 세포 생존력에 대해선 각각 DPPH법과 MMT assay를 이용하여 측정하였다. 아스타잔틴 나노에멀젼에 대한 또 다른 성질은 2D-Page를 이용한 단백질 분석 및 컨포칼, *in-vivo* 테스트를 통해 측정하였다. 본 연구를 통해, 아스타잔틴을 포함하는 나노에멀젼은 MMP발현에 관련한 단백질 억제 및 세포외 기질의 분해를 막고 라디칼의 소거에 매우 우수한 결과를 보였다. 종전의 레시친을 이용한 나노에멀젼 보다는 새로운 베지클을 이용한 아스타잔틴 나노에멀젼의 피부 침투가 매우 효과적임을 CLSM을 통해 측정하였다. 또한 28일 동안의 한국 성인 여성 11명을 통한 보습 및 탄력 인비보 테스트에서 우수한 효과를 확인할 수 있었다.

Abstract: Oil-in-water nanoemulsions of astaxanthin prepared by new vesicle, glyceryl citrate/ lactate/ linoleate/ oleate, were evaluated thoroughly in terms of cosmeceutical properties such as antioxidant effect, cell viability, influence of protein related enzyme, skin penetration, skin hydration and elasticity. Antioxidant effect and cell viability of nanoemulsion of astaxanthin were evaluated by DPPH and MTT assay. Also other properties of nanoemulsions of astaxanthin were measured by proteome analysis using 2D-PAGE, confocal laser scanning microscope and *in-vivo* test. We were able to find that the nanoemulsion of astaxanthin is good at scavenging of radical and inhibits the degradation of dermal extracellular matrix with the down-regulation of MMPs and other proteins related to MMP expression. CLSM was adopted for observing penetration of nanoemulsion of astaxanthin and showed high effective penetration rate compared to the nanoemulsion of astaxanthin prepared by conventional lecithin. *In-vivo* measurement of the nanoemulsions in hydration and elasticity were conducted to 11 Korean female adults for 28 days and showed better results.

Keywords: *nanoemulsion, astaxanthin, antioxidant, 2D-PAGE, confocal laser scanning microscopy*

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1. Introduction

Carotenoids have also received particular attention due to their high provitamin activity and antioxidant capacity. Astaxanthin (AX) is a ketocarotenoid, used as a preferred pigment in aqua-culture feeds. Due to high antioxidant activity[1,2], it can be used as a potential prophylactic agent against skin cancer and as a possible chemo-preventive agent. Despite the availability of synthetic astaxanthin, astaxanthin from natural sources still receives more interest due to its greater antioxidant activity and stability[3]. However, as with most carotenoids, AX is a highly unsaturated molecule and thus it is highly sensitive to high temperature, light, and oxidative conditions which may promote the isomerization of AX into the cis form which possesses less activity than its corresponding trans configuration[4]. In addition, it is not generally used in food and cosmetic applications due to its low solubility, poor photostability and susceptibility to colour change[5]. One approach that can be used to improve the solubility and bioavailability of carotenoids such as β-carotene is to incorporate them in the fine particles of O/W (oil-in water) emulsions. Garti and co-workers[6,7], for example, prepared food grade microemulsions containing carotenoids with considerable success.

During the past two decades nanotechnology has emerged as one of the most interesting and promising research fields[8,9]. The technology offers the potential to significantly improve the solubility and bioavailability of many functional ingredients including carotenoids, polyunsaturated fatty acids, phytosterols and numerous other compounds. Nanoemulsions covering the size range 50 − 200 nm have characteristic properties which can have important application in the personal care, cosmetics and health science fields. The fact that nanoemulsion has improved stability, solubilizing action, delivery and penetration for skin-active materials make nanoemulsion technology attractive to the cosmetics industry. Conventional nanoemulsions are made with hydrogenated lecithin phospholipids (also known as phosphatidyl cholines) which consist of two fatty hydrophobic chains and a hydrophilic head group[10]. Hydrogenated lecithin is readily able to form emulsion systems and liquid crystals since its critical packing parameter is close to 1[10]. However, hydrogenated lecithin has the drawback of being susceptible to oxidation and colour change and can also cause destabilization of anti-bacterial systems. As alternatives to lecithin, certain fatty ester compounds comprising fatty acids esterified with fatty alcohols have also been identified for making emulsions[11]. However, a systematic study using these kinds of emulsifiers for incorporating materials such as carotenoids (e.g. astaxanthin) into nanoemulsions, and subsequent examination of their stabilities, has not been previously reported in the literature.

In our previous study[12], the influence of the following on AX nano dispersions during storage as a very important parameter of emulsions has been studied thoroughly.

1) Emulsifier type and concentration.

2) Homogenization conditions of pressure and time of homogenization.

3) Concentration of AX.

The purpose of this study is to evaluate the cosmeceutical properties such as antioxidant effect, cell viability, influence of protein related enzyme, skin penetration, hydration and elasticity on nanoemulsion of AX.

2. Materials and Experiments

2.1. Materials

Oil-in-water nanoemulsion of AX was prepared using glyceryl citrate/ lactate/ linoleate/ oleate (Imwitor 375, Sasol Co., Ltd., Hong Kong), hydrogenated lecithin (Lipoid 100-3, Lipoid GMBH, Germany), antioxidants, liquid paraffin, propylene glycol, polysorbate 60 and ethanol. AX was extracted by supercritical CO_2[12]. Other commercially available reagents and solvents were used as received. DPPH (2,2-diphenyl-1-picryl hydrazyl, sigma-aldrich, Korea) was used for evaluating radical scavenging property. Human dermal fibroblast cells (ATCC, CRL-1635, American Type Culture Collection,

USA) were purchased to evaluate cell viability. Proteome analysis was performed with IPGphore (Ettan™ 3 IEF, Amersham bioscience, Sweden) and image master (ImageMster™ 2D Elite, Amersham biosciences, Sweden) to evaluate influence of protein related enzyme activity. CLSM (Confocal Laser Scanning Microscopy, Leica microsystem TCS SP5, Germany) was adopted for checking penetration of nanoemulsion of AX. Hydration and elasticity were measured by corneometer (CM 825, Courage+Khazaka electronic GmbH, Germany) and cutometer (MPA 580, Courage+Khazaka electronic GmbH, Germany) respectively.

2.2. Preparation of AX nanoemulsion

Homomixer (Primix Homo mixer, Japan) and high pressure homogenizer (MN250A, Micronox, Korea) were placed for preparing an O/W (oil in water) nanoemulsion of AX. Liquid petrolatum (LP), ceramide, cholesterol and ethanol as dispersed phase and glyceryl citrate /lactate/linoleate/oleate, hydrogenated lecithin, polysorbate 60, propylene glycol and deionized water as continuous phase were heat up to 70 ~ 75 ℃ and mix to form premix. The premix was then homogenized at 3500 rpm for 5 min to pre-emulsify followed by one more homogenization with AX at 3500 rpm for 5 min at the temperature of 50 ℃, and then high pressure homogenization was performed at 1000 bar to give final nanoemulsion of AX ranged from 160 to 190 nm.

2.3. The DPPH radical scavenging assay

DPPH radical scavenging assay was used to compare the anti-oxidative activity on AX and nanoemulsion of AX. Also various co-antioxidants effects to nanoemulsion of AX were measured. DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. Plate 100 μL of AX samples at 50 mg/L into each well of 96-well plate. 100 μL of 0.15 mM methanolic solution of DPPH were added into every well. The plate was shaken vigorously and allowed at room temperature in 30 minutes. The control was prepared as above without any extract and solvent for the baseline correction. Optical density (OD) of the samples was measured at 490 nm using iMark™, microplate absorbance reader (iMark™, Bio-Rad, USA). Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula:

$$\% \text{ radical scavenging activity} = \left[1 - \frac{Sample\ OD - Blank\ OD}{Control\ OD}\right] \times 100$$

The measurement for the radical scavenging effect was conducted after 1 week storage under the sunlight and cycle condition ranged from - 5 ~ 40 ℃. The cycle runs 6 per day.

2.4. MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine cytotoxicity of AX extracts for the viability and growth of human fibroblasts. The fibroblasts were seeded into 96-well plates at 3×10^3 cells/well and allowed 24 hr for surface adhesion in 5% CO_2 incubator.

After treatment of cells for 48 h by AX extracts at 1-100 mg/L in Dulbecco's Modified Eagle Medium (DMEM) containing 1% Antibiotic-Antimycotic (AA), experimental media were removed and the cells were incubated with 50 μL basal medium containing 2.0 mg/mL MTT in CO_2 incubator at 37 ℃ for 3 h. The medium was aspirated, and the remained formazan product in every well was solubilized with 200 μL Dimethyl sulfoxide (DMSO). Absorbance at 595 nm was measured for each well using iMark™ microplate absorbance reader.

2.5. Proteomic analysis

Proteomics is the technology that is able to identify the structure and functionality of all kinds of protein expressed by cell. Commonly used methods are two-dimensional electrophoresis and mass spectrometry. Two-dimensional electrophoresis (2DE), which is able to analyze simultaneously complicated protein complexes, can show the changes of protein caused by cell expression or isomerization. Experiment's stage can be divided into as following.

1) Sample preparation
2) Isoelectric focusing (IEF)
3) Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)
4) Silver staining
5) Image analysis

Mass spectrometry is the technology that is used for identification of protein by mapping between weight of protein and protein database.

2.5.1. Protein extraction

Cells washed in phosphate buffered saline (PBS) were isolated via centrifugation. Proteins were extracted by cell lysis buffer solution. The lysis buffer solution was prepared with 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1propanesulfonate (CHAPS cell extract buffer), 1% dithiothrietol (DTT), 2% carrier ampholyte, 4% Protease inhibitor cocktail (PIC), and 0.002% bromophenol blue (BPB). Cells in lysis buffer were sonicated for 1 min and maintained for 30 min at room temperature. The solution was centrifuged for 5 min at 4,000 ×g. The supernatant was collected and preserved at −20 ℃. The amount of total protein extracted was measured via the modified Bradford method.

2.5.2. 2D-PAGE

2-D PAGE is the separation method for proteins with high resolution. Immobiline Dry strips (24 cm, pI 4-7NL) were used with an Ettan IPGphore fixed-length strip holder. The strip was rehydrated for 12 h with rehydration solution and the sample proteome was injected simultaneously. The rehydration solution was prepared with 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 2% carrier ampholyte, 10% glycerol, 0.002% BPB. Isoelectric focusing was conducted by Ettan IPGphore. After 8 hours of rehydration, stepwise focusing was performed for 60 min at 500 V, 60 min at 2,000 V, and then increased to 8,000 V and maintained until no current change was observed[13]. Focused IPG strips were equilibrated for 15 min in a solution (7 M urea, 2 M thiourea, 2% SDS, 50 mM tris-HCl, 30% glycerol and 1% DTT), and then for an additional 15 min in the same solution containing 2.5% iodoacetamide rather than DTT[14]. After equilibration, the second dimension was run on 12.5% polyacrylamide homogeneous gels (T-13%, C-2.5%, 18 × 24 cm) [15]. The gels were stained with silver nitrate[16]. The stained gel was scanned and the protein spot images were analyzed using 2D Elite image analysis software.

2.6. Measurement of skin penetration

Confocal laser scanning microscope (CLSM) was employed for the measurement of skin penetration. A FITC-dextran was selected as an incorporation medium of nanoemulsion to measure the efficacy of penetration and checked with microscope whether it was incorporated well or not. Pig skin tissue was hydrated with saline for about 30 min before testing. Sample was spreaded on the skin tissue sized 25 × 25 mm after removing moisture. Measurement of skin penetration was conducted in 10, 30, 60, 120 min at 514 nm using confocal laser scanning microscope after sampling on the slide glass by using cryo-microtome (Lecia CM 1850, Germany). Thickness of sample was 20 μm. Light source was argon laser emitting 514 nm and the emission spectrum of 530 nm ~ 585 nm was observed for image visualization.

2.7. Skin hydration and elasticity

In-vivo measurement of hydration and elasticity were conducted by testing 11 Korean female adults for 28 days by using corneometer and cutometer.

Hydration was measured on the right side of the cheek and elasticity was measured on the left side of the cheek. Measurement was performed in constant temperature and humidity chamber(25 ± 1 ℃, 50 ± 5%) after 15 min skin adaptation and took average value among 6 times repeated measurement. The formula which is used for the clinical test is as follow in Table 1.

Table 1. Formulation for AX Nanoemulsion for *in-vivo* Testing

Phase	Ingredients	Formula (wt%)
Water Phase	D.I Water	to 100
	Glycerin	2.0
	Sorbitol	0.5
	1,3-butylene glycol	4.0
	Aloe veragel extract	30.0
	Sodium hyaluronate (1%)	1.0
	Xanthan gum	0.3
	Methyl paraben	0.2
	Phenoxy ethanol	0.3
Oil Phase	Ethylhexyl palmitate	3.0
	Peg-8	2.0
	Polyglyceryl-3 methyl glucose distearate	2.0
	Diisostearate	1.5
	Hydrogenated polyisobutene	1.5
	Cetearyl alcohol	1.2
	Peg-100 stearate	1.0
	Dimethicone	0.3
	Perfume	q.s.
Active	AX-nanoemulsion (Containing 5.5% of AX)	1.0

3. Results and Discussion

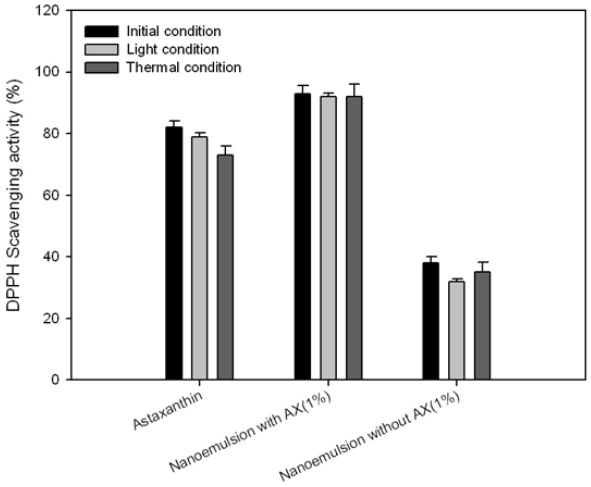
AX is highly effective materials. Its antioxidant activity is approximately 100 times higher than that of β-tocopherol, and can be used as a potential agent against cancer. But AX can easily be degraded by thermal or oxidative processes. So, in our previous study[12], we adopted new vesicle, glyceryl citrate/ lactate/ linoleate/ oleate, and evaluate emulsifier and condition for preparing stable nanoemulsion containing AX. Unimodal nanoemulsion with an average size of 170 nm was obtained when glyceryl citrate/ lactate/ linoleate/ oleate was used. However, a broader particle size distribution was obtained when hydrogenated lecithins were used. In this study, therefore, the cosmetical properties of nanoemulsion containing AX were investigated.

3.1. Effect of radical scavenging

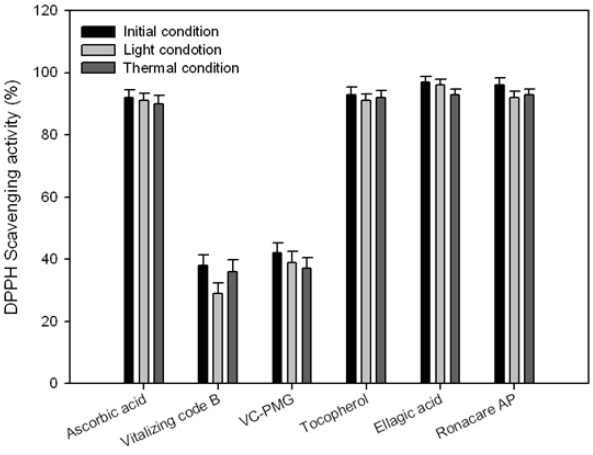
Comparison between nanoemulsions with or without 1% of AX extract for radical scavenging property was performed. And also other antioxidant agents such as ascorbic acid, berry complex (Vitalizing Code B), magnesium ascorbyl phosphate (VC-PMC), tocopherol, ellagic acid, hydroxy dimethoxybenzyl malonate (Ronacare AP) were used as a co-antioxidant for additional comparison. From the result showing in the Figure 1(a), AX encapsulated as nanoemulsion with glyceryl citrate/lactate/linoleate/oleate maintained its antioxidant property while AX itself was degradated from the storage condition which may give light and thermal changes. Figure 1(b) also shows the lipophilic material used as co-antioxidant has better scavenging synergetic effect compared to hydrophilic co-antioxidant.

3.2. Toxicity of AX extract on human fibroblasts

Toxicity of AX extracts extracted by super critical CO_2 at 350 bar, 60 ℃ were investigated against human skin fibroblasts. An MTT assay was conducted with AX extracts at various concentrations, in a dose range of 0 ~ 100 mg/L. According to the results of the MTT assay, AX extract evidenced cell toxicity on fibroblasts at con



(a)



(b)

Figure 1. DPPH scavenging effect (Light condition : 1 week under sunlight, Thermal condition : 1 week cycle test ranged from - 5 ~ 40 ℃, 6 cycle/day).

centrations over 10 mg/L. It shows the figure 2.

3.3. Analysis of proteome

The changes of anti-aging related proteins were observed by comparing the control 2-D gel and sample 2-D gel which was treated by 5 mg/L of AX extracts. On control gel and sample gel, there were 589 and 540 detected spots respectively. Among them, 306 spots were matched as shown in figure 3 and figure 4.

Base on isoelectric point (pI) and molecular weight (MW), 17 proteins which are related to cell turnover and

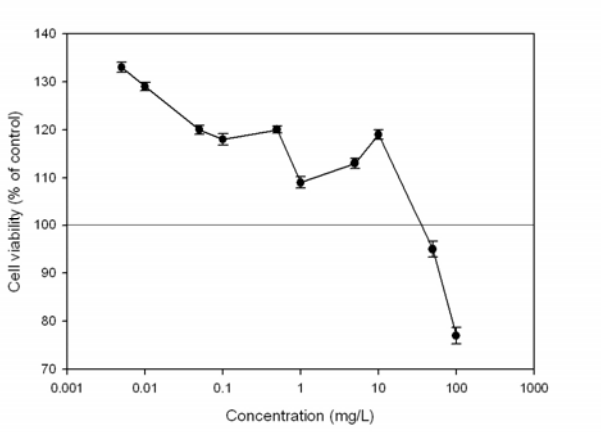


Figure 2. Cell toxicity of fibroblast treated with AX extract.

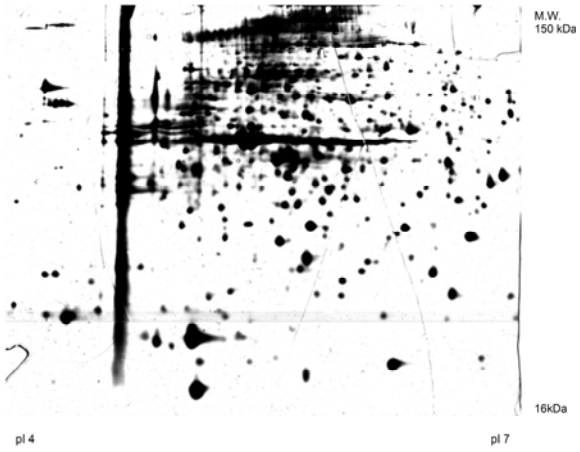


Figure 3. 2D-PAGE image from human fibroblast(control).

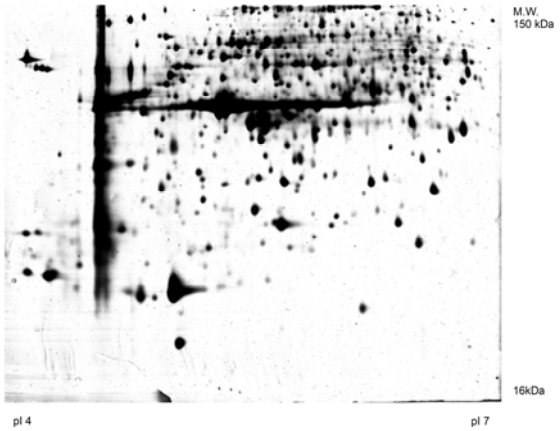


Figure 4. 2D-PAGE image from human fibroblast. Cell treated with AX. Extract.

Table 2. 2D-PAGE Image Spots

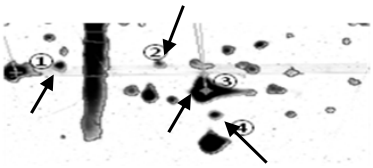
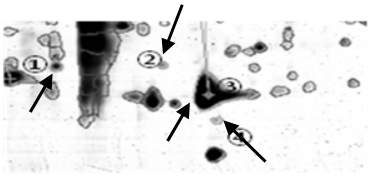
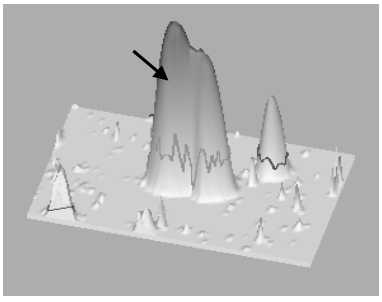
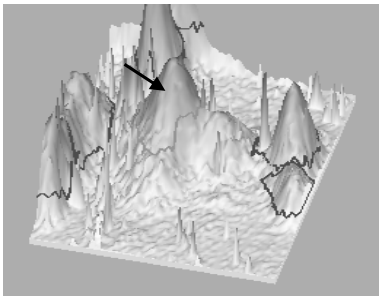
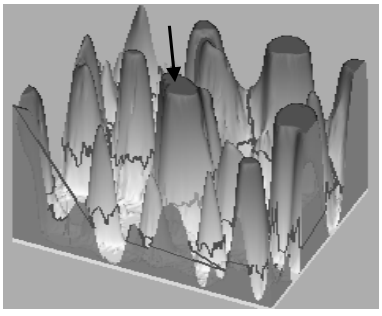
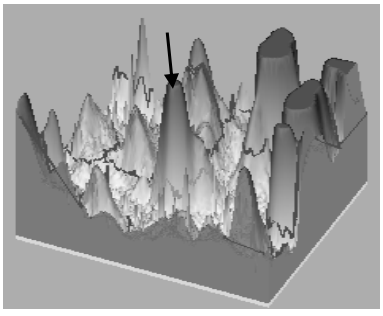
Protein	Control	Treated with AX. extract
① 14-3-3-epsilon ② TNF-alpha ③ fibronectin ④ interleukin 1-alpha		
Cathepsin		
MMP1		
MMP9		

Table 3. 2D-PAGE Image, 3D View of MMP1 and MMP9

Protein	Control	Treated with AX. extract
MMP1		
MMP9		

wrinkle were identified as shown in Table 2, Table 3 and Table 4, respectively.

First, as the extracellular matrix (ECM) composes, type III (alpha 1) collagen and prolyl 4-hydroxylase were identified. Prolyl 4-hydroxylase plays an important role to synthesize the collagen by forming collagen triple helix. The result showed up-regulated of prolyl 4-hydroxylase together with type III (alpha 1) collagen each 4

Table 4. List of Identified Human Fibroblast Cell Treated with AX Extract

	Protein name	NCIBI number	Theoretical		Control Vol.	Sample Vol.	Match ID
			pI	M.W.(kDa)			
ECM components	Type Ⅲ (alpha 1) collagen	P02461	6.11	138.95	914.8	1363.78	299
	Prolyl 4-hydroxylase	P13674	5.70	61.12	2291.6	8896.6	115
Connective protein	Integrin beta-1	P05556	5.27	88.46	296.6	409.9	194
	Vimentin	P08670	4.90	46.80	1912	243.5	67
	Fibronectin	P02751	5.00	15.00	25281	30445	10
Degradation	Cathepsin		5.00	23.00	475.1	769	42
	Cathepsin precursor		6.10	45.00	71.4	400.1	86
	Elastase IIIB	P08861	6.10	30.10	633.3	210.4	61
	MMP1	P03956	6.17	54.01	1344.22	486.88	98
	MMP3	P08254	5.34	53.98	2585.42	600.272	109
	MMP2	P08253	5.02	73.88	6346.43	1593.02	154
	MMP9	P14780	5.44	78.46	4521.49	1761.6	170
	MMP14	P50281	5.76	65.88	1068.7	668.899	101
Cytokines	Interleukin 1-alpha	P01583	5.00	17.00	1857	374	3
	TNF-alpha (tumor necrosis factor)	P01375	5.01	25.90	1470.4	394.7	16
	NF-kappa B	P19838	5.20	105.62	3840	1585	234
etc	14-3-3-epsilon	P62258	4.60	29.20	2496.7	1352.1	15

times and 1.5 times. The increase of type III (alpha 1) collagen was influenced by prolyl 4-hydroxylase which is one of the dermis composite. Normally, the amount of type III (alpha 1) collagen decrease according to the aging process.

Damage to the ECM causes weakness of adhesion between the cell and the ECM. Connective proteins work on adhesion property between the cell and the ECM. In this study, integrin beta-1, vimentin, fibronectin were identified as the connective proteins. Integrin beta-1 and fibronectin which plays a major role in cell adhesion, cell interaction, cellular signal transduction were increased. On the contrary, vimentin found in aged cells was decreased about 8 times. It shows AX extracts induced inhibition of vimentin production.

Normally, Sequential increase of inflammatory cytokine and MMPs can be observed when the immune system has problem. In this study, 20 ~ 50% decrease of interleukin 1-alpha (IL-1α), TNF-α, NFkappa B were observed. While those proteins lead to derepression of MMPs, it also lead to repression of proteoglycan. Also there are enzymes that are playing a very important role to decompose the protein and destructing elastic network by collagenasing. They are interstitial collagenase (MMP1), 72 kDa gelatinase (MMP2), 92kDa gelatinase (MMP9), neutrophil collagenase (MMP8), collagenase-3 (MMP13), and MT1-MMP (MMP14) etc. Among them, the MMP1 has played an important role in the remodeling of connective tissue while MMP2 and MMP3 decompose the elastic quality and matrix protein such as pro

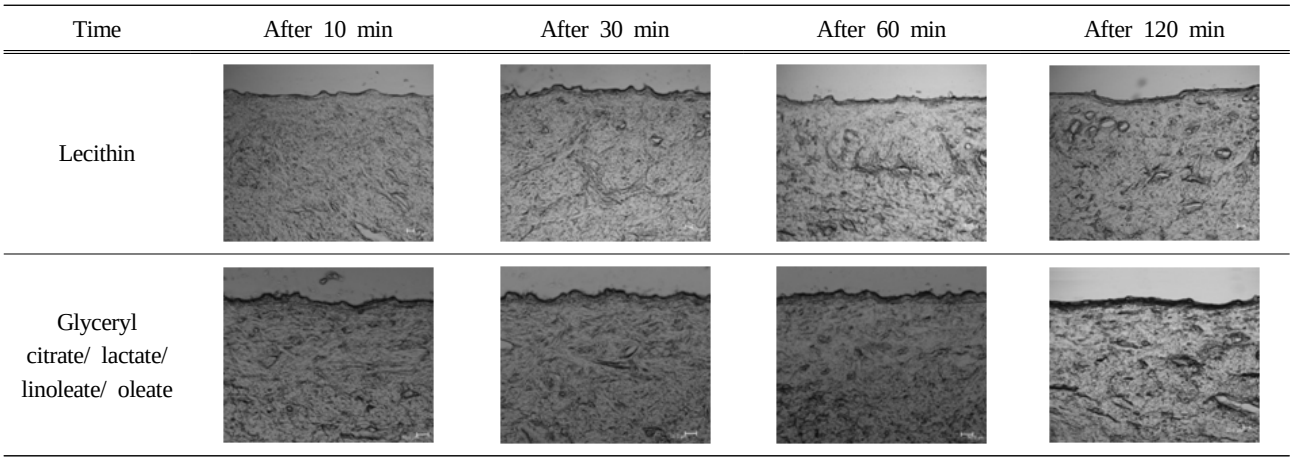


Figure 5. Image showing follicular localization of FITC-nanoparticle sequence.

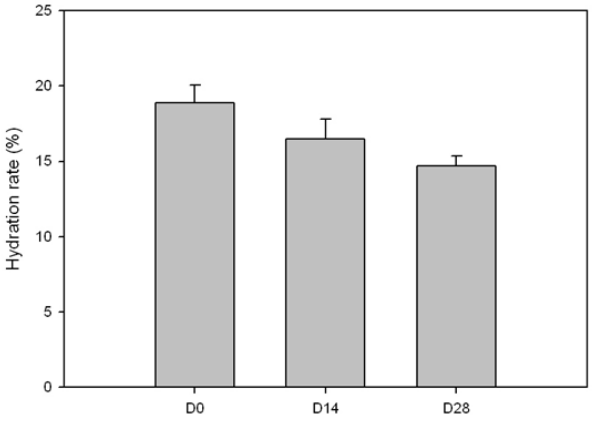


Figure 6. Test result of hydration (Right).

teoglycan, fibronectin and laminin[17]. The decrease of proteins such as MMP1, MMP2, MMP3, MMP9 and MMP14 were found from the analysis and thought to be influenced by MMP expressing proteins. Except them, there are several proteinases such as Cathepsin, cathepsin precursor and elastase IIIB. Also 3 times decrease of elastase IIIB was observed compare to the control and same to other 2 proteins. It is founded that AX extract in the cell has effect of inhibiting the cell destruction, ECM damage and cytokine and strengthening the cell adhesion. Also it was expected that AX extract may affect to facilitation of cell interaction and cell turnover.

3.4. Skin penetration of nanoemulsion of AX

Measurement of skin penetration between nanoemulsion of AX with lecithin and that with glyceryl citrate/lactate/linoleate/oleate was compared through CLSM. The penetration was progressed to the epidermis from the both samples after 30 min of spreading and to the dermis after 60 min of spreading. After the 120 min of spreading, the nanoemulsion was penetrated all area of the dermis form the both samples but nanoemulsion of AX with glyceryl citrate/ lactate/ linoleate/ oleate was more effective and wide spreaded. It shows in the Figure 5.

3.5. Hydration and elasticity test

In-vivo measurement of hydration and elasticity were conducted by testing 11 Korean female adults for 28 days by using corneometer CM825 and cutometer MPA580. Statistical analysis of hydration was conducted using a program named SPSS^{R}14.0. Figure 6 shows increase of hydration on nanoemulsion of AX with glyceryl citrate/lactate/linoleate/oleate formulated by Table 1. After using sample formulated by table I for 4 weeks, it showed statistically significant improvement of skin elasticity. The result shows in the Figure 7.

4. Conclusions

The reactive oxygen radicals from the person's daily life and environmental pollution is inevitable, by changing the lipid and protein which make up the cell mem

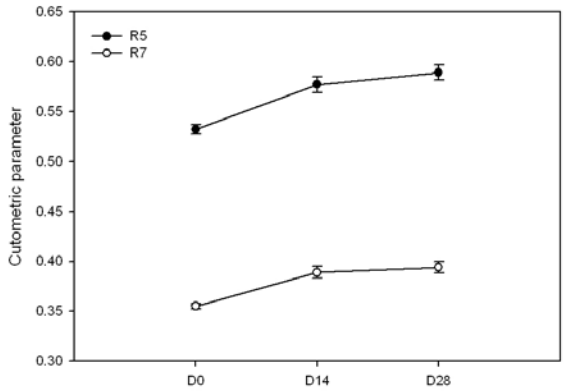


Figure 7. Clinical test result of skin elasticity.

brane, active oxygen radicals result in a loss of elasticity, spots, freckles, and ultimately lead to serious diseases such as skin aging and cancer. It is not easily prevent the occurrence of reactive oxygen species. However, reactive oxygen can be removed by allowing to use of several materials such as vitamin and vitamin derivatives. Specific target substance based on bioactive technology and the formulating technology to facilitate the transdermal absorption of active ingredients is developing continuously in 21st century. In this study, glyceryl citrate/ lactate/ linoleate/ oleate as a novel vesicle was adopted to stabilize the antioxidant AX and optimized to facilitate the skin penetration by nano emulsification. 2D-PAGE analysis was conducted to check protein related to wrinkle and elasticity and identified 17 spots which are related to cell turnover, wrinkle and elasticity. Through this study, it may create a framework for developing functional cosmetics that can be defined as mechanism of an actual effect. Also it is proved as greater value as cosmetic materials by formulating and evaluating cosmetical properties such as radical scavenging effect, safety, hydration, elasticity, and protein analysis.

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