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Research Article

Antimicrobial Effects of Supercritical *Angelica gigas* Extracts and Enhancement of Skin Permeability using Cell Penetrating Peptide

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Abstract: This study aimed to find a way to more efficiently extract the active compounds from Korean *Angelica gigas* (AG) and to maximize the effects by increasing the skin permeability of the optimal AG extract. AG was extracted using hot water extraction method and supercritical carbon dioxide (CO_2) containing ethanol method, the temperature and pressure of extractor were 30°C/350 bar, 45°C/350 bar, 60°C/350 bar. And then optimal extraction condition was found through quantification of decursin and decursinol angelate (D/DA) content, measurement of total polyphenol content, and evaluation of antioxidant activity, tyrosinase inhibition activity, and antimicrobial activity. As a result of the experiments, supercritical CO_2 containing ethanol extraction with the temperature and pressure of extractor were 45°C/350 bar (ASCE45) was selected as the optimal extract. In particular, ASCE45 contained 44.58 % of D/DA, which were not contained in hot water extract (AHW) and also showed an antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Propionibacterium acnes*, which were not showed in AHW. Furthermore, liposome technology and arginine 6 (hexa-D-arginine, R6), one of the cationic cell penetrating peptides (CPPs), were applied to the optimal extract, ASCE45, and as a result skin permeability was increased 1.74 times higher than a control group. In other words, it is inferred very efficient in actually showing effects on the skin because the optimal skin penetration condition was applied to ASCE45 extracted under optimal extraction conditions.

Keywords: *Angelica gigas*, supercritical extracts, antimicrobial, skin permeability, cosmetics

1.Introduction

Supercritical fluid extraction is a technique used to separate components from solids or liquids. The Supercritical fluids have low viscosity as gas, high density as liquid, and its diffusivity is between gas and liquid. These properties allow supercritical fluids to penetrate quickly and deeply into a solid matrix. Furthermore, these properties are tunable and can have the desired solubility and selectivity in supercritical fluids mainly by controlling pressure and temperature (**Pereira & Meireles, 2010**). Carbon dioxide (CO_2) is the most used supercritical fluid because it has an advantageous critical parameter, 31.1°C, 73.8 bar, that can be processed at low temperature and pressure (**Reverchon E., 1997**). Supercritical CO_2 has a restriction of being inappropriate to apply as a solvent for polar components but incorporating an organic solvent such as ethanol can significantly enhance extraction efficiency (**Radzali S.A. et al., 2014**).

The *stratum corneum*, which is the outermost epidermal layer, consists of keratin and intercellular lipids, which serves as a physical barrier preventing permeation of foreign substances. This barrier function makes it difficult for functional materials to penetrate the skin and show efficacy. The application of drug delivery systems (DDSs) is necessary to solve these problems (**Thiele J. et al., 2001**). Liposome, a vesicle that is composed of one or more phospholipid bilayers, is a widely used drug transporter. This is because liposome can trap both hydrophobic and hydrophilic compounds, and also increase drug permeability based on structural and compositional similarity with biological membranes (**Elsayed M.M. et al., 2007**). TAT peptide derived from HIV-1 is one of the most studied cationic cell penetrating peptides (CPPs), which is an active cargo for delivering bioactive molecules into cells. It was reported that TAT peptide contains enriched arginine (R) and lysine (L), which play a key role in cell penetrating capacity. Based on similarity in the structure of lipid bilayer between the cell membranes and intercellular lipids, it is considered that skin permeability of functional substances can be increased by applying CPPs (**Vives E. et al.,1997**; **Lundberg & Langel, 2003**).

Angelica gigas (AG) is a crucial medicinal herb used in Asian countries. It contains many beneficial substances with various effects such as antioxidant, antimicrobial, anti-inflammatory, anticancer, etc. There are differences in biological active compounds contained in AG depending on geographical origin such as Korea, Japan, and China, so the biological effects vary accordingly. The major active compounds of AG are decursin and decursinol angelate

(D/DA), which are pyranocoumarin family. Compared to Japanese and Chinese AG, Korean AG has been reported to contain a large amount of D/DA (**Cho S.K. et al., 2007**; **Ahn K.S. et al., 1996**).

In this study, in order to find a way to more efficiently extract the active compounds from Korean AG, it was extracted using supercritical CO_2 containing ethanol as well as hot water, and optimal extraction condition was found through quantification of D/DA content, measurement of total polyphenol content, and evaluation of physiological activities. Furthermore, liposome technology and arginine 6 (hexa-D-arginine, R6), one of the cationic CPPs, were applied to maximize the effects by increasing the skin permeability of the optimal AG extract.

2. Materials and Methods

2.1. Chemicals and Reagents

Decursin, Folin & Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, tyrosinase from mushroom, L-Tyrosine, and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (USA). hexa-D-arginine (R6) was purchased from Dermafirm Co (Korea).

2.2. Extraction Methods

Dried and crushed AG (Jinbu-myeon, Pyeongchang-gun, Gangwon-do, Korea) was extracted using hot water and supercritical CO_2 containing ethanol as a cosolvent. The hot water extraction was carried out at 80 °C for 4 h with a water bath (Changshin Science, Korea) and the extract was lyophilized by a freeze dryer (Ilshin Bio Base, Korea) after filtration. This extract was marked as AHW. The supercritical CO_2 containing ethanol extraction was conducted using a SC-CO_2 extraction system (ARI instrument, Korea) under three conditions: the temperature and pressure of extractor were 30 °C /350 bar, 45 °C/350 bar, 60 °C/350 bar, respectively. On the other hand, the pressure and temperature of separator, the flow rate of CO_2, and the flow rate of ethanol were conducted in the same conditions as 50 bar/25 °C, 60 mL/min, and 5 mL/min, respectively. The ethanol from the extracts was removed by a rotary vacuum evaporator (EYELA, USA). The extracts were marked as ASCE30, ASCE45, and ASCE60, respectively.

2.3. Decursin and Decursinol Angelate Content Quantification

The content of D/DA contained in AG was quantified using an Agilent 1100 series high performance liquid chromatography (HPLC, Agilent Technologies, USA). The column used in this experiment was a Capcellpak C18 UG120 5 um, 4.6 mm x 250 mm (Shiseido Co., Ltd., Japan) reverse phase HPLC column, and the temperature was 30 °C. The 50 % acetonitrile containing 10 mM sodium dodecyl sulfate and 25 mM sodium phosphate dibasic was used as an isocratic mobile phase, and the flow rate was 0.7 mL/min. 5 μL of samples were injected, and each runtime was 30 min. The UV detection with a diode array detector of analytes was carried out at a wavelength of 230 nm (**Gwon J.H. et al., 2015**). Data acquisition was performed using ChemStation software.

2.4. Total Polyphenol Content Measurement

The total polyphenol content of AG extracts was quantified using the Folin-Ciocalteu method (**Folin & Denis 1912**). Briefly, 10 μl of the extract solution was mixed with 40 μl of 1 N Folin & Ciocalteu's phenol reagent and 150 μl of 20 % Na_2CO_3. The mixture was reacted at room temperature for 5 min in the dark. The absorbance at 760 nm was measured with a microplate reader (Biotek, USA). Gallic acid was used as a standard and the total polyphenol content of the extracts was expressed as mg of gallic acid equivalent (GAE) per g of extract.

2.5. Antioxidant Activity Measurements

2.5.1. Measurement of DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of AG extracts was measured using the method of Blois (**Blois M.S., 1958**). To 10 μL of the extract solution, 190 μL of 0.4 mM DPPH adjusted absorbance at 520 nm to 1.0 ± 0.1was added and reacted at room temperature for 30 min in the dark. The absorbance at 520 nm was measured with a microplate reader. Ascorbic acid served as a positive control.

DPPH radical scavenging activity (%) = $(1\text{-}Abs_{control}/Abs_{sample}) \times 100$

where $Abs_{control}$ and Abs_{sample} represent the absorbance of the control and the sample measured at 520 nm, respectively.

2.5.1. Measurement of ABTS+ Radical Scavenging Activity

The ABTS+ radical scavenging activity of AG extracts was measured using the method of Re et al. (**Re R. et al.,**

1999). ABTS+ working solution was prepared by mixing 7.4 mM ABTS and 2.6 mM potassium persulfate at 1:1 v/v ratio and kept for 16 h at room temperature in the dark. To 10 μL of the extract solution, 190 μL of the ABTS+ working solution adjusted absorbance at 734 nm to 0.7 ± 0.1 was added and reacted at room temperature for 15 min in the dark. The absorbance at 734 nm was measured with a microplate reader. Ascorbic acid served as a positive control.

ABTS+ radical scavenging activity (%) = $(1\text{-}Abs_{control}/Abs_{sample}) \times 100$

where $Abs_{control}$ and Abs_{sample} represent the absorbance of the control and the sample measured at 734 nm, respectively.

2.6. Tyrosinase Iinhibition Activity Measurement

The tyrosinase inhibition activity of AG extracts was evaluated using a variation of the method of Kubo et al. (**Kubo & Kinst-Hori 1999**). To 160 μL of 0.1 M sodium phosphate buffer (pH 6.5), 10 μL of the extract solution, 20 μL of 1.5 mM L-tyrosine, and 10 μL of 2000 U/mL tyrosinase were added and incubated at 37 °C for 15 min. The absorbance at 490 nm was measured with a microplate reader. kojic acid served as a positive control.

Tyrosinase inhibition activity (%) = $(1\text{-}Abs_{control}/Abs_{sample}) \times 100$

where $Abs_{control}$ and Abs_{sample} represent the absorbance of the control and the sample measured at 490 nm, respectively.

2.7. Antimicrobial Activity Measurement

To measure the antimicrobial activity of AG extracts, disk diffusion assay was performed (**Ko M.O. et al., 2018**). Paper disks, 8 mm in diameter, were impregnated with 50 μL of the extract solution and the disks were placed on Mueller-Hinton (MH) or Reinforced Clostridial (RC) agar plates on which bacterial suspensions had been seeded. The MH agar plates with *Staphylococcus aureus* (*S. aureus*, ATCC 6538), *Bacillus subtilis* (*B. subtilis*, ATCC 19659), and *Escherichia coli* (*E. coli*, ATCC 23726) were incubated aerobically for 24 h at 37°C while the RC agar plates with *Propionibacterium acnes* (*P. acnes*, ATCC 6919) was incubated anaerobically for 72 h at 37°C. The antimicrobial activity was expressed as inhibition zone diameters (mm). Methylparaben was used as positive control for *S. aureus*, *B. subtilis*, and *E. coli*. Salicylic acid was used as positive control for *P. acnes*.

2.8. Liposome Manufacturing

ASCE45 was encapsulated in liposomes (F1), and R6 was added to this liposomes (F2). The liposome formulas are shown in Table 1. After melting the [A] and [B] phases at 80 °C, respectively, the [B] phase was injected into the [A] phase and mixed at 3000 rpm for 30 min using a homogenizer (CORETECH, Korea). The pre-emulsion was passed a high pressure homogenizer (Micronox, Korea) three times at 1200 bar to obtain liposomes. ASCE45 0.5 % solution dissolved in 50 % ethanol (F0) was made to be used as a control group.

Table 1. Two types of liposome formulas.

Phase	Component	Content (wt %)	
		F1	**F2**
[A]	Glycerin	60.0	60.0
	Hydrogenated lecithin	5.0	5.0
	DI water	7.5	7.4
	1,2-hexanediol	2.0	2.0
	R6	-	0.1
[B]	Medium chain triglyceride	25.0	25.0
	ASCE45	0.5	0.5

2.9. Particle Size and Zeta Potential Measurements

Particle size and zeta potential of the F1 were measured using Nanotrac Flex particle size analyzer (Microtrac,

USA) and Stabino® particle charge mapping system (Particle Metrix, USA), respectively. The F0 was used as a control group.

2.10. *In vitro* Skin Permeability Experiment

The skin permeability of the F1 and F2 was evaluated using a vertical Franz diffusion cells and systems (PermeGear, USA) (**Alice S. et al., 2016**). Skin (Neoderm®-E, Tegoscience, Korea) was mounted between the donor and receptor chamber with the *stratum corneum* side up and the donor surface area of 1.326665 cm^2. The receptor chamber was filled with 8.5 mL of PBS (pH 7.4) and maintained at 36.0 ± 0.5°C, using a circulating water bath (Lab House, Korea). The receptor phase was continuously stirred at 500rpm using magnetic stirring bar. After 30 min of stabilization, 0.5 mL of the donor samples were applied on the skin. 0.5 mL of the receptor phase was withdrawn after 4, 8 ,12, 16, 18, and 24 h and the receptor phase was immediately refilled with an equal volume of PBS. The receptor samples were analyzed by HPLC using D/DA as an indicator. Because the receptor phase was constantly being removed and replenished, the concentration of D/DA in the receptor samples were corrected and the corrected data were expressed as the cumulative amount permeated of drug per unit of skin surface area. The F0 was used as a control group.

2.11. Statistical Evaluation

All experiments were repeated three times. All values were expressed as mean ± standard deviation (SD). The differences between the values were analyzed by t-test and one-way analysis of variance (ANOVA) using SPSS statistics software version 18.0 (SPSS Inc., USA).

3. Results and Discussions

3.1. Decursin and Decursinol Angelate Content

D/DA constitutional isomers, were quantified together because they were difficult to separate (**Lee J.H. et al., 2006**; **Park K.W. et al., 2007**). As a result of quantifying D/DA, the content of D/DA was 0 % in AHW, but in ASCEs, the content was very high in the order of ASCE45, ASCE30, and ASCE60, with 44.58 %, 41.18 %, and 40.70 %, respectively (Table 2). Therefore, the supercritical CO_2 containing ethanol extraction method is ideal to extract D/DA, the major active compounds of AG, and among them, it is most efficient to set the temperature of the extractor at 45°C when the pressure of the extractor is 350 bar.

Table 2. Decursin and decursinol angelate content according to extraction methods.

Extract	Decursin and decursinol angelate content (%)
AHW	-
ASCE30	41.18 ± 0.05
ASCE45	44.58 ± 0.08
ASCE60	40.70 ± 0.16

3.2. Total Polyphenol Content

The content of total polyphenol was the highest in AGE with 11.65 mg GAE/g, while in ASCEs, the content was in the order of ASCE45, ASCE30, and ASCE60, with 2.69 mg GAE/g, 2.64 mg GAE/g, and 0.92 mg GAE/g, respectively (Table 3).

Table 3. Total polyphenol content according to extraction methods.

Extract	Total polyphenol content (mg GAE/g)
AHW	11.34 ± 1.01
ASCE30	2.64 ± 0.56
ASCE45	2.69 ± 0.05
ASCE60	0.92 ± 0.03

3.3. Antioxidant Activity

3.3.1. DPPH Radical Scavenging Activity

As a result of measuring the DPPH radical scavenging activity, a typical antioxidant effect evaluation method, AHW showed a scavenging activity of 83.82 % at 1.25 μg/mL, while ASCE30, ASCE45, and ASCE60 were 75.72 %, 78.12 %, and 49.57 % at 15 μg/mL, respectively (Figure 1).

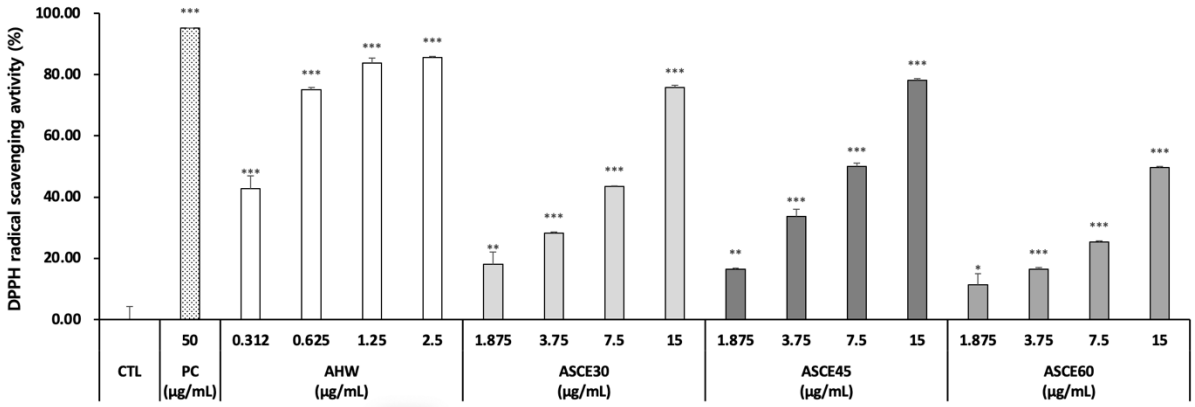

Figure 1. DPPH radical scavenging activity of *Angelica gigas* extracts.

Interpretation of Figure 1.
*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$

3.3.2. ABTS+ Radical Scavenging Activity

According to the results of measuring the ABTS+ radical scavenging activity, another representative method of evaluating antioxidant ability, the concentration of scavenging ABTS+ radical was significantly lower than that of scavenging DPPH radical in all samples. AHW showed a scavenging activity of 85.31 % at 0.25 μg/mL, while ASCE30, ASCE45, and ASCE60 were 79.18 %, 81.35 %, and 54.79 % at 1 μg/mL, respectively (Figure 2).

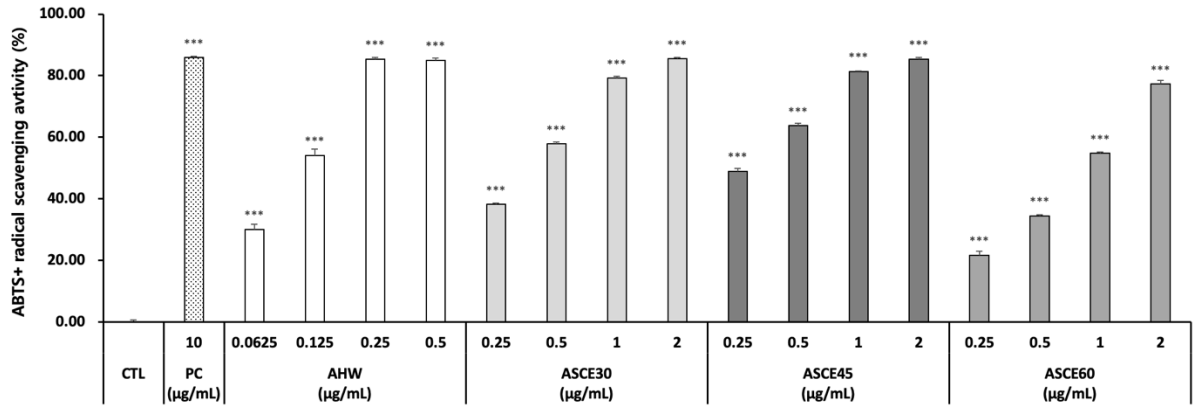

Figure 2. ABTS+ radical scavenging activity of *Angelica gigas* extracts.

Interpretation of Figure 2.
*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$

As a result of measuring the scavenging activity of DPPH radical and ABTS+ radical, AHW showed the highest antioxidant effect. However, ASCEs were also effective at very low concentrations, of which ASCE45 was the best, followed by ASCE30 and ASCE60. These results are in line with the results of total polyphenol content, so it is inferred that the antioxidant activity is greatly affected by polyphenol.

3.4. Tyrosinase Inhibition Activity

Tyrosinase inhibition activity was 26.60 % for AHW, 49.06 % for ASCE30, 46.17 % for ASCE45, and 47.30 % for ASCE60 based on 333 μg/mL. ASCEs showed better activity, and there were no significant differences according to the temperature conditions (Figure 3). This result confirmed the possibility of using AG as a whitening agent.

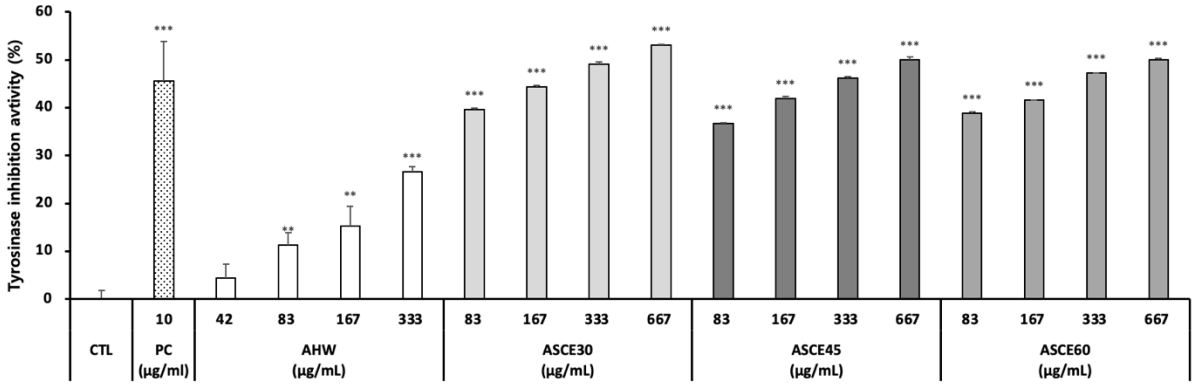

Figure 3. Tyrosinase inhibition activity of *Angelica gigas* extracts.

Interpretation of Figure 3.
*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$

3.5. Antimicrobial Activity

As a result of measuring antimicrobial activity, AHW was inactive for all strains. ASCEs also had no effect on *E. coli*, but had significant effects on *S. aureus*, *B. subtilis*, and *P. acnes*. Although there were no significant differences depending on temperature conditions, ASCE45 was the most effective overall. These results are in line with the results of D/DA content, so it is inferred that the antimicrobial activity is greatly affected by D/DA. Meanwhile, based on strains, the effect on *P. acnes* was the best, followed by *B. subtilis* and *S. aureus*. Detailed clear zone measurement values are indicated in Table 4, Table 5, and Table 6.

Table 4. Antimicrobial activity against *Staphylococcus aureus*

S. aureus	**Diameter of clear zone (mm)**			
Extract	**Concentration (mg/mL)**			
	20	10	5	2.5
AHW	-	-	-	-
ASCE30	12.00 ± 0.10	11.23 ± 0.59	10.20 ± 0.26	9.33 ± 0.29
ASCE45	12.33 ± 0.42	11.53 ± 0.15	11.00 ± 0.50	10.00 ± 0.20
ASCE60	12.07 ± 0.15	11.40 ± 0.44	10.63 ± 0.51	9.50 ± 0.50

Interpretation of Table 4.
Methylparaben 20 mg/mL: 9.90 ± 0.66 mm

Table 5. Antimicrobial activity against *Bacillus subtilis*

B. subtilis	**Diameter of clear zone (mm)**			
Extract	**Concentration (mg/mL)**			
	20	10	5	2.5

AHW	-	-	-	-
ASCE30	12.47 ± 0.15	11.67 ± 0.21	10.83 ± 0.29	9.63 ± 0.45
ASCE45	12.57 ± 0.12	11.73 ± 0.21	11.13 ± 0.12	10.23 ± 0.25
ASCE60	12.67 ± 0.21	11.93 ± 0.12	11.20 ± 0.26	10.10 ± 0.10

Interpretation of Table 5.
Methylparaben 20 mg/mL: 10.80 ± 0.30 mm

Table 6. Antimicrobial activity against *Propionibacterium acnes*

P. acnes	**Diameter of clear zone (mm)**			
Extract	**Concentration (mg/mL)**			
	20	10	5	2.5
AHW	-	-	-	-
ASCE30	13.60 ± 0.46	13.30 ± 0.44	13.00 ± 0.10	12.17 ± 0.29
ASCE45	13.97 ± 0.06	13.37 ± 0.55	13.00 ± 0.10	12.20 ± 0.26
ASCE60	12.80 ± 0.29	13.53 ± 0.35	13.17 ± 0.31	12.40 ± 0.17

Interpretation of Table 6.
Salicylic acid 20 mg/mL: 13.17 ± 0.29 mm

3.6. Particle Size and Zeta Potential

Based on the results of the experiments so far, ASCE45 was selected as the optimal extract. Liposome technology was applied to ASCE45, and as a result, particle size and zeta potential were improved to 103.93 nm and -77.93 mV, respectively, as shown in Table 7. In other words, the solubility and stability of ASCE45 have increased.

Table.7. Particle size and zeta potential of liposome containing ASCE45

Formulation	Particle size (nm)	Zeta potential (mV)
F0	628.00 ± 13.08	-12.43 ± 0.25
F1	103.93 ± 0.91	-77.93 ± 0.60

3.7. *In vitro* skin permeability

Skin permeability of ASCE45 was expected to increase due to liposome technology, but R6, one of the cationic CPPs, was applied to the liposome to maximize effects of ASCE45 by increasing the permeability further. As a result of measuring *in vitro* skin permeability, based on 24h, F1 penetrated 100.10 μg/cm^2, and F2 penetrated 85.81 μg/cm^2, and this is 1.74 times and 1.49 times higher than 57.55 μg/cm^2, cumulative amount permeated of F0 (Figure 4). In other words, applying F2, the optimal skin penetration condition, to ASCE45 extracted under optimal extraction conditions is inferred very efficient in actually showing effects on the skin. This result was statistically significant at all times (p<0.001).

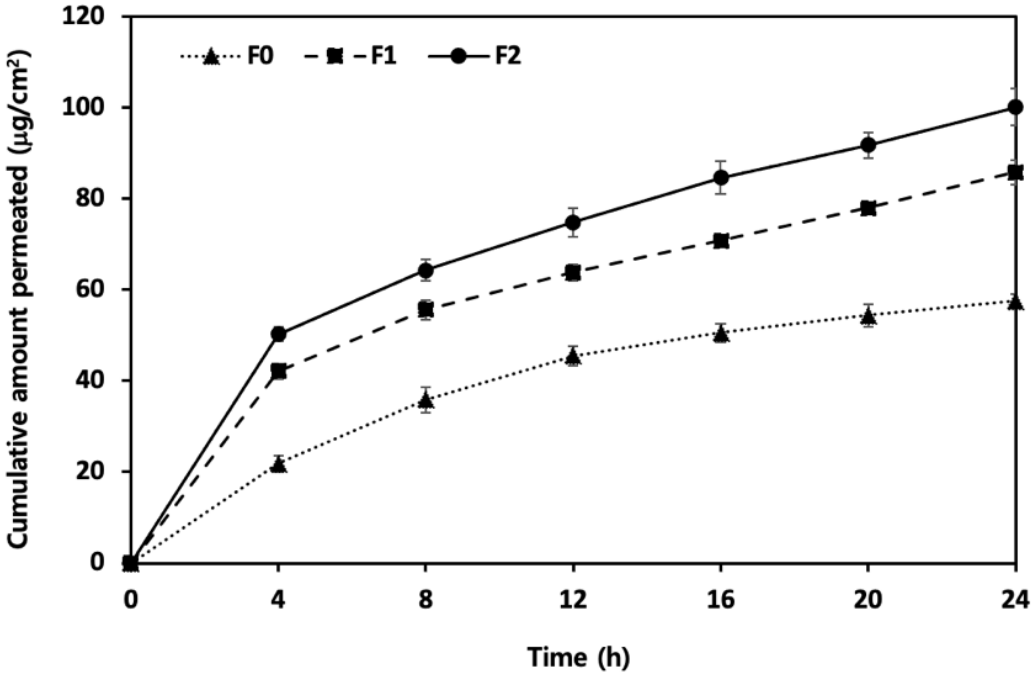

Figure 4. *In vitro* skin permeability of three formulation containing ASCE45

4. Conclusion

This study aimed to find a way to more efficiently extract the active compounds from Korean AG and to maximize the effects by applying DDSs to the optimal extract. To sum up the results, 1) The content of D/DA (decursin and decursinol angelate) was 0 % in AHW, but in ASCEs, the content was very high in the order of ASCE45, ASCE30, and ASCE60, with 44.58 %, 41.18 %, and 40.70 %, respectively. 2) The content of total polyphenol was the highest in AGE with 11.65 mg GAE/g, while in ASCEs, the content was in the order of ASCE45, ASCE30, and ASCE60, with 2.69 mg GAE/g, 2.64 mg GAE/g, and 0.92 mg GAE/g, respectively. 3) The results of DPPH radical and ABTS+ radical scavenging activity were in line with the results of total polyphenol content, so it is inferred that the antioxidant activity is greatly affected by polyphenol. 4) In tyrosinase inhibition activity, ASCEs showed better activity, and there were no significant differences according to the temperature conditions. 5) The results of antimicrobial activity were in line with the results of D/DA content, so it is inferred that the antimicrobial activity is greatly affected by D/DA. 6) Based on the results of the experiments so far, ASCE45 was selected as the optimal extract and liposome technology was applied to it, which result in improving particle size and zeta potential. 7) R6, one of the cationic CPPs, was applied to the liposome to maximize effects of ASCE45 by increasing the permeability further, and as a result of measuring in vitro skin permeability, cumulative amount permeated was increased 1.74 times higher than a control group. Therefore, this study established the optimal conditions to use Korean AG as a natural functional raw material for cosmetics.

Acknowledgment

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