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# Antimicrobial Effect of Supercritical *Robinia pseudo-acacia* Leaf Extracts and Its Transdermal Delivery System with Cell Penetrating Peptide

Soo Hyeon Heo[1], Su In Park[2], Jinseo Lee[1], Miok Kim[3] and Moon Sam Shin[4,*]

[1]*Master Student, Department of Senior Healthcare majoring in Cosmetic Pharmacology, Eulji Univ.*
[2]*Doctorial Student, Department of Senior Healthcare majoring in Cosmetic Pharmacology, Eulji Univ.*
[3]*Professor, Department of Beauty Design, Shin Ansan Univ., Korea.*
[4]*Professor, Department of Senior Healthcare majoring in Cosmetic Pharmacology, Eulji Univ., Korea*
*E-Mail : h_soo_h@naver.com, sooo_30@naver.com, dlwlstj97@naver.com, miok5004@hanmail.net, msshin@eulji.ac.kr*

***Abstract***

*In this paper, we present to evaluate physiological activity of Robinia pseudo-acacia leaf and its skin penetration using liposome and cell penetrating peptide. After extraction with Robinia pseudo-acacia leaf using the distilled water and supercritical, various physiological activities were examined. In antioxidants experiments, the total concentration of polyphenol compounds was determined to be 56.88 mg/g in hydrothermal extract, 45.07 mg/g in supercritical extract. The DPPH radical scavenging ability at 1,000 μg/mL was 33.97% in supercritical extract. The scavenging effect on SOD experiment at 500 μg/mL was 76.41% in supercritical extract. In the antimicrobial experiments, the hydrothermal extract had no effect, but supercritical extract represented maximum clear zone of 14.00 mm in Staphylococcus aureus strain. Liposome containing the RSE (Robinia pseudo-acacia leaf supercritical extract) reduced particle size and stabilized zeta potential. In the epidermal permeability experiment, it was confirmed that the permeation of liposome containing the RSE and cell penetrating peptides was remarkable.*

***Keywords:*** *Robinia pseudo-acacia*, Supercritical, Antimicrobial, Cell Penetrating Peptide, Cosmetics

## 1. Introduction

The modern society has a growing appetite for health and beauty as the economy grows and living standards improve. Also, as we enter into an aging society, the role of cosmetics to cope with aging is rising in demand for functional aspects such as antioxidants and antimicrobial rather than simply cleanliness and moisturizing. In response to these demands, attention is focused on the active ingredient, which is mainly synthetic. They have many problems such as immunity, chronic toxicity, mutagenicity, and carcinogen-causing, and interest in plant resources containing various active ingredients is increasing to compensate for them [1]. In addition, research is underway to develop natural substances that are effective in whitening, acne, antimicrobial, antioxidant, anti-aging, and other materials such as cosmetics, food additives and medicine [2, 3].

Hydrothermal extraction and solvent extraction are mainly used to obtain high purity functional extracts required in the cosmetics and food industry, and solvent extraction methods are used often to obtain various active ingredients. However, there are problems with solvents such as environmental pollution caused by large

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Corresponding Author: msshin@eulji.ac.kr
Tel: +82-31-740-7423, Fax: +82-31-740-7358
Professor, Department of Senior Healthcare majoring in Cosmetic Pharmacology, Eulji University, Korea

amounts of waste solvents and residual solvents in extracts. Interest in supercritical fluid extraction process using supercritical carbon dioxide is increasing as an environment-friendly alternative technology for compensate and improve the shortcomings of existing natural effective extraction process. Extracting supercritical fluids uses carbon dioxide that is harmless to the human body. In addition, when extracting active ingredients with a range of molecular weights from natural materials, there are several application ranges depending on the extraction method. For supercritical carbon dioxide, it is possible to extract the active ingredient for almost any range of molecular weight. Currently, studies are actively conducted on various natural extracts using supercritical carbon dioxide.

In order for these substances to function properly as cosmetics materials, they must be absorbed deep into the skin through the stratum corneum, the outermost layer of the skin. However, the lipids of the stratum corneum consist of ceramide, cholesterol, and fat acid, form a lamellar structure. And as a skin barrier, preventing the transmission of external materials and thus making it difficult to pass the skin permeability of the active ingredients. Since the discovery in the 1960s that phospholipid surfactants of the phospholipid system voluntarily form double layer-like bimolecular lipid membrane in the medium of water surface [4], there has been a lot of research on liposome as a model of biological membrane as well as application of in vivo drug transport to decrease side effects, target orientation, continuous action in the pharmacology field [5, 6]. In order to industrialize liposome, the stability and uniformity of particle distribution must be ensured, because it is a factor that greatly affects the dynamics of the body when the liposome is applied to the clinical practice. As a way to solve these problems, When manufacturing liposome using microfluidizer, high concentrations of lipids that are usually not available in the manufacturing method of multilamellar vesicles (MLV) liposome can be used [7]. The advantage of manufacturing liposome using microfluidizer is that 1) they can produce large quantities of liposomes in a continuous process, which is suitable for industrialization; 2) the ability to control the average particle size of the produced liposome; 3) the ability to use high concentration of lipids to seal the water-soluble drugs with high efficiency; 4) reproducibility is guaranteed in the particle size and encapsulation efficiency of liposome. 5) since the particles are very fine, sterilization by filtration is possible [8].

Cell penetrating peptide (CPP) is a peptide composed of about 10 to 20 short amino acids that can pass through the cell membrane and transfer material inside the cell, and many CPP have been developed. One of the typical CPPs, TAT Peptide is known to easily approach cell membranes due to arginine, a cationic amino acid [9]. CPP is mainly focused on the research of penetrating cell membranes, while intercellular lipid penetrating research is yet insignificant. Although the cell membrane and intercellular lipids differ from each other, it is expected that if arginine oligomer, a key amino acid sequence of cell penetrating peptide, is applied to cosmetics with functional substances, it will be able to maximize the efficacy of functional substances by enhancing skin absorption [10].

In this study, first of all *Robinia pseudo-acacia* leaf were extracted with hydrothermal and supercritical fluid and then antioxidants (total polyphenol content, DPPH radial scavenging activity, SOD-like activity) were measured for verifying physiological activity. In order to stabilize supercritical extracts with excellent antimicrobial effect and enhance its epidermis penetration, liposome similar to lamella liquid crystal of intercellular lipid was made and particle size was reduced and stabilized. In addition, the increase in its epidermis penetration was confirmed by applying CPP (arginine oligomer, R6). As a result, we want to present the possibility of using *Robinia pseudo-acacia* leaf supercritical extract as a functional cosmetics natural new material.

## 2. Materials and methods

### 1. Instruments and reagents

The equipment and reagents used for each experiment are as follows. The solutions used for the polyphenol, DPPH test used in the antioxidant and antibacterial tests were obtained from Sigma Aldrich (USA). R6 (hexa-D-arginine) was obtained from Dermafirm Co. (Seongnam, Korea). The equipment used in the experiment is as follows. Supercritical fluid extraction (ARI instrument, Namyangju, Korea), Absorption spectrophotometer (SYNERGY HTX multi-mode reader, Bio Tek, Seoul, Korea), Centrifugal separator (Supra-25K, Hanil

Scientific Inc., Gimpo, Korea). Thermostat (Changshin Science, Seoul, Korea), High pressure processor (Microfluidizer, Picomax, Seoul, Korea), Particle size analyzer (Nanoctrac Flex, DREAM Co., Suwon, Korea), Particlemetrix (Stabino® Paticle Charge Mapping, DREAM Co., Suwon, Korea), Franz Diffusion Cells and Systems (PermeGear, USA).

**2. Sample extraction**

In the hydrothermal extraction method, purified water was added to the *Robinia pseudo-acacia* leaf powder and extracted for 4 hours in a thermostat at 80℃ and filtered and freeze dried. In the supercritical extraction method, the pressure of the extractor was set to 400 bar and the temperature to 50℃. The pressure of the separator was set to 40 bar, the temperature was set to 40℃, and the flow rate of $CO_2$ was extracted at 60 mL/min for 150 minutes.

**3. Antioxidant activity measurement**

Quantification of polyphenol was measured by Folin-danis [11]. To 100 μL of the Folin-Ciocalteu reagent, add 100 μL of the diluted sample solution and reacted at room temperature for 3 minutes. 100 μL of $Na_2CO_3$ solution was added and the absorbance was measured at 760 nm with an ELISA reader. The average value of polyphenol contents by concentration was calculated. The calibration curves were quantitatively analyzed using garlic acid as a standard.

The effect about DPPH radical scavenging was measured by Blois method [12]. To 100 μL of the extract solution, 120 μL of 0.45 mM 2,2-diphenyl-1-picrylhydrazyl solution was added and reacted in the dark room for 30 minutes. Absorbance was measured at 530 nm with an ELISA reader.

DPPH radical scavenging activity (%) = [(Absorbance of DPPH solution - absorbance of samples) / absorbance of DPPH solution] × 100.

SOD-like activity was performed by modifying Marklund's method [13]. The experiment was carried out using SOD Assay Kit (BCBV5418). 20 μL of buffer solution and 20 μL of enzyme working solution were added to 20 μL of each sample solution, and incubation was carried out at 37℃ for 20 minutes. The absorbance at 420 nm was measured by an ELISA reader.

SOD similar activity (%) = [1-(Absorbance in the sample addition group/absorbance in the no additives)]×100

**4. Antimicrobial experiment**

The disc diffusion test was performed to determine the antimicrobial activity of *Robinia pseudo-acacia* leaf [14]. *Staphylococcus aureus, Escherichia coli, Bacillus subtilis*, and *Propionibacterium acnes* were purchased from KCM and KCTC. The strains *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* were cultured in Muller-Hinton medium at 37℃ for 24 hours, re-cultured once, and then absorbed at 600 nm using a spectrophotometer. *Propionibacterium acnes* was incubated in a sealed container for 72 hours, re-incubated once, and then absorbed at 600 nm using a spectrophotometer. The culture conditions are shown in Table 1.

**Table 1. List of strains and cultivation condition used for antimicrobial experiments**

| Strains | Media | Temperature (℃) | Time (h) |
|---|---|---|---|
| *Staphylococcus aureus* (ATCC6538) | MH | 37 | 24 |
| *Escherichia coli* (ATCC23726) | MH | 37 | 24 |
| *Bacillus subtilis* (ATCC19659) | MH | 37 | 24 |
| *Propionibacterium acnes* (ATCC6919) | RC | 37 | 72 |

Media was used by MH (Muller-Hinton medium) and RC (Reinforced Clostridial medium)

**5. Liposome making method**

First, the composition of the raw materials for manufacturing the liposome is shown in Table 2. Each of the phase was completely dissolved and the B phase was put into A phase. Using homo-mixer, mix evenly for 15 minutes at a speed of 3,000 rpm. Then, by passing through the microfluidizer three times at 1200 bar, the liposome was obtained.

**Table 2. Composition of *Robinia pseudo-acacia* leaf extraction formulation for Liposome**

| Phase | Raw material | Chemical name | Content (wt%) |
|---|---|---|---|
| A | Glycerine | Glycerine | 60.0 |
| | Soya SPL 75H | Hydrogenated Lecithin | 5.0 |
| | Water | Distilled water | 8.0 |
| | 1,2 Hexanediol | 1,2 Hexanediol | 2.0 |
| B | DC 200(100cs) | Dimethicone | Up to 100 |
| | Active | *Robinia pseudo-acacia* leaf supercritical extract | 0.5 |

**6. Measurement of particle size and zeta potential**

We measure of particle size and zeta charge about 0.5% RSE (*Robinia pseudo-acacia* leaf supercritical extract) and MLV containing 0.5% RSE at 3 times using Particlemetrix (Stabino® Paticle Charge Mapping, DREAM CORP, Suwon, Korea)

**7. Skin penetration experiment**

**1) Trans epidermal permeability**

We made 3 formulations for skin permeability experiments. First, *Robinia pseudo-acacia* leaf supercritical extract was dissolved in 99.9%. Second, liposome 0.5% of *Robinia pseudo-acacia* leaf supercritical extract. Third, liposome contains 0.005% R6. Trans epidermal permeability was measured using Franz Diffusion Cells and Systems (PermeGear, USA). The artificial skin is placed on the receptor chamber with the stratum corneum facing up, and the donor chamber is fixed on the stratum corneum. The temperature was maintained at 37℃ in a constant-temperature water bath, and the sample was applied to the skin after stabilization for 30 minutes. Keep the permeated sample uniformly mixed. Then, the receptor medium in which the sample was dissolved was sampled at a fixed time, and the same amount of receptor medium was supplemented. Finally, we compared Tannic acid content among 3 formulations. Skin permeability conditions are shown in Table 3.

**Table 3. Conditions of skin penetration experiment**

| | |
|---|---|
| Skin | Neoderm®-E (Tegoscience, Korea) |
| Receptor medium | PBS (SigmaAldrich, USA) 8.5ml (add 5.0% Tween 80) |
| Sampling aliquot | 500 μL |
| Donor chamber area | 1.326665 $cm^2$ |
| Stirbar speed | 500 |
| Sampling time | 4,8,12,16,20,24 h |
| Temperature | 37℃ |

**2) Tannin acid analyses by HPLC**

Chromatographic separations were achieved using a Shiseido C18 (4.6×250 mm, 5 μm). A reverse phase HPLC assay was carried out using an isocratic elution with a flow rate of 1.0 mL/min, a column temperature of 30℃, a mobile phase of water and methanol (50% : 50% v/v) and detection wavelength of 270 nm. The injection volume was 20 μL of each solutions. The total run time was 5 minutes for each injection. Quantification of the compounds identified was performed using the external standardization method.

**8. Statistical processing**

All experiments were repeated 3 times. All values were expressed as mean and standard deviation and the difference between the values was analyzed by t-test, one-way analysis of variance (ANOVA) with Post hoc(LDS) respectively.

## 3. Result

**1. Yield**

*Robinia pseudo-acacia* leaf was extracted with hydrothermal and supercritical. Each yield was 4.72% in RPH (*Robinia pseudo-acacia* leaf hydrothermal extract) and 3.80% in RPS (*Robinia pseudo-acacia* leaf supercritical extract).

**2. Antioxidant efficacy of *Robinia pseudo-acacia* leaf**

To measure the total polyphenol content, the results of the comparison of the extraction process of *Robinia pseudo-acacia* leaf extract are shown in Table 6. In 250 mg/L, 56.06 ± 1.68 mg/g of polyphenol was extracted from hydrothermal extraction and 46.8 0 ± 2.00 mg/g of polyphenol was detected in supercritical extraction (Table 4).

DPPH radical is a method of measuring the activity of a hydrogen donor. When they get electron from phenolic compounds or aromatic amines, the color is turned purple to yellow by proton-radical scavengers [15]. The antioxidant activity of the extracts was shown between 125 ~ 1,000 mg/L. In 1000 mg/L, the radical scavenging activity was 41.49% in hydrothermal extraction and 33.97% in supercritical extraction (Figure 1).

SOD-like activity assay is an antioxidant activity assay using color development by automatic oxidation [16]. The substances that inhibit superoxide in the samples used in the experiment can inhibit the oxidation by oxidation in the presence of SOD or SOD-like active substances. The highest SOD-like activity of 72.44% in supercritical extract at 250 mg/L (Figure 2).

**Table 4. Total polyphenols of extracts from *Robinia pseudo-acacia* leaf**

| Samples | Method | Total polyphenols (mg/g) |
|---|---|---|
| RPH[a] | Hydrothermal extract | 56.88 ± 8.93 |
| RPS[b] | Supercritical extract | 45.07 ± 2.75 |

Values represent the mean ± SD of three independent experiments.
[a] RPH : *Robinia pseudo-acacia* leaf hydrothermal extract
[b] RPS : *Robinia pseudo-acacia* leaf supercritical extract

**3. Antimicrobial experiment**

The antimicrobial test was conducted three times using the paper disc method [17]. The results of the clear zone measurement are shown in Table 5. As a result of the antimicrobial test, the hydrothermal extract had no effect. In the other hand, the antimicrobial effect was confirmed in three strains among the four strains from

supercritical extract. In the case of *Propionibacterium acnes* strain, the largest clear zone of 10.33 ± 0.58 mm was found at the concentration of 10 mg/mL. In the *Bacillus subtilis* strain, the greatest clear zone of 10.01 ± 0.03 mm was found at the concentration of 10 mg/mL. Supercritical extract represented maximum clear zone of 14.67 ± 0.78 mm in *Staphylococcus aureus* strain. In experiments with the same conditions, *Robinia pseudo-acacia* ethanol extract had no effect in *Staphylococcus aureus* strain [18].

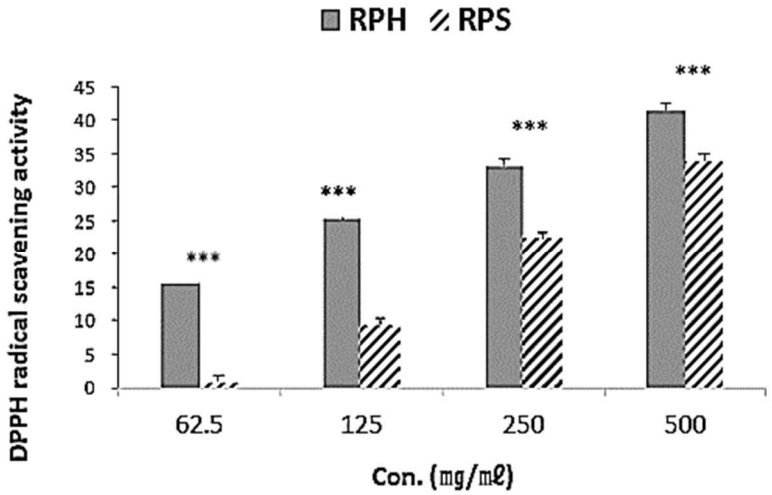


**Figure 1. Scavenging effect of *Robinia pseudo-acacia* leaf on DPPH assays**

Values represent the mean ± SD of three independent experiments. Positive control : Ascorbic acid 100 μg/mL to 97.46%. *p < 0.1, **p < 0.05, ***p < 0.001.

[a] RPH: *Robinia pseudo-acacia* leaf hydrothermal extract

[b] RPS: *Robinia pseudo-acacia* leaf supercritical extract

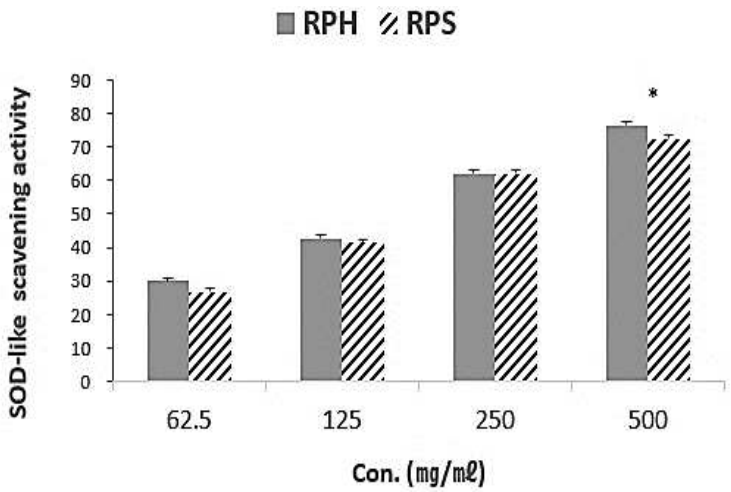


**Figure 2. Scavenging effect of *Robinia pseudo-acacia* leaf on SOD assays**

Values represent the mean ± SD of three independent experiments. *p＜0.1, **p＜0.05, ***p＜0.001.

[a] RPH: *Robinia pseudo-acacia* leaf hydrothermal extract

[b] RPS: *Robinia pseudo-acacia* leaf supercritical extract

**Table 5. The effect of Supercritical *Robinia pseudo-acacia* leaf extract amount on area of clear zone**

| Strain | Clear zone (mm) | | | |
|---|---|---|---|---|
| | 10 mg/mL | 5 mg/mL | 2.5 mg/mL | 1.25 mg/mL |
| *Staphylococcus aureus* | 14.67 ± 0.78 [a] | 14.67 ± 0.58 [a] | 12.33 ± 0.52[a] | - |
| *Escherichia coli* | - | - | - | - |
| *Bacillus subtilis* | 10.01 ± 0.03 [a] | 10.03 ± 0.05 [a] | 10.21 ± 0.10[a] | 10.31 ± 0.10 [a] |
| *Propionibacterium acnes* | 10.33 ± 0.58 [a] | 10.33 ± 0.58 [a] | 9.67 ± 1.15 [a] | 9.67 ± 1.15 [a] |

Positive control: *Staphylococcus aureus, Escherichia coli, Bacillus subtilis* are used methyl paraben, *Propionibacterium acnes* is used salicylic acid.
[a] Growth inhibition line

**4. Measurement of particle size and zeta potential**

The average particle size was compared by measuring 0.5% of RSE and the liposome containing 0.5% RSE at 3 times. The average 0.5% RSE particle size was 3313.33 ± 107.86 nm, and the average particle size of the liposome containing 0.5% RSE was 258.27±8.72 nm, which was reduced approximately by 1/13.

Zeta-potential is 0 ~ ± 5 mV quickly condenses and becomes unstable. When the zeta-potential is ± 30 ~ ± 60 mV, the solution is highly compatible with water, so it is well distributed, the particles are kept stable. And if it is more than ± 61 mV, it can be considered very stable. The average zeta-potential value of 0.5% RSE was 20.30 ± 0.10 mV, and the zeta-potential value of the liposome containing 0.5% RSE was 132.47 ± 1.43 mV. Compared to RSE, the liposome has become very stable.

**5. Skin penetration experiment**

Figure 3 shows the permeation rate of *Robinia pseudo-acacia* leaf extract over time for a given area of skin. The cumulative amount of total *Robonia pseudo-acacia* leaf extract penetrated the skin over time increased with similar tendency for all 24 hours in all three formulations. When the amount of permeation is observed, the amount of permeation is high in the order of Formulation 2, Formulation 1, and Formulation 0, meaning that liposome and cell permeable peptides can increase skin penetration. The results of trans epidermal permeability experiment were as follows (Figure 3). 'Formulation 2' was the most penetrating when the three formulations showed trans epidermal permeability and it had 34.61 μg/cm². All results are shown in Table 6.

**Table 6. Epidermal penetration experiment's result using Franz cell according to formulation with *Robinia pseudo-acacia* leaf**

| | Time (h) | | 0 | 4 | 8 | 12 | 16 | 20 | 24 |
|---|---|---|---|---|---|---|---|---|---|
| Cumulative amount permeated (μg/cm²) | RPS 0.5% in Ethanol | Mean | 0 | 8.35 | 11.30 | 16.06 | 19.78 | 22.70 | 25.72 |
| | | SD | 0 | 0.97 | 0.86 | 0.61 | 1.12 | 0.95 | 1.08 |
| | RPS 0.5% in Liposome | Mean | 0 | 12.71 | 18.31 | 22.63 | 25.47 | 28.22 | 30.15 |
| | | SD | 0 | 1.36 | 0.37 | 0.83 | 0.78 | 0.55 | 0.57 |
| | RPS 0.5% and R6 0.005% | Mean | 0 | 18.42 | 21.76 | 26.20 | 30.02 | 32.36 | 34.61 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| in Liposome | SD | 0 | 0.80 | 1.26 | 0.93 | 1.39 | 1.12 | 1.04 |
| onw-way ANOVA | | - | .000 | .000 | .000 | .000 | .000 | .000 |

RPS : *Robinia pseudo-acacia* leaf supercritical extract

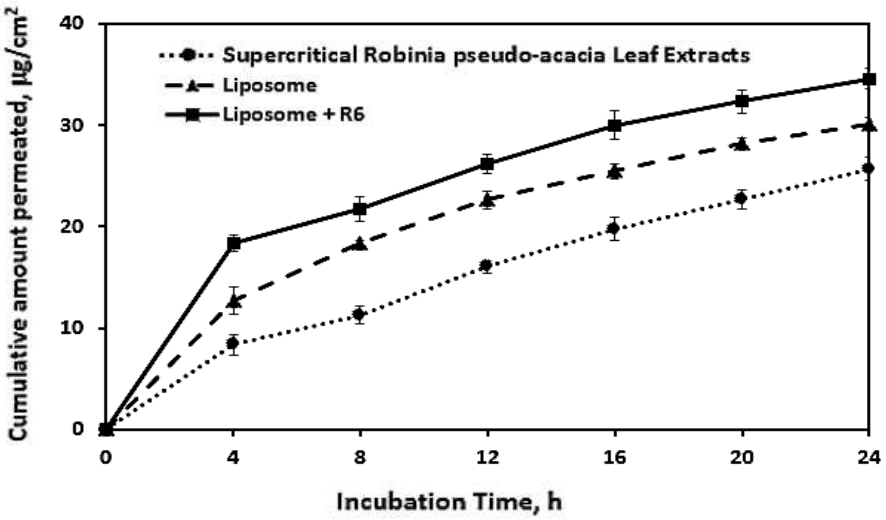


**Figure 3. *In vitro* skin penetration profiles of Supercritical *Robinia pseudo-acacia* leaf extract, Liposome+ R6 through epidermal skin and penetration cumulative amount.**
*Robinia pseudo-acacia* leaf supercritical extract is formulation 0
Liposome is formulation 1
Liposome + R6 is formulation 2

## 4. Conclusions

The purpose of this study was to investigate the efficacy of *Robinia pseudo-acacia* leaf as a cosmetic product and transdermal delivery ability using liposome with cell-penetrating peptide. In antioxidants experiments, the total concentration of polyphenol compounds was determined to be 56.88 mg/g in hydrothermal extract, 45.07 mg/g in supercritical extract. The DPPH radical scavenging ability at 1,000 μg/mL was 33.97% in supercritical extract. The scavenging effect on SOD experiment at 500 μg/mL was 76.41% in supercritical extract. In the antimicrobial experiments, the hydrothermal extract had no effect, but supercritical extract represented maximum clear zone of 14.00 mm in *Staphylococcus aureus* strain. In experiments with the same conditions, the antimicrobial effect of supercritical extract in *Staphylococcus aureus* strain is superior when compared to the fact that it had no effect.

To stabilize RSE (*Robinia pseudo-acacia* leaf supercritical extract) with excellent antimicrobial effect, it was made of liposome. As a result, the average particle size of the liposome containing the RSE was 258.27±8.72 nm, which was reduced approximately by 1/13 and the zeta-potential of the liposome containing RSE was 132.47±1.43 mV. Compared to RSE, the liposome has become very stable. After applying CPP (Cell Penetrating Peptide, R6) to liposome containing RSE, the results of the skin penetration experiment using the three formulations are as follows. Applying CPP to liposome was the most penetrating when the three formulations showed trans epidermal permeability. For 24 hours cumulative amount permeated is as follows. CPP (Cell Penetrating Peptide, R6) to liposome had 34.61 μg/cm², liposome containing RSE had 30.15 μg/cm², RSE had 25.72 μg/cm².

The effect of antioxidant, antimicrobial on *Robinia pseudo-acacia* leaf extract was investigated through this

study so the result of skin penetration through the production of liposome containing *Robinia pseudo-acacia* leaf supercritical extract to be highly likely to be commercialized in the cosmetics industry in the future.

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