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# Cosmetic Efficacy of *Red Pinus densiflora* and Its Epidermis Penetration with Polymer Micelle and Cell Penetrating Peptide

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***Abstract***

This study aimed to investigate the effects and epidermis penetration system with polymer micelle of *Red Pinus densiflora* extract. In the antioxidant test, the total concentration of polyphenol compounds was determined to be 137.5163±7.70 mg/g in ethanol extract, 133.956±1.57 mg/g in hydrothermal extract. The DPPH radical scavenging effects were 95.29±0.15% in ethanol extract at 1,000 mg/L. Elastase inhibition rates were 100.00±2.85% in ethanol extract at 2,000 mg/L. The antimicrobial effect of the ethanol extraction was higher than that of hydrothermal extractions. In the epidermal permeability experiment, it was confirmed that the permeation of the polymer micelle containing the *Red Pinus densiflora*'s ethanol extract and cell penetrating peptides was remarkable. Here, we confirmed that ethanol extract of *Red Pinus densiflora* displayed excellent the effects in antioxidant test and epidermis penetration system with polymer micelle. As a result, *Red Pinus densiflora* extract has potential to be used as a safe and natural cosmetic material in the future.

***Keywords:*** Antioxidant, Cosmetic, Penetration, Polymer micelle, *Red Pinus densiflora*

## 1. INTRODUCTION

As people's interest increases about skin care and the consumption of cosmetics containing natural extracts is increasing. There is also a growing interest raw material for natural products which is used by cosmetics companies [1]. The demand and cost of natural products are important in proportion to the efficacy of natural extracts used in cosmetics. Also, natural extracts are attracting attention because they are easily accessible in real life and reasonable in price. Among them, many studies on *Red Pinus densiflora* which are widely used in Korea and widely used in the fields of forest, food, tea, and cosmetics are underway. *Red Pinus densiflora* is low cost and easy to purchase. Studies have shown that antioxidative and antimutagenic effects, enzyme activities and effects on liver tissue have been reported [2].

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Flavonoids, organic acids, etc. contained in the *Red Pinus densiflora* have lowered cholesterol and have antidiabetic and antioxidant effects. Dehydroabietic acid (DHA) and Trans communic acid (TCA) are typical indicators of *Red Pinus densiflora.* Studies on indicator ingredient include inhibition of UVB-induced MMP-1 protein expression, association with photoaging and antioxidant effects [3].

On the other hand, although the efficacy is excellent, there are natural materials which are difficult to apply to cosmetic formulations due to problems such as insolubility. The *Red Pinus densiflora* have also insoluble component. Various studies are under way to solve these problems, and PCL (polycaprolactone)-PEG (polyethyleneglycol) polymer micelles are part of this research.

PCL-PEG is a copolymer of hydrophobic PCL and hydrophilic PEG, which can form micelles in a hydrophilic solution to collect hydrophobic substances. This can be achieved by variously controlling the compounding ratio of PCL and PEG to solubilize a variety of insoluble materials and is advantageous as a biocompatible polymer [4]. Using this, it is expected that not only the insoluble natural materials apply to the cosmetic formulations but also the trans epidermal permeability will be increased due to the reduction of the particle size.

The stratum corneum, which is the outermost layer of the skin and the core of the transdermal drug delivery, acts as a skin barrier function by forming a lamellar structure between the keratinocyte lipids between the keratin proteins. This interferes with the permeation of the external substance and thus the percutaneous permeation of the functional substance becomes hard [5]. Studies are being conducted to solve these problems and to apply cell penetrating peptides to the skin [6]. It is known that the cell-permeable peptides are easily accessible to the cell membrane due to the cationic amino acid 'arginine' [7]. Both cell membrane and epidermal keratinocyte lipids are similar in lipid bilayer structure. Therefore, if the arginine oligomer, a key amino acid sequence of a cell-penetrating peptide, is applied to a cosmetic product together with a functional substance, it may be possible to maximize the efficacy of the functional substance by enhancing skin absorption.

In this study, we will experiment (1) antioxidant test (Total polyphenol, DPPH, ABTS, SOD), wrinkle improvement test (elastase activity inhibition ability), whitening test (tyrosinase inhibition activity) (2) PCL-PEG polymer micelles were prepared to do emulsifying of *Red Pinus densiflora* extract (3) To enhance the epidermal penetration of *Red Pinus densiflora* extract with the cell permeable peptide 'R6', to confirm the increase in percutaneous permeability. This study suggests the possibility of application as a natural new functional cosmetic ingredient of *Red Pinus densiflora*.

## 2. MATERIALS AND METHODS

### 1. Instruments and reagents

*Red Pinus densiflora* was collected from near Hwaseong and Suwon, Korea. The equipment and reagents used for each experiment are as follows. The solutions used for the polyphenol, DPPH, ABTS, and Elastase test used in the antioxidant and antibacterial tests were obtained from Sigma Aldrich (USA). PCL-PEG copolymer (Mn=2,500, Mw=2,500, ratio of PCL to PEG = 1: 1) was purchased from Sigma Aldrich (USA) and R6 (hexa-D-arginine) was obtained from Dermafirm Co. (Seongnam, Korea). The equipment used in the experiment is as follows. Absorption spectrophotometer (SYNERGY HTX multi-mode reader, Bio Tek, Gangnam-gu, Seoul, Korea), Centrifugal separator (Supra-25K, Hanil Scientific Inc., 16 Arayukro, Gimpo, Korea). Thermostat (Changshin Science, 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**

*Red Pinus densiflora* was extracted with purified water and 70% ethanol respectively. In the hydrothemal extraction method, purified water was added to the *Red Pinus densiflora* powder and extracted for 4 hours in a thermostat at 80 ℃, filtered and freeze dried. Ethanol extracts were prepared by adding 70% ethanol to the *Red Pinus densiflora* powder. The extracts were extracted for 3 days, filtered through a filter and concentration process.

**3. Total polyphenol content measurement**

The amount of polyphenol was quantified using the Folin-denis method [8]. 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 $Na2CO_3$ 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.

**4. Antioxidant activity measurement**

**1) Measurement of DPPH radical scavenging ability**

The antioxidant effect about DPPH radical scavenging was measured by Blois method [9]. 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 (%)

Activity (%) = (Ac-At ) / Ac × 100

At is the absorbance of samples and Ac is the absorbance of DPPH solution

**2) Measurement of ABTS radical scavenging ability**

The antioxidative effects of ABTS radical scavenging were measured by Van den Berg's method [10]. 7 mM 2,2-azinobis (3-ethylbenzothianoline-6-sulfonic acid) and 2.4 mM potassuim persulfate were mixed and allowed to stand for 24 hours at room temperature to form ABTS which was diluted in phosphate buffered saline. 180 μL of ABTS solution was added to 20 μL of each extract solution and incubated in the dark for 7 minutes. Absorbance was measured at 734 nm using an ELISA reader.

ABTS radical scavenging activity (%)

Activity (%) = ($Ac^b$-$At^a$) / Ac × 100

[a] At is the absorbance of samples

[b] Ac is the absorbance of ABTS solution

**3) Measurement of elastase**

Elastase inhibitory activity was measured by Cannell [11]. The experiment was carried out using EnzCheck® elastase Assay Kit (E-12056). 1x Reaction buffer was used to dilute the *Red Pinus densiflora* extract sample and incubated in a 96-well black plate using 100 mg/L DQ elastin solution and 0.2 U/mL elastase for 30 minutes at room temperature. Absorbance was measured at 480 nm excitation and 535 nm emission fluorescence with an ELISA reader.

Inhibition rate of elastase (%)
Inhibition rate (%) = [1-(Absorbance in the sample addition group / absorbance in the no additives)] x 100

**5. Measurement of tyrosinase activity**

Tyrosinase activity was measured using a modification of the Kubo's method [12]. 20 μL of 0.1 M sodium phosphate buffer, 20 μL of each extract solution, 40 μL of 150 mM L-tyrosine, and 20 μL of 2000U tyrosinase were added and incubated at 37℃ for 13 minutes. Absorbance was measured at 490 nm with an ELISA reader.

Inhibition rate of tyrosinase (%)
Inhibition rate (%) = [100-((b-b')/(a-a')) × 100]
a: Absorbance after reaction of blank
b: Absorbance after sample liquid reaction
a',b': Absorbance measured by replacing with buffer solution

**6. Measurement of Superoxide Dismutase (SOD)**

SOD-like activity was performed using a modification of 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 (%)
Similar activity (%)= [1-(Absorbance in the sample addition group / absorbance in the no additives)] x 100

**7. Antibacterial experiment**

The disc diffusion test was performed to determine the antimicrobial activity of *Red Pinus densiflora* [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 Closridial medium)

### 8. Polymer micelle making method

Through previous experiments, polymer micelle formulations were made using the ethanol extracts of *Red Pinus densiflora*, which was relatively effective, compared to use of the hydrothermal extract. And the production process of making polymer micelle is as follows. Add 1% of total *Red Pinus densiflora* ethanol extract to 99.9% ethanol and then mix in maintaining temperature at 65℃. After that, PCL-PEG was added at 5% of the total amount, followed by evaporation of ethanol and add purified water to 100%. The conditions for producing PCL-PEG are shown in Table 2.

**Table 2. Composition of *Red Pinus densiflora* extraction formulation for Polymer micelle**

| Classification | Phase | Component | w/w % |
|---|---|---|---|
| Formulation 1 | Ethanol | 99.9 ethanol | 25.0 |
| | Active | *Red Pinus densiflora* ethanol extract | 1.0 |
| | Polymer micelle | PCL-PEG[a] | 5.0 |
| | Water | Distilled water | up to 100 |
| Formulation 2 | Protein | R6[b] (cell permeable peptide) | 1.0 of total polymer micelle. |

[a] PCL-PEG : Ratio of PCL to PEG = 50 : 50

[b] R6: cell permeable peptide, hexa-D-arginine

### 9. Skin penetration experiment

#### 1) Ingredients of *Red Pinus densiflora* with HPLC analysis

In this experiment, DHA (Dehydroabietic acid) was used as an indicator. Because the TCA (Trans communic acid) is sensitive to light and heat and has difficulty in analyzing it. DHA's HPLC analysis conditions are shown in Table 3.

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

| | |
|---|---|
| HPLC system | Agilent 1200 |
| Detector | PDA |
| Column | Shiseido C18 (4.6×250 mm, 5 μm) |
| Column temperature | 30 ℃ |
| Mobile phase | A : 0.1% Acetic Acid, 20%<br>B : Acetonitrile, 80% |
| Wave length of detection | UV 215 nm |
| Flow rate | 1 mL/min |
| Sample volume | 10 μL |
| Runtime | 10 min |

**2) Trans epidermal permeability**

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 DHA (Dehydroabietic acid) content among three formulations. Skin permeability conditions are shown in Table 4.

**Table 4. Conditions of skin penetration experiment**

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

**3) Formulations**

We made three formulations for skin permeability experiments. First, *Red Pinus densiflora* ethanol extract was dissolved in 99.9% ethanol and purified water at a ratio of 7:3. Second, Polymer micelles contain 1% of *Red Pinus densiflora* ethanol extract. Third, Polymer micelle contains 1% R6. Each condition is indicated in Table 5.

**Table 5. Conditions of formulation**

| Formulation | Composition |
|---|---|
| Formulation 0 (*Red Pinus densiflora* ethanol extract) | 10 mg/L *Red Pinus densiflora* ethanol extract was dissolved in 99.9% ethanol and purified water at a ratio of 7 : 3 |
| Formulation 1 (Polymer micelle) | 1% active (*Red Pinus densiflora* ethanol extract) in water |
| Formulation 2 (Polymer micelle + R6) | 0.1% R6 in Formulation 1(Polymer micelle) |

All formulations were prepared on the basis of 10 mL.

**10. Measurement of particle size**

We measure of particle size about three formulations at three times using Particlemetrix (Stabino® Paticle Charge Mapping, DREAM CORP, Suwon, Korea).

**11. Statistical processing**

All experiments were repeated three 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

*Red Pinus densiflora* was extracted with 70% ethanol and hydrothermal. Each yield was 4.34% in RPE (RPE: *Red Pinus densiflora* ethanol extract) and 4.00% in RPH (RPH: *Red Pinus densiflora* hydrothermal extract).

### 2. Total polyphenol content

To measure the Total polyphenol content, the results of the comparison of the extraction process of *Red Pinus densiflora* extract are shown in Table 6. In 500 mg/L, 137.51±7.70 mg/g of polyphenol was extracted from ethanol extraction and 133.95±1.57 mg/g of polyphenol was detected in hydrothermal extraction.

**Table 6. Total polyphenols of extracts from *Red Pinus densiflora***

| Samples | Method | Total polyphenols (mg/g) |
|---|---|---|
| RPE[a] | Ethanol extract | 137.51±7.70 |
| RPH[b] | Hydrothermal extract | 133.95±1.57 |

Values represent the mean ± SD of three independent experiments.
[a] RPE: *Red Pinus densiflora* ethanol extract
[b] RPH: *Red Pinus densiflora* hydrothermal extract

### 3. Antioxidant efficacy of *Red Pinus densiflora*

### 1) DPPH radical scavenging ability

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. The highest radical scavenging activity is shown the ethanol extract of 95.29±0.153% at 1,000 mg/L (Table 7).

**Table 7. Scavenging effect of *Red Pinus densiflora* on DPPH assays**

| Concentration | Extract | *M* | *SD* | *p* |
|---|---|---|---|---|
| 125ppm | RPE [a] | 26.57 | ±3.338 | 0.578 |
| | RPH [b] | 28.73 | ±5.324 | |
| 250ppm | RPE | 62.02 | ±1.298 | 0.000*** |
| | RPH | 43.34 | ±1.909 | |
| 500ppm | RPE | 93.14 | ±0.716 | 0.000*** |
| | RPH | 54.41 | ±1.193 | |

| | | | | |
|---|---|---|---|---|
| 1000ppm | RPE | 95.29 | ±0.153 | 0.039** |
| | RPH | 94.93 | ±0.133 | |

Values represent the mean ± SD of three independent experiments. Positive control : Ascorbic acid 100 μg/mL to 96.47%. *p < 0.1, **p < 0.05, ***p < 0.01.

[a] RPE: *Red Pinus densiflora* ethanol extract

[b] RPH: *Red Pinus densiflora* hydrothermal extract

**2) Measurement of ABTS radical scavenging ability**

ABTS radical cation is a deep blue-green radical that reacts with antioxidants and is characterized by discoloration by light green. The antioxidant effect of ABTS free radicals produced by the reaction of potassium persulfate is measured by the degree of discoloration of the radical-specific cyan color to light green [16]. As a result, it was found that the concentration dependent of the extracts at 200-800 mg/L. The highest radical scavenging activity is shown the ethanol extract at 800 mg/L (Table 8).

**Table 8. Scavenging effect of *Red Pinus densiflora* on ABTS assays**

| Concentration | Extract | *M* | *SD* | *p* |
|---|---|---|---|---|
| 200ppm | RPE [a] | 53.00 | ±0.607 | 0.000*** |
| | RPH [b] | 36.31 | ±0.230 | |
| 400ppm | RPE | 95.23 | ±0.203 | 0.000*** |
| | RPH | 91.72 | ±0.231 | |
| 800ppm | RPE | 103.33 | ±0.076 | 0.012** |
| | RPH | 99.15 | ±0.812 | |

Values represent the mean ± SD of three independent experiments. Positive control : Ascorbic acid 200 μg/mL to 99.86%. *p < 0.1, **p < 0.05, ***p < 0.01.

[a] RPE: *Red Pinus densiflora* ethanol extract

[b] RPH: *Red Pinus densiflora* hydrothermal extract

**3) Measurement of elastase**

The elastase which is presented in the dermis of the skin is an enzyme capable of degrading various proteins including fibronectin, collagen and elastin which maintains elasticity of the skin in the dermis [17]. The elastase inhibitory effect which is effective for improving the wrinkles of the skin was measured according to the concentration dependent at 250-2,000 mg/L. The highest inhibition rate is shown the ethanol extract at 2,000 mg/L (Table 9).

**Table 9 Scavenging effect of *Red Pinus densiflora* on elastase assays**

| Concentration | Extract | *M* | *SD* | *p* |
|---|---|---|---|---|
| 250ppm | RPE [a] | 40.00 | ±4.000 | 0.512 |

| | | | | |
|---|---|---|---|---|
| | RPH [b] | 1.90 | ±1.527 | |
| 500ppm | RPE | 61.90 | ±0.577 | 0.003*** |
| | RPH | 4.76 | ±2.309 | |
| 1000ppm | RPE | 72.38 | ±0.577 | 0.000*** |
| | RPH | 26.66 | ±1.527 | |
| 2000ppm | RPE | 100.00 | ±2.851 | 0.088* |
| | RPH | 80.00 | ±1.000 | |

Inhibitory effect of RPE, RPH against elastase. Results are expressed as mean ± S.D. of data obtained from three independent experiments. Positive control : N-Succinyl-Ala-Ala-Ala-p-nitroanilide 10 μg/mL to 7.23% (inhibition activity). *p < 0.1, **p < 0.05, ***p < 0.01.

[a] RPE: *Red Pinus densiflora* ethanol extract

[b] RPH: *Red Pinus densiflora* hydrothermal extract

**4. Measurement of tyrosinase activity**

Melanin is a pigment produced by melanomas, one of the cell organelles. Melanin is produced by the action of various enzymes such as tyrosinase, tyrosinase-related protein 1 (TRP1) and tyrosinase-related (TRP2) in melanomas [18]. Among them, tyrosinase is a major regulatory enzyme that plays a role in the oxidation of tyrosine to DOPA quinone after being hydrolyzed with DOPA and is related to melanin. The highest tyrosinase inhibitory activity of 77.80±3.196% was observed at the concentration at 10,000 mg/L of ethanol extract (Table 10).

**Table 10. Scavenging effect of *Red Pinus densiflora* on tyrosinase assays**

| Concentration | Extract | *M* | *SD* | *p* |
|---|---|---|---|---|
| 1250ppm | RPE [a] | 72.39 | ±2.792 | 0.001*** |
| | RPH [b] | 4.84 | ±0.242 | |
| 2500ppm | RPE | 77.96 | ±1.512 | 0.000*** |
| | RPH | 17.35 | ±1.377 | |
| 5000ppm | RPE | 76.51 | ±4.440 | 0.000*** |
| | RPH | 38.90 | ±1.242 | |
| 10000ppm | RPE | 77.80 | ±3.196 | 0.001*** |
| | RPH | 61.50 | ±0.419 | |

Values represent the mean ± SD of three independent experiments. Positive control : Kojic acid 500 μg/mL to 96.85%. *p < 0.1, **p < 0.05, ***p < 0.01.

[a] RPE*: Red Pinus densiflora* ethanol extract

[b] RPH*: Red Pinus densiflora* hydrothermal extrac

**5. Superoxide Dismutase (SOD) measurement**

SOD-like activity assay is an antioxidant activity assay using color development by automatic oxidation [19]. 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 107.60±1.127% in ethanol extract at 2,000 mg/L (Table 11).

**Table 11. Scavenging effect of *Red Pinus densiflora* on SOD assays**

| Concentration | Extract | *M* | *SD* | *p* |
|---|---|---|---|---|
| 125ppm | RPE [a] | 47.77 | ±0.408 | 0.001*** |
| | RPH [b] | 28.03 | ±3.594 | |
| 250ppm | RPE | 75.22 | ±0.226 | 0.003*** |
| | RPH | 53.85 | ±2.040 | |
| 500ppm | RPE | 94.74 | ±0.850 | 0.000*** |
| | RPH | 74.50 | ±1.960 | |
| 1000ppm | RPE | 101.32 | ±0.959 | 0.000*** |
| | RPH | 84.77 | ±1.471 | |
| 2000ppm | RPE | 107.60 | ±1.127 | 0.000*** |
| | RPH | 96.53 | ±1.149 | |

Values represent the mean ± SD of three independent experiments. Positive control : Ascorbic acid 500 μg/mL to 106.27%. *p < 0.1, **p < 0.05, ***p < 0.01.

[a] RPE: *Red Pinus densiflora* ethanol extract

[b] RPH: *Red Pinus densiflora* hydrothermal extract

**6. Antibacterial experiment**

The antimicrobial test was conducted three times using the paper disc method [20]. The results of the clear zone measurement are shown in Table 12. As a result of the antimicrobial test, the antimicrobial effect was confirmed in two strains among the four strains. In the case of *propionibacterium acnes* strain, the largest clear zone of 22.6±0.58 mm was found at the concentration of 50 mg/L ethanol extract. In the Bacillus subtilis strain, the greatest clear zone of 11.3±0.58 mm was found at the concentration of 50 mg/L of hydrothermal extract. Addition to the clear zone, Bacillus subtilis strains showed a wide growth inhibition line of growth of 27,28 mm at the concentration of 50mg/L besides the clear zone each extract.

**Table 12. The effect of *Red Pinus densiflora* extract amount on area of clear zone**

| Strain | Clear zone (mm²) | | | | |
|---|---|---|---|---|---|
| | Positive control 20 mg/L | 50 mg/L | 5 mg/L | 0.5 mg/L | 0.05 mg/L |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Staphylococcus aureus* | 9 | No effect | | | | | | |
| *Escherichia coli* | 10 | No effect | | | | | | |
| *Bacillus subtilis* | 9 | E [a] | H [b] | E | H | E | H | E | H |
| | | 10.0±0.00<br>27.0±0.00[c] | 11.3 ±0.58<br>28.0 ±0.00[d] | - | - | - | - | - | - |
| *Propionibacterium acnes* | 10 | E | H | E | H | E | H | E | H |
| | | 22.6± 0.58 | 19.3 ± 0.58<br>19.6 ± 0.58[e] | 10.3 ± 0.58 | 9.30<br>±0.57 | - | - | - | - |

Positive control: *Staphylococcus aureus, Escherichia coli, Bacillus subtilis* are used methyl paraben, *Propionibacterium acnes* is used salicylic acid.

[a] E: *Red Pinus densiflora* ethanol extract

[b] H*: Red Pinus densiflora* hydrothermal extract

[c.d.e] Growth inhibition line

**7. Skin penetration experiment**

**1) Ingredients of *Red Pinus densiflora* with HPLC analysis**

DHA (Dehydroabietic acid), as an indicator component of *Red Pinus densiflora* was quantified using HPLC (Agilent, USA). For HPLC measurements (Table 3), the mobile phase was made with 20% of 0.1% acetic acid and 80% acetonitrile. The flow rate was set to 1 mL/min and 10 μL of each solution was injected into the chromatograph. Employed stationary phase was Shiseido C18 (4.6×250 mm, 5μm). The UV detector was employed at the wavelength of 215 nm. In later skin penetration experiments, the confirmation of the increase in transdermal permeability was measured and compared with DHA (Dehydroabietic acid) as an indicator component.

**2) Trans epidermal permeability**

Figure 1 shows the permeation rate of *Red Pinus densiflora* extract over time for a given area of skin. The cumulative amount of total *Red Pinus densiflora* 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 polymeric micelles and cell permeable peptides can increase skin penetration. The results of Trans epidermal permeability experiment were as follows. 'Formulation 2' was the most penetrating when the three formulations showed trans epidermal permeability and it had 85.13±3.35 ㎍/㎠. All results are shown in Table 13.

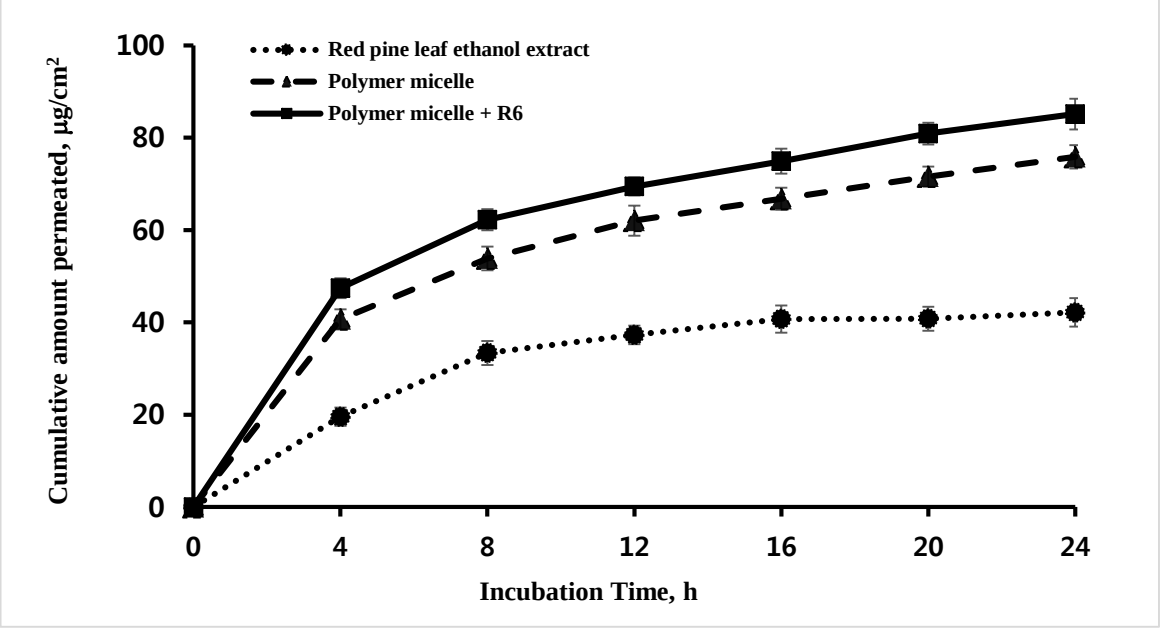



**Fig. 1 *In vitro* skin penetration profiles of *Red Pinus densiflora* extract, Polymer micelle Polymer micelle + R6 through epidermal skin and penetration cumulative amount.**

*Red Pinus densiflora* ethanol extract is formulation 0

Polymer micelle is formulation 1

Polymer micelle + R6 is formulation 2

**Table 13. Epidermal penetration experiment's result using Franz cell according to formulation with *Red Pinus densiflora***

| Classification | | Formulation | | | |
|---|---|---|---|---|---|
| | | Formulation 0 *(Red Pinus densiflora* ethanol extract) | Formulation 1 (Polymer micelle) | Formulation 2 (Polymer micelle + R6) | *F*(p) |
| 4h | M *(SD)* | $19.55^{a}$ (1.96) | $40.64^{b}$ (2.18) | $47.42^{c}$ (2.15) | 144.216(0.000)*** |
| | *post hoc* | *LSD = c > b > a* | | | |
| 8h | M *(SD)* | $33.37^{a}$ (2.59) | $53.85^{b}$ (2.54) | $62.23^{c}$ (2.31) | 107.482(0.000)*** |
| | *post hoc* | LSD = c > b > a | | | |
| 12h | M *(SD)* | $37.28^{a}$ (2.00) | $62.04^{b}$ (3.25) | $69.45^{c}$ (1.92) | 140.293(0.000)*** |
| | *post hoc* | LSD = c > b > a | | | |

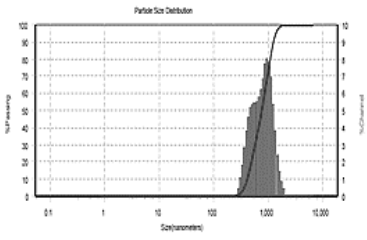
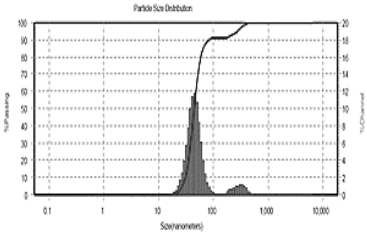
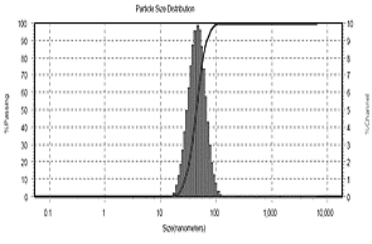
| | | | | | |
|---|---|---|---|---|---|
| 16h | M<br>*(SD)* | 40.71[a]<br>(2.94) | 66.77[b]<br>(2.38) | 74.92[c]<br>(2.68) | 133.799(0.000)*** |
| | *post hoc* | LSD = c > b > a | | | |
| 20h | M<br>*(SD)* | 40.80[a]<br>(2.61) | 71.57[b]<br>(2.19) | 80.91[c]<br>(2.36) | 231.305(0.000)*** |
| | *post hoc* | LSD = c > b > a | | | |
| 24h | M<br>*(SD)* | 42.16[a]<br>(3.10) | 75.86[b]<br>(2.55) | 85.13[c]<br>(3.35) | 168.567(0.000)*** |
| | *post hoc* | LSD = c > b > a | | | |

Each value represents the mean and S.D. Statistical analysis was performed using the one-way ANOVA. *p ＜ 0.1, **p ＜ 0.05, ***p ＜ 0.01.

### 8. Measurement of particle size

We measured three formulations particle size and focus on intensity of particle size. In this study, 'Formulation 2' is the smallest size among three formulations. The reason for this is considered that the shape of the polymer micelle helps the dispersion of the extract to reduce the particle size. Particle size's results are shown in Table 14.

**Table 14 Particle size of *Red Pinus densiflora* ethanol extract, Polymer micelle, Polymer micelle +R6**

| | Formulation 0<br>*(Red Pinus densiflora* ethanol extract) | Formulation 1<br>(Polymer micelle) | Formulation 2<br>(Polymer micelle + R6) |
|---|---|---|---|
| Particle size |  |  |  |
| M | 1318.00 nm | 91.36 nm | 59.86 nm |
| SD | ±593.11 | ±22.13 | ±11.68 |

The average particle size was repeated three times and a weighted average and standard deviation were presented. The distribution of particle size's percentage was calculated as a weighted average.

## 4. CONCLUSION

The purpose of this study was to investigate the efficacy of *Red Pinus densiflora* as a cosmetic product and trans epidermal delivery ability using polymer micelle with cell-penetrating peptide. The effect of antioxidant, antimicrobial, wrinkle and whitening on *Red Pinus densiflora* was investigated through this study. In all experiments, *Red Pinus densiflora* ethanol extract showed better effect than hydrothermal extract.

The results of skin penetration experiment showed that (1) PCL (polycaprolactone) and PEG (polyethyleneglycol) which used when making polymer micelles solubilized insoluble materials of natural extract, resulting in smaller particle size of natural extract and (2) It made the penetration of the skin increased. (3) Added cell penetrating peptide (R6) is similar to the keratinocyte structure, suggesting that it could make the skin penetration rate higher. These results suggest that the natural extract containing the insoluble ingredient can effectively penetrate the skin.

The result of skin penetration through the production of macromolecule micelle containing *Red Pinus densiflora* extract seems to be highly likely to be commercialized in the cosmetics industry in the future. And in the Future study will measure the zeta potential and specify the relationship with particle size.

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