The Beneficial Effects of Extract of *Pinus densiflora* Needles on Skin Health

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**Introduction**

Natural products isolated from plants are used as important resources for medical treatment or health improvement [37]. The natural habit of *Pinus densiflora* Sieb. et Zucc. (red pine) are Korea, Japan, China, and southeastern Russia, various parts of red pine are used as supplementary health food to improve health [39]. Also, the leaves of red pine are consumed as traditional medicine for liver diseases, skin diseases, and hypertension [19, 22]. Volatile chemicals of red pine possess antioxidant activity and growth inhibition activities against human intestinal bacteria [13, 29].

The skin is the primary barrier between the body and the external environment and defenses against external factors such as microbial and chemical agents [18]. Oxidative stress disrupts the protective function of skin and causes roughness and wrinkling [6]. The use of antioxidants is an effective treatment to prevent symptoms associated with the aging of skin [24].

Common skin diseases associated with inflammation such as pityriasis versicolor are caused by a fungus of the genus *Malassezia* [12, 25]. Among the *Malassezia* species, *Malassezia furfur* is associated with pityriasis versicolor.*
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versicolor [15], folliculitis [1], seborrheic dermatitis [2], atopic dermatitis [38], and psoriasis [31]. At high concentrations, this yeast can diminish the normal protective function of skin and affect the body’s ability to regulate inflammation [33]. As such, the goals of treatment are to regulate *M. furfur*’s growth and associated inflammation, and to prevent the outbreak of secondary infections [9, 11].

In the inflammatory response, macrophages recognize the infection and the secreted several pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin(IL)-1β, IL-4, IL-6, and IL-8 induces inflammation response [27]. Among them, TNF-α and IL-1β are important factor to chronic inflammatory disorders [3]. Kesavan [17] investigated the influence of the *Malassezia* on the production of IL-1, IL-6, and TNF-α by peripheral blood mononuclear cells (PBMC). Of these, TNF-α plays a particularly important role in skin diseases related to inflammation [21, 36]. Inflammatory responses to TNF-α are controlled through expression of IL-1, as well as via more pro-inflammatory cytokines [34]. The natural product research has developed a variety of therapeutic agents that are proven to be therapeutically effective against a wide range of diseases [23]. Current interest has led to the search for novel natural products with anti-inflammatory activity [40].

In this study, layers with potential anti-inflammatory action were isolated from the methanolic extract of *P. densiflora* needles. Subsequently, we confirmed their various functional activities relating to skin health: antifungal activity against *M. furfur* (causative factor of seborrheic dermatitis), antioxidant activity directed against aging [24], and tyrosinase inhibition activity associated with the whitening of skin color [35]. Cytotoxicity and anti-inflammatory activity were confirmed from the measurement of IL-1β and TNF-α expression levels in *M. furfur*-activated macrophages.

**Materials and Methods**

**Preparation of needle extracts from *Pinus densiflora***

Fresh needles of red pine were picked in Gokseong province, Jeollanam-Do, South Korea. Harvested needles of red pine were washed clean with tap water (cleaned with 5% charcoal) and dehydrated using a spin-drier. The dehydrated pine needles (100 kg) were dried in the shade. The dried pine needles (49.97 kg) were treated with 500 L of 80% methanol (MeOH) at 69°C for 3 h. The resultant methanolic extracts were concentrated to 20 L. This crude extract was partitioned successively to yield layers of n-hexane (3 × 10 L), EtOAc (3 × 10 L), n-BuOH (3 × 10 L) and water [28].

**2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay**

The DPPH assay was performed according to a method described by Nenadis and Tsimidou [26] with some modifications. Briefly, a methanolic DPPH solution (0.04 mM; 180 μl) was added to 20 μl of different concentrations of extracts and fractions. The mixture was shaken vigorously and a decrease in absorbance was measured at 517 nm after 30 min of incubation in the dark. The blank solution contained the same amount of DPPH reagent and 20 μl of methanol and each test was performed in triplicate. The percentage of DPPH inhibition was calculated as follows:

$$\text{DPPH} = (1 - \frac{A_s}{A_c}) \times 100$$

Where *A_c* and *A_s* are the absorbance of the control and test samples, respectively. Ascorbic acid was used as the reference.

**M. furfur and Raw 264.7 culture conditions**

*M. furfur* strain KCTC7546 was used in all experiments. It was cultured either on modified YPD plates or liquid medium supplemented with Tween 80 (1% yeast extract, 1% peptone, 2% glucose, 1% Tween 80) for 1 day at 30°C under aerobic conditions. The conditions were based on published information [7] regarding nutrients and trace components that promote the growth of *M. furfur*.

Mouse lymphoid microvascular epithelium immortalized cell line, Raw 264.7, was obtained from the ATCC (TIB-71). Raw 264.7 was grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, and 100 μg/ml penicillin-streptomycin at 5% CO₂ and 37°C humidified atmosphere.

**Antifungal activity against M. furfur**

Once grown, *M. furfur* in liquid medium was harvested from a 1 ml culture and suspended in 100 μl PBS.
The suspended solutions were plated onto modified YPD agar medium using a sterile spreader. Plant extracts were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 100 mg/ml. Needle extract of *P. densiflora* (50 µl) and control respectively were impregnated into the sterile paper discs (8 mm diameter) and incubated at 30°C for 1 day. *M. furfur* was visualized and images of the plates were captured. The presence of a clear zone around the discs indicated the sensitivity of *M. furfur* to the *P. densiflora* samples. The total diameter of each clear zone was measured.

**Tyrosinase inhibition analysis**

The activity of tyrosinase was determined by following the L-DOPA assay protocol [15]. The analysis used 1 L of the reaction mixture, which was composed of 1 mM sodium phosphate buffer (pH 6.8, 1 M Na₂HPO₄, 1 M NaH₂PO₄), 10 mM of L-DOPA and 200 unit/ml tyrosinase. Ascorbic acid was used as the positive control.

**Cytotoxicity analysis**

3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay was performed using the method previously described by Boncler et al. [4]. Raw 264.7 cells were seeded at 1 × 10⁵ cells per well in a 96-well plate and these cells were grown in a humidified atmosphere of 5% CO₂ at 37°C. The cells were then exposed to varying concentrations of the pine needle extract (0 to 200 µg/ml) for 24 h. After that, 100 µl of fresh medium was added to the cells. MTT solution (5 mg/ml of PBS) was added and the plate was placed in an optimal atmosphere at 37°C to allow the metabolically active cells to reduce MTT to blue formazan crystals. After 4 h, MTT-formazan crystals were dissolved in 50% ethanol and 50% DMSO and the absorbance was measured at 570 nm on a multifunctional plate reader (Eon, Bio-tek, USA). Comparisons were made with untreated cells.

**Determination of IL-1β and TNF-α mRNA expression**

Raw 264.7 cells (1 × 10⁵ cells/well) were seeded in a 6-well culture plate containing DMEM. The cells were pre-treated with various concentrations of pine needle extracts (0 to 100 µg/ml) for 2 h and then incubated in the absence or presence of *M. furfur* for 6 h. After incubation, they were collected by centrifugation and total RNA was isolated from pine needle extract-treated cells using Hybrid-R (GeneAll, Korea) according to the manufacturer’s protocol. To synthesize cDNA, 2 µg of total RNA was primed with oligo dT and made to react with Hyperscript mix (GeneAll, Korea). To measure the mRNA level of inflammatory cytokines including IL-1β and TNF-α, we designed primers for the target genes (Table 2). cDNA was amplified and the PCR products were visualized using fluorescent dye on a UV transilluminator. mRNA expression of target genes was analyzed by real-time PCR using SYBR Green (Takara, Japan).

**Results**

**Antioxidant activity**

The methanolic extract of *P. densiflora* needles showed stronger antioxidant activity than the water or ethanol extracts. The results of dose-dependent DPPH scavenging activity are presented in Fig. 1. After partitioning the pine needle methanolic extract into n-hexane, EtOAC, n-BuOH, and H₂O layers, the antioxidant activity of the individual layers was measured and was found to be in the order EtOAc layer > n-BuOH layer > water layer (Fig. 2).

**Comparison of *M. furfur* growth in various culture media**

For *M. furfur* culture, variable compositions of liquid and solid medium were tested to achieve optimum conditions. The growth of *M. furfur* in liquid culture was mon-
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Monitored by measuring OD$_{600}$. After about 24 h, the organism was incubated on agar plates for another 24 h. The media compositions have been described in Table 1. Medium B and E did not have yeast extract or glucose. *M. furfur* did not grow well in these media. In liquid Medium A and C containing olive oil, *M. furfur* grew well, but those cultures were extremely oily to spread over the agar plates. Among the five media formulations, Medium D, composed of yeast extract, peptone, and glucose but without olive oil, provided the best conditions for *M. furfur* growth in both agar and liquid culture.

**Antifungal activity of pine needle extracts against *M. furfur***

To determine fungicidal activities of pine needle extracts, the extracts were applied at 100 mg/ml onto paper discs, which were then placed on *M. furfur* culture plates. Fig. 3 shows the inhibitory effect of pine needle extract on *M. furfur* growth. The presence of a clear zone around the disc (8 mm diameter) indicates growth inhibition. The total diameter of the zone of inhibition was measured for each disc and the results are given in Table 3. DMSO was used as an experimental control and to dissolve pine needle extract. The antifungal activity of its individual layers were observed to increase in the order *n*-hexane layer > EtOAc layer > *n*-BuOH layer >

**Table 1. Compositions of *Malassezia furfur* media.**

| Composition                                      | OD$_{600}$
<table>
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<tbody>
<tr>
<td>A 0.3% Yeast extract, 0.3% Malt extract, 0.5% Peptone, 1% D-glucose, 1% Olive oil, 1% Tween 80, 0.5% Glycerol, 0.1% Monosterin</td>
<td>1.759</td>
</tr>
<tr>
<td>B 0.3% Sabouraud dextrose, 0.05% Glycerol monosterate, 0.4% Olive oil, 0.05% Tween 20, 0.1% Glycerol</td>
<td>0.451</td>
</tr>
<tr>
<td>C 0.01% Yeast extract, 1% Peptone, 0.5% Glucose, 0.05% Glycerol monoesterate, 0.4% Olive oil, 0.05% Tween 20, 0.1% Glycerol</td>
<td>1.121</td>
</tr>
<tr>
<td>D$^\text{b}$ 1% Yeast extract, 1% Peptone, 2% Glucose, 1% Tween 80</td>
<td>1.786</td>
</tr>
<tr>
<td>E 0.3% Malt extract, 0.5% Peptone, 0.1% Glycerol monoesterate, 5% Tween 20</td>
<td>0.501</td>
</tr>
</tbody>
</table>

$^a*M. furfur$ was cultured in liquid medium for 24 h at 30°C and the growth of liquid cultured *M. furfur* were monitored by OD$_{600}$.

$^b*D[5]$ possessed the optimal conditions for liquid and solid medium to culture *M. furfur*. 

![Fig. 2. DPPH radical scavenging activity of various layers from *P. densiflora* needle methanolic extract. PME; Ethyl acetate layer of Pine needle methanolic extract, PMB; *n*-utyl alcohol layer of Pine needle methanolic extract, PMH; *n*-hexane layer of pine needle methanolic extract, PW; Water layer of pine needle methanolic extract.](image1)

![Fig. 3. Antifungal activity of *Pinus densiflora* against *M. furfur*. Antifungal activity of *P. densiflora* needle methanolic extract layers. PME; Ethyl acetate layer of pine needle methanolic extract, PMB, *n*-butyl alcohol layer of pine needle methanolic extract, PMH; *n*-hexane layer of pine needle methanolic extract, PW; Water layer of pine needle methanolic extract. DMSO was used as solvent of *P. densiflora*.](image2)
Tyrosinase inhibition activity of pine needle extract

The tyrosinase inhibition activity of the pine needle extract is shown in Fig. 4. All the partitioned layers of the methanolic extract revealed significant tyrosinase inhibitory activity, comparable to that exhibited by ascorbic acid. The layers revealed dose-dependent activity, and the water layer showed the strongest tyrosinase inhibition (Fig. 4).

Cytotoxicity of pine needle extract in Raw 264.7 cell line

The cytotoxic effects of pine needle extract at molecular and cellular levels were investigated in Raw 264.7 cultured cells via the MTT assay. The results indicated that at concentrations of 25 to 100 μg/ml, the n-hexane layer of the methanolic extract of pine needles displayed cytotoxicity while the EtOAc, n-BuOH, and water layers were not cytotoxic (Fig. 5).

Anti-inflammatory activity of pine needle extract

IL-1β and TNF-α are pro-inflammatory cytokines that are released from macrophages upon exposure to M. furfur or other inflammatory conditions. Macrophages were infected by M. furfur (1:30) for 20 hours in the presence or absence of methanolic extract of pine needles, including the EtOAc, n-BuOH and water layers which had no cytotoxicity (100 μg/ml pretreatment for 2 h). Total RNA was then extracted. The effect of the three layers on IL-1β and TNF-α mRNA expression levels in

| Table 2. Sequence of pro-inflammatory gene primer designs. |
|------------------|------------------|------------------|------------------|
| IL-1β            | F 5′-GTG TCT TTC CCG TGG ACC TT-3′ | R 5′-TCG TTG CTT GG ACC TCT CTT-3′ |
| TNF-α            | F 5′-GCG CTC TCT ACC TTT CC-3′ | R 5′-TAG GCG ATT ACA GTA AC GC GC-3′ |
| GAPDH            | F 5′-TCG ACC ACC TGC TTA G-3′ | R 5′-GGA TGC AGG GAT GTT C-3′ |

| Table 3. Diameter of inhibition zones caused by P. densiflora needle methanolic extract layers (100 mg/ml) in M. furfur culture plates. |
|------------------|------------------|------------------|------------------|
| PMH              | PMB              | PME              | PMW              |
| mm               | 1.9              | 1.3              | 1.2              | 1.1              |

M. furfur-induced macrophages was measured by real-time PCR and RT-PCR using mouse IL-1β- and TNF-α-specific sense and antisense primers, as shown in Table 2. The relative intensity of each mRNA quantified was normalized against the mRNA expression of human β-
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actin. The expression levels of IL-1β and TNF-α increased in M. furfur-treated macrophages, which concurs with previously published data [16]. However, as shown Fig. 6A, when macrophages were treated with M. furfur plus EtOAc layer, the EtOAc layer significantly downregulated the M. furfur-induced IL-1β mRNA expression in macrophages. Accordingly, the results of RT-PCR also showed that pre-treatment with pine needle extract strongly inhibited M. furfur-induced production of IL-1β in macrophages (Fig. 6B). Moreover, we determined that the EtOAc layer significantly inhibited M. furfur-induced TNF-α mRNA expression level in macrophages (Fig. 7A). Correspondingly, as shown in Fig. 7B, RT-PCR findings indicate that the M. furfur-induced increase in expression levels of TNF-α is attenuated by EtOAc layer of pine needle extract among the other layers. Then, Macrophages were infected by M. furfur (1:30) for 20 hours in the presence or absence of methanolic extract of pine needles, including the EtOAc layer (0–100 μg/ml pretreatment for 2 h). Total RNA was then extracted. The effect of the EtOAc layer on IL-1β and TNF-α mRNA expression levels in M. furfur-induced macrophages was measured by real-time PCR and RT-PCR. As shown in Fig. 8A, when macrophages were treated with M. furfur plus EtOAc layer, the EtOAc layer significantly downregulated the M. furfur-induced IL-1β mRNA expression in macrophages. Accordingly, the results of RT-PCR also showed that pre-treatment with pine needle extract inhibited M. furfur-induced production of IL-1β in macrophages (Fig. 8B). Moreover, we determined that the EtOAc layer slightly inhibited M. furfur-induced TNF-α mRNA expression level in macrophages (Fig. 9A). Correspondingly, as shown in Fig. 9B, RT-PCR findings indicate that the M. furfur-induced increase in expression levels of TNF-α is slightly attenuated by EtOAc layer of pine needle extract in a dose-dependent manner.

Discussion

The purpose of this study was to evaluate the effects of P. densiflora on skin health and its anti-inflammatory
Fig. 7. Inhibition of *M. furfur*-induced TNF-α expression by layers from *P. densiflora* needle methanolic extract. (A) Treatment of EtOAc layer of *P. densiflora* strongly decreased mRNA expression level of TNF-α, (B) Treatment of EtOAc layer of *P. densiflora* strongly decreased mRNA expression level of TNF-α.

Fig. 8. Inhibition of *M. furfur*-induced IL-1β expression by EtOAc layer from *P. densiflora* needle methanolic extract. (A) Treatment of EtOAc layer of *P. densiflora* dose-dependently reduced mRNA expression level of IL-1β, (B) Treatment of EtOAc layer of *P. densiflora* dose-dependently reduced mRNA expression level of IL-1β.
activity. A wide range of diseases are caused by oxidative stress. Accelerated cell oxidation even leads to wrinkled skin [8]. In recent years, there has been increasing interest in examining natural antioxidants that can protect skin against oxidative stress. In this study, we confirm the antioxidant activity of *P. densiflora* needles, specifically the methanolic extract. We partitioned four layers from the extract and showed that the BuOH and EtOAc layers possess the strongest antioxidant activity. This was in contrast to the findings that the highest scavenging effects are exhibited by the water extract from *P. densiflora* needles in a study by Park et al. [29]. The discrepancy may be attributed to the differences in the extraction method.

The skin is colonized by over 500 commensal microbial species estimated to form over 95% [32]. *Malassezia*, particularly *M. furfur*, a saprophyte occurring widely on human skin, are generally regarded as the causative agents of a number of common dermatological disorders relate to inflammation [20]. In this study, antifungal activity against *M. furfur* was investigated via the clear zone test using paper discs and *n*-BuOH, EtOAc, and *n*-hexane layers of *P. densiflora* needle methanolic extract were shown to possess fungicidal activity.

Tyrosinase is the enzyme responsible for transfer of the substrate tyrosine into melanin by melanocytes [10]. Inhibition of tyrosinase can lead to reduce or no melanin synthesis and whitening of skin. In our study, all layers of the methanolic extract were found to exhibit tyrosinase inhibition activity.

IL-1β and TNF-α are multi-functional cytokines with widely overlapping activities. These inflammatory cytokines have a central role in the pathology of chronic inflammatory diseases [30] through their regulation of the immune response to inflammatory stimuli. The macrophage is one of the major cell types in inflammatory response and influences many chronic inflammatory diseases. IL-1β and TNF-α expression levels in *M. furfur*-treated macrophages, which concurs with previously published data [16]. Thus, it is of therapeutic interest to...
develop an efficient strategy using *P. densiflora* extract to down-regulate the expression of *M. furfur*-induced pro-inflammatory cytokines. The mRNA expression levels of IL-1β and TNF-α in *M. furfur*-stimulated RAW 264.7 cells decreased with increasing concentrations of EtOAc layer in the concentration range of 0–100 μg/mL. We clearly demonstrated significant suppression of IL-1β levels and a slight attenuation of TNF-α levels by the EtOAc layer in *M. furfur*-induced RAW 264.7 murine macrophages.

In conclusion, the EtOAc and *n*-BuOH layer of *P. densiflora* had antioxidant activity and the various layers of the methanolic extract were found to have antifungal activity against the skin pathogen *M. furfur*. Additionally, all layers of the extract had tyrosinase inhibition activity. We have also shown that the EtOAc layer partitioned from the methanolic extract of *P. densiflora* pine needles has dose-dependent anti-inflammatory activity in *M. furfur*-stimulated Raw 264.7 cells through the down-regulation of IL-1β expression levels. Thus, it can be inferred that *P. densiflora* needles and their components have the potential to be used as alternative anti-inflammatory agents and cosmetic materials for skin health improvement.

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