Effects of Galgeungyulpitang on Cellular Production of Melanin and Elastase

Na Young Jo 1, Eun Yong Lee 2, Cham Kyul Lee 2, Jeong Du Roh 1,*

1 Department of Acupuncture & Moxibustion Medicine, Je-Cheon Hospital of Traditional Korean Medicine, Semyung University, Jechoen, Korea
2 Department of Acupuncture & Moxibustion Medicine, Chung-Ju Hospital of Traditional Korean Medicine, Semyung University, Jechoen, Korea

ABSTRACT

Background: This study was designed to investigate the potential effects of Galgeungyulpitang for whitening and elasticity treatment by examining its effect on melanoma cells.

Methods: The effects of Galgeungyulpitang on B16/F10 melanoma cell viability, production of melanin, tyrosinase and elastase, were investigated. Cell viability was measured by colorimetric assay that assesses cell metabolism activity (MTT assay). Melanin was measured by Hosei’s method, tyrosinase was measured by Yogi’s method and elastase was measured by James’s method.

Results: At concentrations higher than 500 µg/mL Galgeungyulpitang, cell viability was significantly reduced (p < 0.05). At concentrations of 500 µg/mL and lower, morphological changes were not observed. The rate of melanin synthesis was significantly reduced to 73.49% ± 2.92% at a concentration of 500 µg/mL. Galgeungyulpitang compared with untreated cells (p < 0.05). Extracellular tyrosinase production was not significantly decreased in vitro, however, intracellular tyrosinase production was significantly reduced to 76.06% ± 2.17% when treated with Galgeungyulpitang at a concentration of 500 µg/mL compared with the control (p < 0.05). Elastase Type 1 production was significantly reduced to 74.98% ± 3.24% and 69.62% ± 4.66% at concentrations of 250 and 500 µg/mL, respectively (p < 0.05). Elastase Type 4 production was significantly reduced to 77.27% ± 3.52% at concentrations of 250 and 500 µg/mL (p < 0.05).

Conclusion: The results in this study showed that Galgeungyulpitang may inhibit melanin and tyrosinase synthesis, and inhibit elastase production, suggesting that Galgeungyulpitang may be potentially beneficial for skin whitening and loss of skin elasticity treatments.

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Introduction

The skin consists of the epidermis and dermis which protects and regulates body temperature, and provides a sensitive surface for touch. The skin is also viewed as a factor of beauty [1]. Skin pigmentation, such as moles, freckles, and darkening of the skin, is mainly due to increased melanin synthesis by melanocytes. Therefore, the inhibition of melanin synthesis, can achieve a whitening effect [2].

In the dermis, collagen and elastin are involved in the elasticity of the skin and form a network structure. When elastin is broken down by elastase it causes the skin to sag and wrinkle. Therefore, inhibiting the activity of elastase can inhibit skin aging [3].

Galgeungyulpitang is a traditional medicine recommended for skin rashes, coughing and chest tightness in the Dongui Bogam [4]. There have been studies on the whitening effects of Pueraariae Radix [5], Fraxini Cortex [6], Armeniacae Semen [7], Anemarrhenae Rhizoma [8], Scutellariae Radix [9], Ephedrae Herba [10], and Glycerhizae Radix [11] which are the constituent components of Galgeungyulpitang. Previous studies suggest that Galgeungyulpitang may also be effective for skin whitening. However, there are no studies on the antioxidant and skin whitening effect of Galgeungyulpitang. Therefore, the inhibitory effect of Galgeungyulpitang on melanin production, wrinkle inhibition, and tyrosinase activity were investigated in a B16/F10 melanoma cell line.
**Materials and Methods**

**Materials**

**Herbs**

The herbs used in the experiment were purchased from Omnitherb (Gyeongssangbukdo, Korea). The composition and content of *Galgeungyulpitang* were based on those in the *DonguiBogam* (Table 1).

**Methods**

**Galgeungyulpitang extract**

Ten packs (280 g) of *Galgeungyulpitang* were boiled at 100°C for 4 hours in 3 L of water. The extract was filtered through non-woven fabric. The extract was centrifuged at 3,000 rpm for 5 minutes. The supernatant was filtered using a Whatman filter (Syringe filter, Whatman). The sterile supernatant was freeze-dried (Yamato, Japan) to produce 59.1 g powder (21.10% yield) which was stored at -20°C immediately before use.

**Cells**

The B16/F10 melanoma cells used in the experiments were purchased from Korean cell line banks (Seoul, Korea).

**Table 1. Compositions and Extracting Yield of *Galgeungyulpitang***

<table>
<thead>
<tr>
<th>Formula</th>
<th>Weight ratio (g)</th>
<th>Yield (%)</th>
</tr>
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<tbody>
<tr>
<td><em>Puerariae Radix</em></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Fraxini Cortex</em></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Armeniacae Semen</em></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>Galgeungyulpitang</em></td>
<td><strong>Anemarrrenae Rhizoma</strong> 4</td>
<td>21.10</td>
</tr>
<tr>
<td></td>
<td><em>Scutellariae Radix</em> 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Ephedrae Herba</em> 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Glycyrrhizae Radix</em> 4</td>
<td></td>
</tr>
</tbody>
</table>

**Cell viability**

The B16/F10 cells were cultured using Dulbecco’s Modified Eagle’s medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) penicillin and streptomycin (Gibco, USA, 100 units/mL, 100 µg/mL). The cells were cultured at 37°C in 5% CO₂.

**Cell viability**

The effect of *Galgeungyulpitang* treatment on cell viability was confirmed by MTT assay [12]. The cells were seeded at a density of 1×10⁴ cells/well/mL in a 96 well plate and were preincubated for 24 hours at 37°C, 5% CO₂. Then, *Galgeungyulpitang* was added at concentrations of 0, 50, 100, 200, 300, 400, and 500 µg/mL and cultured for 24 hours. Thereafter, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and the cells were cultured for 4 hours. The cell culture supernatant was then removed and dimethyl sulfoxide was added to each well containing the cells and allowed to react for 30 minutes. The absorbance was measured at 540 nm using a Microplate Reader (Bio Rad, USA).

**Morphological change**

The B16/F10 cells were cultured for 24 hours for stabilization. Then, *Galgeungyulpitang* was added at a concentration of 500 µg/mL and the cells were cultured for 24 hours. The morphological changes between the *Galgeungyulpitang* untreated cells and the *Galgeungyulpitang* treated cells were observed. The morphological changes of the cells were observed using an Inverted Microscope (Leica, Germany).

**Inhibition of melanin synthesis**

The melanin production was measured using the Hosei method [13]. The B16/F10 cells were cultured and plated at a density of 1×10⁴ cells/well in a 24 well plate. The cells were cultured for 4 hours for stabilization. Then, *Galgeungyulpitang* was used at concentrations of 0, 50, 100, 250 and 500 µg/mL and the cells were cultured for 24 hours. After 24 hours of incubation, each well was washed with PBS and 1 mL of 1 N NaOH solution was added. The cells were then incubated at 60°C for 3 hours. Absorbance was measured at 475 nm using a Microplate Reader (Molecular Devices, USA).

**Inhibition of tyrosinase production in B16/F10 cell supernatant**

Tyrosinase inhibitory activity was measured using the Yagi method [14]. A total of 500 µL of 0.175M sodium phosphate buffer (pH 6.8) and 200 µL of 10mM L-DOPA were mixed. Then, the B16/F10 cells were treated with *Galgeungyulpitang* at concentrations of 50, 100, 250 and 500 µg/mL and cultured for 24 hours. A total of 100 µL supernatant from each concentration of *Galgeungyulpitang* was mixed with 200 µL of mushroom tyrosinase (110 U/mL) and left for 2 minutes at 37°C. The dopachrome that was generated was measured at 475 nm wavelength. The tyrosinase inhibitory activity was expressed as the absorbance reduction rate of the sample solution and the control sample.

**Inhibition of intracellular tyrosinase production in B16/F10 cells**

The B16/F10 cells were divided into 1×10⁴ cells/well in a 24 well plate. The cells were incubated for 24 hours to stabilize them. *Galgeungyulpitang* at 50, 100, 250 and 500 µg/mL concentrations were then cultured with the cells for 24 hours. After incubation, the cells were washed and mixed with 100 µL of 10 mM PBS containing 1% Triton X-100. The cells were vortexed and centrifuged at 1,000 rpm for 5 min. The supernatants were used for the experiments. To measure the tyrosinase inhibitory activity, 100 µL of supernatant was added to each well of the 96 well plate. A total of 200 µL of L-DOPA (2mg/mL) was added, and incubated at 37°C for 1 hour. The absorbance was measured at 475 nm using a Microplate Reader (Molecular Devices, USA). The tyrosinase activity was calculated as a percentage of the absorbance of the control group.

**Inhibition of elastase production**

The elastase inhibitory activity was measured by the James method [15]. First, a 0.2 M Tris-HCl buffer (Sigma, USA) was titrated to pH 8.0. A total of 600 µL of the reagent was transferred to the tube and diluted to 3.3 mM with 180µL of Succ-Ala-Ala-Ala-p-nitroanilide. Then, the *Galgeungyulpitang* was used at 50, 100, 250 and 500 µg/mL concentrations and cultured for 24 hours. 300 µL of the *Galgeungyulpitang* extract diluted by concentration was mixed with it. A total of 60 µL of porcine pancreatic elastase 1 and 2 were diluted to 3.3 mM with 200µL of Succ-Ala-Ala-Ala-p-nitroanilide. Then, the elastase inhibitory activity was calculated according to the following equation: IC₅₀ is the concentration of the sample (µg/mL).
required to inhibit the activity of the elastase by 50%.

**Statistics**
Statistical analysis was performed using SPSS 18 (IBM, USA). The observation was judged to be significant when the p value was less than 0.05 using Student’s t test.

**Results**

**Cell viability**

*Galgeungyulpitang* was incubated with the B16/F10 cells at different concentrations, and cell viability measured. Cell viability rates of 100.00% ± 0.73%, 98.48% ± 1.22%, 97.66% ± 1.65%, 95.15% ± 2.31%, 91.27% ± 2.23%, 75.94% ± 3.54%, and 60.12% ± 2.19% were observed at concentrations of 0, 50, 100, 250, 500, 750 and 1,000 µg/mL, respectively. At concentrations above 500 µg/mL, there was a significant decrease in cell viability, \( p \leq 0.05 \) (Fig. 1).

**Morphological change**

The effect of the *Galgeungyulpitang* extract on the morphology of the B16/F10 cells was observed. As a result, no morphological changes were observed up to a concentration of 500 µg/mL (Fig. 2).

**Inhibition of melanin synthesis**

The B16/F10 cells were treated with *Galgeungyulpitang*, and its effect on melanin synthesis was observed. As a result, the melanin synthesis rates of 100.00% ± 0.54%, 95.24% ± 1.67%, 88.16% ± 2.03%, 83.86% ± 2.85%, and 73.49% ± 2.92% were observed at concentrations of control, 50, 100, 250 and 500 µg/mL, respectively. There was a significant decrease at the 500 µg/mL concentration, \( p < 0.05 \) (Fig. 3).

**Inhibition of tyrosinase production**

Analysis of extracellular B16/F10 cell supernatant
The effects of *Galgeungyulpitang* treatment on extracellular tyrosinase production was analyzed in the cell supernatant. Tyrosinase production rates were 100.00% ± 0.11%, 98.55% ± 2.59%, 97.07% ± 3.47%, 92.52% ± 2.63%, and 89.16% ± 2.09% at concentration of 0, 50, 100, 250 and 500 µg/mL *Galgeungyulpitang*, respectively. *Galgeungyulpitang* inhibited the production of extracellular tyrosinase in a concentration dependent manner, but the decrease was not statistically significant (Fig. 4).
Analysis of intracellular tyrosinase production in B16/F10 cells

The B16/F10 cells were treated with Galgeungyulpitang, and intracellular tyrosinase production analyzed. Tyrosinase production rates were 100.00% ± 0.62%, 96.79% ± 2.44%, 95.81 ± 3.85%, 87.18 ± 3.04%, and 76.06 ± 2.17%, at concentrations of 0, 50, 100, 250 and 500 μg/mL Galgeungyulpitang respectively. There was a significant decrease in tyrosinase production in cells treated with Galgeungyulpitang at a concentration of 500 μg/mL, p < 0.05 (Fig. 4).

Inhibition of extracellular elastase production

Extracellular elastase Type 1 production was analyzed. Elastase Type 1 production rates of 100.00% ± 1.29%, 99.07% ± 3.81%, 83.15% ± 4.67%, 74.98% ± 3.24%, and 69.62% ± 4.66% were observed at concentrations of 0, 50, 100, 250 and 500 μg/mL Galgeungyulpitang, respectively. There was a significant decrease in extracellular elastase Type 1 production in cells at concentrations of 250 and 500 μg/mL Galgeungyulpitang p < 0.05 (Fig. 5).

Analysis of extracellular elastase Type 4 production was performed. Elastase Type 4 production rates were 100.00% ± 0.85%, 98.13% ± 2.87%, 91.58% ± 3.26%, 87.34% ± 4.46%, and 72.77% ± 3.52%, observed at concentrations of 0, 50, 100, 250 and 500 μg/mL, respectively. There was a significant decrease in extracellular elastase Type 4 production at a concentration of 500 μg/mL, p < 0.05 (Fig. 6).

Discussion

The development of functional raw materials with physiological activity is a prerequisite for the development of cosmetics. The desired therapeutic benefits would include whitening, anti-inflammatory responses, wrinkle improvement, anti-aging, and antioxidant effects [16]. Studies to find substances with these therapeutic effects have focused on various plant substances, such as herbal medicine materials. Recently, as the mechanism of skin pigmentation has been elucidated, studies on whitening agents as herbal medicine materials. Recently, as the mechanism of skin pigmentation has been elucidated, studies on whitening agents have been conducted with various substances [17].

Various medicinal herbs such as Codonopsis lanceolata [18], Kaempferia galanga [19], Hominis Placenta [20], and Hyssopus officinalis [21] Have been examined for their effects on skin whitening and wrinkles. In this study, to determine whether

Galgeungyulpitang has inhibitory activity on elastase (which is involved in the maintenance of skin elasticity) and inhibition of melanin biosynthesis was studied. Melanin production, tyrosinase inhibition activity, and elastase production in Galgeungyulpitang-treated B16/F10 cells were assessed and the survival rate and morphological changes were observed. No significant cell cytotoxicity was observed at a concentration of 500 μg/mL Galgeungyulpitang or less.

Human skin color is determined by hemoglobin, carotene and melanin to name a few. However, melanin is the most important determinant of skin color. Melanin is a biopolymer of phenols widely found in animals, plants, and microorganisms. It is a complex of brown pigment and protein mainly found in the skin and hair. It is synthesized from melanocytes, which are mainly in the epidermal basal layer of skin. Melanocytes are transported by keratinocytes to form melanin units. These melanin bodies contain specific enzymes necessary for the normal synthesis of melatonin. The most well-known of these enzymes are the tyrosinases [tyrosinase-related protein-1 (TRP-1)], and TRP-2. The biosynthesis of melanin begins with the amino acid tyrosine which is converted to quinone via DOPA (a type of amino acid with 2 hydroxyl groups in the molecule), then the quinone is changed to indole-5,6-dihydroquinone. Melanin is a polymer of the final compound, indole-5,6-dihydroquinone [22].

In the present study, Galgeungyulpitang significantly inhibited melanin synthesis. This result is an important function in the skin whitening induced by Galgeungyulpitang at the 500 μg/mL concentration, which effectively inhibited melanin production without having a detrimental effect on cytotoxicity. In the cells treated with 500 μg/mL Galgeungyulpitang, the cell viability was decreased to approximately 90% but this was not significantly different to untreated cells, whereas melanin production decreased to approximately 74% and was statistically significantly different to the control (p < 0.005). These results suggest that another mechanism may be involved in inhibiting melanin synthesis (besides cell proliferation), therefore, the effect of tyrosinase activity on melanin synthesis was investigated.

Tyrosinase is an enzyme that acts on the initial reaction, which is the rate-determining step of melanin synthesis. Tyrosine hydroxylase converts tyrosine to 3,4-dihydroxyphenyl alanine (DOPA), and DOPA oxidase oxidizes DOPA to DOPA quinine.
TRP-1 oxidizes 5,6-dihydroxyindole-2-carboxylic acid (DHICA) to indole-5,6-quinone-2-carboxylic acid, and TRP-2 converts DOPA chromophore to DHICA. The tyrosinase inhibitors include hydroquinone, resorcinol, 4-hydroxyanisole, ascorbic acid, and its derivatives kojic acid, arbutin, glucosamine, α-viniferin, and ferulic acid [23].

In this study, treatment of cells with Galgeungyulpitang showed no significant inhibitory effect on extracellular tyrosinase production but showed significant inhibitory activity against intracellular tyrosinase (p < 0.05). These results suggest that Galgeungyulpitang does not directly inhibit tyrosinase activity, but may be a secondary consequence of inhibiting or accelerating intracellular signaling pathways. The tyrosinase inhibitors include antioxidants such as vitamin C and quercetin [24]. These antioxidants have direct inhibitory activity against tyrosinase observed within the treated cells and in the cell culture supernatant. Therefore, Galgeungyulpitang inhibits tyrosinase activity through a different mechanism to these tyrosinase inhibitors. Further studies are needed to determine the effect of Galgeungyulpitang on factors such as microphthalmia associated transcription factor and the cAMP response element, which are known to play important roles in the development of melanin [25].

Elastase is an enzyme that degrades elastin, which is a protein that plays a very important role in maintaining skin elasticity in the dermis. Elastase is also an enzyme capable of degrading collagen. Recent studies have shown that elastase derived from fibroblasts, plays an important role in skin elasticity, and an increase in elastase contributes to the formation of skin wrinkles. Therefore, studies on skin whitening and wrinkle reducing effects through the use of elastase have been conducted [26].

In this study, a statistically significant inhibitory effects of Galgeungyulpitang on the Type 1 and Type 4 elastase were observed, p < 0.05. In this study, treatment of cells with Galgeungyulpitang showed a remarkable inhibitory effect on elastase Type 1.

The levels of tyrosinase detected were used to determine whether melanogenesis was inhibited by Galgeungyulpitang. However, other pathways are known to be involved that include factors such as microphthalmia associated transcription and cyclic adenosine monophosphate response element [27]. Therefore, future studies may focus on these factors. In addition, this study examined the direct effect of Galgeungyulpitang on elastase production detected in the extracellular supernatant, however, further studies should focus on intracellular elastase production. Despite these limitations, Galgeungyulpitang appears to inhibit tyrosinase activity and melanin production directly suggesting that Galgeungyulpitang can be further examined for its therapeutic use in skin whitening and anti-wrinkle products.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

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References

