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The Improvement Effect of *Lespedeza Cuneata* Extract on Oral Health Care

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Abstract

This study was to confirm the antimicrobial effect of *Lespedeza cuneata* extract against *Candida albicans* (*C. albicans*) and to determine its effect on the cells of typical human keratinocytes (HaCaT). Dried *Lespedeza cuneata* was percolated in 70% ethanol, and *Lespedeza cuneata* extract was applied *to C. albicans* at concentrations of 0 mg/ml, 10 mg/ml, 20 mg/ml, and 30 mg/ml. After 6h and 24h, the colony-forming units (CFUs) were evaluated. HaCaT cells were treated and then cultured at 37 °C in 5% CO2 incubator for 6h and 24h. Subsequently, water soluble tetrazolium salt (WST-1) was analyzed. Bacterial proliferation was measured in CFU. The application of *Lespedeza cuneata* extract to *C. albicans* has an antimicrobial effect. The antibacterial effect was evident with increasing concentration and over time. A decrease in CFU was seen in 10 mg/ml, and it was not statistically significant. Meanwhile, there was a statistically significant difference between the 6h and 24h results for 20 mg/ml ($P < 0.05$). After reacting for 2h, the growth rate was measured and cell viability of HaCaT cells decreased as the concentration of *Lespedeza cuneata* extract increased. The half maximal inhibitory concentration (IC50) of 10 mg/ml against HaCaT cells that normally inhabit the oral epithelial mucosa has remedial or preventive effects when it is applied to the oral cavity. *Lespedeza cuneata* extract has shown a significant antibacterial effect when it is applied the 10 mg/ml in the prevention, improvement, or treatment of oral diseases.

Keywords: Lespedeza cuneate; Candida albicans; Epithelial keratinocyte; Antibacterial effect; Oral health care

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Introduction

Oral disease is a condition that occurs in the mouth, such as teeth and gums. The importance of oral healthcare is emphasized since it is not merely limited to a pathological condition in the teeth and gums, and it can affect systematic health (Albandar JM *et al*., 1999). The recent increase in interest for oral health has led to a higher level of prevention against oral disease than other systematic diseases. However, people in many countries are still suffering from oral disease (Watt RG., 2005). In terms of quality of life, oral health plays an important role in providing satisfaction and increasing social confidence (EuroQol Group., 1990).

Oral candidiasis is one of the most common infectious oral mucous diseases that occurs in the tongue, buccal mucosa, or gums in the mouth (Mizugai H *et al*., 2007). It results from the hypergrowth of yeast-like fungus or infection from such in the candida species (Epstein JB., 1990). The important species that occur in the form of an oral opportunistic infection are *Candida albicans* (*C. albicans*), *C. tropicalis*, *C. glabrata*, *C. pseudotropicalis*, *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. stellatoidea* (Akpan A *et al*., 2002). *C. albicans* is found in 80% of the clinical cases and widely known as a cause of oral candidiasis (Odds FC., 1988).

Improvements in oral hygiene, local antifungal agent, or systemic antifungal agent are commonly used as treatment for oral candidiasis (Akpan A *et al*., 2002). Since the problem regarding various side effects of antibiotics has been raised, research on maintaining oral health by using natural medicinal plants (biocompatible antibiotic agents) is ongoing (Chen HL *et al*., 2003) and interest in safe oral medicinal products is increasing. *Lespedeza cuneata*, a natural medicinal plant, has been reported to have anti-oxidizing, anti-aging, and antibacterial effects (Lee HJ *et al*., 2011).

Human keratinocytes (HaCaT) have a significant impact on the growth of the surrounding cells, and keratinocytes exist in most of the epidermis layers, continuously repeating its formation and division (Andreadis ST *et al*., 2001). HaCaT plays an important role in protecting the body against external stimuli and protecting the skin from germs. It also plays a significant role in the oral cavity as mucosal epidermis constitutes most of the oral cavity (Chiller K *et al*., 2001).

As a natural medicinal plant, *Lespedeza cuneata* was extracted in order to identify its antibacterial effect against *C. albicans* and to identify the effect it has on the proliferation of HaCaT cells.

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Materials and Methods

Lespedeza cuneata extraction

The *Lespedeza cuneata* was purchased from Foodsynergy Co., Ltd. (Seoul, South Korea). After adding 70% ethanol to the crushed *Lespedeza cuneata*, an extract was obtained at 60 °C for 12h. *Lespedeza cuneata* extract was filtered by using qualitative filter paper, and the extract was concentrated by using a rotary vacuum evaporator (N-1300E.V.S. EYELA Co., Tokyo, Japan). The *Lespedeza cuneata* was lyophilized by using a freeze dryer at -80 °C (Ilshin Lab Co., South Korea). The sample was prepared in powder and stored at -20 °C after dilution.

Bacterial strains

In this experiment, *C. albicans* (KCTC 7965/ATCC 10231) was used after the subculture in yeast mold broth (YM, Difco, USA). *C. albicans* was incubated in YM broth at 37 °C for 24h, and diluted at a 1×10^5 ratio.

Antimicrobial activity by *Lespedeza cuneata* extract

C. albicans (100 μL, 1×10^5 colony-forming units; CFUs/ml) was inoculated into a 1.5 ml tube containing YM broth in which the *Lespedeza cuneata* extract was added at concentrations of 0 mg/ml, 10 mg/ml, 20 mg/ml, and 30 mg/ml. Each mixture was incubated for 6h and 24h. Each tube was uniformly smeared in a YM agar plate, and then cultured at 37 °C for 6h and 24h in order to check the number of CFUs.

Cell growth

In order to assess the influence on cells, HaCaT cells were diluted in Dulbecco's modified Eagle's medium (DMEM) up to 0 mg/ml, 10 mg/ml, 20 mg/ml, and 30 mg/ml, and incubated at 37 ˚C for 6h and 24h in 5% CO2 incubator. After 3h, 6h, and 24h, water soluble tetrazolium salt (WST-1) assay was performed in order to quantify the effect on cell growth (Lee SN., 2014). They were incubated at 37 ˚C for 2h in 5% CO2 incubator and its 450 nm absorption was measured by using an ELISA reader (Multiskan FC, Thermo Fisher Scientific, Waltham, MA, USA). Cell viability was compared with its optical density (OD). The process was repeated three times.

Statistical analysis

Data analysis was carried out by using Ver. 22.0 (SPSS Inc., Chicago, IL, USA). Student's t-test was conducted in order to identify the changes that could have occurred over time. The difference in each concentration was conducted through a one-way analysis of variance (one-

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way ANOVA), followed by Tukey's test ($p < 0.05$). The significance level to make a decision for the statistical significance was set at 0.05.

Results and Discussion

C. albicans can cause soft tissue disease through opportunistic infection, and it resides in the oral cavity. It does not normally cause problems in healthy individuals (Akpan A et al., 2002). The morbidity rate in general individuals without any symptom is known to be 20% to 75% (Ghannoum MA *et al*., 1990) and the incidence rate of *C. albicans* resulting in oral candidiasis is reported to be 30% to 45% in healthy adults (Lucas VS., 1993). It is especially common in infants and elderly individuals. Not only is the incidence rate high in immune-deprived patients, but it could also be a life-threatening fatal infection (Odds FC., 1988).

Some oral candidiasis conditions are naturally resolved when the contributing cause is removed, as most cases require antifungal agents (Kang SK *et al*., 2010). Applying natural extracts instead of synthetic agents are being recommended in order to control pathogens while maintaining a healthy oral microflora (Cha JD *et al*., 2005). Propolis ethanol extract has shown antifungal effects when applied to oral candidiasis patients (Santos VR *et al*., 2005). Meanwhile, *Acacia nilotica* extract has shown an antifungal effect against *C. albicans* (Khan R *et al*., 2009), and *Broussonetia* extract was also reported to have antifungal effects (Zhang Y *et al*., (2011). The antifungal effect of the Cassia spectabilis leaf against *C. albicans* was also examined (Sangetha S *et al*., (2008). Similarly, a number of researches on the antifungal effect of natural herb extracts against *C. albicans* are ongoing. In this study, *Lespedeza cuneata*, a natural substance, was extracted in order to study its bacteriostatic effect against *C. albicans* and explore how it could be applied to oral diseases. As a result, the antibacterial effect of *Lespedeza cuneata* extract against *C. albicans* was evident with increasing concentration and over time (Fig. 1). A significant change was not observed in 6h as the bacterial growth was insufficient (Fig. 1(a)), but the result of 24h showed significant difference in antibacterial effect, and no bacterial growth was observed in 30 mg/ml (Fig. 1(b)).

Bacterial proliferation was measured in CFU in order to examine the antibacterial effect of *Lespedeza cuneata* extract against *C. albicans*. Table 1 presents the changes in CFUs according to the application of *Lespedeza cuneata* extract after 6h and 24h. The death rate of *C. albicans* was based on the concentration of *Lespedeza cuneata* extract. After 6h, the antibacterial effect of *Lespedeza cuneata* extract was demonstrated, but increasing the concentration did not completely destroy *C. albicans*. On the other hand,20 mg/ml showed 6 CFU and 30 mg/ml showed complete extinction at 24h application. A decrease in CFU was seen in 10 mg/ml, and it

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was not statistically significant. Meanwhile, there was a statistically significant difference between the 6h and 24h results for 20 mg/ml ($P < 0.05$). The antibacterial effect of *Lespedeza cuneata* extract was proportional to its concentration and time applied.

Demonstrations of the antibacterial effect of the extract are ongoing; however, research on its application on HaCaT cells, the oral epithelial cells that constitute most of the oral cavity, is uncommon. This study not only identified the antibacterial effect of the extract, but also its effect on HaCaT cells. In order to check if *Lespedeza cuneata* extract has any cytotoxic effect against HaCaT cells, the extract was applied at 3h, 6h, and 24h in various concentrations, and WST-1 was performed. It was identified that the cell viability of HaCaT cells decreased as the concentration of *Lespedeza cuneata* extract increased (Fig. 2).

After applying 10 mg/ml, 20 mg/ml, and 30 mg/ml for 3h, the cell viability was measured at 51%, 20%, and 2%, respectively. After applying 10 mg/ml, 20 mg/ml, and 30 mg/ml for 6h, the cell viability was measured at 39%, 11%, and 0.2%, respectively. After applying 10 mg/ml, 20 mg/ml, and 30 mg/ml for 24h, the cell viability was measured at 18%, 10% and 0.1%, respectively. *Lespedeza cuneata* has shown a strong bacteriostatic effect against *C. albicans* that was proportional to its concentration and time applied. It was also found that the addition of *Lespedeza cuneata* extract has resulted in cell damage in HaCaT cells. The antibacterial effect increased as the concentration was increased while the survival rate of the cells was decreased. This is similar to the result reported by Shin et al. (2015) wherein *Rubuscoreanus* extract showed bacteriostatic effect against *C. albicans*, which causes oral fungal infection, from 8 mg/ml. However, it also resulted in partial cell damage. Based on the results of this study, although *Lespedeza cuneata* extract resulted in cell damage as it has antifungal effect against *C. albicans*, it did not result in complete damage. For this reason, consideration for the appropriate concentration or timing is deemed necessary.

Lespedeza cuneata extract has shown potential as an oral antifungal agent, and the above results showed that *Lespedeza cuneata* extract has a bacteriostatic effect. In addition, it could be used as a preventive agent against oral fungal infection when it is used 10 mg/ml or above. *Lespedeza cuneata* extract is a natural ingredient that demonstrates selective antibacterial effect and shows significant antibacterial effect against *C. albicans* that affects the oral mucosa. Moreover, its half maximal inhibitory concentration (IC50) of 10 mg/ml against HaCaT cells that normally inhabit the oral epithelial mucosa has remedial or preventive effects when it is applied to the oral cavity

Conclusion

Lespedeza cuneata extract has shown a significant antibacterial effect when it is applied in the prevention, improvement, or treatment of oral diseases. It will serve as a safe and favorable natural antibiotic agent with proper concentration in case it is directly applied to the oral mucosa. Therefore, it is considered that the 10 mg/ml *Lespedeza cuneata* extract can be actively used in clinical practice in the form of oral hygiene products, such as toothpaste and oral cleanser, in order to improve the oral environment.

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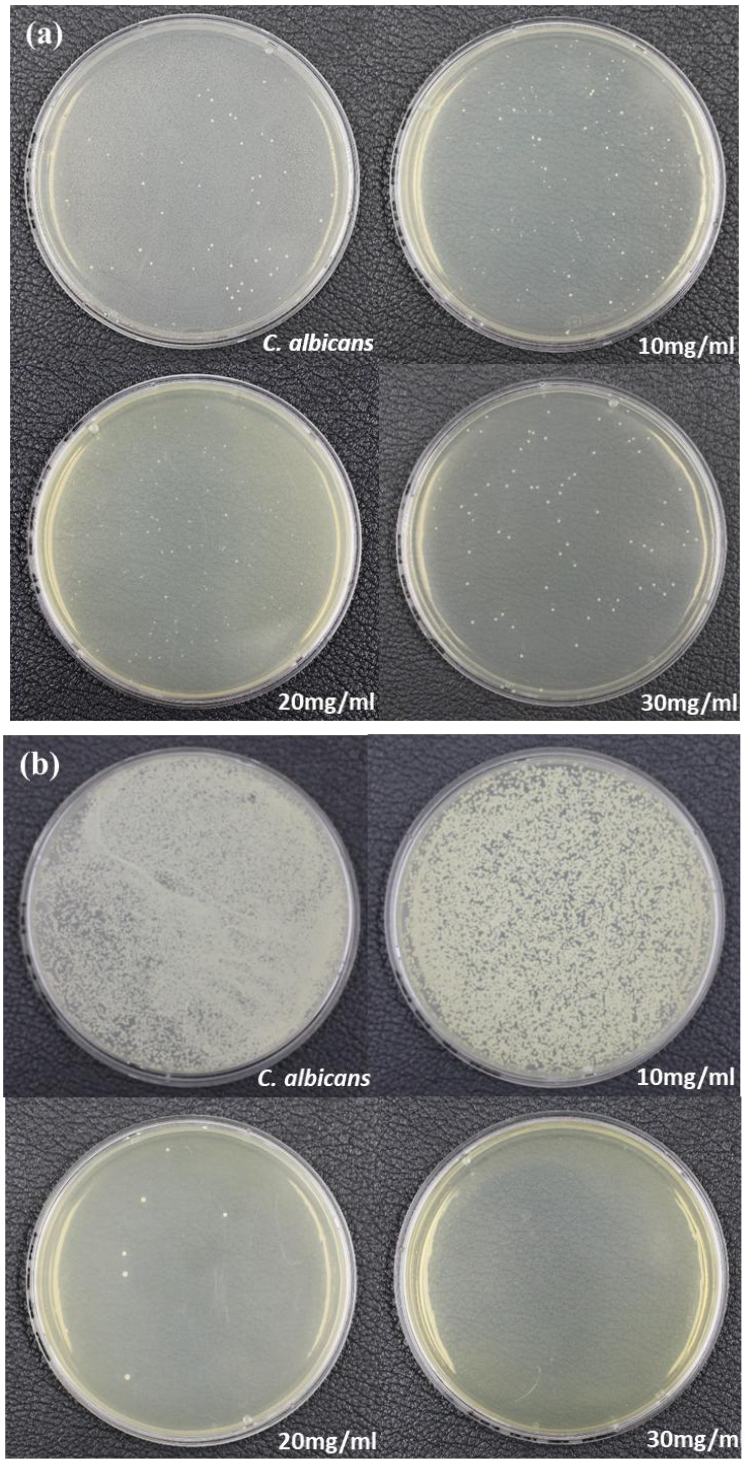

Figure 1: Anticariogenic activity of Lespedeza cuneata extract against C. albicans (a) after 6h and (b) 24h.

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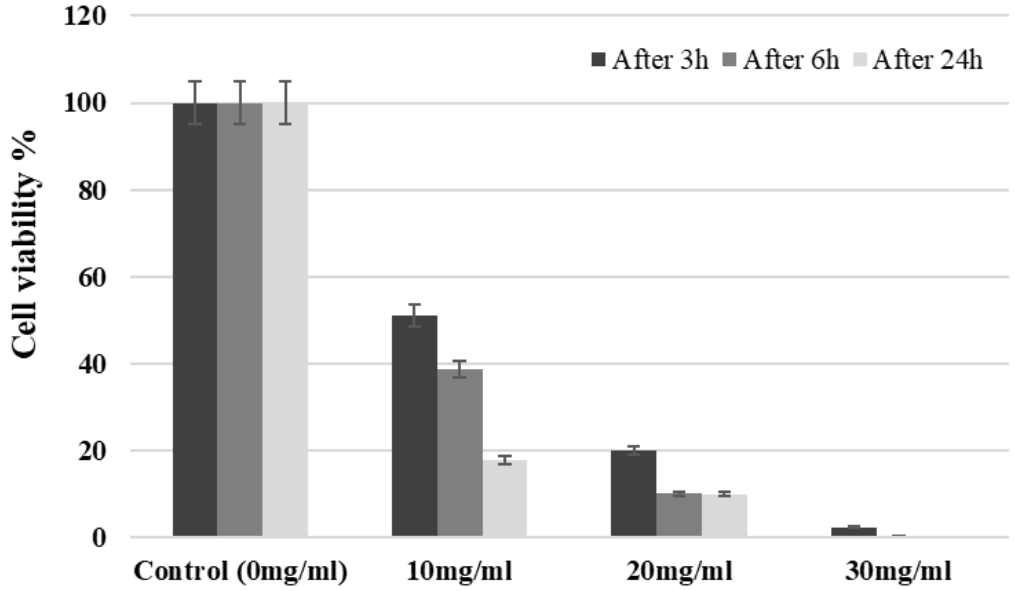

Figure 2. Survival rate of HaCaT cells by*Lespedeza cuneata*extract

Table 1: Changes in CFU according to *Lespedeza Cuneata*extract

Group	*C. albicans* (Control)	t-test P-value	10mg/ml	t-test P-value	20mg/ml	t-test P-value	30mg/ml	t-test P-value	ANOVA P-value
6h	$3.1\pm0.1\ 10^{5a}$	0.189	$1.6\pm0.2\ 10^{2b}$	0.111	$1.3\pm0.1\ 10^{2b}$	0.000	$7.6\pm0.1\ 10^{1b}$	0.000	0.023
24h	$1.3\pm0.1\ 10^{10a}$		$4.9\pm0.1\ 10^{4b}$		6.0 ± 0.1^{b}		0.0 ± 0.0^{b}		0.046

The significant difference for 6h and 24h comparison by student's t-test is shown.

The significant difference among the 4 groups in one-way ANOVA is shown. The different letters are the results presented by posthoc Duncan.