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ANIMAL

Evaluation of the wound healing ability of an *Abeliophyllum distichum* Nakai extract in ICR mouse and of antibacterial activity against human cutaneous flora

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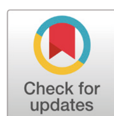
Abstract

In this study, we evaluated the wound healing rate and, inflammatory cells effects of by *Abeliophyllum distichum* Nakai (ADN) extract in mice. We also assessed the stability of the ADN extract upon exposure to sunlight. Treatments were as follows: 1) CON (only saline solution), T1 (CON + 0.0125% ADN extract), T2 (CON + 0.05% ADN extract), and T3 (CON + 0.5% ADN extract). A 4 mm punch was used in the central part of the dorsal area to separate it from the subcutaneous tissue, causing a full-thickness skin wound. An amount of 1 mL of each sample was sprayed onto the treatment section of the wound with a pipette every day from the day of wound creation, with proper application ensured using brush. In the stability test, the pH was measured at 1, 4, and 8 weeks after exposing the samples of each treatment section to sunlight considering, the higher concentrations of the ADN extract. The results of this study indicate that the effectiveness of the wound contraction rate in the mice to which the ADN extract was applied was low. Moreover, the stability of the sample containing a high concentration of the ADN extract could not be verified. In addition, no significant results were obtained in the inflammatory reaction assessment. Therefore, additional research focusing on wound contraction, stability, and inflammatory cell outcomes of the ADN extract is needed.

Keywords: *abeliophyllum distichum* Nakai extract, inflammatory cell, stability, wound contraction



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Introduction

A wound is al loss of functional anatomical continuity of normal living tissue due to damage to the skin and soft tissue. The healing process of the wound tissue occurs through regeneration of the dermis an epithelial tissue. Inflammation phase, proliferation phase, and maturation phase it occurs through the process of remodeling phase (Harper et al., 2014). In the inflammatory phase, hemostatic and inflammatory reactions occur, in the proliferative phase, granulation contraction, and epithelialization occur, and in the maturation phase, scar formation occurs (Hong et al., 2018).

Abeliophyllum distichum Nakai (ADN) is a Korean specialty plants of only one genus in the world, only growing in Korea (Lee et al., 2014). ADN is known for its antioxidant, anti-inflammatory functions (Chang et al., 2018). The leaves of ADN contain a large amount of physiologically active polyphenols and flavonoids (Jeong, 2020). Flavonoids effect antioxidant and anti-inflammatory and constitute a valuable component of skin care (Potapovich et al., 2013; Ko et al., 2019). Experiments using various plant-based natural products are being conducted. However, studies on the effect of ADN extract on wound healing are insufficient, and stability and effectiveness verification through animal testing is required. Therefore, in this experiment, to investigate the effect of ADN on wound healing, stability and antibacterial activity, the purpose of inducing full-thickness wound skin using a wound animal model rat and treating the tissues around the wound with ADN extract to confirm the effect.

Materials and Methods

The experimental protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee of Chungbuk National University, Cheongju, Korea (CBNUA-1533-21-02).

Materials and animals

ADN extract was provided and used by Our Tree Farming Association (Geosan, Korea). For the experiment, foreign substances and soil from the leaves of the ADN were washed with distilled water and then dried to retain a moisture content of 15% using an agricultural product dryer at 40℃. Dried ADN extract was immersed and extracted for 15 minutes at 121℃ and 1.2 atm in an autoclave. ADN extract concentration was divided into 0.0125, 0.05, and 0.5%. The 7-week-old Institute of Cancer Research (ICR) mouse Korea Bio link (Eumseong, Korea) was used as the experimental animal. A 12-hour light/12-hour dark cycle was used. Feed and water were provided *ad libitum*. The composition of the mouse diet is shown in Table 1.

Table 1. Compositions of the basal diets.

Item	Ingredient ($g \cdot kg^{-1}$)
Casein	210.0
L-cystine	3.0
Corn starch	280.0
Maltodextrin	50.0
Sucrose	325.0
Lard	20.0
Soybean oil	20.0
Cellulose	37.15
Mineral mix, AIN-93G-MX (94046)	35.0
Calcium phosphate, dibasic	2.0
Vitamin mix, AIN93-VX (94047)	15.0
Choline bitartrate	2.75
Yellow food color	0.1

Full-thickness skin wound and treatment

The 7-week-old ICR mouse was anesthetised by injecting 0.1 $mg \cdot kg^{-1}$ of Zoletil 50 (Virvac, Seoul, Korea) into the thigh. The hair from the back of the mouse was removed using an electric machine. The skin was completely removed using Bikiro cream (Taegeuk Pharmaceutical, Buyeo, Korea), and then sterilized with 70% ethanol. A 4 mm punch (KEYES, Sialkot, Pakistan) was used in the central portion of the dorsal area to separate it into the subcutaneous tissue, resulting in full-thickness skin wound. To prevent wound infection, the dorsal area was disinfected, and blood and other effluents in the upper portion of the wound were cleaned with phosphate buffer saline (PBS). The mouse carrying the full-thickness skin wound was subjected to four treatments according to the concentration of the ADN extract, and 1 mL was sprayed on the wound with a pipette every day from the day of the wound creation using a brush. Extract treatment was as follows: CON (only saline solution), T1 (CON + 0.0125% ADN extract), T2 (CON + 0.05% ADN extract), and T3 (CON + 0.5% ADN extract).

Stability and antibacterial activity

To test the stability of the extract, each bottle containing ADN extract was exposed to sunlight for eight weeks. The pH of the extract was measured twice during weeks 1, 4, and 8 using a pH meter (Mettller Delta 340, Mettler-tolde Ltd., Cambridge, UK). The sensory evaluation was conducted by 5 individuals based on subjective judgment. The texture, smell, viscosity and color were scored on a 5-point scale: 5, very high preference; 4, high preference; 3, moderate preference; 2, low preference; and 1, very low preference.

The antibacterial activity of the skin bacteria *Staphylococcus aureus* and *Propionibacterium acnes* was tested using the disc method. One colony of each strain was removed, inoculated into each liquid medium, activated for 24 to 36 hours, and then subcultured 3 times. Test bacteria were cultured to an optical density (OD) of 0.8 at 660 nm, suspended at a concentration of 1×10^6 $CFU \cdot mL^{-1}$, and plated on Mueller-Hinton agar and a paper disc. *S. aureus* was incubated for 24 hours and *P. acnes* for 72 hours at 37℃.

Change of wound area

Starting from the day of wound induction, the wound was measured using a camera at 1 pm on days 3, 6, 9, 12, 15, and 18. The wound contraction rate was determined from the measured area.

Inflammatory cell

Five experimental animals were sacrificed in each CON and ADN-treated group on days 3, 6, and 9 after wound induction, according to the concentration. The wound skin tissue was collected and fixed in a 4% neutral formalin solution for 24 hours, and then cut through the center of the wound, dehydrated, and formatted in a paraffin block. The tissue was cut into 4-μm-thick samples. The tissue was stained using Hematoxylin-Eosin (H&E) dye, and observed under an optical microscope (Leica, Wetzlar, Germany). Tissue images were acquired at high magnification (400×) to measure the number of inflammatory cells using Image J Software (https://imagej.nih.gov/ij/download.html).

Hair regeneration evaluation

The extent of hairs grown on the brushed area of the ADN extract in each treatment group was evaluated every 3 days after the wound. Hair growth was scored on a 5-point scale: 5, very good growth; 4, good growth; 3, normal growth; 2, poor growth; and 1, very poor growth.

Statistical analysis

All data were subjected to statistical analysis using a completely randomized design and mixed procedures of SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) with cage as the experimental unit. Orthogonal comparisons were conducted using polynomial regression to determine linear and quadratic effects at the graded levels of 0, 0.025, 0.05, and 0.5% of ADN extract. Differences among treatment means were determined using Tukey's multiple range test. $p < 0.05$ indicated statistical significance.

Results and Discussion

Stability and antibacterial activity

As shown in Table 2, the pH level decreased ($p < 0.001$) as the concentration of ADN extract increased at weeks 1, 4, and 8. The pH levels in the CON group were similar to the values of the group containing the ADN extract. The pH tended to decrease in the T3 group to 5.92, 5.05, and 4.96. In the study evaluating the stability of the extracts of *Chamaecyparis obtusa* leaves and flowers of *Inula britannica*, each sample was fractionated using ethyl acetic acid and then exposed to sunlight at different temperatures (Kim et al., 2011; Lim et al., 2012). However, in this experiment, the pH was measured following exposure to sunlight only regardless of temperature. Therefore, the pH decrease due to temperature change was used to evaluate the stability of the ADN extract. The pH value of the sample treated with the extract was lower, suggesting insufficient stability, and the need for additional pH studies according to temperature. In the case of sensory evaluation, there was no significant difference ($p > 0.05$) between treatments at 1, 4, and 8 weeks. Antimicrobial activities were not affected ($p > 0.05$) by ADN extract in this study, suggesting the absence of a clear zone against the test microorganisms (Fig. 1). Verbascoside in the ADN extract is known to be biologically active and exhibits significant antibacterial activity, especially against Gram-positive bacteria such as *S. aureus* (Avila et al., 1999; Chang et al., 2018). However, this experiment did not demonstrate any antibacterial effect. According to Koh et al. (2005), in the case of pomegranate seed oil extract extracted with water, the antibacterial effect against *Bacillus cereus* and *Escherichia coli* was observed but not against all bacteria when ethanol was used. Thus, the antibacterial effects varied due to differences in the extraction method, and further studies via antibacterial force test are needed.

Table 2. pH value changes and sensory evaluation of *Abeliophyllum distichum* Nakai extract stored at under the sun for 8 weeks.

Item (n = 2)	CON	T1	T2	T3	SE	p-value
	0%	0.0125%	0.05%	0.5%		
pH						
1 weeks[y]	6.89a	6.53b	6.53b	5.92c	0.01	0.001
4 weeks[y]	6.42a	5.82b	5.60b	5.05c	0.01	0.001
8 weeks[y]	6.33a	5.83b	5.64b	4.96c	0.01	0.001
Sensory evaluation[z]						
1 weeks	5.0	5.0	5.0	5.0	0.0	1.000
4 weeks	3.8	4.0	3.3	3.5	0.4	0.316
8 weeks	3.0	3.3	2.8	3.0	0.3	0.529

[y] Linear effect of Adbeliophyllum distichum Nakai extract concentration ($p < 0.05$).

[z] Values were determined as follows: 0 (very bad) - 5 (very good).

a - c: Means different superscripts in same column are differ significantly ($p < 0.05$).

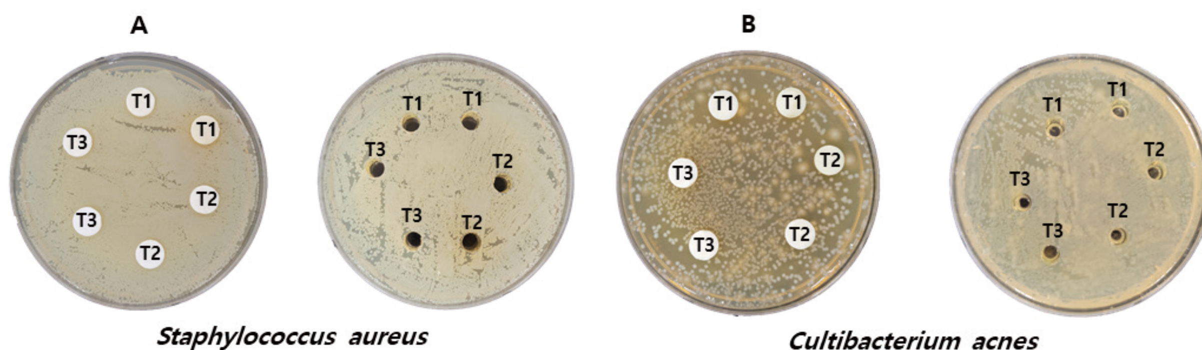

Fig. 1. Antimicrobial activites of *Abeliophyloum distichum* Nakai (ADN) extract against two strains (*Staphylococcus aureus*, and *Cutibacterium acnes*). The inoculum (about 1 × 106) was spread on the different media then, were grown at 37°C at for 24 h (*S. aureus*) and 72 h (*C. acnes*). There was no clear zone against the test microorganisms. T1, CON + 0.0125% ADN extract; T2, CON + 0.05% ADN extract; T3, CON + 0.5% ADN extract.

Hair regeneration evaluation

Table 3 shows the effect of ADN extract on hair regeneration in wounded skins of ICR mouse. The CON and T1 groups showed ($p < 0.05$) scored significantly higher than the other treatment groups in hair regeneration test. The hair regeneration scores of CON group were 4.0, 4.4, 5.0, and 4.2, respectively, indicating effective hair regeneration ability. In the case of T1 group, the hair regeneration scores of 4.5, 5.0, 5.0, and 4.8 suggested treatment effectiveness. However, the T2 and T3 groups treated with relatively high levels of extracts, scored lower on hair regeneration: 2.0, 2.0, 2.2, 2.3 and 1.8, 1.8, 1.6, 20, respectively. The low score suggests differences in mouse feeding environment and methods of extraction.

Table 3. Effect of *Abeliophyllum distichum* Nakai extract on hair regeneration in wounded skins of ICR mouse.

Item	CON	T1	T2	T3	SE	p-value
	0%	0.0125%	0.05%	0.5%		
Day 3	4.0a	4.5a	2.0b	1.8b	0.5	0.001
Day 6	4.4a	5.0a	2.0b	1.8b	0.3	0.012
Day 9	5.0a	5.0a	2.2b	1.6b	0.3	0.023
Day 12	4.2a	4.8a	2.3b	2.0b	0.2	0.013

ICR, Institute of Cancer Research; SE, standard error.

a, b: Means different superscripts in same column are differ significantly ($p < 0.05$).

Change of wound area

Table 4 showed effect of ADN extract on the wound contraction rate in mouse skin. The wound contraction was higher ($p < 0.05$) in the CON and T1 treatment groups than in T2 and T3 on days 6, 9, 12, and 15. However, there was no significant difference between the CON and T1 treatments. According to the study of Pang et al. (2017), the groups exposed to high and medium doses of flavonoids showed significantly accelerated wound contraction and closures compared with untreated groups. The ADN leaves contain a large amount of flavonoids (Kwon et al., 2014). However, in this study, the higher the concentration of the ADN extract, the lower was the wound contraction rate. These differences were attributed to differences in mouse type and extraction method used. Therefore, additional studies are needed to investigate the role of ADN extract in wound contraction. Fig. 2 shows hair regeneration evaluation and change of wound contraction rate.

Inflammatory cell

Table 5 displays the effect of ADN extract on inflammatory cells in wounded skins of ICR mouse. The inflammatory reaction, which is the first step in wound healing, is known to be most active 2 to 3 days after wound induction (Hong et al., 2018). In this experiment, no significant value was detected on day 3 of wound induction. However, after 6 days of wound induction, T1 and T2 groups showed a significant decrease ($p < 0.05$) in the number of inflammatory cells compared to CON- and T3-treated groups. The number of inflammatory cells in the T3 group decreased after 9 days of wound induction ($p < 0.05$) compared with other treatment groups. Therefore, it is difficult to evaluate the inflammatory effects at the cellular level, suggesting the need for additional experiments.

Table 4. Effect of *Abeliophyllum distichum* Nakai extract on the wound contraction rate in mouse skin.

Item	CON	T1	T2	T3	SE	p-value
	0%	0.0125%	0.05%	0.5%		
Day 3	3.5	4.5	5.5	4.5	2.0	0.472
Day 6	39.5a	44.5a	28.5b	25.5b	2.9	0.001
Day 9	55.0a	60.0a	52.0a	38.9b	3.5	0.001
Day 12	87.5a	91.0a	72.0b	68.0b	0.9	0.001
Day 15	99.1a	100.0a	84.5b	85.0b	0.3	0.001
Day 18	100.0	100.0	98.5	94.5	0.3	0.102

SE, standard error.

a, b: Means different superscripts in same column are differ significantly ($p < 0.05$).

Table 5. Effect of *Abeliophyllum distichum* Nakai extract on the inflammatory cells in wounded skins of Institute of Cancer Research (ICR) mouse.

Item (n = 3)	CON	T1	T2	T3	SE	p-value
	0%	0.0125%	0.05%	0.5%		
Day 3	924.3	795.8	871.2	909.6	114.1	0.407
Day 6	1,035.3a	555.2b	499.1b	1,035.0a	115.2	0.003
Day 9	745.9a	846.2a	953.0a	581.0b	89.9	0.041

SE, standard error.

a, b: Means different superscripts in same column are differ significantly ($p < 0.05$).

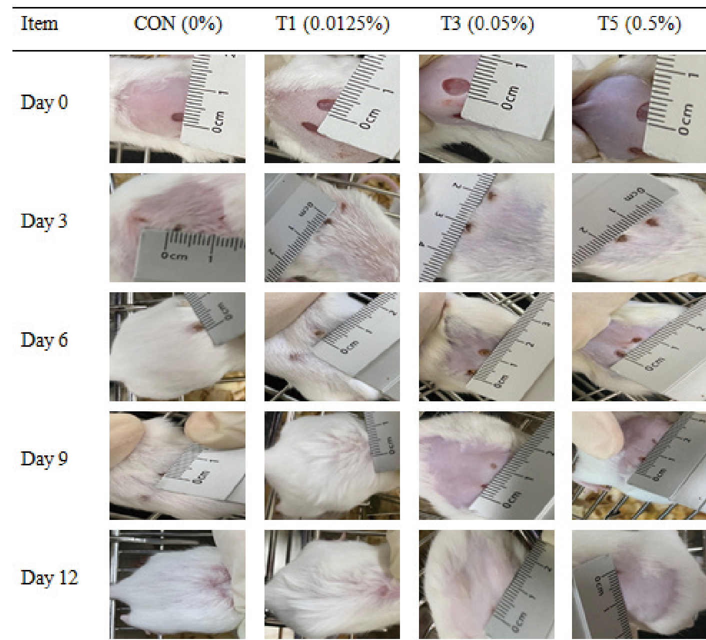

Fig. 2. Hair regeneration evaluation and change of wound contraction rate.

Conclusion

The results of this study indicated that the wound contraction rate of the mice to which ADN extract was applied was low effective, and the stability of the sample containing the high concentration of ADN extract was not verified. In addition, no significant results were obtained in the inflammatory reaction. Therefore, additional research on wound contraction, stability and inflammatory cell of the ADN extract is needed.

Conflict of Interests

No potential conflict of interest relevant to this article was reported.

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