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Effect of Skin Regeneration of Laminariae Thallus Extract in HaCaT Cell and Zebrafish Larvae

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Abstract : Many attempts are being made to find safe and therapeutic materials from natural sources. Laminariae Thallus is said to treat swelling of the face in the Dongui-Bogam and has been used in Traditional Korean Medicine to treat boils and chronic gallstones. Recently Laminariae Thallus has been utilized as a cosmetic material for its moisturizing and whitening properties. In this study, water and ethanolic extracts (30%, 50% and 80%) of Laminariae Thallus were prepared. Their regeneration effects were evaluated by cell proliferation and migration in human keratinocytes (HaCaT cells) and tail fin regeneration assay in zebrafish larvae. An 80% ethanol extract (100 μg/mL) of Laminariae Thallus was found to have the strongest ability on the migration of cells. In the tail fin regeneration experiment using zebrafish larvae, 80% ethanol extract (100 and 50 μg/mL) showed a high regeneration effect compared to the control. In conclusion, this study provides a scientific basis for the use of Laminariae Thallus extract for skin regeneration and suggests that it has great potential as a regenerative material for future natural cosmetics.

Keywords : Skin regeneration, Migration assay, Laminariae Thallus Extract, Dongui-Bogam, Traditional Korean Medicine

Introduction

The skin is an important organ that protects the human body from the outside and accounts for about 7% of an adult's body weight and is composed of dermis, epidermis, and epithelium. The functions of the skin include protection from external environments such as pressure and shock, prevention of skin dryness by controlling evaporation, prevention of microbial penetration, excretion of sebum and sweat, thermoregulation, sensory functions, and synthesis of vitamin D. Due to the recent increase in the elderly population, there is an increasing interest in skin regeneration technology to maintain healthy and elastic skin cells and to treat wounds, ulcers, and scars on the skin.[1] Skin regeneration technology is a convergence of medicine, biology, and engineering that aims to improve health and quality of life by maintaining, restoring, and enhancing the function of tissues and organs, and skin regeneration research is attempted in various ways such as stem cell therapy, biomaterials, and tissue engineering. In particular, in the field of skin regeneration, there is an increasing need for research on developing cosmetic materials to delay skin aging or treatment purposes such as wounds and burns as biomaterials using natural products. As interest and research on natural materials are actively conducted around the world, various natural cosmetics are in the spotlight due to consumer sentiment that explores new functionality from plant and marine materials and prefers new naturalness.[2]

Laminariae Thallus is a marine seaweed in the Laminariaceae with the scientific name *Laminaria japonica* Areschoung. Laminariae Thallus has traditionally been used in Korean medicine treatment, and Dong-uibogam said, "It is cold in nature, salty in taste, and non-venomous.

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It makes urine come out well, calms down swelling of the face, and treats fistulas, spirits, and lumps of energy." Also "It comes from the East Sea, and all seafood should be washed to remove the salty flavor before being added to the medicine".[3] Recently, Laminariae Thallus has been reported to have antitumor activity, increase immune function, lower blood pressure, lower blood sugar, promote excretion of radioactive substances, act as a clotting membrane, and be effective in hyperthyroidism.[4]

Zebrafish (*Danio rerio*) embryos, larvae and adults have been used as a vertebrate model in the toxicity assessment of herbal medicines and drug development.[5] Zebrafish embryos and larvae are small and cheap, producing lots of offspring.[6] Especially, zebrafish larvae are used for skin regeneration for cosmetics.[7]

In preliminary research, we conducted a cellular screen to find natural materials effective for skin regeneration and found that Laminariae Thallus extract were excellent effects on regeneration in human keratinocytes (HaCaT) cells. In this study, we aimed to evaluated the efficacy of Laminariae Thallus ethanol concentration-dependent extracts and determine their efficacy in regenerating zebrafish tail fins.

Experimental

Materials – The Laminariae Thallus used in this experiment was commercially purchased of good quality and carefully selected. The extraction solvent, ethanol, was purchased from Sigma Chemical Co. (St. Louis, USA), and the rest of the reagents were commercially available.

Laminariae Thallus extract – The dried Laminariae Thallus were prepared in four sets of 15 g each and placed in a 500 mL round bottom flask, followed by 150 mL each of purified water, 30% ethanol, 50% ethanol, and 80% ethanol at 10 times the sample weight (w/v) and reflux extraction at 100°C for 3 h. The extract was filtered with filter paper and subjected to reduced pressure concentration under 50°C. For each concentrate, pre-freezing was performed in a deep freezer at −30°C for 12 h, followed by lyophilization in a freeze-dryer (FDU-2100, Eyela, Tokyo, Japan) at −80°C for 72 h to finally obtain four dried Laminariae Thallus extract powders (Fig. 1).

Cell culture – HaCaT cells used in this study were purchased from the American Type Culture Collection (ATCC, USA). Cell cultures were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA) supplemented with 1% penicillin/streptomycin (HycloneTM. USA) and 10% fetal bovine serum (GIBCO, USA) under standard cell culture conditions at 37°C in a 5% CO_2 incubator with passage changes every 2–3 days.

Cell proliferation assay – HaCaT cells were seeded in 96 well plates at 100 μL per well to 1×10^4 cells/mL and allowed to attach for 24 h. Laminariae Thallus water extract and ethanol extract were added in dilutions and incubated for 24 h at 37°C, 5% CO_2 in an incubator. To the cultured cells, 10 μL of CCK-8 assay solution was added per well and incubated for another 4 h under the same incubation conditions. After incubation, the black light intensity was measured at 450 nm in an absorbance meter. Cell viability was expressed as a percentage of the absorbance of the control.

Wound healing assay – HaCaT cells were seeded in a 6 well plate at 6×10^5 cells/well and incubated until confluent in the plate. Scratches were made on the surface of HaCaT cells using a 200p tip, washed once with PBS, treated with Laminariae Thallus water extract or ethanol extract at a concentration of 100 μg/mL, and incubated for 12 h. The degree of healing was observed over time using a microscope, and the area of the remaining scratches was measured to show the relative distance of cell migration compared to the untreated control.

Assay of tail fin regeneration – Zebrafish larvae were incubated for 6 days and their tail fins were cut. Zebrafish larvae were treated with Laminariae Thallus 80% ethanol extract at the concentration of 100 μg/mL, 50 ug/mL, and incubated for 30 h at 28°C (each group, $n = 12$).

Statistical analysis – The difference between the mean of the control group and each experimental group was analyzed by student's t-test, and a p value of less than 0.05 was considered to be statistically significant.

Results and Discussion

Many studies have been conducted to develop various functional cosmetics by utilizing natural products (Korean medicines) that are environmentally friendly. Laminariae Thallus was selected from marine natural products through a functional materials screening study that explored functional cosmetic ingredients from natural products. In this study, in order to provide a scientific basis for the regenerative efficacy of Laminariae Thallus extract in

skin cells, we confirmed that it is an effective material for regeneration in zebrafish fin tail and wound healing through cell migration ability in human skin keratinocytes (HaCaT cells).

In order to compare the efficacy of the extracts according to the ethanol content ratio for the herbal medicine Laminariae Thallus, for each 15 g of dried Laminariae Thallus, 10 times the sample weight (w/v) of purified water, 30% ethanol, 50% ethanol, and 80% ethanol were added, followed by reflux extraction at 100°C for 3 h, and then concentrated and freeze-dried to obtain four types of dried Laminariae Thallus extract powder (Fig. 1). Among them, the extract yield of purified water extraction was the highest at 35.8% (5.38 g), and the extract yield tended to decrease with increasing ethanol content (Table 1).

Laminariae Thallus extracts utilized hydrothermal extracts prepared according to the experimental method and extracts extracted according to ethanol concentration. The water and ethanol extracts were treated at 100 and 200 μg/mL concentrations to determine cytotoxicity and proliferative capacity before evaluating the cell migration capacity. At 100 μg/mL of water and ethanol extract, there was no change in cell toxicity and proliferation, and *N*-acetyl cysteine (NAC, 1 mM), a known antioxidant, showed no significant difference (Fig. 2A). The 80% ethanol extract at 200 μg/mL showed a significant decrease compared to the control (Control, C), while the other extracts showed no change (Fig. 2B).

Previous experiments confirmed the proliferation and toxicity of the extract in HaCaT cells treated with

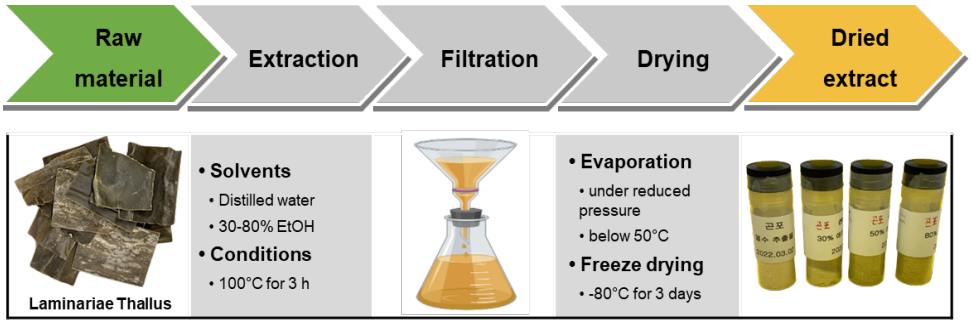

Fig. 1 The production process of Laminariae Thallus extract (LTE) powders

Table 1. Extraction yields of *Laminariae Thallus* by extraction solvents

Raw material (g)	Extraction solvent	Weight of extract (g)	Yield (%)
15 g	Distilled water	5.38	35.8
	30% EtOH	5.30	35.3
	50% EtOH	5.15	34.3
	80% EtOH	5.01	33.4

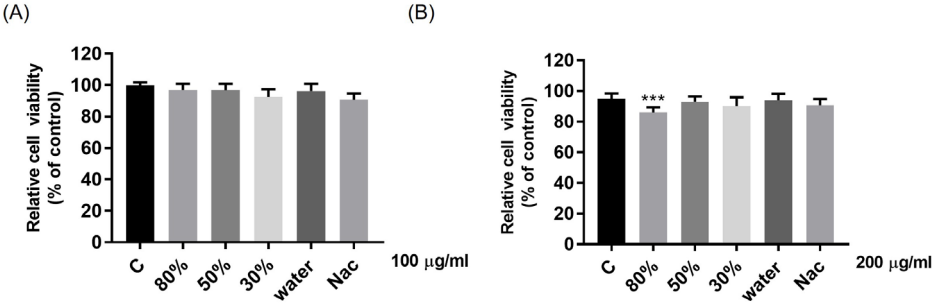

Fig. 2. Effects of Laminariae Thallus extract on cell proliferation assay in HaCaT cells. Cells were treated with 100 μg/mL concentration of Laminariae Thallus extracts as indicated in material and methods. Cell viability was analyzed using the CCK-8 assay. The date indicated the mean ± SD for triplicate experiments ($n = 7$, $p < 0.05$).

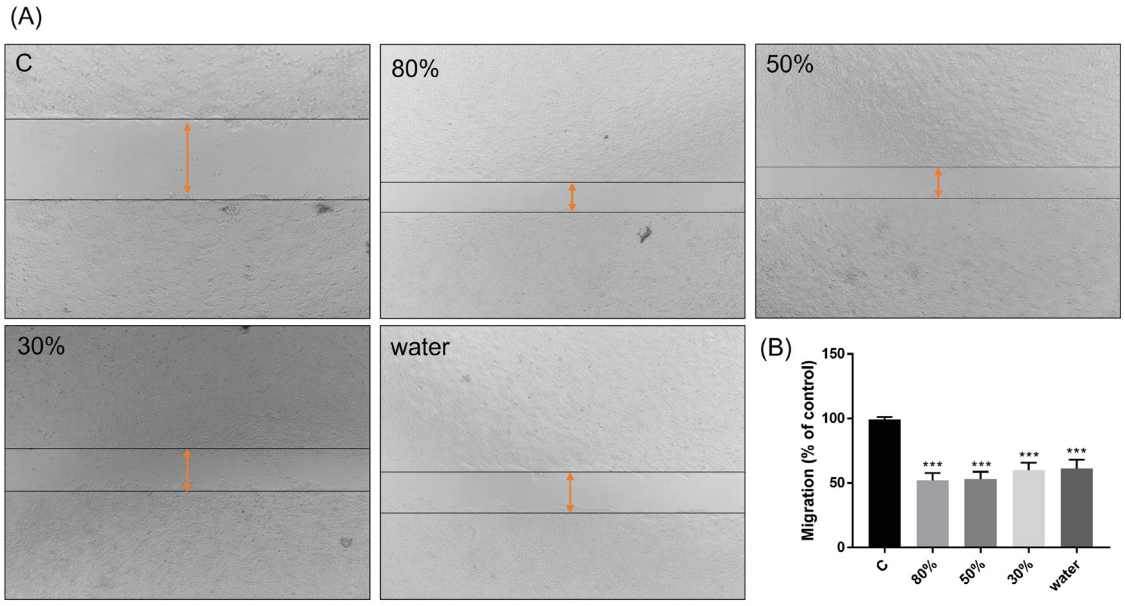

Fig. 3. Effects of Laminariae Thallus extract on wound healing assay in HaCaT cells. Cells were treated with different concentration of Laminariae Thallus extracts as indicated in material and methods. The representative cell images after migration assay (A). Cell migration was analyzed using the Image J program (B). The date indicated the mean ± SD for triplicate experiments ($n = 3$, $p < 0.05$).

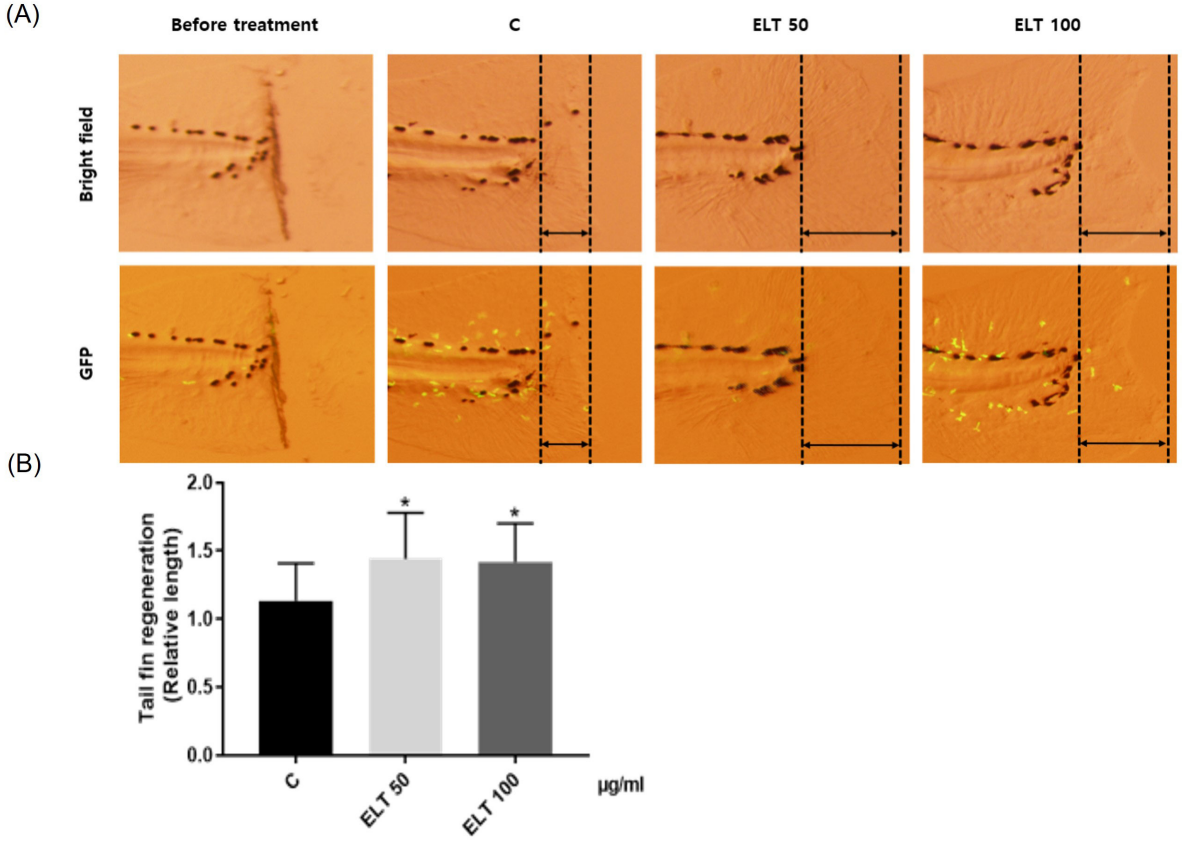

Fig. 4. Effect of Laminariae Thallus extract on tail fin regeneration in zebrafish larvae (A) Zebrafsih larval fin was cut at 6 days post-fertilization (dpf). Zebrafish was treated with ELT 50, ELT 100 for 30 h and observed the fin regeneration. (B) Statistics processing relative length of fin regeneration. The date indicated the mean ± SD for experiment ($n = 12$, $p < 0.05$).

Laminariae Thallus extract. The wound healing assay was performed to determine the degree of wound healing at a concentration (100 μg/mL) that showed no effect on cytotoxicity and proliferation. As shown in Fig 3, we found that the cell migration ability was about 35% faster for the Laminariae Thallus water extract and about 50% faster for the Laminariae Thallus 80% ethanol extract compared to the control (untreated cells).

To determine the zebrafish fin regeneration, we performed cutting tail experiments. Zebrafishes were treated with 50 μg/mL and 100 μg/mL concentrations of Laminariae Thallus 80% ethanol extract (ELT), which was the most effective for wound healing assay of HaCaT cells. As shown by Fig. 4B, the tail fin regeneration compared to the control was 27% and 25% for ELT 50 (Laminariae Thallus 80% ethanol extract 50 μg/mL) and ELT 100 (Laminariae Thallus 80% ethanol extract 100 μg/mL), respectively, but no concentration-dependent differences were found. These data indicate that 80% ethanol extract of Laminaria Thallus has an effect regeneration.

Laminariae Thallus water extract has been reported to inhibit the increased inflammatory response caused by fine dust.[8,9] The present study shows that the treatment of Laminariae Thallus ethanol extract promoted the migration of skin cells and zebrafish tail fin regeneration. In particular, the rapid migration of skin cells with increasing ethanol extract concentration and the regeneration of zebrafish tail fin in 80% ethanol extract were confirmed, and the water extract treatment also confirmed the rapid migration of skin cells compared to the normal group, although later than the ethanol extract.

Among various marine materials, Laminariae Thallus, a Korean medicine of the Laminariaceae, is rich in alginic acid, fucoidan, alginate, and laminaran, which helps skin health. Alginic acid from *Padina boryana* abates inflammatory response in keratocyte.[10] Fucoidan prevents UV-induced skin photoaging.[11] Laminarin also attenuates UV-induced skin damage.[12] In previous HPLC analyses, fucoxanthin, a marine carotenoid present in edible brown algae, was identified as the primary peak in the ethanol extract of *L. japonica*.[13] Additionally, another study quantified the content of indole-3-acetic acid, an auxin-like plant hormone, in *L. japonica* extract using HPLC, revealing concentrations of 90–95 μg/kg fresh weight.[14]

In this study, we found that treatment with Laminariae Thallus extract promoted the migration of skin cells and helped skin regeneration. Therefore, this study suggests that Laminariae Thallus is safe as a food ingredient and is relatively inexpensive, so it has high potential to be used as a skin regeneration material in cosmetics.

Acknowledgments

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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