Protection Convergence

Usefulness of Smilax China Leaves Fermented Product as a Cosmetic Material for Scalp Hair Protection

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Abstract

Purpose: Today, the population with hair loss is decreasing in age given various factors, and due to such, the market for scalp and hair cosmetics is exploding. Due to the growing interest in side effects such as allergies and environmental pollution, recently, many studies have been conducted to find cosmetic raw materials from natural substances or to apply fermentation techniques to the cosmetic manufacturing process. Hence, after enzymatic fermentation with Smilax china, which has been used for various pharmacological functions in the private sector for a long time and has secured clinical safety, the active ingredients and effectiveness (in vitro) were examined.

Method: After preparing a sample by the enzymatic fermentation of Smilax china, which is known to have various pharmacological actions, with malt, the active ingredients and effectiveness (in vitro, HaCat cell protective effect against oxidative stress, antibacterial activity, anti-inflammatory activity, dermal papilla cell proliferation rate) was confirmed.

Results: As a result of the experiment conducted, the fermented Smilax china enzyme had excellent antibacterial activity, HaCat cell protective effect against oxidative stress, anti-inflammatory activity, and dermal papilla cell proliferation rate, respectively.

Conclusion: It was confirmed that the fermented product obtained by fermenting Smilax china malt with malt offers the excellent HaCat cell protection effect against the oxidative stress, antibacterial activity, anti-inflammatory activity, and the dermal papilla cell proliferation rate, and hence, it can be applied as a raw material for various cosmetics. In particular, the usefulness as a cosmetic material for scalp and hair that is effective for the health of the scalp and hair was confirmed. Future research is expected to articulate the pharmacological mechanisms of the Smilax china fermented products through the physiological studies related to the fermentation process and the growth rate of dermal papilla cells.

Keywords: Smilax China, Malt Enzyme Fermentation, Antimicrobial Activity, Hair Loss, Anti-Inflammatory Activity

1. Introduction

1.1. Need and purpose of the study

Given the recently exceptional industrialization and economic growth, the quality of life has significantly improved and the life expectancy has increased. As a result, the interest in the healthy body and beauty is rising over ever before, and the industries such as healthy food, exercise for health, and pharmaceuticals are showing high growth [1]. This trend is also prevailing in the cosmetics industry, and the preference for organic, well-being, eco-friendly, and natural cosmetics is becoming clear [2]. Such a market change has been transformed into an up-
graded stage called the era of bio-cosmetics thanks to the development of life science and technology as a matter of evolving trend[3]. The modern people are suffering from the natural hair loss due to genetic factors or aging, as well as external factors such as increasing pollutants and stress, UV rays, environmental hormones, western eating habits, scalp hair cosmetics that stimulate the scalp, and frequent cosmetic procedures. The scalp has physiologically different characteristics from the normal skin as hair roots are located.

The scalp discharges wastes out of the body through pores, sweat glands, and sebaceous glands, and also plays an important role in balancing the oil-water balance of the skin, and hence, the excessive secretion of sweat and sebum clogs pores and causes bacterial growth. Since such a phenomenon causes scalp diseases such as Tinea capitis and hair loss, scalp management is very important not only in terms of beauty but also in terms of medical care[4]. Accordingly, scalp physiology is very important not only in cosmetic aspects but also in the study of scalp diseases, and while many studies are being conducted in connection with hair loss and hair growth-related hair physiology, there is still no way to completely solve this problem. Furthermore, many studies have been made on scalp and hair care products that can maintain and restore the health of the scalp and hair, but many products irritate the scalp or damage the scalp or cause hair loss, such as causing allergies due to toxic residues, and accordingly, they damage the scalp if not cause side effects[5].

Furthermore, since KINESIOLOGY places importance on muscles and massage areas, various studies are conducted to search for substances useful for scalp care using relatively safe natural products[6][7][8], and not only hair, but also scalp care and massage, etc., are attracting attention in the overall beauty and health fields. Hence, in this study, the main ingredient of Smilax china, which has been used for various pharmacological functions in the private sector for a long time, and has secured clinical safety, can reduce environmental pollution by enzymatic fermentation with malt used in traditional beverage manufacturing, and it was attempted to find the clinically safe cosmetic raw materials.

1.2. Theoretical background

1.2.1. Scalp and hair loss

Skin, which is a membrane covering the outside of the body, is an important part which protects the body from various environmental factors and occupies the position of the primary immune defense system, and the part that covers the head is called the scalp. While the scalp is thin, there is the root of the hair, which is the first line of defense that protects the skull, and has the function of generating, growing, and maintaining hair. Furthermore, since it is a very important part in terms of skin beauty, such as being connected to the skin of the face and neck, and having a great influence on the formation of skin wrinkles[9], and hence, beauty devices that are effective for hair by managing the scalp have also been released[10].

Hair, which operates as a barrier to protect the skull, functions as an excretory organ that discharges wastes through the hair root, and is involved in maintaining body temperature by protecting the head from external stimuli such as physical friction and direct sunlight. Furthermore, as an external organ of the skin, it protects the head from external shocks, ultraviolet rays, cold, friction, etc., and has the function of absorbing and discharging unnecessary heavy metals from the body[11]. It is not always attached to the scalp, but goes through the process of growth and fallout, and this period is very constant[12], which is called the 'hair cycle'. If over 200 hairs fall out per day, it is a condition that requires management due to the abnormal hair loss. Abnormal hair loss occurs when the hair growth cycle is shortened, the number of resting follicles increases, and hair loss occurs excessively, and it is known that it appears for various reasons such as stress, nutritional status, imbalanced action of male hormones, and genetic factors[13]. In particular, it is classified into female pattern hair loss, male pattern hair loss, alopecia areata, and infectious hair loss according to the cause and type[14]. The most cited
causes of hair loss are aging, genetic factors, and blood circulation disorders. Among the substances which perform the function of signal transduction in the body, the chemical that commands the hair follicle to start the growth or resting phase is Superoxide Radical, and the signal that commands the start of the growth phase is Nitrix Oxide, and hence, it can be applied to the hair loss area. In researching the development of therapeutic agents that can be used, many studies are being conducted in the FDA on how to lower the level of Superoxide radical using SOD(Super Oxide Dismutase)[15].

1.2.2. Natural fermented cosmetics

Phenolic compounds, Flavonoids, Carotenoids and Cellulose which are contained in the natural plant extracts are used to improve beauty and health as the nature-friendly physiologically active materials with antioxidant, anticancer and antibacterial properties, and are also used as a material for promotion and for medicine, food and cosmetics[16][17][18]. As the physiologically active ingredients of plants that have been used as medicines in the private sector for a long time, they have been scientifically proven, and are the active ingredients concentrated or separated from the plants and are used as materials for hair cosmetics as reported according to the previous studies[19][20][21].

Fermentation means that humans obtain beneficial effects by using secondary metabolites produced when microorganisms grow[22]. When they undergo the fermentation process, the physiological activities of natural substances are further maximized, the nutrients contained in it are activated, and their absorption is improved, which offer beneficial effects on humans, such as sterilizing or inhibiting the growth of harmful bacteria or neutralizing toxicity. Furthermore, there is a report that anti-inflammatory efficacy and nitrite scavenging ability are increased through fermentation[23], and while Hwangchil extract is fermented, the inhibitory efficacy of pathogenic microorganisms is increased as reported[24]. According to the studies conducted, when secondary metabolites or fermented extracts generated during fermentation were applied to the skin, antioxidant, moisturizing, and cellular activity were improved, while their side effects were relatively few, thereby indicating excellent safety as a cosmetic raw material, and it has been reported that the natural fermented broth exhibited high antioxidant power, increased the efficacy of cosmetic ingredients, and increased transdermal absorption[25]. Fermentation is used in a variety of industries, including food, cosmetics, pharmaceuticals, and feed, due to the results of such preceding studies and the growing interest in natural fermented cosmetics.

1.2.3. Smilax china

*Smilax china* is a deciduous broad-leaved vine shrub affiliated with the Liliaceae family, whose leaves are ovate, and the leaves are heart-shaped, while their tips are round shaped and convex, and has 5-7 veins coming out of the base, and they form a network again. They are gown mainly in the sun at the foot of the mountain, and geographically, it is widely distributed in South East Asia such as Korea, Japan, China, Taiwan, and Manchuria. Depending on the region, it is called variously, such as Myeonggam tree in Gyeongsang-do, bell thorn vine in Jeolla-do, barberry thorn in Hwanghae-do, and berry vine in Gangwon-do, and in the horticultural field, it is known as a sea squirrel or mange tree, and in oriental medicine, it is also called Tobokryeong or Sanguiare[26].

In the ancient times, it has been known that in China or Korea, food was scarce and used as a substitute for old-fashioned food in times of famine. In the private sector, young leaves are eaten as herbs, or large leaves are used as a natural food preservative to preserve rice cakes in summer, and the roots are called Tobokryeong and are used as herbal medicines.

The young shoots and fruits of *Smilax china* are edible, and the roots and wood are known to be effective in antipyretic, detoxification, alleviation of diuresis, physical strength, cystitis, dermatitis, nephritis, arthritis, antibacterial action, and breast cancer[27]. Furthermore, it has been reported that the leaves of Myonggam tree have an antibacterial effect on the skin flora[28],
and there is also a study uncovering the fact that the ethanol extract of the leaves of *Smilax china* maintains high antibacterial activity against *B. cereus*, *V. parahaemolyticus*, *S. typhymurium*, and *S. aureus* even after high-temperature treatment at 65-125°C [29]. As such, various pharmacological actions such as antioxidant and antibacterial action of the leaves of *Smilax china* are known, and is also used for packaging food such as rice cake, and it has been proven to have the effect of not only inhibiting the growth of microorganisms, but also providing a good flavor. Furthermore, it has been reported that bioactive ingredients are effective in preventing aging-related diseases caused by reactive oxygen species[30].

1.2.4. Malt

Malt is a material used to make 'Shikhye', a traditional drink. After sprouting the outer barley, it is dried and ground to a fine powder, which is called malt. It contains a lot of enzymes and minerals such as saccharification enzymes α-Amylase, Glucoamylase, and β-Amylase, and is rich in calcium[31]. In particular, α-Amylase, an enzyme that hydrolyzes α-1,4 bonds in starch, is not present in barley at rest, but is produced during the germination process. At which time, GA3(Gibberellic Acid), a substance similar to Gibberellin, is produced and helps the enzyme activity. Malt is a good source of GABA and is known for its antioxidant properties. Moreover, there is a study which claims that saccharification power differs depending on the length and variety of the first leaf of barley. In addition, there is a research report that the product obtained by saccharification of lactic acid bacteria fermented Chunma using malt demonstrated the best acceptance[32].

2. Research Method

2.1. Fermentation experimental method

The *Smilax china* leaves used in this study were collected from Uiryeong-gun, Gyeongsangnam-do and processed by the Uiryeong-gun Herb Farming Association, purchased from the Nonghyup Oil Farming Cooperative. The leaves delivered in salted state were used after desalting. The first desalting process was carried out in which the salted *Smilax china* leaves were separated into pieces and soaked in running water overnight to remove the base. After the primary desalination process, the leaves of *Smilax china* leaves were placed in a bucket and watered enough to submerge, followed by a secondary desalination process for 24 hours. The second desalting process was repeated 3 times while changing the water. After the desalting process, the leaves of *Smilax china* leaves were dried and extracted. The 250 g of desalted *Smilax china* leaves and 2 L of distilled water were placed in SMART OCOO(Oku, Korea), sealed and extracted by convection for 6 hours. After extraction, it was cooled to room temperature and used for enzyme fermentation.

The 2L of distilled water was added to 200 g of malt, stirred well, and then left at room temperature for 1 hour. After that, knead it well by hand to make a malt liquid, then set it aside to take only the top water. This process was repeated twice and the combined malt juice was used for the enzyme fermentation.

After mixing 300 ml of malt extract with 2 L of *Smilax china* leaves extract, enzymatic fermentation was carried out under 2 conditions. First, it was made to leave stationary fermentation at 25°C for 2 weeks. As another condition, enzyme fermentation was performed in a sealed bath convection method at 60°C for 6 hours in SMART OCOO(Oku, Korea). After the enzyme fermentation was completed, it was filtered through sterile gauze and stored in a refrigerator for use in the experiment.

The experimental method for the enzyme fermentation of *Smilax china* is as follows <Figure 1>.
2.2. Active ingredient analytical method

The Polyphenol content was measured by applying the Folin-Denis method. Polyphenols are representative antioxidants found in plants, and when Pauline reagent reacts with Polyphenols, it turns blue. The concentration of Polyphenols is judged by the intensity of blue, and the darker the color, the higher the concentration. By measuring the absorbance(750nm), a calibration curve was prepared and quantified.

The total sugar content was measured by applying the DNS method. Maltose was used as a standard, and a calibration curve was prepared and quantified by measuring absorbance(470nm).

2.3. Efficacy(in vitro) analytical method

2.3.1. HaCat cytoprotective effect test for the oxidative stress

The HaCat cytoprotective effect on the oxidative stress caused by H$_2$O$_2$ at the cellular level was measured by modifying the CCK-8 method. After culturing the HaCaT cells for 24 hours, a culture solution containing H$_2$O$_2$ was administered to react for 24 hours, and the change in absorbance was measured after reacting by adding a CCK-8 solution, and the cell viability relative to the control was marked and expressed as a percentage.

2.3.2. Antibacterial activation

2.3.2.1. Antibacterial testing method – paper disc method

The strain used in the antibacterial experiment was purchased from the Korean Collection for Type Cultures(KCTC). The antibacterial testing method was the Paper Disc method(Davidson & Parish, 1989). A single colony of each purely isolated strain was inoculated into 10 ml of a bacterial growth liquid medium, and cultured three times for 18 to 24 hours at the appropriate temperature for each strain, and then used as an antibacterial activity test strain. For the preparation of the plate medium for antibacterial test, sterilize the growth medium of each strain with 15% agar added, dispense 15 ml each to Petri-Dish to solidify the medium for the base
layer, and set the concentration of each test bacteria at 650 nm with Optical Density(O.D.) to the value of 0.4(10^6 CFU/ml), which was aseptically added to the medium for layering with 0.7% agar, mixed well, and then was evenly distributed on the medium for the base layer, followed by coagulation to make a medium for inoculation of bacteria. A sterilized 8mm Paper Disc was placed on a sufficiently hardened solid medium, and the sample was absorbed so as to be 5-1.25 mg/disc, and after culturing under suitable conditions for growth of each strain, a clear zone around the disc was observed.

2.3.2.2. Anti-fungal testing method - liquid mixing testing method

After taking a single colony of each purely separated strain, 5 ml of sterilized distilled water was added to Petri-Dish cultured on a plate, scraped with a loop to make a bacterial suspension, and then transferred to a sterilized test tube and this suspension was used as an antifungal activity test strain. In a sterile test tube, 5 ml each of the fungal activity solution and each sample were mixed well and treated at 28°C for 6 hours. Thereafter, 100 μl of each strain was dispensed into the appropriate growth plate medium, loaded and inoculated, and growth inhibition was observed while culturing under appropriate growth conditions for each strain.

2.3.3. Cytotoxicity

Prior to the anti-inflammatory activity, a cytotoxicity test was performed to determine the concentration of the sample that does not cause toxicity to cells. Toxicity for the cells was analyzed by CCK-8 method. Changes in absorbance were measured and cell viability relative to the control was marked and expressed as a percentage.

2.3.4. Anti-inflammatory

The proteins in cultured cells were quantified by using the BCA reagent and electrophoresed on 10% SDS Polyacrylamide Gel. It was transferred to a PVDF membrane, the antibody was reacted, and a photograph of each band was taken after color development.

2.3.5. TNF-α, IL-6 and iNOS expression using the HaCaT cells

TNF-α, IL-6 and iNOS are known to cause inflammatory hair loss[33][34]. An experiment was conducted to find out whether the anti-inflammatory properties of the prepared samples had an inhibitory effect on inflammatory hair loss. In order to compare and analyze the efficacy of inhibiting inflammatory hair loss by co-treating green coffee bean ferment extract and LPS(Lipopolysaccharide) to HaCaT cells cultured for 24 hours, the expression levels of genes causing hair follicle destruction and cell death were compared by relative quantification. The proteins were quantified using the BCA(Bicinchoninic Acid) reagent and electrophoresed in 10% SDS Polyacrylamide Gel. The exposed band was transferred to PVDF membrane, reacted with the antibody, and after color development, the photos of each band were taken using the ChemiDoc.

2.3.6. Dermal papilla cell’s proliferation rate

5x10^4 cells/ml of the HHDPCs cells were dispensed in a 96-well plate, and the samples were diluted 2-4 times and treated for 72 hours in 24 hour units. After adding CCK-8 solution and reacting for 1 hour at 37°C, 5% CO₂ incubator, the change in absorbance was measured and the cell viability relative to the control was expressed as a percentage. For ERK and Akt phosphorylation, HHDPCs cells were cultured for 24 hours and then treated with ferment extract and minoxidil, then absorbance was measured and confirmed with the Western-blot.

3. Results

3.1. Abbreviation
* ME : *Smilax china* extract  
* MEE25 : *Smilax china* fermentation by enzyme at 25 °C  
* MEE60 : *Smilax china* fermentation by enzyme at 60 °C

3.2. Fermentation experimental results  
During the enzymatic fermentation with the malt of *Smilax china* leaves extract, the pattern according to the temperature turned out to be very different. When the enzyme was fermented at 25 °C, the color was light and slightly viscous, and after 7 days, gas was generated, and the stopper had to be opened to release the gas. The recovery rate was 94%. Meanwhile, when the enzyme was fermented at 60 °C, the color was darker, and it became very sticky and thick. The recovery rate was 78%.

3.3. Active ingredient analytical results  
The total Polyphenol content of the *Smilax china* leaves extract was 719 μgTA/ml, and did not change even during enzymatic fermentation, and the difference due to the enzymatic fermentation temperature was not significant. In the enzyme fermented product of *Smilax china* leaves extract, there was no significant change in sugar content, and while there was a difference according to the enzyme fermentation temperature, the difference was not significant <Figure 2>.

![Figure 2](image2.png)

3.4. Efficacy (in vitro) testing results  
3.4.1. HaCaT cell protective effect for the oxidative stress  
As a result of confirming the HaCaT cell protective effect of the sample under the oxidative stress induced by H₂O₂, it was demonstrated that there was a cell protective effect. The enzyme fermented product of *Smilax china* leaves at 25 °C demonstrated significantly superior cell proliferation effect than control Minoxidil <Figure 3>.

![Figure 3](image3.png)
3.4.2. Antibacterial activation

The enzyme fermented products of *Smilax china* leaves extract at 25°C and 60°C demonstrated inhibitory rings (13mm, 13mm) for the *Bacillus subtilis*. Furthermore, the effect of inhibiting the growth of *Microsporum canis* and *Trichophyton mentagrophytes* was excellent. The growth inhibitory effect on *Microsporum canis* was excellent in the order of *Smilax china* leaves extract < *Smilax china* leaves extract at 60°C enzyme ferment << *Smilax china* leaves extract at 25°C enzyme fermented product. In particular, it was excellent in its ability to inhibit the growth of *T. mentagrophytes*, and the enzyme fermented product of *Smilax china* leaves extract 25°C inhibited the growth of *T. mentagrophytes* by over 90% <Figure 4>.

*Figure 4*. Antibacterial effect of fermented *Smilax china* enzyme.

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<th><em>Microsporum canis</em> KCTC 6349</th>
<th><em>Trichophyton mentagrophytes</em> KCTC 6316</th>
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3.4.3. Effects on HaCaT cell proliferation

To determine the effect on the proliferation of HaCaT cells. Minoxidil (10 μM) was treated together as a control, and the enzyme fermented product of *Smilax china* at 25°C demonstrated significantly superior cell proliferation effect than the control Minoxidil <Figure 5>.

*Figure 5*. HaCaT cell’s proliferation effect of *Smilax china* fermented enzyme.
3.4.4. Anti-inflammatory

In the group treated with each sample, it was revealed that TNF-α and IL-6 were inhibited, and it was found that there was an anti-inflammatory effect <Figure 6>.

**Figure 6.** Inhibitive effect on inflammatory cytokines by treatment with *Smilax china* fermented enzyme.

3.4.5. TNF-α, IL-6 and iNOS expression using the HaCaT cells

To compare and analyze the anti-inflammatory efficacy of the HaCaT cells incubated with the sample and LPS, the expression of TNF(Tumor Necrosis Factor)-α and IL-6, which are expressed during inflammation, was confirmed. It was also confirmed that the inflammatory cytokines TNF-α and IL-6 were increased when treated with LPS(400ng/ml), and in the group treated with the sample, TNF-α and IL-6 were inhibited compared to the control treated with LPS confirmed accordingly. In particular, it was confirmed that the TNF-α inhibitory effect was significantly superior to that of Minoxidil, regardless of the presence or absence of fermentation, and hence, the anti-inflammatory effect was significantly superior.

It was sought to confirm the gene expression levels of the inflammatory enzymes iNOS and the inflammatory cytokines TNF-α and IL-6. As shown in the ELISA results, iNOS, TNF-α and IL-6 were increased in the LPS-treated group compared to the control, and decreased when Minoxidil was treated. The expression of TNF-α and IL-6 genes was significantly reduced compared to the LPS-treated group, and in particular, almost no iNOS gene was found <Figure 7>.

**Figure 7.** Inhibitive effect of the inflammatory cytokine gene expression by treatment with *Smilax china* fermented enzyme.
3.4.6. Dermal papilla cell’s proliferation rate

It was sought to confirm the cell proliferation effect on the human dermal papilla cells (HHDPCs). Minoxidil was treated together as a control, and the effect of HDP cell proliferation over time was confirmed. As a result, the cell proliferation effect was increased with time in the cells treated with Minoxidil compared to the control, and the *Smilax china* fermented at 25°C demonstrated a significantly superior cell proliferation effect than the control Minoxidil <Figure 8>.

**Figure 8.** Proliferative effect of human dermal papilla cells (HHDPC) by treatment with *Smilax china* fermented enzyme.

![Figure 8](image)

To investigate the signaling pathways related to the proliferation of dermal papilla cells, the effect on Akt and ERK phosphorylation was confirmed and the Western Blot was performed. As a result of the experiment, the expression level of Akt phosphorylation gene was significantly increased compared to the positive control Minoxidil treatment. In particular, the enzyme fermented product of *Smilax china* at 25°C significantly increased the expression level of ERK phosphorylation gene than Minoxidil <Figure 9>.

**Figure 9.** Effect of Akt and ERK phosphorylation by *Smilax china* fermented enzyme fermented broth treatment.

![Figure 9](image)

4. Conclusion

For this study, the active ingredients and effectiveness (*in vitro*) were analyzed after the enzymatic fermentation of malt used in the manufacture of traditional beverages using *Smilax china*. As a result of the study, the following conclusions were reached.
First, as a result of confirming the protective effect of the HaCaT cells in the oxidative stress induced by H₂O₂, the enzyme fermented product of *Smilax china* at 25°C demonstrated an excellent cell protective effect.

Second, the enzyme fermented extract of *Smilax china* leaves extract was excellent in inhibiting the growth of *Microsporum canis* and *Trichophyton mentagropytes*, which are the causes of Tinea capitis.

Third, the enzyme fermented product at 25°C of *Smilax china* papillae demonstrated an excellent growth rate of dermal papilla cells.

Fourth, the fermented *Smilax china* demonstrated excellent anti-inflammatory properties.

Fifth, the expression levels of Akt and ERK phosphorylation genes significantly increased in the enzyme fermented product of *Smilax china* at 25°C.

As a result of the study conducted, the enzyme fermented product of *Smilax china* has excellent HaCat cell protective effect against oxidative stress, antibacterial activity, anti-inflammatory activity, and dermal papilla cell proliferation rate, and hence, it can improve scalp condition, prevent hair loss and promote hair growth, and it can be applied as an effective material for cosmetics, as it was confirmed.

The difference of this study is that the main raw material is *Smilax china*, a clinically safe natural material that has been used in the private sector for various pharmacological effects for a long period of time, and that it was fermented by enzyme using malt used in traditional beverage manufacturing. Also, By using only pure water as a solvent for extraction and fermentation, a method that is safe for the human body and reduces environmental pollution was presented. A limitation of this study is that dried leaves were not used. Since dried leaves are used for medicinal purposes, further research using dried leaves should be conducted.

It is expected that the studies on the fermented cosmetic materials using natural products will continue in the trend that the population with hair loss is getting younger due to environmental pollution and sensitive scalp, and moving forward, it is also expected that the development of scalp and hair care cosmetics or quasi-drugs using the fermented natural products as materials and applying massage will help the scalp activity and blood circulation of the head muscles.

5. References

5.1. Journal articles


5.2. Thesis degree


5.3. Additional references


6. Appendix

6.1. Author’s contribution

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