Original Article

Whitening Effects of Mori Ramulus, Mori Cortex Radicis and Mori Folium Herbal-acupuncture Solution after Fermentation and Heating

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국문초록

상지, 상백피, 상엽 약침액의 발효 및 열처리 후 미백 효능에 관한 연구

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목적 : 상지, 상백피, 상엽은 모두 뽕나무에서 유래한 것으로 한의학에서 수천 년 동안 사용되어왔다. 최근 이들의 미백효능이 발견되면서 더욱 주목을 받고 있다. 피부의 멜라닌 색소 침착은 자외선에 대한 정상적인 방어기전이다. 그러나 비정상적인 색소침착은 심각한 미용적 문제를 가져온다. 최근 약침이 피부 미백에 이용되고 있다. 또한 발효와 열처리는 미백화장료 가공의 최신 경향이다. 이 연구에서는 상지, 상백피, 상엽 약침액이 발효와 열처리 후에 피부 미백에 어떠한 영향을 미치는지 알아보고자 한다.

방법 : 상지, 상백피, 상엽을 각각 4개의 그룹으로 나누었다. ① 비발효, 비열처리, ② 비발효, 열처리, ③ 발효, 비열처리, ④ 발효, 열처리한 그룹들에 타이로시나제 활성 및 도파 산화 활성, 그리고 멜라닌 함량을 측정하여 비교하였다.

결과 : 타이로시나제 활성 및 도파 산화 활성, 그리고 멜라닌 함량에 있어서, 발효, 열처리가 대체로 효과가 있는 것으로 나타났다. 특히 멜라닌 함량에 있어서 발효, 열처리한 군이 대조군에 비해 유의성 있는 감소를 보였다.

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결론 : 주근깨, 흑색종, 간반(肝斑) 등 비정상적인 색소 침착 질환을 치료하기 위한 상지, 상백피, 상엽 약침액 제조에 있어, 발효 및 열처리 방식을 적용하면 보다 나은 미백효능을 발휘할 수 있을 것으로 생각된다.

핵심단어 : 상지, 상엽, 상백피, 약침액, 발효, 열처리

Ⅰ. Introduction

Fermentation had given many helps to human being from long time ago. Recently, fermented food comes into the spotlight, and fermentation becomes popular in the field of research as many of products created by fermentation show better effect than not fermented products[1-4].

Nowadays interests about esthetics are increasing, among that, whitening is one of the most important interests.

Whitening makes skin white as it sounds. Skin color is dependent on amounts of melanin that produced by melanocyte. So, some of the substances that inhibits melanin formation are main topics of the researchers[5-8].

Melanogenesis is a unique characteristic of melanocytes, and this process is regulated by melanogenic enzymes such as tyrosinase. Tyrosinase is a bifunctional enzyme which plays a pivotal role in the modulation of melanin production, by catalyzing the hydroxylation of tyrosine to DOPA and the subsequent oxidation of DOPA to DOPA quinone[9]. DOPA quinone synthesizes melanin by several pathways.

Arbutin is well known as an inhibitor of melanogenesis[10]. It was used as a positive control in this study.

Mori Ramulus (MR), Mori Cortex Radicis (MCR) and Mori Folium (MF) are known for outstanding whitening materials and these are all originated from Morus alba L. (a mulberry tree)[11]. However there is no study that compares the effects of the each materials and the effects that come from after fermentation or heat-treat of the materials. In this study, I tried to compare the effects of each material after fermentation or heat-treat of the materials.

Ⅱ. Materials and methods

1. Drugs

MR, MCR and MF were bought from Kwangmyungdang Medical herb Korea. Reagents were prepared as follow. 50 g of MR, MCR and MF were extracted in 100 ㎖ of 94 % ethanol for 72h. The extracts were filtered and concentrated by using the rotary evaporator. The reagents were divided in 4 groups ; no-fermentation and no-heating (normal group ; A), no-fermentation and heating (heating group ; B), fermentation and no-heating (fermentation group ; C), and fermentation and heating (fermentation and heating group ; D). Group C and D were fermented by Aspergillus oryzae for 48h. Group B and D were heated in 120℃ for 10 min. Before the cells were treated, all samples were filtered by microfilter. Arbutin is well known as an inhibitor of melanogenesis[10]. It was used as a positive control in this study.

2. Cells

B16 murine melanoma cells (Korean Cell Line Bank, Korea) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Rockville, MD) containing 10% fetal bovine serum, 100U/㎖ penicillin, 0.1㎎/㎖ streptomycin, and 0.25㎎/㎖ amphotericin B at 37℃ in a humidified 95% air / 5% CO2

atmosphere.

3. In vitro tyrosinase inhibition assay

The reaction mixtures were prepared by adding 40 U of mushroom tyrosinase to 20㎕ of MR, MCR and MF dissolved in distilled water (25㎎/㎖ and 50 ㎎/㎖), and then adding 40㎕ of 1.5mM L-tyrosine and 220㎕ of 0.1 M sodium phosphate buffer (pH 6.5). The resulting mixture (300㎕) was incubated for 10 min at 37℃ and then absorbance was measured at 490㎚[12,13]. The same mixture, but without reagents extract, was used as a control.

4. Inhibition of L-DOPA oxidation

The inhibitory effect of MR, MCR and MF on L-DOPA oxidation was determined according to the method of Joshi, P. C. with a slight modification[14]. 50㎕ of reagents dissolved in 0.1 M sodium phosphate buffer (25㎎/㎖ and 50㎎/㎖) were added to 40 U of mushroom tyrosinase in 900㎕ of 0.1 M sodium phosphate buffer (pH 6.5). After 6 min of incubation at 37℃, 3mM of L-DOPA was added. Then the mixture was incubated at 37℃ for 15 min. Activities were quantified by measuring absorbance at 475㎚. The same mixture without reagents extract was used as a control.

5. Melanin contents measurement

The melanin contents were measured by using a modification of a previously described method[15]. B16 cells were seeded onto a 6 well-plate and cultivated in DMEM medium. After 24h of incubation, samples were added with fresh medium (final conc. 300㎍/㎖). Control group was treated with distilled water. Then cells were incubated for 3 days and washed twice with Phosphate Buffer saline(PBS) and collected. Cell pellets were dissolved in 400㎕ of 1 N NaOH containing 10% Dimethyl Sulfoxide(DMSO) at 65℃ for 1h and then absorbance was measured at 490㎚ using an ELISA reader (Synergy HT, Bio-Tek Instruments INC, USA). The melanin levels were corrected by the protein concentration of the samples. Protein concentration was determined by the method of Bradford[16].

6. MTT assay for cell viability

General viability of cultured cells was determined by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan[17]. B16 cells (5×105/㎖) were seeded onto a 24 well-plate and cultivated in DMEM medium for 24h, supplemented with MR, MCR, MF (final conc. 300 ㎍/㎖) and incubation was continued for an additional 24h. MTT (0.5㎎/㎖) was added to each well, and the reaction was allowed to proceed for 1.5h. The precipitated formazan crystals were dissolved in a preset volume of DMSO, and the absorbance was read at 595㎚ with an ELISA reader.

7. Statistical analysis

The results were expressed as means±standard error of the mean (SEM). Significances of changes were evaluated using the Student's t-test. Values of $p<0.05$ were considered significant.

Ⅲ. Results

1. In vitro tyrosinase activity assay

Tyrosinase activities were determined as described in 'Materials and Methods'. The effects of arbutin (a positive control), MR, MCR, and MF on tyrosinase activity were recalculated into 100% of blank group(Fig. 1).

Arbutin groups were treated with 100 (H) or 10 (L)㎎/㎖ of arbutin respectively. MR, MCR and MF groups were treated with 10㎎/㎖ of MR, MCR and MF respectively. In arbutin-H treated group tyrosinase activity was decreased to 2.5±1.3% however, in arbutin-L treated group it was 104.3±4.7%.

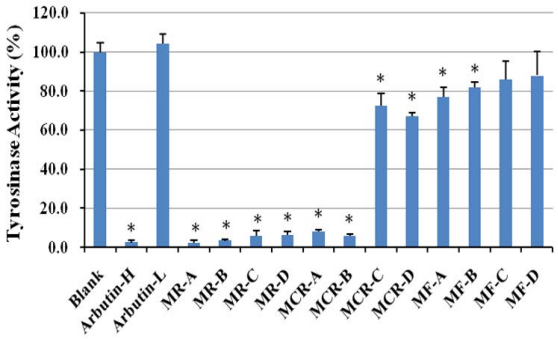

Fig. 1. Effects of Arbutin and MR, MCR and MF on in vitro tyrosinase activity

Blank group was treated with distilled water. Arbutin groups were treated with 100 (H) and 10 (L) ㎎/㎖ of arbutin. MR, MCR and MF groups were treated with 10㎎/㎖ of MR, MCR and MF respectively.

Values represent the means ± S.E.M.

* : significantly different from the blank, $p<0.05$.

Arbutin-H : Group treated with 100㎎/㎖ of arbutin.

Arbutin-L : Group treated with 10㎎/㎖ of arbutin.

MR : Mori Ramulus.

MCR : Mori Cortex Radicis.

MF : Mori Foilum.

A : Normal group. B : Heating group. C : fermentation group, D : Fermentation and heating group.

In MR-A treated group it was decreased to 2.2±1.3%, in MR-B treated group it was decreased to 3.4±0.7%, in MR-C treated group it was decreased to 5.9±2.6%, and in MR-D treated group it was decreased to 6.5±1.9%.

In MCR-A treated group it was decreased to 8.0±1.2%, in MCR-B treated group it was decreased to 5.9±1.1%, in MCR-C treated group it was decreased to 72.5±6.4%, nd in MCR-D treated group it was decreased to 67.3±1.8%.

In MF-A treated group it was decreased to 76.9±5.2%, in MF-B treated group it was decreased to 81.8±2.9%, in MF-C treated group it was 85.8±9.5%, and in MF-D treated group it was decreased to88.0±12.2%. Arbutin-H treated group, MR-A, MR-B, MR-C and MR-D treated groups, MCR-A, MCR-B, MCR-C and MCR-D treated groups and MF-A, MF-B treated groups were significantly different from blank group ($p<0.05$, Figure 1). Arbutin-L treated group, MF-C, MF-D treated groups were not significantly different from blank group.

2. Inhibition of L-DOPA oxidation

The inhibitory effects of MR, MCR and MF on L-DOPA oxidation are determined according to the method described previously. Arbutin was used as a positive control. The effects of MR, MCR and MF on L-DOPA oxidation was recalculated into 100% of blank group(Fig. 2).

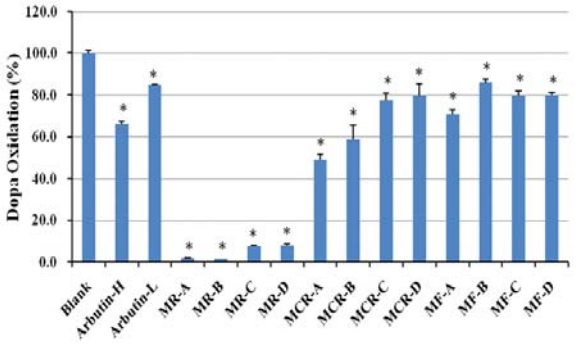

Fig. 2. Effects of Arbutin and MR, MCR, and MF on inhibition of L-DOPA oxidation

Blank group was treated with distilled water. Arbutin groups were treated with 100 (H) or 10 (L) ㎎/㎖ of arbutin. MR, MCR, and MF groups were treated with 10㎎/㎖ of MR, MCR, and MF respectively.

Values represent the means±S.E.M.

* : significantly different from the control, $p<0.05$.

Arbutin-H : Group treated with 100㎎/㎖ of arbutin.

Arbutin-L : Group treated with 10㎎/㎖ of arbutin.

MR : Mori Ramulus.

MCR : Mori Cortex Radicis.

MF : Mori Foilum.

A : Normal group. B : Heating group. C : fermentation group, D: Fermentation and heating group.

Arbutin groups were treated with 100 (H) and 10 (L)㎎/㎖ of arbutin respectively. MR, MCR and MF groups were treated with 10㎎/㎖ of MR, MCR and MF respectively. In arbutin-Htreated group, L-DOPA oxidation was decreased to 66.2±1.2% and in arbutin-L treated group it was decreased to 84.7±0.3%.

In MR-A treated group L-DOPA oxidation was decreased to 1.8±0.2%, in MR-B treated group it was decreased to 1.6±0.1%, in MR-C treated group it was decreased to 7.4±0.4%, and in MR-D treated group it was decreased to 7.9±0.7%.

In MCR-A treated group L-DOPA oxidation was decreased to 49.1±2.5%, in MCR-B treated group it

was decreased to 58.7±6.7%, in MCR-C treated group it was decreased to 77.3±3.6%, and in MCR-D treated group it was decreased to 79.7±5.6%.

In MF-A treated group L-DOPA oxidation was decreased to 70.6±2.4%, in MF-B treated group it was decreased to 85.8±1.6%, in MF-C treated group it was 79.7±2.3%, and in MF-D treated group it was 80.1±1.2%.

In arbutin treated groups (H and L), and all the, MR, MCR and MF treated groups L-DOPA oxidation was significantly different from blank group ($p<0.05$, Fig. 2). Of all, MR treated groups showed superior inhibition activities of L-DOPA oxidation most.

3. Melanin contents level

The effects of Arbutin (a positive control), MR, MCR, and MF on cellular contents of melanin were recalculated into 100% of blank group (Fig. 3). Blank group was treated with distilled water. Arbutin groups were treated with final concentration of 100 (H) or 10 (L)㎍/㎖ of Arbutin. MR, MCR, and MF groups were treated with final concentration of 100㎍/㎖ of MR, MCR, and MF respectively. Arbutin showed decrease of melanin contents. In arbutin-H treated group melanin contents were decreased to 92.4±1.5% and in arbutin-L treated groups those were decreased to 94.4±1.9%. MR-A, MR-C, and MR-D treated groups significantly decreased the melanin contents to 78.5±1.8%, 92.7±1.3%, 83.2±1.5% respectively, compared to blank group (100.0±1.6%). However, MR-B treated group did not show the decrease of the melanin contents (101.0±1.9%). MCR-C and MCR-D treated groups significantly decreased the melanin contents to 85.5±1.3% and 85.1±1.3% compared to blank group (100.0±1.6%), but MCR-A and MCR-B treated groups did not show the decrease of the melanin contents (113.9±2.0% and 111.2±1.4%). MF-A, MF-B, MF-C and MF-D treated groups significantly decreased the melanin contents to 94.4±1.4%, 87.1±1.5%, 82.2±1.2% and 77.9±1.3%, respectively, compared to blank group (100.0±1.6%). For MR, there was no efficiency of heating or fermentation because melanin contents of MR-B, MR-C and MR-D were higher than MR-A. But still those of MR-B, MR-C and MR-D were not higher than blank .

For MCR, fermentation was effective because fermentation groups (MCR-C and MCR-D) significantly decreased the melanin contents while non-fermentation groups (MCR-A and MCR-B) did not decrease the melanin contents compared to blank group.

Comparing all of MF treated groups, fermentation and heating led to the better results. MF-B, a heating group, showed the larger decrease of melanin contents than MF-A, a no-heating group. MF-C, a fermentation group, showed the larger decrease of melanin contents than no-fermentation groups (MF-A and MF-B). Even more, MF-D, a fermentation and heating group, showed the largest decrease of melanin contents than any other MF treated groups.

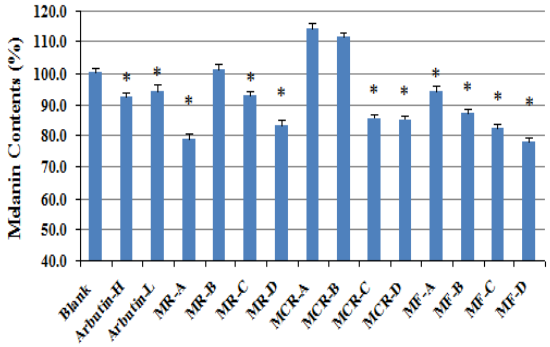

Fig. 3. Effects of MR, MCR and MF on the melanin contents of B16 cells

Most of the MR, MCR and MF treated groups significantly decreased the melanin contents. Data are expressed as a percentage compared to blank and are means±SEM.

* : significantly different from the blank, $p<0.05$.

Arbutin-H : Group treated with 100㎎/㎖ of arbutin.

Arbutin-L : Group treated with 10㎎/㎖ of arbutin.

MR : Mori Ramulus.

MCR : Mori Cortex Radicis.

MF : Mori Foilum.

A : Normal group. B : Heating group. C : fermentation group, D : Fermentation and heating group.

4. Cell viability

Blank (distilled water only), MR, MCR and MF (100㎍/㎖) and arbutin (100 and 10㎍/㎖) were treated on B16 murine melanoma cells for 24 hours. Then the cells were assayed by MTT method. The cell viabilities of blank, arbutin-H, arbutin-L treated groups were 100.0±2.5%, 94.4±0.5% and 112.2±21.2%, respectively. Those of MR-A, MR-B, MR-C and MR-D treated groups were 98.3±0.5%, 103.1±1.3%, 102.6±0.5% and 109.2±5.8%, respectively. Those of MCR-A, MCR-B, MCR-C and MCR-D treated groups were 116.7±4.3%, 114.7±1.0%, 107.6±8.1% and 104.0±0.5%. Those of MF-A, MF-B, MF-C and MF-D treated groups were 114.5±6.3%, 111.0±1.8%, 114.9±4.3% and 114.9±3.8% respectively. There was no statistical difference between groups (Fig. 4).

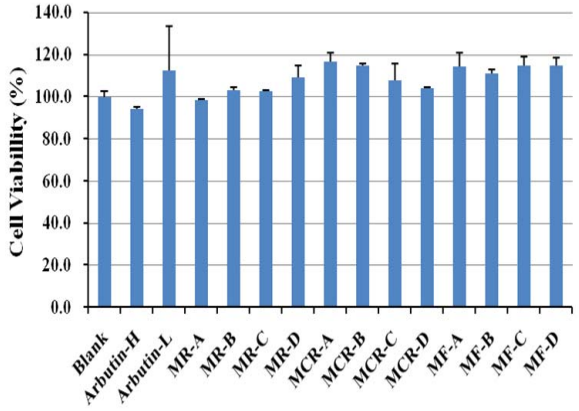

Fig. 4. Cell viability of MR, MCR and MF on B16 murine melanoma cells

There was no statistical difference between groups. The results were indicated by the means±SEM.
Arbutin-H : Group treated with 100㎎/㎖ of arbutin.
Arbutin-L : Group treated with 10㎎/㎖ of arbutin.
MR : Mori Ramulus.
MCR : Mori Cortex Radicis.
MF : Mori Foilum.
A : Normal group. B : Heating group. C : fermentation group, D : Fermentation and heating group.

Ⅳ. Discussion

Mori Ramulus, Mori Cortex Radicis and Mori Folium are all originated from Morus alba (a mulberry tree). They have been used for centuries as useful herbs. Recently, their outstanding whitening effects got attention and now they are used in many cosmetic products[18-22].

Fermentation kinetics and end-point products demonstrated the differences. Fermentation is an old technology to make molecules smaller and to be absorbed well. For example, fermented foods are easy to digest[23]. For the skin, fermented one is also easy to absorb and act more effectively. Fermentable metabolite protected the photoaging skin from solar ultraviolet irradiation[24], improved the wrinkles and scars[25], reduced the prevalence of atopic dermatitis in children[26].

That's why beauty products using this fermentation method are increasing these days. Heating is also a method to convert the nature of the substance. However, the studies on comparing melanogenesis effects of these herbs and the effects that come from fermentation or heating have not been well reported yet. In this study, we tried to compare these things.

As a result, melanin contents of fermented and heated sample groups were significantly decreased comparing blank group. In tyrosinase activity, non fermented sample groups were significantly different from blank group. In DOPA oxidation, normal groups showed superior inhibition activities most. I can suggest that even though its tyrosinase activity and DOPA oxidation were not more effective than other groups, it can reduce melanin contents.

Melanogenesis is regulated by tyrosinase activity and DOPA oxidation. But after DOPA oxidation, DOPA quinone can combine with cysteine by two pathways to benzothiazines and pheomelanins. Alternatively, DOPAquinone can be converted to leucodopachrome and follow two more pathways to the eumelanins[27,28]. So, I think this result is because of these several pathways to synthesize melanin as I stated above.

Ⅴ. Conclusion

Recently, interests of whitening are increasing. To treat the melanin pigmentation disease like freckle, melanoma and liver spots, we can apply Mori Ramulus, Mori Cortex Radicis and Mori Folium herbal acupuncture. Herbal acupuncture is made by combining acupuncture's effect and herb's effect. To produce these herbal acupuncture liquid, it would be better to use heating and fermentation technique studied in this article.

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