

Anti-Inflammatory Effects of Fusion-Fermented *Aralia continentalis Radix* (fACR) on THP-1 cells

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THP-1 세포에서 융합 발효 독활의 항염증 효과

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Abstract This study was to demonstrate the anti-inflammation effect using extracts derived from fusion-fermentation to add the function of *Araliae Continentalis Radix* (fACR). Since there are limitations to the use of ACR, available strains were selected through fermentation using lactobacillus strains, and the increases in total amount of polyphenols and amino acids was confirmed through comparison with Hot Water Extract of ACR. To determine the anti-inflammatory effect of the fACR was measure cytokine inflammation associated with arthritis, the arthritis when cartilage destruction is accompanied mainly MMP-9 activity was confirmed to evaluate the inhibition effect. These results show that fermentation using lactobacillus increases major biological activities and inflammatory response-restraining effects of ACR. This study is expected to be a basis for studying the preventive effect of fACR on arthritis.

Key Words : *Araliae Continentalis Radix*, fusion-fermentation, polyphenol, anti-inflammatory, arthritis prevention

요약 본 연구는 독활의 기능을 부가시키고자 발효과정을 통해 융합-발효 추출물을 얻어 항염증효과를 확인하고자 한다. 독활을 이용하는데 한계가 있어 유산균주들을 활용한 발효과정을 통해 이용 가능한 균주를 선별하고, 독활의 열수 추출물과 비교하여 총 폴리페놀, 아미노산 총량, 주요 생리활성 미네랄 성분 분석 결과 발효과정을 통해 증가함을 확인하였다. 독활의 발효추출물의 항염증 효과를 확인하고자 관절염 염증반응과 관련된 사이토카인들을 측정하였고, 관절염시 주로 동반되는 연골조직 파괴를 억제 효능을 평가하기 위해 MMP-9 활성도를 확인하였다. 이러한 결과들은 유산균을 이용한 발효과정을 통해 독활의 주요 생리활성이 증가하고 염증반응 억제 효과도 증가하는 것으로 보여진다. 이 연구는 독활의 관절염 예방적 효과를 연구하는데 있어 기초 연구가 될 것으로 기대된다.

주제어 : 독활, 융합 발효, 폴리페놀, 항염증, 관절염 예방

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1. Introduction

In an aging society, people increasingly show concerns about chronic diseases stemming from aging. In particular, inflammatory diseases including arthritis have emerged as a major concern to many people regardless of gender. The inflammatory diseases refer to joint inflammations due to several causes such as arthritis, aging, mechanical injury and immune dysfunction. Degenerative arthritis and rheumatoid arthritis are typical of them. As the number of arthritis patients is increasing, different treatments are attempted including a variety of medications, cartilage regeneration, artificial joint replacement and recent stem cell therapy. However, patients cannot help being worried about economic burden and adverse drug reaction of surgical procedure and long-term medication. Accordingly, studies are actively conducted on preventive use of herbal extracts.

Araliae Continentalis Radix is herbaceous perennial plant that belongs to Araliaceae and called *Aralia cordata* Thunb. *Araliae Continentalis Radix* grows wild in the shade of mountainous districts across the nation in Korea and is classified as edible material according to raw material search site of Ministry of Food and Drug Safety. Stem and leaf are used for not only inflammation, fever and cough in folk medicine but also neurasthenia, nephropathy and diabetes, and effective in different strokes. Stem and leaf reportedly contain flavonoid components, and promote antioxidant activity, anti-inflammation and antibacterial activity[1,2,3,4].

Fermentation shows a difference in enhancing and converting biological and pharmacological activities of herbal extracts. In the case of red ginseng, fermented red ginseng is produced, researched and developed. In particular, fermentation technology has developed using micro-organism such as lactobacillus, yeast and bacillus subtilis. Accordingly, fermentation products of natural substances with increased effects in biological activity are being manufactured, and products with

better effectiveness in biological activity due to mutually synergistic effect are being developed[5]. Reportedly, fermented red ginseng using lactobacillus in *Bifidobacterium* has anti-diabetic and anti-hyperlipidemic effects[6]; fermented red-ginseng rich soybean paste (Chungkukjang), which is fermented using the mixture of red ginseng into rich soybean paste mashing fermented through *Bacillus licheniformis* strains, also has anti-diabetic and anti-hyperlipidemic effects[7].

There is little research on biological and pharmacological activity increasing through fermented-*Araliae Continentalis Radix* (fACR). Thus, this study found out fermentation microorganism suitable for enhancing anti-inflammation and antioxidant effect of *Araliae Continentalis Radix*, as natural substance, used for oriental medicine, and also confirmed the increases in biological activities and anti-inflammation effect based on fermentation by comparing *Araliae Continentalis Radix* -Hot Water Extract (ACR) and fACR.

2. Material and Methods

2.1 Production of fACR

Dried *Araliae Continentalis Radix*, which was purchased in Pung-gi Market, Yeongju city, Gyeongbuk province, was washed and used. After hot water extraction at for 10 minutes of 4% *Araliae Continentalis Radix*, the *Araliae Continentalis Radix* was inoculated with each strain, and this strain was cultured. Subsequently, after solid contents were removed, the remainder was refined using UF membrane with MWCO of 50,000da, molecular mass for tea manufacturing, and freeze-dried powder was used for analysis.

2.2 Major analysis

Authorized analysis centers were used for an

objective analysis. In the first place, continentalic acid, which is an index substance of *Araliae Continentalis Radix*, was acquired from Herbal Medicine Research Division under Ministry of Food and Drug Safety, entrusted to Gyeonggi Biocenter for measurement and analyzed quantitatively and qualitatively using SIM-MS chromatogram. Test report about total polyphenol and amino acid contents was entrusted to and prepared by Suwon Women's University Food Analysis Research Center. An analysis of major biologically-active mineral components and heavy metals contents was entrusted to and conducted by Entech Analysis Research Institute Co., Ltd. and subsequently inspection report was prepared.

2.3 Cell viability assay

Each cell with the concentration of 2×10^4 cells/ml was inoculated and incubated at 37°C in 5% CO₂ incubator for 24 hours. After fACR was treated at an appropriate concentration and cultured for 24 hours, 50 μl was taken out of each well and the number of cells per unit area was measured using hemacytometer.

2.4 Cytokines assay

ACR and fACR were each dissolved at a concentration of 10, 30, 50, 100, 300 and 500 μg/ml in triple distilled water, and the sample was treated in THP-1 cell which is a human monocyte cell. Stimulation was provided by treating lipopolysaccharide (LPS) as a model to confirmation inflammatory response-restraining effects. After culture supernatant was separated and ELISA was conducted 24 hours after treatment, the concentrations of cytokine and chemokine were measured.

2.5 Zymography

After the electrophoresis of MMP-9 and MMP-2 related to inflammatory response was carried out in sodium dodecyl sulfate poly-acrylamide gel containing 0.1% gelatin, the extent of decomposition of gelatin was

measured using enzyme action of the above proteins. Based on this, the activities of secreted MMP-9 and MMP-2 were measured.

3. Results

3.1 Selection of lactobacillus from *Araliae Continentalis Radix*

After hot water extract using 4% *Araliae Continentalis Radix* was fermented through inoculation with candidate strains, pH, CFU, total polyphenol content and SOD activity were compared. Candidate strains used in this case include three kinds of lactobacillus such as *Lactobacillus ramninosus* GG (LGG), and *Lactobacillus casei* and *Lactobacillus plantarum* (LP) acquired from College of Agriculture & Life Sciences of Kyungpook National University, two types of *Saccharomyces sp.* as yeast separated from Makgeolli (Rice Wine), and *Bacillus subtilis* separated from rich soybean paste (Cheonggukjang). Finally, fermented liquid mixed with LP and LGG (fACR) was selected as a condition where CFU is highest and total polyphenol content and SOD activity increase the most (data not shown).

3.2 Measurement of Continentalic acid

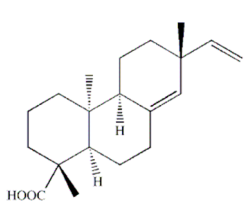
Continentalic acid is known to have a white color with the molecular formula of C₂₀H₃₀O₂ and a molecular weight of 302.45 [Fig. 1]. To analyze the effects of fACR on continentalic acid content, continentalic acid component containing ACR (1) and fACR (2) in the form of liquid samples was primarily analyzed quantitatively and qualitatively. Two types of samples were all checked in conducting a qualitative analysis (report omitted). Notwithstanding a dilution by the same polyploidy for a quantitative analysis, continentalic acid was not measured in ACR, but continentalic acid with a concentration of 0.294±0.028 mg/ml was quantitatively analyzed in fACR. This

analysis result shows that, in *Araliae Continentalis Radix*, the content of continentalic acid, which is index component, somewhat increases during the fermentation of multiple lactobacillus <Table 1>.

3.3 Analysis of major biological activities

3.3.1 Total polyphenol and amino acid contents

An analysis on total polyphenol content was entrusted to and conducted by Food Analysis and Research of Center of Suwon Women’s University, which is an authorized analysis organization. As a result, it is found that total polyphenol content related to antioxidation contained in fACR increased remarkably to 48.63 mg/100g compared to ACR 29.34 mg/100g, which was not fermented, under the same conditions. Also, through a component analysis on major amino acid content, it is found that total amino acid content in fACR increased to 132.78 mg/100g, which are equal to about two and a half times as much as 52.36 mg/100 of ACR that was not fermented. Overall, the contents of 20 types of amino acids were increased by the fermentation of lactobacillus.



[Fig. 1] Continentalic acid.
(-)-pimara-8(14) 15-diene-19-oic acid

<Table 1> The quantitative analysis of continentalic acid in fACR

Sample	Ave. of peak area	Calculated Conc. (mg/ml)	Dilution rate	Conc. (mg/ml)
CA	2355178		1	
ACR	N.D	-	2	-
fACR	3463291	0.147	2	0.294±0.028

3.3.2 Analysis of major mineral components

According to test report prepared by Entech Analysis Research Institute Co. Ltd, it is found that

fACR shows a significant increase in the contents of major minerals, such as Ca, K, Na, P, Mn, Mg, Zn and Cu, which affect biological activity, compared to ACR. In particular, the contents of Ca and P increased remarkably with 7.9 mg/100g and 114.7 mg/100g respectively, compared to 4.04 mg/100g and 68.5 mg/100g of ACR.

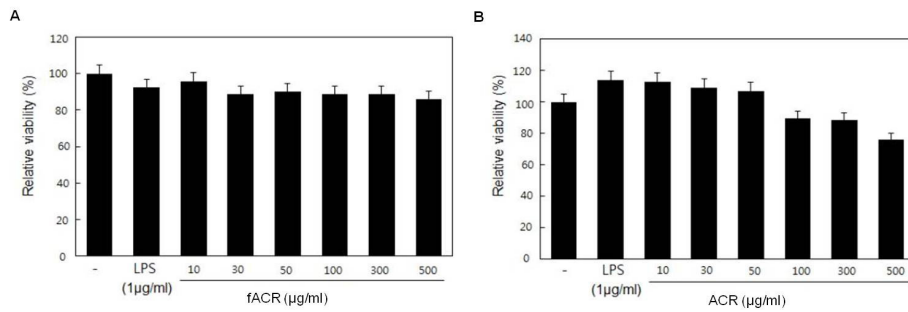
3.4 Cell toxicity

In order to the toxicity of basic cells of *Araliae Continentalis Radix*, cell viability assay was conducted. First, fACR and ACR were treated to a concentration of 500 µg/ml, and then, the number of cells was measured after 24 hours culture. The number of cells somewhat decreased during the treatment of ACR to a concentration of 500 µg/ml, but the treatment of ACR at the same concentration did not greatly affect THP-1 cell viability. This means that fACR is, basically, a little more excellent in terms of cell toxicity compared to ACR [Fig. 2].

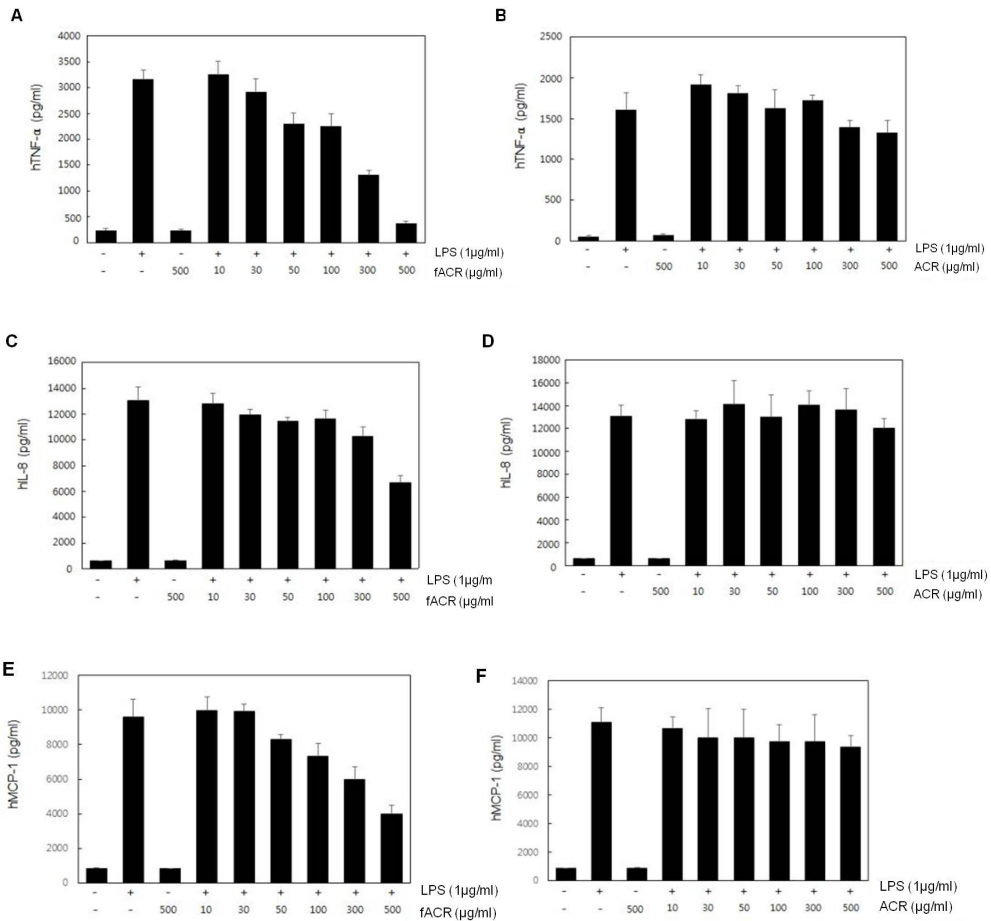
3.5 Effects of anti-inflammatory

Using THP-1 cell line which is a human monocyte cell, the effects of fACR on the expressions of hTNF-alpha and hIL-8 among cytokines related to inflammatory responses in arthritis and hMCP-1, as chemokine, known to be related to the induction of arthritis pain were analyzed to confirmation inflammatory response-restraining effects of fACR. fACR and ACR were each dissolved at a concentration of 10, 30, 50, 100, 300 and 500 µg/ml in triple distilled water, and the sample was treated in THP-1 cells. Stimulation was provided by treating LPS as a model to confirmation inflammatory response-restraining effects. After culture supernatant was separated and ELISA was conducted 24 hours after treatment, the concentrations of cytokine and chemokine were measured.

First, according to the results of checking hTNF-alpha production, it is found that a stimulation



[Fig. 2] Effects of fACR and ACR on the cell viability in THP-1 cells. fACR(A) is, basically, somewhat excellent in terms of cell toxicity compared to ACR(B)



[Fig. 3] Effects of fACR and ACR on the anti-inflammatory in THP-1 cells. (A, B) hTNF-alpha ELISA assay, (C,D) hIL-8 ELISA assay and (E,F) hMCP-1 ELISA assay. (A, C, E) The fACR was suppressed significantly.

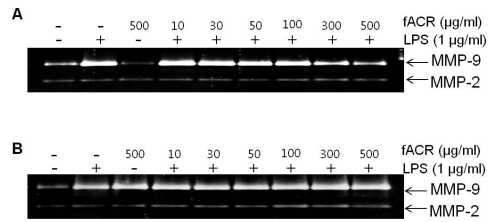
using LPS, which is an inflammation-inducing material, had a tendency to inhibit hTNF-alpha production of fACR at a significant concentration [Fig. 3A]. In contrast, ACR treated at the same concentration had no great effect on hTNF-alpha production [Fig. 3B].

According to the results of checking hIL-8 among cytokines generated in cases where inflammatory response occurs, it is found that fACR stimulated with LPS had a tendency to inhibit hIL-8 expressivity at a significant concentration [Fig. 3C] but that ACR had little effect [Fig. 3D].

An analysis was conducted on the effect of fACR on the expression of hMCP-1 among chemokines generated in cases where inflammatory response occurs. As a result, it is found that the induction of inflammation using LPS had a tendency to inhibit hMCP-1 production at a significant concentration [Fig. 3E] but that ACR had little effect on hMCP-1 production [Fig. 3F].

3.6 Effects on MMP-9 production

To evaluate a basic efficacy of fACR to inhibit the destruction of cartilaginous tissue following arthritis, MMP-9, 2(Matrix metalloproteinase-9, 2) activity was analyzed by zymography using THP-1 cells. After the electrophoresis of MMP-9 and MMP-2 related to inflammatory response was carried out in sodium dodecyl sulfate poly-acrylamide gel containing 0.1% gelatin, the extent of decomposition of gelatin was measured using enzyme action of the above proteins. It is found that the treatment of ACR at a concentration of 300µg/ml and 500µg/ml with a stimulation using LPS had a tendency to inhibit mmp-9 production unlike that of ACR at the same concentration [Fig. 4A] but that had no great effect on MMP-2 activity [Fig. 4B].



[Fig. 4] MMP-9 Expression using zymography in THP-1 cells. MMP-9 production is significantly inhibited in fACR(A) compared to ACR(B).

4. Discussion & Conclusion

In an aging society, people increasingly show concerns about not only chronic diseases stemming from aging but also disease prevention using oriental medicinal herbs or food. Although anti-inflammatory activity of *Araliae Continentalis Radix* is widely known, it has limitations on its use. Thus, fermentation was conducted to enhance biological and pharmacological activities and to contribute to increasing voluptuousness. Candidate strains included three kinds of lactobacillus including LGG, and *L. casei* and LP, two types of *Saccharomyces sp.* as yeast separated from Makgeolli (Rice Wine), and *Bacillus subtilis* separated from rich soybean paste (Chungkukjang). Basically, the optimal conditions were confirmed through inoculation of 4% *Araliae Continentalis Radix* extracts with the changes of the amount of strain and the speed and time of shaking and fermentation was conducted using two kinds of multiple strains such as LGG and LP. Also, in culture using two kinds of mutually different strains, a sequential culture to secure two kinds of culture conditions showed good results than a simultaneous culture. To remove solid contents and to stop unnecessary fermentation, sterilization and refinement were conducted and freeze-dried powder was used for analysis.

In the qualitative analysis of index materials, the continentalic acid was detected in both ACR and fACR.

However, quantitative analysis was measured only fACR in the same condition. This analysis result shows that, in *Araliae Continentalis Radix*, the content of continentalic acid, which is an index component, somewhat increased during the fermentation. Continentalic acid, which is known to have a white color with the molecular formula of $C_{20}H_{30}O_2$ and a molecular weight of 302.45, is generally contained in the material of *Araliae Continentalis Radix*, and an index material that is known to have an effect on different biological activities such as for arthritis[3,8].

A comparison was conducted on total polyphenol content related to anti-oxidation to confirm the increase in continentalic acid, which is an index material, in fACR and the changes in components before and after fermentation. The results show that continentalic acid increased more than 1.6 times in fACR than ACR. It is found that total amino acid content also increased 2.5 times to fACR 132.78 mg/100 g compared to ACR 52.65 mg/100 g. The increase of amino acid facilitates digestion and absorption through fermentation process and affects sweet taste. Some advantages such as the strengthening of muscle protein and anti-oxidation including the improvement of digestion and taste have been highlighted. The fermentation by multiple lactobacillus increases the index components of *Araliae Continentalis Radix* and the total polyphenol and amino acid contents, which is thought to have an effect on the improvement of *Araliae Continentalis Radix* function[9,10,11,12,13]

Inflammation including arthritis is a biological response to various types of infections or stimulating materials among metabolites in a human body, and different mediators such as NO, free radicals, cytokines, PGE2 and lysosomal enzyme are involved in it. Using THP-1 cells, the effects of fACR on the expressions of hTNF-alpha and hIL-8 among cytokines related to inflammatory responses in arthritis and hMCP-1, as chemokine, known to be related to the induction of arthritis pain were analyzed to confirmation

inflammatory response-restraining effects of fACR. The hTNF-alpha represents cell toxicity in tumor cell and has relation to chronic inflammation, and it is an inductive factor of cytokines including IL-8. As the results of a measurement of hTNF-alpha secretion rate using ELISA after inducing inflammation with LPS, it is found that the secretion rates of hTNF-alpha and hIL-8 were significantly inhibited in fACR. The hIL-8 is found in inflammatory diseases including rheumatism, and affects inflammation-related cells such as neutrophil, B lymphocyte and eosinophil. The expression of hMCP-1 also was significantly inhibited in fACR[14,15,16]. Arthritis is accompanied by the destruction of cartilaginous tissue, when the activities of MMP-9, 2(Matrix metalloproteinase-9, 2) are known to increase. Whether the destruction of cartilaginous tissue can be stopped was identified through measurement of the activity of MMP-9, 2 by fACR. This seems not to have a great effect on the activity of MMP-2. However, it is found that MMP-9 production is inhibited by fACR at a concentration of 300 µg/ml and 500 µg/ml. Since this can stop the destruction of cartilaginous tissue, it is expected to contribute to arthritis prevention[17,18,19,20].

To overcome the limitations of useful *Araliae Continentalis Radix* to the use, this study used fermentation. Continentalic acid, an index component, somewhat increased during the fermentation of multiple lactobacillus of LGG and LP. As total polyphenol and amino acid contents increased, taste and flavor were improved. The inhibitory effects of arthritis-related inflammatory factors in cells were confirmed. Thus, these are expected to be the basic results to improve the utilization of *Araliae Continentalis Radix* for arthritis prevention.

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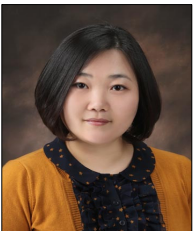
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