

An Experimental Study of the Anti-oxidant and the Anti-inflammatory Effects of *Alum* and Burnt *Alum*

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Key Words

Alum; anti-oxidation; anti-inflammation; Burnt *Alum*; therapeutic effects; external treatments

Abstract

Objectives: The purpose of this study was to compare the anti-oxidant and anti-inflammatory effects of *Alum* (AL) and Burnt *Alum* (BAL), which are commonly used as external ointments.

Methods: Extracts of AL and BAL were classified into three groups: 20, 50, and 100 mg/ μ l. The cytotoxicity was measured by using MTT assays in human keratinocyte cell line (HaCaT). The anti-oxidant effect was measured by using the DPPH [1, 1-diphenyl-2-picryl-hydrazyl-hydrate] radical scavenger. The anti-inflammatory effect was measured by using the inhibitory efficacy for the amount of nitric-oxide (NO) produced in mouse macrophage cell line (RAW 264.7).

Results: BAL showed a higher level of cytotoxicity than AL. The AL groups showed a concentration-dependent scavenging effect on DPPH radicals, but no significant relevance was found. The BAL groups showed a concentration-dependent scavenging effect on DPPH radicals. The scavenging effects of the BAL groups were almost insignificant, but the values for the 20, 50, and 100 μ g/ml trials were different. The BAL groups showed significant concentration-dependent inhibitory effects on NO production, but the AL groups did not.

Conclusions: AL showed an anti-oxidant effect more efficiently than BAL did, which demonstrated a superior anti-inflammatory effect. Therefore, for external usage, AL must be distinguished from BAL.

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1. Introduction

Current Korean medical treatments are not only confined to acupuncture, moxibustion, and herbal medication. Rather, various other treatments have been developed and are already being used clinically, and great challenges seem to be ahead. Dermatology in Korean medicine is no exception. Constant effort should be made to develop new therapies and to renew existing treatments, with a view of expanding the scope of Korean medicine.

Dermatology in Korean medicine is a field that chiefly deals with diseases that develop on the skin and requires the more frequent usage of diverse external ointments than other fields of study do. However, in reality, the present external ointments developed by Korean medical dermatologists are no more than in-house manufactured products. Experimental research on external applications of medicinal herbs is quite insufficient.

Alum (AL) is one of the astringent herbal medicines that features a strong ability to dry dampness. Upon external use, it has the effects of detoxifying and killing worms in addition to an antipruritic effects. Upon internal use, it has a hemostatic effect and can check diarrhea and dispel wind-phlegm. Thus, with external use, it cures eczema, pruritus, and otitis media, while with internally use, it treats chronic diarrhea, bloody stool, flooding and spotting, epilepsy, and delirium [1]. Burnt *Alum* (BAL) heals wounds, has a hemostatic effect, and resolves putridity, curing eczema, otitis media, pruritus vulvae, vaginal discharge, nosebleeds, gum bleeding, and nasal putridity [1]. Generally, AL is used internally, and BAL is used externally [1,2].

However, based on many other Korean medicine clinical records, AL has also been used externally [3]. Furthermore, existing experimental research on AL and BAL has not put

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much emphasis on differentiating these two medicines [4-7]. Thus, finding the obvious differences between AL and BAL through this experimental study of their anti-oxidant and anti-inflammatory effects is worthwhile in order to make them more conducive to Korean medicine clinical practice afterward.

2. Experimental materials and methods

2.1. Materials

2.1.1. Medicinal herbs

The AL and the BAL used in this study were discretely selected from the pharmacy in the Korean medicine hospital affiliated with Sangji University.

2.1.2. Strain and cell line

The human keratinocyte cell line (HaCaT) used for measuring the cytotoxicity was cultured in the Biochemistry Department of Gangwon University, and the mouse macrophage cell line (RAW 264.7) for measuring the anti-inflammatory effects was obtained from Technology Innovation Center of Hanlim University.

2.1.3. Reagents and equipment

Reagents such as 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), N^o-methyl-L-arginine acetate salt (L-NMMA), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), and lipopolysaccharide (LPS) were bought from Sigma (USA); fetal bovine serum (FBS), penicillin-streptomycin and dulbecco's modified Eagle's medium (DMEM)/high glucose were bought from Hyclone (USA); dimethyl sulfoxide (DMSO) and 2-propanol Hueller-Hinton broth were bought from Merck (USA). Equipment such as an ELISA reader (Perkin-Elmer, Foster City, CA), a spectrophotometer (UNICO, USA), and a micro-centrifuge (Hettich, Germany) were also utilized in this study.

2.2. Methods

2.2.1. Reagents

AL and BAL, 25 g each, were mixed with secondary distilled water and boiled for 150 mins at 100°C. After having been filtered through a filter bed (Whatman 4 → ADVANTEC 5C), the filtrate was completely condensed by using a rotary decompressing concentrator. After the H₂O was removed at 70°C, 13.5 g of AL (yield rate: 54%) and 13 g of BAL (yield rate: 52%) were obtained. Lastly, the AL and the BAL were each put into a micro-tube and diluted to 20 mg/ml by using 100% DMSO.

2.2.2. Culturing strain and cell line

HaCaT cells and RAW 264.7 cells were cultured in the DMEM medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 units/ml) under 5% CO₂ at 37°C. The medium was regularly replaced every three or four days and then subcultured after 90% of the cultivation had been completed.

2.2.3. Measuring cytotoxicity

HaCaT cells were placed in the DMEM medium including penicillin (100 units/ml), streptomycin (100 units/ml), and 10% FBS and were cultivated inside the incubator at 37°C under 5% CO₂. Thereafter, the HaCaT cells were diluted to a concentration of 1 × 10⁴/well in a 96-well plate and were cultured for 24 hrs after the inoculation of 100 μl. All the media were removed after cultivation. Ninety μl of serumless medium was put into each well. The AL and the BAL were mixed with 10 μl of diluted reagents by using a serumless medium until their densities reached 20, 50, and 100 μg/ml. After having been cultured for 24 hrs, a 5

μg/ml concentration of dissolved MTT reagent was added to 20 μl each of AL and BAL by using PBS, which were then cultured again for 4 hrs. Then, the MTT reagent and all the media, including other experimental materials, were removed. One hundred μl of acid isopropanol (0.04-N HCl in iso-propanol) were added to each well and stirred for 30 mins. The extinction coefficient was measured at 570 nm by using an ELISA reader [8].

2.2.4. Measuring the anti-inflammatory effect

Ninety-six well plates were cultured for 24 hrs with a 100 μl suspension containing 5 × 10⁴/well RAW 264.7 cells. The medium was removed after cultivation. The AL and the BAL were processed until they reached concentrations of 20, 50, and 100 μg/ml each in a serumless medium; then, 100 ng/ml of LPS, an inflammatory reflex inducing factor, was introduced. The amount of NO produced was measured after a 24-h cultivation [9]. Fifty μl of cell culture liquid was compounded with Griess reagent, a mutual reaction was allowed for 10 mins, and then the extinction coefficient was measured at 570 nm by using an ELISA reader. As a positive control group, NMMA, an inhibitor of NO production, was processed until an amount of 50 uM was obtained in the same way as was done with the other experimental materials, so comparing the inhibitory effect on the NO production was possible.

2.2.5. Measuring the anti-oxidant effect

With the reducing property of DPPH, the DPPH radical scavenging effects of AL and BAL were measured. DPPH was dissolved in methanol and 1 ml of the 0.1 mM DPPH solution was mixed with 1 ml each of AL and BAL taken from the 20, 50, and 100 μg/ml preparations. The extinction coefficient was measured at 565 nm after allowing a chemical reaction for 10 mins at room temperature [10].

2.2.6. Statistical analysis

With SPSS 17.0 for Windows, experimental results were recorded in terms of averages ± standard deviations; the level of significance was p < 0.05. Statistical significance among experimental groups was confirmed by using a one-way ANOVA test, and a subsequent analysis was conducted based on Duncan's multiple comparison test.

3. Experimental results

3.1. Cytotoxicity

This study showed no cytotoxicity for 20 and 50 μg/ml AL. The cell survival rate was 89% in 100 μg/ml AL, 87% in 20 μg/ml BAL, and 82% in 50 μg/ml BAL, while it was 75% in 100 μg/ml BAL (Table 1).

Table 1 The cytotoxicities of AL and BAL extracts for HaCaT cells, found by using MTT assays

Group	Average viability [%]	D	p-value	
Control	100.0 ± 6.8	a	< 0.01	
AL	20 μg/ml	97.0 ± 3.3		a
	50 μg/ml	94.0 ± 4.2		a,b
	100 μg/ml	89.0 ± 3.1		b,c
BAL	20 μg/ml	87.0 ± 2.5		b,c
	50 μg/ml	82.0 ± 2.0		C
	100 μg/ml	75.0 ± 1.6	D	

Statistical significance was tested by using a one-way ANOVA test among

groups.

D: The same letters indicate non-significant differences among groups based on Duncan's multiple comparison test. AL: *Alum*. BAL: *Burnt Alum*.

3.2. Anti-oxidant effect

According to the DPPH radical scavenging experiments for AL and BAL, the concentration-dependent scavenging efficacies were shown to be 12.0 ± 6.0 for $20 \mu\text{g}/\text{mL}$, 14.0 ± 5.2 for $50 \mu\text{g}/\text{mL}$, and 17.0 ± 4.8 for $100 \mu\text{g}/\text{mL}$ in the case of AL. However, no meaningful differences based on concentrations were found. In the case of BAL, the scavenging efficacies were shown to be concentration-dependent: 2.0 ± 0.5 for $20 \mu\text{g}/\text{mL}$, 3.0 ± 0.6 for $50 \mu\text{g}/\text{mL}$, and 4.0 ± 0.2 for $100 \mu\text{g}/\text{mL}$ (Table 2).

Table 2 Scavenging efficacies of AL and BAL on DPPH radicals

Group		Average viability (%)	D	p-value
Quercetin	$20 \mu\text{g}/\text{mL}$	43.0 ± 2.2	a	< 0.01
AL	$20 \mu\text{g}/\text{mL}$	12.0 ± 6.0	b	
	$50 \mu\text{g}/\text{mL}$	14.0 ± 5.2	b	
	$100 \mu\text{g}/\text{mL}$	17.0 ± 4.8	b	
BAL	$20 \mu\text{g}/\text{mL}$	2.0 ± 0.5	d	
	$50 \mu\text{g}/\text{mL}$	3.0 ± 0.6	d	
	$100 \mu\text{g}/\text{mL}$	4.0 ± 0.2	C	

Statistical significance was tested by using the one-way ANOVA test among groups.

D: The same letters indicate non-significant differences among groups based on Duncan's multiple comparison test. AL: *Alum*. BAL: *Burnt Alum*.

3.3. Anti-inflammatory effect

According to the experiments on the inhibitory effects of AL and BAL on NO production conducted on LPS-induced RAW 264.7 cells, no anti-inflammatory effects were observed for 20 and $50 \mu\text{g}/\text{mL}$ extracts of AL, but a 2.0 ± 0.2 degree of anti-inflammatory effect was found for the $100 \mu\text{g}/\text{mL}$ extract. In the case of BAL, a significant concentration-dependent anti-inflammatory effect was seen: 11.0 ± 3.0 for $20 \mu\text{g}/\text{mL}$, 33.0 ± 1.1 for $50 \mu\text{g}/\text{mL}$, and 41.0 ± 4.5 for $100 \mu\text{g}/\text{mL}$ (Table 3).

Table 3 Inhibitory efficacies of AL and BAL on NO production in LPS-induced macrophage RAW 264.7 cells

Group		Average viability (%)	D	p-value
NMMA	$50 \mu\text{M}$	50.0 ± 2.2	a	< 0.01
AL	$20 \mu\text{g}/\text{mL}$	-	e	
	$50 \mu\text{g}/\text{mL}$	-	e	
	$100 \mu\text{g}/\text{mL}$	2.0 ± 0.2	e	
BAL	$20 \mu\text{g}/\text{mL}$	11.0 ± 3.0	d	
	$50 \mu\text{g}/\text{mL}$	33.0 ± 1.1	c	
	$100 \mu\text{g}/\text{mL}$	41.0 ± 4.5	b	

Statistical significance was tested by using a one-way ANOVA test among groups.

D: The same letters indicate non-significant differences among groups based on Duncan's multiple comparison test. NMMA: N^o-methyl-L-arginineacetatesalt. - : No inhibition. AL: *Alum*. BAL: *Burnt Alum*.

4. Discussion

Many herbal medicines have been clinically administered through a unique form of processing called "Xi Zhì," which eliminates toxicity, softens the nature of the medicines, and broadens the range of treatment so that it can maximize the therapeutic effects. Relevant documentary records and methods have been scientifically proven already. Especially, mineral medicines have frequently been processed because of their solid nature, which would otherwise make them unavailable. The most typical processing method for mineral medicines is "DuànFǎ (burning)," which generally burns medicines directly or in a heat-durable container so that the chemical structures break down and become soft and suitable for grinding and boiling, thereby reducing side effects and enhancing therapeutic effects [1].

AL belongs to the group of astringent medicines and is a refined crystal processed from alunite $\text{KAl}_3(\text{SO}_4)_2(\text{OH})_6$, which has antibiotic, anti-trichomonas, and astringent effects. Most of time, it is used externally only because it is highly stimulative if taken internally, except for cases of lead colic [1,11]. Clinically, AL can be used both internally and externally, but it has always been burned before external use. However, it must not be taken internally at doses of over 3 g a day, because of its highly poisonous ingredient, $\text{KAl}(\text{SO}_4)_2$. Therefore, unless otherwise required, it should be replaced with BAL [2].

However, a review of theses on AL, such as "Application of BAL on a patient with cervical erosion" [4], "Application of AL on sinusitis and stomatitis" [5,6], "Oral administration of BAL on white mice with experimentally induced ulcer" [7], and so forth, except for the first thesis mentioned above, shows that all the studies were conducted contrary to the usage guidelines for AL and BAL originally written in the textbook.

Restraint of microbial propagation [12], increases of therapeutic effectiveness [13], changes of index elements [14,15], elimination of toxicity, and the availability of herbs [16] have been experimentally proven to exist for processed medicines such as *Cervus nippon*, *Rheum rhabarbarum* L, *Cinnamomum verum* J. Presl, *Pyrite*, *Magnolia officinalis*, and so forth. Consequently, it is conceivable that many herbal medicines can be more efficiently utilized after appropriate processing. The purpose of this study was to make AL and BAL, which have been commonly used as external treatments, more clinically useful by comparing their anti-oxidant and anti-inflammatory effects.

Prior to the overall experiment, no cytotoxicity of HaCaT cells was found for 20 and $50 \mu\text{g}/\text{mL}$ AL. The cell survival rates were 89% in $100 \mu\text{g}/\text{mL}$ AL, 87% in $20 \mu\text{g}/\text{mL}$ BAL, 82% in $50 \mu\text{g}/\text{mL}$ BAL, and 75% in $100 \mu\text{g}/\text{mL}$ BAL. Thus, BAL had a higher, more concentration-dependent cytotoxicity than AL.

Active oxygen is a term applied to oxygen containing radicals produced by the breakdown of chemical compounds, and it has been reported to cause oxidation reactions on cell membranes or DNA based on its high reactivity. However, it is considered to be an important factor in the research on herbal medicines because it plays essential roles in the immune system related to the anti-inflammatory reflex.

DPPH is a stable free radical that can be reduced to amino acids such as cysteine, glutathione, ascorbic acid, aromatic amine, etc. and that can be utilized for measuring anti-oxidant effects [17]. According to the DPPH radical scavenging experiment for AL, the concentration-dependent scavenging efficacies were shown to be 12.0 ± 6.0 for $20 \mu\text{g}/\text{mL}$, 14.0 ± 5.2 for $50 \mu\text{g}/\text{mL}$, and 17.0 ± 4.8 for $100 \mu\text{g}/\text{mL}$ in the case of AL, but the differences by concentration were not statistically meaningful. In the case of BAL, the scavenging efficacies were shown to be concentration-dependent: 2.0 ± 0.5 for $20 \mu\text{g}/\text{mL}$, 3.0 ± 0.6 for $50 \mu\text{g}/\text{mL}$, and 4.0 ± 0.2 for $100 \mu\text{g}/\text{mL}$. A significant difference was found for

100 $\mu\text{g}/\text{mL}$. AL showed superior effectiveness in terms of anti-oxidant efficacy.

Inflammation is a self-protective attempt by an organism to remove the injurious stimuli. It is a complicated process that is usually triggered by many chemical factors in connective tissues. The amount of iNOS is generally increased by injurious stimuli, such as LPS and cytokine, and bacterial toxin also transforms L-arginine into L-citrulline in order to produce NO, which is a highly reactive free radical, a large amount of which triggers an inflammation reflex. According to the experiment on the inhibitory effects of AL and BAL on NO production conducted on LPS-induced RAW 264.7 cells, no anti-inflammatory effects were seen in the cases of 20 and 50 $\mu\text{g}/\text{mL}$ AL, but a slight 2.0 ± 0.2 anti-inflammatory effect was found in the case of 100 $\mu\text{g}/\text{mL}$ AL. In the case of BAL, a significant concentration-dependent anti-inflammatory effect was seen: 11.0 ± 3.0 for 20 $\mu\text{g}/\text{mL}$, 33.0 ± 1.1 for 50 $\mu\text{g}/\text{mL}$, and 41.0 ± 4.5 for 100 $\mu\text{g}/\text{mL}$. Therefore, BAL has a superior anti-inflammatory effect as compared with AL.

Overall, AL is superior in its anti-oxidant effect, while BAL is superior in its anti-inflammatory effect. Therefore, for efficient external application, it is necessary to differentiate between them.

5. Conclusion

The conclusions drawn from this study are as follows:

1. BAL showed a higher level of cytotoxicity than AL.
2. AL groups showed a concentration-dependent scavenging effect on DPPH radicals, but no significant relevance was found. BAL groups showed a concentration-dependent scavenging effect on DPPH radicals. The scavenging effects of the BAL groups were almost insignificant, but the values for the 20, 50, and 100 $\mu\text{g}/\text{mL}$ trials were different.
3. BAL groups showed meaningful concentration-dependent inhibitory effects on NO production, but AL groups did not.

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