

Lorafocin, a mixture of Eunkyosan and alginate attenuate LPS-induced inflammation in RAW264.7 cells and inhibit growth of infectious bacteria

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ABSTRACT

Influenza is an infectious disease which accompanies systematic inflammation. Although eliminating the primary or secondary infectious agent is an important action in treating influenza, it is still difficult because not only mutations of viruses but also antibiotics-resistance of bacteria. Eunkyosan, is traditionally used herbal formula to symptoms of influenza, have were reported for its pharmacological properties on inflammation. Also, Alginate, a physiologically active acidic polysaccharide obtained from brown algae, alleviates inflammation, exhibits anti-bacterial properties, and shows a collaborative therapeutic effect with an anti-bacterial agent for acute infections. Thus, we investigated anti-inflammatory effects of Lorafocin (a mixture of Eunkyosan and alginate) against lipopolysaccharide (LPS)-induced inflammation in RAW 264.7 macrophage cell line and its underlying mechanisms as well as anti-bacterial effects. In the RAW 264.7 macrophage cell line, Lorafocin treatment reduced the secretion of inflammatory mediators such as nitric oxide (NO) and pro-inflammatory cytokines induced by LPS. To elucidate the anti-inflammatory mechanism of Lorafocin, we investigated the involvement of the mitogen-activated protein kinase (MAPK) and protein kinase B (Akt) signaling pathway. Studies demonstrated that Lorafocin exhibited anti-inflammatory efficacy by regulating Akt, c-Jun N-terminal kinase/stress-activated protein kinase (JNK), and extracellular signal-regulated kinase (ERK) 1/2 in LPS-induced RAW264.7 macrophages. Furthermore, Lorafocin inhibited the bacterial growth of *Staphylococcus aureus* and *Escherichia coli*. Taken together, Lorafocin may possess the capability to effectively modulate inflammatory responses and inhibit the growth of pathogenic bacteria.

Keywords Lorafocin, Eunkyosan, Alginate, Anti-inflammation, Anti-bacteria

INTRODUCTION

Influenza is an infectious disease which accompanying symptoms such as inflammation, pneumonia, high fever, headaches, coughing, fatigue, and muscle aches. Worldwide, influenza patients be annually occurred to 300 to 600 million people, and 1% of them develop severe phase which can lead to complications or death.¹

Viruses are the most important factor in infection of influenza, and they weaken the epithelial barrier function that protects the body from external stimuli and harmful substances, increasing the risk of secondary infection.² Particularly,

respiratory immune system is vulnerable to bacterial infections such as *Staphylococcus aureus*. Respiratory infectious pneumonia caused by bacterial infections has increased the mortality rate of immunocompromised infants, the elderly, and patients.^{3,4}

In treating influenza, eliminating the primary or secondary infectious agent is one of the most important actions. However, developing therapeutics such as vaccines and antibiotics for influenza is still difficult because not only viruses which mutation of antigen frequently occur but also resistance of bacteria on antibiotics.⁵ In addition, the use of some anti-inflammatory drugs for symptom relief has been recently reported to have adverse effects such as hypertension, gastrointestinal issues, kidney, and heart problems.⁶ Therefore, developing natural agents is required for high-efficacy and low-adverse effects for treatments of influenza.

Eunkyosan is an herbal formula composed of *Lonicerae Flos*, *Forsythiae Fructus*, *Menthae Herba*, *Platycodi Radix*,

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Glycyrrhizae Radix, Lophatheri Herba, Schizonepetae Herba, Steamed beans with salt, Arctii Fructus and Saigae Tataricae Cornu. It has been traditionally used for bronchitis, upper respiratory infections, various inflammatory diseases, and respiratory infections in clinic.⁷⁻⁹ Additionally, many studies have reported for pharmacological properties of Eunkyosan components against inflammation, oxidative stress, infectious bacteria and virus infection.¹⁰⁻¹³ Alginate, a physiologically active acidic polysaccharide obtained from brown algae, has also been reported to alleviate inflammation.^{14,15} Specifically, Alginate showed effective antibacterial activity^{16,17}, with a synergistic therapeutic effect from an antibacterial agent for acute infection.¹⁷

In this study, we investigated anti-inflammatory effects of Lorafocin (a mixture of Eunkyosan and alginate) against lipopolysaccharide (LPS)-induced inflammation in RAW 264.7 macrophage cell line and its underlying mechanisms as well as anti-bacterial effects and *Escherichia coli* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Preparation of Lorafocin

Eunkyosan (mixture of ten herbal materials) and alginate were provided from the JBKLAB (Seongnam-si, Korea). To manufacture Lorafocin, the powder of Eunkyosan was mixed with alginate in the optimal ratio. Next, Lorafocin was dissolved in phosphate buffered saline (PBS) or complete media.

Cell culture

The murine macrophage cell line RAW 264.7 was obtained from the Korea Cell Line Bank located in Seoul, Korea. The cells were maintained at a temperature of 37°C within a 5% CO₂ incubator, utilizing complete media composed of Dulbecco's Modified Eagle's Medium (DMEM, Welgene, Seoul, Korea) supplemented with 10% fetal bovine serum (FBS, Welgene, Seoul, Korea) and 100 µg/mL of penicillin and streptomycin (Sigma-Aldrich, MO, USA).

Cell viability assay

Cell viability was determined using WST-8 kit (Biomax Inc., Seoul, Korea). A total 2x10⁴ of RAW 264.7 cells were inoculated into a 96-well plate. Next day, the cells were treated with Lorafocin (50 to 1000 µg/mL) for 24 hours in incubator. After 24 hours, the cells were treated with WST-8 solution for 2 hours and then absorbance at a wavelength of 450 nanometers was recorded utilizing a microplate reader (Thermo Scientific, Waltham, MA, USA).

NO and inflammatory cytokines production assay

RAW 264.7 cells were cultured at a density of 5x10⁴ cells per well for the purpose of nitric oxide (NO) detection, while a density of 2x10⁵ cells per well was utilized for the assessment of

interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) in 48-well plates, respectively. After cells were treated Lorafocin (10 to 1000 µg/mL) for 1 hour, LPS (1 µg/mL) were added and incubated for a total of 24 hours. After centrifuge, the supernatants were used to measure levels of NO, IL-6 and TNF-α. NO detection kit and IL-6 and TNF-α EISA kit purchased from iNtRON BioTechnology (Korea) and Invitrogen (USA), respectively.

Western blot analysis

8x10⁵ of RAW 264.7 cells were seeded in 60-mm dish and incubated at 37°C for 24 hours. After cells were treated Lorafocin (50, 250, 500 or 1000 µg/mL) for 1 hour, LPS (1 µg/mL) were added and incubated for a total of 0.5 or 24 hours. The cells were collected utilizing RIPA buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride, as well as a combination of protease and phosphatase inhibitor cocktail set III (Sigma-Aldrich). Proteins were subjected to electrophoresis utilizing 8-10% SDS-PAGE, followed by transfer to polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ, USA). Subsequently, the membranes were incubated with a blocking solution consisting of 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBS/T buffer) for a duration of 1 hour. The membrane was incubated with specific antibodies against inducible nitric oxide synthase (iNOS), Cyclooxygenase 2 (COX-2), c-jun N-terminal kinase (JNK), phosphate(p)-JNK, protein 38 (p38), p-p38 extracellular signal-regulated kinase (ERK), p-ERK, protein kinase B (Akt), p-Akt (Cell Signaling Technology, Beverly, Ma, USA). The blots were conjugated with a horseradish peroxidase (HRP)-linked secondary antibody. Subsequently, the blots were developed utilizing an enhanced chemiluminescent (ECL) western blotting detection reagent obtained from Amersham Biosciences. The blot presented are representative in two independent experiments and semi-quantified using Image J software. The values of semi-quantification were presented by mean±SD.

Anti-bacterial test

Anti-bacterial test was performed by Allpassbio (Daegu, Korea). Briefly, *Staphylococcus aureus* and *Escherichia coli* (ATCC, USA) were mixed with peptone water (as a control) or Lorafocin (10 or 100 mg/mL) in a shaker (150 rpm, room temperature). After 24 hours, the mixtures were centrifuged at 13000 rpm. And then, supernatants were removed, and the pellets were washed in phosphate buffered saline (PBS). The pellets were diluted in PBS step by step and inoculated in bacterial media. Anti-bacterial activity was calculated by counting colony-forming unit (CFU).

Statistical analysis

All results are expressed as the mean ± standard deviation (SD). Group differences were assessed using one-way analysis of variance (ANOVA). Subsequent to the initial analysis, post hoc multiple comparisons for each group using Tukey's Honestly Significant Difference (HSD) test, performed with

Prism version 7.0. (GraphPad, CA, USA). Statistical significance is expressed as follows by $###p < 0.001$ for vehicle vs. LPS only groups, and $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ for LPS vs. Lorafocin group

RESULT

Cytotoxicity of Lorafocin in RAW264.7

To verify the cytotoxicity of Lorafocin, RAW264.7 cells were treated with Lorafocin at various concentrations, and cell viability was measured after 24 hours. Lorafocin did not cause any cytotoxicity in the concentration range of 50-1000 $\mu\text{g/ml}$. Therefore, subsequent experiments were conducted up to a concentration of 1000 $\mu\text{g/ml}$. (Table 1).

Table 1. Cytotoxicity of Lorafocin in RAW264.7 cells.

Dose ($\mu\text{g/ml}$)	Lorafocin
0	100.00 \pm 1.55
50	110.25 \pm 7.43
250	131.31 \pm 6.01 ^{***}
500	132.09 \pm 4.03 ^{***}
1000	126.17 \pm 3.53 ^{***}

RAW264.7 cells were treated with Lorafocin (0 to 1000 $\mu\text{g/ml}$) for 24 hours. Cell viabilities were assessed using WST-8 assay. Values are expression as mean \pm SD triplicate determinations. ^{***} $p < 0.001$ vs. vehicle group.

Anti-proinflammatory effects of Lorafocin on NO, IL-6 and TNF- α in LPS-induced RAW264.7

NO production, one of the major inflammatory mediators, became dramatic in LPS-induced RAW264.7 cells. While NO production was significantly decreased by Lorafocin treatment and was significantly reduced by approximately 29% and 59% reduction at 500 and 1000 $\mu\text{g/ml}$ concentration, respectively ($p < 0.001$ for 500 and 1000 $\mu\text{g/ml}$, Fig. 1A).

The effects of Lorafocin on pro-inflammatory cytokine production in LPS-induced RAW 264.7 cells are shown in Fig. 1B and C. The treatment of LPS dramatically elevated levels of IL-6 and TNF- α ($p < 0.001$). The treatment of Lorafocin attenuated elevated level of IL-6 ($p < 0.001$ for 500 and 1000 $\mu\text{g/ml}$, Fig. 1B) and TNF- α ($p < 0.01$ or 0.001 for 250 to 1000 $\mu\text{g/ml}$, Fig. 1C).

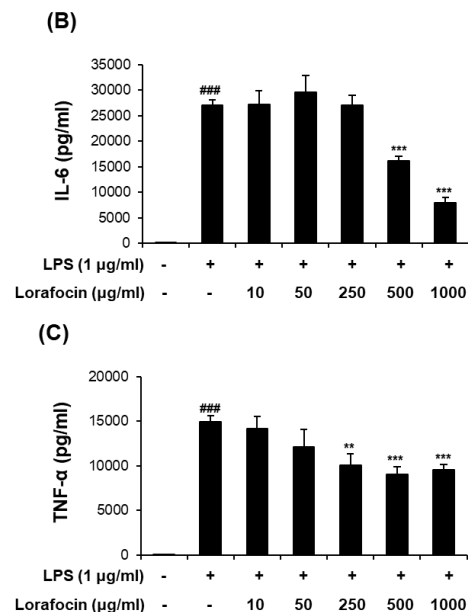
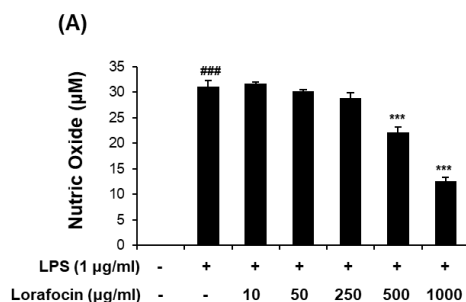
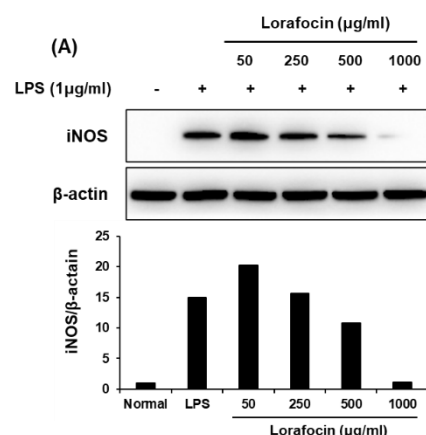


Fig. 1. Effects of Lorafocin on NO, IL-6 and TNF- α production in lipopolysaccharide (LPS)- induced RAW 264.7 cells. Cells were treated LPS (1 $\mu\text{g/ml}$) in the absence or presence of Lorafocin at different concentrations for 24 hours. The level of (A) NO, (B) IL-6, (C) TNF- α in the cell culture supernatant were measured by NO, IL-6, TNF- α detection kit. Values shown in the graphs are mean \pm SD of triplicate determinations. Statistical significance was analyzed by one-way ANOVA test. ^{###} $p < 0.001$ vs. vehicle-treated group. ^{**} $p < 0.01$, ^{***} $p < 0.001$ vs. LPS-treated group.

Inhibitory effects of Lorafocin on iNOS and COX-2 in LPS-induced RAW264.7

The effects of Lorafocin on iNOS and COX-2 protein expression, which mediate the synthesis of NO and prostaglandin E2 (PGE2), were investigated by Western blot. As shown in Fig. 2, LPS remarkably 15- and 25-fold increased protein levels of iNOS and COX-2, respectively. The treatment with Lorafocin significantly suppressed protein expression of iNOS ($p < 0.001$ for 500 and 1000 $\mu\text{g/ml}$, Fig. 2A), but not COX-2 ($p > 0.05$, Fig. 2B).



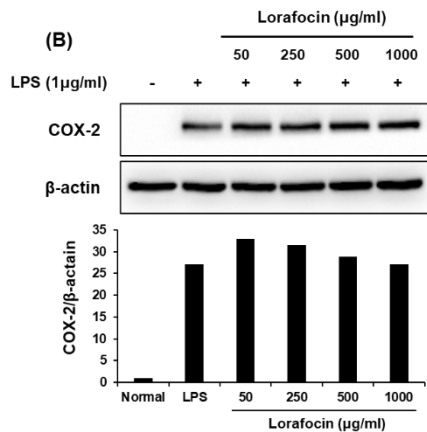


Fig. 2. Effects of Lorafocin on iNOS and COX-2 expression in lipopolysaccharide (LPS)- induced RAW 264.7 cells. Cells were treated with LPS (1 μg/ml) in the absence or presence of Lorafocin at different concentrations for 24 hours. Protein expression of (A) iNOS and (B) COX-2 were determined by western blot analysis. β-actin was used as loading control.

Regulatory effects of Lorafocin on Akt and MAPK signaling pathway in LPS-induced RAW264.7

To verify underlying mechanisms on inflammation, we confirmed the molecular signal pathway including phosphorylation of Akt and mitogen activated protein kinase (MAPK). The treatment of LPS-induced phosphorylation of Akt, JNK, ERK, p38. The phosphorylation of Akt (Fig. 3A), ERK (Fig. 3B) and JNK (Fig. 3D) were inhibited by Lorafocin but did not affect the phosphorylation of p38 (Fig. 3C).

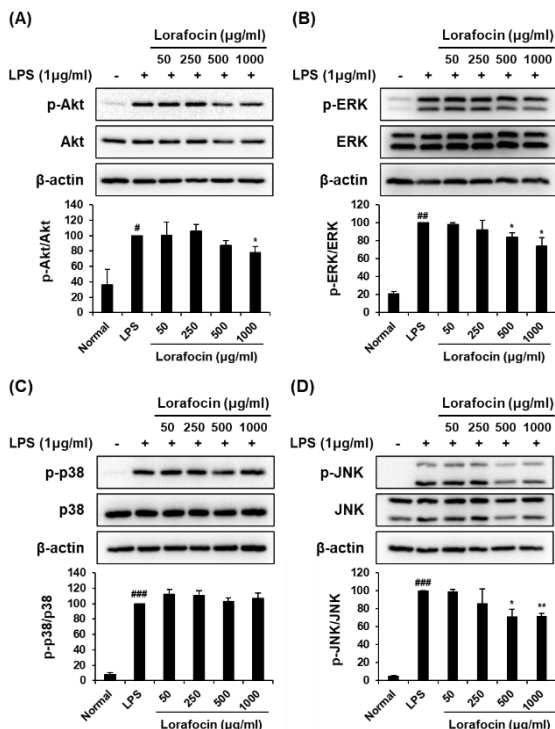


Fig. 3. Effect of Lorafocin on regulation of Akt and MAPK signaling pathways. Cells were treated with LPS (1 μg/ml) in the absence or presence of Lorafocin at different concentrations for 0.5 hours. The phosphorylation of (A) Akt, (B) ERK, (C) p38 and (D) JNK were determined by western blot analysis. β-actin was used as loading control.

Anti-bacterial effects of Lorafocin on *Staphylococcus aureus* and *Escherichia coli*

We incubated two bacterial strains (*Staphylococcus aureus* and *Escherichia coli*) with Lorafocin (10 or 100 mg/mL). The Lorafocin inhibited growth of *Staphylococcus aureus* (65% for 10 mg/mL, 99% for 100 mg/mL) and *Escherichia coli* (99% for 100 mg/mL, Fig. 4).

Sample	Initial inoculum (CFU/mL)	Concentration	Counts after 24hours (CFU/mL)	Inhibition (%)
<i>Staphylococcus aureus</i> ATCC 6538	1.98 x 10 ⁴	10 mg/ml	7.00 x 10 ³	64.7%
		100 mg/ml	4.5	99.9%
<i>Escherichia coli</i> ATCC 10536	2.06 x 10 ⁴	10 mg/ml	4.30 x 10 ⁷	-
		100 mg/ml	ND (No detection)	99.9%

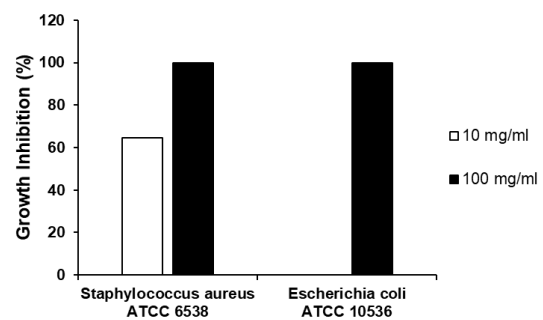


Fig. 4. Effects of Lorafocin on the growth of bacteria. *Staphylococcus aureus* and *Escherichia coli* were incubated with 10 or 100 mg/mL of Lorafocin for 24 hours. The growth inhibition rate was calculated by counting CFU.

DISCUSSION

Humans and microbes have evolved to symbiosis for tens of thousands of years. Among the multitude of microbes that inhabit the earth most microbes are helpful to human metabolism, but there are still concerns about protecting our bodies from many infectious microbes containing pathogens or allergens.¹⁸ Several species of bacteria such as *Staphylococcus aureus* and *pneumoniae* and *Escherichia coli* were classified with harmful bacteria to produce diseases accompanying immune response and inflammation.¹⁹ Thus, it is important to protect our bodies against infection by harmful bacteria or to quickly get rid of them if we do get infected. Based on the above facts, we first investigated the inhibitory effects of Lorafocin on the growth of harmful bacteria. (*Staphylococcus aureus* and *Escherichia coli*, Fig. 4).

The immune system exists to protect the host against pathogenic and allergenic bacteria. There are two major types of responses to bacteria, called innate and adaptive immune systems.²⁰ Innate immune systems continuously respond to infectious conditions using phagocytic cells (neutrophils, monocytes, and macrophages) that release inflammatory mediators (basophils, mast cells, and eosinophils), and natural killer cells. In the present study, we adapted LPS-induced RAW264.7 models which are widely used to evaluate anti-

inflammatory effects. LPS-induced macrophages are activated and release various proinflammatory factors such as NO, COX-2, TNF- α , IL-1 β and IL-6. Excessive inflammation extends to immune response which results in inflammatory cascade and tissue injury.²¹⁻²⁵ Thus, suppressing release of these proinflammatory factors is an important strategy to treat infectious inflammation.²⁶ In our data, we confirmed that Lorafocin suppressed these factors (NO, IL-6, TNF- α) increased by LPS in RAW264.6 macrophage (Fig. 1A to C).

NO is a free radical oxidant with short-term half-life and plays role as proinflammatory mediator in inflammation manner produced by iNOS.²⁷ Besides, the overproduction of NO leading to various systematic inflammation diseases including, rheumatoid arthritis, autoimmune and allograft rejection.²⁸⁻³⁰ In our present study, Lorafocin showed reducible effects of protein levels of NO and iNOS in LPS- induced RAW264.7 macrophage while it did not reduce the protein level of COX-2 (Fig. 1A and 2A and 2B). According to previously reported studies, iNOS and COX-2 partially share molecular mechanisms for the suppression of inflammation.^{31,32} However, some studies have shown that the suppression of either iNOS or COX-2 alone results in anti-inflammatory effects, suggesting that both genes are not necessarily required for inflammation suppression.^{33,34} The anti-inflammatory efficacy of Lorafocin is thought to be due to the inhibition of NO production and pro-inflammatory cytokines associated with the inflammatory response, primarily through the inhibition of iNOS expression rather than COX-2 expression.

Interaction between LPS and macrophage results in the transduction of intracellular signaling through MyD88 and TRIF pathways, leading to MAPK cascades and translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) though Akt signaling pathway.³⁵⁻³⁸ MAPKs, mainly consist of three sub-families with p38, ERK and JNK are serine/threonine-specific protein kinases, and many studies have well reported that these activation produce inflammatory mediators.^{39,40} Additionally, Akt signaling pathway also induce production of inflammatory mediators in response to LPS.⁴¹⁻⁴³ Interestingly, our data indicated that Lorafocin regulated the phosphorylation of Akt, ERK and JNK, but not p38 (Fig. 3A to D).

In summary, our findings demonstrated that Lorafocin exerts anti-inflammatory effects by modulating inflammatory mediators (NO, IL-6, TNF- α , iNOS) in LPS-induced RAW264.7 macrophages. Furthermore, the underlying mechanisms of these effects may involve the regulation of critical signaling pathways, including Akt, JNK, and ERK pathways. These results suggest that Lorafocin has the potential to effectively regulate inflammatory responses and suppress the proliferation of pathogenic bacteria.

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CONFLICT OF INTEREST

The authors have no conflicting financial interests.

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