Phytonecide Extracted from Pinecone Decreases LPS-Induced Inflammatory Responses in Bovine Mammary Epithelial Cells

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Introduction

Bovine mastitis is known to be associated with decreased production and poor quality of milk [33]. Bovine mastitis is caused by many factors, such as microbial infections. In particular, the gram-negative bacterium Escherichia coli causes inflammatory responses in the epithelial cells of bovine mammary glands [37]. During the ensuing inflammatory response, mammary epithelial cells produce pro-inflammatory mediators, such as interleukin (IL)-8, IL-1β, tumor necrosis factor-α, and transforming growth factor β1 [15, 25].

Lipopolysaccharide (LPS) is a component of the outer membrane of gram-negative bacteria such as E. coli and is a major cause of the inflammatory response related to mastitis [26]. In cells, LPS binds Toll-like receptor 4 (TLR4) and activates various intracellular signaling pathways including mitogen-activated protein kinases (MAPK; e.g., extracellular signal receptor-activated kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase) and nuclear factor-kappa B (NF-κB) [21, 29, 31]. MAPKs are mediators of cellular inflammation mainly through activation of NF-κB, and this process can result in upregulation of cyclooxygenase-2 (COX-2) expression [35]. In addition, upon binding of LPS to TLR4, NF-κB is activated and translocates to the nucleus, and in turn activates target genes, such as COX-2 [13, 23]. Another downstream target of the LPS-induced inflammatory signaling molecule is the Akt pathway. Upon stimulation of cells with LPS, TLR4 signaling pathways activate Akt

Keywords: Mastitis, lipopolysaccharide, MAC-T cell, inflammation, COX-2
and NF-κB [36]. Thus, activation of MAPKs, Akt, and NF-κB is important as the signaling components in mastitis [17].

Phytoncides are organic volatile compounds emitted from trees and plants mainly as a self-defense mechanism to protect against insects, microorganisms, and animals [9]. Thus, phytoncide-like compounds have been employed in insect repellents, deodorants, and antibacterial agents. Some studies have shown that phytoncide compounds can attenuate cell damages caused by physical (UVA and UVB) and oxidative stress [5]. However, there is a limited number of publications regarding anti-inflammatory effects of phytoncide in mammalian cells. Recently, a study investigating a similar compound, Perilla leaf extract, showed that the phytoncide in mammalian cells. Recently, a study investigating phytoncide in MAC-T cells that were stimulated with LPS inflammatory effects. Thus, we determined the effect of phytoncide on the COX-2, ERK1/2, p38, Akt, NF-κB, and oxidative stress pathways were attenuated by the leaf extract in macrophage cells, RAW264.7 [11].

In the present study, we hypothesized that a phytoncide extract can modulate inflammatory responses in bovine mammary epithelial cells (MAC-T), due to its anti-inflammatory effects. Thus, we determined the effect of phytoncide in MAC-T cells that were stimulated with LPS in order to simulate a mastitis-like condition. To determine the cellular mechanism of protection, the COX-2, ERK1/2, p38, Akt, NF-κB, and oxidative stress pathways were investigated in cells treated with phytoncide and LPS.

**Materials and Methods**

**Materials**

 Dulbecco’s modified Eagle’s medium (DMEM/high glucose) was obtained from Thermo Scientific (Rockford, IL, USA). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin/streptomycin, and trypsin were purchased from Gibco (Grand Island, NY, USA). Progesterone, insulin, and LPS from in E.coli O111:B4 were purchased from Sigma Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). Antibodies for COX-2, p65, Nrf2, heme oxygenase-1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), lamin B, goat anti-rabbit IgG-HRP, and donkey anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for p-Akt, p38, Akt, and p-p38 were obtained from Cell Signaling Technology (Danvers, MA, USA). Dihydroethidium (DHE) was purchased from Invitrogen (Carlsbad, CA, USA). Inhibitors such as PD98059, LY294002, and SB203580 were purchased from Santa Cruz Biotechnology.

**Extraction of Phytoncide**

Phytoncide solution was provided from Phylus Corporation (Danyang, Korea). Phytoncide solution was extracted from pinecone from nut pine trees using a steam distillation process. Briefly, after removing the pine nuts, the pinecones were cut in 2–3 cm size and 100 g of pinecones was added into a 1 L round flask. The pinecones were mixed with 500 ml of distilled water for 1–2 min, and then subjected to a steam distillation process (100 ± 3°C, 3 h). After cooling the extract (20°C), the pinecone extract (phytoncide essential oil) was separated from water using the difference in specific gravity. Then, the extract was filtrated and concentrated (60°C, 100 mHg). Finally, the extract was sealed and stored at 4°C. To identify the chemical composition of the pinecone extract, GC-MS analysis was completed and a total of 44 individual compounds were determined. The composition of phytoncide extracted from pinecone is shown in Table 1.

**Cell Viability Assay for Phytoncide and LPS**

Cell viability was determined using the MTT assay. Cells seeded in a 96-well plate were treated with phytoncide (0%, 0.01%, 0.02%, 0.04%, 0.08%, and 0.16% (v/v)) for 24 h or LPS (0, 1, and 25 µg/ml) for 12 h. Next, 10 µl of MTT solution (5 mg/ml in PBS) was added to each well and then incubated for 4 h. Acidic isopropanol was added to dissolve any deposited formazan. The optical density was measured at 540 nm with a spectrophotometer (Biotek Instruments, Winooski, VT, USA).

**Cell Culture and Treatments**

The bovine mammary epithelial cells (MAC-T) were a gift from Prof. Hong Gu Lee (Konkuk University, Seoul, Korea). Cells were maintained in DMEM supplemented with 10% FBS, penicillin/streptomycin, 5 µg/ml insulin, and 1 µg/ml progesterone at 37°C in a humidified atmosphere containing 5% CO2. Cells were grown to about 90% confluence and synchronized overnight in medium containing 1% FBS before initiation of cell treatments. The cells were treated with phytoncide at concentrations of 0%, 0.02%, and 0.04% (v/v) for 6 h followed by LPS treatment. Concentrations of phytoncide were chosen based on the cell viability data showing cell protection without cell death. Cells were treated with LPS at concentrations of 0, 1, and 25 µg/ml for various time points depending on each experimental settings. LPS concentrations were chosen based on previous studies by others using mammary epithelial cells [18, 20]. In the experiments for testing the effects of pharmacological inhibitors, cells were pretreated with the inhibitors (PD98059, LY294002, and SB203580) for 20 and 40 µM for 1 h, followed by stimulation with LPS at 0, 1, and 25 µg/ml for 12 h.

**Preparation of Cell Lysate and Western Blot Analysis**

For collecting protein samples after treatments, cells were lysed in RIPA-buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (2 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM PMSE, 5 mM EDTA, 1 mM EGTA, 10 mM sodium fluoride, and 1 mM sodium orthovanadate). Lysed cells were centrifuged at 21,000 × g for 10 min at 4°C. Supernatants were collected and the protein concentration was analyzed using Bradford reagent (Sigma-Aldrich). The cell lysate protein samples
were stored at -80°C until use. For western blot analysis, protein samples (30 µg per treatment) were separated using 10% SDS-PAGE and subsequently were transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk buffer and incubated overnight at 4°C with 1:4,000 primary antibodies. After washing, the membranes were incubated with appropriate secondary antibodies conjugated with horseradish peroxidase at a concentration of 1:4,000 and visualized using ECL detection reagents (Lugen, Seoul, Korea).

### Assessment of Oxidative Stress (Superoxide Levels) in MAC-T Cells

MAC-T cells were grown to confluence on a cover glass in 6-well plates. Cells were treated with phytoncide (0.02% and 0.04% (v/v)) for 6 h followed by LPS treatment (1 µg/ml) for 4 h. Following treatments, the cells were incubated with a final concentration of 1 µM DHE or DMSO (blank) in a 5% CO<sub>2</sub> incubator for 30 min. The cells were washed 3× with PBS, fixed with 4% formaldehyde, and washed again 3× with PBS. The slides were mounted with ProLong Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) to visualize the nuclei. The slides were evaluated under an Olympus IX71 fluorescence microscope and images were captured digitally using an Olympus DP71 camera and DP controller software (Olympus Optical Co. Ltd, Tokyo, Japan). The treatment groups were 1) DMSO (DHE); 2) DMSO (no DHE); 3) LPS 1 µg/ml (DHE); 4) LPS 1 µg/ml (no DHE); 5) phytoncide 0.02% (v/v) (DHE); 6) phytoncide 0.02% (v/v) and LPS 1 µg/ml (DHE); 7) phytoncide 0.04% (v/v) (DHE); and 8) phytoncide 0.04% (v/v) and LPS 1 µg/ml (DHE).

### Nuclear Translocation of NF-κB and Nrf2 in MAC-T Cells

MAC-T cells were treated with phytoncide (0.02% and 0.04% (v/v)) for 6 h followed by LPS treatment (1 µg/ml) for 12 h or 1 h for NF-κB and Nrf2, respectively. For nuclear translocation assay, cells were lysed in hypotonic buffer solution (20 mM Tris (pH 7.4), 10 mM NaCl, and 3 mM MgCl<sub>2</sub>) containing protease inhibitor mixture (same inhibitor mixture as described above). After addition of 10% Triton-X 100, cell lysates were centrifuged at 650 × g for 10 min at 4°C. Pellets were resuspended in cell extraction buffer (100 mM Tris (pH 7.4), 100 mM NaCl, 1% Triton X-100, 10% glycerol, and 0.1% SDS) containing the protease inhibitor mixture. The homogenates were centrifuged at 14,000 × g for 30 min at 4°C and the supernatant was collected as the nuclear fraction. Aliquots of nuclear and cytosolic fractions were stored at -80°C until use. For the nuclear translocation assay, cells were treated with phytoncide (0.02% and 0.04% (v/v)) for 6 h followed by LPS treatment (1 µg/ml) for 4 h. Following treatments, the cells were incubated with a final concentration of 1 µM DHE or DMSO (blank) in a 5% CO<sub>2</sub> incubator for 30 min. The cells were washed 3× with PBS, fixed with 4% formaldehyde, and washed again 3× with PBS. The slides were mounted with ProLong Gold Antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) to visualize the nuclei. The slides were evaluated under an Olympus IX71 fluorescence microscope and images were captured digitally using an Olympus DP71 camera and DP controller software (Olympus Optical Co. Ltd, Tokyo, Japan). The treatment groups were 1) DMSO (DHE); 2) DMSO (no DHE); 3) LPS 1 µg/ml (DHE); 4) LPS 1 µg/ml (no DHE); 5) phytoncide 0.02% (v/v) (DHE); 6) phytoncide 0.02% (v/v) and LPS 1 µg/ml (DHE); 7) phytoncide 0.04% (v/v) (DHE); and 8) phytoncide 0.04% (v/v) and LPS 1 µg/ml (DHE).

### Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined by one-way or two-way ANOVA followed by the Student-Newman-Keuls method.
using Sigma Stat 3.1 software (Systat Software, San Jose, CA, USA). A probability value of $p < 0.05$ was considered statistically significant.

Results

Cytotoxicity of Phytoncide in MAC-T Cells

Results from MTT assay showed that cell viability was not significantly perturbed at concentrations up to 0.04% phytoncide, compared with the control (Fig. 1A). Higher concentrations of phytoncide (i.e., 0.08% and 0.16% (v/v)) markedly decreased cell viability, compared with the control (Fig. 1A). Thus, both 0.02% and 0.04% phytoncide were used in the subsequent experiments. In addition, cells treated with LPS showed decreased but not statistically significant cell viability compared with the control (Fig. 1B). Cell viability was about 80% of the control in cells treated with LPS ($p = 0.066$ and 0.059 for 1 and 25 µg/ml of LPS, respectively). These LPS concentrations were also chosen by others when treating mammary epithelial cells, owing to the capability of cell challenging without significant cell death [18, 20].

Phytoncide Decreases LPS-Induced COX-2 Expression and Activation of ERK1/2, p38, and Akt

Expression of COX-2 is an indicator of inflammatory responses in a variety of cells, including mammary epithelial cells [14]. In order to identify anti-inflammatory effects of phytoncide, cells were treated with phytoncide at concentrations of 0%, 0.02%, and 0.04% (v/v) for 6 h followed by LPS treatment at concentrations of 0, 1, and 25 µg/ml for 12 h. Western blot analysis showed that LPS (1 and 25 µg/ml) significantly increased protein expression of COX-2, compared with the control (Fig. 2A). Pretreatment of phytoncide (0.02% and 0.04% (v/v)) attenuated LPS-induced COX-2 expression in a dose-dependent manner (Fig. 2A). The housekeeping protein GAPDH was used as an internal control for equal protein loading (Fig. 2A). In order to identify intracellular signaling pathways associated with LPS exposure and the effects of phytoncide, the expression levels of multiple signaling proteins related to inflammatory responses were examined. The MAPK and PI3K/Akt signaling pathways are linked to a wide variety of inflammatory responses, including NF-κB activation and COX-2 expression [27]. MAC-T cells were treated with phytoncide for 6 h followed by LPS treatments. The treatment time for LPS was 0.5–1 h (0.5 h for ERK1/2 and p38, and 1 h for Akt). These time points were selected because early activation of these molecules occurred following the LPS exposure, according to our preliminary studies. Phosphorylation/activation of ERK1/2, p38, and Akt was determined by western blot analysis. Treatment of cells with LPS markedly increased phosphorylation of ERK1/2, p38, and Akt (Fig. 2B). Maximum phosphorylation of ERK1/2, p38, and Akt was observed at 0.5–1 h. However, pretreatment with phytoncide (0.02% and 0.04% (v/v)) significantly reduced the LPS-induced phosphorylation of ERK1/2, p38, and Akt (Fig. 2B), suggesting an anti-inflammatory effect of phytoncide.

Pharmacological Inhibitors for ERK1/2, p38, and Akt Decrease Expression of COX-2

To further substantiate the signaling pathways associated with LPS-induced inflammatory responses, the expression of COX-2 in cells treated with pharmacological inhibitors for ERK1/2 (PD98059), p38 (SB203680), and Akt (LY294002) was determined. Cells were pretreated with the inhibitors at 20 and 40 µM for 1 h, followed by stimulation with LPS at 1 and 25 µg/ml for 12 h. COX-2 expression was measured with cell lysates using SDS-PAGE and western blot analysis. Result showed that pretreatment of cells with inhibitors such as PD98059 (an ERK1/2 inhibitor) and
LY294002 (an Akt inhibitor) markedly decreased the LPS-induced COX-2 expression (Figs. 3A and 3B). Treatment of cells with 40 \( \mu \)M SB203680 (a p38 inhibitor) also decreased the LPS-induced COX-2 expression (Fig. 3C).

**Phytoncide Decreases LPS-Induced Oxidative Stress in MAC-T Cells**

Oxidative stress is a major event in cellular inflammatory processes, and reactive oxygen species, particularly, a superoxide anion, can oxidize the fluorescent dye DHE, which stains cells a bright fluorescent red [8]. Results showed that LPS (1 mg/ml) markedly increased superoxide

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**Fig. 2.** Phytoncide decreases LPS-induced COX-2 expression and phosphorylation of Erk, p38, and Akt in MAC-T cells. (A) To measure COX-2 protein expression, cells were pretreated with phytoncide for 6 h followed by LPS treatment for 12 h. Western blot analysis was used to measure protein expression in whole cell lysates. GAPDH was used as the loading control. Results represent the mean ± SEM. Experiments were repeated three times. Different letters indicate there is a significant difference between treatment groups (\( p < 0.05 \)). (B) To identify the phosphorylation of signaling molecules, cells were pretreated with phytoncide for 6 h followed by LPS treatment for 30 min (p-p38 and p-ERK1/2) or 1 h (p-Akt). Western blot analysis was used to measure phosphorylation of the signaling molecules. The western blots shown here are representative images of three independent experiments.

**Fig. 3.** Pharmacological inhibition of signaling molecules results in the decrease of COX-2 expression. MAC-T cells were pretreated with inhibitors for (A) ERK1/2 (PD98059), (B) Akt (LY294002), and (C) p38 (SB203580) at 20 and 40 \( \mu \)M, followed by stimulation with LPS at 1 and 25 \( \mu \)g/ml for 12 h. COX-2 expression was measured in whole cell lysates using western blot analysis. GAPDH was used as the loading control. Results represent the mean ± SEM. Experiments were repeated three times. Different letters indicate there is a significant difference between treatment groups (\( p < 0.05 \)).
production (Fig. 4) in MAC-T cells. LPS-induced superoxide production was attenuated in cells pretreated with phytoncide in a dose-dependent manner, suggesting a strong antioxidant effect of phytoncide in MAC-T cells (Fig. 4).

**Phytoncide Attenuates LPS-Induced NF-κB Activation and Induces Nrf2 Activation**

Phosphorylation of ERK1/2 and Akt can lead to activation of the redox-sensitive transcription factor NF-κB that subsequently increases expression of COX-2 [7, 21, 29]. To determine if treatment with phytoncide can activate NF-κB, nuclear translocation of NF-κB was observed. During the absence of stimulation, NF-κB stays in the cytosol as a heterodimer (p65, p50, and IκBα). However, once stimulated, the p65 subunit is released from the heterodimer and translocates to the nucleus in order to bind to a specific region of the DNA [22]. Results from the nuclear translocation assay showed that LPS could increase the nuclear translocation of NF-κB p65 and this cellular event was attenuated by phytoncide in MAC-T cells (Fig. 5). To determine if treatment with phytoncide can activate the master antioxidant controller Nrf2, the nuclear translocation of Nrf2 was observed. Antioxidant genes, such as heme oxygenase-1 and glutathione S-transferase, are regulated by the transcription factor Nrf2, which translocates from the cytosol to the nucleus [6]. Our results demonstrated that phytoncide significantly increased the nuclear translocation of Nrf2 (Fig. 5). Equal loading of samples was confirmed by the expression of the nuclear housekeeping protein lamin B (Fig. 5).

**Discussion**

Bovine mastitis is an inflammatory event of the mammary gland. The disease is characterized by the infiltration of white blood cells into the mammary gland and is associated with increased levels of cytokines and chemokines, including LPS. These molecules activate NF-κB and other transcription factors that drive the expression of pro-inflammatory genes.

**Fig. 4.** Phytoncide decreases production of superoxide in MAC-T cells.

Cells were treated with phytoncide for 6 h and then stimulated with LPS for 4 h. The cells were stained with DHE to detect superoxide production. The intensity of red fluorescence (superoxide production) was assessed using a fluorescence microscope. The nuclei were stained with DAPI. The images shown here are representatives of three independent experiments. A, DMSO (DHE); B, DMSO (no DHE); C, LPS 1 μg/ml (DHE); D, LPS 1 μg/ml (no DHE); E, phytoncide 0.02% (v/v) (DHE); F, phytoncide 0.02% (v/v) and LPS 1 μg/ml (DHE); G, phytoncide 0.04% (v/v) (DHE); H, phytoncide 0.04% (v/v) and LPS 1 μg/ml (DHE).

**Fig. 5.** Phytoncide decreases the nuclear translocation of NF-κB p65 and increases the nuclear translocation of Nrf2 in MAC-T cells.

To identify the nuclear translocation of NF-κB p65, cells were treated with phytoncide (0.02% and 0.04% (v/v)) for 6 h, followed by LPS treatment (1 μg/ml) for 12 h. To observe the nuclear translocation of Nrf2, cells were treated with phytoncide (0.02% and 0.04% (v/v)) for 6 h followed by LPS treatment (1 μg/ml) for 1 h. Nuclear translocation of NF-κB p65 and Nrf2 was determined in the nuclear fraction using western blot analysis. Lamin B was used as the loading control. The western blots shown here are representative images of three independent experiments.
gland, which negatively affects livestock throughout the world [33]. Mastitis is mostly caused by pathogen infection from *E. coli*, *Staphylococcus aureus*, and *Streptococcus agalactiae* [2], and associated endotoxin (LPS) is closely related to inflammation of the mammary gland. LPS initiates cellular inflammatory events and results in activation of NF-κB and production of pro-inflammatory cytokines such as TNF-α and IL-1β [1, 24, 33].

In the present study, we tested protective effects of phytoncide, particularly COX-2 expression and associated cellular mechanisms in bovine mammary epithelial cells (MAC-T) when exposed to LPS. We have chosen a natural product, phytoncide, since it can be extracted from the pinecone of nut pine trees, which are abundant in Korea and also known to have strong anti-inflammatory effects [12, 19]. We tested the cytotoxicity effects of phytoncide in order to select optimal concentrations for the subsequent experiments. We determined that up to 0.04% was not toxic to MAC-T cells, as determined by MTT assay. In order to examine the anti-inflammatory effects of phytoncide, MAC-T cells were exposed to LPS, which causes inflammatory responses. COX-2 is an enzyme converting arachidonic acid into pro-inflammatory lipid metabolites such as prostaglandin E2. Moreover, COX-2 is regulated by infections, cytokines, growth factors, and stress [3]. COX-2 has also been shown to be upregulated during mastitis [4, 28]. Therefore, COX-2 is a reliable end-point when observing inflammation in cells. Our data demonstrated that phytoncide can inhibit LPS-induced COX-2 expression. Similar observations were reported in bioactive component-treated cells in previous studies. Curcumin, a component of turmeric, decreased COX-2 expression in a breast cell line, MCF-7 [16]. Curcumin also attenuated COX-2 expression in LPS-stimulated human gingival fibroblasts [10]. In this past study, COX-2 attenuation was mediated by inhibition of the NF-κB pathway. In fact, a number of studies have demonstrated that NF-κB is involved in the regulation of COX-2 expression [14]. In addition, several bioactive components (e.g., epigallocatechin gallate, curcumin, and resveratrol) have been shown to suppress NF-κB activation via blocking of nuclear translocation of the NF-κB subunit p65 [30]. In agreement with past work, we observed activation of NF-κB in cells treated with LPS. As expected, nuclear translocation of NF-κB p65 was inhibited by phytoncide, suggesting the NF-κB activation pathway is interfered with by phytoncide. Subsequently, mechanisms and cellular signaling pathways associated with protective effects of phytoncide were studied. Our data demonstrated that LPS increased production of reactive oxygen species (e.g., superoxide anion) and activated cellular signaling molecules (e.g., ERK1/2, p38 and Akt), whereas phytoncide blocked these cellular events. In fact, oxidative stress and activation of such signaling molecules are linked to NF-κB activation [34]. These results were subsequently confirmed in cells pretreated with specific inhibitors such as PD98059, SB203680, and LY294002, which block phosphorylation of ERK1/2, p38, and Akt, respectively. The blocking of these signaling molecules significantly inhibited LPS-induced COX-2 expression. These data suggest that ERK1/2, Akt, and p38 can mediate LPS-induced COX-2 expression in MAC-T cells and phytoncide can block these signaling molecules. Furthermore, inhibition of superoxide production with phytoncide was associated with increased Nrf2 expression. Increased Nrf2 nuclear translocation implies upregulated antioxidant capacity in cells. Previously, it was noted that mechanisms of protection related to plant-derived natural compounds involve the upregulation of multiple antioxidant enzymes that are regulated by the transcription factor Nrf2 [32]. Similarly, our data demonstrated that reduced production of superoxide with phytoncide was associated with increased Nrf2 nuclear translocation. Increase of Nrf2 nuclear translocation due to LPS exposure may be due to the endogenous defensive mechanism in cells.

Taken together, our data suggest that superoxide production, ERK1/2, p38, Akt, and NF-κB are mediators of LPS-induced COX-2 expression in MAC-T cells and phytoncide can attenuate the activation of these signaling molecules. This work highlights the protective effects of the pinecone-derived phytoncide extract against LPS-induced inflammatory responses, but more work needs to be completed to determine if the addition of this mixture in bovine feed or intra-mammary administration may protect the mammary gland against inflammation.

Acknowledgments

This research was supported by the Technology Commercialization Support Program, Ministry of Agriculture, Food and Rural Affairs (Project No. 113041-2).

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