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In vitro induction effects of *Commiphora molmo* (Myrrh) extracts on cell migration through anti-inflammatory activity

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Abstract

Commiphora molmo (Myrrh) contains various biological activities. However, the underlying molecular mechanisms in cells remain unknown. The purpose of this study was to confirm the efficacy of inhibiting nitric oxide (NO) production induced by lipopolysaccharides (LPS) on J774 macrophage cells of myrrh, and additionally regulatory effects of perform wound healing. Further, vascular endothelial growth factor (VEGF), phosphorylation of signal transducer and activator of transcription3 (p-STAT3) and Janus kinase 2 (p-JAK2) levels in macrophage cells were regulated in the presence of myrrh extract. Based on the above results, myrrh is thought to provide basic data on anti-inflammatory properties through interleukin 6 (IL-6) inhibitory activity and regulatory effects of cell migration.

Keywords: *Commiphora molmo* (Myrrh), anti-inflammation, cell migration, wound healing

Introduction

Cell migration and invasion are critical parameters in the metastatic dissemination of macrophage and cancer cells, the major cause of death in some diseases patients include cancer and tumor (Darvin et al., 2015). Wound healing is a complex processes that inflammation, re-epithelialization, tissue and matrix formation (Shan et al., 2014). Signal transducers and activators of transcription (STAT) are a family of transcription factors activated by several inflammatory cytokines and growth factors (Darvin et al., 2015). STAT3 plays a role in response to growth factors and cytokines such as interleukin 6 (IL-6) (Geiger et al., 2007). Also, STAT3 is phosphorylated by receptor-associated Janus kinases (JAK), form homo- or heterodimers, and translocate to the cell nucleus where they act as transcription activators (Kuchipudi, 2015). Recent research have reported the potential of several extract and/or compounds from natural products in inflammation treatment by targeting STAT3.

Commiphora molmo (Myrrh) is a yellow aromatic oleo-gum resin obtained from the stems of plants of the genus Burseraceae

family included *Commiphora abyssinica* and *Commiphora myrrha* (Hanus et al., 2005). These myrrh is already known a noble plant in many country of Asian and Europe, furthermore, it has been used as a traditional medicine that biological activities include antioxidant, antibacterial, anti-nociceptive, and anti-ulcer (Dolara et al., 2000). However, anti-inflammatory activity pertinent to migration of myrrh has little information until now. Therefore, we investigated the suitability of extract of processed myrrh as a functional agent for suppressing the expression of LPS-induced pro-inflammatory mediators. As a result, the myrrh extract has strong biological activity, so it is considered that it can be used as a potential bio-activities material in the industry.

Materials and Methods

Materials

Myrrh obtained from Myrrh Molyac Institute Co. Ltd., (Daejeon, Korea). Insert with μ -dish for cell migration were obtained

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from ibidi GmbH (Martinsried, Germany). Antibodies for JAK2, STAT3, VEGF and actin were purchased from Santa Cruz Biotechnology (Delaware, CA, USA). LPS (serotype 0127:B8) were procured from Sigma-Aldrich (St. Louis, MO, USA). Also, the enhanced chemiluminescence (ECL) advanced detection kit was purchased from Amersham Bioscience (Uppsala, Sweden).

Cell culture

J774 cells were obtained from the ATCC (Rockville, MD, USA). In brief, the cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics (100 U/ml of penicillin, 100 µg/mL of streptomycin). Cells were maintained in 5% CO₂, 95% air and humidified atmosphere at 37°C.

MTT assay for cell viability

Briefly, the cells were seeded in well plates at 2×10^5 in 100 µL/well. After 24 h, these cells were treated with 0, 1, 5, and 10 µg/mL myrrh extracts for 24 h. Afterwards, the OD values were detected at 570 nm under a microplate reader.

Measurement of NO production

The J774 cells (2×10^5 cells/ml) were grown in a 24 well-plate and incubated with sample at various concentrations (5 and 10 µg/mL) in the presence or absence of LPS (50 ng/mL) for 18 h. The obtained culture media at designated time points were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2% phosphoric acid) in a 96-well plate. The optical density at 540 nm was determined with microplate reader (Thermo Electron Corporation, Marietta, OH, USA). Nitrite production was determined by comparing the optical density with the standard curve obtained with NaNO₂.

Wound healing assay

In vitro wound healing modeling was performed using culture-insert in µ-dish (35 mm) assay (Ibidi #80206, Martinsried, Germany). When cells reached confluence, culture inserts were removed and a 500 µm cell-free area was created. After remove of DMEM was added extract of myrrh extracts (10 µg/mL), and cells were incubated for 24 h in a CO₂ incubator. In addition, the closing of the wound induced-insert was measured at 0 to 20 h using optical microscope (GX51, Olympus, Tokyo, Japan).

Western blotting analysis

Total protein from J774 cell lysates was denatured at 95°C for 5 min. Equal amount (20 µg) of denatured proteins was loaded in SDS-PAGE (12%), transferred onto PVDF membranes and blocked with skimmed milk (5%) for 1 h. Ultimately, these PVDF membranes were incubated with specific primary antibodies at 4°C overnight, followed by incubation with secondary antibody at room temperature for 1 h. Finally, target-specific signals were detected using an enhanced chemiluminescence solution (Intron Biotechnology, Seongnam, Korea) (Lee et al., 2012).

Statistical analyses

Data were reported as mean±SD for triplicate determinations. Analysis of variance (ANOVA), accompanied with Tukey's tests (GraphPad Prism 5), were conducted to identify the significant differences between samples ($p < 0.05$).

Results and Discussion

In the present study, the myrrh was selected to investigate anti-inflammatory effects on LPS-induced stimulation, as the myrrh had higher induced migration on macrophage cells. The cells were treated with the myrrh (1, 5, and 10 µg/mL) prior to LPS. All extract fraction of myrrh has no cytotoxicity (Fig. 1). In addition, all extract fraction of myrrh inhibited nitrite contents, ethanol and ethyl acetate extracts which were the highest activity (Fig. 1). To investigate whether some extract fraction such as water, ethanol, ethyl acetate, hexane, and butanol could induce macrophage cell migration, we examined the effects of myrrh extracts on the migration of macrophage cells by wound healing assays. As shown in Fig. 2A and B, myrrh extracts induced the migration of J774 cells. Western blot analysis showed that the LPS induced STAT3 is reduced in myrrh ethanolic extract treated with at 1, 5, and 10 µg/mL on J774 cells (Fig. 2C). Further, we measured the levels of JAK2 and VEGF (Fig. 2C). The phosphorylated STAT3 by IL-6 translocate into the nucleus, where these participate in the expression of pro-inflammatory cytokines such as interferon gamma (IFN-γ), interleukin-17 (IL-17), and interleukin 1 beta (IL-1β) (Kuchipudi, 2015; Darvin et al., 2015). IL-6 is well-known for activating immunity and inflammation through the binding to IL-6 receptor and dimeric gp130, which subsequently phosphorylates JAK2, STAT3, and extracellular-signal-regulated kinase (ERK) (Oh et al., 2014; Neurath and Finotto,

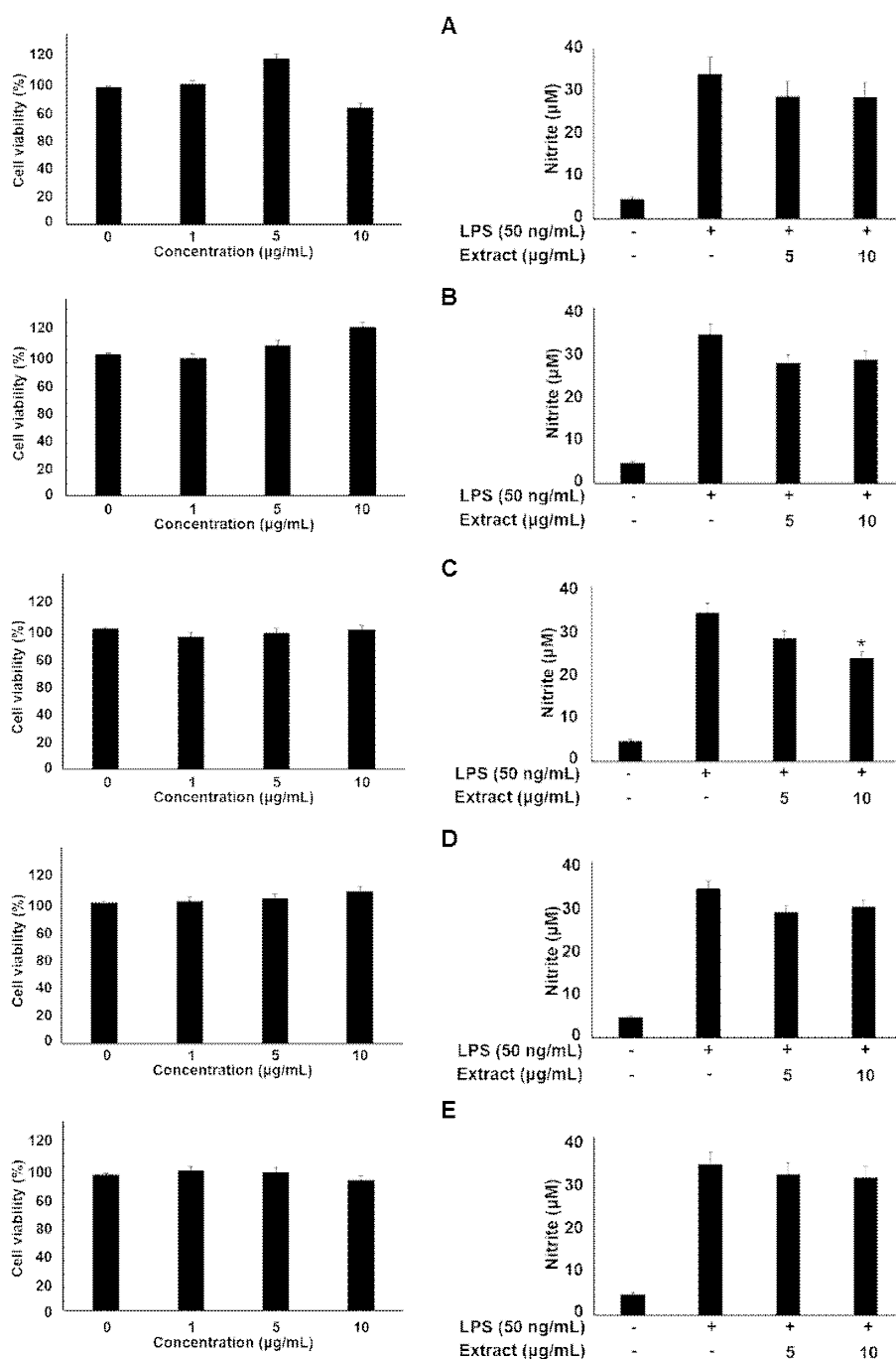


Fig. 1. Inhibitory effects of nitrite production by myrrh in LPS-stimulated macrophage cells. Myrrh on various concentrations (0, 1, 5, and 10 $\mu\text{g/mL}$) for 4 h. Cell viability was measured by MTT assay (left panel). Cells were pretreated for 4 h with different concentrations of myrrh, then treated cells with LPS (50 ng/mL) for 18 h. The concentration of nitrite in the medium was determined by Griess assay (right panel) (A, water extract; B, ethanolic extract; C, ethyl acetate extract; D, hexane extract; E, butanol extract). Results are expressed as mean \pm SD from 3 independent experiments. * $p < 0.05$ indicate to compare with control group. LPS, lipopolysaccharides.

2011).

Based on these data, we inhibitory effect of some protein by inflammatory disease from myrrh, its induced cell migration.

Also, other downstream signaling molecules involved in the LPS pathway may be inhibited by suppression of phosphorylated JAK2, STAT3, and VEGF by myrrh may be responsible.

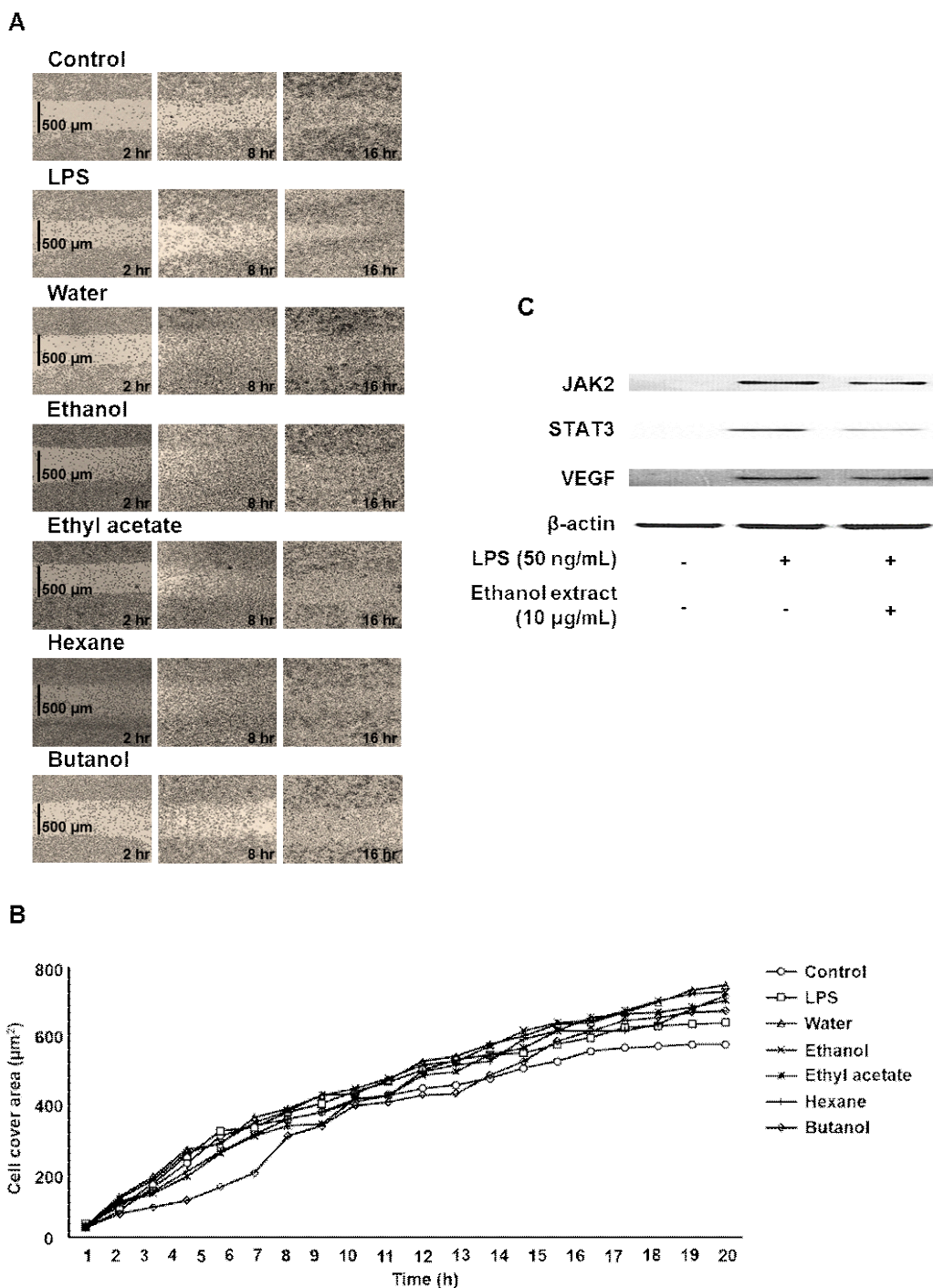


Fig. 2. Effects of myrrh on macrophage cell migration measured by wound healing assay. (A) Cells were plated in μ -dish with insert, and the cell monolayer was then insert removed the next day, a single scratch was made in the confluent monolayer followed by 10 $\mu\text{g/mL}$ myrrh-treatment for 24 h, each scratch was photographed at 2, 8, and 16 h. There were highly significant differences between non-treated group and each soluble fraction of myrrh-treated groups. (B) Effect of STAT3, JAK2, and VEGF activation on LPS-stimulated by myrrh. Cells were pre-incubated with myrrh for 1 h before stimulation by LPS (50 ng/mL) using western blot. (C) The total amount of the corresponding non-phosphorylated protein was used as a loading control for phosphorylated proteins. STAT, signal transducers and activators of transcription; JAK, Janus kinases; VEGF, vascular endothelial growth factor; LPS, lipopolysaccharides.

However further studies are needed to elucidate the precise mechanism *in vitro* and *in vivo*.

Conflicts of Interest

The authors declare no potential conflict of interest.

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Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

Author Contributions

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