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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

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38 **1. Introduction**

39 Cell migration and invasion are critical parameters in the metastatic dissemination of 40 macrophage and cancer cells, the major cause of death in some diseases patients include 41 cancer and tumor (Darvin et al., 2015). Wound healing is a complex processes that 42 inflammation, re-epithelialization, tissue and matrix formation (Shan et al., 2014). Signal 43 transducers and activators of transcription (STAT) are a family of transcription factors 44 activated by several inflammatory cytokines and growth factors (Darvin et al., 2015). STAT3 45 plays a role in response to growth factors and cytokines such as interleukin 6 (IL-6) (Geiger 46 *et al*., 2016). Also, STAT3 is phosphorylated by receptor-associated Janus kinases (JAK), 47 form homo- or heterodimers, and translocate to the cell nucleus where they act as 48 transcription activators (Kuchipudi, 2015). Recent research have reported the potential of 49 several extract and/or compounds from natural products in inflammation treatment by 50 targeting STAT3.

51 *Commiphora molmo* (Myrrh) is a yellow aromatic oleo-gum resin obtained from the stems 52 of plants of the genus *Burseraceae* family included *Commiphora abyssinica* and 53 *Commiphora myrrha* (Hanuš et al., 2005). These myrrh is already known a noble plant in 54 many country of Asian and Europe, furthermore, it has been used as a traditional medicine 55 that biological activities include antioxidant, antibacterial, anti-nociceptive, and anti-ulcer 56 (Dolara et al., 2000). However, anti-inflammatory activity pertinent to migration of myrrh has 57 little information until now. Therefore, we investigated the suitability of extract of processed 58 myrrh as a functional agent for suppressing the expression of LPS-induced pro-inflammatory 59 mediators. As a result, the myrrh extract has strong biological activity, so it is considered that 60 it can be used as a potential bio-activities material in the industry.

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62 **2. Materials and methods**

63 *2.1. Materials*

64 Myrrh obtained from Myrrh Molyac Institute Co. Ltd., (Daejeon, Korea). Insert with μ-
65 dish for cell migration were obtained from ibidi GmbH (Martinsried, Germany). Antibodies
66 for JAK2, STAT3, VEGF and actin were purchased from Santa Cruz Biotechnology
67 (Delaware, CA, USA). LPS (serotype 0127:B8) were procured from Sigma Aldrich (St. Louis,
68 MO, USA). Also, the enhanced chemiluminescence (ECL) advanced detection kit was
69 purchased from Amersham Bioscience (Uppsala, Sweden).

70

71 *2.2. Cell culture*

72 J774 cells were obtained from the ATCC (Rockville, MD, USA). In brief, the cells were
73 grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-
74 inactivated fetal bovine serum (FBS) and antibiotics (100 U/ml of penicillin, 100 μg/ml of
75 streptomycin). Cells were maintained in 5% CO_2, 95% air and humidified atmosphere at
76 37℃.

77

78 *2.3. MTT assay for cell viability*

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

82

83 *2.4. Measurement of NO production*

84 The J774 cells (2×10^5 cells/ml) were grown in a 24 well-plate and incubated with sample
85 at various concentrations (5 and 10 μg/ml) in the presence or absence of LPS (50 ng/ml) for

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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 $NaNO_2$.

2.5. 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 CO_2 incubator. In addition, the closing of the wound induced-insert was measured at 0 to 20 h using optical microscope (GX51, OLYMPUS, Tokyo, Japan).

2.6. 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).

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110 *2.7. Statistical analyses*

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

114

115 **3. Results and discussion**

116 In the present study, the myrrh was selected to investigate anti-inflammatory effects on
117 LPS-induced stimulation, as the myrrh had higher induced migration on macrophage cells.
118 The cells were treated with the myrrh (1, 5, and 10 μg/ml) prior to LPS. All extract fraction of
119 myrrh has no cytotoxicity (Fig. 1). In addition, all extract fraction of myrrh inhibited nitrite
120 contents, ethanol and ethyl acetate extracts which were the highest activity (Fig. 1). To
121 investigate whether some extract fraction such as water, ethanol, ethyl acetate, hexane, and
122 butanol could induce macrophage cell migration, we examined the effects of myrrh extracts
123 on the migration of macrophage cells by wound healing assays. As shown in Fig. 2A and B,
124 myrrh extracts induced the migration of J774 cells. Western blot analysis showed that the
125 LPS induced STAT3 is reduced in myrrh ethanolic extract treated with at 1, 5 and 10 μg/ml
126 on J774 cells (Fig. 2C). Further, we measured the levels of JAK2 and VEGF (Fig. 2C). The
127 phosphorylated STAT3 by IL-6 translocate into the nucleus, where these participate in the
128 expression of pro-inflammatory cytokines such as interferon gamma (IFN-γ), interleukin-17
129 (IL-17), and interleukin 1 beta (IL-1β) (Kuchipudi, 2015; Darvin et al., 2015). IL-6 is well-
130 known for activating immunity and inflammation through the binding to IL-6 receptor and
131 dimeric gp130, which subsequently phosphorylates JAK2, STAT3, and extracellular-signal-
132 regulated kinase (ERK) (Oh et al., 2014; Neurath and Finotto, 2011).

133 Based on these data, we inhibitory effect of some protein by inflammatory disease from

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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. 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

Conceptualization: Cheong SH

Formal analysis and measurement: Ha HJ and Lee SJ

Investigation: Lee SJ and Lee SW

Writing-original draft: Lee SW and Cheong SH

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Figure Captions

Figure 1. Inhibitory effects of nitrite production by myrrh in LPS-stimulated macrophage cells. Myrrh on various concentrations (0, 1, 5 and 10 μg/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 ± SD from 3 independent experiments. $^{*}p < 0.05$ indicate to compare with control group.

Figure 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 μg/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.

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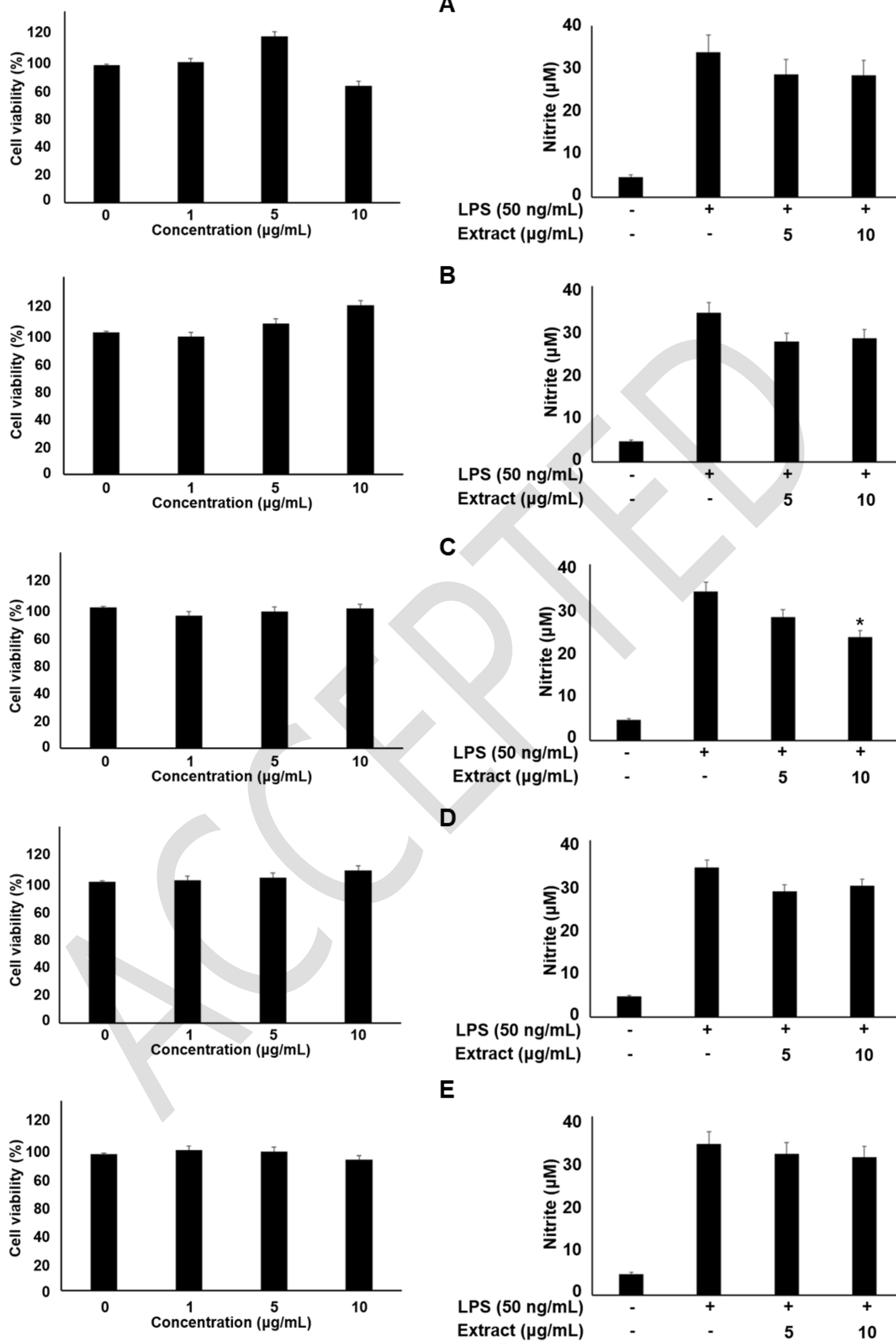

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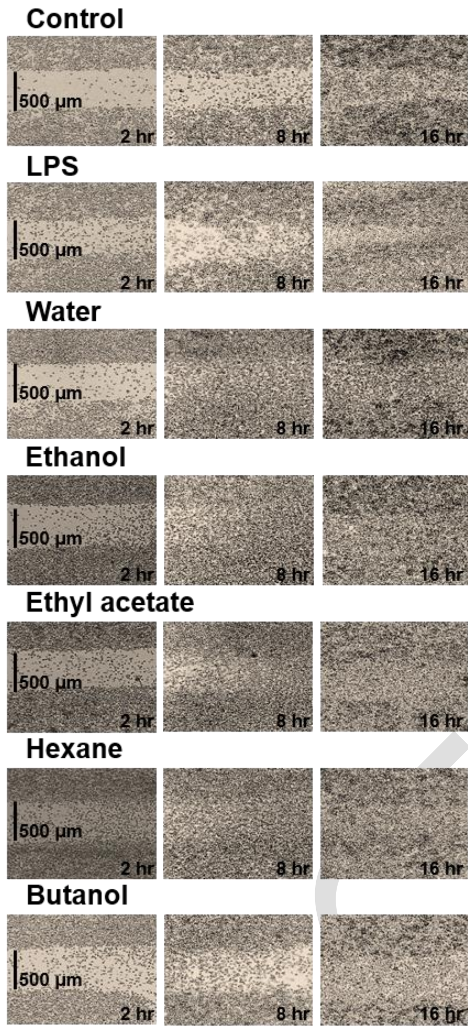
217 **Figure 1. Lee et al.**

A



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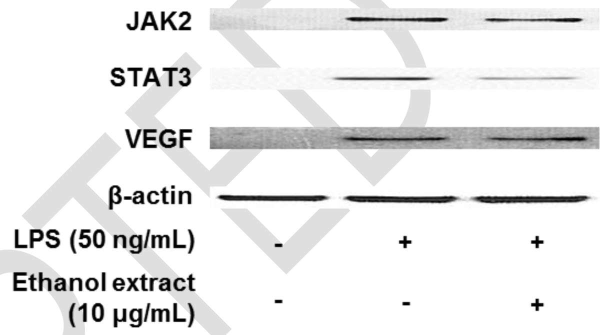

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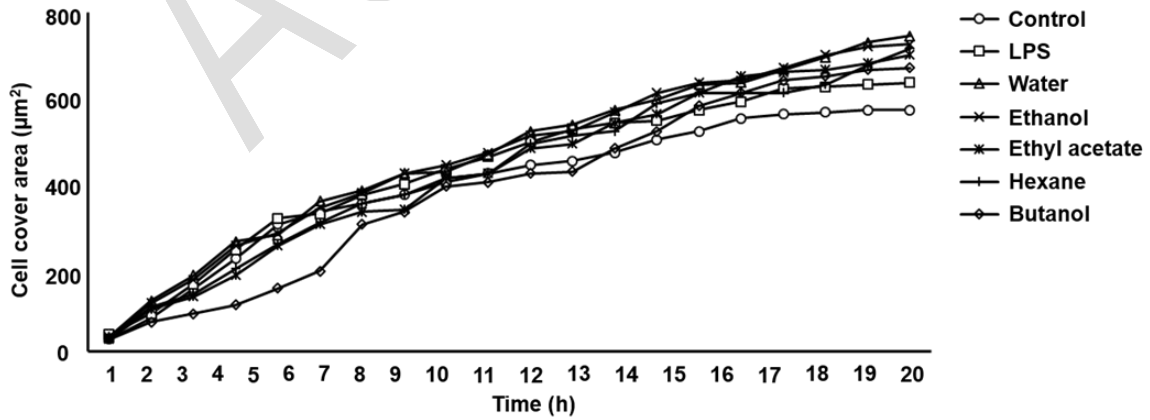

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219 **Figure 2. Lee et al.**

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