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Original Article

Anti-inflammatory effect of cinnamaldehyde and linalool from the leaf essential oil of *Cinnamomum osmophloeum* Kanehira in endotoxin-induced mice

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ABSTRACT

Cinnamomum osmophloeum Kanehira is a Taiwan native plant that belongs to genus *Cinnamomum* and is also known as pseudocinnamomum or indigenous cinnamon. Its leaf is traditionally used by local people in cooking and as folk therapy. We previously demonstrated the chemical composition and anti-inflammatory effect of leaf essential oil of *Cinnamomum osmophloeum* Kanehira of linalool chemotype in streptozotocin-induced diabetic rats and on endotoxin-injected mice. The aim of the present study is to evaluate whether cinnamaldehyde and linalool the active anti-inflammatory compounds in leaf essential oil of *Cinnamomum osmophloeum* Kanehira. Before the injection of endotoxin, C57BL/6 mice of the experimental groups were administered cinnamaldehyde (0.45 or 0.9 mg/kg body weight) or linalool (2.6 or 5.2 mg/kg body weight), mice of the positive control group were administered the leaf essential oil (13 mg/kg body weight), and mice of the negative group were administered vehicle (corn oil, 4 mL/kg body weight) by gavage every other day for two weeks. All mice received endotoxin (i.p. 10 mg/mL/kg body weight) the next day after the final administration and were killed 12 h after the injection. Normal control mice were pretreated with vehicle followed by the injection with saline. None of the treatment found to affect body weight or food or water intake of mice before the injection of endotoxin. Cinnamaldehyde and linalool were found significantly reversed endotoxin-induced body weight loss and lymphoid organ enlargement compared with vehicle ($P < 0.05$). Both compounds also significantly lowered endotoxin-induced levels of peripheral nitrate/nitrite, interleukin (IL)-1 β , IL-18, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and High-mobility group box 1 protein (HMGB-1), and levels of nitrate/nitrite, IL-1 β , TNF- α , and IFN- γ in spleen and mesenteric lymph nodes (MLNs) ($P < 0.05$). Endotoxin-induced expression of toll-like receptor 4 (TLR4), Myeloid differentiation primary response gene 88 (MyD88), myeloid differentiation protein 2 (MD2), Nod-like receptor family, pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein

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containing a caspase-recruitment domain (ASC), and caspase-1 in spleen and mesenteric lymph nodes (MLNs) were inhibited by all tested doses of cinnamaldehyde and linalool ($P < 0.05$). Subsequently, the activation of nuclear factor (NF)- κ B and the activity of caspase-1 in spleen and MLNs were also suppressed by these two compounds ($P < 0.05$). In addition, cinnamaldehyde and linalool at the dose equivalent to their corresponding content in the tested dose of the leaf essential oil, which was 0.9 mg/kg and 5.2 mg/kg, respectively, showed similar or slightly less inhibitory activity for most of these inflammatory parameters compared with that of the leaf essential oil. Our data confirmed the potential use of leaf essential oil of *Cinnamomum osmophloeum* Kanehira as an anti-inflammatory natural product and provide evidence for cinnamaldehyde and linalool as two potent agents for prophylactic use in health problems associated with inflammations that being attributed to over-activated TLR4 and/or NLRP3 signaling pathways.

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1. Introduction

Pattern recognition receptors (PRRs) are proteins expressed mainly by cells of the innate immune system. Upon the recognition of pathogen-associated molecular patterns that associate with microbial pathogens (PAMPs), and damage-associated molecular patterns (DAMPs) that associate with cell components of damaged cell, PRRs initiate cascades of cellular signal transduction that eventually result in the generation of various inflammatory regulators, mainly a series of cytokines which help to conduct a complicated process for sterilizing purpose [1]. However, at time when such inflammatory response is out of control, it leads to tissue/organ damage. Currently, inflammation has been established as a major underlying mechanism of a great variety of acute and chronic clinical conditions associated with infectious and non-infectious problems. Consequently, various approaches with specific strategy to target inflammatory response have been developed for prophylactic and/or therapeutic use, among which the modulation of the activation of PRRs and downstream signaling pathways has raised great interest [2,3].

The membrane bound Toll-like receptors (TLRs) and the cytoplasmic Nod-like receptors (NLRs) are two of the mostly studied PRR families among which TLR4 and NLRP3 have been demonstrated to interact closely and contribute to the progressing of a great variety of inflammatory clinical conditions including several chronic and systemic inflammatory diseases [4,5]. Endotoxin-initiated inflammation is commonly used as a prototypical example to study the priming of such interaction due to the fact that this molecule from Gram-negative bacteria is the most well known TLR4 specific ligand and is recently established to be able to activate NLRP3 inflammasome [5]. When induced by endotoxin, TLR4 activates intracellular signaling pathway through the interaction with the co-receptor, MD2, which subsequently recruits the adaptor protein MyD88 thus activate the downstream signaling molecules [6]. In mice, the induction of either TLR4 mutation or MyD88 dysfunction caused lowered inflammatory response to endotoxin stimulation *in vivo* [7,8]. Although

NLRP3 is not the corresponding receptor for endotoxin; however, the critical role of NLRP3 signaling pathway in endotoxin-induced inflammation has been revealed [5]. The activation of NLRP3 involves in two stages that require NF- κ B to increase the expression of NLRP3 and downstream signal(s) of TLR4 to help deubiquitinate NLRP3 and to phosphorylate the adaptor protein, ASC, to allow the assembling of NLRP3 and ASC which then conjugate with procaspase-1 to transfer this enzyme to its active form, caspase-1 [4]. The activity of caspase-1 is essential for endotoxin-induced generation of IL-1 β and IL-18 through the cleavage of pro-IL-1 β and pro-IL-18 [5]. In either ASC- or caspase-1-deficient mice, it showed resistance to lethal dose of endotoxin-induced shock [9].

In general, the inflammatory status is indicated by level of molecules that elevated as a consequence of the activation of PRRs and commonly include IL-1 β , TNF- α , and interferon IFN- γ . IL-18, the IFN- γ -inducing factor, is relatively rare to be used as a parameter of inflammation status. This may be due to the fact that this cytokine induces the production of IFN- γ by T lymphocytes [10], thus the presence of IFN- γ could well reflect the production of IL-18. Indeed, in murine the induction of endotoxemia elevated levels of IL-18 and IFN- γ in lung while the treatment with anti-IL-18 antibody suppressed leukocyte infiltration in lung with decreased IFN- γ level [11]. High-mobility group protein 1 (HMG-1), also known as HMGB1, is a DAMP that secreted by endotoxin-, IFN- γ -, TNF- α -, or IL-1 β -activated monocytes and macrophages that plays the role as a ligand of TLR4 and has recently been suggested a target for anti-inflammation therapy [12].

A variety of indigenous lauraceous plants in Taiwan has been found to possess anti-inflammatory activity that exerts by both volatile and nonvolatile components from these plants [13]. *Cinnamomum osmophloeum* Kanehira, a species of genus *Cinnamomum* that belongs to the plant family Lauraceae, is native in Taiwan which is also known as pseudo-cinnamomum or indigenous cinnamon and has been cultivated widely on the island and is traditionally used in cooking and as folk therapy to relief fever, arthritis, cold, gout, and general nerve pains. Despite these folk uses of *C. osmophloeum*, scientific evidences for prophylactic and/or

therapeutic use of this plant have only been revealed very recently and reviewed by Rao and Gan [14]. We previously demonstrated that the leaf essential oil of *C. osmophloeum* of linalool chemotype ameliorated tissue content of pro-inflammatory cytokines in streptozotocin-induced diabetes in rats and in endotoxin-induced intestinal damage in mice [15,16]. The present study hypothesized that cinnamaldehyde and linalool two major active constituents in this essential oil for its anti-inflammatory activity due to the fact that linalool is the predominant composition which represent 40.24% of the essential oil and that cinnamaldehyde, although only represent 6.87% of the essential oil, has been demonstrated to be able to modulate peripheral cytokine levels in mice when administered at as low as 1 mg/kg bw/day [15,17]. According to our previous analysis by GC/MS, other compositions in this essential oil include transcinnamyl acetate (11.71%), camphor (9.38%), 3-phenyl-2-propenal (4.06%), caryophyllene (2.65%), coumarin (2.13%), bornyl acetate (1.72%), limonene (1.53%), α -(+)-pinene (1.38%), estragole (1.31%), and caryophyllene oxide (1.00%) [15].

2. Methods

2.1. Chemicals and reagents

Protease inhibitor cocktail was purchased from Roche (Indianapolis, IN); the mouse TNF- α enzyme-linked immunosorbent assay (ELISA) kit and the mouse IFN- γ ELISA kit were purchased from eBioscience (San Diego, CA); the mouse IL-1 β ELISA kit and the rabbit anticaspase-1 antibody were purchased from Invitrogen (Carlsbad, CA); the Mouse HMG-1 ELISA kit and IL-18 ELISA kit were purchased from Usbn (Wuhan, China); the Nitrate/Nitrite Colorimetric Assay Kit, the nuclear extraction kit, and the NF- κ B (p65) transcription factor assay kit were purchased from Cayman (Ann Arbor, MI); the mouse anti- β -actin antibody, the horseradish peroxidase-conjugated goat anti-rabbit antibody, and the goat anti-mouse immunoglobulin G antibody were purchased from Merck Millipore (Darmstadt, Germany); the rabbit anti-TLR4 antibody, the rabbit anti-MD2 antibody, and the rabbit anti-MyD88 antibody were purchased from GeneTex (Irvine, CA); the rabbit anti-NLRP3 antibody and the rabbit anti-ASC antibody were purchased from Biorbyt (San Francisco, CA); the caspase-1 fluorometric assay kit was purchased from Biovision (Meylan, France); the Western Lightning[®] Plus-ECL was purchased from PerkinElmer (Boston, MA); the Bio-Rad protein assay kit was purchased from Bio-Rad Laboratories (Richmond, CA); the endotoxin from *Salmonella typhimurium* and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Plant materials and preparation of essential oil

Fresh *C. osmophloeum* Kaneh. leaves were collected from a research farm at National Chiayi University, in Shekou, a central Taiwan county. The leaves were collected and identified by Dr. Kuen-Yih Ho, Department of Forestry and Natural Resources, National Chiayi University. A voucher specimen (SK-CO-09-M-14) is deposited at the Herbarium of the botanical

garden of National Chiayi University, Chiayi, Taiwan. The preparation and chemical composition of the essential oil of the leaves is the same as described previously [15].

2.3. Animals and experimental procedure

Ten-week-old male C57BL/6J mice were purchased from the National Animal Breeding and Research Center (Taipei, Taiwan). The animals were kept under a 12-h light–dark cycle at an ambient temperature of 23 °C and were given free access to water and standard rodent feed (Rodent Diet 5001; Purina Mills, Richmond, IN). After acclimatization for 1 week, animals were randomly assigned to 6 groups and received by gavage leaf essential oil of *C. osmophloeum* (EO; 13 mg/kg bw), cinnamaldehyde (CA; 0.45 or 0.9 mg/kg bw), linalool (LIN; 2.6 or 5.2 mg/kg bw), or the vehicle (corn oil; 4 mL/kg bw) every other day for 2 weeks. The EO dose selected is according to the effectiveness found in our previous study [16]. The higher doses of cinnamaldehyde and linalool were selected in accordance with the equivalent content of the corresponding compound in EO which were 0.9 and 5.2 mg/kg bw, respectively. The lower doses of these two compounds were designed as half of the higher dose of each to investigate dose-dependent effect.

Endotoxin from *S. typhimurium* (10 mg/kg bw) was injected intraperitoneally 15 days after the first administration of EO, CA, or LIN. The animals' food supply was withdrawn followed by the injection. The control mice, which had received corn oil, were injected with the same volume of sterile saline. The mice were killed by carbon dioxide euthanasia 12 h after the injection. Blood was collected and spleen and the MLNs were removed immediately. Spleen, MLNs and plasma prepared freshly from blood samples were stored at –80 °C until use within 1 week. Housing conditions and experimental procedures were performed according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978), and all protocols were approved by the ethical committee for animal experimentation of Chung Shan Medical University, Taichung, Taiwan.

2.4. Biochemical analysis of plasma, spleen and MLNs

The concentration of IL-1 β , IL-18, TNF- α , IFN- γ , and HMGB-1 in plasma and the content of IL-1 β , TNF- α , and IFN- γ in spleen and MLNs were analyzed with the use of the corresponding ELISA kit. Nitrate/nitrite contents in plasma, spleen, and MLNs were determined with the nitrate/nitrite colorimetric assay kit. The assay procedure was in accordance with the manufacturer's instructions, and the results were analyzed with a microplate reader (VersaMax; Molecular Devices Ltd., Sunnyvale, CA).

2.5. Caspase-1 activity assay

The activity of caspase-1 in spleen and MLNs was determined with the caspase-1 fluorometric assay kit, in accordance with the manufacturer's instructions. The fluorescence intensity of the product generated from the activity from caspase-1 was analyzed by using excitation at 400 nm and emission at 505 nm, with a FlexStation 3 Microplate Reader (Molecular Devices, Silicon Valley, CA). The resultant activity of caspase-1 detected was expressed as percentage of the control.

2.6. Nuclear extraction and NF- κ B determination

NF- κ B typically resides in the cytoplasm of cells as a complex with members of the I κ B inhibitor family of proteins and translocates into the nucleus upon stimulation, such as by endotoxin and certain proinflammatory cytokines [18]. Consequently, the detection of NF- κ B in the nucleus represents the activation of this transcription factor. The present study extracted nuclei from the spleen and MLNs with a nuclear extraction kit according to the manufacturer's instruction. The nuclear extracts were subsequently determined for the content of NF- κ B with the NF- κ B (p65) transcription factor assay kit. The assay procedure was in accordance with the manufacturer's instructions, and the results were analyzed with a microplate reader (VersaMax; Molecular Devices Ltd., Sunnyvale, CA). The resultant nuclear NF- κ B content detected was expressed as percentage of the control.

2.7. Western blotting of spleen and MLNs

Spleen and MLNs were homogenized in radioimmunoprecipitation assay buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.25% deoxycholate) supplemented with a protease inhibitor cocktail. The resultant supernatants were subjected to Bis-Tris gel electrophoresis followed by blotting for the analysis of cellular expression of TLR4, MD2, MyD88, NLRP3, ASC, and caspase-1 were performed as described previously [16].

2.8. Statistical analysis

The data are expressed as the mean \pm SD and were analyzed by one-way analysis of variance. Student's t-test was used to detect differences in means between the control group and the endotoxin-injected mice. Duncan's multiple-comparison test was used to detect differences among the means of the endotoxin-injected groups. P values of <0.05 were considered to be significant. All statistical analyses were performed with commercially available software (SPSS 12 for Windows; SPSS Inc., Chicago, IL).

3. Results

3.1. Animal characteristics

Mice were pretreated with EO, cinnamaldehyde, or linalool for 2 weeks before the injection with endotoxin, in which high dose of cinnamaldehyde and linalool were equivalent to their corresponding contents in the tested EO dose. The body weights of all groups were similar at baseline and at the end of the pretreatment (Table 1). Thus, the tested doses of EO and its active compositions did not significantly affect the growth of these animals. Similarly, at the end of the pretreatment, EO, cinnamaldehyde, and linalool showed no effect on food and water ingestion and urine and fecal excretion when compared with the pretreatment with vehicle (data not shown). After the injection of saline or endotoxin, all groups showed body weight loss which can be attributed partly to the withdrawal of food supply followed by the injection. However, it showed that endotoxin induced a body weight loss more than 2-fold of that in saline injected mice and was significantly ameliorated by the pretreatment with EO ($P < 0.05$, Table 1). Low and high doses of cinnamaldehyde and linalool all significantly reversed endotoxin-induced body weight loss but to a less extent than that by EO. In addition, endotoxin-injection was associated with a significant elevation of the ratios of spleen weight and MLNs weight to body weight ($P < 0.05$), while the administration of EO, cinnamaldehyde, and linalool all significantly ameliorated the enlargement of these lymphoid organ/tissue induced by endotoxin which implicated less inflamed condition in these animals (Table 1).

3.2. Peripheral proinflammatory status

As expected, endotoxin induced dramatic elevation of levels of nitrate/nitrite and several proinflammatory cytokines, including IL-1 β , IL-18, TNF- α , and IFN- γ in peripheral blood ($P < 0.05$, Table 2). Among the endotoxin-injected groups, mice pretreated with EO had slightly but significantly lower level of nitrate/nitrite compared with those pretreated with vehicle

Table 1 – Body weight and spleen or mesenteric lymph nodes weight to body weight ratio of control or endotoxin-injected mice that did or did not receive the leaf essential oil of *Cinnamomum osmophloeum* Kanehira, linalool or cinnamaldehyde^y

	Control-V	Endotoxin-V	Endotoxin-EO	Endotoxin-CAL	Endotoxin-CAH	Endotoxin-LINL	Endotoxin-LINH
Baseline body wt. (g)	23.89 \pm 2.34	23.50 \pm 2.02	24.26 \pm 1.87	23.82 \pm 1.52	23.84 \pm 1.63	24.29 \pm 1.58	24.24 \pm 1.49
Body wt. after treatment and before endotoxin injection (g)	24.71 \pm 2.59	24.12 \pm 2.49	24.61 \pm 2.12	24.16 \pm 1.52	24.50 \pm 1.99	25.37 \pm 1.25	25.11 \pm 1.03
Body wt. difference between before and after injection (g)	-1.14 \pm 0.3	-3.16 \pm 0.34 ^{*a}	-1.59 \pm 0.18 ^d	-1.96 \pm 0.24 ^{bc}	-2.23 \pm 0.32 ^b	-1.99 \pm 0.22 ^{bc}	-1.74 \pm 0.24 ^{cd}
Spleen wt/body wt \times 100 (%)	0.208 \pm 0.015	0.323 \pm 0.014 ^{*a}	0.303 \pm 0.015 ^b	0.301 \pm 0.017 ^b	0.314 \pm 0.025 ^b	0.300 \pm 0.014 ^b	0.307 \pm 0.015 ^b
MLNs wt/body wt \times 100 (%)	0.079 \pm 0.007	0.129 \pm 0.015 ^{*a}	0.109 \pm 0.015 ^{bc}	0.117 \pm 0.015 ^{ab}	0.101 \pm 0.012 ^c	0.113 \pm 0.013 ^{bc}	0.116 \pm 0.012 ^{ab}

^yValues are the mean \pm SD for eight mice per group. control-V, saline-injected mice treated with vehicle; endotoxin-V, endotoxin-injected mice treated with vehicle; endotoxin-EO, endotoxin-injected mice treated with 13 mg/(kg bw) of leaf essential oil of *Cinnamomum osmophloeum* Kanehira; endotoxin-CAL, endotoxin-injected mice treated with 0.45 mg/(kg bw) of cinnamaldehyde; endotoxin-CAH, endotoxin-injected mice treated with 0.9 mg/(kg bw) of cinnamaldehyde; endotoxin-LINL, endotoxin-injected mice treated with 2.6 mg/(kg bw) of linalool; endotoxin-LINH, endotoxin-injected mice treated with 5.2 mg/(kg bw) of linalool. *Significantly different from the control group ($P < 0.05$). a, b, c and d indicate the means within a row not sharing the same superscript letter are significantly different ($P < 0.05$).

($P < 0.05$). High dose of cinnamaldehyde and both doses of linalool also significantly prevented endotoxin-induced elevation of nitrate/nitrite ($P < 0.05$). It is noted that high dose of linalool that is equivalent to its content in the tested EO dose appeared to be more effective than that of EO ($P < 0.05$). As to the levels of proinflammatory parameters, we found that EO dramatically suppressed the elevation of all cytokines and HMGB1 induced by endotoxin ($P < 0.05$). Both cinnamaldehyde and linalool were preventive on the elevation of all the proinflammatory parameters in a dose-dependent manner. Both high doses of cinnamaldehyde and linalool showed to possess similar or slightly less protective effective than EO on the prevention of endotoxin-induced elevation of proinflammatory cytokines (Table 2).

3.3. Inflammatory status in spleen and MLNs

To investigate the inflammatory status induce by endotoxin, the present study also determined levels of proinflammatory parameters in spleen and MLNs. It showed that the levels of

nitrate/nitrite, IL-1 β , TNF- α and IFN- γ in these lymphoid organ/tissue were all elevated by endotoxin while were prevented by EO significantly ($P < 0.05$, Table 3). In spleen and MLNs, cinnamaldehyde prevented endotoxin-induced elevation of these proinflammatory parameters in a dose-dependent manner; however, it was likely that linalool had achieved its plateau phase of protective activity at low dose and showed similar protective effect at low and high doses for most parameters. In addition, high doses of cinnamaldehyde and linalool appeared to possess similar protective effect as that of EO for most proinflammatory parameters (Table 3).

3.4. NF- κ B activation and caspase-1 activity in spleen and MLNs

The induction of inflammation by endotoxin involve in the first signal-TLR4 priming and the second signal-NLRP activation that eventually activate the transcription factor NF- κ B and enzyme caspase-1, respectively. We showed that endotoxin induced the activation of NF- κ B, which reflected by its

Table 2 – Peripheral concentration of proinflammatory cytokines and HMGB-1 of control mice or endotoxin-injected mice that did or did not receive the leaf essential oil of *Cinnamomum osmophloeum* Kanehira, cinnamaldehyde, or linalool^y

	Control-V	Endotoxin-V	Endotoxin-EO	Endotoxin-CAL	Endotoxin-CAH	Endotoxin-LINL	Endotoxin-LINH
Nitrate/nitrite (nmol/ml)	129.9 ± 53.4	1058.5 ± 71.0 ^a	903.8 ± 72.7 ^b	1009.0 ± 74.5 ^a	907.3 ± 88.3 ^b	790.6 ± 51.4 ^c	805.5 ± 45.2 ^c
IL-1 β (pg/ml)	10.63 ± 1.91	74.06 ± 3.37 ^a	24.74 ± 2.69 ^d	48.24 ± 3.58 ^b	32.09 ± 4.77 ^c	50.26 ± 4.55 ^b	34.23 ± 5.13 ^c
IL-18 (pg/ml)	153.2 ± 52.7	1118.1 ± 107.5 ^a	489.0 ± 52.2 ^{cd}	1065.6 ± 73.0 ^a	568.9 ± 42.7 ^c	705.8 ± 72.3 ^b	445.7 ± 38.0 ^d
TNF- α (pg/ml)	3.2 ± 2.0	202.5 ± 12.7 ^a	164.2 ± 19.5 ^{bc}	176.6 ± 11.0 ^b	132.9 ± 12.0 ^d	158.7 ± 13.6 ^{bc}	150.9 ± 12.4 ^{cd}
IFN- γ (ng/ml)	0.34 ± 0.17	45.57 ± 3.83 ^a	11.61 ± 3.84 ^c	13.47 ± 4.19 ^c	11.48 ± 5.10 ^c	22.79 ± 2.08 ^b	9.45 ± 1.60 ^c
HMGB-1 (pg/ml)	24.37 ± 3.47	76.86 ± 5.10 ^a	35.60 ± 3.59 ^d	48.68 ± 2.76 ^b	37.48 ± 3.21 ^d	42.59 ± 3.96 ^c	38.69 ± 3.25 ^{cd}

^yValues are the mean ± SD for eight mice per group. control-V, saline-injected mice treated with vehicle; endotoxin-V, endotoxin-injected mice treated with vehicle; endotoxin-EO, endotoxin-injected mice treated with 13 mg/(kg bw) of leaf essential oil of *Cinnamomum osmophloeum* Kanehira; endotoxin-CAL, endotoxin-injected mice treated with 0.45 mg/(kg bw) of cinnamaldehyde; endotoxin-CAH, endotoxin-injected mice treated with 0.9 mg/(kg bw) of cinnamaldehyde; endotoxin-LINL, endotoxin-injected mice treated with 2.6 mg/(kg bw) of linalool; endotoxin-LINH, endotoxin-injected mice treated with 5.2 mg/(kg bw) of linalool.*Significantly different from the control group ($P < 0.05$). a, b, c and d indicate the means within a row not sharing the same superscript letter are significantly different ($P < 0.05$). HMGB, High-mobility group box 1 protein; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

Table 3 – Proinflammatory Cytokines and nitrate/nitrite content of spleen and mesenteric lymph nodes from mice that did or did not receive the leaf essential oil of *Cinnamomum osmophloeum* Kanehira, cinnamaldehyde, or linalool^y

	Control-V	Endotoxin-V	Endotoxin-EO	Endotoxin-CAL	Endotoxin-CAH	Endotoxin-LINL	Endotoxin-LINH
Nitrate/nitrite (nmol/mg prot)	Spleen	1.15 ± 0.37	10.91 ± 3.02 ^a	3.26 ± 0.69 ^{cd}	5.10 ± 0.60 ^b	2.65 ± 0.72 ^d	4.57 ± 0.89 ^{bc}
	MLNs	1.14 ± 0.49	6.47 ± 0.69 ^a	3.46 ± 0.78 ^c	5.05 ± 0.74 ^b	3.19 ± 1.27 ^c	4.80 ± 0.95 ^b
IL-1 β (pg/mg prot)	Spleen	148.5 ± 33.7	534.0 ± 48.1 ^a	219.0 ± 31.3 ^{bc}	265.2 ± 52.7 ^b	192.7 ± 40.9 ^c	250.9 ± 34.6 ^{bc}
	MLNs	24.18 ± 7.83	81.86 ± 11.11 ^a	32.00 ± 6.40 ^c	61.18 ± 11.81 ^{ab}	38.90 ± 16.16 ^{bc}	17.96 ± 9.27 ^c
TNF- α (pg/mg prot)	Spleen	2.57 ± 1.00	45.53 ± 14.18 ^a	9.53 ± 3.11 ^c	14.94 ± 1.67 ^{bc}	23.17 ± 9.37 ^b	11.16 ± 9.42 ^c
	MLNs	1.37 ± 0.81	13.02 ± 3.67 ^a	4.66 ± 1.69 ^{bc}	6.12 ± 0.48 ^b	4.52 ± 0.99 ^{bc}	2.56 ± 0.54 ^c
IFN- γ (pg/mg prot)	Spleen	31.6 ± 8.5	669.3 ± 30.4 ^a	354.7 ± 27.3 ^d	413.6 ± 11.5 ^c	269.8 ± 44.9 ^e	452.4 ± 24.7 ^b
	MLNs	36.7 ± 9.8	339.5 ± 35.1 ^a	169.5 ± 54.9 ^{bc}	243.9 ± 90.9 ^b	122.0 ± 71.9 ^c	126.5 ± 49.8 ^c

^yValues are the mean ± SD for eight mice per group. control-V, saline-injected mice treated with vehicle; endotoxin-V, endotoxin-injected mice treated with vehicle; endotoxin-EO, endotoxin-injected mice treated with 13 mg/(kg bw) of leaf essential oil of *Cinnamomum osmophloeum* Kanehira; endotoxin-CAL, endotoxin-injected mice treated with 0.45 mg/(kg bw) of cinnamaldehyde; endotoxin-CAH, endotoxin-injected mice treated with 0.9 mg/(kg bw) of cinnamaldehyde; endotoxin-LINL, endotoxin-injected mice treated with 2.6 mg/(kg bw) of linalool; endotoxin-LINH, endotoxin-injected mice treated with 5.2 mg/(kg bw) of linalool.*Significantly different from the control group ($p < 0.05$). a, b, c and d indicate the means within a row not sharing the same superscript letter are significantly different ($p < 0.05$). IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

nuclear content, in both spleen and MLNs and was partly prevented by the pretreatment with EO ($P < 0.05$, Fig. 1). The preventive effect of cinnamaldehyde and linalool on endotoxin-induced NF- κ B activation in spleen and MLNs was not as great as that of EO (Fig. 1). In the other hand, we found that in the experimental condition of the present study, the activation of caspase-1 appeared to be more dramatic than that of NF- κ B by endotoxin. We interpreted this as due to that NF- κ B was mainly activated as the priming phase of the acute immune response induced by bolus endotoxin injection while the activation of caspase-1 was the secondary phase and the biological samples in this study were collected at late stage of this acute inflammatory response. It showed that endotoxin induced elevation of caspase-1 activity by 34.8% and 54.0% in spleen and in MLNs, respectively ($P < 0.05$, Fig. 2). The pretreatment with EO significantly prevent the activation of caspase-1 in these lymphoid organ/tissue and both high and low doses of cinnamaldehyde and linalool showed similar preventive effect as that by EO.

3.5. Expression of molecules of the TLR4 and NLRP3 signaling pathway in spleen and MLNs

Endotoxin dramatically increased the expression of TLR4, MD2, and MyD88 in both spleen and MLNs ($P < 0.05$, Fig. 3A and B, respectively). In mice pretreated with EO, the expression of

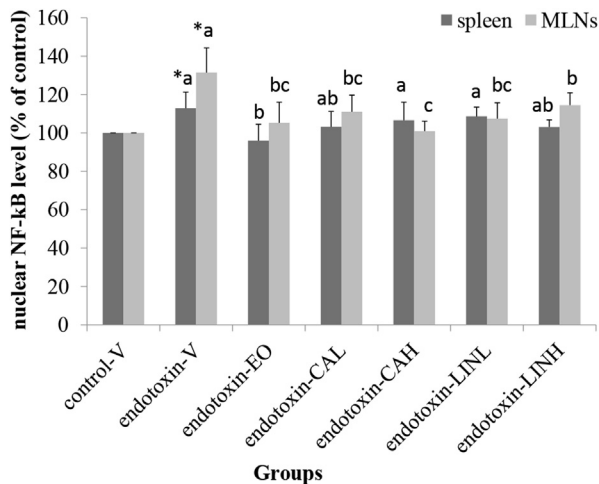


Fig. 1 – Effects of leaf essential oil of *C. osmophloeum*, cinnamaldehyde, and linalool on nuclear factor (NF)- κ B activation in spleen and in mesenteric lymph nodes (MLNs) of endotoxin-injected mice. Mice received by gavage 13 mg/kg leaf essential oil of *C. osmophloeum* (endotoxin-EO), 0.45 mg/kg cinnamaldehyde (endotoxin-CAL), 0.9 mg/kg cinnamaldehyde (endotoxin-CAH), 2.6 mg/kg linalool (endotoxin-LINL), 5.2 mg/kg linalool (endotoxin-LINH), or the vehicle (endotoxin-V) every other day 8 times followed by injection with endotoxin from *S. typhimurium* (i.p., 10 mg/kg). Control mice were pretreated with vehicle (control-V) followed by injection with saline. Data are mean \pm SD for eight mice in each group. *Significantly different from the control ($P < 0.05$). a, b, and c the same tissue not sharing the same letter are significantly different ($P < 0.05$).

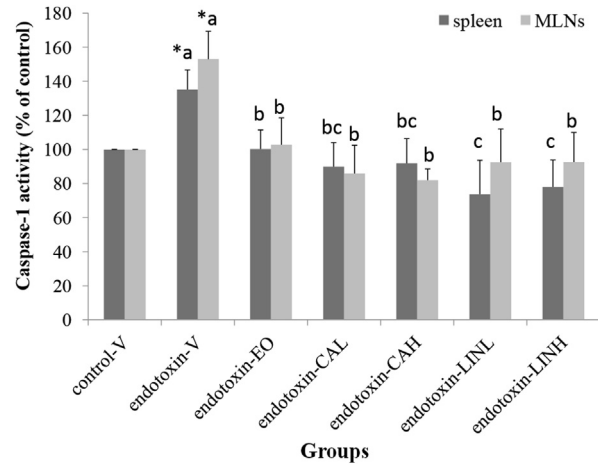


Fig. 2 – Effects of leaf essential oil of *C. osmophloeum*, cinnamaldehyde, and linalool on caspase-1 activity in spleen and in mesenteric lymph nodes (MLNs) of endotoxin-injected mice. Mice received by gavage 13 mg/kg leaf essential oil of *C. osmophloeum* (endotoxin-EO), 0.45 mg/kg cinnamaldehyde (endotoxin-CAL), 0.9 mg/kg cinnamaldehyde (endotoxin-CAH), 2.6 mg/kg linalool (endotoxin-LINL), 5.2 mg/kg linalool (endotoxin-LINH), or the vehicle (endotoxin-V) every other day 8 times followed by injection with endotoxin from *S. typhimurium* (i.p., 10 mg/kg). Control mice were pretreated with vehicle (control-V) followed by injection with saline. Data are mean \pm SD for eight mice in each group. *Significantly different from the control ($P < 0.05$). a, b, and c the same tissue not sharing the same letter are significantly different ($P < 0.05$).

TLR4, MD2, and MyD88 in both spleen and MLNs was significantly suppressed compared with that in the vehicle-treated mice ($P < 0.05$, Fig. 3A and B). Similarly, all tested doses of cinnamaldehyde and linalool significantly prevent endotoxin-induced expression of these key molecules of TLR4 signaling pathway ($P < 0.05$, Fig. 3A and B).

In the other hand, endotoxin also dramatically increased the expression of NLRP3, ASC, and caspase-1 in spleen and MLNs ($P < 0.05$, Fig. 4A and B, respectively). In mice pretreated with EO, the endotoxin-induced expression of NLRP3, ASC, and caspase-1 in both spleen and MLNs was significantly prevented compared with that in the vehicle-treated mice ($P < 0.05$, Fig. 4A and B). Similarly, all tested doses of cinnamaldehyde and linalool significantly prevent endotoxin-induced expression of these key molecules of NLRP3 signaling pathway ($P < 0.05$, Fig. 4A and B).

4. Discussion

We previously reported the composition and protective effect of leaf essential oil of *C. osmophloeum* Kaneh. (EO) on inflammatory status of intestinal mucosa of endotoxin-injected mice [15,16]. The present study intends to investigate whether cinnamaldehyde and linalool the major active

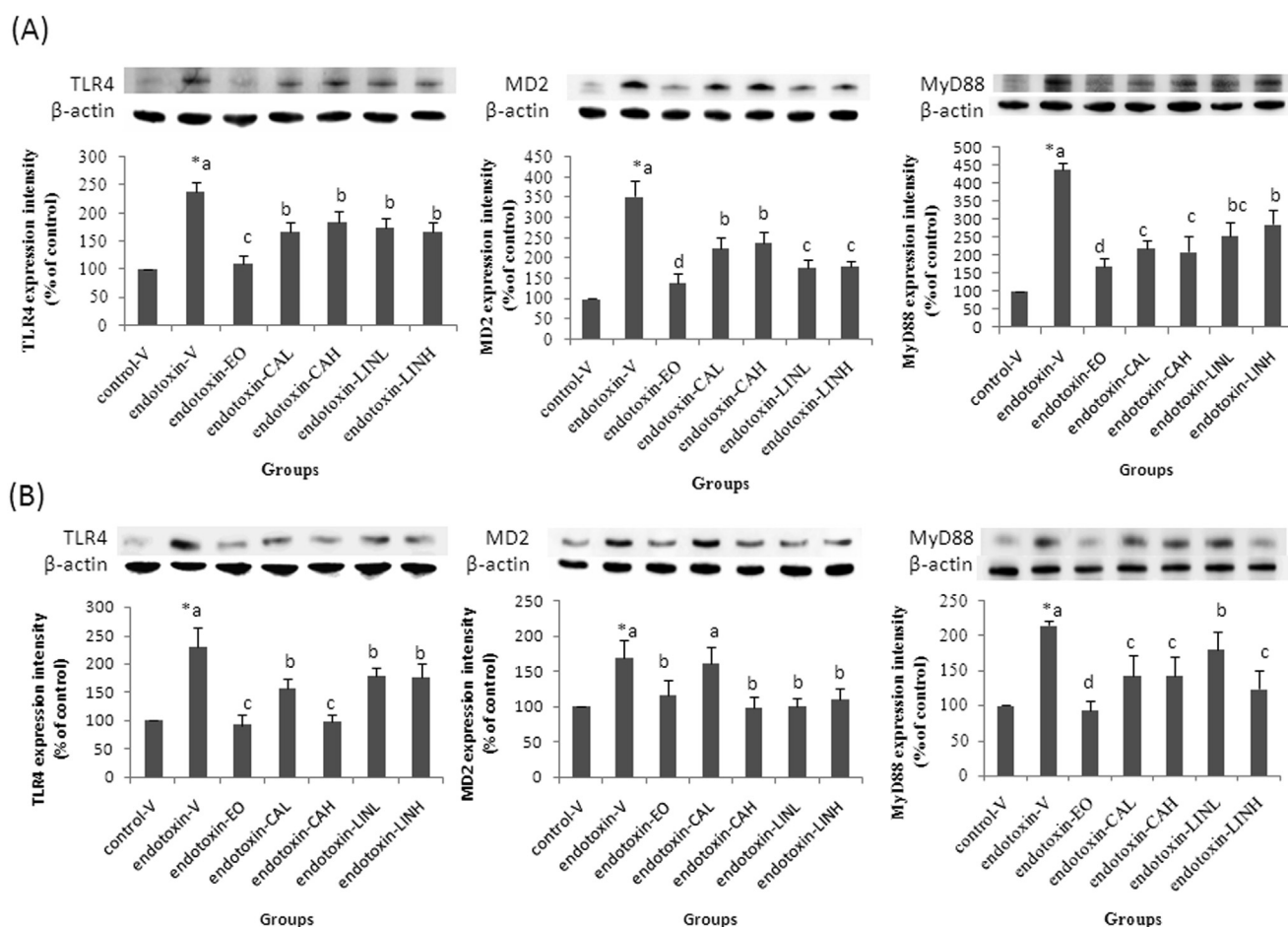


Fig. 3 – Effects of leaf essential oil of *C. osmophloeum*, cinnamaldehyde, and linalool on the expression of TLR4, MD2, and MyD88 in spleen (A) and in mesenteric lymph nodes (MLNs) (B) of endotoxin-injected mice. Mice received by gavage 13 mg/kg leaf essential oil of *C. osmophloeum* (endotoxin-EO), 0.45 mg/kg cinnamaldehyde (endotoxin-CAL), 0.9 mg/kg cinnamaldehyde (endotoxin-CAH), 2.6 mg/kg linalool (endotoxin-LINL), 5.2 mg/kg linalool (endotoxin-LINH), or the vehicle (endotoxin-V) every other day 8 times followed by injection with endotoxin from *S. typhimurium* (i.p., 10 mg/kg). Control mice were pretreated with vehicle (control-V) followed by injection with saline. Data are mean \pm SD for eight mice in each group. *Significantly different from the control ($P < 0.05$). a, b, and c the same tissue not sharing the same letter are significantly different ($P < 0.05$). MD2, myeloid differentiation factor 2; MyD88, Myeloid differentiation primary response gene 88; TLR4, toll-like receptor 4.

constituents responsible for the anti-inflammatory effects of EO and to determine the underlying mechanisms of these two compounds. The present study demonstrated that none of the tested doses of cinnamaldehyde or linalool affected the growth of mice during the pretreatment period. This is consistent with previous data for the safety of cinnamaldehyde and linalool; since these two compounds have been widely used as food additives, the safety of their use has been well established. Both compounds are generally recognized as safe to be used in food by the Flavor and Extract Manufacturers' Association and the Food and Drug Administration (FDA) of the USA. According to the Joint FAO/WHO Expert Committee on Food Additives, Acceptable Daily Intake (ADI) of cinnamaldehyde and linalool are 1.25 mg/kg bw and 0–0.5 mg/kg, respectively [19,20]. In the other hand, according to the US FDA [21], to convert a human dose to equivalent to that in a mouse, the ADI for human should be multiplied by

12.3, accordingly, ADI of cinnamaldehyde and linalool in a mouse are calculated to be 15.4 mg/kg and 6.15 mg/kg, respectively. Thus, the doses 0.45 and 0.9 mg/kg of cinnamaldehyde and the doses 2.6 and 5.2 mg/kg of linalool administered orally to mice in the present study are within the range of the ADI.

After the injection of saline or endotoxin, the body weight of all groups are reduced including the control mice due to that food has been removed after the injection. However, in mice previously received gavage of vehicle, the injection with endotoxin (endotoxin-V group) caused significantly greater body weight loss than the injection with saline (control-V group) which is consistent with the common feature of acute systemic inflammation and can be attributed at least partly to the elevated pro-inflammatory cytokines such as TNF- α and IL-1 β [22]. Furthermore, enlarge of two major lymphoid organs, spleen and MLNs, are observed which is consistent with

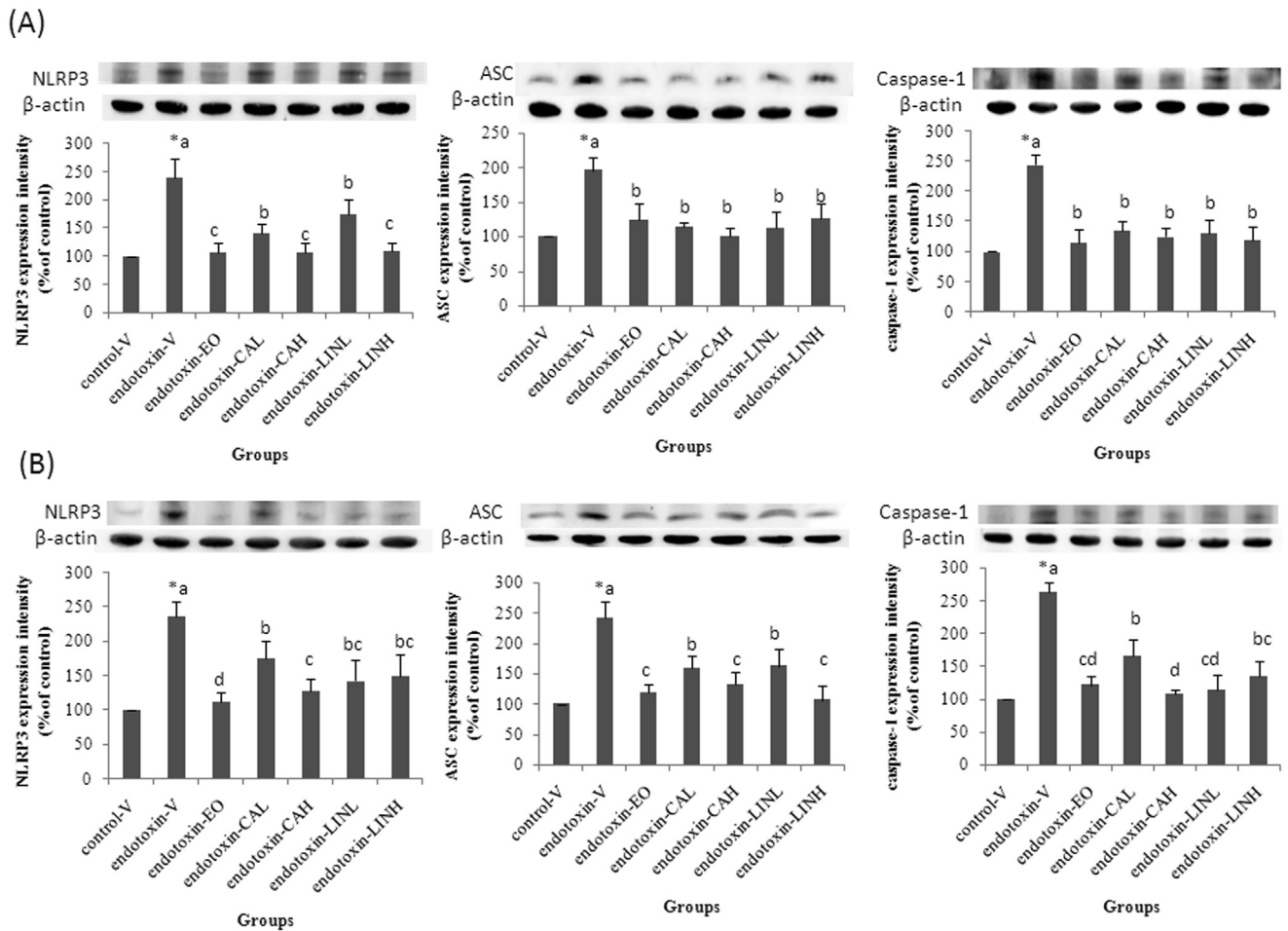


Fig. 4 – Effects of leaf essential oil of *C. osmophloeum*, cinnamaldehyde, and linalool on the expression of NLRP3, ASC, and caspase-1 in spleen (A) and in mesenteric lymph nodes (MLNs) (B) of endotoxin-injected mice. Mice received by gavage 13 mg/kg leaf essential oil of *C. osmophloeum* (endotoxin-EO), 0.45 mg/kg cinnamaldehyde (endotoxin-CAL), 0.9 mg/kg cinnamaldehyde (endotoxin-CAH), 2.6 mg/kg linalool (endotoxin-LINL), 5.2 mg/kg linalool (endotoxin-LINH), or the vehicle (endotoxin-V) every other day 8 times followed by injection with endotoxin from *S. typhimurium* (i.p., 10 mg/kg). Control mice were pretreated with vehicle (control-V) followed by injection with saline. Data are mean \pm SD for eight mice in each group. *Significantly different from the control ($P < 0.05$). a, b, and c the same tissue not sharing the same letter are significantly different ($P < 0.05$). ASC, apoptosis-associated speck-like protein containing a caspase-recruitment domain; NLRP3, Nod-like receptor family, pyrin domain containing 3.

the feature of spleen edema found in endotoxin-induced systemic inflammation in experiment animals [23]. All these general feature of systemic inflammation were prevented by the pretreatment with EO. This protective effect of EO was further confirmed by the significantly lowered levels of peripheral, splenic, and lymphatic nitrate/nitrite and all the proinflammatory cytokines investigated. Both high doses of cinnamaldehyde and linalool showed to ameliorate these parameters significantly and to an extent similar to or slightly less than that of EO suggesting these two compounds the major active anti-inflammatory constituents in EO.

Cinnamaldehyde is a general constituent of cinnamon species and so is it in EO of the present study. Previous *in vitro* study on the anti-inflammatory activity of cinnamaldehyde exhibited its inhibitory effect on endotoxin-induced production of IL-1 β and TNF- α by J774A.1 macrophages and by THP-1

monocytes, and on endotoxin-induced intracellular level of pro-IL-1 β in THP-1 monocytes [24]. Recently, it was also reported that In fructose-induced metabolic syndrome in rats, oral administration of cinnamaldehyde was showed to ameliorate cardiac inflammation reflected by reduced level of IL-1 β in heart tissue [25]. In the present study, cinnamaldehyde was administered orally at doses lower than what was demonstrated to be effective by other researchers in the above mentioned reports and was ranged within ADI of this compound. Thus, data of the present study strengthen the usefulness of cinnamaldehyde as a prophylactic agent for inflammation.

Mechanism of anti-inflammatory activity of cinnamaldehyde has been studied with several *in vitro* and *in vivo* models. In endotoxin-induced J774A.1 macrophages and THP-1 monocytes, the anti-inflammatory act of cinnamaldehyde is

demonstrated to be associated with the inhibition of the TLR4 signaling pathway [24]. In aged rats, the suppression of age-related NF- κ B activation and the subsequent expression of iNOS and cyclooxygenase by the administration of cinnamaldehyde are via the amelioration of oxidative stress-associated signaling pathway [26]. Similarly, it has been shown that the intraperitoneal injection of cinnamaldehyde improved survival of mice induced with viral myocarditis and is associated with inhibited TLR-4-NF- κ B signal transduction pathway [27]. Zhao et al. concluded that compounds with α,β -unsaturated carbonyl groups, such as cinnamaldehyde, inhibit TLR4 activation by interfering with cysteine residue-mediated receptor dimerization, thus suppressing TLR4 signaling pathway [3]. The present study demonstrated that in endotoxin-injected mice, anti-inflammatory role of cinnamaldehyde on the production of pro-inflammation cytokines is associated with inhibited activation of NF- κ B and caspase-1 activity through the suppressed expression of TLR4/MD2, MyD88, NLRP3, ASC, and caspase-1.

Recently, there has been growing interest in the anti-inflammatory activity of linalool. *In vitro*, linalool was reported to inhibit endotoxin-induced production of TNF- α and IL-1 β in RAW264.7 macrophage [28], and TNF- α , IL-1 β , nitric oxide, and prostaglandin E2 in a murine microglial cell line BV2 [29]. With animal models, the administration of linalool intraperitoneally or subcutaneously was demonstrated to ameliorate endotoxin- or a Gram-negative bacteria *Pasteurella multocida*-induced lung injury in mice, respectively, both reflected by reduced lung tissue levels of TNF- α and IL-6 [28,30]. Similarly, in cigarette smoke-induced acute lung inflammation in mice, Ma et al. [31] found a preventive effect of intraperitoneal administration of linalool on the infiltration of macrophages and neutrophils in bronchoalveolar lavage fluid with reduced levels of proinflammatory cytokines including TNF- α and IL-1 β . In consistent with these findings, the present study demonstrated a preventive effect of oral administration of linalool on systemic inflammation induced by endotoxin. It is worth noting that most (if not all) of the *in vivo* study of linalool for its anti-inflammatory effect, with the above mentioned reports as examples, are carried out by giving this compound intraperitoneally or subcutaneously and with higher dose than what we demonstrated in the present study. We for the first time showed that the administration of linalool orally at doses meet safety concern could be a useful prophylactic measure on inflammation associated problem.

The mechanism underlying which linalool acts as an anti-inflammatory agent has currently been revealed in which the inhibition on signaling pathways for the activation of NF- κ B has been suggested to play the central role and was demonstrated with endotoxin-induced-RAW 264.7 macrophages and -BV2 microglial cells *in vitro* [28,29], and with endotoxin- and cigarette smoke-induced lung tissue *in vivo* [28,31]. The activation of nuclear factor erythroid 2-related factor 2 and blockage of mitogen-activated protein kinases signaling pathway has been proposed to attribute the suppressed NF- κ B activity by linalool [29,30]. Similar to the finding of recently published data by other researchers, we demonstrated the inhibitory effect of linalool on the activation of NF- κ B and further showed it to be associated with suppressed expression

of molecules for the signaling pathways of TLR4. In addition, the present study also found suppressed expression of key molecules of NLRP3 inflammasome, NLRP3, ASC, and caspase-1 by linalool. Reduced caspase-1 activity by linalool found in the present study confirmed the suppressed activation of NLRP3 inflammasome by this compound. To the best of the authors' knowledge, this is the first report to show such activity of linalool. Accordingly, previously proposed biological mechanisms of linalool overlap with that of cinnamaldehyde to a certain extent thus may partly explain why when combined together, these two compounds did not show added activity as reflected by that of EO. In the other hand, a possibility that other EO component(s) may disturb the beneficial effect cannot be excluded and remain to be clarified.

In conclusion, the present study confirmed that both cinnamaldehyde and linalool play important role as active constituents in EO for prevention of LPS-induced inflammation and confirmed the potential use of EO as an anti-inflammatory natural product. The novel finding in the present study further include that oral administration of cinnamaldehyde or linalool alone is able to prevent endotoxin-induced systemic inflammation through the inhibition of the expression of molecules in both TLR4 and NLRP3 signaling pathways. Particularly, we showed that such anti-inflammatory effect of cinnamaldehyde and linalool *in vivo* can be exhibited with doses within the proposed range of oral safety. These data provide evidence for cinnamaldehyde and linalool as two potent and safe oral agents for prophylactic use in health problems associated with inflammations that being attributed to over-activated TLR4 and/or NLRP3 signaling pathways.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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