



Research Journal of
**Medicinal
Plant**

ISSN 1819-3455



Academic
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Inhibition of Inflammatory Agent Production by Ethanol Extract and Eugenol of *Syzygium aromaticum* (L.) Flower Bud (Clove) in LPS-Stimulated Raw 264.7 Cells

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ABSTRACT

Various plants and their derived compounds have been used in the treatment of inflammation, including *Syzygium aromaticum* (L.) and eugenol as one of the most active compounds in it. Inflammation is one of an important biological response to injury. Anti-inflammatory is important to treat the danger of acute and chronic inflammation. The objective of the present study was to evaluate anti-inflammatory potential of *S. aromaticum* flower bud (clove) ethanol extract and eugenol from *S. aromaticum* on LPS stimulated-murine macrophage cell line (RAW 264.7). Cell viability assay was performed to evaluate the nontoxic concentration in cell line was performed by MTS assay. The cytotoxic activity which is considered safe on RAW.264.7 cell were 50 and 10 $\mu\text{g mL}^{-1}$ concentration of the *S. aromaticum* flower bud ethanol extract and eugenol. Several measurements were performed including IL-1 β , TNF- α , NO and IL-6 concentration assay by Elisa after treatment compared to non treated cells to determine the anti-inflammatory activity. *Syzygium aromaticum* was inhibited IL-1 β , TNF- α , NO and IL-6 release on LPS stimulated-RAW.264.7. No significant difference was observed in the *S. aromaticum* flower bud extract and eugenol on IL-1 β inhibitory activity but eugenol showed the higher TNF- α inhibitory activity then *S. aromaticum* flower bud extract. The 50 $\mu\text{g mL}^{-1}$ ethanol extract of *S. aromaticum* flower bud showed the highest IL-6 and nitrite-associated NO inhibitory activity. This research revealed that ethanol extract and eugenol of *S. aromaticum* flower bud (clove) possess the anti-inflammatory potential showed by the inhibitory activity of the inflammatory mediator including IL-1 β , TNF- α , NO and IL-6.

Key words: Anti-inflammatory, *Syzygium aromaticum*, eugenol, macrophages, inflammatory mediator

INTRODUCTION

Inflammation is one of an important biological response to injury that relates to various diseases such as rheumatoid arthritis, inflammatory bowel disease, arteriosclerosis, Alzheimer's and has a role in various kinds of cancer (Fang *et al.*, 2008). Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), cytokines (Interleukin (IL)-1 β , IL-6, Tumor Necrosis Factor (TNF)- α) and

NO mediated inflammation and prostaglandin are produced by macrophage during the inflammatory process (Jung *et al.*, 2007). Anti-inflammatory is important to treat the danger of chronic inflammation associated with chronic disease. Anti-inflammatory drugs should have effect on prime causative factors, inhibitory effect or blocking effect on initial reaction set in a biological model by the prime cause and thereby inhibit the established inflammation and effect on end results of established inflammation (Naik and Sheth, 1976).

Bacteria lipopolysaccharide (LPS) exposure is one factor that could increase the cytokines production as inflammation mediator (Kim *et al.*, 2003, 2005). Lipopolysaccharide (LPS) is a pro-inflammatory glycolipid component of the gram negative bacterial cell wall (Boots *et al.*, 2008). Macrophage and inflammatory mediators which are activated by LPS are useful targets for anti-inflammatory drug development (Yoon *et al.*, 2009; Zeilhofer and Brune, 2006).

Natural phytochemicals plays a significant role in drug discovery. Plant extracts are rich of bioactive chemicals and most of them free from adverse effects (Lee *et al.*, 2004). Various plants and their derived compounds have been used in the treatment of inflammation (Agnihotri *et al.*, 2010). *Syzygium* species have anti-inflammatory and antibacterial potential activity (Nassar *et al.*, 2007; Mishra and Sharma, 2014). *Syzygium aromaticum* belongs to Myrtaceae family that widely used for food preservation and medicinal purposes (Amin *et al.*, 2013). *Syzygium aromaticum* were used in folk medicine as diuretic, odontalgic, stomachic, tonic cardiac, aromatic condiment properties and condiment with carminative and stimulant activity (Nassar *et al.*, 2007; Mishra and Sharma, 2014). Phytochemical studies indicate eugenol as one kind of phenylpropanoid class of chemical compound and its derivatives are responsible for the unique aroma of *S. aromaticum* and possess antimicrobial, insecticidal, antioxidant, antitumor, anaesthetic and anti-inflammatory properties (Jung *et al.*, 2012). Eugenol comprising 72-90% of total active constituent in *S. aromaticum* (Amin *et al.*, 2013). Eugenol widely used as a flavoring agent in food product and cosmetic (Krishnaswamy and Raghuramulu, 1998) and utilized as an antiseptic drug, antioxidant and anti-inflammatory activities (Li *et al.*, 2006). The aim of this research is to evaluate anti-inflammatory potential of *S. aromaticum* flower ethanol extract and eugenol extract from *S. aromaticum* on LPS stimulated-murine macrophage cell line (RAW 264.7). The RAW 264.7 cells line is an appropriate model for evaluating and screening of anti-inflammatory agents from plant extract (Yoon *et al.*, 2009).

MATERIALS AND METHODS

Plant extract preparation: The dried flower of *S. aromaticum* was collected from Cimahi-Bandung, West Java, Indonesia in February, 2012. The plants were identified by herbarium staff, Department of Biology, School of Life Science and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. The dried flower of *S. aromaticum* were crushed into fine powder, then extracted with ethanol 70% using maceration technique. Every 24 h the ethanol filtrate was filtered and collected until the filtrate become colorless. Evaporation was performed using 40°C evaporator until the pasta form product was obtained. The extracts were stored at 4°C (Widowati *et al.*, 2013a, b). The *S. aromaticum* extract and eugenol from *S. aromaticum* extract was used as the treatment.

Basic culture of RAW 264.7 cells: The murine macrophage cell line RAW 264.7 (ATCC® TIB-71™) was given by Biomolecular and Biomedical Research Center, Aretha Medika Utama. The RAW 264.7 cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with

10% FBS, 100 U mL⁻¹ penicillin (Biowest), 100 µg mL⁻¹ streptomycin (Biowest) and maintained at 37°C in humidified atmosphere and 5% CO₂ until the cells were confluent. The cells then washed, harvested using trypsin-EDTA (Biowest). The cells then seeded on plates and treated using *S. aromaticum* extract in different concentration (100, 50, 10 µg mL⁻¹) and different concentration of eugenol (100, 50 and 10 µM) (Yoon *et al.*, 2009; Kang *et al.*, 2011).

Viability assay: Cell viability was evaluated by MTS assay. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, WI, USA) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays (Malich *et al.*, 1997). Briefly, 100 µL cells in medium (DMEM supplemented with 10% FBS and 100 U mL⁻¹ penicillin and streptomycin) were plated in 96-well plate (5×10³ cells per well) and incubated for 24 h at 37°C in a humidified atmosphere and 5% CO₂. The medium then washed and added with 99 µL new medium and 1 µL of *S. aromaticum* extract in various concentrations (100, 50 and 10 µg mL⁻¹), eugenol in various concentrations (100, 50 and 10 µM) and DMSO in different plate in triplicate then incubated for 24 h. Untreated cells were served as the control. The 20 µL MTS was added to each well. The plate was incubated in 5% CO₂ at 37°C incubator for 4 h. The absorbance was measured at 490 nm on a microplate reader (MultiSkan Go Thermoscientific). The data is presented as the percentage of viable cells (%) (Widowati *et al.*, 2013a, b). The viability assay was performed to determine the safe and nontoxic concentration for the next assay.

Pro-inflammatory activation of cells: The pro-inflammatory activation of cells was performed based on Yoon *et al.* (2009) and Khan *et al.* (1995) modified method. The cells were seeded in 6 well plate in density of 5×10³ cells per well and incubated for 24 h at 37°C in a humidified atmosphere and 5% CO₂. The medium (DMEM supplemented with 10% FBS and 100U mL⁻¹ penicillin and streptomycin) then washed and supplemented with 1600 µL growth medium and 200 µL extract solution or eugenol in different concentration in 1-2 h prior to the LPS treatment. The 200 µL LPS (1 µg mL⁻¹) was added into the medium and incubated for 24 h at 37°C in a humidified atmosphere and 5% CO₂. The growth medium was taken for the next assay and centrifuged at 2000×g for 10 min. The supernatant was stored at -79°C for the NO, IL-6, IL-1β and TNF-α concentration and inhibitory activity assay.

Determination of IL-1β concentration and inhibitory activity after treatment: The IL-β concentration determination in the cell free supernatant was performed based on the BioLegend (No. Cat 432601) kit protocol. A 100 µL of the capture antibody solution was added into 96-well plate and incubated overnight in 2-8°C one day prior the assay. The cell free supernatant after *S. aromaticum* extract in different concentration (100, 50 and 10 µg mL⁻¹) and different concentration of eugenol (100, 50 and 10 µM) treatment was served as the sample. The plate was washed four times with at least 300 µL of wash buffer then sealed and incubated for 1 h. A 100 µL standard solution was added into the standard well and 100 µL of sample was added into the sample well. The mixture then washed four times after incubated for 2 h with shaking. A 100 µL of detection antibody was added into each well. The mixture then washed four times after incubated for 1 h with shaking. A 100 µL of avidin-HRP solution was added into each well and incubated for 30 min. The plate then washed 5 times and 100 µL of TMB substrate then added. The plate was incubated for 15-30 min until the desired color develops, then the reaction was stopped by adding 100 µL stop solution. The absorbance was read in 450 nm of wavelength. The

LPS-stimulated cells without *S. aromaticum* extract and eugenol was served as positive control. The normal cell was used as negative control (Yoon *et al.*, 2009).

Determination of TNF- α concentration and inhibitory activity after treatment: The determination of TNF- α concentration in the cell free supernatant was performed according to the Biologend Elisa kit (No. Cat 421701). A 100 μ L of capture antibody solution was added into each well in 96 well plate and incubated overnight in 4°C prior to the assay. The plate was washed four times using 300 μ L of Wash Buffer and incubated for one hour in shaker. A 100 μ L of standard solution, then added into each well and 100 μ L of cell free supernatant after *S. aromaticum* extract in different concentration (100, 50 and 10 μ g mL⁻¹) and different concentration of eugenol (100, 50 and 10 μ M) treatment was added into the sample well. The plate then washed four times after 2 h of shaking at room temperature. The 100 μ L of the detection antibody solution, then added to each well and incubated for 1 h with shaking. The plate was washed four times and 100 μ L diluted Avidin-HRP solution was added into each well then incubated with shaking for 30 min. The plate was washed 5 times before 100 μ L addition of TMB substrate solution, then incubated for 15-30 min until the desired color develop. The reaction was stopped by adding 100 μ L of stop solution then the optical density was measured at 450 nm of wavelength. The LPS-stimulated cells without *S. aromaticum* extract and eugenol was served as positive control. The normal cell was used as negative control (Kawamura *et al.*, 2007).

Determination of nitrite associated with NO concentration and inhibitory activity assay after treatment: The determination of nitrite associated with NO production was performed based on Abnova Kit (No cat. KA 1342) protocol. After pre-incubation of RAW 264.7 cells with LPS and *S. aromaticum* extract/eugenol for 24 h, the quantity of nitrite accumulated in the cell free supernatant was measured as an indicator of NO production. A 200 μ L assay buffer was added in the blank well and 100 μ L of standard solution with 100 μ L assay buffer was added into the standard well. Briefly, 100 μ L of cell medium was mixed with 100 μ L assay buffer. The mixture was incubated at room temperature for 10 min. The absorbance at 540 nm was measured in a microplate reader (MultiSkan Go Thermoscientific). The quantity of nitrite was determined from sodium nitrite standard curve. The LPS-stimulated cells without extract or eugenol was used as positive control. The normal cell was used as negative control.

Determination of IL-6 concentration and inhibitory activity assay after treatment: Interleukin-6 (IL-6) was assayed using BioLegend Elisa kit (No. Cat 431301). A 100 μ L capture antibody in coating buffer was added into each well then sealed and incubated in 4°C overnight. The plate was washed using 200 μ L wash buffer four times. A 200 μ L assay diluent was added to each well to block non-specific binding and reduce background and incubated 1 h in 200 rpm shaker. Plate then washed for 4 times using wash buffer. A 100 μ L standard was added into standard well and 100 μ L sample was added into sample well, plate then sealed and incubated in 200 rpm plate shaker for 2 h in room temperature. The plate then washed for 4 times using wash buffer. A 100 μ L detection antibody solution was added to each well, sealed and incubated for 1 h with shaking. The plate then washed for 4 times with wash buffer. A 100 μ L avidin HRP solution was added into each well and kept at room temperature for 30 min in 200 rpm shaker. The plate then washed again for 5 times and 100 μ L TMB substrate was added into each well and incubated

for 15-30 min in the dark until desired color develops. The reaction was stopped by adding 100 μ L stop solution to each well. The absorbance was read by using microplate reader (MultiSkan Go ThermoScientific) at 450 nm of wavelength (Kuroishi *et al.*, 2013).

Statistical analysis: All data was derived from three independent experiments. Statistical analysis was conducted using SPSS software (version 20.0). Value were presented as Mean \pm Standard Deviation. Significant differences between the groups were determined using the Analysis of variance (ANOVA) followed by Tukey HSD *post hoc* Test $p < 0.05$ were considered as statistical significance.

RESULT

Effects of *Syzygium aromaticum* on RAW 264.7 cells viability: The RAW 264.7 cell viability assay was the preliminary study to test the effect of *S. aromaticum* flower extract and eugenol toward RAW 264.7 cell viability. The aim of this assay is to determine the safe and non toxic concentration for the next assay. Viability was measured by MTS assay based on the conversion of yellow tetrazolium salt to form a purple formazan product. The percent of cells viability was determined by comparing the cells viability value of treatments to the control. This viability assays (Table 1) shows that *S. aromaticum* flower extract and eugenol in giving concentration still usable for the normal RAW 264.7 cells except for the concentration of 100 μ g mL⁻¹. At the highest concentration of the sample the RAW 264.7 cells viability was low which, indicate that the concentration is little bit toxic to the cells so that concentration will not used in the next step of the assay.

IL-1 β concentration and inhibitory activity: IL-1 which refers to two proteins (IL-1 α and IL-1 β) is a potent immuno-modulator which mediates a wide range of immune and inflammatory responses including activation of B and T cells (Rapsinski *et al.*, 2015). Inhibiting the production of IL-1 was important in finding the anti-inflammatory agent. *Syzygium aromaticum* flower bud extract and eugenol was showed the inhibitory potential against IL-1 β production with no significance difference (Table 2). The positive control (LPS-stimulated cells free supernatant

Table 1: RAW 264.7 cell viability percentage of various concentration of *Syzygium aromaticum* flower bud extract and eugenol measured in triplicate

Samples	Concentration (μ g mL ⁻¹)		
	100 (100 μ M)	50 (50 μ M)	10 (10 μ M)
<i>Syzygium aromaticum</i> flower bud extracts	89.04 \pm 0.53 ^a	92.15 \pm 0.48 ^a	98.44 \pm 1.67 ^b
Eugenol	90.80 \pm 0.53 ^a	97.09 \pm 8.08 ^a	100.37 \pm 6.63 ^b

Data was presented as Mean \pm Standard Deviation, Different superscript letters in the same row (among concentrations of samples) are significant at $p < 0.05$ Tukey HSD *post hoc* test

Table 2: IL-1 β level and IL-1 β inhibitory activity over the positive control of various concentration *Syzygium aromaticum* flower bud extracts and eugenol measured in triplicate

Samples	IL-1 β level (pg mL ⁻¹)	IL-1 β inhibitory activity (%) over positive control
Positive control	1185.31 \pm 35.34 ^b	0.00 \pm 2.98 ^a
Negative control	844.05 \pm 18.08 ^a	28.79 \pm 1.52 ^b
<i>Syzygium aromaticum</i> flower extracts (50 μ g mL ⁻¹)	898.90 \pm 131.80 ^a	26.44 \pm 7.36 ^b
<i>Syzygium aromaticum</i> flower extract (10 μ g mL ⁻¹)	892.92 \pm 89.81 ^a	26.43 \pm 7.68 ^b
Eugenol (50 μ M)	871.87 \pm 87.20 ^a	24.16 \pm 11.12 ^b
Eugenol (10 μ M)	872.00 \pm 91.05 ^a	24.67 \pm 7.58 ^b

Data was presented as Mean \pm Standard Deviation, Different superscript letters (a, b) in the same column (IL-1 β concentration and inhibitory activity among treatments) are significant at $p < 0.05$, Tukey HSD *post hoc* test

without extract or eugenol) showed the highest IL-1 β concentration it decreased in the treatment was used to determine the inhibitory activity. The negative control showed the lowest IL-1 β concentration because it was the normal cells so the inhibitory activity become high if it measured over the positive control.

TNF- α concentration and inhibitory activity: TNF- α is a multi functional cytokine which can exert regulatory, inflammatory and cytotoxic effects on a wide range of lymphoid and non-lymphoid cells and tumor cells. (Kawamura *et al.*, 2007). *Syzygium aromaticum* flower extract and eugenol was showed the inhibitory activity against TNF- α production based on the lower concentration of TNF- α compared to the positive control (LPS-stimulated cells free supernatant without extract or eugenol). The high TNF- α inhibitory activity that showed by negative control was due to the low concentration of TNF- α in the normal cell that served as negative control. Eugenol was more active in inhibiting TNF- α compared to *S. aromaticum* flower extract (Table 3). No significant difference was observed in inhibitory activity of both of eugenol and *S. aromaticum* flower extract in both concentrations.

Nitrite associated with NO concentration and inhibitory activity: The positive control of this test shows the highest concentration of nitrite concentration compared to the negative control and extract or eugenol treated cells (Table 4). That indicates the LPS successfully induce the inflammation of the RAW 264.7 cells (Sarkar and Fisher, 2006). The percent of inhibitory activity was determined by the value of positive control nitrite concentration minus the nitrite concentration of treatment divided to the nitrite concentration of positive control. A 50 $\mu\text{g mL}^{-1}$ *S. aromaticum* flower ethanol extracts shows the highest nitrite inhibitory activity (Table 5). The negative control shows the highest value of nitrite inhibitory activity too because the normal cell was used. The normal cell did not release much nitrite like LPS-stimulated cells so the inhibitory activity becomes high.

Table 3: TNF- α level and TNF- α inhibitory activity over the positive and negative control of various concentration clove extracts and eugenol measured in triplicate

Samples	TNF- α level ($\mu\text{g mL}^{-1}$)	TNF- α inhibitory activity (%) over positive control
Positive control	475.15 \pm 46.34 ^e	0.00 \pm 9.75 ^a
Negative control	227.49 \pm 10.45 ^a	52.12 \pm 2.20 ^d
<i>Syzygium aromaticum</i> flower extract (50 $\mu\text{g mL}^{-1}$)	384.35 \pm 48.32 ^{bc}	19.11 \pm 10.17 ^{ab}
<i>Syzygium aromaticum</i> flower extract (10 $\mu\text{g mL}^{-1}$)	368.39 \pm 96.47 ^{bc}	22.47 \pm 12.30 ^{ab}
Eugenol (50 μM)	347.01 \pm 17.59 ^{ab}	26.97 \pm 3.70 ^{bc}
Eugenol (10 μM)	340.43 \pm 15.35 ^{ab}	28.35 \pm 3.23 ^{bc}

Data was presented as Mean \pm Standard Deviation, Different superscript letters in the same column (TNF- α concentration and inhibitory activity among treatments) are significant at $p < 0.05$, Tukey HSD *post hoc* test

Table 4: Nitrite content and nitrite inhibitory activity over the positive control of various concentrations of *Syzygium aromaticum* flower extract and eugenol measured in triplicate

Samples	Nitrite concentration (μM)	Nitrite inhibitory activity (%) over positive control
Positive control	35.60 \pm 0.09 ^f	0.00 \pm 0.24 ^a
Negative control	5.18 \pm 1.38 ^a	85.44 \pm 3.89 ^f
<i>Syzygium aromaticum</i> aromaticum flower extract (50 $\mu\text{g mL}^{-1}$)	17.77 \pm 0.11 ^b	50.08 \pm 0.31 ^e
<i>Syzygium aromaticum</i> aromaticum flower extract (10 $\mu\text{g mL}^{-1}$)	23.15 \pm 0.84 ^e	34.96 \pm 0.24 ^c
Eugenol (50 μM)	18.82 \pm 0.09 ^d	47.14 \pm 0.26 ^d
Eugenol (10 μM)	27.28 \pm 0.02 ^e	23.37 \pm 0.05 ^b

Data was presented as Mean \pm Standard Deviation, Different superscript letters in the same column (Nitrite concentrations and inhibitory activity among treatments) are significant at $p < 0.05$, Tukey HSD *post hoc* test

Table 5: IL-6 concentration and IL-6 inhibitory activity over the positive control of various concentration *Syzygium aromaticum* flower extract and eugenol measured in triplicate

Samples	IL-6 concentration (pg mL ⁻¹)	IL-6 inhibitory activity (%) over positive control
Positive control	533.67±17.43 ^f	0.00±3.27 ^a
Negative control	149.33±10.93 ^a	72.02±2.05 ^e
<i>Syzygium aromaticum</i> flower extract (50 µg mL ⁻¹)	145.00±16.28 ^a	72.83±3.05 ^e
<i>Syzygium aromaticum</i> flower extract (10 µg mL ⁻¹)	213.00±30.01 ^b	60.08±5.62 ^d
Eugenol (50 µM)	267.42±24.61 ^c	49.89±4.61 ^c
Eugenol (10 µM)	411.67±32.17 ^d	22.86±6.03 ^b

Data was presented as Mean±Standard Deviation, Different superscript letters in the same column (IL-6 concentrations and inhibitory activity among treatments) are significant at $p < 0.05$, Tukey HSD *post hoc* test

Determination of IL-6 concentration and inhibitory activity: Inhibitory effect toward IL-6 pro-inflammatory cytokine shows that *S. aromaticum* flower extract (50 and 10 µg mL⁻¹) and eugenol (50 and 10 µg mL⁻¹). The *S. aromaticum* flower extract has greater effect on decreasing IL-6 level than eugenol. IL-6 is one of the cytokine that possess biological activities due to acute inflammation (Bertolini *et al.*, 2001).

DISCUSSION

Various substances from plants are playing beneficial role in the prevention and even treatment of different diseases (Luximon-Ramma *et al.*, 2003; Leontowicz *et al.*, 2006). Several studies have reported that phytochemical content of *S. aromaticum* exerts pharmacology actions including antioxidant, hypoglycemic and anti-inflammatory activities (Jung *et al.*, 2012). Eugenol as the main ingredients of *S. aromaticum* have been shown to modulate some immune response, including anti-inflammatory effects (Ko *et al.*, 1995; Dibazar *et al.*, 2014a). In this study, we have evaluated the anti-inflammatory potential of ethanol extract and eugenol of *S. aromaticum* flower bud extract through IL-1 β , TNF- α , NO and IL-6 inhibitory activity assays in LPS stimulated-murine macrophage cell line (RAW 264.7). The LPS is a principle component of the outer membrane of gram negative bacteria as endotoxin that induces the production of proinflammatory mediators such as Nitric Oxide (NO), IL-1, IL-6, TNF- α , interleukins, prostanoids and leukotrienes (Hewett and Roth, 1993; Mahajna *et al.*, 2014).

The ethanol extract and eugenol of *S. aromaticum* in 10 and 50 µg mL⁻¹ of concentration displayed positive result and no toxicity to RAW 264.7. Non toxicity of that substrate was indicated by over 90% of cells was viable in viability test by MTS assay. Viability test is an important aspect of pharmacology that deals with the adverse effect of bioactive substance on living organism prior to the use as drug or chemical in clinical use (Jothy *et al.*, 2011; Lalitha *et al.*, 2012; Rajalakshmi *et al.*, 2014). The ethanol extract and eugenol of *S. aromaticum* inhibited IL-1 β , TNF- α , NO and IL-6 secretion on LPS stimulated-RAW.264.7.

The TNF- α is an important cytokine that involved in inflammatory response by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), cytokine and adhesion molecule inducer (Tak and Firestein, 2001; Libby, 2002; De Cassia da Silveira e Sa *et al.*, 2014). The TNF- α is an important target of anti-inflammatory agent screening (Boots *et al.*, 2008). Blocking one of the cytokine in particular TNF- α can be sufficient as anti-inflammatory since it exists in cascades (Dinarello, 2010). The TNF- α along with IL-1 β and IL-6 serve as endogenous pyrogens that causes fever during inflammation by up regulating the inflammatory responses and stimulating the production of acute phase reactants (Damte *et al.*, 2011). The TNF- α also stimulate additional inflammatory pathways result in additional inflammatory pathways resulting nitric oxide (NO) (De Cassia da Silveira e Sa *et al.*, 2014). Activity of *S. aromaticum* especially due to its major essential oil-eugenol to inhibit the IL-1 β and TNF- α was in accordance to Rodrigues *et al.* (2009) studies in LPS-stimulated human macrophage.

Nitric oxide inhibitory activity is important for anti-inflammatory agent screening. Nitric oxide plays a significant role in host immune defense, vascular regulation, neurotransmission and other system in normal condition. Overproduction of inducible NO Synthase (iNOS) is especially related to various human diseases including inflammation (Kang *et al.*, 2011). This research showed that ethanol extract and eugenol from *S. aromaticum* can inhibit the nitrite production as an indicator of nitric oxide (NO) synthesis. This study is in accordance with study showing that ethanol extract from *S. aromaticum* suppressed the NO release in LPS-stimulated cells. (Dibazar *et al.*, 2014b). Li *et al.* (2006) also confirmed eugenol as the most compound in *S. aromaticum* also have NO inhibitory activity due to the inhibition of protein synthesis of inducible nitrite oxide synthase (iNOS). Eugenol also suppresses nuclear factor κ B (NF- κ B) activation in LPS-stimulated macrophage (Gunawardena *et al.*, 2014). The NF- κ B is a transcription factor that regulates the iNOS expression associated with NO (Li *et al.*, 2006). The LPS can induce iNOS transcription and transduction with subsequent NO production in murine macrophage RAW 264.7 cells. Among the over secreted inflammatory mediator, NO has been strongly implicated the pathogenesis of several diseases (MacMicking *et al.*, 1997).

The IL-6 is a major inducer in the acute phase response of inflammation (Mahajna *et al.*, 2014). In this study, ethanol extract and eugenol of *S. aromaticum* in 10 and 50 $\mu\text{g mL}^{-1}$ of concentration showed IL-6 inhibitory activity. Another study also confirmed that *S. aromaticum* could inhibit IL-1 β and IL-6 production after LPS-stimulated cells (Bachiega *et al.*, 2012; Dibazar *et al.*, 2014a). The eugenol as main component of *S. aromaticum* also could suppress IL-6 in LPS-stimulated macrophage (Rodrigues *et al.*, 2009; Miguel, 2010). The IL-6 production has been detected in many cell types. Macrophages and monocytes at sites of inflammation are the primary source of the cytokine during acute inflammation. IL-6 along with TNF- α and IL-1 is elevated in condition of septic or aseptic inflammation. The NF- κ B also control the IL-6 production (Naugler and Karin, 2008). IL-6 concentration is increased at the site of inflammation and blockade of IL-6 and IL-6 signaling is effective at prevention and treatment in models of inflammatory disease (Gabay, 2006). In addition to IL-1 β , TNF- α , NO and IL-6 inhibitory activity, based on the phytochemical screening, the presence of flavonoid, resins, glycosides, tannins, saponin and alkaloid in *S. aromaticum* ethanol extract was revealed that have potential become anti-inflammatory agent (Tanko *et al.*, 2008). Flavonoid and tannins have been prove to potently inhibit prostaglandins as one of inflammatory mediator (Tanko *et al.*, 2008; Manthey, 2000). Tanko *et al.* (2008) evaluated that the ethanol extract from *S. aromaticum* flower bud also demonstrated anti-inflammatory activity against formalin-induced oedema in rats. Eugenol as majority of *S. aromaticum* essential oil can inhibited Cox-2 expression in a model of inflammation using LPS-stimulated macrophages (Kaczor and Fabno, 2010).

CONCLUSION

This research revealed that ethanol extract and eugenol of *S. aromaticum* flower bud (clove) possess the anti-inflammatory potential showed by the inhibitory activity of the IL-1 β , TNF- α , NO and IL-6 secretion. However, further mode of action test, preclinical and clinical studies should be pursued before pharmaceutical applications.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support of the Research Center and Service Community, Maranatha Christian University, Bandung, Indonesia for research grant 2014. This study was supported by Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, Indonesia for laboratory facilities and research methodology. We are thankful to Pande

Putu Erawijantari from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, West Java, Indonesia for her valuable assistance.

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