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Anti-Inflammatory Potential of Gandarusa (*Gendarussa vulgaris* Nees) and Soursoup (*Annona muricata* L) Extracts in LPS Stimulated-Macrophage Cell (RAW264.7)

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Abstract

Inflammation is one of the important biological responses to injury. Anti-inflammatory is therefore proposed to treat both acute and chronic inflammation. Chemical compounds of various plants are widely used in treatment of inflammation. **Objective:** This study aims to evaluate anti-inflammatory potential of *G. vulgaris* extract (GVE) and *A. muricata* extract (AME) on LPS-stimulated murine macrophage cell line (RAW264.7). Cell viability assay to evaluate nontoxic concentration in cell line was performed with MTS assay. Parameters to determine anti-inflammatory activity between treatment group and non treated cells, were IL-1β, TNF-α, and IL-6 which was measured with Elisa, and NO level which was measured with nitrate/nitrite colorimetric assay. Both GVE and AME of 50 and 10 µg/mL showed high viability (>90%) and it was not significantly different compared to control, makes it suitable for treatment. GVE and AME of 50 µg/mL resulted low TNF-α level in RAW264.7(313.16pg/mL and 264.69 pg/mL respectively), as well as IL-1β level (903.53 pg/mL and 905.00 pg/mL respectively) and IL-6 (175.88 pg/mL and 219.13 pg/mL respectively). Whereas, GVE and AME of 75 µg/mL showed lower NO level (9.76 µM and 9.79 µM respectively) compared to untreated cells. This research revealed that GVE and AME possess the anti-inflammatory potential indicated by inhibition of inflammatory mediators including TNF-α, IL-1β, IL-6 and NO.

Keywords: *Annona muricata* L, Anti-inflammatory, *Gendarussa vulgaris* Nees, RAW264.7 Cell Line

1. Introduction

Inflammation is an important biological response to injury that relates to various diseases such as rheumatoid arthritis, inflammatory bowel disease, artherosclerosis, Alzeimer's disease and cancer[1]. Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), cytokines (Interleukin (IL)-1β, IL-6, Tumor Necrosis Factor (TNF)-α) and Nitric Oxide (NO) mediated inflammation and prostaglandin, are produced by macrophage during the inflammatory process[2]. Anti-inflammatory is proposed to prevent chronic inflammation associated with chronic disease.

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It has been reported that bacterial lipopolysaccharide (LPS) is able to increase cytokines production as inflammation mediator[3,4]. LPS has pro-inflammatory property in its glycolipid which compose gram negative bacterial cell wall[5]. Macrophage and inflammatory mediators activated by LPS are appropriate targets in anti-inflammatory drug development[6,7].

Natural phytochemicals play a significant role in drug discovery. Plant extracts contain bioactive chemicals and most of them found free from adverse effects[8]. These chemical compounds are widely used in treatment of inflammation[9]. Flavonoids found in plants have a great potential as anti-inflammatory agents. *G. vulgaris* and *A. muricata* are common plants to contain such compounds. It has been reported that both plants show significant anti inflammatory activity[10–13].

The aim of this research is to evaluate anti-inflammatory potential of *G.vulgaris* extract (GVE) and *A. muricata* extract (AME) on LPS stimulated-murine macrophage cell line (RAW264.7). The RAW264.7 cell line is an appropriate model for evaluating and screening of anti-inflammatory agents from plant extract[6].

2. Materials and Methods

2.1 Plant Extract Preparation

Leaves of *G. vulgaris* and *A. muricata* were collected from Traditional Medicine Research Center (Balai Penelitian Tanaman Rempah dan Obat), Bogor, West Java, Indonesia. The plants were identified by herbarium staff, Research Center of Biology, Indonesia Institute of Science, West Java, Indonesia. Simplicia of *G. vulgaris* and *A. muricata* of 500 g were extracted with ethanol 96% using maceration technique. Ethanol filtrate was filtered, and wastes were re-macerated in triplicate. Macerates were concentrated using 50°C rotavapor to obtain extract. The extracts were stored at -20°C[14,15]. GVE and AME were used as the experiment.

2.2 Cell Culture

The murine macrophage cell line RAW264.7 (ATCC®TIB-71TM) was given by Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung. The RAW264.7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Biowest L0104) supplemented with 10% Fetal Bovine Serum (FBS) (Biowest S181H), 1% penicillin-streptomycin (Biowest L0022) and maintained at 37°C in humidified atmosphere and 5% CO_2 until the cells were confluent. The cells were then washed, and harvested using trypsin-EDTA (Biowest L0931-500). The cells were seeded on plates and treated using GVE and AME in different concentration (0.4, 2, 10, 50, 150, 250, and 500 µg/mL)[6].

2.3 RAW264.7 Cells Viability Assay

Cell viability was performed with 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.Briefly, 100 µL cells in medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin) were plated in 96-well plate (5×10^3 cells per well) and incubated for 24 h at 37°C in a humidified atmosphere and 5% CO_2. The medium was washed and added with 99 µL of new medium and 1 µL of GVE and AME in various concentrations (0.4, 2, 10, 50, 150, 250, and 500 µg/mL), 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% CO_2 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 (%)[14,15]. The viability assay was conducted to determine the safe and nontoxic concentration for the next assay.

2.4 Pro-Inflammatory Activation of RAW264.7 Cells

The pro-inflammatory activation of cells was performed based on modified method[7,8]. The cells were seeded in 6 well plate in density of 5×10^5 cells per well and incubated for 24 h at 37°C in a humidified atmosphere and 5% CO_2. The medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin) was washed and supplemented with 1600 µL of growth medium and 200 µL of extract in different concentration in 1-2 h prior to LPS treatment. The 200 µL LPS (1 µg/mL) was added into the medium and incubated for 24 h at 37°C in a humidified atmosphere and 5% CO_2. 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.

2.5 Measurement of TNF-α, IL-β, and IL-6, Concentration and Inhibitory Activity

Biolegend ELISA kit was used to measure quantification of TNF-α (430901), IL-6 (431301) and IL-β (432601). Briefly, antibody solution was added into each well of 96 well plate, and then incubated in 4^0C overnight. Cell-free supernatant after treated with GVE and AME in different final concentration (10, 50 and 75 µg/mL), were added and then shaked for 2 h. Antibody solution was added and incubated for 1 h in orbital shaker. Avidin-HRP solution and TMB substrate solution was added to each well. TMB will be oxidated by peroxidase enzymes that indicated performed blue colour. Concentration of cytokines were determined by comparing the OD of the samples to the standard curve. LPS-stimulated cells without GVE and AME, were served as positive control. The normal cell was used as negative control[6,7].

2.6 Measurement of NO Concentration and Inhibitory Activity

The determination of nitrite associated with NO production was performed based on Abnova Kit (No cat. KA 1342) protocol. After pre-incubation of RAW264.7 cells with LPS and GVE and AME for 24 h, the quantity of nitrite accumulated in the cell-free supernatant was measured as an indicator of NO production. Two hundred µL assay buffer was added in the blank well and 100 µL of standard solution with 100 µL assay buffer was added into the standard well. Briefly, 100 µL of cell medium was mixed with 100 µL 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-induced cell without extract was used as positive control. The normal cell was used as negative control[6,7].

2.7 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± Standard Deviation. Significant differences between the groups were determined using the Analysis of variance (ANOVA) followed by Duncan *post hoc* Test.

3. Result

3.1 Effect of *G.vulgaris* and *A. muricata* Extracts on Viability of RAW264.7 Cell Line

The RAW264.7 cell viability assay was the preliminary study to test the effect of GVE and AME toward RAW264.7 cell viability. Viability was measured by MTS assay based on the conversion of yellow tetrazolium salt to form a purple formazan product. Percentage of cells viability was determined by comparing cells viability value of treatments to the control.

As shown in Table 1, cell treated with GVE and AME at concentration of 150, 250, and 500 µg/mL resulted low viability which indicates toxicity to RAW264.7, whereas concentration of 0.4, 0.2, 10 and 50 µg/mL of both GVE and AME showed high viability (>90%). Viable cells obtained at concentration of 10 and 50 µg/mL in both GVE and AME, appeared to reach normal level (control), makes such concentrations suitable for treatment of RAW264.7 cells. Therefore, further analysis

Table 1: Effect of *G. vulgaris* and *A. muricata extracts toward* viability of RAW264.7 cell line

Concentration (µg/mL)	Cell Viability	
	G. vulgaris extract	*A. muricata extract*
Control	100.00±3.45 ^{d}	100.00±3.40 ^{d}
0.4	125.58±0.85 ^{f}	131.16±2.19 ^{f}
2	124.41±2.71 ^{f}	127.53±1.18 ^{ef}
10	119.29±4.27 ^{e}	127.95±3.82 ^{ef}
50	115.69±1.08 ^{e}	125.66±3.67 ^{e}
150	77.24±2.79 ^{c}	68.58±0.89 ^{c}
250	52.26±1.29 ^{b}	55.65±3.22 ^{b}
500	2.45±0.59 ^{a}	6.36±0.64 ^{a}

*Note: Data is presented as average of ± SD from 3 replications. Letter a, b, c, d, e, and f, in each column indicates significance different among concentrations based on Duncan post hoc test with $p < 0.05$ is considered as significantly different.

of GVE and AME uses concentration in range of 10 and 150 µg/mL.

3.2 Effect of *G.vulgaris* and *A. muricata* Extracts on TNF-α Level in LPS-Induced RAW264.7 Cell Line

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. GVE and AME 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). As shown in Table 2, GVE and AME decreased TNF-α level compared to positive control.

Treatment of AME at concentration of 50 µg/mL in RAW264.7 resulted lowest TNF-α level 264.69 pg/mL among other treatments. Level of TNF-α in LPS-induced RAW264.7 treated with AME of 50 µg/mL, was significantly different compared to TNF-α level in positive control (Table 2). These results indicate AME of 50 µg/mL decreased TNF-α level to play its role as anti-inflammatory. Whereas GVE at concentration of 50 µg/mL also generated relatively low TNF-α level (313.16 pg/mL). Both GVE and AME of 50 µg/mL showed significant difference compared to positive control, and resulted good inhibitory activity of TNF-α over positive control (36.46 and 46.82% respectively). AME 50 Ug/mL was the best activity to lower TNF-A level and comparable with negative control.

3.3 Effect of *G.vulgaris* and *A. muricata* Extracts on IL-1β level in LPS-Induced RAW264.7 Cell Line

IL-1 which refers to two proteins (IL-1α and IL-1β), is a potent immuno-modulator which mediates a wide range of immune and inflammatory responses including activation of B and T cells[16]. Inhibiting the production of IL-1 is important in finding the anti-inflammatory agent. GVE and AME showed the inhibitory potential against IL-1β production (Table 3). Effect of GVE and AME level on IL-1β level in LPS-induced RAW264.7 is presented in Table 3.

As shown in Table 3, GVE and AME at concentration of 10, 50 and 75 µg/mL decreased IL-1β level in LPS-induced RAW264.7, which was significant compared to positive control and comparable with negative control. GVE at concentration of 50 µg/mL resulted highest decreased IL-1β level of 903.53 pg/mL.

3.4 Effect of *G.vulgaris* and *A. muricata* Extracts on IL-6 Level in LPS-Induced RAW264.7 Cell Line

IL-6 is one of the cytokines that possess biological activities due to acute inflammation[17]. IL-6 along

Table 2: Effect of *G. vulgaris* and *A. muricata* extracts toward TNF-α level in RAW264.7 cell line

Treatments	TNF-α	
	Level of TNF-α (pg/mL)	Inhibitory activity of TNF-α over positive control (%)
Negative control	236.28±17.25 [a]	52.06±3.50 [d]
Positive control	492.86±28.96 [d]	-0.00±5.88 [a]
G. vulgaris extract 75 µg/mL	403.75±12.71 [c]	18.08±2.57 [b]
G. vulgaris extract 50 µg/mL	313.16±29.65 [b]	36.46±6.01 [c]
G. vulgaris extract 10 µg/mL	343.97±16.72 [b]	30.21±3.39 [c]
A. muricata extract 75 µg/mL	351.97±21.45 [b]	29.29±4.31 [b]
A. muricata extract 50 µg/mL	264.69±11.54 [a]	46.82±2.31 [d]
A. muricata extract 10 µg/mL	316.05±24.02 [b]	36.50±4.82 [c]

*Note: Data is presented as average of ± SD from 3 replications. Letter a, b, c, d in each column indicates significance different among treatments based on Duncan post hoc test with $p < 0.05$ is considered as significantly different.

Table 3: Effect of *G. vulgaris* and *A. muricata extracts toward* IL-1β level in RAW264.7 cell line

Treatments	IL-1β	
	Level of IL-1β (pg/mL)	Inhibitory activity of IL-1β over positive control (%)
Negative control	888.53±8.11 [a]	20.09±0.73 [c]
Positive control	1111.93±4.67 [b]	0.00±0.42 [a]
G.vulgaris extract 75 µg/mL	954.87±16.64 [a]	14.13±1.50 [b]
G.vulgaris extract 50 µg/mL	903.53±11.90 [a]	18.74±1.07 [c]
G.vulgaris extract 10 µg/mL	942.53±12.36 [a]	15.23±1.11 [b]
A. muricata extract 75 µg/mL	950.00±72.33 [a]	17.71±6.27 [b]
A. muricata extract 50 µg/mL	905.00±58.89 [a]	21.60±5.10 [c]
A. muricata extract 10 µg/mL	928.13±42.13 [a]	19.60±3.65 [c]

*Note: Data is presented as average of ± SD from 3 replications. Letter a, b, c, d in each column indicates significance different among treatments based on Duncan post hoc test with $p < 0.05$ is considered as significantly different.

Table 4: Effect of *G. vulgaris* and *A. muricata* extracts toward IL-6 level in RAW264.7 cell line

Treatments	IL-6	
	Level of IL-6 (pg/mL)	Inhibitory activity of IL-6 over positive control (%)
Negative control	171.50±12.05 [a]	71.41±2.01 [a]
Positive control	599.83±7.95 [d]	0.00±1.32 [d]
G.vulgaris extract 75 µg/mL	258.54±31.51 [c]	56.90±5.25 [c]
G.vulgaris extract 50 µg/mL	175.88±6.16 [bc]	70.68±1.03 [d]
G.vulgaris extract 10 µg/mL	195.13±4.23 [ab]	67.47±0.70 [d]
A. muricata extract 75 µg/mL	308.50±28.78 [d]	49.28±4.73 [b]
A. muricata extract 50 µg/mL	219.13±23.35 [bc]	63.89±4.17 [cd]
A. muricata extract 10 µg/mL	263.21±49.29 [c]	56.73±8.10 [c]

*Note: Data is presented as average of ± SD from 3 replications. Letter a, b, c, d in each column indicates significance different among treatments based on Duncan post hoc test with $p < 0.05$ is considered as significantly different.

with TNF-α and IL-1 is elevated in septic or aseptic inflammation, makes it appropriate target in prevention and treatment of inflammatory disease[18]. In this study, GVE and AME decreased IL-6 compared to positive control, as shown in Table 4.

The results showed that LPS induced inflammation and increased IL-6 level in RAW264.7 which was indicated by high level of IL-6 in positive control (599.83 pg/mL) and significantly different compared to negative control. Levels of IL-6 in treatment of GVE and AME were lower and significantly different compared to positive control. These results indicate both GVE and AME are able to decrease IL-6 in inflammation-induced cell. GVE and AME at concentration of 50 µg/mL showed significant decrease in IL-6 level (175.88 pg/mL and 219.13 pg/mL respectively), and significantly different than positive control.

3.5 Effect of *G.vulgaris* and *A. muricata* Extracts on NO Level in LPS-Induced RAW264.7 Cell Line

The positive control of this test showed the highest concentration of NO concentration compared to the negative control and extract treated cells (Table 5). The

Table 5: Effect of *G. vulgaris* and *A. muricata* extracts toward NO level in RAW264.7 cell line

Treatments	NO	
	Level of NO (µM)	Inhibitory activity of NO over positive control (%)
Negative control	6.71±0.30 [a]	79.80±0.91 [d]
Positive control	33.23±1.04 [d]	0.00±3.14 [a]
G.vulgaris extract 75 µg/mL	9.76±0.78 [b]	70.63±2.37 [c]
G.vulgaris extract 50 µg/mL	10.80±1.06 [b]	67.48±3.17 [c]
G.vulgaris extract 10 µg/mL	15.33±0.67 [c]	53.84±2.03 [b]
A. muricata extract 75 µg/mL	9.79±0.78 [b]	70.67±2.35 [c]
A. muricata extract 50 µg/mL	10.84±1.05 [b]	67.53±3.15 [c]
A. muricata extract 10 µg/mL	15.37±0.67 [c]	53.97±2.01 [b]

*Note: Data is presented as average of ± SD from 3 replications. Letter a, b, c, d in each column indicates significance different among treatments based on Duncan post hoc test with $p < 0.05$ is considered as significantly different.

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.

Although NO level of treatment group was higher than negative control, both GVE and AME significantly resulted lower NO than positive control (Table 4), which indicated GVE and AME reduce NO level in inflammation-induced cell. GVE and AME at concentration of 75 µg/mL showed lower NO level (9.76 µM and 9.79 µM respectively). Decrease in NO level by GVE and AME showed both treatments supress inflammation properly, makes it promising in reducing NO to play its role in inflammation[18].

4. Discussion

The result of present study showed both GVE and AME extract showed no toxicity to RAW264.7 at concentration of 0.4, 2, 10, and 50 µg/mL. Non toxicity of substrate performed with MTS assay, was recorded by over 90% of viable cells.Viability test is crucial in pharmacology to determine adverse effect of bioactive substance in living organism prior to clinical use of drug or chemical compounds[19-21].

In this study, LPS was used to induce inflammation in RAW264.7 cell line. It has been reported that bacterial LPS is able to increase cytokines production as inflammation mediator[3,4]. LPS compose outer membrane of gram negative bacteria as endotoxin that induces production of proinflammatory mediators such as NO, IL-1, IL-6, TNF-α, interleukins, prostanoids and leukotrienes [3,4]. LPS induces inflammation via Toll-like receptor 4 (TLR4) binding.TLR4 is a transmembrane protein that recognizes lipopolisaccharide specifically. TLR4 signaling pathway may activates Nuclear Factor Kappa B (NF-κB) and Activation protein 1 (AP-1) which later induces the secretion of proinflammatory mediators such as NO, TNFα, IL-1 and IL-12[22,23].

Anti-inflammatory activities of GVE and AME were observed through markers such as IL-1β, TNF-α, NO and IL-6 inhibitory activity assays in LPS-induced macrophage cell line (RAW264.7). Both GVE and AME extract of 50 µg/mL resulted low TNF-α level in LPS-induced RAW264.7, with lowest level generated from AME. These results indicate both GVA and AME of 50 µg/mL play its role as anti-inflammatory, yet it did not exceed normal level. The TNF-α is an important cytokine involved in inflammatory response via activation of NF-κB, cytokine and adhesion molecule inducer[24,25]. The TNF-α is an important target of anti-inflammatory agent screening[5]. In presence of anti-inflammatory, TNF-α that exists in cascades is blocked[26]. Endogenous pyrogens consisting of TNF-α along with IL-1β and IL-6,

cause fever during inflammation, following up-regulated inflammatory responses that later triggers production of acute phase reactants[27].

GVE and AME at concentration of 50 µg/mL reduced IL-1β level in RAW264.7. IL-1β is prototypic proinflammatory cytokine that exert pleiotrophic effects on a variety of cells and play key roles in acute and chronic inflammatory as well as autoimmune disorders. IL-1β is produced mainly by blood monocytes. IL-1β, TNF-α and IL-6, simultaneously promote fever during inflammation due to up-regulated inflammatory responses that later triggers production of acute phase reactants[5].

In this study, GVE and AME at concentration of 50 µg/mL reduced IL-6 level in RAW264.7, with lowest IL-6 level was obtained in treatment of GVE. The IL-6 production has been detected in many cell types. Macrophages and monocytes are the primary source of cytokine during acute inflammation. IL-6 is pleiotropic cytokine to modulate inflammatory response [26, 27]. IL-6 along with TNF-α and IL-1 is elevated in condition of septic or aseptic inflammation, makes it effective in prevention and treatment of inflammatory disease[18].

The result of present study showed GVE and AME at concentration of 50 µg/mL reduced NO level in RAW264.7 cell, with lowest NO was obtained in treatment of GVE. Additional inflammatory pathways promoted by TNF-α, results nitric oxide (NO)[26, 27]. NO inhibitory activity is frequently used as appropriate target in anti-inflammatory agent screening. NO is responsible in host immune defense, vascular regulation, neurotransmission and other system in normal condition. Excess inducible NO Synthase (iNOS) is especially asssociated with various human diseases including inflammation[15,17].

It has been reported that active compounds from plants play important role in prevention and treatment of various diseases[9,28]. Anti-inflamatory activity of leaf extract of *G. vulgaris* has been documented in previous studies. Phytochemical analysis of *G. vulgaris* extract revealed the presence of flavonoids glycosides, saponins, steroids, tannins and polyphenols. Anti-inflammatory effects are present due to inhibition of mediators in inflammation by glycosides or steroids[9]. According to Jothimanivannan *et al.* (2010), flavonoid content also play key role in anti-inflammatory acitivity of GVE[10]. Kim *et al.* (2004) reported flavonoid contained in plants possess cellular mechanism in anti-inflammatory activity by inhibiting eicosanoid that produces phospholipase A2, cyclooxigenase and lypoxigenase. Inhibition of these enzymes will reduce prostanoid and leucotrien level[11].

AME is effective for both acute and chronic inflammation. It significantly decreases both TNF-α and IL-1β levels in CFA-induced arthritis model[29]. Phytochemical test conducted on ethanolic extract of *A. muricata* indicates presence of alkaloids, saponins, flavonoids, tannins, triterpenes and steroid. Flavonoids have a great potential as anti-inflammatory agents. Flavonoids and tannins have been reported to inhibit prostaglandin synthesis[29-32]. Recent study showed certain flavonoids such as flavon, posses anti-inflammatory properties that regulates pro-inflammatory genes cyclooxigenase-2(COX-2), nitrite oxide synthase (NOS), and cytokines[11]. Other substances present in extract such as tannins, may give the synergistic effect to the flavonoids.

5. Conclusion

This research revealed that ethanol extracts of *G.vulgaris* and *A. muricata* possess the anti-inflammatory potential indicated by inhibition of inflammatory mediators including IL-1β, TNF-α, NO and IL-6.

6. Conflict of Interest

The authors declare that they have no competing interests.

7. Acknowledgement

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Dr. Wahyu Widowati:

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_____

Dear Dr. Wahyu Widowati

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Thank you very much for your review to our manuscript
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Regards

Wahyu Widowati
Faculty of Medicine, Maranatha Christian University
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Dear Journal of Natural Remedies,
We would like to submit an updated manuscript entitled "Anti-inflammatory Potential of Gandarusa (*Gendarussa vulgaris* Nees) and Soursoup (*Annona muricata* L) Extracts in LPS Stimulated-Macrophage Cell (RAW264.7)" for Journal of Natural Remedies consideration.

We have revised grammatical errors addressed by reviewer. However, we have not added the information regarding herbarium number of both plants used in this research. In Indonesia, determination of plants do not usually provide herbarium number.

We are looking forward to hearing the feedback soon.

Best wishes
Wahyu Widowati

Anti-inflammatory Potential of Gandarusa (*Gendarussa vulgaris* Nees) and Soursoup (*Annona muricata* L) Extracts in LPS Stimulated-Macrophage Cell (RAW264.7)

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ABSTRACT

Inflammation is one of ~~an~~ the important biological responses to injury. Anti-inflammatory is therefore proposed to treat both acute and chronic inflammation. Chemical compounds of various plants are widely used in treatment of inflammation. Objective: This study aims to evaluate anti-inflammatory potential of *G. vulgaris* extract (GVE) and *A. muricata* extract (AME) on LPS-~~-~~stimulated ~~-~~murine macrophage cell line (RAW264.7). Cell viability assay to evaluate nontoxic concentration in cell line was performed with MTS assay. Parameters measured to determine anti-inflammatory activity were IL-1β, TNF-α, NO and IL-6 between treatment group and non treated cells, performed with Elisa. Both GVE and AME of 50 and 10 µg/mL showed high viability (>90%) and it was not significantly different compared to control, makes it suitable for treatment. GVE and AME of 50 µg/mL resulted low TNF-α level in RAW264.7 (313.16pg/mL and 264.69 pg/mL respectively), as well as IL-1β level (903.53pg/mL and 905.00 pg/mL respectively) and IL-6 (175.88 pg/mL and 219.13 pg/mL respectively). Whereas, GVE and AME of 75 µg/mL showed lower NO level (9.76 pg/mL and 9.79 pg/mL respectively) compared to untreated cells. This research revealed that GVE and AME possess the anti-inflammatory potential indicated by inhibition of inflammatory mediators including TNF-α, IL-1β, IL-6 and NO.

Keywords: Anti-inflammatory, *Gendarussa vulgaris* Nees, *Annona muricata* L, RAW264.7 cell line.

1. Introduction

Inflammation is an important biological response to injury that relates to various diseases such as rheumatoid arthritis, inflammatory bowel disease, artherosclerosis, Alzeimer's disease and cancer [1]. Reactive Oxygen Species (ROS), Reactive Nitrogen Species (RNS), cytokines (Interleukin (IL)-1β, IL-6, Tumor Necrosis Factor (TNF)-α) and NO mediated inflammation and prostaglandin, are produced by macrophage during the inflammatory process [2]. Anti-inflammatory is proposed to prevent chronic inflammation associated with chronic disease.

It has been reported that bacterial lipopolysaccharide (LPS) is able to increase cytokines production as inflammation mediator [3,4]. LPS has pro-inflammatory property in its glycolipid which compose gram negative bacterial cell wall [5]. Macrophage and inflammatory mediators activated by LPS are appropriate targets in anti-inflammatory drug development [6,7].

Natural phytochemicals plays a significant role in drug discovery. Plant extracts contain bioactive chemicals and most of them found free from adverse effects [8]. These chemical compounds ~~of~~ are widely used in treatment of inflammation [9]. Flavonoids ~~contained~~ found in plants have a great potential as anti-inflammatory agents. *G. vulgaris* ~~Nees~~ and *A. muricata* are common plants to contain such compounds. It has been reported that both plants show significant anti inflammatory activity [10-13].

The aim of this research is to evaluate anti-inflammatory potential of *G. vulgaris* extract (GVE) and *A. muricata* extract (AME) on LPS stimulated-murine macrophage cell line (RAW264.7). The RAW264.7 cells line is an appropriate model for evaluating and screening of anti-inflammatory agents from plant extract [6].

2. MATERIALS AND METHODS

2.1. Plant extract preparation

Leaves of *G. vulgaris* and *A. muricata* were collected from Traditional Medicine Research Center (Balai Penelitian Tanaman Rempah dan Obat), Bogor, West Java, Indonesia. The plants were identified by herbarium staff, Research Center of Biology, Indonesia Institute of Science, West Java, Indonesia. Simplicia of *G. vulgaris* and *A. muricata* of 500 g were extracted with ethanol 96% using maceration technique. Ethanol filtrate was filtered, and wastes were re-macerated in triplicate. Macerates were concentrated using 50°C rotavapor to obtain pasta form. The extracts were stored at -20°C [14-15]. GVE and AME was used as the experiment.

Comment [a1]: Provide herbarium numbers o both plants for their authenticity

2.2. Cell culture

The murine macrophage cell line RAW264.7 (ATCC®TIB-71TM) was given by Biomolecular and Biomedical Researchz Center, Aretha Medika Utama. The RAW264.7 cells were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS, 100 U mL penicillin (Biowest), 100 mL streptomycin (Biowest) and maintained at 37°C in humidified atmosphere and 5% CO_2 until the cells were confluent. The cells then washed, harvested using trypsin-EDTA (Biowest). The cells were seeded on plates and treated using GVE and AME in different concentration (0.4, 2, 10, 50, 150, 250, and 500 µg/mL [6].

2.3. RAW264.7 cells viability assay

Cell viability was performed with 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. Briefly, 100 µL cells in medium (DMEM supplemented with 10% FBS

and 100 U/mL 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 was washed and added with 99 µL of new medium and 1 µL of GVE and AME in various concentrations (0.4, 2, 10, 50, 150, 250, and 500 µg/mL), 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% CO_2 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 (%) [14-15]. The viability assay was conducted to determine the safe and nontoxic concentration for the next assay.

2.4. Pro-inflammatory activation of RAW264.7 cells

The pro-inflammatory activation of cells was performed based on modified method [7-8]. The cells were seeded in 6 well plate in density of 5×10^3 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 100 U/mL penicillin and streptomycin) was washed and supplemented with 1600 µL of growth medium and 200 µL of extract in different concentration in 1-2 h prior to LPS treatment. The 200 µL LPS (1 µg/mL) 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.

2.5. Measurement of TNF-α, IL- β, and IL-6, concentration and inhibitory activity

Quantification of TNF-α, IL-6 and IL-β concentration were performed using Biolegend ELISA kit. Briefly, antibody solution was added into each well of 96 well plate, and then incubated in 4 ^{0}C overnight. After washed supernatant after treated with GVE and AME on RAW264.7 cells in different concentration (10, 50 and 75 µg/mL) and then shaked for 2 h. Antibody solution was added and incubated for 1 h in orbital shaker. Avidin-HRP solution and TMB substrate solution was added to each well. TMB will be oxidated by peroxidase enzymes that indicated performed blue colour. Concentration of cytokines were determined by comparing the OD of the samples to the standard curve. LPS-stimulated cells without GVE and AME, were served as positive control. The normal cell was used as negative control [6-7].

2.6. Measurement of NO concentration and inhibitory activity

The determination of nitrite associated with NO production was performed based on Abnova Kit (No cat. KA 1342) protocol. After pre-incubation of RAW264.7 cells with LPS and GVE and AME for 24 h, the quantity of nitrite accumulated in the cell free supernatant was measured as an indicator of NO production. Two hundred µL assay buffer was added in the blank well and 100 µL of standard solution with 100 µL assay buffer was added into the standard well. Briefly, 100 µL of cell medium was mixed with 100 µL 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-induced cells without extract was used as positive control. The normal cell was used as negative control [6-7].

2.7. 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± Standard

Deviation. Significant differences between the groups were determined using the Analysis of variance (ANOVA) followed by Duncan *post hoc* Test.

3. RESULT

3.1. Effect of ~~*Gendarussa* G.~~ *vulgaris* ~~Nees~~ and ~~*Annona* A.~~ *muricata* ~~L~~ extracts on viability of RAW264.7 cell line

The RAW264.7 cell viability assay was the preliminary study to test the effect of GVE and AME toward RAW264.7 cell viability. ~~This assay aimed to determine the safe and non toxic concentration to be further analyzed.~~ Viability was measured by MTS assay based on the conversion of yellow tetrazolium salt to form a purple formazan product. Percentage of cells viability was determined by comparing cells viability value of treatments to the control.

As shown in Table 1, cell treated with GVE and AME at concentration of 150, 250, and 500 µg/mL resulted low viability which indicates toxicity to RAW264.7 . ~~W~~whereas concentration of 0.4, 0.2, 10 and 50 µg/mL of both GVE and AME showed high viability (>90%). Viable cells obtained at concentration of 10 and 50 µg/mL in both GVE and AME, appeared to reach normal level (control), makes such concentrations suitable for treatment of RAW264.7 cells. Therefore, further analysis of GVE and AME uses concentration in range of 10 and 150 µg/mL.

3.2. Effect of ~~*Gendarussa* G.~~ *vulgaris* ~~Nees~~ and ~~*Annona* A.~~ *muricata* ~~L~~ extracts on TNF-α level in LPS-induced RAW264.7 cell line

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. GVE and AME 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). As shown in Table 2, GVE and AME decreased TNF-α level compared to positive control.

Treatment of GVE at concentration of 50 µg/mL in RAW264.7 resulted lowest TNF-α level (313.16 pg/mL) among other treatments. Level of TNF-α in LPS-induced RAW264.7 treated with GVE of 50 µg/mL, was significantly different compared to TNF-α level in positive control (Table 1). These results indicate GVE of 50 µg/mL decreased TNF-α level to play its role as anti-inflammatory. Whereas AME at concentration of 50 µg/mL also generated relatively low TNF-α level (264.69 pg/mL), yet it was not significant compared to negative control. Both GVE and AME of 50 µg/mL showed no significant difference than positive control, and resulted good inhibitory activity of TNF-α over positive control (36.46 and 46.82% respectively). However, these results did not exceed normal level.

3.3. Effect of ~~*Gendarussa* G.~~ *vulgaris* and ~~*Annona* A.~~ *muricata* extracts on IL-1β level in LPS-induced RAW264.7 cell line

IL-1 which refers to two proteins (IL-1α and IL-1β), is a potent immuno-modulator which mediates a wide range of immune and inflammatory responses including activation of B and T cells [(19)]. Inhibiting the production of IL-1 was important in finding the anti-inflammatory agent. GVE and AME showed the inhibitory potential against IL-1β production (Table 3). Effect of GVE and AME level on IL-1β level in LPS-induced RAW264.7 is presented in Table 3.

As shown in Table 3, GVE and AME at concentration of 50 µg/mL decreased IL-1β level in LPS-induced RAW264.7, which was significant compared to positve control. GVE at concentration of 50 µg/mL resulted highest decreased IL-1β level of 903.53pg/mL, yet it was not significantly different compared to normal cell without LPS induction which resulted IL-1β level of 888.53pg/mL (negative control).

3.4. Effect of ~~*Gendarussa* *G.*~~ *vulgaris* ~~Nees~~ and ~~*Annona* *A.*~~ *muricata* ~~L~~ extracts on IL-6 level in LPS-induced RAW264.7 cells

IL-6 is one of the cytokine that possess biological activities due to acute inflammation [21]. IL-6 along with TNF-α and IL-1 is elevated in septic or aseptic inflammation, makes it appropriate target in prevention and treatment of inflammatory disease [22]. In this study, GVE and AME decreased IL-6 compared to positive control, as shown in Table 4.

Results showed LPS induced inflammation and increased IL-6 level in RAW264.7 which was indicated by high level of IL-6 in positive control (599.83 pg/mL) dan significantly different compared to negative control. Level of IL-6 in treatment of GVE and AME was lower and significantly different compared to positive control. These results indicate both GVE and AME are able to decrease IL-6 in inflammation-induced cell. GVE and AME at concentration of 50 µg/mL showed significant decrease in IL-6 level (175.88 pg/mL and 219.13 pg/mL respectively), and significantly different than that in non treated cell (positive control).

Comment [a2]: ??

3.5.Effect of ~~*Gendarussa* *G.*~~ *vulgaris* and *A*~~*nnona*~~ *muricata* extracts on NO level in LPS-induced RAW264.7 cell line

The positive control of this test shows the highest concentration of nitrite concentration compared to the negative control and extract treated cells (Table 5). 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.

Although NO level of treatment group was higher than negative control, both GVE and AME significantly resulted lower NO than positive control (Table 4), which indicated GVE and AME reduce NO level in inflammation-induced cell. GVE and AME at concentration of 75 µg/mL showed lower NO level (9.76 pg/mL and 9.79 pg/mL respectively). Decrease in NO level by GVE and AME showed both treatments supress inflammation properly, makes it promising in reducing NO to play its role in inflammation.

4. Discussion

The result of present study showed both *G. vulgaris* and *A. muricata* extracts showed no toxicity to RAW264.7 at concentration of 50 µg/mL. Non toxicity of substrate performed with MTS assay, was recorded by over 90% of viable cells. Viability test is crucial in pharmacology to determine adverse effect of bioactive substance in living organism prior to clinical use of drug or chemical compounds [19-21].

In this study, lipopolysaccharide (LPS) was used to induce inflammation in RAW264.7 cell line. It has been reported that bacterial LPS is able to increase cytokines production as inflammation mediator [3-4]. LPS is composed outer membrane of gram negative bacteria as endotoxin that induces production of proinflammatory mediators such as Nitric Oxide (NO), IL-1, IL-6, TNF-α, interleukins, prostanoids and leukotrienes. LPS induces inflammation via Toll-like receptor 4 (TLR4) binding. TLR4 is a transmembrane protein that recognizes lipopolisaccharide specifically. TLR4 signaling pathway may activates Nuclear Factor Kappa B (NF-κB) and Activation protein 1 (AP-1) which later induces the secretion of proinflammatory mediators such as NO, TNFα, IL-1 and IL-12 [22-23].

Anti-inflammatory acitvity of *G. vulgaris* and *A. muricata* was observed through markers such as IL-1β, TNF-α, NO and IL-6 inhibitory activity assays in LPS-induced macrophage cell line (RAW264.7). Both *G. vulgaris* and *A. muricata* extract of 50 µg/mL decreased TNF-α level in LPS-induced RAW264.7, with highest decrease generated from AME. These results indicate *G.vulgaris* extract of 50 µg/mL decreased TNF-α level to play its role as anti-

inflammatory, yet it did not exceed normal level. The TNF-α is an important cytokine involved in inflammatory response via activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), cytokine and adhesion molecule inducer [24-25]. The TNF-α is an important target of anti-inflammatory agent screening [5]. In presence of anti-inflammatory, TNF-α that exists in cascades is blocked [28]. Endogenous pyrogens consisting of TNF-α along with IL-1β and IL-6, cause fever during inflammation, following up-regulated inflammatory responses that later triggers production of acute phase reactants [5].

GVE and AME extract at concentration of 50 µg/mL reduced IL-1β level in RAW264.7. IL-1β are prototypic proinflammatory cytokine that exert pleiotrophic effects on a variety of cells and play key roles in acute and chronic inflammatory as well as autoimmune disorders. IL-1β is produced mainly by blood monocytes. IL-1β, TNF-α and IL-6, simultaneously promote fever during inflammation due to up-regulated inflammatory responses that later triggers production of acute phase reactants [5].

In this study, GVE and AME at concentration of 50 µg/mL reduced IL-6 level in RAW264.7, with lowest IL-6 level was obtained in treatment of GVE. The IL-6 production has been detected in many cell types. Macrophages and monocytes are the primary source of cytokine during acute inflammation. IL-6 is pleiotropic cytokine to modulate inflammatory response [30]. IL-6 along with TNF-α and IL-1 is elevated in condition of septic or aseptic inflammation, makes it effective in prevention and treatment of inflammatory disease [18].

The result of present study showed GVE and AME at concentration of 50 µg/mL reduced NO level in RAW264.7 cell, with lowest NO was obtained in treatment of GVE. Additional inflammatory pathways promoted by TNF-α, results nitric oxide (NO) [30]. NO inhibitory activity is frequently used as appropriate target in anti-inflammatory agent screening. NO is responsible in host immune defense, vascular regulation, neurotransmission and other system in normal condition. Excess inducible NO Synthase (iNOS) is especially asssociated with various human diseases including inflammation [15].

It has been reported that active compounds from plants play important role in prevention and treatment of various diseases [9] [26]. Anti-inflamatory activity of leaf extract of *G. vulgaris* Nees has been documented in previous studies. Phytochemical analysis of *G. vulgaris* Nees extracts reveals the presence of flavonoids glycosides, saponins, steroids, tannins and polyphenols. Anti-inflammatory effects are present due to inhibition of mediators in inflammation by glycosides or steroids [9]. According to Jothimanivannan (2010), flavonoid content also play key role in anti-inflammatory acitivity of GVE [10]. Kim *et al.* (2004) reported flavonoid contained in plants possess cellular mechanism in anti-inflammatory activity by inhibiting eicosanoid that produces phospholipase A2, cyclooxigenase and lypoxigenase. Inhibition of these enzymes will reduce prostanoid and leucotrien level [11].

AME is effective for both acute and chronic inflammation. It significantly decreases both TNF-α and IL-1β levels in CFA-induced arthritis model [29]. Phytochemical test conducted on ethanolic extract of *A. muricata* indicates presence of alkaloids, saponins, flavonoids, tannins, triterpenes and steroid. Flavonoids have a great potential as anti-inflammatory agents. Flavonoids and tannins have been reported to inhibit prostaglandin synthesis [30-32]. Recent study shows certain flavonoids such as flavon, posses anti-inflammatory properties that regulates pro-inflammatory genes cyclooxigenase-2 (COX-2), nitrite oxide synthase (NOS), and cytokines [11]. Other substances present in extract such as tannins, may give the synergistic effect to the flavonoids.

5. Conclusion

This research revealed that ethanol extracts of *G. vulgaris* and *A. muricata* possess the anti-inflammatory potential indicated by inhibition of inflammatory mediators including IL-1β, TNF-α, NO and IL-6.

6. Conflict of interest

The authors declare that they have no competing interests.

~~7.~~ Acknowledgment

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