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Anti-inflammatory effects of *Anredera cordifolia* and *Piper crocatum* extracts on lipopolysaccharide-stimulated macrophage cell line

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Abstract

In this study, the anti-inflammatory potential of *Anredera cordifolia* and *Piper crocatum* extracts on lipopolysaccharide-induced murine macrophage cell line (RAW 264.7) was observed. Cell viability assay was performed with MTS assay. Parameters measured to determine the anti-inflammatory activity were interleukin-1β (IL-1β), tumor necrosis factor (TNF)-α, nitric oxide (NO) and IL-6. Both *A. cordifolia* and *P. crocatum* at concentration of 50 µg/mL in cell line resulted significant decrease in TNF-α level (250.3 and 242.5 pg/mL respectively). *A. cordifolia* showed significant decrease in IL-1β level at 50 µg/mL and IL-6 level at 10 µg/mL, whilst *P. crocatum* showed significant decrease IL-1β level in three concentrations with lowest level at 50 µg/mL. *A. cordifolia* showed lowest decrease in NO level at 50 µg/mL but not comparable with normal cells, whilst *P. crocatum* showed significant decrease in NO level at 50 µg/mL. This research revealed that *A. cordifolia* and *P. crocatum* possess the anti-inflammatory potential indicated by the inhibitory activity of the inflammatory mediators including, TNF-α, IL-1β, IL-6, and NO.

Introduction

Inflammation is an important biological response to injury which has been documented in various diseases such as rheumatoid arthritis, inflammatory bowel disease, artherosclerosis, Alzheimer's disease and cancer (Fang et al., 2008). Several responsible markers are present in macrophage during inflammation such as reactive oxygen species (ROS), reactive nitrogen species (RNS), cytokines [Interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α] and nitric oxide (NO), that mediates inflammation and prostaglandin (Jung et al., 2007).

Bacteria lipopolysaccharide (LPS) is known to play its role in increasing the cytokines level as inflammation mediator (Kim et al., 2005). LPS has pro-inflammatory property in its glycolipid that compose Gram-negative bacteria cell wall (Boots et al., 2008). Thus, macrophage and inflammatory mediators induced by LPS are used as targets in anti-inflammatory drug development (Zeilhofer and Brune, 2006; Dewi et al., 2015; Rusmana et al., 2015).

Anredera cordifolia (madeira-vine) and *Piper crocatum* (red betel) are reported to contain bioactive compounds that possess medicinal properties. *A. cordifolia* possesses antibacterial (Tshikalange et al., 2005), antiobesity and anti-hypoglycemic (Wang et al., 2011), cytotoxic and anti-mutagenic (Yen et al., 2001), antidiabetic (Anh and Kim, 2005), and anti-inflammatory activities (Moura-Letts et al., 2006).

This research was aimed to observe anti-inflammatory

potential of *A. cordifolia* and *P. crocatum* extracts on LPS stimulated-murine macrophage cell line (RAW 264.7). The RAW 264.7 cell line is an appropriate model for evaluating and screening of anti-inflammatory agents from plant extract (Dewi et al., 2015; Rusmana et al., 2015; Widowati et al., 2016).

Materials and Methods

Preparation of plant extract

Leaves of *A. cordifolia* and *P. crocatum* were collected from the Traditional Medicine Research Center, Bogor, West Java, Indonesia. The plants were identified by the Research Center of Biology, Indonesia Institute of Science, West Java, Indonesia. Extraction of *A. cordifolia* and *P. Crocatum* were performed with maceration technique using 96%ethanol. Ethanol filtrate was filtered, and wastes were re-macerated in triplicate. Macerates were concentrated using 50°C evaporator until the pasta form product was obtained. The extracts were stored at -20°C (Widowati et al., 2013a; Widowati et al., 2013b).

Cell culture

The murine macrophage cell line RAW 264.7 (ATCC®TIB-71TM) was given by Biomolecular and Biomedical Research Center, Aretha Medika Utama. The cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) (Biowest L0104) supplemented with 10% fetal bovine serum (FBS) (Biowest S181H), 5 µL penicillin-streptomycin (iLabware l0018-100), and maintained at 37°C in humidified atmosphere and 5% CO_2 until the cells were confluent. The cells were then washed, harvested using trypsin-EDTA (Biowest L0931-500) (Dewi et al., 2015; Rusmana et al., 2015; Widowati et al., 2016).

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, USA). Briefly, 100 µL cells in medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin) were plated (5 × 10^3 cells per well) and incubated for 24 hours at 37°C in a humidified atmosphere and 5% CO_2. The medium was discarded and then added with 90 µL new medium and 10 µL of *A. cordifolia* and *P. crocatum* extracts in DMSO at final concentrations (0.4, 2, 10, 50, 150, 250, and 500 µg/mL) in different plate in triplicate and incubated for 24 hours. 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 hours. The absorbance was measured at 490 nm on a microplate reader (MultiSkan Go Thermoscientific). The data is presented as the percentage of viable cells (%). The viability assay was performed to determine the safe and nontoxic concentration for the next assay (Dewi et al., 2015; Rusmana et al., 2015; Widowati et al., 2016; Laksmitawati et al., 2016).

Pro-inflammatory activation of cells

The pro-inflammatory activation of cells was performed based on Khan et al. (1995) modified method. The cells were seeded in 6-well plates in density of 5 × 10^5 cells per well and incubated for 24 hours at 37°C in a humidified atmosphere and 5% CO_2. The medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin) then washed and supple-mented with 1600 µL growth medium and 200 µL *A. cordifolia* and *P. crocatum* extracts in different concentrations (10, 50 and 75 µg/mL) in 1-2 hours prior to the LPS treatment. The 200 µL LPS (1 µg/mL) was added into the medium and incubated for 24 hours at 37°C in a humidified atmosphere and 5% CO_2. The growth medium was taken for the next assay and centrifuged at 2,000 × 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 (Dewi et al., 2015; Rusmana et al., 2015; Widowati et al., 2016; Laksmitawati et al., 2016).

Measurement of TNF-a, IL- β and IL-6, concentration and inhibitory activity

Biolegend ELISA kit was used to measure TNF-α (430901), IL-6 (431301), and IL-β (432601) concentration. Briefly, antibody solution was added into each well of 96-well plates, and then incubated in 4°C overnight. After washing the plate, cell-free supernatant treated with *A. cordifolia* and *P. crocatum* extracts on cell lines, were added and then shaked for 2 hours. Antibody solution was added and incubated for 1 hour in orbital shaker. Avidin-HRP solution and TMB substrate solution was added to each well. TMB will be oxidated by peroxidase enzymes as indicated by blue color. Concentrations of TNF-α, IL-β, and IL-6 were determined by comparing the OD of the samples to the standard curve. LPS-stimulated cells without *A. cordifolia* and *P. crocatum* extracts, were served as positive control. The normal cell was used as negative control (Dewi et al., 2015; Rusmana et al., 2015; Widowati et al., 2016; Laksmitawati et al., 2016).

Measurement of nitrite associated with NO concentration and inhibitory activity assay

The determination of nitrite associated with NO production was performed based on Abnova Kit (KA 1342) protocol. After pre-incubation of cell lines with LPS and *A. cordifolia* and *P. crocatum* extracts for 24 hours, the quantity of nitrite accumulated in the cell free supernatant was measured as an indicator of NO production. A 200 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-stimulated cells without extract was used as positive control. The normal cell was used as negative control (Dewi et al., 2015; Rusmana et al., 2015; Widowati et al., 2016; Laksmitawati et al., 2016).

Statistical analysis

All data were derived from three independent experiments. Statistical analysis was conducted using SPSS software (version 20.0). Data 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.

Results

Effect on viability of RAW 264.7

The cell line viability assay was the preliminary study to test cytotoxicity of *A. cordifolia* and *P. crocatum* extracts toward cell line. Viability was measured by MTS assay indicated by the conversion of yellow tetrazolium salt to form a purple formazan product. Percentage of viable cells was determined by comparing cells viability value of treatments to the control.

A. cordifolia and *P. crocatum* extracts at concentrations of 150, 250 and 500 µg/mL were toxic to cell line indicated by low viability (Table I). Whereas concentration of 0.4, 2, 10 and 50 µg/mL of both *A. cordifolia* and *P. crocatum* extracts showed high viability (>90%). Viable cells obtained at concentration of 10 and 50 µg/mL in both *A. cordifolia* and *P. crocatum* extracts, that appeared to reach normal level, makes such concentrations suitable for further analysis. Concentration of 50 µg/mL showed viability that close to normal (~100%), and starting at 150 µg/mL showed toxicity (<100%). Thus, range between 50-150 µg/mL was also chosen as safe concentration. Based on linier regression (data are not shown), concentration of 75 µg/mL in both *A. cordifolia* and *P. crocatum* extracts, showed high viability (>90%). Therefore, concentration of *A. cordifolia* and *P. crocatum* extracts used were 10, 50 and 75 µg/mL.

Effect on TNF-a level in LPS-induced cell line

A. cordifolia and *P. crocatum* extracts 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

Table I

Effects of *A. cordifolia* and *P. crocatum* on viability of RAW 264.7 cell line

Concentration (µg/mL)	Cell viability	
	A. cordifolia	*P. crocatum*
Control	100.0 ± 4.8^{d}	100.0 ± 3.2^{d}
0.4	123.6 ± 3.9^{f}	129.6 ± 5.9^{f}
2	129.6 ± 5.9^{f}	126.5 ± 2.5^{f}
10	119.4 ± 0.5^{f}	113.9 ± 12.5^{e}
50	112.4 ± 1.3^{e}	112.0 ± 3.3^{e}
150	82.2 ± 1.1^{c}	65.3 ± 1.5^{c}
250	36.9 ± 3.4^{b}	16.6 ± 0.5^{b}
500	2.9 ± 1.1^{a}	1.6 ± 1.3^{a}

Data are presented as mean ± SD of three replications; Superscript letter ($^{a-f}$), in each column indicates significance different among concentration based on Duncan post hoc test with $p<0.05$ is considered as significantly different

Table II

Effects of *A. cordifolia* and *P. crocatum* on TNF-α, IL-1β, IL-6 and NO levels in RAW 264.7 cell line

Treatment	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)	NO (pg/mL)
Negative control	235.3 ± 16.3^{a}	890.2 ± 24.2^{a}	167.6 ± 40.6^{a}	6.0 ± 1.5^{a}
Positive control	491.5 ± 28.1^{d}	1110.5 ± 18.8^{c}	607.0 ± 46.2^{d}	33.4 ± 1.0^{f}
A. cordifolia extract				
75 µg/mL	328.6 ± 14.0^{b}	987.2 ± 14.0^{b}	330.4 ± 12.8^{c}	33.2 ± 1.6^{f}
50 µg/mL	250.3 ± 22.7^{a}	909.2 ± 19.6^{a}	234.7 ± 13.8^{b}	22.8 ± 2.1^{d}
10 µg/mL	355.3 ± 24.5^{b}	928.2 ± 29.9^{b}	217.8 ± 14.7^{ab}	25.7 ± 0.6^{e}
P. crocatum extract				
75 µg/mL	334.8 ± 23.2^{b}	896.4 ± 88.9^{a}	333.4 ± 37.5^{c}	9.9 ± 0.1^{b}
50 µg/mL	242.5 ± 25.6^{a}	873.4 ± 11.1^{a}	196.0 ± 10.3^{ab}	6.3 ± 1.5^{a}
10 µg/mL	411.7 ± 44.0^{c}	911.5 ± 11.2^{a}	189.0 ± 5.2^{ab}	13.0 ± 1.1^{c}

Data are presented as mean ± SD of three replications; Superscript letter ($^{a-d}$) in each column indicates significance different among treatments based on Duncan pos hoc test with $p<0.05$ is considered as significantly different

without extract).

Both *A. cordifolia* and *P. crocatum* extracts at concentration of 50 µg/mL in cell line resulted significant decrease TNF-α level (250.3 and 242.5 pg/mL). Results of both *A. cordifolia* and *P. crocatum* extracts at 50 µg/mL were comparable with negative control (235.3 pg/mL), which indicates both treatments possess good anti-inflammatory.

Effect on IL-1β level in LPS-induced cell line

Inhibition the production of IL-1 is an important approach in finding the anti-inflammatory agent. *A. cordifolia* and *P. crocatum* extracts showed the inhibitory potential against IL-1β production (Table II).

A. cordifolia and *P. crocatum* extracts decreased the IL-1β level in LPS-induced cell line, which was significantly different compared to positive control (Table II). *A. cordifolia* showed lowest IL-1β level (909.2 pg/mL) at 50 µg/mL, which was comparable to normal cell (890.2 pg/mL). Treatment with *P. crocatum* in three concentrations showed decreasing IL-1β level which was comparable to normal cells. *P. crocatum* showed its lowest level at 873.4 pg/mL.

Effect on IL-6 level in LPS-induced RAW 264.7

Results showed LPS induced inflammation and increased IL-6 level in cell line which was indicated by high level of IL-6 in positive control (607.0 pg/mL) and significantly different compared to negative control (167.6 pg/mL). *A. cordifolia* at 10 µg/mL showed significant decreased IL-6 level (217.8 pg/mL), as well as *P. crocatum* at 10 and 50 µg/mL (189.0 and 196.0 pg/mL respectively). The results were also confirmed by the percentage inhibiton of *A. cordifolia* and *P. crocatum* extracts which peaked at 10 µg/mL. Both extracts were comparable to normal cell.

Effect on NO level in LPS-induced cell line

The positive control shows the highest concentration of nitrite concentration compared to the negative control and extract-treated cells (Table II). The percent of inhibition was determined by the value of positive control nitrite concentration minus the nitrite concentration of treatment divided to the nitrite concentration of positive control. Both *A. cordifolia* and *P. crocatum* extracts resulted lower NO than positive control (Table II). *A. cordifolia* showed the lowest NO level at concentration of 50.0 µg/mL (22.8 pg/mL), yet it was significantly different compared to normal cell (6.0 pg/mL). Whereas *P. crocatum* showed lowest NO level at 50.0 µg/mL (6.3 pg/mL), which was comparable with normal cell.

Discussion

In this study, *A. cordifolia* and *P. crocatum* extracts showed no toxicity to cell line at concentration in range between 10-150 µg/mL. Non toxicity 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 (Dewi et al., 2015; Rusmana et al., 2015; Widowati et al., 2016; Laksmitawati et al., 2016).

LPS successfully induced inflammation of macrophage cell line (RAW 264.7). Cytokines production as inflammation mediator are enhanced by LPS (Kim et al., 2005; Mahajna et al., 2014; Rusmana et al., 2015). LPS is one of components in outer membrane of Gram-negative bacteria as endotoxin that induces production of pro-inflammatory mediators such as NO, IL-1, IL-6, TNF-α, interleukins, prostanoids and leukotrienes (Mahajna et al., 2014; Dewi et al., 2015; Rusmana et al., 2015; Widowati et al., 2016; Laksmitawati et al., 2016).

Potential of *A. cordifolia* and *P. crocatum* extracts were observed through inflammatory markers such as TNF-α, IL-1β, IL-6, and NO inhibitory activity assays in LPS-induced macrophage cell line (RAW 264.7). Both *A. cordifolia* and *P. crocatum* extracts of 50 µg/mL decreased TNF-α level in LPS-induced RAW 264.7, which was comparable to normal cell. 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 (Libby, 2002; de Cassia da Silveira E Sá et al., 2014). TNF-α is therefore used as an important target of anti-inflammatory agent screening (Boots et al., 2008; Dewi et al., 2015; Laksmitawati et al., 2016). TNF-α that exists in cascades, is blocked in presence of anti-inflammatory (Dinarello, 2010). 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 (Damte et al., 2011).

A. cordifolia showed lowest IL-1β level at 50.0 µg/mL, which was comparable to normal cell. Whereas *P. crocatum* in three concentrations showed decreasing IL-1β level which was comparable to normal cells. IL-1β is a key role in acute and chronic inflammatory and autoimmune disorders. IL-1β is prototypic pro-inflammatory cytokine that employs pleiotrophic effects on a variety of cells. IL-1β is produced mainly by blood monocytes (Damte et al., 2011).

In this study, *A. cordifolia* and *P. crocatum* extracts at concentration of 10 µg/mL reduced IL-6 level in cell line that comparable to normal cell, with lowest IL-6 was obtained in treatment of *P. crocatum*. IL-6 is pleiotropic cytokine to modulate inflammatory response (Kostek et al., 2012). IL-6 is present in many cell types. 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.

P. crocatum showed lowest NO level in cell line that almost exceed normal level, that indicates good anti-inflammatory activity. NO originate from additional inflammatory pathways promoted by TNF-α (de Cassia da Silveira E Sá et al., 2014). NO inhibitory activity is often 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 (Kang et al., 2011).

Active compounds from plants is documented to play important role in prevention and treatment of various diseases (Leontowicz et al., 2006). *A. cordifolia* has been reported in previous studies to posses medicinal properties (Moura-Letts et al., 2006; Sukandar et al., 2011; Yuziani et al., 2014; Wahjuni et al., 2014). Phytochemical analysis of *A. cordifolia* indicate that the leaves contain considerable amounts of saponins, alkaloids, and flavonoids (Astuti et al, 2011). It has been reported that flavonoid inhibits inflammatory mediators including TNF-α, IL-1β and IL-6, IL-8, and COX-2 (Tunon et al., 2009; Serafini et al., 2010).

The result of present study showed anti-inflammatory properties of *P. crocatum*. There are only few studies regarding anti-medicinal properties of *P. crocatum*. Wicaksono et al.(2009) reported *P. crocatum* methanol extract inhibits the growth of human breasts cancer (T47D) cells via inhibition of p44/p42 phosphorylation. Recent study shows *P. crocatum* leaves extract to act as anti-inflammation in Wistar rats with atherosclerosis through decrease of TNF-α and IL-6 levels (Wahjuni et al., 2016). Anti-inflammatory properties of *P. crocatum* might be correlated with compounds contained in the plants. Chromatographic analysis of *P. crocatum* shows flavonoids, alkaloids, polyphenolic compounds, tannins and essential oils. These compounds are known to have efficacy as antibacterial, anti-inflammatory, and anti-pyretic (Tunon et al., 2009; Serafini et al., 2010).

Conclusion

Extracts of *A. cordifolia* and *P. crocatum* possess anti-inflammatory potential indicated by inhibition of inflammatory mediators including TNF-α, IL-1β, IL-6, and NO on LPS-induced macrophage cells.

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Conflict of Interest

All authors have completed the ICMJE uniform disclosure form and declare no support from any organization for the submitted work.

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