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The Immunomodulatory Activities of *Saurauia vulcani* Korth Leaves towards RAW 264.7 cell

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Abstract

Saurauia vulcani Korth is an actinidiaceae family plant that is used as traditional medicine by the community. Testing the immunomodulatory effect of *Saurauia vulcani* Korth extract was done in vitro on RAW cells line by stratified extraction using n-hexane, ethyl asetat, and ethanol solvents. Determination of nitric oxide levels by the griess method and inhibition of gene expression TNF-α, IL-6, COX-2, IL-1β, and iNOS by RT-PCR method. *Saurauia vulcani* Korth extract (NESVL, EAESVL, and EESVL) at a concentration of 25 ug / ml was able to reduce the value of nitric oxide in RAW cells 246.7 and was able to inhibit the expression of genes TNF-α, IL-6, COX-2, IL-1β, and iNOS. Where the density value of each band formed by 1.21 ± 0.005; 1.15 ± 0.005; 0.88 ± 0.003 on TNF-α, 1.23 ± 0.003; 1.64 ± 0.005; 0.55 ± 0.003 on IL-6, 0.38 ± 0.003; 0.55 ± 0.003; 0.38 ± 0.003 on COX-2, 0.18 ± 0.003; 0.04 ± 0.003; 0.03 ± 0.003 on IL-1β, and 0.30 ± 0.003; 0.41 ± 0.003; 0.11 ± 0.005 on iNOS.

Keywords: *Saurauia vulcani* Korth , Extract, Immunomodulator, RAW 246.7 Cell Line

INTRODUCTION

Immune system dysfunction is responsible for various diseases like arthritis, ulcerative colitis, asthma, allergy, parasitic diseases, cancer and infectious diseases (Sharma, et al., 2012). One type of cell that plays a role in the process of immune system activity is macrophage cells (Chen, et al., 2014). Macrophages can be activated by microbial components, such as endotoxin, lipopolysaccharides (LPS) and lipoteichoic acids (LTA). Activated macrophages phagocytize micro-organisms, secrete pro-inflammatory cytokines and nitric oxide (NO) and present antigens to helper T cells. These cytokines contribute to defense mechanisms of the host immunity in response to external invasion, but they may induce immuno-pathological disorders when secreted in excess (Chon, et al., 2009).

Saurauia vulcani Korth is a plant that is used as traditional medicine. Pharmacological activities such as antihyperglycemic and traditionally used as anti-inflammatory can not be separated from the content of secondary metabolites in this plant (Hutahaean, et al., 2018). Secondary metabolite compounds such as steroid/triterpenoid, tannin, and flavonoid found in this plant are the basis for their development as immunomodulators (Situmorang, et al., 2015). Testing the immunomodulatory effect is done in vitro on RAW 246.7 cells line. Determination of nitric oxide levels and inhibition of gene expression of TNF-α, IL-6, COX-2, IL-1β, and iNOS become a way to determine the immunomodulatory activity of the extract.

MATERIALS AND METHODS

Materials

Fresh *Saurauia vulcani* Korth leaves were collected from Tiga Lingga village, Dairi regency, Sumatera Utara province, Indonesia. RAW 264.7 cells were obtained from Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum and kept at 37^{0}C with a CO_2 supply of 5%. Lipopolysaccharides are from Escherichia coli O111.B4 (Sigma), Dexamethasone (Harsen), *n*-hexane, ethylacetate, ethanol 96%. TLC Silica gel 60 F_{254} (Merck). All chemicals and reagents used in this work were of analytical grade. Total RNA Mini Kit (Geneaid), ReverTra Ace (Toyobo), GoTaq®Green (Promega), Nuclease-Free Water (Promega), TBE (Vivantis), agarose gel (Promega), Flurosafe (Smobio), DNA ladder 100 bp (Smobio).

Preparation extract of *Saurauia vulcani* Korth leaves

The powder of *Saurauia vulcani* Korth leaves (1 kg) was repeatedly extracted by maceration with *n*-hexane (3×3 day, 10 L) (NESVL). The powder was dried in the air and extracted with ethylacetate (3×3 day, 10 L) (EAESVL) and then the powder was dried in the air and extracted with ethanol (3×3 day, 10 L) (EESVL) at 25-30°C with periodical stirring. The filtrate was collected, and then evaporated to obtain a viscous fraction and then freeze-dried to dry (Satria, et al., 2015; Lestari, et al., 2015; Satria, et al., 2017).

Phytochemical Screening

The Phytochemicals: Alkaloids, Flavonoids, Glycosides, Saponins, Tannins, Steroids were determined using standard procedures (Wargner and Bladt, 1996; Kemkes, RI, 2013; Musa, 2017).

Cell culture and cell viability

RAW 264.7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 100 units/mL of penicillin and 100 μg mL^{-1} of streptomycin as previously described by Chi, et al. 2016. Cells were incubated in the presence of 5% CO_2 at 37°C. The cells (passage 7-12) were seeded at a concentration of $3x10^3$ cells mL^{-1} in 96-well plates and incubation 24 h. The effects of *Saurauia vulcani* Korth leaves extracts on cell viability were evaluated with the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-Tetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich). Extracts of *Saurauia vulcani* Korth leaves were dissolved in 100 % DMSO, and the stock solution of the extract at a concentration of 50.000 μgmL^{-1} was prepared in 10% DMSO. The final concentrations of the extract ranged from 1-200 μgmL^{-1} in the culture media. Dexamethasone and lipopolysaccharides were used as positive and negative controls (Ahamed, et al., 2017; Chi, et al., 2016).

Nitrit oxide (NO) production activity

For the experiment, sodium nitroprusside (10 mM) in phosphate buffered solution (pH = 7.4) was mixed with various concentrations of the extract prepared in 10% DMSO and incubated under light at room temperature for 15 min. The same reaction mixture without the tested extract, but the equal amount of the solvent serves as the control (the last well). After the incubation, 0.05 mL of Griess reagent (1% sulfanilamide, 2% H3PO4 and 0.1% N-(1- naphthyl) ethylenediamine dihydrochloride was added. Ascorbic acid was used as positive control. The absorbance was measured at 546 nm and the percentage of NO radical inhibition by the extract was calculated from the formula equation: $[(A0–A1) /A0] \times 100$. Where A0 is the absorbance of the control, and A1 is the absorbance of the extract/standard [20]. IC50 value was obtained by drawing the equation of line from the graph of concentration (μg/mL) versus percentage of inhibition (Rao, et al., 2016).

Reverse transcription-polymerase chain reaction (RT-PCR)

The genes expression of TNF-α, IL-6, IL-1β, iNOS, and COX-2 were determined by RT-PCR.

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Total RNA from the control cell, LPS, positive control, and treatment groups were extracted using the Total RNA Mini Kit (Geneaid) according to the manufacturer's protocol. The oligonucleotide primers for TNF-α, IL-6, IL-1β, iNOS, COX-2, and β-actin were designed according to a PCR primer selection program at the website of the Virtual Genomic Center from the GenBank database (Table 1).

Table 1. Mouse oligonucleotide primers sequences used for RT-PCR (5-3') and Annealing temperature.

Gen		Primer Sequences	Size (bp)	Temp (°C)
TNF-α	F	5'-TGTGCCGCCGCTGTCTGCTTCACGCT-3'	374	55
	R	5'-GATGAGGAAAGACACCTGGCTGTAGA-3'		
IL-6	F	5'-GATGCTACCAAACTGGATATAATC-3'	269	55
	R	5'-GGTCCTTAGCCACTCCTTCTGTG-3'		
IL-1β	F	5'-CCCTGCAGCTGGAGAGTGTGGA-3'	447	62.5
	R	5'-TGTGCTCTGCTTGTGAGGTGCTG-3'		
iNOS	F	5'-CGAAACGCTTCACTTCCAA-3'	311	60
	R	5'-TGAGCCTATATTGCTGTGGCT-3'		
COX-2	F	5'-CCTGTGTTCCACCAGGAGT-3'	249	55
	R	5'-GTCCCTGGCTAGTGC TTCAG-3'		
β-actin	F	5'- TGGAATCCTGTGGCATCCATGAAAC-3'	349	55
	R	5'- TAAAACGCAGCTCAGTAACAGTCCG-3'		

PCR was consisted of 35 amplification cycles and each cycle carried out for 30 s at 95°C, 1 min at annealing temperature (55°C for TNF-α , IL-6, COX-2 and beta-actin and 60°C for iNOS) and 45 s at 95°C, 1 min at annealing temperature 62.5°C for IL-1β .) and 1 min at 72°C in a thermal cycler (ProFlex™ 3x32-well PCR System, Applied Biosystems). The β -actin was used as an internal control to standardize the relative expression levels for all biomarkers. PCR products were separated electrophoretically on a 2% agarose and fluorosafe (Smobio) with Tris-Borate-EDTA (Vivantis) 0,5x. The stained gel was visualized by using Gel-Doc Quantity One software (Syngene) (Yanti, et al., 2011).

Statistics

Triplicate experiments were performed throughout this study. All data were presented as the mean ± Standard Error Minimum (SEM), which were analyzed using the SPSS 22 software. The significant difference between Lipopolysaccharide and treated groups were analyzed by the paired Turkey HSD ($p<0.05$).

RESULTS

Phytochemicals Screening

The results of phytochemicals screening from extract ethylacetate and ethanol of *Saurauia vulcani* Korth Leaves. contains flavonoids, tannins, saponins, glycosides while extract *n*-hexane only contain steroids which seen on table 1.

Table 1. Phytochemicals Screening Result.

NO	Phytochemicals	NESVL	EAESVL	EESVL
1.	Alkaloids	-	-	-
2.	Flavonoids	-	+	+
3.	Tannins	-	+	+
4.	Saponins	-	+	+
5.	Glycosides	-	+	+
6.	Steroids/Triterpenoids	+	-	+

Notes: (+) positive: contains a class of compounds
(-) negative: does not contain a class of compounds

Nitric Oxide (NO) Production

The results of examination nitric oxide level on RAW 264.7 cell line with LPS (1 μg/mL) induction and administration of ENSVL, EEASVL, and EESVL can be seen on figure 1.

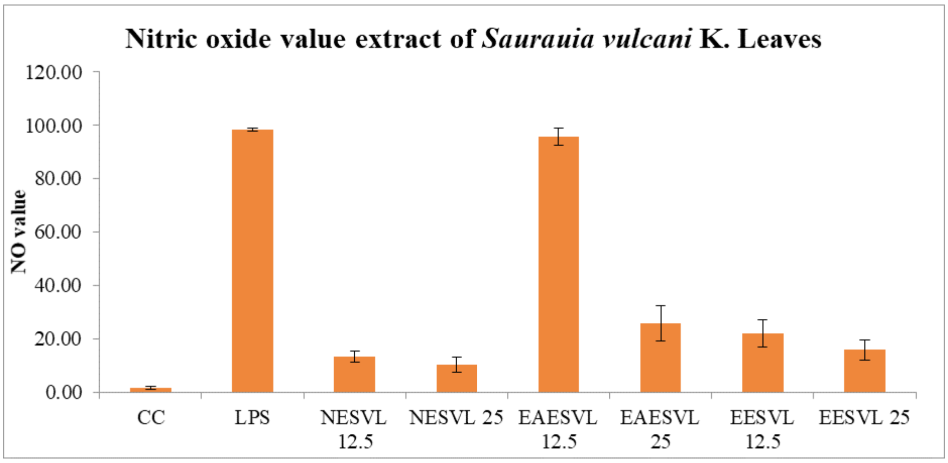

Figure 1. Nitric Oxide Value of extract of *Saurauia vulcani* K. Leaves. CC: Cell Control; LPS: Lipopolysaccharides; NESVL: *n*-Hexana Extract of *Saurauia vulcani* K. Leaves; EAESVL: Ethylacetate Extract of *Saurauia vulcani* K. Leaves; EESVL: Ethanol Extract of *Saurauia vulcani* K. Leaves.

Effects of NESVL, EAESVL, EESVL on the genes expression of cytokines (TNF-α, IL-6, IL-1β), iNOS and COX-2 in LPS-induced macrophages

The results of genes expression which treated with NESVL, EAESVL, EESVL were analyzed using RT-PCR methods and the results were showed in Figure 2.

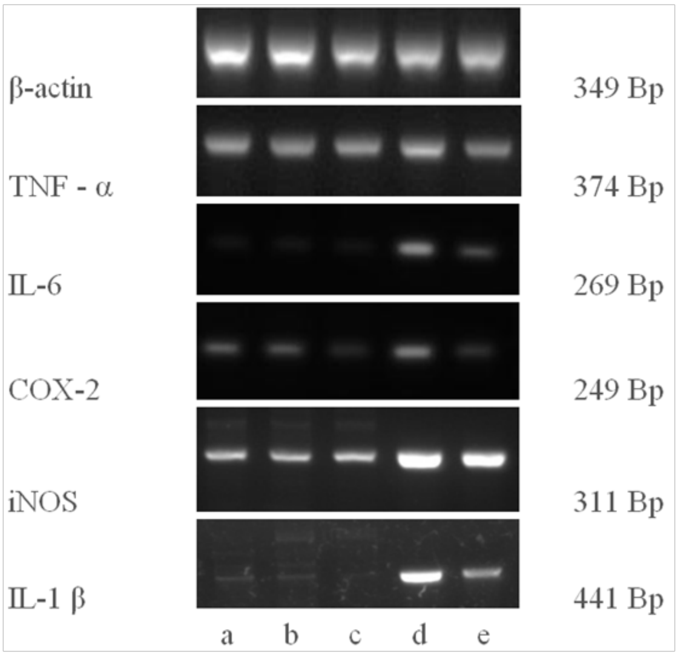

Figure 2. The effect of extracts on the genes expression in RAW 264.7 cell which induced LPS 1 μgmL^{-1} for 6 hours. The total RNAs were isolated, and RT-PCR was performed using the indicated primers in Materials and Methods. EESVL 25 μgmL^{-1} (a), EEASVL 25 μgmL^{-1} (b), NESVL 25 μgmL^{-1} (c), LPS (d), cells control (e). β-actin was used as the internal control. LPS, Lipopolysaccharide; RT-PCR, reverse transcription-PCR; iNOS, Inducible Nitric Oxide Synthase; IL, interleukin; COX-2, Cyclooxygenase-2; Bp, Base Pair.

The result were shown from the value of genes expression from toward LPS showed a significant difference with $P < 0.05$ in Table 2.

Table 2. The value of genes expression in RAW 264.7 cells which induced LPS

Gene	Mean ± SEM				
	EESVL	EAESVL	NESVL	LPS	Control cell
TNF-α	0.88 ± 0.003	1.15 ± 0.005	1.21 ± 0.005	1.64 ± 0.003	1.00 ± 0.00
IL-6	0.55 ± 0.003	1.64 ± 0.005	1.23 ± 0.003	2.12 ± 0.003	1.00 ± 0.00
COX-2	0.38 ± 0.003	0.55 ± 0.003	0.38 ± 0.003	2.14 ± 0.003	1.00 ± 0.00
IL-1β	0.03 ± 0.003	0.04 ± 0.003	0.18 ± 0.003	2.76 ± 0.003	1.00 ± 0.00
iNOS	0.11 ± 0.005	0.41 ± 0.003	0.30 ± 0.003	1.31 ± 0.003	1.00 ± 0.00
β-actin	0.94 ± 0.003	1.12 ± 0.005	1.27 ± 0.003	1.07 ± 0.003	1.00 ± 0.00

DISCUSSION

Saurauia vulcani Korth leaves are a plant with the family *Actinidiaceae* used as traditional medicine. *Saurauia vulcani* Korth Leaves activity as an immunomodulator begins with phytochemical screening tests to determine the content of secondary metabolites contained in simplicia, testing of nitric oxide levels in RAW 264.7 cells line induced by each LPS 1 μg / mL, NESVL (12.5 μg / mL and 25 μg / mL) , EAESVL (12.5 μg / mL and 25 μg / mL), and EESVL (12.5 μg / mL and 25 μg / mL), further testing of TNF-α, IL-6, COX-2, IL-1β, and iNOS gene expression.

The results of phytochemicals screening from extract ethylacetate and ethanol of *Saurauia vulcani* Korth leaves (EAESVL and EESVL) contains flavonoids, tannins, saponins, glycosides while extract *n*-hexane (NESVL) only contain steroids which seen on table 1. *Saurauia vulcani* Korth leaves (NESVL, EAESVL, and EESVL) were tested on RAW 264.7 cell lines to determine NO production. RAW 264.7 cell lines induced using LPS experienced an increase in nitrite levels and in the administration of extracts decreased nitrite levels, seen at concentrations of 12.5 μg / mL greater nitrite levels and at concentrations of 25 μg / mL, nitrite levels decreased significantly which means that *Saurauia vulcani* Korth leaves extract can inhibit NO production. The content of metabolite compounds in leave extract *Saurauia vulcani* Korth such as flavonoids, saponins, tannins, steroids/ triterpenoids and glycosides can inhibit NO production in RAW cells 264.7 (Durga, et al., 2014; Venkatesha, et al., 2016).

Gene expression testing was performed using the RT-PCR method. In this test RAW 264.7 cells were treated with NESVL, EAESVL, and EESVL with a concentration of 25 μg/mL which had been induced by LPS 1 μg/mL. The results of gene expression testing show that NESVL, EAESVL, and EESVL can reduce gene expression compared to LPS. Administration of extract can reduce the expression of TNF-α, IL-6, COX-2, IL-1β, and iNOS in RAW 264.7 cells induced by LPS with a density value of each of 1.21 ± 0.005; 1.15 ± 0.005; 0.88 ± 0.003 on TNF-α, 1.23 ± 0.003; 1.64 ± 0.005; 0.55 ± 0.003 on IL-6, 0.38 ± 0.003; 0.55 ± 0.003; 0.38 ± 0.003 on COX-2, 0.18 ± 0.003; 0.04 ± 0.003; 0.03 ± 0.003 on IL-1β, and 0.30 ± 0.003; 0.41 ± 0.003; 0.11 ± 0.005 on iNOS.

Based on statistical tests conducted using the one way anova method, the significance value of <0.05 was obtained. Which means that there are significant differences between the test groups in the experiments conducted. TNF-α, IL-6, COX-2, IL-1β, and iNOS are genes that influence the performance of macrophages that influence inflammatory events (Yanti, et al., 2011; Adebayo, et al., 2017; Dewi, et al., 2017).. The content of secondary metabolites in extracts such as flavonoids and steroids/triterpenoids is thought to be able to inhibit the expression of these genes (Fachnian, et al., 2017; Tungmunnithum, et al., 2018). This is a strong strategy for the development of extracts as an immunomodulator.

CONCLUSION

Extract A contains secondary metabolite compounds that can reduce NO production and inhibit gene expression such as TNF-α, IL-6, COX-2, IL-1β, and iNOS.

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