Anti-Inflammation Activity of Virgin Coconut Oil In-Vitro Against Raw Cells 264.7

Amin M*,1, Silalahi J1, Harahap U2, Satria D3.

1Department of Chemistry, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia; 2Departemen of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia; 3Departemen of Biology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, Indonesia

ABSTRACT

Objective: This study aims to determine the anti-inflammatory activity of pure coconut oil on Raw Cell 264.7 induced by LPS.

Methods: The anti-inflammatory activity test for Virgin Coconut Oil (VCO) was carried out by testing the expression of TNF-α, IL-6, IL-1β, iNOS, and COX-2 genes on RAW 264.7 cells induced by LPS by the method Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Results: The test results of iNOS, TNF-α, IL-6, IL-1β, and COX-2 gene expression from VCO on RAW 264.7 cells induced by lipopolysaccharide (LPS) decreased the value of VCO density. In iNOS expression, VCO density values (0.90±0.015) showed significantly different effects with positive and negative controls p<0.05. VCO IL-1β density values (2.47±0.010) showed different effects. Significant with normal control, positive control and negative control p<0.05, TNF-α density value on VCO (0.91±0.010) showed significantly different effects from positive control and negative control p<0.05, then the value IL-6 density at VCO (1.23±0.015) showed significantly different effects from normal and positive controls p<0.05, and COX-2 density values at VCO (1.02±0.015) showed significantly different effects with normal and positive controls p<0.05.

Conclusion: Based on the results of this study VCO on RAW 264.7 cells induced by LPS can inhibit the expression of TNF-α, IL-6, IL-1β, iNOS, and COX-2 genes so that VCO effectively has anti-inflammatory activity.

Keywords: VCO, Antiinflammation, RAW 264.7 Cells, Gene Expression, Lipopolysaccharides

INTRODUCTION

Inflammation is the body's defense response to damage or infection due to foreign objects, such as bacteria, parasites and viruses. 1,4 Inflammation functions to reduce, or localize (sekuster) either in damaging agents or damaged tissue, inflammation or inflammation can be triggered by various physical, chemical, or biological agents 2,9.

Lipopolysaccharide (LPS) is the main component of gram-negative bacteria, which plays an important role in triggering inflammation. LPS causes macrophages to secrete molecules that increase the release of inflammatory mediators, as well as the release of cytokines that are mostly released from macrophages 12. Anti-inflammatory is a term for agents or drugs that work against or suppress the inflammatory process, and anti-inflammatory drugs commonly used are divided into two groups, namely steroid anti-inflammatory drugs and nonsteroidal anti-inflammatory drugs. But these drugs have many side effects that are harmful to the body, so we need natural anti-inflammatory drugs that are safe for the body such as VCO 9.

Virgin coconut oil (VCO) contains phytosterols which can provide anti-inflammatory, analgesic and antipyretic effects. 3,8 VCO is a medium chain triglyceride, because it consists of medium chain fatty acids, VCO can be hydrolyzed faster and more complete than long chain triglycerides. Chain fatty acids are being hydrolyzed more rapidly by lipase enzymes in the mouth and in the stomach to produce monoacylglycerol and free fatty acids which are then quickly absorbed through the mucosa and through the portal veins that are transported directly to the liver, one of the tests carried out to determine the anti-inflammatory activity of this VCO by testing gene expression on RAW 264.7 cells LIT-induced in vitro using RT-PCR 5.

MATERIALS AND METHODS

Materials and Tools
Autoclave (Hirayama), conical tube (Thermo Scientific), electrophoresis (BioRad), falcon tube (Thermo Scientific), Gel Doc (Syngene), CO₂ incubator (Heraeus), Laminar Air Flow (Labconco), microwave (Panasonic), nanovue plus (fisher scientific), neubauermehometrometer (Hausscher Scientific), electric balance (Vibra AJ), oven (Memmert), PCR (ProFlex, Applied Biosystems), centrifugator (Eppendorf), rotary evaporator (Stuart), vortex (IKA), 6-well plate (Iwaki), 96-wells plate (Iwaki), VCO (Palm Mustika®, Indonesia).

RAW Cell 264.7, Dulbecco's Modified Eagle's Medium (DMEM) (Biowest) growth media, Fetal Bovine Serum (FBS) 10% (v/v) (Gibco), penicillin-streptomycin 2% (v/v) (Gibco), Fungizone (FBS) Amphoterycin B (Sigma), NaHCO₃, NaN₃, (Sigma), Dexamethason (Harsen), 0.25% Tryptsin-EDTA (Gibco), Lipopolysaccharides (Sigma), MT [3-(4,5-dimethylthiazol-2-il)-2,5 diphenylethrazolium bromide] (Sigma), phosphate buffer saline (PBS) (Irvine Scientific), sodium dodecyl sulfate (SDS) in 0.1 N HCl, Total RNA kit (Geneaid), Tra-Ace Rever (Toyobo), washing and elution of RNA with a strength of 14,000 rpm for 1 minute to elute purified RNA. Calculate the concentration of RNA produced.

RESEARCH METHODS

RNA extraction

Total RNA was isolated using an RNA isolation kit (Geneaid). RNA extraction is carried out by the stages of cell lysis, washing and elution of RNA.

a. Cell Lysis Stage

RAW cells 264.7 (5x10⁵ cells / well) that have been harvested are added 400 μl RB buffer and 4 μl β-mercaptoethanol and the cells are resuspended again. After that the mixture is homogenized, incubate at room temperature for 5 minutes. Add 400 μl with 70% ethanol prepared in ddH₂O (DNaseRNAse free water). Shaken strongly until the mixture is homogeneous. Prepare a 2mL RB tube column, transfer 500 μl mixture to the RB column. Centrifuge with the strength of 14–16,000 rpm for 1 minute, then the filtrate is removed. Move the remaining mixture in the same RB column, centrifuge with a strength of 14–16,000 rpm for 1 minute. Discard the filtrate and place the RB column in the new 2 mL tube.

b. Cell Washing Stage

Added 400 μl WI buffer to the RB column, centrifuge with a strength of 14-16,000 rpm for 30 seconds. The filtrate is removed, then add 600 μl of washing buffer (make sure ethanol has been added) into the RB column. Centrifuge with the strength of 14-16,000 rpm for 30 seconds, then the filtrate is removed. Place the RB column again in a 2 mL tube and centrifuge with a strength of 14-16,000 rpm for 3 minutes to dry the column matrix.

c. RNA Elution Stage

The dried RB column is placed into a new 1,5 mL microcentrifuge tube. Add 50 μl of water-free RNase to the center of the matrix column. Let stand for 1 minute to ensure RNase is free of water absorbed. Then centrifuge with a strength of 14-16,000 rpm for 1 minute to elute purified RNA. Calculate the concentration of RNA produced.

Manufacture of cDNA

The total RNA used 2000 ng was then added DNaseRNase free water to a total volume of 12 μL. A total of 8 μL of mixed solution (5x RT-buffer 4 μL; random primary 1 μL; dNTP 2 μL; ReverTra-Ace 1 μL) were added to each microtube containing RNA, then resuspended and performed PCR under 30°C for 10 minutes, 42°C for 60 minutes, and 99°C for 5 minutes. The concentration of PCO products made from cDNA was measured using a Nanodrop device.

Analysis Gene Expression of iNOS, TNF-α, IL-6, IL-1β, COX-2, and β-actin

The expression of iNOS, TNF-α, IL-6, IL-1β, COX-2 and β-actin genes was examined by taking 100 ng/μL cDNA added to 25 μL PCR Master Mix (GoTaq®Green 12.5 μL; primary forward 1 μL; reverse primer 1 μL; DNaseRNAse free water 9.5 μL).

Table 1: Primary Sequences

| Primer Sequences        | Size (bp) | Annealing Temp (°C) |
|-------------------------|-----------|---------------------|
| iNOS                    |           |                     |
| F 5'-CGAAAACGCTTACCTCCAAC-3' | 311       | 60                  |
| R 5'-TGAGCCTTATATGCTGTCGCC-3' |           |                     |
| IL-1β                   |           |                     |
| F 5'-CCCTGACGGAGGATGGTGGA-3' | 447       | 42                  |
| R 5'-TGTCCTCTCGTGAGGTTGCTG-3' |           |                     |
| TNF-α                   |           |                     |
| F 5'-TGTCGGCCGCGGTGCTCTTACGCT-3' | 374       | 55                  |
| R 5'-GATTAGGAAAGACACCTGGCCTGTAAG-3' |           |                     |
| IL-6                    |           |                     |
| F 5'-GATGTCTACAAACCTGATATATAAC-3' | 269       | 55                  |
| R 5'-GGTCTTTAGACCACCTTTCTTG-3' |           |                     |
| COX-2                   |           |                     |
| F 5'-CCTGCTGTCACCAGAGGT-3' | 249       | 58                  |
| R 5'-GTCCTCTGACTATCGTCCAG-3' |           |                     |
| β-actin                 |           |                     |
| F 5'-TGAATTCCTGCGGATCATGAAAC-3' | 349       | 55                  |
| R 5'-TAAAGGAGCTCAGTAAACGATTCG-3' |           |                     |

PCR consists of 35 amplification cycles and each cycle is carried out for 30 seconds at 95 °C (denaturation), 1 minute at annealing temperature (55°C for TNF-α, IL-6, COX-2 58°C, β-actin and 60°C for iNOS) and 45 seconds at 95°C (denaturation), 1 minute at annealing temperature 62,5°C for IL-1β.) And 1 minute at 72°C (elongation) in a thermal cycler (ProFlexTM 3x32- well PCR System, Applied Biosystems), β-actin is a housekeeping gene that is used as an internal control to standardize the relative expression levels for all biomarkers. PCR products were analyzed by electrophoresis in 2% agarose and Tris-Borate-EDTA (Vivantis), and fluorvue (Smobio) as a dye.
RESULTS AND DISCUSSION

Gene Expression Test

The results of gene expression testing in Figure 1 show that VCO can reduce the expression of TNF-α, IL-6, COX-2, IL-1β and iNOS compared with LPS which experiences the greatest increase. The band image with the treatment (VCO, Dexamethasone) looks thinner compared to the treatment with LPS which produces the thickest band. LPS is the main inducer that can activate macrophages so as to increase gene expression[10,11]. Based on the results of the measurement of gene expression in Figure 1, it was analyzed statistically using One way ANOVA, then continued with the Post Hoc Test in the form of the Tuckey HSD test giving the result that there was a significant difference between the treatment group with cell control as normal control (untreated control), lipopolysaccharides as negative controls (can increase gene expression), and dexamethasone as positive controls (can reduce gene expression).

The effect of TNF-α, IL-6, IL-1β, COX-2, iNOS given (VCO) gene expression was significantly different p <0.05 seen in Table 2.

Table 2: Effect of gene expression density of VCO

| NO | Genes | Treatment Group | Average Density± SEM |
|----|-------|----------------|----------------------|
| 1. | TNF-α | VCO            | 0,91 ± 0,010abc      |
|    |       | Dexamethasone  | 0,70 ± 0,010abc      |
|    |       | LPS            | 1,18 ± 0,010abc      |
|    |       | KS             | 1,00 ± 0,000bc       |
| 2. | IL-6  | VCO            | 1,23 ± 0,015abc      |
|    |       | Dexamethasone  | 0,87 ± 0,015abc      |
|    |       | LPS            | 1,16 ± 0,010abc      |
|    |       | KS             | 1,00 ± 0,000bc       |
| 3. | IL-1β | VCO            | 2,47 ± 0,010abc      |
|    |       | Dexamethasone  | 1,70 ± 0,010abc      |
|    |       | LPS            | 3,38 ± 0,010abc      |
|    |       | KS             | 1,00 ± 0,000bc       |
| 4. | COX-2 | VCO            | 1,02 ± 0,015abc      |
|    |       | Dexamethasone  | 0,77 ± 0,010abc      |
|    |       | LPS            | 1,22 ± 0,015bc       |
|    |       | KS             | 1,00 ± 0,000bc       |
| 5. | iNOS  | VCO            | 0,90 ± 0,015abc      |
|    |       | Dexamethasone  | 0,47 ± 0,015abc      |
|    |       | LPS            | 1,49 ± 0,015bc       |
|    |       | KS             | 1,00 ± 0,000bc       |

Description:

a: Sig (P) <0.05 there is a significant difference with the normal control group (KS), b: Sig (P) <0.05 there is a significant difference with the negative control group (LPS), c: Sig (P) <0.05 there is a significant difference with the positive control group (dexamethasone)

VCO administration can reduce TNF-α, iNOS, IL-1β, COX-2 and IL-6 expression in Lipopolysaccharide-induced RAW cells. In iNOS expression, VCO density values (0,90±0,015) showed significantly different effects with positive and negative controls p<0.05, VCO IL-1β density values (2,47±0,015) showed different effects. In combination with other controls, VCO significantly with normal control, positive control and negative control p<0.05. TNF-α density value on VCO (0,91±0,010) showed significantly different effects from positive control and negative control p<0.05, then the value IL-6 density at VCO (1,23 ± 0,015) showed significantly different effects from normal and positive control.
controls p<0.05, and COX-2 density values at VCO (1.02±0.015) showed significantly different effects with normal and positive controls p<0.05. The effect of gene expression density can be seen in Figure 2 shows that the graph has decreased significantly compared to LPS.

![Figure 2](image_url)

**Figure 2** Graphic Effect of TNF-α, IL-6, IL-1β, COX-2 and iNOS gene expression on VCO. *p<0.05 significantly different from the normal control group (KS) #p<0.05 significantly different from the negative control group (LPS). ^p<0.05 significantly different from the positive control group (dexamethasone).

In TNF-α, IL-6, IL-1β, COX-2, and iNOS genes with VCO, the decrease was greater than VCO. TNF-α, IL-6, IL-1β, COX-2 can be produced from macrophages in response to bacterial LPS stimuli, infections and inflammatory stimuli. These cytokines also play an important role in the immune system by helping cytotoxic and cytostatic effects on infected cells or malignant cells.

**CONCLUSION**

Based on the results of the research the administration of VCO can reduce the density value of TNF-α, iNOS, IL-1β, COX-2 and IL-6 on RAW 264.7 cells induced by lipopolysaccharides.

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