Journal of Research in Pharmacy

Research Article

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The anti-inflammatory activity of hydrolyzed virgin coconut oil towards RAW 264.7 cell

Muhammad Amin Nasution [1,2], Jansen Silalahi [2], Urip Harahap [3], Poppy Anjelisa Zaitun Hasibuan [3], Denny Satria [4*]

1 Faculty of Pharmacy, Universitas Muslim Nusantara Al- Washliyah, Medan, 20147, Indonesia.
2 Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, 20155, Indonesia.
3 Department of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, 20155, Indonesia.
4 Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, 20155, Indonesia.
* Corresponding Author. E-mail: dennysatria@usu.ac.id (D.S); Tel. +6285296458644.

Received: 13 July 2022 / Revised: 18 October 2022 / Accepted: 24 October 2022

ABSTRACT: Inflammation can result from the introduction of foreign things into the body, such as bacteria or viruses. Inflammation activates macrophages and mast cells, which serve as immunological agents. The resultant hydrolysis of virgin coconut oil (HVCO) has an anti-inflammatory effect. This research aimed to determine how HVCO affects anti-inflammatory effects in vitro RAW 264.7 cells were activated against lipopolysaccharide. HVCO has anti-inflammatory effects determined by performing a live-cell viability assay using the MTT method [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide], IL- 6, TNF- α, IL-1β, iNOS, COX-2, and β-actin gene expression have been studied utilizing reverse transcription-polymerase chain reaction (RT-PCR). The HVCO test results on RAW 264.7 cells with the cell viability test at concentrations (62.5 g/mL; 31.5 g/mL) showed the percentage of live cells (> 90%), namely (97.74 ± 0.31; 102.31 ± 1.21) and assays using the expression of iNOS, TNF-α, IL-6, IL-1β, COX-2, and β-actin genes from HVCO in cells induced with LPS decreased the density value of HVCO, the expression of iNOS and IL-1β resulted in density values the best (0.72±0.010) and (2.40±0.015), TNF-α (0.76±0.7633), IL-6 (1.16±0.010), COX-2 (0.98 ± 0.010), and β-actin (1,02± 0,010). This study showed that HVCO has anti-inflammatory actions on RAW 264.7 cells caused by lipopolysaccharide.

KEYWORDS: HVCO; Antiinflammatory; RAW-264.7; Cell-Viability; RT-PCR.

1. INTRODUCTION

The wound healing process involves many hemostasis, inflammation, proliferation, and remodeling of cells. During the inflammatory phase, fibroblasts secrete cytokines and growth factors that stimulate the immune system [1,2]. Inflammation refers to the body's normal condition and defense response against cell damage or infection. Inflammation begins with a stimulus that damages tissue, so macrophages then play an essential role in responding to inflammation by releasing various factors, such as nitric oxide (NO), proinflammatory cytokines (TNF-α, IL-1β, IL-6, and prostaglandin mediators, in response to activating stimulus, e.g., lipopolysaccharide (LPS). Mediator production is utilized in various inflamed tissues and mRNA expression [3-6].

Virgin Coconut Oil (VCO) is extracted from fresh coconut flesh (*Cocos nucifera*), which is treated at low-temperature temperatures or without any heating [7]. Several previous studies have explained that VCO contains phytosterols which can be helpful as an anti-inflammatory and can also be useful as antipyretics, analgesics, antibacterial, antioxidants, and anti-diabetic [8-10]. As a triglyceride, coconut oil lacks antibacterial and antiviral action; however, when partly hydrolyzed, VCO produces free fatty acids and monoglycerides [11]. Fatty acid and monoglyceride combinations are diglycerides that are not antibacterial or antiviral [12,13]. Monolaurin with lactic acid (lauric acid monoglyceride) are antiviral and antibacterial in numerous ways, including viral and bacterial cell membrane lipid layer structural disruption. [14,15].

How to cite this article: Nasution MA, Silalahi J, Harahap U, Hasibuan PAZ, Satria D. The anti-inflamatory activity of hydrolized virgin coconut oil towards RAW 264.7 cell. J Res Pharm. 2023; 27(2): 705-711.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.353

There has not been much research into the anti-inflammatory process utilizing HVCO; HVCO is the outcome of an enzymatic reaction of VCO employing *Rhizomucor miehei lipase* (active at the sn-1,3) location. HVCO will be produced using cell viability and gene expression techniques, which may be employed as an anti-inflammatory test against RAW 264.7 cells [16,17].

2. RESULTS AND DISCUSSION

2.1 Cells Viability Assay in RAW 264.7 Cells

The viability test findings revealed that the cells had been hydrolyzed virgin coconut oil (HVCO), and as a positive control, dexamethasone was employed. The greatest results were obtained with HVCO concentrations of 31.5 g/mL-1 and 62.5 g/mL-1, resulting in the highest viability value. Figures 1 A and 2 B indicate that HVCO and Dexamethasone reduced cell viability in RAW 264.7 cells.

Figure 1 A Cell viability on HVCO in comparison towards RAW 264.7 cells

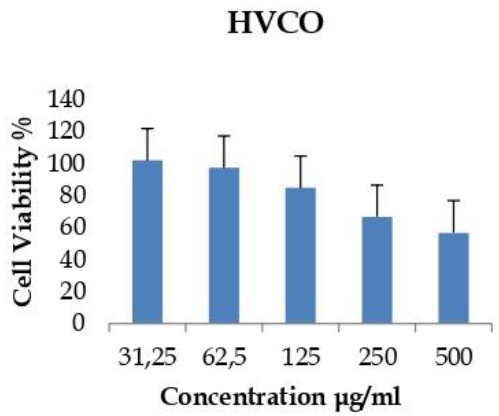
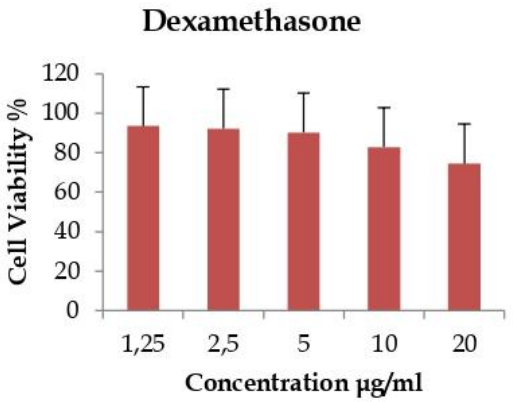


Figure 2 B Cell viability on Dexamethasone towards RAW 264.7 cells



Scientific proof of VCO has been extensively distributed, but in the form of HVCO as an anti-inflammatory, which has received less attention. In this work, VCO was hydrolyzed first to form VCO, and then cell viability was examined to determine the toxicity of HVCO before it was employed in RAW 264.7 cells. RAW 264.7 cells were cultured in HVCO-containing media for 24 hours. The cell viability was then determined using MTT [18,19].

The HVCO treatments plus Dexamethasone as a positive control produced cell viability findings. Figures 1 and 2 indicate that the greater concentration of the viability, the lower or higher the concentration of HVCO than the living cells and less, which implies that the higher concentration of HVCO, the stronger the

cytotoxic impact on cell culture examined. HVCO does not produce toxicity in RAW 264.7 cells, according to cell viability assays [20]. Consequently, the sample concentration that produced the largest percentage of live cells (> 90%) was chosen for the subsequent studies [21, 22].

2.2 HVCO's impact on cytokine IL-6, COX-2 and TNF-α, gene expression, as well as IL-1β, iNOS and β-actin expression, were found in LPS-induced macrophages

The levels of gene expression in HVCO and Dexamethasone-treated genes were assessed by RTPCR, and the findings are displayed in Figure 3.

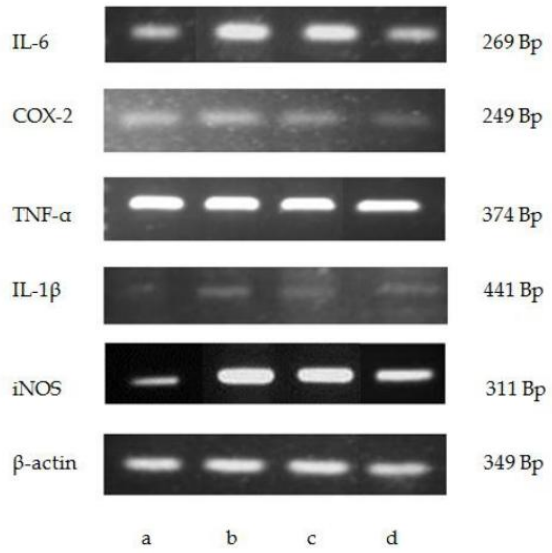


Figure 3. The effect of HVCO on RAW 264 gene expression. seven cells generated one gmL-1 LPS during 6 hours. Total RNA was extracted, and RTPCR was carried out with primers listed in the materials and methods section. The following treatments were used: control cells (a), LPS (b), HVCO 31.25 gmL-1 (c), and Dexamethasone 2.5 gmL-1 (d). β -actin was employed as an internal control. LPS is an abbreviation for lipopolysaccharide; RT-PCR is an abbreviation for reverse transcription-PCR; iNOS is an abbreviation for inducible nitric oxide synthase; IL is an abbreviation for interleukin; COX-2 is an abbreviation for cyclooxygenase-2, and Bp is an abbreviation for base pair. P <0.05 showed a substantial shift in HVCO toward LPS gene expression.

Table 1: The expression of LPS causes changes in genes in RAW 264.7 cell lines.

	Mean ± SEM			
Gene	Dexamethasone	HVCO	LPS	Control Cell
TNF-α	0.70 ± 0.010	0.76± 0.015	1.18 ± 0.010	1.00 ± 0.000
IL-6	0.87 ± 0.015	1.16± 0.010	0.87 ± 0.015	1.00 ± 0.000
IL-1β	1.70 ± 0.010	2.40 ± 0.015	1.16 ± 0.010	1.00 ± 0.000
COX-2	0.77 ± 0.010	0.98± 0.010	3.38 ± 0.010	1.00 ± 0.000
iNOS	0.47 ± 0.015	0.72± 0.010	1.49 ± 0.015	1.00 ± 0.000
β-actin	0.96 ± 0.015	1.02± 0.010	1.12 ± 0.015	1.00 ± 0.000

The gene expression assays with HVCO and Dexamethasone were examined using RT-PCR, displayed in Figure 3. Furthermore, HVCO inhibits the inflammatory cytokine expression in LPS-induced macrophages. As shown in Figure 3, the tests performed using HVCO yielded the best density values for iNOS and IL-1β (0.72±0.010) and (2.40±0.015), TNF- α (0.76±0.7633), IL-6 (1.16±0.010)), COX-2 (0.98 ± 0.010), β-actin (1.02±0.010) are shown in table 1. LPS was employed to stimulate the production of inflammatory cytokines, which then activated iNOS in macrophages during the inflammatory phase [23,24]. In reaction to LPS, macrophages can produce these cytokines, infection, and inflammatory activity. They also contribute to the cytotoxic immune system and cytostatic effects on patients or cancerous individuals. TNF-α, IL-1β and IL-6 as immune cells are

among them; earlier research has shown that VCO can decrease IL-6, TNF-α, iNOS, IL-1β, β-actin, IL-1β, and COX-2 gene production [25-28]. The significant expression of genes from HVCO and control regarding LPS cells revealed P <0.05 indicates a substantial difference.

3. CONCLUSION

Using cell viability and gene expression approaches, the researchers discovered that HVCO had a non-toxic and anti-inflammatory effect on RAW 264.7 cells caused by LPS.

4. MATERIALS AND METHODS

4.1 VCO Enzymatic Hydrolysis

In a 250 ml Erlenmeyer flask, 30 g of oil was put, distilled water 30 mL was added, 25 ml buffer, and 12.5 ml 0.063 M CaCl2. 1 M Tris-HCl pH 8-, and 3-mL R. miehei Lipase. At 50°C, the solution was incubated for ten hours, stirring at 200 rpm every hour. The mixture was transferred to the separating funnel and removed after incubation; N-hexane (50 mL) was added and agitated for 5 minutes. The mixture was allowed to settle until two layers formed. The first extract was the top layer (n-hexane fraction); 50 mL n-hexane was used to extract the bottom layer (water fraction) and split it into two extracts. After combining the first and second extracts, 250g of anhydrous Sulfate of sodium was added to absorb the remaining water. Before filtering, the blended extract was 15 minutes allowed to stand. The acid value was determined after, and In a water bath, the n-hexane was evaporated. HVCO was then used to detect biomarkers in wound healing [29-31].

4.2 Culture of Cell

Mouse myoblast cell line immortalization RAW 264.7 (Parasitology Laboratory, UGM Faculty of Medicine) was employed in this work. Dulbecco's Modified Eagle's Medium (DMEM)/high glucose supplemented with 10% fetal bovine serum (FBS) (Gibco) was used to cultivate the cells. Cells were incubated at 37$^{o}_{C}$ in a humidified environment with 5% CO2 37$^{o}_{C}$.

4.3. LPS and Dexamethasone

LPS from Escherichia coli O111:B4 (Sigma) was dissolved in a 1 g/mL phosphate-buffered solution (PBS). Cell RAW 264.7 LPS was used to grow macrophages in 90% DMEM/high glucose to produce inflammation. Dexamethasone (DEX) (Hersen), routinely used to treat inflammation, was dissolved in double-distilled water at a concentration of 5 g/mL as a positive control.

4.4 Cell Viability Test

RAW 264. In DMEM complete media, seven cells were grown. Seven cells (3x103 cells/well) were grown on a 96-well plate for 24 hours before being treated with HVCO at various doses (500 ug/mL, 250 ug/mL, 125 ug/mL, 62.5 ug/mL, and 31.25 ug/mL), and cell incubated at 37° C in a 5% CO_2 incubator. After incubation, the medium and test solution was removed and rinsed with PBS. MTT was added and incubated for 4-6 hours before being stopped with the stopper reagent (10% SDS in 0.1N HCl) and left to stand overnight at room temperature. A microplate reader was used to observe/read the data at 595 nm. The formula was used to calculate cell viability:

$$\text{Cell Viabilities} = \frac{\text{A (sample)}}{\text{A (control)}} x\ 100\ \%$$

Note: Percentage viability of untreated cells counted 100% [32-34].

4.5 Polymerase chain reaction-reverse transcription (RT-PCR)

IL-6, TNF-α, L-1, COX-2, iNOS, and β-actin gene expression were measured using RT-PCR. RNA total was extracted from the control LPS, cell, positive control, and total RNA Mini Kit (Geneaid) was used in the treatment groups by the manufacturer guidelines. TNF-α, IL-1β, IL-6, iNOS, COX-2, and also β-actin oligonucleotide primers have been created using a PCR primer selection software from the GenBank library on the Virtual Genomic Center website (Table 2).

Table 2. RT-PCR mouse oligonucleotide primer sequences (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 performed in a thermal cycler for thirty seconds at 95°C, 1-minute temperature of annealing (55°C for IL-6, COX-2, TNF-α, and iNOS, and 60oC for β- actin), and 45 seconds at 95°C, 1-minute temperature of annealing (62.5° C for IL- 1) and 1 minute at 72°C (Applied Biosystems ProFlexTM 3x32-well PCR System). To normalize the relative expression levels of all biomarkers, -actin was employed as an internal reference. Electrophoretically separated PCR products 2% agarose Fluorosafe (Smobio) with agarose gel with a 0.5% Gradient of tris-borate-EDTA (Vivantis). Quantity One software from Gel-Doc (Syngene)was used to view the stained gel [35,36].

4.6 Statistics analysis

Throughout this work, triplicate experiments were carried out. The mean was used to represent all data, standard deviation, and minimum SEM, and the SPSS 22 program was used to analyze them. The paired Turkey HSD examined the difference between the Lipopolysaccharide and therapy groups was significant ($P<0.05$).

Author Contributions: J.S, M.A.N, D.S and U.H. were conceived and designed the experiments; P.A.Z.H., M.A.N, and D.S were prepared the manuscript and statistical analysis, Literature Search D.S.; Writing M.A.N.; Critical Reviews M.A.N., J.S., U.H., P.A.Z.H., and D.S.
Conflicts of Interest: The authors declare no conflict of interest.

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