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The Anti-inflammatory Activity of Essential Oil from the Peel of Kaffir Lime (*Citrus hystrix* DC.) In vitro

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ABSTRACT
Inflammation is a defense response of the body to injury or infection caused by foreign substances entering the body. This study aims to evaluate the toxicity and inhibition of nitric oxide (NO) production in RAW 264.7 cells. The essential oil from the peel of Kaffir lime (Citrus hystrix DC.) was isolated using microwave-assisted extraction. The essential oil was tested in vitro for cell viability in RAW 264.7 cells using the MTT assay and for the inhibition of NO production using the Griess assay, which was induced by LPS. The results showed that the viability of RAW 264.7 cells treated with the essential oil from Kaffir lime peel at concentrations of 1.56 and 0.78 µg/mL exhibited a cell viability percentage of >90% (90.29 ± 5.19; 92.71 ± 3.28). The NO production inhibition assay showed that the essential oil at concentrations of 1.56 and 0.87 µg/mL reduced NO levels in RAW 264.7 cells induced by LPS (9.58 ± 0.38; 10.16 ± 0.49). These concentrations of essential oil (1.56 and 0.78 µg/mL) were significantly different from the lipopolysaccharide control. Therefore, the essential oil from Kaffir lime peel demonstrates anti-inflammatory activity.
Keyword: MTT assay, Cell viability, Citrus hystrix, Essential oil, Nitric oxide.

ABSTRAK
Radang merupakan respon pertahanan tubuh jika terjadi cedera atau infeksi yang disebabkan benda asing yang masuk ke dalam tubuh. Penelitian ini bertujuan mengevaluasi toksisitas dan penghambatan produksi nitrit oksida (NO) pada sel RAW 264.7. Isolasi minyak atsiri kulit buah jeruk purut (Citrus hystrix DC.) dilakukan dengan metode microwave-assisted extraction. Pengujian minyak atsiri kulit buah jeruk purut dilakukan secara in vitro pada viabilitas sel RAW 264.7 dengan metode MTT dan pengujian penghambatan nitrit oksida (NO) dengan menggunakan Griess yang diinduksi oleh LPS. Hasil pengujian menunjukan bahwa viabilitas sel RAW 264.7 minyak atsiri kulit buah jeruk purut pada konsentrasi 1.56; 0.78 µg/mL dengan persentase sel hidup >90% sebesar (90.29 ± 5.19; 92.71 ± 3.28). Hasil pemeriksaan penghambatan produksi NO minyak atsiri kulit buah jeruk purut pada konsentrasi (1.56; 0.87 µg/mL) terhadap sel RAW 264.7 yang diinduksi dengan LPS menurunkan kadar NO minyak atsiri kulit buah (9.58 ± 0.38;10.16 ± 0.49). Dimana minyak atsiri konsentrasi 1.56; 0.78 µg/mL berbeda signifikan dengan lipopolisakarida. Sehingga minyak atsiri kulit buah jeruk purut memiliki aktivitas antiinflamasi.
***Kata* kunci**: MTT assay, Viabilitas sel, Citrus hystrix, Minyak atsiri, Nitrit oksida

1. Introduction

Inflammation is the body's defence response in case of injury or infection caused by foreign bodies entering the body, short-term to a disturbance. The body responds by releasing cytokines to signal. The release of prostaglandins, histamine and inflammatory mediators is characterized by redness, heat, swelling, pain and loss of function [1, 2]. The release of cytokines TNF-α, IL-6 from macrophages is induced by lipopolysaccharide (LPS) which activates NF-kB causing *inducible Nitric Oxide Synthase* (iNOS) resulting in increased production of NO and TNF-α, IL-6 inflammation [3,4].

Inflammation is a dangerous problem because it damages normal tissue. For example, chronic inflammation accounts for 50% of global deaths due to autoimmunity, arthritis, diabetes mellitus, cancer, chronic kidney diseas, and stroke. There has been an increase in diseases involving inflammatory processes, namely stroke (56%), diabetes mellitus (23%) and hypertension (32%). The World Health Organization (WHO) has identified chronic inflammatory diseases as the greatest threat to human health [5,6].

People use nonsteroidal anti-inflammatory drugs (NSAIDs) to treat inflammation, inhibiting prostaglandin synthesis by blocking the activity of the cyclooxygenase enzyme. Taking NSAIDs for a long time is known to cause adverse health effects such as kidney, bone, gastrointestinal and cardiovascular problems [7]. Due to the side effects of NSAID drugs, alternatives are needed to find safe ways to treat inflammatory diseases with fewer side effects using plants. Plant chemicals that have anti-inflammatory activity are terpenoids that significantly inhibit the development of chronic joint swelling. Terpenoids have shown therapeutic effects on inflammation [8]. One of the Rutaceae plant families, namely oranges and lemons, contains essential oils that have considerable properties in traditional medicine to treat indigestion, as well as antipyretic and anti-inflammatory properties. Kaffir lime plant *(Citrus hystrix* DC.) has long been used as a herb in conventional medicine [9,10].

Research on the Rutaceae family isolated furanocoumarin from kaffir lime fruit peel (*Citrus hystrix* DC.) was able to inhibit *nitric oxide* induced by lipopolysaccharide in RAW 264.7 cells and inhibition of cyclooxygenase (COX-2) in HT-29 and HCT116 cells [11]. The content of essential oil (*Citrus hystrix* DC.), namely limonene, β-pinene and terpinene-4-ol, is not toxic to normal skin cells in various skin layers such as keratinocytes (HaCat), melanocytes (HEM). Linalool, α-terpineol, and limonene in essential oil *(Citrus aurantium)* or sweet orange were able to inhibit TNF-α, IL-6, decreased *nitric oxide*, COX-2, NF-kB, and MAPK signaling pathways in RAW 264.7 cells stimulated by lipopolysaccharide. Isolation of limonene from (*Citrus latifolia)* or Persian orange is able to inhibit pro-inflammatory mediators in inflammatory exudates and leukocyte chemotaxis with inflammatory cytokines such as TNF-α [12,13].

In this study, anti-inflammatory activity was tested on RAW 264.7 cells induce with lipopolysaccharide (LPS). Lipopolysaccharide is an endotoxin produced by Gram-negative bacteria that induce cytokines through toll-like-receptor (TLR-4), stimulates cytokine production, and activates the macrophage response, resulting in excessive nitric oxide (NO) production [14,15]. So that researchers are interested in evaluating the effect of essential oils of kaffir lime peel (*Citrus hystrix* DC.) on RAW 264.7 cell viability and nitric oxide (NO) levels induced by LPS on anti-inflammatory effects.

2. Materials and Methods

2.1 Materials

Kaffir lime fruit peel was taken from Subusalam Tapak Tuan, Kapa Seusak Village, Trumon Timor District, South Aceh (the plant has been registered in Medanese herbarium with number 2222/MEDA/2024). Lipopolysaccharide (Sigma-Aldrich), Dimethyl sulfoxide (Emsure), RAW 264.7 cells, Dulbecco's Modified Eagle Medium (Gibco), Griess reagent (Biotium), (3-(4,5-Dimethylthiazol-2-yl-2,5-Diphenyltetrazolium Bromide) (Himedia).

2.2 Essential oil extraction Microwave-assisted-extraction (MAE)

Isolation of essential oil by Microwave-Assisted Extraction hydrodistillation method. A 50 gram sample was added to 300 mL of distilled water and heated in a microwave for 30 minutes with a maximum power of 900 Watts [14].

2.3 Determination of essential oil content of kaffir lime fruit peel

Calculation of percent yield is done to show the content of essential oil. Essential oil from kaffir lime peel that has been collected, then calculated the percent yield using the following formula [15].

$$\% \text{ Content} = \frac{Essential\ oil\ produced\ (ml)}{Weight\ of\ raw\ materials\ used\ (gr)} \times 100\%$$

2.4 Determination of refractive index of kaffir lime peel essential oil

Measurement of refractive index of essential oil is done to determine the purity of essential oil obtained. The refractive index value is influenced by the components contained in the essential oil. The refractive index measurement process is carried out by dripping essential oil samples and then reading the scale formed on the Refactometer tool [16].

2.5 Cell culture

For the cell harvesting process, take 500 µL of cell harvest and put it into a culture flask . Add 6 ml of MK, then homogenize. Incubate the cells in a CO_2 incubator, and observe the cell condition the following day. RAW 264.7 macrophage cells were grown in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37^0C with humidity and 5%CO_2 [17,18].

2.6 Cell viability testing (MTT Assay)

RAW 264.7 cells were cultured in DMEM medium containing penicillin, stereptomycin, and fetal bovine serum (FBS) as many as 1×10^4 cells/well were planted in 96-well plates and allowed to grow for 24 hours. After 24 hours, the medium was replaced with new medium, then the test solution of essential oil of kaffir lime peel with concentrations of 100µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL, 3.12 µg/mL, 1.56 µg/mL and 0.78 µg/mL and dexamethasone concentrations of 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL, 0.62 µg/mL, 0.31 µg/mL, 0.15µg/mL, and 0.07µg/mL, then added 1 µg/mL LPS and incubated in a 5%CO_2 incubator at 37^0C for 24 hours. After incubation, the medium and test solution were discarded and the cells were washed with PBS. Each well was then given 100 µL of culture medium and 10 µL of MTT solution (5 mg/mL) to observe cell viability. Incubation was continued for 2-4 hours in a 5%CO_2 incubator at 37^0C, then observed under an inverted microscope. The MTT reaction process was stopped by adding stopper reagent (10% SDS in 0.1 N HCl). The plate was then wrapped with aluminum foil and left at room temperature overnight. Living cells react with MTT to form a purple color. Results were measured using a microplate reader at a wavelength of 595 nm. Cell viability was calculated using the cell viability formula. Concentrations with viable cells (>90%) can be tested subsequently [19-21].

$$\text{Cell viability (\% live cells)} = \frac{(Treatment\ absorbance - Media\ control\ absorbance)}{(Absorbance\ of\ cell\ control - Absorbance\ of\ media\ control)} \times 100\%$$

2.7 Nitric oxide (NO) measurement

RAW 264.7 cells (1×10^4 *cells/well*) were grown on *96-well plates* induced with 1µg/mL LPS. Then incubated for 24 hours and then added the test solution essential oil of kaffir lime peel at a concentration of 1.56 µg/mL, 0.78 µg/mL and dexamethasone at a concentration of 0.15µg/mL, 0.07µg/mL as a positive control, followed by stimulation using LPS as a negative control (1µg/mL) and then incubated again for 24 hours in a 5%CO_2 incubator at a temperature of 370^0C. The amount of nitrite, a stable NO metabolite, was measured using *Griess* reagent. Then 100µL of culture supernatant was added to 100 µL of *Griess* reagent, then incubated for 10 minutes in a dark room. The absorbance was measured at 595 nm on a microplate reader. Standard nitrite concentration was calculated using sodium nitrite standard solution (2000 µM), then reacted with *Griess* reagent and read the absorbance with a microplate reader. NO production in LPS-stimulated RAW 264.7 cells was measured by the accumulation of nitrite in the culture supernatant using the *Griess* reagent system [22,23].

2.8 Statistical analysis

Data were analyzed using the *Statistical Package for Social Sciences* (SPSS) version 22.0. Data are presented as *mean ± standard error means* (SEM). Analysis of variance (ANOVA) for multiple comparisons was used for data analysis $P>0.05$ was considered significantly different.

3. Result and discussion

3.1 Essential oil isolation results

Determination of essential oil content of kaffir lime fruit peel is done by using *microwave-assisted extraction* (MAE) method. This method uses an *aquadest* solvent, so that the *aquadest* and essential oil will evaporate together, where after condensation the essential oil will be collected and separated from the *aquadest* solvent. The volume of essential oil can be measured and calculated levels in the sample [15]. In this study the results of the average content of essential oil of kaffir lime fruit peel with *microwave assisted extraction* (MAE) method is 3.03 ± 0.37%.

Table 1. essential oil isolation results of kaffir lime fruit peel.

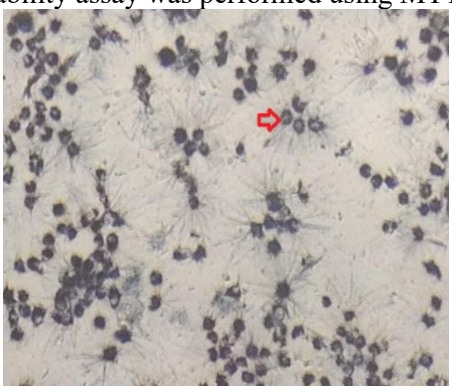
Weight of sample	Essential oil (mL)	Levels
50	1.3	2.6
50	1.6	3.2
50	1.7	3.4
Average		**3.03±0.37%**

3.2 Refractive index of kaffir lime peel essential oil

Refractive index testing is used to identify substances and detect the purity of essential oils. Measurement of the refractive index of kaffir lime peel essential oil in this study was carried out using a refractometer. The working principle of the refractometer is based on the phenomenon of refraction, where light passing through two media with different densities will change direction. The refractive index of kaffir lime peel essential oil is 1.472 ± 0.0001. The reference parameter for the refractive index of kaffir lime peel essential oil is 1.4686-1.4763. So that the refractive index value of kaffir lime peel essential oil is qualified based on reference parameters [24-27].

3.3 Cellular toxicity of kaffir lime (*Citrus hystrix* DC.) fruit peel essential oil

Cell viability assay was performed using MTT assay.



(a)

Figure 1. Formazan crystals: (a) Essential oil

Based on observations in Figure 1. Formazan crystals: (a) essential oil of kaffir lime fruit peel shows the formation of formazan crystals the ability of cells to reduce MTT produces formazan crystals with a purple color indicating that the cells are still alive, while dead cells cannot do so [28].

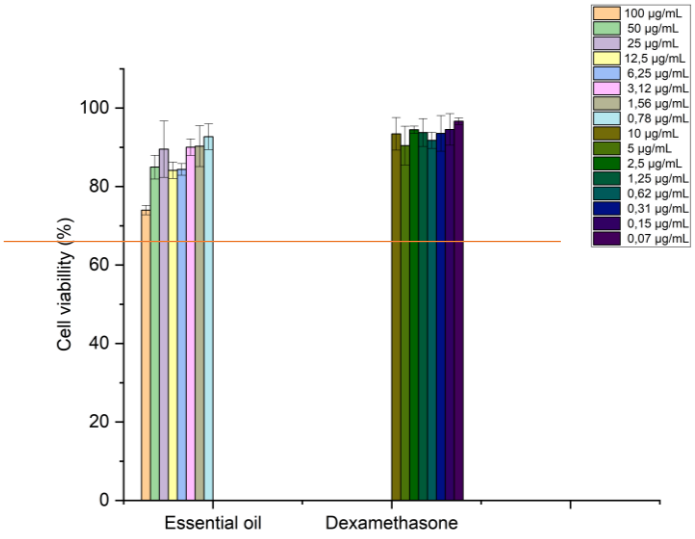


Figure 2. Graph of % of live cells influenced by essential oils of kaffir lime peel.

The average viability value of % live cells in Figure 2. shows that the higher the concentration of essential oil of kaffir lime peel, the lower the cell viability value, indicating that the higher the concentration, the higher the toxicity to the cells. The best results of cell viability test that have the highest % of live cells from essential oil of kaffir lime peel are concentrations of 1.56 and 0.78 µg/mL, while dexamethasone is concentrations of 0.15 and 0.07 µg/mL. In this experiment, the concentration of samples that have an impact on cell viability of >90% indicates that the cells are not toxic and can be continued for the next test [29,30].

3.4 Inhibition of nitric oxide (NO) production

Assaying nitric oxide (NO) levels in cells is used as a marker of inflammatory activity. NO has important physiological functions in the body against microbial immune responses, but excessive production of NO can lead to arthritis, a chronic inflammatory disease. LPS (Lipopolysaccharide) can trigger NO secretion in macrophage cells, which then triggers chronic inflammation. In RAW 264.7 cells, inflammation induced by LPS will experience increased release of pro-inflammatory cytokines such as IL-6, TNF-α and high NO production [31,32]. The nitric oxide assay in this study uses the *Griess* reaction, which is the simplest reaction and has been widely used for sample analysis in cell culture media.

The results of the NO production test in Figure 3 show that cells stimulated by LPS showed higher nitric oxide (NO) levels compared to those not stimulated by LPS ($p<0.05$). This indicates that LPS can induce the production and secretion of NO. LPS is a bacterial endotoxin compound that can trigger the production of NO secretion in macrophage cells [31]. The group given the test solution of essential oil of kaffir lime peel at concentrations of 1.56 and 0.78 µg/mL was able to reduce NO levels (9.58 ± 0.38; 10.16 ± 0.49) compared to LPS. Based on research by Warsito (2017), the analysis of essential oil components of kaffir lime peel with GC-MS contains terpenoid compounds such as β-pinene, citronellal and limonene [33]. The anti-inflammatory activity of terpenoids has methyl and phenol (O-H) groups that play a role in determining the pharmacological activity of terpenoids. In general, activating macrophages induced by LPS will produce pro-inflammatory cytokines or inflammatory mediators. NF-kB and the mitogen-actived protein kinase (MAPK) pathway have an important role in this process. NF-kB is one of the transcription factors that regulates the expression of enzymes and pro-inflammatory cytokines, making it a target for the development of anti-inflammatory drugs. The content of terpenoids such as α-pinene, D-limonene, linalool is able to reduce the release of nitric oxide [34-38].

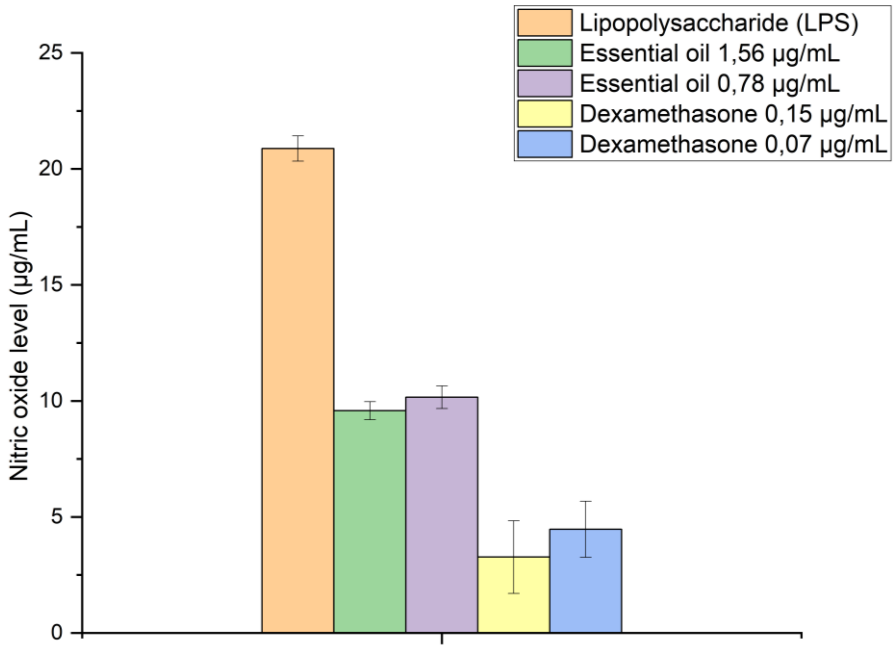


Figure 3. Graph of the effect of nitric oxide on RAW 264.7 cells induced by LPS

4. Conclusion

Based on the results of this study, it was found that essential oils of kaffir lime peel (*Citrus hystrix* DC.) have potential anti-inflammatory activity by inhibiting nitric oxide production in RAW 264.7 cells induced by lipopolysaccharide (LPS). The essential oil of kaffir lime peel at concentrations of 1.56 and 0.78 µg/mL are

non-toxic, able to inhibit nitric oxide (NO) when compared to lipopolysaccharide (LPS). Kaffir lime peel essential oil concentrations of 1.56 and 0.78 µg/mL and significantly different from lipopolysaccharide ($p>0.05$). So it can be concluded that the essential oil of kaffir lime peel have anti-inflammatory activity.

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