

Topical Application of *Citrus limon* Peel Essential Oil Increased Pro-Inflammatory Cytokines Expression on Type IV Hypersensitivity Reaction

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Abstract

Introduction: Citrus limon peels essential oil has the potential to be developed as a topical medicine due to its active components. The essential oil has been demonstrated in numerous studies to have anti-inflammatory, anti-microbial, antifungal, and antioxidant properties. A study is required to determine whether *Citrus limon* peel essential oil can cause a type IV hypersensitivity reaction by examining the expression of the cytokines TNF-, IL-1, and IL-6 as well as the clinical manifestations on an animal skin. In this study, the effect of topically applying the essential oil of *Citrus limon* peel on the expression of TNF- α , IL-1, and IL-6 as well as on type IV hypersensitivity reaction symptoms was examined. **Methods:** This study is based on the guinea-pig maximisation test method in NADFC RI Regulation Number 7 of 2014, with two groups (control group and treatment group) for each set of ten samples. Citrus limon peels essential oil was administered to the treatment group, whereas CMC-Na was given to the control group. Animal models were euthanized after which the tissues were prepared as specimens for immunohistochemical staining. The expression of cytokines was computed and examined using a light microscope. **Results:** There were no clinical signs of type IV hypersensitivity in either group, and the expression of TNF- α , IL-1 and IL-6 in the treatment group was found higher than in the control group. **Conclusion:** After topical administration of *Citrus limon* peels essential oil, there was an increase in the expression of TNF- α , IL-1 and IL-6 without any clinical symptoms of type IV hypersensitivity.

Keywords: *Citrus limon*, Essential Oil, IL-6, TNF- α , IL-1, Type IV Hypersensitivity

1. Introduction

According to the World Health Organization (WHO), plant is one of the best sources of medicine. About 80% of the world's population has used plant-based medicine for health care¹. In Indonesia, it has been common for a long time that herbal plants can be used for therapeutic purposes. WHO has recommended using herbal medicines for the prevention and treatment of diseases, as well as for maintaining public health, because it is known that herbal medicines have comparatively fewer side effects compared to modern medicine². Lemon (*Citrus limon*) is an aromatic plant from the *Rutaceae*

family and can be categorized as a medicinal plant that is rich in vitamin C and citric acid^{3,4}. *Citrus limon* peel consists of two layers, mesocarp and pericarp containing essential oils obtained through steam distillation, solvent extraction, Soxhlet extraction, or cold pressing^{5,6}.

Citrus limon peels essential oil contains more than 200 components of a monoterpene, sesquiterpene hydrocarbon, and its oxygenated derivatives such as ester, alcohol, aliphatic aldehyde, and ketone. *Citrus limon* peels essential oil has the ability as an antiseptic, analgesic, antimicrobial, antifungal, antioxidant, also anti-inflammation^{7,8}. To develop the potential of *Citrus limon* peel essential oil, based on ISO 10993-10-2010 about the

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medical biocompatibility test, it needs to be tested starting from *in vitro*, cytotoxicity, *in vivo*, and hypersensitivity test to confirm that the ingredients are not harmful towards the user. The *in vitro* test results obtained were *Citrus limon* peel essential oil with a concentration of 0.78% was the least concentration that could prevent the growth of *Candida tropicalis*⁹. The *in vivo* test results obtained were *limonene* content in the *Citrus limon* peels essential oil can increase the migration of inflammatory cells such as macrophages to the inflamed area so that the wound healing process is faster¹⁰. The next step is to ensure the biocompatibility of the essential oil through is hypersensitivity reactions test. Since the essential oil is often applied topically, a test is required to ensure that its usage will not trigger hypersensitivity reactions, especially type IV.

Hypersensitivity reactions are divided into four categories according to the Gell and Coombs systems. Type IV reactions are dependent on the interaction of antigens with T cells called delayed-type hypersensitivity. Type IV reaction begins with the sensitization phase when the antigens that are typically external such as drugs entered, will be processed by Langerhans cells which present antigens to the Cluster of Differentiation (CD) 4⁺ T lymphocytes then CD4⁺ T cells will differentiate. After re-exposure to the same antigen, the sensitization phase can switch to the elicitation phase. Activated T lymphocytes release Interferon (IFN) γ which then activates macrophages to secrete inflammatory cytokines, namely Tumor Necrosis Factor (TNF)- α , Interleukin (IL)-1, IL-6.^{11,12-14} Type IV hypersensitivity reactions may take several days to manifest from drug exposure that saw in allergic contact dermatitis. After contact with some compounds that have a small molecular mass, such as herbal components and medications, allergic contact dermatitis may show symptoms of erythema, edema, and occasionally vesicles on the mucosa or skin¹³.

TNF- α , IL-1, and IL-6 are pro-inflammatory cytokines that play a role in the immune response by increasing the activity of the immune system in inflammatory responses and are responsible as mediators in the development of lesions¹⁵. The advantages possessed by essential oil can be made into topical medications. Therefore, the study is required to demonstrate that topical application of *Citrus limon* peels essential oil does not result in hypersensitivity, particularly type IV, by tracking the expression of the relevant cytokines, TNF-, IL-1, and IL-6, as well as the appearance of clinical symptoms on an animal skin.

2. Methods

2.1 Animals

This study is adopted from the guinea-pig maximisation test method in the National Agency for Drug and Food Control of the Republic Indonesia (NADFC RI) Regulation Number 7 of 2014 to identify a substance that can potentially cause skin sensitization. The sample used in this study is *Cavia cobaya* weighted 300-500 grams from Biochemistry Laboratorium, Faculty of Medicine, Universitas Airlangga with a sample size for each group of 10 samples. The ethical clearance has been done in Universitas Airlangga, Faculty of Dental Medicine by the Health Research Ethical Clearance Commission Number 072/HRECC/FODM/VI/2018.

2.2 Citrus limon Peels Essential Oil Gel Making

To obtain 100% pure *Citrus limon* peel essential oil, *Citrus limon* originating from Lawang, East Java was steam-distilled in the Chemistry Laboratory of the Faculty of Science and Technology, Universitas Airlangga. The Gas Chromatography-Mass Spectrometry (GC-MS) test was conducted on *Citrus limon* peel essential oil 100% before the type IV hypersensitivity test to determine the compounds contained therein¹⁶. Using 3% Carboxyl Methyl Cellulose-Sodium (CMC-Na) powder, the Citrus limon peel essential oil 100% is diluted to achieve concentrations of 0.39%, 0.78%, and 1.56% before being made into a gel.

2.3 Preliminary Test

Type IV hypersensitivity test begins with a preliminary test to determine the concentration of *Citrus limon* peel essential oil used as the induce dose and the challenge dose in the main research. Based on the predetermined concentration, nine experimental animals were separated into three groups, each of which had three samples with concentrations of 0.39%, 0.78%, and 1.56%. At 24 hours before treatment, the back of the animal is shaved. Each concentration of gel form is smeared on filter paper and taped on the experimental animal according to the group division, then covered with an occlusive dressing and wrapped using an elastic bandage. The occlusive dressing was opened after 24 hours to observe for any erythema and/or edema, based on the following Table 1.

Table 1. Magnusson and Kligman scale

Topical reaction	Score*
No change	0
Mild erythema	1
Moderate erythema	2
Severe erythema and/or edema	3

*observations were made by ≥ 3 different people and then assessed using the Magnusson and Kligman scale to determine the concentration to be used. Under the regulation set by NADFC RI Number 7 of 2014 and the Organization for Economic Cooperation and Development (OECD) that the induced dose concentration used in the research is the highest concentration that does not cause severe erythema or edema according to the scale, while the challenge dose concentration used in the research is the highest concentration which does not cause any erythema.

2.4 Main Research

According to the standards of ISO 10993-10-2010 and NADFC RI Number 7 of 2014 concerning the In Vivo Non-Clinical Toxicity Test Guidelines, the main study utilized 20 *Cavia cobaya* split into control group and treatment group, with each group consisting of up to 10 *Cavia cobaya*. Based on the result of the preliminary study, it can be determined the concentration of 1.56% was used at the induction stage and the concentration of 0.78% was used at the challenge stage.

At 24 hours before induction, the back of the experimental animal is shaved first. On day 0, intradermal induction is performed, and there are 3 points to be injected; A, B, and C.

- Point A: Located near the head of the animal. Both groups are injected with a 1 ml mixture of Freund's Complete Adjuvant (FCA) + physiological solution.
- Point B: Located in the middle of the animal's back. The control group were injected with physiological solution + CMC-Na 3% and the treatment group was injected with *Citrus limon* peel essential oil 1.56%.
- Point C: Located near the animal's tail. Both groups were injected with the mixture of the solution in points A and B.
- Point D: Located in between points B and C. Topically applied *Citrus limon* peel essential oil 0.78% and the tissue will be biopsied on day 26.

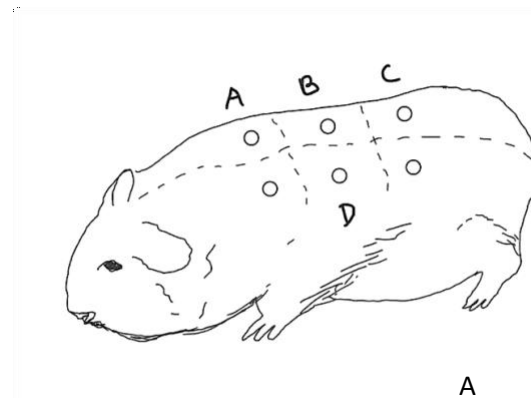


Figure 1. Illustration of induction on the backs of experimental animals.

On day 7, topical induction was carried out. The treatment group received *Citrus limon* peel essential oil 1.56% topically while the control group received diluted CMC-Na 3%. Both groups were subsequently covered for 24 hours with an occlusive covering and an elastic bandage. On day 23, the challenge stage, the treatment group received 0.78% *Citrus limon* peel essential oil topically whereas the control group received diluted CMC-Na 3% topically, then covered with the same material as before. On day 24, occlusive dressing and elastic bandage were released and then observed every 24 hours to 72 hours. On day 26, *Cavia cobaya* was terminated and the tissue at point D was taken using the biopsy method.

Animal tissues model were then made into specimens for Immunohistochemistry (IHC) staining with anti-cytokine antibodies. The expression of TNF- α , IL-1 and IL-6 was observed in macrophage cells from the back skin tissue of experimental animals at the point that was given *Citrus limon* peel essential oil 0.78% topically. The expression of IL-1 was observed using a light microscope at 1000x magnification while TNF- α and IL-6 were observed with 400x magnification, in 5 fields of view, then calculated and averaged.

2.5 Statistical Analysis

The research data were analyzed using the One-Sample Kolmogorov-Smirnov normality test. If data is normally distributed, analysis is followed by an Independent T-test parametric statistical test to see if there are significant differences between the control and treatment groups. According to the Independent T-test results, which showed $p = 0.000$ ($p < 0.05$), it was determined that there were significant differences.

3. Results

3.1 Gas Chromatography-Mass Spectrometry

Based on the GC-MS test conducted, there were 24 compounds contained in the *Citrus limon* peel essential oil. Compounds with the highest percentage include *D-limonene* (75.02%), *Linalool* (5.63%), *E-citral* (4.41%), *Z-citral* (3.68%), and other compounds with a percentage below 1%¹⁶.

3.2 Clinical Features

The clinical observation at 24 hours after topical use of *Citrus limon* peel essential oil 1.56% showed erythema of the *Cavia cobaya* skin while topical use of *Citrus limon* peel essential oil 0.78% and 0.39% did not cause erythema of the *Cavia cobaya* skin. Based on the result of the preliminary study, it can be determined the concentration of 1.56% was used at the induction stage and the concentration of 0.78% was used at the challenge stage.

The clinical observation 24 hours after day 7 showed no clinical changes on an animal's skin. On days 24 and 26, there were no clinical manifestations of type IV hypersensitivity reactions such as inflammation or tissue damage on animal skin (Figure 2) and scored "0" according to Magnusson and Kligman grading scale.

3.3 TNF- α Expression

TNF- α could be seen in macrophage cells as a dark blackish-brown image as shown in Figure 3. TNF- α expression was substantially lower in the control group (3.9 ± 0.737) than in the treatment group (12 ± 0.577).

3.4 IL-1 Expression

The result of the immunohistochemistry examination showed the expression of IL-1 as a round and dark brown image (indicated by arrows in Figure 4). Calculation of IL-1 expression revealed that the treatment group's average IL-1 expression was higher than that of the control group. The average IL-1 expression in the control group was 2.1 ± 1.197 and in the treatment, group was 8.7 ± 4.547 .

3.5 IL-6 Expression

In macrophage cells, IL-6 displays a blackish-brown image as shown in Figure 5. The expression of the control group

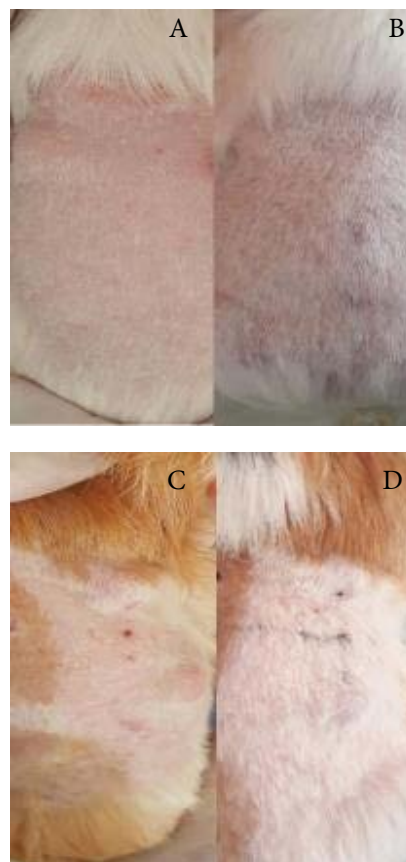


Figure 2. Clinical images of skin from **a.** The control group was on day 24 **b.** The control group on day 26 **c.** Treatment group on day 24 and **d.** The treatment group was on day 26.

seemed lower with an average of 5.3 ± 1.89 compared to the treatment group which had an average of 11.4 ± 3.37 .

3.6 Statistical Analysis

The One-Sample Kolmogorov-Smirnov test result showed that the data were evenly distributed across all groups. On the normally distributed data, analysis is followed by an Independent T-test parametric statistical test. Independent T-test results showed a significance value

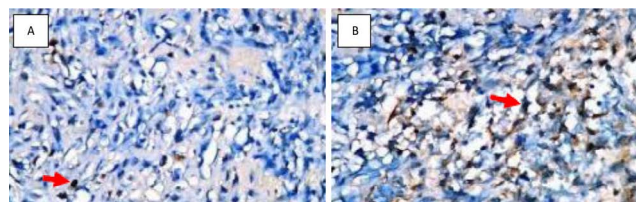


Figure 3. TNF- α expression (red arrow) was seen under a light microscope with 400x magnification. **a.** Control group **b.** Treatment group.

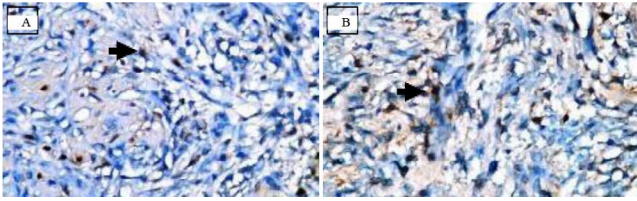


Figure 4. IL-1 expression (black arrow) was seen under a light microscope with 1000x magnification **a.** Control group **b.** Treatment group.

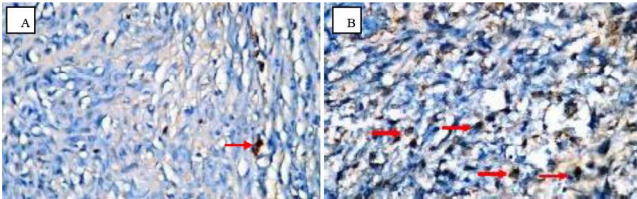


Figure 5. IL-6 expression (red arrow) was seen under a light microscope with 400x magnification **a.** Control group **b.** Treatment group.

of $p < 0.05$ therefore it can be concluded that there were significant differences between the control group and the treatment group in this study (Table 2).

Table 2. Mean and Standard Deviation and Significance Value

	Control Group	Treatment Group	Significance Value
TNF- α expression	3.9 ± 0.737	12 ± 0.577	0.000
IL-1 expression	2.1 ± 1.197	8.7 ± 4.547	0.001
IL-6 expression	5.3 ± 1.89	11.4 ± 3.37	0.000

4. Discussion

The process of this research begins by extracting essential oil from *Citrus limon* peel using the steam distillation method. The advantages of essential oils extracted by the steam distillation method are that they do not change the nature of the oil produced, besides the price is relatively cheap, they are fast, do not require solvents and are safer than other methods¹⁷. The essential oil from *Citrus limon* peel is then mixed with CMC-Na 3% powder so that it becomes a gel. The purpose of making preparations into gel form is to facilitate the application of *Citrus limon*

oil on the skin of experimental animals, more stable, and faster to work on the application site, compared to preparations of creams or ointments¹⁸.

There were 24 compounds contained in the *Citrus limon* peel essential oil of this study with the most compound was *D-limonene* of 75.02% while in the study of Tjahyono et al., there were 81 compounds contained in the *Citrus limon* peel essential oil with the percentage of *D-limonene* was 18.52%. Both internal and external factors contributed to the qualitative and quantitative variance in the composition and quantity of chemical components that make up essential oils. Internal factors are related to plants including the timing of planting and fertilization, interactions with the environment, such as the kind of soil and weather, plant maturity, and the time of harvest. External factors are influenced by the extraction methods, environment, length of the extraction processes, storage techniques, and dehydration processes¹⁹⁻²².

D-limonene and *linalool*, which are terpenoid components in the *Citrus limon* peel essential oil, are known to have biological activity as immunostimulators. Immunostimulators are substances that activate the immune system of animals to increase the body's natural defences. In general, immunostimulators stimulate the major components of the immune system including phagocytosis, the release of IFN γ , and induce the production of certain antibodies and cytokines²³⁻²⁵. The previous study has suggested, if the content of *D-limonene* and *linalool* in the *Citrus limon* peel essential oil oxidized, it could increase skin sensitization in the occurrence of contact allergy. The oxidation products of these compounds react with proteins in the skin thus recognized by the immune system and forming antigens. In animal tests, oxidized compounds are considered strong sensitizers²⁶⁻²⁹. Based on the literature can be said that the *Citrus limon* peel essential oil potentially increase the expression of TNF- α , IL-1, and IL-6.

The delayed-type hypersensitivity response is significantly influenced by the production of TNF-, IL-1, and IL-6, which are primarily made by macrophages. When antigens enter the body, these cytokines are a signal released by APC that activated T lymphocytes and play a role in eliminating antigen in innate immunity. Re-exposure to the same antigen will trigger adaptive immunity which in addition to a role in the similar response to antigen elimination with the first exposure, TNF- α , IL-1, and IL-6 can also act as a pro-inflammatory cytokine that recruits inflammatory cells during the elicitation phase therefore

involved in local inflammation and first formed after tissue damage in type IV hypersensitivity. In addition to hypersensitivity, pro-inflammatory cytokines such as TNF- α , IL-1, and IL-6 also increased in the regulation of host defence against infection or other stimuli by causing an inflammatory response and carrying immune cells to the site of infection to be more effective in destroying pathogens.^{30,31} Therefore, the increase of these cytokines indicates that there are antigens that invade the host's body³².

On day 0 (the induction stage), the treatment group received *Citrus limon* peel essential oil 1.56% intradermally on day 0 and topically on day 7 while the control group received CMC-Na 3% intradermally on day 0 and topically on day 7. Bodies that are never exposed to these substances will respond through innate (non-specific) immunity. Compounds contained in *Citrus limon* peel essential oil will enter and be recognized by the body as an antigen to be presented APC in T cells along with MHC class II molecules in APC. Furthermore, with the help of TNF- α , IL-1, and IL-6 as a signal released by APC, naive CD4⁺ T cells are activated and differentiate into CD4⁺ effector T cells (helper T cells) and memory T cells. This phase lasts for 1-2 weeks and causes animal models to be sensitized. Activated Th1 cells then release IFN γ to increase the role of macrophages in the phagocytosis of antigens. During this process, NF- κ B in the macrophages also activates and induces pro-inflammatory cytokines including TNF- α , IL-1, and IL-6 to mediate the process of antigen elimination. Th1 will also activate cytotoxic T cells to phagocyte antigens directly. After the phagocytosis, the remaining cytotoxic T cells will become memory cells^{8,32-34} The possibility of antigen having been digested and sterilized through the phagocytosis process is proven by no reaction on animal skin observed after day 7.

On day 23 (the challenge stage), *Citrus limon* peel essential oil of 0.78% was applied topically on animal skin. When exposed to the same antigen, the body that has recognized these antigens will respond through adaptive (specific) immunity. Memory T cells that have remembered antigens will accelerate the Th1 cell response in activating macrophages to eliminate antigens through a similar phagocytosis process at the first exposure. However, the pro-inflammatory cytokines released by macrophages namely TNF- α , IL-1, and IL-6 can also increase inflammation and cause tissue damage which are clinical manifestations of type IV hypersensitivity reactions. Cytotoxic T cells that work by killing antigen-

exposed cells directly also cause tissue damage. In some of the body reactions, such as hypersensitivity reactions, the strength of the immune response directed against the antigen can also cause tissue damage^{11,33-35}.

In this study, no clinical manifestations such as inflammation and tissue damage were found on the skin of *Cavia cobaya* showed *Citrus limon* peel essential oil 0.78% did not cause type IV hypersensitivity reactions such as erythema or edema. This condition occurred by the possibility of antigens that have been eliminated and are also caused by the activity of essential oil compounds in both suppressing the inflammatory response to increase the anti-inflammatory effect on the body or as an immunostimulant to increase the body's defence through phagocytosis. This finding is consistent with a previous study, which may be related to the activity of anti-inflammatory cytokines in *Citrus limon* peel essential oil¹⁶.

The results of this study showed an increased expression of TNF- α , IL-1, and IL-6 after topical application of *Citrus limon* peel essential oil. The average of these cytokine expressions in the control group can be inferred as lower than in the treatment group³⁶⁻³⁸. CMC-Na was used in the control group as a placebo to compare the effectiveness of the *Citrus limon* peel essential oil and only functions as a gelling agent. The gelling agent has high stability, and low toxicity, and is used in topical drugs to increase gel viscosity and the effectiveness of gel use. It does not contain active ingredients, is neutral, soluble in water, and also soluble in oil both volatile and non-volatile so that it can blend with oil. CMC- Na which had no therapeutic effect was the cause of lower expression of TNF- α , IL-1, and IL-6 in the control group than in the treatment group. The increased expression of these cytokines in the treatment group after topical use of *Citrus limon* peel essential oil may be caused by the immunostimulatory activity of this essential oil that can enhance the immune response by inducing cytokines.

The increase in TNF- α , IL-1, and IL-6 expression also can be caused by fragrances such as *linalool* and *limonene* that are contained in *Citrus limon* peel essential oil²⁶. In this research, *Cavia cobaya* was shaved before being treated. It is known that shaving with a razor has been shown to increase the risk of contact allergy fragrance³⁹. Because *linalool* and *limonene* are fragrances and in the type of non-oxidizing form, these two ingredients are weak allergens. But, when the two compounds are exposed to air, they will automatically be oxidized and form various

oxidation products, including hyper oxidation which is an allergenic product^{26,40}.

Based on the literature, it can be said that the *Citrus limon* peel essential oil has the potential to increase the expression of pro-inflammatory cytokines including TNF- α , IL-1, and IL-6. In this study, giving *Citrus limon* peel essential oil 0.78% caused a significant difference in the expression of TNF- α , IL-1, and IL-6. After applying *Citrus limon* peel essential oil topically, there was a rise in the expression of TNF- α , IL-1, and IL-6 without any clinical symptoms. It shows *Citrus limon* peel essential oil still influences increasing the type IV hypersensitivity response in *Cavia cobaya*. To confirm the mechanism of *Citrus limon* peel essential oil in type IV hypersensitivity reactions, further research is needed on anti-inflammatory cytokines such as IL-4 and IL-10.

5. Disclosure

No contributing authors have declared conflicts of interest.

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