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Anti-inflammatory Activity of Lemon Pepper Nanoemulsion on Carrageenan-Induced Male Wistar Rat

Linda Chiuman[1], Dwi Putri Agita Sebayang[2], Veranyca Chiuman[3], Suhartomi[4*]

[1] Department of Physiology, Faculty of Medicine, Dentistry, and Health Science, Universitas Prima Indonesia, Medan, Indonesia.
[2] Undergraduate Programme in Medical Sciences, Faculty of Medicine, Dentistry, and Health Science, Universitas Prima Indonesia, Medan, Indonesia.
[3] Department of Dentistry, Faculty of Medicine, Dentistry, and Health Science, Universitas Prima Indonesia, Medan, Indonesia.
[4] Department of Pharmacology, Faculty of Medicine, Dentistry, and Health Science, Universitas Prima Indonesia, Medan, Indonesia.

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Corresponding Author:
Suhartomi
suhartomi@unprimdn.ac.id

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Abstract: Lemon pepper does not only have a unique taste but also has some active compounds, which has anti-inflammatory activity. This study intended to investigate the anti-inflammatory effect of lemon pepper nanoemulsion on carrageenan-induced Wistar rats. This experimental study used Twenty-five male Wistar rats that were grouped into five different groups, including control, standard, lemon pepper nanoemulsion dose of 25, 50, and 75 mg/kgBW. Initially, all rats received air injection in the back area for 5 days to form an air pouch. After that, all rats were injected with 1% carrageenan solution, and received treatment based on the groups for 48 hours via oral route. Then, it counted the number of white blood cells by Giemsa Stain that was expressed as a number of cells per High Power Field (HPF). The increasing of Lemon pepper nanoemulsion dose significantly decreased the number of leucocytes in the carrageenan aspiration after 48 hours of treatment (P-Value < 0.001). The lowest number of leucocytes was found in the Lemon Pepper Nanoemulsion dose of 75 mg/kgBW (2.40 leukocytes per HPF). Hence, it can be deducted that the lemon pepper nanoemulsion has an anti-inflammatory effect, especially at a dose of 50-75 mg/kgBW.

Keywords: Carrageenan; Giemsa; Lemon pepper; Leukocytes; Nanoemulsion

Introduction

Inflammation is a series of an innate immune system as a response to the external noxious stimulus that either enters or invades the body, which potentially damages body tissue. These stimuli are infection, abrasion, burn, or toxin that can be acute or chronic. Some inflammation signs include redness, heat, swelling, pain, and loss of function (Mohan, 2019; Sundari et al., 2021).

Some drugs have been developed to relieve inflammation response, which is grouped into Non-Steroid Anti-Inflammation Drugs (NSAID) and steroid anti-inflammation drugs. Non-steroid anti-inflammation Drugs (NSAIDs) have been widely used as anti-inflammatory drugs, including aspirin, ibuprofen, et cetera. Meanwhile, the steroid anti-inflammation drugs come from the class of corticosteroid hormones drug, including methylprednisolone, dexamethasone, et cetera. Either NSAID or corticosteroid has some adverse drug effects. NSAIDs can cause gastrointestinal discomfort for NSAIDs that inhibit the COX1 enzyme and increase the risk of cardiovascular events for NSAIDs that inhibit the COX2 enzyme. On the other hand, corticosteroid causes more adverse drug, like osteoporosis, gastrointestinal discomfort, opportunity infection, or Cushing's syndrome in the long term (Andayani et al., 2018; Lamontagne et al., 2018; Putri & Anita, 2017).

Due to some adverse drug effects from anti-inflammation drugs, thus it become important to look for newer drugs with milder adverse drug effects. Most

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of the recent studies investigated some natural products for various pharmacologic effects. One of these natural products is lemon pepper, which has the local name *andaliman*. Lemon pepper, *Zanthoxylum acanthopodium DC,* is an herb that comes from the indigenous Toba Samosir District, Sumatera Utara. This herb is widely used as a spice which has a unique taste(Saragih & Arsita, 2019; Sitanggang et al., 2019; Winarti et al., 2018; Worotikan et al., 2017; Yanti et al., 2011).

Although lemon pepper has a unique taste, it also has some bioactive compounds like flavonoids, alkaloids, terpenoids and steroids, which are reported to have anti-inflammatory activity(Asbur, Y., 2018; Sriwichai et al., 2019). Some previous studies have reported the pharmacologic effect of lemon pepper extract. One of these previous studies reported whether lemon pepper extract significantly inhibited the expression of several inflammatory markers at the protein synthesis level, such as TNF-α, COX-2 protein, and MMP-9 and at the gene level, such as TNF-α, IL-6, iNOS, COX-2, and MMP-9 by lipopolysaccharide-induced macrophage in vitro model (Yanti et al., 2011).

Most recent studies only look for the pharmacologic effects of lemon pepper extract. None of these studies developed the pharmacologic effect from the nanoparticle form of lemon pepper extract, while the most recent study looked for the nanoparticle form of an herb. The most common form of nanoparticle, that was investigated is nanoemulsion. A nanoemulsion is a colloid system that forms an emulsion with a nano-sized of less than 100 nm(Ahmad et al., 2021; Jafar et al., 2017). This nanoparticle form has some advantages, including increasing solubility and absorption of the drug, as well as reducing the therapeutic-required dose. Due to these reasons, this study intended to investigate the anti-inflammation activity of lemon pepper nanoemulsion in the carrageenan-induced air pouch model.

Method

This experimental study used a post-test control group design at Laboratorium Terpadu, Universitas Prima Indonesia, in August-September 2023. Moreover, this study evaluated the anti-inflammatory activity by in vivo study from lemon pepper nanoemulsion at the end of intervention and treatment. This study has also been approved by Komite Etik Penelitian Kesehatan (KEPK) Universitas Prima Indonesia with letter no. 031/KEPK/UNPRI/VIII/2023.

Material that was used in this study included lemon pepper fruit, normal saline solution, distilled water, 5% Giemsa solution, propylparaben, PEG 400, methanol, methylparaben, carrageenan, and Tween 80.

Initially, lemon peppers were collected from a traditional market in Medan, Sumatra Utara, Indonesia. The obtained lemon peppers were sorted from the leaves and pedicles. After that, these lemon peppers were washed and dried for 5 hours at 55°C in an oven. The dried lemon peppers were then mashed and sieved by a forty-number mesh sieve. Amount of 700 grams lemon pepper powder was extracted by maceration method for 24 hours, which used Methanol solution as a solvent with ratio of 1:3. The obtained filtrate was collected to concentrate by using an evaporator at a temperature of 55°C (L. Chiuman et al., 2021; V. Chiuman et al., 2022; Gulo et al., 2021; Salim et al., 2021; Suhartomi et al., 2020).

The concentrated lemon pepper extract was formulated into a nanoemulsion form. Initially, Both 100 mg of Propyl Paraben and 200 mg of Methyl Paraben are dissolved into fifty milliliters of warm distilled water. Afterwards, thirty milligrams of Tween 80 was dissolved into distilled water. Then, both mixtures were stirred by a magnetic stirrer at 5000rpm for 30 minutes and labeled as the first mass. Furthermore, five grams of lemon pepper extract and fifteen milligrams of PEG 400 were mixed with a magnetic stirrer at 5000 rpm for 20 minutes and labeled as second mass. At last, the first mass was added gradually into the second mass and mixed it by sonification method to form a 5% lemon pepper nanoemulsion (Tanessa et al., 2023).

Before the lemon pepper nanoemulsion underwent an anti-inflammatory assay, it should ensure the nano-size of emulsion from lemon pepper nanoemulsioin. Hence, this study used Particle Size Analyzer Device to measure the size of the emulsion (Pal et al., 2018; Tanessa et al., 2023).

The obtained lemon pepper nanoemulsion was used for an anti-inflammatory assay by the Carrageenan-Induced air pouch model. In the beginning, this assay was used twenty-five Wistar rats, which grouped into five treatment groups, including control, standard, lemon pepper nanoemulsion dose of 25 mg/ kgBW, 50 mg/ kgBW, and 75 mg/ kgBW. Then, all rats received a twenty milliliters air injection via subcutaneous injection in the back area and then an additional ten milliliters air was subcutaneously injected after 3-6 days to keep the gap open. On the sixth day, two milliliters of 1% carrageenan solution in normal saline was injected into an air pouch that had been formed in the back area via subcutaneous injection. After that, all rats received some treatment based on the groups for 48 hours. The control group received only sodium carboxyl-methyl cellulose as a vehicle. The standard group received 100 mg/ kg BW of sodium diclofenac. Meanwhile, the lemon pepper nanoemulsion dose of 75 mg/ kgBW, 50 mg/ kgBW, and 25 mg/ kgBW were received at 0.225, 0.15, and 0.075 ml/ kg BW, respectively. After 48 hours, all air pouch in back area of rats were aspirated to obtain the carrageenan as a pus for

white blood cells (Leucocytes) counting(Salissou et al., 2022; Yuna et al., 2023).

The white blood cells or leucocyte counting in pus was performed by Giemsa-staining pus swab methods. The carrageenan or pus was swabbed into an object glass and chilled at room temperature until it was dry. Then, it was fixed with a methanol solution for 3 minutes. After that, it was stained with 5% Giemsa solution for 10 minutes. Finally, it was observed under a light microscope in 400x magnification to count the number of white blood cells per HPF(Salissou et al., 2022; Yuna et al., 2023).

Particle size analysis from lemon pepper nanoemulsion was expressed as the Mean, Median, and Mode size of the emulsion. Meanwhile, the number of leucocytes in the pus was expressed as Mean and Standard Deviation (SD). After that, the number of leucocytes from all groups was compared by a parametric statistic, which was one-way ANOVA and followed by Tukey HSD post Hoc Test**.**

Result and Discussion

This study used lemon pepper, which was formulated into nanoemulsion, and the result of particle size analyzed from lemon pepper nanoemulsion was described in Table 1.

Table 1. Particle Size Analysis of Lemon Pepper Nanoemulsion

Parameter	Value
Mean (µm)	0.20
Median (µm)	0.03
Standard Error (µm)	0.89
Min (µm)	0.01
Max (µm)	42.30

Based on Table 1, the mean of lemon pepper emulsion size was 0.20 µm with the smallest emulsion size was 0.01 µm and the largest emulsion size was 42.30 µm. Meanwhile, this study also performed an anti-inflammatory assay by carrageenan -induced air pouch model to evaluate the anti-inflammatory activity from lemon pepper nanoemulsion and the result of this assay was described in Table 2.

Based on Table 2, there was a significant difference in the number of WBC per HPF from pus aspiration among all treatment groups (P-Value < 0.001). The highest number of white blood cells from pus aspiration per HPF was found in the Lemon Pepper Nanoemulsion dose of 25 mg/ kgBW (27.20 ± 1.92 cells per HPF), followed by the Control group, which only received sodium carboxyl methyl cellulose (25.40 ± 5.03 cells per HPF), standard (9.60 ± 2.30 cells per HPF), Lemon pepper nanoemulsion dose of 50 mg/ kgBW (7.00 ± 1.58 cells per HPF), and the lowest one was lemon pepper nanoemulsion of 25 mg/ kgBW (2.40 ± 1.14 cells per HPF). Moreover, this analysis was continued into the Tukey HSD post-Hoc Test which was shown by superscript. According to the Tukey HSD post-test, the Standard group that received sodium diclofenac suspension showed a significant difference number of white blood cells from pus aspiration compared to the lemon pepper nanoemulsion dose of 25 mg/ kgBW and control group. Meanwhile, the highest dose of lemon pepper nanoemulsion (75 mg/ kgBW) significantly reduced the number of white blood cells in pus aspiration compared to the lowest dose of lemon pepper nanoemulsion and control group. However, the lower dose (50 mg/ kgBW) did not show any significant difference number of white blood cells from pus aspiration compared to the highest dose. Finally, the comparison of the number of white blood cells in all groups from pus aspiration is also described as a bar chart in Figure 1.

Table 2. Comparison of the Number of White Blood Cells per HPF from Pus (Carrageenan) Aspiration in All Groups

Groups	Number of WBC per HPF, Mean ± SD*	P-Value*
Control	25.40 ± 5.03^{a}	
Standard	9.60 ± 2.30^{b}	
Lemon Pepper Nanoemulsion dose of 25 mg / kgBW	27.20 ± 1.92^{a}	
Lemon Pepper Nanoemulsion dose of 50 mg/ kgBW	7.00 ± 1.58^{bc}	< 0.001
Lemon Pepper Nanoemulsion dose of 75 mg/ kgBW	2.40 ± 1.14^{c}	

*A different superscript in the same column indicates a significant difference by Tukey HSD Post Hoc Test; **P-Value was obtained from One-Way ANOVA

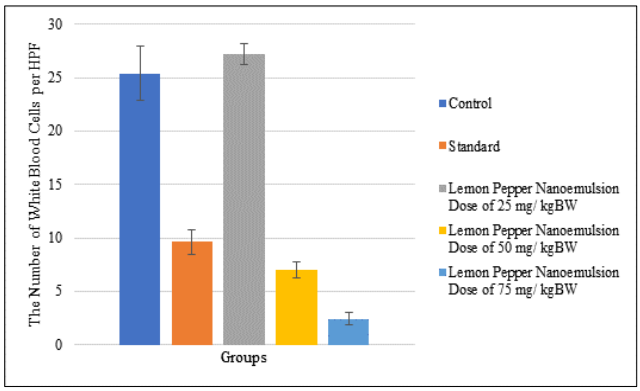

Figure 1. Bar Chart of The Number of White Blood Cells per HPF from Pus Aspiration in All Treatment Groups

According to Figure 1, the highest number of white blood cells per HPF from pus aspiration was found in lemon pepper nanoemulsion dose of 25 mg/ kgBW (gray bar), followed by the control group (dark blue bar), standard group (orange bar), lemon pepper dose of 50 mg/ kgBW (yellow bar), and the lowest number of white blood cells per HPF from pus aspiration was found in lemon pepper nanoemulsion dose of 75 mg/ kg BW (light blue bar). Furthermore, the microscopic view of pus (carrageenan) aspiration swabs that were stained by 5% Giemsa solution was described in Figure 2.

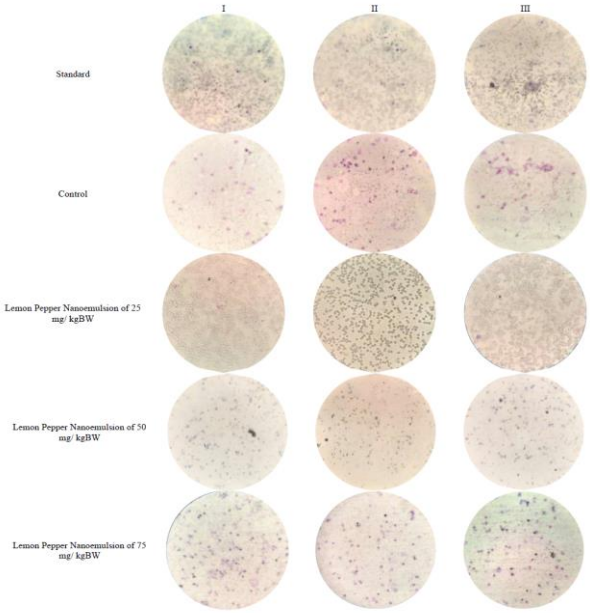

Figure 2. Pus (Carrageenan) Aspiration Smears in All Groups; Magnification: 400x; Stain: Giemsa.

Figure 2 showed some microscopic views of pus (carrageenan) aspiration from all groups, that was stained by 5% Giemsa solution. The white blood cells were indicated by multilobulate nucleus cells (Polymorphonucleated Cells/ PMN). The cytoplasmic of this type of white blood cell was stained by light violet, and the nucleated was dark violet with a multilobulated nucleus.

Nanoemulsion is a colloid system, that is consisted of submicron particle size (emulsion) as a vehicle of drugs (Abdassah, 2017; Ahmad et al., 2021). Nanoemulsion or mini emulsion is a dispersion of the oil phase into the water phase that was used to coat molecules to form some surfactant molecules with an average size range of 20 to 600 nm (Mamillapalli et al., 2016; Tanessa et al., 2023). In the current study, the lemon pepper nanoemulsion had an average emulsion size of 0.20 µm (200 nanometers). Thus, it indicates whether lemon pepper nanoemulsion fulfilled nanoemulsion size, and it can be used for further investigation.

Inflammation is a local response from the host to eliminate some causes of cell injury. Based on the offensive factor of the host, inflammation can be acute or chronic. Acute inflammation is a short-term inflammation (less than 2 weeks) that quickly resolves and is followed by a healing process. When the noxious agents persist for a long term, it leads to chronic inflammation condition. A chronic inflammation dominated by the granulation tissue and, in certain conditions form a granulomatous inflammation. The main features of acute and chronic inflammation are the presence of polymorphonucleate and monomorphonucleate inflamed cells, respectively. The polymorphonucleate inflamed cells can be neutrophils, eosinophils, or basophils in accordance with the cause of inflammation, but neutrophils are the prominent ones.

Meanwhile, the monomorphonucleate inflamed cells are either lymphocytes, plasma cells or macrophages that are part of the adaptive immune system(Kumar et al., 2015; Mohan, 2019; Rodak & Carr, 2017). In a recent study, pus or carrageenan aspiration is dominated by polymorphonucleate inflamed cells, and it indicated an acute inflammation condition. It was in line with the duration of the study. This study kept the carrageenan in an air pouch for 48 hours. Hence, this study investigated the anti-inflammation effect of lemon pepper nanoemulsion in an acute inflammation model. Interestingly, the lemon pepper nanoemulsion also reduced the number of this type of white blood cells, which indicated the anti-inflammation activity.

A limited number of studies investigated the pharmacologic effect of lemon pepper as a nanoparticle form. Most of these studies looked for the anti-inflammation effects of lemon pepper extract. Yanti et al. (2011) reported that lemon pepper extract suppressed inflammation responses by lipopolysaccharide-induced macrophage model(Yanti et al., 2011). Moreover, another study also investigated anti-inflammation activity from another form of lemon pepper nanoparticle. Ahmad et al. (2021) reported whether lemon pepper nanoparticles had an anti-inflammation effect by improving blood vessel structure and function in the atherosclerosis model. Furthermore, Adnan et al. also reported the antioxidant properties of lemon pepper methanol extract due to the presence of flavonoids. This flavonoid is reported to neutralize free radicals by proton-donor, which leads to reduce the free radical impact in the body (Adnan et al., 2021; Ahmad et al., 2021).

The pharmacologic effects of lemon pepper, especially as a nanoemulsion form, are be caused by presence of various phytochemicals, including alkaloids, phenols, and flavonoids. Some studies have reported various pharmacologic effects of these phytochemicals. Flavonoid was reported to inhibits the biosynthesis of

prostaglandins, which are involved in immunologic responses. Prostaglandins are also the final product of cyclooxygenase. In addition, another study also reported whether a flavonoid also affected protein kinase in the cell membrane, which acted as a regulatory enzyme in the inflammation process(Eldahshan & Abdel-Daim, 2015). Gaichu et al. (2017) also reported that other phytochemicals like alkaloids also inhibited the biosynthesis of prostaglandins(Gaichu et al., 2017). Due to this information, it became obvious that both flavonoids and alkaloids in lemon pepper potentially inhibited the biosynthesis of prostaglandins and led to the relief of the inflammation process that had been induced. Moreover, the inhibition of biosynthesis prostaglandins also potentially exhibits analgesic and antipyretic effects.

Conclusion

Overall, it can be deducted that the lemon pepper nanoemulsion that was used in this study has an average emulsion size of 0.20 µm. This lemon pepper nanoemulsion has an anti-inflammation effect by significantly decreasing the number of white blood cells per HPF in pus (carrageenan) aspiration from carrageenan-induced air pouch rat model (P-Value < 0.001). The most effective dose of lemon pepper nanoemulsion was ranged of 50-75 mg/ kgBW.

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Author Contributions
Conceptualization: Linda Chiuman, Dwi Putri Agita Sebayang, and Suhartomi; Methodology: Veranyca Chiuman and Suhartomi; Investigation: Dwi Putri Agita Sebayang; Discussion of results: Linda Chiuman and Suhartomi; Writing – Original Draft: Linda Chiuman, Dwi Putri Agita Sebayang, and Veranyca Chiuman; Writing – Review and Editing: Suhartomi; Supervision: Linda Chiuman and Suhartomi; Approval of the final text: Linda Chiuman, Dwi Putri Agita Sebayang, Veranyca Chiuman, and Suhartomi.

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Conflicts of Interest
The authors declare no conflict of interest.

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