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# Lime Peel Extract Effects in Decreasing Levels of Interleukin 6 in Mice Infected with *Salmonella Typhi*

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**Abstract**

**Background and aim:** Bacterial resistance, especially S. typhi, brings us to new treatments in the form of adjuvant therapy items, namely traditional plants that can be used as antimicrobials. One of them is lime (Citrus aurantifolia). Several studies have shown that the optimum lime peel has antibacterial power to bacteria. Higher concentrations of flavonoids are found in the lime peel compared to other parts such as seeds, fruit, and juice. S. typhi infection can stimulate the activity of innate immunity causing proinflammatory cytokines such as TNFα, IL-1 and IL-6 to be activated. IL-6 mediates the systemic effects of the inflammatory process, Including fever induction, acute phase protein synthesis, and Increased leukocyte production. Other studies have shown that flavonoids reduce regulation of the level of inflammatory genes Including IL-6.

**Methods**: This study is a true experimental pre-post test design to Investigate the effectiveness of lime peel extract (LPE) in calculating the level of IL-6 in serum. Mice were divided into; Group I (LPE 510 mg / kg bw), group II (LPE 750 mg / kg bw), positive control and negative control. The intervention was Carried out for 5 days. After the fifth day, mice were then maintained for 3 weeks to Determine examination of the level of IL-6 in serum.

**Results**: The results of phytochemical screening prove that lime peel contains flavonoids, tannins, saponins, alkaloids, and triterpenoids. Intervention of Lime peel extract for 5 days reduced IL-6 levels in the LPE510 group by 19.63 pg / mL ($p = 0.003$), the LPE750 group decreased 24.53 pg / mL ($p = 0.002$), observations after 30 days, still Decreased IL -6 levels in the LPE510 group ($p = 0.05$), and LPE750 ($p = 0.01$).

**Conclusion**: LPE with a dose of 510 mg / kg bw and 750 mg / kg bw significantly decreases serum levels of IL-6. Lime peel extract as a traditional plant can be used as adjuvant therapy in reducing proinflammatory factors in Salmonella typhi infections.

***Keywords:*** *Lime peel extract, IL-6, Salmonella, Phytochemical screening*

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## Introduction

Typhoid fever is caused by *Salmonella typhi*, is a gram-negative bacterium that is transmitted almost always occurs through contaminated food and drink [1], The most widely used drug in the treatment of typhoid fever is chloramphenicol, a drug used since 1948 and until now it is still used as the drug of choice in Indonesia because its effectiveness against *Salmonella typhi* is still high in addition to the relatively cheap drug prices. From the study of the molecular level suggested that *Salmonella typhi* bacteria are becoming resistant to chloramphenicol due to the enzyme-producing plasmid Chloramphenicol Acetyltransferase (CAT) which activates Chloramphenicol[1], Salmonella typhi bacteria resistance to chloramphenicol has started to increase. The development of antimicrobial resistance in line with the development of the use of antimicrobial drugs is increasing and in line with the discovery of new drugs [2].

Bacterial resistance, especially S. typhi, brings us to new treatments in the form of adjuvant therapy, namely traditional plants that can be used as antimicrobials. One is the plant lime (Citrus aurantifolia) [4]. Lime peel contains active ingredients that supposedly can provide antibacterial effects. These materials include essential oils (essential oils) that can interact and change the permeability of the cell membrane of microorganisms that cause the death of these microorganisms [4]. Based on previous research, lime also contain flavonoids. Higher concentrations of flavonoids are found in the lime peel compared to other parts such as seeds, fruit, juice[5], The content of flavonoids makes lime peels have antibacterial and antioxidant effect. Several studies have shown that lime peel has optimal antibacterial effect[6]. One of them is to test the flavonoid activity that is bacteriostatic or bactericidal by conducting a time-kill study, it was found that flavonoids did not kill bacterial cells but only induced the formation of bacterial aggregates thereby reducing the number of decent numbers of CFU (colony forming units) [7].

During S. typhi bacterial infection, a lipopolysaccharide (LPS) bond occurs from the bacterial wall with a Toll Like Receptor (TLR) 4 from the host which stimulates innate immunity activity. This activity releases a number of proinflammatory cytokines such as TNFα, IL-1 and IL-6. Research conducted by Galdiero et. al, is porins from S. typhi can affect IL-6 gene expression. Interleukin 6 (IL-6) mediates the protective systemic effects of inflammation, including induction of fever, acute phase protein synthesis by the liver, and increased bone marrow leukocyte production. The RT-PCR results in studies conducted in China proved that flavonoids lowered regulation of the level of inflammatory genes including IL-1, IL-6, and TNFα. Further studies showed that hepatoprotection induced by flavonoids inhibits TLR4 / MyD88 and activates the Sirt1 / Nrf2 signaling pathway which inhibits transcription of NF-κB and AP-1 and inhibits inflammatory reactions, one of which is proinflammatory cytokines IL-6 [8]. Therefore, this study aims to look at the effect of Lime peel extract (LPE) on proinflammatory cytokine protein activity IL-6 in mice induced by Salmonella typhi bacteria.

## Material and Methods

This study was a true experimental pre-post test design study using the ELISA examination method to calculate IL-6 antibody levels in 20 male mice with balb / c strain induced by Salmonella typhi bacteria.

### Making of Extracts

Making extracts was carried out at the Hasanuddin University Faculty of Pharmacy Phytochemical

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Laboratory. Lime skin is cut into small pieces and then dried at 50ºC to minimize water content. The dried sample is then put into a glass container / jar to be macerated by adding Ethanol 96% and then let it sit for 3 x 24 hours. Samples which were macerated for 3 x 24 hours were then filtered by the Vacuum Filtration method using a Buchner Funnel. The extracts obtained after the screening process were then evaporated using a Rotary-evaporator until the results were thick extracts.

**Phytochemical Test of Extract**

The phytochemical test of qualitative analysis was conducted at the Phytochemical Laboratory of the Faculty of Pharmacy, Hasanuddin University. Test the content of the active ingredient Flavonoid, with one gram of LPE extracted with 5 ml ethanol then add a few drops of concentrated HCl and 1.5 grams of magnesium metal. The presence of flavonoids, indicated by the formation of pink or red magenta in 3 minutes. For the presence of tannin active ingredients, 1ml of LPE is reacted with a solution of FeCl (3) 10%, if there is a change in color to dark blue or greenish black shows the presence of tannin and polyphenols [9].

Quantitative phytochemical tests were carried out at the Biopharmaceutical Laboratory of the Faculty of Pharmacy, University of Hasanuddin (Unhas Research Center), testing the content of flavonoids, tannins and polyphenols with each standard solution is Quercetin for flavonoids, acids Trees for tannins and acids for polyphenols [10].

**Animal Trial**

Mice balb / c (age 8-12 weeks, weigh 30-40 grams; n = 20) were maintained in the Laboratory of Molecular Biology and Immunology, Department of Microbiology, Faculty of Medicine, Hasanuddin University (Makassar, Indonesia). The rats were acclimatized for 8 days, then divided into four groups (n = 5). All groups were induced intraperitoneally with S. typhi strain Thy1 (3 ml x $10^3$ ml / CFU). After 3 days of induction, each animal tried to be intervened; LPE510 (mice group intervened with LPE dose 510 mg / kg bw), LPE750 (mice group intervened with LPE dose 750 mg / kg bw), postive control group (groups of mice given Levofloxacin antibiotic dose 1.95 mg / kg bw and negative control group (group of placebo mice).

**Elisa**

Serum samples were taken 4 times, baseline (day 0), after induction of S. typhi before intervention (day 5), after intervention (10th day) and after 30 days. Interleukin 6 (IL-6) antibody concentrations are determined by the IL6 ELISA Mouse Sandwich method (User Manual Catalog No. LS-F24855) LSBio, LifeSpan BioSciences, Inc. reader 270, Instrument serial number: 1211006860, measurement mode: Absorbance, measurement wavelength: 450 nm, read mode: normal, unit: OD.

**Statistical Analysis**

Data is expressed as mean ± SE. To assess the difference in VDR levels and the number of bacterial colonies between groups, the paired t-test was applied using SPSS 23 software. The difference between groups was significant when $P \leq 0.05$.

**Statement of Ethics**

This research was approved by the Health Medical Ethics Research Committee at the Faculty of Medicine, Hasanuddin University (Makassar, Indonesia) with registration number 900 / H4.8.4.5.31 / PP36-KOMETIK / 2018 on October 31, 2018.

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# Results

## Phytochemicals Screening

Phytochemical examination of qualitative analysis test, found that Lime peel extract contains active substances such as Flavonoid (+), Tanin (+), Saponin (+), Alkaloid (+), and Triterpenoid (+), shown in Figure 1 and Table 1 below:

Table 1. Phytochemical Screening Results of Lime peel extract

| Phytochemical screening | Samples LPE | Identified |
|---|---|---|
| Flavonoids | + | Red magenta in 3 minutes |
| Saponin | + | Stable emulsion |
| Alkaloids | + | Turbidity and sediment |
| Tannin | + | blackish green |
| Triterpenoid | + | Reddish brown |

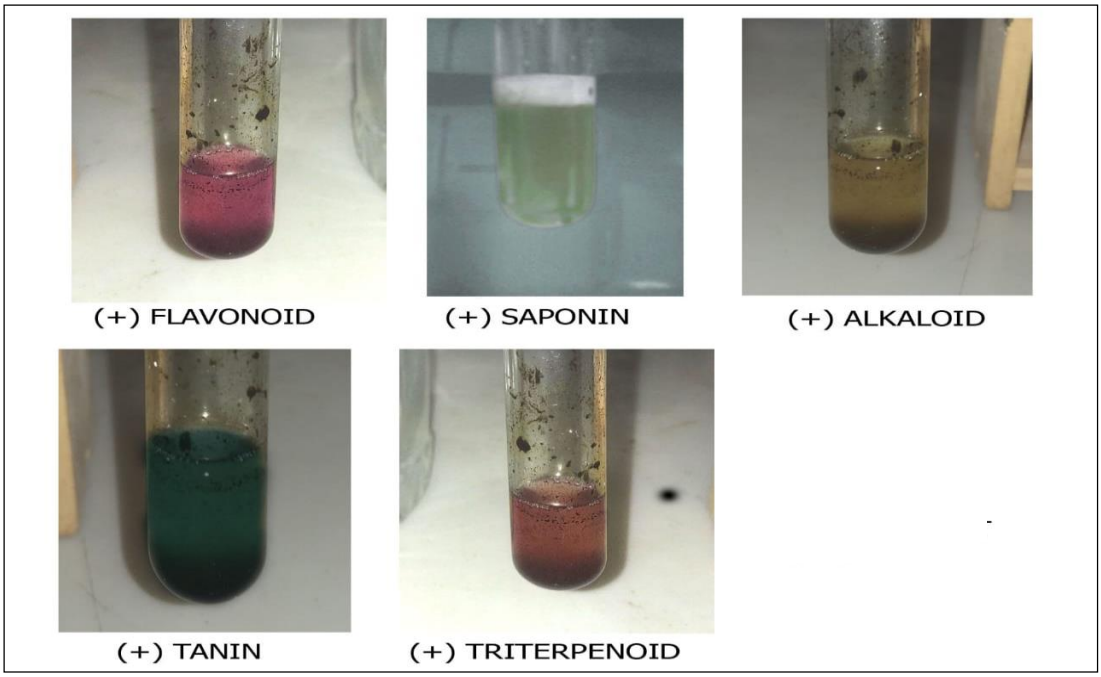


Fig. 1. Phytochemical test for qualitative analysis of active Substances in Lime peel extract.

In table 1. and figure 1. it has been shown that the active substances contained in Lime peel extract based on phytochemical test qualitative analysis is positive containing Flavonoids, Saponins, Alkaloids, Tanins and Triterpenoids. The results of phytochemical tests of quantitative analysis are observed in table 2 and figure 2:

Table 2. The active substance in Lime peel extract

| Active substances in LPE | Absorbance | Concentration (average) |
|---|---|---|
| Total Flavonoids | 0250 | 0260 |
| Polyphenols | 0261 | 2285 |
| Tannin | 0315 | 1,415 |

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In table 2 it can be seen that the total level of flavonoids contained in Lime peel extract is 0.26 µg / mL (A = 0.25), the level of polyphenols in LPE is 2.285 µg / mL (A = 0.261) and tannin level is 1,415 µg / mL (A = 0.315). It can be seen in figure 2 that the level of polyphenols is more than the tannin and total flavonoids.

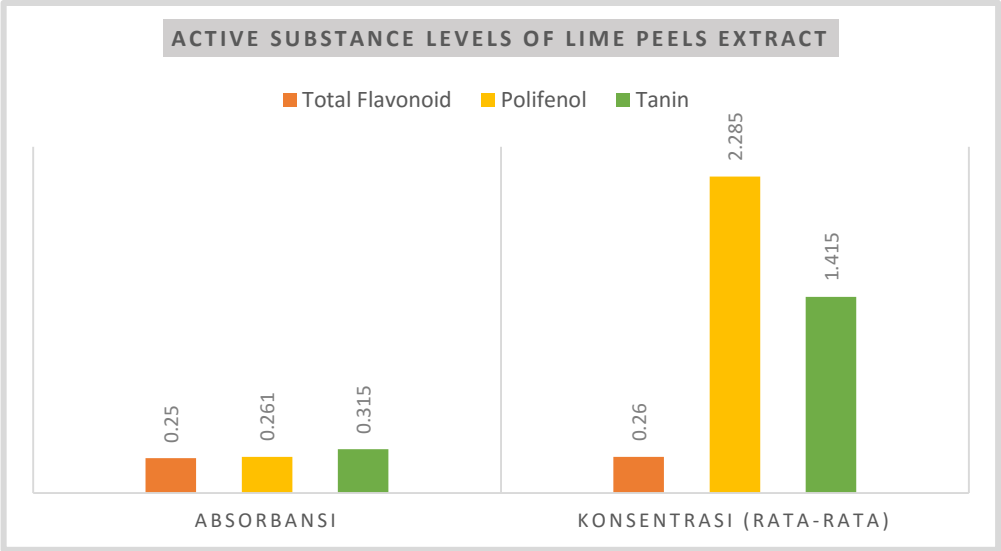


Fig. 2. Levels of flavonoids, polyphenols and tannins in Lime peel extract.

## Interleukin 6 (IL-6)

Serum IL-6 levels at four times were analyzed (table 3).

Table 3. Differences in dynamic levels of serum interleukin 6 (IL-6) between the groups at baseline (H0), once injected the bacteria before the intervention (day 5), 10 days after the intervention (day 10) and 3 weeks after the intervention (day 30).

| Groups | Levels of Interleukin 6 (pg / ml) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **baseline** | **5th day** | ***p value*** | **5th day** | **10th day** | ***p value*** | **10th day** | **30th day** | ***p value*** |
| **LPE510** | 157.26 ± 59.71 | 358.30 ± 59.47 | 0007 | 358.30 ± 59.47 | 338.67 ± 64.89 | 0003 | 338.67 ± 64.89 | 284.68 ± 60.58 | 0:05 |
| **LPE750** | 171.81 ± 58.08 | 434.09 ± 51.26 | 0004 | 434.09 ± 51.26 | 409.56 ± 53.60 | 0002 | 409.56 ± 53.60 | 293.96 ± 34.31 | 0:01 |
| **positive control** | 121.15 ± 35.98 | 502.01 ± 35.42 | 0000 | 502.01 ± 35.42 | 477.95 ± 41.50 | 0006 | 477.95 ± 41.50 | 204.54 ± 40.69 | 0001 |
| **negative control** | 130.28 ± 47.69 | 248.52 ± 27.18 | 0003 | 248.52 ± 27.18 | 236.89 ± 31.78 | 0015 | 236.89 ± 31.78 | 194.99 ± 38.50 | 0008 |

* Values are mean± SD n = 5, p-value of ≤ 0:05 is significant Considered

In Table 3 it can be seen that there are significant differences between serum levels of interleukin 6 in all groups before intervention and after intervention and on the 30th day of observation.

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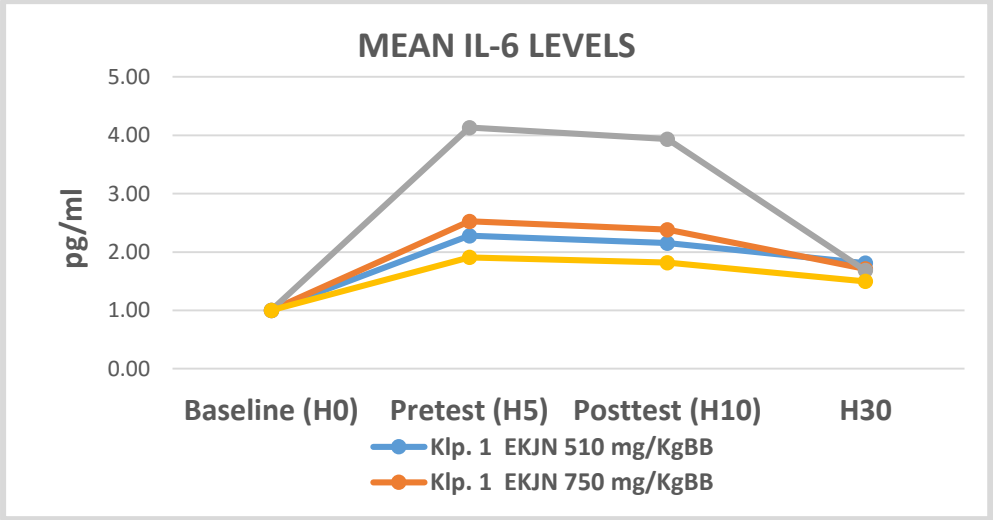


Fig. 3. Dynamics of changes in levels of interleukin 6 (IL-6) during the observation period.

## Discussion

Lime (*Citrus aurantifolia*) contains active ingredients which are thought to provide antibacterial effects. Specifically, in this study, lime peel was taken as an extract because in some previous studies higher concentrations of flavonoids were found in the lime peel compared to other parts such as seeds, fruit, and juice. Based on the phytochemical test qualitative analysis found that Lime peel extract was used in this study positively contained active substances Flavonoids, Tanins, Saponins, Alkaloids and Triterpenoids (Table 1). Based on research from Italy, Loizzo MR (2012), the content of flavonoids in lime peel is Routine (35.1), Apigenin (10.8), Quercetin (5.1), Kaemphenol (13.8), Nobiletin (3.0) 5. Other studies have found that flavonoids have antibacterial activity against E. coli through inhibition of DNA gyrase, inhibiting nucleic acid synthesis and cytoplasmic membrane function [7]. Tanin is one of the chemical compounds belonging to the class of polyphenols which are thought to bind one protein membrane that is owned by bacteria and if this happens it can damage the availability of receptors on the surface of bacterial cells which disrupt the cell's metabolic process. In the writings of Hegermen A, E (2002) tannins have been shown to form irreversible compound complexes with proline, which is a complete protein, which has an inhibitory effect on protein synthesis for cell wall formation [4]. Suparjo (2008) wrote the Saponin mechanism as anti microbes are the bond between saponins and sterols (bacterial proteins) on the surface of bacterial cell membranes which can increase permeability of bacterial cell membranes so that they can change the structure and function of membranes, cause protein denaturation, so that cell membranes will be damaged and lysis is by interfering with the constituent components of peptidoglycan in bacterial cells, so that the cell wall layer is not formed intact and causes cell death. Antibacterial alkaloids are predicted through inhibition of cell wall synthesis which will cause lysis in cells so that cells will die [11].

Based on research from the phytochemical test of quantitative analysis (Table 2) it was found that the levels of Polyphenols in Lime peel extract averaged 2.285 µg / mL using a standard solution of Gallic Acid. For the average Flavonoid level of 0.26 µg / mL with the standard solution Quercetin. Then level the average tanin as much as 1,415 µg / mL with a standard solution of tanic

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acid. Based on research from Iran, Ghasemi K. et.al (2009) conducted a study of antioxidant activity, phenol and flavonoid content on the skin and tissue of 13 citrus species, found that the highest content of flavonoids was on citrus skin with a range of 0.3 - 31.1 µg / mL [12].

Among the components present in Citrus aurantifolia, flavonoids have been suggested as antioxidants that can modulate signaling pathways mediated by TLRs (Toll-Like Receptors). Interactions between host cells and microbes are known as crosstalk where this occurs when certain micro-organism molecules are recognized by TLRs in body cells, especially in intestinal epithelial cells and in immune cells. TLR is the first line of defense against pathogens, plays an important role in innate and adaptive immunity. Flavonoids have been able to modify microbiota composition, to modulate gene expression and TLR proteins, and regulate molecules involved in the TLR pathway [13], [14]. Binding between LPS on bacteria and TLR-4 will give rise to an activation signal that will activate innate immunity cells. These cells will release a number of proinflammatory cytokines such as TNFα, IL-1 and IL-6. Research conducted by Galdiero et. al, where the aim of this study is to verify whether the porins of Salmonella typhimurium can influence the expression of the genes interleukin-1 (IL-1) and interleukin-6 (IL-6) [15].

In this study the serum IL-6 levels studied were based on time response, there were significant differences in baseline (H0) with levels before intervention (H5) in all intervention groups, both LPE510mg ($p = 0.007$), LPE750mg ($p = 0.004$), positive control ($p = 0,000$) and negative control ($p = 0,003$). The mean IL-6 at the baseline was lower than on the fifth day, because on the fourth day injection of $3x10^3$ cfu / mL of Salmonella typhi bacteria in the host was carried out (Table 3). When the body is infected with S. typhi, the body will respond and determine the effect of bacteria on the host. When S. typhi first enters the body, bacteria will be destroyed by macrophages. Bacteria will be known by various receptors located on the surface of phagocytes [16]. Toll-like receptors play a role in observing and destroying S. typhi. Macrophages recognize through identification of components of the lipoarabinomannan (LAM) cell wall. TLR4 plays an important role in the natural immune response by forming signal transcription factors and defense responses. Activation of macrophages by TLR4 results in the production of cytokines that play an important role in granuloma formation [16]. During S. typhi infection, activated proinflammatory cytokines such as IL-1β and IL-6, IFN-γ and TNF-α dysntesis and systemic inflammation occur.

Therefore, IL-6 levels on the fifth day increased compared with IL-6 levels at the start of the study (H0). After cytokine IL-6 is secreted, the activity of helper 1 T cells (Th1) and T helper 2 (Th2) begins. Signals from cytokines injected by host cell and bacterial interactions are crucial in disease progression. The balance between proinflammatory and antinflammatory cytokines will control the prevention of host damage due to excessive inflammation [17].

There were significant differences between IL-6 levels when taking fifth day (pretest) and taking after tenth day intervention (post test) in all intervention groups, both LPE510mg ($p = 0.003$), LPE750mg ($p = 0.002$), positive control ($p = 0.006$) and negative controls ($p = 0.015$). The mean IL-6 on the tenth day (posttest) was lower than the fifth day (pretest). This explains that after treatment in each group there was a decrease in IL-6 levels (Table 3). Giving Lime peel extract can reduce IL-6 levels with a dose of 510mg and a dose of 750mg. In the LPE510mg group there was a decrease in the mean of 19.63 pg / mL, the LPE750mg group decreased by a mean of 24.53 pg / mL. In the positive control group there was a decrease in the mean of 24.06 pg / mL and in the negative control group there was a decrease in the mean of 11.63 pg / mL. The most decrease

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in IL-6 levels was found in the positive control group, followed by the LPE750mg group, then the LPE510mg group and the least decrease in IL-6 levels in the negative control group (Figure 3).

Decreasing IL-6 levels can indicate a strong body defense reaction from the host after the intervention. The flavonoid content in Lime peel extract can modulate the TLR pathway at different levels. Under IEC homeostasis conditions have a low expression of TLR2 and TLR4, and therefore in a healthy context, TLR activation is low. However, in the inflammatory scenario, the TLR expression on IEC increases and then the TLR signal is triggered (De Kivit, S et al, 2014). The best results regarding down-regulatory flavonoid effects have been proven in TLR activation (i.e., LPS as an in vitro TLR4 activator) and have less effect if in non-stimulating conditions (i.e., in healthy animals / subjects) [18].

Modulation of the TLR pathway by Lime peel extract can affect the activation of proinflammatory cytokine factors such as IL-6, which can reduce the amount of the cytokine. If the signaling pathway from TLR is inhibited then there is a decrease in adapter protein recruitment, so that the NFκB transcription factor is not recognized and thus inhibits the synthesis of proinflammatory cytokines such as IL-6, IL-6 triggers an inflammatory effect in the host's body. TNF, IL-1, and IL-6 have several local and systemic inflammatory effects. TNF and IL-1 act on leukocytes and endothelium to induce acute inflammation, and both cytokines induce the expression of IL-6 from other types of leukocytes and cells. TNF, IL-1, and IL-6 mediate the protective systemic effects of inflammation, including induction of fever, acute phase protein synthesis by the liver, and increased leukocyte production by bone marrow [19].

Previous research by Tao Xufeng, et al. (2016), shows the latest evidence showing that TLR4 signaling plays an important role in the development of liver inflammation after cerebral ischemia [20]. The results of western blotting in the study prove that total flavonoids decrease TLR4 and cause downstream protein levels, including MyD88, TRAF6, p-JNK, NFκB, and AP-1. In addition, total flavonoids also inhibit the level and translocation of NFκB. These findings indicate that the total effect of flavonoids on liver damage after cerebral ischemia occurs through inhibition of the inflammatory process through the TLR4 signaling pathway. Further results presented in the study that total flavonoids significantly reduced levels of IL-1 mRNA, IL-6, and TNF-α in the liver. These results prove the potential mechanism of total flavonoids in the process of inhibiting oxidative stress and inflammatory responses in liver damage after cerebral ischemia [8].

Flavonoids can act at three different levels by modulating: (1) the composition of the microbiota, by direct or indirect means (metabolites) that affect growth; (2) Activation of Toll-like receptors (TLR), by acting on receptors and adapter proteins; (3) signal transduction, by interfering with upstream and downstream kinases and transcription factors involved in activation of the inflammatory and immune response [13].

## Conclusion

Lime peel extract contains active substances flavonoids, tannins, saponins, alkaloids and Triterpenoid. Lime peel extract with a dose of 510 mg / kg bw and 750 mg / kg bw significantly reduce serum levels of interleukin 6 receptors. The effect of lime peel extract on Salmonella typhi infection can reduce proinflammatory factors and alternative therapies other than antibiotics in treating typhoid fever.

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## Conflict of Interest

Authors declare that there is no conflict of interest within this research article and publication.

## Abbreviation Used

**LPE:** Lime peel extract, **CFU**: colony forming units, **IL-6**: interleukin 6, **TLR**: Toll Like Receptors

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