Antibacterial and anti-inflammatory effects of lime (Citrus aurantifolia) peel extract in Balb/c mice infected by Salmonella typhi

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Abstract

Lime (Citrus aurantifolia) is a traditional plant that is widely used as antibacterial. This study proves the effect of Lime Peel Extract (LPE) on the colonization and growth, mediated by the activity of IL-6, of bacteria Salmonellatyphi in Balb/c mice. Mice were divided into four groups; LPE 510 mg/kg body weight (bw), LPE 750 mg/kgbw, and positive and negative control. The examination was carried out 3 times, on the 5th day before the intervention, on the 10th day after the intervention and on the 30th day after maintenance. Intervention of LPE for 5 days can decrease the number of S. typhi colonies, even maintenance for 20 days after the intervention showed no bacterial growth. IL-6 pro-inflammatory cytokine activity increased on examination day 5 after S. typhi injection and decreased after intervention on day 10, it was significantly different between pre and post at all groups except for negative controls (p=0.15). The speed of decrease in IL-6 levels was the greatest at the LPE 750 mg/kgbw (velocity=-5.64%). LPE decreased serum levels of IL-6 and inhibited the growth of S. typhi colony in Balb/c mice. LPE has potential for antibacterial and anti-inflammatory.

Introduction

Some antibiotics such as ampicillin, chloramphenicol, tetracycline and co-trimoxazole become ineffective on Salmonella typhi and Salmonella paratyphi,1 Multi-Drug Resistance (MDR) leads to new treatments in the form of adjuvant therapy which is a traditional plant that can be used as an antimicrobial. One of which is the lime plant (Citrus aurantifolia). From many previous studies even to date, all parts of C.aurantifolia have various efficacy.2,3 Specifically, it was emphasized that the peel of lime contains of active ingredients such as polyphenols, flavonoids, tannins, saponins, alkaloids, and triterpenoids.4 Salmonella typhi, is a Gram-negative bacterial pathogen whose transmission almost always occurs through the contaminated food and drinks. From several studies, the resistance of Salmonella typhi has begun to be high. The development of antimicrobial resistance is in line with the increasing use of antimicrobial drugs and in line with the discovery of new drugs.

Bacterial resistance and especially that of Salmonella typhi brings us to a new treatment in the form of adjuvant therapy, one of which is Citrus aurantifolia that can be used as an antimicrobial. Based on the previous research, all parts of Citrus aurantifolia; leaves, root stem, bark, and peel contain useful metabolic
compounds the lime peel has a higher concentration of flavonoids compared to other parts such as seed, fruit and juice. The existence of the content of flavonoids makes the lime peel has antibacterial and antioxidative power. Previous studies tested the bacteria-static or bactericidal activity of flavonoids by conducting a time-kill study found that flavonoids do not kill bacterial cells but only induce the formation of bacterial aggregates thereby reducing the amount of Colony Forming Units (CFU) in a decent amount.

When S. typhi bacterial infection occurs, there is a bond between the lipopolysaccharide (LPS) of the bacterial wall and the Toll-Like Receptor 4 (TLR 4) of the host, which stimulates innate immune activity. The activity releases a number of cytokines such as TNFα, IL-1, and IL-6. Interleukin 6 (IL-6) mediates the protective systemic effects of inflammation, including the effects of fever, acute-phase protein synthesis by the liver, and increased production of leukocytes by the bone marrow. This study discusses the effect of lime peel extract on the amount of bacterial colonization and its effect on the activity of IL-6 pro-inflammatory cytokines in Balb/c mice injected with Salmonella typhi.

Materials and Methods

An experimental design was used to study the effectiveness of LPE in decreasing pro-inflammatory IL-6 levels and bacterial colonization in Balb/c mice strains induced by Salmonella typhi.

Lime peel extract

Lime peel extraction was carried out at the Phytochemical Laboratory, Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia. Lime peel thick extracts were macerated with 96% Ethanol for 72 hours. The Lime Peel Extract (LPE) used in this study was consisted of two doses: 510 mg/kg body weight (bw) and 750 mg/kgbw, both dissolved with Aquades. LPE was obtained as reported in Kasim et al. 4

Balb/c mice

Balb/c mice age ranging from 8 to 12 weeks, and weight from 30 to 40 grams (n=20), were obtained from Laboratory of Molecular Biology and Immunology, Department of Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. Mice were divided into four groups (n=five/group) based on intervention; LPE dose of 510 mg/kgbw, LPE 750 mg/kgbw, positive control (Levofloxacin dose 98 mg/kgbw), negative control (Aquades). All groups were injected intraperitoneally with S. typhi strain Thy1 (3×10^3 CFU/ml). 3 days after the injection of Salmonella typhi, each animal was started to be intervene for 5 days. Animals were treated as reported in Kasim et al. 4

Bacterial colonization

Bacterial colonies were counted from samples taken from peritoneal fluid. Samples were taken three times, on the 5th day after mice were induced by Salmonella typhi (pre-intervention), the 10th post-intervention and 30th day after maintenance without treatment. Samples were taken as much as 0.5 mL and placed in 4.5 mL of physiological saline solution (0.9% NaCl). Dilution was carried out three times so that the culture obtained was not too dense or filled up the cup (the culture is too dense will interfere with observation). One mL of suspension was poured into a sterile Petri dish, then poured warm sterile (nutrient agar) media (45°C) then tightly closed and incubated for 1-2 days at 37°C. The method used was the Plate Count Agar (PCA), a technique to grow microorganisms in agar media by mixing the liquid agar media with bacterial culture stock (agar) so that the cells are evenly distributed and still on the surface of agar or inside of agar.

Interleukin 6 (IL-6) examination

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. IL-6 antibody concentrations were determined by the IL6 ELISA Mouse Sandwich method. IL-6 examination was reported in Kasim et al. 4

Ethics statement

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

Results

Bacterial colonization

The number of bacterial colonies after being given an intervention decreased in all groups, however on the 10th day of the negative control group, the number of bacterial colonies was seen more than other groups, and there were still bacterial colonies (Figure 1) on the 30th day of negative control group. On the 10th day, the number of bacterial colonies decreased after the intervention of lime peel extract which was given for 5 days of intervention. The number of bacterial colonies continued to decrease until the 30th day post-intervention. The decrease in the number of bacterial colonies after LPE administration had almost the same effect as the decrease in the number of colonies after levofloxacin administration (positive control). The effect of quinolone as a bactericidal, in this study showed that the number of bacterial colonies after 30 days continued to decrease even they did not grow in the group given LPE 510, LPE 750, and positive control.

In the LPE group at a dose of 750 mg/kgbw, the number of bacterial colonies on day 10 had no growth, better than positive control group. The average decreased in the number of bacterial colonies in the LPE group 750 mg/kgbw from 1.7×10^7 CFU/mL to 0 or no bacterial growth (p=0.00) whereas in the positive control group decreased from 3.0×10^7 CFU/mL to 1.6×10^6 CFU/mL.

Figure 1. Dynamics of changes in the number of bacterial colonization of S. Typhi by the groups during the observation period; LPE, lime peel extract; CFU, colony forming units.
Interleukin 6 (IL-6)

The level of serum IL-6 at 4 times of data collection was analyzed through paired T-test to assess the dynamics of changes in levels of serum with respect to changes in observation time for each group, as can be seen in Table 1. The mean of IL-6 levels (delta) showed that the largest decrease was found in the LPE 750 intervention group, suggesting that the LPE 750 intervention had a greater impact on decreased IL-6, compared to antibiotics (levofloxacin) intervention. The decrease of IL-6 levels in the LPE 510 intervention group was greater than the placebo group (negative control).

Figure 2 shows the velocity of the decrease of IL-6 averages between the time of observation after the intervention (10th day) and before the intervention (5th day). At the time of observation of the 5th day to the 10th day, it was seen that the velocity of decreasing IL-6 levels was the greatest in the LPE 750: 5.64%, and the smallest velocity of decline was in the negative control group: 4.67%. The velocity of decrease in IL-6 levels in the LPE 750 was more than the positive control group given Levofloxacin. It is assumed that the extract of lime peel in this study is effective as an anti-inflammatory.

Discussion

Bacterial colonization

Various parts of Citrus aurantifolia plant (leaves, stems, roots, barks, and peels) are evaluated through phytochemical screening and it is found out that they contain of various metabolic compounds. Extract of Citrus aurantifolia leaf has an antibacterial effect that has a high inhibitory zone in Gram-positive and Gram-negative bacteria.5,12 When S. typhi first enters the body, the bacteria are destroyed by macrophages. It is recognized by various receptors located on the surface of phagocytes. LPS in the S. typhi cell wall is a signal for macrophages to carry out the activation.13 In previous preliminary studies,4 it was found that the metabolic compounds contained in the extract of lime peel were polyphenols, flavonoids, alkaloids, tannins, saponins, and triterpenoids. Each of these compounds has the property to kill bacteria. These compounds attack bacteria directly, causing bacterial cell death. This is indicated in the calculation of the number of bacterial colonies that are reduced even no growth after 5 days given the intervention of LPE.

Interleukin 6 (IL-6)

One of the earliest responses of the innate immune system to infection and tissue damage is the secretion of cytokines by tissue cells, which is very important for acute inflammation. When S. typhi first enters the body, the bacteria will be destroyed by macrophages. Bacteria will be recognized by various receptors located on the surface of phagocytes.8 Specific marker molecules for Gram-negative bacteria such as S. typhi are LPS, LPS will use TLR-4, a receptor that plays a role in observing and destroying Salmonella typhi. Activated TLR-4 will recruit the MyD88 adapter protein. Then MyD88 recruits IRAK4, IRAK1 and IRAK2. IRAK kinase then phosphorylates and activates the TRAF6 protein, allowing NF-κB to dwell in the cell nucleus and activating transcription and causing induced inflammatory cytokines. Proinflammatory cytokines such as IL-1β and IL-6, IFN-γ and TNF-α are synthesized and systemic inflammation occurs.8,14 It can be said that the inflammatory process by IL-6 cytokines is already running.

The results of this study (Figure 2) showed that LPE therapy gave a greater decrease in IL-6 levels compared to levofloxacin. It is indicated that LPE has an anti-inflammatory effect on IL-6.15 LPE, in addition to having an antibacterial effect, also has an anti-inflammatory effect, LPE has the potential for a more effective therapy. Researchers assume that the inhibitory effect of IL-6 is due to the activity of the polyphenol compounds contained in the extracts of lime peel. In line with this, Haseeb A. (2017) found that the potential inhibitory effect of polyphenols on IL-6 expression and oxidative stress through molecular mechanisms of activation of NF-κB.16

The content of polyphenols and flavonoids contained in LPE of this study reveal that polyphenols are bioactive substances, which are likely to have a potential effect on the inflammatory response. It is known that flavonoids are one of the most extensive groups of secondary metabolite plants, flavonoids are found in many edible fruits and vegetables. The source of polyphenols is mostly contained in citrus fruits.17 The polyphenol content in lime peel extract in this study is 2.29% and flavonoids are 0.26%. The research by Rossano on Citrus aurantifolia in Calabria, Italy revealed that lime peels which were extracted by methanol solvent contained of more phenols (95.6 mg/g) and flavonoids (23.5 mg/g) compared to the leaves. In that study, it was found that there was a significant relationship between phenol and flavonoid with antioxidant activity.18

Table 1. Mean values of IL-6 (delta) differences between all groups at 5th day (pre-intervention), 10th day (post-intervention) and 30th day.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Level of IL-6 (ng/ml)</th>
<th>Delta (mean±SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPE 510</td>
<td>19.63±7.06</td>
<td>53.98±43.38</td>
<td>0.074</td>
</tr>
<tr>
<td>LPE 750</td>
<td>24.54±7.71</td>
<td>115.61±56.92</td>
<td>0.000</td>
</tr>
<tr>
<td>Control (+)</td>
<td>24.07±7.06</td>
<td>273.41±68.26</td>
<td></td>
</tr>
<tr>
<td>Control (-)</td>
<td>11.62±6.33</td>
<td>41.91±18.87</td>
<td></td>
</tr>
</tbody>
</table>

IL-6, interleukin 6; LPE, Lime Peel Extract; Control (+), positive control group; Control (-), negative control group; SD, Standard Deviasi.
Tejada S. (2017) proved that hesperidin (a type of flavonoid) extracted from the citrus genus, has potential as an anti-inflammatory. Hesperidin treatment decreases inflammatory mediators and provides a significant antioxidant effect. Molecular bases for anti-inflammatory effects appear to be mediated by signaling pathways especially the nucleus κβ factor pathway. As for other opinions, a research conducted by Jorge L.A, found that from 3 types of citrus namely *C. limon*, *C. aurantifolia*, *C. limonia*, Essential Oils (EO) contained in the 3 types of citrus have anti-inflammatory effects, especially the limonia content of essential oils from the citrus fruit skin. During the inflammatory event, there is an increase in cytokine production.

**Conclusions**

This study showed that LPE decreased serum levels of IL-6 and bacterial colonization in Balb/c mice induced by *Salmonella typhi*. LPE has potential as antibacterial and anti-inflammatory.

**References**