

Effects of lime (*Citrus aurantifolia*) peel to the expression of mRNA toll-like receptors 4 in balb/c mice-infected *Salmonella typhi*

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ABSTRACT

Lime peel contains metabolic compounds that have lethal effects of bacterial cells, but its effect as an antibacterial modulate innate immunity pathways, especially toll-like receptor 4 (TLR-4) signaling pathway, is unclear. This study examined the effects of lime peel extract (LPE) on the activity of TLR 4 in Balb/c mice induced by *Salmonella typhi*. Mice were induced intraperitoneally and then 3 days after induction, LPE was given orally on two doses (510 and 750 mg/kg BW). The number of bacterial colonization was counted using peritoneal fluid samples by the method of plate count agar. Intervention LPE for 5 days can degrade TLR-4 and the number of colonies of *S. typhi*. On day 3 after was induced *S. typhi*, TLR-4 gene expression of Balb/c mice is increased. Postintervention LPE for 5 days, the expression of TLR-4 gene decreased, significantly different at a dose of 750 mg/kg BW ($P = 0.04$). There was a positive correlation between the expression of TLR-4 gene by the number of bacterial colonization, decreasing gene expression of TLR-4, the number of bacterial colonization is also getting smaller ($P = 0.013, r = 0.408$). LPE can modulate the TLR-4 signaling pathway in host immunity so that the gene TLR-4 is expressed fewer in numbers. This mechanism causes the bacterial colony number to decrease, not even growth.

Key words: Bacterial colonization, *Citrus aurantifolia*, lime peel extract, *Salmonella typhi*, toll-like receptors 4

INTRODUCTION

Many herbal medicines have been explored as antioxidant, antibacterial, and immunomodulator.^[1-6] Typhoid fever is an acute gastrointestinal infection caused by *Salmonella typhi* and still a major problem since endemic in several

countries.^[7] In addition to genetic factors that can affect the multiplication of *S. Typhi*, several immune factors that determine the severity of typhoid fever can also affect *S. Typhi's* multiplication.^[8]

When the body is infected with *S. typhi*, the body will hold out as a response to the reaction of the innate immunity. Special marker molecule of a Gram-negative bacterial is lipopolysaccharide (LPS), which will be instantly recognizable by protein receptors on the host that is toll-like receptor 4 (TLR-4) receptor, TLR-4 binds to LPS.^[9] The body's defense mechanism is elaborated and discussed in this study. The reaction when *S. typhi* first enters the body,

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the bacteria are destroyed by macrophages because the bacteria are recognized by TLR-4 is located on the surface of phagocytes.^[10] TLRs belong to the pattern-recognition receptors and represent the first line of defense against pathogens, playing a pivotal role in both innate and adaptive immunities. Among components present in the diet, flavonoids have been suggested as antioxidant dietary factors able to modulate TLR-mediated signaling pathways.^[11]

Preliminary studies, through qualitative and quantitative phytochemical screening, the metabolic compounds obtained from lime peel extract (LPE) are phenols, flavonoids, tannins, alkaloids, and triterpenoid.^[12] Studies of LPE interventions on TLR4 gene mRNA expression activity in *S. typhi*-infected hosts have not been conducted so far. Therefore, the aim of this study was to know the effect of LPE to mechanism of the host's body defense against *S. typhi* infection through the TLR-4 signaling pathway relationship with the presence of bacterial cell death.

SUBJECTS AND METHODS

Study area and design

This research is a true experimental pre–posttest design and approved by the Research Ethics Committee of Medical Health in the Faculty of Medicine, University of Hasanuddin (Makassar, Indonesia) with registration number 900/H4.8.4.5.31/PP36-KOMETIK/2018 on October 31, 2018.

Lime peel extract

Lime selected with homogeneity, according to the similarity in color and size lemon (diameter 3–5 cm). A lime was separated between the peel and the fruit. One kilogram lime produces approximately 300 g of lime peel. The lime peel separated from the fruit, dried using oven with a temperature 55°C to speed up the drying process until the water content decreases and reaches 10%. And then inserted into a glass container to do the maceration process, dissolved with ethanol 96%. Followed by a filtration process, after macerated and then filtered by vacuum filtration method using Buchner Funnels. Results Extraction if the average weight of 1 lime is 100 g, it can produce ± 30 g of lime peel and has the potential to produce 0.1 g of LPE.

Experimental animals

Balb/c mice (age: 8–12 weeks, weighing: 30–40 g, $n = 20$) placed in a cage made of wire with a floor area size of 30 cm × 50 cm × 15 cm. Adaptation procedures were carried out for 7 days. Further randomization, all the mice were divided into four groups ($n = 5$): Group 1 LPE510 (a group of mice which intervened LPE dose of 510 mg/kgBW), Group 2: LPE750 (a group of mice which intervened LPE dose of 750 mg/kgBW), Group 3: positive control (group of mice was given Levofloxacin 1,95 mg/

kgBW, and Group 4 negative control (group of mice placebo). Animal interventions were carried out for 5 days.

Induction of *Salmonella typhi* bacteria

On day 0 after adaptation, all the mice do baseline blood sampling, and then day 1, mice were induced by the bacteria *S. typhi* as much as 3×10^3 CFU/mL strain thy1 by intraperitoneal injection. On day 2 to day 4, mice were observed for the occurrence of infection process.

Peritoneal fluid intake and investigation bacteria colonies

Peritoneal fluid was take in the positioning body of mice was supine, the abdomen was cleaned with 70% alcohol and NaCl injected as much as 0.8–1 mL into the peritoneal cavity. Then allowed for 1 min while the abdomen was shaken it slowly. Peritoneal fluid removed 0.5 mL is aspirated from the peritoneal cavity of the supine position. Peritoneal fluid collection and examinations were conducted Samples of peritoneal fluid were taken 3 times, pre-intervention (day 5), post-intervention (day 10) and 20 days after intervention (day 30).

Count the number of bacterial colonies using the plate count agar method. 0.5 mL of sample is put into 4.5 ml of saline (0.9% NaCl), then diluted 3 times. 1 ml of suspension is poured into a sterile petri dish, followed by pouring warm sterile nutrient agar (45°C) and then tightly closed and incubated for 24–48 hours at 37°C in an upside-down position. Calculation the number of bacterial colonies based on the growth colonies per ml is equal to the number of colonies per cup divided by dilution factor.^[13]

Toll-like receptor-4

Examination of expression mRNA gene TLR-4 was performed four times: at baseline (day 0), day 5 preintervention, the 10th-day postintervention, and the 30th day after the maintenance of 20 days postintervention. RNA extraction was done using previous methods and stored at a temperature of –80°C. Real-time polymerase chain reaction (PCR) program was run using CFX Connect system, Bio-Rad Laboratories, real-time PCR 96 wells, 0.1 mL, USA.

Primer for mice mRNA TLR4 is TLR4-forward: TGACAGGAAACCCTATCCAGAGTT and TLR4-reverse: TCTCCACAGCCACCAGATTCT.^[14]

The parameter thermal cycle was 30 s at a temperature of 95°C and 40 cycles of denaturation at a temperature of 95°C for 10 s and then annealing at 60°C for 15 s and extension at a temperature of 72°C for 40 s. All PCRs were repeated three times, and the data were analyzed by the instrument detection system of CFX Connect Real-Time PCR Bio-Rad, California, USA using the comparative threshold cycle method. The standard curve was constructed and is an indication of good amplification efficiency (90%–100%).

RESULTS

Bacterial colonies

Analysis of the effect of LPE on the amount of bacterial colonization *S. typhi*, analyzed by using paired *t*-test to assess the dynamics of change in the number of bacterial colonies according to observation time for each group [Table 1]. There was a significant decrease in the intervention group LPE510 and LPE750. The number of bacterial colonies on day 30, kept a decline even in the intervention group LPE, and antibiotics had no bacterial growth. It can be assumed that the effect of LPE can be as bactericidal or can kill the bacteria.

MRNA gene toll-like receptors 4

Figure 1 shows that there are dynamic changes in gene expression of TLR-4 at four times the observation. The expression mRNA gene TLR-4 on day 5, expressed more than on day 1 (before injected bacteria). There are statistically significant differences in all groups: LPE510 ($P = 0.002$), LE750 ($P = 0.001$), positive controls ($P = 0.000$), and negative controls ($P = 0.003$).

Correlation of toll-like receptors 4 with colonization bacteria

Table 2 shows that there is a linear correlation or relationship between the expression of TLR-4 gene mRNA by the number of bacterial colonization, the strength of the correlation is sufficient, and the direction of a positive correlation means that the lower the gene mRNA expression of TLR-4, the smaller the amount of bacterial colonization.

DISCUSSION

Typhoid fever begins with the entry of germs through contaminated food and drink through the oral-fecal pathway, which then the body will carry out defense mechanisms through several processes of an immune response. The latest study showed that *S. Typhi* targets human-specific pathways by inducing host transcriptional changes. These pathways include cytoskeletal rearrangement, polarized cytokine release, and hampering host immune defense system. *Salmonella* is still a dangerous threat to human health without adequate therapeutic options.^[15]

S. typhi infection stimulates macrophage activation.^[16] The content of LPS in the cell wall of *S. typhi* is a signal for macrophages to activate. TLR-4 protein serves as a receptor on the surface of phagocytic cells to recognize LPS *Salmonella sp.*^[17] The TLR-4 receptor protein on the surface of phagocytic cells will capture of LPS of *Salmonella sp.* that managed to penetrate the intestinal wall and intestinal mucosa.

In this study, it was found that TLR-4 at the baseline examination is lower than the TLR-4 after the host bacteria injected, and this indicates the homeostatic condition.^[18] However, if there is inflammation, TLR expression increased and triggered the cytokine pro-inflammation.^[19] In this study, day 5 post injection, TLR-4 mRNA expression in all groups of mice increased. This may explain that LPS which is a marker of *S. typhi* bacteria that are in the cell walls of bacteria, it can stimulate the activation of the receptor TLR-4 on the surface of the host cell.^[17]

Decreased expression of TLR-4 gene mRNA showed a decrease in the amount of LPS in the blood of mice and marked the healing process of *S. typhi* infection in the body of mice. However, this needs to be done further research on the pathomechanism of the number of bacterial colonization, LPS, and TLR4. Previous studies state that if there is a stimulation of LPS *Salmonella*, it can increase the TLR4 signaling pathway.^[20-22] The factor that is important

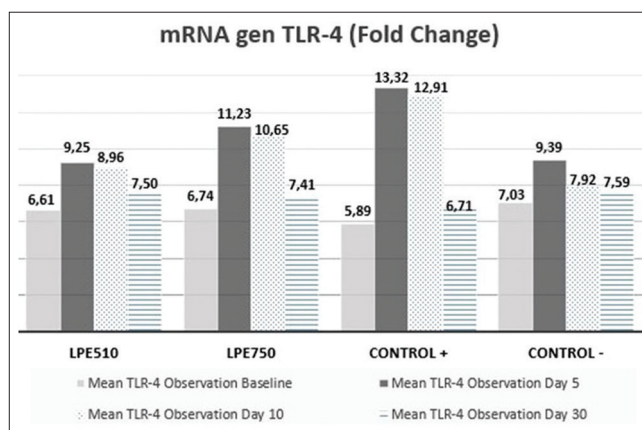


Figure 1: Dynamics changes of toll-like receptor 4 on the time of observation before the intervention (day 5), after the intervention (day 10), and maintenance after the intervention (day 30)

Table 1: Effects of lime peel extract on bacterial colonies based on the time of observation before intervention (day 5), after intervention (day 10), and maintenance after intervention (day 30)

Group	Bacterial colonies <i>S. typhi</i> (CFU/mL)					
	Day 5	Day 10	P	Day 10	Day 30	P
LPE 510	24.60±3.85	3.00±2.92	0.001	3.00±2.92	0.00±0.00	0.830
LPE 750	17.20±2.28	0.00±0.00	0.000	0.00±0.00	0.00±0.00	-a
Control (+)	30.40±7.40	1.60±1.36	0.001	1.60±1.36	0.00±0.00	0.306
Control (-)	22.60±6.07	6.00±2.83	0.009	6.00±2.83	1.20±0.58	0.014

aThe correlation and t cannot be computed because the standard error of the difference is 0. LPE: Lime peel extract, CFU: Colony-forming units

Table 2: Correlation of toll-like receptor-4 and bacterial colonization

Variable	Bacterial colonies (CFU/ mL)	
	Bivariate correlation	
	<i>r</i>	<i>P</i>
mRNA gen TLR-4 (fold change)	0.408	0.013

TLR: Toll-like receptor, CFU: Colony-forming unit

in the nonspecific stimulation of the immune response against LPS is the activity of TLR-4. The presence of LPS stimulates TLR-4 then causes the nuclear translocation of nuclear factor- κ B and cytokines tumor necrosis factor- α and inducible nitric oxide synthase.^[23]

In this study, we assumed that LPE can modulate the TLR-4 signaling pathway in host immunity so that the gene TLR-4 is expressed less and less. TLR-4 recognizes and binds ligand LPS of *S. typhi*. Metabolic compounds contained in the extract can kill the bacteria,^[24-26] LPS is nowhere so that expression of the gene TLR-4 is decreased. This mechanism causes the bacterial colony number to decrease, not even growth. It is recommended for further research to ascertain what compounds are contained in LPE that play a role in the modulation of TLR-4 signaling pathways.

CONCLUSIONS

The LPEs have metabolic compounds such as flavonoids, saponins, tannins, triterpenoids, and alkaloids that can kill the bacteria directly as an antibacterial, decreased the number of bacterial colonization, and decreased the mRNA expression TLR-4 gene.

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Conflicts of interest

There are no conflicts of interest.

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