SUBMIT

Title of the article :

Effects of lime (Citrus aurantifolia) peel to expression of mRNA TLR-4 in Balb/c mice infected Salmonella typhi

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 receptors 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 induced intraperitoneally and then 3 days after induction, LPE given orally on two-dose (510 and 750 mg/kg BW). Count the number of bacterial colonization using peritoneal fluid samples by the method of plate count agar (PCA). Intervention LPE for 5 days can degrade TLR-4 and the number of colonies of Salmonella typhi. TLR-4 gene is expressed increased after 3 days induced *Salmonella typhi*. Post-intervention 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 is a linear relationship with 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 less and less. This mechanism causes the bacterial colony number to decrease even not growth.

Introduction:

Many herbal medicine have been explore as antioxidant, antibacterial, and immunomodulator.[1–6] Typhoid fever is acute gastrointestinal infection cause by *Salmonella typhi* and still major problem since endemic in several countries.[7–9]. In addition to genetic factors that can affect the multiplication of *S. Typhi*, can also be a variety of immune factors that determine the severity of typhoid fever.[10]

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 TLR-4 receptor, TLR-4 binds to LPS.[11,12] The body's defense mechanism will be described in this study, the reaction when *S. typhi* first enters the body, the bacteria are destroyed by macrophages, because the bacteria are recognized by TLR-4 is located on the surface of phagocytes.[13] TLR-4 plays an important role in natural immune response through the pathway of signaling specific transcription factors. Lime peel extract can be as bacteriostatic or bactericide, as well as modulate the pathway of signaling innate immunity. This activity will appear in the examination of gene expression of TLR-4 and the calculation of the amount of bacterial colonization. Therefore the aim of this study was to know the mechanism of the host's body defense against *Salmonella 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-post test 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 originally selected with homogeneity, the benchmark is the similarity in color and size lemon diameter between 3-5 cm, cleaned, then separated between the skin and the fruit. 1 Kg lime produces approximately 300 grams of lime peel. After the lemon rind separate from the fruit, then the skin is dried using oven simplicia with a temperature of 55^0C oven to speed up the drying process until the water content decreases and reaches 10%. And then inserted into a glass container to do maceration process, soaked by adding 96% ethanol and then allowed to stand up to 3x24 hours with occasional stirring so that the solution mix. 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 grams, it can produce ± 30 grams of lime peel and has the potential to produce 0.1 grams of lime peel extract.

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 30 cm x 50 cm x 15 cm. Adaptation procedures carried out for 7 days. Further randomization, all the mice were divided into 4 groups (n = 5) group 1 LPE510 (group of mice which intervened LPE dose of 510 mg/kgBW), Group 2 LPE750 (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 carried out for 5 days.

Induction of *Salmonella typhi* bacteria

On day 0 after adaptation, all the mice do baseline blood sampling, then day 1 mice were induced by the bacteria *Salmonella typhi* as much as 3 x 10^3 CFU/mL Strain Thy1 by intraperitoneal injection. On day 2 to day 4 mice were observed for the occurrence of infections process.[14]

Peritoneal Fluid Intake and Investigation Bacteria Colonies

Peritoneal fluid was take was in the supine position, the abdomen was cleaned with 70% alcohol and NaCl injected as much as 0.8-1 mL into the peritoneal cavity. Then allowed to stand for 1 minute as he rocked slowrly. Peritoneal fluid removed 0.5 mL is aspirated from the peritoneal cavity of the supine position. Peritoneal fluid collection and examinations were conducted three times, on

day 5 after mice were induced by the bacteria *Salmonella typhi* (pre-intervention), the 10th-day post-intervention and the 30th day after maintenance without therapy.

Examination of counting the number of colonies of bacteria by diluting samples of 0.5 mL of peritoneal fluid, put in 4.5 mL of saline (0.9% NaCl). Approximately 1 ml of the suspension was poured into a sterile petri dish, followed by pouring the fertilizer medium (nutrient agar) sterile (45^0C) then sealed and incubated for 1-2 days at a temperature of 37^0C. The method used is the Plate Count Agar (PCA).[15]

Toll-Like Receptor-4

Examination of expression mRNA gen TLR-4 is doing 4 times at baseline (day 0), day 5 pre-intervention, the 10th-day post-intervention and the 30th day after the maintenance of 20 days post-intervention. RNA extraction using previous methods and stored at a temperature of -80^0C.[16–18] Realtime PCR program run by using CFX Connect system, Biorad Laboratories, Real-Time PCR 96 wells, 0.1 mL, USA.[19,20]

Primer for mice mRNA TLR4 is TLR4-Forward: TGACAGGAAACCCTATCCAGAGTT and TLR4-Reverse: TCTCCACAGCCACCAGATTCT.[11]

The parameter thermal cycle was 30 seconds at a temperature of 95^0C and 40 cycles of denaturation at a temperature of 95^0C for 10 seconds and then annealing 60^0C for 15 seconds and extension at a temperature of 72^0C for 40 seconds. All PCR repeated 3 times and the data were analyzed by the instrument detection system of Biorad software 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 lime peel extract to the amount of bacterial colonization *S.typhi* paired T test was used to assess the dynamics of change in the number of colonies of bacteria by a change of observation time for each group (table 1). There was a significant decrease in the intervention group

LPE510 and LPE750. Examination of counting the number of colonies on day 30, also 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 TLR-4

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

Correlation of TLR-4 with Colonization Bacteria

In Table 2, show 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. S. typhi that enters the digestive tract will not always cause infection, because to cause infection S. typhi must be able to reach the small intestine. Gastric acid prevents S. typhi from reaching the small intestine. However, most S. typhi bacteria can survive because they have the ATR (acid tolerance response) gene. Therefore, in this research S. typhi was induced directly into intraperitoneal.[21]

In the small intestine, the germ will penetrate the intestinal mucosa mediated by microbial binding to the epithelium destroying Microfold cells (M cells) so that the epithelial cells undergo desquamation, penetrate the intestinal mucosal epithelium, enter to lamina propria, settle and multiply. Bacterial will multiply in mononuclear cells before they spread into the bloodstream.[21]

S. typhi infection was stimulate macrophage activation. The content of lipopolysaccharide 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. The presence of TLR-4 receptor protein on the surface of phagocytic cells, the cells will facilitate the capture of Salmonella sp. who managed to penetrate the intestinal wall and intestinal mucosa.[22]

In this study it was found that TLR-4 at the baseline examination is lower than the TLR-4 after the host bacteria injected. This indicates that the homeostatic condition, Intestinal Epithelial Cells (IEC) has the gene expression of TLR-4 were low in this case in the context of a healthy, low TLR activation[23]; however, if there is inflammation, the IEC increased TLR expression and then trigger the TLR signal, Then on day 5 post injection of S. typhi day 4, TLR-4 mRNA expression in all groups of mice increased. This may explain the study of TLR-4 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, resulting in an increasing number of LPS captured by the receptor TLR -4 the TLR-4 mRNA expression increased. Visible differences between the mean square of TLR-4 baseline (mq=1.18) with the mean square of TLR-4 day 5 (mq=18.18), is much improved over-expressed compared to prior induced *S. typhi* bacteria.

Decreased expression of TLR-4 gene mRNA showed a decrease in the amount of LPS in the blood of mice and marks the healing process of Salmonella typhi infection in the body of mice. Factor that is important in the non-specific 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 NF-κB and cytokines TNF-α and iNOS.[24]

In this study, we assumed could happen three processes, the extract can kill the bacteria as antibacterial, can modulate the pathways bond LPS with TLR-4 that with the death of bacteria, LPS bacterial cell wall is reduced so that the TLR-4 is not activated.[11] The metabolic compounds in LPE such as flavonoids, saponins, tannins, triterpenoids, and alkaloids, it is assumed that kill the bacteria directly where such compounds as an antibacterial.[6]

Conclusions:

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 Lypopolysacarida (LPS) of *Salmonella typhi*. Metabolic compounds contained in the extract can kill the bacteria, LPS is nowhere so that expresse of the gene TLR-4 is decreased. This mechanism causes the bacterial colony number to decrease even not 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.

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Covering Letter

To,

The Editor

Sub: Submission of Manuscript for publication

Dear Sir,
We intend to publish an article entitled "Effects of lime (Citrus aurantifolia) peel to expression of mRNA TLR-4 in Balb/c Mice infected Salmonella typhi" in your esteemed journal as an Original Article.

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Thanking you,

Yours' sincerely,

Signature 

Corresponding contributor :

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- ❑ Signed by all contributors
- ❑ Previous publication / presentations mentioned
- ❑ Source of funding mentioned
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Running title: Effects of Lime Peel Extract to TLR-4

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Contribution Details (to be ticked marked as applicable):

	Contributor 1	Contributor 2	Contributor 3	Contributor 4	Contributor 5	Contributor 6
Concepts	V	V				
Design	V					
Definition of intellectual content		V				
Literature search			V	V		
Clinical studies	V					
Experimental studies	V	V				
Data acquisition	V	V				
Data analysis	V				V	
Statistical analysis	V			V	V	
Manuscript preparation	V					V
Manuscript editing						V
Manuscript review		V	V	V		
Guarantor						

Title of the article :

Effects of lime (Citrus aurantifolia) peel to expression of mRNA TLR-4 in Balb/c Mice infected Salmonella typhi

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 receptors 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 induced intraperitoneally and then 3 days after induction, LPE given orally on two-dose (510 and 750 mg/kg BW). Count the number of bacterial colonization using peritoneal fluid samples by the method of plate count agar (PCA). Intervention LPE for 5 days can degrade TLR-4 and the number of colonies of Salmonella typhi. TLR-4 gene is expressed increased after 3 days induced *Salmonella typhi*. Post-intervention 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 is a linear relationship with 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 less and less. This mechanism causes the bacterial colony number to decrease even not growth.

Context:

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 receptors 4 (TLR-4) signaling pathway is unclear.

Aims:

This study examined the effects of lime peel extract (LPE) on the activity of TLR 4 in Balb/c mice induced by *Salmonella typhi*

Settings and Design:

This research is a true experimental pre-post test design

Methods and Material:

Mice induced intraperitoneally and then 3 days after induction, LPE given orally on two-dose (510 and 750 mg/kg BW). Count the number of bacterial colonization using peritoneal fluid samples by the method of plate count agar (PCA)

Statistical analysis used:

Paired t-test, bivariate correlation

Results:

Intervention LPE for 5 days can degrade TLR-4 and the number of colonies of Salmonella typhi. TLR-4 gene is expressed increased after 3 days induced *Salmonella typhi*. Post-intervention 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 is a linear relationship with 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 ($p=0.013$, $r=0.408$)

Conclusions:

LPE can modulate the TLR-4 signaling pathway in host immunity so that the gene TLR-4 is expressed less and less. This mechanism causes the bacterial colony number to decrease even not growth.

Key-words:

Bacterial colonization, *Citrus aurantifolia*, Lime peel extract, *Salmonella typhi*, TLR-4

Key Messages:

Lime peel extract can be as bacteriostatic or bactericide, as well as modulate the pathway of signaling innate immunity. This activity will appear in the examination of gene expression of TLR-4 and the calculation of the amount of bacterial colonization.

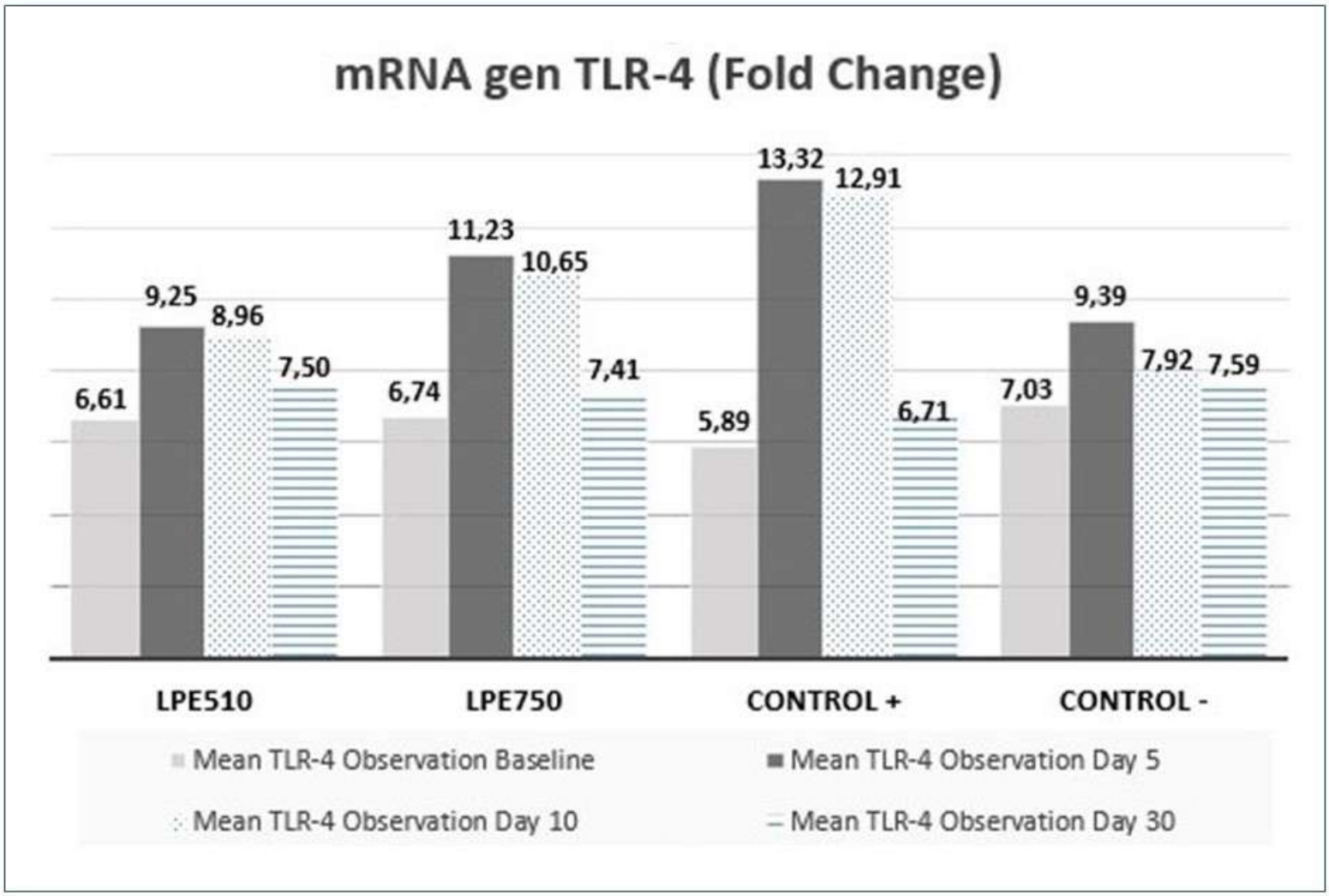
mRNA gen TLR-4 (Fold Change)
6,61
9,25
8,96
7,50
6,74
11,23
10,65
7,41
5,89
13,32
12,91
6,71
7,03
9,39
7,92
7,59
LPE510
LPE750
CONTROL +
CONTROL -
Mean TLR-4 Observation Baseline
Mean TLR-4 Observation Day 5
Mean TLR-4 Observation Day 10
Mean TLR-4 Observation Day 30

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 *S.typhi* (CFU/ml)					
	Day 5	Day 10	*p value*	Day 10	Day 30	*p value*
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

LPE: Lime peel extract, CFU: Colony-forming units

Table 2. Correlation of TLR-4 and bacterial colonization

Variabel	Bacterial Colonies (CFU/ml) *Bivariate Correlation*	
	R	***p value***
mRNA gen TLR-4 (fold change)	0,408	0,013

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

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Journal of Advanced Pharmaceutical Technology & Research <editor@japtr.org> 20 April 2020 20.49
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Dear Dr. Kasim,

Journal of Advanced Pharmaceutical Technology & Research has received your manuscript entitled "Effects of lime (Citrus aurantifolia) peel to expression of mRNA TLR-4 in Balb/c mice infected Salmonella typhi" for consideration for publication. The reference number for this manuscript is "JAPTR_48_20". Kindly quote this in correspondence related to this manuscript.

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Yours sincerely,
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M.Pharm., Ph.D., FSPER
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Dear Dr. Kasim

With reference to your manuscript entitled 'Effects of lime (Citrus aurantifolia) peel to expression of mRNA TLR-4 in Balb/c mice infected Salmonella typhi', please review the comments of the referees from our site https://www.journalonweb.com/japtr. The manuscript would be reconsidered after requisite modifications as per the comments and instructions provided by the journal.

If you wish to continue with the publication process, kindly make the changes according to the comments and upload the revised manuscript from the site along with the point to point clarifications to the comments indicating clearly where in the manuscript the changes have been carried out. Do check the FAQ related to re plying to the comments and uploading a file.

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With warm personal regards,

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Remarks:

Although, author has demonstrated the good piece of work, still, some minor changes are required in the reference section and give some recent relvant reference in the article.

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Reply to the 'remarks'

Reviewer Number	Original comments of the reviewer	Reply by the author(s)	Changes done on page number and line number
-	Although, author has demonstrated the good piece of work, still, some minor changes are required in the reference section and give some recent relvant reference in the article.	Reference; Typhoid fever is acute gastrointestinal infection cause by *Salmonella typhi* and still major problem since endemic in several countries.(7-9) Changed; Reference 7	Page 2, line 33-34
		Reference; In addition to genetic factors that can affect the multiplication of *S. Typhi*, can also be a variety of immune factors that determine the severity of typhoid fever.(10) Changed; Reference 8	Page 2, line 34-36
		Reference; Special marker molecule of a gram-negative bacterial is lipopolysaccharide (LPS), which will be instantly recognizable by protein receptors on the host that is TLR-4 receptor, TLR-4 binds to LPS.(11,12) Changed; Reference 9	Page 2, line 38-40
		Reference; The body's defense mechanism will be described in this study, the reaction when *S. typhi* first enters the body, the bacteria are destroyed by macrophages, because the bacteria are recognized by TLR-4 is located on the surface of phagocytes.(13) Changed; Reference 10	Page 2, line 40-42
		Additional sentences and reference; 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 immunity. Among components present in the diet, flavonoids have been suggested as antioxidant dietary factors able to modulate TLR-mediated signaling pathways.	Page 2, line 42-45

		Reference; 11	
		Additional Statement and reference; Preliminary studies, through qualitative and quantitative phytochemical screening, the metabolic compounds obtained from lime peel extract are phenols, flavonoids, tannins, alkaloids and triterpenoid.(12) Studies of LPE interventions on TLR4 gene mRNA expression activity in salmonella typhi-infected hosts have not been conducted so far. Reference; 12	Page 2, line 46-49
		Aim of study; Therefore the aim of this study was to know the mechanism of the host's body defense against *Salmonella typhi* infection through the TLR-4 signaling pathway relationship with the presence of bacterial cell death. Added a few words; Therefore the aim of this study was to know the effect of LPE to mechanism of the host's body defense against Salmonella typhi infection through the TLR-4 signaling pathway relationship with the presence of bacterial cell death.	Page 2, line 49-51
		Reference; On day 2 to day 4 mice were observed for the occurrence of infections process.(14) Deleted, because not relevant	Page 3, line 79
		Reference; The method used is the Plate Count Agar (PCA).(15) Changed; Reference 13	Page 4, line 91-92
		Reference; RNA extraction using previous methods and stored at a temperature of -80^{0}C.(16-18) Deleted, because not relevant	Page 4, line 96
		Reference; Realtime PCR program run by using CFX Connect system, Biorad Laboratories, Real-Time PCR 96 wells, 0.1 mL, USA.(19,20)	Page 4, line 96-98

		Deleted, because not relevant	
		Reference; Primer for mice mRNA TLR4 is TLR4-Forward: TGACAGGAAACCCTATCCAGAGTT and TLR4-Reverse: TCTCCACAGCCACCAGATTCT.(11) Changed; Reference 14	Page 4, line 99-100
		Sentences and Reference; S. typhi that enters the digestive tract will not always cause infection, because to cause infection S. typhi must be able to reach the small intestine. Gastric acid prevents S. typhi from reaching the small intestine. However, most S. typhi bacteria can survive because they have the ATR (acid tolerance response) gene. Therefore, in this research S. typhi was induced directly into intraperitoneal.(21) Changed; The latest study, *Salmonella 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 interaction with the target human host is to avoid immune response in order to efficiently infect and propagate. *Salmonella* remains a severe human health threat without sufficient therapeutic options.(15)	Page 5-6, line 130-134
		Sentences; In the small intestine, the germ will penetrate the intestinal mucosa mediated by microbial binding to the epithelium destroying Microfold cells (M cells) so that the epithelial cells undergo desquamation, penetrate the intestinal mucosal epithelium, enter to lamina propria, settle and multiply. Bacterial will multiply in mononuclear cells before they spread into the bloodstream. Deleted, because not relevant	-
		Sentence; S. typhi infection was stimulate macrophage	Page 6, line 135

		activation. Additional Reference; 16	
		Sentence; TLR-4 protein serves as a receptor on the surface of phagocytic cells to recognize LPS *Salmonella sp.* Additional Reference; 17	Page 6, line 136-137
		Reference; The presence of TLR-4 receptor protein on the surface of phagocytic cells, the cells will facilitate the capture of *Salmonella sp.* who managed to penetrate the intestinal wall and intestinal mucosa.(22) Deleted, because not relevant	Page 6, line 137-139
		Sentence; This indicates that the homeostatic condition. Additional Reference; 18	Page 6, line 141
		Sentences and reference; Intestinal Epithelial Cells (IEC) has the gene expression of TLR-4 were low in this case in the context of a healthy, low TLR activation.(23) Deleted, because not relevant	-
		Additional sentences and reference; However, if there is inflammation, TLR expression increased and trigger the cytokine pro-inflammation Reference; 19	Page 6, line 141-142
		Sentences; however, if there is inflammation, the IEC increased TLR expression and then trigger the TLR signal, Then on day 5 post injection of S. typhi day 4, TLR-4 mRNA expression in all groups of mice increased. This may explain the study of TLR-4 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, resulting in an increasing number of LPS captured by the	-

		receptor TLR -4 the TLR-4 mRNA expression increased. Visible differences between the mean square of TLR-4 baseline (mq=1.18) with the mean square of TLR-4 day 5 (mq=18.18), is much improved over-expressed compared to prior induced *S. typhi* bacteria. Deleted, because not important in discussion	
		Additional sentences; 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. Reference; 17	Page 6, line 142-145
		Additional sentences and reference; However, this needs to be done further research on the pathomechanism of number of bacterial colonization, LPS and TLR4. Previous studies state that if there is stimulation of LPS salmonella it can be increase the TLR4 signaling pathway. Reference; 20-22	Page 6, line 148-150
		Reference 24; changed into reference 23	Page 6, line 152
		Sentences; In this study, we assumed could happen three processes, the extract can kill the bacteria as antibacterial, can modulate the pathways bond LPS with TLR-4 that with the death of bacteria, LPS bacterial cell wall is reduced so that the TLR-4 is not activated. The metabolic compounds in LPE such as flavonoids, saponins, tannins, triterpenoids, and alkaloids, it is assumed that kill the bacteria directly where such compounds as an antibacterial. Deleted, because not relevant	-
		Additional statement and reference; 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 Lypopolysacarida (LPS) of *Salmonella typhi*. Metabolic compounds contained in the extract can kill the bacteria,(24–26) LPS is	Page 6, line 153-159

		nowhere so that expresse of the gene TLR-4 is decreased. This mechanism causes the bacterial colony number to decrease even not 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. Reference; 25, 26	
		Sentences; 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 Lypopolysacarida (LPS) of *Salmonella typhi*. Metabolic compounds contained in the extract can kill the bacteria, LPS is nowhere so that expresse of the gene TLR-4 is decreased. This mechanism causes the bacterial colony number to decrease even not 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. Changed into; The LPE 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.	Page 7, line 161-163

Title of the article :

Effects of lime (Citrus aurantifolia) peel to expression of mRNA TLR-4 in Balb/c mice infected Salmonella typhi

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 receptors 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 induced intraperitoneally and then 3 days after induction, LPE given orally on two-dose (510 and 750 mg/kg BW). Count the number of bacterial colonization using peritoneal fluid samples by the method of plate count agar (PCA). Intervention LPE for 5 days can degrade TLR-4 and the number of colonies of Salmonella typhi. TLR-4 gene is expressed increased after 3 days induced *Salmonella typhi*. Post-intervention 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 is a linear relationship with 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 less and less. This mechanism causes the bacterial colony number to decrease even not growth.

Introduction:

Many herbal medicine have been explore as antioxidant, antibacterial, and immunomodulator.[1–6] Typhoid fever is acute gastrointestinal infection cause by *Salmonella typhi* and still major problem since endemic in several countries.(7) In addition to genetic factors that can affect the multiplication of *S. Typhi*, can also be a variety of immune factors that determine the severity of typhoid fever.(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 TLR-4 receptor, TLR-4 binds to LPS.(9) The body's defense mechanism will be described in this study, the reaction when *S. typhi* first enters the body, 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 immunity. 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 are phenols, flavonoids, tannins, alkaloids and triterpenoid.(12). Studies of LPE interventions on TLR4 gene mRNA expression activity in salmonella 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 Salmonella 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-post test 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 originally selected with homogeneity, the benchmark is the similarity in color and size lemon diameter between 3-5 cm, cleaned, then separated between the skin and the fruit. 1 Kg lime produces approximately 300 grams of lime peel. After the lemon rind separate from the fruit, then the skin is dried using oven simplicia with a temperature of 55^0C oven to speed up the drying process until the water content decreases and reaches 10%. And then inserted into a glass container to do maceration process, soaked by adding 96% ethanol and then allowed to stand up to 3x24 hours with occasional stirring so that the solution mix. 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 grams, it can produce ± 30 grams of lime peel and has the potential to produce 0.1 grams of lime peel extract.

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 30 cm x 50 cm x 15 cm. Adaptation procedures carried out for 7 days. Further randomization, all the mice were divided into 4 groups (n = 5) group 1 LPE510 (group of mice which intervened LPE dose of 510 mg/kgBW), Group 2 LPE750 (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 carried out for 5 days.

Induction of *Salmonella typhi* bacteria

On day 0 after adaptation, all the mice do baseline blood sampling, then day 1 mice were induced by the bacteria *Salmonella 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 infections process.

Peritoneal Fluid Intake and Investigation Bacteria Colonies

Peritoneal fluid was take was in the supine position, the abdomen was cleaned with 70% alcohol and NaCl injected as much as 0.8-1 mL into the peritoneal cavity. Then allowed to stand for 1 minute as he rocked slowrly. Peritoneal fluid removed 0.5 mL is aspirated from the peritoneal cavity of the supine position. Peritoneal fluid collection and examinations were conducted three times, on day 5 after mice were induced by the bacteria *Salmonella typhi* (pre-intervention), the 10th-day post-intervention and the 30th day after maintenance without therapy.

Examination of counting the number of colonies of bacteria by diluting samples of 0.5 mL of peritoneal fluid, put in 4.5 mL of saline (0.9% NaCl). Approximately 1 ml of the suspension was poured into a sterile petri dish, followed by pouring the fertilizer medium (nutrient agar) sterile (45^0C) then sealed and incubated for 1-2 days at a temperature of 37^0C. The method used is the Plate Count Agar (PCA).(13)

Toll-Like Receptor-4

Examination of expression mRNA gen TLR-4 is doing 4 times at baseline (day 0), day 5 pre-intervention, the 10th-day post-intervention and the 30th day after the maintenance of 20 days post-intervention. RNA extraction using previous methods and stored at a temperature of -80^0C. Realtime PCR program run by using CFX Connect system, Biorad 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 seconds at a temperature of 95^0C and 40 cycles of denaturation at a temperature of 95^0C for 10 seconds and then annealing 60^0C for 15 seconds and extension at a temperature of 72^0C for 40 seconds. All PCR repeated 3 times and the data were analyzed by the instrument detection system of Biorad software 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 lime peel extract to the amount of bacterial colonization *S.typhi* paired T test was used to assess the dynamics of change in the number of colonies of bacteria by a change of observation time for each group (table 1). There was a significant decrease in the intervention group LPE510 and LPE750. Examination of counting the number of colonies on day 30, also 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 TLR-4

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

Correlation of TLR-4 with Colonization Bacteria

In Table 2, show 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, *Salmonella 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 interaction with the target human host is to avoid immune response in order to efficiently infect and propagate. *Salmonella*

remains a severe human health threat without sufficient therapeutic options.(15)

S. typhi infection was stimulate macrophage activation.(16) The content of lipopolysaccharide 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 presence of TLR-4 receptor protein on the surface of phagocytic cells, the cells will facilitate the capture of *Salmonella sp.* who 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, this indicates that the homeostatic condition.(18) However, if there is inflammation, TLR expression increased and trigger 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 marks the healing process of Salmonella typhi infection in the body of mice. However, this needs to be done further research on the pathomechanism of number of bacterial colonization, LPS and TLR4. Previous studies state that if there is stimulation of LPS salmonella it can be increase the TLR4 signaling pathway.(20–22) Factor that is important in the non-specific 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 NF-κB and cytokines TNF-α and iNOS.(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 Lypopolysacarida (LPS) of *Salmonella typhi*. Metabolic compounds contained in the extract can kill the bacteria,(24–26) LPS is nowhere so that expresse of the gene TLR-4 is decreased. This mechanism causes the bacterial colony number to decrease even not 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 LPE 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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Dear Dr. Kasim

Journal of Advanced Pharmaceutical Technology & Research has received your revised manuscript entitled 'Effects of lime (Citrus aurantifolia) peel to expression of mRNA TLR-4 in Balb/c mice infected Salmonella typhi.'
The manuscript will be re-evaluated by concerned referees before final decision on its suitability for publication. We will get back to you within two weeks.

We thank you for submitting your valuable research work to Journal of Advanced Pharmaceutical Technology & Research.

With warm personal regards,

The Editorial Team

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ORIGINAL ARTICLE

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

Vivien Novarina Kasim, Mochammad Hatta[1], Rosdiana Natzir[2], Veni Hadju[3], Ami Febriza[4], Hasta Handayani Idrus[5]

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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*. TLR-4 gene is expressed increased after 3 days induced *S. typhi*. 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 is a linear relationship with 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.

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

<H1>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, 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.

<H1>SUBJECTS AND METHODS

<H2>Study area and design

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<H2>Lime peel extract

With lime originally being selected with homogeneity, the benchmark is the similarity in color and size lemon diameter between 3 and 5 cm, cleaned, then separated between the skin and the fruit. One kilogram lime produces approximately 300 g of lime peel. After the lemon rind separated from the fruit, then the skin is dried using oven simplicia with a temperature of 55°C oven 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, soaked by adding 96% ethanol, and then allowed to stand up to 3 h × 24 h with occasional stirring so that the solution mix. 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.

<H2>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.

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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.

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Peritoneal fluid was take was in the supine position, the abdomen was cleaned with 70% alcohol and NaCl injected as much as 0.8–1 mL into the peritoneal cavity. Then allowed to stand for 1 min as they rocked slowly. Peritoneal fluid removed 0.5 mL is aspirated from the peritoneal cavity of the supine

position. Peritoneal fluid collection and examinations were conducted three times: on day 5 after mice were induced by the bacteria *S. typhi* (preintervention), the 10th-day postintervention, and the 30th day after maintenance without therapy.

Examination of counting the number of colonies of bacteria by diluting samples of 0.5 mL of peritoneal fluid put in 4.5 mL of saline (0.9% NaCl). Approximately 1 mL of the suspension was poured into a sterile petri dish, followed by pouring the fertilizer medium (nutrient agar) sterile (45°C) then sealed and incubated for 1–2 days at a temperature of 37°C. The method used is the plate count agar.[13]

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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 Bio-Rad software using the comparative threshold cycle method. The standard curve was constructed and is an indication of good amplification efficiency (90%–100%).

<H1>RESULTS

<H2>Bacterial colonies

Analysis of the effect of LPE to the amount of bacterial colonization *S. typhi* paired *t*-test was used to assess the dynamics of change in the number of colonies of bacteria by a change of observation time for each group [Table 1]. There was a significant decrease in the intervention group LPE510 and LPE750. Examination of counting the number of colonies on day 30, also 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.

<H2>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 gene TLR-4 on day 5 expressed more than before the injected bacteria (baseline). There are statistically significant differences in all groups: LPE510 ($P = 0.002$), LE750 ($P = 0.001$), positive controls ($P = 0.000$), and negative controls ($P = 0.003$).

<H2>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.

<H1>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* interaction with the target human host is to avoid immune response to efficiently infect and propagate. *Salmonella* remains a severe human health threat without sufficient 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 presence of TLR-4 receptor protein on the surface of phagocytic cells, the cells will facilitate the capture 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 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-κB 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.

<H1>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.

<H2>Acknowledgment

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<H2>Financial support and sponsorship

Nil.

<H2>Conflicts of interest

There are no conflicts of interest.

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Figure Legend

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)

Tables

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 *S. typhi* (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

(−)						

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**	
	r	***P***
mRNA gen TLR-4 (fold change)	0.408	0.013

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

ORIGINAL ARTICLE

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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

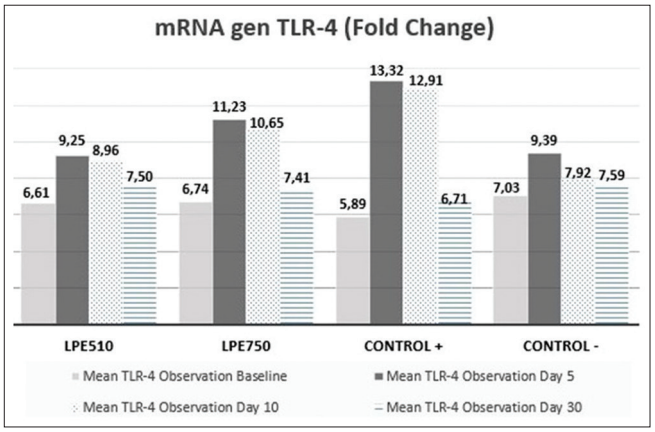

Figure 1: Dynamics changes of toll-like receptor 4 on the time of observation before the intervention (day 5), after the intervention (day 10), and maintenance after the intervention (day 30)

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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	
	r	*P*
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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 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-κB 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.

Acknowledgment

The authors would like to thank Markus, Rommy, Mus, and Wani (Molecular Biology and Immunology Laboratory for Infection Diseases, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia), who helped in the implementation of our research activities. This research was supported by the Ministry of Research and Technology, Indonesia, or Beasiswa Unggulan Dosen Indonesia – Dalam Negeri LDPD.

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ORIGINAL ARTICLE

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

Vivien Novarina Kasim, Mochammad Hatta[1], Rosdiana Natzir[2], Veni Hadju[3], Ami Febriza[4], Hasta Handayani Idrus[5]

Faculty of Medicine, State University of Gorontalo, Gorontalo, [1]Department of Microbiology, Molecular Biology and Immunology Laboratory, Faculty of Medicine, Hasanuddin University, [2]Department of Biochemistry, Faculty of Medicine, Hasanuddin University, [3]Department of Nutrition, Faculty of Public Health, Hasanuddin University, [4]Department of Physiology, Faculty of Medicine, Muhammadiyah University Makassar, [5]Department of Microbiology, Indonesian Muslim University, Makassar, Indonesia

J. Adv. Pharm. Technol. Res.

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°0C 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 370C 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 to the amount of bacterial colonization *S. typhi* paired *t*-test was used to assess the dynamics of change in the number of colonies of bacteria by a change of observation time for each group [Table 1]. There was a significant decrease in the intervention group LPE510 and LPE750. Examination of counting the number of colonies on day 30, also 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 gene TLR-4 on day 5 expressed more than before the injected bacteria (baseline). 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* interaction with the target human host is to avoid immune response to efficiently infect and propagate. *Salmonella* remains a severe human health threat without sufficient therapeutic options.[15]

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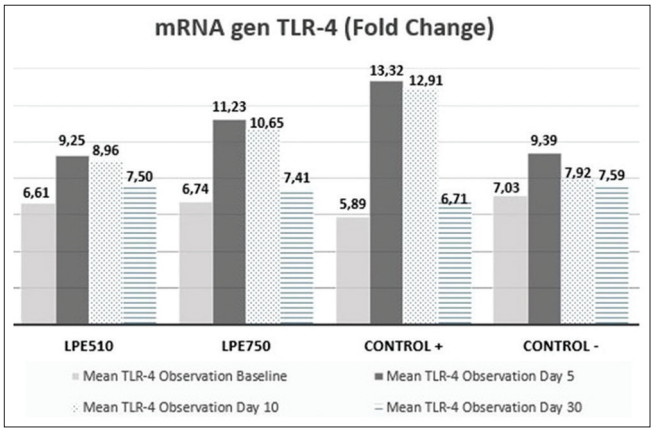

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ORIGINAL ARTICLE

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°0C 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 370C 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

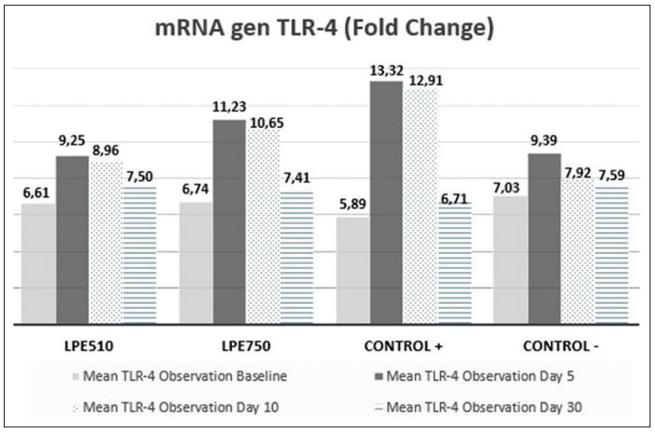

Figure 1: Dynamics changes of toll-like receptor 4 on the time of observation before the intervention (day 5), after the intervention (day 10), and maintenance after the intervention (day 30)

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 *S. typhi* (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	
	r	*P*
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-κB 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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Nil.

Conflicts of interest

There are no conflicts of interest.

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