SUBMIT

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JBR - Journal of Biological Research [paper #8951] - Submission Acknowledgement

Francesca Savio <francesca.savio@pagepress.org> 7 Maret 2020 16.11
Kepada: Vivien Novarina Kasim <viviennovarina@ung.ac.id>

Dear Vivien Novarina Kasim:

Thank you for submitting the manuscript, "Antibacterial and anti-inflammatory effects of lime (citrus aurantifolia) peel extract in mice balb/c induced salmonella typhi" to Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale. Your manuscript will be sent to the peer review phase in a few days and with the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

Manuscript URL: https://www.pagepressjournals.org/index.php/jbr/authorDashboard/submission/8951
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If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Francesca Savio

Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale

I. Title

Antibacterial and anti-inflammatory effects of lime (*citrus aurantifolia*) peel extract in mice balb/c induced *salmonella typhi*

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IV. Acknowledgments:

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VI. Key Words :

Lime peel extract, *Citrus aurantifolia*, Interleukin 6, antibacterial, anti-inflammatory

I. Contributions: The authors contributed equally

II. Conflict of interest: The authors declare no potential conflict of interest

III. Funding: Ministry of Research and Technology, Indonesia or BUDI-DN LDPD.

Antibacterial and anti-inflammatory effects of lime (*citrus aurantifolia*) peel extract in mice balb/c induced *salmonella typhi*

Abstract:

Lime (*Citrus aurantifolia*) is a traditional plant that is widely used as antibacterial. This study to prove the effect of lime peel extract (LPE) on the growth of bacterial colonization of *S. Typhi* medited by activity of IL-6. True experimental pre-post test design, mice were divided into; LPE 510 mg/kgbw, LPE 750 mg/kgbw, positive and negative control. The examination was carried out 3 times, the 5th day before the intervention, the 10th day after the intervention and the 30th day after maintenance. Intervention LPE for 5 days can decreased the number of *S. Typhi* colonies, even maintenance for 20 days after the intervention showed no bacterial growth . IL-6 pro-inflammatory cytokine activity increased on examination day 5 after *S.Typhi* injection and decreased after intervention on day 10, significantly different between pre and post at all groups except negative controls (p=0.15). The speed of decrease in IL-6 levels was greatest at the LPE 750 mg/kgbw (velocity=-5.64%). LPE decreased serum levels of IL-6 and inhibit the growth of *S. Typhi* colony in mice Balb/c. LPE have potential as antibacterial and anti-inflammatory.

Introduction:

Some antibiotics have been resistant to *Salmonella Typhi* and *Salmonella Paratyphi*, such as ampicillin, chloramphenicol, tetracycline and co-trimoxsazole.[1] Multi Drug Resistance (MDR) leads to new treatments in the form of adjuvant therapy,

traditional plants that can be used as antimicrobials. One of them is the lime plant (*Citrus aurantifolia*). From the many previous studies even to date, all parts of the *C. aurantifolia* have various efficacy.[2] Specifically in this study the peel of lime was investigated as having antibacterial properties containing active ingredients such as polyphenols, flavonoids, tannins, saponins, alkaloids and triterpenoids having the effect of reducing the amount of colonization of the *Salmonella Typhi* experiment in Balb/c mice induced by *S. Typhi*.[3] *Salmonella Typhi*, is a gram-negative bacterial whose transmission almost always occurs through contaminated food and drinks. From several studies, resistance to *Salmonella Typhi* has begun to be high. The development of antimicrobial resistance is in line with the increasing use of antimicrobial drugs and in line with the discovery of new drugs.[4]

Bacterial resistance, especially *S. typhi*, brings us to a new treatment in the form of adjuvant therapy, which is a traditional plant that can be used as an antimicrobial, one of which is *Citrus aurantifolia*. Based on previous research, the lime peel has a higher concentration of flavonoids compared to other parts such as seeds, fruit, juice.[5,6] The existence of the content of flavonoids makes the lime peel have antibacterial and antioxidant power. Previous studies tested the bacteriostatic or bactericidal activity of flavonoids by conducting a time-kill study that found that flavonoids do not kill bacterial cells but only induce the formation of bacterial aggregates thereby reducing the amount of colony forming units (CFU) in a decent amount.[7]

When there is an *S. Typhi* bacterial infection, there is a bond between the lipopolysaccharide (LPS) of the bacterial wall and the Toll-Like Receptor 4 (TLR 4) of the host which stimulates innate immune activity. The activity releases a number of cytokines

such as TNFα, IL-1, and IL-6. Interleukin 6 (IL-6) mediates the protective systemic effects of inflammation, including the effects of fever, acute-phase protein synthesis by the liver, and increased production of leukocytes by the bone marrow.[8] This study discusses the effect of lime peel extract on the amount of bacterial colonization and its effect on the activity of IL-6 pro-inflammatory cytokines in Balb/c mice injected with *Salmonella Typhi*.

Material and Methods

An Experimental design was used to studied the effectiveness of lime peel extract in decreasing pro-inflammatory IL-6 levels and bacterial colonization in mice Balb/c strains induced by *Salmonella Typhi*.

Lime peel extract

Making extracts was carried out at the Hasanuddin University Faculty of Pharmacy Phytochemical Laboratory. Lime peel is cut into small pieces and then dried at 50ºC to minimize water content. The dried sample is then put into a glass container or jar to be macerated by adding Ethanol 96% and then let it sit for 3 x 24 hours. Samples that were macerated for 3 x 24 hours were then filtered by the Vacuum Filtration method using a Buchner Funnel. The extracts obtained after the screening process were then evaporated using a Rotary-evaporator until the results were thick extracts.

Experimental Animals

Mice Balb/c (age 8-12 weeks, weigh 30-40 grams; n=20) were maintained in the Laboratory of Molecular Biology and Immunology, Department of Microbiology, Faculty of Medicine, Hasanuddin University (Makassar, Indonesia). The rats were acclimatized for 8 days, then divided into four groups (n=5). All groups were induced intraperitoneally

with *S. Typhi* strain Thy1 (3 x 10^3 CFU/ml). After 3 days of induction, each animal tried to be intervened; LPE 510 (mice group intervened with LPE dose 510 mg/kgbw), LPE 750 (mice group intervened with LPE dose 750 mg/kgbw), postive control group (groups of mice given Levofloxacin dose 98 mg/kgbw and negative control group (group of placebo mice).

Bacterial colonization

Bacterial colonies are counted from samples taken from peritoneal fluid. Samples were taken three times, on the 5th day after mice were induced by *Salmonella Typhi* (pre-intervention), the 10th post-intervention and 30th day after maintenance without treatment. Sampels has been taken as much as 0.5 ml, put in 4.5 ml of physiological saline (0.9% NaCl). Dilution is carried out three times so that the culture obtained is not too dense or fills the cup (the culture is too dense will interfere with observation). 1 ml of suspension was poured into a sterile petri dish, then poured warm sterile (nutrient agar) media (45^0C) then tightly closed and incubated for 1-2 days at 37^0C. The method used is the Plate Count Agar (PCA) is a technique for growing microorganisms in agar media by mixing the liquid agar media with bacterial culture stock (agar) so that the cells are evenly distributed and still on the surface of agar or in in agar.[9]

Interleukin 6 (IL-6) Examination

Serum samples were taken 4 times, baseline (day 0), after induction of *S. Typhi* before intervention (day 5), after intervention (10th day) and after 30 days. IL-6 antibody concentrations are determined by the IL6 ELISA Mouse Sandwich method (User Manual Catalog No. LS-F24855) LSBio, LifeSpan BioSciences, Inc. reader 270, Instrument serial

number: 1211006860, measurement mode: Absorbance, measurement wavelength: 450 nm, read mode: normal, unit: OD.

Ethics statement

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

Results

Bacterial Colonization

The number of bacterial colonies after being given an intervention decreased in all groups, but on the negative control group on the 10th day the number of bacterial colonies was seen more than other groups, and the 30th day negative control group there were still bacterial colonies (Fig-1). On the 10th day, the number of bacterial colonies decreased after the intervention of lime peel extract was given for 5 days of intervention. The number of bacterial colonies continued to decrease until the 30th day post-intervention. Researchers assume that extracts of lime peel can as a bactericidal over be bacteriostatic because the number of bacterial colonies on the 10th and 30th days reaches zero or there is no bacterial growth.

In the LPE group at a dose of 750 mg/kgbw, the number of bacterial colonies on day 10 had no growth, better than positive control group. The average decrease in the number of bacterial colonies in the LPE group 750 mg/kgbw from 17.2 x 10^3 CFU/ml to 0 or no bacterial growth (p=0.00) whereas in the positive control group decreased from 30.4 x 10^3 CFU/ml to 1.6 x 10^3 CFU/ml (p=0.001). In the negative control group, the number

of bacterial colonies decreased from 22.6 x 10^3 CFU/ml to 6 x 10^3 CFU/ml, meaning that the number of bacterial colonies in the negative control group even though it decreased but the number of bacterial colonies was still bigger than the three groups.

Interleukin 6 (IL-6)

The level of serum IL-6 at 4 times the data collection, analyzed using paired T-tests to assess the dynamics of changes in levels of serum with respect to changes in observation time for each group, can be seen in Table 1. The mean of IL-6 level on the 5th day increased in all groups compared at the baseline day, because it is the 4th-day post-injection *Salmonella Typhi*. This indicates that after the injection of *Salmonella Typhi*, all mice in all groups experienced an infection process.

The difference of average of IL-6 levels between groups at the time of observation before the 5th day intervention and after the 10th day intervention, there were significant differences in groups each, LPE 510 (p=0.003), LPE 750 (p=0.002), positive control (p=0.006). Figure 2 shows the velocity of the decrease of IL-6 averages between the time of observation after the intervention (10th day) and before the intervention (5th day). At the time of observation of the 10th day to the 5th day, it was seen that the velocity of decreasing IL-6 levels was greatest in the LPE 750 is 5.64% and the smallest velocity of decline was in the negative control group by 4.67%. The velocity of decrease in IL-6 levels in the LPE 750 was found more than the positive control group given Levofloxacin. It is assumed that the extract of lime peel in this study is effective as an anti-inflammatory.

DISCUSSION

Bacterial Colonization

LPE has an antibacterial effect as previous studies explained that *Citrus aurantifolia* has a high inhibitory zone in gram-positive and gram-negative bacteria.[10] *S. Typhi* injection given intraperitoneal stimulates macrophages to activate and move to the source of infection.[11] The LPS in the *S. Typhi* cell wall as a signal for macrophages to carry out activation. In previous preliminary studies, it was found that the metabolic compounds contained in the extract of lime peel were polyphenols, flavonoids, alkaloids, tannins, saponins, and triterpenoids each having the property to kill bacteria. These compounds attack bacteria directly, causing bacterial cell death. This is indicated in the calculation of the number of bacterial colonies that are reduced even no growth after 5 days given the intervention of LPE.

Interleukin 6 (IL-6)

One of the earliest responses of the innate immune system to infection and tissue damage is the secretion of cytokines by tissue cells, which are very important for acute inflammation. When *S. Typhi* first enters the body, the bacteria will be destroyed by macrophages. Bacteria will be known by various receptors located on the surface of phagocytes.[8] Specific marker molecules for gram-negative bacteria such as *S. Typhi* are LPS, LPS will use TLR-4, which is a receptor that plays a role in observing and destroying *Salmonella Typhi.* Activated TLR-4 will recruit the MyD88 adapter protein. Then MyD88 recruited IRAK4, IRAK1 and IRAK2. IRAQ kinase then phosphorylates and activates the TRAF6 protein, allowing NF-κB to dwell in the cell nucleus and activating transcription and causing induced inflammatory cytokines. Proinflammatory cytokines such as IL-1β and IL-6, IFN-γ and TNF-α are synthesized and systemic inflammation occurs.[8,12] It can be said that the inflammatory process by IL-6 cytokines is already running.

As for the negative control group, there were no significant differences before the intervention (5th day) and after the intervention (10th day), which is p-value 0.15. These results indicate that the extract of lime peel has the same effect as Levofloxacin, which inhibits the activity of pro-inflammatory cytokines IL-6 after administration for 5 days. Researchers assume that the inhibitory effect of IL-6 is due to the activity of the polyphenol compounds contained in extracts of lime peel. In line with this, Haseeb A. (2017) found that the potential inhibitory effect of polyphenols on IL-6 expression and oxidative stress through molecular mechanisms of activation of NF-κB.[13]

Looking from the content of polyphenols and flavonoids contained in LPE of this study, polyphenols are bioactive substances that have the potential to have a potential effect on the inflammatory response. It is known that flavonoids are one of the most extensive groups of secondary metabolite plants, flavonoids are found in many edible fruits and vegetables. The most source of polyphenols is represented by citrus fruits.[14] The polyphenol content in lime peel extract in this study was 2.29% and flavonoids 0.26%, it has been reported that the content of phenols and flavonoids using methanol solvent contained more in the parts of lime peel compared to the leaves, phenol 95.6 mg/g, and flavonoids 23.5 mg/g, are contained in the Rossano *Citrus aurantiolia* bark extract in Calabria, Italy. In that study, it was found that there was a significant relationship between phenol and flavonoid with antioxidant activity.[4]

Tejada S. (2017), proves that hesperidin (a type of flavonoid) extracted from the citrus genus, has potential as an anti-inflammatory. Hesperidin treatment decreases inflammatory mediators and provides a significant antioxidant effect. Molecular bases for anti-inflammatory effects appear to be mediated by signaling pathways especially the

nucleus κβ factor pathway.[15] As for other opinions, research conducted by Jorge L.A, found that from 3 types of citrus namely *C.limon, C.latifolia, C. aurantifolia or C.limonia*, essential oils (EO) contained in the 3 types of citrus have anti-inflammatory effects, especially the limonene content of essential oils from the citrus fruit skin.[16] During the inflammatory event, there is an increase in cytokine production.

CONCLUSION

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

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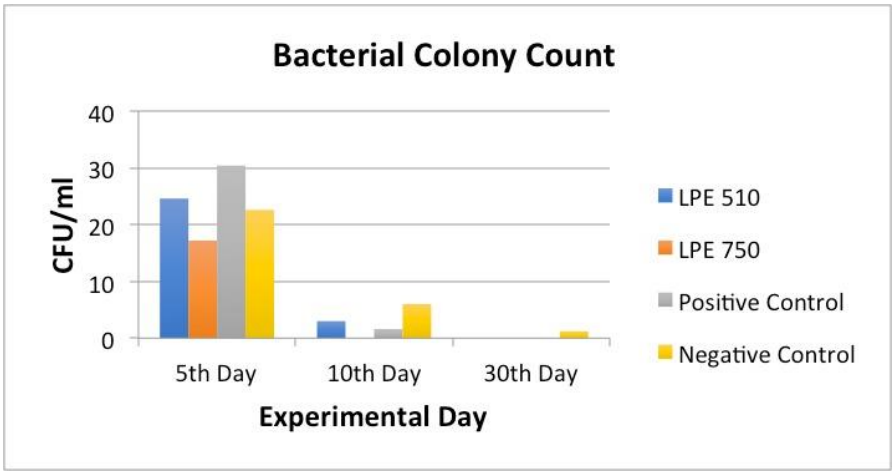

Figure 1. Dynamics of changes in the number of bacterial colonization of *S. Typhi* by the group during the observation period; LPE, lime peel extract; CFU, colony forming units.

Table 1. The differences in the levels of IL-6 serum dynamics between groups at the baseline, 5th day, 10th day and 30th day

Groups	Levels of IL-6 (ρg/ml)								
	Baseline	5th	*p value*	5th	10th	*p value*	10th	30th	*p value*
LPE 510	157.3±59.7	358.5±59.5	0.007	358.5±59.5	338.7±64.9	**0.003**	338.7±64.9	284.7±60.6	0.05
LPE 750	171.8±58.1	434.1±51.3	0.004	434.1±51.3	409.6±53.6	**0.002**	409.6±53.6	293.9±34.3	0.01
Control(+)	121.1±35.9	502.0±35.4	0.000	502.0±35.4	477.9±41.5	**0.006**	477.9±41.5	204.5±40.7	0.001
Control(-)	130.3±47.7	248.5±27.2	0.003	248.5±27.2	236.9±31.8	0.15	236.9±31.8	194.9±38.5	0.08

IL-6, interleukin 6; LPE, lime peel extract; Control(+), positive control group; Control(-), negative control group

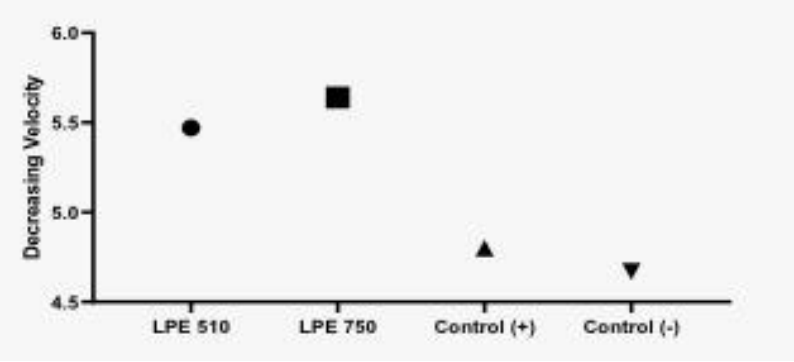

Figure 2. Velocity of decrease (%) the average of IL-6 levels on the day of observation before and after intervention; LPE, lime peel extract; Control(+), positive control group; Control(-), negative control group.

COVER LETTER

Vivien Novarina Kasim
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March 7, 2020

Dear Marco Giammanco
Editor-in-Chief
Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale

Please find our manuscript entitled "Antibacterial and anti-inflammatory effects of lime (*Citrus aurantifolia*) peel extract in mice Balb/c induced *Salmonella Typhi*", which we now would like to submit to Journal of Biological Research. This paper reports the effectiveness of lime peel extract as an antibacterial and reduces levels of interleukin 6.

We think that the content of the manuscript will appeal to the readers of Journal of Biological Research, including clinician and researchers from the field of microbiology, immunology, pharmacy and nutraceutical. Our data provide useful information about the utilization of organic waste such as lime peel which contains compounds that are very effectively used as an adjuvant therapy for handling infections. These results can be considered by doctors and researchers who work on making herbal medicines, preventing and treating infectious diseases.

We suggest the possible reviewers who might be suitable to consider our manuscript:

1. Rosa Tundis, tundis@unical.it, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Nutrition and Health Sciences, University of Calabria, I-87036 Rende (CS), Italy
2. Antoni Sureda, tosugo@hotmail.com, Nutrition and Oxidative Stress, Guillem Colom Bldg, Campus, University of Balearic Islands, E-07122 - Palma de Mallorca, Balearic Islands, Spain

We also believe that this manuscript is appropriate for publication by the Journal of Biological Research because it has a specific link to the journal's aims & scope. This manuscript has not been published and is not under consideration for publication elsewhere. By accepting this manuscript for review, we accept these terms and agree that the terms in this letter are paramount and supersede any provisions in any publication agreement for this article, already signed or to be signed at a later date, that may conflict. We have no conflicts of interest to disclose.

Thank you for your consideration,

Sincerely,
Vivien Kasim, MD

REVISION

Universitas Negeri Gorontalo UNGmail

Vivien Novarina A Kasim <viviennovarina@ung.ac.id>

JBR - Journal of Biological Research [paper #8951] - Editor Decision - Resubmit

Dr. Gian Luigi Mariottini <Gian.Luigi.Mariottini@unige.it> 17 Mei 2020 20.42
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Dear Vivien Novarina Kasim, Mochammad Hatta, Rosdiana Natzir, Veni Hadju, Yusminah Hala, Budu Budu, Gemini Alam, Suryani As'ad, Ami Febriza, Hasta Handayani Idrus,

Your paper entitled "Antibacterial and anti-inflammatory effects of lime (citrus aurantifolia) peel extract in mice balb/c induced salmonella typhi" has been examined by our external reviewers and then re-evaluated inhouse.
Peer reviewers found merit in this paper but raised major, constructive criticisms and do not consider this manuscript acceptable for publication in its current form. The reviewers have raised a number of points, listed on the web site and provided below/attached for your convenience.
The editorial conclusion is that substantial changes should be made to meet the reviewers' criticisms.

Your revised manuscript should be accompanied by a covering letter to explain, point by point, how you have modified your paper in answer to the reviewer's comments.

Important: we recommend that you consult the Authors' guidelines of this journal under Submission, as well as its current contents, to ensure that your revised manuscript is written in accordance to the journal editorial standards (in particular, title page, tables and references style).

The revised manuscript, edited in .DOC format, should be resubmitted electronically within 3 weeks from the date of the Editor Decision message.

In order to resubmit the revised manuscript, please follow this step-by-step procedure:

i) log into the journal using your username and your password;
ii) click on your role as ‘Author’;
iii) click on the correct title;
iv) click on ‘Review’ on the page displayed;
v) under the heading ‘Editor decision’ (bottom-page), upload the revised paper. Use ‘Browse’ to find the files and ‘Upload’ to upload them;
vi) once the files are uploaded, inform the Managing Editor and the Editor-in-Chief of the Journal via e-mail.
Following this procedure, you can upload one or more files (max 8 MB each file). Make sure to click ‘Upload’ for each single file you would like to upload.

Moreover, although we encourage resubmission, please be aware that this is not a statement of acceptance or a promise to accept a revised manuscript. The final decision as to this paper's acceptability for publication will exclusively depend on how our current concerns are met.

Thank you very much for sending this work to our journal: we look forward to receiving a revised manuscript.

With kind regards,

Dr. Gian Luigi Mariottini
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Reviewer C:

The findings from the review of this article, allow us to deduce that this work allows to highlight in vivo the antibacterial effect of lemon zest which shows an originality of the work. However, the part of this work corresponding to a demonstration of the anti-inflammatory effect has already been published in: International Conference on BioMedical Sciences (ICBMS19) September 27-28, 2019 Istanbul (Turkey) Conference Book ISBN 978-600- 98459-5-8: "whose article title is:" Lime Peel Extract Effects in Decreasing Levels of Interleukin 6 in Mice Infected with Salmonella Typhi "the authors are Vivien Novarina Kasim, Ami Febriza, Hasta Handayani Idrus, Mochammad Hatta, Rosdiana Natzir, Veni Hadju.
This article cannot be published as well. Authors must make corrections in accordance with the recommendations indicated in the comments linked to the text.
correction of grammatical and punctuation errors must be taken into account.
The use of bibliographic references must be verified according to the recommendations because the use of any reference does not correspond to the data indicated in the text.
The lack of precision in the methodology must also be reviewed.

Recommendation: Major revisions

Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale

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I. Title

Antibacterial and anti-inflammatory effects of lime (*citrus aurantifolia*) peel extract in mice balb/c induced *salmonella typhi*

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Commented [A2]: Department

IV. Acknowledgments:

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 financially supported by the Ministry of Research and Technology, Indonesia or BUDI-DN LDPD.

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VI. Key Words :

Lime peel extract, *Citrus aurantifolia*, Interleukin 6, antibacterial, anti-inflammatory

I. Contributions: The authors contributed equally

II. Conflict of interest: The authors declare no potential conflict of interest

III. Funding: Ministry of Research and Technology, Indonesia or BUDI-DN LDPD.

Antibacterial and anti-inflammatory effects of lime (*citrus aurantifolia*) peel extract in mice balb/c induced *salmonella typhi*

Abstract:

Lime (*Citrus aurantifolia*) is a traditional plant that is widely used as antibacterial. This study to prove the effect of lime peel extract (LPE) on the growth of bacterial colonization of *S. Typhi* medited by activity of IL-6. True experimental pre-post test design, mice were divided into; LPE 510 mg/kgbw, LPE 750 mg/kgbw, positive and negative control. The examination was carried out 3 times, the 5th day before the intervention, the 10th day after the intervention and the 30th day after maintenance. Intervention LPE for 5 days can decreased the number of *S. Typhi* colonies, even maintenance for 20 days after the intervention showed no bacterial growth . IL-6 pro-inflammatory cytokine activity increased on examination day 5 after *S.Typhi* injection and decreased after intervention on day 10, significantly different between pre and post at all groups except negative controls (p=0.15). The speed of decrease in IL-6 levels was greatest at the LPE 750 mg/kgbw (velocity=-5.64%). LPE decreased serum levels of IL-6 and inhibit the growth of *S. Typhi* colony in mice Balb/c. LPE have potential as antibacterial and anti-inflammatory.

Introduction:

Some antibiotics have been resistant to *Salmonella Typhi* and *Salmonella Paratyphi*, such as ampicillin, chloramphenicol, tetracycline and co-trimoxsazole.[1] Multi

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Drug Resistance (MDR) leads to new treatments in the form of adjuvant therapy, traditional plants that can be used as antimicrobials. One of them is the lime plant (*Citrus aurantifolia*). From the many previous studies even to date, all parts of the *C. aurantifolia* have various efficacy.[2] Specifically in this study the peel of lime was investigated as having antibacterial properties containing active ingredients such as polyphenols, flavonoids, tannins, saponins, alkaloids and triterpenoids having the effect of reducing the amount of colonization of the *Salmonella Typhi* experiment in Balb/c mice induced by *S. Typhi*.[3] *Salmonella Typhi*, is a gram-negative bacterial whose transmission almost always occurs through contaminated food and drinks. From several studies, resistance to *Salmonella Typhi* has begun to be high. The development of antimicrobial resistance is in line with the increasing use of antimicrobial drugs and in line with the discovery of new drugs.[4]

Bacterial resistance, especially *S. typhi*, brings us to a new treatment in the form of adjuvant therapy, which is a traditional plant that can be used as an antimicrobial, one of which is *Citrus aurantifolia*. Based on previous research, the lime peel has a higher concentration of flavonoids compared to other parts such as seeds, fruit, juice.[5,6] The existence of the content of flavonoids makes the lime peel have antibacterial and antioxidant power. Previous studies tested the bacteriostatic or bactericidal activity of flavonoids by conducting a time-kill study that found that flavonoids do not kill bacterial cells but only induce the formation of bacterial aggregates thereby reducing the amount of colony forming units (CFU) in a decent amount.[7]

When there is an *S. Typhi* bacterial infection, there is a bond between the lipopolysaccharide (LPS) of the bacterial wall and the Toll-Like Receptor 4 (TLR 4) of

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the host which stimulates innate immune activity. The activity releases a number of cytokines such as TNFα, IL-1, and IL-6. Interleukin 6 (IL-6) mediates the protective systemic effects of inflammation, including the effects of fever, acute-phase protein synthesis by the liver, and increased production of leukocytes by the bone marrow.[8] This study discusses the effect of lime peel extract on the amount of bacterial colonization and its effect on the activity of IL-6 pro-inflammatory cytokines in Balb/c mice injected with *Salmonella Typhi*.

Material and Methods

An Experimental design was used to studied the effectiveness of lime peel extract in decreasing pro-inflammatory IL-6 levels and bacterial colonization in mice Balb/c strains induced by *Salmonella Typhi*.

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Lime peel extract

Making extracts was carried out at the Hasanuddin University Faculty of Pharmacy Phytochemical Laboratory. Lime peel is cut into small pieces and then dried at 50ºC to minimize water content. The dried sample is then put into a glass container or jar to be macerated by adding Ethanol 96% and then let it sit for 3 x 24 hours. Samples that were macerated for 3 x 24 hours were then filtered by the Vacuum Filtration method using a Buchner Funnel. The extracts, obtained after the screening process, were then evaporated using a Rotary-evaporator until the results were thick extracts.

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Experimental Animals

Mice Balb/c (age 8-12 weeks, weigh 30-40 grams; n=20) were maintained in the Laboratory of Molecular Biology and Immunology, Department of Microbiology, Faculty

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of Medicine, Hasanuddin University (Makassar, Indonesia). The rats were acclimatized for 8 days, then divided into four groups (n=5). All groups were induced intraperitoneally with *S. Typhi* strain Thy1 (3 x 10^3 CFU/ml). After 3 days of induction, each animal tried to be intervened; LPE 510 (mice group intervened with LPE dose 510 mg/kgbw), LPE 750 (mice group intervened with LPE dose 750 mg/kgbw), postive control group (groups of mice given Levofloxacin dose 98 mg/kgbw) and negative control group (group of placebo mice).

Bacterial colonization

Bacterial colonies are counted from samples taken from peritoneal fluid. Samples were taken three times, on the 5th day after mice were induced by *Salmonella Typhi* (pre-intervention), the 10th post-intervention and 30th day after maintenance without treatment. Sampels has been taken as much as 0.5 ml, put in 4.5 ml of physiological saline (0.9% NaCl). Dilution is carried out three times so that the culture obtained is not too dense or fills the cup (the culture is too dense will interfere with observation). 1 ml of suspension was poured into a sterile petri dish, then poured warm sterile (nutrient agar) media (45^{0}C) then tightly closed and incubated for 1-2 days at 37^{0}C. The method used is the Plate Count Agar (PCA) is a technique for growing microorganisms in agar media by mixing the liquid agar media with bacterial culture stock (agar) so that the cells are evenly distributed and still on the surface of agar or in in agar.[9]

Interleukin 6 (IL-6) Examination

Serum samples were taken 4 times, baseline (day 0), after induction of *S. Typhi* before intervention (day 5), after intervention (10th day) and after 30 days. IL-6 antibody concentrations are determined by the IL6 ELISA Mouse Sandwich method (User

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Manual Catalog No. LS-F24855) LSBio, LifeSpan BioSciences, Inc. reader 270, Instrument serial number: 1211006860, measurement mode: Absorbance, measurement wavelength: 450 nm, read mode: normal, unit: OD.

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Ethics statement

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

Results

Bacterial Colonization

The number of bacterial colonies after being given an intervention decreased in all groups, but on the negative control group on the 10th day the number of bacterial colonies was seen more than other groups, and the 30th day negative control group there were still bacterial colonies (Fig-1). On the 10th day, the number of bacterial colonies decreased after the intervention of lime peel extract was given for 5 days of intervention. The number of bacterial colonies continued to decrease until the 30th day post-intervention. Researchers assume that extracts of lime peel can as a bactericidal over be bacteriostatic because the number of bacterial colonies on the 10th and 30th days reaches zero or there is no bacterial growth.

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In the LPE group at a dose of 750 mg/kgbw, the number of bacterial colonies on day 10 had no growth, better than positive control group. The average decrease in the number of bacterial colonies in the LPE group 750 mg/kgbw from 17.2 x 10^3 CFU/ml to 0 or no bacterial growth (p=0.00) whereas in the positive control group decreased from

30.4 x 10^3 CFU/ml to 1.6 x 10^3 CFU/ml (p=0.001). In the negative control group, the number of bacterial colonies decreased from 22.6 x 10^3 CFU/ml to 6 x 10^3 CFU/ml, meaning that the number of bacterial colonies in the negative control group even though it decreased but the number of bacterial colonies was still bigger than the three groups.

Interleukin 6 (IL-6)

The level of serum IL-6 at 4 times the data collection, analyzed using paired T-tests to assess the dynamics of changes in levels of serum with respect to changes in observation time for each group, can be seen in Table 1. The mean of IL-6 level on the 5th day increased in all groups compared at the baseline day, because it is the 4th-day post-injection *Salmonella Typhi*. This indicates that after the injection of *Salmonella Typhi*, all mice in all groups experienced an infection process.

The difference of average of IL-6 levels between groups at the time of observation before the 5th day intervention and after the 10th day intervention, there were significant differences in groups each, LPE 510 (p=0.003), LPE 750 (p=0.002), positive control (p=0.006). Figure 2 shows the velocity of the decrease of IL-6 averages between the time of observation after the intervention (10th day) and before the intervention (5th day). At the time of observation of the 10th day to the 5th day, it was seen that the velocity of decreasing IL-6 levels was greatest in the LPE 750 is 5.64% and the smallest velocity of decline was in the negative control group by 4.67%. The velocity of decrease in IL-6 levels in the LPE 750 was found more than the positive control group given Levofloxacin. It is assumed that the extract of lime peel in this study is effective as an anti-inflammatory.

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DISCUSSION

Bacterial Colonization

LPE has an antibacterial effect as previous studies explained that *Citrus aurantifolia* has a high inhibitory zone in gram-positive and gram-negative bacteria.[10] *S. Typhi* injection given intraperitoneal stimulates macrophages to activate and move to the source of infection.[11] The LPS in the *S. Typhi* cell wall as a signal for macrophages to carry out activation. In previous preliminary studies, it was found that the metabolic compounds contained in the extract of lime peel were polyphenols, flavonoids, alkaloids, tannins, saponins, and triterpenoids each having the property to kill bacteria . These compounds attack bacteria directly, causing bacterial cell death. This is indicated in the calculation of the number of bacterial colonies that are reduced even no growth after 5 days given the intervention of LPE.

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Interleukin 6 (IL-6)

One of the earliest responses of the innate immune system to infection and tissue damage is the secretion of cytokines by tissue cells, which are very important for acute inflammation. When *S. Typhi* first enters the body, the bacteria will be destroyed by macrophages. Bacteria will be known by various receptors located on the surface of phagocytes.[8] Specific marker molecules for Gram-negative bacteria such as *S. Typhi* are LPS, LPS will use TLR-4, which is a receptor that plays a role in observing and destroying *Salmonella Typhi.* Activated TLR-4 will recruit the MyD88 adapter protein. Then MyD88 recruited IRAK4, IRAK1 and IRAK2. IRAQ kinase then phosphorylates and activates the TRAF6 protein, allowing NF-κB to dwell in the cell nucleus and activating transcription and causing induced inflammatory cytokines. Proinflammatory

cytokines such as IL-1β and IL-6, IFN-γ and TNF-α are synthesized and systemic inflammation occurs.[8,12] It can be said that the inflammatory process by IL-6 cytokines is already running.

As for the negative control group, there were no significant differences before the intervention (5th day) and after the intervention (10th day), which is p-value 0.15. These results indicate that the extract of lime peel has the same effect as Levofloxacin, which inhibits the activity of pro-inflammatory cytokines IL-6 after administration for 5 days. Researchers assume that the inhibitory effect of IL-6 is due to the activity of the polyphenol compounds contained in extracts of lime peel. In line with this, Haseeb A. (2017) found that the potential inhibitory effect of polyphenols on IL-6 expression and oxidative stress through molecular mechanisms of activation of NF-κB.[13]

Looking from the content of polyphenols and flavonoids contained in LPE of this study, polyphenols are bioactive substances that have the potential to have a potential effect on the inflammatory response. It is known that flavonoids are one of the most extensive groups of secondary metabolite plants, flavonoids are found in many edible fruits and vegetables. The most source of polyphenols is represented by citrus fruits.[14] The polyphenol content in lime peel extract in this study was 2.29% and flavonoids 0.26%, it has been reported that the content of phenols and flavonoids using methanol solvent contained more in the parts of lime peel compared to the leaves, phenol 95.6 mg/g, and flavonoids 23.5 mg/g, are contained in the Rossano *Citrus aurantiolia* bark extract in Calabria, Italy. In that study, it was found that there was a significant relationship between phenol and flavonoid with antioxidant activity.[4]

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Tejada S. (2017), proves that hesperidin (a type of flavonoid) extracted from the citrus genus, has potential as an anti-inflammatory. Hesperidin treatment decreases inflammatory mediators and provides a significant antioxidant effect. Molecular bases for anti-inflammatory effects appear to be mediated by signaling pathways especially the nucleus κβ factor pathway.[15] As for other opinions, research conducted by Jorge L.A, found that from 3 types of citrus namely *C.limon, C.latifolia, C. aurantifolia or C.limonia*, essential oils (EO) contained in the 3 types of citrus have anti-inflammatory effects, especially the limonene content of essential oils from the citrus fruit skin.[16] During the inflammatory event, there is an increase in cytokine production.

CONCLUSION

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

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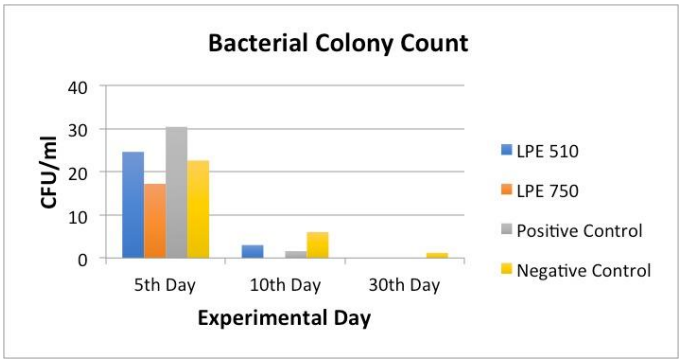

Figure 1. Dynamics of changes in the number of bacterial colonization of *S. Typhi* by the groups during the observation period; LPE, lime peel extract; CFU, colony forming units.

Table 1. The differences in the levels of IL-6 serum dynamics between groups at the baseline, 5th day, 10th day and 30th day

Groups	Levels of IL-6 (pg/ml)								
	Baseline	5th	*p value*	5th	10th	*p value*	10th	30th	*p value*
LPE 510	157.3±59.7	358.5±59.5	0.007	358.5±59.5	338.7±64.9	**0.003**	338.7±64.9	284.7±60.6	0.05
LPE 750	171.8±58.1	434.1±51.3	0.004	434.1±51.3	409.6±53.6	**0.002**	409.6±53.6	293.9±34.3	0.01
Control(+)	121.1±35.9	502.0±35.4	0.000	502.0±35.4	477.9±41.5	**0.006**	477.9±41.5	204.5±40.7	0.001
Control(-)	130.3±47.7	248.5±27.2	0.003	248.5±27.2	236.9±31.8	0.15	236.9±31.8	194.9±38.5	0.08

IL-6, interleukin 6; LPE, lime peel extract; Control(+), positive control group; Control(-), negative control group

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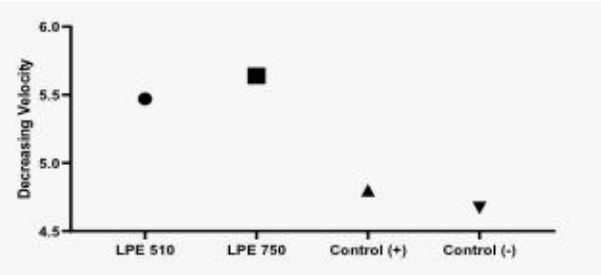

Figure 2. Velocity of decrease (%) the average of IL-6 levels on the day of observation before and after intervention; LPE, lime peel extract; Control(+), positive control group; Control(-), negative control group.

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REBUTTAL LETTER

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May 26, 2020

Dear Gian Luigi Mariottini
Editor-in-Chief
Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale

Thank you for more revision in our manuscript. Here I send our rebuttal letter and revised manuscript. This final revision has already been approved by all authors. I have edited the manuscript to address their concerns, and I will present it point by point as follow below:

Reviewer C :
Recommendation : Major revisions

Comments	Revised in manuscript
Article text	
[1] Respect the writing of the nomenclature of species: the genus begins with a capital letter : *Citrus*	Revised in line 1
[2] *Salmonella*	Revised in line 2
[3] Correct grammatical mistakes in this paragraph	Revised in line 5-7
[4] Correct the meaning of this sentence: It is bacteria that are resistant to antibiotics. Antibiotics become ineffective on resistant bacteria.	Revised in line 21-22
[5] check the punctuation of this sentence	Revised in line 22
[6] Reference 3 (Pathan et al 2012) indicates that the tests were carried out in vitro on Gram-positive and Gram-negative bacteria and not in vivo on mice Balb/c.	Revised in line 29, reference 4
[7] Gram-negative	Revised in line 29
[8] *Salmonella Typhi* resistance or the resistance of *Salmonella Typhi*	Revised in line 31
[9] This reference does not correspond to the idea expressed in this sentence	Revised in line 33, no reference
[10] bacterial resistance and especially that of Salmonella,	Revised in line 34

[11] Reference **6** is not adequate for these data cited in this sentence.	Revised in line 36-39, reference 5,6
[12] to study	Revised in line 56
[13] Add punctuation in this sentence.	Revised in line 65
[14] Correct this sentence: Mices Balb/c, (witch age ranges from 8 **to**12 weeks, and weigh 30 to 40 grams; (n=20),	Revised in line 68-69
[15] Give more details on the type of administration and the substance tested in animals? is it peritoneally? orally or other ??? The substance tested is in the form of an extract ?? or powder ?? Or other??	Revised in line 73-74
[16] 1-Correct: groups: it's group and close the parenthesis. 2-Indicate why the choice of levofloxacin and not of another Drug. (It's antibiotic classed with the quinolone)	1-Revised in line 76 2-Because quinolone antibiotics are currently the best in typhoid fever therapy (drug of choice) and more effective in the case of MDR, especially in levofloxacin which is the S isomer of ofloxacin, ofloxacin which can be used for short-term therapy 5-7 days, and for its preparation levofloxacin has a single dose of 750 mg/day (human therapy), 98 mg/kg body weight is a conversion dose from humans to mice. Revised in line 77, reference 9
[17] mices	Revised in line 81
[18] Samples	Revised in line 83
[19] Physiological saline solution	Revised in line 83
[20] Petri	Revised in line 86
[21] Completely reconstruct the sentence with the verb and the subject instead of putting (:) repeatedly, and checking the punctuation	Revised in line 97-98
[22] These are the results you found in this study, you can compare with other bibliographic data whose references you must cite in discussion.	Revised in line 112-117, reference 11
[23] Review the reconstruction of this sentence	Revised in line 133-135
[24] Review it's : the 5th day to the 10th day	Revised in line 138
[25] Gram-positive and Gram-negative	Revised in line 149
[26] Reference 10 does not describe the activity of *Citrus aurantifolia* on Gram negative and positive bacteria but on alcohol-resistant bacteria *Mycobactrium tuberculosis.*	Revised in line 146-149, references 5,12

[27] Reference 11 does not describe this data	Revised in line 149-152, reference 13
[28] Give adequate references for the data of these studies that you cited in this paragraph.	Revised in line 152-153, reference 4
[29] Compare your results found with those in the literature concerning the antibacterial and anti-inflammatory effect of Levofloxacin.	Revised in line 173-176, reference 15
[30] *aurantifolia*	Revised in line 189
Figures article	
[1] Separate the levels of IL-6 results for each time interval to make your Table more Readable	Revised in figure file (Table 1)
[2] Indicate the time interval	Figure 2 shows the percentage of velocity decrease in IL-6 levels between the time of observation of pre-intervention (day 5) and post-intervention (day 10), Revised in the description of figure 2
Title and authors article	
[1] Completes the name of this author: First name and more name	Revised in title and authors article file
[2] Department	Revised in title and authors article file
The findings from the review of this article, allow us to deduce that this work allows to highlight in vivo the antibacterial effect of lemon zest which shows an originality of the work. However, the part of this work corresponding to a demonstration of the anti-inflammatory effect has already been published in: International Conference on BioMedical Sciences (ICBMS19) September 27-28, 2019 Istanbul (Turkey) Conference Book ISBN 978-600- 98459-5-8: "whose article title is:" Lime Peel Extract Effects in Decreasing Levels of Interleukin 6 in Mice Infected with Salmonella Typhi "the authors are Vivien Novarina Kasim, Ami Febriza, Hasta Handayani Idrus, Mochammad Hatta, Rosdiana Natzir, Veni Hadju.	This paper is our preliminary study, in that paper we found and presented the phytochemical screening of extracts of lime peel (according to the citation in this paper). As for the Interleukin 6 data, in the current paper we have analyzed and will be present the effectiveness of lime peel extract to the velocity of decreasing of interleukin 6 levels. The novelty value is that the rate of decrease in interleukin 6 levels after being given LPE is greater than that of levofloxacin, so we assume that LPE can be as an antibacterial and anti-inflammatory.

I am pleased to submit our revised review article for consideration for publication in Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale.

Thank you for your kind cooperation,

Best Regards,
On behalf of all authors

Vivien Kasim, MD

COVER LETTER

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July 06, 2020

Dear Gian Luigi Mariottini
Editor-in-Chief
Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale

Thank you for re-evaluated our manuscript. I have edited the manuscript according to the evaluated and the recommendation, and I will present it point by point as follow below:

Comments	**Revised in manuscript**
[1] please check the utilization of the name Salmonella tiphy. Indeed, if you refer to the taxonomical species the species name must be written lowercase; otherwise, if you refer to the serovar, the name can be written with capital initial. On the other hand, in your previous paper presented to the Istanbul conference, you used normally "Salmonella typhi".	I have replaced all the words "Typhi" to typhi
You'll find some parts highlighted in yellow; these parts are identical to your conference paper, so to modify these parts is essential. It would be better that you simply refer what was made as, for example, "Lime peel extracts (LPE) were obtained as reported in Kasim et al4". "Animals were trated as reported in Kasim el al4". Similarly for "Interleukin 6 examination". Other small parts highlighted in yellow are to be checked.	
[2] highlighted in yellow : True experimental pre-post test design,	Deleted this sentence
[3] highlighted in yellow : The examination was carried out 3 times: on the 5th day before the intervention, on the 10th day after the intervention and on the 30th day after maintenance.	In abstract of proceeding at Turki, the sentence is: The intervention was Carried out for 5 days. After the fifth day, mice were then maintained for 3 weeks to Determine examination of the level of IL-6 in serum. In abstract of manuscript: The examination was carried out 3 times, on the 5th day before the intervention, on the 10th day after the intervention and on the 30th day after maintenance (line 9-11)
[4] highlighted in yellow : a traditional plant	Deleted this sentence
[5] highlighted in yellow :	Deleted this word

both	
[6] highlighted in yellow : Making extracts was carried out at the Hasanuddin University Faculty of Pharmacy Phytochemical Laboratory. Lime peel is cut into small pieces and then dried at 50ºC to minimize water content. The dried sample is then put into a glass container or jar to be macerated by adding Ethanol 96% and then let it sit for 3 x 24 hours. Samples that were macerated for 3 x 24 hours were then filtered by the Vacuum Filtration method using a Buchner Funnel. The extracts obtained after the screening process were then evaporated using a Rotary-evaporator until the results were thick extracts.	Revised in line 58-62: Lime peel extraction was carried out at the Phytochemical Laboratory, Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia. Lime peel thick extracts macerated with 96% Ethanol for 72 hours. The LPE used in this study was consisted of two doses: 510 mg/kg body weight (bw) and 750 mg/kgbw, both dissolved with Aquades. LPE was obtained as reported in Kasim et.al.[4]
[7] highlighted in yellow : Mice Balb/c, age ranging from 8 to 12 weeks, and weight 30 to 40 grams (n=20), were maintained in the Laboratory of Molecular Biology and Immunology, Department of Microbiology, Faculty of Medicine, Hasanuddin University (Makassar, Indonesia). The rats were acclimatized for 8 days, then divided into four groups (n=5). All groups were injected intraperitoneally with *S. Typhi* strain Thy1 (3 x 10^3 CFU/ml). After 3 days from injection with *Salmonella Typhi*, LPE in form of soluble extract dissolved with Aquadest was given orally once a day for 5 days; group 1 LPE510 (mice group intervened with LPE dose 510 mg/kg body weight (bw), group 2 LPE750 (mice group intervened with LPE dose 750 mg/kgbw), group 3 positive control group (group of mice given Levofloxacin dose 98mg/kgbw,[9] and group 4 negative control group (group of placebo mice).	Revised in line 63-72: Balb/c mice age ranging from 8 to 12 weeks, and weight from 30 to 40 grams (n=20), were obtained from Laboratory of Molecular Biology and Immunology, Department of Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. The mice were divided into four groups (n=five/group) based on intervention; LPE dose of 510 mg/kgbw, LPE 750 mg/kgbw, positive control (Levofloxacin dose 98mg/kgbw,[9]), negative control (Aquades). All groups were injected intraperitoneally with *S. typhi* strain Thy1 (3 x 10^3 CFU/ml). 3 days after the injection of *Salmonella typhi*, each animal was started to be intervened for 5 days. Animals were treated as reported in Kasim et al.[4]
[8] highlighted in yellow : Serum samples were taken 4 times, baseline (day 0), after induction of *S. Typhi* before intervention (day 5), after intervention (10th day) and after 30 days. IL-6 antibody concentrations are determined by the IL6 ELISA Mouse Sandwich method (User Manual Catalog No. LS-F24855) LSBio, LifeSpanBioSciences, Inc. reader 270, Instrument serial number: 1211006860, measurement mode with Absorbance used measurement wavelength of 450 nm, read mode status normal.	Revised in line 87-90: Serum samples were taken 4 times, baseline (day 0), after induction of *S. typhi* before intervention (day 5), after intervention (10th day) and after 30 days. IL-6 antibody concentrations were determined by the IL6 ELISA Mouse Sandwich method. IL-6 examination was reported in Kasim et.al.[4]
[9] highlighted in yellow : Table 1.	Revised in figures article file: Table 1.
[10] highlighted in yellow : *or C. limon*	Deleted this words
[11] Table 1 is already published in your previous paper, so that it is not necessary here and is not possible to re-publish. You can refer to your previous paper for these results, or you can re-evaluate the data	Explanation of a new Table 1 (revised in line 121-125): The mean of IL-6 levels (delta) showed that the greatest decrease was found in the LPE 750 intervention group, this suggests that the LPE 750 intervention had a greater impact on decreased IL-6, compared to antibiotics (levofloxacin) intervention. The decrease of IL-6 levels in

in a different way or re-elaborate them (not reporting the same numbers).	the LPE 510 intervention group was greater than the placebo group (negative control).
[12] Title	Revised in line 1-2 **Antibacterial and anti-inflammatory effects of lime (*Citrus aurantifolia*) peel extract in Balb/c mice infected by *Salmonella typhi***
[13] the English must be extensively revised; the remakrs reported in the file of suggestions are not exhaustive, so that I suggest you ask for help from an English speaking colleague.	all of the remarks are accepted insertion; Revised in line 6-8 Revised in line 21-22, the reference number, subscript Revised in line 25-28, the reference number, subscript Revised in line 36, the reference number, subscript Revised in line 69, the reference number, subscript Revised in line 75 Revised in line 77 Revised in line 140, the reference number, subscript The manuscript has been proofreading from an English speaking colleague.
[14] please, check carefully the references.	All of the remarks in references are accepted insertion; Revised in line 200 Revised in line 206 Revised in line 209-212 Revised in line 217 Revised in line 230 Revised in line 244 Revised in line 247

This final revision has already been approved by all authors. Thank you very much that the referees have been agreeing this manuscript is interesting and potentially acceptable for publication in your Journal, we really appreciate it. We hope this manuscript will be accepted and published in the nearest issue.

Thank you very much for your kindness.

Best Regards,
On behalf of all authors

Vivien Kasim, MD

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

Abstract:

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

Introduction:

Some antibiotics such as ampicillin, chloramphenicol, tetracycline and co-trimoxazole become ineffective on *Salmonella typhi* and *Salmonella paratyphi*,[1] Multi-

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

Bacterial resistance and especially that of *Salmonella typhi* brings us to a new treatment in the form of adjuvant therapy, one of which is *Citrus aurantifolia* that can be used as an antimicrobial. Based on the previous research, all parts of *Citrus aurantifolia*; leaves, root stem, bark, and peel contain of useful metabolic compounds,[5] the lime peel has a higher concentration of flavonoids compared to other parts such as seed, fruit and juice.[6] The existence of the content of flavonoids makes the lime peel has antibacterial and antioxidant power. Previous studies tested the bacteriostatic or bactericidal activity of flavonoids by conducting a time-kill study found that flavonoids do not kill bacterial cells but only induce the formation of bacterial aggregates thereby reducing the amount of colony forming units (CFU) in a decent amount.[7]

When there is an *S. typhi* bacterial infection, there is a bond between the lipopolysaccharide (LPS) of the bacterial wall and the Toll-Like Receptor 4 (TLR 4) of the host which stimulates innate immune activity. The activity releases a number of

cytokines such as TNFα, IL-1, and IL-6. Interleukin 6 (IL-6) mediates the protective systemic effects of inflammation, including the effects of fever, acute-phase protein synthesis by the liver, and increased production of leukocytes by the bone marrow.[8] This study discusses the effect of lime peel extract on the amount of bacterial colonization and its effect on the activity of IL-6 pro-inflammatory cytokines in Balb/c mice injected with *Salmonella typhi*.

Material and Methods

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

Lime peel extract (LPE)

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

Balb/c mice

Balb/c mice age ranging from 8 to 12 weeks, and weight from 30 to 40 grams (n=20), were obtained from Laboratory of Molecular Biology and Immunology, Department of Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. The mice were divided into four groups (n=five/group) based on intervention; LPE dose of 510 mg/kgbw, LPE 750 mg/kgbw, positive control (Levofloxacin dose

98mg/kgbw,[9]), negative control (Aquades). All groups were injected intraperitoneally with *S. typhi* strain Thy1 (3 x 10^3 CFU/ml). 3 days after the injection of *Salmonella typhi*, each animal was started to be intervened for 5 days. Animals were treated as reported in Kasim et al.[4]

Bacterial colonization

Bacterial colonies were counted from samples taken from peritoneal fluid. Samples were taken three times, on the 5th day after mice were induced by *Salmonella typhi* (pre-intervention), the 10th post-intervention and 30th day after maintenance without treatment. Samples were taken as much as 0.5 ml and placed in 4.5 ml of physiological saline solution (0.9% NaCl). Dilution was carried out three times so that the culture obtained was not too dense or filled up the cup (the culture is too dense will interfere with observation). 1 ml of suspension was poured into a sterile Petri dish, then poured warm sterile (nutrient agar) media (45^0C) then tightly closed and incubated for 1-2 days at 37^0C. The method used was the Plate Count Agar (PCA), a technique to grow microorganisms in agar media by mixing the liquid agar media with bacterial culture stock (agar) so that the cells are evenly distributed and still on the surface of agar or inside of agar.[10]

Interleukin 6 (IL-6) Examination

Serum samples were taken 4 times, baseline (day 0), after induction of *S. typhi* before intervention (day 5), after intervention (10th day) and after 30 days. IL-6 antibody concentrations were determined by the IL6 ELISA Mouse Sandwich method. IL-6 examination was reported in Kasim et.al.[4]

Ethics statement

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

Results

Bacterial Colonization

The number of bacterial colonies after being given an intervention decreased in all groups, however on the 10th day ofthe negative control group, the number of bacterial colonies was seen more than other groups, and there were still bacterial colonies (Fig-1) on the 30th day of negative control group. On the 10th day, the number of bacterial colonies decreased after the intervention of lime peel extract which was given for 5 days of intervention. The number of bacterial colonies continued to decrease until the 30th day post-intervention. The decrease in the number of bacterial colonies after LPE administration had almost the same effect as the decrease in the number of colonies after levofloxacin administration (positive control). The effect of quinolone as a bactericidal,[11] in this study showed that the number of bacterial colonies after 30 days continued to decrease even did not grow on the group given LPE 510, LPE 750, and positive control.

In the LPE group at a dose of 750 mg/kgbw, the number of bacterial colonies on day 10 had no growth, better than positive control group. The average decreased in the number of bacterial colonies in the LPE group 750 mg/kgbw from 17.2 x 10^3 CFU/ml to 0 or no bacterial growth (p=0.00) whereas in the positive control group decreased from 30.4 x 10^3 CFU/ml to 1.6 x 10^3 CFU/ml (p=0.001). In the negative control group, the

number of bacterial colonies decreased from 22.6 x 10^3 CFU/ml to 6 x 10^3 CFU/ml, meaning that the number of bacterial colonies in the negative control group was still bigger than the other three groups, even though there was a decrease.

Interleukin 6 (IL-6)

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

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

Discussion

Bacterial Colonization

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

Interleukin 6 (IL-6)

One of the earliest responses of the innate immune system to infection and tissue damage is the secretion of cytokines by tissue cells, which is very important for acute inflammation. When *S. typhi* first enters the body, the bacteria will be destroyed by macrophages. Bacteria will be recognized by various receptors located on the surface of phagocytes.[8] Specific marker molecules for Gram-negative bacteria such as *S. typhi* are LPS, LPS will use TLR-4, a receptor that plays a role in observing and destroying *Salmonella typhi*. Activated TLR-4 will recruit the MyD88 adapter protein. Then MyD88 recruits IRAK4, IRAK1 and IRAK2. IRAQ kinase then phosphorylates and activates the TRAF6 protein, allowing NF-κB to dwell in the cell nucleus and activating

transcription and causing induced inflammatory cytokines. Proinflammatory cytokines such as IL-1β and IL-6, IFN-γ and TNF-α are synthesized and systemic inflammation occurs.[8,14] It can be said that the inflammatory process by IL-6 cytokines is already running.

The results of this study (figure 2) showed that LPE therapy gave a greater decrease in IL-6 levels compared to levofloxacin. It is indicated that LPE has an anti-inflammatory effect on IL-6.[15] LPE, in addition to having an antibacterial effect, also has an anti-inflammatory effect, LPE has the potential for a more effective therapy. Researchers assume that the inhibitory effect of IL-6 is due to the activity of the polyphenol compounds contained in the extracts of lime peel. In line with this, Haseeb A. (2017) found that the potential inhibitory effect of polyphenols on IL-6 expression and oxidative stress through molecular mechanisms of activation of NF-κB.[16]

The content of polyphenols and flavonoids contained in LPE of this study reveal that polyphenols are bioactive substances which are likely to have a potential effect on the inflammatory response. It is known that flavonoids are one of the most extensive groups of secondary metabolite plants, flavonoids are found in many edible fruits and vegetables. The source of polyphenols is mostly contained in citrus fruits.[17] The polyphenol content in lime peel extract in this study is 2.29% and flavonoids are 0.26%. The research by Rossano on *Citrus aurantifolia* in Calabria, Italy revealed that lime peels which were extracted by methanol solvent contained of more phenols (95.6 mg/g) and flavonoids (23.5 mg/g) compared to the leaves. In that study, it was found that there was a significant relationship between phenol and flavonoid with antioxidant activity.[3]

Tejada S. (2017), proved that hesperidin (a type of flavonoid) extracted from the citrus genus, has potential as an anti-inflammatory. Hesperidin treatment decreases inflammatory mediators and provides a significant antioxidant effect. Molecular bases for anti-inflammatory effects appear to be mediated by signaling pathways especially the nucleus κβ factor pathway.[18] As for other opinions, a research conducted by Jorge L.A, found that from 3 types of citrus namely *C. limon, C. aurantifolia C. limonia*, essential oils (EO) contained in the 3 types of citrus have anti-inflammatory effects, especially the limonia content of essential oils from the citrus fruit skin.[15] During the inflammatory event, there is an increase in cytokine production.

Conclusion

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

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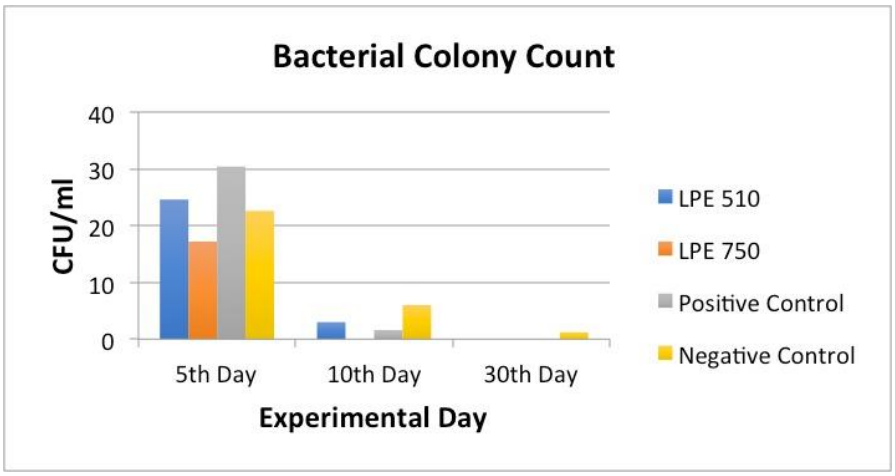

Figure 1. Dynamics of changes in the number of bacterial colonization of *S. Typhi* by the groups during the observation period; LPE, lime peel extract; CFU, colony forming units.

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

Groups	Level of IL-6 (ρg/ml)	
	5^{th} – 10^{th} day **Delta (mean ± SD)**	**10^{th} – 30^{th} day** **Delta (mean ± SD)**
LPE 510	19,63 ± 7,06	53,98 ± 43,38
LPE 750	**24,54** ± 7,71	115,61 ± 56,92
Control (+)	24,07 ± 7,06	273,41 ± 68,26
Control (-)	11,62 ± 6,33	41,91 ± 18,87
P value	0,074	0,000

IL-6, interleukin 6; LPE, lime peel extract; Control(+), positive control group; Control(-), negative control group; SD, standar deviasi

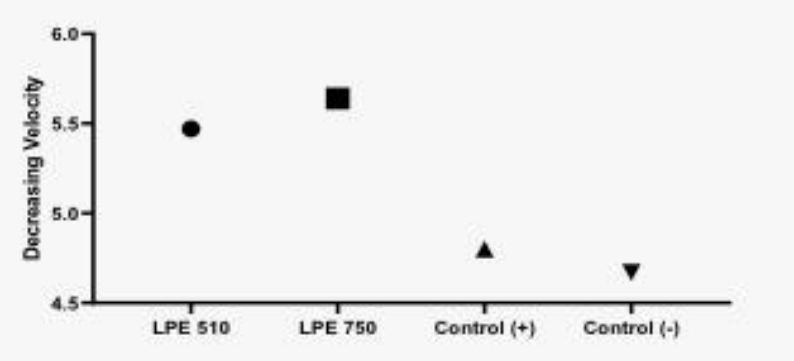

Figure 2. The velocity of decrease (%) of IL-6 levels in each group after the intervention; LPE, lime peel extract; Control (+), positive control group; Control (-), negative control group.

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JBR - Journal of Biological Research [paper #8951] - Editor Decision - Revisions Required

Vivien Novarina A Kasim <viviennovarina@ung.ac.id> 6 Juli 2020 21.29
Kepada: "Dr. Gian Luigi Mariottini" <Gian.Luigi.Mariottini@unige.it>

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Thank you for re-evaluated our manuscript. I have edited the manuscript according to the evaluation and the recommendation. We send 3 files; cover letters, articles, and figures articles.

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Cc: francesca.savio@pagepress.org

Dear Dr. Vivien Kasim, MD,
thank you for the revised version of your manuscript.
A decision will follow soon.
Best regards.
Gian Luigi Mariottini

--
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Dr. Gian Luigi Mariottini <Gian.Luigi.Mariottini@unige.it> 19 Juli 2020 09.20
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There is new activity in the discussion titled "Round 2 of review" regarding the submission "Antibacterial and anti-inflammatory effects of lime (citrus aurantifolia) peel extract in mice balb/c induced salmonella typhi".

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

Abstract:

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

Introduction:

Some antibiotics such as ampicillin, chloramphenicol, tetracycline and co-trimoxazole become ineffective on *Salmonella typhi* and *Salmonella paratyphi*,[1] Multi-

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

Bacterial resistance and especially that of *Salmonella typhi* brings us to a new treatment in the form of adjuvant therapy, one of which is *Citrus aurantifolia* that can be used as an antimicrobial. Based on the previous research, all parts of *Citrus aurantifolia*; leaves, root stem, bark, and peel contain useful metabolic compounds[5] the lime peel has a higher concentration of flavonoids compared to other parts such as seed, fruit and juice.[6] The existence of the content of flavonoids makes the lime peel has antibacterial and antioxidant power. Previous studies tested the bacteriostatic or bactericidal activity of flavonoids by conducting a time-kill study found that flavonoids do not kill bacterial cells but only induce the formation of bacterial aggregates thereby reducing the amount of colony forming units (CFU) in a decent amount.[7]

When *S. typhi* bacterial infection occurs, there is a bond between the lipopolysaccharide (LPS) of the bacterial wall and the Toll-Like Receptor 4 (TLR 4) of the host which stimulates innate immune activity. The activity releases a number of

cytokines such as TNFα, IL-1, and IL-6. Interleukin 6 (IL-6) mediates the protective systemic effects of inflammation, including the effects of fever, acute-phase protein synthesis by the liver, and increased production of leukocytes by the bone marrow.[8] This study discusses the effect of lime peel extract on the amount of bacterial colonization and its effect on the activity of IL-6 pro-inflammatory cytokines in Balb/c mice injected with *Salmonella typhi*.

Material and Methods

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

Lime peelextract (LPE)

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

Balb/c mice

Balb/c mice age ranging from 8 to 12 weeks, and weight from 30 to 40 grams (n=20), were obtained from Laboratory of Molecular Biology and Immunology, Department of Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. Mice were divided into four groups (n=five/group) based on intervention; LPE dose of 510 mg/kgbw, LPE 750 mg/kgbw, positive control (Levofloxacin dose 98

mg/kgbw,[9]), negative control (Aquades). All groups were injected intraperitoneally with *S. typhi* strain Thy1 (3 x 10^3 CFU/ml). 3 days after the injection of *Salmonella typhi*, each animal was started to be intervened for 5 days. Animals were treated as reported in Kasim et al.[4]

Bacterial colonization

Bacterial colonies were counted from samples taken from peritoneal fluid. Samples were taken three times, on the 5th day after mice were induced by *Salmonella typhi* (pre-intervention), the 10th post-intervention and 30th day after maintenance without treatment.Samples were taken as much as 0.5 ml and placed in 4.5 ml of physiological saline solution (0.9% NaCl). Dilution was carried out three times so that the culture obtained was not too dense or filled up the cup (the culture is too dense will interfere with observation). 1 ml of suspension was poured into a sterile Petri dish, then poured warm sterile (nutrient agar) media (45^0C) then tightly closed and incubated for 1-2 days at 37^0C. The method used was the Plate Count Agar (PCA), a technique to grow microorganisms in agar media by mixing the liquid agar media with bacterial culture stock (agar) so that the cells are evenly distributed and still on the surface of agar or inside of agar.[10]

Interleukin 6 (IL-6) Examination

Serum samples were taken 4 times, baseline (day 0), after induction of *S. typhi* before intervention (day 5), after intervention (10th day) and after 30 days. IL-6 antibody concentrations were determined by the IL6 ELISA Mouse Sandwich method. IL-6 examination was reported in Kasim et.al.[4]

Ethics statement

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

Results

Bacterial Colonization

The number of bacterial colonies after being given an intervention decreased in all groups, however on the 10th day of the negative control group, the number of bacterial colonies was seen more than other groups, and there were still bacterial colonies (Fig. 1) on the 30th day of negative control group. On the 10th day, the number of bacterial colonies decreased after the intervention of lime peel extract which was given for 5 days of intervention. The number of bacterial colonies continued to decrease until the 30th day post-intervention. The decrease in the number of bacterial colonies after LPE administration had almost the same effect as the decrease in the number of colonies after levofloxacin administration (positive control). The effect of quinolone as a bactericidal,[11] in this study showed that the number of bacterial colonies after 30 days continued to decrease even they did not grow in the group given LPE 510, LPE 750, and positive control.

In the LPE group at a dose of 750 mg/kgbw, the number of bacterial colonies on day 10 had no growth, better than positive control group. The average decreased in the number of bacterial colonies in the LPE group 750 mg/kgbw from 17.2 x 10^3 CFU/ml to 0 or no bacterial growth (p=0.00) whereas in the positive control group decreased from

30.4 x 10^3 CFU/ml to 1.6 x 10^3 CFU/ml (p=0.001). In the negative control group, the number of bacterial colonies decreased from 22.6 x 10^3 CFU/ml to 6 x 10^3 CFU/ml, meaning that the number of bacterial colonies in the negative control group was still bigger than the other three groups, even though there was a decrease.

Interleukin 6 (IL-6)

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

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

Discussion

Bacterial Colonization

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

Interleukin 6 (IL-6)

One of the earliest responses of the innate immune system to infection and tissue damage is the secretion of cytokines by tissue cells, which is very important for acute inflammation. When *S. typhi* first enters the body, the bacteria will be destroyed by macrophages. Bacteria will be recognized by various receptors located on the surface of phagocytes.[8] Specific marker molecules for Gram-negative bacteria such as *S. typhi* are LPS, LPS will use TLR-4, a receptor that plays a role in observing and destroying *Salmonella typhi*. Activated TLR-4 will recruit the MyD88 adapter protein. Then MyD88 recruits IRAK4, IRAK1 and IRAK2. IRAQ kinase then phosphorylates and activates the TRAF6 protein, allowing NF-κB to dwell in the cell nucleus and activating

transcription and causing induced inflammatory cytokines. Proinflammatory cytokines such as IL-1β and IL-6, IFN-γ and TNF-α are synthesized and systemic inflammation occurs.[8,14] It can be said that the inflammatory process by IL-6 cytokines is already running.

The results of this study (figure 2) showed that LPE therapy gave a greater decrease in IL-6 levels compared to levofloxacin. It is indicated that LPE has an anti-inflammatory effect on IL-6.[15] LPE, in addition to having an antibacterial effect, also has an anti-inflammatory effect, LPE has the potential for a more effective therapy. Researchers assume that the inhibitory effect of IL-6 is due to the activity of the polyphenol compounds contained in the extracts of lime peel. In line with this, Haseeb A. (2017) found that the potential inhibitory effect of polyphenols on IL-6 expression and oxidative stress through molecular mechanisms of activation of NF-κB.[16]

The content of polyphenols and flavonoids contained in LPE of this study reveal that polyphenols are bioactive substances whichare likely to have a potential effect on the inflammatory response. It is known that flavonoids are one of the most extensive groups of secondary metabolite plants, flavonoids are found in many edible fruits and vegetables. The source of polyphenols is mostly contained in citrus fruits.[17] The polyphenol content in lime peel extract in this study is 2.29% and flavonoids are 0.26%.The research by Rossano on *Citrus aurantifolia* in Calabria, Italy revealed that lime peels which were extracted by methanol solvent contained of more phenols (95.6 mg/g) and flavonoids (23.5 mg/g) compared to the leaves. In that study, it was found that there was a significant relationship between phenol and flavonoid with antioxidant activity.[3]

Tejada S. (2017), proved that hesperidin (a type of flavonoid) extracted from the citrus genus, has potential as an anti-inflammatory. Hesperidin treatment decreases inflammatory mediators and provides a significant antioxidant effect. Molecular bases for anti-inflammatory effects appear to be mediated by signaling pathways especially the nucleus κβ factor pathway.[18] As for other opinions, a research conducted by Jorge L.A, found that from 3 types of citrus namely *C. limon, C. aurantifolia, C. limonia*, essential oils (EO) contained in the 3 types of citrus have anti-inflammatory effects, especially the limonia content of essential oils from the citrus fruit skin.[15] During the inflammatory event, there is an increase in cytokine production.

Conclusion

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

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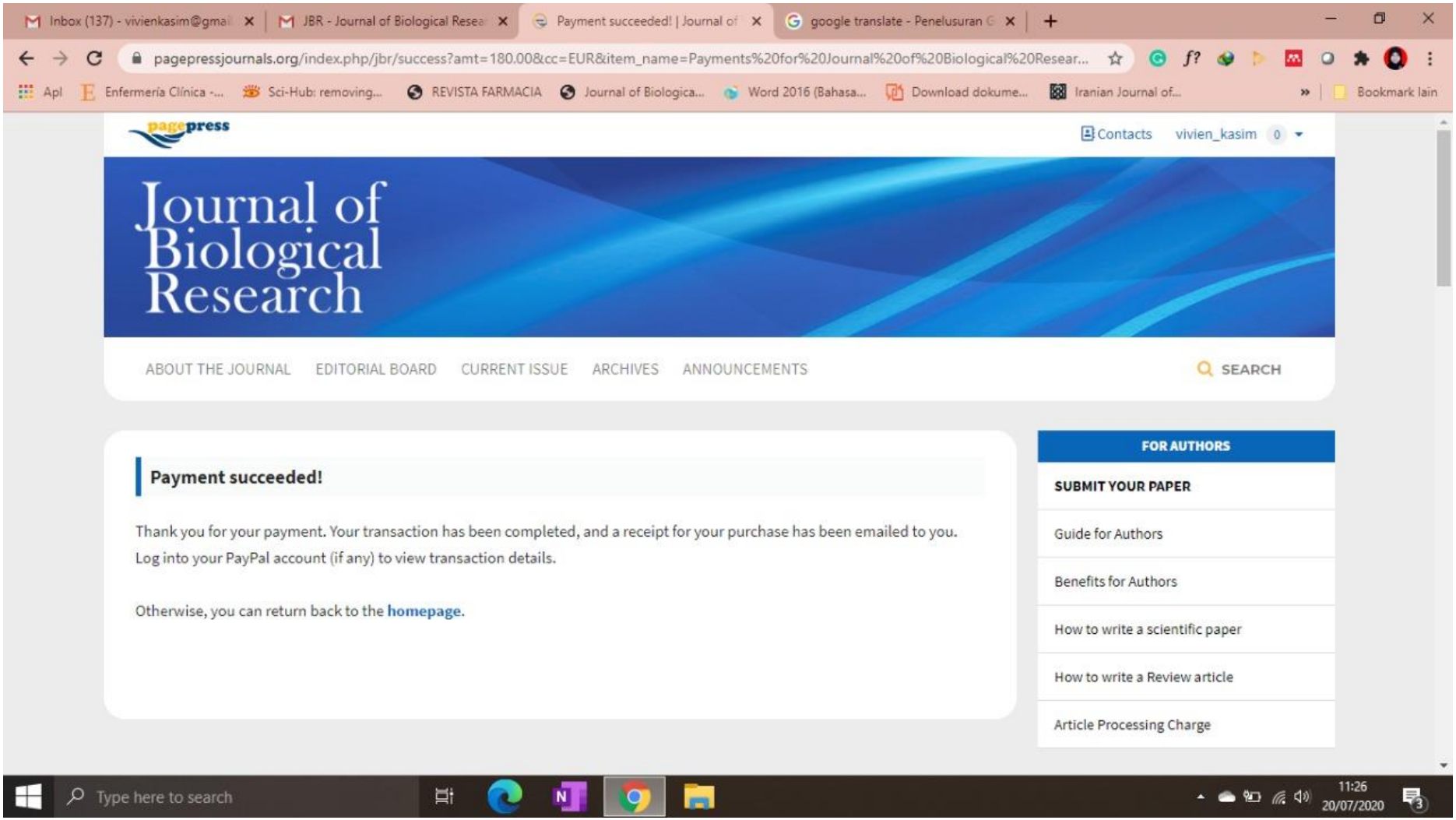
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Antibacterial and anti-inflammatory effects of lime (*Citrus aurantifolia*) peel extract in Balb/c mice infected by *Salmonella typhi*

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Abstract

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

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Key words: Lime peel extract; *Citrus aurantifolia*, Interleukin 6; antibacterial; anti-inflammatory.

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Introduction

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

Bacterial resistance and especially that of *Salmonella typhi* brings us to a new treatment in the form of adjuvant therapy, one of which is *Citrus aurantifolia* that can be used as an antimicrobial. Based on the previous research, all parts of *Citrus aurantifolia*; leaves, root stem, bark, and peel contain useful metabolic

compounds[5] the lime peel has a higher concentration of flavonoids compared to other parts such as seed, fruit and juice.[6] The existence of the content of flavonoids makes the lime peel has antibacterial and antioxidant power. Previous studies tested the bacteriostatic or bactericidal activity of flavonoids by conducting a time-kill study found that flavonoids do not kill bacterial cells but only induce the formation of bacterial aggregates thereby reducing the amount of Colony Forming Units (CFU) in a decent amount.[7]

When *S. typhi* bacterial infection occurs, there is a bond between the lipopolysaccharide (LPS) of the bacterial wall and the Toll-Like Receptor 4 (TLR 4) of the host, which stimulates innate immune activity. The activity releases a number of cytokines such as TNFα, IL-1, and IL-6. Interleukin 6 (IL-6) mediates the protective systemic effects of inflammation, including the effects of fever, acute-phase protein synthesis by the liver, and increased production of leukocytes by the bone marrow.[8] This study discusses the effect of lime peel extract on the amount of bacterial colonization and its effect on the activity of IL-6 pro-inflammatory cytokines in Balb/c mice injected with *Salmonella typhi*.

Materials and Methods

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

Lime peel extract

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

Balb/c mice

Balb/c mice age ranging from 8 to 12 weeks, and weight from 30 to 40 grams (n=20), were obtained from Laboratory of Molecular Biology and Immunology, Department of Microbiology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. Mice were divided into four groups (n=five/group) based on intervention; LPE dose of 510 mg/kgbw, LPE 750 mg/kgbw, positive control (Levofloxacin dose 98 mg/kgbw),[9] negative control (Aquades). All groups were injected intraperitoneally with *S. typhi* strain Thy1 (3×10^3 CFU/ml). 3 days after the injection of *Salmonella typhi*, each animal was started to be intervened for 5 days. Animals were treated as reported in Kasim *et al*.[4]

Bacterial colonization

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

Interleukin 6 (IL-6) examination

Serum samples were taken 4 times, baseline (day 0), after induction of *S. typhi* before intervention (day 5), after intervention (10th day) and after 30 days. IL-6 antibody concentrations were determined by the IL6 ELISA Mouse Sandwich method. IL-6 examination was reported in Kasim *et al*.[4]

Ethics statement

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

Results

Bacterial colonization

The number of bacterial colonies after being given an intervention decreased in all groups, however on the 10th day of the negative control group, the number of bacterial colonies was seen more than other groups, and there were still bacterial colonies (Figure 1) on the 30th day of negative control group. On the 10th day, the number of bacterial colonies decreased after the intervention of lime peel extract which was given for 5 days of intervention. The number of bacterial colonies continued to decrease until the 30th day post-intervention. The decrease in the number of bacterial colonies after LPE administration had almost the same effect as the decrease in the number of colonies after levofloxacin administration (positive control). The effect of quinolone as a bactericidal,[11] in this study showed that the number of bacterial colonies after 30 days continued to decrease even they did not grow in the group given LPE 510, LPE 750, and positive control.

In the LPE group at a dose of 750 mg/kgbw, the number of bacterial colonies on day 10 had no growth, better than positive control group. The average decreased in the number of bacterial colonies in the LPE group 750 mg/kgbw from 17.2×10^3 CFU/mL to 0 or no bacterial growth (p=0.00) whereas in the positive control group decreased from 30.4×10^3 CFU/mL to 1.6×10^3 CFU/mL

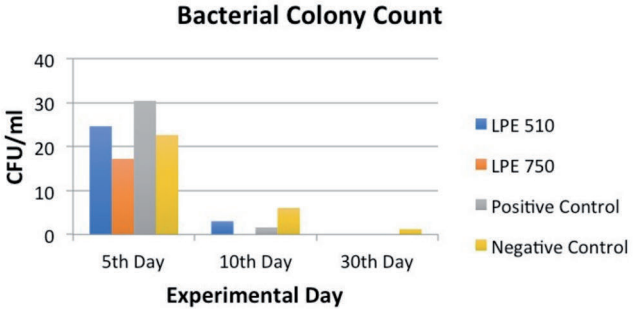

Figure 1. Dynamics of changes in the number of bacterial colonization of *S. Typhi* by the groups during the observation period; LPE, lime peel extract; CFU, colony forming units.

(p=0.001). In the negative control group, the number of bacterial colonies decreased from 22.6×10^3 CFU/mL to 6×10^3 CFU/mL, meaning that the number of bacterial colonies in the negative control group was still bigger than the other three groups, even though there was a decrease.

Interleukin 6 (IL-6)

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

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

Discussion

Bacterial colonization

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

Interleukin 6 (IL-6)

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

The results of this study (Figure 2) showed that LPE therapy gave a greater decrease in IL-6 levels compared to levofloxacin. It is indicated that LPE has an anti-inflammatory effect on IL-6.[15] LPE, in addition to having an antibacterial effect, also has an anti-inflammatory effect, LPE has the potential for a more effective therapy. Researchers assume that the inhibitory effect of IL-6 is due to the activity of the polyphenol compounds contained in the extracts of lime peel. In line with this, Haseeb A. (2017) found that the potential inhibitory effect of polyphenols on IL-6 expression and oxidative stress through molecular mechanisms of activation of NF-κB.[16]

The content of polyphenols and flavonoids contained in LPE of this study reveal that polyphenols are bioactive substances, which are likely to have a potential effect on the inflammatory response. It is known that flavonoids are one of the most extensive groups of secondary metabolite plants, flavonoids are found in many edible fruits and vegetables. The source of polyphenols is mostly contained in citrus fruits.[17] The polyphenol content in lime peel extract in this study is 2.29% and flavonoids are 0.26%. The research by Rossano on *Citrus aurantifolia* in Calabria, Italy revealed that lime peels which were extracted by methanol solvent contained of more phenols (95.6 mg/g) and flavonoids (23.5 mg/g) compared to the leaves. In that study, it was found that there was a significant relationship between phenol and flavonoid with antioxidant activity.[3]

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

Groups	Level of IL-6 (rg/ml)	
	5th-10th day Delta (mean±SD)	10th-30th day Delta (mean±SD)
LPE 510	19,63±7,06	53,98±43,38
LPE 750	24,54±7,71	115,61±56,92
Control (+)	24,07±7,06	273,41±68,26
Control (-)	11,62±6,33	41,91±18,87
P value	0,074	0,000

IL-6, interleukin 6; LPE, Lime Peel Extract; Control(+), positive control group; Control(-), negative control group; SD, Standard Deviasi.

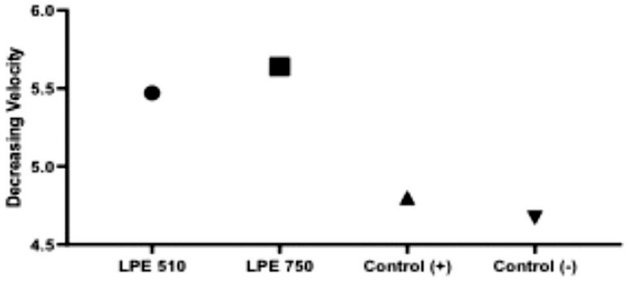

Figure 2. The velocity of decrease (%) of IL-6 levels in each group after the intervention; LPE: Lime Peel Extract; Control (+): positive control group; Control (-): negative control group.

Tejada S. (2017) proved that hesperidin (a type of flavonoid) extracted from the citrus genus, has potential as an anti-inflammatory. Hesperidin treatment decreases inflammatory mediators and provides a significant antioxidant effect. Molecular bases for anti-inflammatory effects appear to be mediated by signaling pathways especially the nucleus κβ factor pathway.[18] As for other opinions, a research conducted by Jorge L.A, found that from 3 types of citrus namely *C. limon, C. aurantifolia, C. limonia*, Essential Oils (EO) contained in the 3 types of citrus have anti-inflammatory effects, especially the limonia content of essential oils from the citrus fruit skin.[15] During the inflammatory event, there is an increase in cytokine production.

Conclusions

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

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