

**SUBMIT**



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

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Kepada: Vivien Novarina Kasim <viviennovarina@ung.ac.id>

7 Maret 2020 16.11

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:

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Francesca Savio

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[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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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

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- II. Conflict of interest: The authors declare no potential conflict of interest
- III. Funding: Ministry of Research and Technology, Indonesia or BUDI-DN LDPD.

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

3

4 **Abstract:**

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

18

19 **Introduction:**

20 Some antibiotics have been resistant to *Salmonella Typhi* and *Salmonella*  
21 *Paratyphi*, such as ampicillin, chloramphenicol, tetracycline and co-trimoxazole.<sup>1</sup> Multi  
22 Drug Resistance (MDR) leads to new treatments in the form of adjuvant therapy,

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

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

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

46 such as TNF $\alpha$ , IL-1, and IL-6. Interleukin 6 (IL-6) mediates the protective systemic effects  
47 of inflammation, including the effects of fever, acute-phase protein synthesis by the liver,  
48 and increased production of leukocytes by the bone marrow.<sup>8</sup> This study discusses the  
49 effect of lime peel extract on the amount of bacterial colonization and its effect on the  
50 activity of IL-6 pro-inflammatory cytokines in Balb/c mice injected with *Salmonella Typhi*.

51

## 52 **Material and Methods**

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

### 56 **Lime peel extract**

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

### 64 **Experimental Animals**

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

69 with *S. Typhi* strain Thy1 ( $3 \times 10^3$  CFU/ml). After 3 days of induction, each animal tried to  
70 be intervened; LPE 510 (mice group intervened with LPE dose 510 mg/kgbw), LPE 750  
71 (mice group intervened with LPE dose 750 mg/kgbw), positive control group (groups of  
72 mice given Levofloxacin dose 98 mg/kgbw and negative control group (group of placebo  
73 mice).

#### 74 **Bacterial colonization**

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

#### 86 **Interleukin 6 (IL-6) Examination**

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



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

### 93 **Ethics statement**

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

97

## 98 **Results**

### 99 **Bacterial Colonization**

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

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

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

### 117 **Interleukin 6 (IL-6)**

118 The level of serum IL-6 at 4 times the data collection, analyzed using paired T-  
119 tests to assess the dynamics of changes in levels of serum with respect to changes in  
120 observation time for each group, can be seen in Table 1. The mean of IL-6 level on the  
121 5th day increased in all groups compared at the baseline day, because it is the 4th-day  
122 post-injection *Salmonella Typhi*. This indicates that after the injection of *Salmonella Typhi*,  
123 all mice in all groups experienced an infection process.

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

134

## 135 **DISCUSSION**

### 136 **Bacterial Colonization**

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

#### 147 **Interleukin 6 (IL-6)**

148 One of the earliest responses of the innate immune system to infection and tissue  
149 damage is the secretion of cytokines by tissue cells, which are very important for acute  
150 inflammation. When *S. Typhi* first enters the body, the bacteria will be destroyed by  
151 macrophages. Bacteria will be known by various receptors located on the surface of  
152 phagocytes.<sup>8</sup> Specific marker molecules for gram-negative bacteria such as *S. Typhi* are  
153 LPS, LPS will use TLR-4, which is a receptor that plays a role in observing and destroying  
154 *Salmonella Typhi*. Activated TLR-4 will recruit the MyD88 adapter protein. Then MyD88  
155 recruited IRAK4, IRAK1 and IRAK2. IRAK kinase then phosphorylates and activates the  
156 TRAF6 protein, allowing NF- $\kappa$ B to dwell in the cell nucleus and activating transcription  
157 and causing induced inflammatory cytokines. Proinflammatory cytokines such as IL-1 $\beta$   
158 and IL-6, IFN- $\gamma$  and TNF- $\alpha$  are synthesized and systemic inflammation occurs.<sup>8,12</sup> It can  
159 be said that the inflammatory process by IL-6 cytokines is already running.

160 As for the negative control group, there were no significant differences before the  
161 intervention (5th day) and after the intervention (10th day), which is p-value 0.15. These  
162 results indicate that the extract of lime peel has the same effect as Levofloxacin, which  
163 inhibits the activity of pro-inflammatory cytokines IL-6 after administration for 5 days.  
164 Researchers assume that the inhibitory effect of IL-6 is due to the activity of the  
165 polyphenol compounds contained in extracts of lime peel. In line with this, Haseeb A.  
166 (2017) found that the potential inhibitory effect of polyphenols on IL-6 expression and  
167 oxidative stress through molecular mechanisms of activation of NF- $\kappa$ B.<sup>13</sup>

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

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

183 nucleus  $\kappa\beta$  factor pathway.<sup>15</sup> As for other opinions, research conducted by Jorge L.A,  
184 found that from 3 types of citrus namely *C.limon*, *C.latifolia*, *C. aurantifolia* or *C.limonia*,  
185 essential oils (EO) contained in the 3 types of citrus have anti-inflammatory effects,  
186 especially the limonene content of essential oils from the citrus fruit skin.<sup>16</sup> During the  
187 inflammatory event, there is an increase in cytokine production.

188

## 189 **CONCLUSION**

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

193

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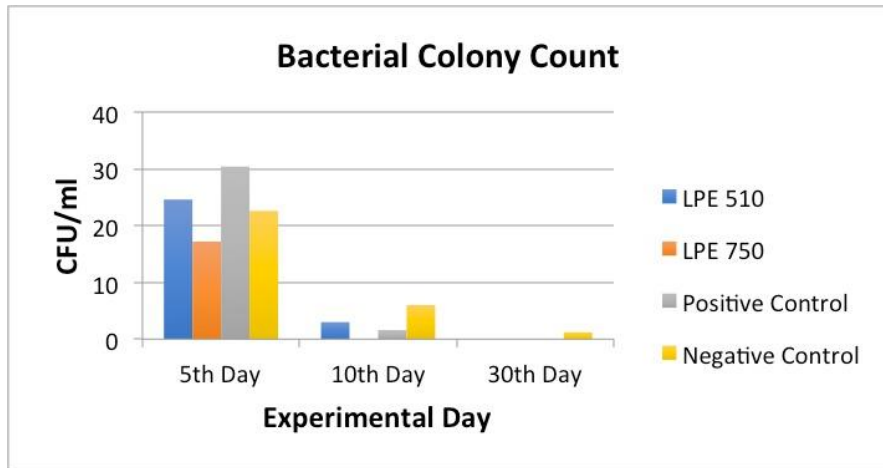


Figure 1. Dynamics of changes in the number of bacterial colonization of *S. Typhi* by the group during the observation period; LPE, lime peel extract; CFU, colony forming units.

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 (pg/ml)								
	Baseline	5th	<i>p</i> value	5th	10th	<i>p</i> value	10th	30th	<i>p</i> value
LPE 510	157.3±59.7	358.5±59.5	0.007	358.5±59.5	338.7±64.9	<b>0.003</b>	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	<b>0.002</b>	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	<b>0.006</b>	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

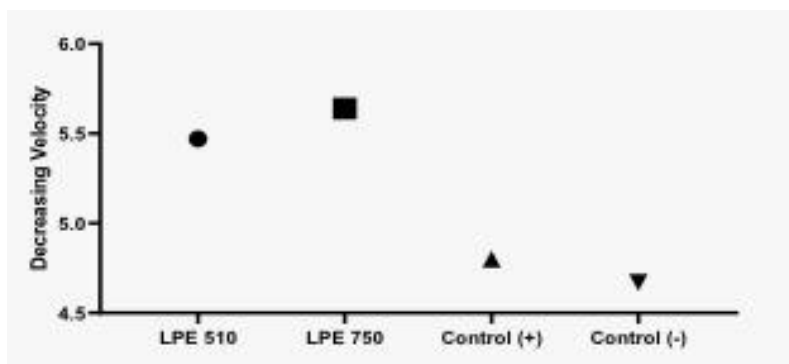


Figure 2. Velocity of decrease (%) the average of IL-6 levels on the day of observation before and after intervention; LPE, lime peel extract; Control(+), positive control group; Control(-), negative control group.



## COVER LETTER

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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](mailto: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](mailto: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**



Vivien Novarina A Kasim &lt;viviennovarina@ung.ac.id&gt;

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

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Dr. Gian Luigi Mariottini &lt;Gian.Luigi.Mariottini@unige.it&gt;

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.

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  - 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

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

II. Authors  
Vivien Novarina Kasim<sup>1\*</sup>, Mochammad Hatta<sup>2</sup>, Rosdiana Natzir<sup>3</sup>, Veni Hadju<sup>4</sup>, Yusminah Hala<sup>5</sup>, Budu<sup>6</sup>, Gemini Alam<sup>7</sup>, Suryani As'ad<sup>8</sup>, Ami Febriza<sup>9</sup>, Hasta Handayani Idrus<sup>10</sup>

Commented [A1]: Completes the name of this author: First name and more name

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Commented [A2]: Department

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.

1 **Antibacterial and anti-inflammatory effects of lime (*Citrus aurantifolia*)**  
2 **peel extract in mice balb/c induced *Salmonella typhi***

**Commented [A1]:** Respect the writing of the nomenclature of species: the genus begins with a capital letter : *Citrus*

**Commented [A2]:** *Salmonella*

3  
4 **Abstract:**

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

**Commented [A3]:** Correct grammatical mistakes in this paragraph

19  
20 **Introduction:**

21 Some antibiotics have been resistant to *Salmonella Typhi* and *Salmonella*  
22 *Paratyphi*, such as ampicillin, chloramphenicol, tetracycline and co-trimoxazole.<sup>1</sup> Multi

**Commented [A4]:** Correct the meaning of this sentence: It is bacteria that are resistant to antibiotics. Antibiotics become ineffective on resistant bacteria.

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

Commented [A5]: check the punctuation of this sentence

Commented [A6]: 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.

Commented [A7]: Gram-negative

Commented [A8]: *Salmonella Typhi* resistance or the resistance of *Salmonella Typhi*

Commented [A9]: This reference does not correspond to the idea expressed in this sentence

Commented [A10]: bacterial resistance and especially that of *Salmonella*, .....

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

Commented [A11]: Reference 6 is not adequate for these data cited in this sentence.

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



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

53

## 54 **Material and Methods**

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

### 58 **Lime peel extract**

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

### 66 **Experimental Animals**

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

Commented [A12]: to study

Commented [A13]: Add punctuation in this sentence.

Commented [A14]: Correct this sentence: Mices Balb/c, ( witch age ranges from 8 to12 weeks, and weigh 30 to 40 grams; (n=20),

69 of Medicine, Hasanuddin University (Makassar, Indonesia). The rats were acclimatized  
70 for 8 days, then divided into four groups (n=5). All groups were induced intraperitoneally  
71 with *S. Typhi* strain Thy1 ( $3 \times 10^3$  CFU/ml). After 3 days of induction, each animal tried  
72 to be **intervened**; LPE 510 (mice group intervened with LPE dose 510 mg/kgbw), LPE  
73 750 (mice group intervened with LPE dose 750 mg/kgbw), positive control group (**groups**  
74 **of mice given Levofloxacin dose 98 mg/kgbw**) and negative control group (group of  
75 placebo mice).

## 76 Bacterial colonization

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

## 88 Interleukin 6 (IL-6) Examination

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

**Commented [A15]:** 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??

**Commented [A16]:**  
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)

**Commented [A17]:** mices

**Commented [A18]:** Samples

**Commented [A19]:** Physiological saline **solution**

**Commented [A20]:** Petri

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

**Commented [A21]:** Completely reconstruct the sentence with the verb and the subject instead of putting (:) repeatedly, and checking the punctuation

## 95 **Ethics statement**

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

99

## 100 **Results**

### 101 **Bacterial Colonization**

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

**Commented [A22]:** These are the results you found in this study, you can compare with other bibliographic data whose references you must cite in discussion.

111 In the LPE group at a dose of 750 mg/kgbw, the number of bacterial colonies on  
112 day 10 had no growth, better than positive control group. The average decrease in the  
113 number of bacterial colonies in the LPE group 750 mg/kgbw from  $17.2 \times 10^3$  CFU/ml to  
114 0 or no bacterial growth ( $p=0.00$ ) whereas in the positive control group decreased from

115 30.4 x 10<sup>3</sup> CFU/ml to 1.6 x 10<sup>3</sup> CFU/ml (p=0.001). In the negative control group, the  
116 number of bacterial colonies decreased from 22.6 x 10<sup>3</sup> CFU/ml to 6 x 10<sup>3</sup> CFU/ml,  
117 meaning that the number of bacterial colonies in the negative control group even though  
118 it decreased but the number of bacterial colonies was still bigger than the three groups.

### 119 Interleukin 6 (IL-6)

120 The level of serum IL-6 at 4 times the data collection, analyzed using paired T-  
121 tests to assess the dynamics of changes in levels of serum with respect to changes in  
122 observation time for each group, can be seen in Table 1. The mean of IL-6 level on the  
123 5th day increased in all groups compared at the baseline day, because it is the 4th-day  
124 post-injection *Salmonella Typhi*. This indicates that after the injection of *Salmonella*  
125 *Typhi*, all mice in all groups experienced an infection process.

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

137

**Commented [A23]:** Review the reconstruction of this sentence

**Commented [A24]:** Review it's : the 5th day to the 10th day

138 **DISCUSSION**

139 **Bacterial Colonization**

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

150 **Interleukin 6 (IL-6)**

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

**Commented [A25]:** Gram-positive and Gram-negative

**Commented [A26]:** Reference 10 does not describe the activity of *Citrus aurantifolia* on Gram negative and positive bacteria but on alcohol-resistant bacteria *Mycobacterium tuberculosis*.

**Commented [A27]:** Reference 11 does not describe this data

**Commented [A28]:** Give adequate references for the data of these studies that you cited in this paragraph.

161 cytokines such as IL-1 $\beta$  and IL-6, IFN- $\gamma$  and TNF- $\alpha$  are synthesized and systemic  
162 inflammation occurs.<sup>8,12</sup> It can be said that the inflammatory process by IL-6 cytokines is  
163 already running.

164 As for the negative control group, there were no significant differences before the  
165 intervention (5th day) and after the intervention (10th day), which is p-value 0.15. These  
166 results indicate that the extract of lime peel has the same effect as Levofloxacin, which  
167 inhibits the activity of pro-inflammatory cytokines IL-6 after administration for 5 days.  
168 Researchers assume that the inhibitory effect of IL-6 is due to the activity of the  
169 polyphenol compounds contained in extracts of lime peel. In line with this, Haseeb A.  
170 (2017) found that the potential inhibitory effect of polyphenols on IL-6 expression and  
171 oxidative stress through molecular mechanisms of activation of NF- $\kappa$ B.<sup>13</sup>

172 Looking from the content of polyphenols and flavonoids contained in LPE of this  
173 study, polyphenols are bioactive substances that have the potential to have a potential  
174 effect on the inflammatory response. It is known that flavonoids are one of the most  
175 extensive groups of secondary metabolite plants, flavonoids are found in many edible  
176 fruits and vegetables. The most source of polyphenols is represented by citrus fruits.<sup>14</sup>  
177 The polyphenol content in lime peel extract in this study was 2.29% and flavonoids  
178 0.26%, it has been reported that the content of phenols and flavonoids using methanol  
179 solvent contained more in the parts of lime peel compared to the leaves, phenol 95.6  
180 mg/g, and flavonoids 23.5 mg/g, are contained in the Rossano *Citrus aurantiolia* bark  
181 extract in Calabria, Italy. In that study, it was found that there was a significant  
182 relationship between phenol and flavonoid with antioxidant activity.<sup>4</sup>

**Commented [A29]:** Compare your results found with those in the literature concerning the antibacterial and anti-inflammatory effect of Levofloxacin.

**Commented [A30]:** *aurantifolia*

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

192

## 193 **CONCLUSION**

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

197

## 198 **References**

199

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244

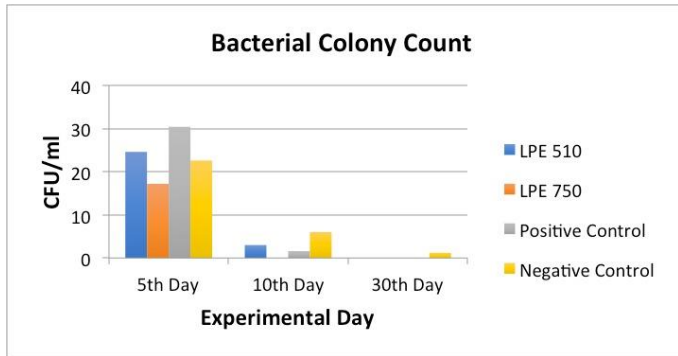


Figure 1. Dynamics of changes in the number of bacterial colonization of *S. Typhi* by the groups during the observation period; LPE, lime peel extract; CFU, colony forming units.

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	<i>p</i> value	5th	10th	<i>p</i> value	10th	30th	<i>p</i> value
LPE 510	157.3±59.7	358.5±59.5	0.007	358.5±59.5	338.7±64.9	<b>0.003</b>	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	<b>0.002</b>	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	<b>0.006</b>	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

**Commented [A1]:** Separate the levels of IL-6 results for each time interval to make your Table more Readable

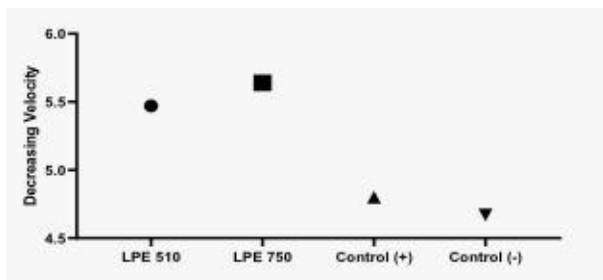


Figure 2. Velocity of decrease (%) the average of IL-6 levels on the day of observation before and after intervention; LPE, lime peel extract; Control(+), positive control group; Control(-), negative control group.

**Commented [A2]:** Indicate the time interval

## 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
<b>Article text</b>	
[1] Respect the writing of the nomenclature of species: the genus begins with a capital letter : <i>Citrus</i>	Revised in line 1
[2] <i>Salmonella</i>	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] <i>Salmonella Typhi</i> resistance or the resistance of <i>Salmonella Typhi</i>	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 <i>Salmonella</i> ,	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 to12 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: group s: 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 <i>Citrus aurantifolia</i> on Gram negative and positive bacteria but on alcohol-resistant bacteria <i>Mycobacterium tuberculosis</i> .	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] <i>aurantifolia</i>	Revised in line 189
<b>Figures article</b>	
[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
<b>Title and authors article</b>	
[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	
<p>[6] highlighted in yellow :</p> <p>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 50°C 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.</p>	<p>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. <u>LPE was obtained as reported in Kasim et.al.<sup>4</sup></u></p>
<p>[7] highlighted in yellow :</p> <p>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 <i>S. Typhi</i> strain Thy1 (3 x 10<sup>3</sup> CFU/ml). After 3 days from injection with <i>Salmonella Typhi</i>, 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,<sup>9</sup> and group 4 negative control group (group of placebo mice).</p>	<p>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,<sup>9</sup>), negative control (Aquades). All groups were injected intraperitoneally with <i>S. typhi</i> strain Thy1 (3 x 10<sup>3</sup> CFU/ml). 3 days after the injection of <i>Salmonella typhi</i>, each animal was started to be intervened for 5 days. <u>Animals were treated as reported in Kasim et al.<sup>4</sup></u></p>
<p>[8] highlighted in yellow :</p> <p>Serum samples were taken 4 times, baseline (day 0), after induction of <i>S. Typhi</i> 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.</p>	<p>Revised in line 87-90: Serum samples were taken 4 times, baseline (day 0), after induction of <i>S. typhi</i> 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. <u>IL-6 examination was reported in Kasim et.al.<sup>4</sup></u></p>
<p>[9] highlighted in yellow :</p> <p>Table 1.</p>	<p>Revised in figures article file: Table 1.</p>
<p>[10] highlighted in yellow :</p> <p><i>or C. limon</i></p>	<p>Deleted this words</p>
<p>[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</p>	<p>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</p>

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 <b>Antibacterial and anti-inflammatory effects of lime (<i>Citrus aurantifolia</i>) peel extract in Balb/c mice infected by <i>Salmonella typhi</i></b>
[13] the English must be extensively revised; the remarks 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



# 1 **Antibacterial and anti-inflammatory effects of lime (*Citrus aurantifolia*)** 2 **peel extract in Balb/c mice infected by *Salmonella typhi***

## 3 4 **Abstract:**

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

## 19 20 **Introduction:**

21 Some antibiotics such as ampicillin, chloramphenicol, tetracycline and co-  
22 trimoxazole become ineffective on *Salmonella typhi* and *Salmonella paratyphi*,<sup>1</sup> Multi-

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

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

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

46 cytokines such as TNF $\alpha$ , IL-1, and IL-6. Interleukin 6 (IL-6) mediates the protective  
47 systemic effects of inflammation, including the effects of fever, acute-phase protein  
48 synthesis by the liver, and increased production of leukocytes by the bone marrow.<sup>8</sup>  
49 This study discusses the effect of lime peel extract on the amount of bacterial  
50 colonization and its effect on the activity of IL-6 pro-inflammatory cytokines in Balb/c  
51 mice injected with *Salmonella typhi*.

## 53 **Material and Methods**

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

### 57 **Lime peel extract (LPE)**

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

### 63 **Balb/c mice**

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

69 98mg/kgbw,<sup>9</sup>), negative control (Aquades). All groups were injected intraperitoneally  
70 with *S. typhi* strain Thy1 ( $3 \times 10^3$  CFU/ml). 3 days after the injection of *Salmonella typhi*,  
71 each animal was started to be intervened for 5 days. Animals were treated as reported  
72 in Kasim et al.<sup>4</sup>

### 73 **Bacterial colonization**

74 Bacterial colonies were counted from samples taken from peritoneal fluid.  
75 Samples were taken three times, on the 5th day after mice were induced by *Salmonella*  
76 *typhi* (pre-intervention), the 10th post-intervention and 30th day after maintenance  
77 without treatment. Samples were taken as much as 0.5 ml and placed in 4.5 ml of  
78 physiological saline solution (0.9% NaCl). Dilution was carried out three times so that  
79 the culture obtained was not too dense or filled up the cup (the culture is too dense will  
80 interfere with observation). 1 ml of suspension was poured into a sterile Petri dish, then  
81 poured warm sterile (nutrient agar) media (45°C) then tightly closed and incubated for 1-  
82 2 days at 37°C. The method used was the Plate Count Agar (PCA), a technique to grow  
83 microorganisms in agar media by mixing the liquid agar media with bacterial culture  
84 stock (agar) so that the cells are evenly distributed and still on the surface of agar or  
85 inside of agar.<sup>10</sup>

### 86 **Interleukin 6 (IL-6) Examination**

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

### 91 **Ethics statement**

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

95

## 96 **Results**

### 97 **Bacterial Colonization**

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

110 In the LPE group at a dose of 750 mg/kgbw, the number of bacterial colonies on  
111 day 10 had no growth, better than positive control group. The average decreased in the  
112 number of bacterial colonies in the LPE group 750 mg/kgbw from  $17.2 \times 10^3$  CFU/ml to  
113 0 or no bacterial growth ( $p=0.00$ ) whereas in the positive control group decreased from  
114  $30.4 \times 10^3$  CFU/ml to  $1.6 \times 10^3$  CFU/ml ( $p=0.001$ ). In the negative control group, the

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

## 118 **Interleukin 6 (IL-6)**

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

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

134

## 135 **Discussion**

### 136 **Bacterial Colonization**

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

#### 150 **Interleukin 6 (IL-6)**

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

160 transcription and causing induced inflammatory cytokines. Proinflammatory cytokines  
161 such as IL-1 $\beta$  and IL-6, IFN- $\gamma$  and TNF- $\alpha$  are synthesized and systemic inflammation  
162 occurs.<sup>8,14</sup> It can be said that the inflammatory process by IL-6 cytokines is already  
163 running.

164 The results of this study (figure 2) showed that LPE therapy gave a greater  
165 decrease in IL-6 levels compared to levofloxacin. It is indicated that LPE has an anti-  
166 inflammatory effect on IL-6.<sup>15</sup> LPE, in addition to having an antibacterial effect, also has  
167 an anti-inflammatory effect, LPE has the potential for a more effective therapy.  
168 Researchers assume that the inhibitory effect of IL-6 is due to the activity of the  
169 polyphenol compounds contained in the extracts of lime peel. In line with this, Haseeb  
170 A. (2017) found that the potential inhibitory effect of polyphenols on IL-6 expression and  
171 oxidative stress through molecular mechanisms of activation of NF- $\kappa$ B.<sup>16</sup>

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



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

191

## 192 **Conclusion**

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

196

## 197 **References**

198

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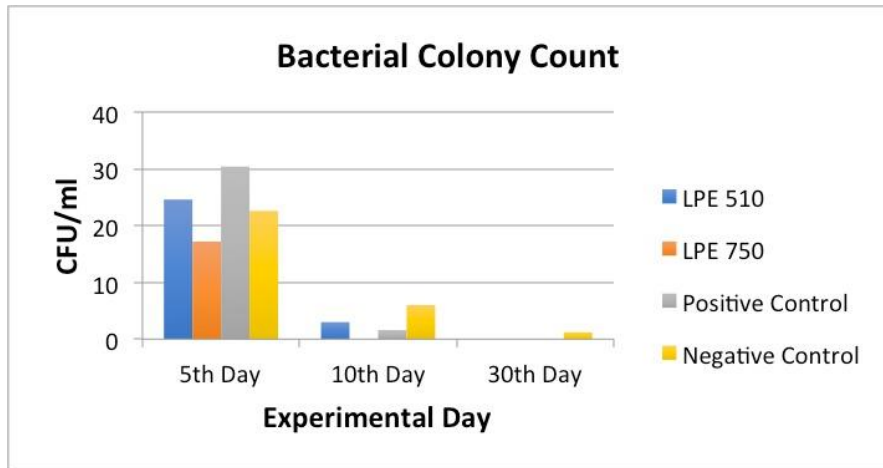


Figure 1. Dynamics of changes in the number of bacterial colonization of *S. Typhi* by the groups during the observation period; LPE, lime peel extract; CFU, colony forming units.

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

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

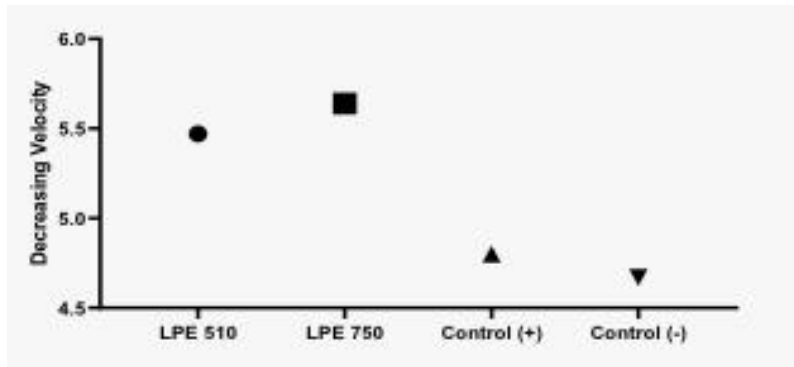


Figure 2. The velocity of decrease (%) of IL-6 levels in each group after the intervention; LPE, lime peel extract; Control (+), positive control group; Control (-), negative control group.



Vivien Novarina A Kasim &lt;viviennovarina@ung.ac.id&gt;

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

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

6 Juli 2020 21.29

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 evaluation and the recommendation. We send 3 files; cover letters, articles, and figures articles.

Thank you very much that the referees have been agreeing on 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,


Vivien Kasim, MD

Pada tanggal Min, 28 Jun 2020 pukul 17.10 Dr. Gian Luigi Mariottini <Gian.Luigi.Mariottini@unige.it> menulis:  
[Kutipan teks disembunyikan]

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

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Gian Luigi Mariottini <Gian.Luigi.Mariottini@unige.it>  
Kepada: Vivien Novarina A Kasim <viviennovarina@ung.ac.id>  
Cc: francesca.savio@pagepress.org

7 Juli 2020 06.22

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

> Dear Gian Luigi Mariottini  
>  
> Editor-in-Chief  
>  
> Journal of Biological Research - Bollettino della Società Italiana di  
> Biologia Sperimentale  
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> Thank you for re-evaluated our manuscript. I have edited the manuscript  
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> Best Regards,  
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> Vivien Kasim, MD  
>  
> Pada tanggal Min, 28 Jun 2020 pukul 17.10 Dr. Gian Luigi Mariottini <  
> [Gian.Luigi.Mariottini@unige.it](mailto:Gian.Luigi.Mariottini@unige.it)> menulis:  
>  
>> Dear Colleagues  
>>

> > 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 referees and then re-evaluated inhouse.  
> > All referees agree that this manuscript is interesting and potentially  
> > acceptable for publication in our journal.  
> > However, it is not acceptable in the present form and changes must be made  
> > before publication.

> >  
> > The file you find in the "discussions" section indicates how your  
> > manuscript must be modified.

> >  
> > Notably:

> >  
> > - 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\*".

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

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## JBR - New notification from Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale

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Dr. Gian Luigi Mariottini <Gian.Luigi.Mariottini@unige.it>  
Balas Ke: Francesca Savio <francesca.savio@pagepress.org>  
Kepada: Vivien Novarina Kasim <viviennovarina@ung.ac.id>

19 Juli 2020 09.20

You have a new notification from Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale:

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".

Link: <https://www.pagepressjournals.org/index.php/jbr/authorDashboard/submission/8951>

Francesca Savio

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[Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale](#)

# 1 **Antibacterial and anti-inflammatory effects of lime (*Citrus aurantifolia*)** 2 **peel extract in Balb/c mice infected by *Salmonella typhi***

## 3 4 **Abstract:**

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

## 19 20 **Introduction:**

21 Some antibiotics such as ampicillin, chloramphenicol, tetracycline and co-  
22 trimoxazole become ineffective on *Salmonella typhi* and *Salmonella paratyphi*,<sup>1</sup> Multi-

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

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

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

46 cytokines such as TNF $\alpha$ , IL-1, and IL-6. Interleukin 6 (IL-6) mediates the protective  
47 systemic effects of inflammation, including the effects of fever, acute-phase protein  
48 synthesis by the liver, and increased production of leukocytes by the bone marrow.<sup>8</sup>  
49 This study discusses the effect of lime peel extract on the amount of bacterial  
50 colonization and its effect on the activity of IL-6 pro-inflammatory cytokines in Balb/c  
51 mice injected with *Salmonella typhi*.

52

## 53 **Material and Methods**

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

### 57 **Lime peelextract (LPE)**

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

### 63 **Balb/c mice**

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

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

### 73 **Bacterial colonization**

74 Bacterial colonies were counted from samples taken from peritoneal fluid.  
75 Samples were taken three times, on the 5th day after mice were induced by *Salmonella*  
76 *typhi* (pre-intervention), the 10th post-intervention and 30th day after maintenance  
77 without treatment. Samples were taken as much as 0.5 ml and placed in 4.5 ml of  
78 physiological saline solution (0.9% NaCl). Dilution was carried out three times so that  
79 the culture obtained was not too dense or filled up the cup (the culture is too dense will  
80 interfere with observation). 1 ml of suspension was poured into a sterile Petri dish, then  
81 poured warm sterile (nutrient agar) media (45<sup>o</sup>C) then tightly closed and incubated for 1-  
82 2 days at 37<sup>o</sup>C. The method used was the Plate Count Agar (PCA), a technique to grow  
83 microorganisms in agar media by mixing the liquid agar media with bacterial culture  
84 stock (agar) so that the cells are evenly distributed and still on the surface of agar or  
85 inside of agar.<sup>10</sup>

### 86 **Interleukin 6 (IL-6) Examination**

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

### 91 **Ethics statement**

92 This research was approved by the Health Medical Ethics Research Committee  
93 at the Faculty of Medicine, Hasanuddin University (Makassar, Indonesia)  
94 with registration number of 900 / H4.8.4.5.31 / PP36-KOMETIK / 2018 on October 31,  
95 2018.

96

## 97 **Results**

### 98 **Bacterial Colonization**

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

111 In the LPE group at a dose of 750 mg/kgbw, the number of bacterial colonies on  
112 day 10 had no growth, better than positive control group. The average decreased in the  
113 number of bacterial colonies in the LPE group 750 mg/kgbw from  $17.2 \times 10^3$  CFU/ml to  
114 0 or no bacterial growth ( $p=0.00$ ) whereas in the positive control group decreased from

115 30.4 x 10<sup>3</sup> CFU/ml to 1.6 x 10<sup>3</sup> CFU/ml (p=0.001). In the negative control group, the  
116 number of bacterial colonies decreased from 22.6 x 10<sup>3</sup> CFU/ml to 6 x 10<sup>3</sup> CFU/ml,  
117 meaning that the number of bacterial colonies in the negative control group was still  
118 bigger than the other three groups, even though there was a decrease.

### 119 **Interleukin 6 (IL-6)**

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

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

135

## 136 **Discussion**

### 137 **Bacterial Colonization**

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

### 151 **Interleukin 6 (IL-6)**

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



161 transcription and causing induced inflammatory cytokines. Proinflammatory cytokines  
162 such as IL-1 $\beta$  and IL-6, IFN- $\gamma$  and TNF- $\alpha$  are synthesized and systemic inflammation  
163 occurs.<sup>8,14</sup> It can be said that the inflammatory process by IL-6 cytokines is already  
164 running.

165 The results of this study (figure 2) showed that LPE therapy gave a greater  
166 decrease in IL-6 levels compared to levofloxacin. It is indicated that LPE has an anti-  
167 inflammatory effect on IL-6.<sup>15</sup> LPE, in addition to having an antibacterial effect, also has  
168 an anti-inflammatory effect, LPE has the potential for a more effective therapy.  
169 Researchers assume that the inhibitory effect of IL-6 is due to the activity of the  
170 polyphenol compounds contained in the extracts of lime peel. In line with this, Haseeb  
171 A. (2017) found that the potential inhibitory effect of polyphenols on IL-6 expression and  
172 oxidative stress through molecular mechanisms of activation of NF- $\kappa$ B.<sup>16</sup>

173 The content of polyphenols and flavonoids contained in LPE of this study reveal  
174 that polyphenols are bioactive substances which are likely to have a potential effect on  
175 the inflammatory response. It is known that flavonoids are one of the most extensive  
176 groups of secondary metabolite plants, flavonoids are found in many edible fruits and  
177 vegetables. The source of polyphenols is mostly contained in citrus fruits.<sup>17</sup> The  
178 polyphenol content in lime peel extract in this study is 2.29% and flavonoids are  
179 0.26%. The research by Rossano on *Citrus aurantifolia* in Calabria, Italy revealed that  
180 lime peels which were extracted by methanol solvent contained more phenols (95.6  
181 mg/g) and flavonoids (23.5 mg/g) compared to the leaves. In that study, it was found  
182 that there was a significant relationship between phenol and flavonoid with antioxidant  
183 activity.<sup>3</sup>

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

193

## 194 **Conclusion**

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

198

## 199 **References**

200

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**ACCEPTED**



Vivien Novarina A Kasim &lt;viviennovarina@ung.ac.id&gt;

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

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Dr. Gian Luigi Mariottini &lt;Gian.Luigi.Mariottini@unige.it&gt;

19 Juli 2020 09.13

Kepada: Vivien Novarina Kasim <viviennovarina@ung.ac.id>, Mochammad Hatta <hattaram@yahoo.com>, Rosdiana Natzir <rosdiananatzir@yahoo.com>, Veni Hadju <phunhas@gmail.com>, Yusminah Hala <yushala@unm.ac.id>, Budu Budu <budu062011@yahoo.com>, Gemini Alam <daengta007@yahoo.com>, Suryani As'ad <suryani\_fkuh@yahoo.com>, Ami Febriza <amifebriza@med.unismuh.ac.id>, Hasta Handayani Idrus <hastahandayani@umi.ac.id>

Dear Dr. Vivien Novarina Kasim, Dr. Mochammad Hatta, Dr. Rosdiana Natzir, Dr. Veni Hadju, Dr. Yusminah Hala, Dr. Budu Budu, Dr. Gemini Alam, Dr. Suryani As'ad, Dr. Ami Febriza, Dr. Hasta Handayani Idrus,

We are pleased to inform you that your paper entitled "Antibacterial and anti-inflammatory effects of lime (citrus aurantifolia) peel extract in mice balb/c induced salmonella typhi" has been accepted for publication in Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale.

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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 *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 5<sup>th</sup> day before the intervention, on the 10<sup>th</sup> day after the intervention and on the 30<sup>th</sup> 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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Ethics approval: 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.

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## Introduction

Some antibiotics such as ampicillin, chloramphenicol, tetracycline and co-trimoxazole become ineffective on *Salmonella typhi* and *Salmonella paratyphi*.<sup>1</sup> 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.<sup>2-3</sup> Specifically, it was emphasized that the peel of lime contains of active ingredients such as polyphenols, flavonoids, tannins, saponins, alkaloids, and triterpenoids.<sup>4</sup> *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<sup>5</sup> the lime peel has a higher concentration of flavonoids compared to other parts such as seed, fruit and juice.<sup>6</sup> 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.<sup>7</sup>

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 TNF $\alpha$ , 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.<sup>8</sup> 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.*<sup>4</sup>

### 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),<sup>9</sup> negative control (Aquades). All groups were injected intraperitoneally with *S. typhi* strain Thy1 ( $3 \times 10^3$  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.*<sup>4</sup>

### Bacterial colonization

Bacterial colonies were counted from samples taken from peritoneal fluid. Samples were taken three times, on the 5<sup>th</sup> day after mice were induced by *Salmonella typhi* (pre-intervention), the 10<sup>th</sup> post-intervention and 30<sup>th</sup> 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.<sup>10</sup>

### 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.*<sup>4</sup>

### 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 30<sup>th</sup> day of negative control group. On the 10<sup>th</sup> 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 30<sup>th</sup> 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,<sup>11</sup> 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  $17.2 \times 10^3$  CFU/mL to 0 or no bacterial growth ( $p=0.00$ ) whereas in the positive control group decreased from  $30.4 \times 10^3$  CFU/mL to  $1.6 \times 10^3$  CFU/mL

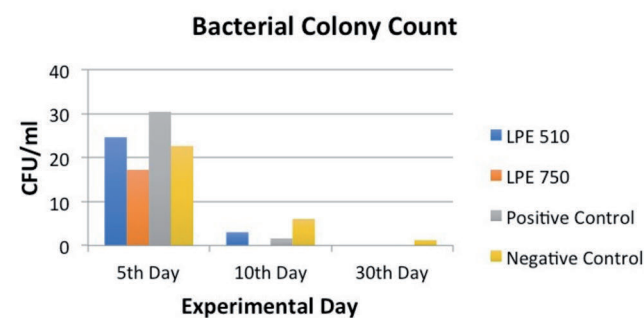


Figure 1. Dynamics of changes in the number of bacterial colonization of *S. Typhi* by the groups during the observation period; LPE, lime peel extract; CFU, colony forming units.

( $p=0.001$ ). In the negative control group, the number of bacterial colonies decreased from  $22.6 \times 10^3$  CFU/mL to  $6 \times 10^3$  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.<sup>5,12</sup> 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.<sup>13</sup> In previous preliminary studies,<sup>4</sup> 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.<sup>8</sup> Specific marker molecules for Gram-negative bacteria such as *S. typhi* are LPS, LPS will use TLR-4, a receptor that plays a role in observing and destroying *Salmonella typhi*. Activated TLR-4 will recruit the MyD88 adapter protein. Then MyD88 recruits IRAK4, IRAK1 and IRAK2. IRAK kinase then phosphorylates and activates the TRAF6 protein, allowing NF- $\kappa$ B to dwell in the cell nucleus and activating transcription and causing induced inflammatory cytokines. Proinflammatory cytokines such as IL-1 $\beta$  and IL-6, IFN- $\gamma$  and TNF- $\alpha$  are synthesized and systemic inflammation occurs.<sup>8,14</sup> It can be said that the inflammatory process by IL-6 cytokines is already running.

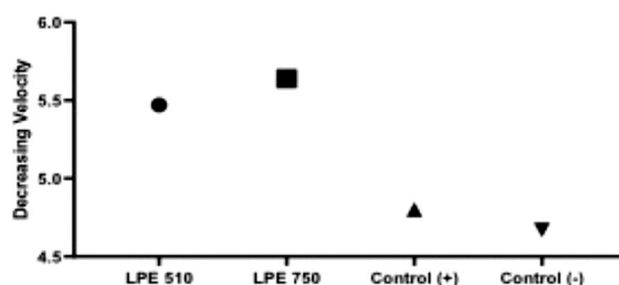
The results of this study (Figure 2) showed that LPE therapy gave a greater decrease in IL-6 levels compared to levofloxacin. It is indicated that LPE has an anti-inflammatory effect on IL-6.<sup>15</sup> LPE, in addition to having an antibacterial effect, also has an anti-inflammatory effect, LPE has 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- $\kappa$ B.<sup>16</sup>

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.<sup>17</sup> 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.<sup>3</sup>

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

Groups	Level of IL-6 (rg/ml)	
	5 <sup>th</sup> -10 <sup>th</sup> day Delta (mean $\pm$ SD)	10 <sup>th</sup> -30 <sup>th</sup> day Delta (mean $\pm$ SD)
LPE 510	19,63 $\pm$ 7,06	53,98 $\pm$ 43,38
LPE 750	24,54 $\pm$ 7,71	115,61 $\pm$ 56,92
Control (+)	24,07 $\pm$ 7,06	273,41 $\pm$ 68,26
Control (-)	11,62 $\pm$ 6,33	41,91 $\pm$ 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  $\kappa\beta$  factor pathway.<sup>18</sup> 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.<sup>15</sup> 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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