Enfermedades Infecciosas y Microbiología Clínica 39 (2021) 283–286

Enfermedades Infecciosas y Microbiología Clínica

www.elsevier.es/eimc

Brief report

Antibacterial activity of *Citrus hystrix* toward *Salmonella* spp. infection

Zulvikar Syambani Ulhaq[a,d,*], Tenta Hartian Hendyatama[b,d], Faizanah Hameed[c,d], Dewi Santosaningsih[d]

[a] *Department of Biochemistry, Faculty of Medicine and Health Sciences, Maulana Malik Ibrahim Islamic State University of Malang, Batu, East Java, Indonesia*
[b] *Department of Internal Medicine, Faculty of Medicine, Airlangga University, Surabaya, East Java, Indonesia*
[c] *Batu Rakit Health Clinic, Trengganu, Malaysia*
[d] *Department of Clinical Microbiology, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia*

ARTICLE INFO

Article history:
Received 24 January 2020
Accepted 15 May 2020

Keywords:
Antibacterial
Citrus hytrix
Salmonella enterica serotype typhimurium
Salmonella typhimurium

ABSTRACT

Introduction: *Citrus hystrix* is widely used by Indonesians as a traditional medicine for gastrointestinal diseases, including *Salmonella* spp. infection. We investigated the antibacterial activity of the ethanolic peel extract of *C. hystrix* against *Salmonella typhimurium.*
Methods: The antibacterial activity was evaluated both *in vitro* and *in vivo*. The minimum inhibitory concentration (MIC) of the extract was determined at a concentration of 0.625% by agar dilution assay. Later, the *in vivo* antibacterial activity was examined by the administration of 16 mg of the extract daily for three consecutive days in a mouse model infected with *S. typhimurium*.
Results: The bacterial loads of *S. typhimurium* in the ileum, liver, and spleen decreased after 24 h of administration of the extract ($p = 0.00008$, $p = 0.00084$, and $p = 0.00003$, respectively).
Conclusion: The ethanolic peel extract of *C. hystrix* shows antibacterial activity against *S. typhimurium*, indicating the potential of *C. hystrix* as an effective treatment for *Salmonella* spp. infection.

Actividad antibacteriana de *Citrus hystrix* frente a la infección por *Salmonella* spp

Palabras clave:
Antibacteriano
Citrus hystrix
Salmonella enterica serotipo *Typhimurium*
Salmonella typhimurium

RESUMEN

Introducción: El *Citrus hystrix* es muy utilizado por los indonesios como medicina tradicional para las enfermedades gastrointestinales, incluida la infección por *Salmonella* spp. Investigamos la actividad antibacteriana del extracto etanólico de piel de *C. hystrix* frente a *Salmonella typhimurium.*
Métodos: La actividad antibacteriana se evaluó tanto *in vitro* como *in vivo*. La concentración mínima inhibitoria (CMI) del extracto se determinó a una concentración del 0,625% mediante un ensayo de dilución en agar. Posteriormente, se evaluó la actividad antibacteriana *in vivo* mediante la administración de 16 mg de extracto de forma diaria durante 3 días consecutivos en un modelo murino infectado con *S. typhimurium*.
Resultados: La carga bacteriana de *S. typhimurium* en el íleo, el hígado y el bazo se redujo 24 h después de la administración del extracto (p = 0,00008, p = 0,00084 y p = 0,00003, respectivamente).
Conclusión: El extracto etanólico de piel de *C. hystrix* muestra actividad antibacteriana frente a *S. typhimurium*, lo que indica el potencial de *C. hystrix* como tratamiento eficaz para la infección por *Salmonella* spp.

* Corresponding author.
E-mail address: zulhaq@kedokteran.uin-malang.ac.id (Z.S. Ulhaq).

https://doi.org/10.1016/j.eimce.2020.05.016

Introduction

Salmonella spp. are gram negative bacteria that are associated with food and water-borne gastroenteritis and typhoid fever in humans.[1] Systemic infections caused by *Salmonella enterica* serotype Typhi (*S. typhi*) remain a public health concern in developing countries, including Indonesia due to a high incidence rate. A study at the slums of Jakarta province, estimated the incidence rate of typhoid at 148.7, 180.3, and 51.2 per 100,000 person-years in the age groups of 2–4, 5–15, and over 16 years of age, respectively.[2] Furthermore, a community-based case-control study conducted at the Jatinegara district, Jakarta, found that 9% and 3% of 1019 patients with fever, presented *S. typhi* and *S. paratyphi* infections, respectively.[3] A study reported that patients receiving appropriate therapy may reduce the case-fatality rate of typhoid fever to 1–4%.[4] Nonetheless, the proportion of multi-drug resistant (MDR) *S. typhi* tends to increase in several areas in Indonesia.[2,5] Thus, MDR typhoid fever may become a serious problem and requires further appropriate treatment in the future.

Citrus hytrix (Indonesian name Jeruk purut) is a member of the genus *Citrus*, family *Rutaceae*, and has been used as a key ingredient in Southeast Asian cuisines. In Indonesia, the use of *Citrus* is not limited to culinary purpose but also as a remedy for respiratory and gastrointestinal disorders, including *Salmonella* spp. infections, although the mechanism of action remains unclear. Interestingly, mice infected with *S. typhimurium* mimic typhoid like-disease.[1] Therefore, this study aims to investigate the antibacterial activity of the ethanolic peel extract of *C. hystrix in vitro* and *in vivo* in a mice model of *Salmonella enterica* serotype Typhimurium *(S. typhimurium)* infection.

Methods

Plant material and extract preparation

The fruit peels of *C. hystrix* were collected from Malang, East Java, Indonesia. The species of *Citrus* was identified and confirmed by a plant taxonomist of the herbarium unit, UPT Materia Medica, Batu, East Java, Indonesia. Duplicate voucher specimens have been deposited in the herbarium unit, UPT Materia Medica, Batu, East Java, Indonesia. The extract was prepared as previously described.[6] Briefly, the fruit peels were shade dried and ground into a fine powder. Thirty grams of fruit peel powder were then macerated in 100 ml absolute ethanol for 48 h by the Soxhlet extraction apparatus. The extract was evaporated to dry at 40 °C using a rotary evaporator and stored at 4 °C for further use.

Bacterial isolate

S. typhimurium was provided by the Department of Clinical Microbiology, Faculty of Medicine, Brawijaya University and identification was confirmed by the Microbact™ Gram-negative system.

Determination of the minimum inhibitory concentration

The agar dilution method was used to determine the MIC of the ethanolic peel extract of *C. hystrix,* as described by Bailey and Scott's Diagnostic Microbiology.[7] Twofold serial dilutions of the extract (0.3125%, 0.625%, and 1.25%) were prepared in Mueller Hinton agar for the agar dilution assay. Ten microliters of bacterial inoculum were delivered onto the agar with the final inoculum of 10^6 CFU/ml. Bacterial plates were incubated at 37 °C and evaluated after 24 h.

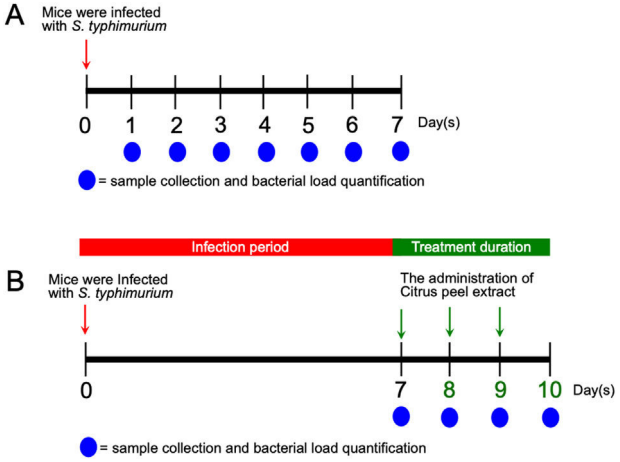

Fig. 1. Experimental timeline. (A) Evaluation of colony counts after mice were infected with *S. typhimurium*; (B) Evaluation of the effectivity of *Citrus* peel extract against *Salmonella* infection.

Animal infection and tissue samples

Female Balb/c mice between 8 and 12 weeks of age were obtained from the Faculty of Medicine, Gadjah Mada University and used in this study. This experiment was divided into two sequential approaches. First, to elucidate the initial stage of transient primary bacteremia in the animal model, thirty-five mice were infected orally with 0.5 ml of 10^8 CFU/ml *S. typhimurium*. Tissue collection procedures were initiated after the mice have been euthanized with isoflurane overdose (mice were placed into a chamber filled with a 5% vapor of the anesthetic isoflurane until respiration ceased, approximately within 2 min). Five mice per day were sacrificed, bacterial counts in the ileum, liver, and spleen were evaluated from day 1 to day 7 post-infection (Fig. 1A). No colonization was observed in the uninfected control (data not shown). One hundred grams of the organs were homogenized and diluted in 2 ml of nutrient broth. Ten-microliters of each samples were then cultured onto solid bismuth sulfite agar (BSA) and incubated at 37 °C overnight. The number of *S. typhimurium* colonies were enumerated in CFU/g. For each sample, the quantification was done in triplicate. Second, to evaluate the antibacterial activity of the ethanolic peel extract of *C. hystrix in vivo*, twenty-five mice were randomly divided into five groups (5 mice each group), which were negative control, positive control, and infected mice treated with the extract orally for one day, two days and three days. The duration of *S. typhimurium* infection was seven days. Five mice of each infected group were sacrificed within 24 h after the last administration of the extract, whereas negative and positive controls were sacrificed at the same time as corresponds to the treatment groups (Fig. 1B). The *in vivo* dose of the extract was quantified by converting % MIC into mg/ml, and then the result was multiplied by a total blood volume of mice.[8] The extract was diluted in distilled water and given orally for 0.5 ml per mouse. The ileum, liver, and spleen were collected and cultured on BSA. Bacterial load was expressed as CFU/g. No colonization was observed in the negative control. The study was approved by the medical ethics committee of the Faculty of Medicine, Brawijaya University, Malang, Indonesia (Reference No. 25-KE).

Statistical analysis

Bacterial load was analyzed by one-way ANOVA, followed by Tukey's *post hoc* test using StatPlus. Significant differences were accepted when $p < 0.05$.

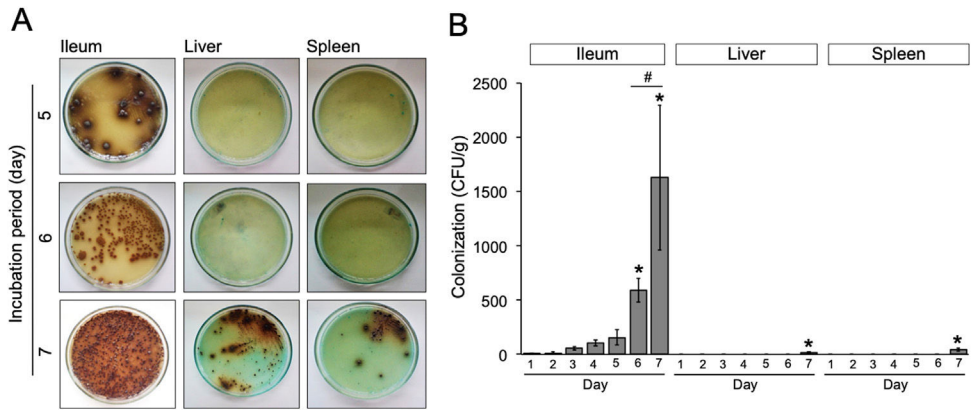

Fig. 2. (A) Growth of *S. typhimurium* colonies on BSA medium. (B) Bacterial load in the ileum, liver, and spleen of *S. typhimurium* infected-model. Data are expressed as a mean ± standard deviation (SD). (*) and (#) indicate a significant difference compared to day-1 post-infection and between two groups indicated in the graphs ($p < 0.05$), respectively.

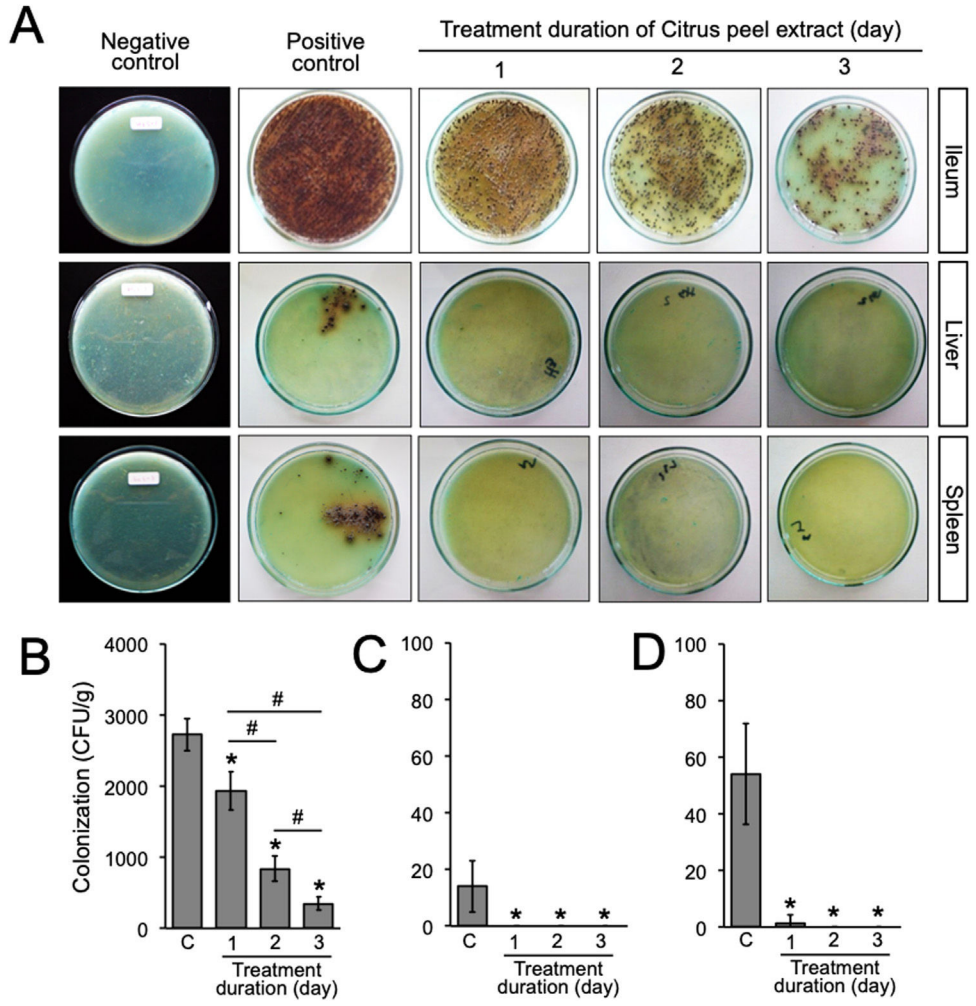

Fig. 3. Bacterial load after the oral administration of *Citrus* peel extract. The colonies were evaluated every 24 h for three days. (A) Growth of *S. typhimurium* colonies on BSA medium; (B–D) Bacterial load in the ileum, liver, and spleen of *S. typhimurium* infected-model, respectively. Data are expressed as a mean ± standard deviation (SD). (*) and (#) indicates a significant difference compared to control and between two groups indicated in the graphs ($p < 0.05$), respectively.

Results and discussion

We evaluated the growth of *S. typhimurium* isolated from the ileum, liver, and spleen on the BSA medium at day-1 to 7 post-infection (Supplementary Table 1). The number of bacterial colonies isolated from ileum was significantly increased at day-6 post-infection ($p = 0.02$) (Fig. 2A) and nearly a 3-folde increased at day-7 post-infection ($p = 0.0001$) (Fig. 2B). In this study, the colonization of *S. typhimurium* in the liver and spleen were found at day-7 post-infection, two more days than previously reported.[9] These differences may be due to individual variation of the immune response among mice model and the inoculum concentration of *S.*

typhimurium. Together, these results suggest that bacteria might be released into the bloodstream between day-5 and 7 post-infection.

A previous report in our laboratory demonstrated that the MIC and minimum bactericidal concentration (MBC) of the ethanolic peel extract of *C. hystrix* against *S. typhi* were 0.625% and 1.25%, respectively.[10] In the same way, our study revealed that the MIC value of the ethanolic peel extract of *C. hystrix* against *S. typhimurium* was 0.625% (data not shown), which then was converted into 16 mg (given orally in 0.5 ml/day for 3 days) for *in vivo* experiments. The administration of the extract was given at day-7 post-infection. Our results showed that the administration of the ethanolic peel extract of *C. hystrix* inhibited significantly the colonization of *S. typhimurium* in the ileum, liver, and spleen after 24 h ($p = 0.00008$, $p = 0.00084$, and $p = 0.00003$, respectively) (Supplementary Table 2, Fig. 3A–D). Thus, our study suggests that daily oral administration of the ethanolic peel extract of *C. hystrix* at 16 mg is effectively inhibiting the colonization of *S. typhimurium in vivo* and might be a potential alternative treatment for *Salmonella* infections.

Several major constituents have been identified in *C. hystrix* peel extract.[11,12] Interestingly, Srifuengfung et al. demonstrated that two active compounds, α-terpineol and terpinene-4-ol, were more concentrated than the other constituents.[12] Moreover, a previous report has shown that α-terpineol displays the most potent antibacterial activity in comparison with the other constituents.[13] Both Gram-positive and Gram-negative bacteria exposed to α-terpineol exhibited morphostructural alterations, mainly causing pores on the outer membrane of *S. typhimurium*.[14] Furthermore, α-terpineol is also able to induce lipid leakage of Gram-negative bacteria.[15] Therefore, it is possible to speculate that the active compounds of *C. hystrix* peels extract might disrupt the membrane integrity of bacteria, which then influence its metabolism. However, further experiments are required to examine the molecular mechanisms of the ethanolic peel extract of *C. hystrix* against bacterial infections, particularly against these caused by *Salmonella* spp.

In conclusion, the ethanolic peel extract of *C. hystrix* exhibits antibacterial activity against *S. typhimurium* both *in vitro* and *in vivo*. These findings imply that peel extract of *C. hystrix* could be an alternative treatment for *Salmonella* infections. Further studies are required to define appropriate *C. hystrix* dosing regimens for *Salmonella* infections and to assess novel treatment strategies, including combination therapies.

Funding statement

None to declare.

Conflict of interest

None to declare.

Acknowledgements

The authors would like to thank Fitri Arkham Fauziah and Siti Wardah for their thoughtful comments toward improving our manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.eimc.2020.05.016.

References

1. Mathur R, Oh H, Zhang D, Park SG, Seo J, Koblansky A, et al. A mouse model of *Salmonella typhi* infection. Cell. 2012;151:590–602, http://dx.doi.org/10.1016/j.cell.2012.08.042.
2. Ochiai RL, Acosta CJ, Danovaro-Holliday MC, Baiqing D, Bhattacharya SK, Agtini MD, et al. A study of typhoid fever in five Asian countries: disease burden and implications for controls. Bull World Health Organ. 2008;86:260–8, http://dx.doi.org/10.2471/blt.06.039818.
3. Vollaard AM, Ali S, van Asten HA, Widjaja S, Visser LG, Surjadi C, et al. Risk factors for typhoid and paratyphoid fever in Jakarta Indonesia. JAMA. 2004;291:2607–15, http://dx.doi.org/10.1001/jama.291.21.2607.
4. Crump JA, Mintz ED. Global trends in typhoid and paratyphoid fever. Clin Infect Dis. 2010;50:241–6, http://dx.doi.org/10.1086/649541.
5. Hatta M, Ratnawati. Enteric fever in endemic areas of Indonesia: an increasing problem of resistance. J Infect Dev Ctries. 2008;2:279–82, http://dx.doi.org/10.3855/jidc.222.
6. Ajithkumar INP, Panneerselvam R. Effect of *Citrus hystrix* and *Citrus limon* extracts on antibacterial activity against human pathogens. Asian Pac J Trop Biomed. 2012;2:1–4.
7. Tille PM, Forbes BA. Bailey & Scott's diagnostic microbiology. Thirteenth edition St. Louis, Missouri: Elsevier; 2014.
8. Fox J, Barthold S, Davisson M, Newcomer C, Quimby F, Smith A. The mouse in biomedical research. Second edition Academic Press; 2007.
9. Chaudhuri D, Roy Chowdhury A, Biswas B, Chakravortty D. *Salmonella* Typhimurium infection leads to colonization of the mouse brain and is not completely cured with antibiotics. Front Microbiol. 2018;9:1632, http://dx.doi.org/10.3389/fmicb.2018.01632.
10. Ellyana I, Santosanigsih D. The effect of the ethanolic peel and leaf extracts of C. hystrix against *Salmonella typhi*. 2008. [Thesis], Faculty of Medicine, Brawijaya University.
11. Kasuan N, Muhammad Z, Yusoff Z, Rahiman MHF, Taib MN, Haiyee ZA. Extraction of *Citrus hystrix* D.C (Kaffir Lime) essential oil using automated steam distillation process: analysis of volatile compounds. Malay J Anal Sci. 2013;17:359–69.
12. Srifuengfung S, Bunyapraphatsara N, Satitpatipan V, Tribuddharat C, Junyaprasert VB, Tungrugsasut W, et al. Antibacterial oral sprays from Kaffir lime (*Citrus hystrix* DC.) fruit peel oil and leaf oil and their activities against respiratory tract pathogens. J Trad Complement Med. 2019., http://dx.doi.org/10.1016/j.jtcme.2019.09.003.
13. Srisukh V, Tribuddharat C, Nukoolkarn V, Bunyapraphatsara N, Chokephaibulkit K, Phoomniyom S, et al. Antibacterial activity of essential oils from *Citrus hystrix* (makrut lime) against respiratory tract pathogens. Sci Asia. 2012;38:7–212, http://dx.doi.org/10.2306/scienceasia1513-1874.2012.38.212.
14. Zengin H, Baysal AH. Antibacterial and antioxidant activity of essential oil terpenes against pathogenic and spoilage-forming bacteria and cell structure-activity relationships evaluated by SEM microscopy. Molecules. 2014;19:17773–98, http://dx.doi.org/10.3390/molecules 319111777.
15. Oyedemi SO, Okoh AI, Mabinya LV, Pirochenva G, Afolayan AJ. The proposed mechanism of bactericidal action of eugenol, α-terpineol and g-terpinene against *Listeria monocytogenes*, *Streptococcus pyogenes*, *Proteus vulgaris* and *Escherichia coli*. Afr J Biotechnol. 2009;8:1280–6.