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Expression of TLR-4 in *Salmonella typhi*-Induced Balb/c Mice Treated by Miana Leaves (*Coleus scutellaroides* (L) Benth)

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ABSTRACT

Introduction: Miana is one of the most widely used medicinal plants in Indonesia because its antibacterial activity, but its mechanisms not clear. This study aims to determine mRNA TLR-4 expression in Balb/c Mice induced by Salmonella typhi after miana leaf extract (MLE) treatment.

Material and Method: Mice were divided into four groups, antibiotic (levofloxacin eight mg/kg body weight) as positive control, placebo (Na CMC), MLE and mixed of MLE and antibiotic. Blood of the mice was taken before (H0) and after induction of 103 CFU/mL Salmonella typhi (H1) and seven days after intervention (H7). The mRNA TLR-4 expression was measured by real time PCR. The results obtained were processed using SPSS.

Results: There were significant difference in H0-H1 for all groups. TLR-4 expression in H1-H7 had a different pattern between placebo and positive control, MLE, and the mixed of MLE and antibiotic. In placebo, there was an increase of TLR-4 expression in H1-H7). In the positive control, MLE treatment group and the mixed of MLE and antibiotic, there was a decrease in TLR-4 mRNA expression.

Conclusions: Miana leaves treatment in Balb/c mice induced by Salmonella typhi significantly gave the same effect as positive control to expression of mRNA TLR-4.

Keywords: *Toll-like Receptor-4 mRNA, Typhoid Fever, Miana, Real-time PCR, CFU.*

INTRODUCTION

Typhoid fever is an acute infection of the digestive tract caused by Salmonella typhi. Typhoid fever is an endemic disease in Indonesia and often cause an outbreak.[1-3] According to WHO data in 2008, patients with typhoid fever in Indonesia recorded 81.7 per 100,000.[4] Typhoid fever in most developing countries is underestimated so that many cases are undiagnosed.[5]

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Salmonella is a gram-negative rod-shaped bacteria, Enterobacteriaceae family, comprising 2463 serovars.[6] Salmonella has a Vi antigen, an acidic polysaccharide polymer present on the surface of the membrane. Salmonella typhi is an intracellular facultative microorganism that can live and even multiply in macrophages, resistant to lysosomes, has the ability to prevent and inhibit fusion of phagolisosomes.[7-9]

One of immunological mechanism against Salmonella typhi is by spurring macrophage function to destroy and eliminate bacteria. By complex of Toll-like receptors (TLR)-5 and TLR-4/MD2/CD-14, macrophages recognize pathogenic molecular patterns

(PAMPs) such as flagella and lipopolysaccharides.[2-3] The bond between LPS and TLR4 that activates MyD88 plays an important role in controlling exponential growth of Salmonella typhi.[10]

The existence of resistance problems of some antibiotics in the treatment of typhoid fever encourages the development of a new paradigm in traditional to treat typhoid fever.[11] Natural products comprise one of the most popular sources of complementary and alternative medicines for treating inflammatory and immune disorders.[12] The facts show that medicinal plants play a vital role in maintenance health because they have many advantages, among others, easy to obtain, very cheap and have very little side effects.[13-15] The use of medicinal plants in most developing countries as a primary means for maintaining health has been considerably observed by UNESCO.[16] Miana leaves contain flavonoid and tannin compounds, in which flavonoid has antibacterial activity because it can denature and coagulate bacterial cell protein.[15]

MATERIALS AND METHOD

Settings and Design

This study was conducted at Laboratory of Molecular Biology and Immunology, Faculty of Medicine, University of Hasanuddin, Makassar, South Sulawesi, Indonesia. This was a laboratory experimental study in Balb/c mice animal model with a simple randomized design.

Balb/c mice

Balb/c mice (35-50 grams) were obtained from the Laboratory of Molecular Biology and Immunology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. They were well kept under both 12-h light and 12-h dark periods, fed and drink sufficiently for seven days. They were divided into four groups (n = five/group) based on intervention, positive control (antibiotics), negative control (placebo), MLE; and mixed of MLE and antibiotics. Blood samplings were performed three times, before Salmonella typhi induction (H0), a day after Salmonella typhi induction (H1) and seven days after intervention (H7).

Miana Leaf Extract (MLE)

Leaves of miana were obtained from Tana Toraja district, South Sulawesi, Indonesia and extracted at Phytochemical Laboratory, Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia. Miana leaves powder were macerated with alcohol 70% for about 48 h occasional shaking. Dose of MLE used ini this study was 510 mg/kg body weight (bw), suspended with natrium CMC 15 w/v.[16]

Salmonella typhi preparation

S. typhi bacteria was from the Molecular Biology and Immunology Laboratory, Faculty of Medicine, University of Hasanuddin, Makassar, Indonesia. The amount was 10^3 CFU/mL (Mc Farland Standard).

Nucleic Acid Extraction

The sample volume of about 100 **µg**/ul blood was fed into 900 **µl** of "L6" solution consisting of 120g of Guanidium thyocyanate (GuSCN) in 100 ml 0.1 M Tris HCl , PH 6.4, 22 ml 0.2 M Ethylene Diamine Tetra Acetate (EDTA) pH 8.0 and 2.6g Triton X-100 (Packard, Instrumens) with final concentration 50 mM Tris HCl, 5 M GuSCN, 20 mM EDTA, 0.1% Triton X-100. Next played at 12,000 rpm. The sediment added a 20 **µl** diatom suspension consisting of 50 ml of H2O and 500 **µl** of 32% (w / v) "Celite" (Jansen Chimica, Beerse, Belgium, 10,846.79), then vortex and centrifuged in a 1.5 ml eppendorf tube at 12,000 rpm for 15 min. The supernatant was removed and the sediment was washed with a solution of "L2" consisting of 120 g of GuSCN in 100 ml 0.1 M Tris HCl, pH 6.4 by adding 1 ml of "L2" solution. Then vortex and centrifuged at 12,000 rpm for 15 min, then washing repeated 2 times using "L2" solution, followed by washing with 1 ml of 70% ethanol twice and 1 ml of acetone. The result was then heated in a water bath at a temperature of 56oC for 10 min and added 60 **µl** of "TE" solution comprising 1 mM EDTA in 10 mM Tris HCL pH 8.0, then vortex and centrifuge followed at 12,000 rpm for 30 s, then incubated in Oven for 10 min at a temperature of 56oC. Then performed vortex and centrifuge again for 30 sec at a speed of 12,000 rpm and taken supernatant. The supernatant of this process will be obtained by nucleotide extraction and stored at -80° C before PCR analysis.[17]

Real Time Polymerase Chain Reaction

Quantitative Real-Time PCR analysis total RNA was extracted from blood using L6 buffer according to the Boom methods. RNA quality and concentration were detected by a NanoDrop 2000 device (Thermo Scientific,

Wilmington, DE, U.S.A.). In a reaction volume of 20 **µL** using M-MLV reverse transcriptase, 2 **µg** RNA was then reverse transcribed to cDNA using a RT-PCR kit. The mRNA level of the target gene was quantified by real-time PCR using a SYBR® Premixed E x Taq kit on a CFX Connect system, Biorad Laboratories, Real Time PCR 96 well 0.1 ml, USA. The standard PCR conditions were as follows: 95ºC (10 min), 40 cycles of 95ºC (15 s) and 60ºC (1 min), followed by a standard denaturation curve. mRNA expression levels of the relevant genes and β-actin were determined using relative quantification by comparison with a standard curve for each gene, which was included in each PCR run generated from the serial dilution of a cDNA pool from the blood samples in the study. The primer pairs TLR-4 For: TGACAGGAAACC CTATCCAGAGTT and TLR4 Rev: TCTCCACAGCCACCAGATTCT and β-actin For: AGA GGGAAATCGTGCGTGAC and β-actin Rev: CAATAGTGATGACCTGGCCGT. Relative mRNA levels were calculated using the 2−ΔΔCt method with data normalized to the Beta actin housekeeping gene.[18-21]

STATISTICAL ANALYSIS

All groups of data are normally distributed with significant values of 0.076-0.200 (Kolmogorov-Spirnov Test) and 0.064-0.964 (Saphiro Wilk Test). Repeated Anova was used to test the mean difference on mRNA expression of TLR-4 between H0, H1 and H7 in each group. All statistics were performed on IBM SPSS version 20 statistical software.

RESULTS

This study showed a significant difference in mRNA TLR-4 expression in H0-H1for all groups with p = 0.000. Differences in mRNA expression of TLR-4 in H1-H7 had a different pattern between negative control and positive control, MLE treatment group and the mixed of antibiotic and MLE treatment group (Figure 1). In the negative control, there was an increase in mRNA expression of TLR-4 from H1 to H7 with significance level p=0.000, mean difference 4.94 and CI95% 4.71-5.17. In the positive control group, MLE treatment group and the mixed of antibiotic and MLE group, there were decrease in mRNA expression of TLR-4 with mean difference in negative values (Figure 1).

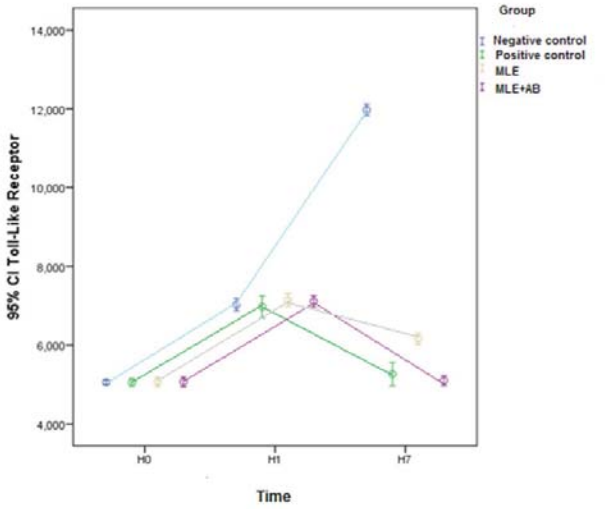

Figure 1: Trend mRNA TLR-4 Expression for placebo, positive control, MLE and Mixed of MLE and Antibiotic in H0, H1 and H7

In the positive control, there was a significant decrease in mRNA expression of TLR-4 with significance level p=0.000, mean difference -1.70 (CI95% (-2.02) - (-1.38), had a greater decrease than the MLE group with mean difference - 0.98(p=0.000, CI 95% (-1.18)-(-0.77)) whereas in the mixed of antibiotic and MLE group had the highest decrease mRNA expression of TLR-4 with mean difference -2.01, significance level p = 0.000 and CI 95 % (-2.27)-(-1.75) (Table 1).

Table 1. Differences Analysis of mRNA expression of TLR-4 Balb/c Mice between before (H0) and 24 hours after (H1) Salmonella typhi induction and Seventh Day After Intervention (H7)

Group	H0	H1	Mean Difference (95%CI)	P*	H7	Mean Difference (95%CI)	P*
	Mean±SD	Mean±SD			Mean±SD		
Negative Control	5.058±0.495	7.025±0.128	1.96(1.82)-(2.11)	0.000	11.971±0.114	4.94(4.71)-(5.17)	0.000
Positive Control	5.057±0.078	6.970±0.225	1.91(1.60)-(2.22)	0.000	5.265±0.238	-1.70(-2.02)-(-1.38)	0.000
MLE	5.071±0.094	7.148±0.132	2.07(1.90)-(2.24)	0.000	6.164±0.118	-0.98(-1.18)-(-0.77)	0.000
MLE+AB	5.069±0.099	7.111±0.118	2.04(1.93)-(2.15)	0.000	5.100±0.102	-2.01(-2.27)-(-1.75)	0.000

DISCUSSION

Significant increase of TLR-4 expression occurred in H0-H1 for all groups. Salmonella typhi has a very immunogenous structure called lipopolysaccharide (LPS). LPS are bound by LPS-binding protein (LBP) in the blood and then activate TLR4. The activated TLR4 recruits the MyD88 adapter protein. Then MyD88 conscripts IRAK4, IRAK1 and IRAK2. The IRA kinase then phosphorylates and activates the TRAF6 protein allowing NFkB to diffuse into the nucleus and activate the transcription and induction of inflammatory cytokines.[3,10,22-23]

At H7, the MLE group showed a significant decrease in TLR-4 expression, as did the positive control and the mixed of antibiotic and MLE group. Alleviation of TLR-4 expression at H7 in the group administered by MLE, positive control and the mixed of antibiotic and MLE group were linear to LPS levels in mice's blood indicating a healing process of Salmonella typhi infection in body of mice. In contrast, TLR-4 expression in negative control continued to increase until H7indicating that LPS remained high. Another study said that the most vital thing in stimulating a non-specific immune response against LPS as a part of Salmonella typhi is activation of TLR-4. The presence of LPS will stimulate TLR-4 then cause nuclear translocation of NFκB and TNF-α cytokines as well as inducible NO synthase (iNOs).[22]

One of the herbal mechanisms as complementary and alternative medicine (CAM) in improving immunity is modulation of pathogen response/T cell regulation. Herbs can enhance immunity by changing the balance between inflammatory and anti-inflammatory cytokines and modifying the level and quality of immune responses of T cells, B cells and cytokines.[24] Miana as one of the medicinal plants family lamiaceae contain among other essential oils, flavonoids, tannins and alkaloids.[15] Administering of MLE and combined antibiotic and MLE had an effect for mRNA expression of TLR-4 subsequently affecting host immunity (mice).

Previous study showed that MLE contains active substances such as alkaloids, saponins, steroids, tannins, triterpenoids, flavonoids and polyphenols that are potential as immunomodulators. MLE contains chemical components as active substances that have antioxidant activity with IC50 MLE value = 34.407 ppm.[15] Another study mentioned that the main active substance of the plant Coleus aromaticus is an essential oil. The potential of Coleus aromaticus therapy can be used as a potential source of bioactive compounds. Coleus aromaticus antioxidant activity is reported mainly due to rosmarinic acid, chlorogenic acid and caffeic acid. Essential oils have a large antimicrobial activity in gram-negative, gram-positive, drug-resistant microorganisms, phytopathogenic microorganisms and fungi.[13,25] According to Kouakou (2013), immunomodulatory activity is determined by knowing the ability of plant extracts to induce NO (nitric oxide), cytokine production and activated mitogen protein kinase fosforilase (MAPK).[26]

CONCLUSIONS

MLE with dosage 510 mg/kg body weight in Balb/c mice induced by Salmonella typhi showed a similar value to positive control on mRNA TLR-4 expression. This study represent that MLE could be a pledging alternative medicine in patients with Salmonella typhi infection.

Conflict of Interest: The authors declare no conflicts of interest regarding the publication of this paper.

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REFERENCES

1. Brusch JL. Typhoid Fever. American College of Physicians Infectious Diseases Society of American, 2016. Avalaible from https://emedicine.medscape.com/article/231135-clinical.
2. Raffatellu M, Chessa D, Wilson RP, Tükel C, Akçelik M, Bäumler AJ. Capsule-mediated immune evasion: a new hypothesis explaining aspects of typhoid fever pathogenesis. Infect Immun, 2006; 74(1):19-27.
3. Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ. Typhoid fever. N Engl J Med. 2002 Nov 28;347(22):1770-82.
4. Depkes RI. Profil Kesehatan Indonesia. Departemen Kesehatan Republik Indonesia. Jakarta, 2010

5. Tarupiwa A, Tapera S, Mtapuri-Zinyowera S, Gumbo P, Ruhanya V, Gudza-Mugabe M, et al. Evaluation of TUBEX-TF and on Site Typhoid IgG/IgM Combo rapid tests to detect Salmonella enteric serovar Typhi infection during a typhoid outbreak in Harare, Zimbabwe. BMC Research Notes, 2015; DOI 10.1186/s13104-015-1015-1.

6. Popoff MY, Bockemuhl J, Brenner FW. Supplement 1998 (no. 42) to the Kauffmann-White scheme. Res. Microbiol, 2000; 151:63–65.

7. Liston SD, Ovchinnikova OG, Whitfield a C. Unique lipid anchor attaches Vi antigen capsule to the surface of Salmonella enterica serovar typhi. Proc Natl Acad Sci U S A 2016 Jun 14; 113(24):6719–6724.doi:10.1073/pnas.1524665113.PMCID: PMC4914157

8. Mohamed E, Dharmana E, Suwondo A, Ausofro M. Effect of Tinospora cordifolia Extract on the Liver Histopathology of Balb/c Mice Infected with Salmonella typhimurium. International Journal of Pharm Tech Research CODEN (USA): IJPRIF, ISSN: 0974-4304 Vol.9, No.4, 2016; pp 344-348.

9. Pui CF, Wong WC, Chai LC, Tunung R, Jeyaletchumi P, Hidayah N, et al. Review Article Salmonella: A foodborne pathogen. Int Food Res J 2011; 18: 465-473.

10. Akira S, Sato S. Toll-like receptors and their signaling mechanisms. Scan J Infect Dis 2003; vol. 35, no. 9, pp. 555–562, 2003.

11. Acar J, Roste B. Antimicrobial resistance: an overview. Rev. Sci. Tech. Off. Int. Epiz 2001; 20(3), 797-810.

12. Huang CF, Lin SS, Liao PH, Young SC, Yang CC. The immunopharmaceutical effects and mechanisms of herb medicine. Cell Mol Immunol,2008;5(1):23-31.10.1038/cmi.2008.3.

13. Bauer N, Vukovic R, Likic S, Jelaska S. Potential of Different Coleus blumei Tissues for Rosmarinic Acid Production. Food Technol Biotechnol, 2015 Mar; 53(1): 3–10. doi: 10.17113/ftb.53.01.15.3661. PMCID: PMC5068421.

14. Verawati, Aria M, Dira, Maisa S, Maharani A. Chemical characterization and anti-inflammatory activity of Piladang Leaf (Coleus Atropurpureus) Extract. Journal of Chemical and Pharmaceutical Sciences ISSN 0974-2115, 2016; vol 6, pp. 2496-2499.

15. Karo M, Hatta M, Salma W, Patellongi I, Natzir R. Effects of Miana (Coleus scutellariodes (L) Benth) to Expression of mRNA IL-37 in Balb/c Mice Infected Candida albicans. Pharmacog J. 2018;10(1):16-9.

16. Syamsuri F, Hatta M, Natzir R, Alam G, Massi N, Dwiyanti R, et al. A Review: Worldwide Medicinal Plants for Typhoid Fever. Indian Journal of Public Health Research and Development, August 2018, Vol.9, No.8; p. 1461-65.

17. Dwiyanti R, Hatta M, Natzir R, Pratiwi S, Sabir M, Yadi Y, Noviyanthi RA, Junita AR, Tandirogang N, Amir M, Fias M, Saning J, Bahar B. Association of Typhoid Fever Severity with Polymorphisms NOD2, VDR and NRAMP1 Genes in Endemic Area, Indonesia. J. Med. Sci., 2017;17 (3): 133-139. DOI: 10.3923/jms.2017.133.139

18. Sirait RH, Hatta M, Arief SK, Simanjuntak TP, Suprayogi B. Profile of HMGB1 mRNA Expression and TLR4 Protein in BALB/c Mice Model Sterile Injury after Systemic Lidocaine Administration. Pharmacog J. 2018;10(3):586-589. DOI : 10.5530/pj.2018.3.96

19. Cezário GA, de Oliveira LR, Peresi E, Nicolete VC, Polettini J, de Lima CR, et al. Analysis of the expression of toll-like receptors 2 and 4 and cytokine production during experimental Leishmania chagasi infection. Mem Inst Oswaldo Cruz. 2011 Aug;106(5):573-83.

20. Yajima T, Yagihashi A, Furuya D, Kameshim H. Quantitative reverse transcription-PCR assay of the RNA component of human telomerase using the TaqMan fluorogenic detection system. Clin Chem. 1998;44(12):2441–5

21. Cui-LT, Zhi Chen. Differential gene expression between asymptomatic HBV carriers and normal adults. Hepatobiliary Pancr Dis Int. 2009;8(4):383-8.

22. Matthew CJ, Royle, Totemeyer S, Louise C, Alldridge, Duncan J, et al. Stimulation of Toll-Like Receptor-4 by Lipopolysaccharide During Cellular Invasion by Live Salmonella typhimurium Is a Critical but Not Exclusive Event Leading to Macrophage Responses. J Immunol 2003,1,170 (11)5445-5454.

23. Fadhilah, Alam G, Hatta M, Natzir R, Bahar B. Expression of Toll-like Receptor-4 (tlr-4) in Balb/c

Mice Induced by Salmonella Typhi. International Journal of Sciences: Basic and Applied Research (IJSBAR) (2017) Volume 36, No 6, pp 42-46.

24. Venkatesha S, Rajaiah R, Berman B. Immunomodulation Autoimmune Arthritis by Herbal CAM. Evidence Based Complementary and Alternative Medicine 2011.
25. Gupta SK, Negi PS. Antibacterial Activity of Indian Borage (Plectranthus amboinicus Benth) Leaf Extracts in Food Systems and Against Natural Microflora in Chicken Meat. Food Technol Biotechnol 2016 Mar; 54(1): 90–1022 (3)
26. Kouakou K, Schepetkin I, Jun S, Kirpotina LN. Immunomodulatory activity of Polysaccharides isolated from Clerodendumsplendes: Beneficial effects in experimental autoimmune encephalomyelitis. BMC Compl and Alter Med 2013; 13:149, 1-19.