Cohort Study

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Decreasing mRNA HMGB1 expression in *Klebsiella pneumoniae* infection treated by Miana (*Coleus scutellarioides* (L.) Benth): a cohort experimental study

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Background: Pneumonia is one of the most common infections caused by the bacterium *Klebsiella pneumoniae*. During the initiation of an infection, the immune system recognizes the pathogen through the release of high mobility group box 1 (HMGB1), thereby triggering the inflammation process. Miana has demonstrated potent inhibitory effects on the inflammatory process during infection in animal models. The aim of this study was to determine the effect of Miana leaf extract on mRNA HMGB1 expression in Balb/c mice infected with *K. pneumoniae*.

Methods: This study comprised a cohort experiment using 20 Balb/c mice divided into four groups. Balb/c mice in each group were intraperitoneally injected with *K. pneumoniae*. Group 1 was given a placebo; Group 2 was given Miana; Group 3 was given levofloxacin; and Group 4 was given both levofloxacin and Miana. The levels of mRNA HMGB1 expression were measured using real-time PCR before, during, and after the infection as well as after the treatments.

Results: The initial examination results showed that the average level of mRNA HMGB1 expression was 5.51 fc. The mRNA HMGB1 expression in mice after being challenged with *K. pneumoniae* was 9.64 fc. Group 1 that was given a placebo had a mean mRNA HMGB1 expression level of 14.99 fc. Group 2 that was given Miana had a mean mRNA HMGB1 expression level of 13.95 fc. Group 3 that was given levofloxacin had an average mRNA HMGB1 expression level of 6.45 fc, and Group 4 that was given levofloxacin and Miana together had an average mRNA HMGB1 expression level of 5.59 fc.

Conclusion: Miana (*Coleus scutellarioides* (L.) Benth) increased mRNA HMGB1 expression at the initial administration via regulation of the immune system. Administration of Miana following *K. pneumoniae* infection inhibited the increase in mRNA HMGB1 expression. Treatment with levofloxacin reduced the level of mRNA HMGB1 expression, and the effect was optimized by the administration of Miana leaf extract as a supplement.

Keywords: high mobility group box 1, *Klebsiella pneumoniae*, Miana (*Coleus scutellarioides* (L.) Benth), mRNA

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Introduction

Infectious diseases remain a massive burden on world health. Pneumonia is one of the most common infectious diseases, and it can be caused by infection with microorganisms such as bacteria, viruses, fungi, and parasites[1]. Pneumonia in Indonesia is often caused by infections with Gram-negative bacteria, including *Klebsiella pneumoniae* (*K. pneumoniae*), *Acinetobacter baumanii*, and *Pseudomonas aeruginosa*[2]. Systemic immunity plays an important role in the early stages of an infection when the immune system recognizes the pathogen through the release high mobility group box 1 (HMGB1). The innate and adaptive immune systems are both involved in the response to pneumonia. In the immune process, dead cells and macrophages will release HMGB1 that will in turn induce the release of proinflammatory cytokines[3]. A previous study examined doxorubicin-induced immune abnormalities and inflammatory responses via HMGB1, HIF1-α, and the VEGF pathway in progressive cardiovascular damage[4]. Furthermore, HMGB1 has been related to head and brain injuries[5,6]. In addition, HMGB1 was increased in closed fracture musculoskeletal

injury through pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP)[7–9].

In a molecular and immunological study of Miana (*Coleus scutellarioides*) used to treat infectious diseases such as typhoid fever, pneumonia, tuberculosis, and candidiasis, the plant extract demonstrated potent inhibitory effects on the inflammatory process in animal models[10–16]. Thus, this study examined the effect of Miana leaf extract on mRNA HMGB1 expression in mice challenged with *K. pneumoniae*.

HIGHLIGHTS

- The immune system recognizes the pathogen through the release of high mobility group box 1 (HMGB1), which increases the inflammation process.
- Miana (*Coleus scutellarioides* (L.) Benth) has demonstrated potent inhibitory effects on the inflammatory process during infection in animal models.
- Miana increased the mRNA HMGB1 expression at the initial administration because it could act as a regulator of the immune system.
- Administration of Miana in *Klebsiella pneumoniae* infection could inhibit the increase in mRNA HMGB1 expression.
- Treatment of levofloxacin reduced the mRNA HMGB1 expression and was optimized by the administration of Miana leaf extract as a supplement.

Materials and methods

Experimental animals

The experiment was designed as a cohort study using Balb/c mice aged 10 weeks and weighing 30–35 g. All mice were in good health and were without disability. The mice were placed in cages of adequate size and were kept under a temperature of 28°C and relative humidity of 40–60%. Mice that died before the end of the study were excluded. Mice were kept for 7 days before being challenged with *K. pneumoniae*. After infection with *K. pneumoniae*, the mice were divided into four groups. Mice in Group 1 were given distilled water as a control. Group 2 mice were treated with Miana. Group 3 mice were treated with levofloxacin as a positive control, and Group 4 mice were treated with levofloxacin and Miana. This study has been reported online following the STROCSS 2021 criteria[17].

mRNA HMGB1 expression

Real-time PCR was used to determine the levels of mRNA HMGB1 expression. HMGB1 mRNA primers for real-time PCR and the PCR conditions were according to a previous research protocol. β-actin was used as a housekeeping gene. The primers used were: HMGB1 Forward: CGTCTGGCTCCCGCTCTCACA; HMGB1 Reverse: GAGTCGCCCAGTGCCCGTC; β-actin Forward: CTGAGAGGGAAATCGTGCGT; and β-actin Reverse: CACAGGGATTCCATACCCAAGA (Macrogen Inc., Seoul, Korea). The qPCR conditions included an initial reverse transcription at a temperature of 48°C for 30 min followed by PCR activation at 95°C for 10 min. This was followed by 45 cycles of 95°C for 15 s and 58°C for 60 s[6–8].

Real-time reverse transcription-PCR (QRT-PCR) was performed using a Green QRT-PCR master mix kit. The protocol was optimized for CFX Biorad instruments. The protocol was adjusted using the instrument by changing dye dilutions based on the instruction manual and the manufacturer's recommendations for the RT-PCR cycle program. A passive dye reference was put in a test tube and diluted to 1:500, and solutions containing dyes were kept away from the light. A 2× dilution of SYBR Green QRT-PCR master mix was prepared and was stored on ice, and the unused portion was stored at 40°C with a note to avoid repeated freeze–thaw cycles. SYBR Green is a fluorescent substance used to stain DNA containing *N′,N′*-dimethyl-*N*-[4-[E-(3-methyl-1,3-benzothiazol-2-ylidene) methyl]-1-phenylquinolin-1-ium-2-yl]-*N*-propylpropane-1,3-diamine (IUPAC). Experimental test tubes contained 12.5 µl of 2 c SYBR Green QRT-PCR master mix, *x* µl of starting primer (optimized concentration), nuclease-free H_2O, *x* µl of final primer (optimized concentration), 0.375 µl of reference dye solution from step 1 (optional), and 1.0 µl of reverse transcriptase (RT) carrying the enzymes modular polymerase and ribonuclease H (RNase H). An enzyme block mixture with 50 µl of the total reaction volume could also be used. The test tubes were mixed slowly (not rotated) so that bubbles did not form, and then the mixture was distributed into the test tubes by adding *x* µl of RNA to each tube. The test tubes were briefly centrifuged, and the PCR program was run using a Real-time PCR machine (CFX Connect system, Biorad Laboratories, USA)[6–8].

Statistical analysis

The data were analyzed using SPSS software version 22.0. The data were tested with Shapiro–Wilks test. The statistical analysis technique using ANOVA test was used to compare the numerical difference in each group. Paired *t*-test and independent *t*-test was used to compare the mRNA HMGB1 expression of each group, before and after experiment. *P* value less than 0.05 was considered significant.

In this study, the authors confirmed that all methods were carried out following the relevant guidelines and regulations of Research ethics committee Medical Faculty of Hasanuddin University, No. 525/UN4.6.5.31/PP36/2021, date 23 August 2021. This manuscript has been reported in line with the STROCSS criteria.

Results

For the first 7 days, all Balb/c mice were adapted to the environment and received the same treatment. After that, *K. pneumoniae* was intraperitoneally injected in all Balb/c mice. The mice in Group 1 were given a placebo. Balb/c mice in Group 2 continued to be given Miana; Group 3 was given levofloxacin, and Group 4 received both levofloxacin and Miana.

The results for mRNA HMGB1 expression in the blood of Balb/c mice in each group are listed in Table 1. Before infection with *K. pneumoniae*, the mice were confirmed to be in good health, and none of the groups had a previous infection. At the initial examination, all mice received Miana. At the first blood test, the average level of HMGB1 mRNA expression was 5.51 fc. Mice were subsequently treated by intraperitoneal injection of *K. pneumoniae*, after which there was a significant increase in the average mRNA HMGB1 expression level to 9.64 fc ($P < 0.05$). The mice that were given placebo or Miana alone showed a significant increase in mRNA HMGB1 expression compared with the initial infection, with the levels being 14.99 fc and 13.95 fc, respectively ($P < 0.05$), but Miana group alone was lower than

Table 1

mRNA HMGB1 expression after administration of Miana leaf extract and levofloxacin in mice induced by *Klebsiella pneumoniae*.

Group	mRNA HMGB1 expression (fc)	*P*
Before infection	5.51	
Infection	9.64	$< 0.05^{*}$
A1 placebo	14.99	$< 0.05^{**}$
A2 Miana	13.95	$< 0.05^{***}$
A3 levofloxacin	6.44	$< 0.05^{****}$
A4 levofloxacin + Miana	5.59	$< 0.05^{*****}$

*Comparison of before infection vs. infection.
**Comparison of placebo vs. infection.
***Comparison of Miana alone vs. infection.
****Comparison of placebo or Miana alone vs. levofloxacin.
*****Comparison of placebo or Miana alone vs. levofloxacin + Miana.

placebo group. The mice that were given levofloxacin alone or levofloxacin plus Miana had significantly decreased compared to placebo or Miana alone with average mRNA HMGB1 expression being 6.44 and 5.59 fc, respectively ($P < 0.05$). The mRNA HMGB1 expression in Group 4 (levofloxacin plus Miana) or Group 3 (levofloxacin alone) was significantly lower compared to initial infection ($P < 0.05$).

The dynamic course of mRNA HMGB1 expression before and during infection and after treatment is shown in Fig. 1. The mRNA HMGB1 expression in all groups was increased after *K. pneumoniae* infection. The mRNA HMGB1 expression in the placebo group continuously increased until the end of the treatment, and this was similar in the Miana group. However, the increase in mRNA HMGB1 expression in the Miana group was less than in the placebo group, while the mRNA HMGB1 expression levels in the groups given levofloxacin or levofloxacin plus Miana decreased after treatment. The level of mRNA HMGB1 expression in the group given levofloxacin plus Miana was lower than with levofloxacin alone.

Discussion

Pneumonia caused by *K. pneumoniae* refers to inflammation of the lung parenchyma due to infection by the microorganisms; the disease usually involves inflammation of the ends of the airways, the alveoli, and the interstitial space of the lungs[12]. HMGB1 is

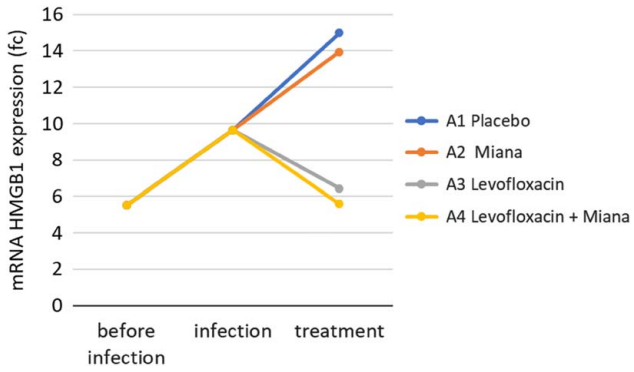

Figure 1. The dynamics of mRNA HMGB1 expression before and during infection and after treatment. HMGB1, high mobility group box 1.

an independent biomarker for pneumonia, for mortality risk in severe pneumonia, for pneumonia due to viral infection, and for acute respiratory distress syndrome (ARDS)[18,19]. HMGB1 also plays a pathogenic role in lung damage due to hypoxia[20]. Leukocyte infiltration promotes HMGB1 secretion due to hypoxia, injury, or inflammatory stimuli[7,8,21]. Extracellularly secreted HMGB1 can stimulate proinflammatory signaling pathways such as the inflammasome and NFKB pathways, resulting in the release of proinflammatory cytokines and acceleration of the inflammatory response[22–24]. Furthermore, HMGB1 can be passively released from damaged cells[4,5]. Extracellular HMGB1, as a DAMP, enables innate immune cells to respond to injury[9,25,26]. Infections may coexist in a general inflammatory process that is regulated by actively or passively released HMGB1[3,27,28]. In this study, there was an increase in HMGB1 mRNA levels in the mice after challenge with *K. pneumoniae*. Due to the increasing resistance of antibiotics in the treatment of pneumonia, it will be important to modulate the immune response in order to deal with infectious diseases in the coming years. Various herbal medicines are known to enhance the immune response at the molecular level. Miana (*Coleus scutellarioides* L. Benth) is an herbal plant from the tropics that can live for many years, and its leaves have various shapes and colors[10,11,13]. The leaves may be yellow, red, cream, or purple-black. The darker the red spots on the leaves, the greater the medicinal benefits. To obtain the benefits of Miana, the leaves need to be extracted. Miana leaf extract contains various compounds such as flavonoids, alkaloids, tannins, saponins, and terpenoids. Flavonoids have antimicrobial, anti-inflammatory, and antioxidant properties[14–16]. In this study, the difference in the increase in mRNA HMGB1 expression in mice given a placebo was greater than in mice given Miana. This was due to the proinflammatory and antioxidant effects of the flavonoid compounds contained in Miana leaf extract[11,13,16]. In mice given the antibiotic levofloxacin, there was a significant decrease in mRNA HMGB1 expression. The decrease in mRNA HMGB1 expression was strengthened when the administration of the antibiotic was combined with Miana leaf extract[13–16]. Flavonoid compounds have antimicrobial effects via inhibiting nucleic acid synthesis and causing damage to bacterial cell walls, microsomes, and lysosomes. In the present study, this led to the effectiveness in reducing mRNA HMGB1 expression by administering a combination of Miana and levofloxacin compared to Miana or levofloxacin alone. Furthermore, the results of this study indicated that although the suppression of mRNA HMGB1 expression after administration of Miana alone was stronger than placebo, it did not show a statistically significant difference. This could be due to the ability of Miana as an anti-inflammatory depending on the duration of the administration of Miana and the number of microorganisms that can cause an increase in HMGB1 expression in the inflammatory process. Therefore, Miana is more appropriate to be used as a supplement together with levofloxacin in the treatment of *K pneumoniae* infection. Besides that, the effect of Miana is not as strongly bactericidal as that of the antibiotic levofloxacin. Thus, future studies are needed in detail about the dynamics and pharmacokinetics of Miana and its relation with mRNA HMGB1 expression in *K. pneumoniae* infection.

Flavonoids in Miana cause increases in T lymphocytes, CD4 T cells, IFN-g, IL-37, TNF-a, TLR4, and NRAMP1. Tannins, saponins, and terpenoids also have antimicrobial activities[10–13].

Thus, Miana leaf extract has a potential role in regulating the immune response against infection. The strength of this study is the apparent effect of Miana alone or levofloxacin + Miana in suppressing HMGB1 mRNA expression. The limitation of this research is that it is still being carried out in animal settings and looking at limited markers. This research cannot be applied directly clinically to humans; it still requires further research to determine the best levels of flavonoids or other substances that can be applied to humans.

Conclusion

There was a significant difference in the mRNA HMGB1 expression in Balb/c mice that received Miana after infection with *K. pneumoniae* compared to the corresponding treatments without Miana. Balb/c mice that received Miana had lower mRNA HMGB1 expression than those that received a placebo. Administration of the antibiotic levofloxacin to mice infected with *K. pneumoniae* reduced the mRNA HMGB1 expression to a level lower than in those of mice receiving a placebo or Miana alone. Administration of the antibiotic levofloxacin reduced the average mRNA HMGB1 expression, and the treatment was optimized by addition of Miana leaf extract.

Ethical approval

This study is an animal experiment. This research has approved the ethical clearance based on the research ethics notification letter from the Faculty of Medicine, Hasanuddin University, on 23 August 2021, with the number 525/UN.4.6.4.5.31/PP36/2021.

Consent

Not applicable.

Sources of funding

Not applicable.

Author contribution

R.A., M.H., M.N.M., I.D., and A.B.: conceived and designed the study, conducted the research, provided the materials, and collected and organized the data; A.A., R.A., R.N., M.H., E.S., A.R. J., A.F., A.A., and A.S.: drafted the manuscript; R.A., A.S., M.R. P., F.M.U., M.F., A.R.J., A.F., A.F., M.H., and B.B.: analyzed and interpreted the data; R.A., R.D., A.S., F.F., A.A., F.M.U., A.R.J., A.F., M.F., and M.H.: wrote the initial and final draft articles, and provided logistical support. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

Conflicts of interest disclosure

The authors declare that there are no conflicts of interest, financial or otherwise.

Research registration unique identifying number (UIN)

Not applicable.

Guarantor

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Data availability statement

Datasets generated and/or analyzed during the current study are publicly available.

Provenance and peer review

Not commissioned, externally peer-reviewed.

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