



Experimental Research

Increasing Natural Resistance Associated Macrophage Protein 1 serum level after Miana treatment in BALB/c induced *Klebsiella pneumoniae* experimental research

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ABSTRACT

Background: Miana (*Coleus Scutellarioides* [L] Benth) inhibits growth of bacterial pathogen inside macrophage. **Objective:** The aim of this study is to determine the protein level of Natural Resistance Associated Macrophage Protein 1 (NRAMP1), after administration of Miana extracts in BALB/c mice induced *Klebsiella pneumoniae*. **Methods:** This is an experimental study using animal model with post test-only controlled group design. Twenty healthy adult male BALB/c mice were randomly divided into four groups, negative control group (distilled water), Levofloxacin 100 mg/kg, injection intraperitoneal, first treatment group (Miana leaves extract/MLE 510 mg/kg) and second treatment group (Miana + levofloxacin). MLE were administered via gastric gavage for ten consecutive days. The blood was drawn from each mice on the first day, on the eight day of experiment (2 h after treatment), and at 10 days. The blood sample was examined by ELISA to determine the NRAMP1 protein level. Analysis of the number of lung tissue bacteria used Plate count agar to see the growth of *Klebsiella pneumoniae*. **Results:** NRAMP1 protein level in BALB/c mice after administration of Miana extract was increased significantly in after 10 days treatment ($p < 0.0001$). The highest increasing in protein levels was found in treatment group (Miana + levofloxacin) with an increase before treatment 3036,07 to 10010,30 pg/ml after treatment $p < 0,0001$. **Conclusion:** NRAMP1 protein level in BALB/c mice were highest increasing in protein levels after administration of Miana extract and Levofloxacin compared Miana or Levofloxacin only and clinical impact proved a comparable effect on suppressing *Klebsiella pneumoniae* growth. The institutional protocol number of this study is 1010/UN4.6.4.5.31/PP36/2019.

1. Introduction

Several study have been reported that herbal medicine could be effective in some diseases [1–5]. Miana (*Coleus scutellarioides*, (L) Benth) is one of herbal medicines used in the Toraja ethnic community, South Sulawesi, Indonesia. Study about traditional medicine in Tana Toraja concluded that the public have been using Miana leaves for tuberculosis treatment. Survey found 74% of tuberculosis patients use traditional

medicine as complementary. Although it has been used empirically for infectious treatment, there is not scientific demonstrations clear yet [6–10]. Therefore, usage of Miana becomes a potential to be studied and developed to understand the mechanisms of Miana leaves.

Previously, study revealed that the effect of immunomodulator and immune responses on Miana leaves for infectious diseases [11–13]. Immunomodulator parameter such as T lymphocyte, CD4 T cell, IFN- γ and TNF- α and other cytokines were measured after herbal medicine

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treated in infection of human and animal model [14–16].

The identification of genetic factors that affect the natural risk regarding infectious disease can give new insight about the defence mechanisms towards infectious disease. The resistance or the vulnerability to intracellular pathogenic infection like *Salmonella* and *Mycobacterium* are controlled by the Natural Resistance associated Macrophage Protein (NRAMP 1) located in chromosome 1, inside macrophage [17,18].

NRAMP1 gene can modulate the function of macrophage and is known to have a relationship with diseases that are linked to immune response. NRAMP1 gene plays an important role as an immune response towards intracellular bacteria. Aside from that, it is possible that NRAMP1 has direct effect with pathogens that survive inside the macrophage, but it may also have a pleiotropic effect including controlling the balance between Th1 and Th2 during the adaptive immune response towards an infection [19].

Mutation in the NRAMP1 gene causes an individual to be more vulnerable towards intracellular pathogenic infection. Within the human blood, polymorphonuclear leukocytes (PMN), expresses messenger RNA NRAMP1 in excess, therefore NRAMP1 plays an important role in these cell's activities. With *northern blot analysis*, mRNA NRAMP1 is mostly only detected in mature neutrophils from the bone marrow, which are neutrophil band and segmented neutrophil. Immunogold study using a cryo-electron microscope with a majority of primary neutrophils (75%) NRAMP1-positive is also positive for gelatinase. The presence of NRAMP1 is in accordance with the presence of mRNA NRAMP1 throughout the maturity of neutrophil in the bone marrow. Immunofluorescence study about *Candida albicans* which contains phagosomes shaped inside the neutrophils shows that NRAMP1 is obtained from the phagosome's membrane during phagocytosis and will later support the role of NRAMP1 during the antimicrobial defence of human neutrophils [20].

Klebsiella pneumoniae stimulates a variety of infection, including pneumonia, urinary tract infection, bacteria and liver abscess. Historically, *K. pneumoniae* has caused serious infectious disease especially on individuals that experience immune disorders, however the incidence and dissemination from the hypervirulent variant expand the amount of individuals which are vulnerable to infection including those who are healthy and immunocompetent. *K. pneumoniae* variant becomes more resistant to antibiotics, causing the treatment for this variant to become more complex. The emergence of hypervirulence and antibiotic resistance has encouraged the development of a handful of new researches. The appearance of *K. pneumoniae* variant that is hard to treat challenges doctors to evaluate the host factor and bacteria throughout the infection. Recently, more researches are identifying the specific virulence factors and innate immune defence. Nevertheless, the idea of the interaction between *K. pneumoniae* with different components of immune response in different tissues is not completed, how the virulence factor cope with the host's defence are also not yet discovered [21].

Although remarkable advances have been made in human medicine, infectious disease still becomes a major public health problem in low and middle income countries, including Indonesia, where lower respiratory tract infections and tuberculosis are the main cause of death. This trend also worsens in Indonesia because the irrational usage of medicine and uncontrolled access towards antibiotics. As a result, infected patients tend to have higher health care costs, longer hospitalizations duration and required second or third line medication treatment that may be less effective, more toxic, and more expensive. In recent years, medicinal plants have attracted the attention of the pharmaceutical and scientific community as a source of antimicrobial substances [22].

The purpose of this study was to determine the expression of NRAMP1 protein level after Miana leaf extract was given to mice infected with *Klebsiella pneumoniae*. Animal models of pneumonia have a pathology identical to that of human pneumonia. Pneumonia must be induced in animals for it to be studied, which mimics human disease in a similar way.

2. Material and methods

2.1. Ethical statement

All experimental protocols employed in this study were approved by the Medical Research Ethics Committee of Hasanuddin University Makassar, Indonesia (1010/UN4.6.4.5.31/PP36/2019). Provenance and peer review are not commissioned, externally peer reviewed.

2.2. Study design

This is an experimental study using 20 healthy adult males BALB/c mice, 8-weeks-old, and weighted approximately 25–35 g. Twenty healthy adult male BALB/c mice were randomly divided into four groups, five for negative control group (distilled water), five for positive control group (Levofloxacin 100 mg/kg body weight), five for first treatment group (Miana extract 510 mg/kg body weight) and five for second treatment group (Miana and levofloxacin). Mice that died or pregnant were dropped out of the study.

2.3. Experimental procedure

Miana leaves that have been collected then dried in the sun for 2 days. Dried miana leaves made into flour using a blender. Miana leaves flour extracted using ethanol solvent with maceration process. Then, the extract is dried using a spinner at 40 °C until crude ethanol extract is formed. Stock solutions of 10% ethanol extract were prepared each by dissolving 10 g of extract in 100 ml of distilled water. Each group was given 10% Miana leaves extract with a single dose, 510 mg/kg body weight. The drugs were administered via gastric gavage with 1 ml syringe.

2.4. Experimental animals

Mice were obtained from the maintenance and development unit of the experimental animal laboratory of Molecular Biology Faculty of Medicine, Hasanuddin University, Makassar, Indonesia. This experiment using 20 healthy adult males BALB/c mice, 8-weeks-old, and weighted approximately 25–35 g.

2.5. Housing and husbandry

The mice were adapted for one week in a room with room temperature of 25 °C, 12-h cycle of light and dark, and were given proper food and drink.

2.6. Experimental outcomes

Mice injected with 0.2 mL *Klebsiella pneumoniae* intraperitoneally (i.p.) at eighth day. The blood was drawn from each mice on the first day (before treatment) and on the eighth day of experiment (2 h after injection intraperitoneally *Klebsiella pneumoniae*) and on the ten day of experiment. The blood sample was examined by enzyme-linked immunosorbent assay (ELISA) Reader 270 (Biomerieux, France) to determine the NRAMP1 protein level. Analysis of the number of lung tissue bacteria (Colony Form Unit) used Plate count agar to see the growth of *Klebsiella pneumoniae*.

2.7. Statistical methods

The data were analysed using SPSS software version 20. The data were tested with Shapiro-Wilks test. The statistical analysis technique using ANOVA test was used to compare numerical difference in each group. Paired T-test and Independent T-test was used to compare the NRAMP1 protein level of each group, before and after experiment. P value < 0.05 was considered significant.

3. Results

Table 1 showed the comparison of NRAMP1 protein levels between before and after treatment from all group using statistical analysis Paired T-test. There was a significant increasing of NRAMP1 protein level from 2782,000 pg/ml before treatment to 7208,078 pg/ml after treatment ($p < 0.0001$).

Statistical analysis using ANOVA test in the four groups (Table 2) showed that the average value of NRAMP1 protein levels before treatment did not show a significant difference between each group ($p = 0,248$), while the contrary was found after treatment ($p < 0.0001$).

Tables 3 and 4 showed the comparison of NRAMP1 protein levels between two groups. There were no significant differences ($p > 0.05$) in NRAMP1 protein level before treatment. However, there were significant differences in NRAMP1 protein level between two groups after treatment ($p < 0.05$), except between Miana and Miana + Levofloxacin 0,0739.

Mean protein level of NRAMP1 before treatment in the control group (distilled water), Miana group, levofloxacin group and Miana + levofloxacin group, although not significant $p = 0,2488$ before treatment did not show a significant difference between each group, after treatment showed a significant difference between each group. Protein level of NRAMP1 showed a significant difference after treatment. Concentration of the NRAMP1 protein level was highest in the Miana + levofloxacin treatment group (mean 10010.3), followed by the Miana group with mean 9305.507, SD 749.52. Then in the levofloxacin group with a mean value 6120.39 and the lowest in the distilled water group (mean 3396.11). No adverse events in each experimental group.

Analysis of the number of lung tissue bacteria used Plate count agar to see the growth of *Klebsiella pneumoniae*. The four groups had a significant difference in the mean comparison. The negative control group was seen the most in *Klebsiella pneumoniae* growth. The group that was given Miana, the average of *Klebsiella pneumoniae* grew 2.8 Colony Forming Unit. In the Levofloxacin and Miana + Levofloxacin groups there was no visible growth of *Klebsiella pneumoniae* as shown as in Table 5.

ANOVA test results on *Klebsiella pneumoniae* growth in the four groups showed a significant difference with a p value of 0.000. The administration of levofloxacin and miana leaf extract to BALB/c mice was proven have an effect on suppressing the growth of *Klebsiella pneumoniae*.

No adverse events in each experimental group.

4. Discussion

The majority of the world's population still uses natural herbal medicines, and Miana is one of the herbal medicines that is often used. Miana leaves extract can inhibit and stop the growth of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Klebsiella pneumoniae*. It also can give a better zone of bacterial growth inhibition than amoxicillin and cefadroxil. Inhibition zone of miana leaves extract is about 25.3–28.7 mm, cefadroxil 16.6–21 mm, whereas amoxicillin 11.6–20 mm. Miana also has a potential as antibacterial for *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Klebsiella pneumoniae* that causes cough [1,5,9].

In this study the NRAMP1 protein level in each mice was analysed two times. The first blood sample was drawn from each mice on the first

day, before drug administration. The second blood sample was drawn from each mice on the tenth day of experiment, after drug administration. The blood sample was examined by enzyme-linked immunosorbent assay.

The NRAMP1 protein levels in the negative control group (distilled water) showed no significant differences with p value of 0.2488, from level of 1971,752 pg/ml before treatment to level of 3396,11 pg/ml after treatment. The NRAMP1 protein levels in the positive control group (levofloxacin) showed an almost significant increment with p value of 0,0001, from level of 3036,065 pg/ml before treatment to level of 6120,396 pg/ml after treatment.

The NRAMP1 protein levels in first treatment group (Miana leaves extract) showed a significant increment with p value of <0.0001 , from level of 2936,676 pg/ml before treatment to level of 9305,507 pg/ml after treatment. The NRAMP1 protein level in the second treatment group (Miana leaves extract + Levofloxacin) showed a significant increment with a p value of <0.0001 , from level of 3185,148 pg/ml before treatment to level of 10010,3 pg/ml after treatment. The results showed a significant increment of NRAMP1 protein levels after administration of Miana leaves extract. Thus, this study proved that Miana leaves extract are associated with changes in NRAMP1 protein levels.

The NRAMP1 protein levels after treatment in the four groups showed a significant difference. The protein in the negative control group (distilled water) was seen to have the lowest level, then followed by a positive control group (Levofloxacin). The highest NRAMP1 protein levels after treatment were obtained in the first treatment group (Miana leaves extract + Levofloxacin) and followed by the second treatment group (Miana leaves extract). The fact showed that the increment of NRAMP1 protein level in the first treatment group, with Miana leaves extract + Levofloxacin, have resulted in higher increment level compared to the second treatment group.

Previous study was conducted a study on animal model of *Salmonella typhi* infection treated with miana leaf extract showing increasing of TLR4 expression [11]. Amsyah et al. conducted a study using miana leaf extract in rat induced *Aggregatibacter actinomycetemcomitans* was found significantly effect had the same effect as levofloxacin on IL-10 mRNA expression [12]. Other study revealed that effects of miana (*Coleus scutellarioides* (L) Benth) to expression of mRNA IL-37 in Balb/c mice infected *Candida albicans* [13].

Gonococcal infection in macrophages induces expression of NRAMP1 (cytosolic iron transport) and iron-carrying protein. These changes in iron homeostasis cause an increase of iron bioavailability which facilitate iron acquisition and increase survival of intracellular gonococcal [23]. Regulation of NRAMP1 in Legionella infection indicates NRAMP1 contributes to phagocyte defence against infections [24]. Mammalian hosts isolate iron, zinc, and manganese ions to limit microbial growth by a process called nutritional immunity, NRAMP1 metal ion transporter can export manganese and iron out of macrophage phagosome to limit the availability of metals to intracellular pathogen [25]. Research conducted by Fritsche et al. showed that NRAMP1 had an effect on *S. typhimurium* infection by increasing the expression of lipocalin 2 [26]. Divalent metal transport through NRAMP1 depends on H^+ . NRAMP1 contributes to defence against infection by extracting divalent cations from the phagosomal space [27]. Because the NRAMP1 protein level is likely to be closely related to the mRNA expression of the NRAMP1 gene, further studies are needed to see whether Miana leaf also affects the mRNA expression of NRAMP1 gene and is associated with the bacterial load of *Klebsiella pneumoniae*.

5. Conclusion

The protein level of Natural Resistance Associated Macrophage Protein 1 (NRAMP1) was increased after administration of Miana leaf extract in BALB/c induced *Klebsiella pneumoniae* mice. Miana leaf extract has been shown have a growth-suppressing effect on *Klebsiella pneumoniae*. This indicates NRAMP1 contributes to phagocyte defence against

Table 1
Comparison of NRAMP1 Protein level before and after treatment Groups.

Variable	N	Mean	SD	P Value
NRAMP1 level before treatment	20	2782,410	1039,000	<0,0001
NRAMP1 level after treatment	20	7208,078	2807,732	

Data were analysed with Independent t -test, p -value of <0.05 was considered significant.

Table 2

Comparison between NRAMP1 protein level before and after treatment of four group.

No	Variable	n	Mean	SD	Min	Max	P Value
1	Before treatment						
	Distilled water	5	1971,752	638,2162	1356,283	2982,723	0,2488
	Miana	5	2936,676	1044,871	1800,807	4498,497	
	Levofloxacin	5	3036,065	929,1524	1907,295	4250,025	
	Miana + Levo	5	3185,148	1277,114	1587,83	4356,513	
2	After treatment						
	Distilled water	5	3396,11	740,1964	2338,924	4168,669	<0,0001
	Miana	5	9305,507	749,5218	8438,073	10200,05	
	Levofloxacin	5	6120,396	1163,12	4643,047	7421,548	
	Miana + Levo	5	10010,3	161,79	9827,32	10233,93	

* Data were analysed with ANOVA test, p-value of < 0.05 was considered significant.

Table 3

Comparison of NRAMP1 protein level before treatment between two groups.

Variable	n	Mean	SD	Min	Max	P Value
1	Distilled water	5	1971,75	638,2162	1356,283	0,1160
	Miana	5	2936,68	1044,871	1800,807	
2	Distilled water	5	1971,75	638,2162	1356,283	0,0677
	Levofloxacin	5	3036,07	929,1524	1907,295	
3	Aquadest	5	1971,75	638,2162	1356,283	0,0939
	Miana + Levofloxacin	5	3036,07	929,1524	1907,295	
4	Miana	5	2936,68	1044,871	1800,807	0,8777
	Levofloxacin	5	3036,07	929,1524	1907,295	
5	Miana	5	2936,68	1044,871	1800,807	0,7450
	Miana + Levofloxacin	5	3036,07	929,1524	1907,295	
6	Levo	5	3036,07	929,1524	1907,295	0,8381
	Miana + Levofloxacin	5	3036,07	929,1524	1907,295	

Data were analysed with Independent t-test, p-value of < 0.05 was considered significant.

Table 4

Comparison of NRAMP1 protein level after treatment between two groups.

No	Variable	N	Mean	SD	Min	Max	P value
1	Distilled water	5	3396,11	740,1964	2338,924	4168,669	0,0000
	Miana	5	9305,51	749,5218	8438,073	10200,05	
2	Distilled water	5	3396,11	740,1964	2338,924	4168,669	0,0022
	Levofloxacin	5	6120,40	1163,12	4643,047	7421,548	
3	Distilled water	5	3396,11	740,1964	2338,924	4168,669	0,0000
	Miana + Levo	5	10010,30	161,7946	9827,324	10233,93	
4	Miana	5	9305,51	749,5218	8438,073	10200,05	0,0009
	Levofloxacin	5	6120,40	1163,12	4643,047	7421,548	
5	Miana	5	9305,51	749,5218	8438,073	10200,05	0,0739
	Miana + Levo	5	10010,30	161,7946	9827,324	10233,93	
6	Levofloxacin	5	6120,40	1163,12	4643,047	7421,548	0,0001
	Miana + Levo	5	10010,30	161,7946	9827,324	10233,93	

Data were analysed with Independent t-test, p-value of < 0.05 was considered significant.

Table 5Comparison of *Klebsiella pneumoniae* colony in lung culture between groups.

No	Variable	n	Mean	SD	Min	Max	P Value
1	Aquadest	5	817,8	405,17	93	999	0,0000
2	Miana	5	2,8	3,89	0	8	
3	Levofloxacin	5	0	0	0	0	
4	Miana + Levofloxacin	5	0	0	0	0	

infection of *Klebsiella pneumoniae*.

These results indicate that clinical impact of the administration of Levofloxacin and miana leaf extract to BALB/c mice proved a comparable effect on suppressing *Klebsiella pneumoniae* growth. Miana leaf extract involves an immune response in the mechanism of reducing infection and potential as a traditional medicine as an antimicrobial.

Ethical approval

All experimental protocols employed in this study were approved by the Medical Research Ethics Committee of Hasanuddin University Makassar, Indonesia (1010/UN4.6.4.5.31/PP36/2019).

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There is no funding on this research.

Author contribution

TDW, MH, AB and AS, MNM initiated and designed the study. TDW, AB, MH and AS, MNM drafted the manuscript. All authors have read and approved the final manuscript.

Registration of research studies

*This study is done on mice.
Name of the registry.
Unique Identifying number or registration ID.
Hyperlink to your specific registration (must be publicly accessible and will be checked).

Guarantor

Mochammad Hatta.

Consent

This experimental study is done on mice. Therefore, there is no written consent.

Declaration of competing interest

There is no conflict of interest or any financial and personal relationship with other people or organisations that could inappropriately influence this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.amsu.2021.102262>.

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