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Original Research Articles

The Effectivity of *Thymus vulgaris* Extract on IL-1 Level and Bacterial Count in The Heart of Balb-c Mice Infected with MRSA

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Article Info

Abstract

Background: The increasing phenomenon resistance of bacteria to various types of antibiotics, for example MRSA (Methicillin Resistant *Staphylococcus aureus*) became a serious problem. Over the last few decades *S. aureus* has become the dominant cause of endocarditis. MRSA data in Indonesia are still rarely reported. This has led to the importance of extracting natural substances that have anti-bacterial effects or that modulate immune response such as *Thymus vulgaris* (TV). It was hypothesized that TV can overcome MRSA infection by increasing IL-1, which is the initial cytokine in a natural immune response stimulated by the presence of *S. aureus*.

Objective: This study was to investigate the effect of *Thymus vulgaris* extract on IL-1 and bacterial counts in the heart of balb-c mice infected with MRSA.

Methods: There were 30 male Balb-c mice randomly divided into six groups. Group K (mice infected with MRSA without treatment),P1(mice treated with TV extract 7 days later infected with MRSA), P2(mice infected and treated with vancomycin), P3(mice infected and treated with TV+vancomycin extract), P4(mice infected and treated with amoxicillin), and P5 (mice infected and treated with TV extract+amoxicillin). IL-1 and bacterial count was assessed by ELISA and microbiological culture.

Results: The result showed that there was a significant increase in IL-1 between groups (p=0.001). The median decrease in the bacterial count in the heart was 0 in almost treatment group except for P4 group which its median was 26.000 (p=0.161). The correlation between IL-1 levels and MRSA count in the heart had a weak negative correlation (r=-0.182).

Conclusion: *Thymus vulgaris* extract was significantly effective to increase IL-1 levels but not proven to decrease MRSA count in the heart of balb-c mice.

Keywords: *Bacterial count; Carditis; IL-1; MRSA; Thymus vulgaris*
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INTRODUCTION

The incidence of infectious diseases including those caused by flora normal in humans such as *Staphylococcus aureus* has increased in recent years. It was usually found in skin infections, however it can also cause bacteraemia, endocarditis, pneumonia and osteomyelitis. *Staphylococcus aureus* was one of the causes of the death in several developing countries including Indonesia.[1,2]

S. aureus infection is a serious problem today due to the increasing resistance of bacteria to various types of antibiotics (Multi Drug Resistance / MDR), such as MRSA (Methicillin Resistant *S. aureus*). There were six reported cases of purulent pericarditis due to MRSA in Jacksonville, Florida, USA in 2019.[1]

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Another unusual case report in New Delhi, India in 2016 of fatal bacterial myocarditis in a seven year old girl, who had a history of cough for a month and fever for two days. Autopsy revealed pyogenic bacterial myocarditis associated with bilateral lobar pneumonia caused by gram positive cocci named *S. aureus*.[3] MRSA data in Indonesia are still rarely reported. However, at this time there was also a discovery on strains of MRSA and *S. aureus* that are resistant to vancomycin (VRSA) in paramedic of RSUD Ratu Zalecha Martapura South Kalimantan.[4] Accessory gene regulator (agr) dysfunction are predictors of VRSA and the presence vancomycin therapy failure.[5] This brought coclusion the importance of extracting natural substances that have anti-bacterial effects or that modulate the immune response.

Research on the anti-bacterial effectiveness of MRSA from natural ingredients (*Althaea officinalis, Ziziphus jujuba, Cordia latifolia,* and *Thymus vulgaris*) has been carried out but only *Thymus vulgaris* was able to reduce the bacterial count in various organs of mice. Thyme (*Thymus vulgaris*) has been used in several countries. According to previous research, *Thymus vulgaris* extract can reduce haematological indicators of infection and reduce the scoring of pathological anatomical lesions.[6] *Thymus vulgaris* oil is a combination of monoterpenes. The most compounds of this oil are thymol and its phenol chemical compound carvacrol.[7] *Thymus vulgaris* contains thymol, carvarcrol, terpenoids, flavonoids, and glycosides so that it has anti-bacterial, anti-viral, antioxidant, immunomodulatory and anti-insecticidal effects.[6,8] The anti-microbial properties of thyme plants are mostly based on in vitro experiments, so it is still necessary to verify the efficacy of thyme plants in vivo.[9] In another in vivo study, *Thymus vulgaris* has the effect of increasing the cytokine IL-12 and decreasing the bacterial count in mice infected with the intracellular bacteria *Salmonella thyphi* but no cytokines has been observed.[10]

The purpose of this study was to prove the effect of *Thymus vulgaris* extract in increasing IL-1 as a pro-inflammatory mediator. IL-1 was able to increase the mobilization of leukocytes and phagocytosis to the site of infection and decrease the bacterial count in the heart of balb-c mice with MRSA infection. The body's defense system is activated by the invasion of *S. aureus* when *S. aureus* as an antigen enters the body, it stimulates phagocytosis by macrophages. Macrophages, dendritic cells, and other cells recognize microbes and respond by producing cytokines. Two of these cytokines, TNF and IL-1, act on the endothelial venules at the site of infection and initiate the migration process of leukocytes into the tissues for the bacteria elimination.[2,10,11]

MATERIALS AND METHODS

This research was an experimental study with the design "Post-test only control group design". The research subjects were 30 male Balb-c mice which were divided randomly into 6 groups infected with MRSA ATCC 43300 0.2 ml (10^7 cfu / ml) intraperitoneal. The research group was divided into 6, namely the control group (K) where the infected mice were not treated; treatment 1 (P1) where the mice were treated with *Thymus vulgaris* extract 7 days later infected with MRSA; treatment 2 (P2) where the mice were infected with MRSA and treated with vancomycin; treatment 3 (P3) where the infected mice were treated with a combination of *Thymus vulgaris* extract and vancomycin; treatment 4 (P4) where the infected mice were treated with amoxicillin, and treatment 5 (P5) where the infected mice were treated with a combination of *Thymus vulgaris* extract and amoxicillin.

The dose of *Thymus vulgaris* extract is 200 mg / kgbb / day or 0,3 ml orally. The dose of amoxicillin was 1 mg/ dose or 0,2 ml/dose. Amoxicillin given 3 times/day. The dose of vancomicin was 5 mg/dose or 0,1 ml/dose given 2 times/day via intraperitoneal injection. The inclusion criteria in this study included: male rats aged 2 months, Balb-c strain infected with MRSA, body weight ± 20-30 grams after acclimatization for a week in individual cages, and no visible anatomical abnormalities. The exclusion criteria in this study were if the mice appeared sick before being infected with MRSA bacteria, while the drop-out criteria were if during induction and treatment, the mice died.

This research was conducted in 3 places, for 8 weeks (July 2018 - August 2018). The process of making *Thymus vulgaris* extract was carried out at Center of Research and Services Universitas Diponegoro (CORES-DU), Semarang. *Thymus vulgaris* were extracted using maceration and vacuum evaporator methods with 96% ethanol solvent, and the results were diluted with *Carboxyl Methyl Cellulose* (until a concentration of 16 mg/ml was reached.; MRSA infection process and treatment (including tissue removal) of experimental animals carried out at the Parasitology Laboratory of Faculty of Medicine, Diponegoro University, Semarang; The process of making and reading the bacterial count preparations was carried out at the Microbiology Laboratory of Faculty of Medicine, Universitas Diponegoro, Semarang.

MRSA ATCC 43300 test bacteria were obtained from the Microbiology Laboratory, Faculty of Medicine, Universitas Diponegoro Semarang with a concentration of 10^7 cfu / ml . The independent variable of this study was the administration of *Thymus vulgaris* extract. The dependent variables in this study were IL-1 levels and bacterial count.

The research data were processed and analysed using SPSS 22.0 for windows. The data analysis included descriptive analysis and hypothesis testing. The data is presented in tables and graphs, then the normality test is carried out using the Saphiro-Wilk test and the homogeneity test. Hypothesis test used in IL-1 is one-way annova test. The hypothesis test used in the bacterial count in the heart of mice is the Kruskall Wallis test. The limit of the degree of significance is if the value p≤0.05 is obtained.

This study used experimental animals by applying animal ethics, which was obtained by submitting approval to the Health Research Ethics Commission of the Faculty of Medicine, Universitas Diponegoro, Semarang, with number 117 / EC / H / FK-RSDK / X / 2018.

RESULTS

Twenty four balb-c mice were adapted for 7 days , there was no sick or dead mice, then randomized into 6

groups. Data analysis of the IL-1 mean levels was presented in Table 1. The normality test with Saphiro-Wilk on the results of measuring IL-1 levels taken 42 hours after the mice were infected with MRSA showed normal data distribution except for groups P5, but after data transformation was carried out, IL-1 levels in all groups were normally distributed.

Further statistical analysis used parametric tests (Table 2). The test for IL-1 levels was carried out using one way Anova. From statistical analysis with the One Way Anova test, it was found that $p < 0.05$ and the homogenicity value > 0.05, which means that there were at least a pair of groups that differ significantly so that it is continued with the Post-Hoc LSD Test (Table 3).
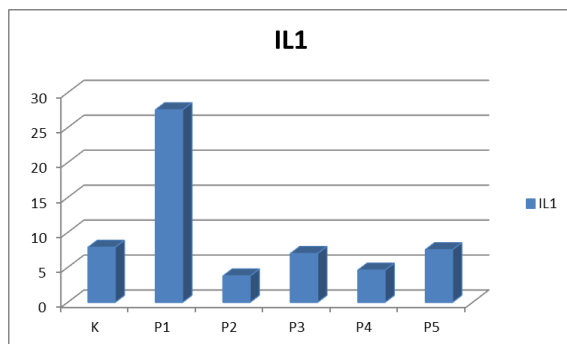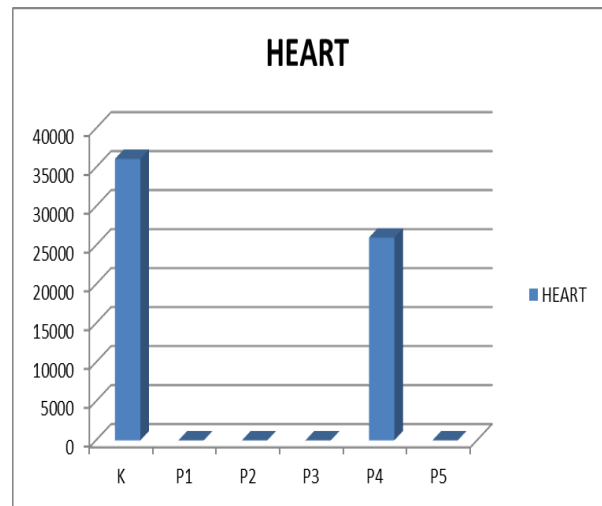

Description : K= mice infected with MRSA and untreated, P1 = mice infected with MRSA + *Thymus vulgaris* extract, P2 = mice infected with MRSA+ vancomycin, P3 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and vancomycin, P4 = infected mice MRSA + amoxicillin, and P5 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and amoxicillin

Figure 1. A bar chart of IL-1 mean levels in each group

Figure 1 shows that the mean of IL-1 levels in the group that received *Thymus vulgaris* therapy tend to be higher than another group who did not get *Thymus vulgaris*. In addition, it can also be seen that the levels of IL-1 in the group that did not receive antibiotics (K and P1) were much higher than the group that received antibiotics (P2, P3, P4, and P5) with or without *Thymus vulgaris*. The difference in mean IL-1 between group K and treatment was statistically significant ($p < 0.05$).

The Saphiro-Wilk normality test (Table 4) on the measurement of the bacterial count in the heart organ taken 42 hours after the mice were infected with MRSA showed insignificant results and it was concluded that the data were not normally distributed, so it was continued with the Kruskal-Wallis analysis test. The results of the Kruskal-Wallis test $p = 0.161$ so it was not followed by other tests. There was no significancy comparison between all group maybe because the small number of the research samples.

In general, it can be seen in figure 2 that the median count of the bacteria in the group that got *Thymus vulgaris* extract was lower than that in the partners group who did not get *Thymus vulgaris*. The highest median value was obtained in the K group, 3.6×10^4 cfu and the lowest median value was 0 cfu which obtained in the treatment group of mice that was only treated by *Thymus vulgaris* (P1), a combination of *Thymus vulgaris* extract and antibiotics both vancomycin (P2) and amoxicillin (P3) and the group that received vancomycin only (P2).

Description : K= mice infected with MRSA and untreated, P1 = mice infected with MRSA + *Thymus vulgaris* extract, P2 = mice infected with MRSA+ vancomycin, P3 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and vancomycin, P4 = infected mice MRSA + amoxicillin, and P5 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and amoxicillin

Figure 2. A bar chart of bacterial count in the heart of each group

From the graph in Figure 2, it can also be seen that there was a decrease in the number of bacterial of the control group compared to the other group that received *Thymus vulgaris* either as a single treatment or in combination with antibiotics.

The correlation test used for IL-1 levels and MRSA counts was the Spearman correlation test. The results of the Spearman correlation test between variables obtained $p=0.395$ and r =-0.182, so it can be concluded that there was a negative correlation between IL-1 and the bacterial count with weak correlation strength.

DISCUSSION

This aims of this study to prove the effect of *Thymus vulgaris* extract to IL-1 and bacterial count in the heart of balb-c mice infected with MRSA. Discussion of the results was carried out sequentially from IL-1 levels and bacterial count in the heart then continued by analyze the relationship between the two variables. In the variable IL-1 levels, there were significant differences between groups. The mean level of IL-1 in the group that got *Thymus vulgaris* was higher than the partner group who did not got *Thymus vulgaris*. The trend of higher IL-1 levels in the group receiving *Thymus vulgaris* compared to their partners supports another study of the pro-inflammatory effects of *Thymus vulgaris* extract in accordance with a previous study conducted by Abousouh et al where *Thymus vulgaris* extract can increase immune response by increasing the proinflammatory cytokine IL-12 in intracellular bacteria *Salmonella thypii*.[10] The higher the IL-1 level, the higher the mobilization of leukocytes and phagocytosis to the infection site for the elimination of MRSA bacteria. This is in accordance with previous studies where *Thymus vulgaris* could significantly decrease MRSA bacterial load of throat, heart, blood and joint of mice.[6]

Table 1 Descriptive and normality of Shapiro-Wilk test of IL-1 level

Group	N	Mean ± SD	Median (min – max)	p	Transf.
K	4	7,85 ± 1,91	8,07 (5,73 – 9,54)	0,233*	0,268*
P 1	4	27,53 ± 12,49	28,19 (11,61 – 42,13)	0,720*	0,382*
P 2	4	3,75 ± 1,38	3,31 (26,3 – 5,73)	0,258*	0,534*
P 3	4	4,59 ± 1,86	4,35 (2,63 – 7,02)	0,450*	0,935*
P 4	4	4,59 ± 1,86	4,35 (2,63 – 7,02)	0,892*	1,000*
P 5	4	7,51 ± 3,40	6,16 (5,16 – 12,57)	0,036	0,104*

Description : * Normal (p > 0,05)
K= mice infected with MRSA and untreated, P1 = mice infected with MRSA + *Thymus vulgaris* extract, P2 = mice infected with MRSA+ vancomycin, P3 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and vancomycin, P4 = infected mice MRSA + amoxicillin, and P5 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and amoxicillin

Table 2 Mean percentage of IL-1 level

Group	Mean ± SD	P	Levene Test
K	7,85 ± 1,91	0,001*	0,139**
P1	27,53 ± 12,49		
P2	3,75 ± 1,38		
P3	4,59 ± 1,86		
P4	4,59 ± 1,86		
P5	7,51 ± 3,40		

Description : * Significant (p < 0,05); ** Homogen (p > 0,05)
K= mice infected with MRSA and untreated, P1 = mice infected with MRSA + *Thymus vulgaris* extract, P2 = mice infected with MRSA+ vancomycin, P3 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and vancomycin, P4 = infected mice MRSA + amoxicillin, and P5 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and amoxicillin.

In the preliminary study, we tested the anti-MRSA effect of thyme and the results from the Minimum Bactericidal Concentration (MBC test) showed no MRSA growth at 1x and 2x concentrations of *Thymus vulgaris* extract. Identification of the content of *Thymus vulgaris* extract based on GCMS analysis in Center of Research and Services Universitas Diponegoro(CORES-DU) obtained peak results of thymol / phenol, 5-methyl-2- (1-methylethyl) (72.95%) and cavacrol / phenol, 2-methyl-5-(1 -methylethyl) (4.23%). Thymol is a naturally phenol monoterpene derivative of cymene and isomer of carvacrol. Thymol also have antimicrobial, antioxidant, antispasmodic, anti-inflammatory, and anti-carcinogenesis activities, as well as immunomodulator. In the present review, these bioactivities have been covered because it can contribute to explain the pharmacology of thymol and its main source, *Thymus vulgaris*.[12] In the in vitro previous study, Oregano oil and thyme red oil and their major common constituents, carvacrol and thymol, significantly inhibited uropathogenic *Escherichia coli* biofilm formation.[13] The free hydroxyl group, hydrophobicity, and the phenol moiety in the carvacrol make its antimicrobial activity higher than the other volatile compounds present in essential oils.[14] The existence of a strong anti-MRSA effect causes the number of bacterial in the host decrease so that the inflammatory response becomes smaller. This explains why IL-1 levels in the group that received no antibiotics and did not receive thyme (K) were much higher than in the group receiving antibiotics either given alone or in combination with *Thymus vulgaris*.

From Figure 2, we could see that the median bacterial count decrease in the group that received additional *Thymus vulgaris* extract therapy. The decrease was greater than the partner group who did not receive *Thymus vulgaris* extract therapy except in the P2-P3 group. This is possible because vancomycin is still a sensitive drug for MRSA therapy. The decreased of difference in the bacterial count occurred in pairs K and P1 and in pairs P4 and P5 although statistically not significant (p = 0.161). The above findings support the proinflammatory effect of *Thymus vulgaris*. The mechanism may be through increased phagocytosis, leukocytes and macrophages mobilization by IL-1 especially seen in the P4 group (only treated with amoxicillin) and the P5 group (*Thymus vulgaris* therapy + amoxicillin) and between the control (no therapy) and P1 groups (only *Thymus vulgaris* treatment.). In the remaining 2 groups there was also a greater reduction in bacterial count when combined with *Thymus vulgaris* although the difference was not statistically significant. This may be because the number of replicated samples is too small to prove this difference statistically. Another factor is the short administration period of *Thymus vulgaris* and the sensitivity of vancomycin as MRSA therapy. Because of its sensitivity, vancomycin and daptomycin are approved as a monotherapy for

Table 3. Post hoc LSD test of IL-1 levels

Group	P1	P2	P3	P4	P5
K	0,004*	0,049*	0,315	0,127	0,816
P1	–	<0,001*	<0,001*	<0,001*	0,003*
P2		–	0,296	0,615	0,077
P3			–	0,579	0,436
P4				–	0,190

Description : * Signifikan (p < 0,05)
K= mice infected with MRSA and untreated, P1 = mice infected with MRSA + *Thymus vulgaris* extract, P2 = mice infected with MRSA+ vancomycin, P3 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and vancomycin, P4 = infected mice MRSA + amoxicillin, and P5 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and amoxicillin

Table 4. Descriptive and normality of Shapiro-Wilk test of bacterial count in the heart of balb-c mice

Group	Mean ± SD	Median (min – max)	P
K	400550 ± 753581,41	36100 (0 – 1530000)	0,004
P1	13500 ± 27000	0 (0 – 54000)	0,001
P2	27000 ± 54000	0 (0 – 108000)	0,001
P3	0,00 ± 0,00	0 (0 – 0)	–
P4	360000 ± 446616,91	26000 (0 – 920000)	0,272*
P5	0,00 ± 0,00	0 (0 – 0)	–

Description : * Normal (p > 0,05)
K= mice infected with MRSA and untreated, P1 = mice infected with MRSA + *Thymus vulgaris* extract, P2 = mice infected with MRSA+ vancomycin, P3 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and vancomycin, P4 = infected mice MRSA + amoxicillin, and P5 = mice infected with MRSA and treated with a combination of *Thymus vulgaris* extract and amoxicillin

methicillin-resistant *S. aureus* (MRSA) bacteraemia.[15] In the previous systematic review and meta-analysis study of evaluation of vancomycin MIC creep in MRSA infection, we have found no evidence of the MIC creep phenomenon (MRSA isolates with high MICs, within the susceptible range).[16]

Analysis of the correlation between IL-1 levels and the number of MRSA bacteria found a negative weak correlation between IL-1 levels and MRSA counts in the heart of balb-c mice. This means that the higher the IL-1 level, the decrease in the bacterial count in the host. This may be because MRSA elimination occurs not only due to increased IL-1-mediated phagocytosis but also due to the anti-bacterial properties of *Thymus vulgaris*. Based on previous study, *Thymus vulgaris* demonstrated significant antibacterial activity against *E. faecalis* and can be used as an alternative therapy.[17] Another study showed that oregano oil, thyme oil, carvacrol and thymol showed good in vitro bactericidal and fungicidal activity against 100 isolates from dogs with otitis externa.[18]

In this study, the mean levels of IL-1 in the combination of antibiotics with *Thymus vulgaris* extract (P3 and P5) were found to be higher than in the treatment group that only received antibiotic therapy either vancomycin (P2) or amoxicillin (P4). Looking at the bar graph (Figure 1 and Figure 2), the increase in IL-1 levels on combination therapy has a greater effect on reducing the number of MRSA germs compared to the partner group who only received antibiotic therapy. This study is in accordance with previous studies where it was reported that there was a synergy between thyme essential oil in combination oxycycline therapy with *Melaleuca armillaris* essential oil in reducing the MIC of *S. aureus*.[9]

This study also showed that *Thymus vulgaris* extract increased potentiation of antibiotics that were known to have been resistant previously. The combination of *Thymus vulgaris* extract with amoxicillin can increase the ability of amoxicillin antibiotics in inhibiting the growth of MRSA bacteria. MRSA has been shown to be resistant to the antibiotic amoxicillin. This can be seen from the Figure 2 in the P4 group. There were still found amount MRSA bacteria in the P4 group who only got amoxicillin therapy. Fewer numbers of MRSA bacteria were found in the P5 treatment group where *Thymus vulgaris* extract was given with amoxicillin antibiotics. This opens up new horizons that *Thymus vulgaris* extract can be used to fight bacterial resistance. These findings support other studies that is reported that carvacrol acts alone or in combination with erythromycin in reducing the number of *erythromycin-resistant Group A Streptococci* so that it can have potentiation as a new therapeutic tool.[19] Other studies suggest that various herbal plants can have resistance-modifying activities found in bacteria. Previous study shown that *Thymus vulgaris* essential oil combined with cefotaxime showed a synergistic action against blaSHV-12 producing *Escherichia coli* and additive effect vs ESBL producing *Enterobacter cloacae*.[20]

CONCLUSION

There are significant IL-1 levels differences between groups. The mean level of IL-1 in the group that get *Thymus vulgaris* is higher than the partner group who do not get *Thymus vulgaris*. There are decrease in bacterial count in the heart of balb-c mice who get *Thymus vulgaris* extract than the partner group who do not receive *Thymus vulgaris* extract therapy although statistically not significant. This is possible because vancomycin is still a sensitive drug for MRSA therapy and amoxicillin found to be resistant to MRSA. There are negative weak correlation between IL-1 levels and MRSA counts in the heart of balb-c mice.

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