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The Changes of HIF-1α and ICAM-1 Expression after Miana (*Coleus Scutellariodes* [L]) Treatment in Balb/C Mice with *Mycobacterium Tuberculosis* Infection

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Increasing resistance to TB drugs raises the challenge of TB eradication. Miana leaves is Indonesian traditional herbal medicine, have antimicrobial, anti-inflammatory, and immunoregulatory action. Not much is known about the effect of Miana on HIF-1a and ICAM-1, the immunoregulators of infection and inflammation. This study aims to elucidate the effect of Miana on HIF-1a and ICAM-1 in M. tuberculosis (Mtb) infected mice. This experimental study used Mtb infected Balb/c mice were divided into 4 groups; group 1 is placebo, group 2 is treated with Rifampicin as Anti TB drug, group 3 is treated with Miana, and group 4 is treated with Miana + Anti TB drug. HIF-1a and ICAM-1 serum levels were analyzed using ELISA. There is a significant difference of mean HIF-1a (p= 0.00, F = 114.21) and ICAM-1 (p= 0.00, F = 113.11) between the four groups after treatment. HIF-1a level is significantly lower in anti TB treatment, Miana, and Miana + anti TB treatment compared to placebo (mean difference (MD) 35,764.67, p=0.00; 29,230.98, p=0.000; 38,489.62, p=0.00, respectively). Furthermore, ICAM-1 level is significantly lower in anti TB treatment, Miana, and Miana + anti TB treatment compared to placebo (MD 95,449.68, p=0.00; 79,509.69, p=0.00; 108,672.83, p=0.00, respectively). HIF-1a and ICAM-1 expression was reduced after Miana administration. Miana can be a potential complement to anti-TB treatment but cannot replace rifampicin as anti-TB drugs.

Keywords: Coleus scutellariodes; HIF-1a; ICAM-1; Miana leaf; M. tuberculosis; Rifampicin.

Tuberculosis (TB) is one of the major global health problem. It is estimated that two thirds of TB burden lies in eight countries including Indonesia which held 8% of the burden. WHO 2018 report estimated there were more than 1 million new TB cases in indonesia.[1,2] Tuberculosis has been increasingly harder to treat because of the growing problem of multi-drug-resistant (MDR) TB. Therefore, there is a need for additional means to antituberculosis drugs to treat TB.[3,4,5,6]

The use of traditional herbal medicine has been a staple alternative and complement to conventional medicine for Indonesian. Miana (*Coleus scutellariodes [L] Benth)* is known as one of the traditional herbal medicine that is often used. It contains flavonoid, tanin, triterpenoid, steroid and atsiri oil that have antibacterial, antiinflammatory and antioxidant properties.[7,8,9,10] In one in vitro study, Miana extract can suppress the growth of *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *M. tuberculosis*.[6,11,12,13,14]Administration of Miana extract also increases the number of CD4 T cells, IFN-ã levels, and TNF-á and also reduces the *M. tuberculosis* colonies in Wistar mice's lungs. Miana extract can affect proliferation of T cells in mice that was given 510mg/kgBW of Miana extract.[15,16,17,18,19]

Miana has an effect on host immune response. HIF-1á and ICAM-1 played an important role in immune response to bacterial infection. *M.tuberculosis* infection triggers the host's immune response and causes inflammation. Local hipoxia caused by the inflammation increases cellular level of HIF-1á.[10,11] HIF-1á induces phagocytotic activities, nitric oxide synthase (NOS) and antimicrobial peptides which have a direct antimicrobial activities. ICAM-1 is also stimulated by inflammation and facilitates the transmigration of leukocytes.[20]

Not much is known about the effect of Miana leaves extract on HIF-1á and ICAM-1 in *M.tuberculosis* infection. This can be useful to find out how the molecular mechanism of Miana against *M. tuberculosis* infection and is a modality for prevention, supplementation and treatment in TB patients.

This study aims to elucidate the effect of Miana on HIF-1á and ICAM-1 in Balb/c mice infected with *M. tuberculosis*.

MATERIAL AND METHODS

Ethical Statement

This study was conducted in accordance with standard guidelines for the use and care of experimental animals. The use of these animals will be reviewed and approved by the ethics committee of the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia, No: 177/UN4.6.4.5.31/ PP36/ 2021, date: 17 March 2021.

Research design

This experimental study used 20 Balbc/c mice and was conducted in July 2020 in Molecular Biology and Immunology Laboratory, Faculty of Medicine, Hasanuddin University (UNHAS), Makassar, Indonesia. The mice were divided into four groups which Group 1 is a negative control group treated with placebo; Group 2 is a positive control group treated with Rifampicin as anti TB drug); Group 3 is an intervention group treated with Miana extract; and Group 4 is an intervention group treated with Miana extract and Rifampicin as anti TB drug.

Balb/c mice

12 weeks old pathogen free Balb/c mice weighing 30-40 grams were used in this experiment. The animals were acclimatized for 7 days before the start of the study, all mice were kept in a room temperature, a 12-hour light and dark cycle, and were given standard natural pellet food and adequate drink[3,4,5,8,9]

Miana Extract

Miana leaves were obtained from Toraja, South Sulawesi, Indonesia. The Miana extract was made using 10 grams of Miana Leaves that were washed and dried in 50°C oven. The dried leaves were grinded and sieved using size 100 mesh sieve to achieve fine powder form. A total of 30 grams of Miana powder was diluted with ethanol with 1:10 ratio and mixed using a shaker for 24 hours in room temperature. The mixture was filtered using Whatman filter with 20 µm of pore size . The filtrate was evaporated using a rotary evaporator in 50°C temperature until concentrated and then dried using a freeze dryer. The concentrated extract was made into a pellet. Keep in black light glass bottle and the pellets were stored in a refrigerator until use. The pellets will be diluted with distilled water (Otsu-WI) and given to the mice using a nasogastric tube for 7 days.[3,4,5,7,9,14,21]

Research Protocol

Shown on Figure 1, all 20 Balb/c mice were put into 4 groups randomly. On day 0, the first blood draw of 0.2 ml of venous blood in tail was drawn (before infection and before treatment blood sample) from all mice in all groups and all mice were infected by *M. tuberculosis*. On day 14 after infection, the second blood draw (after infection before treatment blood sample) was taken. Group 1 was given 10 mg/kgBW/day of distilled water as placebo, group 2 was given 78 mg/kgBW of Rifampicin as anti TB drug, group 3 was given Miana extract, group 4 was given anti TB drug and Miana extract. On day 14 after treatment and day 28 after infection, the final blood draw in tail vena was done[3,7,9,11,13].

Venous blood sample was centrifugated to obtain the blood serum. The serum was stored in -20^{o} C until analysis. HIF-1á and ICAM-1 protein level was measured in pg/ml. HIF-1á and ICAM-1 protein level before induction, before treatment, and after treatment of all groups were done. All sample analysis was done in duplicate to ensure validity of ELISA analysis results.

Statistical Analysis

Normality test was done using Shapiro-Wilk test and Levene's test was done for homogeneity test. Protein levels of each group were presented in means and standard deviation. One-way ANOVA test was used to determine the differences between the means of HIF-1á and ICAM-1 protein levels of the experimental groups. Post-hoc analysis was done to further elucidate the differences of means. P value below 0.05 is determined as statistically significant. All statistical analyses were done using SPSS 20.0 software for Windows.

RESULTS

Table 1 showed HIF-1á and ICAM-1 protein levels in each group. The highest HIF-1á protein levels before infection were found in group 1 (mean ± SD: 16,275.15 ± 3,660.47; 15,287.939 ± 4,346.87; 15,060.39 ± 4,482.69; 14,537.32 ± 3,030.49, respectively), followed by group 2, 3 and 4. HIF-1á protein levels after *M. tuberculosis* infection before treatment were highest in group 3, followed by group 4, 1, and 2 (mean ± SD: 48,996.60 ± 1,880.71; 46,719.28 ± 3,258.97;

Table 1. HIF-1α and ICAM-1 levels in before infection, after M.tb infection and after treatment of each group

	Group	Before infection	After Mtb Infection	After Treatment	F	p value
HIF-1α	Anti-TB drug*	15,287.93 ± 4,346.87	45,388.40 ± 4,205.40	19,643.73 ± 4,229.83		
	Miana	15,060.39 ± 4,482.69	48,996.60 ± 1,880.71	26,177.42 ± 2,647.28		
	Anti-TB drug + Miana	14,537.32 ± 3,030.49	46,719.28 ± 3,258.97	16,918.79 ± 1,967.44	114.21	0.00
	Placebo	16,275.15 ± 3,660.47	45,914.73 ± 3,636.34	55,408.41 ± 5,095.27		
ICAM-1	Anti-TB drug	61,600.63 ± 1,1104.79	141,055.38 ± 10,881.41	74,025.65 ± 13,324.45		
	Miana	65,033.86 ± 10,309.90	148,821.02 ± 10,819.83	89,965.65 ± 6,229.42	113.11	0.00
	Anti-TB drug + Miana	54,353.08 ± 4,494.03	142,790.84 ± 8,440.84	60,802.51 ± 9,207.74		
	Placebo	70,476.65 ± 10,567.54	138,759.95 ± 7,690.45	169,475.34 ± 10,914.71		

*Anti -TB drug : Anti Tuberculosis (Rifampicin)

45,914.73 ± 3,636.34; 45,388.40 ± 4,205.40, respectively). After treatment, the lowest levels of HIF-1á were found in group 4, followed by group 2 and 3 and the highest levels in group 1 (mean ± SD: 16,918.79 ± 1,967.44; 19,643.73 ± 4,229.83; 26,177.42 ± 2,647.28; 55,408.41 ± 5,095.27, respectively).

Before infection, ICAM-1 levels were highest in group 1 (mean ± SD: 70,476.65 ± 10,567.54), then group 3 (mean ± SD: 65,033,86 ± 10309.90), group 2 (mean ± SD: 61,600.63 ± 1,1104.79), Group 4 (mean ± SD: 54,353.08 ± 4,494.03). After infection, ICAM-1 levels increased and were highest in group 3 (mean ±

Table 2. Mean comparison of HIF-1α and ICAM-1 levels in each group after intervention

Group		HIF-1 α		ICAM-1	
		Mean Difference	p value	Mean Difference	p value
Anti-TB drug	Miana	-6,533.69*	.01	-15,939.99*	.02
	Anti-TB drug* Miana	2,724.94	.26	13,223.14	.05
	Placebo	-3,5764.67*	.00	-95,449.68*	.00
Miana	Anti-TB drug	6,533.69*	.01	15,939.99*	.02
	Anti-TB drug Miana	9,258.63*	.00	29,163.14*	.00
	Placebo	-29,230.98*	.00	-79,509.69*	.00
Anti-TB drug + Miana	Anti-TB drug	-2,724.94	.26	-13,223.14	.05
	Miana	-9,258.63*	.00	-29,163.14*	.00
	Placebo	-38,489.62*	.00	-108,672.83*	.00
Placebo	Anti-TB drug	35,764.67*	.00	95,449.68*	.00
	Miana	29,230.98*	.00	79,509.69*	.00
	Anti-TB drug + Miana	38,489.62*	.00	108,672.83*	.00

*Anti -TB drug : Anti Tuberculosis (Rifampicin)

Table 3. Observation of changes in the *M.tuberculosis* count in mice

Group	Mice	Result (AFB/100 field)		
		Before Infection	Post Infection	After treatment
Anti-TB drug	1	0	178	0
	2	0	182	0
	3	0	197	0
	4	0	201	0
	5	0	173	0
Miana	1	0	191	95
	2	0	186	55
	3	0	180	70
	4	0	175	62
	5	0	195	67
Anti-TB drug + Miana	1	0	180	0
	2	0	187	0
	3	0	196	0
	4	0	202	0
	5	0	193	0
Placebo	1	0	176	1321
	2	0	188	1542
	3	0	185	1500
	4	0	200	1278
	5	0	190	1652

SD: 148.821.02 ± 10,819.83), followed by group 4 (mean ± SD: 142,790.84 ± 8,440.84), group 2 (mean ± SD: 141,055.38 ± 10,881.41), and group 1 (mean ± SD: 138,759.95 ± 7,690.45). After treatment, ICAM-1 levels decreased with the lowest ICAM-1 levels found in group 4 (mean ± SD: 60,802.51 ± 9,207.74), then group 2 (mean ± SD: 74,025.65 ± 13,324.45), Group 3 (mean ± SD: 89,965.65 ± 6,229.42), and the highest in group 1 (mean ± SD: 169,475.34 ± 10,914.71).

One-Way ANOVA test was performed to determine whether there was a significant difference between the mean protein levels in each group after being given treatment (table 1). Results showed a significant difference in HIF-1á and ICAM-1 protein levels between four groups (HIF-1á (P value = 0.00, F count = 114.21), ICAM-1 (P value 0.00, F count = 113.11)).

A post-hoc test (table 2) was conducted to further analysed the differences in the levels of HIF-1á and ICAM-1 between groups. HIF-1á level is significantly lower after rifampicin, Miana, and Miana + rifampicin administration compared to placebo (mean difference 35,764.67, p=0.00; 29,230.98, p=0.00). ICAM-1 level is significantly lower after rifampicin, Miana, and Miana + rifampicin administration compared to placebo (mean difference 95,449.68, p=0.00; 79,509.69, p=0.00; 108,672.83, p=0.00, respectively). There was no difference in protein levels of HIF-1á and ICAM-1 levels between the group given anti TB drug + Miana compared to the group that were given anti TB drug alone.

Observation of bacterial load between mice in each group showed a decrease in bacterial load up to no bacteria seen in microscope's field of view after treatment in the group that was given Anti TB drug alone and the group that was given Anti TB drug combined with Miana. In the group that was given Miana alone, the bacterial load also

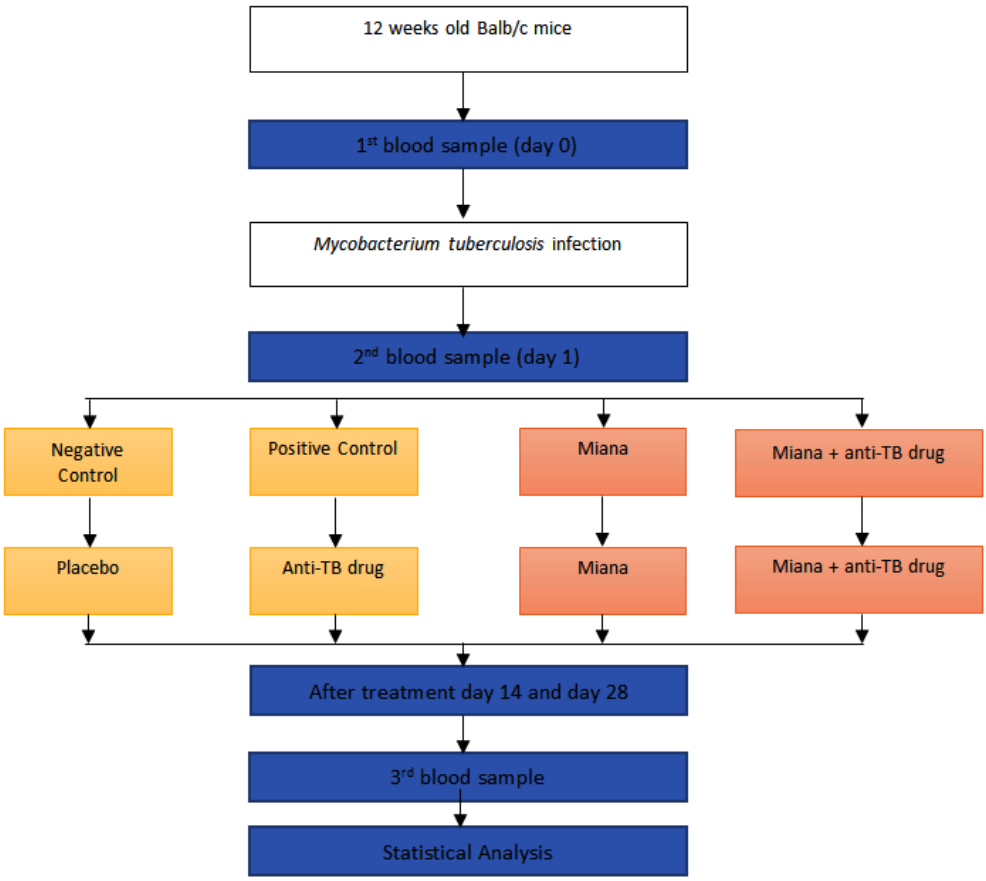

Fig. 1. Research protocol flow chart

decreased dramatically even though there were still few bacteria seen in observation (table 3).

DISCUSSION

The results of this study showed that the pattern of mean protein levels of HIF-1á and ICAM-1 in mice with tuberculosis infection were similar after administration of Rifampicin as anti-TB drug, Miana extract, anti-TB drug and Miana, and distilled water as placebo. The levels of HIF-1á and ICAM-1 in mice with tuberculosis infection were significantly lower after administration of Miana extract than mice given placebo. The same thing happened to infected mice that were given Anti TB drug and Anti TB drug + Miana. However, the levels of HIF-1á and ICAM-1 were still lower in mice given Anti TB drug than in Miana extract alone. There was no significant difference between the levels of HIF-1á and ICAM-1 between mice given Anti TB drug and Miana compared to mice given Anti TB drug.

Various studies have shown HIF-1á levels are increased in *M.tuberculosis* infection in response to local tissue hypoxia.[22,23] Hypoxia-inducible factor 1 alpha (HIF-1á) is induced by pro-inflammatory cytokines, growth factors, and various infections. Its induction is a common component of the body/host response to infection. HIF-1á is required for pro-inflammatory Th17 cell differentiation, activation and regulation of pro-inflammatory cytokine release.[22,23,24,25] HIF-1á levels decreased after administration of antituberculosis drugs and the same thing happened after administration of Miana extract. However, there was no significant decrease in HIF-1 levels between the administration of anti-TB drug alone compared with the administration of anti-TB with Miana extract.[12,14,26]

ICAM-1 levels are known to be elevated in *M.tuberculosis* infection. In this study, ICAM-1 levels were also increased in the serum of Balc/c mice infected with *M.tuberculosis*.[27] ICAM-1 levels decreased after the intervention. Giving Miana extract or anti TB drug alone to mice infected with *M.tuberculosis* caused a decrease in ICAM-1 levels. However, there was no significant decrease in ICAM-1 in mice that were given Anti TB drug with the addition of Miana extract compared to those given Anti TB drug alone. This shows that Miana extract has a similar effect to Anti TB drug on Mtb. However, the mechanism by which Miana reduces ICAM-1 levels in *M.tuberculosis* infection has not been elucidated in this study.[28,29,30]

Based on the results of this study, Miana extract had the same effect as Anti TB drug in mice infected with tuberculosis. However, the mechanism by which Miana can reduce HIF-1á levels has not been elucidated in this study. One theory that can explain this phenomenon is the content of flavonoids, tannins, saponins, and terpenoids in Miana leaves which have antimicrobial, anti-inflammatory and antioxidant properties.[11,31,32]These effects may play a role in reducing inflammation due to infection and leading to improvement of cellular hypoxic conditions, suppressing bacteria, inflammation and overcome ROS and hypoxia results in a decrease in the induction of HIF-1á production.[4,33,34,35,36,37] While ICAM-1 is increased due to proinflammatory cytokine response due to *M.tuberculosis* infection. Miana's effect can decreased inflammation. Thus, the inducer of ICAM-1 activation decreases so that ICAM-1 levels decrease.[21,38,39]

Although the role of Miana extract on HIF-1á and ICAM-1 levels is known by this study, this study cannot explain the exact mechanism of how Miana extract affects HIF-1á and ICAM-1 in *M.tuberculosis* infection. However, Miana extract which contains flavonoids which acts as a type of antibacterial as well as immunoregulatory to enhance host immune response to fight bacteria and anti-inflammatory action. In this study may explain the possible molecular mechanism of Miana on TB infection via HIF-1á and ICAM-1 pathway. Further research is needed to elucidate more specific of the pharmacodynamics of Miana leaf extract on immunoregulation of host.

CONCLUSION

Miana leaf extract can reduce the HIF-1á and ICAM-1 protein levels. There was no significant difference in HIF-1á and ICAM-1 levels between mice given anti TB drug alone and mice given anti TB drug with additional Miana leaf extract. The levels of HIF-1á and ICAM-1 were significantly lower in mice given Rifampicin as anti TB drug and anti TB drug with the addition of Miana leaves. This suggests that the administration

of Miana leaf extract has the same effect as anti TB drug but cannot replace the function of anti TB drug. Miana extract can be a complement or adjuvant to anti TB drug although the effect of anti TB drug with Miana complement on HIF-1á and ICAM-1 is not significant.

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Conflict of interest

There is no conflict of interest.

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