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Original Article

The Increased Superoxide Dismuatase (SOD) in Mice Infected by *Plasmodium Berghei* ANKA Treated with Nanoparticle Extract of Beetroot *(Beta Vulgaris L)*

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Abstract

Background : Malaria infection causes increased free radicals which leads to severity and decreases antioxidant activity, thus increasing the risk of severe malaria complications. Beetroot extract has active compounds that function as anti-inflammatory and antioxidants. Nanoparticles are a technology that can be used to improve drug delivery efficiency in smaller doses. The aims of this study was to prove the effectiveness of beetroot extract nanoparticles on SOD levels in mice infected with malaria and treated with artemisinin

Methods : An experimental study using a post-test-only randomized control group design. The research sample used 30 male Balb/c mice divided into 6 groups. Group 1 was the healthy group, group 2 was the infected group without treatment, group 3 was the infected group with artemisinin treatment, group 4 was the infected group with artemisinin treatment and 50 mg/kgBW/day beetroot extract nanoparticles, group 5 was the infected group with artemisinin treatment and 100 mg/kg BW/day beetroot extract nanoparticles, and group 6 was the infected group with artemisinin treatment and 200 mg/kg BW/day beetroot extract nanoparticles. Beetroot extract and artemisinin supplementation were given after parasitemia index > 1% and given for 4 days. On the 5th day after therapy, serum SOD levels were measured using ELISA.

Results : The measurement of SOD levels in the artemisinin group supplemented with nanoparticle extracts of beetroot at doses 100–200 mg/KgBW were 21,48–21,59 ng/ml. Kruskal Wallis and Mann Whitney test showed that they are significantly higher serum SOD levels compared to the infected mice group (p<0.05).

Conclusion : Supplementation of beetroot extract nanoparticles has an antioxidant effect by increasing SOD levels in mice infected with malaria and receiving artemisinin therapy.

Keywords : malaria; antioxidant; betacyanin, artemisinin

INTRODUCTION

Malaria is a parasitic infectious disease that is a global health priority, with 241 million cases reported worldwide in 2020 in 85 endemic countries. Indonesia is one of the countries with a high incidence of malaria, reaching 254,055 cases in 2020. This has become a concern for the government to gradually implement a malaria elimination program.[1] The formation of free radicals in malaria infection can be caused by two factors, those are the immune response to malaria and produced by malaria itself. Activation of the immune system will increase the production of reactive oxygen species (ROS) during phagocytosis as an effort to eliminate plasmodium. Additionally, during the erythrocytic phase, plasmodium will degrade hemoglobin and release free heme and H_2O_2, which are oxidative to host tissues. Excessive ROS production in malaria infection causes redox imbalance, which triggers the body to combat free radicals using antioxidants. However, there is a lack of antioxidant activity for host defense during infection.[2,3]

Superoxide dismutase (SOD) plays a role in converting free radicals into hydrogen peroxide, which will subsequently be degraded by catalase and glutathione. Several studies have shown a relationship between SOD activity and tissue damage, which SOD level can serve as a marker of malaria severity.[4] This creates an opportunity for the development of antioxidant supplementation in malaria infection.

Beetroot is a plant from the Amaranthaceae family that contains bioactive compounds such as polyphenols, carotenoids, flavonoids, betanin, and betalains. Several studies have shown that beetroot extract has antioxidant and anti-inflammatory effects.[5,6] Beetroot extract has a stronger antioxidant effect than vitamin C due to the presence of betasianin. Beetroot extract supplementation also protects against free radicals in rats induced by a high-fat and fructose diet by increasing the expression of SOD2 and CAT genes.[7] The active compounds in natural materials cannot penetrate cells effectively, which reduces their effectiveness. It can be solved by increasing their effectiveness and absorption, one of which is through the application of nanomedicine technology. Nanoparticles are a technology that can be used to improve drug delivery efficiency in smaller doses by increasing the drug's absorption rate and reducing enzyme biodegradation.[8–10]

Several studies have shown that beetroot extract supplementation at a dose of 100–300 mg/kgBW/day provides anti-inflammatory and antioxidant effects. However, there is no current research on beetroot extract nanoparticles.[11,12] Therefore, this study uses a dose of 50, 100, and 200 mg/kgBW/day for 4 days of beetroot extract nanoparticles. The nanoparticle extracts of beetroot in this study were prepared using the ionic gelation method using chitosan and NaTPP.

This study aims to determine the effect of beetroot extract nanoparticle supplementation on SOD levels in mice infected with malaria and treated with artemisinin.

MATERIALS AND METHODS

An experimental study was done with a post-test-only randomized controlled group design. The study was conducted at the Pharmacology and Parasitology Laboratory, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada Yogyakarta, Indonesia, after obtaining ethical approval from the Health Ethics Committee. The Ethical clearance issued by The Health Research Ethics Committee Faculty of Medicine Universitas Diponegoro.

Animals and Experimental Groups

The study used 30 male Balb/c mice aged 6–8 weeks with a body weight of 25–35 grams, divided into 6 groups. The research group division was as follows:

Baseline group: Healthy mice

Negative control group: Mice inoculated with *Plasmodium berghei* ANKA

Positive control group: Mice inoculated with *Plasmodium berghei* ANKA and treated with artemisinin (ART) at a dose of 0.036 mg/gr BW

Treatment group I: Mice inoculated with *Plasmodium berghei* ANKA and treated with artemisinin (ART) at a dose of 0.036 mg/gr BW + 50 mg/kg BW beetroot extract nanoparticles

Treatment group II: Mice inoculated with *Plasmodium berghei* ANKA and treated with artemisinin (ART) at a dose of 0.036 mg/gr BW + 100 mg/kg BW beetroot extract nanoparticles

Treatment group III: Mice inoculated with *Plasmodium berghei* ANKA and treated with artemisinin (ART) at a dose of 0.036 mg/gr BW + 200 mg/kg BW beetroot extract nanoparticles.

The experimental animals in this study were a malaria model with inoculation using *Plasmodium berghei* ANKA. The *Plasmodium berghei* ANKA isolate was obtained from parent mice with a parasitemia level of 20% and was injected intraperitoneally into infected mice at a volume of 0.2 ml containing 10^7 parasitized erythrocytes. Parasitemia was calculated on day 3 post-inoculation, and after parasitemia reached >1%, artemisinin and nanoparticle extracts of beetroot were administered for 4 days.

Preparation of Extraction and Nanoparticles

The material used in this study was beetroot extract nanoparticles. The beetroot extract was made using the maceration method using 96% ethanol, then filtered and evaporated using a rotary evaporator until a concentrated beetroot extract was obtained. Nanoparticles were made using a formulation of beetroot

extract, chitosan, and Na TPP (ionic gelation method). Chitosan and NaTPP were used in a ratio of 4:1, and the weight of the beetroot extract was adjusted to make doses of 50 mg, 100 mg, and 200 mg. The resulting beetroot extract nanoparticles were then characterized using a Particle Size Analyzer (PSA) to measure particle size and polydispersity index.

Blood Sample Collection and Biochemical Analysis

Specimen collection and termination were performed on day 5 post-therapy, followed by an examination of SOD levels using an ELISA Kit from Bioassay Technology Laboratory (BT Lab) Cat No. E2608Mo. Blood samples were taken through the eye vein using a microhematocrit. Blood serum was separated by centrifugation after 15 minutes. All animals were anesthetized with 0,5 ml ketamin then cervical dislocation was performed.

The measurement of SOD level using a microplate then add 50 standard to standard well. Add 40 µl sample to sample wells and then add 10 µl Mouse SOD1 antibody to sample wells, then add 50 µl streptavidin-HRP to sample wells and standard wells. Mix well then cover the plate with a sealer. Incubate 60 minutes at 37°C. Remove the sealer and wash the plate 5 times with wash buffer. Add 50ul substrate solution A to each well and then add 50 µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark. Add 50 µl Stop Solution to each well, the blue color will change into yellow immediately. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Statistical Analysis

The results of SOD level were then analyzed to determine the significant difference in the supplementation of beetroot extract nanoparticles on SOD levels. The results of the homogeneity test using the Shapiro-Wilk test showed that the data was not normally distributed even after data transformation, so the difference test was analyzed using the Kruskall-Wallis test followed by the Mann-Whitney test to determine the differences between groups in this study. The results of statistical analysis were presented using median (minimum-maximum) and *p-value*, in which a *p-value* <0.05 is considered significantly different.

RESULTS

Characterization of Beetroot Extract Nanoparticles

The characterization of beetroot extract nanoparticles showed that a dose of 50 mg/kgBW had a dark brown color, while at a dose of 100 mg/kgBW was yellowish brown, and at a dose of 200 mg/kg BW was yellow. No sediment in all formulations was found. The characterization of beetroot extract nanoparticles at various doses describes particle size and molecular dispersion index as shown in Table 1 and Figure 1.

TABLE 1
Formulation and Particle Size Analysis

Symptoms AR	Extract Weight	Chitosan Weight	NaTPP Weight	Z Average	Pd Index
Nanoparticle Extract of Beetroot 50 mg/kbBW	150 mg	80 mg	20 mg	282.1 nm	0.381
Nanoparticle Extract of Beetroot 100 mg/kbBW	300 mg	80 mg	20 mg	327.3 nm	0.281
Nanoparticle Extract of Beetroot 200 mg/kbBW	600 mg	80 mg	20 mg	543.7 nm	0.769

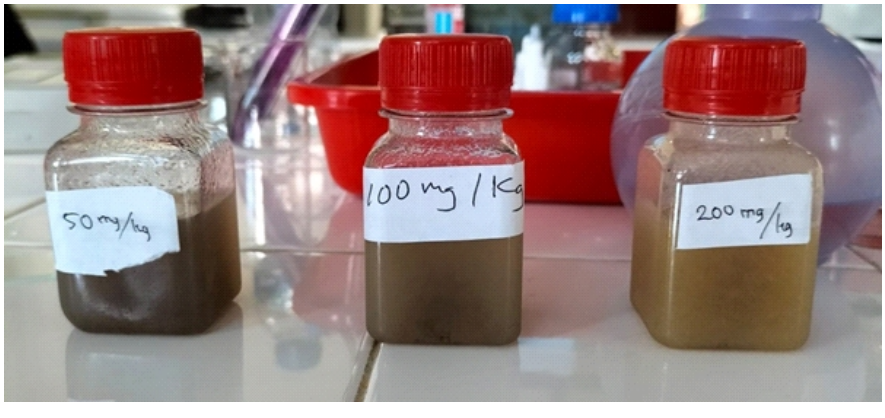

Figure 1. Nanoparticle Extract of Beetroot at doses 50 mg/kgBW(a), 100 mg/kgBW (b) dan 200 mg/kgBW (c)

TABLE 2
Statistical Analysis of SOD Levels in Beetroot Extract Nanoparticle Supplementation

Group	Median (Min–Max) ng/ml	Kruskal-Wallis Test
Baseline	23.89 (21.91 – 41.17)	p = 0.010*)
Negative control	14.44 (13.26 – 19.29)*	
Artemisinin	22.92 (17.32 – 26.22)#	
ART+NEB 50 mg/kgBW	14.67 (13.29 – 21.94)*	
ART+NEB 100 mg/kgBW	21.59 (18.21 – 26.54)#	
ART+NEB 200 mg/kgBW	21.48 (18.89 – 22.77)*#	

Data as presented as median (n=5 for each group)
* p<0.05 compared to baseline;
# p<0.05 compared to negative control

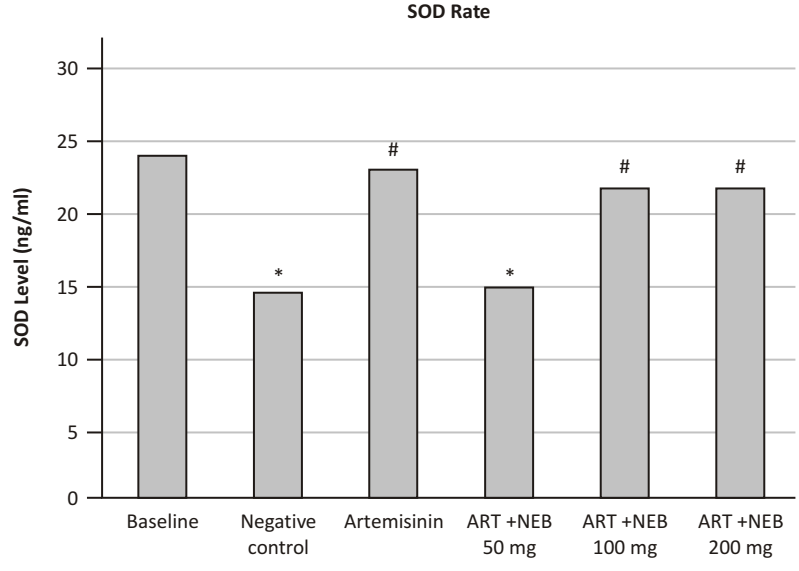

Figure 2. Effect of nanoparticle extract of beetroot in SOD level on mice inoculated with *Plasmodium berghei* ANKA and treated with artemisinin. * p<0.05 compared to baseline; # p<0.05 compared to negative control.

Superoxide Dismutase (SOD) serum Level

Examination of SOD Levels using Serum Specimens with ELISA Method. The examination was read using an ELISA reader at a wavelength of 456 nm and the following results were shown in Table 2 and Figure 2.

The result showed that the SOD level in normal mice is 23.89 ng/ml. Malaria infection affects SOD levels in mice, where the negative control group had the lowest SOD level of all groups, at 14.44 ng/ml. Artemisinin administration in the positive control group showed a higher SOD level than the negative control group, at 22.92 ng/ml. Supplementation with beetroot extract nanoparticles in the treatment groups showed varying results in increasing SOD levels, namely 14.67 ng/ml at a dose of 50 mg/kg BW/day; 21.59 ng/ml at a dose of 100 mg/kg BW/day; and 21.48 ng/ml at a dose of 200 mg/kg BW/day.

Kruskal-Wallis test showed a significant difference between the treatment groups, and the Mann-Whitney test was used to determine which groups were significantly different. Mann Whitney test showed a significant difference in SOD level between the negative control group and the combination group of artemisinin and NEB at 50 mg/kg BW compared to the baseline group, which is the SOD level in these groups was significantly lower than the baseline group. Meanwhile, the negative control group showed a significant difference with the artemisinin group and combination with NEB 100–200 mg/kg BW, in which the SOD level in the combination of artemisinin and NEB were significantly higher. However, there was no difference in SOD level between artemisinin groups and supplementation with NEB.

DISCUSSION

Supplementation with beetroot extract nanoparticles could be a promising future complementary therapy along with artemisinin therapy for malaria infection. Malaria infection by *plasmodium* can lead to oxidative stress and an imbalance in antioxidant activity in the body.[13] This study showed that the lowest SOD levels were found in the group of mice infected with malaria without treatment, which proves that malaria infection causes an increase in the production of free radicals that trigger oxidative stress and inhibit antioxidant activity. Malaria infection triggers the immune system to eliminate parasites, thereby increasing pro-inflammatory cytokines. This causes an increase in the production of free radicals that can harm the body by damaging cells and tissues and causing an imbalance in antioxidant activity in the body. SOD plays an important role in controlling free radicals that can lead to serious complications in malaria patients. SOD acts as an antioxidant by converting free radicals into hydrogen peroxide (H_2O_2), which is then removed by catalase and glutathione. This highlights the importance of supplementation with antioxidant therapy as an adjunct to antimalarial drugs to control malaria infection and prevent the occurrence of serious complications of malaria later on.[4,14,15]

Supplementation with beetroot extract nanoparticles in mice infected with and receiving artemisinin treatment showed higher SOD levels. Beetroot contains active compounds such as betalains, nitrates, and phenolic compounds. Betalains are the main content of beetroot which will be converted into betacyanins and betaxanthins, while betacyanins will be converted into betanin which has antioxidant and anti-inflammatory properties. Betanin has antioxidant properties because it can scavenge radicals, donate hydrogen and electrons, decompose peroxides, or quench singlet oxygen. Betanin has a hydroxyl group from the phenol group that is similar to the phenolic antioxidant ethoxyquin, which gives betanin its properties as a hydrogen donor and free radical reducer.[5,16,17]

Betanin also plays a role in regulating the activity of antioxidant enzymes by modifying cysteine residues and breaking the Kelch-like repressor protein ECH-associated Protein 1 (KEAP1) and Nuclear factor erythroid 2 related factor 2 (Nrf2) bonds so that Nrf2 accumulates and can bind to Antioxidant response element (ARE). This binding will upregulate genes encoding antioxidants and phase II enzymes. In addition, betanin also activates mitogen-activated protein kinase (MAPK) which can lead to phosphorylation and stabilization of Nrf2, thereby increasing the translocation of Nrf2 in the nucleus. Upregulation of Nrf2 target genes will stimulate the activity of antioxidant enzymes and restore the redox balance in the body. This leads to the role of SOD as an antioxidant to convert free radicals into H_2O_2 and converted into H_2O and O_2 by catalase so that cells and tissues are protected from the effects of oxidative stress and prevent the occurrence of serious complications in malaria.[18–21]

The antioxidant activity of beetroot is related to its betalain content. This is also due to nitrates, amino acids, peroxidase enzymes, and β-glucosidase, which act as radical scavengers. The results of this study are consistent with research showing that beetroot juice has potential anti-plasmodial effects by reducing the parasitemia index and potentially serving as an adjuvant therapy for artemisinin. The free radical effects produced during inflammation and artemisinin administration can be balanced with the antioxidant effects of beetroot extract nanoparticles, thereby reducing tissue damage and preventing severe malaria.[22–24]

CONCLUSION

Supplementation with beetroot extract nanoparticles in malaria-infected mice with artemisinin treatment showed antioxidant capacity that can control free radicals by increasing SOD activity. Therefore, supplementation with beetroot extract nanoparticles can be used as an adjuvant therapy for malaria treatment to prevent the occurrence of severe malaria complications.

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