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Anti-Malaria Study of *Nigella sativa* L. Seed Water Extract in *Mus musculus* Mice *Balb* C Strain *In Vivo*

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Abstract

Nigella sativa L. has been reported to exhibit many pharmacological effects, including anti-parasitic properties. This study investigated the anti-malarial effects of a water extract of *N. sativa* seed in *Mus muculus* mice infected with the *Plasmodium berghei* NK65 parasite. The method used was to take a blood parasitaemia count, following the use of Giemsa dye, determining the level of nitric oxide in mice that were infected with *P. berghei* malaria, using the spectrophotometric method, and determining their survival rate after 20 days of being infected with *P. berghei* malaria. The results showed that the decrease in the number of parasitaemia and the level of nitric oxide in subjects treated with doses of *N. sativa* was significant ($p < 0.05$). Further results showed that *P. berghei*-infected mice that were given 100 µg/kg of body weight had a better chance of survival. The conclusion is that the provision of *N. sativa* may reduce the number of malaria parasites and reduce levels of NO. The decrease in the number of parasites may be caused by an immune mechanism, through the regulation of NO levels (lower levels of NO), due to the influence of the anti-oxidant effects of *N. sativa*. Survival rates of the mice did not show significant results with reduced levels of parasitaemia and NO. This is likely to be because the levels of NO in this group were below the threshold levels at which NO can function as an anti-parasitic. It is alleged that, while NO can function as an anti-parasitic at certain levels, at lower levels its function as an antiparasitic is not optimal. On the other hand, if the levels are too high, damage will result, because of the nature of free-radicals.

Abstrak

Pengujian Efek Anti Malaria Ekstrak Air Biji *Nigella sativa* L. Secara *In vivo* pada Mencit (*Mus musculus*) Galur Balb C. *Nigella sativa* L. dilaporkan memiliki berbagai efek farmakologis termasuk sebagai anti parasit. Pengujian efek antimalaria dari ekstrak air biji *N. sativa* secara *invivo* terhadap mencit (*Mus musculus*) yang diinfeksi parasit malaria *Plasmodium berghei NK65* telah dilakukan. Metode yang digunakan adalah pengujian mikroskopik dengan pewarnaan Giemsa terhadap sampel darah hewan uji. Penentuan kadar NO dengan metoda spektrofotometri dilakukan untuk mengetahui efek ekstrak air biji *N. sativa* terhadap kadar NO mencit yang terinfeksi parasit malaria *P. berghei NK65*. Ekstrak air biji *N. sativa* diberikan kepada mencit yang terinfeksi parasit malaria dalam periode 20 hari untuk mengetahui efek pemberian ekstrak air tersebut terhadap kelangsungan hidup (*survival life*) mencit. Hasil penelitian menunjukkan bahwa ekstrak air biji *N. sativa* dapat menurunkan jumlah parasit malaria *P. berghei NK65* dan menurunkan kadar NO pada hewan coba pada dosis yang digunakan. Pemberian ekstrak air biji *N. sativa* pada dosis 100 µg/kgBB memberikan efek relatif lebih baik terhadap kelangsungan hidup mencit yang diinfeksi *P. berghei NK65* dibanding dosis lain. *N. sativa* dapat menghambat jumlah parasit dan kadar NO. NO dapat berfungsi sebagai anti parasit pada kadar tertentu, apabila kadarnya terlalu kecil fungsi sebagai antiparasit tidak optimal, namun jika kadarnya terlalu tinggi akan bersifat merusak karena sifat radikal bebasnya.

Keywords: anti-malaria test, levels of NO, Nigella sativa L.*, parasitaemia, survival rate*

1. Introduction

Malaria remains a major health problem in Indonesia. Almost all provinces in Indonesia are malaria-endemic areas, with varying levels of endemicity. In Indonesia, malaria is estimated to infect about 15 million people per year. Efforts to combat the malaria parasite have long been of concern to the WHO. However, there are still many obstacles. The main obstacle is the resistance of malaria parasites to a variety of anti-malarial drugs.

In addition, invector mosquitoes are resistant to the insecticides that are used [1]. *Plasmodium falciparum* resistance to chloroquin was first reported in Thailand in 1957 [2]. In Indonesia, cases of *P. falciparum* resistance to chloroquin were first discovered in 1974, in eastern Kalimantan. Resistance continues to expand, and by 1996 there were reported cases of malarial resistance to chloroquin found in all provinces of Indonesia. The broad and rapid deployment of malaria parasites that developed resistance in nearly all endemic areas encouraged researchers to discover new anti-malarial drugs, by conducting research into medicinal plants. The success of Chinese researchers, who derived the new anti-malarial drug artemisinin from the medicinal plant *Artemisia annua,* proves the potential for medicinal plants to be used against malaria [3].

In Indonesia, research on the activities of antiplasmodial of *Nigella sativa* fruit extract against *P. falciparum* invitro have been reported by Kurniawan in 2005 [2].

Nigella sativa (*Jinten hitam* in Indonesian language) is known to be one of the medicinal plants that has a variety of pharmacological effects, such as being anti-oxidant, anti-inflammatory, anti-bacterial, anti-viral, anti-allergy, anti-dysentery, and anti-ulcer, as well as being an immunobioregulator, an immune-booster, and having many other properties [4,5]. Extracts from *N. sativa* also contain a variety of different types of alkaloids, which are believed to hold the *P. falciparum* protein synthesis [6]. In addition, it is known to contain phenolic [7] the components of which have anti-carcinogenic, anti-inflammatory, and anti-parasitic properties [8]. *Nigella sativa* water extract as an anti-malarial remedy has been tested in vivo by researchers from Malaysia, using the parasite *P. berghei* [9].

The antioxidant effects of *N. sativa* and its components have been reported to contribute to its efficacy as an anti-malarial remedy. Components of *N. sativa* have been seen to inhibit the production of nitric oxide (NO) in macrophages [10].

This study aims to determine the effect of the water extract of *N. sativa* in mice infected with the malaria parasite *P. berghei* NK 65 in vivo. Decreased parasitaemia was associated with decreased levels of NO, although there needs to be further research to see if there is a connection between decreased levels of parasitaemia, NO, and the survival of test animals.

2. Methods

A water extract of the seeds of *N. sativa* was obtained. The dosage was tested by empirical use in the community. This study was conducted in an an experimental research laboratory. This study used 56 strains of Swiss mice (*Mus musculus*), each of which was two and a half months old, with an average body weight of 25 g. This research used 14 batches of 7 groups for the determination of NO data and parasitaemia (group A), and 7 groups for survival data (group B). Each group consisted of 4 mice. The seven groups for data determination of NO, parasitaemia, and survival rates are as follows: a) Group A1 (negative control group): given oral only 0.85% NaCl, without being infected with *P. berghei* NK65, b) Group A2 (positive control group): infected with 10^6 parasites *P. berghei* NK65, c) Group A3 (the comparison group): infected with10^6 parasites *P. berghei* NK65 + 25 mg/kgBW and given oral chloroquin, d) Group A4 (treatment group 1): infected with10^6 parasite *P. berghei* NK65 + given oral *N. sativa* 100 µg/kgBW, e) Group A5 (treatment group 2): infected with 10^6 parasite *P. berghei* NK65 + *N. sativa* given orally 200 mg/kgBW, f) Group A6 (treatment group 3): infected with 10^6 parasite *P. berghei* NK65 + *N. sativa* given orally 400mg/kgBW, g) Group A7 (treatment group 4): infected with 10^6 parasites *P. berghei* NK65 + *N. sativa* given orally 800mg/ kgBW.

The treatment was administered to each mouse according to the planned research design. On day 4, the mice had blood taken from the tail, after which staining was performed using Giemsa in a phosphate buffer pH 7.2, to calculate the number of malaria parasites that existed at that time. The analysisis was done using a microscope. Parasites were calculated from at least 3000 red bloodcells [11]. The mouse blood was then drawn again, to determine the levels of NO by the spectrophotometric method. The median survival rate was determined during the period of 20 days after the mice were infected.

3. Results and Discussion

Determining % of parasitaemia. From the results of ANOVA $p \leq 0.05$, it can be concluded that there are significant differences between the seven treatments used in this study. In this study, it was found that administration of *N. sativa* may reduce the number of malaria parasites. To determine which treatment had the greatest effect, further testing was done using the Duncan Test. This test indicated that the doses of *N. sativa* are best at preventing the growth of the malaria parasite. *P. berghei* NK65, included in the group treatment A7 *(N. sativa* L = 800 µg/kg BW), are relatively similar to the comparator (group A3) that were given chloroquin 25 mg/kg/BW.

Determination Levels of NO. NO levels in each group can be seen in the Table 1. The differences in treatment that accorded significant impact on the levels of NO were calculated ($p \leq 0.05$). From the graph in Fig. 2, for groups A1-A7, it can be seen that the highest levels of NO are found in the group of mice given parasites, but

not given treatment (positive control group A2). NO levels in all groups that were given *N. sativa* (groups A4-A7) is smaller than the negative control group (A1), the positive control group (A2), and the comparison group that was given chloroquin (A3). This suggests that *N. sativa* can reduce levels of NO, allegedly due to the anti-oxidant properties of *N. sativa*, although in the A5 group there was a slightly greater increase in the levels of NO than in the A3 group.

All treatment groups that were given various doses of *N. sativa* (groups A4-A7) produced NO levels that were lower than the comparison group A3. However, when compared to data of parasitaemia, the decline in parasitaemia in the A4-A7 group is smaller than in the comparison group A3.

The anti-malarial effect of extracts of *N. sativa* is possible because of the content of the active components it contains. Thymoquinone (TQ) is one of the active components having the same effect as *N. sativa*, and is known to be an anti-inflammatory and an anti-oxidant [12].

Extracts from *N. sativa* also contain a variety of different types of alkaloids, which are believed to hold the synthesis of proteins in *P. falciparum* [6]. *N. sativa* is also known to contain phenolic [7], in which the components have anti-carcinogenic, anti-inflammatory, and anti-parasitic effects [8]. Recent research has reported the effects of the antimalarial and anti-oxidant contents of methanol extracts of *N. sativa* compared to *Plasmodium yoelii* [13].

Nitric Oxide (NO) plays an important role in cellular functions in the body [14]. Hossein in 2006 [15], reported that NO (nitric oxide) is one of the important molecules in the immune systemis involved as a mediator in killing the malaria parasite acute, but not an essential contribution to chronic malaria. The combination of NO with various other factors involved in the immune system plays a role in the treatment of

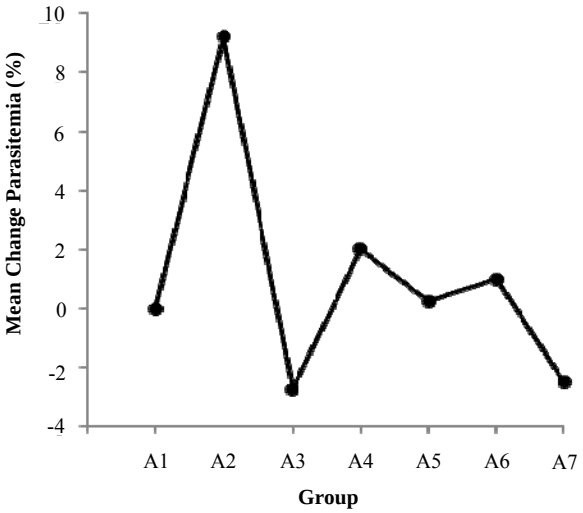

Figure 1. Graph Shows Changes in Parasitaemia

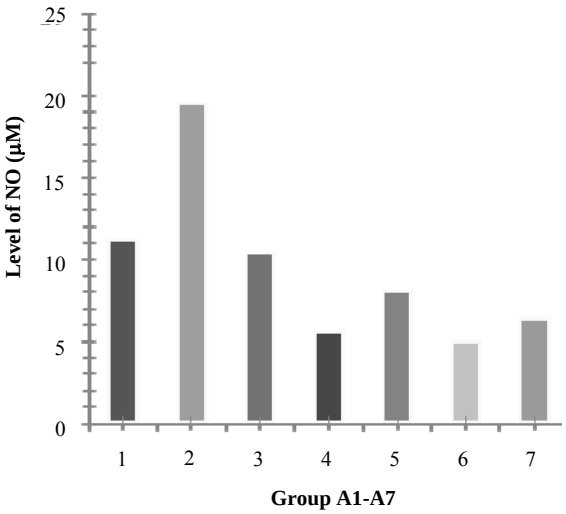

Figure 2. Graph Levels of NO (µM) in Each Group

Table 1. NO Levels in Each Treatment Group

Repeat	NO levels (µM) in each group						
	A1	A2	A3	A4	A5	A6	A7
1	10.9	12.6	12.3	5.6	13.6	6.1	7.6
2	10	4.1	8.5	4.2	5.6	5.6	5.4
3	10.4	53.3	9.2	6.3	5.2	3.9	4.9
4	13.5	8	11.5	6.1	7.6	4.2	7.4
Total	44.8	78	41.5	22.2	32	19.8	25.3
Average	11.2	19.5	10.38	5.55	8	4.95	6.33

malaria. It is well known that NO produced by macrophages stimulated by IFN-γ and TNF-α. They can kill the malaria parasite in the phase eritrositic through a variety of mechanisms, either through the mechanism of phagocytosis and secrete cytotoxic factors. NO, is now known to affect the production of more than 20 cytokin, such asIL-1, IL-6, IL-10, IL-12, IFN-γ, TNF-α, and TGF-β through a varietyof immune cells such a smacrophages, T cells, NK cells, and endothelial cells [16].

The anti-oxidant effects of *N. sativa* and its components have been reported to have an anti-malarial effect. Components of *N. sativa* have been seen to inhibit the production of nitric oxide (NO) in macrophages [10]. NO plays an important role in the mechanism of intra-deadly parasites inside the cell and the natural immune response (innate immunity). Inhibition of this helps to provide a good environment for the multiplication of intra-cellular parasites, and subsequent inhibition of the killing mechanism can lead to increased secondary regulatory mechanisms, from which parasites cannot protect themselves [17]. Inhibition of NO production led to an increase in the degradation of tryptophan, through the induction of the indol amine deoxy genase in human peritoneal macrophages, resulting essential amino acid deficience in parasites.

Determination of Survival Rate. From Table 2, we can see that, on day 20, the number of mice surviving is as follows: group1 100%, 25% of group 2, group 3 50%, and group, 4-7 0%. From the results, it appears that the group given *N. sativa* (groups B4-7) had a survival rate less than that of the negative control group (group B1), the positive control group (group B2), and the comparison group that was given chloroquin (group B3). Until day 15, the B4 group gave the best results when compared to the comparison group B3 (the group that was given 25 mg/ kg of chloroquin).

This study also found that, until day 17, the survival of mice given *N. sativa* for all doses of treatment (group B4-B7) give the same result with the comparison group B3. On day 20, all groups with all doses of treatment (groups B4-B7) had survival rate of 0% (smaller than the groups B1-B3). However, on day 19, the survival rate for group B4, relatively better than for other groups (25%).

Compared with data for parasitaemia turns in the group given *N. sativa* 800 µgr/kg (groupA7), there was no correlation with increasing duration of survival. This can be seen in the B7 group survival data. This is likely to have been caused by the levels of NO in this group being below the threshold levels at which NO can function as an anti-parasitic. It is alleged that, while NO can function as an anti-parasitic to a certain degree, at lower levels its function as anti-parasitic are not optimal, but if levels are too high, damage will result because of the nature of free-radicals. The exact mechanism of the effect of NO levels on parasitaemia levels is unclear, as is the effect on the survival rates of the mice. Currently known to affect the production of NO over 20 cytokin are such compounds as IL-1, IL-6, IL-10, IL-12, IFN-γ, TNF-α, and TGF-β, through a variety of immune cells such as macrophages, T cells, NK cells, and endothelial cells [15]. This indicates the presence of NO is needed as a mediator in the immune mechanisms, but relatively high levels have allegedly worsened its role as an anti-parasitic.

The survival rate of the mice with reduced levels of parasitaemia and NO did not show significant results. This is likely to have been because the levels of NO in this group were below the threshold levels at which NO can function as an anti-parasitic. It is alleged that, while NO can function as ananti-parasitic to a certain degree, at lower levels its function as an anti-parasitic is not optimal, but if the levels are too high, damage will result because of the nature of free-radicals.

Table 2. Table of Survival Rates on Day 20

GROUP	The number of mice on day											
	H1-10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	%
B1	2	2	2	2	2	2	2	2	2	2	2	100
B2	4	4	4	4	4	4	4	4	3	2	1	25
B3	4	4	3	2	2	2	2	2	2	2	2	50
B4	4	4	4	4	4	4	2	2	2	1	0	0
B5	4	3	2	2	2	2	2	2	0	0	0	0
B6	4	3	3	3	3	2	2	2	1	0	0	0
B7	4	3	3	3	2	2	2	2	0	0	0	0

4. Conclusions

The results of this study demonstrate that administration of *N. sativa* may reduce the number of malaria parasites and reduce levels of NO. A decrease in the number of parasites may be caused by an immune mechanism, through the regulation of NO levels (lower levels of NO), as a result of the influence of the anti-oxidant effects of *N. sativa.*

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