

The Effect of Hydroalcoholic Extract of *Nigella sativa* on *Plasmodium berghei*-Infected Mice: An Evaluation of Immune Deviation and Serum Levels of Interferon Gamma (IFN- γ) and Interleukin 4 (IL-4)

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ABSTRACT

Introduction: In tropical countries, malaria is known as one of the most widespread infectious diseases with an estimated 212 million cases globally. Treatment of malaria has become more difficult when the drug resistance appears against parasites. Therefore, safe and effective new drugs are highly required. Because of simplicity, lower cost, low rate of serious complications, and greater tolerability, traditional medicine is an important source for new drugs.

Aim: To evaluate the anti-malaria and immune modulatory effects of *Nigella sativa* against *Plasmodium berghei* in vivo.

Materials and Methods: This study was conducted in the Department of Parasitology, the Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran during January 2014 to January 2015. *N. sativa* powder was macerated in methanol and filtered with Bohnner hopper, and solvent was separated in a rotary evaporator. In the result of toxicity test, the

450 mg/kg was determined as maximum dose with minimum toxicity. Antimalarial efficacy and interferon gamma (IFN- γ) and Interleukin 4 (IL-4) cytokines level were investigated in five groups of *P. berghei*-infected BALB/c mice. The percentage of parasitaemia and surveillance were also evaluated.

Results: The results of our study showed no toxicity, even at high concentration of herbal extract. A significant reduction in the percentage of parasitaemia was not observed in the treatment group. *N. sativa*-treated infected mice showed a significant increase in the IFN- γ but not IL-4 serum level. In addition, no higher surveillance was observed in the mice group treated with *N. sativa* compared to treated mice.

Conclusion: Hydroalcoholic extract of *N. sativa* has the weak efficiency against *P. berghei*. However, there is requirement to evaluate the immune mechanism and also find the major component of this herbal extract by further studies.

Keywords: Drug resistance, Malaria, Parasitaemia, Parasite

INTRODUCTION

Malaria is caused by members of genus *Plasmodium*. It is estimated that about 120 species are able to infect birds, reptiles, and mammals; however, only four of them induce the disease in humans, including *Plasmodium vivax*, *P. malariae*, *P. ovale*, and *P. falciparum*. These species are the reason of most of the deaths since they cause the most severe form of malaria [1]. In recent years, *P. knowlesi*, which normally infects the macaque monkeys, has been reported to infect humans and to be known as the fifth human malaria parasite [2]. Malaria, as the world's most important tropical disease, is transmitted by an Anopheline mosquito vector. Malaria is responsible for killing a million people each year and infecting 212 million individuals worldwide [3]. However in Iran, it infected 3031 cases in 2010, and 1230 cases in 2014 [4-6].

Plasmodium spp., especially *P. falciparum*, is genetically complicated and has ability to cross-mate and produce mutant variants. Genetic mutant is responsible for pathogenicity and deficiency in immune responses. In these conditions, parasite leads to the emergence of resistance against almost all available anti-malarial drugs and helps to spread malaria to new areas and finally causes the recurrence of malaria in the regions where the disease has been eradicated. In such a situation, there is a need for developing new effective anti-malarial drugs [6].

Both humoral and cellular immune responses play a significant role in immunity. In humoral immunity, inflammation produces cytokines

IL-4, IL-5, IL-10, and IL-12, which are stimulated by TH2 cells. In cellular immunity, the parasite enters the macrophages by binding collagen and laminin receptors on the surface of the macrophages, produces penetrating-enhancing factor and then duplicates. Finally, the infected macrophage produces IL-12, which increases the number of T helper 1 (Th1) cells. Th1 cells in turn produce interferon gamma (IFN- γ) [7].

Anti-malarial drug resistance is a major problem that often emerges when a parasite strain survives and/or multiply after exposing to different doses of a drug, equal to or higher than doses recommended with considering the tolerance of the subject [8,9].

Animal malaria species have widely been used for development of anti-malarial drugs. In 1948, *Plasmodium berghei* was introduced as a laboratory mouse strain. Due to high similarities with *P. falciparum*, as the causative agent of severe malaria in human, *P. berghei* has frequently been employed in research as a model for human malaria [10]. New drugs used against malaria should be inexpensive and routinely available to people living in malaria-epidemic areas.

Nigella sativa or black seed, known as Siah Daneh in Persian, mostly grows in the Southern Europe, North Africa, Middle East, and Western Asia. Traditionally, this plant has long been used as a natural medicine for the treatment of many acute and chronic diseases. *Nigella sativa* has various characteristics and features, including antioxidant [11], hepatoprotective [12] and respiratory [13] effects, as well as anti-inflammatory and analgesic [14], anticarcinogenic

and antimutagenic [15], nephroprotective [16], immunomodulatory [17], antibacterial [18], antiulcer [19], and antiparasitic [20] activities. *N. sativa* has also been demonstrated to have some impacts on cardiovascular system and blood [21]. The seeds of this plant are composed of fixed and essential oils, proteins, alkaloids, and saponin. Evidence has shown that most of the biological activity of the seeds is due to thymoquinone, the major component of the essential oil; however, it is found in the fixed oil as well [22]. In spite of broad studies in the field of *N. sativa* seeds, few investigations have been conducted on antimalarial and immunomodulatory activity of *N. sativa* seeds against *P. berghei* ANKA strain in BALB/c mice. To achieve this goal, this study attempted to evaluate the antimalarial and immunomodulatory effects of *N. sativa* against *P. berghei* in vivo.

MATERIALS AND METHODS

Sample Preparation

In this experimental in vivo study, six- to eight-week-old male BALB/c mice were obtained from the Razi Vaccine and Serum Research Institute (Karaj, Iran). The mice were allowed to acclimatize to housing condition for one week prior to the experiment and allowed food and water ad libitum. This study was conducted in the Department of Parasitology, the Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran, during January 2014 to January 2015 in accordance with the animal care and use protocol of Urmia University of Medical Sciences, Urmia, Iran (Ethical code: IR. UMSU.Rec. 1392.69).

The mice (n=50) were divided equally into five groups: Group 1, mice infected with *P. berghei* and treated with chloroquine (P.C.), group 2, mice infected with *P. berghei* and treated with *N. sativa* hydroalcoholic extract (P.E.), group 3, mice infected with *P. berghei* without any treatment (P.N.), group 4, no infection with *P. berghei* but treated with *N. sativa* hydroalcoholic extract (N.E.), and group 5, no infection with *P. berghei* and no treatment (N.N.). From each group, five mice were randomly selected for the measurement of IFN- γ and IL-4 levels, and another five mice were chosen for parasitemia determination and survival test.

Parasite Preparation

P. berghei ANKA 2.34 strain, donated by P.F. Billingsley, was kept in liquid nitrogen, melted in 37°C bath and injected peritoneally into mice. If the parasitemia was 25%, mice blood was diluted 100 times. Since the injected mice had parasitemia about 52%, bloods of all mice were diluted 200 times to achieve appropriate injection dose. Each mice from each group received 0.2 cc of diluted blood peritoneally [23].

Preparation of Hydroalcoholic Extract

N. sativa seeds of Iran origin were purchased from an herbal shop in Piranshahr, West Azerbaijan, Iran. Faculty of Agriculture of Urmia University verified the correctness of plants. The seeds were dried in shade at 35-40°C and crushed into coarse powder. To produce the hydroalcoholic extract, 200 gr of the seed powder was soaked into 800 mL of 70% ethanol. The solution was then shaken once every 24 hours for three days and then filtered through a whatman filter paper number one. Alcohol was removed from the extract solution by evaporation in the vacuum using a rotary device until a liquid gel was formed. This process was performed in order to determine pharmacological and toxicological dose (300, 450, and 600 mg/kg) of extract. Subsequently, each dose was injected into three mice. The general condition of mice was evaluated in 15, 30, 45, 60, 90, and 120 minutes after injection [23]. The movement of mice received the dose of 600 mg/kg decreased, and the mice became lethargic and sleepy, but in two other dose groups, no serious side effects were observed. Therefore, 450 mg/kg was determined as maximum dose with minimum toxicity.

Treatment of Mice

Treatment of mice started at the first day of parasite observation in the peripheral blood. Following the observation of *P. berghei* in blood of mice, treatment was performed with 0.2 cc of extract or 20 mg/kg of chloroquine for four consecutive days intraperitoneally [23].

Parasitemia Determination

Three days after treatment, blood smear (tail blood) was prepared and stained with Giemsa, and the percentage of infected erythrocytes was determined. For this purpose, 1000 RBC were counted, and the percentage of parasitemia was determined.

Measurement of IFN- γ and IL-4 Levels

Levels of IFN- γ and IL-4 were measured in the sera of five mice from each group by using ELISA (mouse IFN-gamma platinum ELISA, BMS 606 and mouse IL-4 platinum ELISA, BMS613, eBioscience, USA) according to the instruction provided by the manufacturer.

The Survival Rate of Mice

Following the observation of *P. berghei* in the blood of five mice in groups P.C., P.E., and P.N. (P.C.: mice infected with *P. berghei* and treated with chloroquine, P.E.: mice infected with *P. berghei* and treated with *N. sativa* hydroalcoholic extract, P.N.: mice infected with *P. berghei* without any treatment), the survival rate of mice was monitored for 20 days.

STATISTICAL ANALYSIS

Data analysis was carried out using Kaplan Meier, ANOVA, and t-test. Significant level was determined statistically as $p < 0.05$.

RESULTS

The serum IFN- γ level in different groups was evaluated, and its result is exhibited in [Table/Fig-1]. The statistical analysis in this section was conducted using ANOVA test. The mean secretion of IFN- γ in P.E. group was significantly more than N.N. group ($p = 0.008$), and that of N.E. group was significantly higher than both P.C. and N.N. groups $p = 0.05$ and $p = 0.003$, respectively. The mean secretion difference between P.N. and N.N. groups were also significant ($p = 0.021$). In spite of differences in IFN- γ rate between P.E. and P.C. groups, it was not statistically significant ($p = 0.89$).

Groups	Number of mice	Mean	Std. Deviation	Std. Error	Min	Max
P.C	5	20.66	13.8132	6.1734	7.64	44.19
P.N	5	28.13	10.2943	4.6083	13.99	38.63
N.E	5	39.10	12.6743	5.6674	21.94	56.11
P.E	5	31.76	10.4687	4.6749	21.94	44.19
N.N	5	13.57	4.9316	2.2052	8.52	21.49

[Table/Fig-1]: Mean of INF- γ secretion in studied mice groups.

The serum rate of IL-4 was elevated in different groups, as illustrated in [Table/Fig-2]. Results showed a significant difference between P.N. and P.C. groups ($p = 0.024$), but no significant difference was found in the secretion mean of IL-4.

Groups	Number of mice	Mean	Std. Deviation	Std. Error	Min	Max
P.C	5	43.4920	13.16312	5.16606	26.22	61.02
P.N	5	74.4756	23.29319	29.0000	57.16	88.61
N.E	5	60.5074	17.223601	7.70818	44.27	88.09
P.E	5	69.7868	31.93453	14.28156	40.40	98.40
N.N	-	-	-	-	-	-

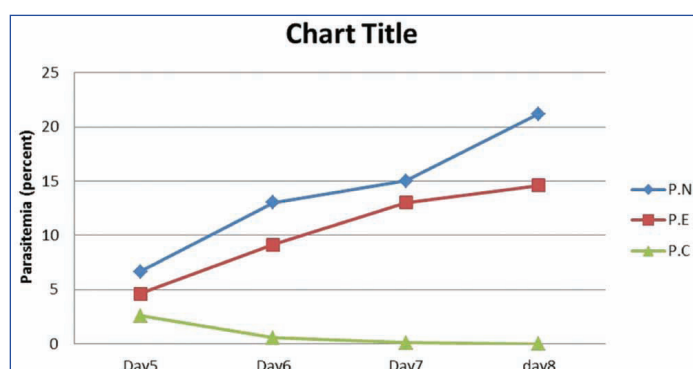
[Table/Fig-2]: Mean of IL-4 secretion in studied mice groups.

Parasitemia and Survival Time

Parasitemia rate in different groups were assessed, and the mean percentage of parasites in 5, 6, 7, and 8 days is shown in [Table/Fig-3,4]. Results demonstrated that parasitemia decreased in P.C. group but increased in both P.N. and P.E. groups. Also, in P.E. group, the parasitemia rate in day four slightly increased compared to P.N. group.

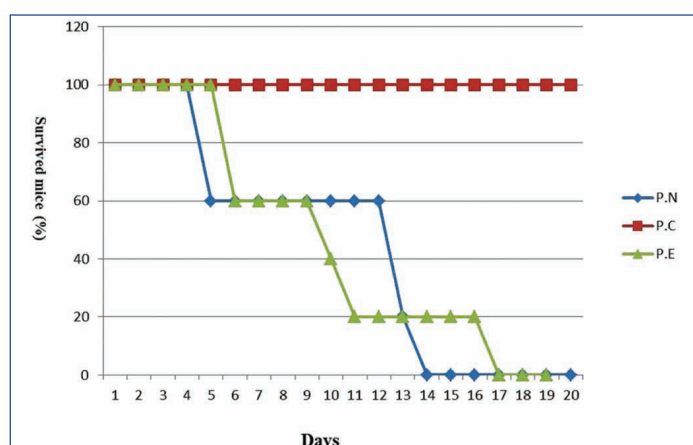
	Day 5	Day 6	Day 7	Day 8
P.N	2.2	5.1	10	20
	11	12	15	21
	12	21	21	29
	7	22	20	24
	1	5	9	12
Average	6.64	13.02	15	21.2
P.E	6.2	9	15.6	17.2
	4.4	7.4	17.6	17.8
	6.2	10	15.4	18.4
	1.4	3.4	11.2	11.4
	5	16	5.3	8.3
Average	4.64	9.16	13.02	14.62
P.C	2.1	0.33	0	0
	2.8	1.33	0.28	0
	4.2	0.21	0	0
	2.2	0.33	0.21	0
	2.6	0.69	0	0
Average	2.58	0.578	0.098	0

[Table/Fig-3]: Mean of parasitemia in infected groups with *P. berghei*.



[Table/Fig-4]: Rate of parasitemia in mice infected with *P. berghei*. Statistical analysis was done with the ANOVA test.

The highest and the lowest survival times were observed in P.C. and control groups, respectively. The last mouse survived in P.N. group died in 14th day, but in P.E. group, the mouse died in 17th day [Table/Fig-5].



[Table/Fig-5]: Survival rates of mice in P.N, P.C, and P.E. Statistical analysis was done with Kaplan Meier test.

The results from parasitemia and survival time in uninfected groups (N.E. and N.N.) are not mentioned in [Table/Fig-4].

DISCUSSION

There are various anti-malarial drugs available in the market such as quinine antifoate, combination drugs, antibiotics, artemisinin compounds, and miscellaneous compounds. The greatest challenge against malaria control program emerges when the parasite shows resistant against antimalarial drugs. Our results suggested that due to the anti-plasmodial activity, *N. sativa* ethanolic extract is able to partly kill or prevent duplication of the parasite. It means that this herbal drug is capable of suppressing the parasite and can lengthen the lifespan of an infected mouse to some extent. Previous studies have also found similar results regarding parasites [24], especially *P. berghei* [23]. Abdulelah H et al., have examined different doses of ethanol aqueous and chloroform extracts of *N. sativa* and concluded that ethanol and chloroform extracts at 100 μ L/kg have the best result in suppression and survival time [23]. Similarly, Emeka PM et al., have revealed that *N. sativa* extract in dose of 10 mg/kg can cause suppression and increases survival time [25]. Their results are partly in agreement with our outcome in terms of increasing the survival time and preventing the duplication of parasites; however, different injection place causes difference in ideal dose. Production of IFN- γ in response to *N. sativa* extract has been mentioned in other studies. In almost all pathogens, responses of immune system starts with innate immune reactions, which initiates with the secretion of TNF- α , activation of acquired immune system induced with Th1 and Th2 cells, and the secretion of IFN- γ , IL-2, IL-6, IL-8, and IL-4, IL-5, IL-9, and IL-13.

Herbal drugs show their immunomodulatory actions with secreting particular cytokines [26]. High secretion of IFN- γ is a favorable outcome in almost all malaria animal models and play a key role in human defense system against plasmodium parasite by producing high levels of nitric oxide [27,28]. In the current work, the extract of *N. sativa* increased the production of IFN- γ , but not as much as P.C. group. Walther M et al., have demonstrated that the excessive levels of proinflammatory cytokines such as IFN- γ may produce cerebral dysfunction in malaria-infected individuals [29]. However, the limited increase in IFN- γ production, as mentioned in the present study, not only restricted malaria infection but also controlled the cerebral disease caused by plasmodium parasite.

IL-4 evaluation exhibited that the effect of *N. sativa* extract between different groups was not statistically significant. This result was in accordance with other studies who evaluated the impact of *N. sativa* extract on human lymphocyte. It was found that *N. sativa* extract significantly increased the levels of IL-1 and IL-3, but it did not have any significant effect on IL-2 and IL-4 [30].

In this study, the same as Laloo DG et al., study, we used chloroquine to evaluate the activity of parasite and choice drug for the treatment of non-falciparum infections and non-severe falciparum infections, which are acquired in the areas of chloroquine sensitivity [31].

LIMITATION

More evaluation is needed regarding the separation of the individual compounds presented in *N. sativa* extract to achieve more effective material. These compounds could contribute to develop new drugs against *Plasmodium* species in the drug resistance situation.

CONCLUSION

We expected that the hydroalcoholic extract of *Nigella sativa* has the same efficiency as chloroquine or even better against *Plasmodium berghei*, but the extract showed a very weak effect. The extract increased the survival time by three days in P.E. group compared to P.N. group, but comparison of P.E. and P.C. groups indicated very weak efficacy of the extract. However, more investigations are needed for the fractionation of *N. sativa* in order to find the active principles responsible for its anti-malarial activity.

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