Potential of Black Seed Oil (*Nigella Sativa*) as Immunomodulator in Mice (*Mus Musculus*) Infected with *Salmonella Typhimurium*

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Abstract: Typhoid fever is a systemic disease mainly caused by *Salmonella typhi* that spreads systemically into blood circulation and leads to bacteria accumulation in the intestine. Black seed oil (*Nigella sativa*) contains a chemical substance called thymoquinone acting as immunomodulator that can enhance immune system to prevent bacteria colonization in the intestine. This study was to determine the potential of black seed oil as immunomodulator to mice infected with *Salmonella typhimurium*. This study is an experimental research using completely randomized design (CRD) with 4 treatments and 6 replications. Animals used were 2-month-old male mice, weighing 25 - 30 g totaling 24. Treatments in this study included K- (normal mice), K+ (mice infected with *Salmonella typhimurium* i.p), P1 (mice orally administered with black seed oil for 7 days and infected with *Salmonella typhimurium* i.p), and P2 (mice orally administered with black seed oil for 14 days and infected with *Salmonella typhimurium* i.p). 7 days after infection, mice intestines were collected and total number of bacteria were counted. Data were analyzed using One-Way ANOVA and Post Hoct test. The result shows ANOVA p-value = 0.006 and p-value of Post Hoc = 0.004, proving that black seed oil has potential as immunomodulator in mice infected with *Salmonella typhimurium*.

1 INTRODUCTION

Salmonella sp is gram-negative bacteria with rod shape from *Enterobacter* famili and pathogenic for animals and human. *Salmonella sp* cause enteritis, sistemical infection, and enterical fever in human. There are four important serotypes of *Salmonella* which mainly cause enterical fever in human, such as *Salmonella typhi*, *Salmonella paratyphi A*, *Salmonella paratyphi B*, and *Salmonella paratyphi C* (Jawetz *et.al*, 2013).

Salmonella typhimurium is usually used in laboratory research as model of typhoid fever in human because there are differences of genetic marker expressed at *Salmonella typhi* and *Salmonella typhimurium* resulting in inability of *Salmonella typhi* to infect mice compared to how *Salmonella typhi* cause typhoid fever in human (Mathur *et.al*, 2013).

Typhoid fever or known as "tifoid, tipes, or typhus" is a systemical acute infection in human gastrointestinal tract caused by *Salmonella enterica*, especially *Salmonella typhi* (Paputungan *et.al*, 2016).

WHO (World Health Organization) predicted that incidence rate of typhoid worldwide increase in average number of 800 cases per 100.000 people every year. One of the reasons of high incidence rate of typhoid fever in Indonesia is because of bad sanitation which causes Salmonella sp can spread easily (Depkes, 2013; Paputungan *et.al*, 2016).

Antibiotics use is one of the way to overcome typhoid fever. Although antibiotics had had good contribution to help fight against infections worldwide, the fact is infection is still one of the cause of human death. It is because bacteria has ability to protect themselves against antibacterial drugs, gradually leading to resistence (Soleha, 2015; Umiana, 2015).

Many cases of antibiotics resistance and use of drugs with specific side effects make a shift in society. Using principal of *Back to Nature*, people think to utilize natural plants as drug. Furthermore, society now thinks about early prevention against infection, for example by consuming supplements made of natural plants. Substances in those natural plants which are able to enhance body immune is

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called immunomodulator (Mardiana, 2012; Sulisti *et.al*, 2014).

Black seed (*Nigella sativa*) or known as Habbatussauda is spices coming from middle east that had been used as medicine. In hadist of Bukhari Muslim, Rasulullah SAW encourages us to use black seed that can cure all diseases, except *Al-Sam* / death (Putra, 2011).

Black seed become more familiar nowadays because there are many thibbun nabawi-based medication which is referred to Al-Qur'an and As-Sunnah. Some of Islamic references believe that black seed is one of herbs used based on sunnah. People now can use black seed easier because they are produced in many practical package such as capsule, oil, or powder. Black seed are empirically used to enhance immunity, stamina, cure diabetes, kolesterol, cancer, athma, inflamation, stroke, ulcer, and many other infections.

There have not been many studies about using of black seed oil (*Nigella sativa*) products to be immunomodulator against typhoid fever. Because of that reason, researcher intended to do experiment to know potential of black seed oil (*Nigella sativa*) as immunomodulator to mice (*Mus musculus*) infected with *Salmonella typhimurium*.

2 MATERIALS AND METHOD

2.1 Research Design

The study used true experiment method with Posttest Only Control Group as research design. This study was aimed to know potential of black seed oil (*Nigella sativa*) as immunomodulator to mice (*Mus musculus*) infected with *Salmonella typhimurium*. Researcher will compare mice acting as positive and negative controls to mice which were given treatments. There were 4 treatments and 6 replications in each treatment.

2.2 Place and Time

This research took place in Microbiology and Zoology Laboratory Faculty of Math and Science, University of Jember starting from May until July 2017.

2.3 Materials

2.3.1 Animal Test

Animal used were male mice of Balb/c strain with age ±2 months weighing 25 – 30 gram, totaling 24 and were divided into four groups such as :

1. K (-) : without any treatment
2. K (+) : *Salmonella typhimurium* 10^8 infection i.p
3. P1 : Black seed oil for 7 days and *Salmonella typhimurium* 10^8 infection i.p
4. P2 : Black seed oil for 14 days and *Salmonella typhimurium* 10^8 infection i.p

2.3.2 Black Seed Oil

Black seed oil used during research was bought from Jalan Nyamplungan Ampel, Surabaya.

2.3.3 Bacteria

Salmonella typhimurium ATCC (*American Type Culture Collection*) 14028 was obtained from Unit Layanan Pengujian/ULP (*Assesment Service Unit*) Faculty of Pharmacy, Universitas Airlangga Surabaya.

2.3.4 Bacterial media

Bacterial media used in this study were Salmonella Shigella Agar (SSA) and Luria Bertani Broth.

2.4 Procedure

Mice were acclimatized in cages for 7 days and given standardized foods and drinks. All laboratory glasswares were sterilized using autoclave at temperature of 121ºC for 15 minutes. Bacterial suspension was made based on infection dose of 10^8.

Black seed oil was administered orally as much as 0,2 ml for 7 days to mice in group P1 and 14 days to mice in group P2. Mice in group K (+), P1, and P2 were infected with *Salmonella typhimurium* via intraperitonal.

Confirmation test was performed on the third day after infection and performed through following steps : First, 0.1 ml blood was collected from mice via intraorbital. Blood then was placed in microtube filled with 0.3 ml EDTA. Blood from microtube was poured inside reaction tube filled with 5 ml *Luria Bertani Broth*. The mixture then was incubated at temperature of 37ºC for 24 hours. During incubation period, the mixture was shaken at 120 rpm. The

mixture was inoculated on petri dish using pour plate method and SSA. Sample was homogenized and incubated at temperature of 37ºC for 24 hours.

Seven days after *Salmonella typhimurium* infection, all mice were knocked out with cloroform, belly was opened and intestine was collected. Intestine was cleaned, weighed, mashed using mortar, diluted in certain volume of saline to create dilution of 10^{-1} and serial dilutions were performed. Using vortex, dilution of intestine was homogenized. The dilution was cultured on SSA media using pour plate method. After incubation period for 24 hours at temperature of 35ºC, number of colony was counted using *colony counter*.

3 RESULTS AND DATA ANALYSIS

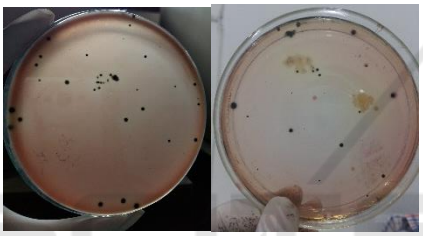

Figure 1 : *Salmonella typhimurium* on SSA media

Table 1: Calculation of *Salmonella typhimurium* colony in mice (*Mus musculus*) intestine of K (-) group.

Treatment	Average number of colony (CFU/gram)
K (-)	0
K (+)	TNTC
P1	6.2×10^8
P2	4.9×10^8

TNTC : Too Numerous To Count

CFU : Colony Forming Unit

Data were further analyzed using post hoc test and showed sig. 0,004 < 0,05, suggesting that there was significant difference between group P1 (7 days consumption of black seed oil) and group P2 (14 days consumption of black seed oil).

Table 2 : Normality test using Kolomogrov Smirnov

One-Sample Kolmogorov-Smirnov Test		
		data
N		17
Normal Parameters[a]	Mean	5.61E7
	Std. Deviation	1.276E7
Most Extreme Differences	Absolute	.238
	Positive	.238
	Negative	-.144
Kolmogorov-Smirnov Z		.981
Asymp. Sig. (2-tailed)		.291
a. Tes distribution is Normal		

Normality test using Kolomogrov Smirnov showed that data of colony number of *Salmonella typhimurium* in mice intestine had normal distribution.

Table 3 : Homogenity test

Test of Homogenity of Variances			
Total Colony			
Bacteria			
Levene Statistic	df1	df2	Sig.
5.900	2	14	.014

Homogenity test using showed that data of colony number of *Salmonella typhimurium* in mice intestine were homogenous with p value of 0.014.

Table 4 : Two-way ANOVA

ANOVA Number of Colony					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.346E15	2	6.728E14	7.477	.006
Within Groups	1.260E15	14	8.999E13		
Total	2.605E15	16			

Data were further analyzed using *One-Way ANOVA* parametric test. From *One-Way ANOVA* parametric test, sig.0,006 < 0,05 was obtained, meaning that there was difference of *Salmonella typhimurium* colony number between P1 (7 days consumption of black seed oil) and P2 (14 days consumption of black seed oil).

Table 5 : Post Hoc Test

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
P1	P2	1.888E7	5.477E6	.004	7136653.12	30630013

						.55
P2	P1	-1.888E7	5.477E6	.004	-3.06E7	-7136653.12

Data were further analyzed using post hoc test and showed sig. 0,004 < 0,05, suggesting that there was significant difference between group P1 (7 days consumption of black seed oil) and group P2 (14 days consumption of black seed oil).

4 DISCUSSIONS

Positive control (not given black seed oil but infected with *Salmonella typhimurium*) showed the highest colony number of *Salmonella typhimurium* among all groups. This is occurred because without any intervention from herbs, *Salmonella typhimurium* could penetrate easily to intestinal epithels as the first process of entering mice body.

Salmonella typhimurium in positive control group penetrated epithelial intestine easily, causing bacterial antigens were trapped by *Peyer's patches*. As the result, local immune response of intestine was triggered. *Antigen-presenting dendritic cells* (DCs) then delivered signal, allowing signal to be recognized by other parts of intestine without interfering reading process of antigens coming from commensal intestinal bacteria or other pathogens. B cells and memory cells were activated by *antigen-presenting cells* at *Peyer's patches*. These activated cells migrated toward exact location of antigen target, associated with multiplication of intestinal immune response. During infection, endotoxin of Salmonella (LPS) triggered macrophage and other phagocyte cells to produce and release various cytokines such as IL-1, TNF-α, and IL-6 (Broz *et.al*, 2012).

Compared to positive control, number of *Salmonella typhimurium* colony of mice orally administered black seed oil for 7 days (group P1) and mice orally administered black seed oil for 14 days (group P2) decreased. It was due to the existence of an active substituent in black seed oil known as thymoquinone. Thymoquinone is believed to serve as immunomodulator. Ability of thymoquinone to be immunmodulator is closely related to its function to enhance immunity in intestine as early barrier to *Salmonella typhimurium* exposure. Thymoquinone in black seed oil helps improving the release of digestive enzymes so that nutritions which are needed to fight pathogens can be absorbed better. Thymoquinone also takes part in decreasing number of coliforms in mice ceccum which is known as stable environment for pathogens and commensal bacteria (Erener *et.al*, 2010).

Thymoquinone in black seed oil stimulated T lymphoctye during the process of bacteria elimination by intestinal macrophage. T cells proliferated inside thymus to become cells involved in expression of specific T cell reseptors. Those specific T cell reseptors were derived into CD4+ or CD8+. CD4+ and CD8+ cells are cell surface proteins which determine the main subpopulation of T cells, CD4 cells (T helper) and CD8 cells (T cytotoxic). Differentiated T cells then migrated to infection site and give systemic protection (Salem *et.al*, 2011; Ahmad, 2013).

The study showed that consuming black seed oil for 14 days were more effective to prevent *Salmonella typhimurium* to grow in mice intestine than black seed oil for 7 days. It was relevant with former research by Musthoza (2017) that the longer period herbs were consumed, better immunity would be formed. This was because herbs take more time to establish and improve whole body system by repairing damaged cells and organs.

5 CONCLUSIONS

The black seed oil is proven to have potential to be immunomodulator in mice infected with *Salmonella typhimurium*.

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