Research Journal of Life Science
APRIL-2018 Volume 5 NO. 1 (14-22)
journal homepage: www.rjls.ub.ac.id

Nigella sativa Extract Increases Antibacterial Activity by Up-Regulating T-reg and Th2 Levels in *Salmonella enterica* subsp. *enterica* serovars Typhimurium-Exposed Balb/c Mice

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KEYWORDS

Bacterial colony
Nigella sativa
Salmonella typhimurium
T-reg
Th-2

Abstract *Nigella sativa* extract (NSE) is known to be an effective antibacterial and immuno-modulating agent. This study aimed to demonstrate that treatment of *Salmonella enterica* subsp. *Enterica* serovar *typhimurium* infected mice with NSE would cause an increase in T-regulatory (T-reg) and Th2 cells and a decrease in the number of spleen bacterial colonies. Mice were randomly divided into five groups [control (T0), mice infected with *S. typhimurium* only (T1), and mice infected with *S. typhimurium* and then treated with NSE 0.52, 5.2, or 52 mg/kg body weight NSE (N1, N2, and N3, respectively). Levels of T-reg and Th2 cells were determined via flow cytometry and the number of spleen bacterial colonies was determined by observation. The results showed that treatment with NSE 0.52, 5.2, and 52 mg/kg significantly increased the number of T-reg and Th2 cells ($p<0.05$) relative to T0. Administration of NSE 5.2 mg/kg increased the number of T-reg cells, while administration of NSE 52 mg/kg caused the greatest increase in the number of Th2 cells in the spleens of *S. typhimurium*-infected mice. Moreover, no *S. typhimurium* colonies were found in the spleens of any NSE treated mice. Our results suggest that NSE has therapeutic potential to ameliorate *S. typhimurium* infection.

Introduction

Salmonella, which is one of the Enterobacteria family belongs to the group of gram-negative facultative anaerobic and intracellular pathogenic bacteria, has caused more than 100 million cases each year that leads to 350,000 deaths (De Jong et al., 2014). *Salmonella* bacteria can be found in food, environment, human body and animals (Santos et al., 2011). *Salmonella enterica* subsp. *enterica* serovars *typhimurium* and *typhi* cause enteric fever, septicemia, gastroenteritis, and typhoid fever when they infect human beings (Santos et al., 2011). Unfortunately, the study of *S. typhi* in humans is restricted; thus, studies of pathogens that causes similar symptoms must be used as a proxy. *S. typhimurium* causes typhoid fever in mice and produces similar symptoms to typhoid fever in humans, therefore, used in this study (Nurjayadi et al., 2016).

Salmonella enters the body through the small intestine and colon which enables in accessing the systemic tissue (usually spleen and liver) through the lymphatic system and the

How to cite this article: Ahmed, Z., Prawiro, S. R., Endharti, A. T. (2018). *Nigella sativa* Extract Increases Antibacterial Activity by Up-Regulating T-reg and Th2 Levels in *Salmonella enterica* subsp. *enterica* serovars Typhimurium-Exposed Balb/c Mice. Research Journal of Life Science 5 (1), 14-22. https://doi.org/10.21776/ub.rjls.2018.005.01.2

Peyer's patches (Santos et al., 2011). When *Salmonella* infects the gastrointestinal tract, the body responds by inducting an array of pro-inflammatory cytokines in epithelial cells and macrophages.

Bacterial cells are rapidly removed from the blood by phagocytes and deposited into the spleen and the liver, where a large fraction of bacterial cells is killed. These first stages of *Salmonella* infection are normally completed within a few hours, and are followed by a phase of several days, during the bacteria multiply within spleen and liver cells, and bacterial titers mightincrease (McArthur et al., 2015).

Salmonella Containing Vacuole (SCV) is a typical intracellular niche of Salmonella that is a modified phago-lysosome, though the way it enters the target cell and its strategy to survive are different depend on the temporal expression of particular genes. Target cells include gut epithelial cells, macrophages, neutrophils, monocytes, granulocytes, dendritic cells, B cells, and T cells (Garai et al., 2012). When the body infected by S. *typhimurium*, the macrophages and dendritic cells as the major cells infected, shift the bacteria to the liver and spleen where the major place of bacteria replications. These two types of cells are the main actors related to the immune response to Salmonella infection, as the macrophages work as the controller of the bacterial growth in the early infection phase and dendritic cells takes care for the initiation of the T cell response (McArthur et al., 2015). T-regulatory (T-reg) cells are $CD4^+$ T-cells responsible for suppressing potentially harmful activities caused by Th cells (Fresnay et al., 2016). It was therefore of interest to assess the stability of $CD4^+$ T cells during a persistent Salmonella infection.

Nigella sativa extract (NSE) contains the active compounds thymoquinone (TQ), dithymoquinone (DTQ), thymohydroquinone, and thymol (THY) and possesses an immuno-modulatory effect. In one study has shown that NSE can increase the immune response in humans by inducing the production of cytokines (Chaieb et al., 2011).

Many studies have explored the uses of NSE, including as a treatment for bone and joint disease, osteoporosis, tumors, bacterial infections, cancer, and as an immunomodulator (Seif, 2014; Khan et al., 2011). Results of these studies show that NSEhas a promising effect on multi-antibiotic resistant organisms including gram-positive and gram-negative bacteria.

Materials and methods

The experiments were done from February 2017 to November 2017. They were obtained from Laboratory Animal. The mice were keep under standard conditions of temperature 25-27°C, relative humidity (55 ±5%) and 12h/12h light/dark cycle. Mice were given normal drinking water ad libitum during the experimental periods. Mice were taken care at the Pharmacology Laboratory in the Faculty of Medicine University of Brawijaya, Malang. All experiments were conducted according to the principles of Guide for the Care and Use of Laboratory Animals in Indonesia and were approved by The Ethical Committee University of Brawijaya, Malang, Indonesia (No.171-KEP-UB)

Animals and Study Groups

The study was conducted using an experimental laboratory with post-test control group design. Experimental design was conducted which consists of four treatment groups of mice and control group. Female Balb/c mice weighing 25-30 g were used. They were obtained from Laboratory Animal. The mice were keep under standard conditions of temperature 25-27°C, relative humidity (55 ±5%) and 12h/12h light/dark cycle. Mice were given normal drinking water ad libitum during the experimental periods. All experiments were conducted according to the principles of Guide for the Care and Use of Laboratory

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Mice were housed 5-6 to a cage and given normal drinking water ad libitum. Twenty fifth male Balb/c mice were randomly divided into five groups (n=5): the vehicle group received Phosphate-Buffered Saline (PBS) (T0), positive control group received S. *typhimurium* only (T1), treatment groups received different concentration of NSE (0.52 (N1), 5.2 (N2), or 52 mg/kg body weight (N3), respectively). The NSE treatment groups were orally administered with different doses once per day.

Nigella sativa Extraction

The seeds were dried and powdered using a mechanical grinder. The seed powder (100 g) were macerated in 800 mL ethanol (80%, v/v), the maceration process was repeated three times in 24 hours, and the crude extracts was subsequently filtered and then, concentrated by evaporating the solvents under reduced pressure to obtain a viscous residue. The crude extracts were filtered and concentrated at ±60°C by evaporating the solvents under reduced pressure. Stock solution of 50 mg/ml was prepared in dimethyl sulfoxide (DMSO) and aliquots stored at 0°C until used. The *Nigella sativa* preparation is done through some procedures as following (Khan & Kour, 2016).

Salmonella typhimurium culture

The bacteria *Salmonella typhimurium* was obtained from Laboratory of Microbiology in the Faculty of Medicine at University of Brawijaya and cultured on Bismuth Sulfite Agar (black colonies) and confirmed as *Salmonella typhimurium* by using Gram staining (Gram negative rods), MacConkey agar, Triple Sugar Iron (acid/base, no gas, H2S positive), IMVIC test, and VITEK2 (BioMérieux). It is made inoculum in 10^8 cfu/mL (with PBS). It is made inoculum in 10^8 cfu/mL (with PBS) for 10^7 cfu/100 µL/mice.

Induction of Salmonella typhimurium and Nigella sativa treatment

Mice were adapted for 1 week in the laboratory with standard feed and fasted over night before S. *typhimurium* inoculation. Mice were received *Salmonella typhimurium* 10^7cfu/100 µL/mice (Seif, 2014). The total cells count was maintained 10^8cfu/mL and was intra-peritoneally injected. To investigate the antibacterial effect of *Nigella sativa* extract, by measuring the number of T-reg and Th-2 cells in spleen of Balb/c mice infected by *Salmonella typhimurium*.

The Levels of T-reg and Th2 using Flowcytometry

For intracellular cytokine measurement, spleen cells ($1x10^6$) were stimulated for 5 h with PMA (1 µg/mL, Sigma Aldrich) and ionomycin (50 µg/mL, BD Biosciences) in the presence of monensin (0.1 mg/mL, Sigma Aldrich) and placed in a 37°C and 5% CO_2. The Levels of T-reg and Th2 in the spleen were determined by Flow cytometry. Spleen cells were washed with PBS and surface-labeled with anti-CD4–FITC (Biolegend, Uithoorn, Netherlands) and anti CD25-PE (BD Biosciences). Spleen were fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences) and stained with anti-IL-4-PE (Biolegend, Uithoorn, Netherlands) and anti-FoxP3-PerCP (BD Biosciences). The stained cells were analyzed using FACS Calibur, and the data were analyzed using Cell Quest Pro software.

Data Analysis

The data analysis of this study was expressed as mean ± standard error of mean. The overall variation in a set of data was analyzed by one-way analysis of variance (ANOVA). A value of $P<0.05$ was considered with four replicates of each concentration tested.

Results and discussion

The Effect of NSE on Number of T-reg in Spleen of Mice Infected by S. typhimurium

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To know the antibacterial effect of NSE mice were administered orally using gavage needle to mice with the doses 0.52 mg/kg BW (N1), 5.2 mg/kg BW (N2), and 52 mg/kg BW (N3) respectively for a week. In the infected *S. typhimurium* group (T1), CD4 levels were lower than control (T0). The other hand, in the groups treated with NSE have more CD4 T cells were produced. However, the percentage of CD4 T cells in the group treated with NSE 0.52 mg/kg BW were still below than control (T0). The highest CD4 T cells was found in the group treated with NSE 5.2 mg/kg BW.

The presence of NSE effect begins when the number of T-reg cells in rats infected with *S. typhimurium* bacteria becomes higher than control. After the treatment of NSE was given starting with a dose of 0.52 mg/kg BW, the number of T-reg cells increased to 15.78%. In addition, the 5.2 mg/kg NSE group showed highest percentage of T-reg cells (17.65%). It shown on Figure 1.

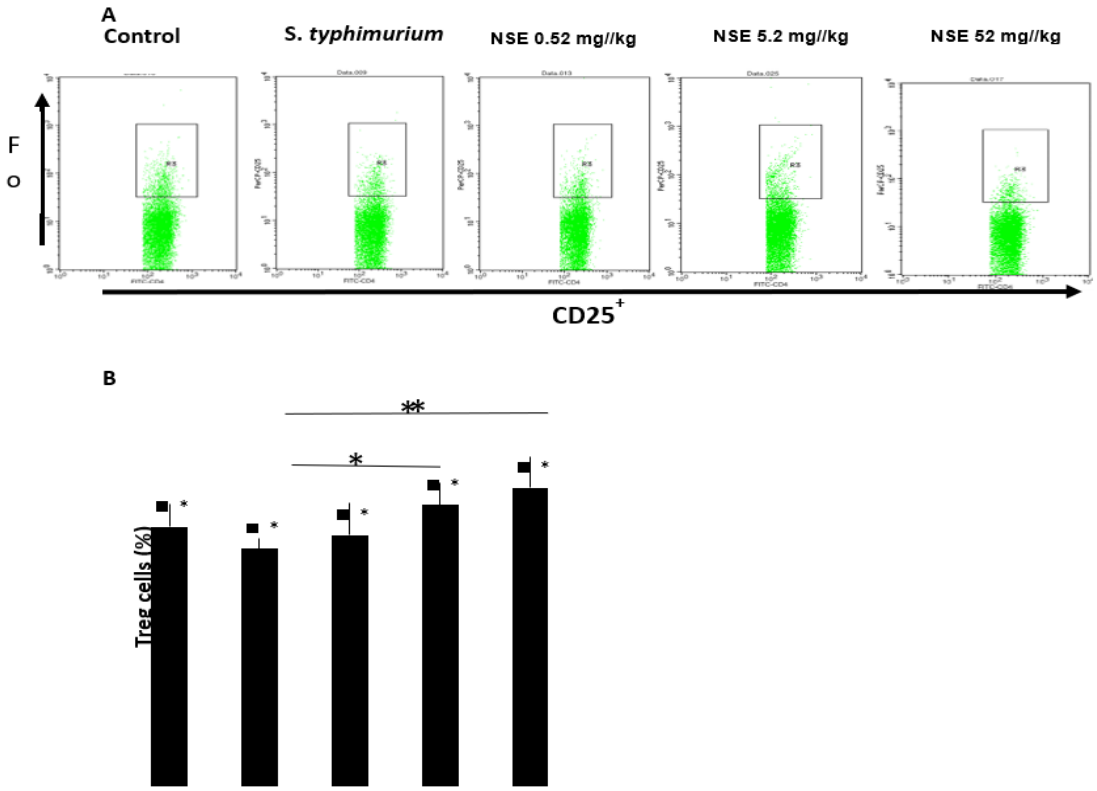

Figure 1. The percentage of Treg cells in spleen of mice infected by *S. Typhimurium.* Mice were injected with *S. typhimurium* (T1) following with or without NSE treatments at doses (0.52 (N1), 5.2 (N2) and 52 mg/kg BW (N3). The percentage of Th2 cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences). Representative results of four replicates in each group are shown. B. Numbers represent the percentages of Treg cells in each histogram are shown. Results shown are mean ± SD, with n =4 replicates in each group. ∗p<0.05, ∗∗p<0. 001.Results shown are mean ± SD, with n = 4 replicates in each group. ∗p<0.05, ∗∗p<0.001.

Figure 1 described the number of T-reg cells in mice infected with *S. typhimurium* with or without treatment of NSE. The number of T-reg cells in T1 were infected with *S. typhimurium* showed a slightly lower than T0. However, when the NSE was administered at a dose of 0.52 mg/kg BW, percentage of T-reg cells increased considerably from the positive control group (T1), but this number was still lower than the negative control (T0). This means that 5.2 mg/kg

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NSE did not show a significant effect on mice suffering from *S. typhimurium* infection (T1) when compared with normal mice (T0).

There were fewer T-reg cells shown of mice infected with *S. typhimurium* (T1) than negative control mice (T0), whereas NSE treatment groups with doses of 0.52, 5.2, or 52 mg/kg (N1, N2, N3, respectively) induced T-reg proliferation. However, there were fewer number of T-reg cells found in N3 group than N2 group. The group treated with NSE 0.52 mg/kg increased slightly higher than T0 and T1 groups. These results indicated that the number of T-reg cells significantly increased of mice infected with *S. typhimurium* compared to the T1 group. NSE treatment at a dose of 5.2 mg/kg further increased the number of T-reg cells, however, treatment with NSE 52 mg/kg increased the number of T-reg cells. Therefore, a dose of NSE 5.2 mg/kg was the most effective at inducing the proliferation of T-reg cells in the spleen of *S. typhimurium*-infected mice. This result supported with other studies showing that NSE improved the immune response by inducing the proliferation of CD4, CD8 and natural killer (NK) cells (Sarwar et al., 2015).

The Effect of NSE on the Number of Th2 in the Spleen of Mice Infected by Salmonella typhimurium

Flow cytometry results showed *S. typhimurium* infected group reduced the number of Th2 cells. The number of $CD4^{+}T$ cells in the spleen reduced post- infection (Figure 2). We suppose that decrease can be attributed to the proportion of lymphocyte subpopulations that have apoptosis after infection. Macrophages that interact with *Salmonella enterica* serotype *typhimurium* that grows under the pressure undergo cell death due to apoptosis (Pham et al., 2015). On the other hand, the group treated with *N. sativa* showed an increase in Th-2-producing cells compared with the infected group. The highest Th2 levels were found in the group treated with *N. sativa* at a dose of 52 mg/kg BW.

According to the results, there was a difference in the number of Th-2 in each group of mice. This indicated that the number of Th2 in the group of mice treated with NSE were significantly higher than the non-treatment groups, the different doses of NSE affected or had different effects on the amount of Th-2. After the treatment of NSE 0.52 mg/kg BW (N1), Th2 number increased to 8.96%, while the treatment group at 5.2 mg/kg NSE (N2) of showed a slightly lower of Th2 at 8.56%. However, the Th2 numbers in both groups were higher than the number of T1 group. Then, Th-2 increased again when mice were given the highest dose of NSE 52 mg/kg BW (N3). This study has showed significantly increase in the number of Th2 cells in mice were infected with *S. typhimurium* compared with T1 group. It suggested that the treatment of NSE at 0.52 (N1), 5.2 (N2) and 52 mg/kg BW (N3) showed a different effect.

Figure 2 described the number of Th-2 cells in mice infected by *S. typhimurium* with or without NSE treatment. The number of Th-2 in T1 showed slightly decrease compared to T0. However, when the extract of NSE was administered at the dose of 0.52 mg/kg BW, the number of Th2 increase moderately from that of the positive control group.

NSE treatment significantly increased the number of Th2 cells in *S. typhimurium*-infected mice. This indicates that NSE 52 mg/kg was the most effective dose to increase the number of Th2 of *S. typhimurium* infected mice. Treatment with NSE 5.2 mg/kg increased T-reg more than Th2 levels, while treatment with NSE 0.52 and 52 mg/kg increased Th2 levels more than T-reg. As shown in another study, T-reg cells can suppress Th2 effector cells, although the mechanism was not evaluated (Inci et al., 2011). Moreover, T-reg cells can decrease effector T lymphocyte IL-2 expression found that diminished expression of the protein FOXP3 caused T-reg cells to revert to effector T cells (particularly Th2-like cells) (Chaieb et al., 2011).

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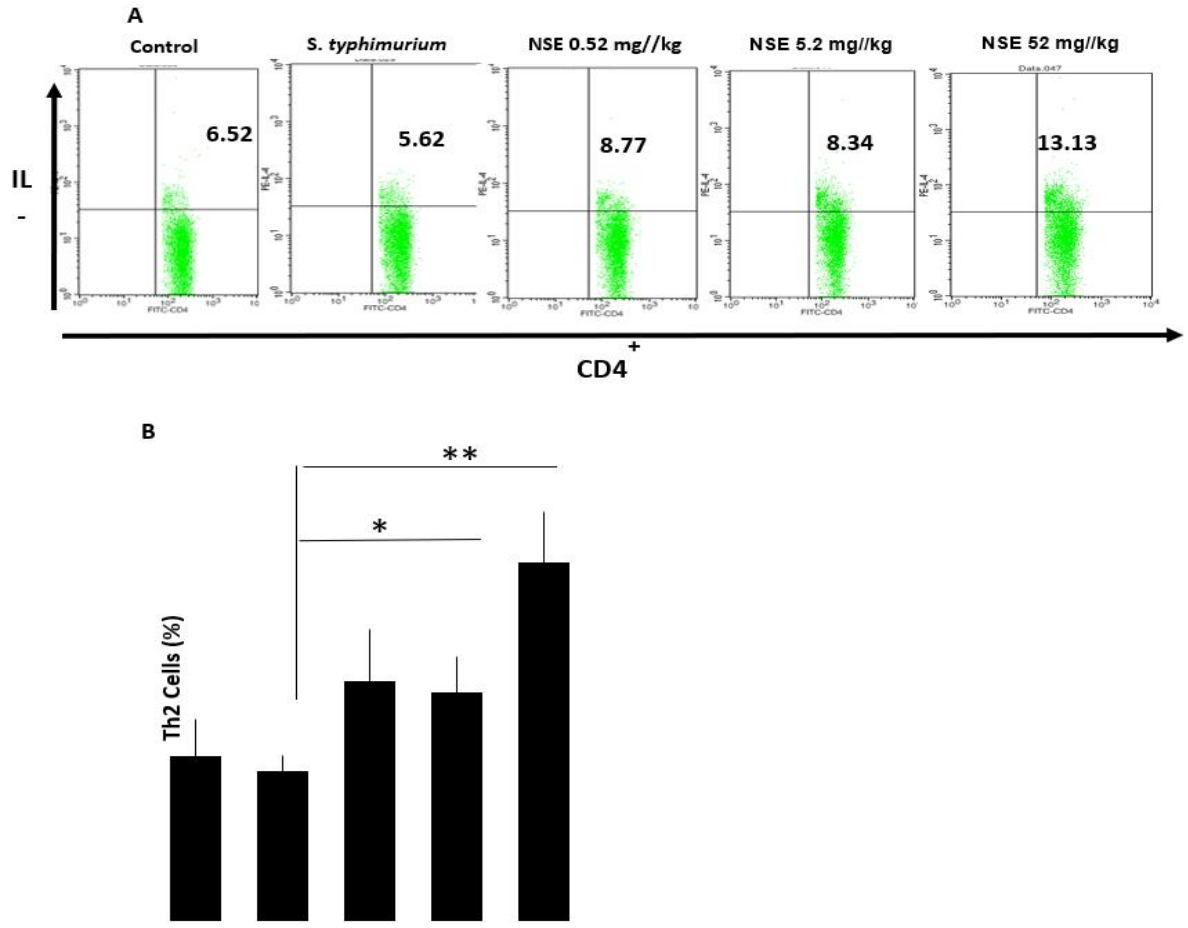

Figure 2. The number of Th-2 cells in spleen of mice infected by *S. Typhimurium.* Mice were injected with *S. typhimurium* (T1) following with or without NSE treatments at doses (0.52 (N1), 5.2 (N2) and 52 mg/kg BW (N3). The percentage of Th2 cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences). Representative results of four replicates in each group are shown. B. Numbers represent the percentages of Th2 cells in each histogram are shown. Results shown are mean ± SD, with n=4 replicates in each group. ∗p<0.05, ∗∗p<0.001.

Our results indicated that T helper cells have been shown to be involved in extracellular bacterial infections, and also play an important protective role against *Salmonella* infection (Pham et al., 2015) shown that Th2 cells activate B cells, which are adapted for defense against microbial. Additionally, Th2 increases mucosal immunity and thereby protects the gastrointestinal tract against the Salmonella (Rahayu et al., 2013) reported that administration of NSE increased the number of $CD4^+$, $CD25^+$, and $FOXP3^+$ T-lymphocytes that regulate the production of Th1.

The Effect of NSE on the Number of Bacterial Colony in the Spleen of Mice Infected by S. typhimurium

Figure 3 showed the different doses of NSE effect on the number of colony of *S. typhimurium* in the spleen of mice. The effect of NSE can be seen by comparing the results of the colony of *S. typhimurium* bacteria induced in mice.

The positive control group (T1) showed the highest number of colony of *S. typhimurium*. The treatment of NSE 0.52 mg/kg BW showed number of *S. typhimurium* colonies significantly decreased compared with T1 group. Similarly, the increasing doses of NSE at 5.2 mg/kg BW and

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52 mg/kg BW also reduced the number of bacterial colonies in the spleen of mice infected by *Salmonella typhimurium.* Thus, the NSE treatment groups shown the number of *S. typhimurium* colonies becomes zero in every group. The NSE effect started once the infected mice were given NSE treatments at any doses. The number of bacterial colony in the group N1 becomes lower to zero than the colony in the positive control group (T1). Moreover, in the group with the highest dose of NSE, *S. typhimurium* colonies were still not found. This indicates that the administration of NSE at 0.52 mg/kg BW, 5.2 mg/kg BW, and 52 mg/kg BW to the mice infected with *S. typhimurium* can kill the bacterial colonies in spleen. There are no bacterial colonies found in other groups including all treated groups. Thus, it can be concluded that NSE effectively kill *S. typhimurium* in spleen of mice. T1 group indicated the highest number of *S. typhimurium* colonies.

The NSE treatment at 0.52, 5.2, or 52 mg/kg caused the number of *S. typhimurium* colonies in spleen decreased to zero (Hasan et al., 2013) reported the effect of DTQ on gram-negative bacteria. Additionally, NSE has shown both synergistic and additive anti-bacterial effects and anti-microbial agents. Thymohydroquinone has an antibacterial effect and decreases the number of bacterial colonies (Paarakh et al., 2010). Moreover, thymoquinone affects gram-negative bacteria by interfering with the bacterial envelope and impeding the bacterial cell from controlling its movement also causes bacterial lysis which leads to bacterial cell death (Ashraf et al., 2016; Sufya et al., 2014). The consumption of antioxidant obtained from N. sativa are found and can stimulate activities of phagocytic cells against Salmonella and regulate both the innate and acquired immune responses.

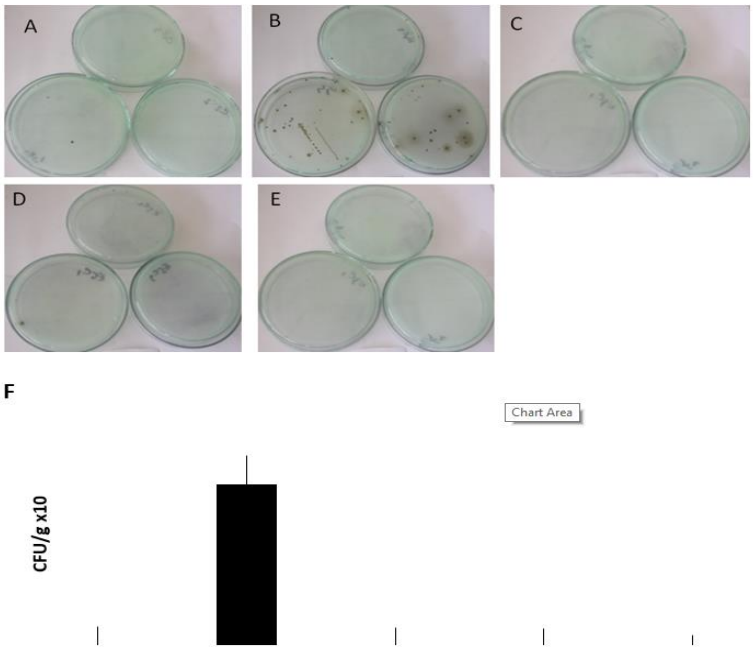

Figure 3. The number of Bacterial colony in spleen of mice were infected with *S. typhimurium* and treated with NSE. A-E. Mice were injected with *S. typhimurium* (T1) group indicated the highest accumulation of bacteria colonies. Mice were injected with *S. Typhimurium* (T1) and NSE different concentration (0.52 (N1), 5.2 (N2) and52 mg/kg BW (N3). F. Results shown are mean ± SD, with n=4 replicates in each group. ∗p<0.05, ∗∗p<0.001.

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Conclusions

In conclusion, *Nigella sativa* extract at a dose of 52 mg/kg BW is useful to enhance the percentage of Treg and Th2 to immune response.

Acknowledgements

We gratefully acknowledge Wahyudha Ngatiril Lady, S.Si. for technical assistance.

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