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Study of Antimicrobial Activity of Black Cumin Seeds (*Nigella sativa* L.) Against *Salmonella typhi In Vitro*

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Abstract

Objective: To determine the effectiveness of extracts of black cumin seeds (*Nigella sativa* L.) as an antimicrobial against *Salmonella typhi in vitro*. And can determine minimum inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) from extracts of black cumin seeds (*Nigella sativa* L.) against *Salmonella typhi*.

Design: This experimental study used post-test only control group design with four time repetition. Step one was cultivating bacteria in liquid medium with various concentration of extract, that was 40%, 42.5%, 45%, 47.5%, 50% with two control, extract control and bacterial control.

Results: The MIC (Minimal Inhibition Concentration) is 45% concentration of extract. Step two was plating in NAP (Nutrient Agar Plate) medium. The MBC (Minimal Bactericidal Concentration) is 47.5% concentration of extract. The result of experiment was knew there are different average of *Salmonella typhi* colony from every group. The result experiment was analyzed by One Way Anova Test. The hypothesis test of MBC show significant differentiation, and then was continued with regression test. The conclusion of this study was the addition of extract concentration occurred reduction average of *Salmonella typhi* colony.

Keywords: Antimicrobial; Extract ethanol; *Nigella sativa* L.; *Salmonella typhi*; *In vitro*

Introduction

Bacterial of the genus *Salmonella* are capable of causing a large number of human infections, including typhoid fever, systemic infections, septicemia, and gastroenteritis varying clinically from watery diarrhoea to dysentery [1].

Typhoid fever is caused by microorganisms *Salmonella enterica* subspecies enterica serotype typhi (abbreviated as *Salmonella typhi*), is a systemic infectious disease, and is still a health problem in Indonesia. Endemic infectious diseases are growing in Asia, Africa, Latin America, the Caribbean and Oceania, including Indonesia, where sanitation is poor. Although WHO declared typhoid fever not including deadly diseases such as pneumonia, tuberculosis, malaria, measles and HIV/AIDS, but the epidemic of typhoid fever can be like in 1989, where 11 countries stricken with typhoid fever. In that year 10% of patients who died of typhoid fever. WHO said there were 20 million deaths each year are caused by typhoid fever. WHO reported incidence of typhoid fever increased by 15.41% in 2008 [2]. Young adult patients often experience severe complications such as bleeding and perforation of the bowel which often end in death [3]. Because of the high morbidity and mortality rates of typhoid fever, the various parties seek to resolve this problem.

In the last five years, clinicians in several countries observe any cases of typhoid fever are severe and even fatal child, which was caused by *Salmonella typhi* strains resistant to chloramphenicol. In the subsequent development of resistance of *Salmonella typhi*, several countries reported a strain of multi-drug resistance (MDR) *Salmonella typhi* that is resistant to two or more antibiotics commonly used are ampicillin, chloramphenicol and cotrimoxazole. The development of MDR *Salmonella typhi* so fast in some countries resulting in mortality cases of typhoid fever in children increases, the experts seek another alternative treatment for typhoid fever that fever down quickly, shorter treatment times and reduced relapse [4].

The continued development of drug resistance of microorganisms to other microorganisms one that makes health organizations in the world were moved. Food Drugs Association (FDA) one of them, implementing an action plan against drug resistance with 4 stages, namely, surveillance, development of a new drug product effective and natural, education, and research on resistance. The fourth stage is already underway FDA since 8 years ago [5].

Currently in Indonesia is developing a new paradigm in the field of health, namely the use of natural herbs and traditional medicines. Medicinal plants as natural resources have not been exploited and developed in depth is still very open to be researched and developed under on finding an effective drug as an anti-microbial, especially in typhoid fever. The use of herbal medicines is expected to be an alternative for the community to prevent the side effects of antibiotic use [6]. Therefore, the need for alternatives to overcome these problems, logical, scientifically appropriate methods of "scientific knowledge". One possible approach is to use plants (herbs) that have known or are still in the form of alleged anti-microbial effects that can be used widely, with a good level of security or no side effects [7].

Since one century, the Greek physician, Dioscorides, it has been reported that black cumin or *Nigella sativa* or Black Seed to treat a variety of ailments such as headaches, nasal congestion, dental pain, and illness internist. During the golden age of Islam, by Al Biruni, the seeds of *Nigella sativa* used nutritional ingredients. In the treatment in Greco-Arab or Greek-Tibb, from Hippocrates, Galen, and Avicenna, *Nigella sativa* is a very valuable remedy in treating gastrointestinal dysfunction and hepatitis described as a stimulant for the different conditions [8]. Religious factors also increase the confidence of his people to take advantage of *Nigella sativa*. In the hadith of Prophet Muhammad SAW said that black cumin can cure all diseases except death. Various studies show that in *Nigella sativa* contains essential oils (volatile oil). Essential oils are generally anti-microbial, anti-inflammatory [9]. Antimicrobials of black cumin seed extract in the form of active compounds such as: alkaloids, saponins, tannins, melantin, derivatives terpenoids, and timokinon [7]. So far, studies have been conducted on the effects of *Nigella sativa* strains of the bacterium Vibrio colera and *E. coli*. The research that published in the journal Pharmaceutical Pakistan showed that more potent volatile oils to kill both bacteria compared antibiotics such as ampicillin and tetracycline [9].

Methodology

Research conducted experimental research laboratory is using the tube dilution method to determine the concentration of extract of black cumin seeds (*Nigella sativa* L.), which can affect the growth of the bacteria *Salmonella typhi* isolates *in vitro*. The tube dilution method is to determine the MIC and MBC. This method includes two phases, phase of testing materials/extracts against isolates of *Salmonella typhi* in the media brain heart infusion broth to determine the MIC and the stage of planting in the medium Nutrient To Plate to determine MBC. There are five groups of treatment, coupled with germ control (0% extract material) and material control extract (100% extract material), with four repetitions.

Result

Extract ethanol of *Nigella sativa* L.

The end result of black cumin seed extract liquid is obtained black. Then extract that has obtained a suspension made by bacteria test (*Salmonella typhi*) that are mixed CMC 0.1% (Cetyl Methyl Cellulose) as emulsifier between the distilled water to extract oil that there are elements, the extract concentration of 40%, 42.5%, 45 %, 47.5% and 50%. As a control material is 100% concentration is the concentration in which the untreated bacteria (pure extract), while the concentration of 0% is a bacterium that controls the concentration of the extract is not given at all. The concentration of each treatment was obtained using the formula dilution with reference based on exploratory study (preliminary investigation) has been done before.

Exploratory research using a concentration of 2.5%, 5%, 10%, 20%, 40%, and 50% by the tube dilution method using serial dilutions. After planting the bacteria in the medium NAP, the exploration results that range from 50% already do not grow bacteria, while at a concentration of 40% was obtained bacterial colonies. Then the researchers explored back with a concentration from 40% to 50%, using a concentration of 40%, 42.5%, 45%, 47.5% and 50%. After planting the bacteria in the medium NAP was found that the concentration of 45% is still a bacterial growth while at a concentration of 47.5% is not contained bacterial growth.

Based on this, researchers took concentration from 40% to 50% to determine the value of MIC and MBC. The final concentration used in this study is 40%, 42.5%, 45%, 47.5% and 50%. Researchers over the difference in the concentration range close up with the aim of MIC and MBC values obtained are more accurate.

The minimum inhibitory concentration (MIC) growth of *Salmonella typhi*

Solution between black cumin seed extract and bacterial extracts were made with a concentration of 40%, 42.5%, 45%, 47.5%, and 50% for the then incubated at 37°C for 18-24 hours, then observed the change of turbidity turbidity which is later will be able to know the level of minimum inhibitory concentration (MIC) black cumin seed extract on the growth of *Salmonella typhi.*

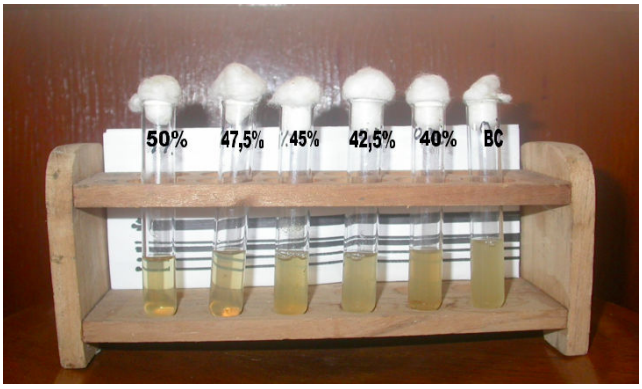

Figure 1: Dilution results for Test Tube MIC against *Salmonella typhi.*

Shown in the Figure 1 that at a dose of 40%, 42.5%, is still turbid media, while at a dose of 45% compared with the media already clear KK. KHM then obtained at a dose 45%.

From Figure 1 it can be seen that at the extract concentration of 0% BC (Bacterial Control) with four repetitions showed turbidity levels are very murky. At a concentration of 40% extract showed turbidity levels are very murky. In the extract concentration of 42.5% showed turbidity levels are murky. At a concentration of extract of 45, 47.5%, 50%, and 100% (Material Control) showed clear. Based on observations of turbidity levels in BHI media can be seen MIC value is the concentration of the extract 45%. The concentration meaning that 45% is the lowest concentration of an antimicrobial that is able to inhibit the growth of bacteria *Salmonella typhi.*

The minimum bactericidal concentration (MBC) *Salmonella typhi*

Once known the value of MIC of observations in liquid media, then planting the NAP with streaking method. These observations were made by counting the number of *Salmonella typhi* colonies that grow on the medium NAP after planting one ose of each concentration to determine the antimicrobial effect of black cumin seed extract on the growth of *Salmonella typhi.* KBM value obtained by counting the number of colonies on the plate using a colony counters "LAB-LINE".

In this study, the dose of the extract obtained KBM 47.5%. At this dose plate, there was no growth of colonies on the plate into four. The following image (Figure 2) on the medium NAP streaking agar plate:

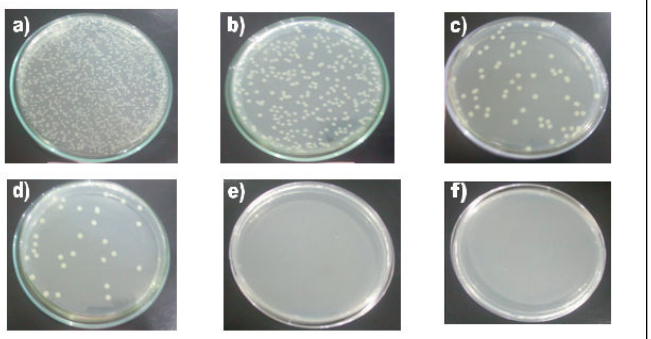

Figure 2: Results *Salmonella typhi* streaking on NA medium for MBC test Caption a) Control of germs b) Dose extract 40% c) Dose extract 42.5% d) Dose extract 45% e) Dose extract 47.5% f) Dose extract 50%.

It appears that at a concentration of 40%, 42.5% and 45%, there are still colonies of bacteria grow that amount> 0.1% of the original inoculum, at a concentration of 47.5% while there was no growth of colonies (colony growth of germs <0.1% of colonies the original inoculum). That is 47.5% doses of the MBC black cumin seed extract.

Then done counting the number of colonies of *Salmonella typhi* at each concentration with four repetitions by using Colony Counter. Counting the number of colonies of *Salmonella typhi* at each NAP. Results planting NAP media of various concentrations of the extract can be seen in Table 1 and Figure 3 below.

Treatment		Repetition			Mean
	1	2	3	4	
0%	8.5×10^7	8.4×10^7	8.9×10^7	7.8×10^7	8.4×10^7
40%	6.2×10^7	6.4×10^7	6.5×10^7	5.8×10^7	6.2×10^7
42.5%	5.1×10^7	4.3×10^7	5.0×10^7	4.2×10^7	4.5×10^7
45%	1.6×10^7	1.1×10^7	2.9×10^7	1.0×10^7	1.25×10^7
47.5%	0	0	0	0	0
50%	0	0	0	0	0
100%	0	0	0	0	0

Table 1: The Results mean colony of *Salmonella typhi* to different treatment.

From Table 1 it can be seen that the decline in the number of bacterial colonies in line with increasing concentrations of black cumin seed extract. In the extract concentration of 40% obtained average colony growth as much as 6.2×10^7, the concentration of the extract contained 42.5% average growth of 4.5×10^7 colony as much as 45% extract concentrations are the average growth of the colony as much as $1{:}25 \times 10^7$; and the extract concentration of 47.5% and 50% had not obtained colony growth. At the bacteria control the growth of bacteria obtained as much as 8.4×10^7 colonies.

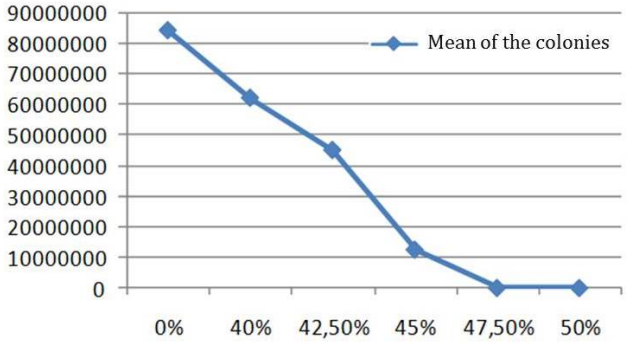

Figure 3: Mean colony of *Salmonella typhi* each deuteronomy inter treatment.

When the number of colonies of each treatment concentration compared to the number of colonies of bacteria control, seen a significant reduction of the number of bacterial colonies. Minimum Bactericidal Concentration (MBC) is the lowest concentration of extract that is capable of killing bacteria, so MBC from black cumin seed extract in this study is the extract concentration of 47.5% due to the concentration had not obtained colony growth on medium NAP (the growth of bacteria colonies <0.1% of the original colony inoculum).

Discussion

Essential oil of black cumin seeds contains active compounds, namely; alkaloids, saponins, tannins, terpenoids and derivatives. Alkaloid class of black cumin seeds are alkaloids isochinolin (nigellimin and nigellimin-N-oxide) and pyrazol alkaloids (nigellidin and nigellicin). Derivatives terpenoids (carvacrol, carvone, limonene, 4-terpineol, citronellol). Active substances such as anti-microbial function [7]. Alkaloids have a mechanism to interact with bacterial DNA by putting themselves between strands of DNA, so that it will interfere with DNA work in organizing all activities of bacteria that cause bacterial lysis [10]. Saponin works by damaging the cytoplasmic membrane and the membrane of bacteria so disturb organelle bacterial cell permeability [11]. Saponins also inhibit the DNA-polymerase so that it can interfere with bacterial nucleic acid synthesis. Tannins can bind a protein which is owned by bacteria, that adhesin and when this happens it can damage the availability of receptors on the cell surface of bacteria. Tannin also can form complexes with compounds irreversible proline, a complete protein, which has a bonding effect of inhibition of protein synthesis for the formation of cell walls [12]. Tannins work by pursed wall/membrane of the cell and disrupt the bacterial cell permeability. Through disruption of the permeability, the cell cannot perform life activities that are stunted or even die. Terpenoids works by forming a complex with the protein extracellular and bacterial cell membrane causing bacterial membrane is damaged.

The mechanism allows one or more ingredients such active substances in inhibiting or killing the bacteria *Salmonella typhi*. Inhibition of the growth of *Salmonella typhi is* observed from the value of MIC (minimum inhibitory concentration) and MBC (Minimum bactericidal concentration). In this study found the MIC at 45%, where

the results of the first dilution tube showed clarity compared to the other doses. In the beginning, before incubation, it was found that the higher the concentration pattern, the result will be more turbid dilution tube, for mixing extract more. However, after incubation for 18-24 hours, the state of being turned away, the higher the concentration, the more lucid colour tube. This is because, during the incubation, the bacteria multiply. At a dose of 40% and 42.5% inhibitory effect was not found. Therefore, the result of the dilution tube looks cloudy. As with the concentration of 45%, 47.5% and 50%, where there is inhibition of growth of bacteria, resulting in a dilution tube visible in the clear.

Research on the *in vitro* test *Nigella sativa* against *Aeromonas hydrophila* done by Tumar and Boimin which concluded that the lowest concentration of *Nigella sativa* extracted, resulting in inhibition zone against *Aeromonas hydrophila* in value of 2%. Sedangkan in *in vitro* studies that performed by Salman et al. proved that *Nigella sativa* extract MIC was 2% against *Pseudomonas aeruginosa* and 1% against *Staphylococcus aureus*. This proves that black cumin seed extract is effective for gram positive and negative bacteria.

MBC obtained from the study of 47.5%, which is not found colonies of *Salmonella typhi* growth at all in order Nutrient Plate or the number of colonies less than 0.1% original inoculum. Means that at a dose of 47.5% is the minimum the concentration of black cumin seed extract can kill *Salmonella typhi*.

Black cumin seeds has anti-microbial effect is of essential oil content or volatile oil. The essential oil contains active substances such as from the class of alkaloids, saponins, tannins, and terpenoids which serves as an anti-microbial. Alkaloids are alkaloids isochinolin (nigellimin and nigellimin-N-oxide) and pyrazol alkaloids (nigellidin and nigellicin). Derivatives terpenoids (carvacrol, carvone, limonene, 4-terpineol, citronellol) [7].

The working mechanism of alkaloids as antimicrobial herein attributed to its ability to berinterkalasi with bacterial DNA by putting themselves between strands of DNA [10]. This causes the genetic encoding process through DNA transcription and protein translation bacteria become impaired and can result in damage to DNA that bacterial cell lysis.

Saponins inhibit DNA polymerase that can interfere with nucleic acid synthesis. Saponins are secondary metabolites that are found in nature, consisting of sugar groups that bind to the aglycone or sapogenin.

Tannin as antimicrobial working on complex formation with proteins, namely through hydrogen bonding and covalent. Works which include the inactivation of microbial adhesion molecules, enzymes, protein transport through the cell membrane, destruction of the substrate and bind to the bacteria cell wall polysaccharides [10]. These compounds form complexes with polysaccharides. Inactivation adhesin by tannin causing bacteria can not stick to the intestinal epithelial cells of the host (human). Terpenoids can destroy bacterial cell membrane through a cluster lypofilic.

Terpenoids are very active in killing the bacteria. Terpenoids can destroy bacterial cell membrane through a cluster lipofiliknya. While other active ingredients such as volatile oil and others have also been known to effect in inhibiting the growth of bacteria.

From other studies that have been conducted, it has been proved that *Nigella sativa* has the effect of anthelmintics against Ascaris suum Goeze at the concentration of 10% [13]. *Nigella sativa* also has antifungal effect on the growth of Aspergillus clavatus Desm at the concentration of 10% [14]. At the end of the writing of this paper the authors hope that this study can provide maximum benefit for all parties who wish to take advantage in it for the good, including for further research, and for all parties aware of the essence of herbal medicine.

Conclusion

The minimum inhibitory concentration (MIC) extract of black cumin seeds (*Nigella sativa* L.) of the bacterium *Salmonella typhi is* obtained at doses 45%. The kill minimal concentration (MBC) extract of black cumin seeds (*Nigella sativa* L.) of the bacterium *Salmonella typhi* obtained at doses 47.5%.

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