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The antibacterial activity of ethanol extracts *Nigella sativa in vitro*

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Abstract
Nigella sativa is commonly used as a traditional medicine since ancient times in which some of its substances are believed to contain anti-oxidants and anti-inflammatory. This research aimed to determine the anti-bacterial activities of ethanol *Nigella sativa* extract from subjects with diabetic foot ulcers *in vitro*. It was an experimental research; *Nigella sativa* extraction was done with n-hexane, ethyl acetate, and ethanol, then antibacterial tests were conducted on the extraction result. Bacterial samples were taken from subjects with diabetic foot ulcers and were cultured in PCA media. Anti-bacterial activities were seen from the diameter of the bacterial inhibitory zone. Data were analyzed using one-way Anova. Results from the culture of diabetic foot ulcers specimens showed apoly microbial growth. The prevalence of Gram-negative bacteria was found to be more than the prevalence of Gram-positive bacteria. The test results revealed that ethanol extracts of *Nigella sativa* were able to inhibit bacterial growth. Ethanol extract of *Nigella sativa* had the greatest effect on bacterial growth with the average inhibitory zone of 257.5 mm2 (p<0.05). We conclude that the ethanol extracts of *Nigella sativa* have powerful anti-bacterial activities.

Keywords: *Nigella sativa*, ethanol, anti-bacterial

1. Introduction
Nigella sativa (*N. sativa*) is known as black cumin in Asia or black seeds in Western countries. In Arabic countries, it is named habbatussauda, and it is known as kalonji in India [1, 2]. *N. sativa* is widely used as a traditional medicine since ancient times in which some of its substances are believed to contain anti-oxidants and anti-inflammatory [3].
Neuropathy in Diabetes mellitus (DM) sufferers will cause diabetic [4]. The diabetic ulcers tend to be wet and damp with a lot of necrotic tissue. The necrotic tissue is a good medium for bacterial growth, either aerobic or anaerobic bacteria [5], an infection that makes diabetic ulcers difficult to be treated. A research result found that the causing-infection bacteria on diabetic ulcers are Pseudomonas aureginosa, Streptococcus, P. Mirabilis, Staphylococcus, Escherichia coli and Klebsiella pneumoniae [6].
This research aimed to find out the antibacterial activity of ethanol *N. sativa* extract from subjects with diabetic ulcers *in vitro*.

2. Materials and methods
2.1 Chemicals and Drugs
The chemicals used, were ethanol 70%, and Dimethyl sulfoxide (DMSO 10%) as a negative control, while the drug used, as a positive control, was chloramphenicol

2.2 Instrument
The tools used in this research were microscope, autoclave, Laminar Air Flow (LAF), beaker, Petri dish, Erlenmeyer flask, beaker cups, test tube, inoculating loop, tube rack, bunsen, vortex, pipette, micropipette, incubator, waterbath, rotary evaporator, spectrophotometer, analytical scales, grinder, stationery, camera, paper disc, MRSA media, Kings B Agar media, Media Plate Count Agar (PCA), Trypticase Soy Broth (TSB) media, Trypticase Soy Agar (TSA).

2.3 Plant Material and Preparation of the Extract
The procedure taken before extraction was to prepare simplicia sample of black cumin seed that has been determined. The dry-black cumin seeds were then grounded by using a grinder so that simplicia powder of black cumin seeds was obtained [7]. The extraction of anti-microbial components by maceration was done by using ethanol (polar) [8, 9]. The filtrate result was evaporated with rotavaporof 40 °C to separate the solvent with the extract.

The extract was then evaporated in the water bath to obtain the pure extract. The evaporated extracts were used as samples for antibacterial analysis and testing.

2.4 Bacterial Preparation and Identification

Specimens were taken directly from patients (subjects) with diabetic ulcers which then were cultured on PCA media that had been made. After that, the colony calculated by using Total Plate Count (TPC) method [10]. The number of colonies less than 30 and more than 300 cannot be used because that statistical count is unreliable. After TPC-standard colonies were obtained, the bacterial culture was performed. The culture was performed in a LAF and was incubated during 24 hours. After bacterial growth occurred in TSB media, the experimental bacteria were made by diluting the TSB media. Then, the bacteria were counted using spectrophotometer of 600 mm wavelength by inserting the TSB media into a cuvette. The spectrophotometer of 600 mm wavelength was set to obtain 0.1 absorption in order to obtain the same turbidity of McFarland standard (1.5x108 bacterial cells/ml) [11]. If the turbidity was too dense, it could be added with 5-10 drops of TSB media by using pipette.

2.5 Experimental Procedure

200 µl of bacteria were taken and dropped on the surface of Petri dishes. After that, they were poured into the warm Mueller Hinton Agar (MHA) media and were homogenized. The dishes that had already contained the solid media were then covered with five disc paper. Each disc paper was dripped with 10µl chloramphenicol, 10µl DMSO 10%, and 10µl concentration variation of the N. sativa by using a micropipette in LAF (Laminar Air Flow). These Petri dishes were then incubated for 24 hours at 37 ºC. Each treatment was repeated nine times. The formed inhibitory zone was measured by using a calliper. The inhibitor zone measurement was conducted on the diameter of inhibitory zone (transparent) [12].

3. Results & Discussion

3.1 Bacterial Charakteriktik

In this research, DM ulcer bacteria were planted on PCA media, as well as Kings B Agar and MRSA experimental media. The planting result showed that the bacteria could grow well in all media. The PCA media were for common bacteria and were used as media for counting bacterial colonies. Kings B Agar media could be used as media for detecting pseudomonas bacteria, while MRSA media were used for growing lactobacillus bacteria. Both of the experimental media could be overgrown with bacteria. This indicated the presence of gram-positive and gram-negative bacteria grown in DM ulcers. The bacterial prevalence on PCA media was 58% gram-negative bacteria and 42% gram-positive bacteria. This was shown in Figure 1.

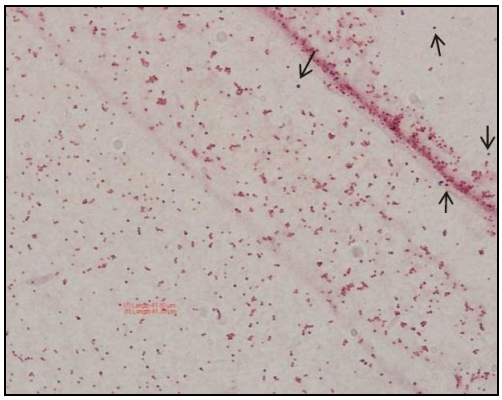

Fig 1: Bacteria isolated from diabetic foot. Gram-negative bacteria were more dominant and were in round shape with red colour. (↑) Gram-positive bacteria were relatively in small numbers and were in round shape with purple/ blue colour.

3.2 Anti-bacterial Test

Anti-bacterial test results of *N. sativa* extracted with ethanol showed the presence of bacterial inhibitory zone, i. e. the averages of bacterial inhibitory zone were 257.5 mm^2 at 100% concentration, 237 mm^2 at 50% concentration, and 10.5 mm^2 at 10% concentration. These results revealed that the ethanol fraction had the greatest effect on bacterial inhibitor zone of diabetic ulcers (Fig 2).

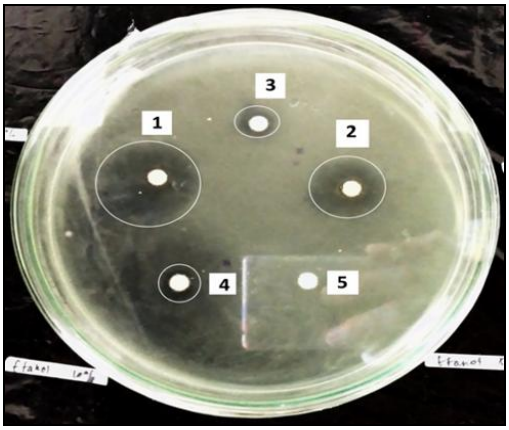

Fig 2: Inhibitory zones of *N. sativa* extract. *N. sativa* extracted with ethanol 70%) (1) 100% concentration; (2) 50% concentration; (3), 10% concentration; (4), chloramphenicol 3% (control positive); (5) DMSO 10% (control negative).

3.3 The inhibitory zone comparison of *N. sativa* extracted ethanol 70%, chloramphenicol 3% and DMSO 10%

The analysis result showed a significant difference from each group. It showed that *N. sativa* which was extracted with ethanol 70% was the most effective extract for inhibiting the diabetic ulcers bacteria compared with chloramphenicol 3% and DMSO 10% (p value <0.05) (Table 1).

Table 1: The comparative measurement results of the inhibitory zones of *N. sative* extracted (100%, 50%, 10%), chloramphenicol 3% and DMSO 10%

Group (I)	Group (J)	Mean Difference (I-J)	Std. Error	Sig.
N. sative 100%	*N. sative* 50%	48.33333*	7.92496	0.000
	N. sative 10%	168.88889*	7.92496	0.000
	Clorampenicol 3%	198.66667*	7.92496	0.000
	DMSO 10%	280.00000*	7.92496	0.000
N. sative 50%	*N. sative* 10%	120.55556*	7.92496	0.000
	Clorampenicol 3%	150.33333*	7.92496	0.000
	DMSO 10%	231.66667*	7.92496	0.000
N. sative 10%	Clorampenicol 3%	29.77778*	7.92496	0.001
	DMSO 10%	111.11111*	7.92496	0.000

The research results revealed that the largest causing-infection bacteria found in diabetic ulcers were the group of gram-negative bacteria seen from Figure 1. The previous research also proved the similar result, i. e. the diabetic ulcers infection was most frequently caused by the gram-negative bacteria [13].
N. sativa which was extracted using ethanol 70% contains the most anti-bacterial active compound compared to the extraction using other solvents. It is estimated that the anti-bacterial content of *N. sativa* extracted with polar solvent (ethanol) can be perfectly extracted, resulting in a high anti-bacterial content [9].
In previous studies, *N. sativa* contains the anti-bacterial compounds include alkaloids, flavonoids, saponins, proteins and thymoquinone [14]. Flavonoid biological activities against bacteria are done by destroying bacteria's cell wall consisting of lipids and amino acids. After that, the compound may enter into bacteria's nucleus which then contacts with bacteria's DNA. Flavonoid can damage the lipid structure of bacteria's DNA, so that the nucleus will get lysis and the bacteria will die [15].

4. Conclusions
The ethanol extracts of *Nigella sativa* have powerful anti-bacterial activities, with the average inhibition zone of 257.5 mm^2

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