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ORIGINAL ARTICLE

Activity of *Nigella Sativa* Oil on Multidrug Resistant Organisms Isolated from Diabetic Foot Patients

[1]Mohamed I. Abd El-Hamid, [1]Marwa M.E. Abd-Elmonsef, [2]Emad M. Sallam, [1]Sara I. Abd El Galil*

[1]Medical Microbiology and Immunology Department, Faculty of Medicine, Tanta University, Tanta, Egypt
[2]Vascular and Endovascular Surgery Department, Faculty of Medicine, Tanta University, Tanta, Egypt

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Corresponding Author:
Sara Ismail Abd El Galil
M.B.B.CH, Medical Microbiology and Immunology Department, Faculty of Medicine, Tanta University, Tanta, Egypt
Tel.: 010 07601714
dr.roka.sara@gmail.com

ABSTRACT

Background: *Multidrug-resistant (MDR) organisms are becoming more prevalent worldwide, which is an issue in poor nations that depend on natural resources, especially traditional and herbal plant sources. Nigella sativa oil (NSO) is a strong antibacterial that could be an excellent option.* ***Objective:*** *The aim of the present work is to determine the in vitro antibacterial efficacy of NSO against MDR microbes isolated from diabetic foot ulcers.* ***Methodology:*** *By using the agar well diffusion method to evaluate the zone of inhibition of NSO, the antibacterial impact of NSO against MDR bacteria of diabetic foot ulcers was ascertained. The tube dilution method was used to find the minimal inhibitory concentration (MIC), and sub-culturing tubes that showed no discernible growth or turbidity in the MIC allowed for the determination of the minimal bactericidal concentration (MBC).* ***Results:*** *NSO produced zone of inhibition ranged from 0 mm. to 28 mm., MIC ranged from 3% to 100% and MBC ranged from 6% to 100% against different MDR bacteria of diabetic foot ulcer. Bacterial outcomes and inhibition zones made by NSO against different isolated organisms were insignificantly different between Gram-positive and Gram-negative organisms.* ***Conclusion:*** *NSO had antibacterial effects against MDR Gram-positive and Gram-negative organisms. It showed significant synergism in combination with different antibiotics.*

INTRODUCTION

In 2000, there were an estimated 171 million individuals globally afflicted with diabetes mellitus (DM), and this figure continues to rise. By 2035, 592 million patients are anticipated to have DM[1].

Many problems arise from diabetes mellitus. One of the most devastating conditions is diabetic foot ulcers (DFUs). According to earlier studies, between 9.1 and 26.1 million people with diabetes mellitus get foot ulcers[2]. Due to their rapid progression to irreversible septic gangrene, which would necessitate the amputation of the afflicted foot, diabetic foot infections (DFIs) are a highly expected consequence of diabetes. People with diabetes account for up to 70% of all limb amputations. Compared to people without diabetes, people with diabetes are 25 times more likely to have their legs amputated[3]. People with diabetes are far more likely than people without diabetes to be admitted to the hospital for soft tissue and bone infections of the foot[4].

Studies of microbiology have revealed that DFIs are polymicrobial, with aerobes, including *Staphylococcus aureus*, and coagulase-negative isolates being the most commonly identified. *Pseudomonas aeruginosa*, *Staphylococcus* spp., *Enterococcus* spp., *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* [5].

The evolution of microbial resistance to several antimicrobial drugs has had harmful consequences on patients and is a major global issue[6]. The emergence of acquired resistance to at least one drug from three or more antimicrobial classes is a characteristic of multidrug-resistant (MDR)[7]. Increased morbidity and mortality rates result from the emergence of MDR. Furthermore, the efficacy of current antibiotics has been compromised due to the escalated treatment expenses, resulting in treatment failure[8].

There is a pressing need to redouble efforts to identify antimicrobial agents that are efficacious against pathogenic bacteria, given the escalating bacterial resistance to conventional antibiotics and the growing public fascination with herbal medicine[9].

It has been documented that *Nigella sativa linn*. (Family Ranunculaceae), it has several pharmacological qualities, including as antibacterial, antifungal, antiparasitic, anti-inflammatory, and antioxidant activities. It is also known as black cumin or black seeds[10]. Antimicrobial activity against various MDR and pathogenic bacteria has been observed in crude extracts and essential oil derived from black seeds[11].

Evaluation of NSO's antibacterial activity against MDR microbes isolated from diabetic foot ulcers was the goal of this study.

METHODOLOGY

From April 2019 to April 2020, this study was conducted at Tanta University's Faculty of Medicine, Department of Microbiology and Immunology. Fifty patients were enrolled in the trial. Patients with diabetic foot ulcer symptoms and indicators of infection are eligible.

Patients who were treated with antibiotics within five days prior to the sample were excluded. The study was conducted with permission from Tanta University's Faculty of Medicine's Ethical Committee in Tanta, Egypt (App No: 33008/03/19). The patients gave their signed, informed permission.

Isolation and identification of the infecting organisms:

On nutritional, blood, and MacConkey's agar plates (Oxoid, UK), all specimens were cultivated aerobically. At 37°C, all plates were incubated for 24 to 48 hours. The organisms were identified by conventional microbiological method.

***Nigella sativa* seed oil:** It was purchased from the local markets, product weight (30 ML). The dosage of *N. sativa* is stored in the dark at 4°C (Elcaptain Company).

Antibiotic sensitivity testing:

Following the requirements of the Clinical and Laboratory Standard Institute (CLSI 2023), the isolates' antimicrobial susceptibility was assessed using the modified Kirby Bauer disc diffusion technique on Muller Hinton agar plates[12]. All discs were obtained from Oxoid, UK.

Penicillin G (10 units), linezolid (30µg), cefoxitin (30µg), clindamycin (2µg), azithromycin (15µg), ciprofloxacin (5µg), and gentamicin (10µg) were the substances that were tested for in Gram-positive organisms. Imipenem (10 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), aztreonam (30 µg), amoxicillin clavulanic acid (20/10 µg), sulfamethoxazole trimethoprim (1.25/23.75 µg), cefotaxime (30 µg), cefipime (30 µg), ceftriaxone (30 µg), piperacillin-Tazobactam (TPZ) (100/10 µg), and meropenem (10 µg) were tested against Gram-negative organisms.

Evaluation of the antibacterial activity of NSO:

Using an inoculating wire loop, three to five pure colonies of the isolated organisms were selected from each isolate, suspended in four to five millilitres of nutritional broth, and cultured for twenty-four hours at 37°C. After that, sterile distilled water was added to the bacterial suspension until its turbidity reached 0.5 McFarland Standards (105–106 CFU/ml). Following the established protocol, the resultant suspensions were further diluted 1:100 in sterile nutritional broth to achieve an inoculum density of 1×104 CFU/ml[13]. The sterilization of NSO was tested by inoculation on blood and Mackonkey agar at 37°C for 24h. No growth was yielded on the inoculated plates.

Determination of MIC of NSO:

The minimum inhibitory concentration (MIC) of *Nigella sativa* oil (NSO) was assessed utilizing the broth tube dilution technique. In this procedure, ten sterile test tubes were arranged in a rack and labelled from 1 to 8, with additional tubes designated for negative control (NC) and growth control (GC) to ensure quality assurance. Each tube received 1 ml of freshly prepared nutrient broth, which was subsequently sterilized and allowed to cool. Using a sterile micropipette and tips, 1 ml of undiluted NSO (100%) was introduced into test tube 1 and the NC. A two-fold serial dilution was then executed by transferring 1 ml of the undiluted oil into the second tube, employing separate sterile tips for each transfer, followed by vortexing to ensure thorough mixing. This process was repeated, transferring 1 ml from tube 2 to tube 3, and so forth, until tube 8 was reached, which represented a dilution of 1:128. At this stage, 1 ml was removed and discarded from tube 8. The GC tube, which did not contain any oil, acted as a growth control, while the NC tube, which lacked bacterial inoculum, served as the oil control. All tubes, except for the NC, were inoculated with 1 ml of the respective organism culture. This entire procedure was replicated for each organism tested with NSO. Following inoculation, the tubes were incubated at 37 °C for 24 hours and subsequently examined visually for signs of growth, indicated by turbidity[14].

Determination of MBC of NSO:

Using the streak plate approach, incubated tubes that showed no discernible growth or turbidity in MIC were sub-cultured onto sterile nutrient agar plates and aerobically incubated for 24 hours at 37 °C in order to estimate the MBC. The MBC was defined as the lowest dose of NSO that prevented test organism development[14].

Determination of the synergistic effect of different antibiotics and NSO on tested organisms:

The disc diffusion method was employed to assess the combined effects of NSO and conventional antibiotics on the organisms under test. After soaking 10 µL droplets of NSO on an antibiotic disc, the plates were left to dry so that the oils could properly diffuse, and they were then incubated for the whole night at 37 °C. The zone of inhibition surrounding each disc was examined the next day, and the outcomes of the NSO and antibiotic combination and NSO alone were compared[15].

Statistical analysis:

SPSS v26 was used for statistical analysis (IBM Inc., Chicago, IL, USA). The ANOVA (F) test with post hoc test (Tukey) was used to compare the three groups'

quantitative variables, which were displayed as mean and standard deviation (SD). The Chi-square test was used to analyse the qualitative variables, which were shown as frequency and percentage (%). It was deemed statistically significant when the P value was less than 0.05.

RESULTS

The type of growth was insignificantly different between the monomicrobial and no-growth groups of samples. Age and sex were insignificantly different among Gram-positive infection, Gram-negative infection, and no growth. Males were higher than females in the 3 groups (Table 1).

Table 1: Type of growth in the samples and demographic data of patients of the study

		Monomicrobial	Polymicrobial	No growth	P
Type of growth		44(88%)	-	6(12%)	0.818
Demographic data					
		Gram-positive infection (n=25)	**Gram-negative infection (n=19)**	**No growth (n=6)**	**P**
Age (years)		57.44±8.94	57.36±9.16	63±10.88	0.914
Sex	**Male**	18(72%)	13(68.42%)	4(66.67%)	0.95
	Female	7(28%)	6(31.58%)	2(33.33%)	

Data are presented as mean ± SD or frequency (%).

Bacterial outcomes and inhibition zones made by NSO against different isolated organisms were insignificantly different between Gram-positive and Gram-negative (Table 2).

Table 2: Bacterial growth outcome, inhibition zone of *Nigella sativa* oil

	Gram-positive		Gram-negative		P
Organisms	**MRSA**	21(84%)	***P. aeruginosa***	13(68.42%)	0.414
	MSSA	2(8%)	***E. coli***	4(21.05%)	
	CONS	2(8%)	***Klebsiella***	2(10.53%)	
Inhibition zone of NSO	**MRSA(n=21)**	19.24±7.23	***P. aeruginosa* (n=13)**	8.32±5.51	0.414
	MSSA (n=2)	17±1.41	***E. coli* (n=4)**	15.75±11.44	
	CONS (n=2)	20±2.82	***Klebsiella* (n=2)**	5.5±7.78	

Data are presented as mean ± SD or range or frequency (%). MRSA; Methicillin-resistant Staphylococcus aureus, MSSA: Meticillin-Sensitive *Staphylococcus*, CONS: Coagulase-negative *Staphylococci aureus*, *P.aeruginosa*: *Pseudomonas aeruginosa*, *E.coli: Escherichia coli*, NSO: *Nigella sativa* oil.

The different ranges of MIC of NSO against MRSA ranged from 3.15%-50%, MSSA ranged from 6.25%-25% and CONS ranged from 12.5%-25%. Regarding Gram-negative organisms, *P. aeruginosa* ranged from 6.25%-50%, *E. coli* ranged from 6.25%-25%, and *Klebsiella spp*. ranged from 50%-100%. The different ranges of MBC of NSO against MRSA ranged from 6.25%-100%, MSSA ranged from 12.5%-50% and CONS ranged from 25%-50%. Regarding Gram-negative organisms, *P. aeruginosa* ranged from 12.5%-100%, *E. coli* ranged from 12.5%-50%, and *Klebsiella spp*. Ranged from 50%-100% (Table 3).

Table 3: Comparative minimal inhibitory concentration and bactericidal concentration value of *Nigella sativa* oil. Tube dilution method:

	Gram-positive	Gram-negative	P
MIC value of NSO	**Gram-positive organisms**		
	MRSA (n=21)	**MSSA (n=2)**	**CONS (n=2)**
	3.15%-50%	6.25%-25%	12.5%-25%
	Gram-negative organisms		
	***P.aeroginosa* (n=13)**	***E. coli* (n=4)**	***Klebsiella* (n=2)**
	6.25%-50%	6.25%-25%	50%-100%
MBC value of NSO	**Gram-positive organisms**		
	MRSA (n=21)	**MSSA (n=2)**	**CONS (n=2)**
	6.25%-100%	12.5%-50%	25%-50%
	Gram-negative organisms		
	***P.aeruginosa* (n=13)**	***E. coli* (n=4)**	***Klebsiella* (n=2)**
	12.5%-100%	12.5%-50%	50%-100%

Data are presented as mean ± SD or range or frequency (%). MRSA; Methicillin-resistant Staphylococcus aureus, MSSA: Meticillin-Sensitive *Staphylococcus*, CONS: Coagulase-negative *Staphylococci aureus*, *P.aeruginosa*: *Pseudomonas aeruginosa*, *E.coli: Escherichia coli*, MIC: minimal inhibitory concentration, NSO: *Nigella sativa* oil.

Antibiotic susceptibility profiles of Gram-positive and Gram-negative isolates are showed in (Table 4).

Table 4: Antibiotic susceptibility profile of Gram-positive and Gram-negative isolates

	Antibiotic Susceptibility among Gram-positive organisms		
	Sensitive	**Intermediate**	**Resistant**
Penicillin	0(0%)	-	25(100%)
Cefoxitin	2(8%)	-	23(92%)
Ciprofloxacin	12(48%)	-	13(52%)
Gentamicin	7(28%)	-	18 (72%)
Linezolid	25(100%)	-	0(0%)
Clindamycin	15(60%)	-	10(40%)
Azithromycin	2(8%)	-	23(92%)
	Antibiotic Susceptibility among Gram-negative organisms		
Ceftazidime	3(15.79%)	-	16(84.21%)
Cefepime	0(0%)	-	19(100%)
Piperacillin-Tazobactam	14(73.68%)	-	5(26.32 %)
Ciprofloxacin	9(47.37%)	-	10(52.63 %)
Imipenem	9(47.37%)	-	10(52.63 %)
Meropenem	9(47.37%)	-	10(52.63 %)
Aztreonam	8(42.11%)	-	11(57.89%)
Cefotaxime	0(0%)	-	6 (100%)
Ceftriaxone	0(0%)	-	6 (100%)
Amoxicillin –Clavunate	1(16.67%)	-	5 (83.33%)
Trimethoprim-Sulfamethoxazole	0(0%)	-	6(100%)

Data are presented as frequency (%). Cefotaxime, Ceftriaxone, Amoxicillin–Clavulanate, Trimethoprim-Sulfamethoxazole were not tested against *Pseudomonas*.

Figure 1 shows that tubes (1-8) had different concentrations of NSO, with tube (6) determining the MIC. Tube (9) is the growth control tube and tube (10) is the NSO control tube.

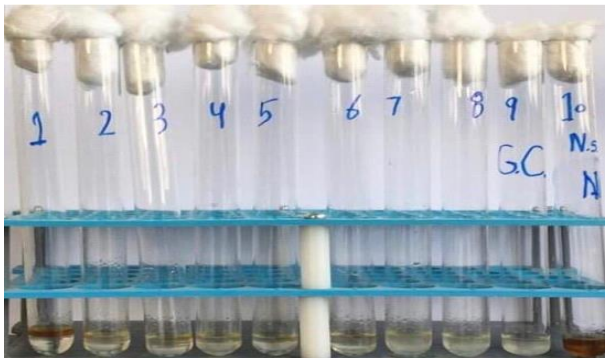

Fig. 1: Minimal inhibitory concentration (MIC) of NSO on an isolate of methicillin-resistant *Staphylococcus aureus* (MRSA) by tube dilution method.

Figure 2 demonstrates the antibiotic synergism test of an isolate showing zones of inhibition around different antibiotic discs: the left plate is showing increased sensitivity (wide zones after the addition of NSO) in comparison to the right plate (resistant zones without the addition of NSO).

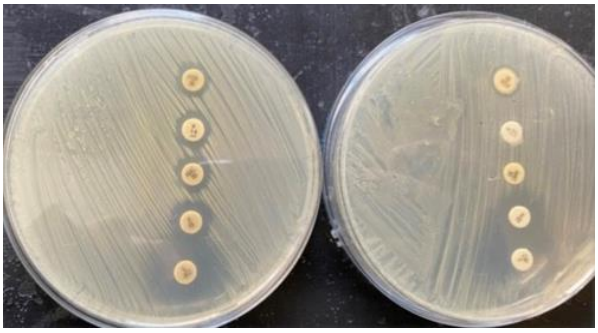

Figure 2: Antibiotic synergism test of an isolate of methicillin-resistant Staphylococcus aureus (MRSA) is showing zones of inhibition around different antibiotic discs.

DISCUSSION

Diabetes complications like DFU, which are linked to prolonged hospital stays and amputations, have become a major burden for patients and society. Bacterial infection is facilitated by the delayed wound closure of ulcer sites brought on by vascular damage and neutrophil dysfunction. Amputation is inevitable if bacterial biofilm or antibiotic resistance develops since traditional treatment usually fails. Therefore, in order to speed up the healing process of wounds and avoid amputation, efficient antibacterial treatment that goes beyond medicines is crucial[16].

In the current study, 25 patients had Gram-positive bacteria (84% MRSA, 8% MSSA, and 8% CONS), while 19 patients had Gram-negative bacteria (68.4% *P. aeruginosa* 21.1% *E. coli* and 10.5% *Klebsiella*).

Between the two groups, there was no statistically significant difference. In a related study, Bady et al.[17] used tissue specimens to detect bacteria in DFU patients and examine how sensitive these bacteria were to NSO and other essential oils. They isolated *P. aeruginosa* 1 (3.2%), *E. coli* 4 (12.5%), *S. aureus* 6 (18.8%), *K. pneumoniae* 6 (18.8%), and *S. epidermidis* 15 (46.9%). The difference between their study and the current study in bacterial types and frequency is due to the difference in the study settings, the conditions of infection in each study, and the prevalent organisms [18].

In the current study, polymicrobial growth wasn't observed in any patient, monomicrobial in 44 (88%) patients, and no growth in 6 (12%) patients. The type of growth was insignificantly different between monomicrobial and no growth. Similarly, Jain and Barman[19] and Kwon and Armstrong[20] reported that polymicrobial infection was found to be lower than monomicrobial infection.

Regarding antibiotic resistance in Gram-positive bacteria isolated in the present study, all isolates were resistant to penicillin (100%). High resistance was found against azithromycin and cefoxitin (92% each). Followed by gentamicin (72%), ciprofloxacin (52%), and finally clindamycin (40%). On the contrary, (100%) of Gram-positive isolates were sensitive to linezolid. Emeka et al.[21] shown that nalidixic acid and ofloxacin resistance to the quinolones remained strong at 74% and 59%, respectively. Of the aminoglycosides, gentamicin and streptomycin resistance were present in 50% and 65% of the isolates, respectively. In general, aminoglycosides shown superior antibacterial efficacy compared to quinolones and beta-lactams. The difference in the antibacterial activity in their study compared to the present study may be related to the fact that antibiotic susceptibility varies in different hospitals and countries. Regarding antibiotic resistance of Gram-negative bacteria isolated in the present study, isolates were resistant to cefepime (100%), followed by ceftazidime (84.21%), aztreonam (57.89%), imipenem, meropenem, and ciprofloxacin (52.63% each) and piperacillin-tazobactam. (26.32%).

Regarding antibiotic the resistance of *E. coli* and *Klebsiella* isolates in the present study, all isolates resistant to cefotaxime (100%), followed by ceftriaxone, trimethoprim-sulfamethoxazole and amoxicillin-clavunate (83.33%each). Similar to our results regarding MIC and MBC values of NSO, Emeka et al.[21] revealed that 42% of their isolates were susceptible to different NSO concentrations, whilst around 27% of their isolates exhibited total resistance to NSO. Remarkably, 79% of their isolates remained resistant in the diluted form (200, 400, and 800 mg/ml), whereas 21% of isolates had a concentration-dependent impact. Nevertheless, 42% demonstrated sensitivity at 1000 mg/ml.

The present study showed that NSO had similar efficacy against sensitive and MDR bacteria with no statistically significant difference. Similarly, Salman et al.[22] reported that out of 13 isolates of *S. epidermidis* tested, all except one were inhibited by NSO. 95% of isolates of other CONS tested, resistant to several antibiotics, were inhibited by NSO.

Limitations of this study include that the sample size was relatively small. The study was in a single centre. So, we recommended that further studies are needed with multicentre cooperation to report the best concentration of NSO to be used in DFU. Further studies are needed for the effect of NSO on different microorganisms. Further studies for the *in vivo* antibacterial effect of NSO are needed. The availability of better drugs and ointments derived from NSO is required.

CONCLUSIONS

The effect of NSO didn't differ between Gram-positive and Gram-negative isolates. NSO had an effect against both sensitive and MDR bacteria. NSO was more effective against *E. coli* and MRSA, while it was less effective against *P. aeruginosa*, *Klebsiella*, MSSA, and CONS. NSO showed highly significant synergism in combination with different antibiotics against both *P. aeruginosa* and MRSA.

Recommendation

From the current study it should be recommended that further studies are needed with multicentre cooperation to report the best concentration of N. Sativa to be used in DFU. Further studies are needed for the effect of NSO on different organisms. Further studies for in vivo antibacterial effect of NSO are needed. Availability of better drugs and ointments derived from NSO is required. Increasing sample size is required for future studies.

Declarations:
Consent for publication: Not applicable
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