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Title: In Vitro Investigation of the Antibacterial Activity of *Nigella sativa* Oil on Some of the Most Commonly Isolated Bacteria in Otitis Media and Externa

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In Vitro Investigation of the Antibacterial Activity of *Nigella sativa* Oil on Some of the Most Commonly Isolated Bacteria in Otitis Media and Externa

ABSTRACT

Objectives: The aim of this study was to evaluate the antibacterial efficacy of *Nigella sativa* (NS) seed oil against the most frequently isolated infectious bacteria of the middle and external ear.

Method: The *in vitro* antibacterial activity of NS oil was evaluated against 34 clinical isolates of *Streptococcus pneumonia*, 32 clinical isolates of *Moraxella catarrhalis*, 32 clinical isolates of *Haemophilus influenzae*, and 32 clinical isolates of *Pseudomonas aeruginosa*. *Staphylococcus aureus*, *Escherichia coli*, and *P. aeruginosa* were also evaluated for their sensitivity to the NS oil. The minimum inhibitory concentration (MIC) of the NS oil was determined via a broth dilution technique. Serial solutions were prepared in a Mueller Hinton-F broth to achieve an ultimate concentration of NS oil within the microplate wells ranging from 256 µg/mL to 0.25 µg/mL. The growth control wells and medium were used for each bacterial strain, and the microplates were incubated at 35°C for 24 hours. Those wells having no visible growth and the lowest concentration of NS oil were accepted as showing the MIC.

Results: In this study, a comparison was made between NS oil and the various antibiotics known to be effective against the bacterial strains mentioned above. The NS was shown to have bactericidal activity against *H. influenzae*, *M. catarrhalis*, and *S. pneumonia.* However, the NS was not found to be effective against *P. aeruginosa* at any concentration.

Discussion: The results of this laboratory-based study support the use of NS oil as an alternative treatment for ear infections. However, it is necessary to conduct clinical studies to evaluate the antibacterial efficacy of NS oil on patients with ear infections.

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Keywords: *Nigella sativa*, Antibacterial activity, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*

1.INTRODUCTION

Acute otitis media (AOM), otitis media with effusion (OME), and otitis externa (OE) are the most commonly seen diseases in otorhinolaryngology clinics [1,2]. *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* are the most frequently isolated bacteria in AOM and OME cases [3-6]. These three bacterial species are responsible for 95% of all AOM cases [7]. OE is an inflammatory disease of the external ear canal and auricula [8]. Fungi and other bacteria may play a role in the etiology, but the two most frequently isolated bacterial species are *Pseudomonas aeruginosa* and *Staphylococcus aureus* [9].

In recent years, due to the increase in bacterial resistance, there has been some debate about the selection and use of antibiotherapy in the treatment of AOM and OME. The use of systemic antibiotics in OME is not recommended, based on the current guidelines [10]. To combat bacterial resistance, it has been suggested that the dose of amoxicillin used as the first step of treatment in AOM should be increased to 80–90 mg/kg instead of the previous 40–50 mg/kg [11,12]. The overall emergence of antibiotic resistance has led to investigations into different options for treatment. Priority in medical treatment of otitis externa is directed to pain. In most cases, full cure is achieved with analgesic and topical agents [13]. Topical fluroquinolone group agents known to be non-toxic with acidifying agents in the external ear canal are recommended for use with steroidal drops [14].

Nigella sativa (NS), which is a member of *Ranunculaceae* family, is also known as black seed or black cummin [15]. It is an annual flowering plant that grows in Southern Asian countries

especially, and it has been used for treatment in various branches of medicine for over 2,000 years [16]. It has been shown that most of the biological activities of the seeds are dependent on thymoquinone, the main component of the volatile oil found in the seed oil. Its antibacterial efficacy against bacteria, especially Gram positive bacteria, has been previously demonstrated [17-19]. Hariharan et al. [20] proved the efficacy of thymoquinone against the frequently seen methicillin-resistant *S. aureus* in their study. However, as far as we know, there are no specific studies in the literature about the most frequently isolated bacteria in AOM, OME, and EO cases. In this study, we aimed to investigate the antibacterial efficacy of NS in a laboratory environment on *S. pneumoniae*, *P. aeruginosa*, *M. catarrhalis*, and *H. influenzae*, which are the most frequently isolated bacteria in AOM, OME, and OE.

2. METHODS

This study began after receiving permission from the local ethics committee (# 4th April, 2017; 2017/0120).

2.1.Bacterial Isolates

Thirty-four strains of *S. pneumoniae*, 32 of *M. catarrhalis*, 32 of *H. influenzae*, and 32 of *P. aeruginosa* were isolated from patients with respiratory tract infections. The identification of isolated bacteria was studied using VITEK 2 automated systems (bioMerieux, France), and they were stored at -80°C until they were used in this study. Before this research began, the *M. catarrhalis* and *H. influenzae* strains were cultivated on chocolate agar, and the *S. pneumoniae* and *P. aeruginosa* were cultivated on 5% Sheep blood agar.

2.2.Antibiotic efficacy and Minimum Inhibitory Concentration Tests

The antibiotic susceptibilities of the strains included in this study were determined via the disk diffusion method and according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards [21]. The used antibiotic discs (Thermo Fisher Scientific Oxoid Ltd., Basingstoke, UK) were stored at -20°C until the time of testing.

The antibiotic susceptibilities were tested after the bacteria were suspended using the 0.5 McFarland turbidity standard. The *S. pneumoniae* sensitivities to penicillin (Oxacillin, 1 µg), moxifloxacin (5 µg), linezolid (30 µg), and cotrimoxazole (1.25/23.75 µg) were investigated. In addition, the *M. catarrhalis* and *H. influenzae* sensitivities to amoxicillin/clavulanic acid (ACA, 2/1 µg), ceftriaxone (30 µg), imipenem (10 µg), meropenem (10 µg), and ciprofloxacin (5 µg) and the *P. aeruginosa* sensitivities to piperacillin/tazobactam (100/10 µg), ceftazidime (30 µg), imipenem (10 µg), meropenem (10 µg), and ciprofloxacin (5 µg) were investigated.

The media (Merck, Darmstadt, Germany) used for the susceptibility testing and to determine the minimum inhibitor concentration (MIC) of the NS on the antimicrobial activity were prepared under laboratory conditions, in accordance with the strains to be studied and the aforementioned standards. The NS MIC was determined via the microdilution method. For this purpose, a stock solution of 512 µg/mL of NS was prepared using dimethyl sulfoxide. The serial dilutions of the NS were prepared from 256 µg/mL to 0.25 µg/mL in a Mueller Hinton-F (MH-F) broth (with 5% (v/v) mechanically defibrinated horse blood and 20 mg/L NAD). Then, 100 µL of the 0.5 McFarland suspension of the clinical isolates and control strains was added to each well. The final NS concentrations of the wells were in the range of 128-0,125. The microplates were incubated at 35°C for 24 hours. The lowest concentration at which no visible reproduction was found was accepted as the MIC value of the NS.

2.3.Quality Control of Strains

S. aureus (ATCC 29213), *Escherichia coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853) strains were used for both the antibiotic susceptibility tests and as control strains in the NS susceptibility studies.

2.4. Statistical evaluation;

Percentages are calculated using the Microsoft Excel 2013 program.

3. RESULTS

The antibiotic susceptibilities of the bacteria used in this study are shown in Table 1. According to our findings, the susceptibilities of *S. pneumoniae* to penicillin and *H. influenzae* to amoxicillin/Clavulonic acid(CA) and meropenem were 100%. The most effective antibiotic for *M. catarrhalis* was ceftriaxone (84.4%) and the least effective was ciprofloxacin (75%). The lowest effective rate for *P. aeruginosa* was obtained with piperacillin/tazobactam at a rate of 56.3%, while the most effective antibiotic was imipenem at a rate of 81.3%.

The NS MIC values for the bacterial strains included in this study are given in Table 2. The quality control strains exhibited MIC values of 0.5 µg/mL for the *S. aureus* (ATCC 29213) and 1 µg/mL for the *E. coli* (ATCC 25922). Because it grew at all the concentrations, the MIC value could not be determined for the *P. aeruginosa* (ATCC 27853). In the current study, the NS was not found to be effective against *P. aeruginosa* at any concentration.

Assuming that the susceptibility limit of the bacteria to NS was an MIC of 2, the bacteria were grouped together as having MIC values of 2 or below and 4 or above. Since 3 strains of *S. pneumoniae* could not be grown, the microdilution tests could not be performed. The NS MIC values of 20 of the strains were 2 or below, while the remaining 11 strains had MIC values of 4 or above. While 10 of the 20 strains with MICs of 2 or below were susceptible to moxifloxacin, 6 of the 11 strains with MICs of

4 or above were susceptible. Moreover, 19 of the 20 strains with MICs of 2 or below were sensitive to linezolid, and all 11 strains with MICs of 4 or above were sensitive. Finally, 10 of the 20 strains with MICs of 2 or below were susceptible to cotrimoxazole, while only 4 of the 11 strains with MICs of 4 or above were susceptible (Table 3).

The NS MICs of 19 of the *H. influenzae* strains were 2 or below, while the remaining 13 strains had MICs of 4 or above. Of the 19 strains with MICs of 2 or below, 12, all 19, and 17 were susceptible to ceftriaxone, meropenem, and ciprofloxacin, respectively. Of the 13 strains with MICs of 4 or above, 10, 4, and 9 were susceptible to ceftriaxone, meropenem, and ciprofloxacin, respectively (Table 3).

The NS MICs of 7 strains of *M. catarrhalis* were 2 or below, with MICs of 4 or above found in the other 25 strains. Of the 7 strains with MICs of 2 or below, 6, all 7, 5, 6, and 4 were susceptible to amoxicillin/CA, ceftriaxone, imipenem, meropenem, and ciprofloxacin, respectively, while 20, 20, 21, 19, and 20 of the 25 strains with MICs of 4 or above were susceptible, respectively (Table 3).

Multiple drug resistance (MDR) was detected in 8 strains of *S. pneumoniae* and *M. catarrhalis*, and in 5 *H. influenzae* strains. The susceptibility rates of the strains to NS, with or without MDR and/or susceptibility to all antibiotics, are shown in Table 4. If an MIC of 2 was used as the cut-off value, 50% of the *S. pneumoniae* strains with MDR, 40% of the *H. influenzae* strains, and 25% of the *M. catarrhalis* strains were found to be susceptible to NS. While 74% of the *S. pneumoniae* and 63% of the *H. influenzae* strains without MDR and with sensitivity to all antibiotics were found to be susceptible to NS, only 21% of the *M. catarrhalis* strains were susceptible.

4.DISCUSSION

NS oil, which is used for the treatment of many health problems in traditional alternative medicine, has been proven to be effective against many bacteria [17-19]. For example, Hariharan et al.

[20] reported the antibacterial activity of NS against methicillin-resistant *S. aureus*. In another study, Salman et al. [22] reported that NS was more effective against Gram positive bacteria than Gram negative bacteria. Specifically, they noted that NS oil was effective against Gram positive bacteria, *Staphylococcus epidermidis*, other coagulase negative staphylococci, and *Streptococcus pyogenes*. They also reported that *Enterococcus faecalis* and *Streptococcus agalactiae* were resistant to NS. Efficacy was only detected for *P. aeruginosa* in the Gram negative bacterial group [22]. In another study performed by Chaieb et al. [19] the efficacy of thymoquinone, which is the active ingredient of NS, on 11 human pathogenic bacteria was evaluated, with reported efficacy, especially against *S. epidermidis* and *S. aureus*. All these studies suggest that NS may possess antibacterial properties that can be used in various fields.

Antibiotic resistance is an extremely important problem. In particular, the use of inappropriate and unnecessary antibiotics increases the number of resistant bacterial strains and their resistance rates. The fact that this resistance becomes more and more evident each day increases concerns for the future. Therefore, research into new molecules that may be effective against infectious agents continues throughout the world.

Some common infections of the ear, nose, and throat area are middle ear and external ear canal infections, such as AOM, OME, and OE. Some of the most frequently found pathogens in these infections are *S. pneumoniae*, *M. catarrhalis*, *H. influenzae*, and *P. aeruginosa* [3-6]. Many studies have shown that these pathogens have developed resistance to certain antibiotics at specific rates.

There has been a steady decline in the susceptibility of *S. pneumoniae* to the most commonly used beta-lactams. In a current study, Cherazard et al. [23] found *S. pneumoniae* isolate resistance rates of 20%–40%, 22%, and 35% against macrolides, clindamycin, and trimethoprim/sulfamethoxazole (TMP/SMX), respectively. In another study, Rosenblut et al. [24] reported resistance rates of 5% against penicillin and 33% against erythromycin. According to

Sillanpää et al. [25] 80% of *S. pneumoniae* isolates were penicillin-sensitive and 20% were moderately resistant. They also reported that none of these resistant strains were susceptible to erythromycin or TMP/SMX [25]. Additionally, the authors reported that the resistance to erythromycin was 40% and the resistance to TMP/SMX was 20% in the overall evaluation of all the isolates. According to Sillanpää et al.[25], the antibiotic resistance of *H. influenzae* was evaluated, and moderate resistance to erythromycin [47%] was found, with full resistance to TMP/SMX and ampicillin reported as 29% and 24%, respectively. Additionally, the authors reported that *M. catarrhalis* resistance, it was found that all the strains were resistant to ampicillin and sensitive to the other antibiotics [25]. Rosenblut et al. [24] studied *H. influenzae* and reported the antibiotic resistance against ampicillin as 14%, cefuroxime as 2%, and cefotaxime as 2%. In another study, *P. aeruginosa* was reported to be highly resistant to various antibiotics, including ciprofloxacin and tobramycin [26]. The common results of these and similar studies show that antibiotic resistance has reached important levels. These results are clear signs of the need for new and different molecules to fight infection.

In our study, the antibacterial efficacy of NS oil against the most frequently found bacterial isolates in AOM, OME, and OE was evaluated in the laboratory. MIC values were obtained in all the bacteria except *P. aeruginosa*. When the efficacy of the NS oil were compared with the other known antibiotics against the pathogens in our study, the NS oil was found to be ineffective against *P. aeruginosa*, as opposed to the Salman et al. report [22]. These contrary results for the same bacteria should be clarified by different studies.

The limitation of the study is the low number of strains. Further studies with more strains may contribute.

To our knowledge, there have been no studies about the effect of NS against the bacteria known to be particularly active in AOM and OME, such as *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae*. For this research, a comparative study was conducted between NS and various antibiotics

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known to be effective against these bacteria, and the NS was found to have effects similar to some of these antibiotics, and in some cases, stronger effects.

Because of the increase in bacterial resistance to the antibiotics used in daily practice, it appears that NS oil may be an alternative for the treatment of AOM and OME. More detailed studies will contribute to the clarification of this issue in the future.

Conflict of interest: None

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Table 1: Some antibiotic susceptibility rates of bacterial isolates

	Streptococcus pneumoniae (n=34)		*Haemophilus influenzae* (n=32)		*Moraxella catarrhalis* (n=32)		*Pseudomonas aeruginosa* (n=32)	
	n	%	n	%	n	%	n	%
Penicillin	34	100						
Amoxicillin-Clavulanic Acid			32	100	26	81,3		
Piperacillin-Tazobactam							18	56,3
Ceftazidime							19	59,4
Ceftriaxone			22	68,8	27	84,4		
Imipenem			32	100	26	81,3	26	81,3
Meropenem			31	96,9	25	78,1	22	68,8
Ciprofloxacin			26	81,3	24	75	25	78,1
Moxifloxacin	19	55,9						
Linezolid	33	97						
Co-trimoxazole	15	44						

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Table 2: *MIC values of Nigella sativa oil in bacterial isolates*

Strains MIC values (µg/mL)	*Streptococcus pneumoniae* (n=31)	*Haemophilus influenzae* (n=32)	*Moraxella catarrhalis* (n=32)	*Pseudomonas aeruginosa* (n=32)
≤0,25	6	4	0	0
0,5	5	6	1	0
1	7	5	2	0
2	2	4	4	0
4	4	5	8	0
8	2	2	7	0
16	-	2	4	0
32	3	1	2	0
64	1	3	3	0
128	-	0	1	0
≥256	1	0	0	0

MIC: Minimum inhibitory concentration

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Table 3: Number of strains susceptible to antibiotics according to MIC values determined for Nigella sativa

	Streptococcus pneumoniae (n=31)				*Haemophilus influenzae* (n=32)				*Moraxella catarrhalis* (n=32)			
Nigella sativa MIC values	**2 or less (20)**		**4 or more (11)**		**2 or less (19)**		**4 or more (13)**		**2 or less (7)**		**4 or more (25)**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Penicillin	20	100	11	100								
Amoxicillin Clavulanic Acid					19	100	13	100	6	85	20	80
Ceftriaxone					12	63	10	77	7	100	20	80
Imipenem					19	100	13	100	5	71	21	84
Meropenem					19	100	12	92	6	85	19	76
Ciprofloxacin					17	89	9	69	4	57	20	80
Moxifloxacin	10	50	6	55								
Linezolid	19	95	11	100								
Co-trimoxazole	10	50	4	36								

MIC: Minimum inhibitory concentration

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	Streptococcus pneumoniae		*Haemophilus influenzae*		*Moraxella catarrhalis*	
	n*	%	n*	%	n*	%
MDR, Yes	8	50	5	40	8	25
MDR, No	23	74	27	63	24	21
Total	31		32		32	

Tablo 4: Susceptibility rates of Nigella sativa strains with and without multiple drug resistance

* Number of susceptible strains to Nigella sativa, MDR: Multiple Drug Resistance

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