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Susceptibility of Clinical Bacterial Isolates and Control Strains to *Nigella Sativa* Oil

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ABSTRACT

The clinical bacteria of multi-drug resistant to some antibiotics are considered a common problem in the world wide. Alternative antibacterial strategies are urgently needed, and thus this situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as plants and plant-based products, including *Nigella sativa* (*N. sativa*). This study aimed to evaluate the antibacterial activity of Yemeni *N. sativa* oil against some Clinical Bacterial Isolates (CBI) and Control Strains of Bacteria (CSB) in comparison with Augmentin[®] (Amoxicillin and Clavulanic Acid). This study was carried out in Sana'a city during the period of one year from May 2012 to April 2013. The antibacterial activity of oil with different concentrations was determined by using disc agar diffusion technique against CBI (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) and CSB (*Staphylococcus aureus* ATCC25619, *Pseudomonas aeruginosa* ATCC29737), also the Minimum Inhibition Concentration (MIC) of oil extract was determined. The results indicated that, the highest significant antibacterial activity was at the maximum concentration of the oil against *Staphylococcus aureus* and *Pseudomonas aeruginosa* in comparison with Augmentin[®] discs ($p < 0.05$) respectively. This study observed that, there was strong positive correlation between the diameter zone (DZ-mm) and oil concentrations (%). In addition, the DZ (mm) of the oil for CSB was higher than CBI. Also the oil activity was found to be more effective against *Staphylococcus aureus* than *Pseudomonas aeruginosa*. On the other hand, the MIC of the oil against CBI were ranged between $\geq 0.5\%$ and 4% . Whereas, the CSB particularly *Staphylococcus aureus* were sensitive to low concentrations of oil. It could be concluded that, the oil had high antibacterial effect against CSB and CBI with high concentration and more effective than Augmentin[®] discs.



Introduction

Infectious diseases still represent an important cause of morbidity and mortality among humans, especially in developing countries¹. Human pathogenic microorganisms have developed resistance in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases². Therefore, alternative antimicrobial strategies are urgently needed, and thus this situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as plants and plant-based products, including *Nigella sativa* (*N. sativa*)³. It also known as black cummin is an annual herbaceous plant belonging to the Ranunculaceae family⁴. The plant is indigenous to Mediterranean areas, although it is grown in other parts of the world as well⁵. Its seeds have played an important role over the years in ancient Islamic system of herbal medicine, where they have been traditionally used in folk medicine. Traditionally, it is used as a natural remedy for a number of illnesses that include asthma, cough, hypertension, bronchitis, diabetes, headache, eczema, fever, inflammations, and other diseases⁶. Different crude extracts of *N. sativa* have shown effectiveness against multi-antibiotic resistance bacterial isolates⁷. The potential use of alternative antibiotics in drug-resistant bacteria from various plant extracts have been studied by many researchers. Study performed by⁴ Ali *et al.*, 2011 was investigated the anti-microbial effects *N. sativa* oil seeds against different pathogenic microbes. The importance of *N. sativa* cannot be over-emphasized as regards their rule in health remedy. Therefore, the antibacterial activity of *N. sativa* has been demonstrated against gram positive and

negative bacteria, and it confirmed by numerous scientific studies⁸⁻¹⁰.

Materials and Methods

Extraction Assay

The seeds were purchased from a local herbal shop in Hadhramaut, Yemen. The seeds were grounded using an electric grinder. Then, The *N. sativa* oil was prepared based on steam distillation method. The crude oil samples were combined and stored at 4°C until used.

Study Design

This study included Yemeni *N. sativa* oil was which tested for their activity against 100 clinical bacterial isolates (CBI), divided in to two groups (50 *Staphylococcus aureus* and 50 *Pseudomonas aeruginosa*), and two Control Strains of Bacteria (CSB) namely *Staphylococcus aureus* ATCC 25619 and *Pseudomonas aeruginosa* ATCC 29737.

Collection of bacterial samples

The control strains of bacteria were obtained from Microbiology Laboratory of Supreme Board of Drugs and Medical Appliances, and the CBI, obtained from different Department of Microbiology in Al-Thawra General Hospital, Atypical Police Hospital and National Center for Central Public Health Laboratories in Sana'a city, were isolated from 100 patient swabs and collected from pus, ear discharge, urine, nasal, semen and throat.

Cultivation assay

The isolates were identified based on standard microbiological techniques, and sub-cultured in nutrient agar slope at 37°C under aerobic condition for 24 hour and stored in refrigerator at 4°C until used^{11,12}.

Preparation of bacterial strains

Active cultures for experiments were prepared by isolating a loop full of cells from each stock culture of tested bacteria and emulsify in 3-4 ml of sterile physiological saline to a turbidity that matches 0.5 McFarland standard (106 Colony Forming Unit (CFU)/ml)^{11,12}.

Antibacterial Susceptibility

Antibacterial activity was measured using a well disk diffusion method according to the National Committee for Clinical Laboratory Standard¹³. Whatman filter paper was used to prepare discs (6 mm diameter). The filter paper discs were sterilized by autoclaving and impregnated in different concentrations of oil (The oil extraction was diluted in 95 % alcohol to obtain 0.5%, 1%, 2% and 4%). After that, prepared discs were stored at 4 °C in the refrigerator until use. To avoid any condensation the discs were kept at room temperature for one hour before use. A loop full of the prepared bacterial suspensions were separately applied to the center of a sterile Mueller-Hinton plate and spread evenly using a sterile dry cotton wool. The discs were placed and the plates were incubated at 37°C for 24hr. The antibacterial activity of the oil was assessed by measuring the diameter zone (DZ). The positive control (Augmentin® 30µg disc – Himedia , India) and negative control (disc impregnated with 95 % alcohol) were also included for each experiment^{11,12}.

Minimum inhibitory concentration measurement of *N. sativa* oil

The MICs of the oil was determined by tube dilution techniques in Müller-Hinton broth. The oil concentrations were incorporated into Müller-Hinton broth media to test their efficiency against CSB (*Staphylococcus aureus* ATCC 25619 and *Pseudomonas. aeruginosa* ATCC 29737) and CBI (*Staphylococcus aureus* and *Pseudo-*

monas aeruginosa). Each series of dilutions was inoculated with 10⁶ (CFU/ml) of the tested bacteria and incubated at 37°C for 24hr before determining the lowest concentration that inhibited the appearance of visible growth. The highest dilution that exhibited no visible growth was recorded as the MIC. The broth without growth from the MIC procedure was streaked onto subculture of each tested oil concentration^{11,12}.

Statistical analysis

Data were analyzed by using the SPSS Version 15 (Social Package of Statistical Science) computer program by LEAD Technologies; Inc. USA (1991-2000). Data were checked for normally distribution, and were expressed as Mean ± SD (Standard deviation). Differences in variables were tested by using Independent sample T-test. The significant interrelationships between parameters were analyzed by Pearson Correlation coefficient (R²) test. The significant differences were indicated if the probability value (p) < 0.05.

Results

In this study, antibacterial activity of oil against some clinical and control strains bacteria was evaluated. Fifth samples were collected from different Department of Microbiology in Al-Thawra General Hospital, Atypical Police Hospital and National Center for Central Public Health Laboratories in Sana'a city. The basic information of the collected samples is shown in Table 1 and Figure 1. The bacteria were isolated by different human specimens (pus, ear, sputum, nasal, semen and urine); the most bacteria isolates were given in the Table 1 namely *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

The activity of the oil concentration maximum against CBI (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) was measured by using DZ and the results were

shown in Table 2 and Figure 2. However, this parameter reduced gradually with low concentration of oil and raised with high concentration. In addition, the oil concentration minimum was 0.5 % for *Staphylococcus aureus* and the response was not available for *Pseudomonas aeruginosa* at the same concentration. On the other mean, there was strong positive correlation between the DZ and concentrations of oil concentration. As regard as, the DZ for Augmentin® in CSB was higher than in CBI. Furthermore, the DZ for Augmentin® in *Staphylococcus aureus* was higher than in *Pseudomonas aeruginosa* for both CSB and CBI.

Also, the results showed that the oil maximum concentration has higher significant antibacterial activity against *Staphylococcus aureus* in compared to Augmentin® and other oil extract concentrations (Table 3) and all results of this table were statistical significance except at 2%. In addition, the results of the Table 4 proved that the maximum concentration of the oil has obvious significant antibacterial activity against *Pseudomonas aeruginosa* in compared to Augmentin® and other oil concentrations. All results of this table were statistical significant except at minimum concentration of oil extract. Finally, n Table 5 showed that the highest percentage of MIC for oil was 1% against *Pseudomonas aeruginosa* then it reduced gradually with decrease of concentration. In contrast, the lowest percentage of MIC for oil was 0.5 % against *Staphylococcus aureus* then it raised gradually with increase of concentration. In addition, the response of *Pseudomonas aeruginosa* for oil concentrations was lower than the response of *Staphylococcus aureus* isolates.

Discussion

Over the years, the World Health Organization advocated that countries should

interact with traditional medicine with a view to identifying and exploiting aspects that provide safe and effective remedies and ailments of both microbial and non-microbial origins. Among strategies to combat bacteria, the search for new antibacterial drugs including those derived from plants appears to be apriority. Investigations of plants of various genera have provided strong evidence for several compounds with potent antibacterial activity¹⁴. *N. sativa* supplemented manually prepared diet had been confirmed to suppress harmful intestinal bacteria in animals and humans. *N. sativa* oil might have potential as an alternative to hazardous antibiotic to formulate low cost and environment-friendly diet for humans and animals³. In this study, the *N. sativa* oil has difference inhibitory effect against CSB and CBI according to its concentrations. This results were agreed to that reported by¹⁵ El-Kamali *et al.*, (1998). However the results of this study showed that the activity of the *N. sativa* oil was found to be more effective on *Staphylococcus aureus* than *Pseudomonas aeruginosa* bacteria and these results were similar to previous studies^{16,17} in which they found antibacterial activity of *N. sativa* oil against the Gram positive bacteria as *Staphylococcus aureus* than gram negative bacteria as *Pseudomonas aeruginosa*. In comparison with previous studies, a number of compounds derived from plants often show considerable effect against Gram positive bacteria but less against Gram negative bacteria due different of cell wall permeability for both bacteria¹⁸.

N. sativa against has antibacterial activity against *N. sativa* against clinical isolates of methicillin resistant staphylococcus aureus that was recorded Hannan *et al* with MIC range (0.2 – 0.5)¹⁹. In other study by Bessedik *et al*, antibacterial effect of essential oil of *N. sativa* was estimated, this study proved that the essential oil of black cumin possesses a very interesting

antimicrobial effect against the pathogenic bacteria and fungi studied so far²⁰. In addition, the study was performed by Faraydoon *et al*, inhibition of *Staphylococcus aureus* by *N. sativa* products using different extraction method was carried out and the DZ in this study raised with increase of oil amount²¹.

The positive inhibition may be attributed to the many important active ingredients of *N. sativa* oil namely thymoquinone and thymohydroquinone^{16,22}. This study was supported by different authors that, the antibacterial principle present in the extract was isolated and characterized found to be thymoquinone²³. Also, these components in *N. sativa* in other study that have antibacterial activity and their activity could be potentiated by antibiotics especially in case of *Staphylococcus aureus*²⁴. In oldest study, the antimicrobial principle has been isolated, identified as thymohydroquinone, and found to be active against gram-positive bacteria better than gram -negative bacteria²⁵.

The lowest concentration namely 0.5% of *N. sativa* oil was inactive against *Pseudomonas aeruginosa*, while this concentration has the highest percentage of *Staphylococcus aureus* for MIC. This result was similar to that reported by El-Fatary²⁶ who found that the low concentration of *N. sativa* oil had an inhibitory effect against Gram positive such as *Staphylococcus aureus*. Also, MIC of *Staphylococcus aureus* was 0.5 % and *Pseudomonas aeruginosa* 1%, the different in the activity due to different in amount of active ingredient (thymohydroquinone) of *N. sativa*, direct results were described by Toma *et al* where MIC of *Staphylococcus aureus* was 8 µl/ml and MIC of *Pseudomonas aeruginosa* was 124 µl/ml²⁵.

On the other hand, Augmentin is included in guidelines and recommendations for the treatment of bacterial sinusitis, acute otitis media, community-acquired pneumonia

and acute exacerbations of chronic bronchitis. Augmentin continues to be an important agent in the treatment of community-acquired respiratory tract infections, both now and in the future²⁷. In vitro evaluation of Augmentin by broth microdilution and disk diffusion susceptibility testing was performed and staphylococci was susceptible to Augmentin²⁸. Also the clinical development and launch of Augmentin for the treatment of a range of community-acquired infections was reported by Ball and this therapy had high level of clinical efficacy²⁹.

Conclusion

The study concluded that, the *N. sativa* oil was potent antibacterial agents against CSB and CBI. The highest antibacterial activity of this oil was at the maximum concentrations but reduces with decreasing the dilution percentage against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. There was strong positive correlation between the DZ (mm) and oil extract concentrations (%). The activity of the oil was found to be more effective against *Staphylococcus aureus* than *Pseudomonas aeruginosa* and at the highest concentration was more effective than Augmentin® discs.

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Table 1. Tested CBI and their sources

Bacterial isolated	Source and number of tested strains						
	Urine	Pus	Ear discharge	Nasal	Semen	Sputum	Total
<i>S. aureus</i>	2	19	17	4	3	5	50
<i>P. aeruginosa</i>	5	26	12	0	0	7	50
CBI : Clinical Bacterial Isolates ; <i>S. aureus</i> : <i>Staphylococcus aureus</i> ; <i>P. aeruginosa</i> : <i>Pseudomonas aeruginosa</i>							

Table 2. Correlation between DZ (mm) and oil concentrations by Pearson correlation coefficient

Name of Bacteria	DZ (mm) ± SD					R ²
	0.5%	1%	2%	4%	Augmentin® 30µg	
<i>S. aureus</i>	13.8±8.3	20.7±9.2	27.9±6.5	33.5±4.4	27.1±6.0	0.944
<i>P. aeruginosa</i>	0	10.7±5.6	16.1±5.4	24.2±4.3	20.3±3.6	0.949
<i>S. aureus</i> ATCC 25619	18±6.1	28±4.1	34±4.3	40±5.1	35±3.2	0.903
<i>P. aeruginosa</i> ATCC 29737	10±3.5	15±5.1	20±3.3	27±6.1	25±3.3	0.979
R ² : Pearson's correlation coefficient (> 0.995) ; DZ : Diameter Zone						

Table 3. Comparison of antibacterial activity between oil extract concentrations and Augmentin® against *S. aureus* according to DZ (mm), (n = 50)

Oil extract	DZ (mm) ±SD		p value
	<i>S. aureus</i>	Augmentin® 30µg	
0.5%	13.8±8.3	27.1±6.0	0.000*
1%	20.7±9.2		0.01*
2%	27.9±6.5		0.314
4%	33.5±4.4		0.000*
*: Statistically Significant			

Table 4. Comparison of antibacterial activity against between oil extract concentrations and Augmentin® against CBI (*P. aeruginosa*) according to DZ (mm) ; (n = 50)

Oil Concentration	DZ (mm) ±SD		p value
	<i>P. aeruginosa</i>	Augmentin® 30µg	
0.5%	0	20.3±3.6	undefined
1%	10.7±5.6		0.000*
2%	16.1±5.4		0.002*
4%	24.2±4.3		0.001*
*: Statistically Significant			

Table 5. MIC of oil for CSB and CBI

Name of Bacteria	Oil Concentration (%)	DZ (mm)
<i>S. aureus</i>	0.5	13.8±8.3
<i>P. aeruginosa</i>	1	10.7±5.6
<i>S. aureus</i> ATCC 25619	0.5	18±6.1
<i>P. aeruginosa</i> ATCC 29737	0.5	10± 3.5
CSB : Control Strains of Bacteria ; CBI : Clinical Bacterial Isolates ; MIC : Minimum Inhibitor Concentration		

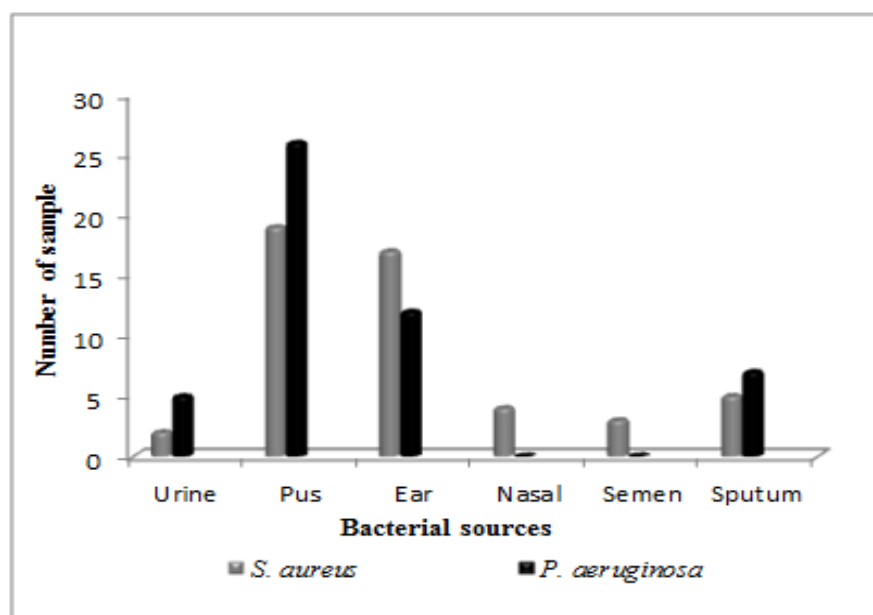


Figure 1. Tested CBI and their sources

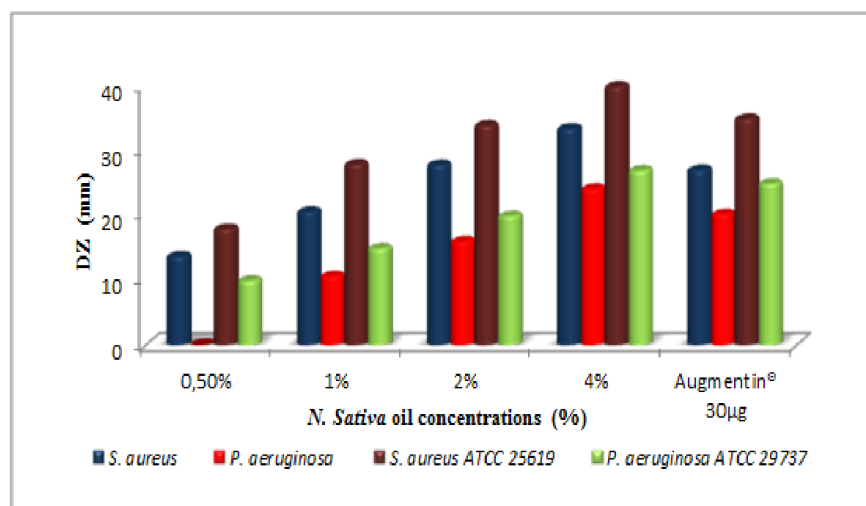


Figure 2. Comparison of antibacterial activity between oil extract and Augmentin® according to DZ (mm)