Research Article

Alteration of Multi-drug Resistance Activities by Ethanolic Extracts of *Nigella sativa* Against Urinary Pathogens

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Abstract

**Background and Objective:** Most of the urinary pathogens cause urinary tract infection (UTI) have developed multidrug-antibiotics resistance (MDR) and had forewarned the interest in researching natural products to increase the usage of medicinal plants as alternative therapies for infectious diseases. The study was aimed to investigate the alteration of multidrug-resistant properties of ethanolic extract from *Nigella sativa* against clinically isolated MDR urinary pathogens. **Materials and Methods:** Antimicrobial activity of ethanolic extract from *Nigella sativa* was investigated against clinical isolates of *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Acinetobacter baumannii* using agar disc diffusion technique, minimal bactericidal concentration (MBC) and minimal inhibitory concentration (MIC) assays. The data was analyzed using Microsoft Excel. **Results:** Ethanol extract of *Nigella sativa* possesses strong microbial growth inhibitory effects in a dose-dependent manner. The minimum MIC values were observed for *Proteus mirabilis* (1.5 mg mL⁻¹), followed by *Pseudomonas aeruginosa* (2 mg mL⁻¹), extended-spectrum β-lactamase (ESBL) producing *Klebsiella pneumoniae* (2.5 mg mL⁻¹), ESBL producing *Escherichia coli* (2.75 mg mL⁻¹) and *Acinetobacter baumannii* (2.5 mg mL⁻¹). The MBC inhibitory effects of ethanolic extracts were greater than that of corresponding MIC results for all clinical isolated urinary pathogens. The bactericidal concentration of ethanol extracts was slightly increased than the corresponding MIC. **Conclusion:** This study augmented the effective alteration of MDR properties by the ethanolic extract of *Nigella sativa* seeds. Thus, it might be used as an effective natural source of a safe antimicrobial agent against emerging MDR clinical isolates in the future.

**Key words:** *Nigella sativa* seeds, antibacterial activity, multidrug-resistant pathogens, extended-spectrum β-lactamases producers, medicinal plant, urinary tract infection, antibiotic resistance

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**Competing Interest:** The author has declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.
INTRODUCTION

The most widespread of bacterial infections are urinary tract infections (UTI) that affecting people all the way through their lifespan. The pathogenesis of UTIs is complex and altered by host behavioral factors and characteristic features of the infecting urinary pathogens. The most common and leading urinary pathogens include Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Enterococcus faecalis, Proteus mirabilis, Pseudomonas aeruginosa and Candida albicans. The occurrence of acute UTI (uncomplicated) is supposed to exceed 0.5 episodes annum of a young female between 18-30 years. Currently, most of the pathogenic microorganisms have developed resistance against commonly used commercial antimicrobial drugs due to their indiscriminate use in the treatment. In spite of the subsistence of strong antibiotics or MDR bacterial strains gradually appearing, there is an impressive demand for a permanent solution and development of novel drugs without any side effects. Therefore, alternative therapeutic approaches are promptly necessary and thus this circumstance has directed to a reconsideration of the treatment regimens used in olden medications include herbal plants and its products.

Nigella sativa seeds known as black cumin have been used for therapeutic purposes for centuries in the form of herb and oil in Asia, Africa and Middle East. Nigella sativa seeds are used to treat intestinal health and stomach, respiratory health, immune system support and circulatory, liver and kidney function and common health issues. Studies in the last 4-5 decades about Nigella sativa seeds have widely reported that it possesses various numbers of medicinal properties. The essential oil and crude extracts of Nigella sativa seeds possess antimicrobial activity against various clinical isolates. The essential oil has antimicrobial properties against various Gram-positive and Gram-negative bacteria on dose-dependent manner. Many bioactive components have been isolated from Nigella sativa seeds but quinone constituents such as thymoquinone is responsible for its pharmacological effects.

Antibacterial agents are one of the major significant arms in fighting against infectious diseases, the emergence of resistant against various microorganisms pave the way to investigate for newer drugs. This study designed to screen and investigate the alteration multidrug resistance action of urinary tract clinical isolates by ethanolic extracts of Nigella sativa seeds.

MATERIALS AND METHODS

This descriptive analysis was performed at General Hospital, Wadi Al-Dawasir, Kingdom of Saudi Arabia during the 8 month period from September, 2017 to March, 2018. A total of 55 urinary tract isolates including 34 multidrug resistance bacterial strains were included in this analysis. All urinary tract isolates used throughout this study were maintained on nutrient agar slants were maintained. The cultures were stored at 4°C, with the regular transfer at monthly intervals and their morphological characteristics confirmed by macroscopic and microscopic examination.

Clinical bacterial isolates: In this study, 5 MDR Gram-negative bacteria include E. coli, P. mirabilis, K. pneumoniae, A. baumannii and P. aeruginosa were isolated. All isolates were isolated from urine samples of UTI patients attending the hospital identified in accordance with the Centers for Disease Control and Prevention/National Healthcare Safety Network (CDC/NHSN) criteria. All urinary tract isolates were identified using standard biochemical methods and confirmed by the VITEK-2 automated system (bio-Merieux, France) in accordance with the manufacturer’s instructions. The MDR patterns of urinary tract isolates were identified and determined by the microdilution method (reference broth) according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Carbapenem resistance (ESBL) was identified and confirmed by the Hodge test according to the CLSI guidelines.

Nigella sativa seeds collection and extraction: The selection of medicinal plants such as Nigella sativa was used based on their reported traditional use and purchased from the local market from Al-Khamasim (Wadi Al-Dawasir city) and air-dried. Using milling machine the Nigella sativa seeds were blended to powder and kept in sterile plastic bags in a cool dry place until further used (extraction).

About 100 g of Nigella sativa seeds powered were used for ethanolic extraction by a Soxhlet extractor based on the polarity the extraction was done. The extract was concentrated by rotary evaporator at 40 °C the solvents were evaporated from the extract and the excess solvent was evaporated to dryness by using a water bath and stored at 2-8 °C until used. The dried ethanolic extract was measured, 50 mg of ethanolic extract mL-1 in 2% dimethyl sulfoxide (DMSO) solution, with distilled water.
**Bacterial inoculum preparation:** All the clinical isolated bacterial strains were grown to be inoculated into 2.0 mL sterilized normal saline and compared the inoculum density with McFarland standard solution.

**Antimicrobial activity of Nigella sativa:** The antimicrobial sensitivity tests of ethanolic extracts of Nigella sativa seeds was performed using disc diffusion (Kirby-Bauer’s) method and followed by standard minimal bactericidal concentration (MBC) and minimal inhibitory concentration (MIC) methods.

**Minimum inhibitory concentration (MIC) of Nigella sativa:** The MIC of Nigella sativa seeds ethanolic extracts was performed by the broth micro-dilution method according to CLSI guidelines. All clinical isolates were sub-cultured in Muller Hinton Agar plates (MHA) and incubated at 37°C for 24 h prior to MIC determination. All test organisms inoculum were made in 0.84% sterile saline and an inoculums density was corresponding to 0.5 McFarland standard solution. Dispensed 100 μL of double strength MH broth which containing 5% DMSO into 96-well microritter plates. One row line in each plate was used with tobramycin (BioMerieux, France) as a positive control (in a serial dilution of 36-0.015 μg mL⁻¹) for Gram-negative isolates. The stock ethanolic extract solution was diluted (50 mg mL⁻¹) and transferred into the first well and serially diluted, concentrations of ethanolic extract were range from 50-1.56 mg mL⁻¹ (i.e., 50, 25, 12.5, 6.25, 3.12 and 1.56 mg mL⁻¹). To each well 15 μL of each test organism inoculums (equivalent to 0.5 McFarland standards) was added and incubated at 37°C for 24 h. The MIC for each bacterial strain was performed 3 times in triplicates. An inhibited visible growth at the lowest concentration of ethanolic extract of Nigella sativa seeds was defined as MIC.

**Minimal bactericidal concentration (MBC) of Nigella sativa:** The ethanolic extract of Nigella sativa seeds treated MIC wells with no discernible growth was chosen to determine the MBC according to Hayes and Markovic. Briefly, after homogenization, a loop (~10 μL) of each bacterial suspension was inoculated on MHA and incubated at 37°C for overnight. The MBC of ethanolic extract of Nigella sativa seeds was determined in which no discernible growth was observed. The MBC for each bacterial strain was performed three times in triplicates.

**Statistical analysis:** Data recorded were expressed diagrammatically and as tables, was done using Microsoft Excel.

**RESULTS**

**Selection of Nigella sativa seeds:** Islamic literature and ethnomedicinal information explain about uses of Nigella sativa seeds in different frameworks of medicines and food forms of therapeutics.

**Antibiotic sensitivity test of bacteria:** The commercial antibiotic report of all clinical isolates used in this study was determined using selective specified antibiotic discs. Among the 5 urinary pathogens, K. pneumoniae were resistant to 5 antibiotics out of 15 A. baumannii and E. coli were resistant to 10 antibiotics and intermediate to 1 out of 15, P. mirabilis were resistant to 4 and intermediate to 6 out of 15 antibiotics and P. aeruginosa were resistant to 4 antibiotics out of 15. The details of specific antibiotics resistant profiles of clinically isolated pathogens are shown in Table 1 and concluded that isolate urinary pathogens were multidrug-resistant.

**Antibacterial activity measurement by filter paper impregnation method:** Ethanolic extracts of Nigella sativa seed were tested against various MDR strains of urinary pathogens with various concentrations (10, 20, 30, 40 and 50 μL). The antimicrobial activity of the ethanolic extract of Nigella sativa is represented in Fig. 1 and 2. The results showed varying degrees of antimicrobial activity on the dose-dependent manner with less effect than the cork borer method. The antimicrobial

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Specimen</th>
<th>ESBL</th>
<th>AMP</th>
<th>AMO</th>
<th>PIP</th>
<th>CEL</th>
<th>CEX</th>
<th>CET</th>
<th>CEF</th>
<th>IMP</th>
<th>MER</th>
<th>AMI</th>
<th>GEN</th>
<th>CIP</th>
<th>TIG</th>
<th>NIY</th>
<th>TRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Urine</td>
<td>Positive</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>Urine</td>
<td>Positive</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>R</td>
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<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>Urine</td>
<td>Positive</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>Urine</td>
<td>Positive</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Urine</td>
<td>MDR</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>S</td>
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<td>S</td>
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<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
</tbody>
</table>


Table: 1 Pattern of antimicrobial resistant among the urinary pathogens used in this study
activity of *Nigella sativa* ethanolic extract was impregnation with sterile Whatman filter paper was higher at 50 µL especially competent against *Proteus mirabilis* (18±1.2) followed by *Pseudomonas aeruginosa* (13±0.8), *Klebsiella pneumoniae* (12±0.8), *Escherichia coli* (15±1.3), *Acinetobacter baumannii* (12±0.8) and ATCC (35218) *E. coli* (15±0.8). This *Nigella sativa* seeds ethanolic extract was less effective than the cork borer techniques with the same concentration. The results were the mean and standard deviation (SD) of triplicate results and were the diameter of the test-the diameter of the control (solvent).
Fig. 3: Zone of inhibition (mm) of ethanolic extract of *Nigella sativa* seeds against clinically isolated urinary pathogens by cork borer technique

ESBL: Extended spectrum β-lactamase producer, MDR: Multidrug resistant, ATCC: American type culture collection

**Antimicrobial activity measurement by cork borer disk diffusion method:** The antimicrobial activity of the ethanolic extract of *Nigella sativa* is represented in Fig. 3 and 4. The results showed that the alcoholic extract of black cumin seeds (*Nigella sativa*) showed varying degrees of antimicrobial activity in dose-dependent manner. The antimicrobial activity of *Nigella sativa* ethanolic extract was significantly higher and 30 µL especially competent against *Proteus mirabilis* (38±1.2) followed by *Pseudomonas aeruginosa* (26±0.9), *Klebsiella pneumoniae* (32±0.8), *Escherichia coli* (25±1.3) and *Acinetobacter baumannii* (24±1.2). *Nigella sativa* seeds ethanolic extract were effective besides the selected clinical isolates of ESBL-producing *E. coli* and MDR *Acinetobacter baumannii*. The results were the mean and standard deviation (SD) of triplicate results and were the diameter of the test-The diameter of the control (solvent).
**MBC of ethanolic extract of *Nigella sativa seeds*:** Minimum bactericidal concentration (MBC) values of ethanolic extracts of *Nigella sativa* seeds against 5 MDR urinary pathogens are shown in Table 2. The MBC concentration for most of the urinary pathogenic organisms was confirmed by the absence of bacterial growth of the tested strains streaked form inhibition zone corresponding to their lowest MIC’s. The extract showed potentially bactericidal activity against the tested pathogenic bacteria (Proteus mirabilis with MBC of 0.5 mg mL⁻¹, while *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Escherichia coli* with MBC of 1 mg mL⁻¹ while MBC of *Acinetobacter baumannii* extract reached to 2 mg mL⁻¹ and its minimal bactericidal concentration reached to 25 mg mL⁻¹. The results of MIC and MBC of the effective plant extracts suggested that *Nigella sativa* can be effectively used to control the multidrug-resistant pathogens. The MBC values signified that the minimal concentration of ethanolic extracts of *Nigella sativa* seeds is used, whereas, a greater concentration of ethanolic extracts suggested the exploit of the control of any bacterium.

**DISCUSSION**

Multidrug resistance is an emerging problem at an alarming pace, negotiation with ability to control and treat much pathogenic infection, as well as destabilization many other signs of progress in health issues and medication. Health awareness and economic consequences of MDR represents a rising heavy burden worldwide (low, middle and high-income countries), necessitate serious action at regional, national and global levels, predominantly in point of restricted improvement of newly emerging antibacterial drugs¹⁶,¹⁷. Thus, there is an urgent requirement for the finding of novel substances or potent bioactive compounds from natural sources (plant material/extract) could be used for therapeutic purpose as antimicrobial substances¹⁸. Most of the pathogenic microorganisms cause serious infection to human well-being and reveal multi-drug resistance (MDR) owing to misuse and insufficient use of antibiotics. Therefore, the alternative approaches to overcome multidrug-resistant organisms by using natural bioactive components include plant essential oils, plant extracts and plant bioactive compounds¹⁹.

Present investigation actively exhibited the persuasive antibacterial activity of ethanolic extracts of *Nigella sativa* seeds against various clinically isolated urinary pathogens with special reference to MDR pathogens like ESBL *E. coli, Proteus mirabilis, Pseudomonas aeruginosa* and *K. pneumonia and MDR A. baumannii*. These data are consistent with some earlier reports. *Nigella sativa* seeds ethanolic extract was found to be active against MDR bacterial strains as previously reported by Emeka et al.²⁰. Present data were similar to previous reports which showed significant antimicrobial effects of *Nigella sativa* against *Proteus mirabilis, Klebsiella pneumonia* and *E. coli*²¹,²². The preliminary assessment of ethanolic extracts of *Nigella sativa* seeds illustrated effective inhibitory action against Gram-negative clinically isolated urinary pathogens based on the dose-dependent manner (Fig. 1-4).

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**Table 2: MIC and MBC values ethanolic extract of *Nigella sativa* seeds and tobramycin as positive control against bacteria**

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>MIC values (mg mL⁻¹)</th>
<th>MBC values (mg mL⁻¹)</th>
<th>MIC values (mg mL⁻¹) [Positive control]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>1.500</td>
<td>0.5</td>
<td>125</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>3.125</td>
<td>1.0</td>
<td>125</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>25.000</td>
<td>1.0</td>
<td>25.0</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>6.250</td>
<td>1.0</td>
<td>25.0</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>25.000</td>
<td>2.0</td>
<td>50.0</td>
</tr>
</tbody>
</table>
Emeka et al.\textsuperscript{20} proved that \textit{Nigella sativa} seeds essential oil possess antibacterial effects. The MIC was 2\% against \textit{Pseudomonas aeruginosa} and 1\% against \textit{Staphylococcus aureus}. This evidence proved that black cumin seed oil is effective for both Gram-positive and Gram-negative bacteria. This study reports revealed a better alternative complementary therapy of common antibiotic drugs. \textit{Nigella sativa} is conventional medicine used for a very long period. The study proved that \textit{Nigella sativa} possesses an effective antimicrobial response as compared with various commonly available antibiotics and better to rely on these traditional herbs as they possess the very least side effects as well as chances of developing resistance against commonly used antibiotics\textsuperscript{23}.

Singh et al.\textsuperscript{24} reported that the bioactive components from \textit{Nigella sativa} possess strong antibacterial action, indicating that \textit{Nigella sativa} seeds extract consists of effective bio-active constituents responsible for altering the growth and cell division of bacterial strains which helps to eliminate pathogens. The antimicrobial efficacy was expressed at varying degrees with a dose-dependent manner (Fig. 1, 3). In addition, distinctions in \textit{Nigella sativa} antimicrobial action might be owing to a variation in the bioactive components of the oils/extract collected from various region\textsuperscript{25} as well as the different profiles of clinically isolated pathogens collected from different parts of the world.

The strong antimicrobial action of \textit{Nigella sativa} was seen for \textit{Proteus mirabilis} and \textit{Pseudomonas aeruginosa}, as well as weak antimicrobial activity, was seen on \textit{Acinetobacter baumannii}, due to its resistance to a wide range of antimicrobial agents. The MDR-characteristic features of resistance include the production of antibiotic-modifying enzymes, impaired entry through the bacterial cell wall, active efflux mechanism and effectively target mutations that diminish antibacterial affinity\textsuperscript{26}. The antimicrobial activity of ethanolic extract of \textit{Nigella sativa} seed exerts by its active phytoconstituents of thymoquinone and thymol (phenolic alcohol)\textsuperscript{21}. The presence of secondary metabolites in ethanolic extract of \textit{Nigella sativa} seeds could act by altering changes in membrane structure, inhibition of cell wall synthesis, by binding/inhibiting protein synthesis through binding to 50S subunit ribosomal molecules and finally interrupting with the peptidyl transferase activity\textsuperscript{27}.

The present investigation has to expand future research to prove the antimicrobial activity from \textit{Nigella sativa} extracts against a wide range MDR nosocomial strains, antiviral and antifungal activities. Furthermore, elucidate the exact alteration of the drug resistance mechanism by \textit{Nigella sativa} extracts seeds.

CONCLUSION

The present study highlighted the efficacy of potent antimicrobial activity for ethanol extracts of \textit{Nigella sativa} seeds against various clinically isolated urinary pathogens. Ethanolic extracts of \textit{Nigella sativa} seeds could effectively enhance place in the treatment of some microbial infections in topical applications. Furthermore, in vitro and in vivo studies on a huge number of clinical isolates are necessary for further analysis which helps and standardize the effective inhibitory action of \textit{Nigella sativa} extracts against these emerging pathogens. Furthermore, the promising results in this present study may pave the way for complementary medicine as a potential new antibacterial against most multidrug-resistant pathogens.

SIGNIFICANT STATEMENT

This study discovered the beneficial effects of ethanolic extract of \textit{Nigella sativa} against multidrug-resistant urinary pathogens via antimicrobial activity. The study will help the future researcher to uncover the critical areas of antibiotic resistance, multidrug resistance organism and study of isolated components from \textit{Nigella sativa} that many researchers were not able to explore to date. This may be a promising finding for future treatment for drug-resistant bacteria and in the prevention of side effects using natural plants.

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REFERENCES


