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Antibacterial and Alteration of Drug Resistance Activities of Black Cumin Seed (Nigella Sativa) Extracts against Urinary Pathogens

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ABSTRACT

Most urinary pathogens that cause urinary tract infection (UTI) have developed multidrug-antibiotic resistance (MDR) and forewarned our research interest in investigating natural products to increase medicinal plant usage as alternative therapies for infectious diseases. This study was carried out to investigate the antibacterial activities of ethanolic extract from black cumin seeds (Nigella sativa), which were evaluated by minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays against clinically isolated MDR urinary pathogens and compared with E.coli ATCC 35218. In this study, ethanol extract of Nigella sativa possesses strong microbial growth inhibitory effects against the tested pathogens. The inhibitory activity of ethanolic extract (EE) was more robust with dose-dependent inhibitory effects. The minimum MIC values were observed for Proteus mirabilis (1.5 mg/ml), followed by Pseudomonas aeruginosa (2 mg/ml), extended-spectrum β-lactamase (ESBL) producing Klebsiella pneumonia (2.5 mg/ml), ESBL producing Escherichia coli(2.75 mg/ml)and Acinetobacter baumannii(2.5 mg/ml).MBC inhibitory effects of ethanolic extracts were more significant than those of corresponding MIC results for all clinically isolated urinary pathogens. The bactericidal values of ethanol extracts were slightly increased than the related MIC. All the data was compared with E.coli ATCC 35218. This study augmented the effective antibacterial activity of Niofla sativa seed extract against carbapenem-resistant E. coli and Klebsiella pneumonia clinical isolates. Thus, it might be an effective potential source of safe and natural antimicrobial agents against emerging ESBL-producing clinical isolates.

Keywords
Antibacterial activity
Extended Spectrum Beta-Lactamase
Multidrug-resistant pathogens
Nigella sativa seeds

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Introduction

The most widespread bacterial infections are urinary tract infections (UTI), affecting people throughout their lifespan [Foxman, 2002; Barnett and Stephens, 1997]. The pathogenesis of UTI is complex and altered by host behavioural factors and characteristic features of the infecting urinary pathogens. The most common and leading urinary pathogens include Klebsiella pneumoniae Escherichia coli, Proteus mirabilis,

Enterococcus faecalis, Pseudomonas aeruginosa, and Candida albicans [1]. The acute UTI (uncomplicated) is supposed to exceed 0.5 episodes/ annum between 18-30 years of females [2]. Currently, most pathogenic microorganisms have developed resistance against commonly used commercial antimicrobial drugs due to their indiscriminate use in treating infectious diseases. Despite the subsistence of strong antibiotics or MDR, bacterial strains are gradually appearing, with impressive demand for a permanent solution and the development of novel drugs without any side effects. Therefore, alternative antimicrobial approaches are promptly needed, and thus, this circumstance has led to reconsidering the therapeutic regimens used in ancient remedies, including plants and plant-derived products [3].

The seeds of Nigella sativa, generally known as black cumin, have been used for medicinal purposes for centuries in the form of herb and oil in the Middle East, Asia, and Africa [4]. It has been used as a traditional medicine to treat stomach and intestinal health, respiratory health, circulatory and immune system support, kidney and liver function, and general health issues [5]. Studies in the last 4-5 decades about Nigella sativa seeds have widely reported that they possess various medicinal properties [5][6]. Essential oil [7] and crude extracts [8] have been reported to possess antimicrobial activity against diverse clinical isolates. The Nigella sativa seeds essential oil has dependent anti-bacterial effects against various Gram-positive and Gram-negative bacteria. Many bioactive components have been isolated from Nigella sativa seeds, but quinine constituents such as thymoquinone are responsible for its pharmacological effects.



Fig. 1. Flower and seeds of Nigella sativa

Antibacterial agents are one of the major significant arms in fighting against infectious diseases; the emergence of resistance against various microorganisms paves the way to investigate newer drugs. The present study was designed to screen and explore the antibacterial activity of ethanolic extracts of Nigella sativa seeds on selected urinary pathogenic bacteria isolated from urine, especially ESBL-producing clinical isolates.

Material And Methods

This descriptive analysis was performed at General Hospital, Wadi Al Dawaser, Kingdom of Saudi Arabia, for eight months, from September 2017 to March 2018. A total of 30 clinical isolates, including 20 MDR bacterial isolates, were included in this study. All bacterial strains used throughout the present investigation maintained on nutrient agar slants were maintained. The cultures were stored at four °C, with regular transfer at monthly intervals, and their morphological characteristics were confirmed by macroscopic and microscopic examination.

A. Clinical Bacterial Isolates

Five Gram-negative bacteria, including A. baumannii, E. coli, K. pneumoniae, P. mirabilis, and P. aeruginosa, were used in this study. All clinical bacterial isolates were isolated from urine samples of UTI patients attending the hospital identified by the Centers for Disease Control and Prevention/National Healthcare Safety Network (CDC/NHSN) criteria. The clinically isolated bacterial strains, namel: Proteus mirabilis, Pseudomonas aeruginosa, and Acinetobacter baumannii carbapenem-resistant Gram-negative bacilli, include Klebsiella pneumonia and Escherichia coli. All clinical isolates were identified using standard biochemical methods and confirmed using the VITEK-2 automated system (bio-Merieux, France) by the manufacturer's instructions. MDR patterns of all clinical isolates were identified and determined by the micro-dilution method (reference broth) according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Carbapenem resistance (ESBL) was identified and confirmed by the Hodge test according to the CLSI guidelines.

B. Nigella sativaseedsCollection and Extraction

Medicinal plants such as Nigella sativa were selected based on their reported traditional use. Nigella sativa seeds were purchased from the local market from Al-Khamasin (Wadi Al Dawaser city) and air dried. Using a 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 Nigella sativa seeds were used for ethanolic extraction by a Soxhlet extractor based on the polarity of the extraction. The extract was concentrated by rota ary evaporator at 40°C. The solvents were evaporated from the extract, and the excess solvent was evaporated to dryness using a water bath and stored at 2–8°C until used. The dried ethanolic extract was measured, 50 mg of ethanolic extract/ml in 2% dimethyl sulfoxide (DMSO) solution with distilled water.

C. Bacterial Inoculum Preparation

All the clinically isolated bacterial strains were grown to be inoculated into 2.0 ml of sterilised normal saline and the inoculum density with McFawas rthe land standard solution. The antimicrobial sensitivity tests of ethanolic extracts of Nigella sativa seeds were performed using Kirby-Bauer's disc diffusion method and followed by standard minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) methods.

D. Determinations of Minimum Inhibitory Concentration (MIC) of Nigella sativa

The MIC of Nigella sativa seeds ethanolic extracts was performed by broth microdilution method according to the guidelines of the CLSI. All clinically isolated strains were sub-cultured in Muller Hinton Agar (MHA) (Ministry of Health, KSA), and plates were incubated for 24 h at 37 °C before MIC determination. All test organisms' inoculums were prepared in sterile saline (0.84% NaCl), and an inoculum density corresponded to 0.5 McFarland standard solution. Dispensed 100 µl of double-strength MH broth containing 5% DMSO into 96-well microtiter 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) for Gram-negative isolates. The stock ethanolic extract solution was diluted (50mg/ml) and transferred into the first well and serially diluted; concentrations of ethanolic extract ranged from 50–1.56 mg/ml (i.e. 50, 25, 12.5, 6.25, 3.12, 1.56 mg/ml). 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 three times in triplicates. An inhibited visible growth at the lowest concentration of Nigella sativa seeds ethanolic extract was defined as MIC.

E. The Minimal Bactericidal Concentration (MBC) of Nigella sativa

The ethanolic extract of Nigella sativa seeds treated in MIC wells with no discernible growth was chosen to determine the MBC [9]. After homogenisation, each bacterial loop (≈10µl) suspension was inoculated on MHA and incubated at 37°C overnight. MBC of Nigella sativa seeds ethanolic extract was determined, and no discernible growth was observed. The MBC for each bacterial strain was performed three times in triplicates.

Results

A. Antibiotic sensitivity test of bacteria

Islamic literature and ethnomedicinal information explain the uses of Nigella sativa seeds in different frameworks of medicines and food therapeutics. The commercial antibiotic report of all clinical isolates used in this study was determined using selectively specified antibiotic discs, as shown in Table 1. Among the five urinary pathogens, K. pneumoniae were resistant to 5 antibiotics out of 15, A. baumannii and E. coli were resistant to 11 antibiotics 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 MRD.

Table 1. Effect of antibiotics used on clinical isolates urinary pathogens by antimicrobial sensitivity testing.

Bacteria	Specimen	ESBL	Antibiotics															
			AMP	AMO	PIP	CEL	CEX	CET	CEF	CEP	IMP	MER	AMI	GEN	CIP	TIG	NIY	TRI
K. pneumoniae	Urine	Positive	R	S	S	S	S	S	S	I	R	I	S	R	R	S	S	R
E. coli	Urine	Positive	R	S	S	R	R	I	S	S	R	R	R	R	R	S	R	R
P. mirabilis	Urine	Negative	R	S	S	R	R	R	I	I	S	I	I	I	I	S	S	S
A. baumannii	Urine	MDR	R	R	S	R	R	S	S	S	R	R	R	R	R	S	I	R
P. aeruginosa	Urine	Negative	R	S	S	R	R	S	S	I	S	I	I	R	R	S	S	S

AMP Ampicillin, AMO Amoxicillin, PIP Piperacillin, CEL Cefalotin, CEX Cefoxitin, CET Ceftazidime, CEF Cefriaxon, CEP Cefepime, IMP Imipenem, MER Meropenem, AMI Amikacin, GEN Gentamicin, CIP Ciprofloxacin, TIG Tigecycline, NIY Nitrofurantion, TRI Trimethoprim.

B. Antibacterial activity against the ethanolic extract of Nigella sativa seed by filter paper impregnation disk diffusion

Ethanolic extracts of Nigella sativa seed when tested against various MDR strains of urinary pathogens with different concentrations (10, 20, 30, 40, 50µl). The antimicrobial activity of the ethanolic extract of Nigella sativa is represented in Fig.s 2 and 3. The results showed that the alcoholic extract of black cumin seeds (Nigella sativa) showed varying degrees of antimicrobial activity in a dose-dependent manner with less effect than the cork borer method. Nigella sativa ethanolic extract's antimicrobial activity was impregnated with sterile

Whatman filter paper. It was higher at 50µl exceedingly competent against Proteus mirabilis (18 ± 1.2), followed by Pseudomonas aeruginosa (15 ± 0.8), Klebsiella pneumoniae (14 ± 0.8), Escherichia coli (13 ± 1.3) and Acinetobacter baumannii (13 ± 0.8). These Nigella sativa seeds' ethanolic extracts were less effective than the cork borer techniques with the same concentration. The mean and standard deviation (SD) of triplicate results were the diameter of the test–The diameter of the control (solvent).

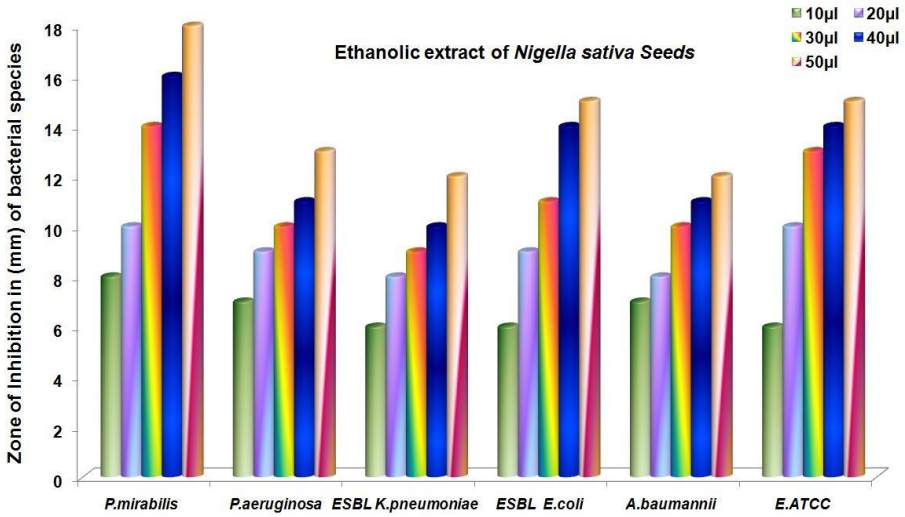


Fig. 2. The zone of inhibition diameter in millimetres (mm) of ethanolic extract of Nigella sativa seeds against clinically isolated urinary pathogens.

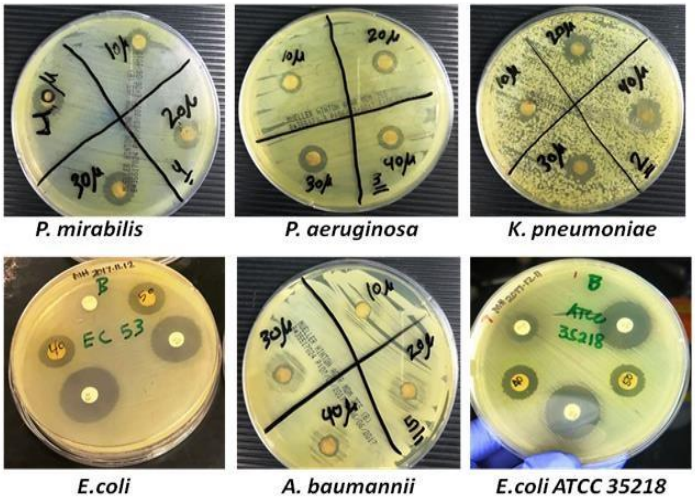


Fig. 3. Representative profile of antibacterial activity of ethanolic extract of Nigella sativa seeds against clinically isolated urinary pathogens.

C. Antimicrobial activity measurement by cork borer disk diffusion method

The antimicrobial activity of the ethanolic extract of Nigella sativa is represented in Fig. 4 and Fig. 5**.** The results showed that the alcoholic extract of black cumin seeds (Nigella sativa) showed varying degrees of antimicrobial activity in a dose-dependent manner. The antimicrobial activity of Nigella sativa ethanolic extract was significantly higher and 30µl exceedingly competent against Proteus mirabilis (38 ± 1.2), followed by Pseudomonas aeruginosa (26 ± 0.9), Klebsiella pneumoniae (32 ± 0.8), Escherichia coli (24 ± 1.3 to 22 ± 1.1) and Acinetobacter baumannii (24 ± 1.2). Nigella sativa seeds ethanolic extract was effective besides the selected clinical isolates of ESBL-producing E. coli and MDR A. baumannii. The mean and standard deviation (SD) of triplicate results were the diameter of the test–The diameter of the control (solvent).

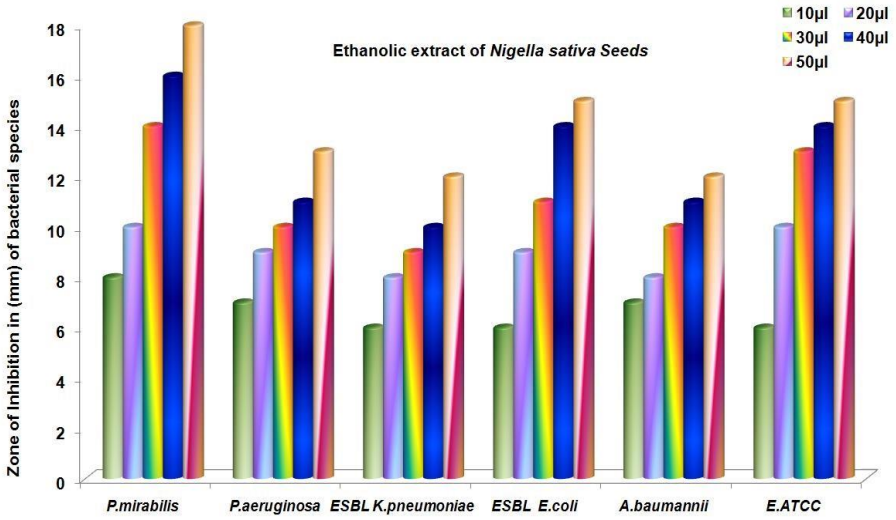


Fig. 4. The zone of inhibition diameter in millimetres (mm) of ethanolic extract of Nigella sativa seeds against clinically isolated urinary pathogens.

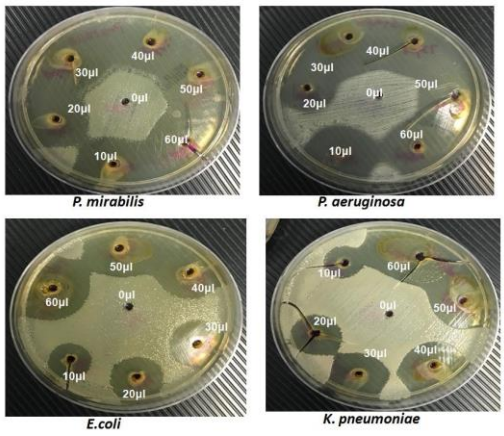


Fig. 5. Representative profile of antibacterial activity of ethanolic extract of Nigella sativa against seeds against clinically isolated urinary pathogens by cork borer technique.

D. MIC of ethanolic extract of Nigella sativa seeds

The lowest minimum inhibitory concentration (MIC) values of ethanolic extracts of Nigella sativa seeds against 5 MDR urinary pathogenic organisms are shown in Table 2. The ethanolic extract of Nigella sativa seeds has strong growth inhibitory effects on all tested urinary pathogens. However, ethanolic extracts inhibitory activity against all pathogens, the Proteus mirabilis (1.5 mg/ml) was recorded as the lowest concentration of MIC value fol,lowed by Pseudomonas aeruginosa (3.125 mg/ml). The MIC valvaluesr ESBL-producing Klebsiella pneumoniae (6.25 mg/ml) and Escherichia coli (6.25mg/ml) were observed. The highest MIC was observed for Acinetobacter baumannii (25 mg/ml). Lower MIC values signify that the least amount of ethanolic extracts of Nigella sativa seeds is used. In contrast, a higher value represents exploiting more ethanolic extracts to control any bacterium.

Table 2. MIC and MBC value ethanolic extract of Nigella sativa seeds and tobramycin as a positive control against bacteria

Bacterium	Ethanolic extract of *Nigelle sative* seeds		MIC values (mg/ ml)
	MIC values (mg/ ml)	MBC values (mg/ ml)	(Positive control)
Proteus mirabilis	1.5	0.5	12.5
Pseudomonas aeruginosa	3.125	1.0	12.5
ESBL-producing *Klebsiella pneumoniae*	6.25	1.0	25
ESBL-producing *Escherichia coli*	6.25	1.0	25
Acinetobacer baumannii	25	2.0	50

E. MBC of ethanolic extract of Nigella sativa seeds

Minimum bactericidal concentration (MBC) values of ethanolic extracts of Nigellasativa seeds against 5 MDR urinary pathogenic organisms are shown in Table 2. MBC values for most urinary pathogenic organisms exceeded the MIC values. However, the MBC values were identical to the MIC values for A. baumannii. MBC values signify that the least amount of ethanolic extracts of Nigella sativa seeds is used. In contrast, a higher value means the exploitation of moderately more ethanolic extracts for controlling any bacterium.

Discussion

Multidrug resistance is an emerging problem at an alarming pace, negotiating our ability to treat and control many pathogenic infections, as well as undermining many other progresses in health issues and medicine. Health awareness and economic consequences of MDR represent a rising heavy burden worldwide (low-, middle- and high-income countries), necessitating urgent action at regional, national, and global levels, predominantly in point of restricted improvement of newly emerging antimicrobial agents [10][11]. Thus, there is an urgent requirement to find novel substances or potent bioactive compounds from natural

sources (plant material/extract) that could be used for therapeutic purposes as antimicrobial substances for synthesising useful drugs [12]. Most pathogenic microorganism causes severe infection to human well-being and reveals multi-drug resistance (MDR) due to misuse and insufficient use of antibiotics. Therefore, the alternative approaches to overcoming multidrug-resistant bacteria are using natural bioactive components for antimicrobial substances, including plant essential oils, plant extracts, and bioactive components [13].

Our study actively exhibits the persuasive antimicrobial activity of ethanolic extracts of Nigella sativa seeds against various clinically isolated urinary pathogens with particular reference to MDR pathogens like carbapenem-resistant E. coli 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 Ref. [14]. Our data were similar to previous data reported by Ref. [15], which show significant antimicrobial effects of Nigella sativa against Proteus mirabilis, Klebsiella pneumonia, and E. coli. The preliminary assessment of ethanolic extracts of Nigella sativa seeds illustrated effective inhibitory action against Gram-negative clinically isolated urinary pathogens based in a dose-dependent manner (Fig. 2 and Fig. 5).

Ref. [14] proved that the oil of Nigella sativa seeds MIC was 2% against Pseudomonas aeruginosa and 1% against Staphylococcus aureus. This evidence demonstrates that black cumin seed oil is effective for Gram-positive and Gram-negative bacteria. Our reports reveal a better alternative, complementary therapy to standard antibiotic drugs. Nigella sativa is a conventional medicine used for a very long period. Our study proves that Nigella sativa has an effective antimicrobial response compared to various commonly available antibiotics. It is better to rely on these traditional herbs as they possess minor side effects and chances of developing resistance against commonly used antibiotics.

Ref. [16] reported that the bioactive compounds from Nigella sativa possess potent antimicrobial action, indicating that Nigella sativa seed extracts consist of effective bio-active constituents responsible for altering bacterial strains' growth and cell division, which helps to eliminate pathogens. The antimicrobial activity was expressed at varying degrees in a dose-dependent manner (Fig. 2 and Fig. 4). In addition, distinctions in N. sativa antimicrobial action might be due to a variation in the chemical composition of the oils/extract collected from various regions and different profiles of clinically isolated pathogens collected from other parts of the world.

The antimicrobial solid action of Nigella sativa was seen for P. mirabilis and P. aeruginosa. Weak antimicrobial activity was seen in A. baumannii due to its resistance to various antimicrobial agents. Characteristic resistance features include the production of

antibiotic-modifying enzymes, impaired entry through the bacterial outer membrane, active efflux mechanism, and target mutations that diminish antimicrobial affinity [17]. The antimicrobial activity of N.satN. sativated by the ethanolic extract may be due to early reported active phytoconstituents such as thymoquinone and thymol. A phenolic alcohol molecule called thymol is present in the essential oil of N. sativa [6]. The presence of secondary metabolites in the ethanolic extract of N. sativa seeds could act by altering changes in membrane structure, inhibiting cell wall synthesis, binding /inhibiting protein synthesis by binding to 50S subunit ribosomal molecules, and finally interrupting the peptidyl transferase activity.

Our study is most advantageous in examining and analysing the antibacterial action of N.sativa extract against these MDR-urinary pathogens. Our research finding is of significant concern. These emerging infectious pathogens are well known for their drug resistance (innate and acquired) to multiple aantibioticclasses and tofor enduringn nosocomial environments [18][19]. This present study has some limitations, including:

- Clinical strains were representative of MDR pathogens isolated from patients
- Investigation of antimicrobial activity against only those clinically isolated bacterial strains
- It is also likely to expand future research to prove the antibacterial activity from N.sativa extracts against a wide range of MDR nosocomial strains, antiviral and antifungal activities
- Illustration of the exact molecular mechanism of bacterial growth inhibition by the extracts needs further investigation.

Conclusion

The present study highlights the efficacy of potent antimicrobial activity for ethanolic extracts of Nigella sativa against various clinically isolated urinary pathogens, suggesting that, with future research and enhancement. Nigella sativa seed extracts could effectively enhance a place in the treatment of some microbial infections in topical applications. Furthermore, in vitro and in vivo studies on many clinical isolates are necessary for further analysis, which helps standardise the effective inhibitory action of Nigella sativa extracts against these emerging pathogens. Furthermore, this study's promising results may pave the way for complementary medicine as a potential new antibacterial against most MDR UTI-causing bacteria.

Conflict of Interest

The authors declare that there is no conflict of interest.

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