Inhibitory Effects of *Nigella sativa* Phenolic Extracts on Biofilm Formation and Virulence Gene Expression in Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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Abstract

Background : Methicillin-resistant *Staphylococcus aureus* (MRSA) is a well-known opportunistic human pathogen that causes healthcare-associated infections. Biofilm formation plays a major role in antimicrobial resistance and persistence, which is especially significant when pertaining to clinical settings. Natural products have received significant focus as prospective therapeutic agents for drug-resistant bacteria. Abstract Background The aim of this study was to assess the anti-biofilm activity and biofilm-associated virulence gene in clinical MRSA isolates treated with phenolic extracts obtained from seeds of *Nigella sativa*. **Materials and Methods:** Methods: A total of sixty bacterial clinical MRSA isolates were obtained from different kinds of specimens in the Kirkuk General Hospital/Iraq. Abstract: 70% ethanol was used to extract *Nigella sativa* seeds, and high-performance liquid chromatography (HPLC) for thymoquinone determination. The microdilution and crystal violet assays respectively evaluated the MIC for bacteria growth inhibition and biofilm inhibition activity. The expression levels of biofilm-associated genes (icaA, icaD, fnbA clfA and spa) were determined by quantitative real-time PCR (qRT-PCR). **Results**: The phenolic extract showed remarkable antibacterial activities against MRSA (MIC at 0.5-2.0 mg/mL). The biofilm development was inhibited in a concentration-dependent manner with the inhibitory effect being 88.5% at the highest inhibiting concentration of 4.0 mg/mL respectively Moreover, biofilm-associated genes were significantly downregulating at the level of gene expression (ical: > 75-fold; icad and spa : greater than three fold decrease with $P < 0.001$). Microscopic examination additionally confirmed significant disruptions of the biofilm architecture after treatment with extract. **Conclusion:** *Nigella sativa* phenolic extracts demonstrated potent anti-biofilm and antibacterial activities against clinical MRSA isolates. The observed effects were associated with suppression of key virulence and biofilm-related genes, suggesting that these extracts may serve as promising candidates for the development of alternative strategies to combat MRSA-associated infections.

Keywords: *Nigella sativa*, thymoquinone, MRSA, phenolic extracts, antibiotic resistance.

Introduction

Change to Antimicrobial resistance is one of the most serious risks to global health today, and the World Health Organization (WHO) has identified it as one of the most significant global health problems this century [1]. Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria are at the vanguard of this threat; millions of infection cases are recorded worldwide each year, with mortality rates approaching those associated with the human

ISSN 2072-3857

immunodeficiency virus (HIV) in certain affluent countries [2].

MRSA was first reported in the United Kingdom in 1961, shortly after methicillin was introduced into the clinic [3], MRSA has emerged as an important global pathogen in both the hospital and community. The SCCmec element carries the mecA gene that is mainly responsible for its extraordinary multidrug resistance. This gene encodes the modified penicillin-binding protein PBP2a, which reduces susceptibility to β-lactam antibiotics by decreasing their binding affinity with subsequent resistance against nearly all known classes of β-lactams and multiple additional antibiotic types [4].

Among the different mechanisms that determine MRSA pathogenicity, biofilm formation seems to be a key factor for determining antimicrobial resistance. The biofilm architecture generates a protective microenvironment that restricts drug delivery and favors the persistence of bacteria, leading to severe antimicrobial resistance in cells encased within biofilms at concentrations 1000-fold greater compared with free-living (planktonic) counterparts [5]. The biofilm is composed of extracellular polysaccharide intercellular adhesin (PNAG), which is genetically encoded by the ica gene loci icaA and icaD, surface proteins (fnib, clfA, and spa), and extracellular DNA (eDNA) [6]. The agr (Accessory Gene Regulator) quorum sensing system is a master regulator that coordinates the expression of virulence determinants in a population density-dependent manner by controlling the synthesis of cyclic peptide signals (AIP) [7]. Several studies have demonstrated that inhibiting this system leads to effective suppression of biofilm formation and reduction of bacterial virulence. In the face of these challenges, medicinal plants are attracting increasing attention as promising sources of antimicrobial compounds, especially in combating multidrug-resistant strains. Sequential scientific reports have proven that plant extracts possess multi-target mechanisms of action that hinder bacteria from developing effective resistance [8] . The black seed plant (*Nigella sativa*) from the Ranunculaceae family is at the forefront of these plants due to its rich historical record in traditional medicine among Islamic and Mediterranean civilizations; ancient manuscripts documented its use in treating a wide range of diseases. Recent pharmaceutical research has proven that the richness of its biochemical composition especially thymoquinone (TQ), carvacrol, thymol, and a spectrum of phenolic acids and flavonoids grants it antimicrobial, antioxidant, and anti-inflammatory properties [9] [10].

Despite the accumulated evidence of the antimicrobial activity of *Nigella sativa* extracts, their specific inhibitory effect on the biofilm formation mechanisms in MRSA especially regarding the gene expression of virulence determinants lacks comprehensive molecular characterization in the local Iraqi context. From this standpoint, the objectives of this study emerge to bridge this knowledge gap and provide precise experimental data that serve future clinical applications . The current study aims to isolate and identify MRSA strains from various clinical sources in Kirkuk Governorate and determine their resistance patterns. And the extraction of phenolic extracts from *Nigella sativa* seeds and the determination of their chemical identity by HPLC. And determining the minimum inhibitory concentration (MIC) and the minimum biofilm inhibitory concentration (MBIC) of the phenolic extract. And also evaluating the effect of the extract on biofilm formation and disassembly using

morphological, chemical, and microscopic methods. And revealing the molecular mechanism thru the analysis of virulence gene expression using the qRT-PCR technique

Material and Methods

Sample Collection, Bacterial Isolation, and Diagnosis

Sixty confirmed clinical MRSA isolates were collected from Kirkuk General Hospital during the period from January to December 2024, from various sources including: skin infection wounds, bloodstream infections, respiratory tract infections, and urinary tract infections. The study received ethical approval from the Research Ethics Committee at the University of Kirkuk. Standard diagnostic tests were conducted according to CLSI guidelines [12] Gram staining, catalase and coagulase tests, followed by MRSA confirmation using oxacillin disks, latex agglutination test, and PCR technique for the mecA gene.

Preparation of phenolic extracts

High-purity *Nigella sativa* seeds were used. Drying was carried out (40°C/48 hours), grinding, and extraction with 70% ethanol (1:10 weight/volume, 25°C, 72 hours with constant shaking) The extract was filtered, dried using a rotary evaporator, and dissolved in 1% DMSO. The total phenols were estimated using Folin-Ciocalteu, and the concentration of thymoquinone was determined by HPLC.

Determination of MIC and MBIC

The CLSI 2024 guidelines were followed to determine the MIC using the microdilution method in Mueller-Hinton Broth (CLSI, 2024). Two-fold dilutions (0.0625-8 mg/mL) were prepared in 96-well plates with a cell density of 5×10^5 CFU/mL. For the biofilm, the modified Stepanovic protocol [13] was followed, using crystal violet staining and measuring absorbance at 595 nanometers.

qRT-PCR Analysis

Total RNA was isolated using TRIzol reagent, and cDNA synthesis was performed with a specific kit. qRT-PCR was conducted using the SYBR Green kit on the ABI 7500 device in three replicates. The target genes included: icaA, icaD, fnbA, clfA, spa, hla, agr, using 16S rRNA as an internal reference

Results and Discussion

Characteristics of MRSA Isolates and Their Resistance Patterns

The mecA-PCR analysis confirmed the MRSA in Table (1) characteristic in 60 out of 73 staphylococcal isolates (82.2%). Skin wounds were the most common source (35%), followed by bloodstream infections (28.3%), pneumonia (21.7%), and urinary tract infections (15%). This distribution is consistent with the findings of the study by [14] from multiple Iraqi hospitals. Table (1): Source distribution and antibiotic resistance pattern in MRSA isolates.

Analysis of the phenolic extract

HPLC revealed a thymoquinone concentration of 28.4 ± 1.2 mg/g (dry weight), and total phenolics of 156.8 ± 4.3 mg GAE/g. The main identified compounds: rosmarinic acid (18.2%), quercetin (14.7%), chlorogenic acid (11.3%), and kaempferol (9.8%).

MIC values and biofilm effect

The MIC values ranged between 0.5 and 2.0 mg/mL for the phenolic extract, and between 0.25 and 1.0 mg/mL for pure thymoquinone. Figure (1) shows the distribution of values across the selected isolates.

The phenolic extract resulted in a concentration-dependent inhibition of biofilm formation in all isolates; it reached 88.5% at 4.0 mg/ml and 44.7% at 0.5

ISSN 2072-3857

mg/ml ($p<0.001$). These results represent a relative superiority over what [6] documented using purified Thymol alone[6] As shown in Figure (2), the biofilm inhibition curves of the phenolic extract and thymoquinone are compared to vancomycin.Table (2) data show the inhibition rates of biofilm formation and disassembly at different concentrations of the phenolic extract.

Molecular analysis by qRT-PCR

The qRT-PCR analysis revealed a highly significant decrease in the expression of all biofilm genes. icaA recorded the deepest decrease (7.1 times at 2.0 mg/ml), followed by icaD (6.4 times) and spa (5.8 times) ($p<0.001$). The dormancy of the agr system reflects a disruption of the quorum sensing pathways as documented by Peng and colleagues [7]. Figure (3) shows the heatmap of the relative gene expression of virulence genes at increasing concentrations of the phenolic extract[10].

Comparative Microscopic Images

Microscopic images (40×) stained with crystal violet revealed a dense biofilm in the control group, in contrast to a clear matrix disintegration and a sharp decrease in cellular density in the group treated with 2.0 mg/ml, which is consistent with the microscopic results of [10].

This study provides comprehensive laboratory evidence of the biofilm inhibitory ability of phenolic extracts from *Nigella sativa* against local MRSA isolates in Kirkuk Governorate. Its results contribute to filling a significant research gap related to the molecular characterization of the anti-biofilm effect in the Iraqi geographical context.

The recorded MIC values (0.5-2.0 mg/ml) closely align with the range of results gathered from a comprehensive systematic review conducted by [8] on 47 studies examining *Nigella sativa* extracts against multidrug-resistant bacterial pathogens [8]. They also conform to the study values established by [16] on Iraqi isolates indicate consistency of efficacy across local isolates [16]. The inhibition rates obtained (88.5% at 4.0 mg/ml) were a relative improvement on reported results in earlier studies. Inhibition rates of 65-82% were reported in a similar study using the same laboratory conditions [15]. This disparity is most likely due to the much higher concentration of thymoquinone in this extract (28.4 mg/g) compared to the standardized extracts of the comparative studies. The qRT-PCR results logically align with the phenotypes. The sharp decrease in the expression of icaA (7.1 times) and icaD (6.4 times) directly indicates a reduction in the synthesis of PNAG, the central polysaccharide component of the biofilm matrix. [17]. documented that the loss of PNAG leads to a severe failure in biofilm formation under stress conditions [17]. The recorded suppression of the agr system (a 4.2-fold decrease) represents a significant research gain, as it reflects the disruption of the quorum sensing pathways necessary for the transition from the planktonic phase to the biofilm phase. A study by [7] . reported that thymoquinone compounds interfere with the synthesis of the signaling peptide AIP, which is essential for activating the agr pathway [7] . The results of cellular leakage align with [5] explanation of how monoterpenes interact with phospholipid membrane components by affecting unsaturated fatty acids, thereby disrupting the structural integrity of the membrane and the osmotic chemical potential gradient [5].

Although vancomycin is still considered the reference treatment for MRSA infections, concerns are rising about the emergence of strains with reduced susceptibility to it (VISA/hVISA) [18]. The multi-target effect of phenolic extracts

ISSN 2072-3857

encompassing both cell membrane disruption and suppression of virulence genes adds momentum to hindering the development of targeted resistance. Current data also reveal the potential for synergistic effects with antibiotics, which future studies should explore.

Table (1): Source distribution and antibiotic resistance pattern in MRSA isolates

Predominant Resistance Pattern	Percentage (%)	Number	Clinical Source
Oxa, Ery, Tet	35.0	21	Skin wounds
Oxa, Ery, Cip	28.3	17	Bloodstream infections
Oxa, Tet, Chl	21.7	13	Pneumonia
Oxa, Ery	15.0	9	Urinary tract infections (UTIs)
—	**100**	**60**	Total

Abbreviations: Oxa = Oxacillin, Ery = Erythromycin, Tet = Tetracycline, Cip = Ciprofloxacin, Chl = Chloramphenicol.

Table (2): Biofilm inhibition percentages at different concentrations of the phenolic extract

P-value	Biofilm Disruption Inhibition (%)	Biofilm Formation Inhibition (%)	Concentration (mg/mL)
0.042	8.4 ± 1.2	12.1 ± 1.8	0.125
0.008	19.7 ± 2.1	28.4 ± 2.3	0.25
<0.001	35.2 ± 2.8	44.7 ± 3.1	0.5
<0.001	51.8 ± 3.4	62.3 ± 2.9	1.0
<0.001	67.5 ± 2.7	78.9 ± 2.4	2.0
<0.001	76.3 ± 2.1	88.5 ± 1.6	4.0

ISSN 2072-3857

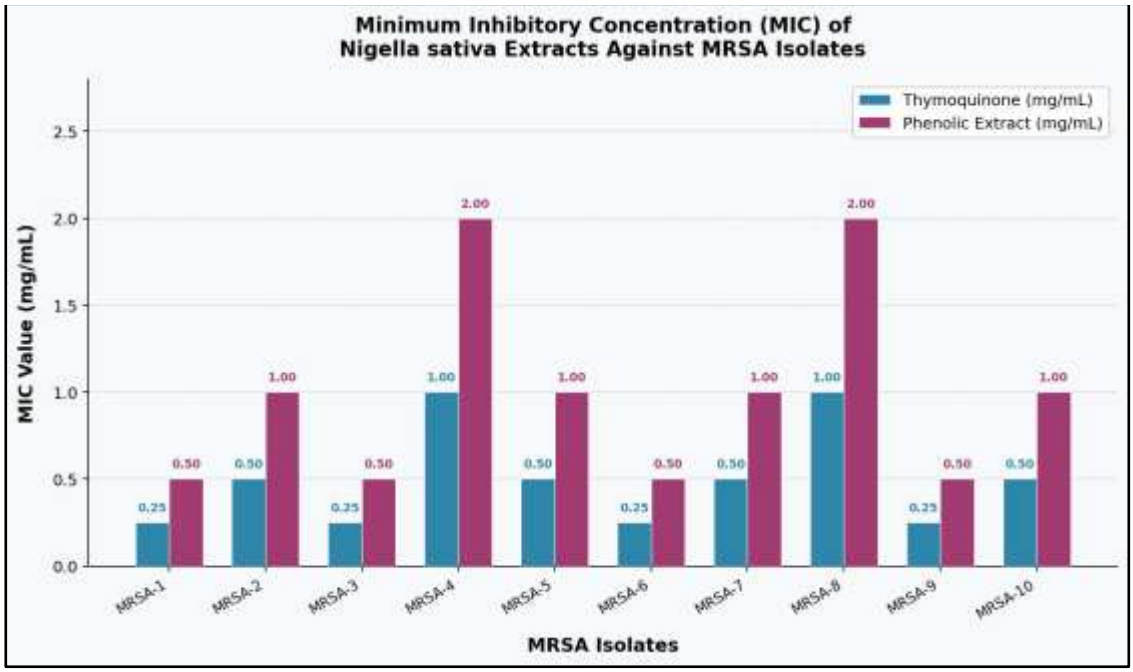

Figure (1): MIC values of the phenolic extract and pure thymoquinone against 10 selected MRSA Isolates.

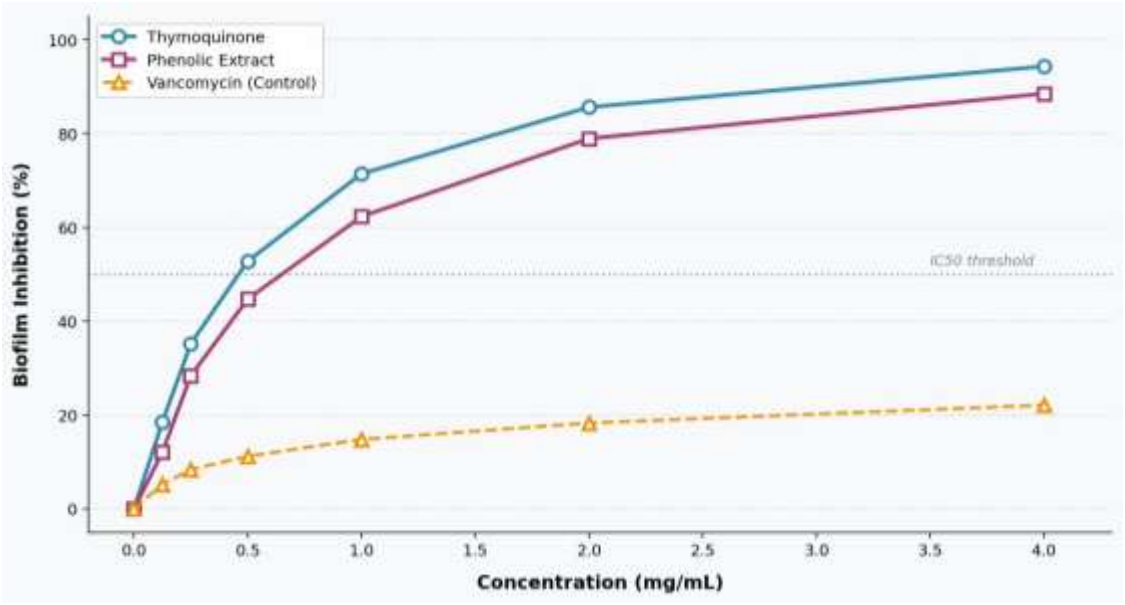

Figure (2): Biofilm inhibition curves of phenolic extract and thymoquinone compared to vancomycin.

ISSN 2072-3857

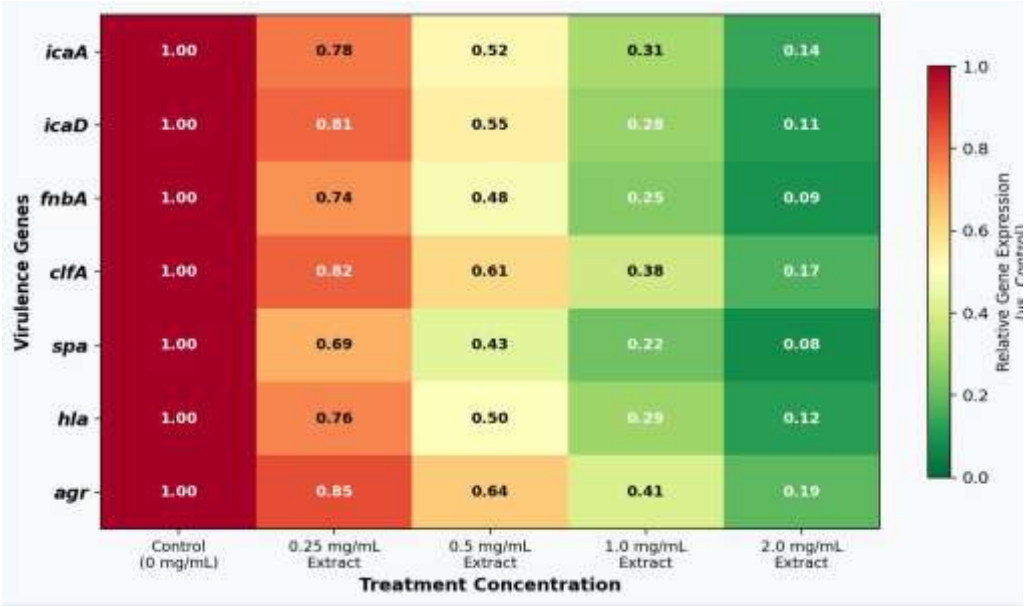

Figure (3): Heatmap of relative gene expression of virulence genes at increasing concentrations of the extract.

Figure (5): Comparative microscopic images showing the dense biofilm in the control group (left) versus the disintegration in the treatment (right)

Conclusion

The phenolic extracts of *Nigella sativa* possess a strong inhibitory activity against MRSA with MIC values (0.5-2.0 mg/ml). The efficacy is characterized by its dose-response nature in inhibiting the biofilm (88.5% at 4.0 mg/ml) and disrupting the mature one. The mechanism of action involves the regulation of the expression of the icaA, icaD, and spa genes, as well as the agr system, in a significant manner.

Acknowledgment: The authors would like to thank the Northern Technical University and Kirkuk General Hospital for their support and cooperation in conducting this study.

Funding: No external funding was received for this research.

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