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Thymoquinone, a potential candidate to treat biofilm-producing methicillin-resistant *Staphylococcus aureus* (MRSA) infections

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Abstract

Multidrug resistance is a leading public health challenge that is causing a significant increase in mortality and morbidity. If antimicrobial resistance (AMR) remains unsolved, it may cause 10 million deaths every year. Along with a public health concern, it is also a financial concern that would cause 2-3.5% reduction in Gross Domestic Product (GDP) and a 100 trillion USD loss to the world. One of the ways to combat AMR is to discover new antibiotics. This study was aimed to evaluate the antibiofilm and antibacterial potencies of essential oil of *Nigella sativa*. Standard microbiological guidelines (CLSI) were used for the identification and antibiogram of selected strains of MRSA. Moreover, a time-kill assay of MRSA against Thymoquinone extracted from *Nigella Sativa* was also performed. Five strains, including four MRSA strains from implants related infections and one standard strain ATCC 25923, were examined. GC-MS identified components of essential oil of Nigella Sativa. Thymoquinone and p-cymene, major compounds of essential oil, were subjected to antibacterial and antibiofilm activities. Thymoquinone revealed strong inhibitory activities against MRSA strains. Zone inhibition measured 22 to 44 mm, and MIC values ranged from 26 to 43 Ul/mL. Thymoquinone also exhibited strong antibiofilm activity against biofilm producer MDR strains of *Staphylococcus aureus.*

1. Introduction

Difficult to treat infections include implant-related infections, and *Staphylococcus aureus* is the major contributor to these chronic infections. Especially those having the potential to form a biofilm of medical devices. The rapid emergence of antibiotic resistance is making the situation more complicated. Resistance to antibacterial drugs diverted research into new antibiotics, especially plant-derived compounds. Significantly decreased toxicity, the diverse application against a variety of pathogens, and cost efficiency make medical plants an ideal candidate for antimicrobial agents **(Chouhan, Sharma *et al.* 2017)**. Essential oils of medicinal plants are proved to have excellent antibacterial activity and antioxidant function **(Memarzadeh, Gholami *et al.* 2020)**. Cells attributed to biotic and abiotic surfaces and exceptional resistance to antibiotics are aggregated as a Biofilm. Rigorous treatment strategies are required to successfully remove the pathogens from prosthetic devices implanted in the human body. Some plants and essential oils against *Staphylococcus aureus* are recently reported to remove biofilm effectively. Some studies have shown that combining essential oils and antibiotics to eliminate *S. aureus* is very successful **(Memarzadeh, Gholami *et al.* 2020)**.
Nigella sativa has reported efficacy against infectious diseases worldwide for 2000 years, especially in Asia and middle east regions **(Ahmad, Ahmad *et al.* 2021)** . Infection causing gram-positive and gram-negative bacteria have been successfully treated with *Nigella sativa* throughout the world **(El-Fatatry 1975, Chaieb, Kouidhi *et al.* 2011, Ahmad, Ahmad *et al.* 2021)**. The current study is designed to investigate the antibacterial and antibiofilm potential of Thymoquinone against MRSA clinical strains isolated from catheter-related infections.

2. Material and methods

2.1 Ethyl acetate extraction of *Nigella sativa*

Nigella sativa seeds were ground, soaked in ethyl acetate and oil was obtained after the evaporation of ethyl acetate in the rotary vacuum evaporator **(Sarwar and Latif 2015)**. Oil fractioning was done by GC-MS. Major constituents of the essential oil of *Nigella sativa* *p*-Cymene (9%) and Thymoquinone (8%) were subjected to evaluation.

2.2 Selection of Bacterial Strains

Four Methicillin-resistant and biofilm-forming Staphylococcus aureus (MRSA) isolated from prosthetic device mediated infections. It was characterized microbiologically, biochemically and phylogenetically **(Sohail and Latif 2018)** and a reference strain (ATCC® 25923™) was purchased from the American Type

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Culture Collection, Virginia USA, were selected for antibacterial and antibiofilm studies.

2.3 Antibacterial activity analysis

Antibacterial activity of essential oil of Nigella sativa and its fractions Thymoquinone (TQ) and p-cymene was measured against selected strains **(Mottaghiyan, Aghazadeh et al. 2019)**. The susceptibility was measured by the disc diffusion method as described by **Shunying et al. (2005) (Shunying, Yang et al. 2005)** with minor modifications. Briefly, 6 mm Whatman filter paper was impregnated with 10µL oil of Nigella sativa oil in six dilutions (100%, 80%, 50%, 40%, 30% 20% and 10%). Freshly prepared bacterial suspension of 108 CFU/mL was inoculated on MHA (Mueller Hinton Agar) plates, and discs containing Nigella sativa essential oil concentrations were placed on it. After 24 hours of incubation at 37C°, the inhibition zones were measured. Likewise, the antimicrobial activity of Thymoquinone and p-cymene was also determined. The antimicrobial activity demonstrated by p-cymene was not significant so, it was not considered for further studies. Thymoquinone inhibited MRSA at low concentration, and it is selected for further studies,

2.4 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) measurement

The minimum inhibitory concentration (MIC) of Thymoquinone (0 to 256 µg/mL) was measured by the broth microdilution procedure in 96-well round-bottom polystyrene plates, following the standard CLSI 2020 guidelines.
MRSA strains were grown on enriched broth media overnight. The overnight culture was diluted ten times in tryptic soy broth (TSB; Oxoid, UK) and incubated at 37C° for two to three hours as per their exponential growth period. TQ in Mueller Hinton (MH; Oxoid, UK) was serially diluted two folds, and 190µL was poured into 96-well plates wells. Standard inocula containing 5X106 CFU / mL of MRSA was prepared, and 10µl of this inocula was added to the microtiter plate along with Thymoquinone, except for the sterility and negative control wells. The bacterial concentration of 5X105 CFU / mL achieved by adding 10µL of resazurin (0.015%), which is an indicator of bacterial growth. On the shaking incubator, the microtiter plate was incubated at 37C°. Bacterial growth was evaluated visually by a change in colour from purple to pink and turbidity in the wells. Thymoquinone concentration that completely inhibited the bacteria growth was considered as MIC. Minimum bactericidal concentrating was determined by inoculating 10µL suspension from wells with no visible growth on MH agar and incubated at 37C° for 24 hours. All tests were performed in triplicate.

2.5 Antibiofilm activity

The antibiofilm potential of Thymoquinone was assayed on selected strains as described by **Nostro et al. (2007) (Nostro, Roccaro et al. 2007)**, using 1 MIC, 2 MIC and 4 MIC on 96 well polystyrene microtiter plates. The bacterial culture was grown overnight in MH broth with 2% glucose, and bacterial concentration was adjusted to 108 CFU/mL. 100µL bacterial suspension in MH broth with 2% glucose was added in a 96-well plate along with 100µL of Thymoquinone. The bacterial suspension was not added in sterility control, while the negative control well was without Thymoquinone. Plate incubated at 37C° for 24 hours, and suspension from each well was discarded carefully, followed by washing with sterile PBS three times. The plate was air-dried and stained by adding 200µL of 4% crystal violet stain for 4 minutes, followed by washing with water. 200 µL of ethanol (95%) was added to stained biofilm for one hour, and OD (Optical Density) was measured using a microplate reader (Rayto, RT-6100). Biofilm inhibition potential (%) of Thymoquinone was assayed by [(OD growth control) – (OD sample) / (OD of growth control)] × 100. Each experiment was repeated three times, and values were taken as average.

2.6 Statistical analysis

For statistical analysis, SPSS version 20 was used. ANOVA and Pearson simple tests were conducted, and results were recorded as linear correlation coefficient (r) and significance level (p).

3. Results

3.1 Antibiogram

Four selected strains of MRSA named ZS35, ZS41, ZS46, and ZS47 were multidrug-resistant. ZS35 and ZS47 were 72% resistant to the tested antibiotics, while ZS41 and ZS46 demonstrated 62% resistance described previously **(Sohail and Latif 2017)**. The antibiogram of these selected strains showed in Table 1.

3.2 Antimicrobial activity of Essential oil of Nigella sativa

The essential oil of Nigella sativa (Dilutions 100%, 80%, 50%) demonstrated good antibacterial activity against selected strains (Figure 1), and these were subjected to HPLC and GC-MS for fractioning. Multiple compounds were isolated with GC-MS; Thymoquinone and cymene were prominent and selected for further analysis. There was no antibacterial activity in cymene, so only Thymoquinone (274666, Sigma-Aldrich) was chosen for further investigations.

3.3 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

Microdilution assay for MIC determination revealed that MRSA is extremely sensitive to the Thymoquinone with MIC values from 8 to 64 µg/mL. Similarly, MBC values were 2 to 4 times higher than MIC values, respectively (Table 2).

3.4 Anti-Biofilm Activity

Crystal violet assay was used to assess the potential of Thymoquinone to inhibit the biofilm formation of selected strains of MRSA isolated from prosthetic device-related infections and control strains. The effect of Thymoquinone on biofilm development was expressed as a percentage. Thymoquinone significantly inhibited biofilm development depending upon the challenged dose and resistance profile of tested pathogens. The biofilm formation potential of ATCC 25923 was inhibited significantly by Thymoquinone, depending upon the exposed dosage. Results illustrated that Thymoquinone inhibited 85% biofilm formation of ZS35 and ZS47 at a concentration of 4 times MIC against the respective planktonic form of these strains. This was not effective against one strain of MRSA (ZS46), where it inhibited 65% biofilm at the maximum dose used (Figure 2).

Table 1 Antibiogram of selected MRSA strains

	AK (30µg)	CN (10µg)	TOB (10µg)	AZ (30µg)	DO (30µg)	CIP (5µg)	OFL (5µg)	SXT (1.25/23.75µg)	DA (30µg)	LZD (30µg)	C (30µg)
ZS35	R	R	R	R	S	R	R	R	R	S	S
ZS41	R	R	R	R	S	R	R	R	S	S	R
ZS46	S	R	R	R	R	R	R	R	R	S	S
ZS47	R	R	R	R	R	R	R	S	R	S	R

AK(Amikacin), CN(Gentamicin), TOB(Tobramycin), AZ(Azithromycin), DO(Doxycycline), CIP(Ciprofloxacin), OFL(Ofloxacin), SXT(Trime/Sulphamethoxazole), DA(Clindamycin), LZD (Linezolid), C (Chloramphenicol)

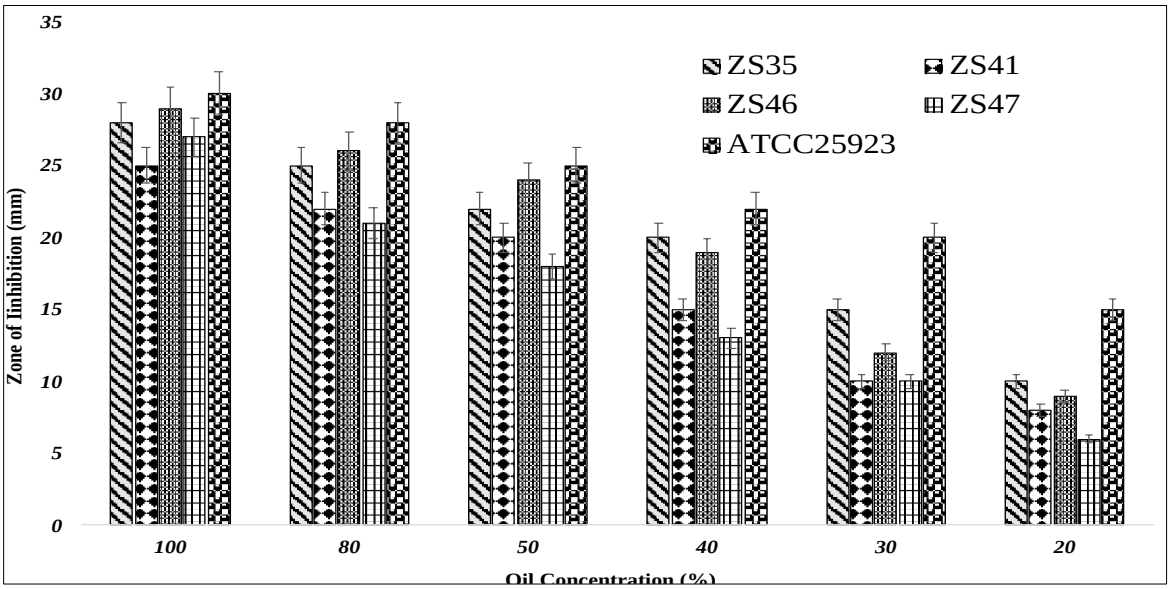

Figure 1 Inhibitory effect of Thymoquinone on MRSA by disc diffusion method

Table 2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of MRSA against Thymoquinone

	MRSA (ZS35)	MRSA (ZS41)	MRSA (ZS46)	MRSA (ZS47)	Control (ATCC25923)
MIC (mg/mL)	8	16	32	16	16
MBC (mg/mL)	16	32	64	32	32

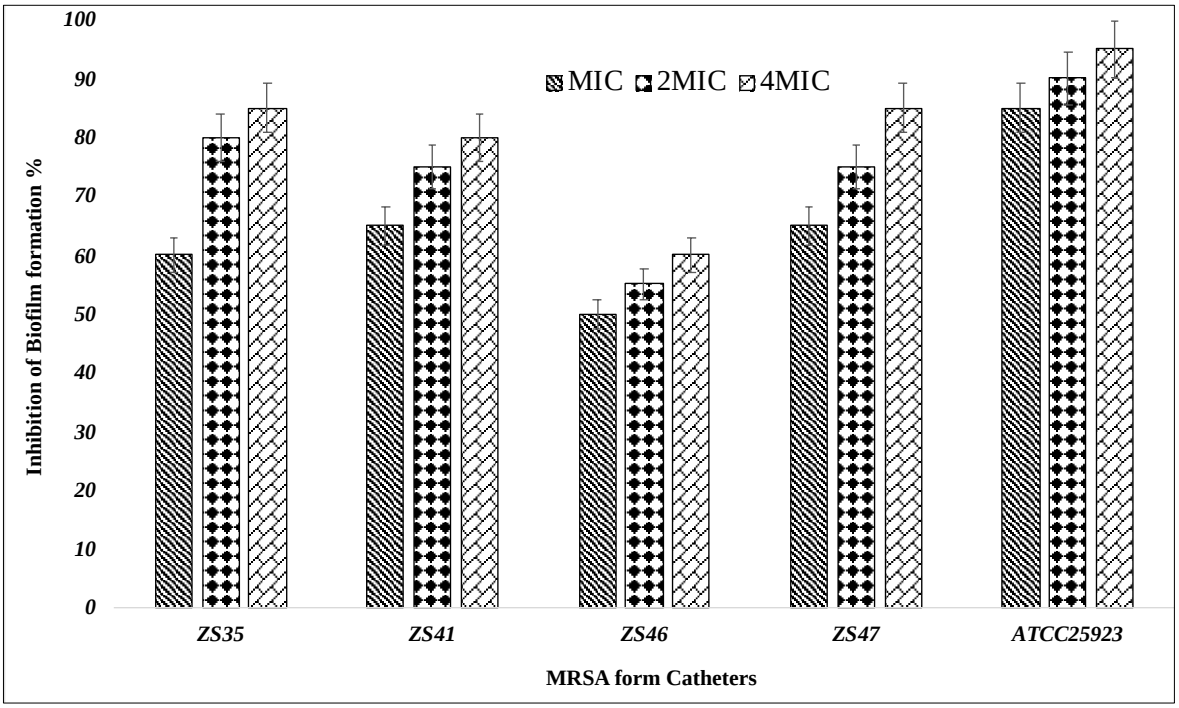

Figure 2 Percent (%) inhibition of biofilm synthesized by MRSA by usage sublethal concentrations of Thymoquinone

4. Discussion

Antimicrobial resistance (AMR) is a worldwide challenge that can enormously affect the world. MRSA medical devices mediated infections are among the leading infections which are becoming untreatable over time **(Lam, Panlilio et al. 2020)**. Continuous work is required in finding new drugs to combat the AMR challenge. Nigella sativa is a medicinal plant and thought to be involved in the cure of infections before discovering antibiotics **(Ahmad, Ahmad et al. 2020)**. There is a dire need to evaluate the antimicrobial potential of the bioactive compounds of the Nigella sativa against different multidrug-resistant bacteria.

Thymoquinone antimicrobial and antibiofilm activity was measured against MRSA isolated from infections associated with medical devices. Thymoquinone, a biologically active constituent of Nigella sativa, has exhibited marvelous antibacterial and antibiofilm activity against selected MRSA strains isolated from prosthetic device-related infections. This study indicated that Thymoquinone is a potential candidate for treating MRSA, which is responsible for difficult to treat infection; these results are in accordance with the previous study conducted on gram-positive bacteria **(Mottaghiyan, Aghazadeh et al. 2019)** The current study reported MIC of Thymoquinone ranging from 8 to 64 mg/mL, which is the same as reported earlier for various Multidrug resistance pathogens, especially gram-positive **(Intorasoot, Chornchoem et al. 2017, Sychrová, Kolářiková et al. 2020)**.

Biofilm bacteria are 1000 times more resistant to antibiotics than planktonic forms and very difficult to remove from biotic or abiotic surfaces **(Chaieb, Kouidhi et al. 2011, Asli, Brouillette et al. 2017)**. Thymoquinone was effective against MRSA's planktonic form and biofilm form, with a two to four times increase in minimum inhibitory concentration. Thymoquinone at a level of MIC eradicated 85% biofilm synthesized by control strain. TQ possessed a statistically significant inhibition ($p<0.05$) of the potential for biofilm formation of selected MRSA strains isolated from infections linked to prosthetic devices. MRSA (ZS35) was inhibited at 60% when MIC concentration of Thymoquinone was applied against it, and Thymoquinone inhibited 80% and 85% biofilm synthesis capacity when 2MIC and 4MIC concentration are applied, respectively. A similar pattern was demonstrated by other MRSA strains like ZS41 and ZS47.

On the other hand, ZS46 having comparatively high MIC and MBC, like 32 and 64 mg/mL, respectively, demonstrated 50% biofilm inhibition at MIC concentration. 2MIC and 4MIC inhibited 55% and 60% biofilm formation potential of ZS46. The other significant fraction of Nigella sativa oil p-cymene did not show satisfactory antimicrobial activity.

AMR is a leading threat to the public health system, which should be addressed in multiple ways; one is to find new antibiotics sources. Thymoquinone is a good candidate against biofilm-producing MRSA, this should be evaluated against other bacteria, and its activity against these infections in vivo would be a good addition.

4. Conclusion

This study concluded that the active component of Nigella sativa (Thymoquinone) inhibited the planktonic form of MRSA and reduced biofilm formation potential, which indicates an efficient biofilm control agent on biotic and abiotic surfaces. The mechanism of action of Thymoquinone needs to be evaluated.

Declaration of interest

There is no conflict of interest regarding this study.

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