

# Synergistic efficacies of thymoquinone and standard antibiotics against multi-drug resistant isolates

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## ABSTRACT

**الأهداف:** هذه الدراسة تستكشف النشاط المضاد للبكتيريا للثايموكينون وهو المستخلص من حبة البركة.

**المنهجية:** أجريت هذه الدراسة في الفترة من مايو 2019 إلى مارس 2020 في قسم علوم المختبرات الإكلينيكية، كلية العلوم الطبية التطبيقية، جامعة الملك خالد، أبها، المملكة العربية السعودية. تم تحديد النشاط المضاد للميكروبات، والتركيز المثبط الأدنى، والحد الأدنى من تركيز مبيد الجراثيم للثايموكينون باستخدام نشر الآجار ومقاييس التخفيف الدقيق، وتم تقسيم التأثيرات باستخدام المضادات الحيوية بالتوازي. تم تحديد التأثير التثبيتي للثايموكينون على أغشية الخلايا البكتيرية باستخدام المجهر الإلكتروني. كما تم فحص الخصائص المضادة للفوعة باستخدام الثايموكينون والتي تشمل الالتصاق وتكوين الأغشية الحيوية باستخدام فحوصات الالتصاق وتكوين الأغشية الحيوية على التوالي.

**النتائج:** أظهر الثايموكينون فعالية مبيد للجراثيم ضد السلالات البكتيرية 4/14 مع نطاقات التركيز المثبط الأدنى والحد الأدنى من تركيز مبيد الجراثيم من 1.04–8.3 ميكروغرام/مل و10.41–66.66 ميكروغرام/مل. كما أظهر الثايموكينون تآزراً مع الكلينيسية الرئوية، المكورة العنقودية الذهبية، والعنقودية بَشْرَوِيَّة والعنقودية الذهبية بالاشتراك مع المضادات الحيوية المختبرة. الثايموكينون يثبط الالتصاق البكتيري بنسبة 39%–54%، 48%–68%، و61%–81% عند 0.5، 1، 2 من التركيز المثبط الأدنى على التوالي. السلالات البكتيرية المختبرة تثبط بشكل كبير تكوين البايوفيلم بعد المخالطة بتركيزات مختلفة من الثايموكينون لمدة 24 و48 ساعة.

**الخلاصة:** يجب مراعاة التأثير التوليفي للثايموكينون مع مضادات الميكروبات عند تطوير نظم علاج جديدة بمضادات الميكروبات للتغلب على عزلات مقاومة الأدوية المتعددة.

**Objectives:** To explore the antibacterial activity of thymoquinone (TQ), a quinone extracted from *Nigella sativa*.

**Methods:** This study was conducted from May 2019 to March 2020 at the Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, Abha, Saudi Arabia. The antimicrobial activity, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) of TQ were determined using an agar well diffusion method and broth microdilution assays, and the synergistic effect was evaluated using antibiotics in parallel. The disruptive effect of TQ on bacterial cell membranes was determined using scanning electron microscopy. The antivirulence

properties of TQ, which include adherence and biofilm formation, were also investigated using adherence and biofilm formation assays, respectively.

**Results:** Thymoquinone demonstrated bactericidal efficacy against 4/14 bacterial strains, with MIC range of 1.04–8.3 µg/mL and MBC range of 10.41–66.66 µg/mL. Thymoquinone showed synergism against *Klebsiella pneumoniae*, *Staphylococcus epidermidis* (American Type Culture Collection 12228), *Staphylococcus aureus*, and *Staphylococcus epidermidis* in combination with the tested antibiotics. Thymoquinone inhibited bacterial adhesion by 39%–54%, 48%–68%, and 61%–81% at 0.5 × MIC, 1 × MIC, and 2 × MIC, respectively. The tested bacterial strains significantly inhibited biofilm formation after treatment with various concentrations of TQ for 24 and 48 hours.

**Conclusion:** The combinatory effect of TQ with antimicrobials should be considered when developing new antimicrobial therapy regimens to overcome multidrug-resistant.

**Keywords:** thymoquinone, antibacterial, adhesion, biofilm formation, synergism

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Antibiotic resistance has become a major health issue over the past decade due to the rapid growth of multidrug-resistant (MDR) microbes.<sup>1</sup> Antimicrobial drugs are effective in bacterial infection control. However, microbes have the ability to develop resistance to several active antimicrobials.<sup>2</sup> Due to the emergence of multidrug resistance and the insufficiency of new antimicrobials, novel and innovative approaches to combat MDR bacteria are crucial.<sup>3,4</sup> However, bacteria efficiently develop resistance to antimicrobials soon after they have been introduced, and the majority of antibiotics have side effects.<sup>5</sup> Therefore, searches for new drugs or combinations with less toxicity and uncompromised efficacy against resistant strains are continually evolving.<sup>6,7</sup> Nonetheless, the issue of bacterial resistance to antibiotics was resolved by the uncovering of new antibiotics, like glycopeptides, aminoglycosides, and macrolides, as well as by chemically modifying existing medicines.<sup>1</sup>

A potential strategy to minimize microbial resistance is the administration of antimicrobials in combination with naturally available compounds.<sup>1,8</sup> Phytochemicals synergistically enhance the effect of traditional antimicrobials.<sup>9</sup> Thymoquinone (TQ; 2-isopropyl-5-methyl-1,4-benzoquinone) is an essential active compound of *Nigella sativa* (*N. sativa*) L. seeds (black seed), which have been traditionally used for decades in the Middle East, Northern Africa, and India as a natural remedy for many contagious diseases such as bronchitis, pulmonary infection, cough, influenza, and fever.<sup>10-11</sup> Thymoquinone exerts various pharmacological effects and has antiinflammatory, anticancer, antidiabetic, antiasthmatic, hypolipidemic, antihypertensive, nephroprotective, and antimicrobial activities.<sup>12-16</sup>

The antibacterial effects of TQ and other *N. sativa* extracts have been determined against *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Listeria monocytogenes*, and *Escherichia coli* (*E. coli*).<sup>17-19</sup> Thymoquinone exerts inhibitory effects against gram-negative and gram-positive bacteria.<sup>20</sup> A diethyl ether extract of *N. sativa* showed synergy with streptomycin and gentamicin and an additive effect with erythromycin, streptomycin, doxycycline, tobramycin, nalidixic acid, chloramphenicol, ampicillin, cotrimoxazole, and lincomycin.<sup>21</sup> Furthermore, both

antibiotic-sensitive and MDR gram-positive and gram-negative bacterial isolates are susceptible to *N. sativa* extracts.<sup>22</sup> This study aimed to further elucidate the potential effect of TQ against clinically significant bacteria by investigating the antibacterial effect of TQ alone and in combination with different antibiotics against drug-resistant isolates.

**Methods.** This is an experimental study which was conducted from May 2019 to March 2020 in the Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, Abha, Saudi Arabia.

**Organisms and chemicals.** The antibacterial effect of TQ was examined using pathogenic strains, including the gram-negative bacteria *P. aeruginosa* (American Type Culture Collection [ATCC] 27853), *E. coli* (ATCC 25922), *E. coli*, *Salmonella typhi*, *Pseudomonas sp.*, *Klebsiella pneumoniae* (*K. pneumoniae*), and *Shigella sp.*, and the gram-positive bacteria *Enterococcus faecalis* (ATCC 29212), *S. epidermidis* (ATCC 12228), *S. aureus*, *S. saprophyticus*, *S. epidermidis*, *Streptococcus pyogenes*, and *Mycobacterium smegmatis*. Gentamycin, ofloxacin, tetracycline, penicillin, chloramphenicol, nalidixic acid, and TQ were purchased from Sigma (Sigma-Aldrich, Switzerland).

**Antimicrobial susceptibility assays.** The antimicrobial activity of the compounds was evaluated using an agar well diffusion method. The bacterial culture was inoculated in lysogeny broth and incubated at 37°C for 3 h (hours). The turbidity of the inoculum was consequently adjusted using phosphate-buffered saline (PBS) to be equivalent to 0.5 McFarland's standard. Then, 20 µL of TQ (50 µg/mL) was transferred into each well of the Petri dishes, which were then incubated aerobically at 37°C for 24 hours (h). The width of the zone of inhibition of bacterial growth surrounding each well was calculated in millimeters, as previously described.<sup>23</sup>

**Determination of minimum inhibitory concentration (MIC).** The MICs of TQ against the bacterial strains were determined by broth microdilution assays using TQ concentrations of 0.024-50 µg/mL. Two-fold dilutions of TQ (100 µL) were pipetted into the wells of a sterile flat-bottomed 96-well plate for each strain under test. The initial inoculum concentration for each strain was  $1.5 \times 10^5$  colony-forming units/mL. The control wells contained the bacterial inoculum only. The plate was incubated aerobically 37°C for 24 h. The lowest TQ concentration that exhibited neither turbidity nor visible bacterial growth after the 24-h incubation was considered the MIC.

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**Determination of minimum bactericidal concentration (MBC).** After the 24-h incubation, 100  $\mu$ L of inoculum from each well of the broth microdilution assay plate was sub-cultured on Mueller–Hinton agar (MHA) plates, which were then incubated for 24 h. The minimum concentration of TQ that resulted in no bacterial growth was considered the MBC.

**Synergistic antimicrobial assays.** The synergistic antimicrobial activity of antibiotics (gentamycin, ofloxacin, tetracycline, penicillin, chloramphenicol, and nalidixic acid) in combination with TQ was investigated. Bacterial strains at a turbidity of 0.5 McFarland's standard were spread on MHA plates. The specific antibiotic discs, which had been kept aerobically at 37°C for 24 h, were separately soaked in 5  $\mu$ L of TQ (at the MBC value) and placed on the inoculated MHA plates. Then, the plates were incubated aerobically at 37°C for 24 h. The inhibition zones formed by TQ in combination with the selected antibiotics after overnight incubation were calculated, as previously described.<sup>24</sup>

**Scanning electron microscopy (SEM).** Scanning electron microscopy was used to investigate the effect of TQ on cell morphology.<sup>25</sup> Scanning electron microscopy sample preparation was initially carried out in centrifuge tubes. Then, the bacterial specimens were instantly fixed in 2.5% (wt/vol) phosphate-buffered glutaraldehyde (pH 7.4) at 4°C for 4 h, followed by post-fixation in 1% phosphate-buffered osmium tetroxide (pH 7.4) for 1 h. After washing and dehydration in ascending grades of ethanol, critical-point drying was performed using an EMitech K850 critical-point dryer. The samples were mounted on aluminum mounts with silver glue and then sputtered with a gold coat using a BOC Edwards Scancoat sputter coater. The specimens were examined using a JSM-6390LV scanning electron microscope (Jeol Ltd., Japan).<sup>26</sup>

**Adherence assay.** Bacterial adherence was assessed as described elsewhere.<sup>27</sup> Briefly, 100  $\mu$ L of the bacterial cells under test (optical density at 610 nm [ $OD_{610\text{ nm}}$ ]=0.01) grown in Roswell Park Memorial Institute (RPMI), New York, United States in America (USA) 1640 media and buffered with 0.165 M morpholinepropanesulphonic acid (MOPS) (Sigma-Aldrich, MO, USA) at pH 7.0 were loaded into all wells of a 96-well plate. Then, the bacterial cells were treated with different concentrations of TQ (0.5  $\times$  MIC, 1  $\times$  MIC, and 2  $\times$  MIC) and incubated for 6 h at 37°C. Untreated bacterial cells were used in each set of experiments as negative controls. Following incubation, the media were discarded, and each well was rinsed twice with 200  $\mu$ L PBS to eliminate the non-adherent bacterial cells. Then, 100  $\mu$ L of Alamar blue at an absolute concentration of

5% in RPMI 1640 media was loaded into each well, followed by incubation at 37°C for 6 h. The amount of fluorescence was measured at 555 and 585 nm for excitation and emission, respectively, using a Synergy HT microplate reader (BioTek Instruments, VT, USA).

**Biofilm formation assay.** Biofilm production was assessed by the inoculating the tested bacterial cells suspensions ( $OD_{610\text{ nm}}=0.01$ ) grown in RPMI 1640 media buffered with 0.165 M MOPS (pH 7.0) into the wells of a sterile flat-bottomed 96-well plate, and the plate was incubated for 6 h at 37°C.<sup>28</sup> Thereafter, the culture medium was carefully removed without disturbing the formation of biofilm. Then, different TQ concentrations (0.5  $\times$  MIC, 1  $\times$  MIC, and 2  $\times$  MIC) were prepared in fresh RPMI 1640 medium and loaded into the wells. Untreated bacterial cells were used as negative controls in each set of experiments. The 96-well plate was further incubated at 37°C, and the effect of TQ on biofilm formation was assessed after 24 h and 48 h, as previously described.<sup>29</sup>

**Statistical analysis.** Each investigation was conducted in triplicate, and the results were presented as means  $\pm$  standard deviation (SD). GraphPad Prism software v6.0 (GraphPad, CA, USA) was used to perform the statistical analyses. One-way analysis of variance with Dunnett's multiple comparison test was used to compare the mean between different groups with that of the control. *P*-values <0.05 were considered statistically significant.

**Results. Antibacterial activity of TQ.** Thymoquinone exerted a bactericidal effect on 4/14 strains, with MICs of 1.04–8.3  $\mu$ g/mL and MBCs of 10.41–66.66  $\mu$ g/mL (Table 1 & Figure 1). This effect was almost indistinguishable from the tested antibiotics (ofloxacin, gentamycin, tetracycline, penicillin, chloramphenicol, and nalidixic acid). The gram-negative bacteria *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Pseudomonas sp.*, *Salmonella typhi*, *Shigella sp.*, and *E. coli* were resistant to TQ. However, for gram-positive bacteria, only *Enterococcus faecalis* ATCC 29212, *M. smegmatis*, *S. saprophyticus*, and *S. pyogenes* appeared resistant to TQ.

**Synergism action.** For gram-positive bacteria, TQ showed synergism in combination with gentamicin and penicillin against *S. epidermidis* ATCC 12228; with ofloxacin, tetracycline, and chloramphenicol against *S. aureus*; and with ofloxacin and penicillin against *S. epidermidis*. For gram-negative bacteria, TQ showed synergism with gentamicin, ofloxacin, penicillin, and nalidixic acid against *K. pneumoniae* (Table 2).

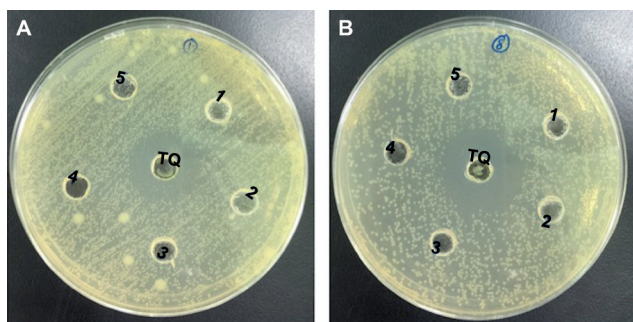
**Antagonism action.** For gram-positive bacteria, TQ showed antagonism against *S. epidermidis* ATCC



**Table 1** - Antibacterial activity of thymoquinone against pathogenic bacterial strains.

Organism	Origin	Thymoquinone		
		Zone of inhibition (50 µg/ml)	µg/ml	
			mm Mean (SD)	MIC Mean (SD)
<b>Gram negative bacteria</b>				
<i>Pseudomonas aeruginosa</i> (ATCC 27853)	ATCC	-	-	-
<i>Escherichia coli</i> (ATCC 25922)	ATCC	-	-	-
<i>Pseudomonas</i>	Clinical	-	-	-
<i>Salmonella typhi</i>	Clinical	-	-	-
<i>Klebsiella pneumoniae</i>	Clinical	15 (2)	1.30 (0.45)	33.33 (14.43)
<i>Shigella</i>	Clinical	-	-	-
<i>Escherichia coli</i>	Clinical	-	-	-
<b>Gram positive bacteria</b>				
<i>Enterococcus faecalis</i> (ATCC 29212)	ATCC	-	-	-
<i>Staphylococcus epidermidis</i> (ATCC 12228)	ATCC	17 (1.73)	1.04 (0.45)	10.41 (3.60)
<i>Mycobacterium smegmatis</i>	Clinical	-	-	-
<i>Staphylococcus aureus</i>	Clinical	15 (1)	2.60 (0.90)	41 (14.43)
<i>Staphylococcus saprophyticus</i>	Clinical	-	-	-
<i>Staphylococcus epidermidis</i>	Clinical	13 (1.73)	8.3 (3.60)	66.66 (28.86)
<i>Streptococcus pyogenes</i>	Clinical	-	-	-

-: no effect, MIC: minimum inhibitory concentration, MBC: minimum bactericidal concentration,  
ATCC: American Type Culture Collection

**Figure 1** - Antibacterial activity of TQ. A) Antibacterial activity of thymoquinone (TQ) against *Klebsiella pneumoniae*. B) Antibacterial activity of TQ against *Staphylococcus epidermidis* (ATCC 12228).

12228 when combined with ofloxacin, tetracycline, and chloramphenicol; *S. aureus* with penicillin; and *S. epidermidis* with tetracycline and chloramphenicol. However, for gram-negative bacteria, TQ antagonism was not detected against *K. pneumoniae* in combination with any of the tested antibiotics (Table 2).

**Additive action.** For gram-positive bacteria, TQ showed additive action with nalidixic acid against *S. epidermidis* ATCC 12228 and with gentamicin and nalidixic acid against *S. aureus* and *S. epidermidis*.

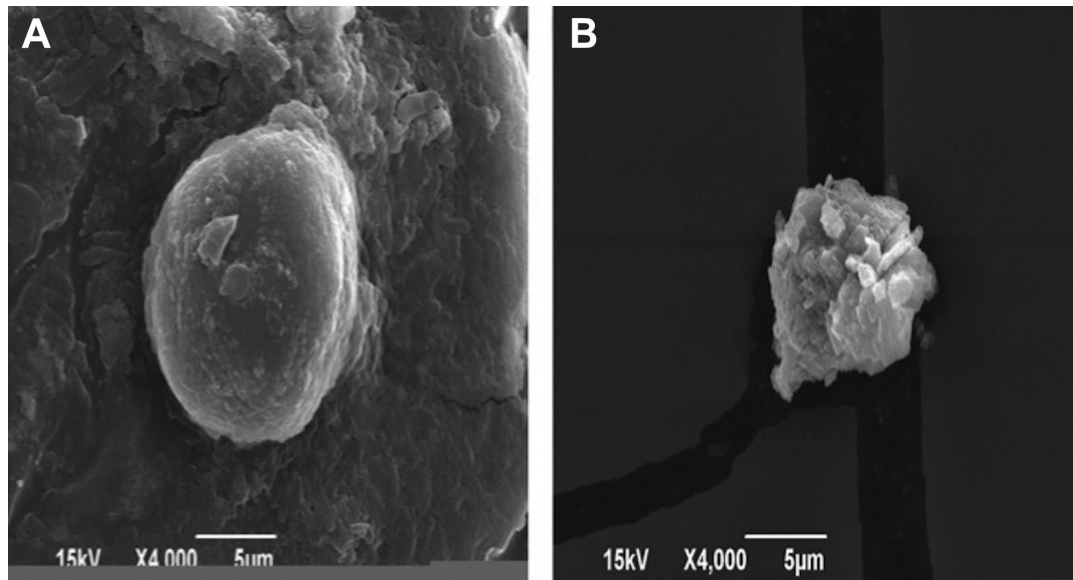
For gram-negative bacteria, TQ showed additive action with tetracycline and chloramphenicol against *K. pneumoniae*. Most of the results are same during each trial and there were no any significant difference observed with  $\alpha=0.05$ .

**Disruption of bacterial cells visualized by SEM.** Scanning electron microscopy was used to observed changes in the structural morphology of *S. epidermidis* in response to TQ (Figure 2). In the absence of TQ (control), the cells showed characteristic normal morphology (Figure 2A). Unlike the control, the cells that were treated with TQ had damaged cell membranes, showing that TQ treatment reduced the synthesis of extracellular polysaccharides (Figure 2B).

**Thymoquinone inhibits bacterial adhesion.** Adhesion assays were performed using Alamar blue dye to assess the effect of TQ on bacterial adherence. The bacterial cells showed inconsistent inhibition of adherence following exposure to various TQ concentrations, and this was concentration-dependent. For *K. pneumoniae*, *S. epidermidis* (ATCC 12228), *S. aureus*, and *S. epidermidis*, TQ inhibited bacterial adhesion by 39%-54% at  $0.5 \times$  MIC, 48%-68% at  $1 \times$  MIC, and 61%-81% at  $2 \times$  MIC (Figure 3), indicating that TQ was extremely efficient in diminishing adhesion of the tested bacteria, even at subinhibitory concentrations.

**Table 2** - A synergistic antimicrobial activity of thymoquinone (TQ) with different antimicrobial agents.

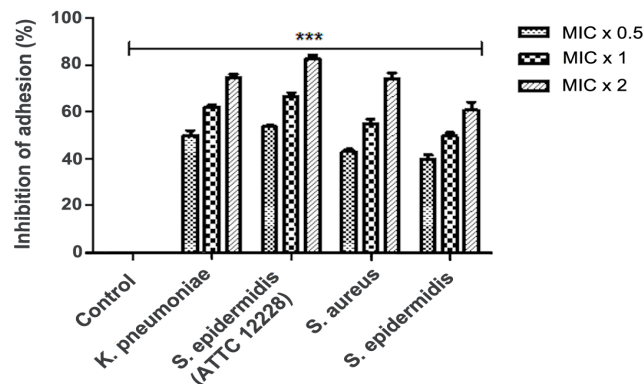
Bacteria	Antimicrobial agent	Mean of inhibition zone with antibiotics (mm) Mean (SD)	Mean of inhibition zone with Tq (mm) Mean (SD)	Inhibition zone with synergism Mean (SD)	Outcome	P-value
<i>Klebsiella pneumoniae</i>	Gentamicin	20 (1.73)	15 (2)	37 (3.60)	Synergism	0.458
	Ofloxacin	17 (1.0)		34 (1.73)	Synergism	0.638
	Tetracycline	0		15 (1)	Additive	≤ 0.001
	Penicillin	15 (2)		34 (2)	Synergism	0.485
	Chloramphenicol	0		15 (1.73)	Additive	≤ 0.001
	Nalidixic acid	3 (1)		20 (1)	Synergism	≤ 0.001
<i>Staphylococcus epidermidis</i> (ATCC 12228)	Gentamicin	18 (2)	17 (1.73)	36 (1)	Synergism	0.254
	Ofloxacin	25 (3)		34 (2)	Antagonism	1.258
	Tetracycline	0		15 (2)	Antagonism	0.458
	Penicillin	18 (1.73)		37 (3.6)	Synergism	0.471
	Chloramphenicol	25 (1)		36 (1)	Antagonism	0.689
	Nalidixic acid	0		17 (2.64)	Additive	≤ 0.001
<i>Staphylococcus aureus</i>	Gentamicin	0	15 (1)	15 (2)	Additive	≤ 0.001
	Ofloxacin	13 (01)		30 (1.73)	Synergism	0.356
	Tetracycline	4 (2)		21 (1)	Synergism	0.095
	Penicillin	16 (1.73)		27 (1.73)	Antagonism	0.245
	Chloramphenicol	18 (2)		35 (2)	Synergism	0.325
	Nalidixic acid	2 (1)		17 (1)	Additive	≤ 0.001
<i>Staphylococcus epidermidis</i>	Gentamicin	18 (1.73)	13 (1.73)	31 (3.6)	Additive	0.471
	Ofloxacin	17 (2.64)		32 (2)	Synergism	0.247
	Tetracycline	18 (1)		29 (1)	Antagonism	1.784
	Penicillin	3 (1)		21 (1)	Synergism	≤ 0.001
	Chloramphenicol	20 (2)		26 (1.73)	Antagonism	0.478
	Nalidixic acid	0		13 (2.64)	Additive	≤ 0.001

**Figure 2** - Effect of thymoquinone (TQ) on cell morphology of *Staphylococcus epidermidis*. Changes in the cellular morphology were observed by scanning electron microscopy. **A)** Control cell **B)** shows bacterial cell treated with TQ.

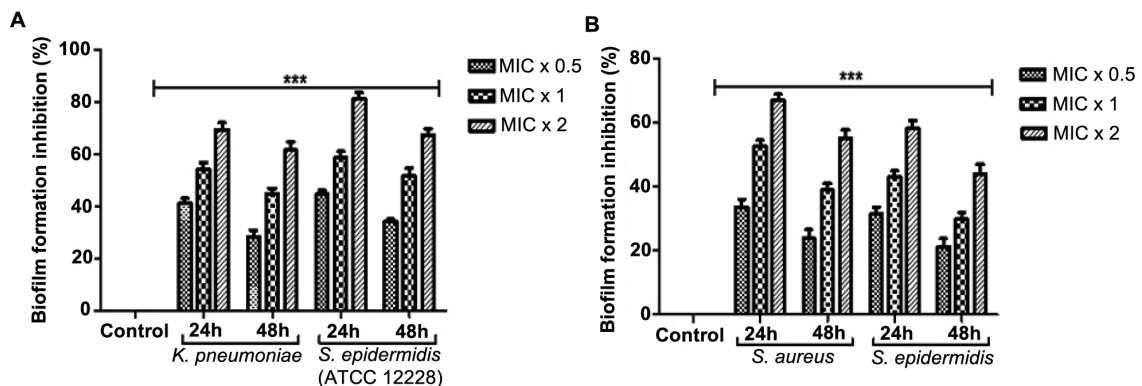
**Thymoquinone inhibits bacterial biofilm formation.** TQ treatment at 0.5 × MIC, 1 × MIC, and 2 × MIC significantly inhibited biofilm formation in *K. pneumoniae*, *S. epidermidis* (ATCC 12228), *S. aureus*, and *S. epidermidis* by 31%-45%, 41%-59%, and 58%-81%, respectively, after 24 h and by 21%-34%, 30%-51%, and 44%-67%, respectively, after 48 h (Figure 4). The rate of biofilm formation inhibition by TQ was concentration- and time-dependent.

**Discussion.** Thymoquinone is one of the most bioactive components of *N. sativa* and has diverse

beneficial properties. Regarding antimicrobial effects, TQ has a broad-spectrum efficacy encompassing gram-negative and gram-positive bacteria.<sup>27</sup> Thymoquinone also exhibits a synergistic effect with gentamicin and streptomycin and an additive effect with spectinomycin, doxycycline, tobramycin, erythromycin, chloramphenicol, ampicillin, lincomycin, nalidixic acid, and a combination of sulfamethoxazole and trimethoprim.<sup>21,27</sup> In our study, TQ exhibited synergism in combination with gentamicin ( $p=0.254$ ) and penicillin ( $p=0.471$ ) against gram-positive *S. epidermidis* (ATCC 12228). Other studies have reported a similar effect



**Figure 3 -** Thymoquinone (TQ) inhibits bacterial adhesion. Alamar Blue based polystyrene adhesion assay was used to evaluate the effect of TQ on *Klebsiella pneumoniae*, *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* and *Staphylococcus epidermidis* adherence. All the tested bacterial strains were exposed to MIC x 0.5, MIC x 1 and MIC x 2 values of TQ for 6 h at 37°C. Control bars indicate all untreated bacterial strains, accepted as 0% inhibition. Results are presented from three independent experiments using means ± SD \*\*\* $p<0.0001$ .



**Figure 4 -** Thymoquinone (TQ) reduces biofilm formation. A) *Klebsiella pneumoniae* and *Staphylococcus epidermidis* (ATCC 12228), B) *Staphylococcus aureus* and *Staphylococcus epidermidis* were incubated with MIC x 0.5, MIC x 1 and MIC x 2 values of TQ under biofilm growing conditions for 24 and 48 h. Control bars indicate all untreated bacterial strains, accepted as 0% inhibition. Results are presented from 3 independent experiments using means±SD \*\*\* $p<0.0001$ .

against *S. epidermidis* in combination with ofloxacin and penicillin. Furthermore, *S. epidermidis* strains were reported as resistant to more than one antibiotic, and *S. epidermidis* isolates were also more sensitive to ofloxacin and gentamycin. Combined with antibiotics, these natural extracts showed significant synergy, and the maximum synergistic effect was detected with cephalexin and erythromycin.<sup>30-32</sup>

Methicillin-resistant *S. aureus* is the most common pathogen encountered in clinical and laboratory settings.<sup>27</sup> Previous studies have suggested that TQ in combination with antibiotics, such as cephalexin, ampicillin, tetracycline, gentamicin, and chloramphenicol, may show synergistic activity against *S. aureus*.<sup>23</sup> Our observations agreed with the literature, as TQ showed a synergistic effect with ofloxacin, tetracycline, and chloramphenicol against *S. aureus*.<sup>7,27</sup> Furthermore, our observations are supported by a report of TQ increasing the anti-staphylococcal effect of penicillin, tetracycline, and oxacillin against resistant bacterial strains.<sup>33</sup>

The MIC values that we observed for TQ against *K. pneumoniae* were much higher than those in wild-type *E. coli*. Therefore, it is evident that *K. pneumoniae* is highly resistant to several antimicrobial agents. Multidrug-resistant efflux pumps are the most important mechanisms through which bacteria can resist antibiotics, most notably tetracyclines.<sup>33</sup> This pump expels multiple drugs and could play a role in multidrug resistance.<sup>34</sup> To the best of our knowledge, there is a lack of literature on TQ synergistic studies with antibiotics against *K. pneumoniae*. In our study, TQ was synergistic with gentamicin ( $p=0.458$ ), ofloxacin ( $p=0.638$ ), penicillin ( $p=0.485$ ), and nalidixic acid ( $p\leq 0.001$ ) against gram-negative *K. pneumoniae*. Thymoquinone has been reported to exert antibacterial activity against both gram-positive and gram-negative bacteria. Furthermore, MDR clinical strains of *Shigella flexneri*, *S. typhimurium*, *S. aureus*, and *S. enteritidis* were reported with TQ sensitivity similar to that of non-MDR strains, and the susceptibility of resistant bacterial isolates to TQ has been reported.<sup>35</sup> Clinical isolates of *S. aureus* are often resistant to nearly all of the frequently used antimicrobials, including macrolides, aminoglycosides, chloramphenicol, fluoroquinolones, and tetracycline.<sup>36</sup> Our study demonstrated that TQ exerts synergistic lethal effects against both gram-negative and gram-positive bacteria when combined with antibiotics. Therefore, TQ could be used as an antibacterial drug.

Efflux pumps are a bacterial defense mechanism that eliminates antibiotics from the cell. The action

of the efflux pumps often predisposes the progression of microorganisms toward being highly resistant to antimicrobials.<sup>37,38</sup> Some investigations have demonstrated the vital function of phytochemistry to explore the effectiveness of efflux pump inhibitors, particularly against gram-positive bacteria such as *S. epidermidis* and *S. aureus*.<sup>30</sup> It was also reported that TQ could inhibit efflux pumps, consequently enhancing the concentration of 4,6-diamidino-2-phenylindole in the cells.<sup>38</sup> Hence, we hypothesize that this potential mechanism could also play a role in the anti-staphylococcal activity of TQ-tetracycline in combination with antibiotics, which is consistent with our study results. In contrast, -lactams are generally responsible for staphylococcal resistance by producing modified penicillin-binding proteins (PBPs) with decreased binding properties to antimicrobial agents.<sup>39</sup> Therefore, it has been suggested that TQ may play a role in inhibiting PBPs responsible for staphylococcal resistance.<sup>34</sup> Previous findings demonstrated that TQ could be an effective compound in combination with other antimicrobials against many bacterial strains, especially *Staphylococcus sp.* However, further investigations are necessary to examine their practical application.<sup>40</sup>

The capability of bacteria to adhere and form biofilm is responsible for their virulence. Our study revealed that TQ inhibited bacterial adhesion and biofilm formation. Adhesion properties are directly related to biofilm formation and lead to plaque formation. Bacterial cells have specialized proteins (adhesins) that encode host cell surface glycoproteins, enabling bacteria to adhere to host cells.<sup>41</sup> We found that TQ significantly inhibited adherence by 39%-81% in a dose-dependent manner in *K. pneumoniae*, *S. epidermidis* (ATCC 12228), *S. aureus*, and *S. epidermidis*, and adherence to host tissue lead to biofilm formation in these strains. Biofilm is a vital virulence factor in periodontal bacteria. It enhances resistance to most conventional antibacterial drugs.<sup>42</sup> Furthermore, there are numerous reports that biofilms are more resistant than planktonic cells to existing antimicrobials.<sup>43</sup> In our study, TQ treatment significantly suppressed biofilm formation in a concentration-dependent manner by 31%-81% for 24 h and 21%-67% for 48 h. Our findings support the assumption that TQ targets membrane adhesion proteins to inhibit adherence to host cells, subsequently inhibiting biofilm formation.

In conclusion, our study demonstrates that TQ can enhance the antibacterial activity of some antimicrobials. As demonstrated in **Table 1**, TQ was found more efficient anti-bacterial activities particularly



against gram-positive bacteria. Although our findings provide new insights into possible mechanistic scenarios associated with the combinatory effect of TQ with antimicrobials. Further investigations are necessary to facilitate the development of novel therapeutic strategies using TQ against resistant microbes.

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