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Original research paper

Antibacterial and antibiofilm activities of naturally occurring naphthoquinones 2-hydroxy-1,4-naphthoquinone and 2-methoxy-1,4-naphthoquinone against *Escherichia coli*

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ABSTRACT

The aim of this study was to evaluate the antibacterial and antibiofilm potential of two naturally occurring naphthoquinones, 2-hydroxy-1,4-naphthoquinone (2-HNQ) and 2-methoxy-1,4-naphthoquinone (2-MNQ), against the Gram-negative bacterium *Escherichia coli* strain ATCC 25922. In the first step of the study, the minimum inhibitory concentrations (MICs) of 2-HNQ and 2-MNQ were determined using the microdilution method. Subsequently, possible mechanisms underlying the antibacterial activity of 2-HNQ and 2-MNQ against *E. coli* were investigated by assessing intracellular reactive oxygen species (ROS) production and membrane permeability. Finally, the impact of 2-HNQ and 2-MNQ on swarming motility and on pre- and post-biofilm formation of *E. coli* was evaluated. The MIC of 2-HNQ against *E. coli* was 500 μg $mL^{-1,}$ while that of 2-MNQ was 100 μg mL^{-1}. Both compounds increased intracellular ROS production and altered the membrane permeability of *E. coli*. Moreover, 2-HNQ and 2-MNQ reduced swarming motility and inhibited both pre- and post-biofilm formation. The results of this study indicate that both naphthoquinones possess antibacterial potential, with 2-MNQ showing greater potency against *E. coli*. Their antibacterial activity appears to involve ROS generation and disruption of membrane permeability. Importantly, both tested naphthoquinones also impaired *E. coli* motility and biofilm formation, suggesting potential applications in the treatment of infections caused by *E. coli*.

Keywords: natural compounds, antibacterial activity, swarming motility, biofilm formation, ROS production, membrane permeability

INTRODUCTION

Escherichia coli is a Gram-negative bacterium and a common human and animal pathogen (1, 2). Among other diseases, *E. coli* can cause diarrhoea, enteritis, urinary tract infections (UTIs), pneumonia, peritonitis and neonatal meningitis (1–3). The worldwide increase in multidrug-resistant *E. coli* strains makes the treatment of diseases caused by *E. coli*

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challenging and, consequently, a public health concern (3–5). Therefore, there is a need to explore alternative strategies to combat pathogenic bacteria.

Naphthoquinones (NQs) are natural compounds synthesised by certain plants, fungi, algae and bacteria, and are characterised by bicyclic structures with two carbonyl groups on one nucleus (6, 7). The most widely distributed and most extensively studied NQs are 1,4-NQs. Owing to their chemical structure, NQs are highly reactive and possess various properties and applications (7–9).

One of the simplest naturally occurring derivatives of 1,4-NQs is 2-hydroxy-1,4-naphthoquinone (2-HNQ; Fig. 1a). It is found in the leaves of the henna plant (*Lawsonia* spp.), as well as in the microscopic fungus *Quambalaria cyanescens*. It has been demonstrated that 2-HNQ exhibits various biological effects, including antifungal, antiviral, antibacterial and anticancer activities (10, 11). 2-Methoxy-1,4-naphthoquinone (2-MNQ; Fig. 1b) is another 1,4-NQ that is produced by plants from the genus *Impatiens* (*I. balsamina* L., *I. glandulifera* Royle) as well as by the plant *Swertia calycina* and the microorganism *Nocardiopsis alba*. Studies indicate that the biological activities of 2-MNQ include anti-inflammatory, antifungal, antibacterial and anticancer potentials (12, 13).

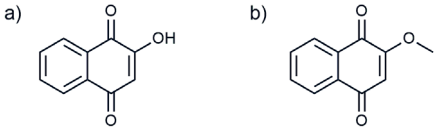

Fig. 1. Chemical structure of: a) 2-hydroxy-1,4-naphthoquinone (2-HNQ); b) 2-metoxy-1,4-naphthoquinone (2-MNQ).

Although studies indicate that NQs have antibacterial potential, information on the antibacterial and antibiofilm effects of the naturally occurring NQs, 2-HNQ and 2-MNQ, against *E. coli* are missing. Therefore, the aim of this study was to test the possible antibacterial potential and mechanisms of antibacterial activity of 2-HNQ and 2-MNQ against *E. coli*, as well as to evaluate their impact on swarming motility and biofilm formation by *E. coli*.

EXPERIMENTAL

Bacteria, culture conditions and tested compounds

The reference strain *Escherichia coli* ATCC 25922, obtained from the microbial collection of the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, was used as the model organism in this study. The strain was routinely cultured on Mueller-Hinton agar (MHA; Merck, Germany) at 37 °C under aerobic conditions.

Stock solutions of 2-HNQ (Sigma-Aldrich, USA) at a concentration of 200 mg mL^{-1} and 2-MNQ (Sigma-Aldrich) at a concentration of 20 mg mL^{-1} were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich). For experimental use, the stock solutions were diluted at least 100 × with Mueller-Hinton broth (MHB), resulting in a final DMSO concentration below 1 % in all treatment solutions.

Minimum inhibitory concentration assay

The minimum inhibitory concentrations (MICs) of 2-HNQ and 2-MNQ were determined using the microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (14). An overnight culture of *E. coli* was diluted in MHB to a final concentration of 5×10^5 CFU mL^{-1}. Subsequently, 100 μL of naphthoquinone solutions prepared in MHB were dispensed into the wells of a 96-well round-bottom microtiter plate, providing final concentration ranges of 2000–200 μg mL^{-1} for 2-HNQ and 200–20 μg mL^{-1} for 2-MNQ. Thereafter, 100 μL of the bacterial suspension was added to each well. Wells containing only the bacterial suspension and wells containing only MHB were included. The microtiter plates were incubated at 37 °C for 24 h. Following incubation, 20 μL of 2,3,5-triphenyltetrazolium chloride (TTC, 50 μg mL^{-1}) was added to each well, and the plates were further incubated for 1 h at 37 °C. The MIC was defined as the lowest concentration of the tested compound that completely inhibited visible bacterial growth. Each concentration was tested in quadruplicate, and all experiments were performed at least three times independently.

Reactive oxygen species assay

Intracellular reactive oxygen species (ROS) production in *E. coli* following exposure to 2-HNQ or 2-MNQ was assessed using the fluorescent probe dihydroethidium (DHE, Sigma-Aldrich). DHE is a redox-sensitive fluorescent dye commonly employed for the detection of intracellular ROS generation (15). A bacterial suspension prepared in MHB at a concentration of 5×10^5 CFU mL^{-1} was aliquoted into sterile Falcon tubes and treated with 2-HNQ or 2-MNQ at concentrations corresponding to 0.25 × MIC or 0.5 × MIC. An untreated bacterial suspension served as the control. The samples were incubated at 37 °C with constant shaking (100 rpm) for 24 h. After incubation, the bacterial suspensions were centrifuged (5000 × *g*, 5 min), and the pellets were resuspended in DHE solution (10 μmol L^{-1}). The samples were incubated at room temperature in the dark for 15 min and subsequently washed with phosphate-buffered saline (PBS) to remove excess dye. Aliquots (100 μL) of each suspension were transferred to opaque 96-well microtiter plates, and fluorescence intensity was measured using a microplate reader (SpectraMax i3x, Molecular Devices, USA) at an excitation wavelength of 535 nm and an emission wavelength of 635 nm. To normalise fluorescence data to cell density, the absorbance of each sample was measured at 600 nm. All treatments were performed in triplicate, and each experiment was independently repeated at least twice.

Crystal violet assay

Alterations in membrane permeability were evaluated using the crystal violet (CV) assay (16). A bacterial suspension (10^8 CFU mL^{-1}) was centrifugated (5000 × *g*, 5 min) and resuspended in PBS buffer to eliminate potential interference from growth medium components. Afterwards, the bacterial suspension was aliquoted into sterile Falcon tubes and treated with 2-HNQ or 2-MNQ at 0.25 × MIC or 0.5 × MIC concentration for 30 min, 60 min or 90 min. An untreated bacterial suspension served as the control. After incubation (at 37 °C with constant shaking at 100 rpm for the indicated times), bacterial suspensions were centrifuged (5000 × *g*, 5 min) and the pellets were resuspended in CV solution (10 mg mL^{-1}).

After incubation at 37 °C for 15 min, the cells were again centrifuged (5000 × *g*, 5 min), and the absorbance (Abs) of the supernatant was measured at 590 nm using a microplate reader (SpectraMaxi3x, Molecular Devices, USA). The percentage of CV uptake was calculated as:

Percentage of crystal violet uptake = Abs_{590} sample / Abs_{590} CV solution × 100

All treatments were performed in triplicate, and each experiment was independently repeated at least twice. Throughout the experiments, cell numbers were monitored; however, the number of cells remained unchanged during the experimental procedure, most likely due to the absence of growth medium (the cells were in PBS).

Swarming motility assay

The swarming motility assay was performed according to the method described by Aygul *et al.* (17), with minor modifications. Swarming motility was assessed on semi-solid Ball and Sellers' motility medium (Biolife, Italy). Plates containing only motility medium (control) and plates supplemented with 2-HNQ or 2-MNQ at concentrations corresponding to 0.25 × MIC or 0.5 × MIC were prepared. Each plate was inoculated at the centre with 5 μL of an overnight *E. coli* culture (10^8 CFU mL^{-1}). The plates were incubated at 37 °C for 24 h, after which the diameters of the swarm colonies were measured and expressed in millimetres (mm). All treatments were performed in triplicate, and each experiment was independently repeated at least twice.

Pre-biofilm inhibition assay

The effect of 2-HNQ and 2-MNQ on *E. coli* biofilm formation was evaluated according to the method described by Lemos *et al.* (18), with slight modifications. Briefly, 100 μL of a bacterial suspension (5×10^5 CFU mL^{-1} in MHB) was added to each well of a round-bottom 96-well microtiter plate. The suspensions were then treated with 2-HNQ or 2-MNQ at concentrations corresponding to 0.25 × MIC or 0.5 × MIC, while untreated wells served as the control. The plates were incubated at 37 °C for 24 h to allow biofilm formation. After incubation, the culture medium containing planktonic (free-floating) cells was carefully removed, and the wells were gently washed with 200 μL of PBS to remove unattached bacteria. The formed biofilms were fixed by heat for 30 min and subsequently stained with 200 μL of 0.1 % CV solution for 30 min at room temperature. Excess stain was removed by washing with PBS, and the bound stain was solubilised by adding 30 % acetic acid to each well. The absorbance of the solubilised stain was measured at 590 nm using a microplate reader (SpectraMax i3x, Molecular Devices, USA). All treatments were performed in quadruplicate, and each experiment was independently repeated at least twice.

Post-biofilm inhibition assay

The effect of 2-HNQ and 2-MNQ on established *E. coli* biofilm was evaluated using a post-biofilm inhibition assay, as described by Lemos *et al.* (18), with slight modifications. Briefly, 100 μL of a bacterial suspension (5×10^5 CFU mL^{-1} in MHB) was added to each well of a round-bottom 96-well microtiter plate and incubated at 37 °C for 24 h to allow biofilm formation. Following incubation, the culture medium containing unattached (planktonic)

cells was carefully removed, and the wells were gently washed with PBS to eliminate non-adherent bacteria. Subsequently, 2-HNQ or 2-MNQ was added to the wells at concentrations corresponding to 0.25 × MIC or 0.5 × MIC, and the plates were incubated again at 37 °C for 24 h. After treatment, the residual biofilm mass was quantified using CV staining as described for the pre-biofilm inhibition assay. All treatments were performed in quadruplicate, and each experiment was independently repeated twice.

Biofilm imaging assay

The effect of 2-HNQ or 2-MNQ on *E. coli* biofilm formation was visualised microscopically. First, glass coverslips were pre-sterilised by UV light and placed in 6-well microtiter plates. A bacterial inoculum (400 μL) was added to each well, followed by treatment with 2-HNQ or 2-MNQ at 0.25 × MIC or 0.5 × MIC concentration. In the experiments, an untreated control was included. The plates were incubated at 37 °C for 24 h to allow biofilm development. Following incubation, the culture medium containing free-floating bacteria was removed, and the wells were washed with PBS to remove unattached cells. The formed biofilms were fixed onto the coverslips by heat treatment for 30 min, stained with 0.1 % CV solution, and incubated at room temperature for 15 min. Afterwards, the coverslips were placed on slides and observed under a light microscope (CX22, Olympus, Japan).

Statistics

Results on the levels of ROS, CV uptake, swarming motility, pre-biofilm formation and post-biofilm formation were presented as mean ± standard deviation (SD). Statistical significance between the treated groups and the control was determined using a *t*-test. Statistical significance was set at $p \leq 0.05$. Between different parameters, Pearson's correlation coefficients (R) were calculated. Statistical analysis was performed using STATISTICA 12.0 (StatSoft, Inc., USA).

RESULTS AND DISCUSSION

Antibacterial activity of 2-HNQ and 2-MNQ

E. coli is, among other diseases, responsible for urinary tract infections, enteritis, and neonatal meningitis, which are attributed to multidrug-resistant *E. coli* strains that are difficult to treat (1–5). Therefore, it was reasonable to investigate the potential antibacterial and antibiofilm activities of 2-HNQ and 2-MNQ against *E. coli*.

In the first step of this study, the MICs of 2-HNQ and 2-MNQ were determined. The results demonstrated that both compounds exhibited antibacterial activity against *E. coli;* however, 2-MNQ displayed greater efficacy than 2-HNQ. The MIC value of 2-MNQ against *E. coli* was 100 μg mL^{-1}, whereas the MIC value of 2-HNQ was 500 μg mL^{-1} (Table I).

Table I. Minimum inhibitory concentrations (MICs) of 2-hydroxy-1,4-naphthoquinone (2-HNQ) and 2-methoxy-1,4-naphtoquinone (2-MNQ) against Escherichia coli (E. coli)

	2-HNQ	2-MNQ
E. coli strain 25922	500 μg mL^{-1}	100 μg mL^{-1}

Available literature contains only one study that reports the MIC value of 2-HNQ against *E. coli* (19). In the mentioned study, experiments were conducted on the same *E. coli* strain (ATCC 25922), and the reported MIC value was equal to or greater than 500 μg mL^{-1}, which aligns with our findings. Although studies determining the MIC value of 2-MNQ against *E. coli* are missing, there is a study evaluating the MIC value of 2-MNQ against a Gram-negative bacterium, *Helicobacter pylori* (12). In that study, 2-MNQ showed significant potency against several strains of *H. pylori*, with MIC values ranging from 0.15 to 0.62 μg mL^{-1}.

Mechanism of antibacterial activity of 2-HNQ and 2-MNQ

To elucidate the possible mechanisms underlying the antibacterial activity of 2-HNQ and 2-MNQ against *E. coli*, the impact of NQs on intracellular ROS generation and membrane permeability was investigated using the fluorescent probe DHE and the CV assay, respectively.

Exposure of *E. coli* to NQs increased intracellular ROS levels compared to untreated control cells (Fig. 2). After 24 h of exposure to 2-HNQ even at the lowest applied concentration

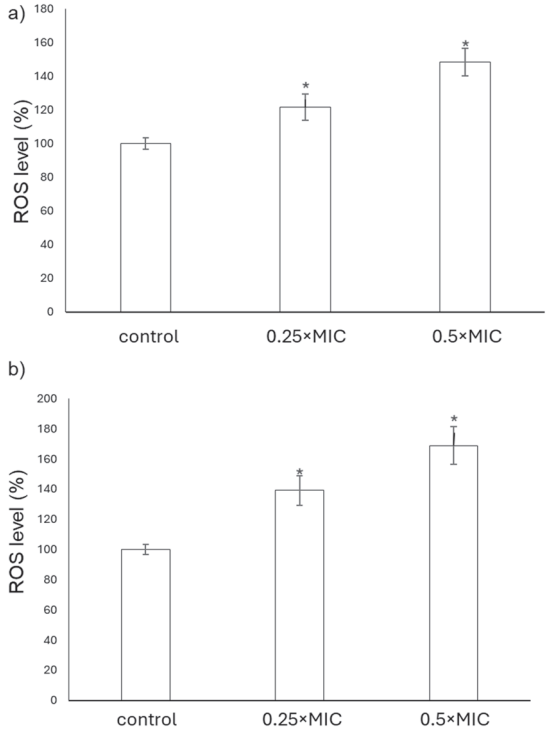

Fig. 2. Intracellular level of reactive oxygen species (ROS) in *E. coli* strain ATCC 25922 after 24 h exposure to: a) 2-hydroxy-1,4-naphthoquinone (2-HNQ); b) 2-methoxy-1,4-naphthoquinone (2-MNQ) in 0.25 × MIC or 0.5 × MIC concentration. * Significantly different from control ($p \leq 0.05$).

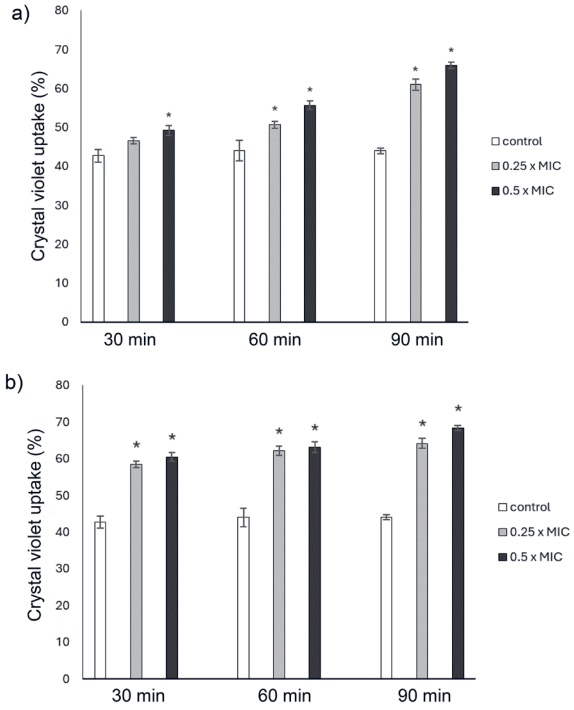

Fig. 3. Change of *E. coli* strain ATCC 25922 membrane permeability assessed by the crystal violet uptake assay after 30 min, 60 min or 90 min exposure to: a) 2-hydroxy-1,4-naphthoquinone (2-HNQ); b) 2-methoxy-1,4-naphthoquinone (2-MNQ) in 0.25 × MIC or 0.5 × MIC concentration. * Significantly different from control ($p \leq 0.05$).

(0.25 × MIC) an increase in ROS production relative to untreated (control) *E. coli* was observed (121.6 ± 7.8 % *vs.* 100 ± 3.5 %; $p \leq 0.05$; Fig. 2a). Similarly, 24 h exposure to 2-MNQ at the lowest applied concentration (0.25 × MIC) elevated intracellular ROS levels in *E. coli* compared to control cells (139.2 ± 9.8 *vs.* 100 ± 3.5 %; $p \leq 0.05$; Fig. 2b).

The observed increase in intracellular ROS levels following exposure to 2-HNQ and 2-MNQ can be explained by the chemical structure of NQs. Due to their quinonoid structure, NQs can undergo the stepwise one-electron reduction to form semiquinone and then hydroquinone, activating redox cycles between NADH and molecular oxygen. This process results in the generation of intracellular ROS, which, in turn, can lead to damage of cellular macromolecules and finally contribute to microbial death (8–10, 12).

In the next step of the study, membrane permeability was assessed using the CV assay. The CV assay is commonly employed to detect membrane damage, as CV easily enters cells with impaired membranes. However, it poorly enters cells with intact/undamaged membranes (16). Compared to control bacteria, *E. coli* treated with 2-HNQ or 2-MNQ had higher CV uptake, indicating that 2-HNQ and 2-MNQ affected the membrane permeability of *E. coli* (Fig. 3). However, this effect was more pronounced for 2-MNQ. 2-MNQ significantly affected membrane permeability after 30 min of exposure at both concentra-

tions (0.25 × MIC and 0.5 × MIC), whereas 2-HNQ at the same exposure time only affected membrane permeability at the highest applied concentration (0.5 × MIC).

The change in membrane permeability can be attributed to the overproduction of intracellular ROS induced by NQs, as ROS can attack membrane macromolecules and, by altering membrane composition, affect membrane permeability. In addition to inducing ROS production, NQs can bind to proteins by forming irreversible complexes with amino acids, leading to protein inactivation. Due to this property, NQs target surface adhesins, membrane polypeptides and membrane-bound enzymes, which further alter membrane permeability (6, 9). Furthermore, NQs can bind to nucleophiles such as nitrogen atoms in thiol groups of proteins, which can also contribute to changes in membrane permeability (6).

Effect of 2-HNQ and 2-MNQ on swarming motility

Swarming motility represents a coordinated form of collective movement of a bacterial population across a surface to access nutrients, colonise new environments, and establish infections (17, 20). In pathogenic bacteria, swarming motility contributes to successful host colonisation, invasion and increased resistance to antimicrobial agents (17). *E. coli* is a rod-shaped bacterium that utilises multiple flagella for movement (21). During swarming, cells undergo morphological changes characterised by an increase in the number of flagella and the elongation of the cells (17, 21). To our knowledge, the effect of NQs on *E. coli* swarming motility has not been previously investigated. In the present study, the impact of 24 h of exposure to 2-HNQ and 2-MNQ on *E. coli* swarming motility was evaluated. The results demonstrated that both 2-HNQ and 2-MNQ inhibited the swarming motility of bacterial colonies (Fig. 4). 2-MNQ was effective in inhibiting the swarming motility of *E. coli* colonies at both applied concentrations, 0.25 × MIC and 0.5 × MIC. The swarming diameter of the control colonies was 6.63 ± 0.48 mm, while exposure to 2-MNQ at 0.25 × MIC and 0.5 × MIC resulted in diameters of 5.50 ± 0.34 mm ($p \leq 0.05$) and 5.00 ± 0.42 mm ($p \leq 0.05$), respectively (Fig. 4b). In contrast, 2-HNQ showed no significant effect at 0.25 × MIC (5.90 ± 0.63 mm *vs*. 6.63 ± 0.48 mm, $p > 0.05$; Fig. 4a). However, at the higher concentration (0.5 × MIC) 2-HNQ effectively inhibited swarming motility, reducing the swarming diameter to 4.12 ± 0.25 mm compared to the control (6.63 ± 0.48 mm, $p \leq 0.05$).

Antibiofilm effects of 2-HNQ and 2-MNQ

Bacterial biofilms are densely packed communities of microbial cells surrounded by a self-produced extracellular polymeric matrix (18, 22). This biofilm structure provides protection against various physico-chemical stressors, including UV light, heavy metals, acidity and phagocytosis. Bacterial biofilms present significant clinical concerns, as they can lead to chronic and persistent infections (18). Bacteria growing within biofilms exhibit enhanced resistance to host immune responses and to antimicrobial treatments; therefore, biofilm-mediated infections often require much higher concentrations of antibiotics for effective treatment compared to infections caused by planktonic bacteria (18, 22).

In this study, the impact of 2-HNQ and 2-MNQ on biofilm adhesion (pre-biofilm formation) and on already formed biofilms (post-biofilm formation) of *E. coli* was evaluated. Both compounds inhibited biofilm development and disrupted established biofilms (Fig. 5).

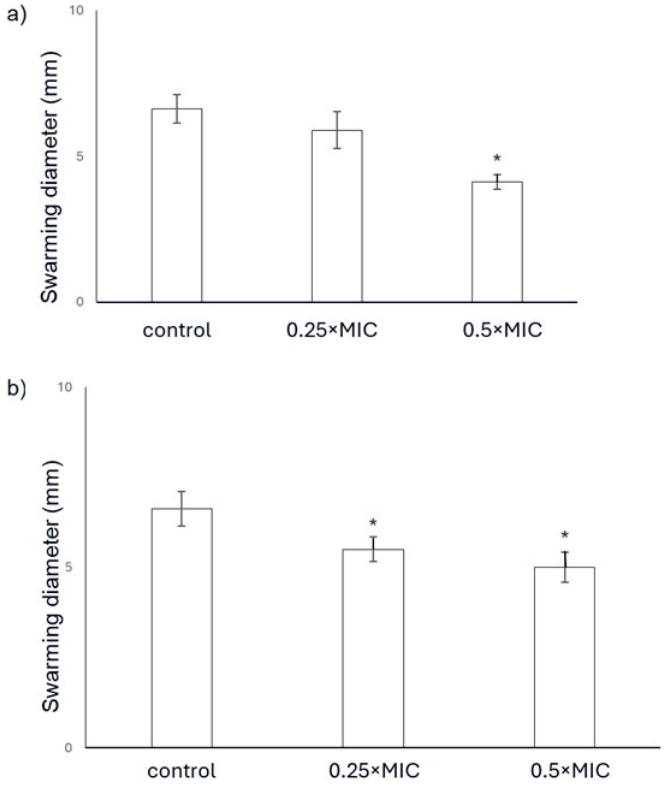

Fig. 4. Swarming motility of *E. coli* strain ATCC 25922 after 24 h exposure to: a) 2-hydroxy-1,4-naphthoquinone (2-HNQ); b) 2-methoxy-1,4-naphthoquinone (2-MNQ) in 0.25 × MIC or 0.5 × MIC concentration. * Significantly different from control ($p \leq 0.05$).

As expected, 2-MNQ exhibited a stronger inhibitory effect than 2-HNQ in both assays. Interestingly, the impact of NQs on *E. coli* pre-biofilm formation and post-biofilm formation was comparable. In comparison to the control where no inhibition of pre- or post-biofilm formation was observed (biofilm formation was set on 100 %), 2-HNQ at a concentration 0.25 × MIC reduced pre-biofilm formation by 29.5 ± 12 % ($p \leq 0.05$; Fig. 5a), and post-biofilm formation by 36.2 ± 9.02 % ($p \leq 0.05$; Fig. 5b). On the other hand, treatment with 2-MNQ at 0.25 × MIC inhibited pre-biofilm formation by 65.2 ± 8.6 % ($p \leq 0.05$; Fig. 5c) and post-biofilm formation by 61.3 ± 9.2 % ($p \leq 0.05$; Fig. 5d). The effects of NQs on *E. coli* biofilm formation were visualised using a light microscope (Fig. 6). The control bacteria formed a robust biofilm (Fig. 6a). However, the inhibitory effect of NQs on biofilm formation was evident (Fig. 6b–d).

The observed decrease in both pre- and post-biofilm formation by NQs may be partially attributed to the inhibition of swarming motility, since swarming motility can influence the initial stages of biofilm attachment (20). A strong positive correlation between swarming motility and both pre- and post-biofilm formation following exposure to 2-HNQ

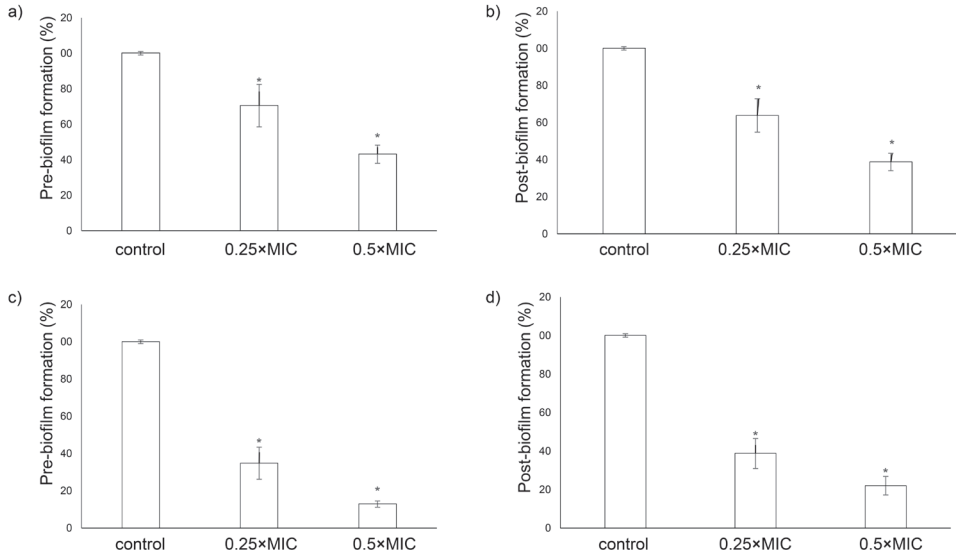

Fig. 5. Pre-biofilm formation and post-biofilm formation of *E. coli* strain ATCC 25922 after 24 h exposure to naphthoquinones. a) Impact of 2-hydroxy-1,4-naphthoquinone (2-HNQ) on pre-biofilm formation; b) impact of 2-HNQ on post-biofilm formation; c) impact of 2-methoxy-1,4-naphthoquinone (2-MNQ) on pre-biofilm formation; d) impact of 2-MNQ on post-biofilm formation in 0.25 × MIC or 0.5 × MIC concentration. * Significantly different from control ($p \leq 0.05$).

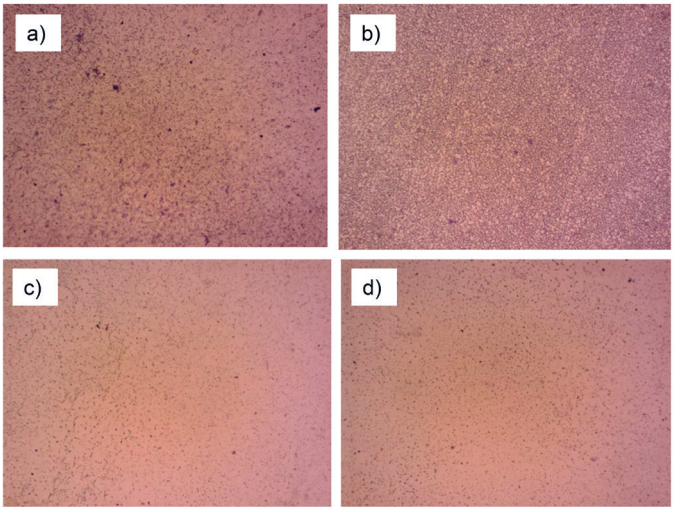

Fig. 6. Pre-biofilm formation of *E. coli* strain ATCC 25922: a) control (unexposed); b) 24 h exposed to 0.25 × MIC 2-hydroxy-1,4-naphthoquinone (2-HNQ); c) 24 h exposed to 0.25 × MIC 2-methoxy-1,4-naphthoquinone (2-MNQ); or d) 24 h 0.5 × MIC 2-MNQ (light microscopy images, 400 ×).

and 2-MNQ (correlation coefficients, *R*, ranged from 0.9424 to 0.9982) confirmed the link between swarming motility and biofilm formation (Suppl. Tables I and II). Additionally, the ability of NQs to induce intracellular ROS generation and to alter membrane permeability may contribute to the destabilisation of biofilm structure (6, 9). In this study, a strong negative correlation between: (*i*) the overproduction of ROS and swarming motility, (*ii*) the overproduction of ROS and biofilm formation, (*iii*) change in membrane permeability and swarming motility and (*iv*) change in membrane permeability and biofilm production following exposure to 2-HNQ and 2-MNQ was observed (Suppl. Tables I and II). These correlations indicate that ROS generation and destabilisation of membrane permeability contribute to the decrease in swarming motility and biofilm production. The binding of NQs to membrane proteins, in particular surface adhesins, may disrupt the adhesion of bacteria, leading to a decrease in swarming motility and biofilm formation. Moreover, due to their ability to interact with proteins and nucleophilic biomolecules, NQs can affect the composition of the biofilm matrix, resulting in altered motility and formation of biofilm.

CONCLUSIONS

This study demonstrated that both 2-HNQ and 2-MNQ exhibit antibacterial activity against *E. coli*, with 2-MNQ showing greater potency than 2-HNQ. The results indicate that NQs exert their antimicrobial effects through multiple mechanisms, including increased intracellular ROS production and disruption of membrane permeability. In addition, both compounds reduced swarming motility and inhibited biofilm formation of *E. coli* as well as decreased the mass of established biofilms of *E. coli*. These findings are particularly relevant considering the global rise of multidrug-resistant *E. coli* strains and the clinical challenges associated with biofilm-associated infections. The demonstrated antibiofilm and antibacterial properties of 2-HNQ and 2-MNQ highlight their potential as promising candidates for the development of new therapeutic strategies targeting *E. coli* infections, especially those involving biofilm formation.

Supplementary material is available upon request.

Conflict of interest. – The authors declare no conflict of interest.

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Authors contributions. – Conceptualisation, D.S.P. and A-M.D.; methodology, D.S.P. and A.-M.D.; investigation and analysis, D.S.P., D.K.B., and A-M.D.; writing, original draft preparation, D.S.P. and A-M.D.; writing, review and editing, D.S.P., D.K.B., and A-M.D. All authors have read and agreed to the published version of the manuscript.

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Supplementary material

S*upplementary Table I. Correlation coefficients (r) between assessed parameters after exposure E. coli to 2-hydroxy-1,4-naphthoquinone*

	CV30	CV60	CV90	ROS	Motility	Pre-biofilm	Post-biofilm
CV30		0.9995	0.9813	0.9846	-0.9387	-0.9956	-0.9999
CV60			0.9748	0.9896	-0.9490	-0.9980	-0.9998
CV90				0.9325	-0.8547	-0.9589	-0.9791
ROS					-0.9845	-0.9967	-0.9864
Motility						0.9669	0.9424
Pre-biofilm							0.9965
Post-biofilm							

CV30- crystal violet uptake following 30 min exposure; CV60-crystal violet uptake following 60 min exposure; CV90-crystal violet uptake following 90 min exposure; ROS-reactive oxygen species; motility - swarming motility; pre-biofilm – pre-biofilm formation; post-biofilm - post-biofilm formation.

Supplementary Table II. Correlation coefficients (r) between assessed parameters after exposure E. coli to 2-methoxy-1,4-naphthoquinone

	CV30	CV60	CV90	ROS	Motility	Pre-biofilm	Post-biofilm
CV30		0.9981	0.9983	0.9423	-0.9798	-0.9899	-0.9948
CV60			0.9928	0.9201	-0.9658	-0.9796	-0.9868
CV90				0.9604	-0.9899	-0.9966	-0.9991
ROS					-0.9902	-0.9801	-0.9715
Motility						0.9982	0.9951
Pre-biofilm							0.9992
Post-biofilm							

CV30- crystal violet uptake following 30 min exposure; CV60-crystal violet uptake following 60 min exposure; CV90-crystal violet uptake following 90 min exposure; ROS-reactive oxygen species; motility - swarming motility; pre-biofilm – pre-biofilm formation; post-biofilm - post-biofilm formation.