Evaluation of 1,4-naphthoquinone derivatives as antibacterial agents: activity and mechanistic studies

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A B S T R A C T
The diverse and large-scale application of disinfectants posed potential health risks and caused ecological damage during the 2019-nCoV pandemic, thereby increasing the demands for the development of disinfectants based on natural products, with low health risks and low aquatic toxicity. In the present study, a few natural naphthoquinones and their derivatives bearing the 1,4-naphthoquinone skeleton were synthesized, and their antibacterial activity against selected bacterial strains was evaluated. In vitro antibacterial activities of the compounds were investigated against Escherichia coli and Staphylococcus aureus. Under the minimum inhibitory concentration (MIC) of ≤ 0.125 μmol/L for juglone (1a), 5,8-dimethoxy-1,4-naphthoquinone (1f), and 7-methyl-5-acetoxy-1,4-naphthoquinone (3c), a strong antibacterial activity against S. aureus was observed. All 1,4-naphthoquinone derivatives exhibited a strong antibacterial activity, with MIC values ranging between 15.625 and 500 μmol/L and EC₅₀ values ranging between 10.56 and 248.42 μmol/L. Most of the synthesized compounds exhibited strong antibacterial activities against S. aureus. Among these compounds, juglone (1a) showed the strongest antibacterial activity. The results from mechanistic investigations indicated that juglone, a natural naphthoquinone, caused cell death by inducing reactive oxygen species production in bacterial cells, leading to DNA damage. In addition, juglone could reduce the self-repair ability of bacterial DNA by inhibiting RecA expression. In addition to having a potent antibacterial activity, juglone exhibited low cytotoxicity in cell-based investigations. In conclusion, juglone is a strong antibacterial agent with low toxicity, indicating that its application as a bactericidal agent may be associated with low health risks and aquatic toxicity.

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1 Introduction

The 2019-nCoV pandemic period witnessed the widespread and large-scale application of disinfectants, which led to potential health risks and ecological damage, raising widespread concerns from the public. Spraying disinfectants containing chlorine dioxide, sodium hypochlorite, hydrogen peroxide, peroxyacetic acid, and quaternary ammoniums led to the cumulative accumulation of the chemically reactive agents in the environment, and the amount of disinfectant by-products (DBPs) also increased gradually (Cui and Jia, 2021). Disinfectants can easily enter the atmosphere directly through evaporation and pollute the soil and water. The presence of disinfectants and DBPs was detected in lakes, rivers,
Large-scale application of disinfectants can also cause potential ecotoxicity (Cui and Jia, 2021). Some disinfectants including quaternary ammonium compounds, glutaraldehyde, methylisothiazolinone (MIT), octylisothiazolinone (OIT), and benzalkonium chloride cause ecotoxicity and have been reported to impact the survival, development, and endocrine system of aquatic organisms (Christen et al., 2017; Le Roux et al., 2017). More importantly, DBPs are abundantly present in the environment, over, DBPs are abundantly present in the environment, and stored in 25% water (DeLeo et al., 2020). DPBs were also detected in the atmosphere and precipitation (Kimura and Ortega-Hernandez, 2019; Li et al., 2019).

Therefore, the development of disinfectants having low health risks and low aquatic toxicity is highly warranted. Natural products are a well-spring of antimicrobial agents, which have been used for thousands of years (Hashiguchi et al., 2017; Ye et al., 2020; Ozma et al., 2021). The number of clinical therapeutics derived from natural products so far is remarkable. Regarding the development of novel antimicrobial drugs, natural naphthoquinones have garnered considerable research attention due to their diverse functions and clinical applications. Natural naphthoquinones, such as plumbagin and lapachol, exhibit strong cytotoxicity, as well as insecticidal (Pavela, 2013), anti-inflammatory, and antipyretic activities (Janečko et al., 2016). A few natural naphthoquinones, such as 1,4-naphthoquinone, 2-amino-1,4-naphthoquinones, and lawsone, have been used as anticancer, antimalarial and antibacterial agents in folk medicine (Prati et al., 2015). 1,4-Naphthoquinone and its derivatives have attracted extensive attention owing to their strong antibacterial and antitumor activities; 1,4-naphthoquinone can kill S. aureus by promoting the accumulation of reactive oxygen species (ROS) in S. aureus (Paul et al., 2021). Likewise, 2-methoxy-1,4-naphthoquinone can enhance the host defense ability against Penicillium italicum (Wu et al., 2022). In addition, 1,4-naphthoquinone and its natural derivatives (plumbagin, juglone, lawsone, menadione, lapachol, and β-lapachone) exhibit potential as redox modifiers, along with anti-inflammatory, anticancer, antidiabetic and antimicrobial activities, for implications in therapeutic settings (Kapoor et al., 2021).

The main objective of the present study was to synthesize a series of 1,4-naphthoquinone derivatives, including naturally occurring juglone and its derivatives, and examine their antibacterial activity to develop novel antibacterial drugs, with promising antibacterial activity, based on the structure of 1,4-naphthoquinone. Juglone, as the most potent compound among the synthesized derivatives, was evaluated for its antibacterial action mechanism and potential use as a natural disinfectant with low health risks and low aquatic toxicity.

2 Materials and methods

2.1 Synthesis of 1,4-naphthoquinone derivatives

The synthesis methods for the tested 1,4-naphthoquinone derivatives are described in the Supporting Information. According to HPLC analysis, the purity of the tested compounds was more than 98%.

2.2 Evaluation of the antibacterial activity of 1,4-naphthoquinone derivatives

2.2.1 Microbial strains

The reference bacterial strains used in the present study are as follows: Gram-positive bacteria: E. coli (reference strain GDMCC 1.115) and Gram-negative bacteria: S. aureus (reference strain GDMCC 1.2442). Both strains were purchased from the Institute of Microbiology, Guangdong Academy of Sciences (Guangzhou, China), and stored in 25% glycerol at −80°C. All E. coli and S.
aureus cultures were initiated in LB media and shaken overnight at 37 °C until the log phase of growth was reached.

2.2.2 Minimum inhibitory concentration of 1,4-naphthoquinone derivatives against E. coli and S. aureus

A modified microplate bioassay method was used to evaluate the antibacterial activity of the synthesized 1,4-naphthoquinones (Zeng et al., 2018). First, 100 μL gradiently diluted solution of 1,4-naphthoquinones was added into each vertical row well in turn; LB medium was used for all dilutions. Three repetitions were set for each concentration gradient. Subsequently, day-old cultures of bacteria were diluted to 0.8 McFarland Standard. Then, 100 μL bacterial suspension was added to each well, which resulted in the reaction concentrations (500 μmol/L, 250 μmol/L, 125 μmol/L, 62.5 μmol/L, 31.25 μmol/L, 15.625 μmol/L) of synthesized 1,4-naphthoquinones to become half of the set concentration. Then, the plates were incubated at 37 °C for 24 h. Each plate was covered with a sealing membrane before incubation to reduce evaporation and allow free air exchange. After 24 h of incubation, the lowest concentration without bacterial growth in all samples was considered as the minimum inhibitory concentration (MIC) of the agent against the tested bacterial strain.

2.2.3 IC₅₀ of 1,4-naphthoquinone derivatives against E. coli and S. aureus

Bacterial suspension, collected after 1-day culture in the presence of different concentrations (500 μmol/L, 250 μmol/L, 125 μmol/L, 62.5 μmol/L, 31.25 μmol/L, 15.625 μmol/L, and 7.8125 μmol/L) of synthesized 1,4-naphthoquinones in a 96-well plate, was washed with PBS for three times, resuspended in NaCl (0.85%), and diluted to OD₅₀₀ = 0.5 after centrifugation. The bacterial suspension was stained using the Live & Dead Bacterial Staining Kit for 15 min at room temperature. All specimens were examined through FACS Calibur Flow cytometry. Fluorescence signals were recorded using the FL1 and FL3 channels (logarithmic amplification). The data were finally analyzed using FlowJo10.0 to determine the bacterial survival rate under different concentrations, and the IC₅₀ value was fitted using Prism (v. 7.0).

2.3 E. coli cellular response to juglone

2.3.1 Increased ROS production in E. coli

ROS levels in the bacterial cells were measured using the dihydrouethidium (DHE) assay kit. In cells, ROS can oxidize DHE to ethidium, which then combines with RNA or DNA to produce red fluorescence. After treating the bacteria with different concentrations of juglone for 4 h, the cells were incubated with DHE solution (1 μL, in PBS) at 37 °C for 30 min. The cells were washed with PBS, and all specimens were examined using the FACS Calibur Flow cytometer (BD, USA). Fluorescence signals were recorded using FL3 channels (logarithmic amplification).

2.3.2 DNA damage in E. coli

DNA damage was measured using the TUNEL Assay Kit. When genomic DNA breaks, the exposed 3'-OH can be catalyzed by terminal deoxynucleotidyl transferase (TDT) and ligated to fluorescein dUTP (dUTP labeled by the green fluorescent probe fluorescein isothiocyanate [FITC]). Bacterial suspension, collected after a 4-h culture in the presence of different concentrations of juglone or the PBS control, was resuspended in PBS and diluted to 5 × 10⁵–10 × 10⁵ cells/mL after centrifugation. All specimens were examined using the LSR Fortessa Flow cytometer (BD, USA); fluorescence signals were recorded using FITC channels (logarithmic amplification).

2.4 Effect of juglone on the transcription level and protein expression of specific genes

2.4.1 Quantitative real-time PCR

The changes in the transcription level of specific genes in cells can be determined through quantitative real-time PCR. In brief, we used TRIzol reagent to extract the total RNA of the bacteria treated with different agents. Subsequently, we used the First Strand cDNA Synthesis Kit (RNase H minus) to reverse transcribe mRNA into cDNA. The reverse transcription of mRNA was performed at 42 °C for 1 h, followed by inactivation of M-MuLV reverse transcriptase at 80 °C for 10 min. Finally, the first strand of cDNA was obtained, and then, quantitative real-time PCR was performed for quantitative analysis. The PCR cycle for the amplification of cDNA was as follows: 95.0 °C for 10 s, 58.0 °C for 30 s, and 72 °C for 20 s. The primer sequences are shown in Table 1.

2.4.2 Western blot

Changes in the expression of specific proteins in the cells were measured through western blotting. Bacterial suspension, collected after 4-h culture in the presence of different reagents, was resuspended in PBS and diluted to 5 × 10⁸ –10 × 10⁸ cells/mL after centrifugation. Bacteria cells were lysed by RIPA cell lysate to collect protein solution. Protein concentration was measured using the BCA method. The total protein extracted from the cells was separated through SDS-PAGE. Subsequently, these proteins were transferred to the nitrocellulose membrane...
by electrophoresis. After blocking miscellaneous proteins, the nitrocellulose membrane was incubated with the anti-RecA primary antibody (rabbit anti E. coli) and HRP-labeled secondary antibody (Goat anti rabbit antibody). HRP coupled to the secondary antibody chemically reacted with the ECL chemiluminescence kit to emit fluorescence, which allowed the detection of the sample using chemiluminescence imaging equipment. GADPH was used as an internal reference protein in western blotting.

2.5 Cell lines and MTT assay

The human normal human liver cell line HL-7702 cell line was purchased from ScienCell (California, USA) and cultured in RPMI-1640 medium (Beyotime, Shanghai, China) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin Solution (100X) (Beyotime, Shanghai, China). The HFF-1 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA), and cultured in Dulbecco’s modified Eagle’s medium (Beyotime, Shanghai, China) supplemented with 15% FBS and 1% Penicillin Streptomycin Solution (100X) (Beyotime, Shanghai, China). The cells were incubated at 37 °C with 5% CO₂ in a humidified chamber.

Cytotoxicity of juglone was measured using the standard MTT assay. Cell suspension (5 × 10⁴ cells/mL, 100 μL) was added to each well of a 96-well plate, and then, the cells were incubated at 37 °C and 5% CO₂ for 24 h. Then, 100 μL of juglone with different concentrations (between 1 and 100 μmol/L, diluted with LB medium) was added to each well. After incubating the cells at 37 °C and 5% CO₂ for 48 h, 20 μL MTT was added to each well. MTT can combine with living cells to produce purple formazan. After 4 h, the supernatant was discarded from the 96-well plate, 150 μL of DMSO was added to each well to dissolve formazan, and finally, the absorbance of DMSO solution was determined through a multifunctional microplate reader; the cell survival rate was calculated using the following formula: inhibitory rate = (Abs₄₉₀ control cells − Abs₄₉₀ treated cells)/Abs₄₉₀ control cells × 100%.

2.6 In vivo exposure of zebrafish embryo to juglone

Stock solutions of juglone with different concentrations of juglone were prepared in fish water (32.4 mg/L NaHCO₃, 61.6 mg/L MgSO₄·7H₂O, 2.9 mg/L KCl, and 147.0 mg/L CaCl₂·2H₂O). Zebrafish embryos were obtained through self-breeding of zebrafish.

For each substance, one control group (fish water) and the experimental groups exposed to different concentrations of juglone, with four replicates each, were used: 1 μmol/L, 5 μmol/L, 10 μmol/L, 25 μmol/L, and 50 μmol/L juglone. In addition, we used 84 disinfectant (a type of disinfectant containing sodium hypochlorite) as the positive control and expressed the disinfectant concentration as the concentration of sodium hypochlorite. During the exposure, the water temperature was between 27 °C and 29 °C and the photoperiod was maintained at the light/dark period of 14:10. The fish water must be changed every day.

After 96 hours of exposure, 20 larvae from each treatment group were transferred to 48-well plates for the swimming behavior analysis. The behavioral parameters (swimming frequency, swimming/still time ratio, total distance, and average swimming speed of zebrafish) were measured. In brief, we transferred zebrafish to a 48-well plate and recorded their movement effects with a camera. Following, the behavioral parameters were then measured using the ImageJ software.

3 Results and discussion

3.1 Chemistry

As shown in Scheme 1, the starting material for the synthesis of most 1,4-naphthoquinones was 1,5-naphthalenedioli. The oxidation of 1,5-naphthalenedioli by freshly prepared peroxyacetic acid under mild conditions resulted in the production of juglone (1a) in high yield. The reaction temperature was maintained below 60 °C because a large number of by-products are formed at temperatures higher than 60 °C due to excessive oxidation of the desired product.

Juglone methyl ether (Scheme 1, 1b) was prepared through the reduction and methylation of juglone (1a) and further CAN-mediated oxidation according to a previously described procedure (Cui et al., 2022). Acylation of juglone using acetic acid anhydride and a

| Gene      | Primer forward | Primer reverse       |
|-----------|----------------|---------------------|
| GAPDH     | TCCCAGAAACATCATCCGGTCCTC | AACGCCATACCGATGCTTTGCC |
| RecA      | GAAGGGGAATCCGGCACTCTC | TGAAGATCAGCGGTTGGAC |
| umuD      | TTGTCTTCAAGCCTGGATCTCC | AACGTAATCTGCTGCGGTAAG |
| dinB      | ACTTTCCGGCCGTATTCAGTGC | TCCCCAGTTTCCCTGGAGAATTTCAC |

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A catalytic amount of sulfuric acid led to the production of acetyl juglone (1c). Then, propionyl juglone (1d) was synthesized using a similar procedure, with propionyl anhydride as the reactant. Benzylolation of the chelated phenolic hydroxyl group of juglone produced benzyl juglone (1g) in high yield.

The natural naphthoquinone shikonin (2e) and the synthetic 5,8-dimethoxy-1,4-naphthoquinone were prepared, with 1,5-naphthalenediol as the starting material, according to a previously reported procedure (Cui and Jia, 2021).

7-Methyl juglone (3b) and its derivatives were synthesized through Stobbe condensation between 2,5-dimethoxy benzaldehyde and diethyl succinate (Scheme 2), intramolecular cyclization, reduction, acid-facilitated debenzylation, and further cerium (IV) ammonium nitrate-mediated oxidation according to the reported procedures (Cui and Jia, 2021).

6-Methyl-1,4-naphthoquinone (2c) was prepared through Diels–Alder cycloaddition and further oxidation (Scheme 3). The cycloaddition of 1,4-benzoquinone to isoprene resulted in the production of 6-methyl-γ-4a,5,8,8a-tetrahydro-1,4-naphthoquinone (Scheme 3) in high yield. The conjugate addition proceeded smoothly at room temperature without any catalyst or pressurizing equipment. However, a large amount of raw material was left when the reaction time was < 48 h. When the reaction time was extended to one week, the amount of anthraquinones as by-products greatly increased. The optimal reaction time was 72-96 h. Generally, the reaction should be conducted at ambient temperature because anthraquinones and tar-like impurities are generated at temperatures higher than 40 °C. The enol-keto tautomerization and further regioselective oxidation produced the target compound 2c. The structures of the prepared compounds were characterized through 1H and 13C NMR spectra. The occurrence of the signal with chemical shifts (δ) ranging from 7.0 to 6.8 mg/L in the 1H NMR spectra is the most remarkable feature of this series of compounds, which should be ascribed to the resonance of protons on the quinone ring in a common magnetic field. In addition, the signals of quinone carbonyl carbons with chemical shifts of higher than 160 mg/L in 13C NMR spectra were observed for the synthesized naphthoquinones.

3.2 Antibacterial activity evaluation

All the synthesized compounds were screened using the high-throughput microwell method to evaluate their MIC and EC50 against S. aureus and E. coli according to the standard method described previously (Lee et al., 2015).
using broth microdilution method, which has been frequently used for the evaluation of antibacterial efficacy of naphthoquinones (Ibis et al., 2011; Novais et al., 2018). Studies have confirmed that this method does not affect the stability of naphthoquinones. The degradation of tested compounds was negligible at low microbial concentrations 24 h after incubation (Lee et al., 2015; Novais et al., 2018). The results of a previous study indicated that juglone, being the preventative of prepared 1,4-naphthoquinones, was stable in solutions under pH values ranging from 2.0 to 8.0 (Sharma et al., 2009) within 24 h. To evaluate the MIC values, 1,4-naphthoquinones derivatives were incubated in LB culture medium under neutral pH conditions (pH = 7.4). In addition, free air exchange in the incubation system prevented the production of acidic metabolites of bacteria, which led to a decrease in pH values of the culture medium. Additionally, DMSO as the solvent with concentrations less than 0.5% (v/v) in each well did not show antibacterial activities against any of the test microorganisms.

*E. coli* and *S. aureus* are the well-known gram-negative and gram-positive bacteria, respectively. The peptidoglycan layer in the cell wall of gram-positive bacteria is thick, has phosphatic acid, and lacks the outer membrane, whereas the peptidoglycan layer in the cell wall of gram-negative bacteria is thin, lacks phosphatic acid, and has an outer membrane. The structural characteristics determine the sensitivity of gram-positive bacteria to most antibacterial agents based on natural products, which was also reflected in the experimental results of our study.

Table 2 summarizes the MIC and EC$_{50}$ values for all the tested compounds. Shikonin, which has the antibacterial activity, was used as the positive control.

As shown in Table 2, the tested 1,4-naphthoquinones exhibited the antimicrobial activity, with MIC values of 15.625–500 μmol/L and EC$_{50}$ values of 10.56–248.42 μmol/L. For each compound, the MIC values were consistent with the corresponding EC$_{50}$ values. Nearly all the tested compounds exhibited a strong antiproliferative activity against *S. aureus*, while showing less potent antibacterial efficacy against *E. coli*. Among the tested compounds, juglone (1a) and its derivatives (1c, 1e, 1f and 1g), synthetic 1,4-naphthoquinone (2d), and 7-methyl juglone derivatives (3b and 3c) exhibited the highest potency against *S. aureus*, with EC$_{50}$ values ranging between 10.56 and 29.35 μmol/L. Furthermore, among all the compounds, juglone exhibited the highest antibac-
3.3 Structure-activity relationship studies

To investigate the structural requirements for the antibacterial activity of natural 1,4-naphthoquinones such as juglone and 7-methyl juglone, we prepared several 1,4-naphthoquinone derivatives containing different functional groups on scaffolds of juglone and 7-methyl juglone and determined their antibacterial efficacy. The structure-activity relationship studies for the synthesized compounds are as follows:

1) Influence of substitutions on the hydroxyl group of juglone

Protection of the chelated phenolic hydroxyl group of juglone with either a methyl group or an acetyl moiety led to a decrease in the activity. The methylated juglone (1b) showed a more than 6-fold higher EC$_{50}$ value compared with the parent compound juglone against the gram-negative bacteria _E. coli_. A four-fold increase in the EC$_{50}$ value against _S. aureus_ was also observed upon the methylation of juglone. Benzylation of the C (5) hydroxyl group of juglone had detrimental effects on antibacterial activity (1g). Methylation of the C (5) hydroxyl group and ethoxylation at the C (8) of juglone produced 5,8-dimethoxy-1,4-naphthoquinone (1f), which was inactive against _E. coli_ but still active against _S. aureus_. Overall, the results demonstrated that the presence of a hydroxyl group on the aromatic B ring of juglone was crucial for its antibacterial activity.

Conversely, for 7-methyl juglone derivatives, the methylation and acetylation of the phenolic hydroxyl group led to an increase in the antibacterial activity against both _E. coli_ and _S. aureus_. The results suggested that the methyl group on the aromatic B-ring and the substituents on the phenolic hydroxyl group may show some positive or negative interactions, with certain bacterial cellular targets, and their synergistic effect can influence the bactericidal activity of this series of 1,4-naphthoquinones. However, propionylation of the phenolic hydroxyl group of 7-methyl juglone or the formation of methoxy methyl ether was not preferred.

2) Influence of hydroxylation/methylation of 1,4-naphthoquinones

The introduction of a methyl group on C (2) of the quinone ring (1e) produced detrimental effects. For example, methylation of 1,4-naphthoquinone on C (6) caused a reduction in the activity, and methylation of the quinone ring of 1,4-naphthoquinone at the C (2) position remarkably reduced the activity against _E. coli_, although the activity against _S. aureus_ remained unchanged with
these modifications. The aforementioned results demonstrated that the methylation of certain carbons on the quinone scaffold might not improve the antibacterial activity. Similarly, hydroxylation of the quinone ring of 1,4-naphthoquinone led to a decrease in the activity.

3.4 Antibacterial mechanism of juglone

We further focused on exploring the mechanisms of cell death caused by juglone, including DNA oxidative damage and DNA repair. The SOS response is a well-known example of DNA damage of response (DDR) that was reported for the time in *E. coli* (Zgur-Bertok, 2013; Memar et al., 2020). Therefore, the SOS response in *E. coli* is more clearly understood than that in other bacteria. Hence, we selected *E. coli* to study the antibacterial mechanism of juglone.

3.4.1 ROS generation after treatment with the Juglone in *E. coli*

We explored the antibacterial mechanism of juglone because it exhibited the strongest antibacterial activity among all compounds. Many chemicals such as anticancer agents, antibiotics, insecticides, anesthetics, and aromatic hydrocarbons can induce ROS generation (Liang et al., 2019). Therefore, the generation of ROS was studied in the cells treated with juglone.

In the present study, DHE assay was employed to monitor the production of ROS in the *E. coli* cells after treatment of the cells with different concentrations of juglone. As shown in Fig. 1, compared with the control cells (Red histogram), juglone induced a rapid increase in fluorescence in a dose-dependent manner. This phenomenon proved that juglone can promote the production of intracellular ROS.

Abundant ROS are produced under stress. ROS are highly reactive molecules that can damage cell structures such as carbohydrates, nucleic acids, lipids, and proteins and alter their functions at high concentrations, leading to oxidative stress and eventually to apoptosis and necrosis (Ahmed et al., 2022).

A study reported that juglone can increase the production of ROS and cause cell death of *A. Castellani* (Jha et al., 2015) and *S. aureus* (Wang et al., 2016). In the present study, increased fluorescence, as detected by the DHE assay, was found in the juglone-treated cells. The result is consistent with that of a previous report. Studies have proposed that juglone causes cell death in *E. coli* by inducing oxidative damage (Wu et al., 2012).

3.4.2 DNA damage after treatment with the Juglone in *E. coli*

During oxidative stress, ROS cause DNA damage by attacking the DNA molecular chain in cells, leading to the generation of various oxidative products. Therefore, we determined the extent of damage to the intracellular DNA of bacterial cells treated with juglone.

Increased fluorescence, detected by TUNEL, was found in the juglone-treated cells in our experiment (Fig. 2). The percentage of FITC-positive cells in each group was as follows: blank control group, 0.62%; 5-μmol/L juglone group, 42.53%; 25-μmol/L juglone, 61.38%; and 50-μmol/L juglone, 69.52%. The results indicated that with an increase in the treatment concentration of juglone, the proportion of FITC-positive cells increased gradually, reflecting an increase in the extent of DNA damage to the cells. These data proved that the excess ROS produced by the treatment with the natural disinfectant causes DNA damage.

These results indicated that excess ROS produced by juglone are the key inducer of the apoptosis. Juglone showed a strong antibacterial activity by disrupting the cell membrane structure through ROS generation and DNA fragmentation.
3.4.3 Expression of RecA protein in *E. coli* and its effect on SOS response

Most bacteria have evolved a coordinated system of response to DNA damage. In *E. coli*, this self-healing system is called the SOS response. When DNA damage occurs, RecA induces, upon ATP binding, self-cleavage of LexA. The self-cleavage of LexA induces the expression of more than 40 genes involved in various functions to induce DNA repair.

SOS response can not only repair damaged DNA but also induce *E. coli* to undergo gene mutation and further acquire resistance. The three low fidelity DNA polymerases in SOS response, namely Pol II, Pol IV, and Pol V, allow translation and DNA replication, thereby introducing mutations into the bacterial genome. Furthermore, the activation of the SOS response is directly related to the bacterial ability to acquire resistance through horizontal gene transfer. (Kohanski et al., 2007; Nautiyal et al., 2014; Pavlopoulou, 2018; Ojha and Patil, 2019; Ji et al., 2020; Kiran and Patil, 2022). Accordingly, RecA has become an important therapeutic target against antimicrobial resistance.

In this study, we used norfloxacin as a positive control, which is a traditional antibiotic that can impede bacterial DNA synthesis by affecting the incision-sealing function during DNA synthesis and interfering with the normal replication and transcription of DNA, thereby inducing oxidative damage, and consequently, cell death (Yang et al., 2021).

As shown in Fig. 3, RecA expression was significantly downregulated when *E. coli* cells were treated with juglone. The inhibitory effect was more remarkable with an increase in the treatment dose of juglone. Moreover, we observed a significant downregulation of RecA expression when the cells were treated with a combination of norfloxacin and juglone (Fig. 4). This finding preliminarily proved that the inhibitory effect of juglone on RecA protein expression is the functional route through which juglone aggravates DNA damage.

To confirm this hypothesis, we measured the transcription level of key genes (such as umuD and dinB) in the SOS response follow-up, and the levels were found to be similar to the RecA protein expression level (Fig. 5). These phenomena proved that the SOS response is inhibited by juglone.

Furthermore, western blotting was used to measure RecA protein expression. To ensure the clarity of protein bands, only five samples are generally loaded onto each gel; however, we had to assess seven samples. Therefore, we used two pieces of gel. To ensure the reliability of the results, the same concentration of standard protein was used as the internal reference protein for each gel, and the operation of the two gels was also performed in parallel under similar conditions. In the data processing stage, we normalized the concentration of the two internal reference protein samples to eliminate the error between two gels caused by operation. As shown in Fig. 5, the expression of RecA protein in *E. coli* increased significantly after norfloxacin treatment. By contrast, in the experimental group treated with juglone, the RecA protein expression was downregulated compared with that in the blank control. In addition, RecA expression in the experimental group treated with a combination of juglone and norfloxacin was also significantly lower than that in the experimental group treated with norfloxacin alone. These results confirmed the inhibitory effect of juglone on RecA protein expression. The results of these experiments and transcriptome experiments were also consistent.

The findings proved that juglone can not only produce a large amount of ROS to cause DNA damage but also
reduce the DNA self-healing ability of bacteria by inhibiting the expression of RecA protein. Notably, a few studies have investigated the antibacterial agents targeting RecA expression. Based on the findings of this research, juglone may be a new antibacterial agent targeting RecA, similar to p-coumaric (Ojha and Patil, 2019) and suramin (Nautiyal et al., 2014).

3.5 Cytotoxicity of juglone

To confirm the potential of juglone as an antibacterial agent, we determined the cytotoxicity of juglone by using human liver HL-7702 cells and human fibroblast HFF-1 cells. The experimental results of the MTT assay are shown in Fig. 6. As shown in the figure, the IC$_{50}$ of juglone was 20.58 μmol/L in HL-7702 cells and 23.43 μmol/L in HFF-1 cells, which are significantly higher than that against the two bacterial strains used in the study. The results suggested that the natural naphthoquinone does not exhibit significant cytotoxicity under the MIC. The two cells tested in this study, namely human liver cells and skin fibroblasts, are the most susceptible cells that are affected after human beings come in direct contact with or ingest disinfectants. Results of the MTT assay suggested that juglone poses a low health risk to humans.

3.6 Effects of juglone on zebrafish embryo development

Rivers possess high biodiversity. In the rivers present even in the most densely populated urban regions, up to 30 fish species can be found. In addition, river water is one of the places where disinfectants can be most easily detected. Thus, to assess the aquatic toxicity and potential ecotoxicological effects of juglone, we selected zebrafish as the animal model because of the high similarity of its genome to that of humans (Zhang et al., 2011). Zebrafish were treated with 1 μmol/L, 5 μmol/L, 10 μmol/L, 25 μmol/L and 50 μmol/L of juglone and 1 μmol/L, 5 μmol/L, 10 μmol/L, 25 μmol/L, and 50 μmol/L of sodium hypochlorite (84 disinfectant) to observe whether the behavior of zebrafish was affected.

Among the experimental groups treated with 1 μmol/L, 5 μmol/L, and 10 μmol/L of juglone, we did not observe significant changes in behavioral parameters. However, at higher concentrations (25 μmol/L and 50 μmol/L), the behavior of zebrafish was affected.

Fig. 5 Expression level of RecA protein in E. coli cells. E. coli exposed to different compounds.

Fig. 6 Cytotoxicity of juglone and effects of juglone on zebrafish: (a) Cytotoxicity of HL-7702; (b) Cytotoxicity of HFF-2; (c) Swimming/still time ratio of zebrafish; (d) Average swimming speed of zebrafish; (e) Total distance of zebrafish; (f) Swimming freq of zebrafish.
swimming frequency, swimming/resting ratio, average swimming speed, and total distance decreased. The experimental groups treated with 25 μmol/L and 50 μmol/L juglone showed significant differences in the swimming frequency and swimming/resting ratio compared with the blank control group. However, zebrafish embryos treated with 1 μmol/L, 5 μmol/L, 10 μmol/L, 25 μmol/L, and 50 μmol/L sodium hypochlorite (84 disinfectant) had all died, and their body had been heavily decomposed, which may be related to the strong oxidative effect of sodium hypochlorite (84 disinfectant).

The experimental results showed that juglone had a sedative effect on zebrafish at high concentrations; however, determining the effect of juglone on zebrafish behavior at the MIC was challenging. Therefore, juglone can be considered to be less toxic to zebrafish at the MIC, which implies that juglone has low aquatic toxicity. The experiment to compare the effects of juglone and sodium hypochlorite (84 disinfectant) also showed that the aquatic toxicity of juglone was lower than that of sodium hypochlorite (84 disinfectant).

Juglone is an isomer of lawsone (2-hydroxy-1,4 naphthoquinone), which is a coloring agent and one of the oldest dyes used for dyeing hair throughout history (Nazari, 2017). A study showed that juglone may be used as a natural dye in the coloring of natural and synthetic fibers (Na et al.). Shells of walnut are an important source of juglone. Some reported uses of juglone include the preparation of skin-coloring agents (Kurz et al., 1996) and hair dyes (Boga et al., 2013). The walnut liquor containing juglone at concentrations of approximately 380 μg/mL was a popular drink in a certain area of East Europe (Jakopic et al., 2007). These results indicate that juglone, a natural dye, is not only safe for consumption but also has several medicinal effects. To date, no study has reported the poisoning effect of juglone with its widespread use as a hair dyeing agent or dyed products by humans.

Most importantly, the present study showed that juglone can produce excessive ROS. DNA damage was also observed in E. coli cells, and the enhancement of ROS production is one of the important mechanisms, leading to bacterial DNA damage. In addition, the inhibitory effect of juglone on RecA protein expression could reduce the probability of bacterial gene mutation, thus indirectly preventing the emergence of drug-resistant bacteria. Collectively, due to the strong antibacterial efficacy, low toxicity, and less allergenic properties of the naturally occurring 1,4-naphthoquinone, juglone, it has the potential to be used as a new bactericidal agent with low health risks and low ecotoxicity.

### 4 Conclusions

In this study, we synthesized a few natural naphthoquinones and derivatives bearing the 1,4-naphthoquinone skeleton and evaluated their antimicrobial activity against the selected bacterial strains. Among the tested compounds, juglone showed the strongest antibacterial activity against both E. coli and S. aureus. In the follow-up study, we found that juglone can cause oxidative damage to E. coli DNA by promoting ROS production. In addition, we found that juglone can effectively inhibit RecA protein expression in E. coli, thereby inhibiting SOS response and reducing the ability of E. coli to repair damaged DNA. The study findings related to the antibacterial targets of juglone may contribute to the therapeutic application of a combination of juglone and antibiotics against the diseases caused by MDR bacteria in clinical practice. Overall, the present study suggests that juglone is a strong antibacterial agent with low toxicity and less allergenic properties, and thus, its application as a bactericidal agent may reduce health risks and ecotoxicity.

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