

# Investigation of Antimicrobial, Antibiofilm, and Cytotoxic Effects of Straight-Chained Sulfanyl Members of Arylamino-1,4-naphthoquinones as Potential Antimicrobial Agents

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## ABSTRACT

**Objective:** Naphthoquinone derivatives are known to have antibacterial activity and are likely to succeed a new class of compound that can be applied as antimicrobial agents.

**Materials and Methods:** The purpose of this experiment was to evaluate the potential antimicrobial, antibiofilm, anticancer, and cytotoxic activities of six naphthoquinone compounds previously reported in the literature.

**Results:** According to our studies, 2-(4-(trifluoromethyl)phenylamino)-3-(propylthio)naphthalene-1,4-dione (5a) and 2-(4-(trifluoromethyl)phenylamino)-3-(pentylthio)naphthalene-1,4-dione (5b) were found to have good antimicrobial activity against *Staphylococcus aureus* ATCC 29213 with 1.22 and 19.53 µg/mL MIC values, respectively. When we carried out the test against biofilm, the most effective agent, 5a, showed up to 40% inhibition of the *S. aureus*'s biofilm at the 1 x MIC concentration. However, when we investigated the cytotoxic effect of 5a on the cancer and non-cancer cell lines, we found that 5a showed higher toxicity to cancer cell lines.

**Conclusion:** The findings of our study suggest that further studies to develop these compounds and investigate its pharmacological properties could be useful to define the functionality of them as antimicrobial or anticancer agents.

**Keywords:** Sulfanyl 1,4-naphthoquinone, Arylamine, Antimicrobial activity, Cytotoxicity, Biofilm

## INTRODUCTION

Discovering the potential of naphthoquinone compounds as antimicrobial agents increased the efforts towards synthesis new molecules and investigation of their antimicrobial properties by scientists (1, 2). Structure-

activity relationship studies performed to determine structural features or functional groups of the naphthoquinone derivatives that increase or decrease antimicrobial activity pointed out that the incorporation of substituted aromatic ring and sulphur (S) atom in the quinone skeleton was an important factor for enhancing



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the biological activities (3). The increase in the number of studies on this subject is not surprising. In this context, it is no surprise that recent studies contain these activity increasing substituents (4, 5). Recently Errante et al. reported that some thio derivatives of naphthoquinones exhibited better activities than amphotericin B against some fungi (6). In a previous study, phenylamino derivatives of naphthoquinone that also bear straight chain thiol groups in the structure as substituents were obtained. Infrared, NMR (<sup>1</sup>H, <sup>13</sup>C), and mass spectrometry were first used by Bayrak to identify their structures as original compounds (Figure 1) (7).

In the present study, we evaluated the potential antimicrobial, antibiofilm, and bactericidal efficacies of previously synthesized (7) thio derivatives of phenylamino-naphthoquinones against several pathogen microorganisms. Moreover, the cytotoxic activity of compound **5a** (which has the strongest antimicrobial activity in diverse cancer cell lines in comparison to non-cancerous cell lines) was examined.

## MATERIALS AND METHODS

### Microorganisms

The proposed routine quality control strains used in order to screen test performance with synthesized compounds in test panels are shown in Table 1. *Staphylococcus aureus* (ATCC 25923) was included in the experiment as a reference strain to confirm the biofilm forming bacteria to ensure antibiofilm activity of the

**Table 1.** The proposed routine quality control strains used to screen test performance with synthesized compounds in test panels.

Organisms	Culture Collection Numbers
<i>Escherichia coli</i>	ATCC 25922
<i>Staphylococcus aureus</i>	ATCC 29213
<i>Staphylococcus epidermidis</i>	ATCC 12228
<i>Enterococcus faecalis</i>	ATCC 29212
<i>Pseudomonas aeruginosa</i>	ATCC 27853
<i>Proteus mirabilis</i>	ATCC 14153
<i>Klebsiella pneumoniae</i>	ATCC 4352
<i>Candida albicans</i>	ATCC 10231
<i>Candida parapsilosis</i>	ATCC 22019
<i>Candida tropicalis</i>	ATCC 750

ATCC: American Type Culture Collection, 12301, Parklawn Drive, Rockville, MD 20852, USA.

compound. Inoculums of bacteria and yeasts were prepared with overnight cultures to cultivate a concentration of  $1 \times 10^8$  colony forming units (CFU/mL) and  $1 \times 10^7$  CFU/mL, respectively.

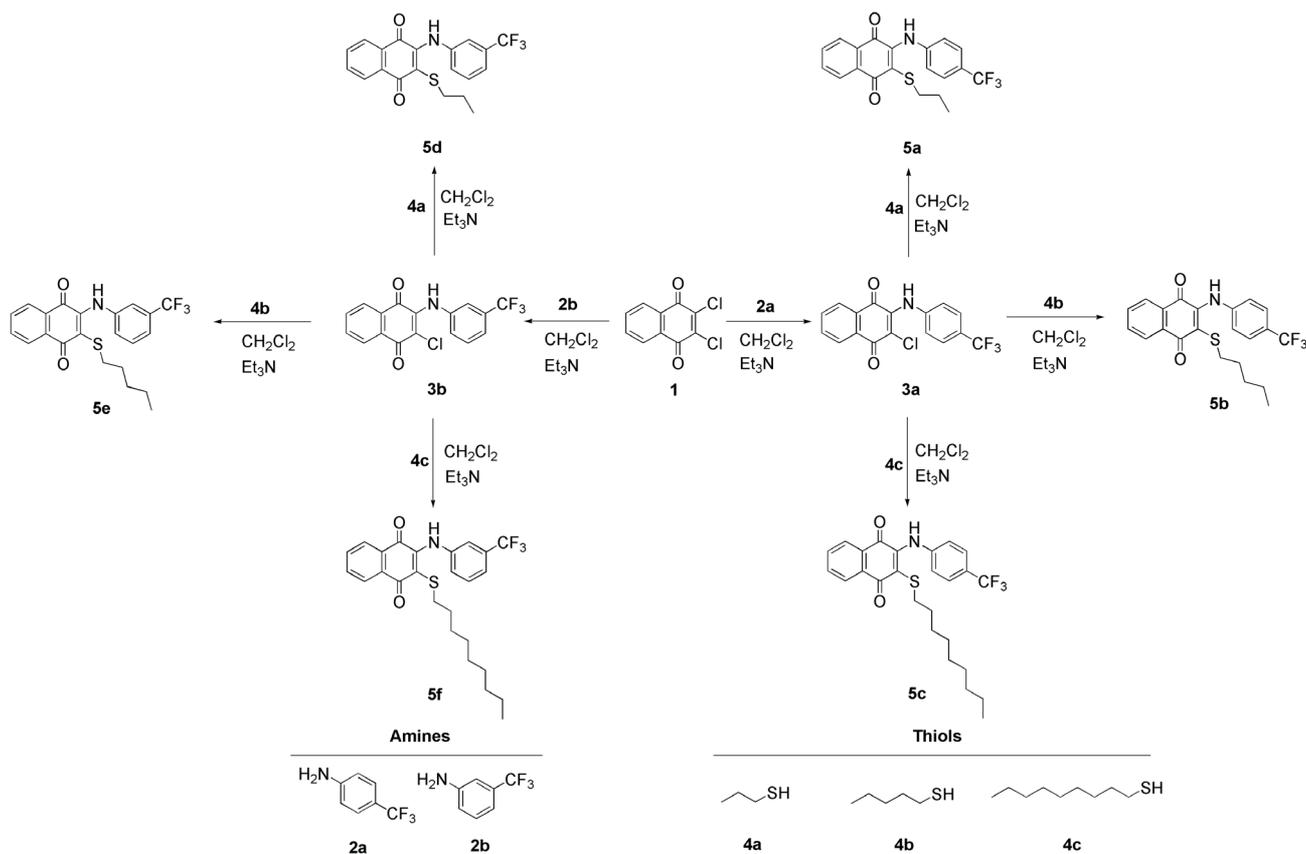


Figure 1. Straight-chained thio phenylamino-1,4-naphthoquinone derivatives.

## Media

Tryptic soy broth (TSB- Difco Laboratories) plus 1% glucose was used for the biofilm production and antibiofilm activities assays. Mueller-Hinton broth (MHB, Oxoid) was used to identify the minimum inhibitory concentration (MIC) and time-kill curve; and Tryptic Soy agar (TSA, Difco Laboratories) was used for vital growth colony counts.

## Antimicrobial Activity Assessment

### Minimum Inhibitory Concentration Assay

This assay comprises of the determination of the synthesized compounds' spectrum of antimicrobial susceptibility in compliance with the resistance of studied Gram positive/negative bacteria and yeasts by the CLSI broth microdilution reference method (8, 9). The MIC was defined as the lowest concentration of the molecules causing complete inhibition in visible growth. The antimicrobial effect of the solvents was determined as a control and the test results were evaluated accordingly.

The conclusion from the antimicrobial activity tests prompted our research to investigate in vitro antimicrobial activities of compound **5a** contrary to 20 clinically obtained strains of *Staphylococcus aureus* by the CLSI broth microdilution reference method (8).

### Antibiofilm Activity Assessment

The initial biofilm attachment assay and inhibition of biofilm formation tests were performed by using a slightly modified version of the method by Mataraci et al. (10) that was previously explained. 1/10 x MICs of the compound **5a** were appended to the 24 h biofilm and the plates were incubated for 1, 2 and 4 h for *S. aureus* ATCC 25923 (biofilm forming bacteria) at 37°C; molecules at 1x, 1/10x and 1/100 x MIC concentrations were appended to the 24 h biofilm and the wells were incubated for 24 h at 37 °C, respectively. Six wells were used for the tested compound. Sterile TSB-glucose was used for the positive controls. Then the plates were washed with PBS and evaluated at OD595 nm (BioTek EON Microplate Reader).

### Determination of Bactericidal Effects by Time-Kill Curves

Time-kill curve analyses were performed by culturing *S. aureus* ATCC 29213 in MHB medium, in the presence of **5a** at 1 x MIC. An assessment of the dynamic bactericidal activity of compound **5a** was made with the time-kill curve method by testing at 1x times the MIC against *S. aureus* ATCC 29213 as described previously. Solvent containing the control was included in the test for the tested strain. The inoculum was quantified spectrophotometrically and added to the flasks. The antimicrobial amount was checked by the inhibition of viable colony growth at the site of the initial inoculum in accordance with NCCLS recommendations (11). Bactericidal activity was described as a decrease of  $\geq 3 \log_{10}$  CFU/mL from the initial inoculum at 24 h.

### Cell Cultures

Three non-cancer cell lines were used in this study: Mouse embryonic fibroblast (BALB/3T3), human umbilical vein endothelial cell (HUVEC), and human keratinocyte (HaCaT). The three cancer cell lines used were human hepatocellular carcinoma (HepG2),

human neuroblastoma (SH-SY5Y), and human prostate cancer cell (PC-3). All cells were grown with DMEM medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic solution in a 37 °C, 5% CO<sub>2</sub> humidified incubator. The cells were passaged routinely at a confluence of 90% by trypsinization.

### Cell Treatments and Cytotoxicity Assay

For the cytotoxicity assay, the cells were seeded in 96 well plates 1x10<sup>4</sup> cells/well and incubated overnight for cell attachment. Subsequently, the media were replaced with fresh media and the cells were treated with compound final concentrations 100-6.25 µg/mL and vehicle control 1% DMSO for 24 h at 37 °C. After 24 hours, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The final volume of the 5 mg/mL MTT reagent was added to the wells and the plates were incubated in the dark for 3 h.

The media was then removed and the formazan precipitates were dissolved in 100 µL DMSO. Optical density was measured using a microplate reader (Biotek Instruments, Inc., Vermont, USA) at 590 nm. Cell viability was expressed as a percentage of the absorbance recorded for vehicle control.

### Statistical Analysis

All tests were performed in three independent assays. One-way ANOVA, Bonferroni's multiple comparison test was used to compare the differences between the control and compound-treated biofilms and time-kill kinetics. A *p* value < 0.001 was considered as statistically significant.

## RESULTS

### Molecules

In this study, we used six molecules (2-(4-(trifluoromethyl)phenylamino)-3-(propylthio)naphthalene-1,4-dione (**5a**), 2-(4-(trifluoromethyl)phenylamino)-3-(pentylthio)naphthalene-1,4-dione (**5b**), 2-(4-(trifluoromethyl)phenylamino)-3-(nonylthio)naphthalene-1,4-dione (**5c**), 2-(3-(trifluoromethyl)phenylamino)-3-(propylthio)naphthalene-1,4-dione (**5d**), 2-(3-(trifluoromethyl)phenylamino)-3-(pentylthio)naphthalene-1,4-dione (**5e**), 2-(3-(trifluoromethyl)phenylamino)-3-(nonylthio)naphthalene-1,4-dione (**5f**)) that had been previously synthesized by Bayrak (7). Bayrak reported that the thiol derivatives of phenylamino-1,4-naphthoquinones (**5a-f**) were obtained by a substitution reaction with phenylamino-chloro-1,4-naphthoquinone derivatives (**3a-b**) that synthesized by the chemical reaction of 2,3-dichloro-1,4-naphthoquinone with trifluoro substituted phenyl amines and the appropriate straight-chained thiol in dichloromethane were mixed at room temperature by applying Et<sub>3</sub>N (7). The reactions of 2-(4-(trifluoromethyl)phenylamino)-3-chloronaphthalene-1,4-dione (**3a**) and 2-(3-(trifluoromethyl)phenylamino)-3-chloronaphthalene-1,4-dione (**3b**) with aliphatic thiol compounds (propane, pentane, and nonane thiol) proceeded *via* the substitution of a chlorine atom with a sulfur atom to form phenylamino-1,4-naphthoquinones with straight chain thio group (**5a-f**). The structures of **5a-f** were also clarified by IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR, and MS data (Figure 1).

### Antimicrobial Activity

The *in vitro* antimicrobial activity of six thio phenylamino-1,4-naphthoquinone derivatives (**5a-f**) against three Gram-positive bacteria, four Gram-negative bacteria, and three fungi by the microbroth dilutions technique using the CLSI recommendations (8, 9).

The antimicrobial experiment results of all the six sulfanyl derivatives of phenylamino-1,4-naphthoquinone (**5a-f**) are given in Table 2. The test-cultures *E. coli*, *P. mirabilis*, and *K. pneumoniae* appeared to be resistant to the all synthesized compounds. None of the studied molecules showed any antibacterial activity against the Gram-negative bacteria except for **5f**. Concerning the antibacterial activity, the Gram-positive bacteria were more susceptible to the sulfanyl derivatives of phenylamino-1,4-naphthoquinone than the Gram-negative ones. Generally, the findings showed that some compounds displayed varying effects on the growth of the tested Gram-positive bacterial strains. The results showed that all thio-phenylamino-1,4-naphthoquinone derivatives exhibited antimicrobial activity against *S. aureus*. **5a** and **5b** showed good activity against *S. aureus* with an MIC value of 1.22 and 19.53 µg/mL, respectively. Notably, **5a** had the same inhibitory activity against *S. aureus* as that of Cefuroxime-Na (MIC = 1.22µg/mL). An evaluation of the antifungal activity of the thio- phenylamino-1,4-naphthoquinone derivatives exhibited no antifungal activity against *C. albicans*, *C. parapsilosis*, and *C. tropicalis* except **5d**. The **5d** was active analog against *C. tropicalis* (MIC = 312.5µg/mL) (Table 2). According to our results, **5a** was found active against the standard *S. aureus*, so we investigated the potential antimicrobial activity of this compound against 20 clinically obtained *Staphylococcus aureus* (Table 3). Susceptibility testing demonstrated that the MIC ranges for **5a** were 1250- >2500 µg/mL, for these clinically obtained strains.

### Antibiofilm Activities

Because of the its potent activity, only **5a** was used in the antibiofilm activities assays. When we carried out these tests, the agent inhibited the biofilm attachment according to time, and it showed an important inhibitor activity against biofilm formation at 24 h depending on concentration (Figure 2).

### Time-kill Kinetics

Time-kill kinetic studies showed that the naphthoquinone compounds used in this study displayed concentration-dependent bactericidal activity. When **5a** was used at 1 × MIC, bactericidal activity was not seen for the studied strain *S. aureus* ATCC 29213 at 24 h (Figure 3). However, in our study **5a** only showed approximately 2 log<sub>10</sub> reduction in bacterial cell count at the 1 × MIC concentration used.

### Cytotoxicity Assay

The cytotoxicity of **5a** was screened in three different non-cancer cell lines, mouse embryonic fibroblast (BALB/3T3), human umbilical vein endothelial cell (HUVEC), and human keratinocyte (HaCaT), together with three cancer cell lines, human hepatocellular carcinoma (HepG2), human neuroblastoma (SH-

**Table 2.** *In vitro* antimicrobial activity results of thio-phenylamino-1,4-naphthoquinone derivatives (5a-f).

ID	Gram-negative Bacteria (MIC, µg/mL)				Gram-positive Bacteria (MIC, µg/mL)			Yeast (MIC, µg/mL)		
	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. mirabilis</i>	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>E. faecalis</i>	<i>C. albicans</i>	<i>C. parapsilosis</i>	<i>C. tropicalis</i>
5e	-	-	-	-	625	1250	-	-	-	-
5f	625	-	-	-	1250	1250	-	-	-	-
5b	-	-	-	-	19.53	1250	-	-	-	-
5c	-	-	-	-	1250	-	-	-	-	-
5d	-	-	-	-	312.5	-	-	-	-	312.5
5a	-	-	-	-	1.22	1250	625	-	-	-
Reference	2.4	4.9	4.9	2.4	1.2	9.8	128	4.9	0.5	1
antimicrobials	Ceftazidime	Cefuroxime-Na	Cefuroxime-Na	Cefuroxime-Na	Cefuroxime-Na	Cefuroxime	Amikacin	Clotrimazole	Amphotericin B	Amphotericin B

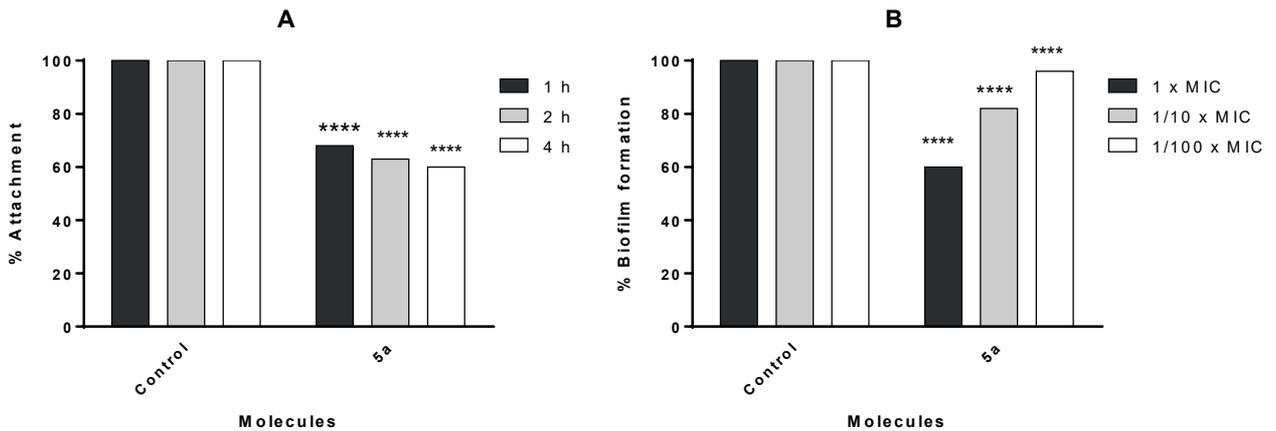


Figure 2. Inhibition of *S. aureus*. **A**: surface attachment to the wells contained 1/10 x MIC of molecule and an inoculum of  $1 \times 10^7$  CFU/200  $\mu$ l, incubated for 1, 2, or 4 h at 37°C; **B**: biofilm formation in each well contained 1 x, 1/10 x, or 1/100 x MIC of molecule and an inoculum of  $5 \times 10^5$  CFU/200  $\mu$ l, incubated for 24 h at 37°C. Control bars indicate bacterium without molecule accepted as 100%. Six wells were used for the tested molecule. Each experiment is representative of three independent tests. All differences between the control and molecule treated biofilms were statistically significant ( $p < 0.001$ ).

SY5Y), and human prostate cancer cell (PC-3). In all cell lines **5a** decreased cell proliferation significantly after 24 h. The most significant effect was seen in the HepG2 cell line with an  $IC_{50}$  value of 21.96  $\mu$ g/mL. **5a** showed a similar cytotoxic effect in the SH-SY5Y and PC-3 cells with  $IC_{50}$  values of 31.94  $\mu$ g/mL and 31.95  $\mu$ g/mL, respectively (Figure 4). **5a** had  $IC_{50}$  values of 60.72  $\mu$ g/mL, 5727  $\mu$ g/mL, and 38.8  $\mu$ g/mL against non-cancer cells, HaCaT, 3T3, and HUVEC cells respectively (Figure 5), implies that higher concentrations of compound exhibit toxicity to non-cancer cells.

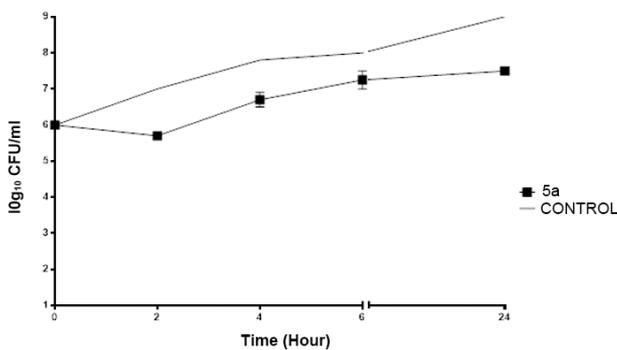


Figure 3. Time kill determinations for *S. aureus* ATCC 29213 strain after treatment with **5a** alone at 1 x MIC. The x-axis represents the killing time, and the y-axis represents the logarithmic *S. aureus* survival.

**Table 3.** *In vitro* activities of **5a** against 20 clinically obtained strains of *S.aureus*.

Molecule	Staphylococcus aureus		
	MIC range	MIC50	MIC90
5a	1250- >2500	1250	2500

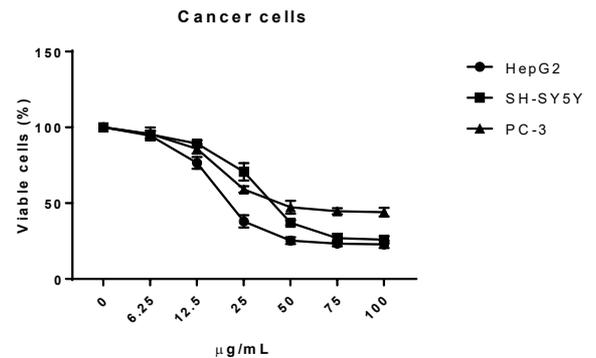


Figure 4. Cytotoxic effect of **5a** against HepG2, SH-SY5Y and PC-3 cells determined by MTT assay. Each point representing the mean of three separate biological experiments  $\pm$  S.D.

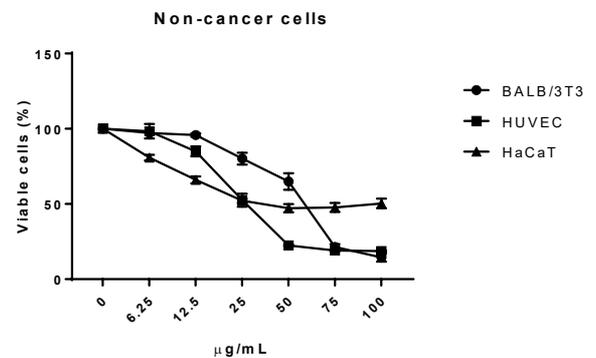


Figure 5. Cytotoxic effect of **5a** against BALB/3T3, HUVEC and HaCaT cells determined by MTT assay. Each point representing the mean of three separate biological experiments  $\pm$  S.D.

## DISCUSSION

The increase and spread of antimicrobial resistance among the various microorganisms is now one of the world's major health problems. Antibiotic resistance is increasing both in the community and in hospitals, multidrug resistant (MDR) and even pan resistant strains (resistant to all common antibiotic groups for therapeutic use), which lead to failure of antibiotic treatment, increased mortality and morbidity and have a huge increase on the cost of medical treatment and prevention of bacterial infectious diseases (12, 13). For these reasons, researchers have produced many synthetic or semi-synthetic molecules as candidates for new and beneficial drugs (13-15). The discovery of new antibacterial agents or multidrug agents for reversing is critical as we may not have any effective medicines to treat bacterial infections caused by the emerging superbugs that are resistant to the majority of clinically available antibiotics (16).

In our present study, the *in vitro* antimicrobial activities of known phenylamino derivatives of naphthoquinone having straight chain thiol groups were evaluated and two molecules (**5a** and **5b**) were found to have a potent antibacterial efficacy against *S.aureus* human-pathogenic strains and causes not only community acquired but also immortal nosocomial infections (10).

The communities of microorganisms attached to a surface were termed 'biofilm'. Bacteria within the biofilm require a 100 to 1000 times greater antibiotic concentration to achieve destruction versus planktonic bacteria. Standard intravenous therapy does not reach a high enough concentration to reduce the bacterial burden within the biofilm. It is a fact that this is the case for up to 60% of the infections which are usually associated with microorganisms that have settled in the microbial biofilms. Not all antimicrobial agents are the same in terms of biofilm eradication. The agent's mechanism of action, its interaction with the biofilm matrix and the effect of biofilm related parameters such as oxygen concentration, biofilm and growth rate should be considered (17-19). To this end, we researched the inhibition of surface bacterial adhesion and the inhibition of biofilm production by MIC or sub-MIC values of the **5a** compound. **5a** significantly inhibited the attachment of bacteria at 1/10 x MIC in 1-4 h and 24 h biofilm formation up to 40%, in particular at 1 x MIC ( $p < 0.001$ ). Although the inhibition of mature biofilm is very difficult, the inhibition of biofilm formation seems to be more applicable in early critical stages.

Although **5a** has limited bactericidal activity against *S. aureus* at 1 x MIC, it could be considered for future studies since its combination with antibiotics as an adjuvant could cause synergism, thus lowering **5a**'s potential toxic effects and preventing the development of resistance. Moreover, comparing the cytotoxic effect of the compound on non-cancer and cancer cell lines, we noticed that **5a** showed higher toxicity to cancer cell lines. Human hepatocellular cancer HepG2 cells in particular were the most effected cells. This result may suggest potential anticancer use against human hepatocellular cancer.

HaCaT cells are immortalized human keratinocytes and it is a useful model for studying dermal toxicity. **5a** showed the least toxicity to

the HaCaT cells and  $IC_{50}$  value was over 60  $\mu\text{g/mL}$ . Also, increasing doses of the compound did not cause more cytotoxic effect in the HaCaT cells. Additionally, the compound showed a 2-fold higher  $IC_{50}$  value to the mouse embryonic fibroblast BALB/3T3 cells than the cancer cells. These results may indicate potential antibacterial dermal use of the compound in low concentrations without causing significant dermal toxicity. Follow-up research is essential to understand the compound's mechanism of action and define detailed the activity-structure relationship.

## CONCLUSION

With respect to the antimicrobial, anticancer, and cytotoxic activities of the phenylamino derivatives of naphthoquinone that contain straight chain thiol groups in the structure as substituents which had been previously synthesized, further studies to establish their pharmacological properties would be helpful to define their functionality as antimicrobial or anticancer agents.

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**Conflict of Interest:** The authors declare that they have no conflicts of interest.

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