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Original article

# ANTIMICROBIAL ACTIVITY OF COMPOSITE PREPARATIONS BASED ON BIOSURFACTANTS AND HETEROCYCLIC AMINO-CONTAINING 1,4-NAPHTOQUINONE DERIVATIVES

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**Background.** The development of highly effective and environmentally safe composite drugs with antimicrobial properties is an important challenge in biotechnology and pharmacy.
**Objective.** To determine the antimicrobial activity of new composite preparations based on rhamnolipids combined with heterocyclic amine-containing derivatives of 1,4-naphthoquinone against the test-bacteria *Escherichia coli* B-906, *Staphylococcus aureus* 209-P, *Mycobacterium luteum* B-917, and fungi *Candida tenuis* UCM Y-70 and *Aspergillus niger* UCM F-1119.
**Methods.** The *in vitro* antimicrobial activity of heterocyclic amine-containing derivatives of 1,4-naphthoquinone, biosurfactants, and composite preparations based on them was tested against cultures of *Escherichia coli* B-906, *Staphylococcus aureus* 209-P, *Mycobacterium luteum* B-917, and fungi *Candida tenuis* UCM Y-70 and *Aspergillus niger* UCM F-1119 using the agar diffusion method on solid nutrient media, as well as the serial dilution method (minimum inhibitory and bactericidal concentrations). The toxicity of the heterocyclic amine-containing derivatives of 1,4-naphthoquinone was predicted using *in silico* methodology via the ProTox-II software.
**Results.** It was found that 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene-1,4-dione 3.2 and 2-chloro-3-((3-(*n*-tolyl)- 1H-pyrazol-5-yl) amino) naphthalene-1,4-dione 3.3 exhibit antimicrobial activity. Their composite preparations, due to the combination with rhamnolipids, demonstrated improved solubility and enhanced antimicrobial effects. Based on the results of the calculated assessment, the studied heterocyclic amine-containing derivatives of 1,4-naphthoquinone are predicted to fall into toxicity class IV.
**Conclusions.** The antimicrobial activity of 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene-1,4-dione 3.2 and 2-chloro-3-(3-( 3-(p-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione 3.3 is enhanced. This suggests the potential of the proposed biotechnological approaches in the development of new biocidal preparations.

**Keywords:** biosurfactants; rhamnolipids; amino-containing heterocyclic derivatives; 1,4-naphthoquinone; composite preparations; antibacterial activity.

## Introduction

It is known that excessive and unjustified use of antibiotics leads to an increase in the resistance to them of many pathogenic microorganisms. In fact, new modifications of infectious agents are emerging that cannot be cured by existing antimicrobial agents. Therefore, for now, the search for new antimicrobial drugs is an important area of research for scientists. Interesting and promising are 1,4-naphthoquinones, which consist of two ketone groups as vital chromophores and can donate or accept electrons in various biological systems. A high degree of bioavailability and significant distribution in living organisms contribute to their use as a basis for the development of effective medicines [1]. Known substances of natural and synthetic origin, which include a quinoid fragment. Among the natural compounds, the following antibiotics are known: kinamycin D, KD (1,4-benzo[b]carbazoloquinone cyanamide), (produced by *Streptomyces murayamaensis*), juglone derivatives: frenocillin and granaticin (produced by *Streptomyces roseofulvus* and *Streptomyces oliavaceus*), calafungin, nanaomycin, which are included in the group of antibiotics – derivatives of pyranonaphthoquinone griseusin A and B (produced by *Streptomyces griseus*) and others [2, 3]. One of the prominent representatives of 1,4-naphthoquinone derivatives is Daunorubicin, isolated from *Streptomyces peucetius*, which is used for the treatment of acute myeloid leukemia, and is also the basis for the synthesis of such quinoid com-

pounds as: doxorubicin, epirubicin, idarubicin, and zorubicin [4]. Taking into account the above, the purposeful obtaining of new compounds with antimicrobial activity – structural analogues of naphthoquinone will be a significant contribution to solving the problem of antibiotic resistance. However, the low solubility in water complicates the use of naphthoquinones. Biosurfactants are surface-active substances of microbial origin, low toxicity, with stable physicochemical properties and the ability to increase the permeability of cell membranes and enhance the effect of other substances when used together [5–7]. In compositions with biologically active compounds, biosurfactants have the ability to improve their water solubility, bioavailability and enhance their effect [8–10]. There are known studies of compositions of rhamnolipids with antimicrobial agents. In combination with nisin, rhamnolipids demonstrate a synergistic effect and demonstrate antimicrobial activity against *L. monocytogenes* [9]. The literature describes a therapeutic composition containing syringopeptin and rhamnolipid, which act synergistically, have antibacterial, antifungal, and antitumor activity and may be a promising antimicrobial or anticancer agent [10]. Today, there is no information about compositions of rhamnolipids with pyrazole or 1,2,3-triazine derivatives of 1,4-naphthoquinone with antimicrobial effect. Therefore, obtaining and researching such objects is quite promising. The main purpose of the combined use of synthetic naphthoquinone derivatives and biosurfactants is to improve solubility in water and reduce the dose (inhibitory concentration) of the drug. New composite preparations based on biosurfactants and biologically active substances are potential means that have high activity against harmful microorganisms, are available, relatively inexpensive and safe for the environment.

## Materials and Methods

The work uses rhamnolipids RL – products of microbial synthesis of *Pseudomonas* sp. PS-17 were obtained at the Department of Physical Chemistry of Fossil Fuels of the Institute of Physical-Organic Chemistry and Coal Chemistry named after L.M. Lytvynenko of the National Academy of Sciences of Ukraine and amine-containing heterocyclic derivatives of 1,4-naphthoquinone: pyrazolo-, 1,2,4-triazino- and 1,2,4-triazole-containing derivatives of 1,4-naphthoquinone (3.1-3.14), the preparation of which is described in previous works [9–11]. Synthesis scheme of heterocyclic amine-containing derivatives of 1,4-naphthoquinone (Figure).

Composite preparations based on rhamnolipids with heterocyclic amine-containing derivatives of 1,4-naphthoquinone were developed, which demonstrated the best activity against the studied test cultures of bacteria and fungi, and their antimicrobial activity was investigated.

*Toxicity studies in silico*

Toxicity prediction is an integral element in the design of newly synthesized compounds. In our work, the software ProTox-II was used, which is available at the web link https://tox-new.charite.de/protox_II. With the help of this program, the class of toxicity according to the average lethal dose $LD_{50}$, hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, immunotoxicity of the studied compounds was determined.

*Study of antibacterial and antifungal activity*

Biosurfactants – rhamnolipids (RL), amino-containing derivatives of 1,4-naphthoquinone (3.1-3.14) and composite preparations based on them (3.2-RL, 3.3-RL) were evaluted for their antibacterial and antifungal activity using diffusion method and serial dilution method at the Department of Technology of Biologically Active Compounds, Pharmacy and Biotechnology of the Lviv Polytechnic National University. Research was conducted on test cultures of bacteria: *Escherichia coli* B-906, *Staphylococcus aureus* 209-P, *Mycobacterium luteum* B-917, *Candida tenuis* UCM Y-70, *Aspergillus niger* UCM F-1119. Reagents: Vancomycin hydrochloride, Nystatin were obtained from Sigma Aldrich (St Louis, MO, USA).

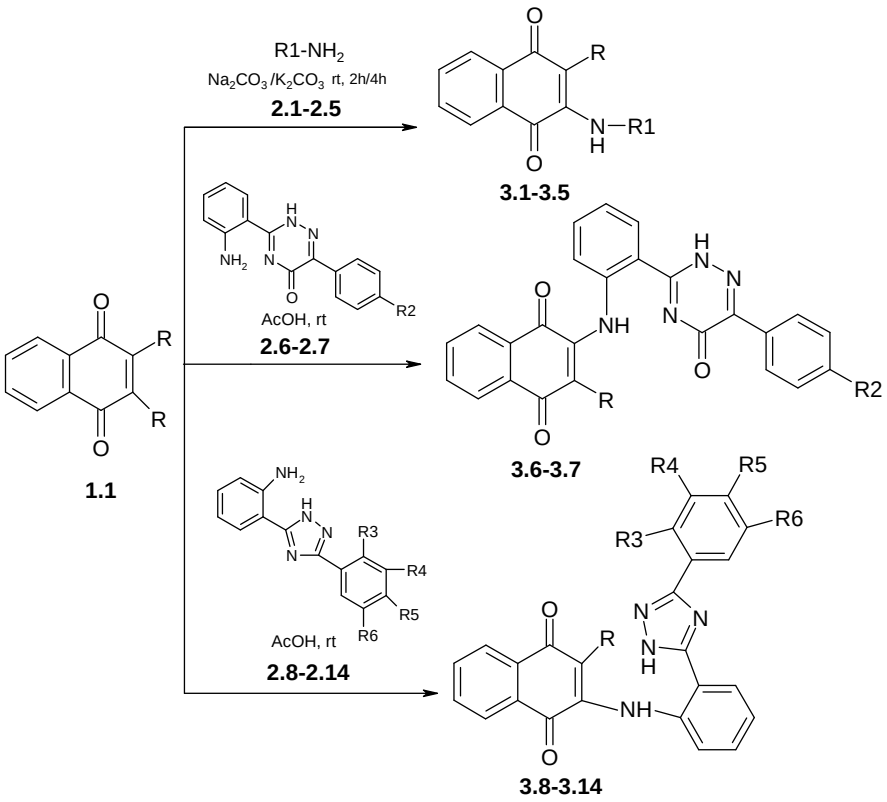
These are standard test microorganisms used to evaluate the antimicrobial activity of drugs (considered opportunistic).

Antibacterial substance vancomycin and antifungal substance nystatin were used as a reference control for comparative evaluation of the action of the studied compounds.

*Method* 1. *Determination of antimicrobial activity by the method of substance diffusion in agar*

Antimicrobial activity was studied by diffusion in agar on a solid nutrient medium (meat peptone agar for bacteria, wort agar for fungi). Petri dishes containing 20 ml of nutrient medium were used for all studied microorganisms. The microbial load was $10^9$ CFU in 1 mL (to make a suspension of bacteria, a turbidity standard of 0.5 according to McFarland was used; the counting of fungal cells (spores) was carried out in the Hemocytometer). The duration of incubation was 24 hours at 35 °C

for bacteria and 48–72 hours at 28–30 °C for fungi. The antimicrobial effect and the degree of activity of the studied substances were evaluated by measuring the diameters of the zones of inhibition of the growth of microorganisms. Each experiment was repeated three times. The degree of activity of the studied compounds was assessed by the diameter of the zones of inhibition of the growth of test cultures of microorganisms, considering that with a diameter of 11–15 mm, the microorganism is not very sensitive to the drug, with a diameter of 16–25 mm, it is sensitive, and with a diameter of more than 25 mm, it is highly sensitive.



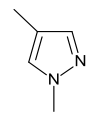
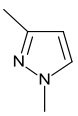
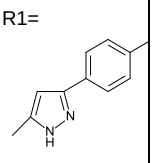
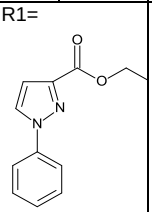
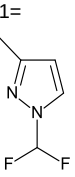

| 1.1 | 1.2 | 2.1 | 3.1 | 2.2 | 3.2 | 2.3 | 3.3 | 2.4 | 3.4 | 2.5 | 3.5 | 2.6 | 3.6 | 2.7 | 3.7 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| R=Cl | R=H | | R=Cl | | R=Cl | | R=Cl | | R=Cl | | R=Cl | | R=H | | R=H |
| | | R1=  | | R1=  | | R1=  | | R1=  | | R1=  | | R2=F | | R2=CH(CH3)2 | |
| **2.8** | **3.8** | **2.9** | **3.9** | **2.10** | **3.10** | **2.11** | **3.11** | **2.12** | **3.12** | **2.13** | **3.13** | **2.14** | **3.14** | | |
| | R=H | | R=H | | R=H | | R=H | | R=H | | R=H | | R=H | | |
| R3=H R4=F R5=H R6=H | | R3=F R4=H R5=H R6=H | | R3=Cl R4=H R5=H R6=H | | R3=Br R4=H R5=H R6=H | | R3=H R4=Br R5=H R6=H | | R3=H R4=H R5=H R6=OCH3 | | R3=H R4=H R5=OCH3 R6=H | | | |

**Figure:** Synthesis scheme of heterocyclic amine-containing derivatives of 1,4-naphthoquinone

*Method 2. Determination of antimicrobial activity by the method of serial dilutions*

The minimum inhibitory (MIC), bactericidal (MBC) and fungicidal (MFC) concentration was determined by the method of serial dilutions of the substance in a liquid nutrient medium (meat-peptone broth for bacteria and unhopped beer wort 6-80B for fungi) in the range of 0.9–500 μg/ml using a previously prepared working solution of the compound in DMSO at a concentration of 10,000 μg/ml. The culture medium was inoculated with inoculum of bacteria and fungi (microbial load of $10^6$ CFU per 1 ml, using a McFarland turbidity standard of 0.5). The inoculated tubes were kept in a thermostat at the appropriate temperature (37 °C for bacteria, 30 °C for fungi) for 24–72 hours The results were evaluated by the presence or absence of the growth of microorganisms, performing visual control in transmitted light, comparing the degree of microbial turbidity of the nutrient medium with the "negative control". To determine the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) from test tubes, in which the medium solutions turned out to be visually transparent, 0.02 ml of the medium were taken and sterile MPA (for bacteria) or CA (for fungi) were applied in sterile Petri dishes, which were incubated in a thermostat. The results were evaluated for test bacteria after 24 hours, for test fungi after 48–72 hours. Based on the lack of growth of colonies of microorganisms on the incubated Petri dishes, the MBC of the substance under study was determined. https://www.nelsonlabs.com/testing/mic-mbc/.

*Statistical analysis*

Each experiment was repeated three times. Statistical processing of experimental data was carried out using the Microsoft Excel 2010 software package, determining the arithmetic mean, standard error. In addition, the differences between the experimental data were statistically analyzed using the Statistica software package version 12.0 (StatSoft Inc., Tulsa, OK, USA). Differences were considered statistically significant at $p < 0.05$.

## Results

To predict the toxicity of synthesized compounds, which is an important indicator for the introduction of new drugs, *in silico* modeling of acute toxicity was carried out in the modern ProTox-II software [12]. The results are presented in Table 1.

**Table 1:** Prediction of the toxicity of substances

| Compounds | Oral toxicity* | | | Prediction: active, probability of 1 | | | | |
|---|---|---|---|---|---|---|---|---|
| | Toxicity Index | $LD_{50}$, mg/kg | Toxicity prediction, % | Hepato-toxicity | Carcino-genicity | Immuno-toxicity | Muta-genicity | Cyto-toxicity |
| **3.1** | 4 | 1260 | 54.26 | 0.51 | 0.60 | 0.90 | 0.68 | 0.59 |
| **3.2** | 6 | 11700 | 54.26 | 0.60 | 0.58 | 0.78 | 0.59 | 0.58 |
| **3.3** | 4 | 560 | 54.26 | 0.61 | 0.58 | 0.89 | 0.52 | 0.62 |
| **3.4** | 4 | 800 | 54.26 | 0.56 | 0.64 | 0.81 | 0.50 | 0.57 |
| **3.5** | 6 | 11700 | 54.26 | 0.64 | 0.56 | 0.88 | 0,58 | 0.68 |
| **3.6** | 4 | 2000 | 54.26 | 0.66 | 0.55 | 0.63 | 0.61 | 0.79 |
| **3.7** | 4 | 2000 | 54.26 | 0.57 | 0.50 | 0.64 | 0.60 | 0.83 |
| **3.8** | 4 | 1000 | 54.26 | 0.68 | 0.53 | 0.59 | 0.54 | 0.70 |
| **3.9** | 4 | 1000 | 54.26 | 0.68 | 0.53 | 0.90 | 0.54 | 0.70 |
| **3.10** | 4 | 1000 | 54.26 | 0.64 | 0.57 | 0.95 | 0.50 | 0.63 |
| **3.11** | 4 | 1000 | 54.26 | 0.66 | 0.54 | 0.84 | 0.53 | 0.54 |
| **3.12** | 4 | 1000 | 54.26 | 0.66 | 0.54 | 0.54 | 0.53 | 0.54 |
| **3.13** | 4 | 1000 | 67.38 | 0.63 | 0.50 | 0.55 | 0.62 | 0.55 |
| **3.14** | 4 | 1000 | 54.26 | 0.63 | 0.50 | 0.88 | 0.62 | 0.55 |
| **Griseusin A** | 4 | 620 | 72.35 | 0.65 | 0.50 | 0.99 | 0.67 | 0.68 |
| **Granaticin** | 5 | 5000 | 68.07 | 0.66 | 0.59 | 0.93 | 0.60 | 0.72 |
| **Ampicillin** | 5 | 5000 | 100.00 | 0.87 | 0.83 | 0.98 | 0.94 | 0.60 |
| **Fluconazole** | 4 | 1271 | 100.00 | 0.84 | 0.62 | 0.83 | 0.52 | 0.76 |

*Notes.* * – Class I: fatal if swallowed ($LD_{50} \le 5$); Class II: fatal if swallowed ($5 < LD_{50} \le 50$); Class III: Toxic if swallowed ($50 < LD_{50} \le 300$); Class IV: harmful if swallowed ($300 < LD_{50} \le 2000$); Class V: may be harmful if swallowed ($2000 < LD_{50} \le 5000$); Class VI: not toxic ($LD_{50} > 5000$).

As can be seen from the results, the compounds 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene1,4-dione 3.2 and 2-chloro-3-((1-(difluoromethyl)-1-*H*-pyrazol-3-yl)-amino)-naphthalene-1,4-dione 3.5 belong to the VI class of toxicity ($LD_{50}$ = 11700 mg/kg). The rest of the studied compounds belong to the IV toxicity class, which also indicates the possibility of their further pharmacological research. According to the prediction results, the hepatotoxicity (immunotoxicity, cytotoxicity) and carcinogenicity (mutagenicity) of amine-containing heterocyclic derivatives of 1,4-naphthoquinone are similar to natural quinones (grizeusin A and the juglone derivative granaticin), as well as to the well-known antimicrobial drugs ampicillin and fluconazole.

*Study of the antimicrobial activity of amine-containing heterocyclic derivatives of 1,4-naphthoquinone*

For the development of composite drugs, we searched for leader compounds among the studied amine-containing heterocyclic derivatives of 1,4-naphthoquinone. For this, new pyrazole-containing, 1,2,4-triazino- and 1,2,4-triazole-containing derivatives of 1,4-naphthoquinone were selected and their effect on bacteria *E. coli*, *S. aureus*, *M. luteum* and fungi *C. tenuis*, *A. niger* was investigated. The main results of the study of their antimicrobial activity by the method of serial dilutions are shown in Table 2.

When analyzing the antimicrobial activity data of the synthesized amine-containing heterocyclic derivatives of 1,4-naphthoquinone 3.1-3.14, it was established that the pyrazole derivatives 3.1-3.5 exhibit antimicrobial activity, as evidenced by the data (Table 2). It was fiund that *E. coli* (gram-negative) bacteria is resistant to the action of all synthesized compounds in the studied concentration. Compounds 3.2, 3.3, and 3.5 demonstrated the highest activity against *S. aureus*. The most active compounds against *C. tenuis*, determined by the method of serial dilutions, are 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene-1,4-dione 3.1 (MIC = 7.8 μg/ml, MBC = 15.6 μg/ml), 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene-1,4-dione 3.2 (MIC = 15.6 μg/ml, MFC = 62.5 μg/ml) and 2-chloro-3-((3-(n-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione 3.3 (MIC = 0.9 μg/ml, MFC = 1.9 μg/ml). It was also found that all investigated aminopyrazole heterocyclic derivatives of 1,4-naphthoquinone 3.1-3.5 demonstrated high activity against *M. luteum* bacteria. Studies by the method of serial dilutions showed

**Table 2:** Antimicrobial activity of pyrazolo-, 1,2,4-triazino- and 1,2,4-triazole-containing derivatives of 1,4-naphthoquinone (3.1-3.14) by serial dilutions method (Method 1)

| Compounds | *Escherichia coli* | | *Staphylococcus aureus* | | *Mycobacterium luteum* | | *Candida tenuis* | | *Aspergillus niger* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MIC, μg/ml | MBC, μg/ml | MIC, μg/ml | MBC, μg/ml | MIC, μg/ml | MBC, μg/ml | MIC, μg/ml | MFC, μg/ml | MIC, μg/ml | MFC, μg/ml |
| 3.1 | – | – | 125.0 | 250.0 | 7.8 | 125.0 | 15.6 | 31.2 | 62.5 | - |
| 3.2 | 500.0 | – | 15.6 | 62.5 | 7.8 | 15.6 | 15.6 | 62.5 | 250.0 | 500.0 |
| 3.3 | – | – | 7.8 | 15.6 | 7.8 | 15.6 | <0.9 | <0.9 | 31.2 | 62.5 |
| 3.4 | – | – | 125.0 | 250.0 | 15.6 | 31.2 | 250.0 | 500.0 | 62.5 | 250.0 |
| 3.5 | 250.0 | – | 7.8 | 15.6 | 15.6 | 31.2 | 250.0 | 500.0 | 250.0 | – |
| 3.6 | – | – | – | – | 125.0 | 250.0 | 250.0 | 500.0 | – | – |
| 3.7 | – | – | – | – | – | – | – | – | – | – |
| 3.8 | 500.0 | – | 125.0 | 250.0 | – | – | 250.0 | 500.0 | – | – |
| 3.9 | – | – | – | + | – | – | 31.2 | 62.5 | – | – |
| 3.10 | – | – | 250.0 | 500.0 | – | – | – | – | – | – |
| 3.11 | – | – | 250.0 | 500.0 | – | – | – | – | – | – |
| 3.12 | – | – | – | – | – | – | – | – | – | – |
| 3.13 | – | – | 250.0 | 500.0 | 125.0 | 250.0 | 125.0 | 250.0 | – | – |
| 3.14 | – | – | – | – | – | – | 125.0 | 250.0 | – | – |

*Notes.* "–" – no antimicrobial effect was observed in the tested concentrations.

that *A. niger* are insensitive to 2-[2-(6-aryl)-5-oxo-2,5-dihydro-1,2,4-triazin-3-yl)phenyl)amino] naphthalene-1,4-diones and 2-[2-(3-aryl-1H-1,2,4-triazol-5-yl)phenyl)amino]-naphthalene-1,4-diones 3.6-3.7, 3.8-3.14 in the range of studied concentrations (Table 2).

Insignificant activity of compounds 3.8, 3.10, 3.11 and 3.13 against *S. aureus* was determined by the method of serial dilutions: for compound 3.8 MIC = 125.0 μg/ml, MBC = 250 μg/ml, and for 3.10, 3.11, and 3.13 – MIC = 250.0 μg/ml, MBC = 500.0 μg/ml. It was found that compounds 3.6 and 3.7 show little activity against *M. luteum* – MIC = 125.0 μg/ml, MBC = 250.0 μg/ml, on the other hand, 3.7 and 3.8 did not affect the growth of cultures of *C. tenuis*, *M. luteum* and *A. niger*. The moderate activity of compound 3.9 against *C. tenuis* was determined by the method of serial dilutions – MIC = 31.2 μg/ml, MFC = 62.5 μg/ml, 3.6, 3.8, 3.13 and 3.14 also demonstrated little activity against *C. tenuis*.

Thus, it was established that only part of the pyrazole-containing derivatives of 1,4-naphthoquinone showed antimicrobial activity. The obtained results indicate the selective bacteriostatic and fungistatic activity of the synthesized compounds. Preparations based on 1,2,4-triazine and 1,2,4-triazole derivatives of 1,4-naphthoquinone did not demonstrate significant antimicrobial activity against the selected test cultures.

The lead compounds 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene-1,4-dione 3.2 and 2-chloro-3-((3-(*p*-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione 3.3, which showed bacteriostatic and fungistatic activity against selected microorganisms, which were used to form composite preparations with biosurfactants – rhamnolipids.

*Study of the antimicrobial activity of composite preparations based on rhamnolipids and amine-containing derivatives of 1,4-naphthoquinone*

The main idea of the combined use of biosurfactants with new amine-containing heterocyclic derivatives of 1,4-naphthoquinone is to partially increase their solubility, thus reducing the dose. The antimicrobial activity of amino-containing derivatives of 1,4-naphthoquinone, rhamnolipids and composite preparations based on them was determined against the bacteria *E. coli*, *S. aureus*, *M. luteum* and the fungi *C. tenuis*, *A. niger* by the method of diffusion in agar (method 1) and by the method of serial dilutions (method 2). The antimicrobial activity of aminopyrazole, 1,2,4-triazine and 1,2,4-triazole derivatives of 1,4-naphthoquinone was determined by the method of diffusion in agar (method 1) and is presented in Table 3.

It was determined that *E. coli* are resistant to the action of both composite drugs RL-3.2, RL-3.3, and rhamnolipids and compounds 3.2, 3.3 in the studied concentration. Activities against *S. aureus*

**Table 3:** Antimicrobial activity of synthesized compounds and their composite preparations with rhamnolipids by the diffusion method in agar (method 1)

| Compounds | Concentration, % | The diameter of the zones of inhibition of growth of microorganisms, mm; Mean ± SD | | | | |
|---|---|---|---|---|---|---|
| | | *Escherichia coli* | *Staphylococcus aureus* | *Mycobacterium luteum* | *Candida tenuis* | *Aspergillus niger* |
| RL-3.2 | 0.25:0.25 | 0 | 20.0 ± 0.2 | 18.0 ± 0.3 | 0 | 18.0 ± 0.2 |
| | 0.05:0.05 | 0 | 18.0 ± 0.2 | 12.0 ± 0.2 | 0 | 0 |
| RL | 0.5 | 0 | 0 | 12.0 ± 0.3 | 0 | 0 |
| | 0.1 | 0 | 0 | 0 | 0 | 0 |
| 3.2 | 0.5 | 0 | 20.0 ± 0.2 | 17.0 ± 0.2 | 21.0 ± 0.4 | 7.0 ± 0.2 |
| | 0.1 | 0 | 18.0 ± 0.2 | 12.0 ± 0.2 | 0 | 0 |
| 3.3 | 0.5 | 0 | 20.0 ± 0.3 | 25.0 ± 0.2 | >40.0 | 12.0 ± 0.4 |
| | 0.1 | 0 | 12.0 ± 0.2 | 10.0 ± 0.3 | >40.0 | 0 |
| RL-3.3 2:1 | 0.5:0.25 | 0 | 10.0 ± 0.2 | 17.0 ± 0.2 | 10.0 ± 0.4 | 10.0 ± 0.2 |
| | 0.1:0.05 | 0 | 0 | 9.0 ± 0.2 | 10.0 ± 0.4 | 0 |
| RL-3.3 1:1 | 0.5:0.5 | 0 | 15.0 ± 0.3 | 20.0 ± 0.2 | >40.0 | 11.0 ± 0.2 |
| | 0.1:0.1 | 0 | 0 | 12.0 ± 0.3 | >40.0 | 0 |
| Vancomycin | 0.1 | 14.0 ± 0.3 | 15.0 ± 0.2 | 18.0 ± 0.2 | – | – |
| Nystatin | 0.1 | – | – | – | 19.0 ± 0.4 | 20.0 ± 0.2 |

*Notes.* "–" – the study was not conducted; all analyses were carried out in triplicate, and results are reported as the mean ± standard deviation (SD).

at 0.5% and 0.1% 3.2 20.0 mm and 18.0 mm, respectively, for 3.3 20.0 mm and 12.0 mm, for rhamnolipids at these concentrations not established, growth retardation diameters 20.0 and 18.0 mm of the composite preparation RL-3.2, 15.0 mm for RL-3.3 (1:1) in a concentration of 0.5%:0.5%. When compared with the drug Vancomycin (15.0 mm), RL-3.2 both at 0.5% and 0.1% shows moderate activity against staphylococcus. The highest sensitivity of 2-chloro-3-((3-(*p*-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione 3.3 and the composite preparation with RL-3.3 (1:1) was established with respect to *C. tenuis*, diameters of growth retardation in given concentrations of 0.5% and 0.1% > 40.0 mm, which is greater than for the comparator drug Nystatin (19.0 mm) and indicates their promise as a fungicidal agent.

Antimicrobial activity of rhamnolipids against *E. coli*, *S. aureus* and *C. tenuis* was not detected, however, rhamnolipids are active against *M. luteum* – MIC = 62.5 μg/ml and MBC = 125.0 μg/ml, respectively, and for *A. niger* MIC = 250.0 μg/ml, MFC was not detected (Table 4). Antimicrobial activity against *M. luteum* for the composition of RL-3.3 rhamnolipids and 2-chloro-3-(3-(3-(*p*-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione 3.3 (ratio 1:1) was: MIC = 7.8:7.8 μg/ml and MBC = 15.6:15.6 μg/ml and is better than Vancomycin (comparator); against *A. niger* fungus for individual compound 3.3 it was: MIC = 31.2 μg/ml and MFC = 62.5 μg/ml; for composition RL-3.3 in a ratio of 1:1 it was: MIC = 15.6:7.8 μg/ml and MFC = 62.5:31.2 μg/ml (below the control). In the ratio (2:1) for RL-3.3: MIC = 7.8:7.8 μg/ml and MFC = 15.6:15.6 μg/ml. This indicates an increase in activity in the composition. The composite RL-3.2 demonstrates a high antimicrobial effect against *M. luteum* (MIC = 15.6:15.6 μg/ml and MBC = 31.2:31.2 μg/ml). 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene-1,4-dione 3.2 demonstrates little activity against *A. niger* (MIC = 250.0 μg/ml and MFC = 0), however, in the composition with rhamnolipids, the activity improves, as indicated by MIC = 62.5:62.5 μg/ml and MFC = 250.0:250.0 μg/ml. So, it was found that the antimicrobial activity of composite preparations of 2-chloro-3-(3-(3-(*p*-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione 3.3 and of 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene1,4-dione 3.2 with rhamnolipids increased compared to synthesized drugs. In addition, the composite preparation RL-3.3(2:1) was distinguished by the best indicators of antifungal activity against the test culture *C. tenuis* (MIC < 0.9:0.9, MFC < 0.9:0.9 μg/ml), than the comparator Nystatin, indicating its potential for further study as an antifungal agent. The obtained results indicate the need for further research into the antimicrobial potential of composite drugs RL-3.2 and RL-3.3.

**Table 4:** Indicators of minimum inhibitory (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentrations (MFC) of compounds by the method of serial dilutions

| Compound | *Escherichia coli* | | *Staphylococcus aureus* | | *Mycobacterium luteum* | | *Candida tenuis* | | *Aspergillus niger* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MIC, μg/ml | MBC, μg/ml | MIC, μg/ml | MBC, μg/ml | MIC, μg/ml | MBC, μg/ml | MIC, μg/ml | MBC, μg/ml | MIC, μg/ml | MFC, μg/ml |
| RL-3.2 | – | – | 62.5: 62.5 | 125.0: 125.0 | 15.6: 15.6 | 31.2: 31.2 | – | – | 62.5: 62.5 | 250.0: 250.0 |
| RL | – | – | – | – | 62.5 | 125.0 | – | – | 250.0 | – |
| 3.2 | 500.0 | – | 15.6 | 62.5 | 7.8 | 15.6 | 15.6 | 62.5 | 250.0 | 500.0 |
| 3.3 | – | – | 7.8 | 15.6 | 7.8 | 15.6 | <0.9 | <0.9 | 31.2 | 62.5 |
| RL-3.3 1:1 | 500.0: 500.0 | – | 31.2: 31.2 | 62.5: 62.5 | 7.8: 7.8 | 15.6: 15.6 | 3.9:1.9 | 250.0: 125.0 | 15.6: 7.8 | 62.5: 31.2 |
| RL-3.3 2:1 | – | – | 62.5: 31.2 | 125.0: 62.5 | 15.6: 7.8 | 31.2: 15.6 | <0.9: 0.9 | <0.9: 0.9 | 7.8:7.8 | 15.6: 15.6 |
| Vancomycin | 3.9 | 31.2 | 7.8 | 31.2 | 7.8 | 31.2 | – | – | – | – |
| Nystatin | – | – | – | – | – | – | 15.6 | 31.2 | 3.9 | 31.2 |

*Notes.* "+" – in the tested concentrations (0.9–500 μg/ml) no biocidal effect was observed (microorganism growth was observed); "*" – in the studied concentrations (0.9–500 μg/ml) indicators of the biocidal effect were not established; "–" – the study was not conducted.

## Discussion

Acute toxicity, which is determined in various ways, is an important characteristic for determining the prospects of new antimicrobial drugs. Toxicity modeling using the modern ProTox 3.0 web service was used as the most appropriate approach [14]. Acute oral toxicity prediction results are based on the analysis and recognition of toxic fragments of 38,000 unique compounds with known oral $LD_{50}$ values determined for rodents. ProTox 3.0 includes molecular similarity (CLUSTER cross-validation based on fragment similarity) based on 61 models for predicting toxicity indicators: acute toxicity, hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, immunotoxicity [14]. In addition, the toxicity class of the compound is determined from I to VI (according to the Globally Harmonized System of Classification of Chemical Labeling, GHS, United Nations, First Revised Edition, 2005). Thus, the ProTox-II hepatotoxicity prediction model has an accuracy of about 80.00% [15]. It was determined that compounds 3.2 and 3.5 belong to the VI class of toxicity ($LD_{50}$ = 11700 mg/kg), and the rest belong to the classes V-IV. Hepatotoxicity (immunotoxicity, cytotoxicity) and carcinogenicity (mutagenicity) have been predicted for amine-containing heterocyclic derivatives of 1,4-naphthoquinone, natural quinones (griseusin A and granaticin), ampicillin and fluconazole. It was established that compounds 3.1-3.14 have similar results to widely used drugs, which indicates the expediency of their further pharmacological studies.

The antimicrobial activity of the obtained composite preparations of heterocyclic derivatives of aminopyrazole 1,4-naphthoquinone with rhamnolipids was determined. Biosurfactants – products of microbial synthesis – have practical potential due to special physico-chemical and biological properties, in particular, the effect on the permeability of cell membranes [13]. Therefore, composite preparations of biosurfactants with biologically active compounds can increase their effectiveness.

Compositions of biologically active compounds with biosurfactants that have a fungicidal effect are known from literary sources [17–19]. The addition of biosurfactants to the composition with thiosulfonates contributed to the reduction of the minimum inhibitory and biocidal concentrations of thiosulfonates: for example, when acting on *M. luteum*, the MIC is reduced by 50%, the MIC is reduced by 4 times, which is explained by the increase in the permeability of the cell membranes of microorganisms under the action of biosurfactants [14]. Compositions of allylthiosulfanilate and methylthiosulfanilate with a rhamnolipid biocomplex have a higher antimicrobial effect against *A. tumefaciens* and *C. michiganensis* microorganisms compared to thiosulfonates [15]. In our previous works, composite preparations of biosurfactants (rhamnolipids and trehalolipids) with aminopyrazole and 1,2,4-triazine-, 1,2,4-triazole derivatives of 1,4-naphthoquinone were obtained and their anticonvulsant activity was investigated [11]. This indicates the expediency of their further research for use as a highly effective anticonvulsant.

The antibacterial effect of compositions of rhamnolipids with thiosulfonate derivatives of naphthoquinone on *P. aeruginosa*, *B. subtilis* and *A. faecalis* was also determined. The combined use of naphthoquinones and rhamnolipids showed synergistic activity of these combinations against experimental strains of bacteria [16]. There is no information in the literature about the use of compositions of rhamnolipids with aminopyrazole derivatives of 1,4-naphthoquinone as antimicrobial agents.

To study the antimicrobial action, we chosed test cultures *E. coli* B-906, *S. aureus* 209-P, *M. luteum* B-917, *C. tenuis* UCM Y-70, and *A. niger* UCM F-1119, because they (considered), according to WHO, are opportunistic pathogens and pose the greatest threat to human health. *E. coli* strains that cause diarrheal diseases are among the most important among the various etiological agents of diarrhea. *E. coli* strains that cause diarrheal diseases are among the most important etiological agents of diarrhea [20]. When the skin, mucous membranes and soft tissues are damaged (wounds, areas of surgical intervention), *S. aureus* are among the first to settle in the wound. In 80–90% of cases of pustular infections of the skin and soft tissues (impetigo, boils, phlegmons), staphylococci are found to be the causative agents of the inflammatory process [21]. Among the main clinical manifestations of mycobacteriosis, lung damage is most common, mainly in elderly people with chronic non-specific lung diseases, silicosis, as well as in patients with tuberculosis and respiratory mycoses [22]. HIV-infected and AIDS patients mainly develop disseminated diseases with an unfavorable prognosis. Candidiasis is an infection caused by *Candida species* (most often *C. albicans*), which manifests itself in lesions of the skin and mucous membranes, fungemia, sometimes focal infection with the involvement of various organs in the process. Symptoms depend on the site of infection and include

dysphagia, skin and mucosal lesions, blindness, vulvovaginal symptoms (pruritus, burning, discharge), fever, shock, oliguria, renal failure, and disseminated intravascular coagulation [23]. Fungi of the genus *Aspergillus* tend to affect open areas, such as cavities in the lungs, caused by previous lung disease (eg, bronchiectasis, tumor, tuberculosis), nasal sinuses, or external auditory canals. Such infections are usually locally invasive and destructive, although systemic spread occasionally occurs, particularly in immunocompromised patients with neutropenia or immunosuppression on corticosteroids. Aspergillosis can also occur in patients with HIV infection/AIDS [24].

To study the antimicrobial effect, we chosed test cultures *E. coli* B-906, *S. aureus* 209-P, *M. luteum* B-917, *C. tenuis* UCM Y-70, and *A. niger* UCM F-1119, because, according to WHO, they are conditionally pathogenic. *E. coli* strains are one of the most important etiological agents of diarrhea [20]. *Staphylococci* are causative agents of the inflammatory process in lesions of the skin, mucous membranes, and soft tissues (impetigo, boils, and phlegmons) [21]. *Mycobacteriosis* causes lung damage [22]. Candidiasis is the most common pathogenic fungal infection of humans, manifested by skin and mucous membrane lesions with various symptoms [23]. Fungi of the genus *Aspergillus* are causative agents of opportunistic infections, especially in patients with HIV infection [24].

Considering the need to find new antimicrobial drugs effective against the strains described above, we obtained composite drugs based on rhamnolipids and heterocyclic amine-containing derivatives of 1,4-naphthoquinone and investigated their effect on test bacteria *E. coli* B-906, *S. aureus* 209-P, *M. luteum* B-917 and fungi *C. tenuis* UCM Y-70, *A. niger* UCM F-1119. An increase in the activity of the obtained compositions compared to naphthoquinones was established. This is explained by the permeabilization of cells by biosurfactants, which increases the sensitivity of cells to biocides [21].

## Conclusions

Heterocyclic amine-containing derivatives of 1,4-naphthoquinone belong to toxicity classes IV-VI according to *in silico* prediction, which indicates their promise for further biological research. Composite preparations based on 2-chloro-3-(3-(3-(*p*-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione and 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene-1,4-dione with rhamnolipids was obtained. On the basis of studies conducted by *in silico* and *in vitro* methods, promising compounds were created, namely composite preparations 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene-1,4-dione (RL-3.2) and 2-chloro-3-(3-(3-(p-tolyl)-1H-pyrazol-5-yl)amino) naphthalene-1,4-dione (RL-3.3) with rhamnolipids with increased antimicrobial activity against the test cultures of *C. tenuis*, *M. luteum* and *A. niger* compared to 1,4-naphthoquinones. The composite drug RL-3.3(2:1) demonstrated better indicators of antifungal activity against the test culture *C. tenuis* (MIC < 0.9:0.9, MPC < 0.9:0.9 μg/ml) than comparison drug Nystatin. The obtained results indicate the prospect of an in-depth study of their antimicrobial action against test cultures *C. tenuis*, *M. luteum* and *A. niger*.

## Interests disclosure

Olena Karpenko is the member of the Editorial Council of *Innovative Biosystems and Bioengineering* and was not involved in the editorial evaluation or decision to accept this article for publication. The other authors have no conflicts of interest to declare.

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**АНТИМІКРОБНА АКТИВНІСТЬ КОМПОЗИЦІЙНИХ ПРЕПАРАТІВ НА ОСНОВІ БІОСУРФАКТАНТІВ І ГЕТЕРОЦИКЛІЧНИХ АМІНОВМІСНИХ ПОХІДНИХ 1,4-НАФТОХІНОНУ**

**Проблематика.** Розроблення високоефективних та екологічно безпечних композиційних препаратів з антимікробною активністю є актуальною проблемою біотехнології та фармації.
**Мета.** Визначення антимікробної активності нових композиційних препаратів на основі рамноліпідів із гетероциклічними аміновмісними похідними 1,4-нафтохінону щодо тест-бактерій *Escherichia coli* B-906, *Staphylococcus aureus* 209-P, *Mycobacterium luteum* B-917 і грибів *Candida tenuis* UCM Y-70, *Aspergillus niger* UCM F-1119.
**Методика реалізації.** Дослідження *in vitro* антимікробної дії гетероциклічних аміновмісних похідних 1,4-нафтохінону, біосурфактантів і композиційних препаратів на їх основі щодо культур бактерій *Escherichia coli* B-906, *Staphylococcus aureus* 209-P, *Mycobacterium luteum* B-917 і грибів *Candida tenuis* UCM Y-70, *Aspergillus niger* UCM F-1119 шляхом дифузії речовини в агар на твердому живильному середовищі та методом серійних розведень. Прогнозування токсичності гетероциклічних аміновмісних похідних 1,4-нафтохінону з використанням методології *in silico* за допомогою програмного забезпечення ProTox-II.
**Результати.** Встановлено, що 2-хлоро-3-((1-метил-1H-піразол-3-іл)аміно)нафтален-1,4-діон 3.2 та 2-хлоро-3-((3-(*п*-толіл)-1H-піразол-5-іл) аміно) нафтален-1,4-діон 3.3 виявляють антимікробну активність щодо тестових мікроорганізмів, а в їх композиційних препаратах із рамноліпідами-біосурфактантами антимікробна дія підсилюється. За результатами розрахункової оцінки спрогнозовано, що досліджені гетероциклічні аміновмісні похідні 1,4-нафтохінону можна віднести до IV класу токсичності.
**Висновки.** Визначено, що антимікробна активність композиційних препаратів 2-хлоро-3-((1-метил-1H-піразол-3-іл)аміно)нафтален1,4-діону 3.2 та 2-хлор-3-(3-(3-(*p*-толіл)-1*H*-піразол-5-іл)аміно)нафталін-1,4-діону 3.3 із рамноліпідами підвищилась порівняно із синтезованими препаратами.

**Ключові слова:** рамноліпіди; аміновмісні гетероциклічні похідні; 1,4-нафтохінон; композиційні препарати; антимікробна активність.