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PHARMACOLOGICAL AND PHYSICO-CHEMICAL PROPERTIES OF COMPOSITIONS BASED ON BIOSURFACTANTS AND N-CONTAINING HETEROCYCLIC DERIVATIVES OF 1,4-NAPHTHOQUINONE

Nataliia Polish[*1], *Mariia Nesterkina*[2], *Andriy Karkhut*[1], *Nataliia Marintsova*[1], *Lesia Zhurakhivska*[1], *Kateryna Volianiuk*[3], *Tetyana Pokynbroda*[4], *Iryna Kravchenko*[2,5], *Elena Karpenko*[1,4]

Address(es):
[1] Lviv Polytechnic National University, Department of Technology Biologically Compounds, Pharmacy and Biotechnology, 3/4 St. George Square, Lviv, Ukraine, 79013.
[2] Odessa International Medical University, Department of Pharmacology and Pharmacy, Kanatna Street 99, Odessa, Ukraine, 65000.
[3]Lviv Polytechnic National University, Department of Organic Chemistry, 3/4 St. George Square, Lviv, Ukraine, 79013.
[4] 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, Str. Naukova, 3a, Lviv, Ukraine, 79060.
[5] State Enterprise Ukrainian Research Institute for Medicine of Transport, Ministry of Health of Ukraine, 65039 Odesa, Kanatna Street 92, Ukraine.

*Corresponding author: polishn@ukr.net

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ABSTRACT

Composite preparations based on rhamnolipid biosurfactants and N-containing heterocyclic 1,4-naphthoquinone derivatives were obtained. The formation of the compositions was confirmed by UV spectroscopy, dynamic light scattering and changes in the surface tension of the compositions in comparison with the original solutions. Physicochemical and biological properties of composite drugs have been studied. The anticonvulsant effect was assessed using the pentylenetetrazole (PTZ) model. Doses of PTZ for induction of clonic-tonic seizures (DCTC) and tonic extension (DTE) were calculated relative to control. The effect of rhamnolipid on increasing the permeability of cell membranes for compounds **1a-e** was evaluated indirectly by studying anticonvulsant activity. Compound (2-chloro-3-((3-(*p*-tolyl)-1H-pyrazol-5-yl)amino) naphthalene-1,4-dione) (**1b**) in combination with rhamnolipid was found to have a higher anticonvulsant activity DCTC and DTE averaged 239% and 244%, respectively, which may indicate an improvement of the penetration of the compound when in complex with rhamnolipid into the cell and as a result, an increased anticonvulsant activity of the composite drug.

Keywords: N-containing heterocyclic derivatives of 1,4-naphthoquinone, rhamnolipid biosurfactants, anticonvulsant activity, acute toxicity, physico-chemical properties

INTRODUCTION

Today one of the important areas of pharmacy is the search for new effective and safe neurotropic drugs. Depression is known to be a comorbidity in patients with epilepsy, and antidepressants increase the risk of seizures (**Olson**, *et. al.*, **1999**). Therefore, the search for new active low-toxic compounds with a combined and prolonged effect on the central nervous system is an urgent need of modern pharmacology.

Previously synthesized N-containing heterocyclic derivatives of 1,4-naphthoquinone (**Polish** *et. al*., **2020**) were found to demonstrate high anticonvulsant activity. However, it is known that they are characterized by low solubility in water and that complicates their use. Due to their ability to regulate the permeability of cell membranes, biosurfactants enhance the action of biologically active substances and increase the bioavailability of sparingly soluble substances (**Banat *et al.*, 2010**; **Salihu**, ***et al.*, 2009**). This allows creating new drugs with improved functional properties. Therefore, composite preparations of biosurfactants with N-containing heterocyclic derivatives of 1,4-naphthoquinone have been developed.

Biogenic surfactants (bioSAR), in particular rhamnolipids, are products of the biosynthesis of bacteria of the genus *Pseudomonas* and are characterized by low values of surface tension of solutions, high emulsifying and wetting ability. Physico-chemical properties of biosurfactants are able to increase the effectiveness and stabilize the functional properties of various drugs. In addition, biosurfactants have antimicrobial effects against bacteria, fungi and viruses, as well as antiproliferative action against cancer cells (**Haba**, *et. al*., **2014**). Their addition to biologically active substances allows increasing the activity of the latter and reduce their effective concentration. The synergistic effect of rhamnolipids with thiosulfonates (**Lubenets** *et. al.*, **2013**), antibiotics, in particular ramoplanin for nosocomial superinfections, with nisin, essential oils (**Xihou**, *et. al*., **2014**) has been shown. The effectiveness of clarithromycin and amoxicillin compositions with rhamnolipids relative to *H. pylori* biofilm has been shown (**Chen** *et. al*., **2019**). Biosurfactants are not inferior to synthetic surfactants and are active at low concentrations, stable at different pH, temperature, biodegradable, low-toxic, environmentally friendly (**Shekhar *et al.*, 2015**). Therefore, to enhance the pharmacological action of low-soluble substances, it is advisable to use biosurfactants as permeation enhancers (**Naughton, *et al.*, 2019; Ceresa, *et al.*, 2021**). The main purpose of the combined use of synthetic derivatives of naphthoquinone and biosurfactants is to improve water solubility, bioavailability and reduce the therapeutic dose (inhibitory concentration) of the drug (**Sotirova *et al.*, 2012; Koretska *et al.*, 2020**). So the aim of our work was to study the effect of rhamnolipids on the bioavailability of N-containing heterocyclic derivatives of 1,4-naphthoquinone and their anticonvulsant activity.

MATERIALS AND METHODS

Materials

In this study the products of microbial synthesis of *Pseudomonas* sp. PS-17, containing surfactant rhamnolipids were used. N-containing heterocyclic derivatives of 1,4-naphthoquinone (**1a-e**): 2-chloro-3-((1-methyl-1H-pyrazol-3-yl) amino) naphthalene-1,4-dione (**1a**), 2-chloro-3-((3- (*p*-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione (**1b**), ethyl-4-(3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-yl)amino)-1-phenyl-1H-pyrazol-3-carboxylate (**1c**) were synthesized by the nucleophilic substitution of the 2,3-dichloro-1,4-naphthoquinone chlorine atom with the corresponding amine. 2-((2-(3-(3-(3-Bromophenyl)-1H-1,2,4-triazol-5-yl)phenyl)amino)naphthalene-1,4-dione (**1d**) was obtained by the interaction of 1,4-naphthoquinone with a 1,2,4-triazine derivative by the Michael reaction and 2-(((2-(5-(2-bromophenyl)-1H-1,2,4-triazol-3-yl)phenyl)amino)-3-hydroxynaphthalene-1,4-dione (**1e**) – by the nucleophilic substitution with parallel hydrolysis of the second chlorine atom.

Methods for obtaining heterocyclic amino derivatives of naphthoquinone are described in our previous works (**Polish *et al.*, 2020**, **Polish *et al.*, 2021**). The surface tension of the compositions was determined by the Rebinder method, which is based on the measurement of the maximum pressure in the bubble on the

device PPNL-1 (**Fainerman *et al.*, 2011**). Generally accepted methods of variation statistics were applied for the statistical analysis of the reliability of experimental data used generally accepted methods of variation statistics (**Lakin *et al.*, 1990**). The surface tension of aqueous solutions of heterocyclic amine-containing naphthoquinone derivatives at concentrations of 10^{-2}–10^{-1} g/l and their compositions with rhamnolipids was measured. The value of the maximum pressure in the middle of the bubble was recorded at the time point 5 ± 1 s from the formation of the bubble. UV spectra were recorded on a ULAB 108UV spectrophotometer in the range of 200-700 nm at a concentration of 0.01 g / l in distilled water.

Dynamic Light Scattering (DLS) study of micelle-like structures (MLS). Hydrodynamic dimensions in aqueous solution and of the formed MLS were measured by DLS on a DynaProNanoStar instrument (Wyatt Technology, Santa Barbara, USA) using 298 K non-invasive backlight scattering (NIBS) technology. Samples for DLS measurements were prepared by dissolving a surfactant of microbial origin in distilled water at pH 7.0, the concentration of rhamnolipid $1x10^{-3}$ g / ml, added to them pre-dissolved in 1 ml dimethyl sulfoxide (DMSO) N-containing heterocyclic naphthoquinones, mixed, then they were inserted into a pre-washed capillary cell to measure the zeta potential. At least three measurements were made for each sample. DLS study of micelles containing N-containing heterocyclic 1,4-naphthoquinone derivatives at concentrations of 10^{-2} – 10^{-1} g /l. Solutions of compositions for DLS study were prepared 24 hours before measurement.

Anticonvulsant activity

Anticonvulsant effects of compounds **1a-e** (100 mg/kg) co-administered with rhamnolipid were evaluated at 3 h after their administration. 1,4-Naphtoquinone derivatives were mixed with rhamnolipid in a mass ratio 1:1, dissolved in 1,2-propylene glycol followed by oral administration to mice. The anticonvulsant action of compositions was estimated by pentylenetetrazole model (PTZ) as described in (**Nesterkina *et al.*, 2021**). Doses of PTZ for inducing clonic-tonic convulsions (DCTC) and tonic extension (DTE) were calculated relative to control. Anticonvulsant action was evaluated from the increase of pentylenetetrazole minimum effective dose (MED) compared with a control group. MED in percent was calculated using the formula:

$MED = V/m \times 10^4$

where MED – minimum effective dose of PTZ inducing DCTC or DTE; V – volume of PTZ solution, ml; m – animal weight, g.

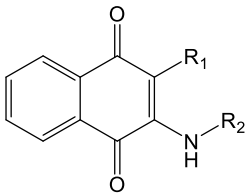
Statistical analysis

All results are expressed as mean ± standard error mean (SEM). One-way analysis of variance (ANOVA) was performed to determine the statistical significance of the results followed by Tukey's *post hoc* comparison. ** $p < 0.01$ and * $p < 0.05$ was considered as significant.

Statistical analysis. All experiments were repeated three times with three parallels in each variant. All data were expressed as a mean ± SD. Statistical analysis was performed using two sided Student`s t-test. P-value of < 0.05 was considered as statistically significant.

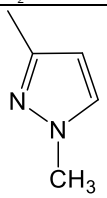
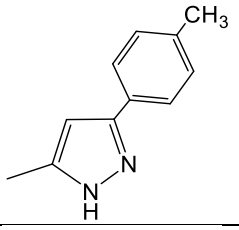
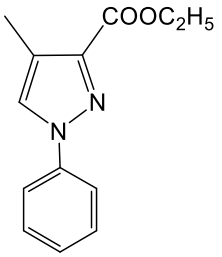
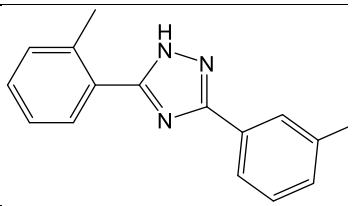
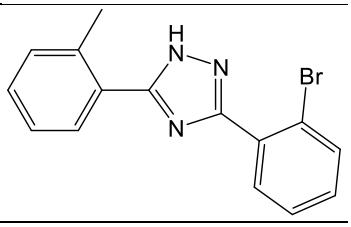
RESULTS AND DISCUSSION

Compositions based on amine-containing heterocyclic derivatives of 1,4-naphthoquinone and rhamnolipids were performed according to the method described in a previous publication (**Polish, *et al.*, 2021**).



1a-e

Table 1 Substituents in compounds **1a-e**

Compound	R_1	R_2
1a	Cl	
1b	Cl	
1c	Cl	
1d	H	
1e	OH	

1a – 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene-1,4-dione, 2-chloro-3-((3-(*p*-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione (**1b**), ethyl-4-((3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-yl)amino)-1-phenyl-1H-pyrazol-3-carboxylate (**1c**), 2-((2-(3-(3-bromphenyl)-1H-1,2,4-triazol-5-yl)phenyl)amino)naphtalen-1,4-dione (**1d**), 2-((2-(5-(2-bromophenyl)-1H-1,2,4-triazol-3-yl)phenyl)amino)-3-hydroxynaphthalene-1,4-dione (**1e**).

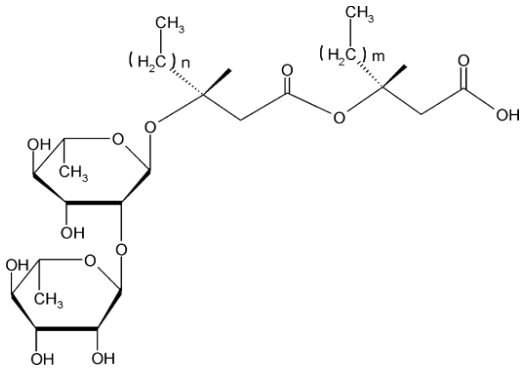


Figure 1 Structure of dirhamnolipid.

Ultraviolet - Visible Spectroscopy

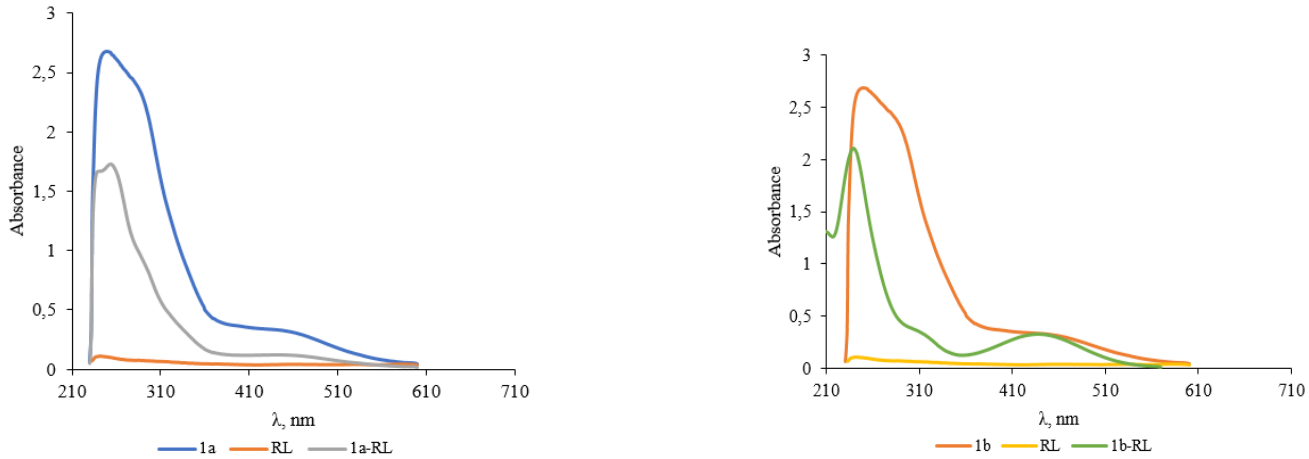


Figure 2 UV spectra in the range of 200 - 700 nm at a concentration of 0.01 g / l rhamnolipid complexes **1c** - RL, **1d** - RL, ethyl-4-((3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-yl)amino)-1-phenyl-1H-pyrazole-3-carboxylate (**1c**), 2 - ((2- (3- (3-bromophenyl) -1H-1,2,4-triazol-5-yl) phenyl) amino) naphthalene-1,4-dione (**1d**) and RL (rhamnolipid).

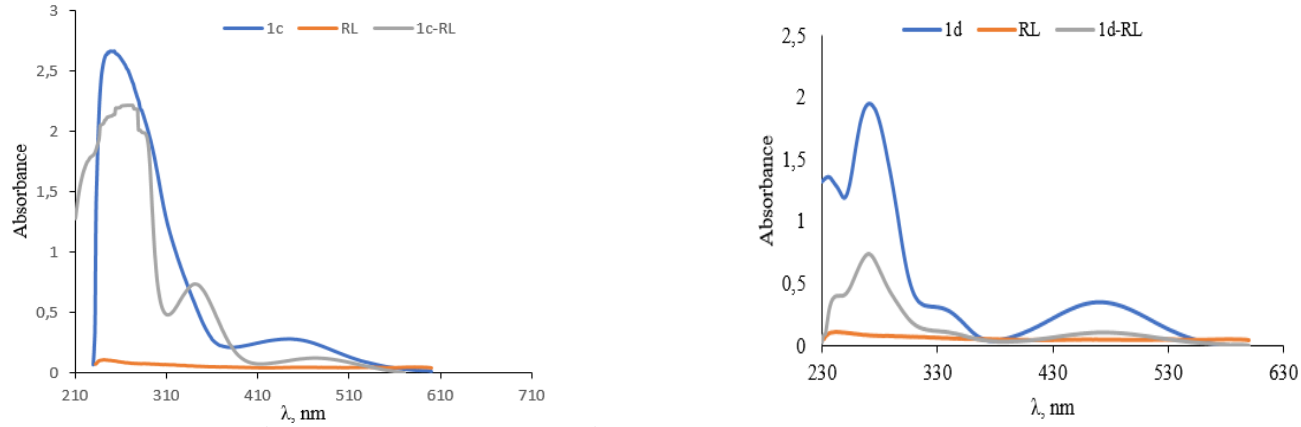


Figure 3 UV spectra in the range of 200 - 700 nm at a concentration of 0.01 g / l rhamnolipid complexes **1c** - RL, **1d** - RL, ethyl-4-((3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-yl)amino)-1-phenyl-1H-pyrazole-3-carboxylate (**1c**), 2-((2-(3-(3-bromophenyl)-1H-1,2,4-triazol-5-yl)phenyl)amino)naphthalene-1,4-dione (**1d**) and RL (rhamnolipid).

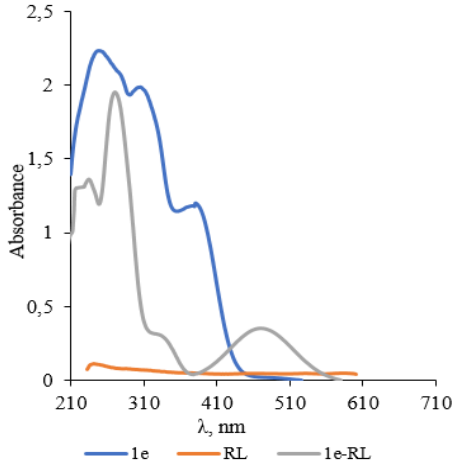


Figure 4 UV spectra in the range of 200 - 700 nm at a concentration of 0.01 g / l rhamnolipid complex **1e** - RL, 2-((2-(5-(2-bromophenyl)-1H-1,2,4-triazol-3-yl) phenyl)amino)-3-hydroxynaphthalene-1,4-dione (**1e**) and rhamnolipid (RL).

According to the obtained results of UV spectroscopy in the range of 200 - 700 nm (Fig. 2-4) for amine-containing heterocyclic derivatives of 1,4-naphthoquinone - **1a, 1b, 1c, 1d, 1e**, the spectra are characterized by bands with analytical maxima in the range of 252 - 274 nm. No peaks were recorded for RL spectra in this UV region. Hypsochromic shift of approximately 40 nm is observed on the spectra of composite preparations **1a** - RL, **1b** - RL, **1e** - RL. For **1b** - RL, **1c** - RL and **1e** - RL, new peaks are formed with a maximum of 343 nm, 448 nm and 274 nm, respectively, which may indicate the formation of intermolecular bonds between these compounds. At the same time, for compositions **1c** – RL and **1d** – RL, the characteristic band undergoes a hypsochromic shift of about 20 nm.

Physico-chemical parameters of the obtained compositions

Rhamnolipid biosurfactants are capable of causing a considerable decrease in the surface and interfacial tensions to values of 27.5 –29.8 and 0.04 - 0.07 mN/m respectively, and are capable of forming stable highly dispersed emulsions of vegetable oils, hydrocarbons, fats etc. The parameters for their surface and interfacial tensions, as well as their critical concentrations for micelle formation CMC (20-80 mg/l) and emulsifying index indicate their high surface activity.

In water solutions in mixtures with other substances, they form micelle-like structures. Different particle sizes are probably associated with the formation of such micellar structures between rhamnolipids (biosurfactants) and amine-containing heterocyclic derivatives. In our work on the synthesis of silver nanoparticles, rhamnolipids were both reductants of silver ions and stabilizers of silver nanoparticles. Due to the surface-active properties of rhamnolipids coagulation of silver particles did not take place **(Kuntyi *et. al.*, 2020).**

In this work, the hydrodynamic properties of the obtained micelle-like structures by the method of DLS were studied. The measurement results are shown in Fig. 5 and in table 3.

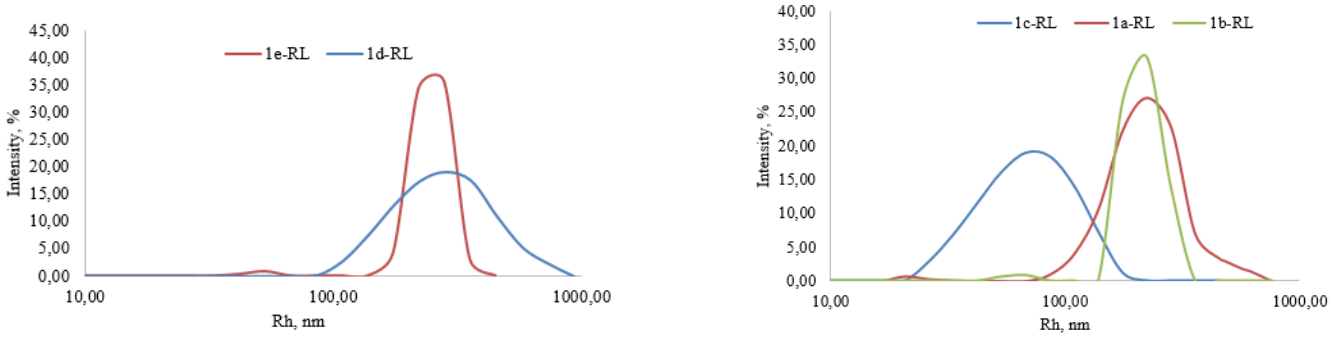


Figure 5 Hydrodynamic dimensions of micellar structures formed by solutions of rhamnolipid and **1a** - 2-chloro-3-((1-methyl-1H-pyrazol-3-yl)amino)naphthalene-1,4-dione and 2-chloro-3-(3-(*p*-tolyl)-1H-pyrazol-5-yl) amino) naphthalene-1,4-dione (**1b**), ethyl-4-((3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-yl) amino)-1-phenyl-1H-pyrazol-3-carboxylate (**1c**), 2-((2-(3-(3-(3-bromophenyl)-1H-1,2,4-triazol-5-yl)phenyl)amino)naphthalene-1,4-dione (**1d**), 2-((2-(5- (2-bromophenyl)-1H-1,2,4-triazol-3-yl)phenyl)amino)-3-hydroxynaphthalene-1,4-dione **1e** respectively (at pH = 7).

As can be seen from Figures 5, the value of the hydrodynamic dimensions of micelle-like structures confirms the existence of self-organized micelle-like structures of different sizes in solution depending on the structure of microbial surfactants.

Table 3 Hydrodynamic dimensions of micelle-like structures of compositions **1a-RL, 1b-RL, 1c-RL, 1d-RL and 1e-RL**

composite preparations	d , nm
1a-RL	223
1b-RL	220
1c-RL	68
1d-RL	283
1e-RL	280

As can be seen in Table 3, the particle size of the MLS is affected by the structure of the corresponding N-containing heterocyclic derivative of 1,4-naphthoquinone. MLS based on the composite preparation **1c-RL**, containing in its structure an ester group, has the smallest dimensions in the range of about 68 nm. This may indicate the compactification of the hydrophobic fragment in the MLS. Other preparations contain larger MLS. MLS based on composite preparation **1d-RL** and **1e-RL** contains particles of 283 nm and 280 nm respectively. This means that a larger structure has formed in the aqueous medium, which is probably due to the fact that macromolecules are more difficult to form a more compact structure due to the sufficiently high rigidity of the triazole fragment.

Anticonvulsant activity

The effect of rhamnolipid to increase the permeability increasing of compounds **1a-e** through biological membranes was evaluated indirectly by investigating the anticonvulsant activity. For this purpose, 1,4-naphthoquinone derivatives **1a-e** (100 mg/kg) were combined with rhamnolipid and administered orally into mice. In order to estimate the anticonvulsant effect, PTZ-induced model of epileptic seizures was applied. In the present study, technique of intravenous PTZ infusion (i.v.PTZ) was used whereby chemoconvulsant is injected into the tail vein of mice with constant flow rate (0.01 ml/s). PTZ dose that provoke clonic-tonic convulsions (DCTC) and tonic extension (DTE) were registered. As shown in Fig. 3, all heterocyclic compounds **1a-e** were found to possess antiseizure effect at 3 h after their administration as confirmed by increasing of DCTC and DTE values ($p < 0.01$ *vs* control).

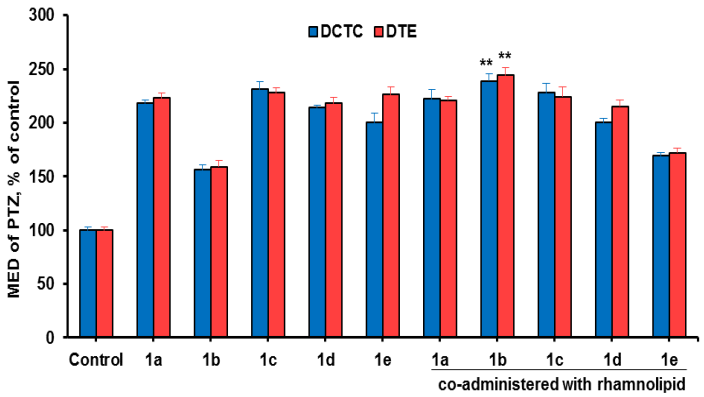


Figure 3 Anticonvulsant activity of compounds **1a-e** co-administered with rhamnolipid at 3 h after oral administration. Values are given as mean ± SEM, n = 5 mice; for all groups $p < 0.01$ compared with control. ** $p < 0.01$ for **1b** *vs* **1b** co-administered with rhamnolipid

1,4-Naphthoquinone derivatives **1a**, **1c-1e** at this time point demonstrated antiseizure effect with average DCTC and DTE values at 215% and 224%, respectively, whereas compound **1b** was inferior in activity (156% for DCTC and 159% for DTE). When co-administered with rhamnolipid derivatives **1a**, **1c-1e** possessed similar activity compared to those typical for pure compounds indicating no significant influence of rhamnolipid on membrane permeation. By contrast, co-administration of naphthoquinone **1b** with rhamnolipid was found to enhance the protection against PTZ-induced seizures in experimental animals leading to DCTC and DTE increase (239% and 244%, accordingly). The obtained results might be explained by the slightly activity of initial compound **1b** and its complex formation with rhamnolipid resulted in improved permeability through the membranes and, in turn, enhancement of pharmacological properties.

CONCLUSIONS

Physicochemical properties of composite preparations based on N-containing heterocyclic derivatives of 1,4-naphthoquinone and rhamnolipid were studied. The hydrodynamic dimensions of micelle-like structures were determined, which confirmed the formation of compositions. The anticonvulsant properties of the studied composite preparations were determined by oral administration of the studied 1,4-naphthoquinone derivatives and compositions based on them (100 mg / kg) 3 h after administration. Indicators of DCTC and DTE for **1a**, **1c-1e** averaged 215% and 224%, for 1b 156%, 159%, respectively, compared with controls (100%), indicating the presence of anticonvulsant effect in synthesized compounds, which is manifested in short periods of time. Concomitant use of compound **1b** (2-chloro-3-(3-(3-(*p*-tolyl)-1H-pyrazol-5-yl)amino)naphthalene-1,4-dione) in combination with rhamnolipid revealed a significant increase in the anticonvulsant effect DCTC and DTE averaged 239% and 244%, respectively), probably due to improved membrane permeability and, in turn, to enhanced pharmacological properties.

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