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Antibacterial Potential of Novel Synthetic Derivatives of 1,4-Naphthoquinone and Their Complexes with Biosurfactants.

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ABSTRACT

The need of new antimicrobial agents has become important in the last decade due to emerging resistance to a number of conventional drugs. In this study we tried to implement an approach, using the combined impact of novel synthetic derivatives of 1,4-naphthoquinone and biosurfactants (rhamnolipid, trehalosolipid). The synthetic naphthoquinone derivatives comprise different substituents, which probably determine the difference in their antimicrobial activity. Minimal inhibitory concentrations of naphthoqiunones (Lub4, Lub5 and Lub6) were determined against model bacterial strains: *Pseudomonas aeruginosa, Bacillus subtilis, Alcaligenes faecalis* and *Escherichia coli.* Strongest activity against all tested bacteria demonstrated Lub5, containing (4-acetaminophenyl) substituents. Lower was the antibacterial effect of Lub6 and especially of Lub4, comprising (4-aminophenyl) or (*p*-tolyl) substituents, respectively. The combination of trehalosolipid and naphthoquinones determined the biosurfactant inability to potentiate antibacterial activity of naphthoquinone derivatives. The combined application of naphthoquinones and rhamnolipid increased the antimicrobial potential of the inhibitors and proved these combinations synergistically active against the test bacterial strains. The enhancement of the antibacterial activity of naphthoquinones in presence of rhamnolipid significantly increased the therapeutic potential of the synthetic derivatives. The implementation of this approach was found perspective for application in biomedicine for new antimicrobial drug therapy and may represent another productive antimicrobial strategy.

Keywords: naphthoquinones, biosurfactants, *Pseudomonas aeruginosa, Bacillus subtilis, Alcaligenes faecalis*, *Escherichia coli*

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INTRODUCTION

The discovery and development of antibiotics and other antimicrobial agents dramatically reduced the threat of the diseases caused by microbial infections. However, the emergence of resistance to antibiotics in many pathogenic strains over the past 40 years has now become a serious problem of public health and could undermine the achievements in the treatment of infection diseases [1, 2]. Therefore, the need for discovery and development of novel pharmaceutical preparations with wide spectrum of activity for application in human and veterinary medicine or for plant protection has become important. The main requirements of those pharmaceuticals are high efficiency, low toxicity and cost-effectiveness.

In searching novel biologically active compounds it was proved that substances with 1,4-naphthoquinone structure are unique reagents in organic chemistry, demonstrated high reactivity and can be used as substrates for development of novel synthetic naphthoquinone derivatives [3]. The mechanisms of action of compounds with naphthoquinone structure should be of interest. The naphthoquinones show pharmacological properties as antibacterial, antifungal, antitumoral, or antiprotozoal agents [4-6]. Such naphthoquinone of natural origin is Lawsone ((2-hydroxy-1,4-naphthoquinone), contained in the leaves of henna (Lawsonia inermis). The reported tuberculostatic and antimicrobial activity of henna is probably due to lawsone, known to be the major bioactive constituent. [6, 7]. The structure-activity relationship of nitrogen and sulfur containing derivatives of 1,4-naphthoquinones is studied and the results show that they possess stronger antiviral, antibacterial, antifungal, antimalarial and anticancer activity compared to the starting compounds [8-10].

A new approach in development of novel complex antibacterial preparations is the application of surfactants with microbial origin (biosurfactants). The biosurfactants are surface-active compounds with low toxicity and high biodegradability. They could be applied alone or in combination with antibacterial agents as additives that improve the efficacy of the biologically active compounds [11, 12]. The main purpose of concomitant use of the synthetic naphthoquinone derivatives and biosurfactants is to decrease the therapeutic dose (inhibitory concentrations) of the tested antimicrobial agents.

The increased interest to trehalolipids is due to their ability to lower interfacial tension and increase pseudosolubility of hydrophobic compounds that make them potential candidates for practical applications. The investigationon of the interactions between a trehalose lipid from Rhodococcus sp. and dimyristoylphosphatidylglycerol membranes showed that biosurfactant incorporates into the phosphatidylserine bilayers thus suppose structural perturbations of the membrane affecting their function [13]. These results suggest that trehalose lipids may have potential application in the healthcare industry [14].

The low parameters for surface and interfacial tensions and critical micelle concentration (CMC) of rhamnolipid –biosurfactant indicated its high surface activity and it was an object of our previous investigations [15-17].

The aim of the present work was to determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of three novel nitrogen and sulfur containing derivatives of 1,4-naphtoquinone in absence and in presence of biosurfactants (rhamnolipid and trehalosolipid) and to assess their potential for use in biomedicine as complex antimicrobial preparations.

MATERIALS AND METHODS

Microorganisms

The model bacterial strains used in this study were Pseudomonas aeruginosa NBIMCC 1390; Bacillus subtilis 168; Alcaligenes faecalis 6132 and Escherichia coli W 1655 (National Bank of Industrial Microorganisms and Cell Cultures). The cultures were maintained at 4ºC on Bacto agar (Difco) slants and were transferred monthly.

Culture Medium and Growth Condition.

The bacterial strains were grown on mineral salt medium (MSM) Spizizen, [18] supplemented with $CaCl_2$, 2 mM; casein hydrolysate (Fluka), 0.5%; maltose, 0.5%; pH 7.2. Inoculums were prepared by transferring the cells from agar slants to 10 ml of MSM medium in 100 ml flasks and cultivated at 37ºC with agitation at 200 rpm.

The experimental cultures of 10 ml were inoculated with 1% (v/v) inoculum and incubated in 100 ml flasks until late exponential phase. Growth conditions were the same as those used for preparing the inoculum. Growth was monitored by measuring the absorption at 570 nm. Protein was determined by the method of Bradford [19].

Inhibitors (Antimicrobial Agents)

The synthetic derivatives of 1,4-naphthoquinone used in the study:

S,S-di-p-tolyl 1,4-dioxo-dihydronaphthalene-2,3-bis(sulfonothioate) – Lub 4
S,S-bis(4-acetamidophenyl)1,4-dioxo-dihydronaphthalene-2,3bis(sulfonothioate)–Lub 5
S,S-bis(4-aminophenyl) 1,4-dioxo-dihydronaphthalene-2,3-bis(sulfonothioate) – Lub 6 were synthesized in the Laboratory of Biotechnology, Ukrainian Academy of Sciences (Lviv town), and were provided by Dr. V. Lubenets. The tested concentrations were between 2 and 120 µg ml^{-1}.

Biosurfactants

The rhamnolipid-biosurfactant (RL) and trehalosolipid-biosurfactant (TL) used in the study were isolated from Pseudomonas sp. PS-17 and Rhodoccocus sp., respectively in the Laboratory of Biotechnology, Ukrainian Academy of Sciences (Lviv town), and was provided by Dr. E. Karpenko. The rhamnolipid and trehalosolipid were used in concentrations below CMC – 10 µg ml^{-1}.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

Antimicrobial activity was evaluated according to the minimum inhibitory concentration (MIC), the lowest concentration of an antimicrobial agent that inhibits development of visible microbial growth after incubation at 37^{o}C for 20 h. To determine the MBC, subcultures were made on inhibitors-free agar plates from each clear tube in the MIC test series, followed by incubation at 37^{o}C for 20 h.

Statistical Analysis

All experiments were carried out in triplicate, the reported results are the averages of at least three measurements, and the coefficients of variations, expressed as the percentage ratio between standard deviations (SD) and the mean values, were found to be<10 in all cases.

RESULTS

Studies on Antimicrobial Activity of Novel Synthetic Compounds and Biosurfactants

Effect of Trehalosolipid (TL) on the Growth and Cell Surface Properties of Model Gram (-) and Gram (+) Bacteria.

Bacillus subtilis 168 and Pseudomonas aeruginosa NBIMCC 1390 were used as model strains for tracking changes in growth and cell surface properties caused by the presence of TL. The results presented on Fig.1 showed that the presence of TL in the culture medium of Ps aeruginosa at concentrations below (10 μg ml^{-1} and 30 μg ml^{-1}), close to (50 μg ml^{-1}) and above (100 μg ml^{-1}, 300 μg ml^{-1}and 500 μg ml^{-1}) CMC did not alter significantly the growth compared to the control. The biosurfactant TL had not antibacterial activity at all tested concentrations. A decrease in the amount of extracellular protein was recorded at all concentrations of TL, which was more significant at concentration 500 μg ml^{-1} (Fig. 1). This lowering in cell permeability was dose dependent on the concentration range investigated, showing a general decrease in protein relese (compared to the control) with increasing TL concentration. Study on cell permeability of B. subtilis proved permeabilization effect of TL at concentrations below CMC (10 и 30 μg ml^{-1}), while at concentrations above CMC it was less pronounced.

Study on the Effect of Synthetic Derivatives of 1, 4-Naphthoquinone (Lub4, Lub5, Lub6) and Their Complexes with TL and RL on the Growth of Model Bacterial Strains

Antibacterial Activity of Naphthoquinone Lub 4 and its Complexes with TL and RL towards the Model Bacterial Strains

The investigation of growth ability of the model bacterial pathogens in presence of naphthoquinone derivative Lub4, containing two p-tolyl substituents was conducted to determine its minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The MIC determined for P. aeruginosa was 60μg ml^{-1}, while that for B. subtilis was

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120µg ml^{-1}. At these concentrations, Lub4 revealed bacteriostatic effect. MBCs were not established even at concentrations exceeding 120µg ml^{-1} (Fig.2).

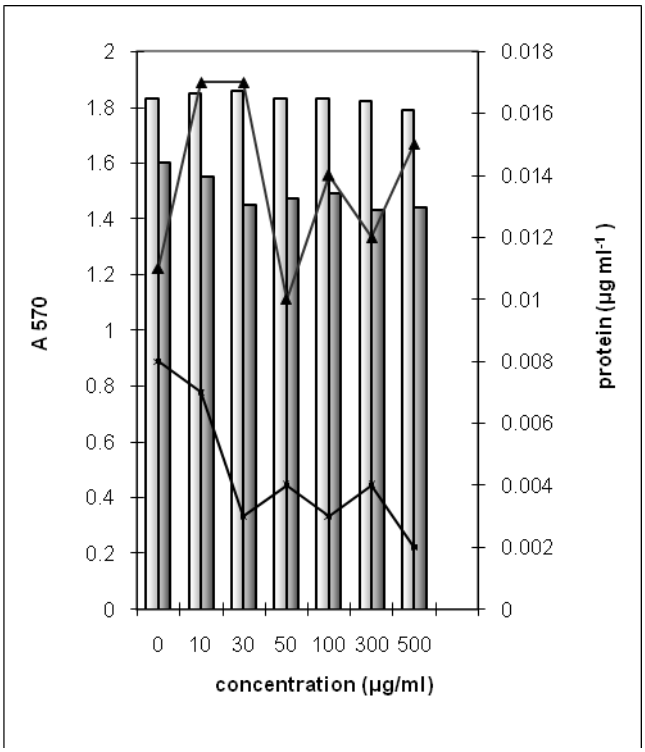
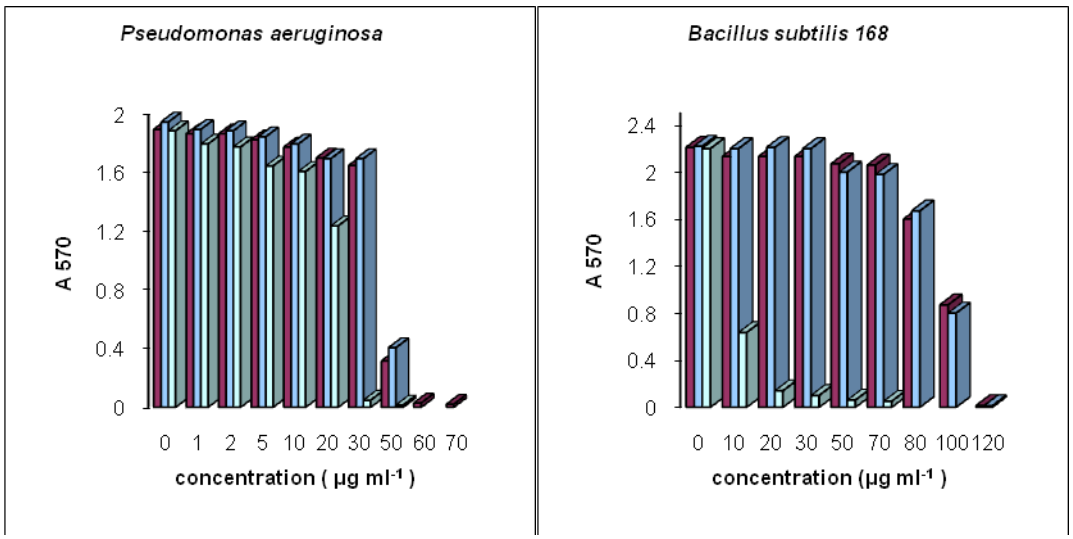


Figure 1: Effect of TL on growth and cell permeability of *B. subtilis* (□, ▲) and *P. aeruginosa* (▮, ■)



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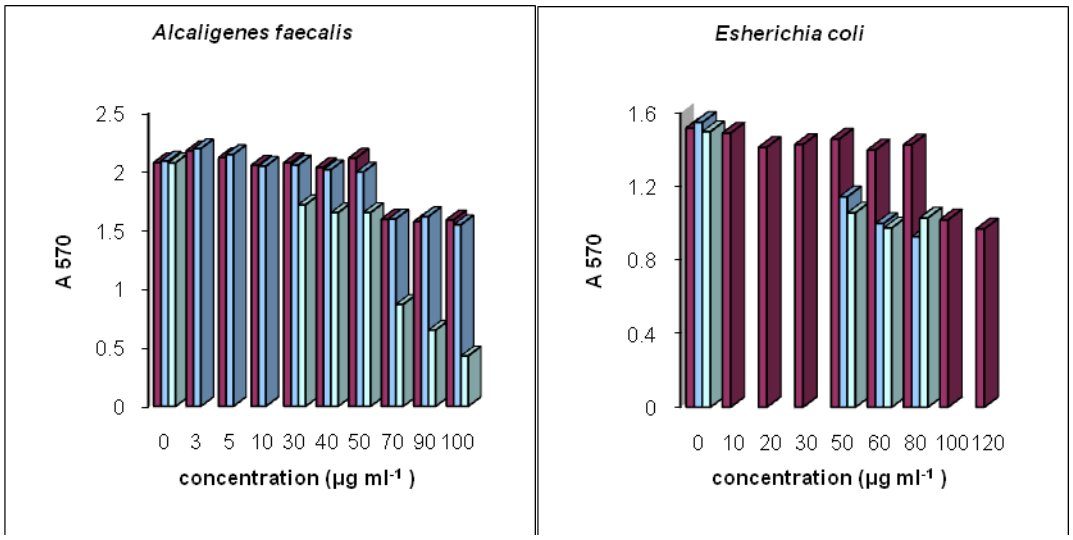

Figure 2: Effect of Lub4 – ■ **and its complexes with TL-** ■ **and RL-** ■ **on the growth of *P. aeruginosa, B. subtilis, A. faecalis* and *E. coli***

The higher MIC value, determined for B. subtilis could be supposed that the tested naphthoquinone had low sporicidal effect. Results on Fig. 2 indicated that MICs for A. faecalis and E. coli were not determined up to 100 µg ml^{-1} and 120 µg ml^{-1}, respectively. Both strains proved resistance to the tested substance at all concentrations.

The experimental data on the antibacterial activity of the complexes Lub4/RL and Lub4/TL showed a significant decrease of MIC for P. aeruginosa (30µg ml^{-1}) and for B. subtilis (70 µg ml^{-1}) in the presence of RL (10 µg ml^{-1}), while the addition of TL (10 µg ml^{-1}) had no effect (Fig.2). There was evidence for a synergistic-like effect of RL with Lub 4 but bactericidal activity of this combination was not established.

Antibacterial Activity of Naphthoquinone Lub5 and its Complexes with TL and RL against the Model Bacterial Strains

The results presented on Fig. 3 indicated that the antimicrobial activity of Lub5 comprising two 4-acetamidophenyl substituents was revealed at relatively low naphthoquinone concentrations. MIC for P. aeruginosa was 30µg ml^{-1}, for B. subtilis - 10 µg ml^{-1} and for A. faecalis- 5µg ml^{-1}.MIC for E.coli was determined to be above 80µg ml^{-1}.

The addition of RL led to an increase of antibacterial activity of naphthoquinone Lub5 but only against P. aeruginosa and B. subtilis. The minimal inhibitory concentrations decreased to 10 µg ml^{-1} and 2 µg ml^{-1}, respectively (Fig.3). The combination of naphthoquinone Lub5 with TL had no effect on the activity of the antibacterial agent. Only against E. coli, the ability of TL to potentiate the effect of Lub 5 was established. In this case a significant decrease in MIC was determined.

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Antibacterial Activity of Naphthoquinone Lub6 and its Complexes with TL and RL against the Model Bacterial Strains

Bactericidal activity of Lub6, containing two 4-aminophenyl substituents, and of its complexes with RL and TL was not found. The MICs of naphthoquinone Lub6 applied alone in the culture medium were 70 µg ml^{-1} and 80 µg ml^{-1} for P. aeruginosa and B. subtilis respectively. While of A. faecalis the growth inhibition was registered at concentration 10 µg ml^{-1}. The model strain E. coli was resistant to Lub6 at all tested concentrations. Furthermore, the addition of Lub6 in concentration of 10 µg ml^{-1} even stimulated the bacterial growth to certain extent. Slight inhibition of the growth was observed at concentrations of 20 to 80 µg ml^{-1} but at 100 and 120 µg ml^{-1}, the values were close to those of the control (Fig.4.)

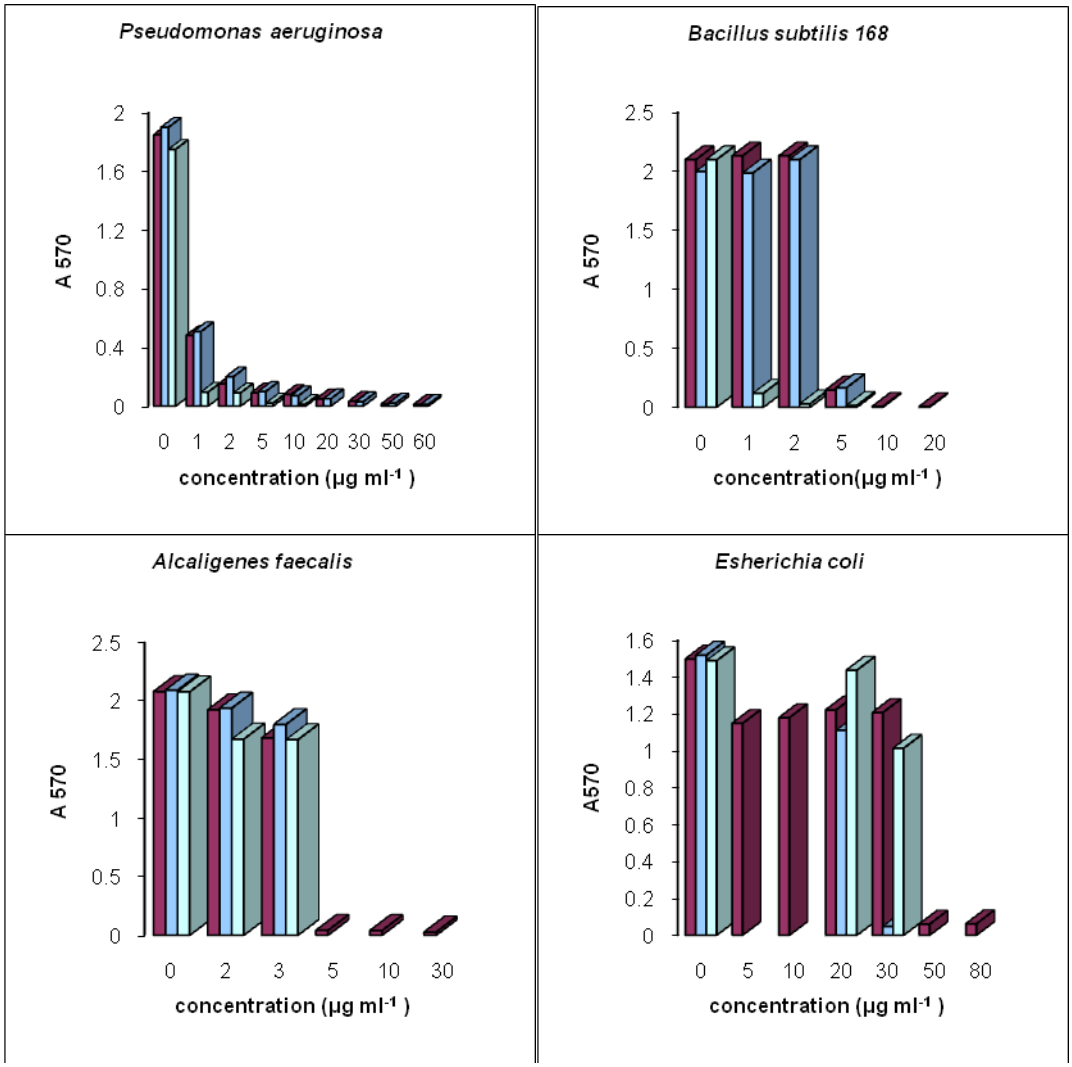

Figure 3: Effect of Lub5 and its complexes with TL- and RL- on the growth of *P. aeruginosa, B. subtilis, A. faecalis* and *E. coli*

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The results presented on Fig.4 did not reveal any significant difference in the antibacterial activity of Lub6 used alone and in combination with TL.

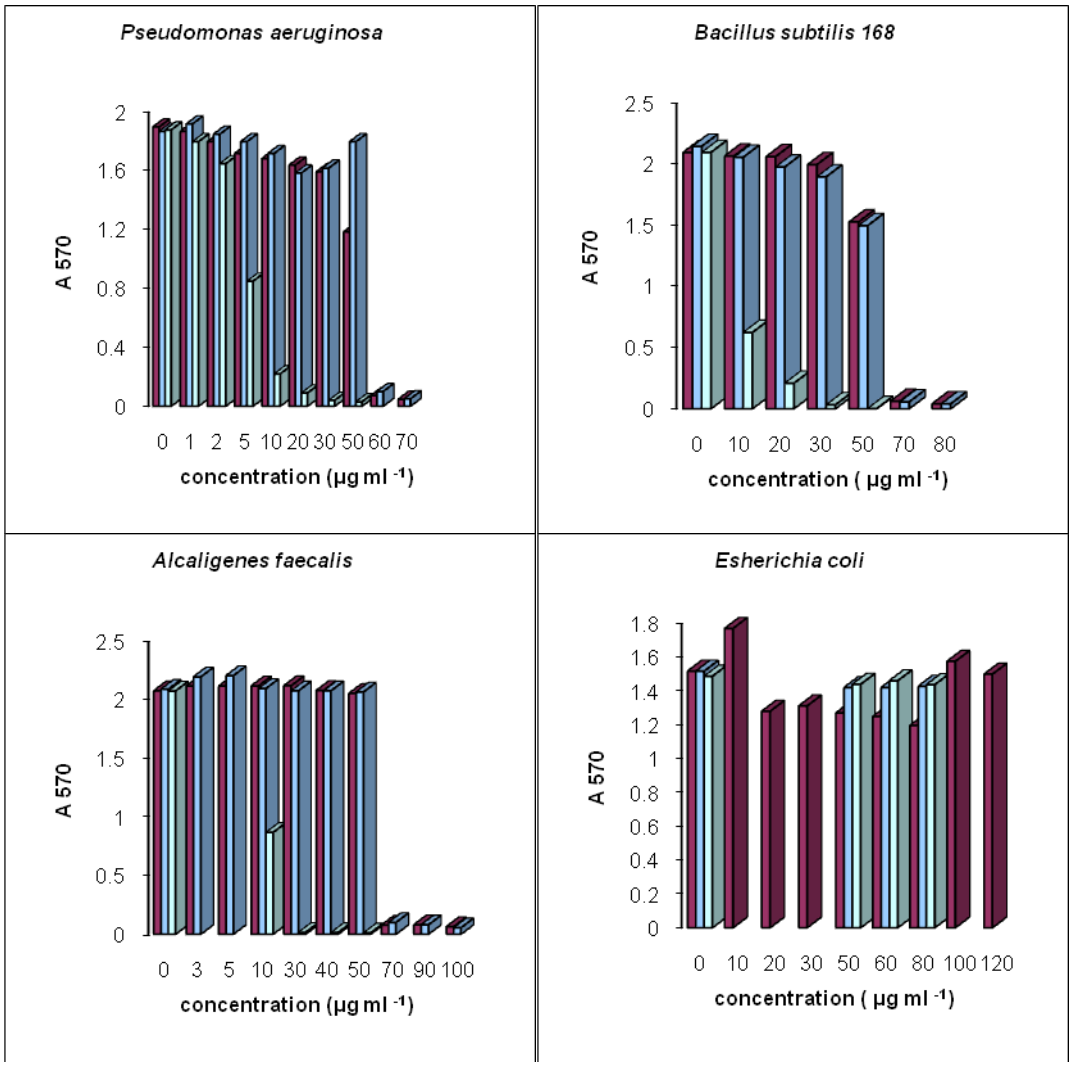

Figure 4: Effect of Lub6 and its complexes with TL- and RL- on the growth of *P. aeruginosa, B. subtilis, A. faecalis* and *E. coli*

The results obtained from the experiments with the complex Lub6/RL confirmed the assumption of its higher antibacterial activity compare with Lub6 applied alone but only against P. aeruginosa, B. subtilis and A. faecalis. The MICs of Lub6/RL were considerably lower for these strains (Fig.4). Complete inhibition of A. faecalis growth was not observed in presence of Lub6 alone, while MIC of the combination Lub6/RL was 30µg ml^{-1}. The MICs values against P. aeruginosa and B. subtilis were 50 µg ml^{-1} and 30 µg ml^{-1}, respectively. E. coli was found to be resistant toward the complex Lub6/RL. The bacterial growth of the model strain at concentrations of the complex up to 80 µg ml^{-1} was unaffected compared to the controls (Fig.4).

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DISCUSSION

The antimicrobial potential of the naphthoquinone derivatives was studied against model bacterial strains, selected according to their ability to damage human and animal health, agriculture or industrial manufacturing: **Pseudomonas aeruginosa** is known not only as an important opportunistic human but as plant pathogen as well. It inhabits soils and aqueous environments and is highly resistant to a large number disinfectants and antibiotics. Moreover, P. aeruginosa can grow well on variety of surfaces and is often the cause of medical device contamination and nosocomial infections; **Bacillus subtilis** was adopted as a model Gram (+) bacterium for laboratory studies. B. subtilis is not a human pathogen. It may contaminate food and rarely causes food poisoning. B. subtilis spores can survive in extreme conditions thus making this strain suitable (appropriate) for determining the bactericidal effect of the tested compounds**; Alcaligenes faecalis** usually is found in the environment. In humans the strain exists in latency but may cause urinary tract infections and peritonitis, meningitis etc in immunocompromised patients. A. faecalis as well may cause damages in some technological processes due to its ability to decolourize synthetic dyes and thus to spoil the quality of the final product [20]; **Escherichia coli** inhabit the gastro-intestinal tract of humans and of warm-blooded animals and in some circumstances may cause severe gastro-intestinal or urinary infections.

The surface structure of microbial cells provide an effective protective barrier to antimicrobial agents. Cell permeabilization with different agents helps to overcome the barrier and increases cell susceptibility to antibiotics [21]. It is known that surface-active compounds from microbial origin, biosurfactants, have a permeabilizing effect on a number of microbial cells [11, 15, 16, 22]. We hypothesized that the addition of biosurfactants to the growth medium in concentrations below critical concentrations for micelle formation (CMC) would influence the membrane permeability – as a result, the antibacterial effect of the inhibitors will be developed at lower concentrations. In our previous investigations, it was proved that the addition of rhamnolipid contributed to the decrease of the minimal bactericidal and fungicidal concentration of the tested antimicrobial agents – alkyl esters of thiosulfonic acid [12].

In this study we tried to implement the same approach in the search for antimicrobials, using the combined impact of novel synthetic derivatives of 1,4-naphthoquinones and biosurfactants (rhamnolipid and trehalosolipid). The three naphthoquinone derivatives used in this study have different substituents, which probably determine the difference in their antimicrobial activity. Naphthoquinone Lub5 possesses acetylated amino-group in the substituents (4-acetamidophenyl) and exhibits the highest inhibitory effect against the model bacterial strains, even toward E. coli (resistant to Lub4 and Lub6). In addition, multidrug resistance is not a congenital feature of E. coli, and time of use of drugs and resistance are closely related, which requires the constant search for new antibacterial agents. naphthoquinone Lub5.

The presence of acetamido group in the side chain probably enhances the inhibitory activity of naphthoquinone Lub5 to all test bacterial strains. Tandon et al argued that the

inhibitory activity of the naphthoquinone derivatives depend on the number and location of the nitrogen atom or a carboximido group in the structure of the naphthoquinone [8, 9, 10]. In the present study sensitivity to Lub6, comprising comprising two 4-aminophenyl substituents was specified for P. aeruginosa, B. subtilis and A. faecalis. Lub4, containing p-tolyl substituents, inhibits only the growth of P. aeruginosa and B. subtilis at relatively high inhibitor concentrations. It can be presumed that the presence of nitrogen (amino group) as in naphthoquinone Lub6, or acetamido group in the side chain as in naphthoquinone Lub5 increases the antibacterial activity of the corresponding compound.

The aim of the research was to investigate the bioactive and synergistic like properties of mixtures of the synthetic naphthoquinone derivatives and biosurfactants. In the case of TL, the results demonstrated an absence of antimicrobial activity and luck of permeabilizing effect. Furthermore, with the increase of TL concentration, a decrease of membrane permeability of P. aeruginosa cells was registered. These results determine its inability to increase antibacterial effect of the naphthoquinone derivatives. Only effective combination of Lub 5 and TL was proved for E. coli strain, but to explain this result, further research is needed.

Among the glycolipid biosurfactants, rhamnolipids are the most studied. Rhamnolipids possess antimicrobial activities and have strong permeabilizing effect [15, 16, 22, 23]. There are researches demonstrating that some antimicrobial agents and rhamnolipids are synergistically active against variety of microorganisms, including yeast and fungi [12, 24, 25]. The biosurfactant provokes changes in the bacterial cell surface and affects different components of the outer and inner membrane [17, 26, 27]. The alteration in the bacterial membrane and surface properties that causes it probably facilitates the access of inhibitors to the bacterial cells. As a result, it leads to reduction of the concentrations of studied naphthoquinone derivatives that are able to suppress the bacterial growth. Obviously, the combined application of naphthoquinones and rhamnolipid PS-17 increases the antimicrobial potential of tested inhibitors and proved this combination synergistically active against the test bacterial strains.

The use of rhamnolipids or other biosurfactants active against variety of microorganisms, in combination with antibiotic treatment, or antimicrobials may represent another productive antimicrobial strategy. The therapeutic strategies implemented biosurfactant/antimicrobials combination are therefore likely to be more effective against various microbial infections that cause dangerous diseases.

In conclusion, it was found that the studied naphthoquinone derivatives according to the substituents in their structure have well pronounced antibacterial effect on P. aeruginosa, B. subtilis and A. faecalis. Only E. coli behaved resistant to the inhibitors. We propose a new promising approach to generate novel and effective antimicrobials, namely, synergistic- like combination between rhamnolipid and the novel synthetic derivatives of 1,4-naphthoquinone, containing different substituents in the quinone structure. The addition of RL in concentrations below CMC affects the membrane composition of bacteria and as a result, the antibacterial effect of inhibitors is developed at lower concentration, reducing the therapeutic dosage of the complex antimicrobial preparations.

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REFERENCES

[1] Yoneyama H, Katsumata K. Biosci Biotechnol Biochem 2006; 70: 1060-1075.
[2] Simmons KJ, Chopra I, Fishwick CWG. Microbiology 2010; 8: 501-510.
[3] Ibis C, Tuyun AF, Ozsoy-Gunes Z, Bahar H, Stasevych MV, Rostyslav Ya. Musyanovych Rd, Komarovska-Porokhnyavets O, Novikov V. Eur J Med Chem 2011; 46: 5861-5867.
[4] Morello A, Pavani M, Garbarino JA, Chamy MC, Frey F, Mantilla J. Comp Biochem Physiol C: Pharmacol Toxicol Endocrinol 1995;112C:119–28.
[5] Zhang L, Arnold LD. Natural Products: Drug Discovery and Therapeutic Medicine. Humana Press Inc, 2005.
[6] Rahmoun MN, Benabdallah M, Villemin D, Boucherit K, Mostefa-Kara B, Ziani-Cherif C. Der Pharma Chemica 2010; 2: 320–326.
[7] Sharma VK. Tubercle 1990; 71:293–295.
[8] Tandon VK, Vadav DB, Chaturvedi AK, Shukla PK. Bioorg Med Chem Lett 2005; 15: 3288–3291
[9] Tandon VK, Maurya HK, Mishra MM, Shukla PK. Eur J Med Chem 2009; 44: 3130–3137
[10] Tandon VK, Maurya HK, Verma MK, Kumar R, Shukla PK. Eur J Med Chem 2010; 45: 2418-2426.
[11] Ivanova A, Karpenko E, Galabov A.S., Remichkova M. CR Acad Bulg Sci 2011; 64: 157-160.
[12] Sotirova A., Avramova T., Lazarkevich I., Lubenets V., Karpenko E., Galabova D. CR Acad Bulg Sci 2010; 63: 1307-1314.
[13] Ortiz A, Teruel JA, Espuny MJ, Marqués A, Ángeles Manresa A, Aranda FJ. Biochim Biophys Acta 2008; 1778: 2806–2813.
[14] Ryll R, Kumazawa Y, Yano I. Microbiol Immunol 2001; 45:801–811.
[15] Sotirova A, Spasova D, Galabova D, Karpenko E, Shulga A. Curr Microbiol 2008; 56: 639–644.
[16] Sotirova A, Spasova D, Vasileva-Tonkova E, Galabova D. Microbiol Res 2009; 164: 297–303.
[17] Sotirova A, Avramova T , Stoitsova S, Lazarkevich I, Lubenets V, Karpenko E, Galabova D. Curr Microbiol 2012; 65:534-541
[18] Spizizen J. Proceed. Nat. Acad. Sci. USA. 1958; 44: 1072–1078.
[19] Bradford M M. Analyt Biochem 1976; 72: 248–254.
[20] Mutafov S., T. Avramova, L. Stefanova, B. Angelova. World J. Microbiol.Biotechnol. 2007; 23: 417–422.
[21] Ayres H. M., J. R. Furr, A. D. Rusell. Lett. Appl. Microbiol 1999; 28: 13–16.
[22] Al-Tahhan RA, Sandrin TR, Bodour AA, Maier RM. Appl Environ Microbiol 2000; 66: 3262–3268.

[23] Haba, E, A. P., Jauregui O, Espuny M J, Infante M R, Manresa A. Biotechnol Bioengineer 2002; 81: 316 – 322.
[24] Bensaci MF. The Bioactive Properties of Syringomycin E – Rhamnolipid Mixtures and Syringopeptins. 2009; PhD thesis, Utah State University
[25] Samadi N[1], Abadian N, Ahmadkhaniha R, Amini F, Dalili D, Rastkari N, Safaripour E, Mohseni FA. Folia Microbiol 2012; 57: 501-508.
[26] King AT, Davey MR, Mellor IR, Mulligan BJ, K. C. Lowe BJ. Enzyme Microb Technol 1991; 131: 148–153.
[27] Spasova D, Sotirova A, Avramova T, Galabova D. CR Acad Bulg Sci 2010; 63: 1449-1454.