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ORIGINAL ARTICLE

Activity of selected salicylamides against intestinal sulfate-reducing bacteria

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Abstract

OBJECTIVES: The aim of our work was to evaluate effect of selected salicylamides on cell viability of sulfate-reducing bacterium *Desulfovibrio piger* Vib-7 isolated from the human large intestine, as well as to assess antimicrobial activity and biological properties of these compounds.
METHODS: Microbiological, biochemical, biophysical methods, and statistical processing of the results were used.
RESULTS: An antimicrobial activity and biological properties of salicylamides against intestinal sulfate-reducing bacteria was studied. Primary in vitro screening of the synthesized selected salicylamides was performed against *D. piger* Vib-7. Adding 0.37–1.10 μmol.L^{-1} (N-(4-bromophenyl)-5-chloro-2-hydroxybenzamide, 5-chloro-2-hydroxy-N-[4-(trifluoromethyl)phenyl]benzamide, 5-chloro-N-(3,4-dichlorophenyl)-2-hydroxybenzamide, 5-chloro-2-hydroxy-N-(4-nitrophenyl) benzamide and 4-chloro-N-(3,4-dichlorophenyl)-2-hydroxybenzamide) caused decrease in biomass accumulation by 8–53, 64–66, 49–50, 82–90, 43–46% compared to control, respectively. The studied compounds completely inhibited the growth of *D. piger* Vib-7 under the effect of 30 μmol.L^{-1}. Moreover, addition of the compounds in the culture medium inhibited the process of dissimilation sulfate dose dependently. Treatment with salicylamides led to the bacterial growth inhibition which correlated with the level of inhibition of sulfate reduction. The data on relative survival of *D. piger* Vib-7 cells and cytotoxicity of salicylamides are consistent to our research in previous series of the biomass accumulation experiments.
CONCLUSIONS: A significant cytotoxic activity under the influence of salicylamides was determined. These results are consistent with a data on bacterial growth and inhibition process of dissimilation sulfate. The strongest cytotoxic effect of the derivatives was observed in compounds of 5-chloro-2-hydroxy-N-[4-(trifluoromethyl)phenyl]benzamide and 5-chloro-2-hydroxy-N-(4-nitrophenyl) benzamide which showed low survival and high toxicity rates.

Abbreviations
CFU - colony-forming unit
DMSO - dimethyl sulfoxide
IC_{50} - inhibitory concentration
KS - Kravtsov-Sorokin medium
MIC - minimal inhibitory concentration
OD - optical density
PBS - phosphate-buffered saline
SRB - sulfate-reducing bacteria
UC - ulcerative colitis

INTRODUCTION

The increased number of sulfate-reducing bacteria (SRB) and intense process of dissimilatory sulfate reduction in the gut is thought to be a significant risk factor of inflammatory bowel diseases in both humans and animals (Gibson *et al.* 1991, 1993; Kushkevych 2012a). These bacteria are often found in persons with rheumatic diseases, and with ankylosing spondylitis, etc. (Barton & Hamilton 2010). There is also an assumption that SRB can be responsible for some forms of cancer of the rectum through the formation of hydrogen sulfide, which affects the metabolism of intestinal cells and give rise to various inflammatory bowel diseases. The species of *Desulfovibrio* genus can cause bloody diarrhea, weight loss, anorexia, epithelial hyperplasia, abscesses and inflammatory infiltrates in animals and humans (Loubinoux *et al.* 2000, 2002). The increased number of SRB was found in feces from people with ulcerative colitis in comparison with healthy individuals (Cummings *et al.* 2003). The injection of these bacteria in hamster intestine caused infection clinically similar to human colitis (Pitcher *et al.* 1996; Cummings *et al.* 2003).

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon that affects up to 12 per 100,000 people in Western countries, mostly between 15 and 30 years of age (Rowan *et al.* 2009). The treatment of mild to moderate UC includes, in a first approach, sulfasalazine and mainly 5-aminosalicylate containing drugs which type and dosage depend on the location and severity of the disease. Other options of treatment include corticosteroids and immunosuppressants (for moderate to severe UC, with a high mortality disease) or probiotics (for improving the microbial balance) (Cummings *et al.* 2003; Kushkevych 2012b). Despite the bacterial nature of the disease, antibiotics failed in the treatment of UC so far. However, new antibacterial compounds with high specific effect against SRB could yield better efficiency in the treatment of this disease. At present, the effects of salicylamides on the intestinal sulfate-reducing bacteria *D. piger* have never been well-characterized and have not been studied yet.

The aim of our work was to evaluate effect of selected salicylamides on cell viability of sulfate-reducing bacterium *D. piger* Vib-7 isolated from the human large intestine, as well as to assess antimicrobial activity and biological properties of these compounds.

MATERIALS AND METHODS

Tested compounds

The studied salicylamides **1**–**5** were synthesized by means of microwave-assisted synthesis described in literature. The compounds were isolated and fully characterized (melting point, elemental analysis, infrared as well as ^{1}H and ^{13}C NMR spectroscopy) (Pauk *et al.* 2013). The compounds were kept in microtubes dissolved in dimethyl sulfoxide (DMSO) solution. The quantity of DMSO necessary to dissolve each compound was calculated previously so that the concentration of the component would be 30 $mmol.L^{-1}$. Afterwards it was diluted 4 times in a proportion 1:3 so that there would be 5 different concentrations of chemical compound: 0.37; 1.1; 3.3; 10 and 30 $mmol.L^{-1}$. The maximum concentration of DMSO in the assays never exceeded 0.1%.

Bacterial culture

The sulfate-reducing bacteria *D. piger* Vib-7 were isolated from the healthy human large intestine as described previously (Kushkevych 2013; Kushkevych *et al.* 2014). The strain has been kept in the collection of microorganisms at the Department of Molecular Biology and Pharmaceutical Biotechnology, Faculty of Pharmacy at the University of Veterinary and Pharmaceutical Sciences Brno (Czech Republic).

Bacterial cultivation

The bacteria were grown for 72 hours at 37 °C under anaerobic conditions in nutrition modified Kravtsov-Sorokin's (KS) liquid medium (Kushkevych & Moroz 2012). Before bacterial passage in the medium, 0.05 $ml.L^{-1}$ of sterile solution of $Na_2S{\times}9H_2O$ (1%) was added. The sterile 10 $mol.L^{-1}$ solution of NaOH (0.9 $ml.L^{-1}$) in the medium was used to provide the final pH 7.2. The medium was heated in boiling water for 30 min in order to obtain an oxygen-free medium, and cooled to 30 °C. The tubes were brim-filled with medium and closed to provide anaerobic conditions.

Assay of bacterial cell concentration

About 1 mL of liquid medium without Mohr's salt (blank) was transferred into a plastic cuvette and taken to a biophotometer (Eppendorf®) for taring. Subsequently, 1 mL of bacterial suspension was transferred into another cuvette and taken again to the biophotometer for measuring at OD_{340}. Before SRB were used for the experiments, optical density (OD_{340}) was always measured to assure there was approximately the same amount of bacteria in each experiment. If it was necessary proper dilutions were made in order to obtain the desired concentration of the bacteria.

The best concentration of *D. piger* Vib-7 was assessed to be $5{\times}10^5$ $CFU.mL^{-1}$. Based on our previous work a correlation between OD_{340} and the amount of cells in the solutions measured in the biophotometer was concluded as:

$$y = 1.0\times10^{9}\,\chi - 6.0\times10^{6},$$

where y means the bacterial concentration and χ means the OD_{340} measured (Figure 1).

The biomass of the *D. piger* Vib-7 cells was calculated by the formula:

$$C = \frac{E \times n}{K},$$

where C – bacterial biomass ($mg.mL^{-1}$); E – extinction; n – dilution factor, times; K – coefficient of conversion, obtained gravimetrically.

Assay of sulfate, lactate sulfide and acetate in cultivation medium

The sulfate ion concentration in the medium was determined by the turbidimetric method after it had been precipitated by barium chloride. To stabilize the suspension, glycerol was used (Kolmert *et al.* 2000). Lactate concentration was measured through the dehydrogenation reaction using Lactate Assay Kit (Sigma-Aldrich, Catalog Number MAK064). Sulfide concentration in the culture medium was assayed by the spectrophotometric method as was described (Cline 1969). Accumulation of acetate ions in process of bacterial growth in the medium was determined using Acetate Assay Kit (Colorimetric, Catalog Number KA3764).

Tratment of bacterial culture

Bacterial culture of stationary phase of growth was centrifuged for 3 min at a rotation speed of 3,500 rpm. Supernatant was removed and replaced by new and fresh KS liquid medium where the bacterial precipitate was diluted. The bacterial suspension was mixed and OD_{340} was measured. Numbers and viabilities of the bacterial cells were determined by counting with a haemocytometer after staining with erythrosine B [0.1% erythrosine B ($w{\cdot}v^{-1}$) in phosphate-buffered saline (PBS), pH 7.2–7.4]. Unstained cells were considered to be viable.

The bacterial suspension (initial concentration $0.5\,mg.mL^{-1}$) was poured to the top in microtubes (280 µL) which contained samples + solvent control (DMSO) + sample control + blanks; it was prepared 3 times each sample as well as the controls so that the average of the results did not had a great discrepancy from the results independently (except in the 3 blanks). The sample controls contained only bacterial suspension and medium (free of tested compounds); and for the 3 blanks only medium.

Calculations were made to assess how much bacterial solution should be in each, based on the OD. Determination of biomass and concentrations of sulfate, lactate, acetate and sulfide in the culture medium under the treatment of 0.37; 1.1; 3.3; 10 and 30 $\mu mol.L^{-1}$ compounds after 12, 24, 36, 48, 60, 72 hours was carried out. During experiments, bacteria were grown at 37 °C under anaerobic conditions.

Analysis of viability of D. piger Vib-7 and cytotoxicity of the compounds

The bacterial suspension (5×10^{4} cells·well^{-1} in 200 µL culture medium) was filled to the top in 96-well plates in triplicate in the KS liquid medium (without Mohr's salt), treated with 0.37; 1.1; 3.3; 10 and 30 $\mu mol.L^{-1}$ compounds and incubated at 37 °C. Relative survival of *D. piger* Vib-7 cells and cytotoxicity of the compounds was determined by 36th hour of cultivation using a WST-1 assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions (Zídek *et al.* 2014). The relative survival rate was calculated by the following equation:

$$\frac{(A_{Sample} - A_{Blank})}{A_{Control} - A_{Blank}},$$

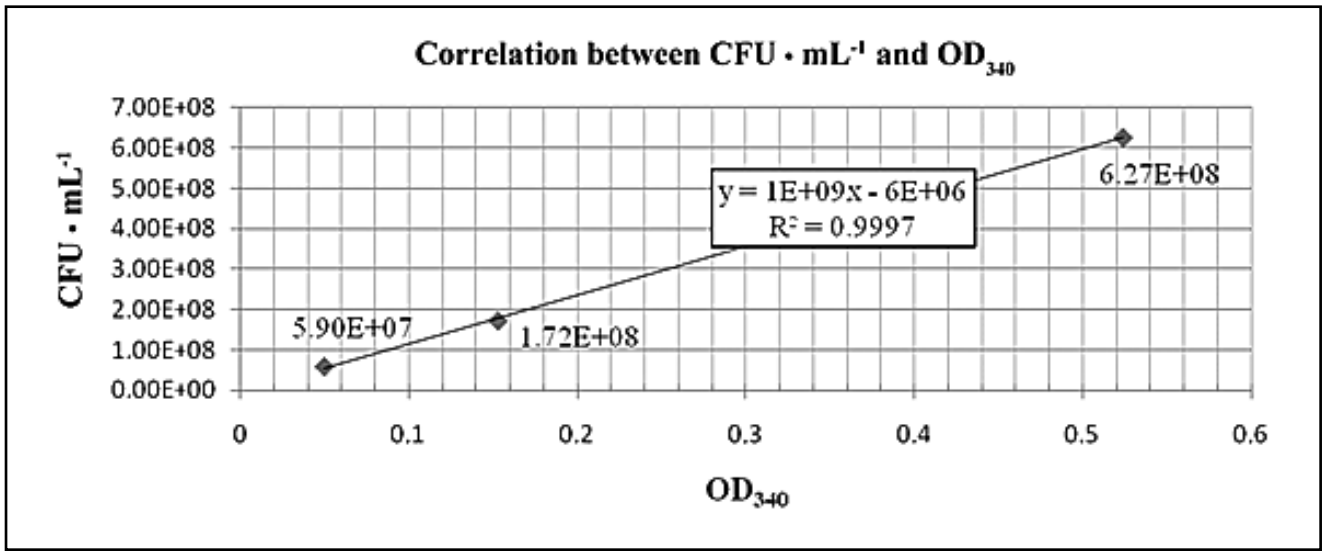

Fig. 1. The correlation between optical density and the amount of bacterial cells.

and multiplied by 100 for the result in percentage. The relative cytotoxicity rate was determined as described previously (Tengler *et al.* 2013). All data were evaluated using GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com).

Statistical analysis

The statistical calculations of the results were carried out using the software MS Office and Origin computer programs. The research results were treated by methods of variation statistics using Student *t*-test. Statistical significance was tested using the one-way analysis of variance with Dunnett's test and Tukey post-test for comparisons between the means, and differences between two conditions were retained for $p \leq 0.05$. Statistical significance was determined at levels of $p<0.05$, $p<0.01$, and $p<0.001$ (Bailey 1995).

RESULTS

The structure of the discussed salicylamides, their molecular weight and position of the lateral group are shown in Table 1.

The effect of the selected salicylamides in increasing concentrations on the growth of sulfate-reducing bacteria *D. piger* Vib-7 was studied. As shown in Figure 2, the highest level of bacterial biomass accumulation was found in the control KS medium (compounds-free group). Maximum biomass (3.88±0.36 mg.mL^{-1}) of the bacteria *D. piger* Vib-7 on the 60th hour of cultivation was accumulated. Exponential growth phase started from the beginning of bacterial cells seeding in the medium and continued until 48th hour, subsequently, the stationary growth phase started. Treatment with salicylamides in the concentration range between 0.37 to 30 μmol.L^{-1} led to decrease in biomass accumulation (Figure 2).

Under the effect of 0.37–1.1 μmol.L^{-1} **1**–**5** compounds, the growth on the 48th hour of cultivation was inhibited by 8–53, 64–66, 49–50, 82–90, 43–46% compared to control, respectively. The highest level of growth inhibition was observed at higher concentrations (3.3–10 μmol.L^{-1}) of the compounds. Treatment with 30 μmol.L^{-1} of studied compounds completely inhibited the growth of *D. piger* Vib-7. Most likely, this concentration of salicylamides was the most toxic for bacterial cells, as a complete lack of the growth and statistically significant reduction in the initial biomass of the cells (initial seeding) was observed. Based on these data, we can assume that these compounds in concentration of 30 μmol.L^{-1} can cause the lysis of bacterial cells.

Sulfate-reducing bacteria consume the sulfate ions as electron acceptor in the process of dissimilatory sulfate reduction (Kushkevych 2012a, 2012b). The final metabolite of such a process is hydrogen sulfide, which accumulates in the medium. These bacteria also need organic compounds as electron donor (Kushkevych & Moroz 2012). In our previous studies, it was shown that the bacteria *D. piger* Vib-7 consumed sulfate and accumulated hydrogen sulfide in concentration of 2.31±0.21 mmol.L^{-1}. They use lactate as electron donor which oxides to acetate (Kushkevych 2013).

The effect of different concentration of salicylamides on the process of dissimilatory sulfate reduction in *D. piger* Vib-7 cells on the 36th of cultivation was studied. As shown in Figure 3, the addition of the compounds in the culture medium inhibits the process of dissimilation sulfate directly proportional to the increase in concentrations (0.37–30 μmol.L^{-1}).

Under these conditions, the utilization of sulfate and lactate was inhibited, hence the level of accumulation of hydrogen sulfide and acetate was reduced. These data are consistent with our research in previous series of the experiments. The percentage inhibition of sulfate reduction process correlates with the percentage of bacterial growth inhibition under the salicylamides treatment.

Tab. 1. Structure of the discussed salicylamides (Pauk *et al.* 2013); and minimal inhibitory concentration (MIC) and inhibitory concentration (IC_{50}) of compounds **1**–**5** and ciprofloxacin standard against intestinal sulfate-reducing bacteria *D. piger* Vib-7.

Comp.	R^1	R^2	MW	Name	MIC (μmol.L^{-1})	IC_{50} (μmol.L^{-1})
1	5-Cl	4-Br	326.57	*N*-(4-bromophenyl)-5-chloro-2-hydroxybenzamide	<0.37	>1.1
2	5-Cl	4-CF_3	315.67	5-chloro-2-hydroxy-*N*-[4-(trifluoromethyl)phenyl]benzamide	<0.16	>0.28
3	5-Cl	3,4-Cl	316.57	5-chloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide	<0.25	>0.37
4	5-Cl	4-NO_2	292.68	5-chloro-2-hydroxy-*N*-(4-nitrophenyl)benzamide	<0.27	>0.32
5	4-Cl	3,4-Cl	316.57	4-chloro-*N*-(3,4-dichlorophenyl)-2-hydroxybenzamide	<0.24	>1.1
CPX	–	–	–	ciprofloxacin	>41	>68

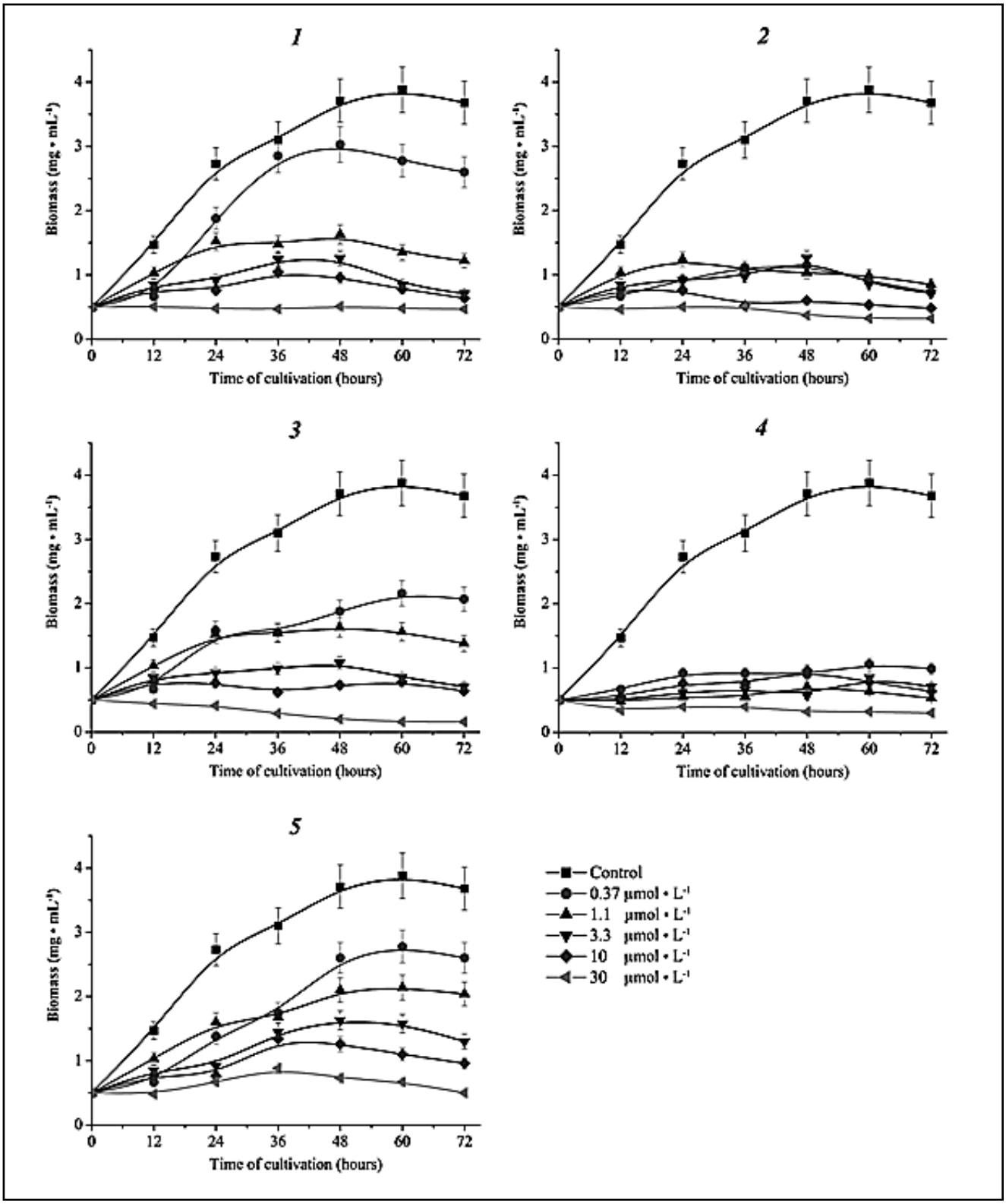

Fig. 2. Growth of *D. piger* Vib-7 cells under the effect of salicylamides in indicated concentrations.

The relative survival of *D. piger* Vib-7 cells and cytotoxicity of salicylamides using WST-1 method was studied. In this series of experiments, compounds **1**–**5** exerted cytotoxicity against these bacteria already in low (0.37–1.1 μmol.L^{-1}) concentrations (Figure 4).

The data on relative survival of *D. piger* Vib-7 cells and relative cytotoxicity under the salicylamides effect were in concert with the previously obtained results of bacterial growth and inhibition level of dissilitatory sulfate reduction process. Treatment with all tested com-

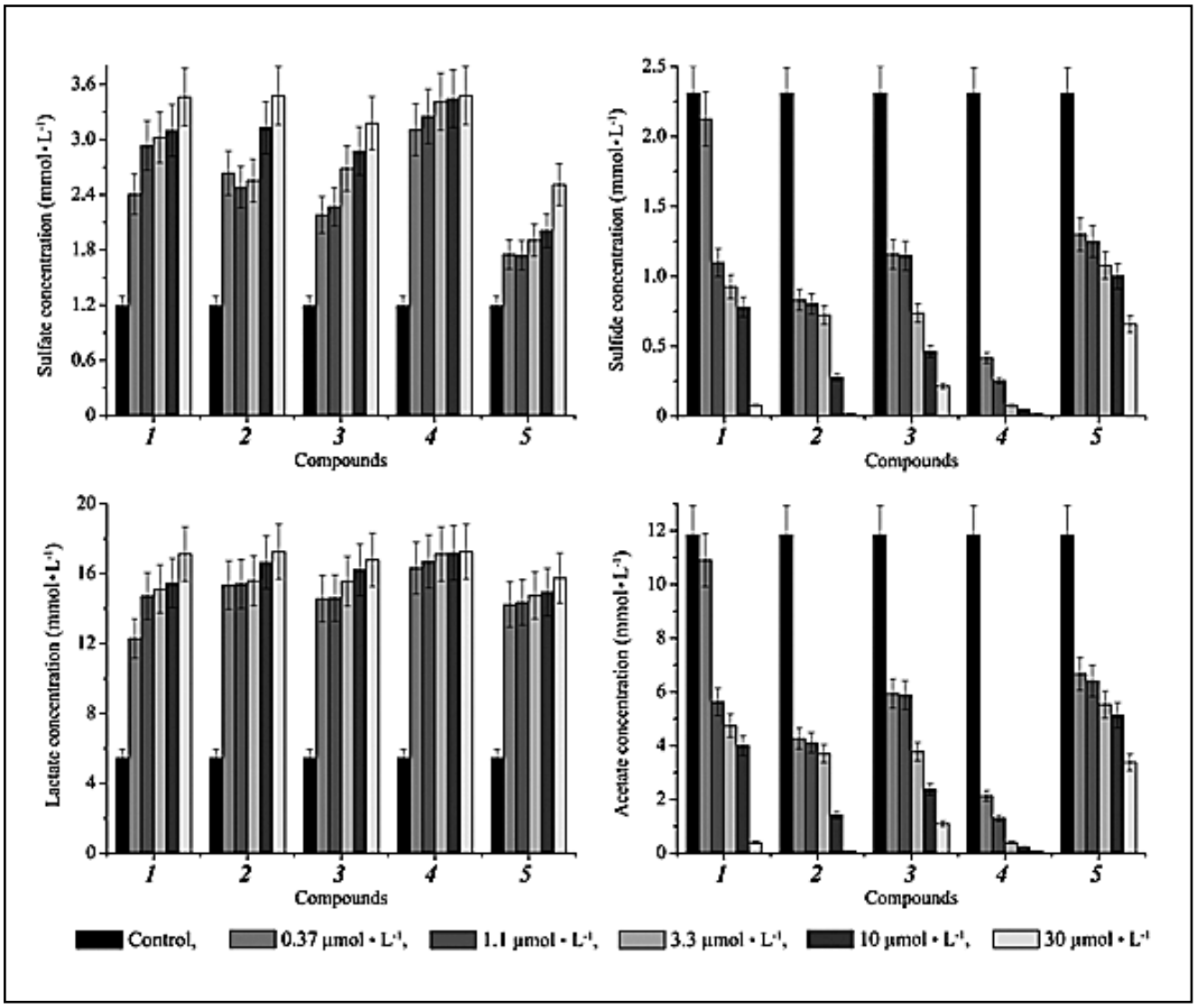

Fig. 3. Sulfate reduction of *D. piger* Vib-7 cells under the treatment of indicated doses of salicylamides.

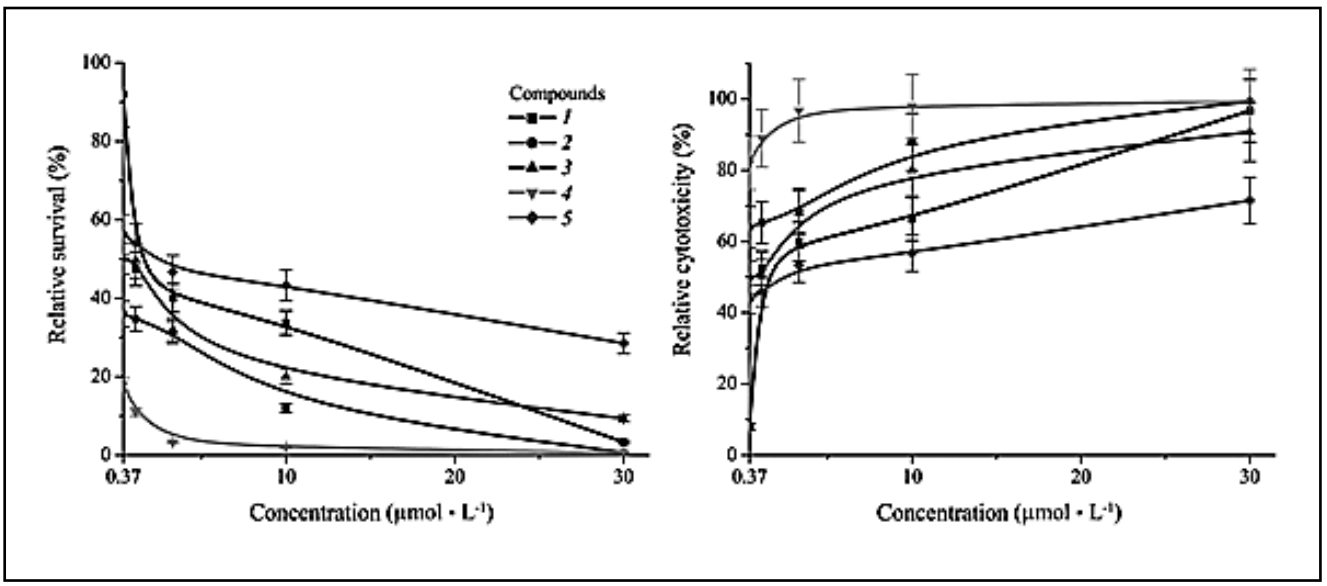

Fig. 4. Relative survival of *D. piger* Vib-7 cells and cytotoxicity of salicylamides.

pounds led to a significant dose-dependent cytotoxicity, with the strongest effect observed in compounds **4** and **5**.

Since some of salicylamides (such as **4**, **2**, or **3**) have a pronounced antimicrobial effect against *D. piger* Vib-7 (combination of low survival and high toxicity rates) they could be considered as promising agents against the growth of this type of bacteria.

Based on obtained results, the minimal inhibitory concentration (MIC) and inhibitory concentration (IC_{50}) of salicylamides against intestinal sulfate-reducing bacteria were established. As shown in Table 1, the MIC for all compounds was lesser 0.37 μmol.L^{-1}.

Regarding IC_{50}, for the compounds **1** and **5**, it was over 1.1 μmol.L^{-1}. On the other hand, for the compounds **3** and **4** the IC_{50} was over 0.37 and 0.32 μmol.L^{-1} respectively. Concerning IC_{50}, only for the compound **2** was over 0.28 μmol.L^{-1}, while all other compounds had shown IC_{50} over 30 μmol.L^{-1}.

DISCUSSION

Sulfate-reducing bacteria *Desulfovibrio* genus belongs to the intestinal microbiota of humans and animals (Kushkevych 2012b). They are anaerobic microorganisms, dissimilating sulfate as an electron acceptor and organic compounds as an electron donor and carbon source in the process of "dissimilatory sulfate reduction" (also known as "dissimilatory anaerobic sulfate respiration") (Kushkevych 2012a). Lactate is the most common substrate used by the species belonging to the intestinal sulfate-reducing bacteria. The species of *Desulfovibrio* oxidize lactate incompletely to acetate. Lactate oxidation to acetate occurs together with the concurrent reduction of sulfate to sulfide (Barton & Hamilton 2010). The presence of lactate and sulfate in the human intestine contributes to the intensive bacteria growth and the accumulation of their final metabolic products, acetate and hydrogen sulfide, which are toxic, mutagenic and cancerogenic to epithelial intestinal cells (Pitcher *et al.* 1996; Rowan *et al.* 2009). There is also an assumption that sulfate-reducing bacteria can cause some forms of cancer of the rectum through the formation of hydrogen sulfide.

In the light of our results it can be stated that almost all of salicylamides in concentrations above 10 μmol.L^{-1} have an interesting antimicrobial activity against *Desulfovibrio piger*. This activity was concentration-dependent, with the strongest effect in 30 μmol.L^{-1} concentrations. Tested compounds can be considered as good alternative for the treatment of colitis or colorectal cancer although it should be taken in account that these compounds can be aggressive also to commensally bacteria and even to other parts of the human body. This should be a concern to be clarified in the near future with complementary assays.

Based on all obtained results in our study we can conclude that compounds **1**–**5** in concentration 0.37–1.1 μmol.L^{-1} inhibited the growth on the 48th hour of cultivation by 8–53, 64–66, 49–50, 82–90, 43–46% compared to control, respectively. The highest level of growth inhibition was observed at higher concentrations (3.3–10 μmol.L^{-1}) of the compounds. These results are consistent with a data on inhibition process of dissimilation sulfate. A significant cytotoxic activity under the influence of salicylamides was determined. The strongest cytotoxic effect was observed in compounds **4** and **5**. Derivatives **4**, **2**, and **3** showed low survival and high toxicity rates, and thus are interesting for further studies.

Understanding the role of sulfate-reducing bacteria in colonic conditions would be enhanced by the ability to inhibit the number of the sulfate-reducing bacteria and/or reduce the production of sulfide and acetate. This would help to clarify the factors influencing sulfide production in the human and animal colon.

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