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Anticancer and antimicrobial properties of imidazolium based ionic liquids with salicylate anion

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Abstract: Ionic liquids (ILs) are well known for their physico-chemical properties which recommend them for many purposes. Still, many of ILs are not environmentally friendly. Having in minds these facts, we designed a series of imidazolium-and salicylate-based ILs with low general toxicity and studied their pharmacological potential. Here we present their antiproliferative effect against human cancer cell lines and antimicrobial activity on selected Gram-positive and Gram-negative bacterial and Candida strains. ILs with 1-butyl-3-methylimidazolium or imidazolium cation (ILs 1 and 5), with the lowest dipole moments and highest lipophilicity, exerted highest cytotoxicity against colon and/or lung cancer cells, manifesting high selectivity to the normal cells. The most non-polar IL with 1-butyl-3-methylimidazolium cation expressed the strongest anticancer potential, but it was toxic against normal cells as well, although its cytotoxicity was less than the cytotoxic effect of commercially used chemotherapeutic agents. The same compounds (ILs 1 and 5) expressed modest effect on the bacterial strains which causes serious lung diseases and pulmonary infections (S. aureus) or which are included in colon cancer formation (E. coli and E. faecalis). Salicylate itself was toxic against lung cancer cell line A549 and affected some Candida strains.

Keywords: ionic liquids; imidazolium; salicylate; cytotoxicity, antibacterial activity, antifungal activity

INTRODUCTION

Ionic liquids (ILs) express wide range of physico-chemical features that can find many different applications, including biomedicine. Therefore, it is not...
surprising that they have drawn the attention of biomedical researchers as convenient catalytic media for drug synthesis, as well as potential components of drug formulations. Several notable reviews have emphasized the advantages of using ionic liquids in medicinal chemistry. Improvement of water-solubility and thus bioavailability, by conversion of active pharmaceutical ingredients (API) to ionic liquid represents a common way for improving drug formulations. Physical, chemical and biological properties of these compounds, such as lipophilicity or toxicity, are connected to their bioavailability as well. Furthermore, there are many well-known examples where combined biologically active cations and anions result in salts that exhibit therapeutic effects of both of its ingredients. Moreover, today is emphasized and should be taken into account that many ILs have not been declared as environmentally friendly chemicals, so, before medical use, toxicity has to be tested; and vice versa, thanks to their toxicity, a plethora of distinct new opportunities for their application, especially in the area of medicine, are being created.

There are no many studies presenting cytotoxic potential of salicylate-based ILs against human or animal cell lines or against microorganisms, while imidazolium-based ILs are more studied. The particular mechanism of biological activity of ILs may vary among the different organisms, but water seems to be of great importance for most mechanisms in the living systems. Water-solubility and interactions with water are crucial factors determining biological, pharmacological, but also environmental activity of ILs. Biological activity of ILs was shown to depend on their hydrophilicity and hydration state/hydration number. Starting from the assumption ‘more polar–less toxic’, we presumed lower cytotoxicity of the ILs with polar groups and thus tested it. According to that, the aim of our study was to assess the anticancer potential of imidazolium-based ionic liquids with salicylate anion in the human cancer cell lines using the MTT cell viability assay, as well as antimicrobial potential of these compounds against several bacterial and Candida strains by standard double diluted antimicrobial procedure.

EXPERIMENTAL

Tested imidazolium based ILs with salicylate anion: Imidazolium-based ionic liquids 1–4 and salt 5 with salicylate anion were prepared according to the procedure published before. Their structures and names are presented in the Table 1. Since ILs are water-soluble compounds, stock solutions of each were prepared by solubilisation in distilled water, as well as diluted solutions for tests.

In order to obtain values of dipole moments for tested compounds, DFT calculations were performed using Maestro program, as a part of Schrödinger Suite 2015-2 package. All structures were geometrically optimized firstly, using conformational search and later DFT calculations were performed using functional B3LYP-d3 with basis set 6-31+G(d,p). Dipole moments were calculated from the obtained results, using a method developed by Rashin et al.
Table I. Structures and names of the tested ILs (1–4), salt 5 and reference sodium salicylate (6)

<table>
<thead>
<tr>
<th>Structure of cation</th>
<th>Structure of anion</th>
<th>Name of compound/Code</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Butyl-3-methylimidazolium salicylate" /></td>
<td><img src="image2" alt="COO" /></td>
<td>1-Butyl-3-methylimidazolium salicylate (1)</td>
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<tr>
<td><img src="image3" alt="1-(4-Hydroxy-2-oxobutyl)-3-methylimidazolium salicylate" /></td>
<td><img src="image2" alt="COO" /></td>
<td>1-(4-Hydroxy-2-oxobutyl)-3-methylimidazolium salicylate (2)</td>
</tr>
<tr>
<td><img src="image4" alt="1-(2-Oxobutyl)-3-methylimidazolium salicylate" /></td>
<td><img src="image2" alt="COO" /></td>
<td>1-(2-Oxobutyl)-3-methylimidazolium salicylate (3)</td>
</tr>
<tr>
<td><img src="image5" alt="1-(3-Hydroxypropyl)-3-methylimidazolium salicylate" /></td>
<td><img src="image2" alt="COO" /></td>
<td>1-(3-Hydroxypropyl)-3-methylimidazolium salicylate (4)</td>
</tr>
<tr>
<td><img src="image6" alt="Imidazolium salicylate" /></td>
<td><img src="image2" alt="COO" /></td>
<td>Imidazolium salicylate (5)</td>
</tr>
<tr>
<td><img src="image7" alt="Na⁺" /></td>
<td><img src="image2" alt="COO" /></td>
<td>Sodium salicylate (6)</td>
</tr>
</tbody>
</table>

**Determination of antiproliferative activity:** Antiproliferative activity of the imidazolium based ILs with salicylate anion was tested against six human cancer cell lines: two types of human breast adenocarcinoma, thus the estrogen receptor positive (ER+) MCF-7 (American Type Culture Collection–ATCC HTB22) and triple negative MDA-MB-231 (ATCC HTB26), prostate cancer PC-3 (ATCC CRL 1435), cervix adenocarcinoma HeLa (ATCC CCL2), colon cancer HT-29 (ATCC HTB38) and lung cancer A549 (ATCC CCL 185) cell lines, as well as normal fetal lung fibroblast cell line MRC-5 (ATCC CCL 171). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% glucose, supplemented with 10% of fetal calf serum (Sigma) and antibiotics: 100 IU/mL penicillin and 100 µg/mL streptomycin.
Cells were cultured in flasks (Costar, 25 cm$^2$) at 37 °C in high humidity with 5% CO$_2$. Only viable cells were used in the assays, and cell viability was determined by trypan blue dye exclusion test.

Antiproliferative activity of the imidazolium-salicylate-based ILs was evaluated by tetrazolium colorimetric MTT assay, as previously described. To measure the number of viable cells in microwell plates, cells were exposed to test compounds for 72 h at five concentrations ranging from 0.01 to 100 μM (0.01; 0.1; 1; 10 and 100 μM). Reference compounds used in this assay were cisplatin (Cis) and doxorubicin (Dox), as nonselective anticancer agents, and sodium salicylate to test salicylate toxicity, respectively. The IC$_{50}$ value, defined as a dose of compound that inhibits the cell growth by 50% related to control (untreated) cells, was determined for each tested compound by median effect analysis.

Antimicrobial activity and data analysis: Six bacterial strains including three Gram-positive (G$^+$) bacteria: S. aureus $h$ (human), B. subtilis ATCC 6633 and E. faecalis ATCC 19433 and three Gram-negative (G$^-$) bacteria: P. mirabilis $h$, E. coli ATCC 11229 and P. aeruginosa ATCC 15692, and four yeast strains: two of them (C. albicans L and C. albicans ATCC 10231) were obtained from the culture collection of microorganisms from Department of Biology and Ecology, University of Novi Sad, while two human yeast isolates (C. albicans III $h$ and Candida IV $h$) were obtained from the Faculty of Medicine, Clinical Centre of Vojvodina. All human isolates of microorganisms were obtained from the Faculty of Medicine, Department of Obstetrics and Gynecology, University of Novi Sad, were protocol was approved by the Institutional Ethical Board of the same Institution. The antibacterial activity of ILs was evaluated as minimum inhibitory concentrations (MICs) and minimum bactericidal/fungicidal concentrations (MBCs, i.e. MFCs), by double-microdilution method according to the CLSI procedure.

Statistical analysis: Data were subjected to nonparametric analysis (principal component analysis—PCA and hierarchical cluster analysis—HCA) by using software STATISTICA 13.3 (Statsoft, Tulsa, Oklahoma, USA) based on the obtained MIC and MBC/MFC values on the tested microbial strains.

RESULTS AND DISCUSSION

It is postulated and proved that ionic liquids with polar ether, hydroxyl or nitrile functional groups within the side chains exhibit lower cytotoxicity compared to their structural analogues with non-polar alkyl side chains. Those functional groups were thought to impede cellular uptake by membrane diffusion and reduce lipophilicity-based interactions with the cell membrane. Otherwise, the study of the cytotoxicity of imidazolium-based ionic liquids showed that the chain length of alkyl substitution at N-3 position of imidazole ring plays crucial role towards their anti-cancer activity.

In our previous report synthesis and general toxicity of ionic liquids were presented. Among them was 1-butyl-3-methylimidazolium salicylate (1), salicylate analogue of well-known BMIM$^+$ cation-based ionic liquid, which expresses very good physico-chemical properties and, accordingly, have a wide range of applications. Compared to compound 1 which possesses non-polar n-butyl substituent at N-1, ILs with more polar substituents (compounds 2-5) showed lower general toxicity (Table II).
In this study we have tested the effect of imidazolium based ILs with salicylate anion 1-5 and salt 6 against human cancer cell lines, as well as against selected bacteria or fungal strains. The most sensitive cell line was colon cancer cell line HT-29. The most cytotoxic against these cells was ionic liquid 2, with IC_{50} concentration 9.26 μM. Substituent on N-1 position of this compound is quite polar, containing both ether and hydroxyl functions. Salicylate 5, with non-substituted imidazolium moiety as cation, showed modest cytotoxicity against HT-29 cells, with IC_{50} 39.94 μM, while other ILs did not decrease numbers of HT-29 cells significantly, i.e. their effects were very weak, even in the highest concentrations tested (Table II). Similar effect as 2 expressed compound 1 (IC_{50} 14.89 μM), with the most non-polar group (n-butyl), though compound 1 showed general toxicity: it was also toxic against normal human lung fibroblasts (MRC-5 cell line, IC_{50} 27.54 μM). Beside colon carcinoma cell line, lung adenocarcinoma cell line A549 was sensitive to the tested salicylate ILs as well, although in a more modest manner. Against this cell line modest effect expressed compounds 3 and 5 (IC_{50}s 57.31 μM and 23.03 μM), carrying ether or hydroxyl function, respectively. Reference sodium salicylate also expressed moderate antiproliferative effect against these cells (IC_{50} 44.39 μM).

Structure-activity relationship study could be based on the polarity of the tested ionic liquid compounds, studied via dipole moment vectors (Table III). ILs with the larger dipole moments (2, 3 and 4) induced lower effect on cancer cells, and vice versa, ILs with lower dipole moments expressed stronger effect against cancer cell lines.

Widely used anticancer agents cisplatin (Cis) and doxorubicin (Dox) were used as reference compounds in this study. They are no selective. On the contrary, they were similarly effective in the lowering of number of both normal and cancer cells (Table II), which is in accordance to other studies.\(^{16,17}\)

Table II. Antiproliferative effect, estimated by MTT test and presented as IC_{50} values, of the tested imidazolium- based ILs and salt 1-5 and salicylate- and reference compounds: sodium salicylate, doxorubicin and cisplatin after 72 h treatment

<table>
<thead>
<tr>
<th>Comp.</th>
<th>IC_{50} μM</th>
<th>MCF-7</th>
<th>MDA-MB-231</th>
<th>PC-3</th>
<th>HeLa</th>
<th>HT-29</th>
<th>A549</th>
<th>MRC-5</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>&gt;100</td>
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<td>&gt;100</td>
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<tr>
<td>Dox</td>
<td>0.29</td>
<td>0.09</td>
<td>89.90</td>
<td>1.68</td>
<td>0.10</td>
<td>7.52</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Cis</td>
<td>1.60</td>
<td>2.64</td>
<td>4.56</td>
<td>2.10</td>
<td>4.10</td>
<td>3.20</td>
<td>0.24</td>
<td></td>
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</tbody>
</table>

\(^{N/A—IC_{50}}\text{ value was not available due to very low effect}\)
TABLE III. Dipole moments of the cations and anion of the tested imidazolium-salicylate-based compounds 1-5

<table>
<thead>
<tr>
<th>Comp. label of cation / anion</th>
<th>Visualization of dipole moment</th>
<th>Dipole moment, D</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 $n$-butyl</td>
<td><img src="image1" alt="Visualization of dipole moment" /></td>
<td>5.40</td>
</tr>
<tr>
<td>2 4-Hydroxy-2-oxobutyl</td>
<td><img src="image2" alt="Visualization of dipole moment" /></td>
<td>8.14</td>
</tr>
<tr>
<td>3 2-Oxobutyl</td>
<td><img src="image3" alt="Visualization of dipole moment" /></td>
<td>8.62</td>
</tr>
<tr>
<td>4 3-Hydroxypropyl</td>
<td><img src="image4" alt="Visualization of dipole moment" /></td>
<td>7.14</td>
</tr>
<tr>
<td>5 H</td>
<td><img src="image5" alt="Visualization of dipole moment" /></td>
<td>3.92</td>
</tr>
<tr>
<td>6 Salicylate</td>
<td><img src="image6" alt="Visualization of dipole moment" /></td>
<td>6.68</td>
</tr>
</tbody>
</table>
The most non-polar IL from the group of tested ILs, n-butyl derivative 1 was the only IL that decreased the number of normal cells, which is in agreement with other results on reducing toxicity with increasing polarity.\textsuperscript{21-23} Selectivity indexes for tested ILs 1-5 could not be calculated and thus selectivity of ILs quantified, except for compound 1, because IC\textsubscript{50} values for compounds 2-5 are too high (though IC\textsubscript{50} values in Table II are presented simply as values higher than 100 μM), \textit{i.e.} cytotoxicity of these compounds, even in the highest tested concentration, is very low.

On the other hand, salicylic acid (which is also metabolically obtained from acetylsalicylic acid/Aspirin) is long since recognized in \textit{in vitro} tests as an anticancer agent,\textsuperscript{30} that induces apoptosis by activating caspases. Still, it also could be used in preadministration, and thus increase the chemosensitivity of some cancer cells, because it suppresses NF-κB activation, and is included in the proapoptotic signal modulation therapy.\textsuperscript{31} Considering the fact that ionic liquids contain salicylate anion, the overall effect of ionic liquids could be attributed partly to the effect of the salicylate anion itself, although in our study reference compound sodium salicylate (6) expressed modest antiproliferative effect only against A549 lung adenocarcinoma cell line (Table II).

In the antimicrobial testing (Table SI), the most resisting strains (R\textsuperscript{*}) towards common antibiotics streptomycin, kanamycin and ampicillin were \textit{P. aeruginosa} ATCC 15692 and \textit{P. mirabilis} h. Two ATCC strains \textit{B. subtilis}, \textit{E. coli} and one human isolate, \textit{S. aureus} h, were sensitive towards all antibiotics at the lowest concentrations applied (0.01 mg/ml). On the contrary, tested ionic liquids expressed weak antimicrobial potential towards all treated bacterial strains, except compounds 1 and 5 that were the most effective, exerting modest antimicrobial activity against \textit{S. aureus} (both compounds) and \textit{P. aeruginosa} (5). Since \textit{P. aeruginosa}, a Gram-negative opportunistic pathogen, was resistant towards almost all tested antibiotics, this result is getting in importance, especially bearing in mind that this bacterial species often causes acute pneumonia with a high mortality rate, especially in the compromised host.\textsuperscript{32} Compound 5 was effective in decreasing the number of lung carcinoma cells A549 as well.

Furthermore, both compounds 1 and 5 affected \textit{S. aureus}, for which connection with lung diseases is also postulated, since it causes serious pulmonary infections in adults with concomitant illnesses that are typically nosocomial or in patients with cystic fibrosis.\textsuperscript{33} Human isolates such the strain \textit{S. aureus} h, are well known to be more resistant to common antibiotics due to general phenomena of antibiotic resistance that is accelerated by the misuse or overuse of antibiotics, as well as poor infection prevention and control.\textsuperscript{34} Hence, modest effect obtained for both compounds 1 and 5 on tested human strains could implicate these ILs as new pharmacons with antimicrobial potential.
*E. coli* is a common minor component of the colonic microbiota, while some bacterial strains, such as tested *E. coli* and *E. faecalis*, present pathogens that are responsible for the colon cancer initiative process and promotion of tumorigenesis via genotoxic effect. Although in the present study tested ILs exerted quite low antibacterial effect against these two strains compared to the antibiotics, it should be kept in mind that these compounds, namely 1 and 5, affected colon adenocarcinoma cells HT-29, which make them worthy of attention in further drug development.

All tested ionic liquids or salts (1-5), possessing both imidazolium-based cation and salicylate anion, were effective against *Candida* strains. Still, compounds 4 and 5 expressed the strongest effects against *Candida* strains (Table SI). Salicylate salt with non-substituted imidazolium cation (IL 5) was the most active in testing of anticancer and antibacterial potential. The role of water in living organisms is recognized long time ago, but water’s roles in sustaining life are not understood well yet, though many researchers are working in this research area. Still, in protic surroundings of the living cells, protons from N atoms of imidazolium ring of ionic liquid 5 looks to play an important role in the biological activity of this compound.

According to the antimicrobial activity of salicylate anion (compound 6) tested in this study, its antibacterial effect was low, while its antifungal property was more exerted: it was effective towards eukaryotic cells of all four *Candida* strains, including both human isolates (III h, IV h) of gynaecological origin. Salicylic acid (salicylate) is already known for some time as good antifungal agent, useful in treatment of *Candida* strains, especially against *Candida* biofilm formation. It is reported previously that salicylic acid suppresses growth of *C. albicans* via suppressing 3(R)-hydroxyoxylipins, selectively located in *Candida* hyphae and other filamentous structures. Moreover, *C. albicans* stimulates prostaglandin E2 (PGE2) production in HeLa cells. All tested ILs contain salicylate anion, hence it can be assumed that expressed anti-candida properties are derived therefrom, as well as connection to the effect of salicylate-based ionic liquid 1 against HeLa cells.

According to hierarchical cluster analysis (HCA) presented in Fig. 1a it can be seen that all investigated ionic liquids are grouped into two clusters and one outlier due to a different alkyl chain length on the imidazolium ring in the position N-1. The first cluster is formed by the ionic liquids with alkyl chain with four carbon atoms: IL 1, IL 2 and IL 3. Ionic liquids IL 5 and IL 6 without any alkyl chain form the second cluster, and the IL 4 with propyl-derived substituent is an outlier. Thus, it can be concluded that the antimicrobial activity of studied ionic liquids mainly depends on the alkyl chain length in the cation structure.
Fig. 1. HCA and PCA: a. Dendrogram of examined ionic liquids according to their antimicrobial activity; b. Score plot as a PCA result of PC1 variation with PC2 for investigated ILs; c. Dependence of PC3 with dipole moments (D) of studied imidazolium based ILs (1-5). Correlation coefficient R = 0.946; d. Dendrogram of examined bacterial and yeast strains; e. Score plot as a PCA result of PC1 variation with PC2 for investigated bacterial and yeast strains: 20 – Candida IV h MIC; 19 – C. albicans IV h MBC/MFC; 17 – C. albicans III h MBC/MFC; 15 – C. albicans ATCC10231 MBC/MFC; 14 – C. albicans sp. L MIC; 12 – P. aeruginosa ATCC15692MIC; 2 – S. aureus h MIC; circled – all other.
In our case, three principal components describe 94% of the total variance in the data (PC1 64.26%, PC2 22.56% and PC3 7.21%). The results of the principal component analysis (PCA) are in a good agreement with those obtained using HCA and the same trend is observed in the Fig. 1b, where the first two PCs separate studied ionic liquids according to the alkyl chain length in the position N-1 of the imidazolium ion.

Obtained PC3 represented by 7.21% can be related by the polarity of investigated ILs. Namely, variation of the PC3 with calculated dipole moments of ILs is linear, as shown in Fig. 1c. The obtained linear dependence means that PC3 carries important information on the influence of the dipole moment on the antimicrobial activity of the investigated ILs.

The same analyses (HCA and PCA) were performed using MIC and MBC/MFC values obtained for all investigated bacterial and yeast strains. Presented dendrogram obtained using HCA in Fig. 1d shows two main clusters, dominantly separating Candida strains into one cluster and bacterial strains into the second one. In the second cluster, two subclusters can be observed, where the bacterial strains are divided according to their MIC and MBC/MFC values. Also, the results obtained by PCA (Fig. 1e), indicate the same trend since the PC1 separates Candida strains from the studied bacterial strains. In addition, PC2 separates procaryotic cells bacteria P. aeruginosa ATCC 15692 and S. aureus h from all other as indicated in Fig. 1e. Among the eucaryotic Candida strains, it can be seen that Candida sp. L is slightly distant, which is based on its highest sensitivity.

CONCLUSION

Ionic liquids, as water soluble, are usable for many purposes, so as pharmaceuticals. Studied imidazolium-based ILs possess different substituents at N-1 position, containing alkyl chain with different polarity, and salicylate anion. IL 2, with alkyl chain containing both ether and hydroxyl functions, expressed the best anticancer potential against colon cancer cells. Compound 5 with non-substituted imidazolium cation has a large dipole moment as well, and it showed modest anticancer potential against both lung and colon cancer cells. These compounds were selective towards cancer cell lines only. IL with the most non-polar cation, IL 1, expressed the highest cytotoxicity, but with no selectivity. Further experiments towards ILs for pharmacological purposes are in progress.

SUPPLEMENTARY MATERIAL

Supplementary Material are available electronically from http://www.shd.org.rs/JSCS/.

Acknowledgment. This research was supported by the Ministry of Education, Science and Technological Development, Republic of Serbia (Grant No. 172012 and 172058).
ИЗВОД
АНТИКАНЦЕРСКЕ И АНТИМИКРОБНЕ ОСОБИНЕ ЈОНСКИХ ТЕЧНОСТИ НА БАЗИ ИМИДАЗОЛА СА САЛИЦИЛАТИМ АНЈОНОМ

СУЗАНА ЈОВАНОВИЋ-ШАНТА, ВЕСНА КОЈИЋ1, КРИСТИНА АТЛАГИЋ2, АЛЕКСАНДРА ТОТ, МИЛАН ВРАНЕЋ, СЛОБОДАН ГАДУРИЋ И МАЈА КАРАМАН1

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Јонске течности (ЈТ) су добро познате по својим физичко-хемијским својствима која их препоручују за употребу у многе сврхе. Ипак, многе ЈГ нису еколошки прихватљиве. Имајући у виду ове чињенице, дизајнирали смо низ имидазолима резких ЈТ са салицилатним анјоном ниске опште токсичности и прочувале њихов фармаколошки потенцијал. У раду је приказан њихов антиксиперферативни ефект на ћелијске линије хуманских кациера и антимикробна активност на одабраним Gram-позитивним и Gram-позитивним бактеријским и Candida сојевима. ЈТ са 1-бутил-3-метилимидазолимским катионом (ЈГ 1 и 5), са највишим депоним моментима и највишим цитотоксичности, показали су највећу цитотоксичност на ћелије кациера колона и/или плућа, испољавајући високу селективност према нормалним ћелијама. Најинтензивнија ЈТ, са 1-бутил-3-метилимидазолимским катионом, показала је највиши антиканцеренти потенцијал, али је такође била токсична и на нормалне ћелије, мада је њена цитотоксичност била мања од цитотоксичног ефекта комерцијално коришћених хемотерапеутских средстава. Иста једињења (ЈГ 1 и 5) испољавала су и мањи ефект на бактеријске сојеве који узрокују забиљне плућне болести и плућне инфекције (S. aureus), као и на сојеве укључене у настанак кациера колона (E. coli и E. faecalis). Сам салицилата анјон је био токсичан за ћелијску линију плућа плућа A549, а деловао је и на неке Candidа сојеве.

(Примање 22, мај; ревирирано 12. септембра; прихваћено 12. септембра 2019)

REFERENCES
1. K. S. Egorova, E. G. Gordeev, V. P. Ananikov, Chem. Rev.117 (2017) 7132 (http://dx.doi.10.1021/acs.chemrev.6b00562)
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**Determination of antiproliferative activity**

**Cell lines and cell culture:** Antiproliferative activity of the imidazolium-and salicylate-based ILs was tested against six human cancer cell lines: two types of human breast adenocarcinoma, thus the estrogen receptor positive (ER+) MCF-7 (American Type Culture Collection–ATCC HTB22) and triple negative MDA-MB-231 (ATCC HTB26), prostate cancer PC-3 (ATCC CRL 1435), cervix adenocarcinoma HeLa (ATCC CCL2), colon cancer HT-29 (ATCC HTB38) and lung cancer A549 (ATCC CCL 185) cell lines, as well as normal fetal lung fibroblast cell line MRC-5 (ATCC CCL 171). Cells were grown in Dulbecco’s modified Eagle's medium (DMEM) with 4.5 % glucose, supplemented with 10 % of fetal calf serum (Sigma) and antibiotics: 100 IU/mL penicillin and 100 μg/mL streptomycin (Sigma). Cells were cultured in flasks (Costar, 25 cm²) at 37 °C in high humidity with 5 % CO₂. Only viable cells were used in the assays, and cell viability was determined by trypan blue dye exclusion test.

**Antiproliferative activity and data analysis:** Antiproliferative activity of the imidazolium-and salicylate-based ILs was evaluated by tetrazolium colorimetric MTT assay,¹⁴ as previously described.¹⁵ To measure the number of viable cells in microwell plates, cells were exposed to test compounds for 72 h at five concentrations ranging from 0.01 to 100 μM (0.01; 0.1; 1; 10 and 100 μM). Reference compounds used in this assay were cisplatin (Cis) and doxorubicin (Dox), as nonselective anticancer agents¹⁶,¹⁷ and sodium salicylate to test salicylate toxicity, respectively. Exponentially growing cells were harvested, seeded into 96-well plates at a density of 5000 cells/well and allowed to stand overnight in complete medium at 37 °C, after which the medium containing the test compound was added (10 μL/well) in all wells except in negative controls. After 72 h treatment, 10 mL of MTT solution (5 mg/mL), and, after 3 h, acidified 2-propanol were added to each well. After a few minutes incubation at room temperature absorbance was read on a spectrophotometric plate reader (Multiscan MCC340, Labsystems) at 540/690 nm. Wells without cells, containing complete medium and MTT only, were used as a blank. Absorbances of samples (A_sample) and control (A_control) were measured and antiproliferative effect, presented as percent of cytotoxicity, was calculated according to the formula:

\[
CI, \% = (1 – A_{sample}/A_{control})100
\]

The antiproliferative activity of compounds (expressed as a percentage of cytotoxicity) was obtained by averaging values from two independent experiments conducted in quadruplicate for each administrated concentration. The IC₅₀ value, defined as a dose of compound that inhibits the cell growth by 50 % related to control (untreated) cells, was determined for each tested compound by median effect analysis.¹⁸
Antimicrobial activity and data analysis

**Bacterial and Candida strains:** Six bacterial strains including three Gram-positive (G⁺) bacteria: *S. aureus* h (human), *B. subtilis* ATCC 6633 and *E. faecalis* ATCC 19433 and three Gram-negative (G⁻) bacteria: *P. mirabilis* h, *E. coli* ATCC 11229 and *P. aeruginosa* ATCC 15692, and four yeast strains: two of them (*C. albicans* L and *C. albicans* ATCC10231) were obtained from the culture collection of microorganisms from Department of Biology and Ecology, University of Novi Sad, while two human yeast isolates (*C. albicans* III h and *Candida* IV h) were obtained from the Faculty of Medicine, Clinical Centre of Vojvodina. All human isolates of microorganisms were obtained from the Faculty of Medicine, Department of Obstetrics and Gynecology, University of Novi Sad, were protocol was approved by the Institutional Ethical Board of the same Institution.

**Antimicrobial assay:** The antibacterial activity of ILs was evaluated as minimum inhibitory concentrations (MICs) and minimum bactericidal/fungicidal concentrations (MBCs i.e. MFCs), by double-microdilution method according to the CLSI procedure. The strains of bacteria were obtained from the overnight cultures, grown at 37 °C on the Müller-Hinton agar (MHA, Torlak, Belgrade, Serbia), while yeasts strains were grown on the Sabouraud agar (SA, Torlak, Belgrade, Serbia) during 48 h. McFarland inoculum of bacteria and yeasts were prepared in the sterile saline solution; reaching the final 1.5×10⁶ CFU/mL for bacteria and 1.5×10⁵ for yeasts. Müeller Hinton broth (MHB, Torlak, Belgrade, Serbia) and Sabouraud broth (SB, Torlak, Belgrade, Serbia) were used for the antimicrobial screening. Double dilution test was performed in a 96-well microtitre plate (Spektar, Čačak, Serbia) with MHB or SB and different concentration of ILs, diluted in sterile distilled water. The final concentrations of ILs ranged from 0.01 – 11 mg / mL. After incubation, during 24 or 48 h for bacteria or yeast, respectively, MICs were determined visually. MBCs and MFCs were confirmed after inoculation of MHA and SA plates with 100 µL of broth, where turbidity was absent (MIC point). Nystatin, the antifungal drug (Hemofarm, Vršac, Serbia), and antibiotics streptomycin, kanamycin, ampicillin and chloramphenicol (Sigma), were used as positive controls (in final concentrations ranging from 0.01 – 0.45 mg / mL), while distilled water without ILs was used as negative control. Test was performed in triplicate for each compound and the average was used for getting MIC, MBC or MFC values.
Table SI MIC, MBC and MFC values of tested ILs and selected antibiotics/antimicotics towards bacterial and Candida strains

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Str</th>
<th>Kan</th>
<th>Amp</th>
<th>Chlo</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/mL</td>
<td></td>
<td></td>
<td></td>
<td>mg/mL</td>
<td></td>
<td></td>
<td></td>
<td>mg/mL</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> h</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
<td>0.03</td>
<td>4.50</td>
<td>9.01</td>
<td>9.60</td>
<td>9.60</td>
<td>8.65</td>
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</tr>
<tr>
<td><em>B. subtilis</em> ATCC 6633</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>9.01</td>
<td>9.01</td>
<td>9.60</td>
<td>9.60</td>
<td>8.65</td>
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<tr>
<td><em>E. faecalis</em> ATCC 19433</td>
<td>0.12</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
<td>9.01</td>
<td>9.01</td>
<td>9.60</td>
<td>9.60</td>
<td>8.65</td>
<td>8.65</td>
</tr>
<tr>
<td><em>P. mirabilis</em> h</td>
<td>R*</td>
<td>R*</td>
<td>0.23</td>
<td>9.01</td>
<td>9.01</td>
<td>9.60</td>
<td>9.60</td>
<td>8.65</td>
<td>8.65</td>
<td>11.03</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 11229</td>
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<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>9.01</td>
<td>9.01</td>
<td>9.60</td>
<td>9.60</td>
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</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 15692</td>
<td>R*</td>
<td>R*</td>
<td>0.12</td>
<td>9.01</td>
<td>9.01</td>
<td>9.60</td>
<td>9.60</td>
<td>8.65</td>
<td>8.65</td>
<td>11.03</td>
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</table>

<table>
<thead>
<tr>
<th>Fungal strains</th>
<th>Nystatin, mg/mL</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida L.</em></td>
<td>0.06</td>
<td>4.50</td>
<td>9.01</td>
<td>9.60</td>
<td>9.60</td>
<td>8.65</td>
<td>8.65</td>
</tr>
<tr>
<td><em>C. albicans</em> ATCC 10231</td>
<td>0.25</td>
<td>4.50</td>
<td>9.01</td>
<td>4.80</td>
<td>9.60</td>
<td>4.32</td>
<td>8.65</td>
</tr>
<tr>
<td><em>C. albicans</em> III h</td>
<td>0.25</td>
<td>4.50</td>
<td>9.01</td>
<td>4.80</td>
<td>9.60</td>
<td>4.32</td>
<td>8.65</td>
</tr>
<tr>
<td><em>Candida IV</em> h</td>
<td>0.25</td>
<td>4.50</td>
<td>9.01</td>
<td>4.80</td>
<td>9.60</td>
<td>4.32</td>
<td>9.65</td>
</tr>
</tbody>
</table>

| Str – streptomycin; Kan – kanamycin; Amp – ampicillin; Chlo – Chloramphenicol; R* – resistant; ↑ - the MBC/MFC value is higher than the highest tested concentration |