European Journal of Pharmaceutical Sciences 143 (2020) 105162

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Comparative biocompatibility and antimicrobial studies of sorbic acid derivates

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ARTICLE INFO

Keywords:
Sorbates
Preservatives
Biocompatibility
G. mellonella
Caco-2 cells
Antimicrobials

ABSTRACT

Nowadays, the sorbates are the third largest group of antimicrobial preservatives in food and pharmaceutical industries, following the parabens and benzoates whose safety is questioned by recent publications. A disadvantage of sorbates is their pH dependence, as their antimicrobial effect is greatly reduced in alkaline environment. The main, widely used sorbate derivatives are sorbic acid and potassium sorbate, no sorbic acid esters are involved in current industrial application. We aimed to test whether the esters of sorbic acid are capable to extend the antimicrobial spectrum of the original molecule while maintaining its advantageous biocompatibility profile. A comparative biocompatibility study of different derivatives (sorbic acid, potassium sorbate, isopropyl sorbate and ethyl sorbate) was carried out. In vitro cell viability assays of MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide), Neutral Red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) and flow cytometry with propidium iodide and annexin were performed on Caco-2 cells. In case of in vivo toxicity study, *G. mellonella* larvae were injected with different concentrations of the test compounds. Time-kill tests were executed on reference strains of *C. albicans, E. coli*, and *S. aureus*. According to the MTT-assay, the IC_{50} values were the following: ethyl sorbate, sorbic acid $<0.045\%\ ^{w}/_{w}$, isopropyl sorbate $0.32\%\ ^{w}/_{w}$, potassium sorbate $>0.75\%\ ^{w}/_{w}$, while Neutral Red values were $>0.75\%\ ^{w}/_{w}$ for the esters and potassium sorbate and $0.66\%\ ^{w}/_{w}$ for sorbic acid. Flow cytometry results indicated the higher cell damage in case of isopropyl sorbate. However, the cytotoxic results of isopropyl sorbate, in vivo toxicity study on *G. mellonella* larvae did not show significant mortality. It was found, that the antimicrobial properties of isopropyl sorbate were outstanding compared to sorbic acid and potassium sorbate. These results indicate, that the use of sorbate esters can be advantageous, hence, further toxicity studies are needed to prove their safety.

1. Introduction

Many commonly used excipients are presented in pharmaceutical and food industries. One such jointly used group of compounds are the antimicrobial preservatives. As both liquid, oral pharmaceutical preparations and certain beverages and drinks can be opened and closed multiple times until their expiration date, every interaction with the outer environment risks the contamination of the product. The alkyl esters of 4-hydroxybenzoic acid, the parabens are the most commonly used group of pharmaceutical preservatives. However, recent studies indicated that they could actively promote the proliferation of estrogen dependent cell lines (Roszak et al., 2017). Their interaction with human

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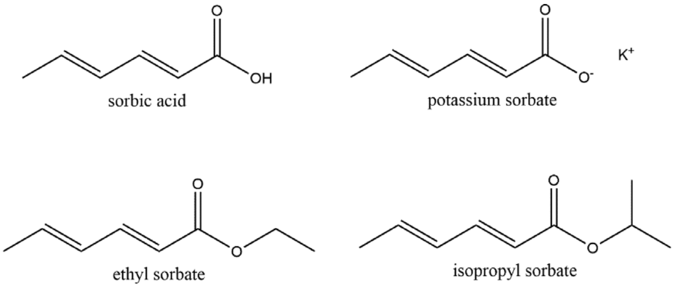
https://doi.org/10.1016/j.ejps.2019.105162
Received 15 July 2019; Received in revised form 24 October 2019; Accepted 18 November 2019
Available online 20 November 2019



Based on these previous studies, our aim was to test the alkyl esters of sorbic acid compared to potassium sorbate and sorbic acid. Antimicrobial properties of the tested

Fig. 1. Substances involved in our experiments.

endocrine system was also described (Nishihama et al., 2016). These results question their safe use and numerous governments limit the utilization of these materials (European Commission Regulation (EU) No 1004/2014). Accordingly, preservatives performing favourable biocompatibility profiles combined with reliable antimicrobial activity may replace parabens in food, pharmaceutical and cosmetics industry.

2,4-Hexadienoic acid, better known as sorbic acid and its potassium salt are alternatives of parabens. They are already widespread throughout the food and pharmaceutical industries, their application is well-established. Originally extracted from rowanberry, sorbic acid can also be found in various other plants (Shabir et al., 2011; Esquivel-Ferriño et al., 2012), although nowadays it is synthetically produced for commercial purposes. Aqueous solutions, o/w emulsions, suspensions, gels or any other product with high water content in pharmaceutical or food industries can be preserved by these compounds considering the chemical or physical interactions. Sorbic acid can be applied in more lipophilic environment such as ointments as well. Nowadays, potassium sorbate and sorbic acid with the sole purpose of antimicrobial preservation in concentration range of 0.1–0.2% are widely used in pharmaceutical industry (Rowe et al., 2009). Generally, they are accepted to be safe for human use, although toxicity and biocompatibility data profiles are incomplete and fragmentary. Rats, fed by the ten times of acceptable daily intake (ADI) for 60 days developed medium levels of toxicity (Abo-EL-Sooud et al., 2018), while the liver tissue of mice, fed with lower concentrations of potassium sorbate, than ADI showed no elevation in inflammatory genes (Raposa et al., 2016). Other publications revealed low ciliary toxicity in rabbits (Wang et al., 2012) and improved growth performance in swine through the increase of IGF-I (Lou et al., 2011). Also, the inhibition of gastrointestinal endoproteinases was reported (Esimbekova et al., 2017). Cell line investigations include low toxicity on HL7702 hepatocyte cells and high toxicity in acidic conditions on *D. tertiocleta*(Chen et al., 2017), and no toxicity on human primary nasal ciliary epithelial cells compared to benzalkonium-chloride (Jiao et al., 2014; Ho et al., 2008) and low genotoxicity on human lymphocytes (Mamur et al., 2010). These various data show, that sorbates do not express serious toxicity and if the regulatory concentrations are not exceeded, the human health risk is minimal (Mpountoukas et al., 2008). Acceptable human daily intake of sorbates (sorbic acid and potassium sorbate) is a maximum of 25 mg/kg but the official regulations vary in different countries (Dehghan et al., 2018).

The antimicrobial action of sorbates is not well understood, yet it is considered to be basically based on the intracellular acidification of microbes (Bagar et al., 2009; Plumridge et al., 2004). After penetrating the cell membrane, at the pH level of the cytosol, as a weak carboxylic acid, it releases a proton, which acidifies the cytosol, thus leading to the disruption of catabolic pathways (Mira et al., 2010). One possible resistance mechanism is the preventive acidification of cytosol and the adaption to it, in order to decrease the uptake of sorbic acid (Stratford et al., 2014). Another method is the decarboxylation of sorbic acid to 1,3-pentadiene (Plumridge et al., 2008, 2010). It was also proved, that with the increase of extracellular pH, the antimicrobial action of sorbates decreases, as only the nonionized form can enter the cells (Wang et al., 2018). Also, there are signaling pathways, that sense the intracellular presence of sorbate ion and upregulates certain specific defense mechanisms (Kim et al., 2019) and these mechanisms does not provide general resistance against all weak acids (Creamer et al., 2017). Sorbates can also effectively reduce bacterial biofilm formation (Al-Ahmad et al., 2008; Arzweiler et al., 2008;). Cellular stress caused by sorbates can also result in increased toxin production (Fodil et al., 2018). In theory the antimicrobial effect is increased, if a more lipophilic compound enters the cell more easily. In this case, the ester derivatives of sorbic acid act as a prodrug, as further enzymatic activation is needed, to release the carboxylic group (Larsen et Johnson, 2019).

Based on these previous studies, our aim was to test the alkyl esters of sorbic acid compared to potassium sorbate and sorbic acid. Antimicrobial properties of the tested substances (Fig. 1.) were studied on frequent pathogens, *C. albicans, S. aureus* and *E. coli* with time-kill method.

Meanwhile, cytocompatibility was assessed by MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide) and Neutral Red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) assays on Caco-2 human colon adenocarcinoma cell line and *G. mellonella* larve survivability tests. Caco-2 cells can model the susceptibility of the gastrointestinal tract as they morphologically represent the intestinal epithelium (Mao et al., 2016; Medrano-Padial et al., 2019). MTT and Neutral Red assays are rapid cytotoxicity methods which complement each other, because their mechanisms of action are different (Fotakis et Timbrell 2006). *G. mellonella* larvae is a recent, emerging method for in vivo toxicity testing. (Maguire et al., 2016)

2. Materials and methods

2.1. Materials

Ph. Eur. 9. quality sorbic acid was purchased from Hungaropharma (Budapest, Hungary). Potassium sorbate and Neutral Red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) was obtained from Alfa Aesar (Karlsruhe, Germany) and ethyl-sorbate from TCI (Zwijndrecht, Belgium). The MTT (2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide)) dye, Dulbecco's Modified Eagle's Medium with high glucose and L-glutamin (DMEM), phosphate buffered saline (PBS), trypsin from porcine, ethylene-diamine-tetra-acetic acid (EDTA), heat-inactivated fetal bovine serum (FBS), Roswell Park Memorial Institute-1640 (RPMI-1640) and Mueller-Hinton broth, sorbic chloride and propidium iodide were purchased from Sigma-Aldrich (Budapest, Hungary). Non-essential amino acids solution and penicillin-streptomycin mix, GlutaMax™ supplement, cell culture flasks and Annexin V, Alexa Fluor™ 647 conjugate were obtained from Thermo-Fisher (Darmstadt, Germany). Propan-2-ol, pyridine, dichloromethane were purchased from Molar Chemicals (Halásztelek, Hungary).

2.2. Cell culture

Caco-2 (COlon adenoCArcinoma) cell line was obtained from the European Collection of Cell Cultures (ECACC, No. 86010202). Cells were grown in Nunc™ EasyFlask™ (Thermo-Fisher, Darmstadt, Germany) surface-treated plastic cell culture flasks in Dulbecco's Modified Eagle's Medium, supplemented with 3,7 g/l $NaHCO_3$, 10% ($^v/_v$) heat-inactivated fetal bovine serum (FBS), 1% ($^v/_v$) non-essential amino acids solution, 0.584 g/ L-glutamine, 4.5 g/L D-glucose, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37 °C in an atmosphere of 5% CO_2. The cells were routinely maintained by regular passaging and glutamine was supplemented by GlutaMax™. The cells used for cytotoxic experiments were between passage numbers 20 and 40.

2.3. Cell viability tests

The cytotoxic effects of the various solutions were evaluated using the MTT and Neutral Red methods. Caco-2 cells in complete medium were seeded on 96-well plates at a final density of 10.000 cells/well. After 7 days, the medium was removed, and the cells were incubated for 30 min with the test solutions. In case of MTT-assay, the samples were removed, and a 5 mg/mL MTT solution (MTT salt solved in PBS) was added to each well. The plates were incubated for 3 h, then the MTT solution was removed and 0.1 mL of a solution of isopropanol – 1 M hydrochloride acid (25:1) was added to each well to dissolve the formed formazan crystals. In case of Netural Red assay, the test solutions were removed and a 33,3 mg/mL NR solution (NR solved in cell culture medium) was added to each well. The cells were incubated for 2 h then, the NR solution was removed and 0.1 mL of a solution of isopropanol – 1 M hydrochloride acid (25:1) was added to each well to dissolve the cells. The absorbance compounds were measured at 565 nm for MTT-assay and 540 nm for NR-assay. We used empty wells of the plate as reference and all the measurements were carried out with a Thermo-Fisher Multiskan Go (Thermo-Fisher, USA) microplate reader. Cell viability was expressed as a percent of the cell viability of the untreated control cells, which were incubated with PBS for 30 min.

2.4. G. mellonella larvae survivability tests

Larvae of the sixth developmental stage of *G. mellonella* were obtained from Bugs World Inc. (Budapest, Hungary). Larvae were at 10 °C and in a dark environment prior to use. Larvae size was between 2 and 3 cm and they showed no sign of melanization. For each treatment, 20 healthy larvae were placed in sterile vented Petri dishes. The test compounds were dissolved in PBS 20 µl of each sample was injected into the *G. mellonella haemocoel* through the last pro-leg using a 29 G needle. The injected larvae were incubated at 30 °C for 96 h in dark environment. For the assessment of larval viability, larvae were gently probed with a blunt-ended needle and if no response was observed, the larvae were considered to be dead. Viability was observed at 24 h, 48 h, 72 h, and 96 h.

2.5. In vitro time-kill antimicrobial tests

In killing studies, we tested *E. coli* (American Type Culture Collection® 25,922™), *S. aureus* (ATCC® 43,300™) and *C. albicans* (ATCC® 10,231™) reference strains. The activity of sorbates was determined against *C. albicans* and bacterium strains in RPMI-1640 and Mueller-Hinton broth at 0.045%, 0.09%, 0.18%, 0.375%, 0.75% $^w/_w$ concentrations using a starting inoculum of 1×10^5 cells/mL and 1×10^6–10^7 cells/mL, respectively, in a final volume of 5 mL, pH set to 7 (Nagy et al., 2019). In case of *C. albicans*, aliquots of 100 µl were removed after 0, 4, 8, 12 and 24 h of incubation, tenfold serial dilutions were prepared, and samples of dilutions (4 × 30 µl) were plated onto a single Sabouraud dextrose agar plate and incubated at 35 °C for 48 h. In case of *E. coli* and *S. aureus*, aliquots of 100 µl were removed after 0, 2, 4, 6, 8, 10, 12 and 24 h of incubation, tenfold serial dilutions were prepared, and samples of dilutions (4 × 30 µl) were plated onto a single Mueller–Hinton plate and incubated at 35 °C for 48 h. Tests were carried out in duplicates and mean values were presented. In any give concentration, were results differed from each other more than 5%, a third experiment was carried out.

2.6. Synthesis of isopropyl sorbate

Isopropyl sorbate was synthetized in situ for our experiments. To a stirred solution of isopropyl alcohol (11.7 mL, 0.15 mmol) in dry dichloromethane (100 mL) under inert argon atmosphere and cooled to 0 °C, 2.0 equivalent (24.2 mL, 0.3 mmol) of dry pyridine and 1.0 equivalent (20 mL, 0.15 mmol) of sorbic chloride was added. The reaction was stirred at room temperature overnight. After completion, 2 mL of water was added, and the reaction mixture was stirred for 1 h. Then, the reaction mixture was diluted with dichloromethane (300 mL) and was washed with saturated solution of $NaHSO_4$ twice, and Na_2CO_3 twice as well. The organic layer was then separated, dried over $MgSO_4$, filtered and distilled under vacuum to give isopropyl sorbate (9 g, 40%), yellow, fruity smell liquid.

2.7. Flow cytometry measurements

For the flow cytometry measurements, a BD FACSArray (BD Biosciences, Germany) flow cytometer were used. 5 × 3 million Caco-2 cells were harvested from cell culture flasks with trypsin-EDTA solution and were treated with 0.75% $^w/_w$ solutions of the tested compounds, dissolved in cell culture media. After 30 min, the cells were centrifuged, the culture media was removed and the cells were gently washed with cold PBS and centrifuged again. Supernatant was removed and with annexin-binding puffer, 1×10^6 cells/mL cell suspension was created. 100 µl of this suspension was treated with 5 µl of Alexa Fluror™ 647 and 1 µl of 100 µg/mL propidium iodide solution. The cell suspension was stained for 15 min on ice then immediately analyzed with the flow cytometer. The propidium iodide were excited with the 532 nm laser line and detected between 564–606 (yellow parameter). The Alexa Fluor™ 647 were excited with the 635 nm laser line and detected between 653–669 nm (red parameter). The evaluation was made with FCS Express 6 (De Novo Software, USA). On the FSC SSC scatterplot the noncellular events were excluded. On FSC-A-FSC-W scatterplot the duplets were excluded. The remaining events (8000–10.000) were analysed on a propidium iodide-Alexa Fluor 647 scatterplot, the quadrant gates were determined on non-labeled samples. The double positive cells regarded as necrotic/late apoptotic cells. The annexin V positive population was regarded as early apoptotic, the double negative population regarded as viable cells.

2.8. Statistical analysis

All data were analysed using GraphPad Prism (version 6; GrapPad Software, San Diego, California, USA). In case of MTT-assay and NR-assay results, the data was presented as means ± SEM. Each cell viability value represents the mean of twelve independent, parallel wells, with the highest and lowest absorbance values were excluded when calculating the mean. After that, at each concentration, the means of different solutions where compared with Kruskal–Wallis test followed by Dunn's test when all solutions were compared to each other. Previously, all data groups were analysed with Shapiro–Wilk test for Gaussian distribution and Bartlett's test for equal variances. In each case we used significance level $p < 0.05$. In vivo survival curves of *G. mellonella* larvae were plotted according to the Kaplan-Meier analysis, the survival curves were compared with Mantel-Cox log-rank test, GraphPad's Logrank test for trend and Gehan–Breslow–Wilcoxon test.

Table 1
Results of Dunn's multiple comparison test generated by the data of MTT and NR assay. * = $p < 0.05$; ** =$p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$.

Compared data sets MTT assay0.045%	Level of significance
Sorbic acid vs. Potassium sorbate	****
Sorbic acid vs. Ethyl sorbate	ns
Sorbic acid vs. Isopropyl sorbate	***
Potassium sorbate vs. Ethyl sorbate	***
Potassium sorbate vs. Isopropyl sorbate	**
Ethyl sorbate vs. Isopropyl sorbate	**
0.09%	
Sorbic acid vs. Potassium sorbate	****
Sorbic acid vs. Ethyl sorbate	ns
Sorbic acid vs. Isopropyl sorbate	***
Potassium sorbate vs. Ethyl sorbate	***
Potassium sorbate vs. Isopropyl sorbate	**
Ethyl sorbate vs. Isopropyl sorbate	**
0.18%	
Sorbic acid vs. Potassium sorbate	****
Sorbic acid vs. Ethyl sorbate	ns
Sorbic acid vs. Isopropyl sorbate	***
Potassium sorbate vs. Ethyl sorbate	****
Potassium sorbate vs. Isopropyl sorbate	**
Ethyl sorbate vs. Isopropyl sorbate	**
0.375%	
Sorbic acid vs. Potassium sorbate	****
Sorbic acid vs. Ethyl sorbate	ns
Sorbic acid vs. Isopropyl sorbate	**
Potassium sorbate vs. Ethyl sorbate	****
Potassium sorbate vs. Isopropyl sorbate	**
Ethyl sorbate vs. Isopropyl sorbate	**
0.75%	
Sorbic acid vs. Potassium sorbate	***
Sorbic acid vs. Ethyl sorbate	ns
Sorbic acid vs. Isopropyl sorbate	ns
Potassium sorbate vs. Ethyl sorbate	**
Potassium sorbate vs. Isopropyl sorbate	****
Ethyl sorbate vs. Isopropyl sorbate	ns
NR assay	
0.045%	
Sorbic acid vs. Potassium sorbate	ns
Sorbic acid vs. Ethyl sorbate	ns
Sorbic acid vs. Isopropyl sorbate	ns
Potassium sorbate vs. Ethyl sorbate	ns
Potassium sorbate vs. Isopropyl sorbate	ns
Ethyl sorbate vs. Isopropyl sorbate	ns
0.09%	
Sorbic acid vs. Potassium sorbate	ns
Sorbic acid vs. Ethyl sorbate	ns
Sorbic acid vs. Isopropyl sorbate	*
Potassium sorbate vs. Ethyl sorbate	*
Potassium sorbate vs. Isopropyl sorbate	*
Ethyl sorbate vs. Isopropyl sorbate	ns
0.18%	
Sorbic acid vs. Potassium sorbate	ns
Sorbic acid vs. Ethyl sorbate	ns
Sorbic acid vs. Isopropyl sorbate	*
Potassium sorbate vs. Ethyl sorbate	*
Potassium sorbate vs. Isopropyl sorbate	*
Ethyl sorbate vs. Isopropyl sorbate	ns
0.375%	
Sorbic acid vs. Potassium sorbate	****
Sorbic acid vs. Ethyl sorbate	ns
Sorbic acid vs. Isopropyl sorbate	ns
Potassium sorbate vs. Ethyl sorbate	***
Potassium sorbate vs. Isopropyl sorbate	**
Ethyl sorbate vs. Isopropyl sorbate	ns
0.75%	
Sorbic acid vs. Potassium sorbate	****
Sorbic acid vs. Ethyl sorbate	***
Sorbic acid vs. Isopropyl sorbate	**
Potassium sorbate vs. Ethyl sorbate	****
Potassium sorbate vs. Isopropyl sorbate	****
Ethyl sorbate vs. Isopropyl sorbate	ns

Table 1. shows the statistical analysis of the results of the MTT and NR assays. Flow cytometry tests were carried out as triplicates.

3. Results

3.1. Cell viability tests

Preservatives have high concentrations in the pharmaceutical product, to ensure the absolute inhibition of microbial growth. However, they are diluted in the stomach and later parts of the gastrointestinal tract. In order to compare both the antimicrobial and the biocompatibility tests, all compounds were tested in a wide range, setting 0.75% $^{w}/_{w}$ as maximum value and halving the concentration of every further solution. According to the regulation of Hungarian pharmaceutical compounding formulation, the maximum applied dose of sorbic acid (only slightly soluble in water, but moderately in hot water) and potassium sorbate is 1% $^{w}/_{w}$. and tolerable according to Hungarian regulations. Also, according to the European regulations, sorbic acid and potassium sorbate as food additives can be used from 0.02% (200 ppm) to 0.5% (5000 ppm) (Commission Regulation (EU) No 1129/2011). We aimed to investigate the biocompatibility and antimicrobial properties of the tested compounds above this approved range in order to get a more detailed view of such properties. Therefore, our concentrations were 0.045%, 0.09%, 0.18%, 0.375% and 0.75% $^{w}/_{w}$ which cover the whole range of application. All of the sorbates for cytotoxicity tests were all diluted in PBS.

MTT assay (Fig. 2.) showed a dose-dependent toxicity of sorbates, where potassium sorbate was the least toxic compound, followed by isopropyl sorbate and ethyl sorbate, while sorbic acid had the lowest cell viability results. However, at the highest concentration, isopropyl sorbate, ethyl sorbate and the sorbic acid caused nearly total cell death. Calculated IC_{50} values are $<0.045\%$ $^{w}/_{w}$ for ethyl sorbate and sorbic acid, 0.32% $^{w}/_{w}$ for isopropyl sorbate and $>0.75\%$ $^{w}/_{w}$ for potassium sorbate.

The lower concentrations of sorbates had only a minor impact on the viability of Caco-2 cells measured by Neutral Red assay (Fig. 3.). Meanwhile, 0.375% and 0.75% drastically increased the toxicity of the test substances. Compared to the results of MTT assay, sorbic acid was

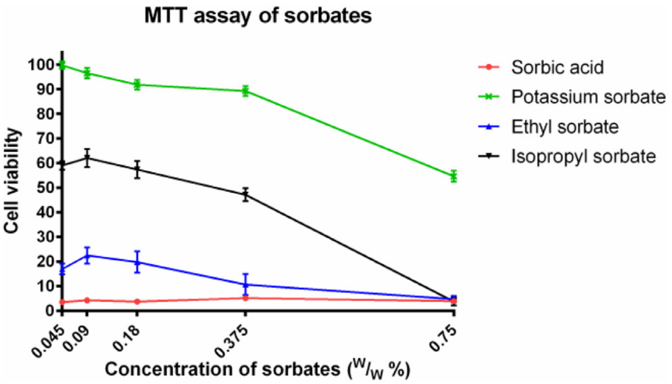


Fig. 2. Cytotoxicity of sorbates measured by MTT assay. Cell viability expressed as the percentage of the absorbance of the untreated control cells. Data expressed as mean ± SEM, $n = 12$.
Cell viability of the test samples at 0.045%, 0.09%, 0.18%, 0.375% and 0.75% ($^{w}/_{w}$)%:
Sorbic acid: 3.6% ± 0.18%; 4.3% ± 0.1%; 3.8% ± 0.1%; 5.1% ± 0.3%; 4.0% ± 0.3%.
Potassium sorbate: 99.8% ± 1.5%; 96.6% ± 2.1%; 91.9% ± 2%; 89.3% ± 2%; 54.7% ± 2.2%.
Ethyl sorbate: 17.0% ± 2.2%; 22.6% ± 3.3%; 19.9% ± 4.3%; 10.7% ± 4.3%; 4.8% ± 1.2%.
Isopropyl sorbate: 59.1% ± 1.7%; 62.1% ± 3.7%; 57.4% ± 3.5%; 47.2% ± 2.6%; 3.8% ± 1.6%.

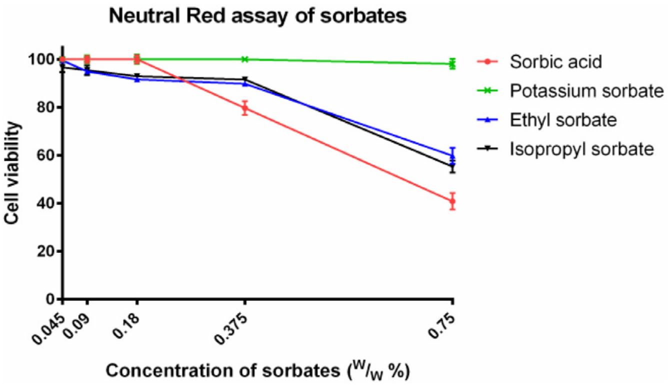


Fig. 3. Cytotoxicity of sorbates measured by Neutral Red assay. Cell viability expressed as the percentage of the absorbance of the untreated control cells. Data expressed as mean ± SEM, $n = 12$.
Cell viability of the test samples at 0.045%, 0.09%, 0.18%, 0.375% and 0.75% ($^{w}/_{w}$)%:
Sorbic acid: 100% ± 1%; 100% ± 1.4%; 100% ± 1.6%; 79.7% ± 2.9%; 40.8% ± 3.4%.
Potassium sorbate: 100% ± 1.1%; 100% ± 1.6%; 100% ± 1.9%; 100% ± 0.5%; 98.2% ± 2%.
Ethyl sorbate: 99.6% ± 0.4%; 94.9% ± 0.7%; 91.7% ± 0.9%; 89.9% ± 0.9%; 59.8% ± 3.3%.
Isopropyl sorbate: 96.7% ± 2.1%; 95.5% ± 2%; 93% ± 1%; 91.6% ± 1%; 55.4% ± 2.5%.

the most toxic compound in this experiment too. Calculated IC_{50} values are above 0.75% $^{w}/_{w}$ for the esters and potassium sorbate and 0.66% $^{w}/_{w}$ for sorbic acid.

3.2. In vivo toxicity tests

G. mellonella larvae were injected with 20 µl of the four test substances, dissolved in PBS. Throughout the 4 days of the experiment, their viability was observed every 24 h. Two concentrations of sorbates were used, 0.18% and 0.018% $^{w}/_{w}$. Each group consisted of 20 healthy larvae. Only a minor number of specimens died during the experiment and overall, the larvae showed no sign of melanisation or increased mortality (Fig. 4.). According to the statistical analysis, no curves were significantly different from PBS control and from each other.

3.3. In vitro antimicrobial time-kill experiments

Time-kill tests were carried out, in order to study the antimicrobial effect of sorbates. *C. albicans, E. coli* and *S. aureus* were inoculated in RPMI-1640 or Mueller-Hinton broth at 0.045%, 0.09%, 0.18%, 0.375%, 0.75% $^{w}/_{w}$ concentrations of the different compounds. At given time-points, 100 µl of aliquots were plated on agar plates and counted. Killing activity was determined by a threshold of 99.9% ($\log_{10}$ CFU = 2.24) extermination of initial CFU.

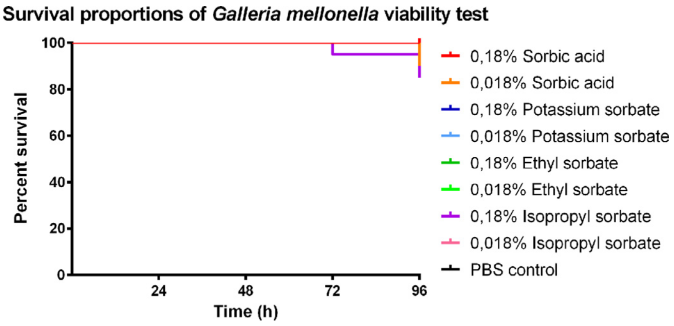


Fig. 4. Survival curve of *G. mellonella* larvae. Larvae were injected with 20 µl of test samples, each group had 20 larvae in it.
Death events of the experiment:
24 h: 0.
48 h: 0.
72 h: 1–0.18% isopropyl sorbate; 1–0.018% isopropyl sorbate.
96 h: 2–0.018% sorbic acid; 1–0.018% ethyl sorbate; 2–0.18% isopropyl sorbate; 2–0.018% isopropyl sorbate.

In case of the lowest concentration, *C. albicans* (Fig. 5A–D) was resistant to every tested compound. At 0.09% $^{w}/_{w}$ concentration isopropyl sorbate (Fig. 5D) had a slight fungistatic effect, inhibiting the further growth of fungal cells. At 0.18% $^{w}/_{w}$, isopropyl sorbate terminated all pathogens after 12 h. No other tested substance had any effect on *C. albicans* at these concentrations. Potassium sorbate (Fig. 5B) had fungistatic effect at 0.375% $^{w}/_{w}$ and above, while sorbic acid (Fig. 5A) and ethyl sorbate (Fig. 5C) could prevent the germination only at the highest tested concentration. Meanwhile, isopropyl sorbate had an increased killing effect above 0.18% $^{w}/_{w}$, as both higher concentrations identically eliminated all cells after 8 h.

S. aureus was totally resistant to potassium sorbate, ethyl sorbate and sorbic acid (Fig. 6. A–C) as the inoculum size increased with time in case of every concentration. As such, *S. aureus* was the least sensitive organism in our experiment. Isopropyl sorbate (Fig. 6D) had a bacteriostatic effect at 0.375% $^{w}/_{w}$ concentration and above.

The growth of *E. coli* was heavily affected by isopropyl sorbate (Fig. 7D), as after twelve hours, no antimicrobial activity could be detected at 0.375% $^{w}/_{w}$ concentration and above. 0.18% $^{w}/_{w}$ concentration of isopropyl sorbate was bacteriostatic. Sorbic acid and potassium sorbate (Fig. 7A and B) were totally ineffective against this species. Meanwhile the results of ethyl sorbate (Fig. 7C) are contradictory, as 0.375% $^{w}/_{w}$ had a stable static effect, while 0.75% $^{w}/_{w}$ proved to be ineffective.

3.4. Flow cytometry measurements

Caco-2 cells were treated with 0.75% $^{w}/_{w}$ solutions of the tested substances for 30 min and stained with propidium iodide and annexin V. Figs. 8A–E shows the results the distribution of the gated cells. The double positive cells regarded as necrotic/late apoptotic cells, the annexin V positive population was regarded as early apoptotic, the double negative population regarded as viable cells. Propidium iodide negative and annexin positive cells were negligible. Isopropyl sorbate had increased cytotoxic effect, compared to the other compounds which had increased dead cell percentage than the untreated control.

4. Discussion

2,4-hexadienoic acid, as known as sorbic acid, is widely used as an antimicrobial preservative for food, cosmetic and pharmaceutical industry. Its mechanism of action is stated to be based on the diffusion through the cell membrane and intracellular acidification of the targeted microbe (Stratford et al., 2013). As the sorbates can only enter the cell in unionized form, low pH greatly enhances their action, as they can be mostly found in that state at such conditions (Bayan, 2010). If the pH of a given product cannot be adjusted to acidic range, due to its stability, the effect of sorbates is reduced (Wang et al., 2018). The alkyl esters or sorbic acid might be the solution for the pH-dependency issue. Thus, ethyl and isopropyl sorbates were involved in our study.

Tzatzarakis et al. (2000) and Charvalos et al. (2001) previously formulated different polyvinylpyrrolidone based polymers, to which sorbic acid was covalently bonded, and tested it against several fungi species. The inhibitory concentrations were promising, yet, no toxicity data is available, connected to the newly formed compounds. Moreover, they were not tested against bacterial strains either. Narasimhan et al. synthesized 42 different sorbic acid esters and analysed their antimicrobial potential (Narasimhan et al., 2007). This publication suggested, that the increase of lipophilicity enhances the antibacterial and antifungal actions of the given compound. However, a disadvantage of

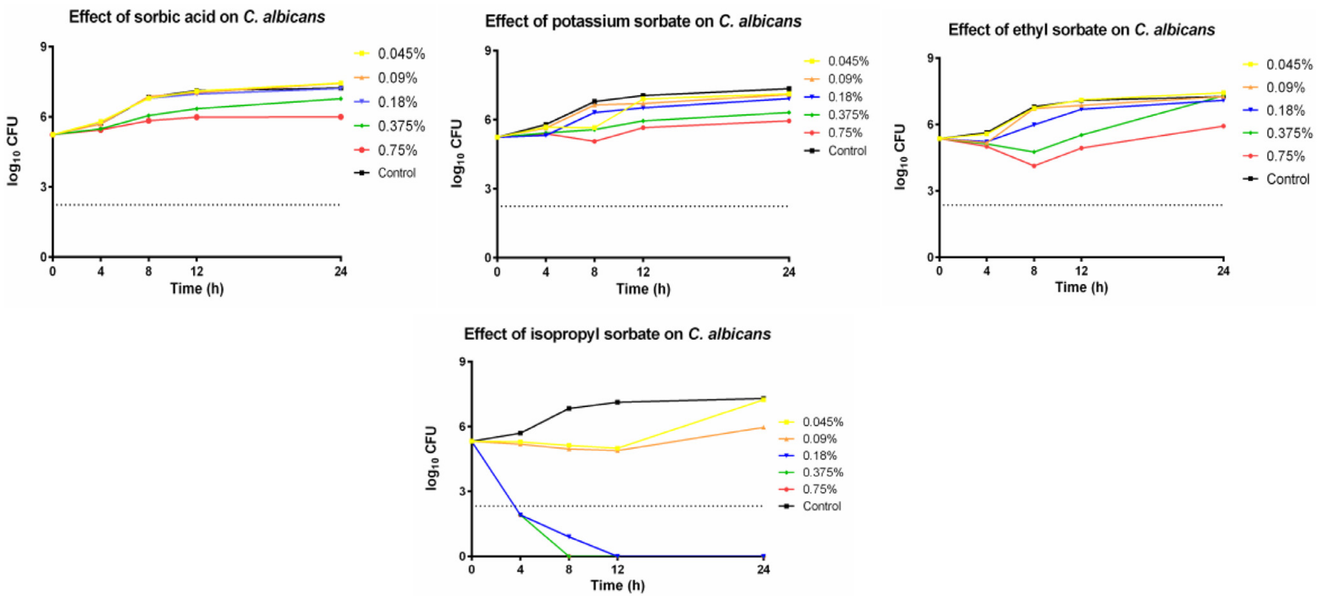


Fig. 5. A-D Antimicrobial effect of sorbates on *C. albicans.*

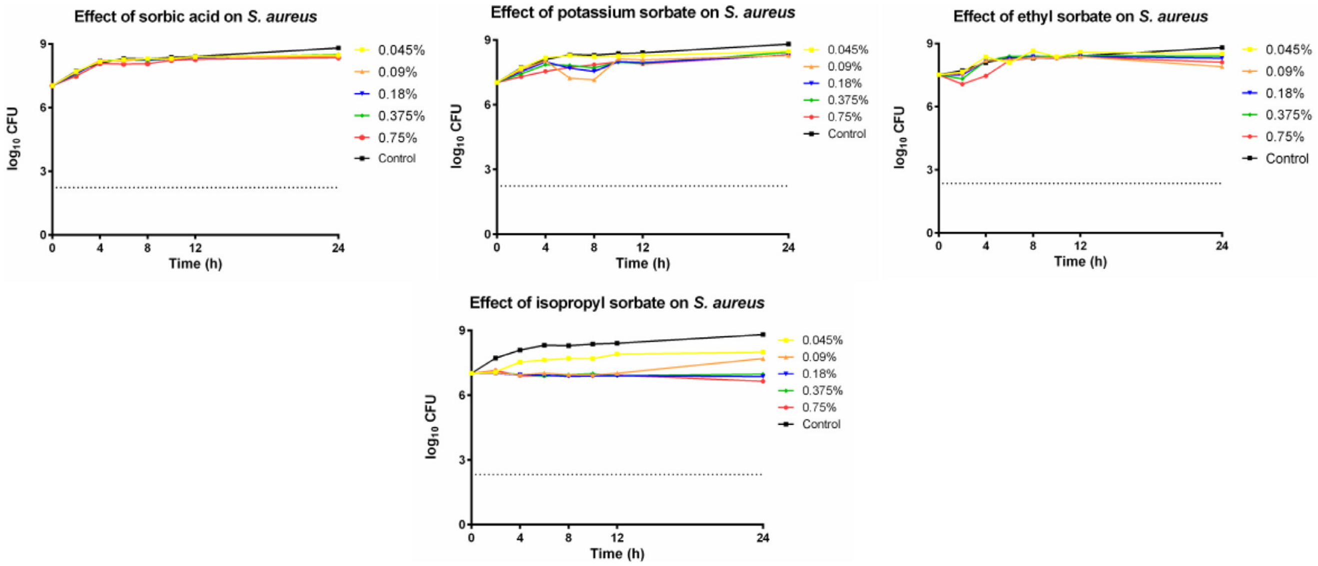


Fig. 6. A-D Antimicrobial effect of sorbates on *S. aureus.*

these derivatives was the poor water solubility which limits their application in water-based systems. Our test substances were two esters with short alkyl chains, performing moderate water solubility.

The literature revealed, that all tested compounds are generally well tolerated. Qu et al. (2019) reported, that potassium sorbate had an IC_{50} value of 1.25 g/L after 24 h of incubation on HepG2 human liver cell line measured by MTT, while HUVEC cell line showed an 659.96 µM IC_{50} value of after 24 h of incubation measured by MTT (Mohammadzadeh-Agdash et al., 2018). These results match our findings (Fig. 2.), as after 30 min of incubation, 0.18% potassium sorbate concentration lowered the cell viability to 91.9%. The cell viability difference between potassium sorbate and sorbic acid might be explained by the acidifying nature of the latter.

Smith et al. measured the cytotoxicity of potassium sorbate on Balb/C 3T3 clone A31 embryonic mouse cells with Neutral Red and found that it was toxic only in extremely high concentrations, far over the generally applied concentrations (Smith et al., 2005). Our results (Fig. 3.) well correlates with this, as only the highest concentrations decreased cell viability. The high correlation of Neutral Red and MTT cytotoxitcy tests was reported (Fotakis et Timbrell 2006). However, the differences between the assays in our experiments, were based on the acidification of the cytosol of Caco-2 cells. As the change of intracellular pH disrupted the metabolism of the cell, the enzymatic conversion of MTT is highly decreased (Berridge et al., 2005), but the lysosomal staining by Neutral Red was not inhibited (Elliott et Auersperg, 1993). Another possible explanation of the cytotoxicity profile differences of sorbates is their binding to proteins, as it was proved that relatively similar molecules as carboxylic acids have various binding sites (Mohammadzadeh-Agdash, Akbari, Esazadeh and Dolatabadi, 2019).

Flow cytometry measurements revealed that compared to the control, potassium sorbate, sorbic acid and ethyl sorbate could increase the amount of propidium iodide and annexin positive cells with 10%. However, isopropyl sorbate was significantly more cytotoxic (68% compared to the 28% of other tested substances), than any other derivatives. We suspected that this can be explained by the non-pH dependent mechanism of action and the higher membrane permeability of the isopropyl sorbate, which greatly exceeds the less lipophilic ethyl sorbate. MTT and NR assays did not certify that difference, as both

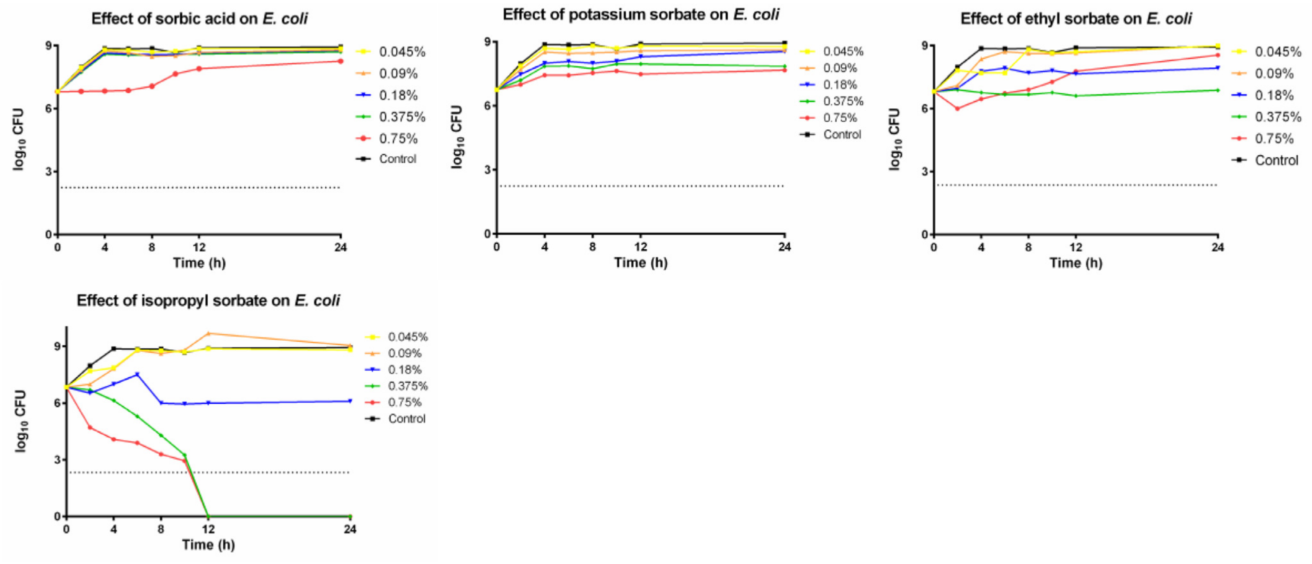


Fig. 7. A-D Antimicrobial effect of sorbates on *E. coli.*

substances showed similar cytotoxic effects. However, it was proved that the minimum change in the length of alkyl chain greatly modifies the biological activity and membrane passage in the case of salicylic acid derivatives (Li et al., 2019). Further investigation is needed to explain particularly the modification of sorbate esters membrane permeability with different lengths of alkyl chains.

The use of *G. mellonella* larvae as a biocompatibility model organism is relatively new. However, Maguire et al. found that the correlation between LD$_{50}$ values observed on this species and the results of previous rat feeding toxicity and cytotoxicity results was linear (Maguire et al., 2016). Several recent publications concluded, that the use of *G. mellonellae* larvae not just complemented to cell culture studies in toxicity experiments, (Allegra et al., 2018; Bombarda et al., 2019) might be a good substitute of rodent model systems (Ignasiak et

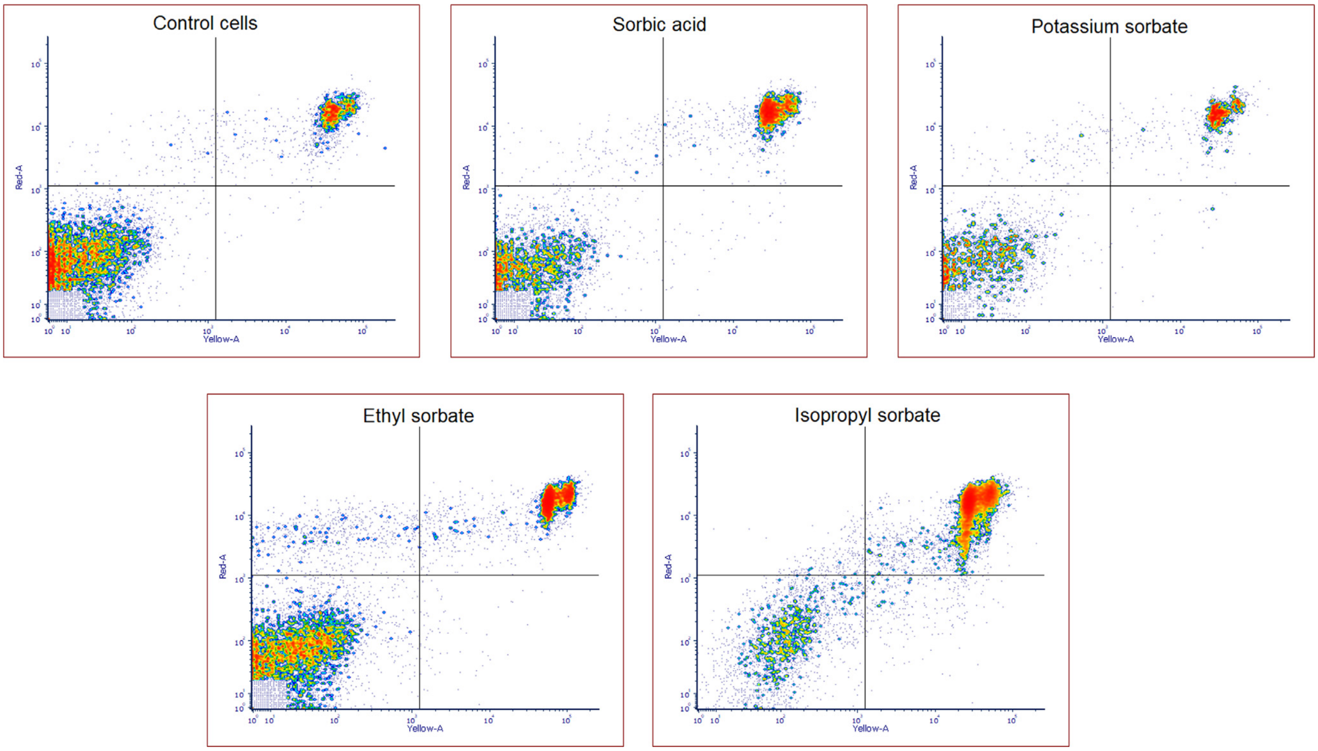


Fig. 8. A-D. Flow cytometric measurement of Caco-2 cells, treated with 0.75% $^{w}/_{w}$ solutions of the test compounds, stained with propidium iodide (PI) and annexin (A). Data is represented as mean of triplicates. Mean percentage distribution of cells between the upper left (PI+, A-), upper right (PI+, A+), lower left (PI-, A-) and lower right (PI-, A+) quadrant, ± SEM:
control: 1.4% ± 0.1%, 18.1% ± 1.2%, 80.3% ± 1.3%, 0.3% ± 0.0%.
sorbic acid: 1.9% ± 0.1%, 29.3% ± 0.4%, 68.2% ± 0.4%, 0.5% ± 0.0%.
potassium sorbate: 4% ± 0.2%, 27.9% ± 2.1%, 67.5% ± 2.2%, 0.5% ± 0.1%.
ethyl sorbate: 6.5% ± 0.1%, 28.5% ± 0.3%, 64.3% ± 0.5%, 0.6% ± 0.0%.
isopropyl sorbate: 1.9% ± 0.1%, 67.8% ± 0.4%, 27.3% ± 0.4%, 3% ± 0.1%.

Maxwell, 2017), thus the prediction of human toxicity of tested compounds can be greatly enhanced. In our experiments, there was no significant difference between the mortality of different treated groups (Fig. 4), the larvae showed no sign of toxicity. As the injectable liquid volume is limited and 0.18% is a higher concentration, than sorbates are generally used at, we found that the further increase of the dose in not necessary.

Our results match the findings of Narasimhan et al. who reported, that isopropyl sorbate was significantly more active against *S. aureus, E. coli* and *C. albicans*, than ethyl sorbate, which exceeded the original molecule only against *E. coli* and *C. albicans* (Narasimhan et al., 2007). In our experiment, MIC value was reached neither in the case of sorbic acid, nor with potassium sorbate (Figs. 5–7A and B) against the tested microbes. In many previous publications, it was found, that, the efficacy of sorbic acid and potassium sorbate decreases with the elevation of pH (Lues et Theron, 2012; Hwang et al., 2015). Wang et al. found, that potassium sorbate had a MIC value of 0.4 $^{w}/_{w}$% against *E. coli* and *S. aureus* at pH 5, but 1.6 and 3.2 $^{w}/_{w}$% if the pH was adjusted to 7 (Wang et al., 2018). Isopropyl sorbate could actively kill *E. coli* (Fig. 7D) and *C. albicans* (Fig. 5D) cells and inhibit the growth of *S. aureus* (Fig. 6D) at pH 7, which is a remarkable feat compared to other sorbates. Lipophilicity and long-term acidification of the cytosol are critical in the antimicrobial mechanism of weak acids (Ullah et al., 2012) and we suspect, that isopropyl sorbate could more effectively pass through the cell membranes without the need of specific proteins (Piper, 2011), than the other tested compounds. Bacterial esterases are known to be part of antibiotic resistance in several species and thus (Egorov et al., 2018), they could possibly cleave the sorbate esters, as known in the case of parabens (Valkova et al., 2003).

Two generally accepted and applied preservatives, sorbic acid and potassium sorbate and two lipophilic sorbate derivates were tested on human colorectal cells, *G. mellonella* larvae and various pathogens in order to test their biocompatibility and antimicrobial properties. Further studies are needed, to specifically describe, the mechanism of action of sorbate esters, whether they have antimicrobial action on their own, or they act as prodrugs and can only be effective after enzymatic conversion to sorbic acid. While ethyl sorbate had no significant inhibitory activity against the tested bacteria and fungi, isopropyl sorbate demonstrated a significant bactericide and fungicide potential. As there is only one methyl group difference between the ethyl sorbate, which had limited effect against the tested microbes based on our experiments, an antimicrobial study with more sorbate esters would be able to clarify the lipophilicity-antimicrobial action correlations. Our results indicate, that the more lipophilic sorbate derivates could be promising antimicrobial preservatives, but their low water solubility can limit their application. In order to properly assess the safety biocompatibility profiles of these compounds, beside our study, different in vitro and in vivo genotoxicity and toxicity studies are also required including vertebrates and human cell lines.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

The project was financed by the Gedeon Richter's Talentum Foundation (1103 Budapest, Gyömrői street 19-21.). The published work was also supported by EFOP-3.6.1-16-2016-00022 and EFOP-3.6.3-VEKOP-16-2017-00009 projects of the European Union and ÚNKP-18-3 New National Excellence Program of the Ministry of Human Capacities of Hungary. The research was co-financed by the Higher Education Institutional Excellence Programmeof The Ministry of Human Capacities in Hungary, within the framework of the Research and Development on Therapeutic purposes thematic programme of the University of Debrecen (NKFIH-1150-6/2019).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2019.105162.

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