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ANTIMICROBIAL ACTIVITY AND CYTOTOXICITY OF PICOLINIC ACID AND SELECTED PICOLINATES AS NEW POTENTIAL FOOD PRESERVATIVES

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In this study antimicrobial activity of picolinic acid (PA), sodium picolinate (PS), potassium picolinate (PP), benzoic acid (BA), sodium benzoate (BS) and potassium benzoate (BP) against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Candida albicans* were measured using microbroth dilution method. Cytotoxicity assay of PS and BS was estimated in the human skin fibroblasts using Thiazolyl Blue Tetrazolium Bromide (MTT) test and DNA-binding assay. PA and PS showed antimicrobial activity at concentrations 0.02 to 0.78 mg/mL at pH=5.0 and 0.19 to 3.13 mg/mL at pH=7.0. Minimal inhibitory concentration (MIC) of BA on tested microorganisms was found at 0.13-0.50 mg/mL (pH=5.0) and about 1 mg/mL at pH=7.0. PA and PS showed strong antimicrobial activity, without significant influence on DNA synthesis and survival of human fibroblasts. The data suggest that PA and PS may represent new potential food preservatives.

INTRODUCTION

Several chemical compounds are able to inhibit the growth of microorganisms, however, only few of them grant claims for preservatives, which is why the search for ideal antimicrobial agent is continued. Picolinic acid (PA) is a catabolite of L-tryptophan detected in the human body [Cai *et al.,* 2006; Dazzi *et al*., 2001]. Picolinates are used in dietary supplements. PA is a metal ion chelator and, as a complex group with chromium, is used as a dietary supplement for obese people, since it has a beneficial effect on reducing diabetes risk [Komorowski *et al.,* 2008]. Zinc picolinate (as a source of zinc) has an antioxidant effect and may be used in patients with chronic obstructive pulmonary disease [Kirkil *et al.,* 2008]. PA is also a metabolite of fungi (*e.g. Fusarium* spp.) and is known as an inhibitor of phenoloxidase [Dowd, 1999]. The study of Fernandez-Pol *et al.* [2001] indicated the antiviral activity of PA. PA at 0.185 mg/mL and 0.369 mg/mL was active against human HIV-1 and HSV-2-infected cells. Although the antiviral mechanism of its action is unknown, the antimicrobial activity of PA may be due to the effect on the iron metabolism inside the macrophage as well as to other regulatory effects [Cai *et al.,* 2006]. Musk & Hergenrother [2008] show that ferric picolinate (also ferric acetohydroxamate) is effective against *Pseudomonas aeruginosa*. Microbiological study on alkaline picolinates showed that some of them inhibit the growth of *Escherichia coli* [Koczoń *et al.,* 2005, 2006].

Antimicrobial activity of PA, PS and PP against several strains of microorganisms was reported earlier; however, there is no information on cytotoxicity and inhibitory effect on the growth of *Staphylococcus aureus*, *P. aeruginosa* and *Candida albicans*. Proving the antimicrobial properties and low cytotoxicity of the studied compound is the first step to estimate its usefulness as a food preservative. The antimicrobial properties exert many phenols and benzoic acid derivatives [Cai *et al.,* 2006; Kalinowska *et al.*, 2008; Nihei *et al*., 2003; Park *et al.*, 2001]. Benzoic acid (BA) and its sodium (SB), and potassium (PB) salts are widely used as food and cosmetic preservatives [WHO, 2000]. These chemical compounds are used in beverages, fruit products, sauces and condiments, preferably in a range below pH=4.5. The average concentrations allowed in food in different countries are between 0.15% and 0.25%.

The aim of this study was to estimate antimicrobial activity of PA, PS and PP and cytotoxicity of PS in comparison to the respective benzoic acid derivatives.

MATERIALS AND METHODS

Test solutions

BA, PA and BS were Sigma analytical reagents. Complexes of alkaline metals were prepared by dissolving appropriate acid in an aqueous solution of metal hydroxide (Merk) in a stoichiometric ratio. Water was evaporated at 90°C in a dryer. Afterwards, the complex was dissolved and crystallized from the demineralized water. Elementary analysis was performed after drying.

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Microorganisms

Four microorganisms were used in this work. They were as follows, Gram-positive: *S. aureus* (NCTC 4163) and *B. subtilis* (NCTC 10400), Gram-negative *P. aeruginosa* (NCTC 6749) and fungi – *C. albicans* (ATCC 10231).

Antimicrobial activity

An antimicrobial effect was tested using a microbroth dilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) and applied by other authors as well [Espinel–Ingroff *et al.*, 1995; Speciale *et al.*, 2002; Woods *et al.*, 1995]. Colonies from overnight growth on an agar base plate were suspended in 0.9% sterile saline to match the turbidity of 0.5 (*S. aureus, P. aeruginosa*) or 1.0 (*B. subtilis*) McFarland standard – approximately 10^8 CFU/mL. The suspension was diluted at 1:1000, with Mueller-Hinton broth (Becton, Dickinson & Company, USA). The final inoculum was approximately $1\text{-}5\times10^5$ CFU/mL. The inoculum of *C. albicans* was prepared by picking colonies from 48 h-old cultures and suspending the material in 0.85% sterile saline to much turbidity of 0.5 McFarland standard ($1\text{-}5\times10^6$CFU/mL). Cell suspension was diluted at 1:2000 with RPMI 1640 medium (Biomed, Poland) to provide a testing inoculum of approximately 10^3 CFU/mL. Chemical compounds were dissolved in ultra pure water for to reach the concentration of 100 mg/mL. BA was dissolved to the concentration of 2 mg/mL. Serial double dilutions of agents were executed in 96-well flat-bottom microtiter plates. Afterwards, 100 μL of inoculum of microorganisms was added to each hollow microtiter plate. Plates with *S. aureus, P. aeruginosa and B. subtilis* were incubated in the air at 35°C for 24 h and plates with *C. albicans* were incubated at 25°C for 48 h. The growth of microorganisms was read visually. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of an agent that yielded no growth. Wells were plated to proper agar growth media and incubated at previously mentioned times and temperatures. The complete absence of growth was considered as the minimal bactericidal concentration (MBC) or for *C. albicans* – minimal fungicidal concentration (MFC). The experiment was repeated 6 times for each concentration.

Cell culture and treatment

Studies were performed on normal human skin fibroblasts (NHSF) [(CRL-1474), which were purchased from American Type Culture Collection, Rockville, MD]. NHSF were maintained in DMEM (glucose 4.5 g/L, L-glutamine, pyruvate – Gibco, Paisley, Scotland) supplemented with 10% fetal bovine serum (FBS – Gibco, Paisley, Scotland), 50 U/mL penicillin, and 50 μg/mL streptomycin (Sigma). Cells were used in the 8th to 14th passages. Sub-confluent cells were detached with Trypsin-EDTA solution (Sigma) in calcium-free phosphate buffered saline (PBS), counted in hemocytometer and plated at 5×10^5 cells per well of 24-well plates in 0.5 mL of growth medium (DMEM with FBS). Cells reached confluence at day 6 and confluent cells were used for assays.

Cell viability assay

Cell viability was measured by means of the quantitative colorimetric assay with MTT from Sigma [Carmichael *et al.*, 1987]. Confluent cells were treated with the studied agents added to the growth medium and incubated for 24 h at 37°C in an atmosphere of 5% CO_2 in the air. The last row contained medium only. After that, 50 μL of MTT solution (5 mg MTT/1mL PBS) was added to each well. Plates were incubated for 4 h. Medium and MTT were next removed from the wells, and 1 mL of 0.1 mol/L HCl with 2-propanol was added to each well. The absorbance was recorded in a spectrophotometer (U-2001, Hitachi, Japan) at a wavelength of 570 nm. Toxicity was presented as per cent of control. The estimate of cytotoxicity was based on the percent for survival of cells: non cytotoxic – >90% cells viability, slightly cytotoxic – 60-90% cells viability, moderately cytotoxic – 30-59% cells viability and strongly cytotoxic – below 30% cells viability [Dahl *et al.*, 2006].

Assessment of DNA-binding

To examine the effect of the studied compounds on fibroblasts proliferation, the cells were seeded in 24-well tissue culture dishes at 1×10^5 cells/well with 1 mL of growth medium (DMEM with FBS). After 48 h, the plates were incubated with estimated chemicals and 0.5 μCi of [^{3}H] thymidine for 24 h at 37°C in 5% CO_2. Afterwards, the cells were rinsed three times with 0.05 mol/L Tris-HCl and two times with 5% TCA. The cells were solubilized with 1 mL of 0.1 mol/L sodium hydroxide containing 1% SDS. Scintillation fluid was added and radioactivity incorporation into DNA was measured in a scintillation counter.

RESULTS AND DISCUSSION

The MICs and MBCs were determined for *S. aureus, P. aeruginosa, B. subtilis* and fungi – *C. albicans* (MIC and MFC). The results of the antimicrobial activity of PA, PS and

TABLE 1. MIC (mg/mL) of picolinic acid, benzoic acid and their sodium and potassium salts.

Compounds	*Staphylococcus aureus*		*Pseudomonas aeruginosa*		*Bacillus subtilis*		*Candida albicans*	
	pH=5.0	pH=7.0	pH=5.0	pH=7.0	pH=5.0	pH=7.0	pH=5.0	pH=7.0
Benzoic acid	0.50	1.00	0.25	1.00	0.13	1.00	0.13	>1.0
Picolinic acid	0.05	1.56	0.19	1.56	0.02	1.56	0.39	1.56
Sodium benzoate	0.39	6.25	1.56	25.00	0.19	6.25	12.50	25.00
Sodium picolinate	0.10	1.56	0.78	3.13	0.10	0.78	0.78	0.19
Potassium benzoate	12.50	12.50	12.50	12.50	0.39	12.50	50.00	12.50
Potassium picolinate	0.10	3.13	0.78	12.50	0.02	1.56	1.56	1.56

MIC – minimal inhibitory concentration.

TABLE 2. MBC and MFC (mg/mL) of picolinic acid, benzoic acid and their sodium and potassium salts.

Compounds	*Staphylococcus aureus*		*Pseudomonas aeruginosa*		*Bacillus subtilis*		*Candida albicans*	
	pH=5.0	pH=7.0	pH=5.0	pH=7.0	pH=5.0	pH=7.0	pH=5.0	pH=7.0
Benzoic acid	0.50	1.00	0.25	>1.0	0.13	1.00	0.25	>1.0
Picolinic acid	0.78	3.13	0.39	3.13	6.25	3.13	0.39	1.56
Sodium benzoate	>50.0	>50.0	6.25	25.00	0.39	50.00	25.00	>50.0
Sodium picolinate	>50.0	50.0	3.13	12.50	0.19	1.56	1.56	0.78
Potassium benzoate	>50.0	>50.0	>50.0	>50.0	50.00	25.00	50.00	>50.0
Potassium picolinate	>50.0	>50.0	50.00	12.50	25.00	6.25	3.13	3.13

MBC – minimal bactericidal concentration; MFC – minimal fungicidal concentration.

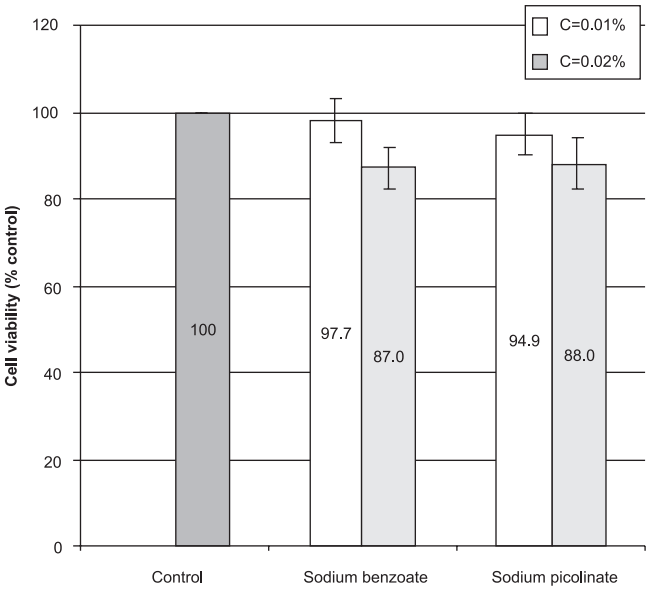

FIGURE 1. Viability of NHSF incubated for 24 h in medium with variable concentrations of sodium benzoate and sodium picolinate.

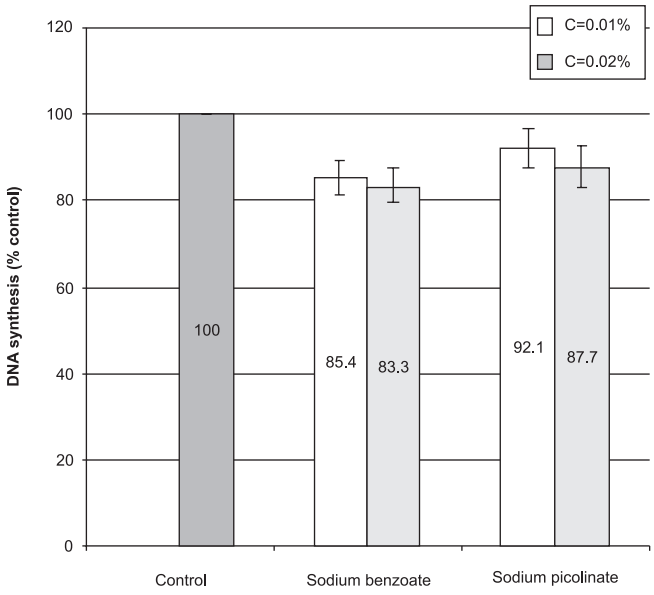

FIGURE 2. DNA synthesis in NHSF incubated for 24 h in medium with variable concentrations of sodium benzoate and sodium picolinate.

PP are shown in Table 1 and Table 2. Benzoic acid alkaline salts were determined as a control group. The MICs for all the tested microorganisms at variable pH are shown in Table 1. PA and its sodium salt showed activity against microorganisms at pH=5.0 at levels between 0.02 to 0.78 mg/mL and 0.19 to 3.13 mg/mL at pH=7.0. PP evoked strong antimicrobial activity against the Gram-positive species and *C. albicans* at a level of 0.02–3.13 mg/mL, but lower activity against *P. aeruginosa* at pH=7.0 (12.5 mg/mL). MICs of BA on tested microorganisms at pH=5.0 were between 0.13-0.50 mg/mL and at pH=7.0 were 1.0 mg/mL or over 1.0 mg/mL (for *C. albicans*). Generally, stronger antimicrobial activities of agents were observed at pH 5.0 than at pH 7.0. Growth inhibition of *Fusarium oxasporium* measured after incubation with BA (610 mg/L) was 23.7% at pH 7.2 and 83.5% at pH 4 [WHO, 2000]. Sodium benzoate inhibited the growth of yeast *(S. cerevisiae*) or mould (*Penicillium glaucum)* in concentrations of 120–600 mg/L, at pH 2.6; 1000–4000 mg/L at pH 5; or 20000 – 60000 mg/L at pH 7 [WHO, 2000]. The MBCs and MFCs at variable pH are shown in Table 2. PA and BA had the best bactericidal properties from all the studied chemical compounds. Picolinates had stronger antimicrobial activity than benzoates.

The upper concentration of BA and BS accepted in food is 0.5% (5 mg/mL), however, it is usually used at concentrations between 0.015 and 0.2% [WHO, 2000]. PA and PS showed activity in that concentration at pH 5.0–7.0.

The results of the influence of PS and BS on NHSF viability are shown in Figure 1. The concentrations of the studied sodium salts were 0.01% and 0.02% because most of food preservatives were used in these amounts. PS and BS showed a similar effect on the growth of human fibroblasts and both of them were non-cytotoxic at the concentration of 0.01%. A slightly cytotoxic effect was observed at the concentration of 0.02%. Cell viability at this concentration was below 90% (for PS – 88% and BS – 87%). PS and BS did not exert any significant influence on the synthesis of DNA in NHSF (Figure 2).

CONCLUSIONS

1. Picolinic acid and sodium picolinate show a high antimicrobial activity at pH=5.0 and 7.0.

2. Sodium picolinate and sodium benzoate do not show any cytotoxic effect on NHSF at the concentration of 0.01%, but are slightly cytotoxic at 0.02%.

3. Picolonic acids and their salts may represent new potential food or cosmetic preservatives.

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