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Antimicrobial properties of phenolic acid alkyl esters

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Abstract: The series of phenolic acid (2-, 3-, 4-monohydroxy- and 2,4-, 2,5-dihydroxy) alkyl esters (methyl, ethyl, propyl, and butyl) were prepared, and their antimicrobial activities were determined. The antimicrobial activity against the tested microorganisms *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus brasiliensis* was investigated and expressed by minimum inhibitory concentration (MIC) in the range of 1.2–20 mM. The inhibitory activity of higher esters of phenolic acids was found to be higher than that of methyl esters and acids. The minimum inhibitory concentration (MIC) of tested compounds was compared with that of 4-hydroxybenzoic acid and its esters (parabens).

Keywords: phenolic acid; antioxidant properties; antimicrobial effect; parabens

Phenolic acid derivatives are compounds present in plant materials, including food products of plant origin. These compounds show antimicrobial, antiatherogenic, anticarcinogenic and radical scavenging effects.

Esters of *p*-hydroxybenzoic acid (parabens) have been used as antimicrobial agents for over 70 years. Antimicrobial activity has been found to increase with the increasing alkyl length of ester group. However, esters with longer alkyl chains have limited use due to their low solubility in water (Saad et al. 2005).

Parabens are stable in air and resistant to hydrolysis in hot and cold water (Andersen 2008). Antimicrobial activity of parabens has been observed in several Gram-negative and Gram-positive bacteria. Many studies (Charnock and Finsrud 2007; Kosová et al. 2015; Crovetto et al. 2017) have been conducted with various bacterial strains and under different incubation conditions, so comparison of available results of these studies is difficult. However, from the minimum inhibitory concentration (MIC) data it is obvious that with the increasing length of the alkyl chain the inhibitory activity also increases.

Salicylic acid as a naturally occurring derivative of benzoic acid is a plant hormone (Trivedi et al. 2015), and it is an important signal molecule in plant defence (Shah 2003). The *in vitro* spore germination of *Penicillium expansum* was significantly inhibited when its concentration was increased to 7.24 mmol L^{-1} (Ting and Xiao 2006). Si et al. (2006) reported antimicrobial properties of 3-hydroxybenzoic acid and other phenolic acids against *Escherichia coli* O157 : H7 and *Salmonella typhimurium* DT104.

The phenolic compounds 2,4-dihydroxybenzoic and 3,4-dihydroxybenzoic acids showed high activity against the majority of Gram-negative and Gram-positive bacteria. Furthermore, phenolic compounds inhibited more

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MRSA (methicillin-resistant *Staphylococcus aureus*) than methicillin-sensitive *Staphylococcus aureus* (Alves et al. 2013). Ethyl β-resorcylate (ethyl 2,4-dihydroxybenzoate), isolated from dried root bark of the mulberry tree (*Morus alba* L.), has the antimicrobial activity against 28 species of fungi and 5 species of bacteria (Uno et al. 1981).

This work is a follow-up study of the frequently quoted publication Merkl et al. (2010). The aim of this study was to determine antimicrobial properties of the next series of phenolic acids and their (2-, 3-, 4- monohydroxy- and 2,4-, 2,5-dihydroxy) alkyl esters of selected aromatic hydroxy acids.

MATERIAL AND METHODS

Material. Salicylic acid (2-hydroxybenzoic acid), m-hydroxybenzoic acid (3-hydroxybenzoic acid), p-hydroxybenzoic acid (4-hydroxybenzoic acid), β-resorcylic acid (2,4-dihydroxybenzoic acid), gentisic acid (2,5-dihydroxybenzoic acid), ethylparaben, p-toluenesulphonic acid monohydrate were obtained from Merck (St. Louis, USA); chloroform – stabilized with 1% EtOH, ethanol, ethyl acetate, methanol, magnesium sulphate anhydrous, n-butanol, n-hexane, n-propanol, sodium bicarbonate, sodium chloride, sulphuric acid were furnished by Penta (Czech Republic); methyl salicylate (98%) was supplied by Alfa Aesar (Germany); Silica gel 60 was prepared by Merck (Germany).

Synthesis of derivatives of salicylic acid, m-hydroxybenzoic acid, p-hydroxybenzoic acid, β-resorcylic acid and gentisic acid. All derivatives were prepared by esterification of the appropriate acid and alcohol by catalysis with 96% sulphuric acid or p-toluenesulphonic acid. A mixture of the appropriate acid and alcohol was stirred to achieve a complete dissolution, then the catalyst (96% sulphuric acid or p-toluenesulphonic acid) was added. The mixture was stirred and refluxed. Ethyl acetate was added to the cooled reaction mixture and washed with $NaHCO_3$ solution (5 wt%, 2 × 50 mL) and saturated NaCl solution (2 × 50 mL). The solution was dried with anhydrous Na_2SO_4 (Penta, Czech Republic) overnight. The next day the solution was filtered and evaporated (Rotavapor R-300; BÜCHI Labortechnik AG, Switzerland) under vacuum. The crystalline residue was recrystallized from an appropriate alcohol and some products were purified by flash chromatography. Exact quantities of the used chemicals, reaction conditions for the preparation of alkyl esters and other pertinent data are shown in Table 1.

The purity of synthetized derivatives was confirmed by thin-layer chromatography, determination of melting point and by gas chromatograph with mass spectrometric detector (GC/MSD) (Agilent Technologies 7860 A, 5975 C, USA). A sample (1 µL) was directly injected to a capillary column (HP-5 MS; Agilent Technologies, USA) 30 m × 250 µm, film thickness 0.25 µm. The conditions of the analysis were split injection (1 : 25) at the temperature of 300 °C, carrier gas (He) with flow 1.1 mL min^{-1} and the column program was 80 °C for 2 min, the increase of temperature was 15 °C min^{-1} to 320 °C with the holding time of 30 min.

Table 1. Reaction conditions for the preparation of selected esters

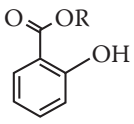
Compound	R	Formula / Molecular weight (g mol^{-1})	Acid (mmol) / Alcohol (mol)	Catalyst / Weight (mmol)	Condition of reaction (°C) / Time (h)	Melting point literature (°C) / Melting point determined (°C)	Purity (%) / Purity (%)	Appearance
salicylic acid	CH_3	$C_8H_8O_3$ 152.15	Merck	x	x	–9[1)] liquid	Merck 98	colorless liquid
	C_2H_5	$C_9H_{10}O_3$ 166.17	109.0 3.0	H_2SO_4* 54	80 6	1.3[2)] liquid	50 ≥ 99	light beige liquid
	C_3H_7	$C_{10}H_{12}O_3$ 180.20	109.0 3.0	H_2SO_4* 90	110 7	–13.2[3)] liquid	57 99.6	light beige liquid
	C_4H_9	$C_{11}H_{14}O_3$ 194.23	109.0 3.0	H_2SO_4* 90	120 8	–7.4[4)] liquid	73 ≥ 99	light beige liquid

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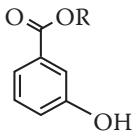
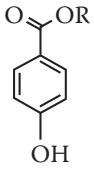
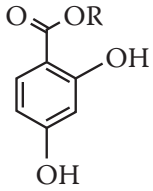
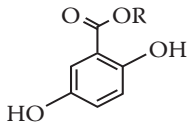
Table 1. To be continued

Compound	R	Formula / Molecular weight (g mol^{-1})	Acid (mmol) / Alcohol (mol)	Catalyst / Weight (mmol)	Condition of reaction (°C) / Time (h)	Melting point literature (°C) / Melting point determined (°C)	Purity (%) / Purity (%)	Appearance
m-hydroxybenzoic acid	CH_3	$C_8H_8O_3$	145.0	H_2SO_4*	90	66.9–68[5]	39	white
		152.15	3.0	54	4	65–71	≥ 99	crystals
	C_2H_5	$C_9H_{10}O_3$	109.0	H_2SO_4*	110	70–72[6]	11	white
		166.17	3.0	54	5	68–71	≥ 99	crystals
	C_3H_7	$C_{10}H_{12}O_3$	72.0	H_2SO_4*	120	32–33[7]	35	light beige
		180.20	3.0	90	6	24–27	≥ 99	crystals
	C_4H_9	$C_{11}H_{14}O_3$	72.0	*p*–TSA**	130	40–41[8]	5	light beige
		194.23	3.0	20	7	34–38	≥ 99	crystals
p-hydroxybenzoic acid	CH_3	$C_8H_8O_3$	109.0	H_2SO_4*	80	122–124[9]	68	white
		152.15	3.0	54	5	123–124	≥ 99	crystals
	C_2H_5	$C_9H_{10}O_3$	Merck	x	x	116–118[10]	Merck	white
		166.17				x	99	crystals
	C_3H_7	$C_{10}H_{12}O_3$	72.0	H_2SO_4*	110	95–97[11]	45	white
		180.20	3.0	90	7	92–95	99.5	crystals
	C_4H_9	$C_{11}H_{14}O_3$	72.0	H_2SO_4*	120	65–67[12]	87	white
		194.23	3.0	90	8	66–67	99.7	crystals
β-resorcylic acid	CH_3	$C_8H_8O_4$	65.0	H_2SO_4*	90	114–115[13]	23	light beige
		168.15	2.0	54	4	114–116	≥ 99	crystals
	C_2H_5	$C_9H_{10}O_4$	130.0	H_2SO_4*	110	73[14]	45	beige
		182.17	3.0	54	5	57–68	99	crystals
	C_3H_7	$C_{10}H_{12}O_4$	65.0	H_2SO_4*	120	32–34[15]	40	beige
		196.20	3.0	90	6	31–34	97	crystals
	C_4H_9	$C_{11}H_{14}O_4$	97.0	H_2SO_4*	130	44–45[16]	36	brownish
		210.23	3.0	90	7	43–45	95	crystals
gentisic acid	CH_3	$C_8H_8O_4$	130.0	H_2SO_4*	95	85–86[17]	55	white
		168.15	2.0	54	3	83–85	≥ 99	crystals
	C_2H_5	$C_9H_{10}O_4$	65.0	*p*-TSA**	99	78–80[18]	17	light beige
		182.17	2.0	20	5.5	72–76	≥ 99	crystals
	C_3H_7	$C_{10}H_{12}O_4$	65.0	*p*–TSA**	132	60[19]	17	light beige
		196.20	3.0	20	6	60–62	≥ 99	crystals
	C_4H_9	$C_{11}H_{14}O_4$	97.0	H_2SO_4*	130	65–67[20]	41	light beige
		210.23	3.0	90	6.5	60–62	≥ 99	crystals

* H_2SO_4 (96%); ** *p*-TSA – *p*-toluenesulfonic acid monohydrate; R – methyl, ethyl, propyl, butyl

[1] Friedman (2018); [2] Schneider (1896); [3], [4] Rodionov (1969); [5] Zhu et al. (2018); [6] Magano et al. (2006); [7] Moore and Vernsten (1956); [8] Inouye et al. (1999); [9] Sang et al. (2018); 10) Liu et al. (2018); [11], [12] Bayguzina et al. (2018); [13] Zhang et al. (2018); [14] Takenaka and Yamazaki (1994); [15] Katritzky et al. (2006); [16] Ahad et al. (1991); [17] Chen et al. (2012); [18] Carta et al. (2013); [19] Sabalitschka (1931), [20] Roushdi (1976)

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The detection was done in a scan mode (*m/z* range 30–1 000). The temperature of the source was 230 °C and that of the quadrupole was 150 °C.

Used microorganisms. Single strains of the five following microorganisms were obtained from the American Type Culture Collection (ATCC, USA): *Escherichia coli* ATCC 8739 (gram-negative bacteria), *Pseudomonas aeruginosa* ATCC 9027 (gram-negative bacteria), *Staphylococcus aureus* ATCC 6538 (gram-positive bacteria), *Candida albicans* ATCC 10231 (yeast) and *Aspergillus brasiliensis* ATCC 16404 (mould). Antimicrobial activity of prepared esters against these test microorganisms was compared with commercial and synthesized parabens. Culture medium and cultivation conditions for individual microorganisms are listed in Table 2.

Preparation of the initial inoculum of microorganisms. The initial inoculum of tested bacteria *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538 and yeast *C. albicans* ATCC 10231 was prepared by the dilution of fresh culture in the physiological saline solution to a final absorbance of 0.5 ± 0.05. Absorbance was measured at 650 nm for bacteria and at 630 nm for yeast on the Cary 50 Conc spectrophotometer (Agilent Technologies, Santa Clara, USA).

The inoculum of the mould *A. brasiliensis* ATCC 16404 was prepared by pipetting 7–10 mL of a 1% polysorbate Tween 80 solution on agar plate with grown mould and slightly removed with sterile loop. The resulting suspension was filtered through a sterile frit into a sterile tube to separate the spores..The suspension was applied to the Bürker counting chamber and observed under a microscope. The number of spores was determined in 1 mL, and the suspension was then diluted with saline solution to an initial value of 10^6–10^7 spores mL^{-1} (Equation 1).

$$x = \frac{1}{4} \times \frac{n}{p} \times 10^6 \text{ (number of spores mL}^{-1}\text{)} \quad (1)$$

where: x – the number of spores in the medium; n – the total number of spores in all squares; p – the number of calculated squares.

Determination of inhibitory activity of tested compounds. For all acids and their derivatives the same concentration series (1.25, 2.50, 5.00, 10.00, 20.00 mmol L^{-1}) were prepared by dilution of substances in the appropriate culture media (see Table 2) and sterilized (121 °C, 15 min, 0.15 MPa) in autoclave (PS20A; Chirana, Slovakia). The tested media, inoculated with 1% (v/v) inoculum of the selected microorganism, were applied to the wells (200 µL) of microtitration plate (Nunc™; Roskilde, Denmark). A blank was prepared by the same procedure, except that there was no addition of the tested compound. Outer wells of the microtitration plate were filled with sterile demineralized water to prevent drying of the samples during cultivation of plates. Plates were analyzed on the PowerWave XS spectrophotometer (BioTek; Winooski, USA), which is an automated device that provides heating, mixing and spectrophotometric detection of microbial growth inside the wells of the microtitration plate during the selected time period. The data were collected in each two-hour interval. From these data the minimum inhibitory concentration (MIC) was determined.

RESULTS AND DISCUSSION

The antimicrobial efficacy of tested compounds observed for the 5 strains of microorganisms is graphically illustrated by the 5 intervals in Table 3.

Table 3 shows that salicylic acid was more effective than all its derivatives, in MIC 5.00 mmol L^{-1} against the bacterium *Staphylococcus Aureus* and in MIC 2.5 mmol L^{-1} against the remaining tested microorganisms. It is further evident from the results that the antimicrobial activity of salicylates is generally very low, as previously shown by Oloyede (2016).

By comparing the results of salicylates with *p*-hydroxybenzoates (parabens) it is clear that the antimicrobial activity of the hydroxyl group at the *o*-position was lower than at the *p*-position. This may be due to the fact that in the case of salicylates it is easier to create hydrogen

Table 2. Conditions of cultivation and measurement

Used microorganisms	Time (h)	Temperature (°C)	Culture medium	Absorbance (nm)
Escherichia coli ATCC 8739	24	37	nutrient broth	650
Pseudomonas aeruginosa ATCC 9027	24	37	nutrient broth	650
Staphylococcus aureus ATCC 6538	24	37	nutrient broth	650
Candida albicans ATCC 10231	48	25	malt extract broth	630
Aspergillus brasiliensis ATCC 16404	72–120	25	malt extract broth	–

ATCC – American Type Culture Collection

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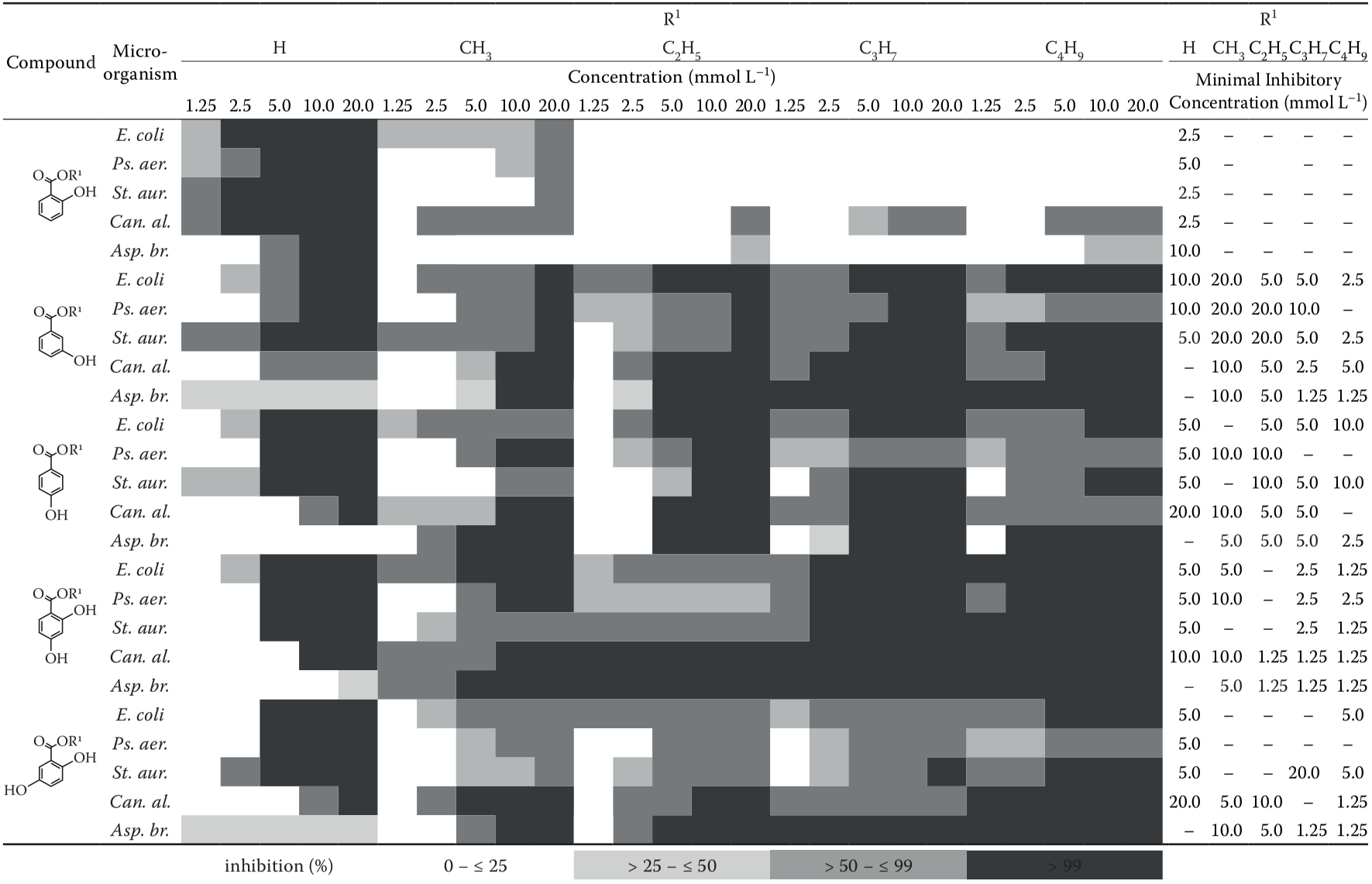
Table 3. Inhibitory activity of selected preservatives



Compound	Micro-organism	Minimal Inhibitory Concentration (mmol L^{-1})				
		H	CH_3	C_2H_5	C_3H_7	C_4H_9
	E. coli	2.5	–	–	–	–
	Ps. aer.	5.0	–		–	–
	St. aur.	2.5	–	–	–	–
	Can. al.	2.5	–	–	–	–
	Asp. br.	10.0	–	–	–	–
	E. coli	10.0	20.0	5.0	5.0	2.5
	Ps. aer.	10.0	20.0	20.0	10.0	–
	St. aur.	5.0	20.0	20.0	5.0	2.5
	Can. al.	–	10.0	5.0	2.5	5.0
	Asp. br.	–	10.0	5.0	1.25	1.25
	E. coli	5.0	–	5.0	5.0	10.0
	Ps. aer.	5.0	10.0	10.0	–	–
	St. aur.	5.0	–	10.0	5.0	10.0
	Can. al.	20.0	10.0	5.0	5.0	–
	Asp. br.	–	5.0	5.0	5.0	2.5
	E. coli	5.0	5.0	–	2.5	1.25
	Ps. aer.	5.0	10.0	–	2.5	2.5
	St. aur.	5.0	–	–	2.5	1.25
	Can. al.	10.0	10.0	1.25	1.25	1.25
	Asp. br.	–	5.0	1.25	1.25	1.25
	E. coli	5.0	–	–	–	5.0
	Ps. aer.	5.0	–	–	–	–
	St. aur.	5.0	–	–	20.0	5.0
	Can. al.	20.0	5.0	10.0	–	1.25
	Asp. br.	–	10.0	5.0	1.25	1.25

E. coli – *Escherichia coli* ATCC 8739; *Ps. aer.* – *Pseudomonas aeruginosa* ATCC 9027; *St. aur.* – *Staphylococcus aureus* ATCC 6538; *Can. al.* – *Candida albicans* ATCC 10231; *Asp. br.* – *Aspergillus brasiliensis* ATCC 16404; R^1 – methyl, ethyl, propyl, butyl

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bonds, altering the internal structure and thus reducing their resulting inhibitory activity of these substances.

Good antimicrobial effects on bacteria were observed for m-hydroxybenzoic acid, p-hydroxybenzoic acid, β-resorcylic acid and gentisic acid with the MIC being 5.00 mmol L^{-1}, (10.00 mmol L^{-1} for m-dihydroxybenzoic acid). The acids did not exhibit any mould inhibitory properties (MIC ≥ 20.00 mmol L^{-1}).

Methyl esters of gentisic acid were weaker inhibitors than methylparaben. Methyl ester of β-resorcylic acid showed higher antimicrobial activity than methylparaben. Ethyl, propyl and butyl esters of m-hydroxybenzoic acid, β-resorcylic acid and of gentisic acid had a higher antimicrobial effect on yeast and mould. Butyl esters had the lowest antimicrobial activity on Pseudomonas aeruginosa. The best antimicrobial activity from all tested compounds was shown by esters of β-resorcylic acid, especially propyl and butyl ester of β-resorcylic acid.

Minimum inhibitory concentrations (MIC) of tested compounds were compared with 4-hydroxybenzoic acid and its esters (parabens are frequently used as preservatives in food and cosmetic formulations).

CONCLUSION

The antimicrobial efficacy of esters of four aromatic hydroxy acids against 5 strains of microorganisms was investigated in a model system.

While salicylic acid was effective against all tested microorganisms, derivatives of this acid showed very low activity. The remaining tested acids (m-hydroxybenzoic acid, β-resorcylic acid and gentisic acid) showed better antimicrobial effects on bacteria than on yeast and mould. Ethyl, propyl and butyl esters of m-hydroxybenzoic acid, β-resorcylic acid and gentisic acid had higher antimicrobial effects on yeast and mould. Compounds with the best antimicrobial activity were esters of β-resorcylic acid, especially propyl and butyl ester of β-resorcylic acid.

The results indicate that the tested compounds could find the use in food, pharmaceutical and cosmetic products. We recommend further studies in a real system to understand better the antimicrobial properties of tested compounds.

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