Antimicrobial and Antioxidant Properties of Phenolic Acids Alkyl Esters

Roman MERKL, Iveta HRÁDKOVÁ, Vladimír FILIP and Jan ŠMIDRKAL

Department of Dairy and Fat Technology, Faculty of Food and Biochemical Technology, Institute of Chemical Technology in Prague, Prague, Czech Republic

Abstract

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Some phenolic acids alkyl esters (methyl, ethyl, propyl, butyl and hexyl) and determine their antioxidant and antimicrobial activities were prepared. The antimicrobial activity against the tested microorganisms *Escherichia coli* DMF 7503, *Bacillus cereus* DMF 2001, *Listeria monocytogenes* DMF 5776, *Fusarium culmorum* DMF 0103, and *Saccharomyces cerevisiae* DMF 1017 was investigated and expressed by minimum inhibitory concentration (MIC) in the range of 1.2–20mM. The inhibitory activity of phenolic acids butyl esters was found to be higher than that of methyl esters (MIC below 1.25mM). The antioxidant activity of the selected phenolic acids alkyl esters was investigated by Rancimat method. The esters of 3,4-dihydroxyphenolic acids (protocatechuic and caffeic acids) exhibited higher antioxidant activities in comparison with the respective phenolic acids. The highest antioxidant activity was found in the case of caffeic alkyl esters.

Keywords: phenolic acid; antioxidant properties; antimicrobial properties

The selected phenolic compounds are plant secondary metabolites naturally present in almost all plant materials, including food products of plant origin and other substances such as propolis. Many biological effects of these compounds, such as anti-inflammatory, antiviral, antibacterial, antiatherogenic, and anticarcinogenic properties have already been reported. These compounds are considered to be an integral part of human food (Psomiadou & Tsimidou 2002). Phenolic acid derivatives are often isolated and applied as a blend of plant species extracts. An exception is phenethyl ester of caffeic acid which has been identified as one of the major components of honeybee propolis. Biological activities are well known in the group of alkyl esters for *p*-hydroxybenzoic acids (parabens) (Grunberger *et al.* 1988). They are used widely as antimicrobial preservatives in pharmaceuticals, cosmetics, foods, and beverages and their potential toxicity and pharmacological activities have been evaluated. Each phenolic derivative has a low acute toxicity, which increases with the increasing length of the alkyl chain. Butyl ester is approximately three times more toxic than methyl ester. It appears that the methyl, ethyl, and propyl esters of *p*-hydroxybenzoic acid can be safely applied in food and drug preservatives, which have been recommended by Matthews *et al.* (1956). The maximum daily intake of parabens is 0.42 mg/kg, as reported by Soni *et al.* (2005). Carcinogenicity and estrogenicity of phenolic derivatives, however, have been little studied (Soto *et al.* 1991). The

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correlation between the molecular structure and antioxidant activity of phenolic substances has been described by Cuvelier *et al.* (1992). Monophenols were found by them to be less effective than polyphenols. Moreover, they found that the second hydroxyl group at either *ortho* or *para* position increases the antioxidant activity, and the activity of monophenols increases considerably with one or two methoxylic substituents.

In this work, the alkyl esters studied were synthesised from pure acids to reduce the polarity of the final substances, which increases the solubility in oil and also facilitates the access to the lipophilic cell wall of microorganism.

MATERIALS AND METHODS

Chemicals. Sigma-Aldrich Chemie GmbH (Steinheim, Germany): protocatechuic acid (3,4-dihydroxybenzoic acid), ≥ 97%; gentisic acid (2,5-dihydroxybenzoic acid), ≥ 99%; *p*-hydroxybenzoic acid (4-hydroxybenzoic acid), 99%; Merck (Hohenbrunn, Germany): vanillic acid (4-hydroxy-3-methoxybenzoic acid), ≥ 98%; ferulic acid (4-hydroxy-3-methoxycinnamic acid), ≥ 98%; *p*-toluenesulfonic acid, ≥ 98%; ethanol, 96%; Alfa Aesar (Karslruhe, Germany): caffeic acid (3,4-dihydroxycinnamic acid), 99%; Penta (Strakonice, Czech Republic): propanol, ≥ 99.5%, methanol ≥ 99.8%; Lachema (Strakonice, Czech Republic): butanol, ≥ 99.5%; Fluka (Steinheim, Germany): hexanol, ≥ 98%

Phenolic acid alkyl esters. Phenolic acid alkyl esters were obtained by the reaction of phenolic acid (commercial source) with the respective alcohols. The acid was firstly dissolved in alcohol and the catalyst (*p*-toluenesulfonic acid in the ratio of 0.3–1:1 w/w of phenolic acid) was then added. The reaction was carried out continuously under reflux (2–6 h; 65–95°C) (Etzenhouser *et al.* 2001). The isolation of the phenolic acid derivatives was performed by the method described by Silva *et al.* (2000) with a little modification. After cooling, the solvent was evaporated. The mixture was dissolved in ethyl acetate and neutralised with 8.4% w/w Na_2CO_3 and subsequently washed with 1% w/w NaCl solution. The organic phase was separated and dried overnight over Na_2SO_4. The products were purified by flash-chromatography (silica gel, hexane/ethyl acetate 7:3) or by crystallisation in benzene with a small addition of the appropriate alcohol. All compounds were obtained with a high yield and their purity was confirmed by TLC-FID. The purity of the compounds was greater than 98%. Thin-layer chromatography (TLC) was carried out on silica gel 60 F254 and cellulose plates (Merck, Hohenbrunn, Germany). The mobile phase was chloroform/methanol (9:1). The spots were visualised under UV (254 nm) (Nagaoka *et al.* 2002).

Antioxidant activity. The antioxidant activities of the phenolic acid derivatives (36mM) prepared were determined using the Rancimat apparatus (Rancimat 743, Metrohm, Ltd., Herisau, Switzerland). The principle of this method is to bubble air through heated sunflower oil (Table 1) and monitor continuously the conductivity of demineralised water containing volatile secondary oxidative products. The analysis was performed at the temperature of 120°C with air flow of 20 l/h. The protection factor of antioxidants was calculated using Rancimat software according to the equation:

$$PF\ (\%) = IP\ (oil + antioxidant)/IP\ (oil) \times 100$$

where:

PF – protection factor

IP – duration of the induction period (Hrádková *et al.* 2009)

Antimicrobial activity. Antimicrobial activity of phenolic derivatives against Gram-positive and Gram-negative bacteria, yeast, and fungi was measured by means of the minimum inhibitory concentration (MIC). The MIC was defined as the lowest concentration of phenolic acid alkyl esters which would inhibit the visible growth of the microorganism after the respective incubation (Table 2) (Andrews 2001).

The antibacterial effect of phenolic acid derivatives was followed in microtitration plates. The

Table 1. Characterisation of sunflower oil

AV (mg KOH/g)			0.16	
PV (meq. act. O/kg)			3.28	
IV (g I_2/100 g)			122.06	
Tocopherol content (mg/kg)			120.00	
Composition of fatty acids (% w/w)	C14:0	0.07	C18:3	0.04
	C16:0	6.12	C20:0	0.30
	C18:0	4.05	C20:1	0.02
	C18:1	26.62	C22:0	0.82
	C18:2	61.71	C24:0	0.25

Table 2. Conditions of incubation

Organism	Incubation conditions
Escherichia coli DMF 7503	37°C in air for 20 h
Bacillus cereus DMF 2001	30°C in air for 20 h
Listeria monocytogenes DMF 5776	37°C in air for 20 h
Saccharomyces cerevisiae DMF 1017	25°C in air for 48 h
Fusarium culmorum DMF 0103	20–23°C in air for 72–120 h

culture grew at the corresponding temperature under aerobic conditions for 20–120 hours. The bacterial cultures used in the experiment were prepared freshly in nutrient broth, while yeast and fungi were prepared in malt extract. The initial density of bacterial strains was approximately 10^7 CFU/ml (CFU = colony-forming unit). In the case of *Fusarium* species, their spores were diluted to 10^4 CFU/ml. In each well of the microtitration plates, a volume of 50 µl suspensions of the microorganisms was mixed with 200 µl nutrient broth containing phenolic acid alkyl esters. The amount of antimicrobial agent was diluted with 30% ethanol. The final concentration of ethanol in the test broth was not above 2.5% because at this level the concentration has no influence on the growth of microorganism. The controls inoculated without antimicrobial agent were processed simultaneously (Bartošová *et al.* 2004).

Statistical analysis. All experiments were performed in three replications. The results were expressed as mean values ± SD (the corresponding error bars were displayed in the graphical plots). All statistical tests were performed at a confidence level of 95% (P = 0.05).

RESULTS AND DISCUSSION

The MIC values of the phenolic acids alkyl esters prepared are shown for each microorganism strains tested in Table 3. The MIC values of phenolic acids and alkyl esters determined for yeast (E) and fungi (D) were significantly different.

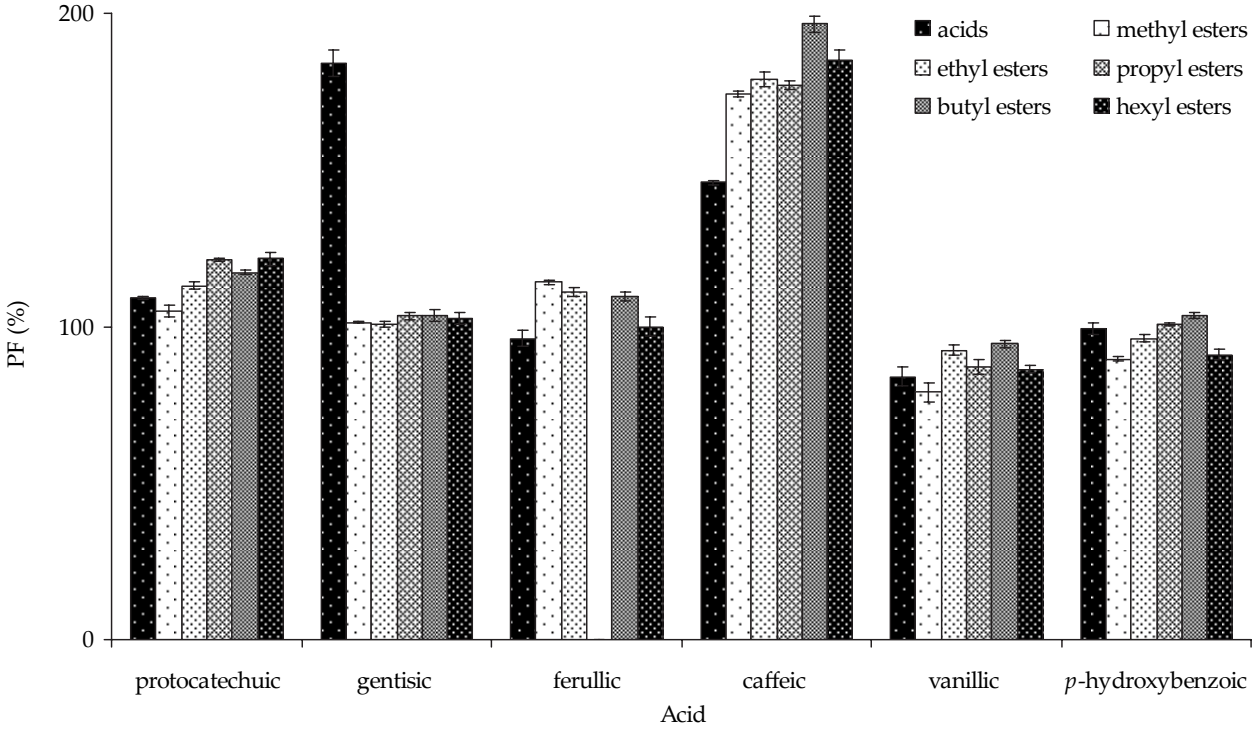
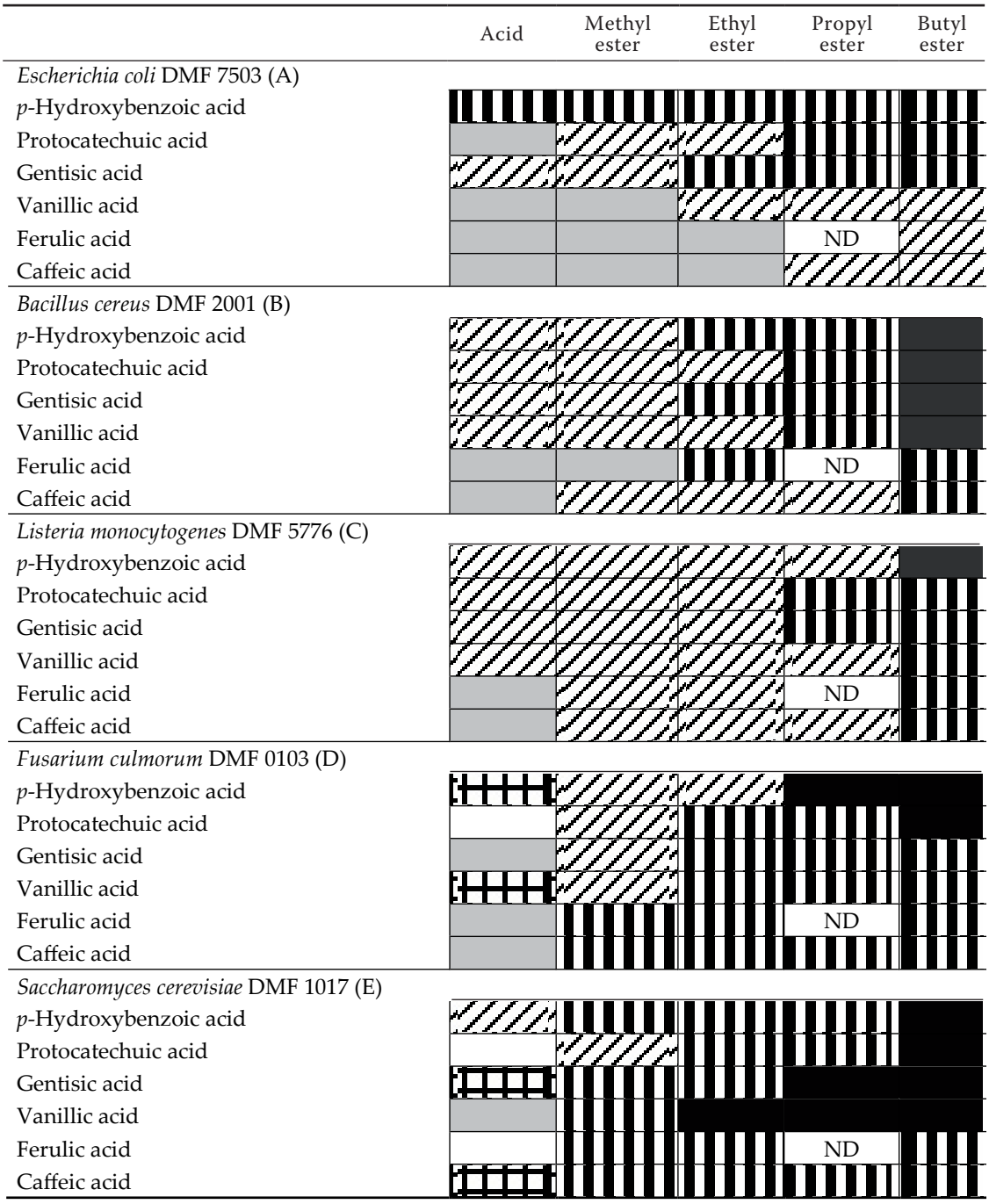


Figure 1. Protection factor (PF) of sunflower oil with phenolic acid and their alkyl esters (36mM), Rancimat method

Table 3. The minimum inhibitory concentrations (MIC) in mM of phenolic acid and their alkyl esters against tested microorganisms (A) *Escherichia coli* DMF 7503, (B) *Bacillus cereus* DMF 2001, (C) *Listeria monocytogenes* DMF 5776, (D) *Fusarium culmorum* DMF 0103, and (E) *Saccharomyces cerevisiae* DMF 1017



Possible explanation may reside in the higher proportions of lipids and phospholipids contained in the cell walls of the former. It can be seen most evidently in the case *Fusarium*. Significant differences between MIC values for Gram-negative (*Escherichia*) and Gram-positive (*Bacillus* and *Listeria*) bacteria were observed. Moreover, the sensitivity of Gram-positive bacteria was higher even in the case of phenolic acids and their methyl or ethyl esters.

Table 3 is arranged in the order from the lightest shade (the highest amount of the tested substance)

to the darkest one (the lowest amount of tested substance). Propyl ester of ferulic acid was not obtained in suitable purity, therefore its antioxidant activity was not determined.

Antioxidant properties of the substances, expressed as protective factors (PF), are shown in Figure 1. It is obvious that some compounds show pro-oxidation activity (ferullic acid, all derivatives of vanillic acid and some derivatives of *p*-hydroxybenzoic acid). Gentisic acid exhibited approximately doubled PF, whereas its ester forms exhibited considerably low PF as a result of losing their antioxidant properties. Masuda *et al.* (2008) described the antioxidant mechanisms of polyphenols (caffeic acid) as quinone form of dihydroxylbenzene that is much more easily oxidisable than the biological material.

CONCLUSION

Generally, the antimicrobial effect of phenolic acids derivatives increases with the increasing length of the alkyl chain. Butyl esters of phenolic acids effectively inhibit the growth of *Bacillus cereus* DMF 2001 and *Saccharomyces cerevisiae* DMF 1017.

Caffeic acid, its esters, and gentisic acid (only) show significant PFs (higher than 150%). Protocatechuic acid and its esters also possess antioxidant activity but their protection factor does not exceed 120%.

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Corresponding author:

Ing. Roman Merkl, Vysoká škola chemicko-technologická v Praze. Fakulta potravinářské a biochemické technologie, Ústav technologie mléka a tuků, Technická 5, 160 28 Praha 6, Česká republika
tel.: + 420 220 443 266, e-mail: roman.merkl@vscht.cz