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Phenolic Acids from Plant Foods Can Increase or Decrease the Mutation Frequency to Antibiotic Resistance

LUCIA BIROŠOVÁ,[†] MÁRIA MIKULÁŠOVÁ,*[,†] AND ŠTEFÁNIA VAVERKOVÁ[‡]

Department of Biochemistry and Microbiology, Faculty of Chemical and Food Technology, Slovak University of Technology, Bratislava, Slovakia, and Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University, Bratislava, Slovakia

Phenolic compounds are ubiquitous in plant foods, and they have been reported to have multiple biological effects. This study investigates the ability of derivatives of hydroxybenzoic and hydroxycinnamic acids to influence the development of ciprofloxacin resistance in the antibiotic-sensitive strain *Salmonella enterica* subsp. *enterica* serovar Typhimurium. We have found that cinnamic acid and its hydroxy derivatives increase the frequency of mutations leading to ciprofloxacin resistance in comparison with spontaneous mutagenesis. Derivatives of hydroxybenzoic acid showed no or very little effect. Interestingly, gallic acid caused only phenotype resistance but not arising of mutant strains. Vanillic acid decreased spontaneous mutation frequency, leading to resistance not only to ciprofloxacin but also to tetracycline and gentamicin, and also decreased mutation frequency induced by 3-(5-nitro-2-furyl)acrylic acid, sodium azide, and hydrogen peroxide leading to ciprofloxacin resistance.

KEYWORDS: Phenolic acids; vanillic acid; ciprofloxacin; antibiotic resistance; antimutagen

INTRODUCTION

Phenolic compounds are ubiquitous in plant foods, and they have been reported to have multiple biological effects including antioxidant (*1, 2*) and antimutagenic (*3–7*) activity. In recent years researchers and food manufacturers have become increasingly interested in these compounds, which may be exploited for the development of health foods or nutraceuticals (*8, 9*).

Phenolic acids form a diverse group that includes the widely distributed hydroxycinnamic acids, most frequently as simple esters with hydroxycarboxylic acid or glucose, and hydroxybenzoic acid compounds which are present mainly in the form of glucosides. The presence of the −CH═CH−COOH group in hydroxycinnamic acids is considered to be key for the significantly higher antioxidative efficiency than the −COOH in the hydroxybenzoic acids (*10*). The main source of these compounds are several types of plant material such as vegetables, fruits, leaves, oilseeds, cereal crops, spices, and herbs (*11*). A significant amount of phenolic acids such as ferulic, caffeic, *p*-hydroxybenzoic, protocatechuic, *p*-coumaric, vanillic, and syringic is typically found in cereals (*10*). In berries and fruits hydroxylated derivatives of benzoic acid and cinnamic acid are present (*12*). Hydrolyzable tannins in tea contain either ellagitannins or gallotannins; the latter yield glucose and gallic acid upon hydrolysis by certain enzymes (*13*).

Besides plant foods many types of nutraceuticals are known to contain phenolic compounds. The therapeutic effect of *Echinacea* plants has been assigned to the presence of caffeic acid derivatives such as cichoric and chlorogenic acid together with other compounds found in the hydroalcoholic extracts (*14*). However, not all phenolic compounds and not all of their actions are beneficial. Some of them have mutagenic (*15*) and/or prooxidant effect, and they may interact with essential biochemical pathways (*16*).

Food-borne pathogens like *Salmonella* spp. in the human gastrointestinal tract come into contact with these compounds. An issue has been raised as to how these compounds influence the bacteria from the point of view of antibiotic resistance development. Fluoroquinolones like ciprofloxacin are a group of synthetic antimicrobial agents, which are quite active against Gram-positive and Gram-negative bacteria, but there is growing evidence of clinical isolates with decreased susceptibilities associated with quinolone resistance (*17*). The aim of this work was to determine the effect of hydroxybenzoic and cinnamic acid derivatives on the mutation frequency leading to ciprofloxacin resistance.

MATERIALS AND METHODS

Compounds Tested. Caffeic acid [3-(3,4-dihydroxyphenyl)prop-2-enoic acid], chlorogenic acid [(1*R*,3*R*,4*S*,5*R*)-3-[(*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid], cichoric acid [(2*R*,3*R*)-2,3-bis[[(*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy]butanedioic acid], ferulic acid [(*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoic acid], and rosmarinic acid [[*R*-(+)]-α-[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-3,4-dihydroxybenzenepro-

* Corresponding author. Tel: +421-2-59325528. Fax: +421-2-52926090. E-mail: maria.mikulasova@stuba.sk.
[†] Slovak University of Technology.
[‡] Comenius University.

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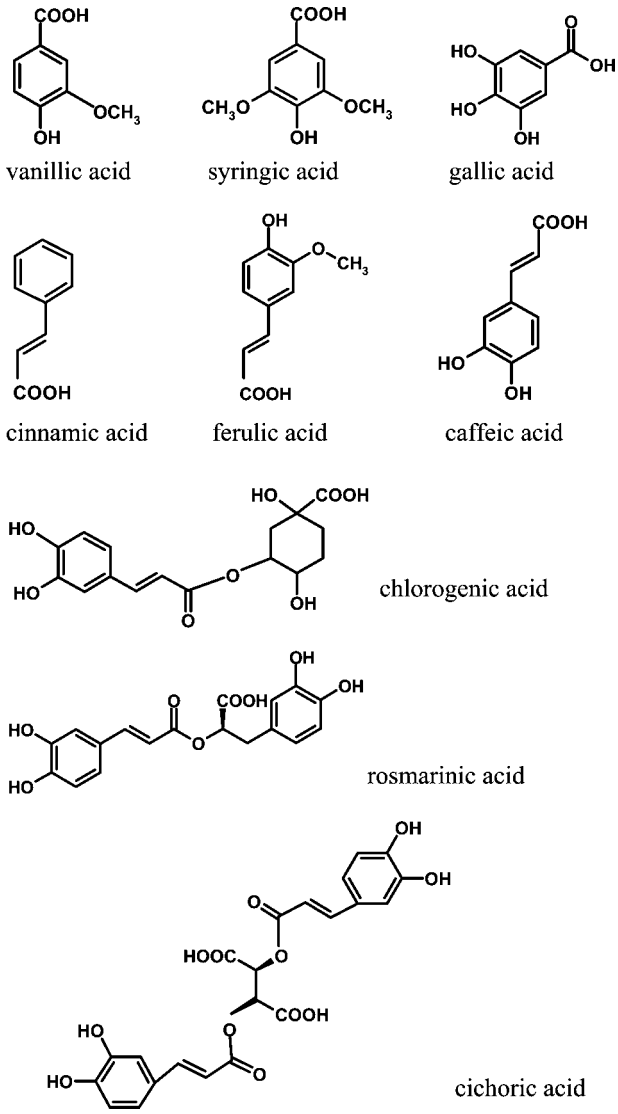

Figure 1. Structures of studied phenolic acids.

panoic acid] were extracted, isolated (*18*), and identified according to published methods (*19, 20*). Gallic acid (3,4,5-trihydroxybenzoic acid), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), and vanillic acid (4-hydroxy-3-methoxybenzoic acid) were purchased from Merck (Germany). Structures of phenolic acids used in this study are depicted in **Figure 1**.

Bacterial Strains and Antibiotics. The bacterial strain used in this study, *Salmonella enterica* subsp. *enterica* serovar Typhimurium CCM 4763, was received from the Collection of Microorganisms, Masaryk University, Brno (Czech Republic).

Ciprofloxacin, tetracycline, gentamicin, and chloramphenicol were purchased from Merck (Germany).

Antimicrobial Susceptibility Determination. The minimal inhibitory concentration (MIC) of an antibiotic was determined by the agar dilution method using agar plates containing serial 2-fold dilutions of a drug and by microsuspenssion assay in 96-well microplates. The MIC was defined as the lowest concentration of drug that inhibits visible growth after 24 h of incubation at 37 °C.

Determination of Mutation Frequencies Leading to Antibiotic Resistance. *S. enterica* ser. Typhimurium strain was grown overnight in antibiotic-free broth with the viable cell number being around 10^9/mL. This culture was divided into 0.1 mL aliquots, to which 0.1 mL of phenolic acid and 0.5 mL of phosphate buffer (pH 7.4) were added, and cultures were treated with the tested compound for 30 min. Then fresh Luria–Bertani medium was added, and cultures were incubated for 3 h at 37 °C to allow a few cell divisions and protein expression to occur. The number of ciprofloxacin-resistant mutants that emerged in each culture was determined by plating the entire culture on nutrient agar plates containing a selective concentration of antibiotic. The total number of viable cells was determined by plating an appropriate dilution of cultures on nonselective medium. Colonies on both selective and nonselective plates were counted after incubation for 2 days. The frequency of resistant mutants [resistance index (RI)] was expressed as a mean number of resistant cells divided by the total number of viable cells per culture.

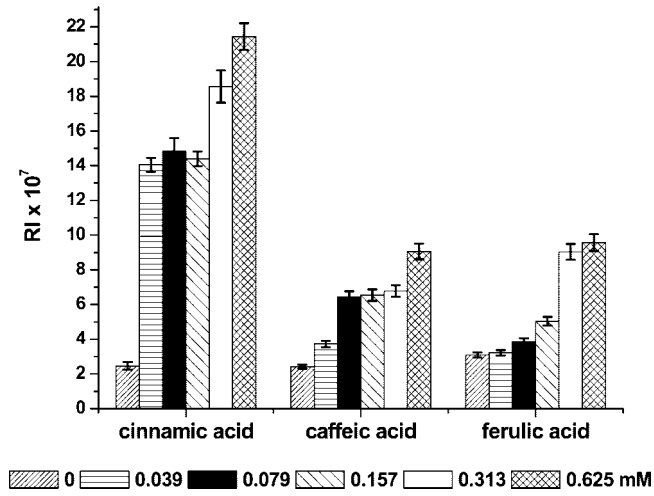

Figure 2. Effect of cinnamic, caffeic, and ferulic acids on the mutation frequency leading to ciprofloxacin resistance. RI (resistance index) = number of resistant cells divided by the number of viable cells.

Subculturing of Mutant Isolates. From each culture growing at selective antibiotic concentration one random mutant colony was picked and passaged in media without antibiotics for 10 consecutive days. Following serial culture, the ciprofloxacin MIC of cells collected after the first and last subculture to antibiotics was determined. Strains which maintained increased values of MIC are considered to be resistant mutants.

Statistical Analysis of Data. Data shown in this study represent the mean of three independent experiments; each experiment was made in five parallel determinations and statistically evaluated by Student's *t*-test.

RESULTS AND DISCUSSION

For comparison of their effects, all phenolic acids were tested in the same concentrations from 0.039 to 0.625 mM. According to the mode of action we can divide these compounds into three groups: compounds with no effect on mutation frequency and compounds which increase or decrease mutation frequency to antibiotic resistance.

Cinnamic acid and their derivatives slightly increase mutation frequency. Depending on concentration, cinnamic acid increases mutation frequency to ciprofloxacin resistance 14–22-fold. Caffeic acid and ferulic acid increase the resistance index less, 9- and 10-fold, respectively, in the highest tested concentration (**Figure 2**). These phenolic acids are considered as antioxidants and antimutagenic compounds in other tests (*21*), but in the case of mutagenesis to antibiotic resistance they have a very small but still mutagenic effect, as obvious from **Figure 2**. Cichoric and rosmarinic acids with two aromatic rings have a similar effect; in lower concentrations they slightly increase the mutation frequency, but using higher concentrations this frequency decreases to the spontaneous mutability level (**Figure 3**). Chlorogenic acid increases the resistance index almost 10 times. This phenolic acid is a known antioxidant (*22*), but in our previous study (*15*), we have confirmed a moderate genotoxic effect of chlorogenic acid on testing strain *Salmonella typhimurium* TA102 using the classical plate incorporation Ames

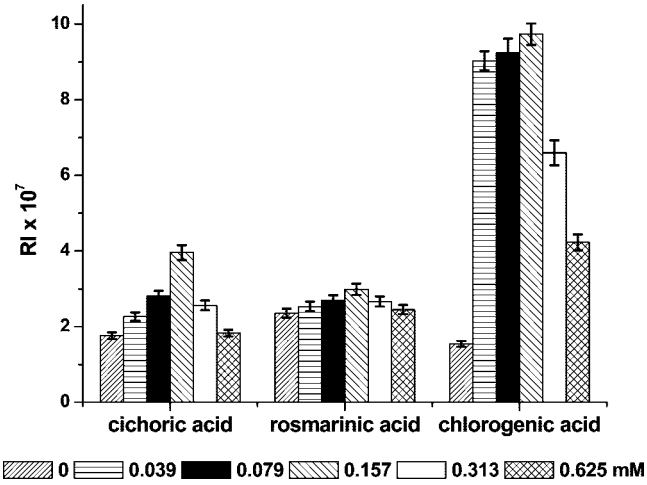

Figure 3. Effect of cichoric, rosmarinic, and chlorogenic acids on the mutation frequency leading to ciprofloxacin resistance.

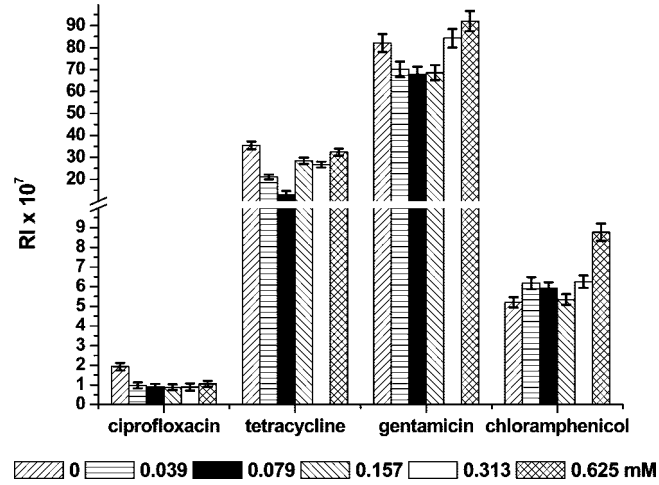

Figure 4. Effect of vanillic acid on the frequency of mutations leading to resistance to antibiotics with different modes of action.

test. This strain is sensitive to oxidative mutagens and is suitable for the detection of compounds causing oxidative damage of DNA. Cichoric acid has a significant effect on strain *S. typhimurium* TA98, which indicates frameshift mutations (*15*). This phenolic acid increases the mutation frequency to ciprofloxacin resistance very slightly. Mutations in the *gyrA* gene that results in ciprofloxacin resistance are base substitution mutations (*23*). Therefore, compounds which appear to be frameshift mutagens do not have a significant effect on the development of resistance.

Resistant strains were identified following their growth on plates with selective agar (2 × MIC of ciprofloxacin). From each plate one random mutant colony was picked and subcultivated in media without antibiotics for 10 consecutive days. After this process susceptibility to ciprofloxacin of cells collected after the first and last subculture was determined. Cultures growing on plates supplemented with antibiotic are considered to be mutants, while strains that were not able to survive after subculturing in antibiotic-free medium showed only phenotype changes due to the influence of phenolic compounds. The majority of isolated strains were able to grow in the presence of ciprofloxacin at selective and even substantially higher concentration; in some cases we obtained clinically relevant values of ciprofloxacin MIC.

Derivatives of benzoic acid did not increase mutation frequency leading to ciprofloxacin resistance. From this point of view it is interesting that although gallic acid raised the number of colonies growing on selective plates, no cells were able to survive at selective concentrations of ciprofloxacin after the growth in antibiotic-free medium. Benzoate and sodium salicylate have been shown to induce the *marRAB* operon in *Escherichia coli*, which exerts overlapping effects on the regulation of efflux pumps and porin synthesis (*24*). Induction of the *marRAB* operon leads to a decrease of intracellular antibiotic concentration and to elevation of minimal inhibitory concentration. Gallic acid apparently gives rise to such phenotype changes.

Syringic acid did not influence the number of resistant mutants either in a positive or in a negative way. However, in the case of vanillic acid we detected a considerable dose-dependent decrease in mutation frequency leading to ciprofloxacin resistance. Using the lowest tested concentration of vanillic acid, mutation frequency decreased to 50%, and with increasing concentration this response slightly increased.

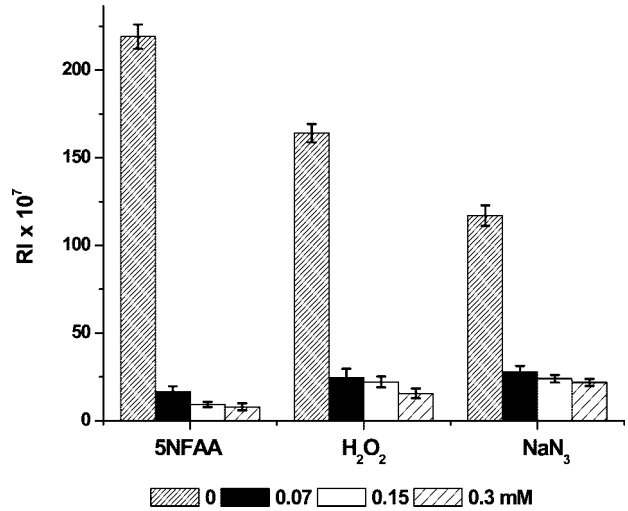

Figure 5. Effect of vanillic acid on the frequency of mutations leading to ciprofloxacin resistance induced by positive mutagens.

Consequently, we have verified the ability of vanillic acid to inhibit development of mutations leading to resistance to other antibiotics with a different mode of action. The favorable effect on mutation frequency leading to tetracycline resistance is evident from **Figure 4**; vanillic acid decreased resistance index by 63%. We determined less substantial action of this phenolic compound on mutation frequency leading to gentamicin resistance (only by 17%) and no effect on the development of chloramphenicol resistance. The beneficial response of vanillic acid vanished when using the two highest tested concentrations.

Following determination of this beneficial effect of vanillic acid on the frequency of spontaneous mutations leading to ciprofloxacin resistance, we also determined the ability of this compound to decrease the frequency of mutations induced by positive mutagens (**Figure 5**): 3-(5-nitro-2-furyl)acrylic acid (5NFAA) (16.4 μM), sodium azide (NaN_3) (77 μM), and hydrogen peroxide (H_2O_2) (0.8 mM). Vanillic acid significantly inhibited the frequency of induced mutations leading to ciprofloxacin resistance in all tested concentrations. In the case of 5NFAA we determined the reduction of mutation frequency in the range from 93% to 95%. The resistance index to ciprofloxacin resistance induced by hydrogen peroxide decreased in the range of 85–90% and RI induced by sodium azide in the range of 76–81%. Results represent the mean value from three independent experiments made in five parallel assays. These

results indicate that protective effects shown by vanillic acid could be due not only to its antioxidant properties but also to its property to initiate and promote DNA repair, similar to its derivative, vanillin (4-hydroxy-3-methoxybenzaldehyde) (*25*).

The strong inhibitory effect of vanillic acid on the development of antibiotic-resistant mutants suggests a new possibility to promote lowering of resistance development: the use of compounds with antimutagenic and antioxidant properties. Pillai et al. (*26*) showed that green tea catechins possess the potential to prevent or delay the development of resistance. Synthetic and mainly natural compounds with antimutagenic activity are intensively studied in light of the possibility to use them in the chemoprevention of cancer. Our results suggest that they have an advantage also in the prevention of antibiotic resistance development.

Another result of our work shows that some phenolic compounds may increase the development of resistance. Drugs are usually taken with a cup of tea. We confirmed that gallic acid present in tea allowed bacterial cells to grow at higher concentrations of antibiotic. To our knowledge, very few compounds present in tea were tested for their effect on the development of antibiotic resistance. Nutraceuticals are widely used, and we know only a little about their effect on the development of drug resistance. It is known that the flavonoid quercetin is a competitive inhibitor of gyrase, the primary target of fluoroquinolones like ciprofloxacin. Therefore, in case of usage of ciprofloxacin it is not recommended to take preparations with quercetin (*27*). Ward et al. (*28*) showed an increase of ampicillin MIC in the presence of garlic, echinacea, and zinc products.

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