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ARTICLE

Phenolic Compound Combinations on *Escherichia coli* Viability in a Meat System

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The aim of this work was to investigate the antibacterial activity of flavonoid and nonflavonoid phenolic compound combinations and the synergistic antibacterial effects against *Escherichia coli* ATCC 35218. In nutrient medium, the combinations of gallic and protocatechuic acids, gallic and caffeic acids, and rutin and quercetin were the best antibacterial agents, with synergistic effects, and were selected to test their activity in a meat model system. All combinations diminished the bacterial growth, without cellular death at 20 °C. The combinations of gallic and caffeic acids and rutin and quercetin were the most effective at 4 °C; no viable cells were detected with 100 or 200 mg/L at 21 or 14 days of incubations, respectively. The lowest decimal reduction times were found with the rutin−quercetin combination. These results demonstrate a synergistic effect of the selected combination of flavonoid or nonflavonoid compounds with an important antibacterial effect in meat, using low concentrations.

KEYWORDS: Phenolic compounds; antibacterial; *E. coli*; meat; synergistic effect

INTRODUCTION

Escherichia coli is a natural inhabitant in the intestinal tracts of humans and warm-blooded animals. Animal fecal matter is the primary source of pathogenic bacteria on meat products (*1*), and the presence of this species provides absolute proof of fecal contamination. Some strains of *E. coli* can cause diarrhea, urinary tract infections, inflammations, and peritonitis in immunesuppressed patients such as children and the elderly (*2*, *3*). As a consequence, the absence of *E. coli* in foods can be used to assess the sanitary quality (*4*).

Unfortunately, there is a dramatic increase throughout the world in the number of reported cases of foodborne illness that result from the consumption of food contaminated with pathogenic bacteria (*5*). Fresh meat and derivative products can be easily contaminated with microorganisms and, if not properly handled and preserved, support growth of spoilage and pathogen bacteria, leading to loss of quality and potential public health problems. Refrigeration storage is usually the most common preservative method of fresh meat and meat products. To extend refrigerated storage time, antimicrobial and antioxidant additives especially of synthetic origin, are added to muscle foods (*6*). The use of chemical additives is perceived by consumers as a health risk. Thus, the exploration of natural antimicrobials for food preservation receives increased attention due to consumer awareness of natural food products and a growing concern of microbial resistance toward conventional preservatives (*7*). Therefore, the development of new natural preservatives is essential in the fields of food industry to ensure food safety. Phenolic compounds represent a common constituent of the human diet; they are found in fruits, vegetables, nuts, seeds, stems, and flowers as well as tea, wine (*8*), propolis, and honey (*9*). They have a variety of beneficial effects on human health, including anti-inflammatory activity, enzyme inhibition, antiallergic activity, antioxidant activity, vascular activity, and cytotoxic antitumor activity (*10*). Phenolic compounds are subdivided into three groups: nonflavonoids (e.g., gallic, protocatechuic, vanillic, and caffeic acids), flavonoids (e.g., quercetin, rutin, and catechin), and tannins (*11*).

It is known that when two chemical compounds are used together, the interaction between them can modify their individual effect and phenomena of synergism or antagonism can take place.

In pervious works, we demonstrated the effect of phenolic compounds on the viability of *E. coli* (*12*). This bacterium being commonly responsible of foodborne illness, the possibility of using phenolic compounds as natural preservatives in food is very interesting.

At the present, little information is available about the synergistic effect of phenolic compounds in relation with antibacterial activity.

With the aim of improveing the antibacterial efficiency of these compounds, synergistic antibacterial effects of flavonoid and nonflavonoid phenolic compound combinations were investigated using *E. coli* as test bacteria. The combinations were assayed in nutrient complex medium to select the most effective with synergistic effect. The behaviors of the selected combinations were then investigated in a meat model system.

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MATERIALS AND METHODS

Microorganism and Medium. The bacterium used as test organism was *E. coli* ATCC 35218 (American Type Culture Collection). The strain was grown at 37 °C in nutrient broth and agar medium. Before experimental use, cultures from solid medium were subcultured in liquid media, incubated for 24 h, and used as the source of inocula for each experiment.

Differential Medium. The medium used for enumeration of *E. coli* in meat was McConkey medium (Britania, Argentina) that contained (g/L) peptone, 17.0; plurypeptone, 3.0; lactose, 10.0; bile salts mixture, 1.5; sodium chloride, 5.0; neutral red, 0.03; crystal violet, 0.001; and agar, 13.5.

Pure Phenolic Compounds. Catechin was obtained from Sigma (St. Louis, MO), gallic acid was obtained from Merck (Darmstadt, Germany), vanillic acid, protocatechuic acid, caffeic acid, quercetin, and rutin were purchased from ICN (Cleveland, OH). The purity level of all phenolic compounds was >98%. All phenolic compounds were dissolved in ethanol 99.8% (Merck) and filter-sterilized through a 0.22 μm membrane filter (Durapore, EM PVDF, Millipore).

Table 1. Individual Antibacterial Effect of Phenolic Compound in Nutrient Medium

	50 mg/L		100 mg/L	
	total growth[a]	growth reduction[a]	total growth	growth reduction
control	2.35 ± 0.1		2.35 ± 0.1	
gallic acid (G)	1.77 ± 0.08	0.58	1.4 ± 0.09	0.95
protocatechuic acid (P)	2.05 ± 0.09	0.3	1.8 ± 0.08	0.55
vanillic acid (V)	2.2 ± 0.07	0.15	2.08 ± 0.05	0.27
caffeic acid (Ca)	0.85 ± 0.05	1.5	0.2 ± 0.03	2.15
rutin (R)	1.12 ± 0.07	1.23	0.7 ± 0.05	1.65
quercetin (Q)	0.44 ± 0.05	1.91	0.19 ± 0.02	2.16
catechin (C)	1.4 ± 0.09	0.95	1.31 ± 0.09	1.04

[a] Log cfu/mL.

Antibacterial Activity. *Antibacterial Effect of Pure Phenolic Compound Mixtures in Nutrient Medium.* Phenolic compounds for the different combinations were selected by considering the antibacterial effect previously obtained (**Table 1**) when assayed as individual compounds in nutrient medium (*12*). Individual phenolic compounds were added to the medium to obtain in the mixture a final concentration of 100 mg/L (50 mg/L of each one) or 200 mg/L (100 mg/L of each one). These concentrations were selected by considering their concentrations naturally found in fruits and wines. The following combinations of nonflavonoids were utilized: gallic−protocatechuic, gallic−vanillic, gallic−caffeic, protocatechuic−vanillic, and protocatechuic−caffeic acids. The combinations of flavonoids utilized were quercetin−rutin, rutin−catechin, and quercetin−catechin. Ethanol was added to all media to obtain a final concentration of 5% v/v. The media were inoculated with an overnight bacterial culture to obtain 2.0×10^7 cfu/mL. The control was carried out with the media added with 5% ethanol, without phenolic compounds and inoculated with an overnight culture. *E. coli* in nutrient broth medium with different phenolic compound combinations was incubated for 18 h at 37 °C, and growth was followed in a tunable microplate reader (Versamax, Molecular Devices). The plates used were microtiter plate flat form. The samples were serially diluted with isotonic solution, subsequently; 0.1 mL of each dilution was spread on nutrient agar. Plates were incubated for 24 h before enumeration. Each experiment was repeated at least three times.

Theoretical and Experimental Inhibitory Effect of Combined Phenolic Compounds. Experimental values (VE) represent the

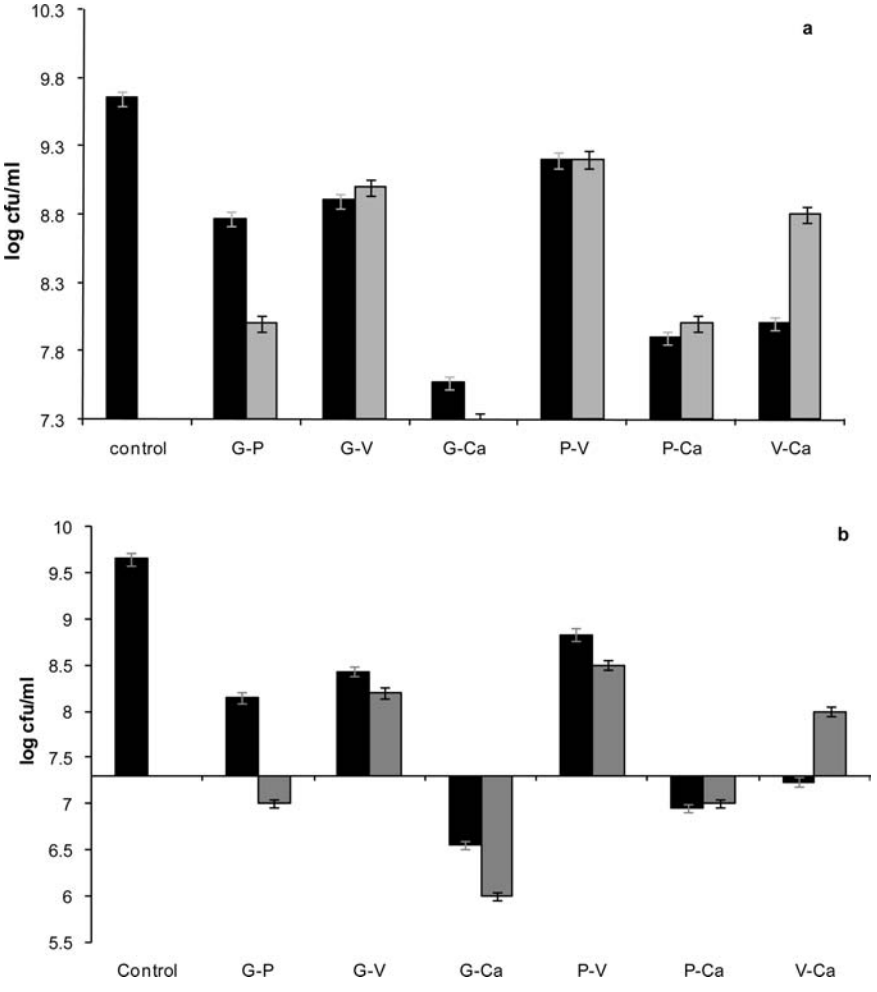

Figure 1. Number of viable cells of *E. coli* in media supplemented with 100 mg/L (**a**) or 200 mg/L (**b**) of different nonflavonoid compound combinations: (black bars) theoretical values; (gray bars) experimental values. Each point represents the average value of three determinations.

viable cells (log cfu/mL) obtained after 18 h of incubation in the presence of phenolic compound combinations.

Theoretical values (VT) represent the expected value considering the sum of these individual values for each combination (**Table 1**) and expressed as viable cells (log cfu/mL).

$$VT = \text{final growth (log cfu/mL) in control medium} - (\Sigma \text{ growth reduction (log cfu/mL) of individual compounds})$$

The effect could be synergistic, additive, or antagonistic. In the first effect, the experimental value was higher with respect to the theoretical value. In the additive effect, there were no differences between theoretical and experimental values. An antagonistic effect is observed when the theoretical value was higher than the experimental value.

Effect of Selected Phenolic Compound Combinations in Meat. Lean meat, obtained from a local commercial outlet, was stored at −20 °C. Ten grams of meat was aseptically placed in stomacher bags, and 10 mL of isotonic solution with phenolic compound combinations was added to the food to obtain a final concentration of 100 or 200 mg/L in a ratio of 1:1. From results in culture medium, the selected combinations for this experiment were gallic−protocatechuic acids, gallic−caffeic acids, and quercetin−rutin. The stomacher bags were inoculated with 10^9 cfu/mL of *E. coli* and were homogenized for 3 min. Stomacher bags were stored at 4 or 20 °C for 21 days. The control was inoculated meat in the stomacher bag with 10 mL of isotonic solution with 5% ethanol added.

Decimal Reduction Time. The decimal reductino time is the time to reduce by 90% the viable cells of *E. coli*. It was calculated graphically for each sample at 4 °C.

Statistical Analysis. All experiments were carried out at least in triplicate. Experimental data were analyzed by ANOVA. Growth experimental data means were compared using Student's *t* test.

RESULTS

Pure Phenolic Compounds Mixtures in Nutrient Medium. In control medium without phenolic compounds, the number of viable cells increased from 2.0×10^7 to 4.47×10^9 cfu/mL at the end of incubation time (18 h). **Figure 1** shows the number of viable cells of *E. coli* at 18 h of incubation in nutrient medium supplemented with 100 (**a**) or 200 (**b**) mg/L of nonflavonoid mixtures. With 100 mg/L (**Figure 1a**), gallic−protocatechuic and gallic−caffeic acid combinations were the most effective, demonstrating a synergistic effect. In these cases, the microorganism viability diminished 0.77 and 0.30 log cycle, respectively, with respect to the corresponding theoretical value. With the addition of 200 mg/L (**Figure 1b**), the combinations of gallic−protocatechuic and gallic−caffeic acids produced cell death, decreasing the counts 0.3 and 1.3 log cycles in the inoculated cells, respectively. At the two concentrations, gallic−vanillic acids, protocatechuic−vanillic acids, and protocatechuic−caffeic acids produced an additive effect with respect to theoretical values. The only combination that produced an antagonist effect was vanillic−caffeic acids (**Figure 1**).

Figure 2 shows the inhibition of *E. coli* viable cells at 18 h of incubation in media supplemented with 100 (**a**) or 200 (**b**) mg/L of

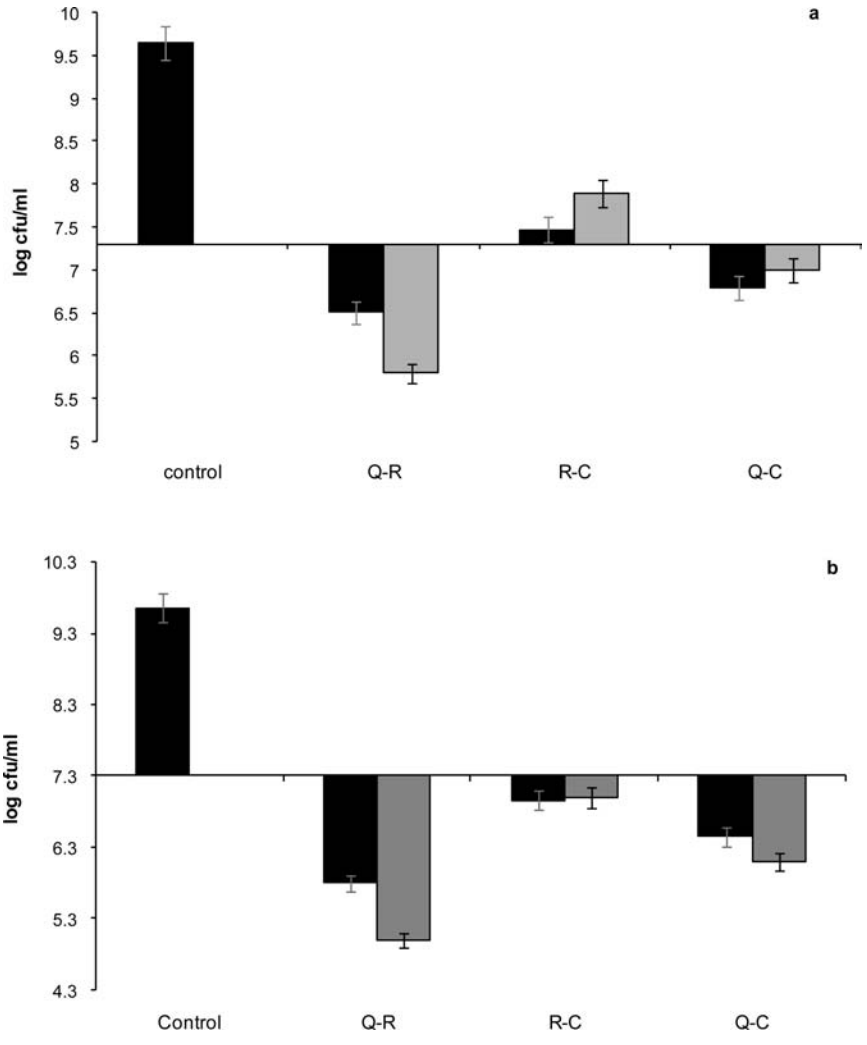

Figure 2. Number of viable cells of *E. coli* in media supplemented with 100 mg/L (**a**) or 200 mg/L (**b**) of different flavonoid compound combinations: (black bars) theoretical values; (gray bars) experimental values. Each point represents the average value of three determinations.

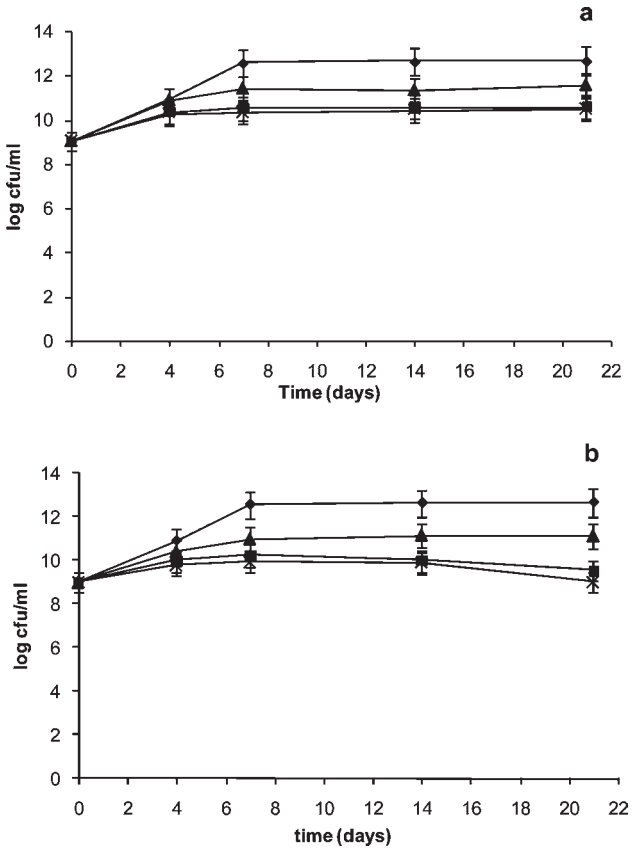

Figure 3. Survey of *E. coli* in meat supplemented with phenolic compound combinations at 20 °C: (**a**) 100 mg/L; (**b**) 200 mg/L; (◆) control; (▲) gallic−protocatechuic acids; (■) gallic−caffeic acids; (×) rutin−quercetin. Each point represents the average value of three determinations.

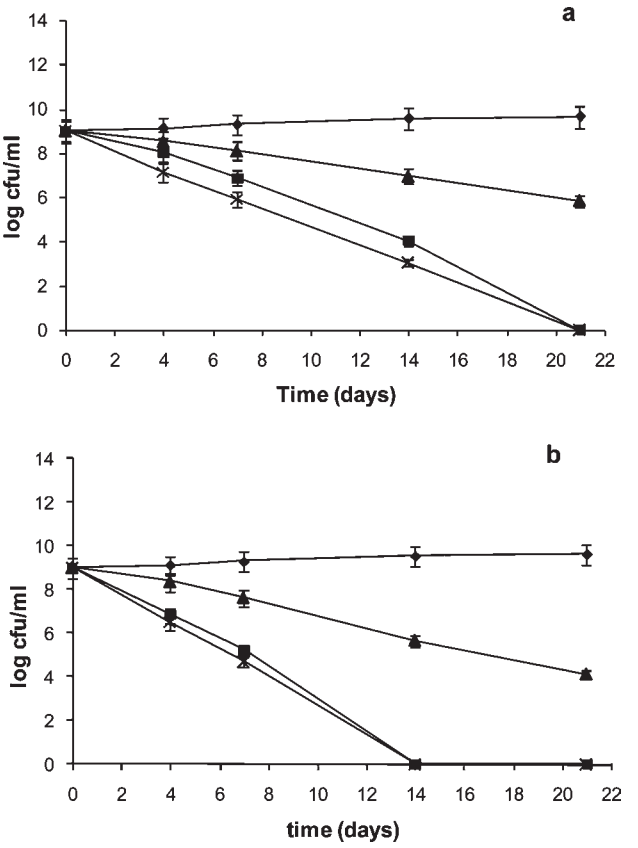

Figure 4. Survey of *E. coli* in meat supplemented with phenolic compound combinations at 4 °C: (**a**) 100 mg/L; (**b**) 200 mg/L; (◆) control; (▲) gallic−protocatechuic acids; (■) gallic−caffeic acids; (×) rutin−quercetin. Each point represents the average value of three determinations.

flavonoid mixtures. With 100 mg/L combinations (**Figure 2a**) only the quercetin−rutin mixture produces a synergistic effect with cellular death, reducing the number of viable cells by 1.5 log cycles with respect to the inoculated cells. Rutin−catechin and quercetin−catechin combinations produce an additive effect. With the addition of 200 mg/L combinations (**Figure 2b**), the quercetin−rutin combination increased the inhibitory effect and cellular death, decreasing by 2.3 log cycles the inoculated cells. At the same concentration, rutin−catechin and quercetin−catechin combinations produce an additive inhibitory effect with cellular death.

Of the nonflavonoid compounds, gallic acid combined with protocatechuic or caffeic acid produced a synergistic inhibitory effect against *E. coli* at two assayed concentrations. Cellular death was observed only in the presence of 200 mg/L of each combination, the gallic−caffeic acid combination being the more effective with the highest inhibitory effect against *E. coli*. The better combination of flavonoids as antibacterial agent was quercetin−rutin, which produces a synergistic effect in the inhibition of *E. coli* with cellular death at two assayed concentrations.

Survey of *E.coli* in Meat. The three best phenolic compound combinations assayed against *E. coli* in nutrient medium were selected to be tested in a meat system.

Figure 3 shows the growth response of *E. coli* in meat supplemented with the selected phenolic compound combinations during storage at 20 °C. In control meat, without phenolic compounds, microorganism growth increased 3.98 log cycles with the inoculated cells at 21 days of incubation. With 100 mg/L of mixtures (**Figure 3a**), gallic−protocatechuic acids, gallic−caffeic acids, and rutin−quercetin combinations decreased the growth of *E. coli* 30.2, 57.7, and 59.3%, respectively, with respect to control meat. The addition of 200 mg/L (**Figure 3b**) of gallic−protocatechuic acids, gallic−caffeic acids, and rutin−quercetin combinations increased the inhibitory effect 42.3, 86.0, and 100%, respectively.

Table 2. Decimal Reduction Times Calculated Graphically for Each Phenolic Compound Combination at 4 °C

	decimal reduction time of *E. coli* (days)	
phenolic compound combination	100 mg/L	200 mg/L
gallic−protocatechuic acids	6.0	3.75
gallic−caffeic acids	2.6	1.9
rutin−quercetin	2.2	1.6

The growth response of *E. coli* in meat supplemented with phenolic compound combinations at 4 °C is shown in **Figure 4**. In control meat the microorganism growth was 0.63 logaritmic cycles at 21 days of incubation. With 100 mg/L of mixtures (**Figure 4a**), a diminished 3.17 log cycles of inoculated cells was found with gallic−protocatechuic acids. No viable cells were detected with the addition of gallic−caffeic acids and rutin−quercetin combinations at 21 days of incubation. The addition of 200 mg/L (**Figure 4b**) of these combinations increased these lethal effects. Gallic−protocatechuic acids combination reduced by 4.89 log cycles the inoculated cells at 21 days. Gallic−caffeic acids and rutin−quercetin were the most effective combinations because at 14 days of incubations no viable cells were detected.

Table 2 shows the decimal reduction times calculated graphically from **Figure 4** for each phenolic compound combination at 4 °C. The lowest decimal reduction times corresponding to

1.6 and 1.9 days were found for rutin−quercetin and gallic−caffeic acids combinations, respectively.

DISCUSSION

Our results indicated that same mixtures of flavonoid and nonflavonoid phenolic compounds, either in nutrient medium or in a model meat system, enhanced their antibacterial activity when using *E. coli* as the test bacteria. The combinations showing the best antibacterial effect, gallic−protocatechuic acids, gallic−caffeic acids, and rutin−quercetin, were selected to test their activity in a model meat system at 20 and 4 °C.

As expected, even at 20 °C there was an important inhibitory effect; the combinations of phenolic compounds were more effective at 4 °C, producing cellular death at the two concentrations assayed, as evidenced by the values of decimal reduction times, the lowest corresponding to the rutin−quercetin combination. Similar results were reported by Arima et al. (*13*), with respect to the antibacterial activity of flavonoids in culture media, which was enhanced by combining or mixing them. They found that the combinations of quercetin−quercitrin, quercetin−morin, and quercetin−rutin were much more active than any of these flavonoids alone.

Flavonoids have been reported to enhance the anticancer or antiviral activity of drugs: the anticancer activity of tamoxifen was enhanced by combination with tangeretin (*14*), and the antiviral activity of acyclovir was enhanced by combination with quercetin in a clinical application (*15*). Flavonoids have been reported to enhance the antibacterial activities of naringenin (*16*).

Lin et al. (*17*) studied the antimicrobial activity of different extracts of oregano and cranberry, and they observed that synergistic effects of plant extracts provide a wide range of phenolic diversity, significantly increasing or decreasing the antimicrobial efficacy.

To our knowledge, there is no information on the effect of nonflavonoid combinations. Further studies need to be done to clarify the mechanisms involved in these interactions conducive to synergistic effects.

The more important finding of this work is the description of the synergistic effect of the selected combination of flavonoid or nonflavonoid mixtures and the efficiency of these mixtures in a meat model system. The advantage of such a synergistic effect is not only stronger activity but also reduction in the concentration employed. These results can be used to formulate natural preservative products for the food industry, with additional human health benefits inherent to polyphenols' properties.

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