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Antimicrobial Activity of Cinnamate-eugenol: Synergistic Potential, Evidence of Efflux Pumps and Amino Acid Effects

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ABSTRACT

Food safety is achieved mainly by the combination of preservation factors which results in additive or synergistic effect. Mixtures of cinnamate and eugenol were tested to determine bacteriostatic and bactericidal doses against *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, *Listeria monocytogenes and Staphylococcus aureus*. A combination of the two compounds was used to challenge these four bacteria, yielding a dose-dependent bactericidal or bacteriostatic effect. The synergistic effect was demonstrated in the three rod-shaped bacteria, *L. monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium, while for *S. aureus*, the mixture showed no synergistic activity. Notably, in the case of *S. aureus*, the interaction term was not significant ($p>0.05$), consistent with an additive but not synergistic effect between cinnamate and eugenol. The optimal combination of 1.35 mM cinnamate and 0.12 mM eugenol was bactericidal. Data showed that in response to this antimicrobial activity, these strains expressed efflux pumps. Additionally, the effect of three aminoacids on the bactericidal activity of these compounds was investigated. The bactericidal activity was interrupted by the presence of cysteine and proline, but not tyrosine. This effect was reversed when larger doses of eugenol and cinnamate were tested. We conclude that combining cinnamate and eugenol produces a synergistic bactericidal effect against *L. monocytogenes*, *E. coli* O157:H7 and *Salmonella* Typhimurium. This knowledge may be useful in the development of food products.

Key words: Natural-antimicrobials, *Salmonella* Typhimurium, *E. coli* O157:H7, *Listeria monocytogenes, Staphylococcus aureus*

INTRODUCTION

Foodborne illnesses resulting from consuming food contaminated with pathogenic bacteria have been of vital concern to public health (Lemya *et al*., 2006). *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7 account for the largest number of outbreaks, cases and deaths (Behravesh *et al*., 2011). *Listeria monocytogenes* may be found in different food products (Awaisheh, 2009), is the causative agent of histeriosis, and may cause a 20% mortality rate (Churchill *et al*., 2006; Brandt *et al*., 2010) *Staphylococcus aureus* causes a foodborne disease that is a frequent problem in the food sector of many countries (Biswas *et al*., 2011; Todd *et al*., 2008). Various extracts and essential oils from plants have shown antibacterial activity (Timothy *et al*.,

2008; El-Abed *et al.*, 2011; Mishra and Mishra, 2011; Sundaram *et al.*, 2011). Some of them are recognized as safe (GRASS) according to the FDA and can thus be used as antimicrobials in food to ensure its ultimate safety (Zaitoun *et al.*, 2012). Further, these natural products constitute an alternative source of antimicrobial chemicals (Babu *et al.*, 2011; Ponce *et al.*, 2011). There is interest in studying the properties of eugenol and cinnamic acid to potentially be used as natural food preservatives, as they have already demonstrated antimicrobial activity against bacteria and fungi (Acero-Ortega *et al.*, 2005; Perez-Sanchez *et al.*, 2007; Garcia-Garcia *et al.*, 2011).

A caveat of using active compounds in food products is limiting their effective antimicrobial concentrations as they may exceed acceptable levels in food sensory (Ponce *et al.*, 2011). To avoid high concentrations, spice-derived compounds, based on several bacterial cell targets, should be combined with antimicrobial agents as a cornerstone technique in hurdle technology (Hinton and Ingram, 2011; Gill and Holley, 2004).

The aim of this study was to discover a natural compound combination that produces a synergistic bactericidal effect. Eugenol and cinnamate were selected because of their chemical structures. Since their inhibitory mechanisms are different, present study proposed that their mixing would have potent activity against pathogenic bacteria. Moreover, the consequence of amino acids cysteine, proline and tyrosine in the efficacy of this antimicrobial combination was additionally investigated.

MATERIALS AND METHODS

Materials: *L. monocytogenes* ATCC 19115, *E. coli* O157:H7 ATCC 43895, *S. aureus* ATCC 25923 and *S.* Typhimurium ATCC 14028 were maintained in slant tubes containing trypticase soy agar (TSA, BD Bioxon, México) at 4°C until use. The strains were cultured monthly from a reserve to ensure characteristics.

Antimicrobial compounds: Eugenol was obtained from Merck (Germany); a 0.09% solution was prepared by dissolving the stock in absolute ethanol and distilled water (Nazer *et al.*, 2005). Cinnamic acid was obtained from Sigma® Chemical Company (USA); a 0.9% stock solution was prepared by dissolving the salt in an equimolar solution of sodium hydroxide.

Experimental design: In order to ascertain the effects of the individual mixture components on bacterial growth, a Design of Experiments (DOE) was performed to determine the statistical significance of the individual compounds and their possible interaction. This interaction could be synergistic, antagonistic, or null. A central composite design was performed with Statgraphics Plus® v 5.1 (Statpoint Technologies, Inc., Warrenton, VA, USA). In the present study, the effect of cinnamic acid and eugenol concentration was tested at the confidence limit of 95% (corresponding to a p-value of 0.05). The p-values that were less than 0.05 were regarded as statistically significant. A graphical display of the standardized effect ordered (estimated effect divided by its standard error) of each factor was provided in a Pareto chart which analyzed the magnitude and importance of each variable. The length of bars in the chart was proportional to the standardized effect. A factor was considered statistically significant if its standardized effect exceeded the threshold of 0.05. The mean is the average of all responses obtained for a particular level and was used for plotting the marginal means. The plot of marginal means offered an important understanding of the relationship between a quantitative response variable and independent variables indicating whether a parameter has either a decreasing or increasing role. A plot for one

independent variable (factor) with two levels was obtained by placing the levels of that factor on the abscissa axis and the total values of the dependent variable on the ordinate axis. The final plot was obtained by connecting the mean values of the dependent variable with two levels of the independent variable. The extent of the variable effect was determined from the line slope (Sadat-Shojai *et al.*, 2011).

Antimicrobial activity of the cinnamic-eugenol combination based on an experimental design. To study the growth inhibition of *E. coli* O157:H7, *L. monocytogenes*, *S. aureus* and *S.* Typhimurium, different concentrations of the active compounds were combined according to data provided by the experimental design. A series of tubes was made for each bacterium by adding 10 mL trypticase soy broth (TSB) with differing concentrations of cinnamate, sterilized at 121°C for 15 min. These were finalized by adding differing concentrations of eugenol. Each tube was inoculated with 50 μL of a 10^4 CFU mL^{-1} suspension of bacteria and incubated at 37°C for 24 h. The count of surviving bacteria was performed by the method described by Miles *et al.* (1938). A tube containing no antimicrobial agent was used as a control (Acero-Ortega *et al.*, 2005).

Determination of the minimum inhibitory concentration (MIC) and detection of efflux pumps: MIC of eugenol and cinnamate was determined using the broth microdilution method recommended by the Clinical and Laboratory Standard Institute (CLSI/NCCLS, 2005). Microplates with 96 round bottom wells were prepared by making dilutions of each antimicrobial compound in a final volume of 50 μL Finally, bacterial inoculums of 50 μL were added to each well. A blank was included with every plate. The microplates were incubated at 37°C for 18-24 h. Detection of the involvement of efflux pumps to detoxify the bacteria from the two antimicrobials was performed by the same method of microdilution in broth in the absence or presence of 50 mg mL^{-1} of the inhibitor phenyl-arginine-β-naphthylamide (PAβN, Sigma® Chemical Company, USA). Efflux pumps were considered the resistance mechanism when the MIC for each substrate was reduced by two dilutions in the presence of the inhibitor(Mesaros *et al.*, 2007).

Effect of antimicrobial activity by adding cysteine, proline, or tyrosine: The optimal bactericidal combination was selected and the inoculation procedure, explained previously, was used to test the inhibitory effect. One millimeter proline (Sigma-Aldrich® Co., USA), or cysteine (Merck, Germany), or tyrosine (Sigma® Chemical Company, USA) was added to each combination. The method of Miles *et al.* (1938) was followed to count bacterial colonies in AST plates incubated at 37°C for 24 h (Apostolidis *et al.*, 2008).

RESULTS AND DISCUSSION

Evidence of synergy: The ranking of standardized effects of independent variables and possible interactions for the responses are shown on the Pareto charts in Fig. 1. The bars extending over the vertical line indicate that the effects were statistically significant with a $p<0.05$. The synergistic effect was demonstrated in the three rod-shaped bacteria, *L. monocytogenes*, *E. coli* O157: H7 and *S.* Typhimurium, while for *S. aureus*, the mixture showed no synergistic activity (Table 1). Notably, in the case of *S. aureus*, the interaction term was not significant ($p>0.05$), consistent with an additive but not synergistic effect between cinnamate and eugenol. This was also highlighted in the Pareto chart for *S. aureus* (Fig. 1c).

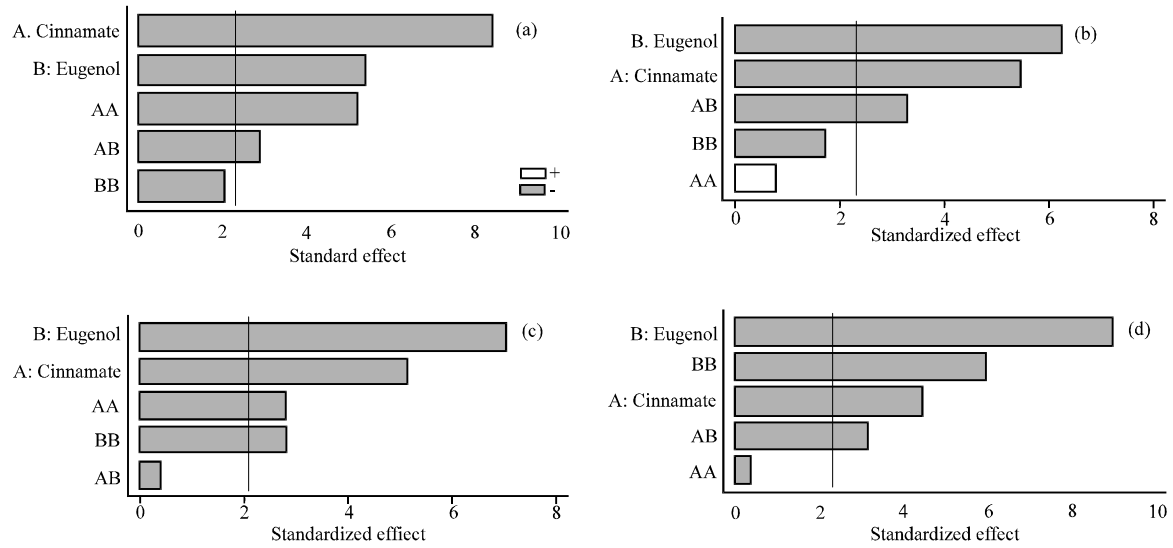

Fig. 1(a-d): Synergism of the combination cinnamate-eugenol on pathogenic bacteria on (a) *L. monocytogenes*, (b) *E. coli* O157: H7, (c) *S. aureus* and (d) *S.* Typhimurium, using Pareto charts with p = 0.05

Table 1: Quadratic equations of the response surfaces for the growth of four different bacteria in the presence of cinnamate and eugenol

Microorganism	Equation	R^2 (%)
L. monocytogenes	X = 8.014+22.824A-3.904B-63.764A^2-435.722AB	91.2
E. coli O157:H7	X = 8.536+0.973A-7.909B-583.541AB	85.7
S. aureus	X = 8.842+8.655A+38.423B-46.475A^2-4637.92B^2	86.9
S. Typhimurium	X = 8.276 -1.017A+191.744B-6166.6B^2-326.1AB	91.5

X: Bacterial growth in log (CFU mL^{-1}), A: Cinnamate concentration in % and B: Eugenol concentration in %

The plots of marginal means resulting from the statistical analysis are shown in Fig. 2. As highlighted in Fig. 2a and g, cinnamate was bactericidal against *L. monocytogenes* and *S. aureus,* while eugenol showed this effect against *E. coli*, *S. aureus* and S. Typhimurium at the concentrations indicated in the experiments (Fig. 2e, h and k). Slopes for eugenol graphs were higher than those for cinnamate, indicating a stronger effect. Eugenol concentrations were approximately ten times smaller than the cinnamate ones, logically explaining this difference. Figure 2c, f, i and l indicate a potential synergistic effect between both compounds. The slope of these graphs are greater than the slopes of the graphs for single compounds; they also indicate that a bactericidal effect can be obtained against the four bacteria using both compounds concurrently. Moreover, these trends are observed in the surface response shown in Fig. 3. The combination of 0.4% cinnamate and 0.04% eugenol showed bactericidal implications for all the tested microorganisms resulting in no survival under these conditions, a combination considered optimal. These results are modeled by quadratic equations which are shown in Table 2.

The combination of cinnamate and eugenol demonstrated a bactericidal effect in four pathogenic bacteria and importantly, showed a synergistic effect in three. Previously, De Oliveira *et al.* (2010) reported a synergistic effect similar to that observed in this study using the combination of the phenolic compound carvacrol with an organic acid. The bactericidal effect exerted by eugenol could be attributed to cell membrane targeting since it has been reported to

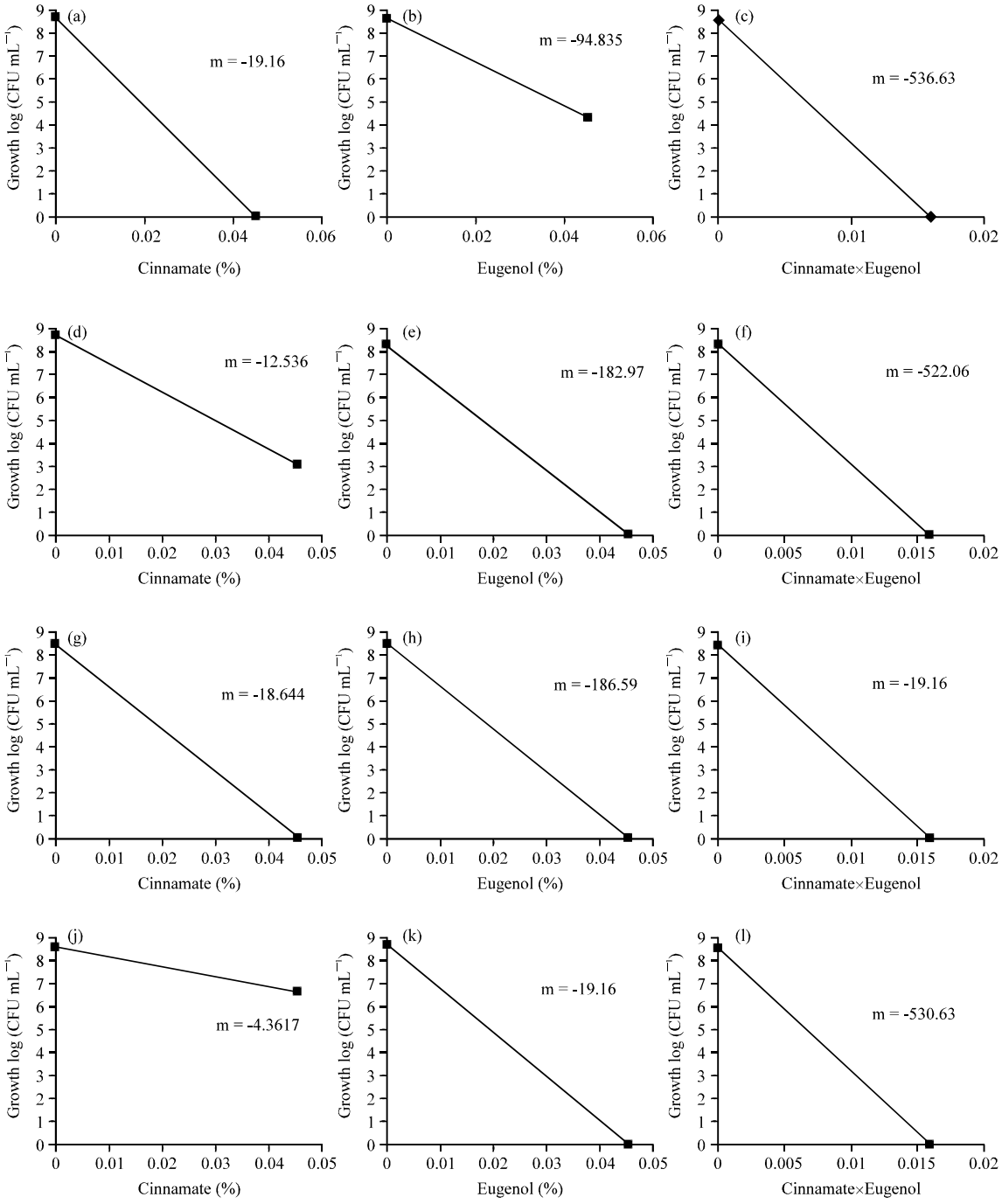

Fig. 2(a-l): Plots of marginal means for the growth of (a, b, c) *L. monocytogenes*, (d, e, f) *E. coli*, (g, h, i) *S. aureus* and (j, k, l) *S.* Typhimurium in the presence of cinnamate, eugenol and the binary mixture

change the composition of fatty acids (Di Pasqua *et al.*, 2006); Cinnamic acid may act on the sulfhydryl groups of enzymes involved in the production of ATP and glucose intake

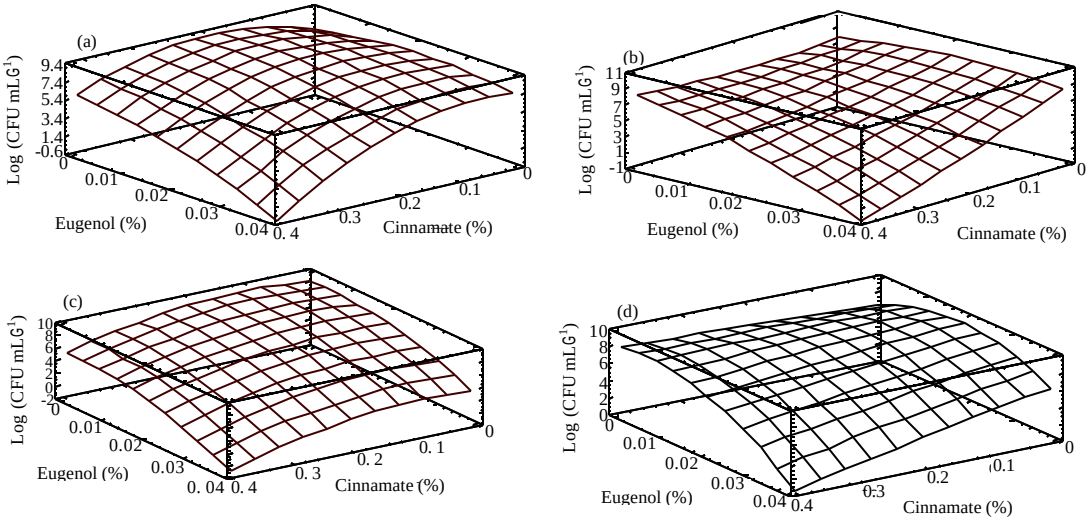

Fig. 3(a-d): Different growth behavior of pathogenic bacteria in the presence of eugenol-cinnamate combination, (a) *L. monocytogenes*, (b) *E. coli* O157:H7 (c) *S. aureus*, (d) *S.* Typhimurium

Table 2: Effect of combined cinnamate and eugenol on four pathogenic bacteria

			Bacteria count (Log. CFU mL^{-1}) after 24 h incubation			
*N	Cinnamate (mM)	Eugenol (mM)	*L. monocytogenes*	*E. coli* O157:H7	*S. aureus*	*S.* Typhimurium
Control	0.0	0	8.8	9.0	8.6	9.0
1	0.76	0.07	6.25	4.22	7.43	7.9
2	0.76	0.07	8.42	5.4	7.07	8.0
3	1.5	0.07	0.0	3.0	0.0	6.6
4	0.18	0.01	8.69	8.4	8.47	8.5
5	1.3	0.12	0.0	0.0	0.0	0.0
6	0.76	6.6×10^{-5}	8.35	7.65	8.38	8.3
7	0.18	0.12	7.33	7.84	3.17	6.5
8	5.04×10^{-4}	0.07	8.56	8.41	8.37	8.17
9	0.76	0.14	4.26	0.0	0.0	0.0
10	0.76	0.07	8.39	6.92	7.53	7.6
11	0.76	0.07	8.31	6.13	6.33	7.2
12	0.76	0.07	8.39	6.5	7.54	7.6
13	0.76	0.07	8.29	5.8	5.33	7.4
14	1.3	0.01	6.544	7.5	6.24	7.14

*N: Experiment number

(Kouassi and Shelef, 1998). Thus, the combination of these discrete actions could result in a synergistic effect, eliminating a high concentration of each if used independently to reach a bactericidal effect.

Determination of minimal inhibitory concentration (MIC): The minimal inhibitory concentration was evaluated for eugenol and cinnamate using a microplate following

Burt (2004).Thus, different concentrations of eugenol were presented against the four pathogenic bacteria and an MIC of 0.25% was determined.

We found similar results to those reported by Moreira *et al.* (2005)that tested the essential oil of cloves in *E. coli* and obtained an MIC of 0.25%. Other researchers have reported lower MIC results for *E. coli* (0.16%) using essential oil of cloves (Prabuseenivasan *et al.*, 2006), or by using 0.041% eugenol in a turbidity method (Palaniappan and Holley, 2010). Higher values of 0.4% (Oussalah *et al.*, 2007) and 0.318% (Di Pasqua *et al.*, 2006) were also reported. Other studies with eugenol and clove oil against *S. aureus* led to a determined MIC of 0.64% for eugenol and 2.5% for clove oil (Prabuseenivasan *et al.*, 2006; Joseph and Sujatha, 2011) and a sublethal concentration (MSC) of 0.212% (Di Pasqua *et al.*, 2006), while other authors reported lower MIC values of 0.041% (Palaniappan and Holley, 2010). Reports of eugenol and *L. monocytogenes* present data of a 0.4% MIC (Oussalah *et al.*, 2007).

We found an MIC value of 0.25% for *S.* Typhimurium which coincides with the four bacteria that were treated. Palaniappan and Holley (2010) reported an MIC of 0.041% which uses 99% pure eugenol in 5 mL of BHI. Oussalah *et al.* (2007) reported an MIC of 0.4% for *S.* Typhimurium. Our MIC results for the four pathogenic bacteria parallel the numbers obtained by Palaniappan and Holley (2010). Any differences can be attributed to media type or essential oil source.

The minimal inhibitory concentration obtained for cinnamate was 1% for the four bacterial strains. Similar results were reported by Acero-Ortega *et al.* (2005) that tested cinnamic acid against *L. monocytogenes* and reported that concentrations ranging from 0.5 to 1% generated a bacteriostatic effect. Cinnamic acid antilisterial activity was also reported by Kouassi and Shelef (1998), they reported no live cell recovery after exposure to 1% of cinnamic acid at pH 5.5 and a bacteriostatic effect at pH 7.

Evaluation of the presence of efflux pumps in pathogenic bacteria as a defense mechanism: To determine potential involvement of efflux pumps as a resistance mechanism to the action of natural antimicrobials, we designed a study of susceptibility to cinnamic acid and eugenol in the presence of an efflux pump inhibitor. Differences in MIC in the presence and absence of PAβN were more evident in *L. monocytogenes*, as it reduced the MIC of 0.81 mM to 0.05 mM. The other three bacterial species also exhibited this trend in MIC shift, as the presence of FAβN generated a decrease in the MIC of eugenol or cinnamate required to inhibit the bacteria in at least two dilutions (Table 3) (Mesaros *et al.*, 2007).

The target site of antibiotics or natural antimicrobials are typically the cell wall, cytoplasmic membrane, ribosomes, transcription and DNA replication, all of which are essential for bacterial growth (Fernandes *et al.*, 2003). Bacteria have efflux pumps that rid the organisms of compounds obtained during cellular processes, as well as antibiotics (Abdi-Ali *et al.*, 2007). These systems, when overexpressed, can help select strains resistant to several antibiotics (Ramalhete *et al.*, 2011; Maripandi and Al-Salamah, 2010). Therefore, by adding PAβN, we obtained decreases in the MIC

Table 3: Minimum inhibitory concentration of eugenol and cinnamate in the presence of efflux pumps inhibitors

Bacteria	Eugenol (mM)	Eugenol (mM)+inhibitor	Cinnamate (mM)	Cinnamate (mM)+inhibitor
E. coli O157:H7	0.81	0.101	6.74	0.840
L. monocytogenes	0.81	0.050	6.74	0.052
S. Typhimurium	0.81	0.202	6.74	0.840
S. aureus	0.81	0.202	6.74	1.680

for cinnamate and eugenol for all bacteria in question, indicating that these organisms have efflux systems to eliminate these toxic antimicrobials.

Modification of the bactericidal effect via a combination eugenol-cinnamate in the presence of three amino acids: Changes in bactericidal activity in the presence of the amino acids proline, cysteine and tyrosine were investigated (Table 4, 5). Proline is associated with energy production; its oxidation is catalyzed by proline dehydrogenase at the plasma membrane in prokaryotes. Previous studies on *Helicobacter pylori* (Lin *et al*., 2005) and *L. monocytogenes* (Apostolidis *et al*., 2008) showed that the addition of proline decreased the inhibitory effect of various phenolic phytochemicals and lactate against these bacteria. This was explained by considering the potential of small phenolics and lactate to act as proline analogs with their aromatic ring structures and lactate radical forming capability, respectively. In our study, the antibacterial effect of 0.19 mM eugenol and the combination of 0.12 mM eugenol and 1.35 mM cinnamate was significantly reduced by the addition of proline (Table 4, 5). This may allude to eugenol having a similar mechanism of action as the phenolic phytochemicals described before, specifically via inhibition of proline dehydrogenase. This is likely because of eugenol's nature as a substituted aromatic compound, similar to ferulic and caffeic acids (Apostolidis *et al*., 2008).

Addition of cysteine caused a reversal of the bactericidal effect (to bacteriostatic) when 0.19 mM eugenol and a combination of 0.12 mM eugenol and 1.35 mM cinnamate were used (Table 4, 5). The large reduction in efficiency of this combination may be due to the reaction

Table 4: Modification of bactericidal effect on *L. monocytogenes* and *S. aureus* of the combination cinnamate-eugenol in the presence of three aminoacids

	Bacteria connt (log CFU mL^{-1}) after 24 h incubation					
	L. monocytogenes			*S. aureus*		
Amino acid (mM)	Proline	Cysteine	Tyrosine	Proline	Cysteine	Tyrosine
Control	8.26	8.26	7.31	9.07	8.9	8.9
Control	7.98	7.98	8.9	9.04	8.7	9
Aminoacid 1						
Eugenol 0.19	0	0	0	0	0	0
Eugenol 0.19	4.38	4.38	0	5.9	4.9	0
Aminoacid 1						
Cinnamate 3.37	3.97	3.97	3.1	5.97	4.93	4.77
Cinnamate 3.37	3.95	3.95	3.5	5.4	4.97	4.54
Aminoacid 1						
Eugenol 0.19	0	0	0	0	0	0
Cinnamate 3.37						
Eugenol 0.19	0	0	0	0	0	0
Cinnamate 3.37						
Aminoacid 1						
Eugenol 0.12	0	0	0	0	0	0
Cinnamate 1.35						
Eugenol 0.12	3.17	3.17	0	5.3	5.2	0
Cinnamate 1.35						
Aminoacid 1						

Table 5: Modification of bactericidal effect on *E. coli* O157:H7 and *S.* Typhimurium of the combination cinnamate-eugenol in the presence of aminoacids

	Bacteria count (log CFU mL^{-1}) after 24 h incubation					
	E. coli O157:H7			*S.* Typhimurium		
Amino acid (mM)	Proline	Cysteine	Tyrosine	Proline	Cysteine	Tyrosine
Control	8.69	8.65	9.07	9.3	8.9	8.65
Control	8.59	8.9	8.9	9.1	8.8	9.06
Aminoacid 1						
Eugenol 0.19	0	0	0	0	0	0
Eugenol 0.19	5.05	5.23	0	5.92	5.61	0
Aminoacid 1						
Cinnamate 3.37	5.83	4.95	5.54	4.84	5.3	4.97
Cinnamate 3.37	5.17	5.3	4.1	5.29	5.12	4.90
Aminoacid 1						
Eugenol 0.19	0	0	0	0	0	0
Cinnamate 3.37						
Eugenol 0.19	0	0	0	0	0	0
Cinnamate 3.37						
Aminoacid 1						
Eugenol 0.12	0	0	0	0	0	0
Cinnamate 1.35						
Eugenol 0.12	5	4.01	0	5.76	5.87	0
Cinnamate 1.35						
Aminoacid 1						

of cysteine and cinnamic acid, resulting in neturalization of the antibacterial effect of this phenylpropanoid. This probable mechanism of action has been described by Kouassi and Shelef (1998). Cysteine is used as an additive in some foods which may interfere with antimicrobial activity. Effects of 1 mM proline and 1 mM cysteine were overcome, however, when eugenol and cinnamate concentrations were increased from 0.12 to 0.19 mM and from 1.35 to 3.37 mM, respectively. Lastly, bactericidal activity of the eugenol-cinnamate combination in the presence of tyrosine was also tested. Unlike proline and cysteine, tyrosine did not alter the antimicrobial effect, potentially a result of its side chain phenolic structure (Table 4, 5). The effect of cysteine and proline was reversed when larger doses of eugenol (0.19 mM) and cinnamate (3.37 mM) were tested.

CONCLUSIONS

The synergistic bactericidal effect of the natural antimicrobials cinnamate and eugenol was determined on four pathogenic bacteria and this may be useful in food development. We concluded that efflux pumps in the bacteria mediate the resistance level to these antimicrobial agents. We also observed that the presence of cysteine and proline in the growth media caused shift from bactericidal to bacteriostatic activity when these amino acids were used as supplements to the two compounds. This effect was reversed when larger doses of eugenol and cinnamate were tested. So we conclude that the survival of these four pathogenic bacteria may be prevented by the use of mixtures of cinnamate and eugenol, since a dose-dependent bactericidal or bacteriostatic effect was obtained in this work. However, future research should be performed in the specific food product to be developed.

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