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Antibacterial and anti-staphylococcal enterotoxin activities of phenolic compounds

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ABSTRACT

Food safety has been an established research field for many decades. This report describes the antibacterial and anti-staphylococcal enterotoxin properties of major phenolic compounds found in essential oils (cinnamaldehyde, citronellol, eugenol, geraniol and terpineol). The determination of minimum inhibitory concentrations (MIC) against Gram-negative and Gram-positive strains, including methicillin-resistant *Staphylococcus aureus* (MRSA) and enterotoxin-producing *S. aureus* (enterotoxins A, B, C and D) were evaluated. Cinnamaldehyde displayed MIC values ranging from 100 to 400 μg/mL against Gram- positive and Gram-negative bacteria, while the other four compounds showed similar values only against Gram-negative bacteria. For Gram-positive strains, MIC values ranged from approximately 800–1750 μg/mL. Interactions between the compounds and antibacterial drugs were evaluated by disc diffusion and time-kill curve assays against MRSA. Combinations of phenolic compounds that included gentamicin showed the greatest synergistic effect. In vitro treatments with subinhibitory concentrations of phenolic compounds resulted in a decreased production of enterotoxins B and C (SEB and SEC). Transmission electron microscopy was performed to evaluate mechanisms of action for cinnamaldehyde and geraniol against *E. coli* and MRSA. Cells treated with compounds showed complete loss of membrane integrity, separation of the cytoplasmic membrane from the cell wall, cytoplasmic content leakage and cytoplasmic polarization. Thereby, this work showed in vitro potential of using combinations of phenolic compounds and antimicrobial drugs against *S. aureus* and the virulence of *S. aureus* enterotoxin producers.

Industrial relevance: Antimicrobial compounds derived from plants are a focus of renewed interest as potential substitutes for artificial food preservatives. In our study subinhibitory concentrations of phenolic compounds had a significant effect on the quantity of enterotoxins produced by *Staphylococcus aureus* and inhibited the growth of *Escherichia coli*, *Salmonella* Enteritidis and other bacteria in microbiological media.

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1. Introduction

Foodborne diseases are a major concern worldwide. Approximately 250 different types of foodborne diseases are described and bacteria are responsible for 2/3 of the outbreaks (Le Loir, Baron, & Gautier, 2003). Staphylococcal food poisoning is one of the most common foodborne illnesses and results from ingestion of preformed enterotoxin in food, produced and released by strains of coagulase-positive staphylococci, particularly *S. aureus* (Hennekinne et al., 2009). Thereby, the impact of *S. aureus* resistance on clinical outcomes stimulates the search for new antimicrobial drugs aiming the treatment of foodborne diseases caused by these bacteria.

Volatiles compounds from plant sources could provide alternative therapies since many possess excellent therapeutic properties and do not cause bacterial resistance (Mitić-Ćulafić, Vuković-Gačić, Knežević-Vukčević, Stanković, & Simić, 2005). Thus, a new alternative strategy against *S. aureus* is to target bacterial virulence factors (e.g. hemolysins, enterotoxins, adhesins) (Song et al., 2009) to minimize the effects of the presence of such bacteria in food.

Antibacterial drugs or antibiotics have been used to treat infectious diseases, however, bacteria have responded by increasing the level and complexity of their mechanisms of resistance (Tenover, 2006). Multidrug resistance is a result of antibiotic misuse and selection pressure (Tohidpour, Sattari, Omidbaigi, Yadegar, & Nazemi, 2010). Therefore, the use of broad-spectrum antibiotics, rather than narrow-spectrum drugs, increases antibiotic resistance (Barbosa & Levy, 2000). A wide range of bacteria can be listed as resistant to several antibacterial drugs in hospital environments and community. Staphylococci are of

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great epidemiological concern for contemporary invasive medicine and *S. aureus* has the strongest virulence potential among all staphylococcal species. Since 1960 methicillin-resistant *S. aureus* (MRSA) has become one key pathogen responsible for healthcare-associated infections, which are usually difficult to treat (Bigos & Denys, 2008).

In this context, essential oils (EOs) have been screened for their potential use as alternatives to drugs, for treatment in numerous infectious processes, as well as natural food preservatives (Schuenzel & Harrison, 2002; Tepe, Daferera, Sökmen, Polissiou, & Sökmen, 2004). EOs can comprise more than 70 compounds, principally polyphenols, terpenes, monoterpenes and sesquiterpenes, and some of these compounds may represent more than 85% of the total content in the EOs (Cowan, 1999; Nascimento et al., 2007). EOs are considered the most important antimicrobial agents present in plants and have been studied for their antibacterial (Mourey & Canillac, 2002; Oussalah, Caillet, Saucier & Lacroix, 2007; Silva, Barbosa, Seito & Fernandes Júnior, 2012; Barbosa et al., 2015; Moritz, Barbosa, Saeki & Fernandes Júnior, 2015), antiparasitic (George, Smith, Shiel, Sparagano, & Guy, 2009), antifungal (Fitzgerald, Stratford, & Narbad, 2003), antiviral (Astani, Reichling, & Schnitzler, 2011), insecticidal (Enan, 2001; Kim, Roh, Kim, Lee, & Ahn, 2003), antioxidant (Brenes & Roura, 2010) and anti-inflammatory properties (Andrade, Conti, Santiago, Fernandes Júnior, & Sforcin, 2014; Burt, 2004; Delamare, Moschen-Pistorello, Artico, Atti-Serafini, & Echeverrigaray, 2007; Kordali et al., 2005).

Considering the importance of *S. aureus* in food and medical microbiology, several studies have been conducted to enhance the action of antimicrobials drugs. Indeed, synergism between natural antimicrobials and conventional antimicrobial drugs has been reported for *S. aureus* (Fernandes Júnior, Balestrin, Betoni, Orsi, Cunha and Montelli, 2005; Mantovani, Rall, Batalha, Fernandes, & Fernandes Júnior, 2008; Zago, Ushimaru, Barbosa, & Fernandes Junior, 2009; Silva et al., 2013; Qian et al., 2015). Therefore, in this study, the antibacterial and anti-enterotoxin properties of five phenolic compounds found in EOs were evaluated against *S. aureus* strains, with emphasis on MRSA.

2. Materials and methods

2.1. Bacterial strains and growth culture conditions

The staphylococcal strains that were used included: MRSA (ATCC 33591), methicillin-sensitive *S. aureus* (MSSA) (ATCC 25923) and enterotoxigenic *S. aureus* strains (ATCC 13565, ATCC 14558, ATCC 19095 and ATCC 23235). The Gram-negative strains used were: *Salmonella* Enteritidis (ATCC 13076), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* serotype O157:H7 (ATCC 43895). MRSA and MSSA strains also were isolated from human specimens at the University Hospital of Botucatu Medical School, São Paulo, Brazil (Universidade Estadual Paulista "Júlio de Mesquita Filho") and enterotoxigenic *S. aureus* strains were isolated from food samples. Stock cultures were frozen at −80°C in culture medium plus glycerol. For experimental use, strains were maintained in brain heart infusion (BHI) (Difco, Becton Dickinson and Company, Franklin Lakes, NJ) agar slopes at 4°C. The use of bacterial strains from human specimens was approved by our Institutional Committee on Human Research (document 4375-2012), according to the ethical principles for medical research involving human subjects.

2.2. Antimicrobial compounds

Five phenolic compounds, normally found in EOs, were tested: geraniol (Ger) (*Cymbopogon martinii)*, cinnamaldehyde (Cin) (*Cinnamomum* sp.), β-citronellol (Cit) (*Cymbopogon* sp.), eugenol (Eug) (*Syzygium aromaticum*) and terpineol (Ter) (*Melaleuca alternifolia*). All compounds used were obtained from Sigma-Aldrich (St. Louis, MO). The working solutions were prepared in a 1:1 ratio of dimethyl sulfoxide (Sigma-Aldrich) and sterile water to standardize the compounds to 1000 μg/mL.

2.3. Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) of the compounds were measured by resazurin microtiter assay (REMA) (Martin, Camacho, Portaels, & Palomino, 2003) with modifications for MRSA, MSSA, *S. aureus* enterotoxin A producer (SEA), *S.* Enteritidis, *P. aeruginosa* and *E. coli*. Seven clinical isolates and one standard strain (ATCC) of each species were used. Various concentrations of the compounds (from 100 to 1800 μg/mL) were placed in 96-well sterile microtiter plates containing Mueller-Hinton Broth (MHB) (Difco, Becton Dickinson and Company, Franklin Lakes, NJ). Inocula were prepared by diluting overnight cultures (37°C/18–24h) in saline solution until a turbidity equivalent to 0.5 McFarland standard (approximately 10^8 colony-forming units (CFU)/mL) was obtained. Aliquots of 100 μL were then added to each well, resulting in a final volume of 200 μL and approximately 10^5 CFU/mL per well. The negative control consisted of 100 μL of MHB and 100 μL of cell suspension. Plates were incubated at 37°C/18–24h, prior to enumeration. MIC was defined as the lowest concentration of the compound that inhibited the visible microorganism growth after incubation. Fifty μL of resazurin (0.01%) was added to each well and a color change from blue to pink was indicative of viable bacterial cells. The corresponding values of $MIC_{90\%}$ for each group of microorganisms were calculated. All experiments were performed in duplicate.

2.4. Synergism assays according to the Kirby-Bauer protocol

Assays were performed using the Kirby-Bauer disc diffusion method adapted by Stepanovic, Antic, Dakic & Svabic-Vlahovic (2003) in cation-adjusted Mueller-Hinton II agar (MHA) (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ), with the addition of 0.5% Tween 80. Amounts corresponding to 25% MIC of each compound were mixed individually with the medium and poured into Petri dishes. Bacterial density was adjusted to 0.5 McFarland standard. Using sterile cotton swabs, MRSA and MSSA were inoculated on the medium. Discs of oxacillin (Oxa, 1 μg), gentamicin (Gen, 10 μg), erythromycin (Ery, 15 μg), sulfazotrin (Sul, 25 μg), vancomycin (Van, 30 μg), penicillin G (Pen, 10 U) levofloxacin (Lev, 5 μg), tetracycline (Tet, 30 μg) and linezolid (Lin, 30 μg) were placed on the surface of inoculated MHA. Culture medium prepared without the compounds was used as the control. The plates were incubated at 37°C/24h. After incubation, the zone of inhibition formed was measured in millimeters (mm), and synergy was considered positive when the halo of the culture media containing compounds showed an increase in size compared to the control.

2.5. Time-kill assay

An assay was performed to identify synergistic interactions among the compounds and antimicrobial drugs, according to the National Committee for Clinical Laboratory Standard guidelines (Clinical and Laboratory Standards Institute, CLSI, 2012). MRSA strain (ATCC 33591) (10^5 CFU/mL) was incubated with either the compounds alone at 25% MIC or in combination with antimicrobial drugs in MHB (Difco, Becton Dickinson and Company, Franklin Lakes, NJ) (Mahon & Manuselis, 1995). Culture media prepared without compounds and/or antimicrobial drugs were treated similarly as the controls. Aliquots were taken from each tube and diluted serially using sterile saline at intervals of 0, 2, 4, 6, 8 and 24h, and inoculated onto agar plates, which were then incubated at 37°C/24h. Subsequently colonies were enumerated and expressed as CFU/mL (Hamoud, Sporer, Reichling, & Wink, 2012). After 18–24h incubation, an antimicrobial agent was considered: bactericidal, when it caused a reduction $\geq 3 \log_{10}$ CFU/mL; and bacteriostatic when it caused a reduction in the bacterial count of $< 3 \log_{10}$ CFU/mL. A combination was considered synergistic when it caused a reduction

Table 1.
Minimum inhibitory concentrations (MIC - μg/mL) and subinhibitory concentrations (60 and 80% of the MIC) of antimicrobial compounds against *S. aureus* strains producing enterotoxins A (ATCC 13565 - SEA), B (ATCC 14558 - SEB), C (ATCC 19095 - SEC) and D (ATCC 23235 - SED).

MIC (μg/mL)															
Strain	Eugenol			Terpineol			Cinnamaldehyde			Citronellol			Geraniol		
	MIC	60%	80%	MIC	60%	80%	MIC	60%	80%	MIC	60%	80%	MIC	60%	80%
13565	1200	720	960	1100	660	880	200	120	160	900	540	720	800	480	640
14558	900	540	720	700	420	560	500	300	400	1100	660	880	500	300	400
19095	900	540	720	1300	780	1040	300	180	240	1000	600	800	900	540	720
23235	1100	660	880	900	540	720	500	300	400	1200	720	960	1000	600	800

≥2 $\log_{10}$ CFU/mL after 18–24 h incubation (Rochon-Edouard, Pestel-Caron, Lemeland, & Caron, 2000).

2.6. Influence of phenolic compounds on staphylococcal enterotoxin production

The effects of the phenolic compounds on enterotoxin production were evaluated. *S. aureus* strains producing enterotoxins A (SEA), B (SEB), C (SEC) and D (SED) (ATCC 13565, ATCC 14558, ATCC 19095 and ATCC 23235, respectively) were incubated in tryptic soy broth (TSB) (Difco, Becton Dickinson and Company, Franklin Lakes, NJ) (37°C/24h) in the presence of 60% and 80% MIC of each compound (Table 1), obtained using the REMA method (Section 2.3). Controls containing TSB and either compound or bacteria only were treated similarly. After incubation, each sample was centrifuged (9000g, 4°C, 30 min), and the supernatants were examined for enterotoxin production, using a reversed passive latex agglutination kit (SET-RPLA) (Oxoid, Japan). Assays were conducted, according to manufacturer's instructions. Serial dilutions of the supernatants were performed to determine the concentration of enterotoxin produced. Latex controls showed no interference in enterotoxin detection by all compounds tested.

2.7. Effect of cinnamaldehyde and geraniol on bacterial morphology

To elucidate the mechanism of action of Cin and Ger against bacterial cells, transmission electron microscopy (TEM) was carried out, using a modified method of Moosavy et al. (2008). Overnight cultures of MRSA (ATCC 33591) and *E. coli* (ATCC 43895) strains (BHI at 37°C/24h) were treated with MIC and 2×MIC of each compound. Contact between the bacteria and phenolic compounds was maintained for 2h. Subsequently, 3 mL of Karnowski solution was added and the samples then centrifuged (1500g/4°C/20 min). The supernatants were discarded and Karnowski solution was added to cover the pellet. The samples were then incubated for 2h at room temperature and maintained at 4°C for 24h. Postfixation was performed using osmium tetroxide solution (1%) in 0.1M phosphate buffer (pH 7.3) for 2h, followed by material dehydration in acetone and impregnation into araldite blocks to obtain ultrafine sections. The sections were subsequently stained with uranyl acetate and lead citrate. Finally, samples were analyzed and photographed (digital imaging) with a transmission electron microscope (CM 100, Philips) operated at 80 kV.

2.8. Statistical analysis

Data obtained from the REMA and time-kill assays were analyzed by Kruskal-Wallis one-way analysis of variance (ANOVA) and Student-Newman-Keuls method (multiple comparisons) with a significance level of 5%. Disc diffusion results were compared using Mann-Whitney test ($p < 0.05$), and enterotoxin production using non-parametric Kruskal-Wallis test with scores relating to subinhibitory concentrations of the compounds used to compare between treatments and controls.

3. Results and discussion

3.1. Determination of minimum inhibitory concentrations

The $MIC_{90\%}$ values of the five phenolic compounds evaluated are shown in Table 2. Cin revealed the greatest antibacterial activity among all the bacteria tested, with $MIC_{90\%}$ ranging from 100 to 400 μg/mL. Moreover, previous studies have reported its antimicrobial (Kaskatepe et al., 2016; Ooi et al., 2006; Zhu, Du, Fox, & Zhu, 2016), antioxidant and anticancer properties (Li, Kong, & Wu, 2013; Thomas & Kuruvilla, 2012; Wang, Wang, & Yang, 2009). In particular, Budri et al. (2015) reported the effect of cinnamon (*Cinnamomum zeylanicum*) EO and its major phenolic compound (Cin) against *S. aureus* biofilms on various surfaces, with significant inhibition on polystyrene and stainless steel surfaces. In the current study, Gram-negative bacteria were more susceptible to the phenolic compounds investigated than Gram-positive bacteria. The $MIC_{90\%}$ ranged from 200 to 1750 μg/mL against Gram-positive bacteria. Among the bacterial strains investigated, MRSA was most resistant to the compounds, while *P. aeruginosa* strains were most susceptible. Other compounds showed also similar values against the others Gram-negative bacteria.

Eug has an excellent bactericidal activity against a wide range of organisms, including *E. coli*, *P. aeruginosa* (Walsh et al., 2003) and *Listeria monocytogenes* (Filgueiras & Vanetti, 2006). The principal antibacterial mechanism of Eug is its disruption of the bacterial cytoplasmic membrane, which increases its non-specific permeability (Li et al., 2015). Moreover, the hydrophobic nature of Eug enables it to penetrate into the outer lipopolysaccharide membrane of Gram-negative bacteria, altering the cell wall structure, and subsequently resulting in the leakage of intracellular constituents (Burt, 2004).

3.2. Synergism

Synergism between the phenolic compounds and antibacterial drugs could assist in the elimination of MRSA and other pathogenic bacteria. The compound and antimicrobial drug combinations used against MRSA showed a synergistic effect between Eug/Gen, Eug/Van, Eug/Lin, Cit/Gen, Cit/Van, Cit/Lev Cit/Lin and Ter/Lin, whereas there were no synergistic interactions with Ger (Table 2).

Table 2.
Minimum inhibitory concentrations ($MIC_{90\%}$) of antimicrobial compounds against bacterial strains.

	$MIC_{90\%}$ (μg/mL)				
Bacteria	Eugenol	Terpineol	Cinnamaldehyde	Citronellol	Geraniol
MSSA	1300^{a}	1550^{b}	300^{c}	1500^{d}	800^{e}
MRSA	1600^{a}	1700^{b}	400^{c}	1750^{b}	1450^{a}
Enterotoxigenic *S. aureus*	1350^{a}	1000^{b}	200^{c}	900^{d}	900^{d}
S. Enteritidis	250^{a}	250^{a}	100^{b}	200^{c}	150^{c}
P. aeruginosa	200^{a}	200^{b}	150^{a}	200^{b}	200^{b}
E. coli	250^{a}	250^{a}	100^{b}	250^{a}	200^{c}

Different letters in the same row indicate a significant difference at $p \le 0.05$.

Table 3.
Synergism between antibacterial drugs and antimicrobial compounds against methicillin-resistant *S. aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA) isolated from human specimens by Kirby&Bauer protocol (Stepanovic et al., 2003).

	Eugenol		Terpineol		Citronellol		Cinnamaldehyde		Geraniol	
	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA	MRSA	MSSA
Oxa	−	−	−	−	−	−	−	−	−	−
Gen	+	+	+	+	+	+	−	+	−	−
Ery	−	−	−	−	−	−	−	+	−	−
Sul	−	−	−	−	−	+	−	+	−	−
Van	+	−	−	−	+	+	−	+	−	−
Pen G	−	−	−	−	−	−	−	−	−	−
Lev	−	−	+	−	+	+	−	+	−	−
Tet	−	−	−	−	−	+	−	+	−	−
Lin	+	−	+	−	+	+	−	+	−	−

(+) means positive synergism, i.e., statistical difference in halo size between control and treatment groups. Eugenol (Eug), terpineol (Ter), citronellol (Cit), cinnamaldehyde (Cin), geraniol (Ger), oxacillin (Oxa), gentamicin (Gen); erythromycin (Ery), sulfazotrin (Sul), vancomycin (Van); penicillin G (Pen G), levofloxacin (Lev), tetracycline (Tet), linezolid (Lin).

Compared to the disc diffusion assay data, which showed numerous synergistic interactions between the antimicrobial phenolics and antibacterial drugs (10 synergistic interactions against MRSA strains - Table 3) only four combinations were confirmed by time-kill curve (Fig. 1). The time-kill curve assay is a more refined and accurate method to study interactions between antimicrobial substances. The antimicrobial compounds that showed the most promising results in the time-kill assay (Eug, Cit and Ter) were chosen for further investigation. As observed in Fig. 1, all three combinations with Gen showed a synergistic and bactericidal effect. For the first 2h of MRSA contact with Eug and drugs, the Eug/Gen combination showed a greater decrease in colony count compared to the MIC of Eug and Gen, alone. Similar results were observed for Gen/Cit and Gen/Ter, although the number of colonies remained higher than that of Eug/Gen, for up to 6 h of contact. Eug/Van, Eug/Lin and Cit/Lev showed bacteriostatic effects, whereas only Cit/Lev was synergistic.

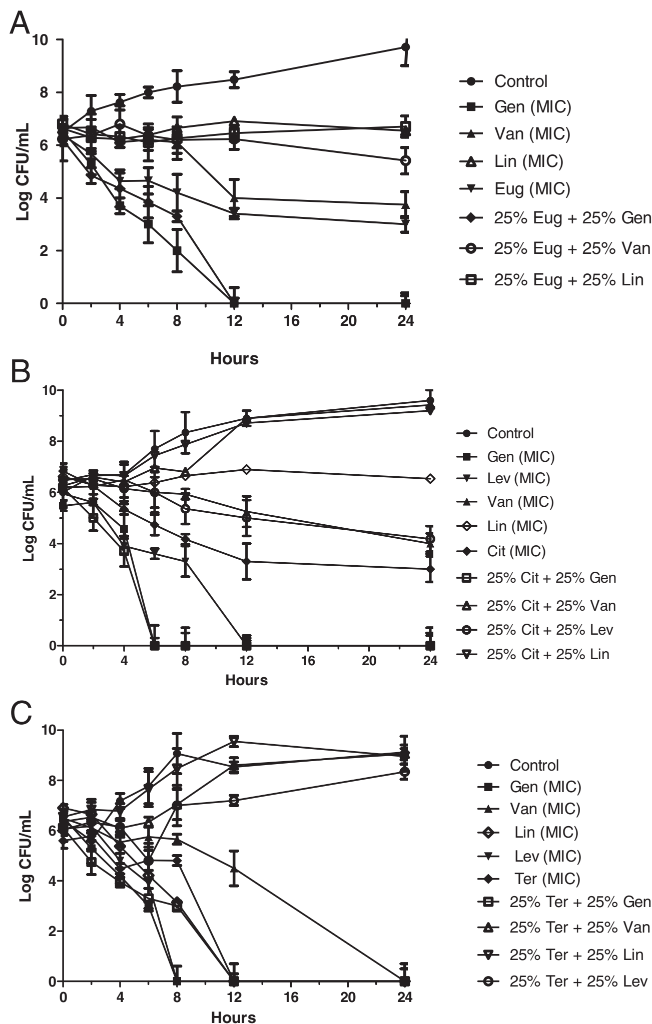

Fig. 1. Time-kill curve of compounds and antibacterial drugs against MRSA (ATCC 33591). (A) Time-kill curves of MIC of eugenol (Eug), gentamicin (Gen), vancomycin (Van) and linezolid (Lin), alone, and in combination with 25% of MIC of each substance; (B) Time-kill curves of MIC of citronellol (Cit), gentamicin (Gen), vancomycin (Van), levofloxacin (Lev) and linezolid (Lin), alone, and in combination with 25% of MIC of each substance; (C) Time-kill curves of MIC of terpineol (Ter), gentamicin (Gen), vancomycin (Van), levofloxacin (Lev) and linezolid (Lin), alone, and in combination with 25% of MIC of each compound.

Alves et al. (2016) demonstrated a synergistic bactericidal effect of nisin when combined with phenolic compounds (carvacrol, thymol, Cin and Eug) against *S. aureus* and *L. monocytogenes*. Palaniappan and Holley (2010) found that thymol, carvacrol and Cin acted synergistically with Pen, ampicillin and bacitracin against *S. aureus* resistant to these antibiotics. The exact mechanism for the decreased antibiotic resistance by natural compounds is unknown but has been attributed to a structural change in the bacteria. Indeed, the natural compounds may facilitate penetration of the drug through the outer layers of the bacterial cell wall, act to block the inhibitory effects of protective enzymes, or interfere with single or multiple metabolic targets of the antibiotic (Hemaiswarya, Kruthiventi, & Doble, 2008). Zhao, Hu, Hara, and Shimamura (2002) found that the green tea polyphenol, epigallocatechin gallate (EGCg), inhibited the activity of penicillinase produced by *S. aureus*, restoring the activity of Pen. Similar results were reported by Hu et al. (2002), showing that EGCg synergistically enhanced the activity of carbapenems against MRSA. Shiota et al. (2000) reported that tellimagrandin I, a polyphenol extracted from red rose (*Rosa canina* L.) petal, substantially decreased the MIC of β-lactam antibiotics against MRSA. Similarly, corilagin, an active compound extracted from *Aretostaphylos uva-ursi*, decreased the MIC of β-lactam antibiotics (oxacillin and cefmetazole) against MRSA (Shimizu et al., 2001).

3.3. Influence of phenolic compounds on staphylococcal enterotoxin production

Subinhibitory concentrations of the phenolic compounds had a significant effect on the total quantity of enterotoxin produced (Tables 4 to 7). For example, Eug, Cit and Ger ($p < 0.001$) had a significant influence on SEA production (Table 4). Ter and Eug completely inhibited SEB production at both concentrations tested (Table 5). SEC production was the most sensitive to the phenolics (Table 6), while the opposite effect was observed for SED production, which was not influenced by any of the compounds (Table 7).

Extracellular protein production can be modulated by subinhibitory concentrations of EOs (e.g. oils of bay, cinnamon and clove) and,

Table 4.
Production of staphylococcal enterotoxin type A (SEA) by *S. aureus* ATCC 13565 cultured with subinhibitory concentrations of phenolic compounds.

		Eugenol		Terpineol		Cinnamaldehyde		Citronellol		Geraniol	
Dilution	Control	60%	80%	60%	80%	60%	80%	60%	80%	60%	80%
1:1	+++	++*	++*	+++	+++	+++	+++	++*	++*	++*	++*
1:10	+++	++*	++*	+++	+++	+++	+++	++*	++*	++*	++*
1:100	+++	++*	++*	+++	++	+++	+++	++*	++*	++*	++*
1:1000	+++	++*	+*	++	+	+++	+++	++*	+*	+*	+*

* $p < 0.001$ significant difference from control.

Table 5.
Production of staphylococcal enterotoxin type B (SEB) by *S. aureus* ATCC 14558 cultured with subinhibitory concentrations of phenolic compounds.

		Eugenol		Terpineol		Cinnamaldehyde		Citronellol		Geraniol	
Dilution	Control	60%	80%	60%	80%	60%	80%	60%	80%	60%	80%
1:1	+++	−*	−*	−*	−*	+++	+++	+++	+++	+++	+++
1:10	+++	−*	−*	−*	−*	+++	+++	+++	+++	+++	+++
1:100	+++	−*	−*	−*	−*	+++	+++	+++	+++	+++	+++
1:1000	+++	−*	−*	−*	−*	+++	+++	++	++	+++	+++

* $p < 0.001$ significant difference from control.

consequently, influence enterotoxin production (Smith-Palmer, Stewart, & Fyfe, 2004; Tranter, Tassou, & Nychas, 1993). Treatment with subinhibitory concentrations of tea tree oil (Ter, a major compound) led to a dose-dependent decrease in SEA and SEB production, and a downregulation of exoprotein in *S. aureus* (Shi et al., 2016). The tea tree oil inhibited SEB production more effectively than SEA production (Shi et al., 2016). This trend was similar to that obtained in the current study. Qiu et al. (2010a) reported a dose-dependent suppression of SEA and SEB secretion by licochalcone A.

This phenotypic change possibly is due to the secretion of offside-related proteins to physical changes in bacterial plasmatic membrane caused by compounds (Nostro, Cannatelli, Musolino, Procopio, & Alonzo, 2002; Shah, Stapleton, & Taylor, 2008). Physical alterations in the plasma membrane can interfere with transmembrane transport processes, resulting in changes to protein secretions associated with *S. aureus* virulence (Ikigai, Toda, Okubo, Hara, & Shimamura, 1990). Moreover, when antibiotics are used in subinhibitory concentrations, which have little or no effect on overall microbial growth, bacterial enzyme expression can still be affected (Souza, de Barros, de Oliveira, & da Conceição, 2010). For example, protein synthesis inhibitors, such as linezolide, decreased the expression of *S. aureus* virulence factors, including SEA and SEB at subinhibitory concentrations (Bernardo et al., 2004). Some studies with phenolic compounds have shown suppression of protein secretions, such as α- and γ-hemolysin, DNAse, lipase, coagulase and toxic shock syndrome toxin-1 (TSST-1) (Gemmell, 1995; Ohlsen et al., 1998; Shah et al., 2008).

The effects on enterotoxin production by phenolic compounds could have occurred at a number of points, including translation, transcription, export from the cell or direct inactivation of the toxin. Furthermore, EO compounds have a natural image and are more readily accepted by consumers than synthetic antimicrobial agents (Smith-Palmer et al., 2004).

The clinical performance of antibiotics used to treat *S. aureus* infections depends not only on their bacteriostatic or bactericidal effects but also on their ability to prevent the release of virulence factors by dying or stressed bacteria (Bernardo et al., 2004). Many genes encoding virulence factors are coordinately regulated in response to a variety of intracellular and extracellular signals. It has been shown that subinhibitory concentrations of antibiotics may interfere with the translation of one or more regulatory gene products in *S. aureus*, which in turn affects transcription of exoprotein-encoding genes. The expression levels of TSST-1, SEB and α-hemolysin are positively controlled by *agr* (accessory gene regulator), a locus that controls the expression of most of the exoprotein genes (Arvidson & Tegmark, 2001; Peng, Novick,

Table 6.
Production of staphylococcal enterotoxin type C (SEC) by *S. aureus* ATCC 19095 cultured with subinhibitory concentrations of phenolic compounds.

		Eugenol		Terpineol		Cinnamaldehyde		Citronellol		Geraniol	
Dilution	Control	60%	80%	60%	80%	60%	80%	60%	80%	60%	80%
1:1	+++	+*	−*	+*	−*	+*	−*	+*	−*	−*	−*
1:10	+++	−*	−*	−*	−*	+*	−*	−*	−*	−*	−*
1:100	+++	−*	−*	−*	−*	−*	−*	−*	−*	−*	−*
1:1000	+++	−*	−*	−*	−*	−*	−*	−*	−*	−*	−*

* $p < 0.001$ significant difference from control.

Table 7.
Production of staphylococcal enterotoxin type D (SED) by *S. aureus* and ATCC 23235 cultured with subinhibitory concentrations of phenolic compounds.

		Eugenol		Terpineol		Cinnamaldehyde		Citronellol		Geraniol	
Dilution	Control	60%	80%	60%	80%	60%	80%	60%	80%	60%	80%
1:1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1:10	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1:100	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
1:1000	+++	++*	++	+++	+++	+++	+++	++	++	+++	+++

* $p < 0.001$ significant difference from control.

Kreiswirth, Kornblum, & Schlievert, 1988). However, *agr* has no effect on SEA expression (Novick, 2003). Therefore, it is possible that the influence of subinhibitory concentrations of compounds on SEB and SEC depend on compound-induced inhibition of the *agr* system (Qiu et al., 2010b). Nevertheless, the mechanisms by which *S. aureus* regulates virulence gene expression are extremely complicated. This regulation involves an interactive, hierarchical regulatory cascade among the *agr*, *sar* and other regulatory gene products (Chan & Foster, 1998).

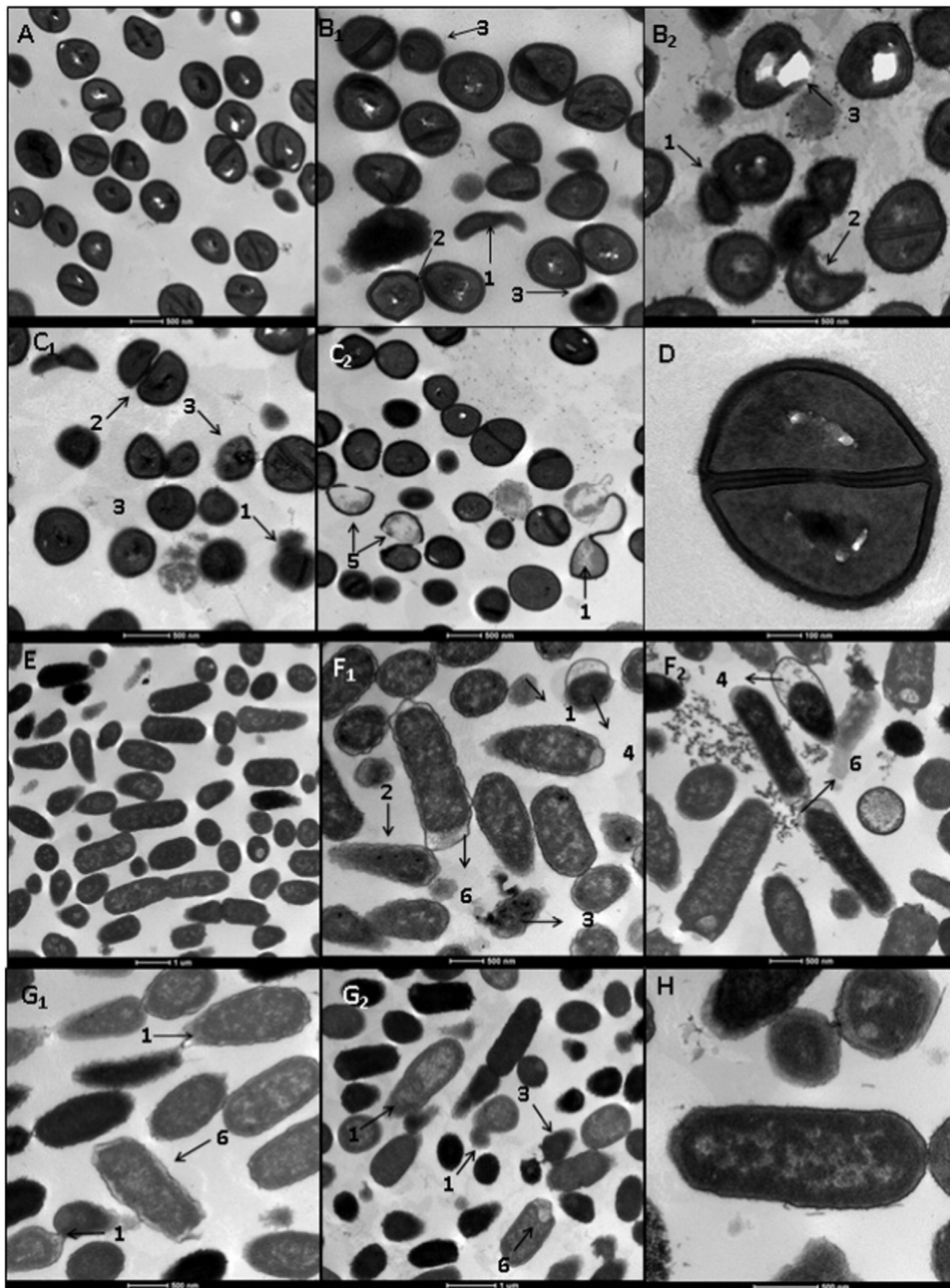

Fig. 2. Transmission electron microscopy (TEM) of MRSA (ATCC 33591) and *E. coli* (ATCC 43895) treated without Cin or Ger (control) and with Cin or Ger at MIC. (A) MRSA cells grown in the absence of EO compounds (control) (10,000×). (B_1 and B_2) MRSA cells treated with Cin at MIC (10,000×). (C_1 and C_2) MRSA cells treated with Ger at MIC (10,000×). (D) Detail of MRSA cell (30,000×). (E) *E. coli* cells grown in the absence of EO compounds (control) (10,000×). (F_1 and F_2) *E. coli* cells treated with Cin at MIC (10,000×). (G_1 and G_2) *E. coli* cells treated with Ger at MIC (10,000×). (H) Detail of *E. coli* cell (30,000×). 1, disintegration of the cell with leakage of cytoplasmic content; 2, distortion of the cell; 3, complete lysis of the cell; 4, polarization of cytoplasmic content; 5, lack of cytoplasm; 6, cytoplasmic membrane separated from the cell wall.

3.4. Effect of cinnamaldehyde and geraniol on bacterial morphology

The morphologies of MRSA and *E. coli* cell ultrastructures treated with Cin and Ger were visualized by TEM (Fig. 2). Relative to the untreated cells of MRSA and *E. coli* (Fig. 2A and E), deformation of the bacterial cell membrane occurred on the addition of Cin and Ger. Both strains exhibited cell wall damage, and a complete loss of membrane integrity was evident (Fig. 2B, C, F and G). Treated MRSA cells exhibited several morphological changes, including separation of the cytoplasmic membrane from the cell wall, cell wall and cell membrane lysis, cytoplasmic content leakage, cytoplasmic content polarization, and cell distortion (Fig. 2B and C). Similar changes occurred in treated *E. coli* (Fig. 2F and G). Additionally, the cytoplasmic content condensed (Fig. 2G_1 and G_2) due to abnormal protein precipitation.

According to Shen et al. (2015), *E. coli* and *S. aureus* suffered similar damages to those observed in this study, when exposed to MIC of Cin, including a loss of cell wall integrity. Chemical constituents of plant-derived EOs, such as monoterpenes, as tested in the current study, are associated with cell membrane damage due to their hydrophobic nature; these compounds accumulate in the lipid-rich environments of cell membrane structures, causing structural and functional damage (Cox et al., 2000; Lambert, Skandamis, Coote, & Nychas, 2001; Sikkema, De Bont, & Poolman, 1995). Furthermore, they can dissolve in biomembranes and interact with lipophilic side chains of phospholipids (Wink, 2008). Although the exact mechanism remains unclear, evidence of physicochemical and physiological changes to cell structure and components has been reported, and more than one mechanism may be involved in the activity of phenolic compounds. However, the relevance of alternate mechanisms can be discounted if rapid inhibition of energy generation occurs. The possible mechanisms of inhibition of energy generation are inhibition of glucose uptake or utilization of glucose and effects on membrane permeability (Gill & Holley, 2004).

4. Conclusions

This study illustrated the potential of phenolic compounds to interact synergistically with antimicrobial drugs against MRSA and to significantly decrease enterotoxin production by *S. aureus*. This is potentially of considerable importance in the food and pharmaceutical industries, and a promising area for further development.

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