Phytochemical profiling as a solution to palliate disinfectant limitations

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Abstract

The indiscriminate use of biocides for general disinfection has contributed to increased incidence of antimicrobial tolerant microorganisms. This study aims to assess the potential of seven phytochemicals (tyrosol, caffeic acid, ferulic acid, cinnamaldehyde, coumaric acid, cinnamic acid and eugenol) in the control of planktonic and sessile cells of *Staphylococcus aureus* and *Escherichia coli*. Cinnamaldehyde and eugenol showed antimicrobial properties, minimum inhibitory concentration of 3-5 and 5-12 mM and minimum bactericidal concentration of 10-12 and 10-14 mM against *S. aureus* and *E. coli*, respectively. Cinnamic acid was able to completely control adhered bacteria with effects comparable to peracetic acid and sodium hypochlorite and it was more effective than hydrogen peroxide (all at 10 mM). This phytochemical caused significant changes on bacterial membrane hydrophilicity. The observed effectiveness of phytochemicals makes them interesting alternatives and/or complements to commonly used biocidal products. Cinnamic acid is of particular interest for the control of sessile cells.

Keywords: biocides, disinfection, *Escherichia coli*, phytochemicals, sessile cells, *Staphylococcus aureus*

Introduction

Effective disinfection is crucial to prevent and control microbial proliferation in hospital, industrial and domiciliary settings. The World Health Organization (WHO) defines hospital-acquired infections (HAI) as those infections developed after 48 hours of hospitalization or visit that were not incubating at admission (Kelly and Monson 2012). In the USA around 1.7 million HAI are reported every year with 16% involving microorganisms resistant to commonly used antibiotics (Kallen et al. 2010). The WHO also considers food safety a top priority. Forty eight million people suffer from foodborne disease in the USA every year (Stein et al. 2007, Scallan et al. 2011, Jahid and Ha 2012). Billions of dollars are imposed annually as a result of microbial contamination (van Rijen et al. 2008, Kuehn et al. 2010, Van Houdt and Michiels 2010, Kelly and Monson 2012).

Chemical disinfectants, such as hydrogen peroxide, peracetic acid and chlorine-releasing agents (*e.g*. sodium hypochlorite solutions), are widely used both in hospital and industrial environments (Russell 1997, 2002, DeQueiroz and Day 2007, Van Houdt

and Michiels 2010). Although the mechanism of action of this type of agents is not fully understood some of these disinfectants are active oxidizing agents interacting with biological components, including proteins, lipids and nucleic acids (Chapman 2003, Kitis 2004). In addition, hydrogen peroxide, peracetic acid and chlorine releasing agents suffer from a number of drawbacks that include chemical instability, environmental toxicity, human toxicity and corrosion (Kitis 2004, Ferraris et al. 2005, Ronco and Mishkin 2007, Park et al. 2008, Jahid and Ha 2012, Linley et al. 2012).

The increasing number of resistant microorganisms to commonly used benchmark disinfectants along with their side-effects has led to the search for new biocidal strategies (Fraise 2002). Therefore, the interest in environmentally friendly, non-toxic and degradable yet potent biocides has never been so high. Several plant secondary metabolites, normally referred as phytochemicals, have been biosynthesized to protect the plant against microbial infections and other external stress conditions (Liu 2004). Consequently, over the years a significant number of these biological active phytochemicals have been explored for a number of purposes especially as pharmaceutical agents or excipients (Cowan 1999, Simões et al. 2009, Doughari 2012). Secondary metabolites largely fall into three classes of compounds: alkaloids, terpenoids, and phenolics (Cowan 1999). Phenolic compounds are one of the most numerous and ubiquitous group of phytochemicals, including simple phenols and their derivatives, flavonoids and tannins among others (Manach et al. 2004). They are produced via the so-called phenylpropanoid pathway, in which phenylalanine ammonia lyase (PAL) deaminates phenylalanine or tyrosine yielding cinnamic acid and related compounds (Figure 1). The aromatic amino acids are synthesized via the shikimate pathway followed by the branched aromatic amino acid metabolic pathway, with chorismate serving as a major branch point intermediate metabolite (Dewick 2001, Boerjan et al. 2003, Zhang et al. 2011). This group of phytochemicals exhibits a wide range of biological properties, including antibacterial, anti-inflammatory, anti-allergic, hepatoprotective, antithrombotic, antiviral, anticarcinogenic and vasodilatory actions (Saavedra et al. 2010, Borges et al. 2012).

The purpose of this study was the assessment of the biocidal efficacy of selected phytochemicals (molecules from the plant secondary metabolism). The phytochemicals were cinnamic derivatives and analogues derived from aromatic amino acids through phenylpropanoid pathway and so related with each other (Figure 1). Their effects in controlling the growth of planktonic cells of *S. aureus* and *E. coli* was characterized and

compared with the selected benchmarked biocides: hydrogen peroxide, peracetic acid and sodium hypochlorite. The efficacy to remove monolayer sessile bacteria from surfaces as well as the possibility to interfere with bacterial surface properties was also evaluated.

Materials and Methods

Chemicals

Cinnamaldehyde, coumaric acid, caffeic acid, ferulic acid, tyrosol, eugenol and peracetic acid were purchased from Sigma (Portugal). Cinnamic acid and hydrogen peroxide were purchased from Merck (VWR, Portugal). Sodium hypochlorite was purchased from Acros Organics (Portugal).

Microorganisms, culture conditions and test solutions

Test suspensions of *Staphylococcus aureus* CECT 976 and *Escherichia coli* CECT 434 (from the Spanish Type Culture Collection) used in the study were obtained from overnight cultures in 250 mL flasks with 100 mL of Mueller-Hinton broth (MHB, Merck, Germany) incubated at 30 °C and under 150 rpm agitation. Phytochemical solutions were prepared using dimethyl sulfoxide (DMSO, Sigma) and were always added as 10% (v/v) of the test medium/solution. Hydrogen peroxide, peracetic acid and sodium hypochlorite were prepared using sterile distilled water. All chemicals were neutralized by dilution to sub-inhibitory concentrations according to Johnston et al. (2002). The initial pH of bacterial suspensions with phytochemicals were 7.0 ± 0.2 and 6.0 ± 0.2 if the test solution were in MHB or NaCl (8.5 g/L), respectively.

Antibacterial susceptibility testing

The minimum inhibitory concentration (MIC) of each chemical was determined by the microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2012). Bacteria from an overnight culture (≈16 hours) were adjusted to a density of 10^8 colony forming units (CFU) per mL with fresh culture medium. A maximum volume of 200 µL/well was used in 96-well microtiter plates, containing the bacterial test suspension in growth medium and the different concentrations of the chemicals (10% v/v). The bacterial growth was measured at 600 nm using a microplate reader (Spectramax M2e, Molecular Devices, Inc.). The MIC was determined as the lowest concentration that inhibited microbial growth (Ferreira et

al. 2011). To determine the minimum bactericidal concentration (MBC) a volume of 10 µL/well was plated in Plate Count Agar (PCA, Merck, Germany) and incubated overnight at 30 ± 3°C. The MBC (minimum bactericidal concentration) was considered the lowest concentration of chemical were no growth was detected on the solid medium (Ferreira et al. 2011). Three independent experiments were performed for each chemical.

Bacterial adhesion

Bacterial suspensions (≈ 10^8 CFU/mL) were dispersed into 96-well polystyrene plates (200 µL/well) and their adhesion to the surface was measured following (Simões et al. 2007) in which an adhesion period occurred for 2 hours at 30 °C under agitation at 150 rpm. After the adhesion period non-adhered bacteria were discarded by washing the plates with a NaCl (8.5 g/L) solution prior to exposure to biocides or phytochemicals. Biocides and phytochemicals were tested at 10 mM for 1 hour at 30 °C under agitation (150 rpm). This concentration was selected as it was the lowest MBC obtained for the phytochemicals. Afterwards, sessile bacteria were washed with NaCl solution (8.5 g/L) to reduce the concentration of the chemicals to sub-inhibitory levels (Johnston et al. 2002). Sessile cells were scraped with a pipette tip for 1 minute, ressuspended in NaCl solution and their viability was assessed after plating on Mueller-Hinton Agar (MHA, Merck, Portugal). CFU were determined after 24 h at 30 °C incubation and presented as log CFU/cm^2. Three independent experiments were performed for each condition tested.

Physicochemical characterization of bacterial surfaces

The physicochemical properties of *S. aureus* and *E. coli* surfaces were assessed by the sessile drop contact angle measurement on bacteria lawns as described by Busscher et al. (1984). Contact angles were determined using an OCA 15 Plus (DATAPHYSICS) video-based optical measuring instrument, allowing image acquisition and data analysis. Measurements (≥ 15 per liquid and chemical) were performed according to Simões et al. (2007) after bacteria incubation (1 h) with the biocides or phytochemicals (all at 10 mM). The liquid's surface tension components reference values were obtained from the literature (Janczuk et al. 1993). Hydrophobicity was assessed after contact angle measurement following the van Oss approach (van Oss et al. 1987, 1988, 1989). The degree of hydrophobicity of a given surface (s) is expressed as the free energy of interaction between two entities of that surface when immersed in water (w) $-(\Delta G_{sws} -$

mJ/cm^2). The surface is considered hydrophobic if the interaction between two entities is stronger than the interaction of each with water ($\Delta G_{sws} < 0$). On the other hand, if $\Delta G_{sws} > 0$ the material is considered hydrophilic. ΔG_{sws} can be calculated using the surface tension components of the interacting entities by the following equation:

$$\Delta G_{sws} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^+\gamma_w^-}\sqrt{\sqrt{\gamma_s^-\gamma_w^+} - \sqrt{\gamma_s^+\gamma_s^-} - \sqrt{\gamma_w^+\gamma_w^-}}\right); \quad (1)$$

γ^{LW} represents the Lifshitz-van der Waals component of the free energy of the surface and γ^+ and γ^- are the electron acceptor and donor parameters, respectively, of the Lewis acid-based component (γ^{AB}), where $\gamma^{AB} = 2\sqrt{\gamma^+\gamma^-}$. The surface tension components of a solid material have been obtained by measuring the contact angles of three liquids with different polarities and known surface tension components (1): α-bromonaphtalene (apolar), formamide (polar), and water (polar). Upon obtaining the data, the following equation can be solved:

$$(1 + \cos\theta)\gamma_w^{Tot} = 2\left(\sqrt{\gamma_s^{LW}\gamma_w^{LW}} + \sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+}\right); \quad (2)$$

θ is the contact angle and $\gamma^{Tot} = \gamma^{LW} + \gamma^{AB}$.

Statistical analysis

Data were analyzed applying the t-test using SPSS (Statistical Package for the Social Sciences) version 22.0. The average and standard deviation (SD) within samples were calculated for all cases (three independent experiments were performed for each condition). Statistical calculations were based on confidence level $\geq 95\%$ ($p < 0.05$) which was considered statistically significant.

Results

This study was performed with seven biosynthetically related phytochemicals (Figure 1) in order to ascertain their biocidal potential. Three commonly used disinfectants (hydrogen peroxide, peracetic acid and sodium hypochlorite) were used for comparison. *S. aureus* and *E. coli* were the selected microorganisms and the MIC and MBC of disinfectants and phytochemicals were assessed (Table 1).

Hydrogen peroxide had MIC and MBC values more than 20 times higher for *S. aureus* (400 and 450 mM) than for *E. coli* (16 to 20 mM for MIC and MBC). Peracetic acid and sodium hypochlorite were the disinfectants with the lowest MIC and MBC regardless of

the bacteria tested. The most efficient phytochemicals were cinnamaldehyde and eugenol, showing the lowest MIC and MBC against both bacteria. Moreover, cinnamaldehyde and eugenol exhibited MIC similar to sodium hypochlorite (except MIC of eugenol for *S. aureus*) and MIC and MBC comparable to peracetic acid ($p > 0.05$). Cinnamaldehyde and eugenol MIC and MBC were lower than for hydrogen peroxide ($p < 0.05$). Caffeic, ferulic, coumaric and cinnamic acids showed similar MIC when tested against *S. aureus* ($p > 0.05$). Coumaric and cinnamic acids had also similar MIC against *E. coli*. Some phytochemicals shown MIC or MBC above 25 mM. Tyrosol had the lowest antimicrobial activity (MIC and MBC > 25 mM against both bacteria).

Additional tests were performed with sessile bacteria on polystyrene surfaces to evaluate the efficacy of the disinfectants and phytochemicals in the removal of monolayer adhered bacteria. After a 2 h adhesion period, 5.21 log CFU/cm^2 of *S. aureus* and 4.89 log CFU/cm^2 of *E. coli* adhered on the polystyrene surface. The polystyrene-adhered bacteria were exposed to the selected disinfectants and phytochemicals for 1 h and the CFU of adhered bacteria are presented in Figure 2. Exposure to hydrogen peroxide only caused CFU reduction of adhered *E. coli*. Peracetic acid and sodium hypochlorite were the most efficient disinfectants causing total CFU reduction of both bacteria ($p > 0.05$). Considering the selected phytochemicals it was observed that cinnamic acid promoted a drastic CFU reduction of *S. aureus* and *E. coli* from polystyrene at a concentration 2.5 times lower than the MBC (concentration used: 10 mM). This phytochemical displays an activity comparable to peracetic acid and sodium hypochlorite ($p > 0.05$) and it was more efficient than hydrogen peroxide against *S. aureus* sessile bacteria ($p < 0.05$). The phytochemicals with poor activity (≤1 log CFU/cm^2 reduction from surfaces) against *S. aureus* were cinnamaldehyde, coumaric, caffeic and ferulic acids, tyrosol and eugenol. Tyrosol and eugenol were the least efficient against *E .coli* with reduction from surfaces lower than 1 log CFU/cm^2, followed by ferulic acid (1< log CFU/cm^2 reduction from surfaces ≤ 2), caffeic acid (2 < log CFU/cm^2 reduction from surfaces ≤ 3), cinnamaldehyde, coumaric acid and cinnamic acid (3 < log CFU/cm^2 reduction from surfaces ≤ 4).

The possibility of changes on membrane hydrophobicity of *S. aureus* and *E. coli* following exposure to the selected disinfectants and phytochemicals was also assessed (Table 2). Sodium hypochlorite was able to enhance the hydrophilicity (ΔG_{sws}) of both bacteria ($p < 0.05$). Peracetic acid had no significant effects on the membrane

hydrophilicity of both bacteria ($p > 0.05$). Hydrogen peroxide was able to increase the ΔG_{sws} of *E. coli*. Considering the phytochemicals, cinnamic acid was found to reduce the hydrophilicity of *S. aureus* and increased hydrophilicity of *E. coli* ($p < 0.05$). The remaining phytochemicals increased the hydrophilicity of *S. aureus*, with the exception of tyrosol ($p < 0.05$). In fact, tyrosol did not influence the membrane properties of *S. aureus* or *E. coli* ($p > 0.05$). Caffeic, *p*-coumaric and ferulic acids, and cinnamaldehyde increased the hydrophilicity of ($p < 0.05$). Eugenol increased membrane hydrophilicity, however, the effect on *E. coli* was not as evident as it was against *S. aureus* ($p < 0.05$).

Discussion

Over the years natural products have assumed an important role as alternative sources of novel bioactive molecules. In this study seven phytochemicals were selected based on their related chemical structures. Their effects were assessed against planktonic and sessile cells of two strains of *S. aureus* and *E. coli* previously used in diverse antimicrobial screening studies (Simões et al. 2008, Borges et al. 2013). For comparison, three commonly used disinfectants (hydrogen peroxide, peracetic acid and sodium hypochlorite) were also tested. The selected disinfectants are recognized for their broad antimicrobial spectrum (Rutala and Weber 1997, McDonnell and Russell 1999, Pericone et al. 2000, Rasmussen et al. 2013). An initial screening was performed with the selected disinfectants and phytochemicals to ascertain their MIC and MBC against *S. aureus* and *E. coli*. Hydrogen peroxide was the least effective benchmark disinfectant. The lower susceptibility of *S. aureus* to hydrogen peroxide in the concentration used in this study, compared to *E. coli* could be explained with the expression of catalase by *S. aureus* (Park et al. 2008); although this was not ascertained in our study. Peracetic acid and sodium hypochlorite are powerful oxidizing agents that are effective against both Gram-positive and Gram-negative bacteria (Penna et al. 2001). The data attained in the present study (Table 1) confirmed their reported microbicidal efficacy (Penna et al. 2001, Spoering and Lewis 2001). Despite a high efficacy against bacteria, they present distinct advantages and disadvantages that influence their use (McDonnell and Russell 1999, Estrela et al. 2002, Kitis 2004, Ferraris et al. 2005).

Although some of the selected phytochemicals presented high (≈ 25 mM) MIC and MBC values, cinnamaldehyde and eugenol presented MIC and MBC comparable to benchmark disinfectants. Differences on the MIC and MBC of the phytochemicals

against *S. aureus* and *E. coli* were observed. In general, *S. aureus* was more resistant than *E. coli*, contrarily to what is commonly observed. Gram-negative bacteria are more tolerant than Gram-positive bacteria to biocides due to the presence of an outer membrane (Livermore 2012). The higher resistance of Gram-positive bacteria can be related with phytochemicals selectivity. Cinnamic acid derivatives are organic acids (pKa $\approx$ 4.2) and their efficacy as antimicrobials is thought to be dependent on the concentration of undissociated acid (Johnston et al. 2003, Campos et al. 2009). In fact, this small lipophilic molecules can cross the cell membrane by passive diffusion as undissociated chemicals, disturb or even disrupt the cell membrane structure, acidify the cytoplasm and cause denaturation of proteins as well as increase bacterial permeability (Johnston et al. 2003, Campos et al. 2009). Therefore, the presence of a thinner peptidoglycan layer in Gram-negative bacteria may facilitate the antimicrobial action of phytochemicals.

Considering the promising antibacterial activities observed, their activity as quorum sensing inhibitors was also assessed since several phytochemicals shown to have anti-quorum sensing properties which can confer them an importance role in biofilm control (Borges et al. 2014). However, in this study only eugenol demonstrated a slight anti-quorum sensing activity against *Chromobacterium violaceum* (supplementary information). This characteristic cannot be discarded for the other phytochemicals tested since several authors observed inhibition of quorum sensing with some phytochemicals: eugenol, cinnamaldehyde, curcumin and *p*-coumaric acid (Bodini et al. 2009, Brackman et al. 2011, Zhou et al. 2013). In this study only the quorum sensing system of *C. violaceum*, homologs of LuxI/LuxR system, was studied (Borges et al. 2014). Therefore, the possibility of inhibition of other quorum sensing systems cannot be discarded. Despite the absence of anti-quorum sensing activity, the phytochemicals were assessed for their ability to control adhered cells and their effects were compared with the disinfectants. Monolayer adhered bacteria were used in this study rather than three-dimensional biofilm structures. According to previous studies, contaminated hospital surfaces are mostly colonized by monolayer adhered cells with densities of 10^4 - 10^6 CFU/cm^2 (values in the range of those found in this study for *Escherichia coli* and *Staphylococcus aureus*) (Dancer, 2004; Wren et al. 2008; Otter et al. 2015). Moreover, it was found that the effects of selected disinfectants were similar on CFU reduction of monolayer adhered cells (2 h adhesion) and biofilms (24 h-old) (Meireles et al. 2015).

Hydrogen peroxide was the least efficient disinfectant. Its biocidal activity is based on a bimodal killing pattern where the first mode occurs when *E. coli* is exposed to low concentrations of hydrogen peroxide that damages DNA. The second mode occurs when *E. coli* is exposed to higher concentrations and cell membrane damage can be observed (Imlay and Linn 1986, Linley et al. 2012). The influence of hydrogen peroxide on *E. coli* surface properties was observed in this study with an increase in the surface hydrophilicity. The high effectiveness of peracetic acid and sodium hypochlorite can be explained by their mode of action. Peracetic acid action includes disruption of cell wall permeability, proteins denaturation, and oxidation of sulfhydryl and sulfur bonds in proteins (Kitis 2004, Al-Adham et al. 2013). Furthermore, it was hypothesized that it can disrupt the chemiosmotic function of the lipoprotein from cytoplasmic membrane and transport function through dislocation or even rupture of cell walls (Kitis 2004). This is reinforced by the increase of the hydrophilic character of *S. aureus* and the slight decrease of the hydrophilic character of *E. coli*. The microbicidal activity of sodium hypochlorite can be largely attributed to undissociated hypochlorous acid (HOCl) and to its dissociate form hypochlorite ion (OCl^-), whose formation is pH dependent. Hypochlorous acid can penetrate the bacteria, cross the cell wall and membranes, inhibiting the activity of essential enzymes that modulates growth, damaging the membrane and DNA and causing damage in the membrane transport system (Estrela et al. 2002, Fukuzaki 2006). The hydrophobicity data attained in this work also support this hypothesis. The exposure of *S. aureus* and *E. coli* to sodium hypochlorite led to a significant increase on their surface hydrophilicity. The data is in accordance with the findings of Gottardi and Nagl (2005) where the action of active chlorine (hypochlorous acid) in bacteria can be divided in two effects: non-lethal and lethal. In the first stage reversible chlorination of the bacterial surface occurs; in the second stage penetration into the bacteria combined with irreversible cell changes occurs. In another study it was found that bacterial membrane damage was related to changes in membrane hydrophilicity (Borges et al. 2013).

In general, phytochemicals were highly efficient in causing sessile bacteria reduction from surfaces, with the exception of tyrosol and eugenol. Although tyrosol has been described as an antimicrobial agent it can be also converted to phenolic intermediates by bacteria reducing its antimicrobial activity (Brooks et al. 2006, Liebgott et al. 2007, Liebgott et al. 2008). On the other hand, eugenol demonstrated antimicrobial effectiveness at low concentrations (10 mM); this was also observed by Ali and

coworkers (2005) with eugenol and cinnamaldehyde against *Helicobacter pylori*. However, in this study eugenol was not effective in the control of sessile bacteria, even if other studies were able to observe antibiofilm potential of this phytochemical against *Pseudomonas* spp., *Candida albicans* and oral bacteria (Niu and Gilbert 2004, Magesh et al. 2013, de Paula et al. 2014). These observations propose that the efficacy of eugenol to control sessile bacteria appears to be species dependent.

Cinnamaldehyde, *p*-coumaric, caffeic and ferulic acids exhibited similar activities against the sessile cells, which supports the fact that these phytochemicals are known to have similarities in their mode of action, regarding bacterial surface interaction (Johnston et al. 2003, Campos et al. 2009, Lou et al. 2012). Ghosh and coworkers (2013) demonstrated that cinnamaldehyde is able to promote bacterial surface disruption especially in association with silver nanoparticles. Cinnamaldehyde was also described as being capable to control *Pseudomonas* spp. biofilms (Niu and Gilbert 2004). The observed increase in hydrophilicity of bacteria surface after the exposure to eugenol, caffeic, *p*-coumaric and ferulic acids as well as cinnamaldehyde for both bacteria supports the accepted mechanism of action for the generality of phytochemicals that includes membrane disturbance with increase in permeability (Gill and Holley 2004, Campos et al. 2009, Lou et al. 2012).

Interestingly, the action of cinnamic acid on the control of sessile bacteria was comparable to that of benchmark disinfectants and its efficiency was similar against both bacteria. In fact, it was the only phytochemical that demonstrated a high efficiency in the control of sessile bacteria. The results on the assessment of the bacterial physicochemical surface properties shown that cinnamic acid acts on bacterial surface hydrophilicity, an effect that was more noticeable against *S. aureus.* This results corroborates previous studies performed with cinnamic acid against *Listeria monocytogenes, E. coli* and *Pseudomonas aeruginosa* (Ramos-Nino et al. 1996, Chambel et al. 1999) and the yeast *Saccharomyces cerevisiae*, proposing that cinnamic acid can change the membrane properties of bacteria. Since the phytochemicals were chosen based on rational structure differences it is possible to hypothesize that the effects of cinnamic acid on the bacterial surface properties can be related to the absence of moieties in the benzene ring and the presence of the carboxylic function in its structure (Johnston et al. 2003, Campos et al. 2009). Although this phytochemical is recognized by several authors for its bioactive properties such as anticancer, antidiabetic, antimicrobial, antifungal and antiviral, the antibacterial mode of action of

cinnamic acid is not yet completely understood (Sharma 2011, Korošec et al. 2014, Zhang et al. 2014). This study provides further results and demonstrates the potential of cinnamic acid to control sessile *E. coli* and *S. aureus*.

In conclusion, new biocides are required for general disinfection practices, both in hospital settings and industry. This has led to the search for new and alternative molecules to be used as biocides or as adjuvants/potentiators to commonly used disinfectants. In this context phytochemicals emerged as a sustainable source of new and environmentally friendly molecules. In this study it was observed that cinnamaldehyde and eugenol can be considered antimicrobials as their MIC and MBC are comparable to the selected disinfectants. Moreover, it was also found that phytochemicals, despite the absence of evident antimicrobial properties, could be used as dispersing agents of sessile cells, particularly cinnamic acid which caused total reduction of sessile *E. coli* and *S. aureus* after exposure to sub-MIC/MBC. The efficacy of cinnamic acid was similar to peracetic acid and sodium hypochlorite and higher than that of hydrogen peroxide, especially in the control of *S. aureus*. This phytochemical was able to modify the bacteria surface properties by decreasing their hydrophilic character. The results achieved in this study and the accepted status of environmentally friendly and low cytotoxic of phytochemicals (Fresco et al. 2006, Abreu et al. 2012) reinforce their potential as new biocides and/or adjuvants of biocidal formulations for daily disinfection.

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601

Tables and Figures

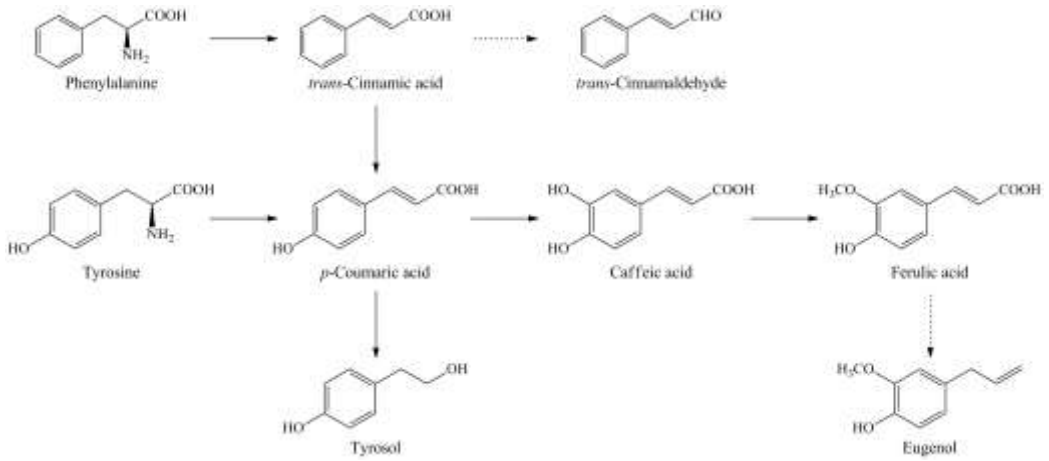


Figure 1 – Biosynthetic relationship of the phytochemicals used in the study.

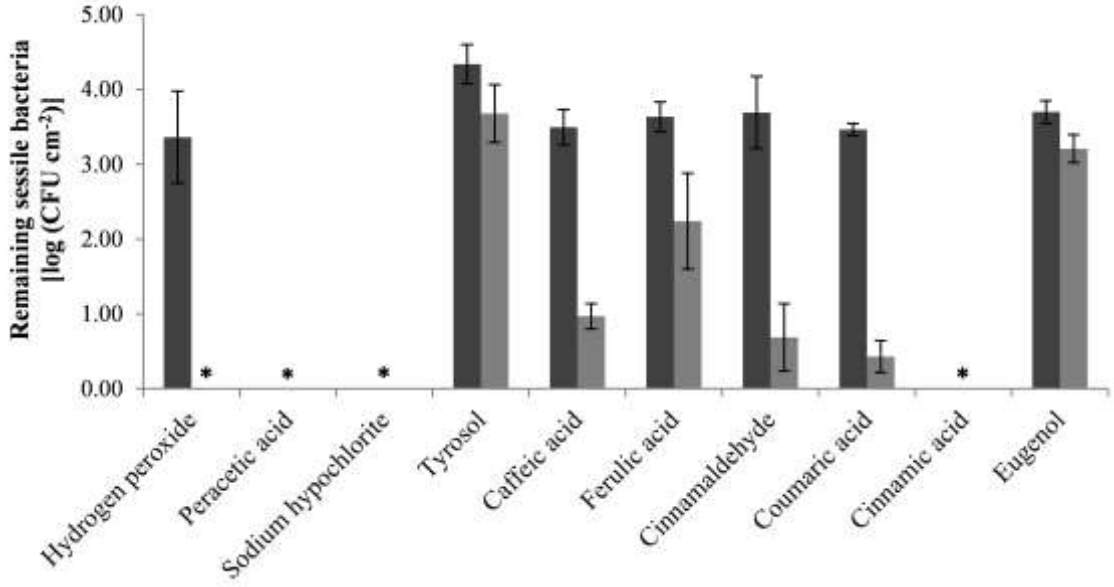


Figure 2 – Effects of the selected disinfectants and phytochemicals on the control of sessile *S. aureus* (■) and *E. coli* (■). The figure presents the remaining CFU of sessile bacteria after 1 hour exposure to the selected chemicals. Values are mean ± SD of three experiments. *****- No CFU were detected.

Table 1 – Properties of the selected phytochemicals and MIC and MBC of the chemicals against *S. aureus* and *E. coli*

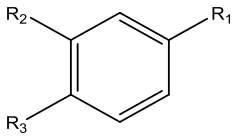
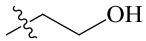
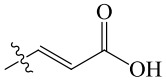
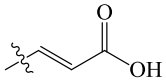
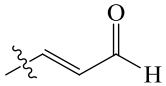
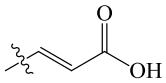
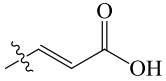
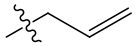
				*S. aureus*		*E. coli*	
	R_1	R_2	R_3	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)
Hydrogen peroxide				400	450	16	20
Peracetic acid				9	10	5	7
Sodium hypochlorite				4	5	3	3
Tyrosol		-	OH	> 25	> 25	> 25	> 25
Caffeic acid		OH	OH	23	> 25	25	> 25
Ferulic acid		OCH_3	OH	25	> 25	> 25	> 25
Cinnamaldehyde		-	-	5	12	3	10
Coumaric acid		-	OH	25	25	15	> 25
Cinnamic acid		-	-	25	25	15	> 25
Eugenol		OCH_3	OH	12	14	5	10

Table 2 – Effects of the selected disinfectants and phytochemicals on the hydrophobicity of *S. aureus* and *E. coli*

	Hydrophobicity (mJ/m^2) - ΔG_{sws}^{TOT}	
	S. aureus	*E. coli*
Control (Water)	20.78 ± 5.45	25.22 ± 5.22
Hydrogen peroxide	21.50 ± 4.69	42.38 ± 3.80
Peracetic acid	27.93 ± 4.94	21.05 ± 2.51
Sodium hypochloride	42.45 ± 4.79	33.81 ± 3.96
Control (DMSO)	23.28 ± 5.77	28.14 ± 4.30
Tyrosol	23.81 ± 1.99	29.39 ± 0.48
Caffeic acid	28.77 ± 2.08	37.67 ± 8.78
Ferulic acid	26.81 ± 5.02	32.26 ± 3.35
Cinnamaldehyde	27.98 ± 2.43	34.03 ± 4.98
Coumaric acid	27.73 ± 4.26	32.58 ± 3.65
Cinnamic acid	10.09 ± 5.75	31.68 ± 6.76
Eugenol	30.17 ± 5.14	27.94 ± 0.97