Comparison of the efficacy of natural-based and synthetic biocides to disinfect silicone and stainless steel surfaces

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Abstract

New biocidal solutions are needed to combat effectively the evolution of microbes developing antibiotic resistance while having a low or no environmental toxicity impact. This work aims to assess the efficacy of commonly used biocides and natural-based compounds on the disinfection of silicone and stainless steel (SS) surfaces seeded with different *Staphylococcus aureus* strains. Minimum inhibitory concentration was determined for synthetic (benzalkonium chloride-BAC, glutaraldehyde-GTA, *ortho*-phthalaldehyde-OPA and peracetic acid-PAA) and natural-based (cuminaldehyde-CUM), eugenol-EUG and indole-3-carbinol-I3C) biocides by the microdilution method. The efficacy of selected biocides at MIC, 10×MIC and 5500 mg/L (representative in-use concentration) on the disinfection of sessile *S. aureus* on silicone and SS was assessed by viable counting. Silicone surfaces were harder to disinfect than SS. GTA, OPA and PAA yielded complete CFU reduction of sessile cells for all test concentrations as well as BAC at 10×MIC and 5500 mg/L. CUM was the least efficient compound. EUG was efficient for SS disinfection, regardless of strains and concentrations tested. I3C at 10×MIC and 5500 mg/L was able to cause total CFU reduction of silicone and SS deposited bacteria. Although not so efficient as synthetic compounds, the natural-based biocides are promising to be used in disinfectant formulations, particularly I3C and EUG.

Keywords: Biocides, disinfection, phytochemicals, *Staphylococcus aureus*

Introduction

The role of contaminated environmental surfaces and medical devices in the transmission of healthcare-associated pathogens has been well reported (Kramer *et al.* 2006, Boyce 2007, Weber *et al.* 2010; Otter *et al.* 2015). A number of studies suggests that microbial contamination of those surfaces and devices plays an important role in the spread of pathogens (Weinstein and Hota 2004, Gebel *et al.* 2013; Otter *et al.* 2015). Effective pathogen transmission depends on several factors including the ability of microorganisms to remain viable on dry surfaces, their resistance to disinfectants and the frequency that contaminated surfaces or devices are in contact with patients and healthcare workers (Boyce 2007, Weber *et al.* 2010). In order to prevent the acquisition and the spread of healthcare associated infections (HAIs) it is important to implement adequate and efficient cleaning and disinfection protocols. HAIs represent high morbidity and mortality costs for patients and financial burden for healthcare units. Therefore, effective strategies are required to disinfect hospital surfaces and devices (Abreu *et al.* 2013). However, bacterial resistance to disinfectants is an important factor in the control of HAIs. Microorganisms may have intrinsic/innate resistance to disinfectants which is commonly related with cellular impermeability. However, the continuous exposure to disinfectants may increase microbial resistance by cellular mutations or acquisition genetic elements (Abreu *et al.* 2013; Russel 1998). Quaternary ammonium compounds, biguanides and phenolics, particularly used in a number of biocidal products in healthcare, have been associated with emerging bacterial resistance *in vitro* (Russell *et al.* 1999, Maillard 2005, SCENIHR 2009). For example, studies reported *Pseudomonas aeruginosa, Listeria monocytogenes* and *Staphylococcus aureus* with low susceptibility to benzalkonium chloride (BAC) (Sakagami *et al.* 1989, Akimitsu *et al.* 1999, To *et al.* 2002, Bridier *et al.* 2011, Ibusquiza *et al.* 2011).

Concerns of bacterial resistance to high-level disinfectants such as oxidising and alkylating agents have also been reported, for example, resistance to peracetic acid (PAA) in *L. monocytogenes* (Bridier *et al.* 2011, Ibusquiza *et al.* 2011) and vegetative *Bacillus subtilis* (Bridier *et al.* 2012), *Mycobacterium avium* and *Mycobacterium terrae* (Bridier *et al.* 2011), and glutaraldehyde (GTA) resistance in atypical mycobacteria (Griffiths *et al.* 1997). Bacterial resistance to OPA was reported by Fisher *et al.* (2012) who isolated *Mycobacterium gordonae* and *M. avium* from endoscopes disinfected with OPA. This clearly proposes that the development of new disinfectant solutions is of utmost importance to prevent effectively HAIs.

In this work, three natural-based biocides derived from the plant secondary metabolism (cuminaldehyde – CUM presented in *Cuminum cyminum* (Morshedi *et al.* 2015), eugenol - EUG presented in *Syzygium aromaticum* - clove (Just *et al.* 2015) and indole-3-carbinol - I3C presented in some vegetables of the *Brassica* genus, including cabbage, cauliflower, and brussels sprouts (Bjeldanes *et al*. 1991)) were evaluated for their potential bactericidal efficacy against four *S. aureus* strains seeded on silicone or stainless steel, two surface materials commonly found in hospital settings (Kovaleva *et al.* 2013, Gastmeier and Vonberg 2014). BAC, GTA, OPA and PAA were used for comparison of activity.

Materials and methods

Bacterial strains

S. aureus SA1199B, which overexpresses the NorA MDR efflux pump, *S. aureus* RN4220, which contains plasmid pU5054 (that carries the gene encoding the MsrA macrolide efflux protein), and *S. aureus* XU212, which possesses the TetK efflux pump and is also an MRSA strain, were kindly provided by S. Gibbons (University College London, UCL)

(Oluwatuyi *et al.* 2004, Smith *et al.* 2007). The collection strain *S. aureus* CECT 976, already used as model microorganism for antimicrobial tests with phytochemical compounds (Abreu *et al.* 2012b, Saavedra *et al.* 2010) was included as a quality control strain.

Biocides

The selected biocides were purchased from Sigma (Sintra, Portugal). BAC, GTA, PAA and OPA solutions were prepared in sterile distilled water. CUM, EUG and I3C solutions were prepared in dimethyl sulfoxide (DMSO, Sigma). DMSO was used as negative control and at the concentration used (10% v/v) did not inhibit bacterial growth neither reduced the number of CFU in all four test strains.

Test surfaces

Stainless steel ASI 316 (SS) and silicone coupons (1 × 1 cm) were acquired from (Neves & Neves, Muro, Portugal) and used as test surfaces. Prior to use, the coupons were washed with commercial detergent (Sonasol, Henkel) for 30 min and then rinsed with distilled water to remove the residual detergent. The coupons were then immersed in ethanol at 70% (v/v), for 1 h to kill potential microbial contaminants. Finally, the coupons were rinsed with sterile distilled water for three consecutive times and were stored until required. In order to ascertain the absence of microbial contaminants from surfaces 400 μL of 4,6-diamino-2-phenylindole (DAPI) (Sigma) at 0.5 μg/mL were spread on the test surface and left in the dark for 5 min (Lemos *et al.* 2015). The surfaces were visualized under an epifluorescence microscope (Leica DMLB2 with a mercury lamp HBO/100W/3) incorporating a CCD camera to acquire images using IM50 software (Leica), using a ×100 oil immersion

fluorescence objective, and a filter sensitive to DAPI fluorescence (359-nm excitation filter in combination with a 461-nm emission filter).

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of each biocide was determined by the broth microdilution method according to CLSI (2012).

Manolayer bacterial adhesion and surface disinfection

S. aureus was grown overnight in Muller Hinton (MH) broth at 30 ºC under 150 rpm agitation (AGITORB 200, Aralab, Portugal). Bacterial suspensions were then centrifuged (Eppendorf centrifuge 5810R) at 3777 *g* for 10 min, washed twice with saline solution (NaCl, 8.5 g/L) and ressuspended in saline to a final concentration of 3×10^8 CFU/mL. Monolayer bacterial adhesion in 48-well microtiter plates was performed for 2 h according to Simões et al. (2007) and Meireles et al. (2015). Briefly, coupons of silicone or SS were inserted vertically in each well and 1 mL of bacterial suspension was added. Microtiter plates were then incubated at 30 ºC and 150 rpm. After 2 h incubation the coupons were transferred to new microtiter plates with NaCl (8.5 g/L) to remove non-adherent and weakly adherent bacteria (Meireles *et al.* 2015). In order to evaluate if bacteria adhered on the surfaces, coupons were microscopically analysed with DAPI according to Lemos *et al.* (2015). The coupons were inserted in new microtiter plates with the selected biocide. Biocides were tested at different concentrations: MIC, 10×MIC and a concentration representing those actually applied in hospital disinfection (5500 mg/L, a concentration higher than the MIC of the selected biocides, corresponding to the in-use concentration of OPA (Rutala *et al.* 2008)). The bacteria adhered on the surfaces were exposed to biocides

for 30 min. According to the CDC guidelines for the disinfection of healthcare facilities the exposure time for high-level disinfection should be at least 12 min, depending of the compound (Rutala *et al.* 2008). Taking into account that natural-based compounds are not as efficient as high-level disinfectants (higher MIC values were obtained) 30 min exposure was used following previous studies with phytochemicals and synthetic biocides (Lemos *et al.* 2015; Simões *et al.* 2006). After biocide exposure coupons were carefully rinsed in another microtiter plate with saline solution. This procedure was repeated twice to reduce the levels of biocide to sub-lethal concentrations (Johnston *et al.* 2002). Chemical neutralizers were not used as there is no data on antimicrobial quenchers for the selected phytochemicals. However, the dilution to sub-lethal concentrations showed to be as efficient as the application of neutralizers for BAC, GTA, OPA and PAA, using the methods described by Walsh *et al.* (1999) and Furi *et al.* (2013). Adhered bacteria were scraped with a metal scalpel from the surface of the coupons and resuspended in saline solution. The coupons were also inserted in saline solution and vortexed (Heidolph reax-top) for 1 min in order to improve bacterial detachment and to disaggregate cell clusters (Meireles *et al.* 2015). The viability of bacteria was assessed in MH agar plates. The number of colony forming units (CFU) was evaluated after 24 h incubation at 30 °C. Results are presented as log CFU *per* cm^2 of surface. All the experiments were performed in triplicate with three repeats.

Statistical analysis

The data were analyzed using the statistical program SPSS version 20.0 (Statistical Package for the Social Sciences). Results were analyzed using a One-Way ANOVA test. Statistical calculations were based on a confidence level ≥ 95 % ($P < 0.05$ was considered statistically

significant).

Results and Discussion

The use of disinfectants in hospital environments is a first line defense in infection prevention and control. Recent bacterial outbreaks have highlighted the importance of infection prevention and control illustrating just how quickly disease can spread at both national and global level (Duarte *et al.* 2009; Gebel *et al.* 2013). The increasing use of biocides is also a concern for emerging bacterial resistance and exacerbating environmental toxicity (SCENIHR 2009, Davin-Regli and Pagés 2012).

Phytochemicals are an attractive source of environmental friendly, relatively inexpensive and widely available new broad-spectrum antimicrobials with low levels of cutaneous cytotoxicity, corrosion and environmental toxicity. In terms of antimicrobial potential, phytochemicals have already demonstrated activity when used alone and when combined with other compounds as antimicrobial potentiators or as resistance-modifying agents of less effective products (Abreu *et al.* 2012a, Saavedra *et al.* 2010). Nevertheless, bacterial resistance to phytochemicals has not been studied yet probably due to their modest use for microbial growth control (Abreu *et al.* 2013; Simões *et al.* 2009).

Three phytochemical products were selected for this study based on their different chemical structures (CUM is a benzaldehyde with a isopropyl group, EUG is a phenylpropanoid and I3C an indole) and on the existence of previous evidences of their antimicrobial activity against planktonic bacteria (Gill and Holley 2006, Sung and Lee 2008, Mandal 2011). Gill and Holley (2006) tested the ability of membrane disruption by some plant aromatic oil compounds. They evaluated the action of eugenol against *Escherichia coli, Listeria monocytogenes* and *Lactobacillus sakei*, and observed a non-specific antimicrobial action

of this compound apparently due to ATPase inhibition. Mandal (2011) tested the antimicrobial activity of three different ethanolic extracts from three different plants. They found antimicrobial action of cumin extracts, containing CUM, against methicillin-resistant *S. aureus* (MRSA), with a MIC range of 128-512 µg/ml. Sung and Lee (2008) assessed the antimicrobial activity of I3C against Gram negative (*E. coli* and *P. aeruginosa* strains) and Gram positive (*S. aureus* and *E. faecium* strains) bacteria finding MIC between 34 and 544 µM.

The transmission of pathogens through environmental contaminated surfaces depends on their ability to survive on dry environments. *S. aureus* is able to survive on dry surfaces for several months (Boyce 1997, Neely and Maley, 2000, Wagenvoort *et al.* 2000, Weinstein and Hota 2004, Kramer *et al.* 2006). In this study, four distinct strains of *S. aureus*, three of them expressing characterized efflux pumps, were used. The expression of efflux has been recognized as a major driver in antimicrobial resistance and cross-resistance in bacteria (SCENHIR, 2009). Those strains were exposed to the natural-based and the synthetic biocides. BAC had the lowest MIC while EUG had the highest MIC for all the strains tested (Table 1). Among the synthetic biocides GTA had the highest overall MIC (for all strains tested). CUM, OPA and PAA showed similar MIC values ($P > 0.05$). However, OPA and CUM had lower MIC against CECT976 and SA1199b strains, while PAA had lower MIC against XU212 and RN4220 strains. I3C showed values of MIC lower than those obtained by the high level disinfectants (GTA, OPA and PAA) for all the strains ($P < 0.05$). The MIC values clearly show variability in susceptibility of the diverse *S. aureus* strains: *S. aureus* expressing efflux pump were less susceptible to phytochemicals ($P < 0.05$). This behavior was also verified for BAC and EUG.

The extent of membrane damage induced by a compound can be related to its hydrophobicity, which can be determined by its partition coefficient in octanol/water (Log P) (Nostro *et al.*, 2007). This parameter was calculated for the selected compounds as cLog P using ChemDraw Ultra 12.0 software. I3C had the lowest cLog P (1.094), followed by CUM (1.993) and EUG (2.397). In this study, the natural-based compounds with lower cLog P were those with lower MIC. In fact, Kubo *et al.* (2002) observed that lipophilicity of compounds is important for antimicrobial action, even if lipophilicity cannot be considered the most important parameter to determine antimicrobial compound activity against MRSA. In fact, cLog P of the synthetic biocides (2.930 for BAC, 1.358 for OPA, -0.924 for PAA and -1.205 for GTA) do not allow to ascertain their antimicrobial potential.

MIC values were used as guide to choose concentration against adhered bacteria on surfaces. Biocides were used at MIC, 10×MIC and 5500 mg/L recognizing that biocide efficacy of antimicrobials against planktonic bacteria is better than against sessile bacteria on surfaces (Chavant *et al.*, 2004). Significant variability on the adhesion ability of the selected *S. aureus* strains ($P < 0.05$) was observed (Figure 1). Those adhered bacteria were exposed to the selected biocides for 30 min. As depicted in Figure 1, no bacteria were recovered when exposed to GTA, OPA and PAA regardless the biocide concentration, the test surface and the strain used. BAC at its MIC was not able to completely eliminate adhered bacteria, except CECT976 and SA1199b strains on SS. No *S. aureus* were recovered from the surface when applied at 10×MIC and at 5500 mg/L on silicone and SS ($P > 0.05$). No bacteria were recovered with BAC at MIC against CECT976 and SA1199b on SS. BAC at MIC against RN4220 adhered on silicone did not reduce the number of CFU/cm^2. Nevertheless, the MIC for BAC was around 1000 times lower than the in-use concentration for hospital disinfection (Table 1) (Al-Adhan, 2013). These results confirmed

that antimicrobial planktonic tests are not reliable predictor on the action of BAC against adhered bacteria. Previous studies showed the role of efflux pumps in antimicrobial resistance, particularly to BAC (Huet *et al.* 2008, Pagedar *et al.* 2012, Costa 2013). This is a probable reason why strains carrying efflux pumps were less susceptible to BAC at MIC level. Smith and Hunter (2008) also found that BAC at 10000 mg/L could not completely inactivate biofilms formed by MRSA and *P. aeruginosa* on SS, Teflon and polyethylene surfaces. In the present work BAC at 5500 mg/L was efficient in eliminating monolayer adhered bacteria from SS and silicone surfaces.

Concerning the natural-based biocides, CUM and EUG showed similar performances on the control of monolayer bacteria adhered on silicone. No bacteria were recovered following CUM exposure at 10×MIC and at 5500 mg/L for CECT976 and SA1199b on SS, and at 5500 mg/L on SS for RN4220. There was no bacterial survivor for CECT976 and RN4220 on silicone surface with CUM treatment at 10×MIC. Less than 2 log CFU/cm^2 reductions were obtained with CUM at MIC against CECT976, RN4220 and SA1199b adhered on SS. No significant reduction was observed following CUM treatment against XU212 on SS for all tested concentrations ($P > 0.05$). CUM at all concentrations tested had no biocidal effect on silicone against SA1199b and at MIC and 10×MIC against XU212. For the other conditions tested, CUM produced less than 2 log CFU/cm^2. The antimicrobial activity of *Cuminum cyminum* extracts against planktonic cells has been demonstrated elsewhere (Shetty *et al.*,1994). Our results demonstrates the inadequacy of CUM to control sessile *S. aureus* when adhered on silicone surfaces. A possible limitation on the disinfecting efficacy of CUM can be related to its specific mode of action. Mandal (2011) proposed that *C. cyminum* extracts may affect the synthesis of the peptidoglycan layer of the cell wall, indicating the need for active growth. Although *C. cyminum* extracts can be a

promising alternative and/or complement for antibiotic chemotherapy, there are no studies describing the action of CUM on sessile bacteria.

EUG is a clove essential oil commonly used as antiseptic on oral infections (Nuñez and D' Aquino, 2012). No bacteria (all strains) were recovered following exposure to EUG at MIC, 10×MIC and at 5500 mg/L on SS. However, there was no CFU reduction when EUG was used against SA1199b and XU212 adhered on silicone, and when EUG at the MIC was used against RN4220 on silicone. No bacteria were recovered when treating CECT976 strain on silicone with EUG at 10×MIC (around 9500 mg/L) a concentration significantly higher than the in-use one. EUG at 10×MIC caused a reduction > 2.5 log CFU/cm^2 against RN4220 on silicone. The other treatments on silicone caused CFU reduction < 2.5 log CFU/cm^2. Gill and Holley (2006) demonstrated that EUG antimicrobial activity is caused by membrane disruption and by non-specific permeabilization of cytoplasmic membrane, which correlate favorably with its cLog P (2.397). This possible non-specific action of EUG makes it interesting to apply on disinfection processes, despite its high MIC. Yadav *et al.* (2015) also demonstrated the efficiency of EUG to inhibit and eradicate biofilms of MRSA and MSSA clinical strains. These authors found that EUG was able to damage cell membrane, to disrupt the cell-to-cell connections in biofilms, to kill *S. aureus* within biofilms and to interfere in the expression of some biofilm-related genes, decreasing accumulation of polysaccharides and bacterial adhesion (Yadav *et al.* 2015). The common use of EUG in toothpaste at concentrations between 100 to 100 000 mg/L (Banerjee *et al.* 2013) as well as its effectiveness on removal *in vitro* and *in vivo* biofilms (Yadav *et al.* 2015) demonstrates the non-toxic effects of EUG and its possible efficiency on hospital disinfection, even against antibiotic resistant bacteria.

I3C showed to be efficient in reducing bacteria from both surfaces, particularly at 10×MIC and at 5500 mg/L, for which no bacteria were recovered. CECT976 were not recovered when I3C was applied at MIC, 10×MIC and 5500 mg/L on silicone and SS. Likewise there was no bacteria recovery on SS for RN4220, and silicone for SA1199b at 10×MIC, and at 10×MIC, and at 5500 mg/L against XU212 on silicone and SS. No bacteria recovery was also achieved with SA199b on SS and with RN4220 on silicone. However, there was no CFU reduction when I3C was used at MIC against RN4220 and XU212 on silicone. For the remaining treatments < 2 log CFU/cm^2 reduction was observed. I3C showed high efficiency (total CFU reduction) for the disinfection of silicone and SS contaminated with *S. aureus* strains, including strains expressing efflux pumps, using concentrations lower than 5500 mg/L (the value assumed in this study as a concentration normally applied in hospitals, particularly of OPA – Rutala *et al.* (2008)). Lee *et al.* (2011) observed that I3C at 100 µg/mL was able to decrease the ability of *E. coli* O157:H7 to form biofilms. Monte *et al.* (2014) also showed that I3C was able to inactivate biofilms of *S. aureus* and *E. coli*. This phytochemical is also of potential interest on the reversal of antibiotic resistance, as a previous study demonstrated the synergistic effects on the combination of I3C with diverse antibiotics (Sung and Lee 2008). For those cases where phytochemicals demonstrate therapeutic potential as effective antibiotic resistance modifiers, it is unlikely their use as hospital disinfectants. However, there is no present therapeutic strategy using I3C as antibiotic resistance modifiers as well as no previous studies are available on the role of I3C as surface disinfectant.

Our data on silicone and SS disinfection demonstrated that the surface material can affect significantly the antimicrobial efficacy of biocides. Bacteria adhered on silicone appeared to be less susceptible to the action of natural-based biocides than those adhered on SS. It is

known that porous surfaces can confer higher protection to microorganisms (Rogers *et al.* 2005, Grand *et al.* 2010) and this may help to explain the results obtained in this study using silicone. I3C was the only natural-based biocide able to completely disinfect silicone surfaces. *S. aureus* CECT976, the collection strain, was the most susceptible to the action of natural-based biocides. Total CFU reduction from SS surfaces was observed for all the tested compounds at 10×MIC and at 5500 mg/L, and even at MIC for EUG and I3C. Moreover, CUM, EUG and I3C were not efficient for all tested conditions, remaining some viable bacteria after treatment, particularly of those strains expressing efflux pumps and adhered on silicone. This result can be related with a possible resistance mechanism. Nevertheless, specific experiments need to be performed in order to assess putative mechanisms of resistance to the phytochemicals.

Our study demonstrated that under the test conditions applied, BAC, GTA, OPA and PAA caused total CFU reduction of *S. aureus* adhered on silicone and SS. The use of natural-based compounds at concentrations close to those in-use for traditional biocides were efficient in the disinfection of SS surfaces, although their modest efficiency for lower concentrations. CUM and EUG showed similar behavior on silicone disinfection. I3C was the natural biocide with the most promising disinfection potential. This investigation adds support for the use of natural-based biocides in disinfectant formulations, helping the development of green-based antimicrobial strategies and contributing to the potential recycling of older biocides through the combination of active molecules.

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Conflict of interests

None to declare.

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Figure legend

Fig. 1. *S. aureus* adhered on silicone and SS surfaces after 30 min of treatment with the natural-based (CUM - cuminaldehyde; EUG - eugenol; I3C - indole-3-carbinol) and synthetic (BAC - benzalkonium chloride; GTA - glutaraldehyde; OPA - *ortho*-phthalaldehyde; PAA - peracetic acid) biocides. The means ± SD for at least three replicates are represented. ▨ - Untreated coupons (Control: DMSO 10% v/v), ■ - MIC, ▩ - 10×MIC, □ - 5500 mg/L. * - No CFU detected, Limit of detection: 2.8 Log CFU/cm^2.

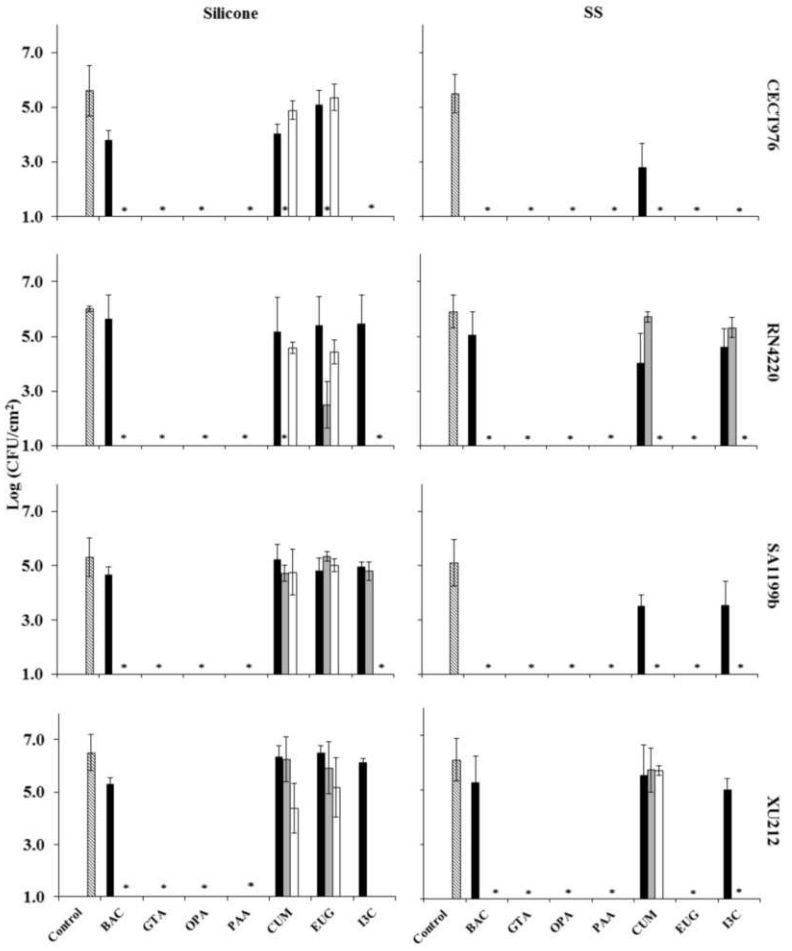

Table 1. Minimum inhibitory concentrations (mg/L) of natural-based and synthetic biocides (mean ± SD of three independent experiments). The in-use concentrations (mg/L) of synthetic biocides for hospital disinfection is provided (Rutala *et al.* 2008; Al-Adhan *et al.* 2013). BAC - benzalkonium chloride; GTA - glutaraldehyde; OPA - *ortho*-phthalaldehyde; PAA - peracetic acid; CUM - cuminaldehyde; EUG - eugenol; I3C - indole-3-carbinol

	BAC	GTA	OPA	PAA	CUM	EUG	I3C
S. aureus CECT976	1.5 ± 0.5	750 ±41	620 ± 21	750 ± 29	612 ± 25	950 ± 43	156 ± 43
S. aureus RN4220	3.0 ± 0.8	750 ± 29	700 ± 44	600 ± 20	700 ± 41	1000 ± 66	300 ± 59
S. aureus SA1199b	4 ± 0.4	800 ± 58	500 ± 61	750 ±47	600 ± 43	1300 ± 82	400 ± 80
S. aureus XU212	3 ± 0.7	750 ± 20	700 ± 34	600 ± 42	700 ± 32	1200 ± 90	400 ± 65
In-use concentration	1000-2000	20000	5500	2000	-	-	-