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BIOFOULING, 2016
VOL. 32, NO. 9, 1103–1114
http://dx.doi.org/10.1080/08927014.2016.1232402

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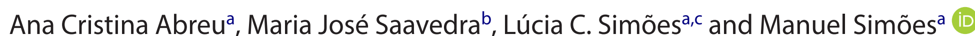
Combinatorial approaches with selected phytochemicals to increase antibiotic efficacy against *Staphylococcus aureus* biofilms



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ABSTRACT

Combinations of selected phytochemicals (reserpine, pyrrolidine, quinine, morin and quercetin) with antibiotics (ciprofloxacin, tetracycline and erythromycin) were tested on the prevention and control of *Staphylococcus aureus* biofilms. The phytochemicals were also studied for their ability to avoid antibiotic adaptation and to inhibit antibiotic efflux pumps. Morin, pyrrolidine and quercetin at subinhibitory concentrations had significant effects in biofilm prevention and/or control when applied alone and combined with antibiotics. Synergism between antibiotics and phytochemicals was found especially against biofilms of NorA overexpressing strain *S. aureus* SA1199B. This strain when growing with subinhibitory concentrations of ciprofloxacin developed increased tolerance to this antibiotic. However, this was successfully reversed by quinine and morin. In addition, reserpine and quercetin showed significant efflux pump inhibition. The overall results demonstrate the role of phytochemicals in co-therapies to promote more efficient treatments and decrease antimicrobial resistance to antibiotics, with substantial effects against *S. aureus* in both planktonic and biofilm states.

ARTICLE HISTORY
Received 10 May 2016
Accepted 31 August 2016

KEYWORDS
Antibiotic adaptation; antibiotic resistance; biofilms; efflux pump; phytochemicals; *Staphylococcus aureus*

Introduction

The widespread use of antibiotic-resistance elements among bacterial pathogens has made the treatment of some infections a serious concern. Indeed, many diseases that were once easily treatable have become deadly again (Brown & Wright 2016). It is clear from the past two decades of efforts that developing new, highly effective, safe and broad spectrum antibiotics has been an impossible task (IDSA 2011). New strategies are emerging to control microbial growth, including the use of combinations of therapeutic agents. The use of molecules antagonizing bacterial resistance mechanisms is a promising way to ensure the efficacy of antibiotics. Augmentin® (amoxicillin-clavulanic acid), Timentin® (ticarcillin-clavulanic acid), Unasyn® (ampicillin-sulbactam) and Tazocin® (piperacillin-tazobactam) are successful examples of combinatorial therapies effective against multidrug resistant strains (Abreu et al. 2013; Lewis 2013). More recently, combined treatments between antibiotics and plant secondary metabolites showed promising results and represent an increasingly active research topic (Abreu et al. 2012, 2014, 2015). Plant-based systems continue to play an essential role in healthcare, and their ethnopharmacological use has been extensively documented (Cragg & Newman 2013). Numerous reports can be found on non-antimicrobial plant secondary metabolites that act as adjuvants in therapy, especially due to the inhibition of microbial resistance mechanisms, but also due to an overall improved solubility and stability of the active compounds (Gibbons et al. 2003; Mullin et al. 2004; Oluwatuyi et al. 2004; Abreu et al. 2012). In previous studies, three alkaloids – reserpine, pyrrolidine and quinine – and two flavonoids – morin and quercetin – at sub-inhibitory concentrations have shown to potentiate ciprofloxacin, tetracycline and erythromycin against diverse antibiotic resistant strains of *S. aureus* in the planktonic state, including methicillin-resistant *S. aureus* (MRSA), presenting acceptable cytotoxicity (Abreu et al. 2014, 2015). Reserpine has been receiving much attention as an efflux pump inhibitor (EPI), despite some toxicity issues (Schmitz et al. 1998; Markham et al. 1999; Gibbons & Udo 2000). However, to the authors' knowledge, its effect was never tested against biofilms. Indeed, antimicrobial resistance is increased when microorganisms form biofilms (Olson et al. 2002; Burmolle

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et al. 2006; Verstraeten et al. 2008). It is estimated that > 80% of bacterial infections in humans involve the formation of biofilms (Brooun et al. 2000; Simoes et al. 2008; Busetti et al. 2010). Moreover, antimicrobial studies using planktonic cells despite having provided extensive information describing the mechanisms inhibiting bacterial growth may not be enough to predict therapeutic success (Pratt & Kolter 1998). In this work, combinations of phytochemicals and antibiotics were evaluated with respect to the prevention and control of *Staphylococcus aureus* biofilms. It may be expected that by combining antibiotics with an adjuvant, acting as biofilm inhibitor, as resistance-modifying agent, or even as a cell permeabilizer, the outcome would be an improved therapeutic benefit (Abreu et al. 2013). Further experiments were performed in order to understand the mode of action of these phytochemicals and whether the synergic combinations of phytochemicals and antibiotics were due to resistance modifying activity.

Materials and methods

Bacteria and culture conditions

S. aureus SA1199B, *S. aureus* RN4220 and *S. aureus* XU212, which overexpress the NorA, TetK and MsrA efflux pumps, respectively, were kindly provided by S. Gibbons (University College London, UCL) (Gibbons & Udo 2000; Gibbons et al. 2003; Oluwatuyi et al. 2004; Smith et al. 2007). The collection strain *S. aureus* CECT 976 was included as a quality control strain. The strains were stored at −80°C. Prior use, they were transferred to a Mueller–Hinton (MH, Merck Millipore, Germany) agar plate grown overnight and inoculated in MH broth at 37°C and under agitation (150 rpm). Bacterial susceptibility was assessed following the Clinical and Laboratory Standards Institute (CLSI 2003) guidelines as shown in Table 1.

Preparation of antibiotics and phytochemicals

Stock solutions of ciprofloxacin, erythromycin and tetracycline (Sigma, Sintra, Portugal) were prepared (at 10 g l^{-1}) according to the manufacturer's recommendations. Reserpine, pyrrolidine, quinine, morin and quercetin (Sigma) were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Stock concentrations were prepared at 10 g l^{-1} for quinine and reserpine and 50 g l^{-1} for morin, quercetin and pyrroldine. The compounds were stored at −4°C.

Table 1. Characteristics of the *S. aureus* strains.

Strains	Antibiotic	MIC (mg l^{-1})	Description
CECT 976	CIP TET ERY	1 (S) 0.96 (S) 0.24 (S)	From the Spanish Type Culture Collection; no antibiotic resistance described
SA1199B	CIP	128 (R)	Overexpresses NorA efflux pump
XU212	TET	128 (R)	Overexpresses the TetK efflux pump
RN4220	ERY	256 (R)	Contains plasmid pU5054 (that carries the gene encoding the MsrA efflux protein)

Notes: MIC were determined for each strain according to CLSI guidelines (CLSI 2003) and classified as resistant (R) or susceptible (S) to ciprofloxacin (CIP), tetracycline (TET) and erythromycin (ERY).

Biofilm formation, prevention and control

The minimal inhibitory concentration (MIC) of the antibiotics and phytochemicals were first determined against planktonic cells according to CLSI guidelines (CLSI 2003) and as described by Abreu et al. (2014, 2015). Antibiotics were applied at MIC, 10 × MIC and 50 × MIC against biofilms. The phytochemicals were found to have no antimicrobial activity for the concentrations tested (up to 1,500 mg l^{-1}, data not shown). Consequently, the concentrations applied corresponded to the optimal concentrations previously found to potentiate the antibiotics against the tested bacteria in planktonic state: 100 mg l^{-1} for reserpine and quinine and 500 mg l^{-1} for pyrrolidine, quercetin and morin (Abreu et al. 2014, 2015).

Antibiotics and phytochemicals were tested individually and in combination in order to assess their ability to control biofilms and prevent their formation. Overnight cultures adjusted to a cell density of 10^6 cells ml^{-1} were added to sterile 96-well polystyrene microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium) to form biofilms during 24 h at 37°C and 150 rpm. Afterwards, the medium was removed and the biofilms were exposed to the antibiotics, phytochemicals and their combinations (at 5% (v v^{-1}) of the well for a final volume of 200 μ l^{-1}), for 1 h at 37°C and 150 rpm. The same protocol was performed for an exposure time of 24 h. These assays tested the effects of two exposure times in the control of established biofilms.

In order to assess the combinations towards the prevention of biofilm formation, overnight cell suspensions were added to microtiter plates along with antibiotics, phytochemicals and their combinations (5% v v^{-1} of the well). The plates were incubated for 24 h at 37°C and 150 rpm. After incubation, biofilms were scraped and diluted in saline solution (0.85% NaCl). The numbers of colony forming units per unit of adhesion area (CFU cm^{-2}) were assessed in MH agar for both biofilm control and prevention experiments. The results are presented as a $\log_{10}$ CFU cm^{-2} reduction from the DMSO control.

A phytochemical-antibiotic synergy assay was performed according to Monzon et al. (2001). The combination was considered synergic when the $\log_{10}$ reduction CFU cm^{-2} caused by a combination was significantly higher ($p < 0.05$) than the sum of reductions by the

individual treatments. An antagonistic combination was characterized when the $\log_{10}$ reduction in CFU cm^{-2} of the combination was significantly lower ($p < 0.05$) than that obtained with the more active product.

Antibiotic adaptation assay

An antibiotic adaptation assay was performed with *S. aureus* SA1199B growing with increasing subinhibitory concentrations of ciprofloxacin for 15 days, according to Figure 1. The most promising phytochemical-antibiotic synergy results were obtained with this strain, and therefore it was selected for the antibiotic adaptation assay aiming to understand whether the phytochemicals were effective in reversing bacterial resistance or avoiding the development of resistance due to prolonged exposure. *S. aureus* SA1199B was grown overnight in MH broth at 37°C, adjusted to a cell density of 10^6 cells ml^{-1} and incubated with ciprofloxacin at 1/32 × MIC (4 mg l^{-1}, phase 1) for 24 h (at 37°C and 150 rpm agitation) in the presence of each phytochemical. After 24 h, the bacterium was refreshed: centrifugation was performed at 3,999 *g* for 10 min, the supernatant discarded, the pellet was washed twice and resuspended in MH broth with both molecules (at the same concentrations). This procedure was repeated after 48 h. On the fourth day, after washing the cultures, the concentration of the antibiotic was increased to 1/16 × MIC (8 mg l^{-1}, phase 2), and consecutively, for a total of 15 days, until a final concentration of 1/2 × MIC was reached. Controls were performed in the absence of phytochemicals or ciprofloxacin and with DMSO (5% v v^{-1}). Bacterial samples were taken every three days, washed as previously described and susceptibility profiles of bacterial populations to ciprofloxacin were evaluated by the disk diffusion method according to CLSI guidelines (CLSI 2003). The final population grown in the presence of only ciprofloxacin (named SA1199B(r)) was recovered after 15 days, washed and stocks were prepared for further

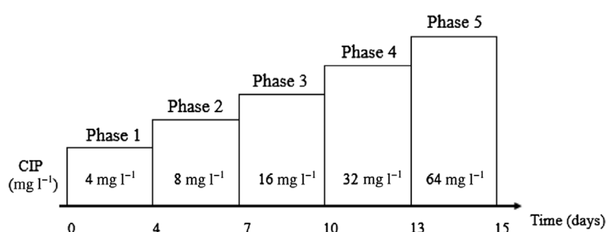

Figure 1. Scheme of ciprofloxacin treatment applied to *S. aureus* SA1199B for 15 days in order to assess bacterial adaptation. Bacterial cultures were incubated with defined subinhibitory concentrations of ciprofloxacin increasing every three days (from 1/32 × MIC to 1/2 × MIC) for a total of 15 days. Bacterial susceptibility to ciprofloxacin was determined in the end of each cycle by disk diffusion method (CLSI 2003).

experiments to assess efflux pump inhibition with ethidium bromide (EtBr). Triplicates of each combination were performed.

Disk diffusion method

Bacterial suspensions were adjusted to 0.5 McFarland standards and seeded over hardened MH agar in Petri dishes using a sterilized cotton swab. Sterile blank disks (6 mm diameter; Oxoid, Madrid, Spain) were placed on the agar plate seeded with the respective bacteria. A volume of 10 μl of ciprofloxacin prepared according to CLSI (2003) (ciprofloxacin −5 μg $disk^{-1}$) was added to the blank disks. Disks with 10 μl of DMSO were used as negative control. The plates were incubated at 37°C for 24 h. After incubation each inhibition zone diameter (IZD) was recorded and antibiotic susceptibility was analyzed according to CLSI guidelines (CLSI 2003). No inhibition zone was obtained with DMSO (data not shown). All tests were performed in triplicate.

Ethidium bromide accumulation assay

Ethidium bromide (EtBr) accumulation was tested in order to assess the putative action of phytochemicals as NorA inhibitors. First, the MIC of EtBr (prepared in a stock of 10 g l^{-1}) was first determined according to CLSI guidelines (CLSI 2003) and described by Abreu et al. (2014, 2015). The detection of EtBr accumulation by *S. aureus* strains was performed using a fluorometric method according to Rodrigues et al. (2013) and Jin et al. (2011). Briefly, bacteria were grown in MH broth at 37°C until mid-log phase (OD_{600} of 0.6 to 0.7). Bacterial suspensions were centrifuged at 3,999 *g* for 10 min, the supernatant was discarded, the pellet was washed in phosphate buffered saline (PBS; pH 7.4), and bacterial suspension adjusted to 10^8 CFU ml^{-1} with PBS. Aliquots of 100 μl of the bacterial suspensions were transferred to wells of a 96-well plate containing serial dilutions of EtBr at concentrations ranging from 80 to 0.06 mg l^{-1}. In order to assess the effect of phytochemicals on EtBr accumulation, EtBr (at ½ MIC, in order not to compromise the bacterial viability) was applied in the absence or presence of each phytochemical: reserpine and quinine at 100 mg l^{-1}; pyrrolidine, quercetin and morin at 500 mg l^{-1} (Abreu et al. 2014, 2015). The negative control was performed with 5% (v v^{-1}) DMSO. Also, controls for each phytochemical with EtBr in PBS were performed in order to detect possible fluorescence emitted by the products themselves. Fluorescence was acquired every 1 min for 60 min at 37°C in a microplate reader (Spectramax M2e, Molecular Devices, Inc., Sunnyvale, CA, USA) using 530 and 590 nm as excitation and detection wavelengths, respectively.

Statistical analysis

The results were analyzed by Student's *t*-test using SPSS (Statistical Package for the Social Sciences) version 19.0 (IBM Corp., Armonk, NY, USA). Statistical calculations were based on a confidence level 95% ($p < 0.05$) which was considered statistically significant. All *p*-values reported were two-tailed.

Results and discussion

Biofilms are recognized for their insusceptibility to current therapeutic approaches (Smith 2005; Römling & Balsalobre 2012). This is due to the different characteristics of biofilm cells compared to their planktonic counterparts (Simões et al. 2009). For instance, *S. aureus* in biofilms expresses a different set of genes such as *ica* types, and *bap* and *agr* types, with an increased potential to induce the development of recurrent infections (Gogoi-Tiwari et al. 2015). Therefore, new biofilm control strategies are required. Phytochemicals have been extensively studied as antibacterial products and, more recently, as potentiators/adjuvants of antibiotics (Markham et al. 1999; Abreu et al. 2012). However, few data can be found on their effects against biofilms, even though most infections are biofilm-related. The ability of phytochemicals and antibiotics to control 24 h old biofilms formed in sterile 96-well polystyrene microtiter plates within 1 and 24 h of exposure was evaluated. Antibiotics were first applied alone at MIC, 10 × MIC and 50 × MIC. As no significant differences ($p > 0.05$) in biofilm $\log_{10}$ CFU cm^{-2} reduction were obtained (data not shown), the combinations were

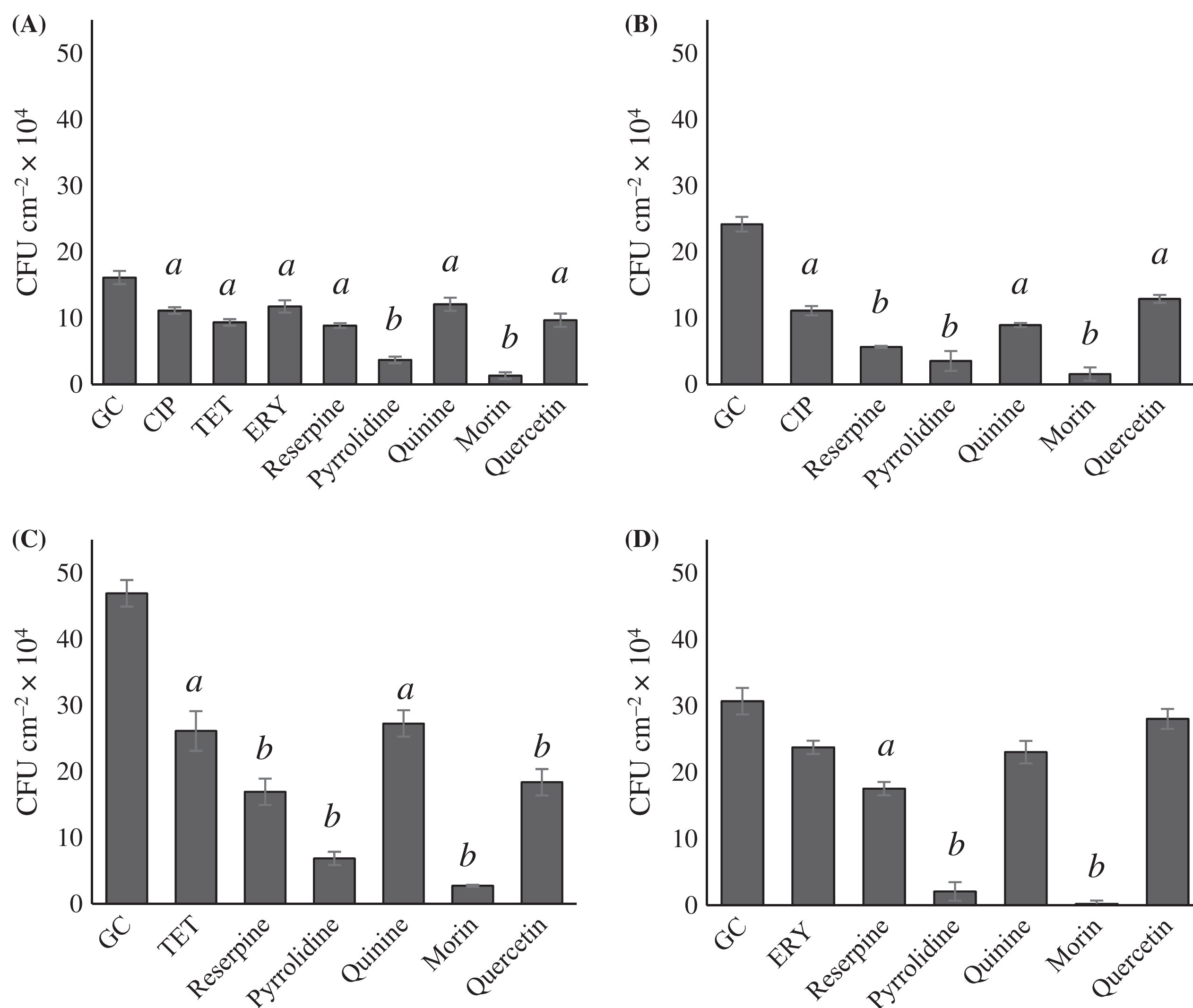

Figure 2. CFU cm^{-2} of biofilms after exposure to antibiotics and phytochemicals for 1 h: *S. aureus* CECT976 (A), SA1199B (B), XU212 (C) and RN4220 (D). Strains SA1199B, XU212 and RN4220 were only exposed to the antibiotics to which they are resistant: CIP, TET and ERY, respectively. Antibiotics were applied at MIC; reserpine and quinine were applied at 100 mg l^{-1}; pyrrolidine, morin and quercetin were applied at 500 mg l^{-1}. *a*, when statistically lower than GC ($p < 0.05$); *b*, when statistically lower than GC and the antibiotics ($p < 0.05$). GC, growth control (5%, v v^{-1} DMSO); CIP, ciprofloxacin; TET, tetracycline; ERY, erythromycin.

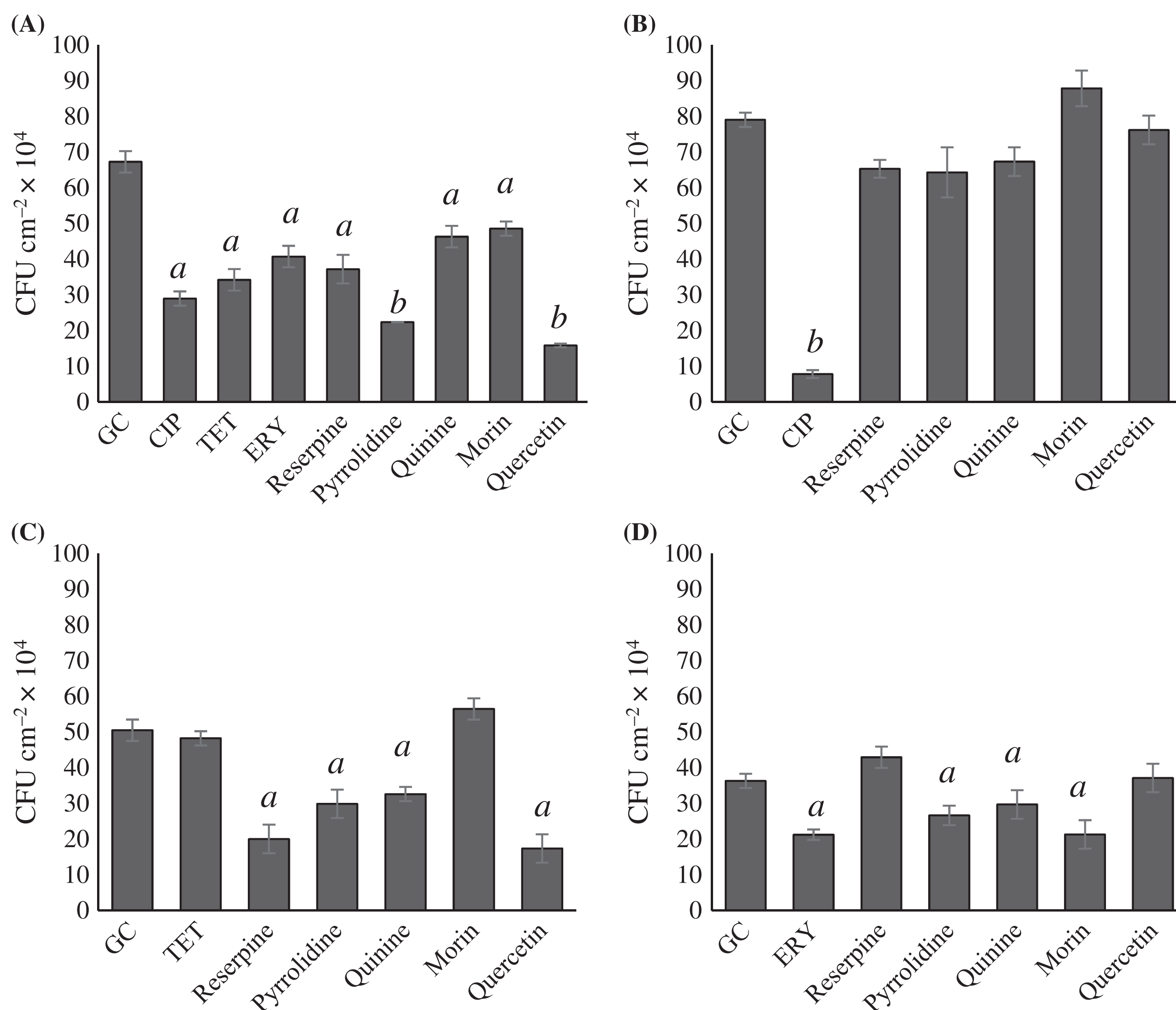

Figure 3. CFU cm^{-2} of biofilms after exposure to antibiotics and phytochemicals for 24 h. *S. aureus* CECT976 (A), SA1199B (B), XU212 (C) and RN4220 (D). Strains SA1199B, XU212 and RN4220 were only exposed to the antibiotics to which they are resistant: CIP, TET and ERY, respectively. Antibiotics were applied at MIC; reserpine and quinine were applied at 100 mg l^{-1}; pyrrolidine, morin and quercetin were applied at 500 mg l^{-1}. *a*, when statistically lower than GC ($p < 0.05$); *b*, when statistically lower than GC and the antibiotics ($p < 0.05$). GC, growth control (5%, v v^{-1} DMSO); CIP, ciprofloxacin; TET, tetracycline; ERY, erythromycin.

performed with antibiotics at their MIC. Figures 2 and 3 present the number of CFU cm^{-2} in the biofilm after incubation with antibiotics at their MIC and phytochemicals individually for 1 and 24 h, respectively. Phytochemicals were applied at subinhibitory concentrations that were previously found to cause antibiotic-potentiation (Abreu et al. 2014, 2015). Morin at 500 mg l^{-1} applied for 1 h (Figure 2) caused the highest CFU reductions ($\log_{10}$ CFU cm^{-2} reductions of 1.2 - 2.1 for all strains), followed by pyrrolidine (at 500 mg l^{-1}, $\log_{10}$ CFU cm^{-2} reductions of 0.7 - 1.1). These reductions were higher ($p < 0.05$) than those obtained with antibiotics applied at MIC ($\log_{10}$ CFU cm^{-2} reductions of 0.08–0.4). These results proposed that the selected phytochemicals caused biofilm disruption as they did not show antimicrobial activity by themselves. However, morin and pyrrolidine were not effective ($p < 0.05$) in disturbing biofilms for a 24 h exposure period, for which the maximal $\log_{10}$ CFU cm^{-2} reductions were 0.3 and 0.5, respectively (Figure 3). The decreased susceptibility to prolonged exposure periods was generally observed for almost all the strains. This is probably due to the effects of longer incubation periods. The fast growing cells will die readily when exposed to the antibiotics but the dormant cells fraction can survive and replicate (Shafahi & Vafai 2010). The exception was verified with strain SA1199B when exposed to ciprofloxacin, where the $\log_{10}$ CFU cm^{-2} reduction increased with the longer exposure from 0.4 to 1.1.

Additionally, in order to assess the ability of the phytochemicals to prevent bacterial adhesion and biofilm

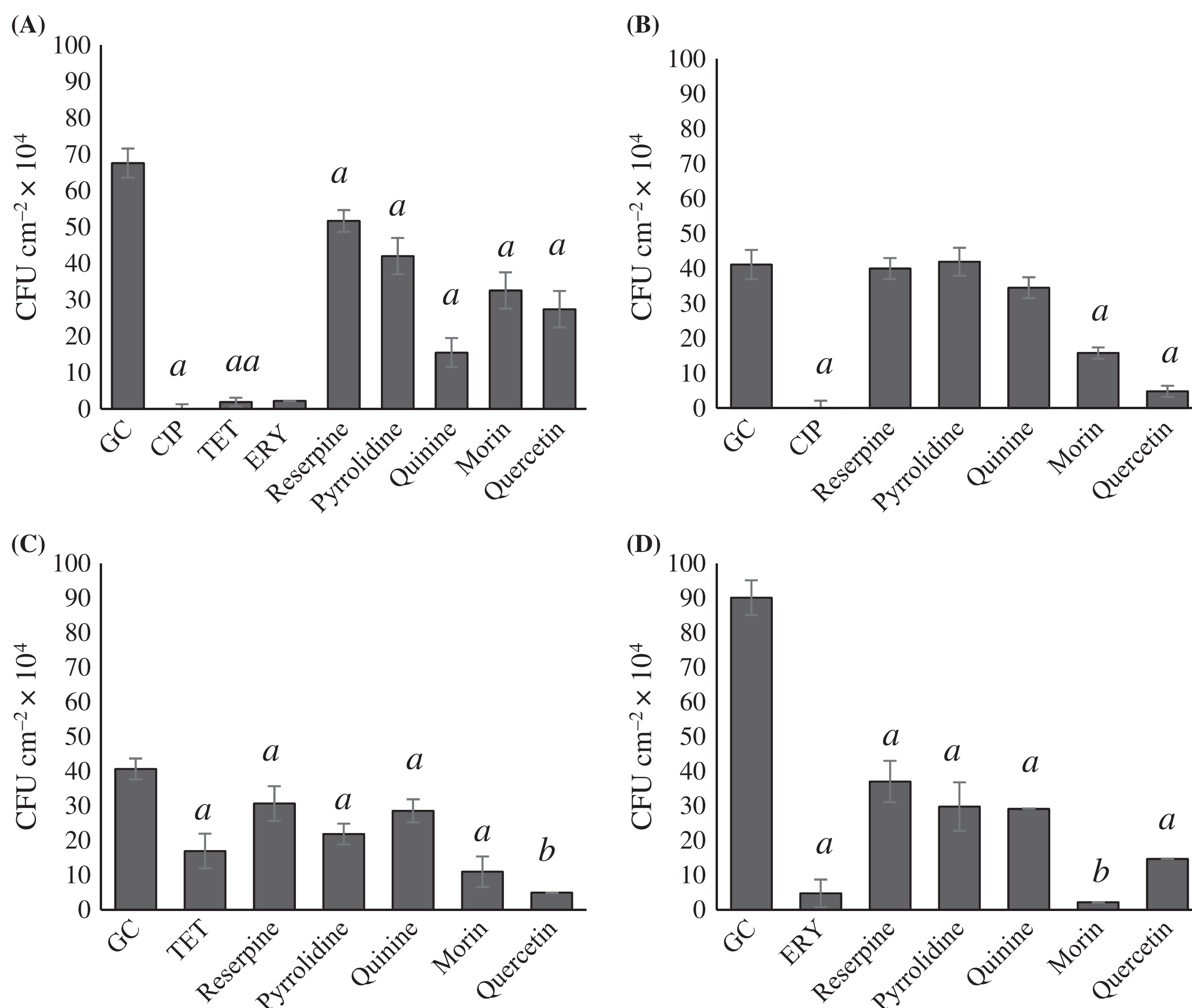

Figure 4. CFU cm^{-2} of *S. aureus* CECT976 (A), SA1199B (B), XU212 (C) and RN4220 (D) biofilms after 24 h growth in the presence of antibiotics and phytochemicals. Strains SA1199B, XU212 and RN4220 strains were only exposed to the antibiotics to which they are resistant to: CIP, TET and ERY, respectively. Antibiotics were applied at MIC; reserpine and quinine were applied at 100 mg l^{-1}; pyrrolidine, morin and quercetin were applied at 500 mg l^{-1}. *a*, when statistically lower than GC ($p < 0.05$); *b*, when statistically lower than GC and the antibiotics ($p < 0.05$). GC, growth control (5%, v v^{-1} DMSO); CIP, ciprofloxacin; TET, tetracycline; ERY, erythromycin.

formation, tests with sessile cells were performed in the presence of the phytochemicals for 24 h. Figure 4 presents the number of CFU cm^{-2} that was able to adhere to polystyrene when grown in the presence of the antibiotics and phytochemicals for 24 h. All antibiotics (at MIC) caused significant prevention of biofilm formation – a reduced number of CFU cm^{-2} compared to growth control ($p < 0.05$). Morin was able to reduce biofilm formation of all strains ($p < 0.05$), especially of RN4220 (1.4 log_{10} CFU cm^{-2} reduction). Additionally, quercetin at 500 mg l^{-1} also had a considerable effect in preventing biofilm formation ($p < 0.05$), particularly of strains SA1199B and XU212 (~1 log_{10} CFU cm^{-2} reduction). Pyrrolidine was unable to prevent biofilm formation, as it did not affect biofilm removal when applied for 24 h, despite showing significant effects when applied for 1 h ($p < 0.05$). No antibiotic or phytochemical caused total biofilm control or prevention. This reinforces the higher tolerance of biofilm cells compared to their planktonic counterparts. Studies report that even cells that are merely adhered to a surface have more resistant phenotypes (Bridier et al. 2011). However, it seems that among all the phytochemicals, morin was particularly effective in controlling (within 1 h of incubation) and inhibiting biofilm formation. Morin can therefore be added to the reduced list of phytochemicals with potential to interfere with the process of biofilm formation and development (Simões et al. 2009).

Table 2 presents the log_{10} CFU cm^{-2} reductions caused by the phytochemical–antibiotic combinations applied to all strains. The maximum CFU reduction was obtained after 24 h exposure to the synergic combination of ciprofloxacin and quinine (2.1 log_{10} CFU cm^{-2} reduction) against SA1199B biofilms. Additionally, a combination of

Table 2. Log_{10} CFU cm^{-2} reductions after biofilm exposure to antibiotic-phytochemical combinations.

		Log_{10} CFU cm^{-2} reductions														
		A - Biofilm control (1 h)					B - Biofilm control (24 h)					C - Biofilm prevention				
Strains	Antib.	Res	Pyr	Qui	Mor	Querc	Res	Pyr	Qui	Mor	Querc	Res	Pyr	Qui	Mor	Querc
CECT 976	CIP	0.29	0.91	0.36	1.29	0.45	0.33	0.52	0.48	0.05 (A)	0.03 (A)	3.21	3.34	3.51	1.42 (A)	1.13 (A)
	TET	0.49	0.81	0.42	0.99	0.05 (A)	0.28	0.24 (A)	0.25	0.04 (A)	0.04 (A)	1.43	0.97 (A)	1.41	1.33	1.06 (A)
	ERY	0.28	**1.44 (S)**	0.26	0.64 (A)	0.28	0.49	0.47	0.48	0.18	0.28 (A)	1.51	**2.09 (S)**	1.50	1.17	1.18
SA1199B	CIP	0.37	1.10	0.53	0.98 (A)	0.57	**1.30 (S)**	**1.71 (S)**	**2.10 (S)**	1.05	0.92	**3.52 (S)**	2.7	3.05	3.43	2.11
XU212	TET	0.33	0.93	0.29	0.92 (A)	0.37	0.23	0.16	0.22	0.33 (S)	0.11	0.70(S)	**1.55 (S)**	0.48	**1.12 (S)**	1.16
RN4220	ERY	0.01	1.08	0.24 (S)	1.03 (A)	0.47 (S)	0.36	0.39	0.49	0.29	0.05 (A)	1.08	1.03	1.07	1.26	0.84

A – biofilm exposure to antibiotics and phytochemicals for 1 h; B – biofilm exposure to antibiotics and phytochemicals for 24 h; C – biofilm grown in the presence of antibiotics and phytochemical/biofilm prevention. The combination was considered synergic (S) when a log_{10} CFU cm^{-2} reduction by the combination was significantly higher ($p < 0.05$) than the sum of log_{10} CFU cm^{-2} reductions in individual treatments (Monzon et al. 2001). An antagonistic (A) combination was obtained when the log_{10} CFU cm^{-2} reduction in the combination was significantly lower ($p < 0.05$) than that obtained with the most effective product. Bold values represent synergic interactions with a log_{10} CFU cm^{-2} reduction ≥ 1.0.

*Pyr, Mor, Querc: 500 mg l^{-1}; Res, Quin: 100 mg l^{-1}. CIP, ciprofloxacin; TET, tetracycline; ERY, erythromycin; Res, reserpine; Pyr, pyrrolidine; Quin, quinine, Mor, morin; Querc, quercetin.

ciprofloxacin with reserpine, also considered synergic, was the most efficient in preventing biofilm formation (a 3.5 log_{10} CFU cm^{-2} reduction) of strain SA1199B. Other synergic antibiotic–phytochemical combinations were found, with the following causing ≥ 1 log_{10} CFU cm^{-2} reduction: ciprofloxacin combined with pyrrolidine and reserpine (a log_{10} CFU cm^{-2} reduction of 1.7 and 1.3, respectively) to control SA1199B biofilms exposed for 24 h; erythromycin combined with pyrrolidine to control biofilms of CECT976 exposed for 1 h (a 1.4 log_{10} CFU cm^{-2} reduction) and also to prevent their formation (a 2.1 log_{10} CFU cm^{-2} reduction); and tetracycline combined with pyrrolidine and morin to prevent biofilm formation of XU212 (a log_{10} CFU cm^{-2} reduction of 1.6 and 1.1, respectively). The majority of these synergic results corroborate previous studies with planktonic cells (Abreu et al. 2014, 2015). Some antagonistic results were obtained, especially for combinations involving the phenolic compounds. It seems that the presence of antibiotics disturbs the activity of morin, which was found to be effective when applied individually for 1 h. Indeed, biofilms facilitate the spread of antibiotic resistance by promoting horizontal gene transfer and cells can switch to more tolerant phenotypes upon environmental stress (Fux et al. 2005). Antagonistic combinations were also found with quercetin when combined with antibiotics mainly against CECT976 biofilms. Other discrepancies in the effects against the diverse strains were found, such as the fact that combinations of tetracycline with morin and tetracycline with pyrrolidine were synergic against XU212 but antagonistic against CECT976 in a biofilm control for 24 h and biofilm prevention, respectively. This strain-dependent susceptibility can be related to the impact of combined stresses on their susceptibility/resistance patterns and it would need a more integrated approach in order to be fully understood. Antibiotic-mediated interactions may trigger multicellular behavior in bacteria, which makes it impossible to predict cell responses. The remaining combinations tested had indifferent effects on biofilm prevention and control.

Since most synergic interactions were obtained against strain SA1199B (a NorA overexpressing strain), a further experiment was conducted to induce adaptation of SA1199B to ciprofloxacin. The phytochemicals were also used to understand their effects in reversing bacterial adaptation to ciprofloxacin after long exposure times. Figure 5 shows the results obtained in the adaptation assay with SA1199B to ciprofloxacin (the treatment was performed as previously indicated in Figure 1). By growing this strain with subinhibitory concentrations of ciprofloxacin (control assay) it was possible to observe a reduction in the IZD until day 9 (phase 3), and then no IZD was detected (phases 4 and 5), indicating that bacteria became more resistant to ciprofloxacin. Biological responses induced in bacteria when antibiotics are applied at subinhibitory concentrations can affect various cellular responses or alter gene expression leading to different adaptive responses impacting antibiotic resistance/tolerance (Kaplan et al. 2012; Bernier & Surette 2013). A potential increased NorA overexpression could explain the improved tolerance to ciprofloxacin observed by strain SA1199B. In phases 2 and 3, it was possible to observe that the populations growing in the presence of all phytochemicals were more susceptible, since IZD promoted by ciprofloxacin were significantly higher ($p < 0.05$) than IZD obtained when bacteria were growing only in the presence of ciprofloxacin. According to CLSI guidelines (CLSI 2003) IZD obtained in phase 3 allows characterization of the bacterial cultures as susceptible to ciprofloxacin. This susceptibility was observed earlier for quercetin growing-population (in phase 1). This means that until day 9, all the phytochemicals were able to reverse the bacterial resistance mechanisms. However, by increasing ciprofloxacin concentrations to ¼ and ½ MIC (phases 4 and 5, respectively), all populations were resistant to

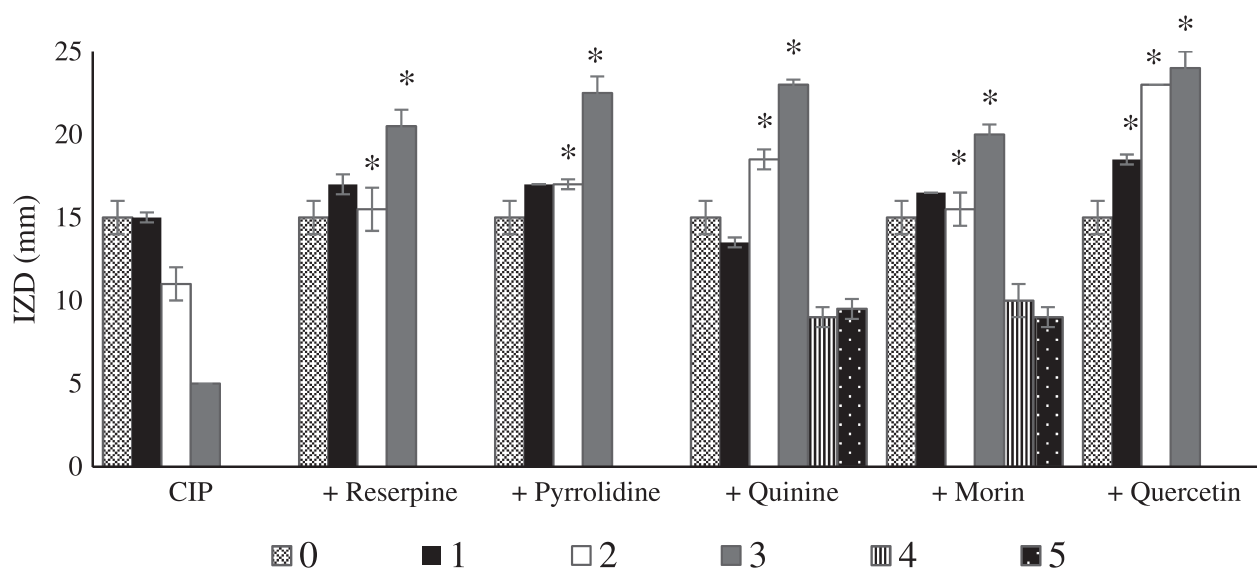

Figure 5. Inhibition zone diameter (IZD) promoted by ciprofloxacin against *S. aureus* SA1199B grown in the presence of increasing subinhibitory concentrations of ciprofloxacin (CIP) alone and in the presence of phytochemicals. Reserpine and quinine were applied at 100 mg l^{-1}; pyrrolidine, morin and quercetin at 500 mg l^{-1}. Results are given for the five different phases of this assay by increasing ciprofloxacin concentrations every three days (from 1/32 MIC to 1/2 MIC) over a total of 15 days, according to Figure 1. Bars with (*) presented significantly higher inhibition zone diameters ($p < 0.05$) compared to the ciprofloxacin control population, for each phase.

ciprofloxacin similar to the control (no IZD detected), with the exception of bacteria growing in the presence of quinine and morin. The results indicate that both phytochemicals were successful in preventing tolerance to ciprofloxacin by strain SA1199B ($p < 0.05$).

The potential of the phytochemicals to inhibit NorA was tested using EtBr, a substrate widely applied for detecting efflux activity in *S. aureus* strains (Costa et al. 2013). EtBr form complexes with double stranded DNA and RNA by intercalating between base pairs (Walberg et al. 1999). Several products, such as reserpine, behave as if they inhibit efflux pumps and hence have become known EPI (Holler et al. 2012). Accumulation of EtBr inside the bacterial cells can be increased in the presence of an EPI (Mullin et al. 2004; Holler et al. 2012) and can be measured fluorometrically in SA1199B cells due to the retention of fluorescence over time. Also, in order to understand if overexpression of NorA is the reason for the increased tolerance of bacterial cells growing with ciprofloxacin for 15 days, this population, named SA1199B(r), was included in the experiments. The susceptible strain CECT976 was also tested as negative control. The MIC of EtBr was first determined for each strain: 5 mg l^{-1} for CECT976 and 40 mg l^{-1} for SA1199B and SA1199B(r). Due to the good sensitivity of the fluorometric method, the demonstration of the effects of reserpine on the accumulation of EtBr on strain SA1199B over time was readily made. Figure 6A shows the comparison between EtBr accumulation in strains CECT976, SA1199B and SA1199B(r). CECT976 accumulated more EtBr contrarily to SA1199B and SA1199B(r), both overexpressing NorA. There were no significant differences between accumulation by SA1199B and SA1199B(r) ($p > 0.05$), suggesting that the higher resistance of this strain to ciprofloxacin may be due to the expression of resistance mechanisms other than overexpression of NorA efflux pump. Figure 6B shows the effect of several concentrations of reserpine on EtBr accumulation. Reserpine at 20 mg l^{-1} is usually used as reference (Schmitz et al. 1998; Couto et al. 2008). Figure 6C shows the accumulation of EtBr by SA1199B in the presence of the phytochemicals. Quercetin (at 500 mg l^{-1}) showed the best accumulation results ($p < 0.05$). Quinine (at 100 mg l^{-1}) also improved EtBr accumulation ($p < 0.05$), suggesting that this compound inhibited the NorA efflux pump, apparently inducing higher tolerance of SA1199B to ciprofloxacin. Morin (at 500 mg l^{-1}) had no effect on EtBr accumulation, but it was able to prevent bacterial adaptation to ciprofloxacin, suggesting that this product is apparently involved in the inhibition of other mechanism, excluding the NorA efflux pump. Interestingly, the difference in the structures between morin and quercetin is that the first is 2′,4′-dihydroxylated in B ring while the second is 3′,4′-dyhydroxylated. However, it is already known that different substitutions on phenolic rings can promote significantly different activities (Cushnie & Lamb 2005; Kumar & Pandey 2013). Indeed, Tsuchiya et al. (1996) indicated that 2′,4′- or 2′,6′-dihydroxylation of the B ring and 5,7-dihydroxylation of the A ring in the flavanone structure was important for anti-MRSA activity. Previous findings using flavonols, such as fisetin, quercetin, and kaempferol described their anti-biofilm activity against *S. aureus* (Lee et al. 2013). In addition, it was found previously that several

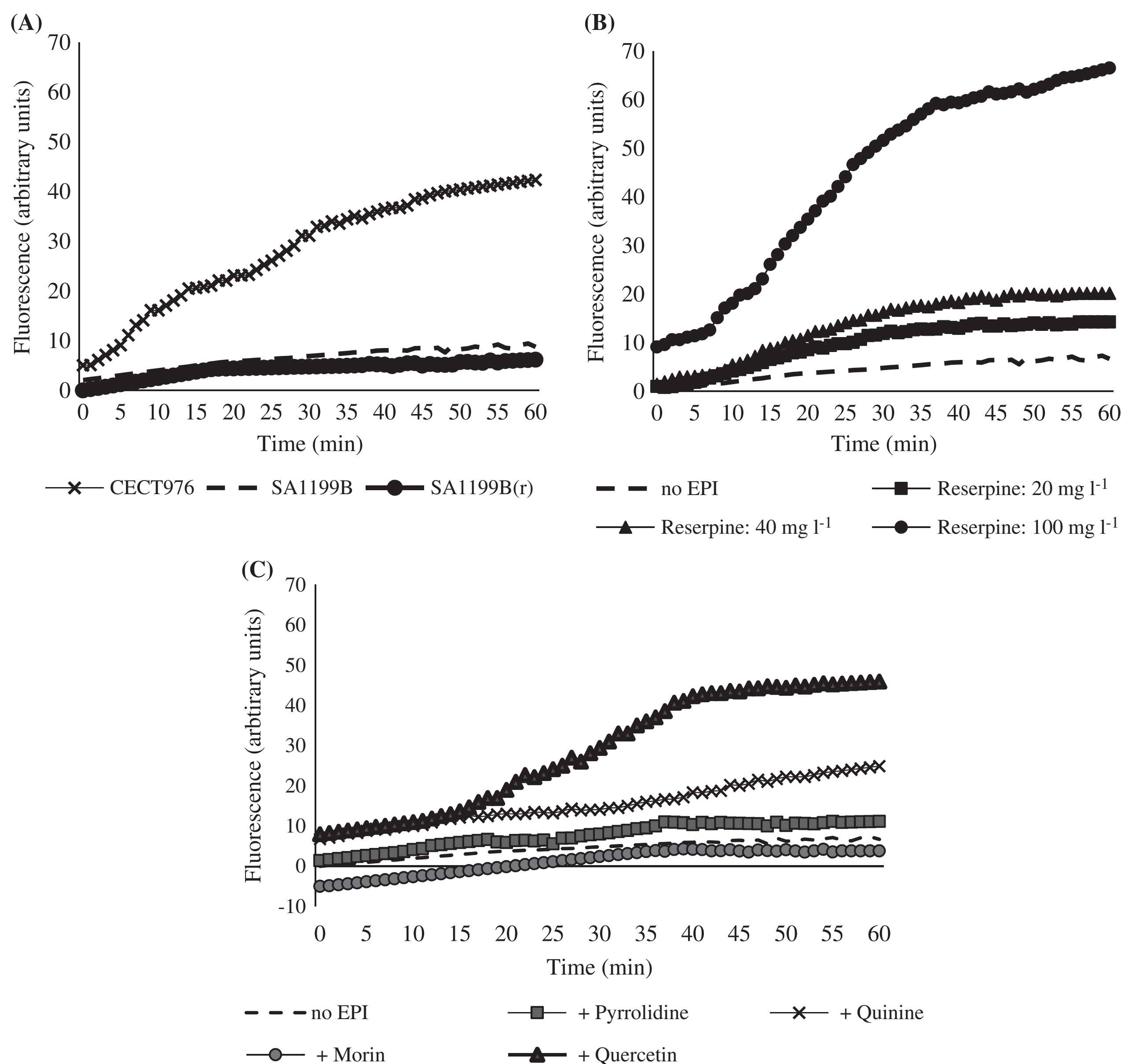

Figure 6. Fluorescence as a measure of EtBr accumulation. (A) EtBr accumulation in *S. aureus* CECT976, SA1199B and SA1199B(r); (B) effects of reserpine at 20, 40 and 100 mg l^{-1} on EtBr accumulation; (C) effects of phytochemicals on EtBr accumulation in *S. aureus* SA1199B. The bacteria were loaded with EtBr at ½ MIC in the presence of different phytochemicals for a period of 1 h at 37°C. Mean values of three replicates are shown.

flavonoids, particularly quercetin, reduced hemolysis by *S. aureus* which would reduce its virulence and ability to form biofilms (Caiazza & O'Toole 2003). Pyrrolidine did not improve EtBr accumulation neither was able to avoid or increase resistant of SA1199B strain ($p > 0.05$). However, it caused biofilm removal when applied for 1 h.

Over the last decade there has been a resurgence of interest in the search for products that will restore the activity of licensed antimicrobial agents (Abreu et al. 2012). The prospects of finding useful plant-derived products are enormous. Many resistant mechanisms to antimicrobials, such as the ability to pump antibiotics out of cells, are shared between clinical and environmental bacteria (Wright 2010). Therefore, products that interfere with efflux of active inhibitors from the cell or other resistance mechanisms may be easily found in nature. Studies on plants allowed the discovery of interesting efflux pump inhibitors. This is an attractive strategy for the design of novel therapeutic approaches. With this study, known phytochemicals are highlighted for their important activities in co-therapies with known antibiotics against *S. aureus* biofilms.

In conclusion, despite being structurally different and presenting diverse activities, all the phytochemicals

tested showed interesting results, highlighting the use of these substances as antibiotic adjuvants and resistance modifying agents for antimicrobial therapy against planktonic and sessile *S. aureus*. Morin applied for 1 h showed the best biofilm reduction and significantly inhibited biofilm formation of all the strains tested. Interestingly, morin has been studied for other medical applications as an inhibitor of amyloid β-peptide aggregation, whereas other hydroxyflavones such as quercetin failed to be effective (Lemkul & Bevan 2012; Noor et al. 2012). Pyrrolidine had a significant effect in controlling biofilms with a 1 h exposure, while quercetin was highlighted for preventing biofilm formation. Also, several significant synergic combinations of antibiotics with the phytochemicals were obtained, notably the combinations between ciprofloxacin and quinine to control SA1199B biofilms (24 h exposure with a 2.1 $\log_{10}$ CFU cm^{-2} reduction) and between ciprofloxacin with reserpine to prevent SA1199B biofilm formation (a 3.2 $\log_{10}$ CFU cm^{-2} reduction). These effects were dependent on the incubation time as longer exposure times and at non-lethal antibiotic doses, bacteria showed increased antibiotic tolerance. All the phytochemicals were able to increase the susceptibility of SA1199B to ciprofloxacin for nine days incubation, but after that only quinine and morin were successful in reducing antibiotic tolerance. Quercetin promoted the highest EPI activity in the SA1199B strain. In addition to the promising results promoted by selected phytochemicals in biofilm prevention and control, due to their extensive functional group chemistry and chirality those products are potentially interesting scaffolds for the discovery and development of antibacterial therapeutic approaches.

Acknowledgements

The authors are grateful to Professor Simon Gibbons (Department of Pharmaceutical and Biological Chemistry, The School of Pharmacy, UCL School of Pharmacy, London) for providing the bacterial strains.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was financially supported by: Laboratory for Process Engineering, Environment, Biotechnology and Energy (LEPABE) funded by FEDER funds through COMPETE2020-Programa Operacional Competitividade e Internacionalização (POCI) [project POCI-01-0145-FEDER-006939]; and by national funds through Fundação para a Ciência e a Tecnologia (FCT)/MEC [grant numbers SFRH/BD/84393/2012 and SFRH/BPD/81982/2011].

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