Full paper

Antimicrobial and anti-biofilm properties of novel synthetic lignan-like compounds.
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Running title: antimicrobial properties of a lignan-like furan

Summary
Antibiotic resistance and biofilm tolerance are among the principal factors involved in the persistence of chronic infections. The need for new antimicrobials is an ever-increasing challenge in clinical environments and in the control of global health. Arylfurans form a set of structures that have been identified in many natural products, e.g. lignans. Lignans are a sub-group of non-flavonoid polyphenols that play an active role in plants’ defense against bacteria and fungi infections. The aim of this study was to identify novel synthetic arylfurans and lignan-like arylbenzylfurans exhibiting antimicrobial properties. The molecules synthetized were tested against Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and S. epidermidis. We found that among tested compounds, arylbenzylfuran 11 was active against S. aureus and S. epidermidis with an MIC of 4 µg ml⁻¹. Compound 11 was also active on methicillin-resistant S. aureus and S. epidermidis. By confocal laser scanning microscopy, we showed that 32 µg ml⁻¹ of compound 11 was able to induce a significant reduction in S. aureus and S. epidermidis biofilms viability. Finally, we demonstrated that compound 11 was not cytotoxic on HaCat cells up to 128 µg ml⁻¹. This work shows the antimicrobial and anti-biofilm potential of a synthetic lignan-like furan.

Keywords: furan, lignan, Staphylococcus aureus, Staphylococcus epidermidis, MIC, biofilm.
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Introduction
The incidence of infections associated with skin, soft tissues and/or the use of medical devices is increasing significantly (Gutierrez et al., 2013). For this reason, the scientific community is actively searching for novel alternatives to prevent or solve this problem (Buommino et al., 2014; Barra et al., 2015). *Staphylococci*, especially *S. aureus* and *S. epidermidis*, are the bacteria most frequently isolated from skin and soft tissue infections following trauma, burns, surgery (Watanabe et al., 2017), blood-borne infections (Tong et al., 2015) and/or implant surfaces due to their ability to form biofilms (Otto, 2017). Bacterial biofilms are highly organized surface-associated communities of bacteria cocooned within a self-produced extracellular matrix. The nature of the matrix and the metabolic status of bacteria embedded in it make most of the currently used antimicrobial drugs ineffective. Several *in vivo* studies on wound models, or *in vitro* studies using keratinocyte cultures, have shown that biofilm formation severely delays the healing process and promotes chronicization of the lesion (Hall et al., 2017; Tankersley et al., 2014; Zhao et al., 2012). The cogent problem of drug-resistant bacteria highlights the need to discover new compounds with improved activity against planktonic as well as sessile microorganisms. To provide alternative molecular scaffolds with the potential to inhibit the formation of bacterial biofilms, we considered lignans, a class of natural antimicrobial products, as a source for structural insight to guide the molecular design of new synthetic compounds. Lignans are characterized by a broad range of biological activities, including antimicrobial, anticancer, antioxidant, and anti-inflammatory. There is a currently renewed interest in this class of compounds due to the continuous discovery of novel pharmacologically active derivatives, either natural or synthetic (Teponno, 2016). Although there is wide structural diversity, the basic lignan backbone consists of two aryl propanoidic (C6-C3) units, generally linked by a β,β’ bond. The structural motif we investigated is based on furan-containing lignan-like compounds. The use of this five-membered heterocyclic ring was motivated by the presence of the furan ring in a variety of natural products and by the synthetic versatility of this system (Li, 2013; Wong et al. 2008; Merino et al., 2007). Furans undergo a wide range of reactions that allow the introduction of various functional groups and/or their conversion into reduced forms such as tetrahydrofurans, or oxidated forms such as furanones or enediones (Merino et al., 2007; Montagnon et al. 2008). Some furans and derivatives have been found to have antibiotic activity (Monasterios et al. 2006, Wang et al. 2008; Alaby and Owolabi, 2012; Kayumov et al., 2015) and, in particular, aryl furan moieties are already present in some pharmaceuticals (Zhou et al., 2004). In this study, arylfurans, namely methyl 2-arylfurans, namely methyl 2-arylfurans, namely methyl 2-aryl-4-hydroxymethyl-3-carboxylates, were used as a suitable scaffold to obtain arylbenzylfurans with a typical lignan backbone. The antimicrobial properties of lignan-like compounds and of the starting arylfurans were investigated.
Materials and Methods

Preparation and characterization of furan derivatives

Arylfurans 1-3 and arylbenzylfurans 4-11 were synthesized as reported in Iesce et al. (Iesce et al., 2016) while new derivatives 12-14 were prepared as follows. All reagents (triphenylphosphine oxide (Ph3PO), trifluoromethylsulfonic anhydride (Tf2O), phenol) and solvents used in the following were obtained from commercial suppliers (Sigma-Aldrich). Trifluoromethylsulfonic anhydride (Tf2O) (0.1 ml, 0.6 mmol) was added to a solution of triphenylphosphine oxide (Ph3PO) (0.6 mmol) in dry dichloromethane (CH2Cl2) (1 ml) at 0°C and the mixture was stirred for 15 min at r.t. Then, phenol (0.5 mmol) and furan 2 (0.5 mmol in 1 ml of dry CH2Cl2) were added and the mixture was stirred. Upon completion of the reaction (1 h), the organic solvent was evaporated and the residue was obtained by preparative silica gel thin-layer chromatography (TLC) using ethyl acetate-CH2Cl2, 10% as eluent, to give pure 12 (28%) (spectral data are reported in supporting information S1). Using furan 3 and phenol, the above procedure gave a mixture of 13 and 14 that was further subjected to preparative TLC (ethyl acetate- CH2Cl2, 10% as eluent) to give pure 13. (spectral data are reported in supporting information S1).

Bacterial strains and culture

P. aeruginosa ATCC 27853, E. coli ATCC 25922, S. aureus ATCC 12600, and S. epidermidis ATCC 35984 were obtained from the American Type Culture Collection (Rockville, MD). Clinical strains of methicillin-resistant S. aureus (MRSA), and methicillin-resistant S. epidermidis (MRSE) were obtained from specimens of patients admitted to the University Hospital Federico II, Naples. Identification was performed by sub-culturing on Tryptic Soy Agar (TSA, Becton Dickinson) and by biochemical characterization using the Vitek II system (Biomerieux). The antimicrobial susceptibility testing of all isolates was determined by using the Vitek II system and results were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST version 7.1, 7 June 2017). P. aeruginosa, E. coli and S. epidermidis were cultured in Luria-Bertani broth (LB, OXOID), and S. aureus was cultured in tryptic soya broth (TSB, OXOID), under aerobic conditions at 37°C, for 24 h on an orbital shaker at 200 rpm. Each tested compound was dissolved in dimethylsulfoxide (DMSO, Sigma, Milan) and diluted in LB or TSB broth to give a stock solution.

Susceptibility assays on planktonic cells

Minimal inhibitory concentrations (MICs) of the tested compounds were determined in LB and TSB medium by the broth micro-dilution assay, according to the European Committee on Antimicrobial Susceptibility Testing. Bacterial suspensions were diluted to yield an optical density (OD) around 0.5 at 595 nm and further diluted to a final concentration of 1 × 10^6 CFU ml⁻¹. The compounds were added to bacterial suspension in each well, yielding a final cell concentration of 5 × 10^5 CFU ml⁻¹ and a final compound concentration ranging from 2 μg ml⁻¹ to 128 μg ml⁻¹. Negative control wells
were set to contain bacteria in LB or TSB plus the amount of DMSO used to dilute each compound. Positive controls included tobramycin and vancomycin (ranging from 0.25 μg ml\(^{-1}\) to 4 μg ml\(^{-1}\)). Medium turbidity was measured by a microtiter plate reader (Tecan, Milan, Italy) at 595 nm. Absorbance was proportional to bacterial growth.

**Cytotoxicity on eukaryotic cells (MTT assay)**

Cytotoxicity assays were performed on human, immortalized keratinocytes (HaCat), cultured in DMEM supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine, 100 IU ml\(^{-1}\) penicillin, and 100 μg ml\(^{-1}\) streptomycin, at 37°C in a humidified atmosphere of 5% CO\(_2\) and 95% air. Semi-confluent HaCat cells (10\(^3\)/well), were treated or not with compound 11 or compound 13 at 2, 4, 8, 16, 32, 64 and 128 μg ml\(^{-1}\). Cells were grown in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 μl DMEM. After 8, 24, and 48 h, 10 μl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) labelling reagent (Roche Diagnostics, Basel, Switzerland; final concentration 0.5 mg ml\(^{-1}\)) was added to each well. Four hours later, 100 μl of a solubilization solution (10% SDS in 0.01 M HCl) was added and the plates were incubated overnight. Absorption was measured using a microplate ELISA reader (Biorad) at 570 nm. HaCat control cells were incubated in DMSO-PBS. One millimolar of hydrogen peroxide (H\(_2\)O\(_2\)) was used as positive control (Mosmann, 1983).

**Killing rate**

Time kill assays for compound 11 on *S. aureus* ATCC 12600 were carried out as previously described in Olajuyigbe, et al. (Olajuyigbe, et al., 2012) with minor modifications. Bacterial suspension (10\(^5\) CFU ml\(^{-1}\)) was added to the microplates along with compound 11 (4 μg ml\(^{-1}\)). The plates were incubated at 37°C on an orbital shaker at 120 rpm. Viability assessments were performed at 0, 2, 4, 6, and 24 h at 37°C by plating 0.1 ml undiluted and 10-fold serially diluted samples onto LB plates in triplicate. After incubation, bacterial colonies were counted and compared with counts from control cultures.

**Biofilm formation assay**

Biofilm formation was evaluated by measuring the ability of cells to adhere to sterile 96-well polystyrene flat-bottom microtiter plate (BD Falcon, Mississauga, Ontario Canada) as described by Stepanović with minor modifications (Stepanović et al., 2007). Briefly, a suspension of *S. aureus* or *S. epidermidis* (TSB supplemented with 0.5% glucose) at the final density of 10\(^5\) CFU ml\(^{-1}\) was treated with compound 11 at sub-MIC concentrations ranging from 0.5 μg ml\(^{-1}\) to 2 μg ml\(^{-1}\). As positive controls, bacteria were incubated with 100 μl of medium alone. After 24 h at 37°C, the cell suspension was removed and the wells were washed twice with phosphate-buffered saline (PBS) and dried at 60°C for 30 min. Crystal violet solution (150 μl at 0.1%) was added to each well and the plates were incubated at room temperature for 30 min. The wells were then washed with PBS and discolored with
200 μl of 99% ethanol for 20 min. Absorbance was measured at 595 nm using a microtiter plate reader. The percentage of biofilm mass reduction was calculated using the formula: 

\[
\frac{(Ac−At)}{Ac} \times 100
\]

where Ac is the OD$_{595}$ for control wells and At is OD$_{595}$ in the presence of a compound.

**Pre-formed biofilm assay**

Biofilms were allowed to form in each well of a 96-well microtiter plate, as described above. After 24 h planktonic cells were removed and the wells were rinsed with 200 μl of PBS. Cells stuck to the polystyrene wells were exposed to 200 μl of broth containing the compound under investigation at a concentration ranging from 8 μg ml$^{-1}$ to 32 μg ml$^{-1}$. The plate was further incubated for 24 h at 37°C. Positive controls were untreated cells incubated with 200 μl of broth. Control wells were prepared by growing bacteria in broth plus the amount of DMSO used to dilute each compound. Biofilm mass was assessed by crystal violet-staining as described above.

**Quantitation of metabolic activity of mature biofilm by XTT assay**

To quantify the metabolic activity of mature biofilms of *S. aureus* and *S. epidermidis* we used XTT [2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (Roche Diagnostics) reduction assay. After treatment, planktonic cells were removed and the wells were washed twice with PBS. Subsequently, 150 μl of XTT was added to each well and the plate incubated in the dark for 40 min at 37°C, then the signal was photometrically quantitated at 490 nm. Negative controls were prepared using wells containing medium alone.

**Confocal laser scanning microscopy (CLSM)**

*S. aureus* and *S. epidermidis* were grown for 24 h in static condition in chambered cover glasses (μSlide 4 well; ibidi GmbH, Munich, Germany) suitable for confocal microscopy. Compounds under investigation were added to 1-day-old biofilms at 32 μl ml$^{-1}$. Untreated bacterial suspensions were used as positive control. After 24 h, biofilms were rinsed with PBS and stained with LIVE/DEAD® BacLight Bacteria Viability stains (Life Technologies, Monza, Italy). Briefly, 200 μl of a 1:1 aqueous solution of SYTO9 and propidium iodide (PI) were added to the well and incubated at room temperature for 15 min in the dark. After incubation, stain was removed, and wells were washed with water. Images were observed using an LSM 700 inverted confocal laser-scanning microscope (Zeiss, Arese, Milan, Italy). Three different areas of each well were scanned using a 10X lens. Signals were recorded in the green channel for Syto9 (excitation 488 nm, emission 500–525 nm) and in the red channel for PI (excitation 500-550 nm, emission 610-650 nm).

**Statistical analysis**

Each experiment was repeated at least three times on different days. Arithmetic means and standard deviations were used to statistically analyze continuous variables. Student’s t test was used to determine statistical differences between two means.
Results

Synthesis of furans

Arylfurans 1-3 (Figure 1) were prepared as reported in our previous paper (Iesce et al., 2016) by NaBH₄ reduction of the corresponding dimethyl 2-aryluran-3,4-dicarboxylates (Fan et al., 2005). Compounds 1-3 were used as alkylating reagents in a variant of the Friedel-Crafts reaction with arenes (anisole, 1,2-dimethoxybenzene and phenol) (Iesce et al., 2016) to obtain arylbenzylfurans 4-14. The reaction uses a combination of Tf₂O and Ph₃PO and represents an environmentally conscious methodology for lignan-like derivatives 4-14 preparation (Figure 1).

Antimicrobial activity against Gram-positive and Gram-negative bacteria.

The potential antimicrobial activity of aryl furans 1-3 and lignan-like furans 4-14 was evaluated by dilution assay against P. aeruginosa, E. coli, S. aureus and S. epidermidis. Compounds concentration ranged from 2 µg ml⁻¹ to 128 µg ml⁻¹. As shown in Table 1, only compound 11 was active against S. aureus and S. epidermidis with an MIC value of 4 µg ml⁻¹. A MIC of 64 µg ml⁻¹ was obtained with compound 13 against tested Gram-positive bacteria. Compounds 9 and 12 were effective at 128 µg ml⁻¹. None of the tested compounds could affect P. aeruginosa and E. coli growth, thus resulting inefficacious. The antimicrobial activity of compound 11 was further investigated against methicillin resistant S. aureus (MRSA) and S. epidermidis (MRSE). Compound 11 resulted active at 4 µg ml⁻¹ against both clinical strains, reducing cell growth by 80% (table 1). The solvent DMSO alone was not effective (negative control). Therefore, the MIC value of 4 µg ml⁻¹ was used in the time kill assay, while sub-MIC concentrations (0.5 - 2 µg ml⁻¹) were used in biofilm formation assay, and 2 × MIC (8 µg ml⁻¹), 4 × MIC (16 µg ml⁻¹) and 8 × MIC (32 µg ml⁻¹) were tested in mature biofilm assay.

Effect of compound 11 on HaCat cells viability.

Cytotoxic effects of compounds 11 and 13 were tested on HaCat cells. Cells were treated with different concentrations of compounds (ranging from 2 µg/ml to 128 µg/ml) and viability was recorded at 8, 24 and 48 h. As reported in Figure 2A, compound 11 did not modified cell viability at any tested concentration. In addition, compound 11 neither displayed cytotoxic activity nor induced modification of cell morphology or evident sign of cell death at any tested concentration. On the contrary, compound 13 reduced HaCaT cells viability by 30% at 32 µg ml⁻¹ (Fig 2B). Thus, subsequent studies were performed only for compound 11.

Time kill assay

The time-kill assay of compound 11 at the MIC value (4 µg ml⁻¹) is shown in Table 2. The results showing changes of the log₈ CFU ml⁻¹ of viable colonies indicate that compound 11 inhibited bacterial growth already at 2 h, with a 40% CFU reduction, compared to untreated bacteria (5.39 versus 8.77 log₈ CFU ml⁻¹, for S. aureus treated and untreated cells, respectively; 4.95 versus 7.34
log_{10} CFU ml\(^{-1}\) for \textit{S. epidermidis}, treated and untreated cells, respectively). The inhibitory effect of compound \(11\) was clearly visible up to 6 h. After 24 h of incubation an increase in cell growth was observed, confirming that compound \(11\) has a bacteriostatic effect on \textit{S. aureus} and \textit{S. epidermidis}.

Effect of compound \(11\) on biofilm

Further experiments were focused on the ability of compound \(11\) to inhibit \textit{S. aureus} and \textit{S. epidermidis} biofilm formation. Compound \(11\) was tested at sub-MIC concentrations, ranging from 0.5 µg ml\(^{-1}\) to 2 µg ml\(^{-1}\) for 24 h. Quantification of crystal violet staining by spectrophotometric measurements (OD\(_{595}\)) showed that compound \(11\) does not have any appreciable effect on \textit{S. aureus} and \textit{S. epidermidis} biofilm formation at any of the used concentrations (data not shown). Successively, the activity of compound \(11\) on mature biofilms was evaluated by crystal violet assay and rapid colorimetric XTT (Stepanović et al., 2007). The latter gives evidence on the viability of the remaining biofilm cells, which cannot be established by crystal violet staining. \textit{S. aureus} and \textit{S. epidermidis} were allowed to grow and form biofilm for 24 h. Compound \(11\) was then added to one-day-old-biofilm at a concentration of 2 × MIC (8 µg ml\(^{-1}\)), 4 × MIC (16 µg ml\(^{-1}\)) and 8 × MIC (32 µg ml\(^{-1}\)) for 24 h. Crystal violet assay revealed that compound \(11\) had no effect on the biomass of treated biofilms; on the contrary, XTT assay showed that compound \(11\) caused a significant reduction (T-student test, \(p < 0.05\)) of mature biofilm viability compared to untreated controls (Figure 3). The minimal concentration which reduced \textit{S. aureus} and \textit{S. epidermidis} preformed biofilm (MBEC\(_{90}\)) was 32 µg ml\(^{-1}\) and 8 µg ml\(^{-1}\), respectively. Additional evidence for the anti-biofilm property of compound \(11\) was provided by studies of Confocal Laser Scanning Microscopy, using a BacLight LIVE/DEAD staining kit that allows differential staining of live cells (green) and dead/damaged cells (red-orange). As shown in Figure 4, the addition of compound \(11\) at the concentration of 32 µg ml\(^{-1}\) induced an increased level of PI intensity and reduced intensity of SYTO9 green fluorophore, which clearly confirmed the killing efficacy of compound \(11\) on \textit{S. aureus} and \textit{S. epidermidis} mature biofilms.

Discussion

\textit{S. aureus} and \textit{S. epidermidis} are common inhabitants of human skin and mucous surfaces and, as such, are most frequently associated with skin and soft tissue infections following trauma, burns, surgery, and/or catheter-related, as well as blood-borne infections. Biofilms play an important role in device-related infections and, in recent years, their relevance has been recognized as a complicating element of lesions such as those listed above. Several studies of wound models \textit{in vivo}, \textit{or in vitro} using keratinocyte cultures, have shown that biofilm formation severely delays the healing process and promotes chronicization of the lesion (Tankersley, 2014; Zhao, 2012). Biofilms are impermeable to most antimicrobials and are recognized as primary elements of persistent infections. Because there are few reports on the antimicrobial activity of lignans and their derivatives (Yanti et al., 2008;
Raghavendra et al., 2016), in this study we tested the effects of synthetic lignans and aryl furans against *S. aureus* and *S. epidermidis*. Many of the compounds tested showed modest *in vitro* activity, with the notable exception of compounds 11 and 13. The main difference of furan structures is the aryl substitutes, typical of natural lignans, such as methoxyl and hydroxyl. The presence of a hydroxyl on the aromatic moiety of compounds 11 and 13 enhances their activity, as is the case for natural lignans (Teponno, 2016). The position of the hydroxyl moiety appears to be important, as suggested by the remarkable difference in potency of compounds 13 and 14. Compound 11, although inactive on Gram-negative species, proves to be the most potent against *S. aureus* and *S. epidermidis*, with an MIC50 of 4 µg/ml. However, based on the results here reported we cannot affirm what the mode of action of compound 11 is. It is generally difficult to establish a valid correlation between structural features and antibacterial properties, even in a serial of very similar compounds (Monasterios et al, 2007). In our case, the lack of activity of compounds 1-10 could be attributed to their low polarity, which prevents the crossing of both the Gram-positive and Gram-negative bacterial cell wall. The slight increase in polarity of compounds 11-14 due to the presence of a phenol group may enable them to cross only the Gram-positive bacterial wall. The presence of other groups, such as bromine and methoxyl, reduce (compound 13) or abrogate (compounds 12 and 14) the activity compared to compound 11. Of interest, Maruyama et al. reported the antibacterial activity of lignan only on Gram-positive bacteria (Maruyama et al, 2007). However, more data need to be produced to confirm our hypothesis. Compound 11 proves to be active also against methicillin resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE), causing strong inhibition of growth (~80%). This result is relevant because infections involving *S. aureus* and *S. epidermidis* are very common and very difficult to treat due to the induction of methicillin resistance, which has become a serious clinical problem. It is worth noting that compound 11 acts rapidly, inhibiting *S. aureus* and *S. epidermidis* cell growth at 2 h; however, recovery of cell growth after 24 h of incubation suggests the bacteriostatic action of the drug.

A new compound with antimicrobial activity needs to be tested on eukaryotic cells and/or tissues to evaluate its cytotoxicity before being considered for therapeutic use. Compound 11 has been tested on an established cell line of human keratinocytes (HaCat). Our results show that compound 11 does not affect cell morphology or cell viability at bactericidal concentrations.

The first requirement for successful bacterial colonization is attachment to surfaces of medical devices or host tissues (Simões, 2011). This can be non-specific in the case of abiotic medical devices or mediated through specific interactions with receptors of host biological tissues. In either case, this step is fundamental for the formation and spatial organization of biofilms. Many recent studies have indicated that microorganisms of a pathogenic nature live as aggregates and thrive in biofilms as the preferred form of growth; moreover, it is generally believed that aggregation and biofilm formation
enhance the virulence of the pathogenic phenotype. Thus, from a medical standpoint, molecules perturbing the early phase of biofilm formation are of great interest in terms of prevention and prophylaxis. Of equally great interest would be molecules capable of disrupting preformed biofilms, which could be used as more effective therapeutic remedies and an additional weapon to fight antimicrobial resistance (Chua et al., 2014). This prompted us to investigate whether compound 11 could inhibit biofilm formation as well as act on preformed biofilms. Our results showed that compound 11 was unable to inhibit bacterial attachment and biofilm formation. As for its activity on mature biofilm, compound 11 did not disrupt the biofilm structure, as shown by crystal violet assay, but was effective in reducing cell viability of one-day-old-biofilms. Confocal laser scanning microscopy results confirmed that compound 11 strongly reduced *S. aureus* and *S. epidermidis* mature biofilm viability, without affecting biofilm biomass. Molecules that can act on preformed biofilms may have a greater therapeutic potential. Biofilm formation can take as little as a few hours. Antimicrobial treatment often starts when biofilm organization has already occurred, and most antimicrobials are unable to cross the barrier. Compound 11 seems to be able to penetrate the biofilm matrix and to interact with bacterial cells and kill them. This is a noteworthy feature, since many antimicrobials are able to disrupt the biofilm but not to eliminate bacteria embedded in the biofilm, causing the release and the spread of live planktonic bacteria from the biofilm site (Uppu et al., 2017). This result is of the utmost importance, since our antimicrobial can be active on both planktonic and sessile bacteria.

In conclusion, all of these findings suggest that compound 11 can reduce the viability of both planktonic and biofilm embedded bacteria, thus representing a lead for the development of new antimicrobial agents. However, further studies of these compounds and optimization of their structures, leading to novel analogues with superior biological properties, are indispensable.
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Author Disclosure Statement
The authors have no conflict of interest to declare.
References

Alabi K A and Owolabi BJ. (2012). Synthesis and antimicrobial property of 2-(2-nitrovinyl)furan
*J Pure Appl Microbio.* **6:** 131-134.

therapy in combination to fight biofilms and resistant surface bacterial infections. *Int J Mol Sci.* **16:**
20417-20430.

advances in natural product-based anti-biofilm approaches to control infections. *Mini Rev Med Chem.* **14:**
1169-1182.

Chua SL, Liu Y, Yam JK, Chen Y, Vejborg RM et al. (2014). Dispersed cells represent a distinct
stage in the transition from bacterial biofilm to planktonic lifestyles. *Nat Commun.* **5:** 4462.


Gutierrez, K., Halpern, M.S., Sarnquist C., et al. (2013). Staphylococcal infections in children,

Hall CW and Mah TF. (2017). Molecular mechanisms of biofilm-based antibiotic resistance and

Iesce MR, Sferruzza R, Cermola F and DellaGreca M. (2016). Furanyl alcohols as alkylating reagents
in Friedel-Crafts Reaction of arenes. *Helv Chim Acta.* **99:** 296-301.

Kayumov AR, Khakimullina EN, Sharafutdinov IS. Inhibition of biofilm formation in *Bacillus subtilis*
by new halogenated furanones. *J Antibiot.* **68:** 297-301.

*Comprehensive Heterocyclic Chemistry III,* ed. Katritzky AR, Ramsden CA, Scriven FV, Taylor


advances and applications. *Curr Org Chem*** **11:** 1076-1091.


Figures

**Figure 1.** Structures of arylfurans 1-3 and lignan-like arylbenzylfurans 4-14.
Figure 2. Time course assessment of treatment with different concentrations of compound 11 (A) and 13 (B) on HaCat cells viability assessed by MTT assay. On the x axis are reported the tested concentrations ($\mu$g ml$^{-1}$), while the y axis reports the observed percentage viability relative to untreated samples. Data represent means ± standard deviations from three independent experiments, each performed in triplicate. * indicates significant differences (T-student test, $p < 0.05$) compared with untreated controls.
Figure 3. Upper panel reports biofilm biomass assessed by crystal violet; bottom panel reports biofilm viability obtained by XTT reduction assay. The tested concentrations (µg ml$^{-1}$) are reported on the x axis; the observed absorbance at 595 and 490 nm are reported on the y axis. Data represent means ± standard deviations from three independent experiments, each performed in triplicate. * indicates significant differences (T-student test, $p < 0.05$) compared with untreated controls.
Figure 4. Confocal laser scanner microscopy micrographs. A) and C) *S. aureus* ATCC 12600 and *S. epidermidis* ATCC 35984 biofilm untreated; B) and D) *S. aureus* ATCC 12600 and *S. epidermidis* ATCC 35984 biofilm treated with Compound 11 at 32 µg ml⁻¹. Left panels contain three-dimensional images, right panels contain orthogonal images of biofilm. Green and red fluorescence is associated with live and dead cells, respectively.
Table 1. In vitro antibacterial activity. MIC values (μg ml⁻¹) for compounds 1-14 against *E. coli*, *P. aeruginosa*, *S. epidermidis*, *S. aureus*.

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<th>Compounds</th>
<th><em>E. coli</em> ATCC 25922</th>
<th><em>P. aeruginosa</em> ATCC 27853</th>
<th><em>S. epidermidis</em> ATCC 35984</th>
<th><em>S. aureus</em> ATCC 12600</th>
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<td>&gt;128</td>
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<td>NT</td>
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</tbody>
</table>

*MIC values (μg ml⁻¹)

**NT, not tested
Table 2. In vitro time-kill assay. *S. aureus* ATCC 12600 and *S. epidermidis* ATCC 35984 were exposed to 4 μg ml⁻¹ compound 11 for 2 h, 4 h, 6 h and 24 h. The inhibitory effect on bacterial growth was assessed by measuring the number of CFUs obtained after the treatment. Data represent means ± standard deviations from three independent experiments, each performed in triplicate.

<table>
<thead>
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<th>Compounds</th>
<th>Log₁₀ CFU ml⁻¹</th>
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<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td><em>S. aureus</em> untreated</td>
<td>5.51±0.31</td>
</tr>
<tr>
<td><em>S. aureus</em> + compound 11</td>
<td>5.45±0.25</td>
</tr>
<tr>
<td><em>S. epidermidis</em> untreated</td>
<td>5.11±0.28</td>
</tr>
<tr>
<td><em>S. epidermidis</em> + compound 11</td>
<td>4.89±0.27</td>
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</tbody>
</table>