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ROYAL SOCIETY OF CHEMISTRY

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View Article Online
DOI: 10.1039/C5RA05840F

RSC Advances

PAPER

Fine-tuning of the hydrophobicity of caffeic acid: studies on the antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*

Received 00th April 20xx,
Accepted 00th April 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Mafalda Andrade[a¥], Sofia Benfeito[b¥], Pedro Soares[b], Diogo Magalhães e Silva[b], Joana Loureiro[a], Anabela Borges[a,b,c], Fernanda Borges[b*] and Manuel Simões[a*]

The increased bacterial multidrug resistance caused by inappropriate use and overuse of antimicrobials is a global concern. To circumvent this issue, a quest for the development of new active agents has been widely recognized. Some phytochemical products, produced by plants as part of their chemical defense strategies, are regarded as new stimulus to develop novel antimicrobials that are not as vulnerable as current drugs to bacterial resistance mechanisms. In this study, the antimicrobial activity and mode of action of caffeic acid (CAF) and a series of CAF alkyl esters was assessed against *Escherichia coli* and *Staphylococcus aureus*, with the aim of analyzing the influence of the alkyl ester side chain length on the activity. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), changes in physicochemical surface properties and intracellular potassium leakage were used as physiological indices for the antimicrobial mode of action. CAF alkyl esters were found to be effective antimicrobial agents against both bacteria. Their activity was directly dependent on their lipophilicity, which affected bacterial susceptibility, the physicochemical properties of the bacteria and the integrity of the membranes. *E. coli* was less susceptible than *S. aureus* to the action of the compounds. Longer alkyl side chains were more effective against the Gram-positive bacterium, while medium length alkyl side chain compounds were more effective against the Gram-negative bacterium. Caffeic acid derivatives are proposed to act as cell permeabilizers, inducing membrane alterations, causing rupture with potassium leakage, particularly on the Gram positive bacterium, and consequent cell death.

1 Introduction

Control of microbial growth is required in many microbiologically sensitive environments, especially when the conditions for their proliferation are favorable.[1] For this purpose, antibacterial agents such as biocides and antibiotics are usually employed [2,3] and their selection differ by the extent of their pharmacological specificity and of their degree of mammalian toxicity. Antibiotics usually have a single biochemical target (i.e., a selective toxicity) and are used against bacterial infections in human beings and animals. On the other hand, biocides generally possess several distinct targets, with diverse susceptibilities (i.e., a broad spectrum of usage) and are often regarded as antiseptics (used externally on human skin), disinfectants (for surface sanitizing), and/or preservatives (incorporated in pharmaceutical, cosmetic or other types of products to prevent microbial contamination).[1,2,4] However, these agents have been widely recognized as being used inappropriately, and constantly subjected to overuse, underuse and general misuse over the years. On bacteria, these type of mishandlings create a selective pressure for the development of bacterial resistance and/or multidrug resistance to these compounds, with resistance being transmitted within and between individuals.[2,5–8] The emergence of resistant microorganisms is a global concern and has led to a quest for the search and development of new alternative antimicrobial products not so vulnerable as current drugs to bacterial resistance mechanisms.[7–10]

Plants produce a vast array of secondary metabolites (phytochemicals), a number of which are commonly believed to be involved in chemical strategies to protect themselves against pathogen microbial attack of fungi, yeasts and bacteria.[11,12] The use of phytochemicals as antimicrobial agents is at present considered a strategic approach to overpass the mentioned drawbacks because they not only have multiple and different modes of action from current antibiotics (posing a low risk for the development of resistance), but are also derived from natural sources presenting a green and safe status.[13,14] Bacterial susceptibility to phytochemicals, especially phenolic compounds, have been studied by several authors and, in general, they present a promising antimicrobial profile.[8,9,15–22] Polyphenols are the most important and abundant group of phytochemicals and can be found in diverse dietary products like vegetables, fruits, chocolates and beverages (as

[a] *LEPABE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, s/n, 4200-465 Porto, Portugal.*
[b] *CIQUP, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, Rua do Campo Alegre s/n, 4169-007, Porto, Portugal.*
[c] *CECAV-Veterinary and Animal Science Research Center, Department of Veterinary Science, University of Trás-os-Montes e Alto Douro, 5001-801 Vila Real, Portugal.*
¥ Both authors contributed equally to the work.
*Authors to whom correspondence should be addressed; e-mail: fborges@fc.up.pt; mvs@fe.up.pt

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DOI: 10.1039/C5RA05840F

coffee, tea or wine).[23–25] Phenolic compounds can be divided into two main groups: flavonoids and non-flavonoids that include several chemical classes, such as flavonols, isoflavones, anthocyanins, phenolic acids.[24,26] Phenolic acids are the most common non-flavonoid naturally occurring phenolics which contain two distinguishing constitutive carbon frameworks: the hydroxycinnamic (C_6C_3) and hydroxybenzoic (C_6C_1) structure. Only a minor fraction of phenolic acids exists in the free form. Instead, the majority are linked through ester, ether or acetal bonds either to structural components of the plant, larger polyphenols or smaller organic molecules (e.g., glucose, quinic acid). Particular attention is at present given to hydroxycinnamic acids due to their remarkable biological properties, including antimicrobial activity of broad spectrum.[24,26,27] The antimicrobial mode of action of phenolic acids can be due to their ability to destabilize and permeabilize the cytoplasmatic membrane, inhibition of enzymes involved in radical generation and also the inhibition of the synthesis of nucleic acids of both Gram-negative and -positive bacteria.[8] Caffeic acid (Fig. 1) is generally the most abundant hydroxycinnamic acid, representing between 75% and 100% of the total hydroxycinnamic acid content of diverse fruits and beverages.[24,28,29]

The efficacy of phytochemicals as antimicrobials as well as their diversity provides a renewed interest towards the discovery of new antibacterial drugs, being a new hope to overcome the bacterial resistance problem[8,16]. They can constitute new scaffolds for drug discovery and development programs as they can be tailored for fine-tuning their drug-like properties and so replenish the antibiotic pipeline.[30-33] In this context, a structure-antimicrobial activity relationship (SAR) study was carry out focused on caffeic acid (*trans*-3,4-dihydroxycinnamic acid, CAF) (Fig. 1), a phytochemical that has been shown to display interesting antibacterial activity,[9] and an homologous series (C2 to C10) of alkyl esters derivatives (Schemes 1 and 2). In addition, the study of the effects of the increment of CAF lipophilicity on the planktonic bacterial growth of *Staphylococcus aureus* and *Escherichia coli* was performed. Their effects on bacterial physicochemical properties and membrane integrity was also evaluated.

2 Materials and methods

2.1 Chemistry

2.1.1 Reagents. Caffeic acid and all the other reagents were purchased from Sigma–Aldrich Química S.A. (Sintra, Portugal) and used without additional purification. The solvents were pro analysis grade and were acquired from Merck (Lisbon, Portugal). Thin-layer chromatography (TLC) was carried out on precoated silica gel 60 F254 (Merck) with layer thickness of 0.2 mm. For analytical control, the following elution systems were used: dichloromethane, ethyl acetate, dichloromethane/methanol and petroleum ether/ethyl acetate in several proportions. The spots were visualized under UV detection (254 and 366 nm). Flash column chromatography was performed using silica gel 60 (0.040–0.063 mm) (Merck). Following the workup and after extraction, the organic phases were always dried over Na_2SO_4. Solutions were decolorized with activated charcoal, when necessary. Solvents were evaporated in a Buchi Rotavapor.

2.1.2 Apparatus. ^{1}H and ^{13}C NMR spectra were acquired, at room temperature, on a Brüker Biospin GmbH 400 spectrometer operating at 400 and 100 MHz, respectively. Chemical shifts were expressed in δ (ppm) values relative to tetramethylsilane (TMS) as internal reference; coupling constants (*J*) were given in Hz. Assignments were also made from DEPT (Distortionless Enhancement by Polarization Transfer) (underlined values). Electron impact mass spectra (EI-MS) were acquired on a VG AutoSpec instrument; data were reported as *m/z* (% of relative intensity of the most important fragments). Microwave-assisted synthesis was performed in Biotage® Initiator Microwave Synthesizer.

2.2 Synthesis of alkyl caffeates

2.2.1 Synthesis of alkyl caffeates with short chain length. The synthetic procedure was adapted from Garrido *et al*.[34] The cinnamic acid (1.0 g), the ethanol or butanol (30 mmol) and 2 drops of concentrated sulphuric acid were added to a glass vial (2-5 mL) that was then sealed. The system was heated 20 ^{0}C above the boiling point of the alcohol for 10 min in microwave reactor cavity under mechanical stirring. After cooling to room temperature, the crude products were extracted with ethyl acetate (3 × 10 mL) and washed with water (3 × 10 mL). The combined organic layers were dried with Na_2SO_4, filtered and solvent was evaporated. The resulting residues were purified by flash chromatography using silica gel as stationary phase and petroleum ether/ethyl acetate as eluent.

trans*-Ethyl 3-(3,4-dihydroxyphenyl)propenoate (CAFC2):** Yield: 78%, ***^{1}H NMR (DMSO-d6): 1.23 (3H, *t*, *J* = 8.0 H(2')), 4.14 (2H, *m*, H(1')), 6.25 (1H, *d*, *J* = 16.0 H(β)), 6.76 (1H, *d*, *J* = 8.0 H(5)), 6.99 (1H, *dd*, *J* = 8.0; 2.0 H(6)), 7.04 (1H, *d*, *J* = 2.0 H(2)), 7.46 (1H, *d*, *J* = 16.0 H(α)). ***^{13}C NMR (DMSO):*** 14.3 (C(2')), 59.7 (C(1')), 114.1 (C(2)), 114.8 (C(α)), 115.7 (C(5)), 121.4 (C(6)), 125.5 (C(1)), 144.9 (C(β)), 145.6 (C(3)), 148.4 (C(4)), 166.5 (CO). ***MS/EI m/z:*** 209 ($M^{+\bullet}$, 30), 208 (100), 180 (33), 164 (23), 163 (96), 136 (55), 135 (40), 134 (45), 117 (21), 89 (47), 77 (25), 63 (24), 51 (23).

trans*-Butyl 3-(3,4-dihydroxyphenyl)propenoate (CAFC4):** Yield: 80%, ***^{1}H NMR ($CDCl_3$): 0.96 (3H, *t*, *J* = 7.4, H(4')), 1.43 (2H, *m*, H(3')), 1.69 (2H, *m*, H(2')), 4.21 (2H, *t*, *J* = 6.6, H(1')), 6.26 (1H, *d*, *J* = 16.0 H(β)), 6.88 (1H, *d*, *J* = 8.4 H(5)), 7.00 (1H, *dd*, *J* = 2.0; 8.4 H(6)), 7.11 (1H, *d*, *J* = 2.0 H(2)), 7.58 (1H, *d*, *J*=16.0 H(α)). ***^{13}C NMR ($CDCl_3$):*** 13.8 (C(4')), 19.2 (C(3')), 30.8 (C(2')), 64.8 (C(1')), 114.4 (C(2)), 115.4 (C(α)), 115.5 (C(5)), 122.4 (C(6)), 127.4 (C(1)), 144.0 (C(3)), 145.3 (C(β)), 146.6 (C(4)), 168.4 (CO). ***MS/EI m/z:*** 236 ($M^{+\bullet}$, 78), 180 (98), 163 (100), 136 (43), 135 (26), 134 (30), 89 (27).

2.2.2 Synthesis of alkyl caffeates with long chain length

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2.2.2.1 Synthesis of malonic acid half esters. The synthetic procedure was adapted from Menezes *et al.*[35] Equimolar quantities (10 mmol) of Meldrum's acid and the appropriate long chain alcohols were refluxed in toluene (5 mL) for 4 h. After cooling the reaction to room temperature 10 mL of a saturated NaHCO3 solution was added. The formation of white foam is instantly observed. The compounds were extracted with diethyl ether (3 × 10 mL) acidified with HCl, washed with water (3 × 10 mL). The combined organic layers were dried with Na_2SO_4, filtered and solvent was evaporated. The malonic acid half esters were characterized and used in the next reaction without further purification.

Monohexyl malonate (1): Yield 61%. ***^{1}H NMR($CDCl_3$):*** 0.89 (3H, *t*, *J* = 7.0 H(6')), 1.33 (6H, *m*, H(3')-H(5')), 1.65 (2H, *m*, H(2')), 3.43 (2H, *s*, H(2)), 4.16 (2H, *t*, *J* = 6.6 H(1')). ***^{13}C NMR($CDCl_3$):*** 14.2 (C(6')), 22.4 (C(5')), 25.8 (C(4')), 29.1 (C(3')), 31.6 (C(2')), 41.5 (C(2)), 64.8 (C(1')), 166.9 (C(3)), 170.5 (C(1)).

Monooctyl malonate (2): Yield 48%. ***^{1}H NMR($CDCl_3$):*** 0.89 (3H, *t*, *J* = 7.0 H(8')), 1.30 (10H, *m*, H(3')-H(7')), 1.65 (2H, *m*, H(2')), 3.43 (2H, *s*, H(2)), 4.16 (2H, *t*, *J* = 6.6 H(1')). ***^{13}C NMR($CDCl_3$):*** 14.3 (C(8')), 22.4 (C(7')), 29.3 (C(5')), 29.4 (C(4')), 26.9 (C(3')), 29.2 (C(2')), 32.3 (C(6')), 41. 2 (C(2)), 64.7 (C(1')), 166.8 (C(3)), 169.9 (C(1)).

Monodecyl malonate (3): Yield 55%. ***^{1}H NMR($CDCl_3$):*** 0.88 (3H, *t*, *J* = 6.8 H(10')), 1.30 (14H, *m*, H(3')-H(9')), 1.65 (2H, *m*, H(2')), 3.43 (2H, *s*, H(2)), 4.16 (2H, *t*, *J* = 6.6 H(1')). ***^{13}C NMR($CDCl_3$):*** 14.0 (C(10')), 22.7 (C(9')), 25.7 (C(3')), 28.8 (C(2')), 29.2 (C(7')), 29.5 (C(4')), 29.7 (C(5')), 29.9 (C(6')), 32.1 (C(8')), 40.8 (C(2)), 64.6 (C(1')), 166.6 (C(3)), 168.4 (C(1)).

2.2.2.2 Condensation of malonic acid half esters with 3,4-dihydroxybenzaldehyde. The synthetic procedure was adapted from Menezes et al.[35] Equimolar quantities (1.0 mmol) of the malonic acid half esters and the 3,4-dihydroxybenzaldehyde were added to cyclohexane (3-5 mL). Then, anhydrous pyridine (1 mmol) and β-alanine (1.6 mmol) were added and the mixture was refluxed for 6-10 h. After cooling the mixture in an ice bath, concentrated HCl was added dropwise. After pH neutralization the mixture was extracted with diethyl ether (3 × 10 mL) and water (3 × 10 mL). The organic layer was dried with Na_2SO_4, filtered and solvent was evaporated under vacuum. The residues were purified by flash chromatography using gradient elution (petroleum ether/ethyl acetate).

trans*-Hexyl 3-(3,4-dihydroxyphenyl)propenoate (CAFC6):** Yield 69%. ***^{1}H NMR (DMSO-d_6): 0.87 (3H, *t*, *J* = 7.0, H(6')), 1.31 (6H, *m*, H(3')-H(5')), 1.62 (2H, *m*, H(2')), 4.10 (2H, *t*, *J* = 6.6 H(1')), 6.26 (1H, *d*, *J* = 15.6 H(β)), 6.76 (1H, *d*, *J* = 8.4 H(5)), 6.94 (1H, *dd*, *J* = 2.0; 8.4 H(6)), 7.04 (1H, *d*, *J* = 2.0 H(2)), 7.47 (1H, *d*, *J* = 15.6 H(α)), 9.12 (1H, *s*, OH), 9.57 (1H, *s*, OH). ***^{13}C NMR (DMSO-d_6):*** 14.4 (C(6')), 22.5 (C(5')), 25.6 (C(4')), 28.7 (C(3')), 31.4 (C(2')), 64.2 (C(1')), 114.5 (C(2)), 115.3 (C(α)), 116.2 (C(5)), 121.8 (C(6)), 126.0 (C(1)), 145.5 (C(β)), 146.0 (C(3)), 148.8 (C(4)), 167.1 (CO). ***MS/EI m/z (%):*** 264 ($M^{+\bullet}$, 80), 181 (30), 180 (100), 163 (90), 136 (49), 135 (34), 89 (38).

trans*-Octyl 3-(3,4-dihydroxyphenyl)propenoate (CAFC8):** Yield 53%. ***^{1}H NMR (DMSO-d_6): 0.86 (3H, *t*, *J* = 7.0 H(8')), 1.30 (10H, *m*, H(3')-H(7')), 1.62 (2H, *m*, H(2')), 4.10 (2H, *t*, *J* = 6.6 H(1')), 6.26 (1H, *d*, *J* = 15.6 H(β)), 6.76 (1H, *d*, *J* = 8.4 H(5)), 6.94 (1H, *dd*, *J* = 2.0; 8.4 H(6)), 7.04 (1H, *d*, *J* = 2.0 H(2)), 7.47 (1H, *d*, *J* = 15.6 H(α)), 9.40 (2H, 2x s, 2xOH). ***^{13}C NMR (DMSO-d_6):*** 15.3 (C(8')), 23.5 (C(7')), 26.8 (C(6')), 29.7 (C(5')), 30.0 (C(4'), C(3')), 32.6 (C(2')), 65.1 (C(1')), 115.4 (C(2)), 116.2 (C(α)), 117.1 (C(5)), 122.7 (C(6)), 126.7 (C(1)), 146.4 (C(β)), 146.9 (C(3)), 149.7 (C(4)), 168.0 (CO). ***MS/EI m/z:*** 292 ($M^{+\bullet}$, 50), 181 (25), 180 (100), 163 (70), 136 (30).

trans*-Decyl 3-(3,4-dihydroxyphenyl)propenoate (CAFC10):** Yield 42%. ***^{1}H NMR ($CDCl_3$): 0.88 (3H, *t*, *J* = 6.8 H(10')), 1.32 (14H, *m*, H(3')-H(9')), 1.70 (2H, *m*, H(2')), 4.20 (2H, *t*, *J* = 6.6 H(1')), 6.27 (1H, *d*, *J* = 15.6 H(β)), 6.88 (1H, *d*, *J* = 8.4 H(5)), 7.01 (1H, *dd*, *J* = 8.4; 2.0 H(6)), 7.12 (1H, *d*, *J* = 2.0 H(2)), 7.59 (1H, *d*, *J* = 15.6 H(α)). ***^{13}C NMR ($CDCl_3$):*** 14.1 (C(10')), 22.7 (C(9')), 26.0 (C(8')), 28.7 (C(7')), 29.3 (C(6'), C(5')), 29.6 (C(4'), C(3')), 31.9 (C(2')), 65.0 (C(1')), 114.5 (C(2)), 115.5 (C(5), 115.6 (C(α)), 122.4 (C(6)), 127.5 (C(1)), 143.9 (C(3)), 145.0 (C(β)), 146.4 (C(4)), 168.1 (CO). ***MS/EI m/z (%):*** 320 ($M^{+\bullet}$, 28), 180 (100), 163 (47), 134 (22), 98 (24), 84 (24), 57 (40), 55 (34).

2.3 Microbiological studies

2.3.1 Microorganisms and chemical solutions. *S. aureus* CECT 976 and *E. coli* CECT 434, obtained from the Spanish Type Culture Collection, were selected for this study. The bacteria were cryopreserved at -80 °C, in a mixture of Mueller-Hinton broth (MHB, Merck) and 30% (v/v) glycerol and subcultured in Mueller-Hinton agar (MHA), at 30 °C for 24 h, before testing. Stock solutions of all tested compounds were prepared in dimethyl sulfoxide (DMSO, Fisher), under sterile conditions, and kept in the dark, at room temperature, for a maximum of two weeks. Serial dilutions of the stock solutions were prepared in DMSO, when needed.

2.3.2 Evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Bacterial cells were grown overnight in MHB at 30 °C and 120 rpm in a Sartorius Certomat® BS-1 (Portugal) incubator. Afterwards, an inoculum was taken and adjusted to an optical density (OD) of 0.1 ± 0.02 ($OD_{600\ nm}$). MIC values were determined in sterile 96-well flat-bottomed polystyrene tissue culture microtiter plates (Orange Scientific, Belgium). In each well, a volume of 20 µL of compound's solution was added to 180 µL of cell culture. All compounds were tested in a range of different concentrations to reach the MIC. Cell suspensions with DMSO and cell suspensions without caffeic acid and derivatives were used as negative controls. Ciprofloxacin (Sigma–Aldrich Química S.A.) was used as positive control for MIC and MBC determination. The OD_{600} was measured at t = 0 h and at t = 24 h,

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DOI: 10.1039/C5RA05840F

using a microtiter plate absorbance reader (Biotek Synergy HT), after incubation at 30 °C and 120 rpm. The MIC was defined as the lowest concentration of tested compound at which no bacterial growth was detected.[20,37] After MIC determination, a volume of 10 µL of each concentration tested for MIC assessment was plated out on MHA. Plates were incubated at 30 °C for 24 h and growth was visually inspected. The MBC was determined as the lowest concentration of compound in which total inhibition of growth on solid medium was observed (no colony forming units - CFU - were detected).[1]

2.3.3 Characterization of bacterial surface hydrophobicity. Overnight cultures grown in MHB were centrifuged at 3202 *g* for 10 min and washed twice with sterile saline solution (0.85% (w/v) NaCl, BDH Prolabo). Subsequently, the $OD_{640\ nm}$ of the cell suspension was set to 0.4 ± 0.04. A volume of 45 mL of the cell suspension was added to 5 mL of each test compound (to a final concentration of 0.1 mM) and incubated for 1 h at 30 °C and 120 rpm. All compounds were tested at the same concentration, regardless their MIC and MBC. A negative control was prepared with DMSO. Bacterial lawns (i.e. homogeneous layers of cells) were then prepared according to Busscher *et al.*[38] After exposure to the compounds, cell cultures were filtered into nitrocellulose sterile filters (47 mm of diameter and pore size of 0.45 µm; Advantec) and contact angle measurements were carried out according to Simões *et al.*[39], by using the sessile drop contact angle method to determine the surface tension of the bacterial surfaces (at least 20 determinations for each liquid and for each microorganism). The measurements were performed at room temperature using three different liquids of well-known surface tension components (two polar - ultrapure water and formamide - and one apolar - α-bromonaphtalene; Sigma). Contact angles were automatically determined using an OCA 15 Plus (Dataphysics) video-based optical measurement instrument, which allowed image acquisition and data analysis. The values of the liquids surface tension components were taken from the literature.[40] Hydrophobicity was assessed after contact angle measurements using the approach of van Oss *et al.*[41], where the degree of hydrophobicity of a given surface (s) is expressed as the free energy of interaction between two entities of that surface, when immersed in water (w): ΔG_{sws}. If the interaction between two entities is stronger than the interaction of each one of the entities with water, then $\Delta G_{sws}>0$ mJ m^{-2} and the surface of bacterial cells are considered hydrophobic. Contrariwise, if $\Delta G_{sws}<0$ mJ m^{-2} the bacterial cell surfaces are hydrophilic. ΔG_{sws} can be calculated from the surface tension components of the interacting entities:

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

where γ^{LW} is the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively of the Lewis acid-base component (given by $\gamma^{AB} = 2 \times \sqrt{\gamma^+\gamma^-}$). The surface tension components can be determined by simultaneous resolution of three equations (accounting for the three different liquids used for measuring the contact angles) of the form:

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

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

2.3.4 Evaluation of zeta potential. *S. aureus* and *E. coli* were incubated overnight in MHB at 30 °C and under 120 rpm agitation. The cells were centrifuged twice at 3202 *g* for 10 min (at 25 °C) and washed with sterile distilled water. The cell suspensions were adjusted to $OD_{640\ nm}$ = 0.2 ± 0.02. A volume of 1.8 mL of this culture was added to 200 µL of test compound (to a final concentration of 0.1 mM) and incubated for 1 h at 30 °C and 120 rpm. A negative control was prepared with DMSO. The zeta potential of the bacterial suspensions was determined, according to the procedure described by Borges *et al.*[8], using a Nano Zetasizer (Malvern Instruments) equipment, in carefully filled zeta potential cells (DTS1060, Malvern), at room temperature.

2.3.5 Evaluation of potassium leakage. Bacterial suspensions of *S. aureus* and *E. coli* were prepared in MHB and incubated at 30 °C under 120 rpm agitation. After overnight grow, the cells were centrifuged at 3202 *g* for 15 min and washed twice with sterile distilled water. The cell suspensions were adjusted to $OD_{640\ nm}$ = 0.2 ± 0.02 and incubated for 1 h at 30 °C and 120 rpm, in contact with each test compound (at a final concentration of 0.1 mM). A negative control was prepared with DMSO. A positive control was performed with benzyldimethyldodecylammonium chloride (Sigma–Aldrich Química S.A.) according to Ferreira *et al.*[1] The bacterial suspensions were then filtered in sterile cellulose nitrate membrane filters (pore size 0.2 µm) (Whatman, UK). Atomic absorption spectroscopy (conditions: flame air-acetylene; wavelength 769.9 nm) were used for K^+ titration in the filtered bacterial solutions, using a GBC ASS 932plus device with the GBC Avante 1.33 software.[1]

2.4 Statistical analysis.

All experiments were carried out in triplicate with at least three repeats. The data was analyzed using the *GraphPad Prism 5* software. The mean and standard deviation within samples was calculated for all cases. To assess the statistical significance of the data, an unpaired Student's t-test was used (confidence level ≥ 95%); $p<0.05$ was considered statistically significant.

2.5. Calculation of drug-likeness properties

The parameters for drug-likeness were evaluated according to the Lipinski's 'rule-of-five', using the MolinspirationWebME Editor [http://www.molinspiration.com].

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DOI: 10.1039/C5RA05840F

3 Results and discussion

3.1 Chemistry

The alkyl caffeates with short chain length (Scheme 1) were synthesized by a nucleophilic acyl substitution reaction often called Fischer esterification. Esterification of carboxylic acids by primary or secondary alcohols is conventionally carried out under catalytic conditions, namely with sulfuric acid, tosylic acid and Lewis acids. Despite being a reaction widely used in (bio)organic synthesis it has some disadvantages, including long reaction times, safety and hazardous environmental problems, tedious purification processes and average to low reaction yields.[34,42] The use of microwave-assisted organic synthesis over conventional reactions may offer a convenient solution to overpass some of the mentioned problems, allowing a reduction of reaction time and/or the amount of reagent/solvent. In our case, yields around 80% (20 min of reaction time) have been obtained for CAFC2 and CAFC4 using caffeic acid (CAF) as starting material and ethanol and butanol as reagent, respectively (Scheme 1). Compared with the classic Fisher esterification, microwave conditions significantly reduced the reaction time (10 min versus 5 hours to 5 days, if conducted at room temperature), increased the yields and simplified the purification process.[43,44] The same reaction was used to obtain the alkyl caffeates with long chain length (Scheme 2). However, due to purification problems, mainly related to the higher boiling points of the primary alcohols and low yields, a different synthetic strategy was envisaged. Alkyl caffeates (CAFC6, CAFC8 and CAFC10) have been synthesized in moderate yields by synthetic strategy encompassing two steps: a) the first one consists in the formation of monomalonate compounds (**1**-**3**) by heating Meldrum's acid with the appropriate alcohols in toluene; b) in the second the condensation of malonate half esters to 3,4-dihydroxybenzaldehyde, in the presence of dry pyridine and *β*-alanine, was performed by a Verley-Doebner modification of Knoevenagel reaction.[45]

3.2 Antibacterial activity of caffeic acid and its alkyl esters

CAF and its alkyl esters (C2 to C10) were screened towards *S. aureus* CECT 976 and *E. coli* CECT 434 strains. Although esters with a higher chain length (C12, C14 and C16) have been synthesized, water solubility problems preclude their use. So, the study was performed only with the compounds that present drug-like properties (Tables 1 and 2). According to the Lipinski's 'rule-of-five' most "drug-like" molecules must have logP ≤ 5, molecular weight ≤ 500, number of hydrogen bond acceptors ≤10, and number of hydrogen bond donors ≤ 5. Molecules violating more than one of these parameters may have problems with bioavailability, namely poor oral absorption or membrane permeability.[46,47]

In general cafeic alkyl esters present drug-like properties, only CAF10 has its partion coefficient on boundary, possessing an adequate number of proton acceptor and proton donor groups to ensure efficient interaction with the hydrogen bonding groups of the receptors. The predictive topological polar surface area (TPSA) data allow concluding that they could have a good capacity for penetrating cell membranes (Tables 1 and 2). From the data one can notice that caffeic acid has not satisfactory properties to cross membranes effectively.

The MIC and MBC data of CAF and its alkyl ester derivatives (C2-C10) gathered along the study are shown in Table 2. Considering that lipophilicity is an important property for data interpretation the theoretical partition coefficients (clogP) were also calculated (Table 2). In general, for each bacterial strain, a clear tendency was observed: MIC or MBC values decreased with increasing length of the alkyl ester chain. A simple modification of the lipophilicity of CAF (see CAF *vs* CAFC2) caused a relevant MIC decrease. It must be stressed that the esterification process also preclude the ionization process of CAF to a carboxylate ion (-COOH pk_{a1}= 4.36) at the working pH. The data is in accordance with the results obtained by Merkl *et al.*[20] using similar systems.

In *S. aureus*, octyl (CAFC8) and decyl caffeates (CAFC10) presented the lowest MIC values. As observed in Fig. 2 (A and B) these alkyl lengths seem to represent the maximum threshold of the parabolic curve of antimicrobial activity *vs* lipophilicity described by Kubo *et al.*[17,18] for gallic acid esters. The same tendency was observed from MBC data, as the maximum of antibacterial activity is observed for CAFC8 (Fig. 3A and 3B).

The antibacterial activity of the compounds towards *E. coli* followed the same tendency as observed for *S. aureus*. MIC values decreased with the increasing alkyl length of the compounds. The maximum threshold of antimicrobial activity was also observed for CAFC8 and CAFC10 (Fig. 2A and 2B). MBC differed in several orders of magnitude from the MIC and showed a more prominent parabolic behavior than the one observed for *S. aureus*, with maximum antibacterial activity being achieved for CAFC4 (Fig. 3A and 3B). These data propose that *E. coli* viability (bactericidal effects) is more affected by the length of the alkyl side chain than growth inhibition (bacteriostatic effects). In general, *E. coli* was less susceptible than *S. aureus* to the action of caffeate alkyl esters, which is in accordance with the observations made by several authors for similar systems.[7,11,16] This behavior is most likely explained by the fact that Gram-negative bacteria possess an outer membrane with a hydrophilic coating of lipopolysaccharides (LPS)[48], creating a greater barrier to antimicrobial agents. Contrarily, Gram-positive bacteria do not have an outer membrane and the cell wall consists almost entirely of peptidoglycan.[49] The activity seems to be also reliant on the presence of a catechol moiety as the analogues based on ferulic (*trans*-3-methoxy-4-hydroxycinnamic acid) or sinapic (*trans*-3,5-dimethoxy-4-hydroxycinnamic acid) acids did not display remarkable activity (data not shown). This particular behavior can be related with the antioxidant/prooxidant and iron chelating properties displayed by catechol cinnamic systems.[50]

The MIC values obtained in this study are in the range of those described in other works with CAF and related compounds as well as reviewed by Guzman *et al*.[31] These compounds showed a broad spectrum of antimicrobial activity, with MIC values between 0.49-8 mM and 0.12-6 mM against *E. coli* and *S. aureus*, respectivly. For instance, in a work performed by Parkar *et al*.[51] the MIC of CAF against *S. aureus* and *E. coli* was 0.69 mM and 2.78 mM, respectively. In other studies, MIC values in the range of 0.16 to >5.5 mM against *E. coli* were found.[52-55] In the same way, other natural cinnamic acids, such as ferulic and sinapic, showed significant activity against *S. aureus* with MIC between 0.64 and 0.55 mM, respectively. Similar values were found against *E. coli* with these compounds.[56] Interesting antimicrobial activity was found in a study performed with caffeic acid phenethyl ester and

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derivatives, where it was obtained MIC values ranging from 41 to 86 μM against *S. aureus*, including strains of MRSA.[57]

The MIC and MBC values of CAF and its alkyl esters were significantly higher than those of the positive control, ciprofloxacin (MIC = 7.5×10^{-4} mM and MBC = 1.8×10^{-2} mM for *E. coli*; MIC = 1.5×10^{-3} mM and MBC = 1.8×10^{-2} mM for *S. aureus*) and comparable to the susceptibilities previous reported for *E. coli* and *S. aureus*.[58,59] Neverthless, CAF and its alkyl esters can be considered antimicrobials. In fact, plant-derived compounds are routinely classified as antimicrobias on the basis of susceptibility tests that produce MIC in the range of 100 to 1000 μg/mL, orders of magnitude weaker than those of typical antibiotics produced by bacteria and fungi (MIC in the range of 0.01 to 10 μg/mL).[9,11]

3.3 Effects of caffeic acid and alkyl esters on the physicochemical surface properties of bacterial cells

The results on the hydrophobicity and other surface tension parameters (polar, apolar, electron acceptor and donor components) of *S. aureus* and *E. coli* strains in the absence and presence of CAF and its alkyl esters are depicted in Table 3. Both bacterial surfaces presented hydrophilic properties (ΔG^{TOT}>0 mJ/m^2) and this parameter was slightly changed due to the exposure to CAF and its alkyl esters. *S. aureus* surface hydrophilicity decreased in the presence of alkyl caffeates with exception of CAFC2 (p<0.05), where an increase was found. No significant variation of hydrophilicity values was observed for CAFC4 and CAFC6, when comparing with untreated cells (p>0.05). This reduction in the hydrophilic character was significant (p<0.05) for *S. aureus* exposed to CAFC8 and CAFC10.

The surface of *E. coli* cells was changed with the exposure to the compounds tested. Their hydrophilic character decreased with the ester caffeates, with the minimum values attained for cells treated with CAFC6 and CAFC8. Exposure to CAFC10 did not show a significant effect on the cell surface hydrophobicity, when compared with the control.

The apolar character of both bacteria (γ^{LW}) was reduced with the increase of the lipophilicity of the compounds. The polar component (γ^{AB}) showed a parabolic behavior for *E. coli*, increasing with the lipophilicity of the compounds tested, being the maximum obtained for cells treated with CAFC6. However, no significant changes in this parameter was found for *S. aureus* (p>0.05). The electron acceptor properties were also maximized for *E. coli* exposed to CAFC6 and minimized for *S. aureus* exposed to CAFC2, while CAFC8 and CAFC10 caused a reduction of the electron donor properties of both bacteria (p<0.05). The overall data on hydrophobicity and its components clearly demonstrate that the CAF and its alkyl esters derivatives interact with the bacterial surface. A similar mode of action was found by Borges *et al.*[8] for ferulic and gallic acids.

The surface charge of cells is frequently determined based on their zeta potential, which is calculated from their electrophoretic motility in the presence of an electric field, under defined pH and salt concentrations.[60] When applying an electric field across a bacterial suspension, bacteria with non-zero zeta potential migrate towards the electrode of the opposite charge, with a velocity proportional to the magnitude of their zeta potential.[1] The results obtained from zeta potential measurements with *S. aureus* and *E. coli* in the absence and presence of CAF and its alkyl esters are shown in Table 4. Bacterial cells normally present a negative surface charge, due to the presence of anionic groups in their membranes, such as carboxylate and phosphate groups.[61,62] In the present study, zeta potential values of -30.9 mV and -22.6 mV were obtained for *S. aureus* and *E. coli*, respectively, without exposure to the compounds. Changes in the surface charge of *S. aureus* to less negative values were obtained after exposure to CAF and CAFC2. The highest change on surface charge, for *E. coli,* was caused by CAF. This effect was probably due to its ionization to a carboxylate ion (-COOH pk_{a1}= 4.36) at the working pH.[63] In general, the other CAF alkyl esters did not change significantly the surface charge (p>0.05) of the selected bacteria.

3.4 Effects of caffeic acid and its alkyl esters on the cell membrane integrity

Potassium leakage is considered to be a good indicator of microbial cytoplasmatic membrane damage.[64] In fact, the internal ionic environment of prokaryotic and eukaryotic cells is rich in potassium, which means that membranolytic events in bacteria (i.e., any impairment to the barrier between cytoplasm and the extracellular medium) generally cause release of this ion.[8,64] Table 5 shows the effects of CAF and its alkyl esters on K^+ release by *E. coli* and *S. aureus*. A significant increase in K^+ release was detected for *S. aureus* exposed to CAFC6, CAFC8 and CAFC10. This indicates an alteration in the cytoplasmic membrane permeability. However, no significant K^+ release was found due to exposure to CAF and alkyl ester derivatives (CAFC2 and CAFC4). K^+ release by *E. coli* was found for cells treated with CAF, CAFC4, CAFC6 and CAFC10, even if at lower extents than for *S. aureus*, when comparing to the control. The application of benzyldimethyldodecylammonium chloride (positive control) at 0.2 mM caused K^+ release of 1.6 ± 0.28 μg/mL and 2.8 ± 0.18 μg/mL for *E. coli* and *S. aureus*, respectively. This biocide is known to cause cellular disruption and loss of membrane integrity with consequent leakage of essential intracellular constituents, including K^+.[1] Therefore, comparing with the positive control, the overall data propose that alkyl caffeates inflicted damage on the bacterial membranes of both bacteria, with remarkable effects on *S. aureus*. Therefore, one can propose that these compounds may act as membrane permeabilizers, *i.e.* as antimicrobial agents that weaken the outer membrane of the cells, inducing changes on its permeability and, consequently, in the bacterial physicochemical characteristics.[63,64] It is possible that the antimicrobial activity of alkyl caffeates is associated with a fine balance between affinity for the lipid bilayers of cell membranes and the ability to cause disruption of the membrane, which visibly differs from Gram-positive to Gram-negative bacteria (due to their different cell wall structure and composition) and is dependent on the length of the alkyl ester side chain. Therefore, it is proposed that in Gram-negative bacteria, smaller length alkyl ester chains might have a better lipophilicity balance, and thus, be more effective in crossing the LPS layer of the outer membrane.[48] This in accordance with the results obtained for MBC and K^+ leakage, where medium length CAF alkyl esters, such as CAFC4 and CAFC6,

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were more effective as bactericidal agents and proven to cause more damage to the membrane integrity. It is also noteworthy that lower susceptibilities observed for Gram-negative bacteria, might also be credited to the fact that the outer membrane is an effective barrier.[17,49] On the Gram-positive bacterium longer alkyl side chain compounds, such as CAFC8 and CAFC10, reveal to be the best bacterial growth inhibitors and the compounds that have a greater impact on destabilizing the membrane based on the results of MIC/MBC, hydrophobicity and K^+ leakage.

The selected compounds possess a head-and-tail structure, similar to an amphiphile. Amphiphiles are molecules which present both hydrophilic and hydrophobic parts, usually a long-chain alkyl group.[48] In this case, the amphiphile properties of caffeic acid esters are associated with the presence of the phenolic groups (hydrophilic moiety) and the alkyl side chain (lipophilic tail)[15], which contributes to the parabolic function attained when antimicrobial activity is plotted against lipophilicity.[15–18] This biological behavior could be correlated with the cutoff phenomenon, very distinctive of amphiphilic substances: antimicrobial properties of amphiphiles tend to increase with increasing alkyl chain length till a limit (the cutoff effect). However, it must be stressed that the length increasing lead to a concomitant shrinkage of compounds solubility in aqueous media a fact that can be also related with the decrease of their antimicrobial activity.[48]

4 Conclusions

Plant-derived molecules may offer a groundbreaking green approach to the discovery of broad-spectrum antimicrobials. This study shows that CAF is an interesting scaffold for the development of antimicrobial agents. CAF alkyl esters were more effective than CAF in controlling bacterial growth of *S. aureus* and *E. coli*. The lipophilicity of the compounds influenced its activity. Longer alkyl side chain compounds appeared to be more effective in inhibiting bacterial growth of the Gram-positive bacterium, particularly CAFC8 and CAFC10. Medium length alkyl side chain compounds were more effective against the Gram-negative bacterium, particularly CAFC4. These molecules had broad spectrum activity causing significant changes in cell surface hydrophobicity, charge and induced K^+ leakage, an effect more significant for *S. aureus*. Moreover, as some cinnamic derivatives, have demonstrated good efficacy in the treatment of patients with tuberculosis[65], the CAF alkyl esters are of potential interest to be used against *Mycobacterium tuberculosis*. Indeed, cinnamic derivatives already revelead high potency and selectivity against this bacterium, with MIC only at micromolars.[66,67]

In summary, the study reinforce the idea that phytochemicals can operate as scaffolds for the development of new chemical entities with antimicrobial activity. The results of the present study suggests that this class of compounds is worthy for further studies, namely the evaluation of their additive or synergistic action with the antibiotics currently in use for antimicrobial therapy.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper. The authors alone are responsible for the content and writing of the article.

Acknowledgements

This work was supported by Operational Programme for Competitiveness Factors–COMPETE and by FCT–Portuguese Foundation for Science and Technology through Projects FCUP-CIQ-UP-NORTE-07-0124-FEDER-000065 and PTDC/DTP-SAP/1078/2012 and the grants awarded to S. Benfeito (SFRH/BD/99189/2013) and A. Borges (SFRH/BPD/98684/2013). Further financial support was obtained from Project UID/EQU/00511/2013-LEPABE, by the FCT/MEC with national funds and co-funded by FEDER in the scope of the P2020 Partnership Agreement; Project NORTE-07-0124-FEDER-000025-RL2_ Environment&Health, by FEDER funds through Programa Operacional Factores de Competitividade – COMPETE, by the Programa Operacional do Norte (ON2) program and by national funds through FCT - Fundação para a Ciência e a Tecnologia. M. Andrade acknowledges the grant provided by the European Research Project SusClean (Contract n_ FP7-KBBE-2011-5, project number: 287514). The work was undertaken as part of the COST Actions FA1202 and TD1305.

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Figures, Tables and Schemes

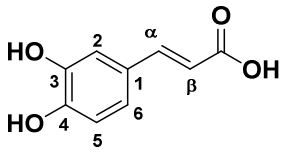

Fig. 1 Chemical structure and IUPAC numbering of caffeic acid (**CAF**).

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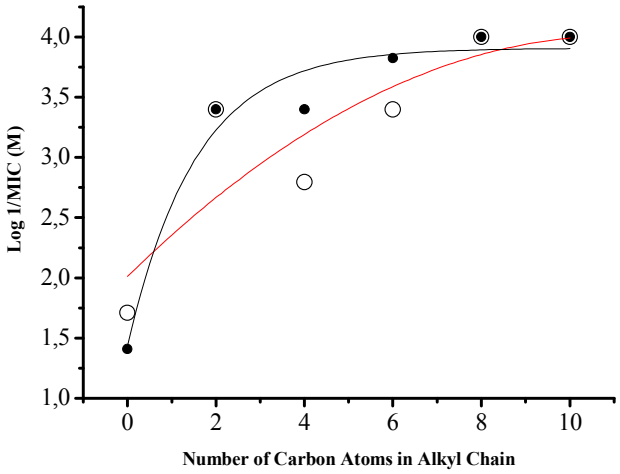
A



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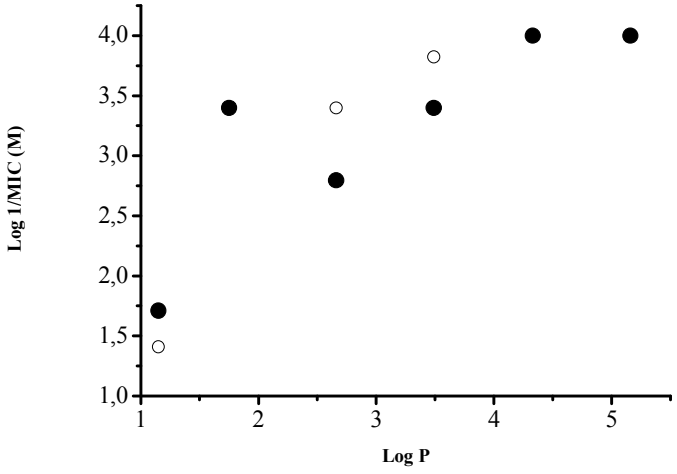

Fig. 2 (A) Comparison of antimicrobial activity of CAF and alkyl esters against *S. aureus* (○) *and E. coli* (●). The best curve fit was obtained with the Boltzmann equation y =3.9 – 6811.03/(1+exp (x+12.28)/1.55), R^2 = 0.9197) by *S. aureus* and polynomial equation y = 2.011+0.359x-0.016 x^2), R^2= 0.6256) by *E. coli.* (B) Regression plots of log(1/MIC) towards *S. aureus* (○) and *E. coli* (●) for CAF and alkyl esters derivatives tested.

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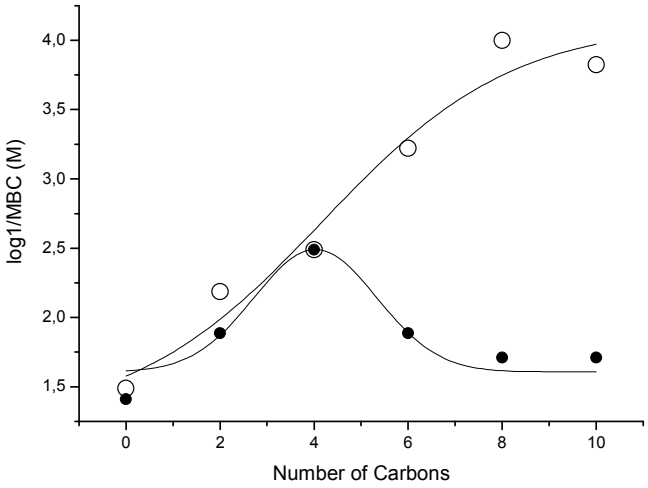
A



B

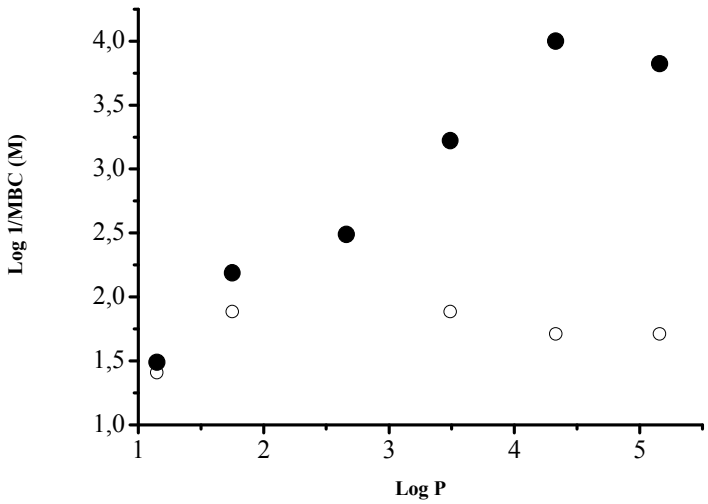


Figure 3 - (A) Comparison of antimicrobial activity of CAF and alkyl esters against *S. aureus* (○) and *E. coli* (●). The best curve fit was obtained with the Boltzmann equation $y = 4.13 – 2.88/(1+\exp(x-4.16)/2.03)$, $R^2 = 0.9176$) by *S. aureus* and Gaussian equation $y = 1.607+ 0.883 \exp(-0.5(x-4.027)/1.305)^2$, $R^2 = 0.7591$) by *E. coli*. Regression plots of log(1/MBC) towards *S. aureus* (○) and *E. coli* (●) for CAF and alkyl esters derivatives tested.

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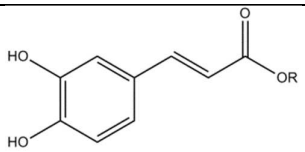
Table 1 Structural properties of caffeic acid and alkyl esters.

		R	Molecular weight (g/mol)	n-ROTB	TPSA (Å2)	n-ON acceptors	n-OHNH donors	Volume (Å3)
Caffeic acid	**CAF**	H	180.16	4	77.76	4	3	154.50
Caffeic alkyl esters	**CAFC2**	C_2H_5	208.21	4	66.76	4	2	188.83
	CAFC4	C_4H_9	236.27	6	66.76	4	2	222.43
	CAFC6	C_6H_{13}	264.32	8	66.76	4	2	256.03
	CAFC8	C_8H_{17}	292.38	10	66.76	4	2	289.64
	CAFC10	$C_{10}H_{21}$	320.43	12	66.76	4	2	323.24

[a] n-ROTB, number of rotable bonds; TPSA, topological polar surface area; n-ON, number of hydrogen bond acceptors; n-OHNH, number of hydrogen bond donors. The data was determined with Molinspiration calculation software

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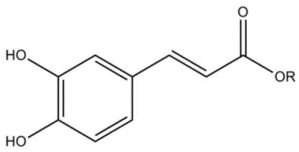
Table 2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of caffeic acid (CAF) and its alkyl ester derivatives (CAFC2-C10) against *S. aureus* and *E. coli*.

		R	*S. aureus*		*E. coli*		cLog[a]
			MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	
Caffeic acid	**CAF**	H	19.5	32.5	39	39	1.15
Caffeic alkyl esters	**CAFC2**	C_2H_5	0.4	6.5	0.4	13	1.75
	CAFC4	C_4H_9	1.6	3.25	0.4	3.25	2.66
	CAFC6	C_6H_{13}	0.4	0.6	0.15	> 13	3.49
	CAFC8	C_8H_{17}	0.1	0.1	0.1	> 19.5	4.33
	CAFC10	$C_{10}H_{21}$	0.1	0.15	0.1	> 19.5	5.16

[a]Theoretical estimated using ChemBioDraw Ultra 13.0 program.

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Table 3 Surface tension parameters and hydrophobicity of *E. coli* and *S. aureus* exposed to caffeic acid (CAF) and its alkyl ester derivatives (CAFC2-C10) at 0.1 mM, for 1 h

		Surface tension parameters (mJ/m^2)				ΔG^{TOT} (mJ/m^2)
		γ^{LW}	γ^{AB}	γ^{+}	γ^{-}	
S. aureus	Control	34.3 ± 1.2	21.5 ± 1.2	2.4 ± 0.3	47.6 ± 3.0	23.0 ± 3.5
	CAF	32.4 ± 2.2	23.5 ± 2.8	2.8 ± 0.6	49.2 ± 1.4	24.3 ± 0.7
	CAFC2	34.8 ± 0.7	18.0 ± 1.3	1.6 ± 0.2	50.0 ± 1.2	27.5 ± 1.4
	CAFC4	34.1 ± 1.6	20.8 ± 2.3	2.3 ± 0.5	47.9 ± 1.1	23.8 ± 1.8
	CAFC6	31.7 ± 0.3	22.0 ± 2.1	2.7 ± 0.5	46.2 ± 1.4	22.1 ± 2.0
	CAFC8	25.6 ± 4.4	17.2 ± 4.0	2.1 ± 0.9	38.0 ± 4.4	15.5 ± 4.9
	CAFC10	24.8 ± 2.0	18.9 ± 2.0	2.3 ± 0.7	39.7 ± 4.1	17.6 ± 5.5
E. coli	Control	32.7 ± 1.4	21.6 ± 2.6	2,2 ± 0.5	54.5 ± 1.8	31.3 ± 2.7
	CAF	31.1 ± 1.4	25.5 ± 2.1	3.1 ± 0.5	53.8 ± 0.4	28.6 ± 1.1
	CAFC2	30.1 ± 0.8	26.2 ± 1.5	3.3 ± 0.3	51.9 ± 1.0	26.4 ± 0.4
	CAFC4	29.9 ± 0.9	26.9 ± 1.3	3.4 ± 0.3	52.8 ± 0.2	27.1 ± 0.3
	CAFC6	24.0 ± 1.6	32.3 ± 1.3	5.1 ± 0.5	50.9 ± 1.0	23.1 ± 1.5
	CAFC8	22.6 ± 1.4	22.4 ± 4.8	3.2 ± 1.5	43.3 ± 4.4	16.8 ± 2.4
	CAFC10	24.0 ± 2.3	19.9 ± 6.0	2.2 ± 1.5	51.6 ± 4.0	34.7 ± 4.3

$\Delta G^{TOT} > 0$ mJ/m^2 – Hydrophilic; $\Delta G^{TOT} < 0$ mJ/m^2 - Hydrophobic

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Table 4 Effects of caffeic acid (CAF) and its alkyl ester derivatives (CAFC2-C10) in the surface charge of *S. aureus* and *E. coli* cells at 0.1 mM, for 1 h.

	Zeta Potential (mV)	
	S. aureus	*E. coli*
Control	-30.9 ±3.3	-22.6±6.2
CAF	-10.2±4.5	-11.8±3.0
CAFC2	-16.4±2.8	-28.1±4.7
CAFC4	-24.7±3.7	-17.4±3.2
CAFC6	-28.3±1.3	-18.4±0.9
CAFC8	-25.5±4.5	-20.5±0.8
CAFC10	-27.0±5.6	-19.6±2.1

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Table 5. K^+ concentration in the solution after exposure of *E. coli* and *S. aureus* cells to CAF and its alkyl ester derivatives (CAFC2-10) at 0.1 mM, for 1 h.

	Concentration of K^+ in solution (μg/mL)	
	S. aureus	***E. coli***
Control	0.904 ± 0.272	0.263 ± 0.001
CAF	1.177 ± 0.305	0.417 ± 0.010
CAFC2	0.845 ± 0.344	0.356 ± 0.092
CAFC4	0.927 ± 0.280	0.447 ± 0.021
CAFC6	2.770 ± 0.095	0.566 ± 0.081
CAFC8	2.745 ± 0.035	0.348 ± 0.071
CAFC10	2.789 ± 0.004	0.502 ± 0.370

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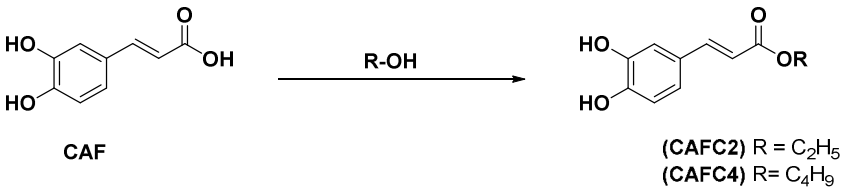
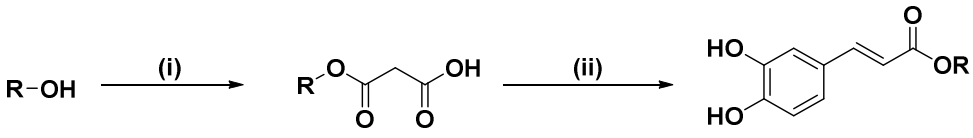


CAF

(CAFC2) R = C_2H_5
(CAFC4) R= C_4H_9

Scheme 1 Synthesis of ethyl (**CAFC2**) and butyl (**CAFC4**) caffeate from caffeic acid (**CAF**). Reagents and conditions: alkyl alcohol, H_2SO_4, microwave irradiation, 20min.



1-3

(CAFC6) R = C_6H_{13}
(CAFC8) R = C_8H_{17}
(CAFC10) R = $C_{10}H_{21}$

Scheme 2 Synthesis of long alkyl chain cinnamates (CAFC6, CAFC8, CAFC10) from monomalonates **1-3.** (i) Meldrum's acid, toluene, reflux, 4h; (ii) 3,4-dihydroxybenzaldehyde, pyridine, β-aniline, cyclohexane, reflux 6-10h.