International Journal of Food Science +Technology

International Journal of Food Science and Technology 2017, **52**, 679–686

Original article

Antimicrobial and antibiofilm activities of procyanidins extracted from laurel wood against a selection of foodborne microorganisms

Alfonso Alejo-Armijo,[1] Nicolás Glibota,[2] María P. Frías,[3] Joaquín Altarejos,[1] Antonio Gálvez,[2] Elena Ortega-Morente[2]* & Sofía Salido[1]

1 Departamento de Química Inorgánica y Orgánica, Facultad de Ciencias Experimentales, Universidad de Jaén, Campus de Excelencia Internacional Agroalimentario (ceiA3), Jaén 23071, Spain
2 Departamento de Ciencias de la Salud, Facultad de Ciencias Experimentales, Universidad de Jaén, Campus de Excelencia Internacional Agroalimentario (ceiA3), Jaén 23071, Spain
3 Departamento de Estadística e Investigación Operativa, Facultad de Ciencias Experimentales, Universidad de Jaén, Campus de Excelencia Internacional Agroalimentario (ceiA3), Jaén 23071, Spain

(Received 21 September 2016; Accepted in revised form 8 November 2016)

Summary This study aimed to evaluate the antimicrobial and antibiofilm activities of two procyanidins isolated from an ethyl acetate extract of laurel wood against a selection of foodborne pathogens. The analysis of the extract by HPLC–DAD/ESI–MS allowed us to detect the presence of two procyanidins, which were selectively isolated and identified by chromatographic and spectroscopic means as cinnamtannin B-1 (**1**) and procyanidin B-2 (**2**). Procyanidins **1** and **2** exhibited two biological activities: inhibition of bacterial growth at high concentrations and prevention of biofilm formation at lower concentrations. Synergistic effect was also detected when both compounds were tested in combination against *Listeria monocytogenes*. Significant effects were also detected on disruption of preformed biofilm. The ability of procyanidins to inhibit microbial growth and biofilm formation and to synergistically work with each other may stimulate a market as natural food preservatives, and/or natural sanitisers for processing equipment where foodborne pathogens reside.

Keywords Antimicrobial activity, biofilm, cinnamtannin B-1, *Laurus nobilis*, procyanidin B-2, procyanidins, synergism.

Introduction

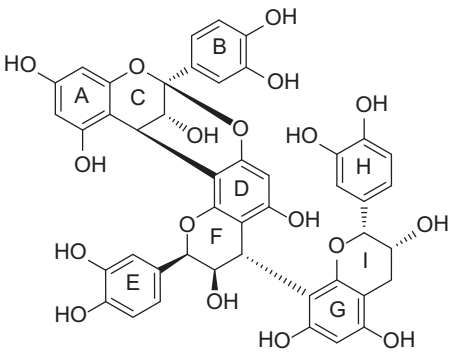
Foodborne disease is a global issue with significant impact on human health. The high capacity of different foodborne pathogens to survive in the presence of several environmental stressors such as heat, cold, salt and acidic conditions, and its capacity to form biofilms on biotic or abiotic surfaces are main factors for these bacteria to be continuously introduced into the processing environment, and cross-contaminate food contact surfaces, equipment, floors, drains and other locations. In biofilm formation, quorum sensing allows a phenotypic change in the bacteria, so sessile biofilm bacteria show increased resistance to many biocides, disinfectants and antibiotics (Amaral *et al*., 2015).

Plant extracts as well as pure plant-derived compounds (mainly phenolic compounds) have received increasing attention as natural antimicrobial and antibiofilm agents against important foodborne pathogens in the recent years (Elhariry *et al*., 2014; Phillips, 2016). Cranberry juice, elaborated from fruits of *Vaccinium macrocarpon* Ait., has also long been used to prevent infections of the urinary tract, which are often related to biofilm formation and numerous studies have found that A-type proanthocyanidins (PACs) (oligomers with at least a double interflavanyl linkage between two flavan-3-ol units) from cranberries have antibiofilm properties against many bacterial and fungal pathogens (Rane *et al*., 2014; Ulrey *et al*., 2014).

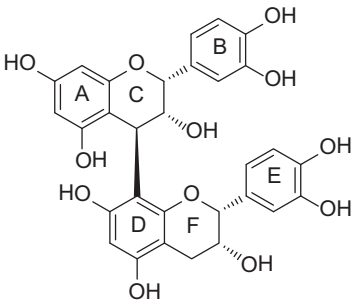
Laurus nobilis L. (Lauraceae), known as laurel, is a tree currently cultivated for its flavoured leaves. Although laurel leaves have been studied extensively from a chemical and antimicrobial viewpoint (García-Díez *et al*., 2016), little is comparatively known on the composition and antimicrobial activity of the woody parts of the laurel tree (Bouaziz *et al*., 2007; Al-Hussaini & Mahasneh, 2009).

*Correspondent: E-mail: eortega@ujaen.es

doi:10.1111/ijfs.13321



Cinnamtannin B-1 (**1**)



Procyanidin B-2 (**2**)

Figure 1 Structures of the procyanidins isolated from a laurel wood EtOAc extract.

In the course of our studies on the recovery of valuable compounds from agri-food by-products (Pérez-Bonilla *et al*., 2014), the polyphenol cinnamtannin B-1 (**1**) was isolated from laurel wood (Fig. 1) (Bouaziz *et al*., 2007). It is a trimeric procyanidin with high antioxidant power and potential interest to prevent thrombotic disorders (Ben Amor *et al*., 2007). This compound is an A-type PAC like those found in cranberries (Foo *et al*., 2000). The presence of three catechol groups (rings B, E and H) in the compound (Fig. 1), suitable to bind iron (Andjelkovic *et al*., 2006), seems to point that compound **1** would be a potential agent to prevent adhesion and/or reduce biofilm production originated by microorganisms as cranberry PACs do. Thus, the purpose of this work was to study the antimicrobial and antibiofilm activities of **1** as well as that of other procyanidins present in laurel wood against a selection of foodborne microorganisms. This is one of the scarce reports on evaluation of biological activities of pure A-type and B-type procyanidins, as most of works previously reported have been conducted with PACs-enriched fractions.

Materials and methods

Chemicals

Solvents used for laurel wood extraction and chromatographic separations of procyanidins (dichloromethane, ethyl acetate, methanol, ethanol) were glass-distilled prior to use. Methanol and acetonitrile used for high-performance liquid chromatography (HPLC) analyses were of HPLC grade. Deuterated methanol (CD_3OD) was used to prepare solutions of purified compounds for nuclear magnetic resonance (NMR) analysis.

Plant material and extraction

The laurel wood sample used in this work (kindly provided by laurel's owner Fernando Altarejos-Gil) was collected in June 2013 in the village of Mogón (Jaén province, Spain) from a male tree. The sample was dried at room temperature with passive ventilation. Just before starting the extraction process, the wood piece was shaved. Wood shavings (1 kg) were extracted for 2 h at reflux with dichloromethane (DCM) and then (the residual shavings) for 2 h at reflux with ethyl acetate (EtOAc) (15 L of each). The solvents were evaporated under reduced pressure to give the corresponding DCM (16.80 g, 1.7%) and EtOAc (7.95 g, 0.8%) extracts. DCM extract was discarded, whereas the EtOAc extract was stored under argon at −20 °C until analysis.

Detection of procyanidins in the laurel extract

The EtOAc extract was analysed by HPLC–DAD and HPLC–DAD/ESI–MS techniques to detect (epi)catechin oligomers (procyanidins). It showed the presence of one A-type procyanidin trimer (**1**) and one B-type procyanidin dimer (**2**) (Figs 1, 2 and S1). The HPLC–DAD analyses and separations were conducted on a Waters 600E instrument as described before (Salido *et al*., 2015). The HPLC–DAD/ESI–MS analyses were performed on an Agilent 1100 HPLC instrument connected to an Esquire 6000 ion mass spectrometer as described before (Salido *et al*., 2015). Analyses were carried out using scan from m/z 50 to 1200. MS^2 fragment-targeted experiments were performed to focus only on compounds producing in MS experiments a pseudomolecular ion at m/z 575, 577, 863 or 865 (Figure S1).

Isolation of detected procyanidins

The purification of the compounds contained in the target HPLC peaks was carried out by a combination of chromatographic techniques such as thin-layer chromatography (TLC), silica gel column chromatography (CC), size-exclusion chromatography (SEC) and

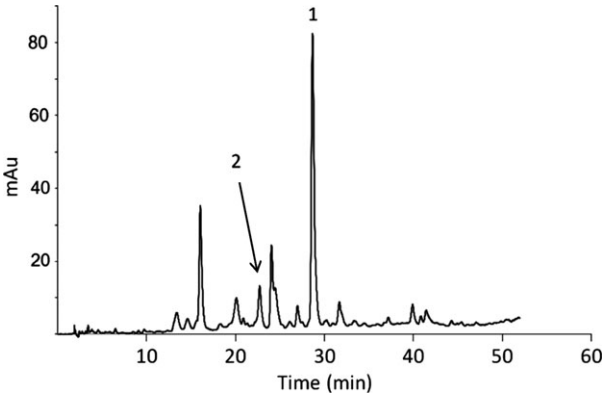


Figure 2 Detection of cinnamtannin B-1 (**1**) (t_R 28.5) and procyanidin B-2 (**2**) (t_R 22.3) in the HPLC chromatogram at 280 nm of the laurel wood EtOAc extract using the HPLC–DAD/ESI–MS technique in negative ion mode.

semipreparative HPLC as shown in Figure S2. Silica gel 60 F_{254} precoated aluminium sheets (0.25 mm, Merck Chemicals, Darmstadt, Germany) were employed for TLC. Silica gel 60 (Merck), particle size 0.200–0.06 mm, was employed for CC. Sephadex LH-20 (Sigma-Aldrich Chemie, Steinheim, Germany) was used for SEC. Semipreparative HPLC separations were performed using a RP-HPLC column (Spherisorb ODS-2 column, 250 mm × 10 mm i.d., 5 mm, Waters Chromatography Division, Milford, MA, USA) on the instrument described above, at flow rate of 5 mL min^{-1}. Fraction D_2 contained pure compound **1** (129 mg; purity of 95% according to HPLC) and from fraction C2 was purified by semipreparative RP-HPLC compound **2** (19 mg; purity of 93% according to HPLC) (Figure S2).

Identification of isolated procyanidins

Structures of the isolated compounds were determined using physical and spectroscopic techniques, such as optical rotation ($[\alpha]_D$) measurements (Jasco P-2000 automatic polarimeter; Jasco Analytical Instruments, Easton, MD, USA), ultraviolet (UV) spectra (Varian Cary 4000 UV/Vis spectrophotometer; Varian Inc., Palo Alto, CA, USA), and infrared (IR) spectra (Bruker Tensor 27 Fourier transform infrared spectrometer; Bruker Optik GmbH, Ettlingen, Germany). NMR spectra were recorded as described before (Pérez-Bonilla *et al.*, 2014). High-resolution mass spectra (HRESIMS) were recorded on an Agilent 6520B Quadrupole time-of-flight (QTOF) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The analysis of all these data allowed us to identify compound **1** as cinnamtannin B-1 (epicatechin-(4β→8,2β→*O*-7)-epicatechin-(4β→8)-epicatechin) and compound **2** as procyanidin B-2 (epicatechin-(4β→8)-epicatechin) (Fig. 1).

Antimicrobial activity

The antimicrobial activity of the compounds was measured on strains from the Spanish Type Culture Collection (CECT) and the Culture Collection of the University of Göteborg (CCUG), as well as on a biocide-resistant strain identified as *Enterococcus faecalis* UJA27t and previously isolated in our laboratory from organic eggplant (Fernández-Fuentes *et al.*, 2012). *Candida albicans* was also evaluated to study the effect of these compounds on eukaryotic cells. The compounds were dissolved in dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Madrid, Spain) and serially diluted in tryptic soya broth (TSB) (Scharlab, Barcelona, Spain) for antimicrobial and antibiofilm assays. Antimicrobial activity of procyanidins **1** and **2** was initially screened by the standard agar diffusion method and then subjected to the minimal inhibitory concentration (MIC) test.

Minimal inhibitory concentration test

Preliminary screenings of antimicrobial activity in standard agar diffusion tests were performed to determine the appropriate dilutions of each substance to be used in MIC tests.

Minimal inhibitory concentration values were determined by the broth microdilution method in 96-well microtitre plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2012). The minimal bactericidal concentration (MBC) corresponded to the lowest compound concentration that prevented any CFU after plating 100 μL from the wells onto nutrient agar plates. The reported MIC and MBC data result from three independent assays. Those strains with highest sensitivity against compounds **1** and **2** were selected for subsequent studies on synergism and biofilm formation.

Checkerboard titre tests

The possible synergism between procyanidins **1** and **2** was evaluated by the checkerboard method and expressed as the sum of the fractional inhibitory concentration (FIC) index for each agent, calculated as the MIC of this agent in combination divided by the MIC of this agent alone. The FIC value of the most effective combination is used in calculating the fractional inhibitory concentration index (FICI) by adding both FICs: $FICI = FICA + FICB = C_A^{comb}/MIC_A^{alone} + C_B^{comb}/MIC_B^{alone}$ where MIC_A^{alone} and MIC_B^{alone}

are the MICs of drugs A and B when acting alone and C_A^{comb} and C_B^{comb} are concentrations of drugs A and B at the isoeffective combinations, respectively. The FICI was interpreted as synergistic when it was ≤0.5, antagonistic when it was >4.0, and any value in between was interpreted as indifferent (Rukayadi *et al*., 2009; Guo *et al*., 2010). Each isolate was tested in triplicate on different days.

Biofilm formation inhibition assay

10-fold serially diluted purified compounds, ranging from 0.01 μg mL^{-1} to 10 or 100 μg mL^{-1} depending on the MIC value, as previously described by Ulrey *et al*. (2014), were tested in both biofilm formation inhibition assay and disruption of preformed biofilm assay.

Inhibition of biofilm formation by compounds **1** and **2** was measured by the crystal violet stain method described by Djordjevic *et al*. (2002) with some modifications. Briefly, bacterial and fungal suspensions (10^5 CFU in TSB) were incubated with increasing concentrations of each compound, or without treatment, in a 96-well plate (24 h; 30 °C). Wells were washed with tap water, and the biofilm fixed with methanol. The plate was stained with 0.3% crystal violet for 5 min, washed and reconstituted with absolute ethanol. The plate was read on an iMarkMicroplate Reader (Bio-Rad, Madrid, Spain) OD595.

Disruption of preformed biofilm

The disruption of preformed biofilm was assayed as previously described (Dean *et al*., 2011; Ulrey *et al*., 2014). After biofilm formation (24 h, 30 °C) in a 96-well plate, contents were aspirated from all experimental and control wells, wells were gently washed twice with sterile physiological saline, and 100 μL of the appropriate diluted compound were added. Wells without treatment served as the positive control. After an additional incubation (24 h, 30 °C), the crystal violet stain was performed as described in the biofilm production inhibition assay.

Statistical analysis

All experiments were carried out in triplicate. The average data ± standard deviations from absorbances at OD595 were determined with Excel program (Microsoft Corp., Redmond, WA, USA). A *t*-test was performed at the 95% confidence level with Statgraphics Plus version 5.1 (Statistical Graphics Corp., Rockville, MD, USA), to determine the statistical significance of data corresponding to biofilm production inhibition and disruption of preformed biofilm.

Results and discussion

Isolation and identification of cinnamtannin B-1 (1) and procyanidin B-2 (2) from laurel wood

To detect the presence of procyanidins in laurel wood, an ethyl acetate extract was analysed by the HPLC–DAD and HPLC–DAD/ESI–MS techniques. It allowed us to pinpoint target HPLC peaks and, therefore, to focus the subsequent effort in the preparative separation and identification of exclusively the desired components.

When procyanidins were searched in the ethyl acetate extract by extracted ion chromatogram analysis only two HPLC peaks were located with characteristic m/z values: peak at 22.3 min ($[M–H]^-$ ion at m/z 577), which could be indicative of a dimeric B-type procyanidin, and peak at 28.5 min ($[M–H]^-$ ion at m/z 863), which could be indicative of a trimeric A-type procyanidin (Fig. 2). Both compounds have in their MS and MS^2 spectra (Figure S1) characteristic fragment ions that support the tentative identification (Gu *et al*., 2003). The compounds contained in those HPLC peaks were isolated, to know their real identity, by a combination of silica gel column chromatography, size-exclusion chromatography, and final semipreparative HPLC purification. Both pure compounds were characterised and identified by spectroscopic means and specific optical rotation measurements as the A-type trimer cinnamtannin B-1 (**1**) (t_R 28.5) and the B-type dimer procyanidin B-2 (**2**) (t_R 22.3) (Figs 1, 2 and S1). Spectral and physical data of both compounds are in agreement with earlier published data (Bouaziz *et al*., 2007; Mohri *et al*., 2009). Compound **1** was isolated for the first time from laurel tree wood (Bouaziz *et al*., 2007), and later on it was also isolated from laurel leaves (Dall'Acqua *et al*., 2009). However, compound **2** was early found in laurel leaves (Sakar & Engelshowe, 1985) but never detected in any other organ of the tree. Both compounds are present in some common foods and spices (Killday *et al*., 2011; Bordiga *et al*., 2015).

Antimicrobial activity

Minimal inhibitory concentration values of procyanidins **1** and **2** against twelve selected foodborne microorganisms are shown in Table 1. Preliminary agar diffusion tests showed no antimicrobial activity when concentrations lower than 0.1 mg mL^{-1} of both compounds were tested (data not shown), so concentrations of the substances in MIC tests ranged from 0.1 to 2 mg mL^{-1}. Procyanidin B-2 (**2**) showed low antimicrobial activity against the strains tested, with MIC values of 1 mg mL^{-1} for all strains excluding some Gram-positive strains (*Staphylococcus aureus* CECT 828, *S. aureus* CECT 4465 and *E. faecalis* UJA27t), which required

Table 1 Antimicrobial activity of procyanidins **1** and **2** against target microorganisms

	MIC (mg mL^{-1})	
Target strain	**Procyanidin B-2 (2)**	**Cinnamtannin B-1 (1)**
S. enterica CECT 4395	1	0.5
S. enterica CECT 409	1	1
S. enterica CECT 4300	1	1
S. enterica CECT 915	1	1
E. coli CCUG 47553	1	1
E. coli CCUG 47557	1	1
L. monocytogenes CECT 4032	1[†]	1
S. aureus CECT 828	1.5	0.1
S. aureus CECT 4465	1.5	0.1
S. aureus CECT 976	1	0.1
E. faecalis UJA27t	1.5	1.5
C. albicans CECT 1001	1	1

[†]This concentration was also described as minimal bactericidal concentration (MBC).
MBC values were higher than 1 mg mL^{-1} for remaining strains.

concentrations even higher to inhibit their growth. Cinnamtannin B-1 (**1**) showed better antimicrobial effect against the three *S. aureus* strains tested, with MIC values of 0.1 mg mL^{-1}, and 0.5 mg mL^{-1} for *S. enterica* CECT 4395. Remaining strains required a concentration of 1 mg mL^{-1} or higher to inhibit their growth. MBC values were higher than 1 mg mL^{-1} for all strains tested, excluding *Listeria monocytogenes*, which showed a MBC of 1 mg mL^{-1}.

Checkerboard titre tests

The MIC for each agent singly or in combination and the corresponding FIC and FICI values is shown in Table 2. Results showed that when *E. coli* CCUG 47553, *L. monocytogenes* CECT 4032 or *S. aureus* CECT 976 were used as target strains, complete growth inhibition was obtained using lower concentrations of both compounds in combination, compared with their MICs in single treatments. However, only when combined treatments were applied on *L. monocytogenes*, outcome of synergy with eightfold and fourfold reductions in MIC of compounds **1** and **2**, respectively, was obtained (FICI < 0.5). Remaining target strains showed indifferent results (FICIs between 0.5 and 4). Phenolics have been previously described as useful in a synergistic approach with conventional antibiotics against antibiotic-resistant forms of pathogens in clinical medicine (Xu *et al.*, 2014). Results about synergistic activity of cinnamtannin B-1 and procyanidin B-2 against *L. monocytogenes* suggest that the use of proanthocyanidins should be further explored as natural preservatives or to potentiate the action of biocides and reduce bacterial colonisation in food industries.

Effect of procyanidins on biofilm formation

Effects of subinhibitory concentrations of procyanidins **1** and **2** on biofilm formation by six target strains are shown in Fig. 3a. Biofilm formation by target strains was not significantly inhibited ($P > 0.05$) in the presence of procyanidin B-2 (**2**) at any of the concentrations tested, showing minor increases or decreases in the capacity of biofilm formation depending upon the concentration tested and the target strain. Cinnamtannin B-1 (**1**) induced a 30% inhibition ($P < 0.05$) on the formation of biofilm by *E. coli* at a concentration as low as 0.1 μg mL^{-1}. However, as the concentration of **1** was increased up to 100 μg mL^{-1}, the inhibitory

Table 2 Checkerboard assay of procyanidins **1** and **2** against target strains

	MIC of each agent (μg mL^{-1})					
Target strain	**Agent**	**Alone**	**Combination**	**FIC**	**FICI**	**Outcome**
S. enterica CECT 4395	Cinnamtannin B-1 (**1**)	500	500	1		
	Procyanidin B-2 (**2**)	1000	1000	1	2	IND
E. coli CCUG 47553	Cinnamtannin B-1 (**1**)	1000	500	0.5	1.5	IND
	Procyanidin B-2 (**2**)	1000	1000	1		
L. monocytogenes CECT 4032	Cinnamtannin B-1 (**1**)	1000	125	0.125		
	Procyanidin B-2 (**2**)	1000	250	0.25	0.375	SYN
S. aureus CECT 976	Cinnamtannin B-1 (**1**)	100	50	0.5	1.5	IND
	Procyanidin B-2 (**2**)	1000	1000	1		
E. faecalis UJA 27t	Cinnamtannin B-1 (**1**)	1500	1500	1		
	Procyanidin B-2 (**2**)	1500	1500	1	2	IND
C. albicans CECT 1001	Cinnamtannin B-1 (**1**)	1000	1000	1	2	IND
	Procyanidin B-2 (**2**)	1000	1000	1		

MIC, minimal inhibitory concentration; FIC, fractional inhibitory concentration (FIC = MIC combination/MIC alone); FICI = FIC(**1**) + FIC(**2**); SYN, synergy; IND, indifferent.

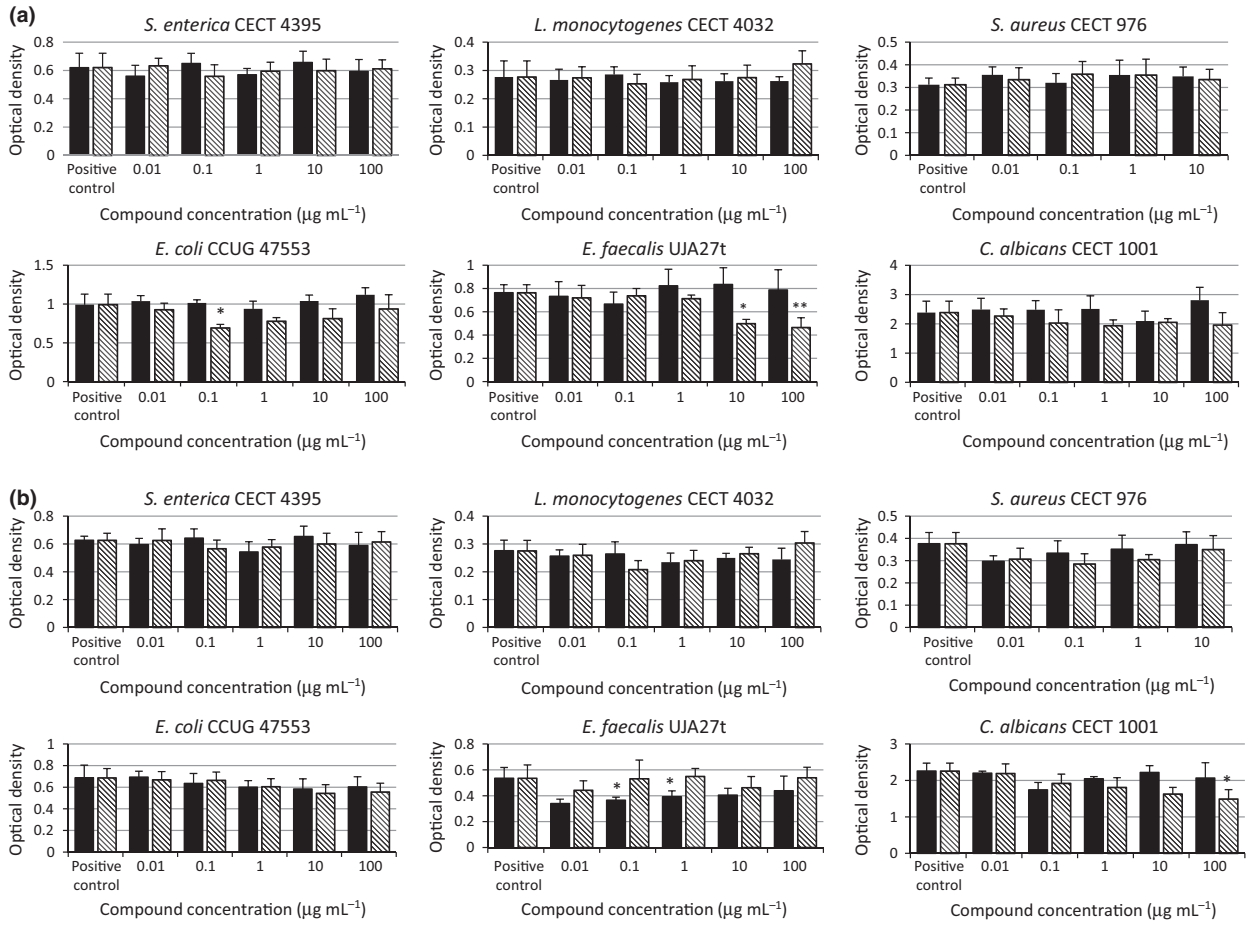


Figure 3 Effects of procyanidins **1** and **2** on biofilm formation (a) and on disruption of preformed biofilms (b). Black bars: Procyanidin B-2 (**2**); Striped bars: Cinnamtannin B-1 (**1**). $*P < 0.05$; $**P < 0.01$.

effect was lower. On the contrary, the effect of **1** on the biofilm formation by *E. faecalis* was dose dependent, so it induced a decrease from 7% to 39% as the concentration was increased from 0.1 to 100 μg mL^{-1}. Cell differences between Gram-positive and Gram-negative bacteria may be responsible for these differences. On the other hand, when *C. albicans* was incubated in the presence of cinnamtannin B-1, a reduction of 20% in the capacity of biofilm formation compared with untreated control was observed at concentrations between 0.1 and 100 μg mL^{-1}, although differences were not statistically significant.

Remaining target strains (*S. enterica*, *L. monocytogenes* and *S. aureus*) were not modified in their ability to produce biofilm in the presence of this procyanidin.

The dose-independent effect of procyanidins found on biofilm formation by foodborne microorganisms in our study had been previously described by Rane *et al.* (2014) when studying the effect of cranberry PACs on *C. albicans* biofilm formation as a paradoxical effect, such that high doses of cranberry PACs had no significant effect ($P > 0.05$) on metabolic activity of *C. albicans* despite a statistically significant reduction ($P < 0.05$) in metabolic activity at moderate doses. A strain- and drug-dependent paradoxical effect had also been noted upon treatment of various *Candida* species with echinocandins (Melo *et al.*, 2007). This behaviour could probably be linked to the known tendency of some polyphenols to self-associate in aqueous solutions when the concentration increases (Goto *et al.*, 1990) and/or to form aggregates with peptides and proteins (Charlton *et al.*, 2002). Mainly antimicrobial peptides known to be synthesised by all bacterial species to inhibit the growth of closely related species (Cavera *et al.*, 2015) may be responsible for the formation of these aggregates in our model. Both binding processes should necessarily lead to a lower effective concentration of the polyphenol in the medium. Highly different external structures distinctive of Gram-positive and Gram-negative bacteria and, to a

greater extent of fungal cells, may be responsible for the dose-dependent effect of cinnamtannin B-1 on the biofilm formation by *E. faecalis*, in contrast to the paradoxical effect found in *E. coli* and previously defined in fungal pathogens.

Effect of procyanidins on disruption of preformed biofilms

When we allowed bacteria to attach and form biofilm for 24 h before treatment, exposure to procyanidin B-2 for an additional 24 h resulted in a 20% reduction of preformed biofilm, compared with untreated control, in *C. albicans* at 0.1 μg mL^{-1} and *S. aureus* at 0.01 μg mL^{-1} (Fig. 3b). Reductions of 15% of preformed biofilm were also detected for *L. monocytogenes* at 1 μg mL^{-1} and *E. coli* at 10 μg mL^{-1}. Assays on *S. aureus* and resistant *E. faecalis* showed that concentration of procyanidin B-2 and disruption of preformed biofilm varied inversely. No effect on disruption of preformed biofilm by *S. enterica* was found when the procyanidins were tested on this target strain (Fig. 3b). As to cinnamtannin B-1, best results were found on *C. albicans* at the highest concentration tested (100 μg mL^{-1}), which induced a 34% reduction ($P < 0.05$) on preformed biofilm compared with untreated control. Minor reductions were also detected at concentrations from 0.1 to 10 μg mL^{-1}. When cinnamtannin B-1 was added at a concentration of 0.1 μg mL^{-1} to *L. monocytogenes* preformed biofilm, a 25% reduction was observed compared with untreated control, and an inverse relationship was again found when higher concentrations of this procyanidin were tested. Similar results were found when *S. aureus* was used as target strain. Reductions of 20% in preformed biofilm were induced by the highest concentrations of cinnamtannin B-1 tested on *E. coli* (10 and 100 μg mL^{-1}). Previous research has also revealed the ability of PACs to prevent the P-fimbriae adhesion of *E. coli in vitro* and *in vivo* (Amer *et al.*, 2010), so multiple mechanisms may be responsible for their effect on microbial viability and biofilm formation. On the other hand, no effect was observed when cinnamtannin B-1 was tested on *E. faecalis* preformed biofilm. The higher molecular size of **1** compared with that of **2** may probably hinder its access to the sessile bacteria within the preformed biofilm, what would account for these results.

Proanthocyanidins have been shown to have an effect on a wide variety of bacterial systems, as their primary mechanism is iron chelation. Fe^{3+} is required for bacterial biofilms to fully mature into a large structure and it has been found that in iron-limiting conditions, *P. aeruginosa* will only form flat, thin biofilms. Bacteria in these thin biofilms may be much less resistant to antibiotics than bacteria in a fully developed biofilm. Thus, the use of PACs at low concentrations should be further explored to potentiate the reduction in bacterial colonisation and biofilm development of other antimicrobial compounds, as biocides or chemical preservatives, in food industries.

Conclusions

The two procyanidins isolated from a laurel wood extract exhibited two biological activities: inhibition of bacterial growth at high concentrations and prevention of biofilm formation at lower concentrations. Higher antimicrobial activity of A-type trimer cinnamtannin B-1 (**1**) as compared to the B-type dimer procyanidin B-2 (**2**) was found, in particular on the three *S. aureus* strains analysed. Results about the synergistic activity between both compounds also suggest that the use of procyanidins should be further explored as natural preservatives or to potentiate the action of biocides and reduce bacterial colonisation and biofilm development (even disruption of preformed biofilm) in food environments. The ability of procyanidins to inhibit microbial growth and biofilm formation and to synergistically work with each other may stimulate a market as natural food preservatives, and/or natural sanitisers for processing equipment where foodborne pathogens reside. However, further studies are necessary, as effects of procyanidins have been shown to be dependent on the cell structure (Gram-positive or Gram-negative bacteria, or fungal cells) and not always dose dependent.

Acknowledgments

This study was supported by the University of Jaén (UJA_08_16_05). A. A.-A. was the recipient of a predoctoral fellowship granted by the University of Jaén. Part of the work was supported by the *Centro de Instrumentación Científico-Técnica* of the University of Jaén.

Conflict of interest

The authors declare no conflict of interests.

References

Al-Hussaini, R. & Mahasneh, A.M. (2009). Microbial growth and quorum sensing antagonist activities of herbal plants extracts. *Molecules*, **14**, 3425–3435.

Amaral, V.C.S., Santos, P.R., daSilva, A.F., Santos, A.R., Manchinski, M. & Mikcha, J.M.G. (2015). Effect of carvacrol and thymol on *Salmonella* spp. biofilms on polypropylene. *International Journal of Food Science and Technology*, **50**, 2639–2643.

Amer, L.S., Bishop, B.M. & van Hoek, M.L. (2010). Antimicrobial and antibiofilm activity of cathelicidins and short, synthetic peptides against Francisella. *Biochemical and Biophysical Research Communications*, **396**, 246–251.

Andjelkovic, M., Van Camp, J., De Meulenaer, B. *et al.* (2006). Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chemistry*, **98**, 23–31.

Ben Amor, N., Bouaziz, A., Romera-Castillo, C. *et al.* (2007). Characterization of the intracellular mechanisms involved in the antiaggregant properties of cinnamtannin B-1 from bay wood in human platelets. *Journal of Medicinal Chemistry*, **50**, 3937–3944.

Bordiga, M., Locatelli, M., Travaglia, F., Coisson, J.D., Mazza, G. & Arlorio, M. (2015). Evaluation of the effect of processing on cocoa polyphenols: antiradical activity, anthocyanins and procyanidins profiling from raw beans to chocolate. *International Journal of Food Science & Technology*, **50**, 840–848.

Bouaziz, A., Romera-Castillo, C., Salido, S. *et al.* (2007). Cinnamtannin B-1 from bay wood exhibits antiapoptotic effects in human platelets. *Apoptosis*, **12**, 489–498.

Cavera, V.L., Arthur, T.D., Kashtanov, D. & Chikindas, M.L. (2015). Bacteriocins and their position in the next wave of conventional antibiotics. *International Journal of Antimicrobial Agents*, **46**, 494–501.

Charlton, A.J., Baxter, N.J., Khan, M.L. *et al.* (2002). Polyphenol/peptide binding and precipitation. *Journal of Agricultural and Food Chemistry*, **50**, 1593–1601.

Clinical and Laboratory Standards Institute. (2012). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, 9th ed. Wayne, PA: Clinical and Laboratory Standards Institute; CLSI publication M7-A9.

Dall'Acqua, S., Cervellati, R., Speroni, E. *et al.* (2009). Phytochemical composition and antioxidant activity of *Laurus nobilis* L. leaf infusion. *Journal of Medicinal Food*, **12**, 869–876.

Dean, S.N., Bishop, B.M. & van Hoek, M.L. (2011). Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against *Staphylococcus aureus*. *BMC Microbiology*, **11**, 114–126.

Djordjevic, D., Wiedmann, M. & McLandsborough, L.A. (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology*, **68**, 2950–2958.

Elhariry, H., Abuzaid, A.A., Khiralla, G.M. & Gherbawy, Y. (2014). Antibiofilm formation and anti-adhesive (to HEp-2 cells) effects of rosemary water extract against some food-related pathogens. *International Journal of Food Science and Technology*, **49**, 1132–1141.

Fernández-Fuentes, M.A., Ortega-Morente, E., Abriouel, H., Pérez-Pulido, R. & Gálvez, A. (2012). Isolation and identification of bacteria from organic foods: sensitivity to biocides and antibiotics. *Food Control*, **26**, 73–78.

Foo, L.Y., Lu, Y., Howell, A.B. & Vorsa, N. (2000). A-type proanthocyanidin trimers from cranberry that inhibit adherence of uropathogenic P-fimbriated *Escherichia coli*. *Journal of Natural Products*, **63**, 1225–1228.

García-Díez, J., Alheiro, J., Pinto, A.L. *et al.* (2016). Behaviour of food-borne pathogens on dry cured sausage manufactured with herbs and spices essential oils and their sensorial acceptability. *Food Control*, **59**, 262–270.

Goto, T., Yoshida, K., Yoshikane, M. & Kondo, T. (1990). Chiral stacking of a natural flavone, flavocommelin, in aqueous solutions. *Tetrahedron Letters*, **31**, 713–716.

Gu, L., Kelm, M.A., Hammerstone, J.F. *et al.* (2003). Liquid chromatographic/electrospray ionization mass spectrometric studies of proanthocyanidins in foods. *Journal of Mass Spectrometry*, **38**, 1272–1280.

Guo, N., Wu, X., Yu, L. *et al.* (2010). *In vitro* and *in vivo* interactions between fluconazole and allicin against clinical isolates of fluconazole-resistant *Candida albicans* determined by alternative methods. *FEMS Immunology and Medical Microbiology*, **58**, 193–201.

Killday, K.B., Davey, M.H., Glinski, J.A. *et al.* (2011). Bioactive A-type proanthocyanidins from *Cinnamomum cassia*. *Journal of Natural Products*, **74**, 1833–1841.

Melo, A.S., Colombo, A.L. & Arthington-Skaggs, B.A. (2007). Paradoxical growth effect of caspofungin observed on biofilms and planktonic cells of five different *Candida* species. *Antimicrobial Agents and Chemotherapy*, **51**, 3081–3088.

Mohri, Y., Sagehashi, M., Yamada, T. *et al.* (2009). An efficient synthesis of procyanidins using equimolar condensation of catechin and/or epicatechin catalyzed by ytterbium triflate. *Heterocycles*, **79**, 549–563.

Pérez-Bonilla, M., Salido, S., van Beek, T.A. & Altarejos, J. (2014). Radical-scavenging compounds from olive tree (*Olea europaea* L.) wood. *Journal of Agricultural and Food Chemistry*, **62**, 144–151.

Phillips, C.A. (2016). Bacterial biofilms in food processing environments: a review of recent developments in chemical and biological control. *International Journal of Food Science and Technology*, **51**, 1731–1743.

Rane, H.S., Bernardo, S.M., Howell, A.B. & Lee, S.A. (2014). Cranberry-derived proanthocyanidins prevent formation of *Candida albicans* biofilms in artificial urine through biofilm- and adherence-specific mechanisms. *Journal of Antimicrobial Chemotherapy*, **69**, 428–436.

Rukayadi, Y., Lee, K., Lee, M.-S., Yong, D. & Hwang, J.-K. (2009). Synergistic anticandidal activity of xanthorrhizol in combination with ketoconazole or amphotericin B. *FEMS Yeast Research*, **9**, 1302–1311.

Sakar, M.K. & Engelshowe, R. (1985). Tannin producing monomeric and dimeric substances in bay leaves (*Laurus nobilis* L.). *Zeitschrift Fuer Lebensmittel-Untersuchung und –Forschung*, **180**, 494–495.

Salido, S., Pérez-Bonilla, M., Adams, R.P. & Altarejos, J. (2015). Phenolic components and antioxidant activity of wood extracts from 10 main Spanish olive cultivars. *Journal of Agricultural and Food Chemistry*, **63**, 6493–6500.

Ulrey, R.K., Barksdale, S.M., Zhou, W. & van Hoek, M.L. (2014). Cranberry proanthocyanidins have anti-biofilm properties against *Pseudomonas aeruginosa*. *BMC Complementary and Alternative Medicine*, **14**, 499–511.

Xu, C., Yagiz, Y., Hsu, W.-Y., Simonne, A., Lu, J. & Marshall, M.R. (2014). Antioxidant, antibacterial, and antibiofilm properties of polyphenols from muscadine grape (*Vitis rotundifolia* Michx.) pomace against selected foodborne pathogens. *Journal of Agricultural and Food Chemistry*, **62**, 6640–6649.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) MS spectrum of cinnamtannin B-1 (**1**) (HPLC peak at t_R 28.5). (b) MS^2 spectrum of **1** at m/z 863. (c) MS spectrum of procyanidin B-2 (**2**) (HPLC peak at t_R 22.3). (d) MS^2 spectrum of **2** at m/z 577.

Figure S2. MS-guided chromatographic fractionation of the laurel wood EtOAc extract focused on the isolation of procyanidins.

Data S1. Experimental details.