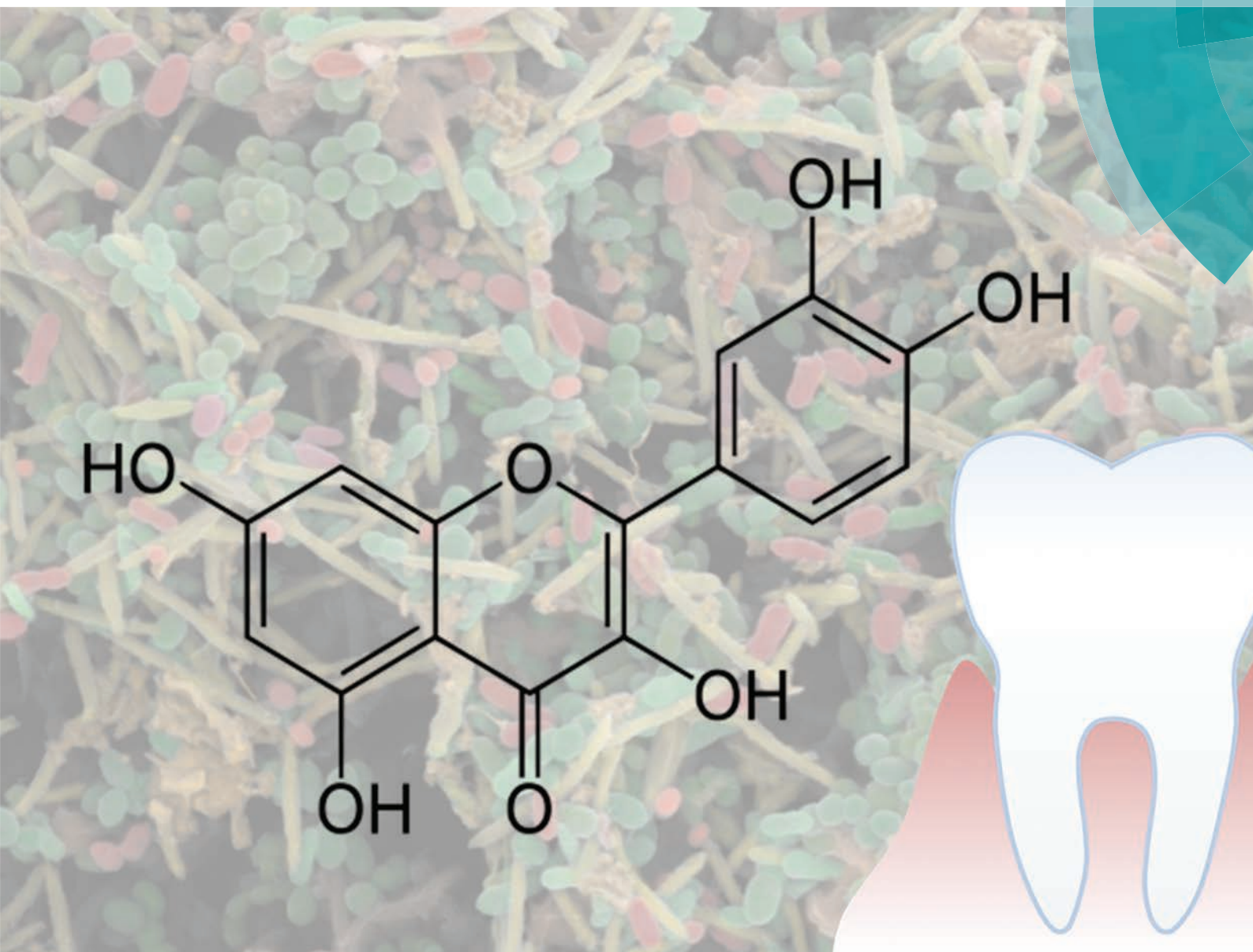


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**PAPER**

Emilie Combet, Gordon Ramage *et al.*  
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biofilm formation



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# Selected dietary (poly)phenols inhibit periodontal pathogen growth and biofilm formation

Muhammad Shahzad,<sup>a</sup> Emma Millhouse,<sup>b</sup> Shauna Culshaw,<sup>b</sup> Christine A. Edwards,<sup>a</sup> Gordon Ramage<sup>b</sup> and Emilie Combet<sup>\*a</sup>

Periodontitis (PD) is a chronic infectious disease mediated by bacteria in the oral cavity. (Poly)phenols (PPs), ubiquitous in plant foods, possess antimicrobial activities and may be useful in the prevention and management of periodontitis. The objective of this study was to test the antibacterial effects of selected PPs on periodontal pathogens, on both planktonic and biofilm modes of growth. Selected PPs ( $n = 48$ ) were screened against *Streptococcus mitis* (*S. mitis*), *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*), *Fusobacterium nucleatum* (*F. nucleatum*) and *Porphyromonas gingivalis* (*P. gingivalis*). The antibacterial potential of each compound was evaluated in terms of planktonic minimum inhibitory concentration (PMIC) and planktonic minimum bactericidal concentration (PMBC) using standardized broth microdilution assays. The most active PPs were further tested for their effect on mono-species and multi-species biofilms using a colorimetric resazurin-based viability assay and scanning electron microscopy. Of the 48 PPs tested, 43 showed effective inhibition of planktonic growth of one or more test strains, of which curcumin was the most potent (PMIC range =  $7.8\text{--}62.5\text{ }\mu\text{g mL}^{-1}$ ), followed by pyrogallol (PMIC range =  $2.4\text{--}2500\text{ }\mu\text{g mL}^{-1}$ ), pyrocatechol (MIC range =  $4.9\text{--}312.5\text{ }\mu\text{g mL}^{-1}$ ) and quercetin (PMIC range =  $31.2\text{--}500\text{ }\mu\text{g mL}^{-1}$ ). At this concentration, adhesion of curcumin and quercetin to the substrate also inhibited adhesion of *S. mitis*, and biofilm formation and maturation. While both curcumin and quercetin were able to alter architecture of mature multi-species biofilms, only curcumin-treated biofilms displayed a significantly reduced metabolic activity. Overall, PPs possess antibacterial activities against periodontopathic bacteria in both planktonic and biofilm modes of growth. Further cellular and *in vivo* studies are necessary to confirm their beneficial activities and potential use in the prevention and or treatment of periodontal diseases.

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## Introduction

Periodontitis (PD) is a common chronic disease triggered by the bacterial biofilm of dental plaque, resulting in inflammatory loss of tooth supporting tissues.<sup>1</sup> It is a major public health issue of significant economic impact, affecting 10–15% of the world adult population<sup>2</sup> and is the most common cause of tooth loss in adults.<sup>3</sup> Moreover, a number of clinical and animal studies have identified positive and independent relationships between chronic periodontitis and other systemic conditions, including diabetes, cardiovascular and respiratory diseases.<sup>4–6</sup>

In humans, PD is commonly associated with a dental plaque biofilm that accumulates on the tooth surfaces. It is one of the most complex and diverse microbial ecosystems within the human body, encompassing some 700 different reported bacterial species.<sup>7</sup> However, initiation and causation of periodontitis is associated with a few pathogenic species, including *P. gingivalis* and *P. intermedia*, and *A. actinomycetemcomitans* in aggressive periodontitis.<sup>8</sup>

Current management approaches include periodontal surgery, scaling and root planning along with adjunctive antibiotic therapy.<sup>9</sup> However, these treatment options not only have limited effectiveness in high risk populations and in those with advanced periodontal disease,<sup>10</sup> but can also be associated with adverse side effects and antibiotic resistance.<sup>11</sup> Therefore, alternative therapeutic and preventive measures which are safe, effective and free of side effects, are highly desirable.<sup>12</sup>

In recent years, an increasing interest has been observed in the use of natural compounds (of dietary origin) for the management of oral infectious diseases, including caries,<sup>13</sup> oral

<sup>a</sup>Human Nutrition, School of Medicine, Glasgow Royal Infirmary, College of Medical, Veterinary and Life Sciences, The University of Glasgow, Glasgow G3 7ER, UK.

E-mail: Emilie.CombetAspray@glasgow.ac.uk; Tel: +44 (0) 141 201 8527

<sup>b</sup>Infection and Immunity Research Group, Glasgow Dental School, College of Medical, Veterinary and Life Sciences, The University of Glasgow, Glasgow G2 3JZ, UK



candidosis<sup>14</sup> and periodontitis.<sup>15</sup> While plant-derived phytochemicals, especially the (poly)phenols (PPs), have been studied for their antioxidant and anti-inflammatory properties,<sup>16</sup> the antimicrobial properties of PPs have received limited exposure in the context of prevention and treatment of PD.<sup>17</sup> Nevertheless, several studies have reported the inhibitory activities of certain PP extracts, including cranberries, lotus, seaweed and perilla seeds against a range of planktonic periodontal pathogens.<sup>18–21</sup> Moreover, the antibacterial activity of flavan-3-ol-rich green tea extracts has been reported against planktonic *S. mitis*, *P. gingivalis* and *F. nucleatum*.<sup>22–25</sup> Others have quantified the antibacterial activity of specific PP molecules (including gallic acid, naringin and quercetin) on planktonic *P. gingivalis* and *F. nucleatum*.<sup>21,26–28</sup> In addition, there are reports that the antimicrobial potential of PPs depends on their chemical structure.<sup>29,30</sup> Given that periodontal pathogens in dental plaque exist in the form of biofilm, there has been very limited research investigating the effect of PPs on biofilm development and formation. Therefore, the aim of the present study was to evaluate the potential of a panel of PPs in inhibiting growth and biofilm formation of PD pathogens, with emphasis on structure–function relationship, if any.

## Materials and methods

### Bacterial strains, media and culture conditions

The bacterial strains used in the study were *S. mitis* NCTC 12261, *A. actinomycetemcomitans* ATCC 43718, *P. gingivalis* ATCC 33277 and *F. nucleatum* ATCC 10953. Prior to each experiment, working stocks were prepared by plating bacterial cells from frozen stocks (Microbank vials, Pro-Lab Diagnostics, Cheshire, UK). *S. mitis* and *A. actinomycetemcomitans* were maintained on Columbia agar plates with 5% defibrinated horse blood (E & O laboratories, UK; Ref DHB 100), then placed in a 5% CO<sub>2</sub> incubator for 24 h at 37 °C. *P. gingivalis* and *F. nucleatum* were inoculated on fastidious blood agar supplemented with 5% defibrinated horse blood and incubated in an anaerobic chamber containing 85% N<sub>2</sub>, 10% CO<sub>2</sub> and 5% H<sub>2</sub> (Don Whitley Scientific Limited, UK) at 37 °C for 72 h. Prior to each experiment, the test strains were grown to stationary phase using appropriate nutrient broth: tryptic soy broth (Sigma, Poole, UK) supplemented with 0.8% glucose and 0.6% yeast extract for *S. mitis* and *A. actinomycetemcomitans*, and Schaeffer anaerobic broth (Oxoid, Cambridge, UK) for *F. nucleatum* and *P. gingivalis* and respective growth conditions. Bacterial cultures were washed by centrifugation at 3000 rpm for 5 min in PBS and the cell count estimated using spectrophotometer, and confirmed by plate counting (CFU mL<sup>-1</sup>).

### (Poly)phenolic compounds

Forty eight purified (HPLC grade) PP compounds (Sigma Aldrich, Poole, UK) were used in this study. Depending on solubility, a fresh stock concentration of each PP was prepared either in water or dimethyl sulfoxide (DMSO, Sigma Aldrich, Poole, UK) and stored at –20 °C until required. Dilutions of

the stock were made in respective growth media for each strain. The final concentration of DMSO was adjusted to less than 3% (v/v), which was shown to have no discernible effect on the growth of test strains (data not shown). All solution preparatory steps were carried out in a laminar flow hood (Microflow, Hampshire, England, UK).

### Planktonic susceptibility testing

The inhibitory potential of PPs ( $n = 48$ ) against planktonic growth of the test strains ( $n = 4$ ) was assessed in terms of planktonic minimum inhibitory concentration (PMIC) and planktonic minimum bactericidal concentration (PMBC) using broth microdilution technique following standard CLSI guidelines.<sup>31</sup> Briefly, standardized ( $1 \times 10^5$  CFU mL<sup>-1</sup>) inoculums of the test strain were prepared in appropriate nutrient broth. The inoculum was loaded into 96-well, round bottom, polystyrene microtitre plates (Corning Incorporated, NY, USA) containing serial double dilutions of each PP (within their soluble range and triplicates). After incubation, the plates were visually inspected for any visible growth. The maximum dilution of the test compound that inhibited visible growth was recorded as PMIC, and the PMBC was determined by inoculating from those wells at concentrations  $\geq$ PMIC and assessing growth thereafter on their respective agar plate. The first concentration with no growth was recorded as the PMBC.

### Manufacture of hydroxyapatite coated plates

The effect of PPs on biofilm formation and maturation was assessed using flat bottom, 96-well polystyrene microtitre plates (Corning Incorporated, NY, USA), coated with hydroxyapatite (HA, Sigma Aldrich, Poole, UK; Product no 289396) by modification of the method described by Schilling *et al.*<sup>32</sup> Briefly, a uniform (10% w/v) HA suspension was prepared in acetone. It was homogenized prior to transfer (60  $\mu$ L) into each well of the microtitre plate. The plates were maintained on an orbital shaker set at 200 rpm and in 37 °C incubator for 15 min. After drying, loose HA powder was removed by tapping the plate, which was then washed twice in PBS and dried overnight at 37 °C. Before use, the plates were sterilized by soaking in 70% ethanol for 10 min, washed, dried and placed in an ultraviolet cabinet for 30 min.

### Adsorption of (poly)phenols to the substrate HA

Adsorption affinity of PPs was assessed following the method of Shellis *et al.*,<sup>33</sup> with slight modifications. Briefly, 1.5 mL stock solutions (50  $\mu$ g mL<sup>-1</sup>) of selected PPs ( $n = 10$ ) were added into Eppendorf tubes containing 200 mg of fresh HA powder. Appropriate controls (PPs without HA) and blanks (HA and water) were also included. PPs were allowed to adsorb onto HA by incubating for 1 h at room temperature with occasional inversion and protection from light. After incubation, PP/HA suspensions were centrifuged at 5000 g for 5 min and the supernatants analysed for total phenol content using the Folin–Ciocalteu method.<sup>34</sup> The total amount of PPs adsorbed per gram of HA ( $\mu$ g g<sup>-1</sup> of HA) was determined by measuring the difference in PP concentration between sample





and control. All experiments were carried out on two different occasions using samples and controls in triplicate.

### Effect of selected (poly)phenols adsorption to HA on adhesion of *S. mitis*

A viability assay based on reduction of resazurin by metabolically active cells<sup>35</sup> was used to assess the effect of PP adsorption on HA-coated plates (biofilm substrate), prior to adhesion of *S. mitis* and biofilm formation. First, artificial saliva (AS) was prepared by adding porcine stomach mucin (0.25% w/v), sodium chloride (0.35 w/v), potassium chloride (0.02 w/v), calcium chloride dihydrate (0.02 w/v), yeast extract (0.2 w/v), Lab-Lemco powder; meat extract made from specially selected raw materials to enhance bacterial growth in media (0.1 w/v; Oxoid, Cambridge, UK; product code LP0029), proteose peptone (0.5 w/v) into ddH<sub>2</sub>O (Sigma, Poole, UK).<sup>36</sup> This was sterilised by autoclaving prior to addition of urea (0.05% v/v), with a final pH of 7.4. Subsequently, AS containing PMIC concentration of selected PPs was added into each well of a HA-coated 96 well plate. Plates were sealed with adhesive lids and stored at 4 °C overnight to allow adsorption. The following day, PP solutions were removed by pipetting, and excess media allowed to evaporate in a laminar flow cabinet. Thereafter, standardized ( $1 \times 10^7$  cells mL<sup>-1</sup>) *S. mitis* suspension in AS was added to the wells. The bacteria were allowed to adhere for 4 h at 37 °C. After incubation, non-adherent cells were removed by rinsing the wells twice with PBS before adding TSB containing 10% (v/v) AlamarBlue® (Life Technologies Ltd, Paisley, UK).<sup>37</sup> The plate was incubated for 2 h at 37 °C, after which the AlamarBlue® suspension was transferred into a clean microtitre plate, and the absorbance measured at 570 and 600 nm using a microplate reader (FluoStar Omega, BMG Labtech, UK). A percentage reduction in biofilm viability was calculated as described previously.<sup>38</sup> The experiment was performed on two different occasions using six replicates.

### Determining the effect of (poly)phenols on maturation of single-species *S. mitis* biofilms

In order to evaluate the inhibitory effects of PPs on freshly adhered sessile cells, a standardized inoculum suspension ( $1 \times 10^7$  CFU mL<sup>-1</sup>) of *S. mitis* was prepared in artificial saliva (AS). An aliquot (200 µL) was then transferred into each well of a HA-coated 96-well plate. Following 4 h of adhesion, the media containing non-adhered cells was removed by pipetting and the biofilms washed twice with PBS. Subsequently, AS containing PMIC concentrations of selected PPs were added into each well, and further incubated for 20 h at 37 °C. Appropriate positive and negative (growth and sterility) controls were also included. Following incubation, biofilm containing wells were washed with PBS and viability of the biofilms assessed using AlamarBlue® as described in the previous section.<sup>37</sup> The experiment was performed on two independent time points using six replicates.

### Effect of (poly)phenols on metabolic activity and ultra-structure changes in a mature, multi-species, pathogenic biofilm

The effect of selected PPs on the metabolic activity of a mature multi-species biofilm was assessed using an *in vitro* biofilm model developed by our group.<sup>38</sup> Biofilms were formed on pre-sterilised, HA discs (Clarkson Chromatography Products Inc., Williamson, PA, USA). To produce multi-species biofilm, 500 µL of standardized ( $1 \times 10^7$  CFU mL<sup>-1</sup>) *S. mitis* (pioneer species) inoculum in AS was added to 24-well plates containing HA discs and incubated at 37 °C, in 5% CO<sub>2</sub> for 24 h. Following incubation, the supernatant was removed and fresh AS containing standardized *F. nucleatum* added, which was further incubated anaerobically for 24 h at 37 °C. The supernatant was removed and standardised *P. gingivalis* and *A. actinomycetemcomitans* added. The plates were incubated anaerobically for a further four days with fresh AS replaced daily. On the seventh day, the biofilms were washed twice with PBS before addition of fresh AS containing PMIC concentration of the selected PPs. After 24 h incubation, the metabolic activity of the cells was assessed using the AlamarBlue® assay, as described above.

To assess ultra-structural change of biofilms following PP treatment, scanning electron microscopy (SEM) was performed on PP-treated, mature, multispecies biofilms and untreated controls. Briefly, cells were standardised as described above, and grown directly onto Thermanox™ coverslips (Nunc, Roskilde, Denmark) to allow biofilm formation. Following maturation, biofilms were carefully washed with PBS and treated with PMIC concentrations of the selected PPs for 24 h. Following treatment, biofilms were again washed with PBS and then fixed in 2% *para*-formaldehyde, 2% glutaraldehyde and 0.15 M sodium cacodylate, and 0.15% w/v Alcian Blue, pH 7.4, and prepared for SEM as previously described.<sup>39</sup> The specimens were sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope. Images were assembled using Photoshop (Adobe, San Jose, CA, USA).

### Statistical analysis

Data distribution and statistical analysis was performed using GraphPad Prism (Version 5; LaJolla, CA, USA). Unpaired *t*-tests were used to assess differences between two independent samples. Statistical significance was achieved if *p* < 0.05.

## Results

### Effect of (poly)phenols on planktonic growth of test strains

We first set out to screen a panel of PPs on our test organisms to determine if any showed antimicrobial activity. An indicative selection of 48 PPs from two major groups, flavonoids (*n* = 27) and non-flavonoids (*n* = 21) were selected. Based on solubility, a range of serial double dilutions of each PP was tested, and the PMIC and PMBC determined (Table 1). All PPs tested displayed antimicrobial activity against one or more test strains, except for kaempferol-3-glucuronide, epicatechin, malvin



Table 1 Planktonic antimicrobial activity of test (poly)phenols acids against periodontal pathogens

	Range tested <sup>a</sup>	<i>S. mitis</i>		<i>A. actinom.</i>		<i>P. gingivalis</i>		<i>F. nucleatum</i>	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<b>Hydroxybenzoic acids</b>									
Benzoic acid	4.88–2500	1250	>2500	625	1250	625	>2500	625	625
3-Hydroxybenzoic acid	4.88–2500	2500	2500	625	1250	1250	>2500	1250	>2500
4-Hydroxybenzoic acid	4.88–2500	1250	2500	625	1250	2500	>2500	1250	>2500
3,4-Dihydroxybenzoic acid	4.88–2500	1250	2500	312.5	312.5	1250	2500	1250	1250
Gallic acid	4.88–2500	312.5	1250	9.6	9.6	1250	2500	2500	2500
Hippuric acid	4.88–2500	2500	2500	1250	1250	1250	>2500	2500	>2500
Syringic acid	1.46–750	>750	>750	750	750	375	>750	750	1500
Vanillic acid	2.92–1500	750	>1500	325	650	1500	>1500	1500	1500
<b>Hydroxycinnamic acids</b>									
Chlorogenic acid	4.88–2500	>2500	>2500	312.5	312.5	312.5	>2500	525	>2500
Coumaric acid	2.92–1500	1500	1500	375	375	750	>1500	750	1500
Ferulic acid	1.46–750	>750	>750	375	750	750	>750	375	>750
Caffeic acid	0.97–500	>500	>500	62.5	62.5	62.5	>500	62.5	500
<b>Hydroxyphenylacetic acid</b>									
Phenylacetic acid	4.88–2500	1250	2500	625	1250	2500	2500	1250	>2500
3-Dihydroxyphenylacetic acid	4.88–2500	2500	2500	1250	1250	2500	2500	2500	>2500
3,4-Dihydroxyphenylacetic acid	4.88–2500	312.5	2500	4.88	4.88	2500	2500	2500	2500
3-Methoxy-4-hydroxyphenylacetic acid	4.88–2500	2500	2500	1250	1250	2500	2500	2500	>2500
<b>Flavonols</b>									
Quercetin	0.97–500	250	>500	31.25	31.25	62.5	>500	500	>500
Quercetin 3- <i>O</i> -glucoside	0.488–250	>250	>250	325	325	125	>250	250	>250
Quercetin 3- <i>O</i> -glucuronide	0.488–250	>250	>250	>250	>250	>250	>250	250	>250
Kaempferol 3- <i>O</i> -rutinoside	0.24–125	>125	>125	>125	>125	125	>125	125	>125
Kaempferol 3- <i>O</i> -glucuronide	0.488–250	>250	>250	>250	>250	>250	>250	>250	>250
Kaempferol 7- <i>O</i> -neohesperidoside	0.24–125	>125	>125	>125	>125	125	>125	125	>125
<b>Flavanols</b>									
Theaflavin extract	0.488–250	>250	>250	>250	>250	>250	>250	>250	>250
Procyanidin B2	0.488–250	>250	>250	>250	>250	>250	>250	>250	>250
Catechin	0.488–250	>250	>250	250	250	250	>250	>250	>250
Epicatechin	0.488–250	>250	>250	>250	>250	>250	>250	>250	>250
Epigallocatechin	0.488–250	250	>250	31.25	31.25	250	>250	125	>250
Epigallocatechin 3- <i>O</i> -gallate	4.88–2500	312.5	625	39.06	39.06	156.25	156.25	312.5	312.5
Epicatechin 3- <i>O</i> -gallate	0.488–250	>250	>250	250	250	125	250	>250	>250
Catechin 3- <i>O</i> -gallate	0.24–125	>250	>250	>250	>250	125	125	>250	>250
<b>Flavanones</b>									
Naringenin	2.92–1500	162.5	325	162.5	162.5	46.87	375	162.5	325
Naringin	2.92–1500	>1500	>1500	>1500	>1500	750	1500	1500	>1500
Rutin hydrate	0.97–500	500	>500	250	>500	>500	>500	500	>500
Hesperitin	0.97–500	125	125	62.5	62.5	15.62	>500	125	125
Hesperidin	0.97–500	>500	>500	31.25	31.25	500	>500	>500	>500
<b>Anthocyanins</b>									
Malvidin	0.488–250	>250	>250	62.5	62.5	31.25	62.5	125	>250
Malvin	0.488–250	>250	>250	>250	>250	>250	>250	>250	>250
Malvidin 3- <i>O</i> -galactoside	0.488–250	>250	>250	125	125	125	>250	>250	>250
Pelargonidin	0.97–500	>500	>500	62.5	62.5	62.5	>500	>500	>500
Oenin	0.488–250	>250	>250	125	125	>250	>250	>250	>250
Pelargonin	0.488–250	>250	>250	>250	>250	125	>250	>250	>250
<b>Flavones</b>									
Apigenin	0.488–250	>250	>250	>250	>250	15.62	>250	125	>250
<b>Isoflavonoids</b>									
Daidzein	0.488–250	>250	>250	>250	>250	500	>250	500	>250
<b>Phenolics</b>									
Pyrocatechol	4.88–2500	312.5	312.5	4.88	4.88	312.5	312.5	312.5	312.5
Resorcinol	4.88–2500	2500	2500	1250	1250	1250	2500	2500	2500
Phloroglucinol	2.92–1500	1500	>1500	137.50	137.50	375	750	750	750
Pyrogallol	4.88–2500	9.76	19.5	2.4	2.4	312.5	312.5	2500	2500
Curcumin	0.390–200	62.5	>200	15.62	15.62	7.81	15.62	31.25	62.5

<sup>a</sup> Range of test concentration, All MIC values are in  $\mu\text{g mL}^{-1}$ . *A. actinom.* = *A. actinomycetemcomitans*.

**Table 2** Comparison of mean minimum inhibitory concentration (PMIC) of (poly)phenols for different test strains<sup>a</sup>

Parameter	Test strain			
	<i>S. mitis</i>	<i>A. actinomycetemcomitans</i>	<i>P. gingivalis</i>	<i>F. nucleatum</i>
No of effective PPs	23/48	35/48	40/48	35/48
Range	9.76–2500	2.4–1250	7.81–2500	31.25–2500
MIC	1027 ± 916***	341 ± 393	691 ± 811*	941 ± 895***

<sup>a</sup> All MIC Values are given as Mean ± S.D. ( $\mu\text{g mL}^{-1}$ ). Significant difference between MIC of *A. actinomycetemcomitans* compared with other test strains. \* $p < 0.05$ , \*\*\* $p < 0.001$  (*T* test).

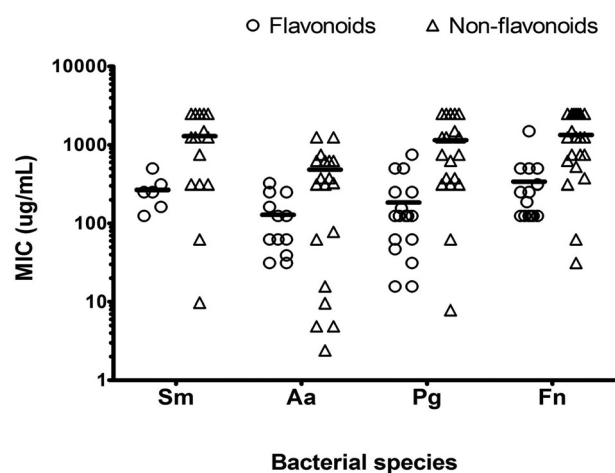
(highest concentration tested:  $250 \mu\text{g mL}^{-1}$ ), theaflavin and procyanidin B2 (highest concentration tested:  $125 \mu\text{g mL}^{-1}$ ). Among all the PPs tested, curcumin was the most potent inhibitor of planktonic growth, with an MIC range of  $7.81$ – $62.5 \mu\text{g mL}^{-1}$ , against all test strains. The highest activity was shown against *P. gingivalis* (PMIC =  $7.81 \mu\text{g mL}^{-1}$ ) and *A. actinomycetemcomitans* (PMIC =  $15.62 \mu\text{g mL}^{-1}$ ). Curcumin was followed by pyrogallol (very wide PMIC range;  $2.4$ – $2500 \mu\text{g mL}^{-1}$ ), pyrocatechol (PMIC range  $4.88$ – $312.5 \mu\text{g mL}^{-1}$ ) and quercetin (PMIC range  $31.25$ – $500 \mu\text{g mL}^{-1}$ ). Apigenin and daidzein were effective only against anaerobic bacteria (PMIC range  $15.6$ – $125 \mu\text{g mL}^{-1}$  and  $500 \mu\text{g mL}^{-1}$ , respectively) at the concentrations tested.

The PMBC for the majority of PPs was either the same or  $2 \times \text{PMIC}$ . Wide variations in the susceptibility (mean MIC ± SD) to PPs were observed among the test strains (Table 2 and Fig. 1). *A. actinomycetemcomitans* showed highest susceptibility ( $p < 0.05$ ) to test PPs, with a mean PMIC of  $341 \pm 393 \mu\text{g mL}^{-1}$  compared with *S. mitis* ( $1027 \pm 916 \mu\text{g mL}^{-1}$ ,  $p < 0.001$ ), *P. gingivalis* ( $691 \pm 811 \mu\text{g mL}^{-1}$ ,  $p < 0.05$ ) and *F. nucleatum* ( $941 \pm 895 \mu\text{g mL}^{-1}$ ,  $p < 0.001$ ) (Table 2). The order of PPs susceptibility of the test strain was *A. actinomycetemcomitans* > *P. gingivalis* > *F. nucleatum* > *S. mitis*.

### Relationship between (poly)phenols structure and antibacterial activity

In order to assess structure–function relationship, the organism most susceptible to a broad range of PPs (*A. actinomycetemcomitans*) was studied.

Antimicrobial activity increased considerably (lower MICs) with increasing number of OH groups attached to the benzene ring (Table 3). The antibacterial activity was also dependent on the location of the OH group. This was reflected in the higher antibacterial potential (lower PMICs) of pyrogallol (1,2,3-trihydroxybenzene; PMIC  $2.4 \mu\text{g mL}^{-1}$ ) and pyrocatechol (1,2-dihydroxybenzene; PMIC  $4.8 \mu\text{g mL}^{-1}$ ), having the same structure as phloroglucinol (1,3,5-trihydroxybenzene; PMIC  $137.5 \mu\text{g mL}^{-1}$ ) and resorcinol (1,3-dihydroxybenzene; PMIC  $1250 \mu\text{g mL}^{-1}$ ) respectively, but different hydroxylation pattern and higher PMIC values. A similar trend was observed for other PPs (Table 3). The presence of pyrogallol (3,4,5-trihydroxybenzoyl) group in the chemical structure of PPs also increased the antibacterial activity. For example, flavan-3-ols containing a pyrogallol (1,2,3-trihydroxybenzene) moiety in their structure



**Fig. 1** Scatter dot plot with individual MICs of individual effective PPs in each subgroup (flavonoids  $n = 27$ , non-flavonoids  $n = 21$ ). Line represent mean of all PP against the test strain. Aa = *A. actinomycetemcomitans*, Sm = *S. mitis*, Pg = *P. gingivalis*, Fn = *F. nucleatum*.

(epigallocatechin, epigallocatechin 3-*O*-gallate, epicatechin 3-*O*-gallate) had lower PMICs ( $31.2$ ,  $39.0$ ,  $250 \mu\text{g mL}^{-1}$  respectively) and higher antibacterial activity than epicatechin and procyanidin B2 containing catechol group (1,2-dihydroxybenzene) which possessed higher PMICs ( $>250 \mu\text{g mL}^{-1}$ ).

Aglycones (PP with no sugar group attached) were stronger (lower MICs) at inhibiting the growth of *A. actinomycetemcomitans* than corresponding glycosides (PPs with attached glucose moiety). An example is quercetin, an aglycone, which had a much lower MIC ( $31.25 \mu\text{g mL}^{-1}$ ) than its glycoside, quercetin-3-*O*-glucoside (MIC =  $325 \mu\text{g mL}^{-1}$ ). The same was true for other aglycones and corresponding glycosides (Table 4).

The PMIC data was thoroughly reviewed and only a subset of the most effective PPs (those with PMIC values of  $<100 \mu\text{g mL}^{-1}$  against one or more of the test strains) were carried forward for subsequent assays. Selected PPs ( $n = 10$ ) included curcumin, quercetin, EGC, EGCG, pyrogallol, gallic acid, 3,4-dihydroxyphenyl acetic acid, naringenin, hesperetin and pyrocatechol.

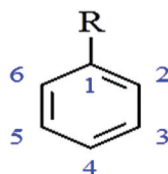
### Effect of selected (poly)phenols on single-species *S. mitis* biofilms

Given that *S. mitis* is one of the key pioneer species in dental plaque biofilm and is required for subsequent complex



**Table 3** Effect of hydroxylation of non-flavonoid phenolic compounds on planktonic growth of *A. actinomycetemcomitans*

Basic structure of non-flavonoids



PP Compound (chemical name)	Common name	Hydroxylation pattern	Substitutions R	MIC ( $\mu\text{g mL}^{-1}$ )
1,2-Dihydroxybenzene	Pyrocatechol	1,2	OH	4.88
1,3-Dihydroxybenzene	Resorcinol	1,3	OH	1250
1,2,3-Trihydroxybenzene	Pyrogallol	1,2,3	OH	2.4
1,3,5-Trihydroxybenzene	Phloroglucinol	1,3,5	OH	137.50
4-Hydroxybenzoic acid		4	COOH	625
3,4-Dihydroxybenzoic acid	Protocatechuic acid	3,4	COOH	312.5
3,4,5-Trihydroxybenzoic acid	Gallic acid	3,4,5	COOH	9.6
3-Hydroxyphenylacetic acid		3	$\text{CH}_3\text{COOH}$	1250
3,4-Dihydroxyphenylacetic acid	Homoprotocatechuic acid	3,4	$\text{CH}_3\text{COOH}$	4.88
4-Hydroxycinnamic acid	Coumaric acid	4	$\text{CH}=\text{CH}-\text{COOH}$	375
3,4-Dihydroxy-cinnamic acid	Caffeic acid	3,4	$\text{CH}=\text{CH}-\text{COOH}$	62.5

**Table 4** Effect of hydroxylation of flavonoid glycosides and corresponding aglycones on growth of *A. actinomycetemcomitans*

Basic structure of flavonoids



PP compound (chemical name)	Common name	Hydroxylation pattern	Substitutions R	MIC ( $\mu\text{g mL}^{-1}$ )
Quercetin 3-O-glucoside	Isoquercetin	5,7,3,4	3-O-Glucoside	325
Quercetin-3-O-rutinoside	Rutin	5,7,4',5'	3-O-Rutinoside	250
3,5,7,3',4'-Penta-hydroxyXavone	Quercetin	3,5,7,3',4'	Nil	31.25
Naringenin 7-O-neohesperidoside	Naringenin	5,7,4'	7-Rhamnoglucoside	>1500
5,7,4'-Trihydroxyflavanone	Naringenin	5,7,4'	Nil	162.5
Malvidin 3,5-O-diglucoside	Malvin	7,4'	3,5-Diglucoside	>250
Malvidin 3-O-glucoside	Oenin	5,7,4'	3-O-Glucoside	125
Malvidin 3-O-galactoside		5,7,4'	3-O-Galactoside	125
3,5,7,4'-Tetrahydroxy-3',5'-dimethoxyflavylium	Malvidin	3,5,7,4'	Nil	62.5
Pelargonidin 3,5-O-diglucoside	Pelargonin	7,4'	3,5-Diglucoside	>250
3,5,7,4'-Tetrahydroxyflavylium	Pelargonidin	3,5,7,4'	Nil	62.5
Hesperetin 7-O-rutinoside	Hesperidin	5,3'	7-O-Rutinoside	62.5
5,7,3'-Trihydroxy-4'-methoxyflavanone	Hesperetin	5,7,3'	Nil	31.25

biofilm formation and maturation, we wanted to determine if these PPs had the capacity to inhibit different stages of *S. mitis* biofilm.

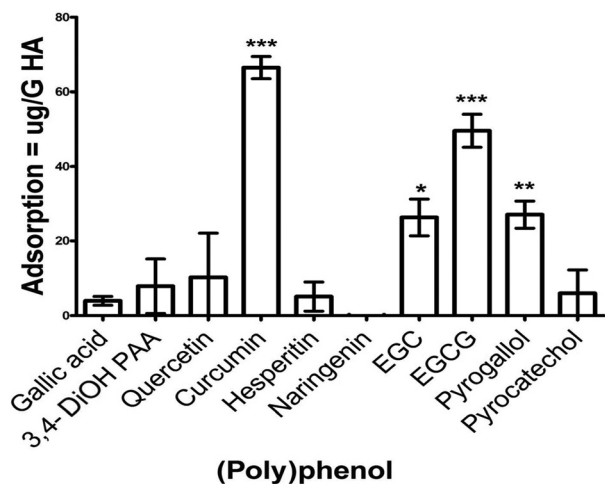
The potential for PPs to adsorb onto HA, a key constituent of tooth enamel was tested. After 1 h incubation, the amount of PP adsorbed ranged from 1–66.5  $\mu\text{g g}^{-1}$  HA. Compared with the control, a significant adsorption was observed for curcumin ( $p < 0.001$ ), EGC ( $p < 0.05$ ), EGCG ( $p < 0.001$ ), and pyrogallol ( $p < 0.01$ ) (Fig. 2). Other PPs (gallic acid, 3,4-dihydroxyphenyl acetic acid, naringenin, quercetin, hesperetin and pyrocatechol) did not show any significant adsorption onto HA.

Adsorption of PP onto substrate (HA coated, 96-well plate) with the ten selected PPs (at PMIC) resulted in differential suppression of *S. mitis* biofilms (Fig. 3). Biofilm suppression ranged from 1 to 12% of the untreated control, with significant ( $p < 0.05$ ) inhibitory activity shown by quercetin only (12%).

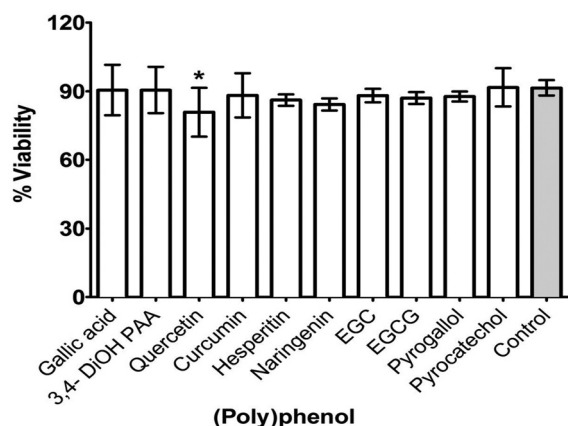
The ten selected PPs were also tested at their PMIC against maturation of *S. mitis* biofilms. Decreased viability (3–10%) in biofilms was observed following treatment with gallic acid ( $p < 0.01$ ), curcumin ( $p < 0.001$ ), hesperetin ( $p < 0.001$ ), and pyrocatechol ( $p < 0.001$ ) compared with untreated controls





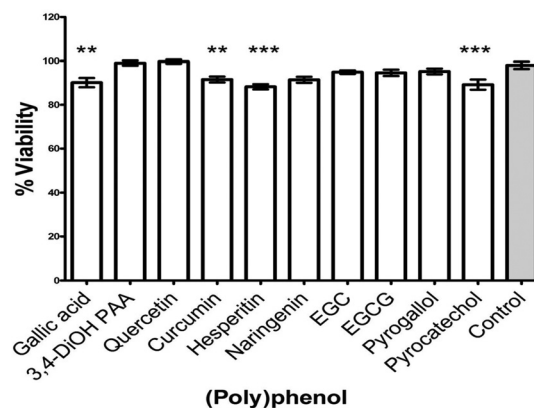


**Fig. 2** Adsorption of polyphenols onto hydroxyapatite. Standardized PPs solution ( $50 \mu\text{g mL}^{-1}$ ) was incubated in the presence and absence of 200 mg HA powder for one hour followed by centrifugation at 5000g for 5 minutes. Total PPs concentration of the supernatants was determined using the Folin–Ciocalteu method. The assay was performed on two independent occasions, each with triplicate. Unpaired *t*-test was used to compare the difference between sample and control. 3,4-diOH PAA = 3,4-dihydroxyphenylacetic acid, EGC = epigallocatechin, EGCG = epigallocatechin 3-*O*-gallate. Error bars represent SEM (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).



**Fig. 3** Effect of selected (poly)phenols adsorption on formation of *S. mitis* biofilm. Selected PPs (PMIC) were allowed to coat surface of the substrate (HA coated, 96-well plates) overnight. The solution was removed by pipetting and the substrate is air-dried before adding standardized ( $1 \times 10^7$  CFU  $\text{mL}^{-1}$ ) inoculum of *S. mitis* prepared in artificial saliva. After 4 h incubation, media was removed and the well were gently washed with PBS. Biofilm was then quantified by adding Alamar-Blue® for 2 h and measuring absorbance at 570 and 600 nm using a microplate reader. The assay was performed on two independent occasions using six replicates. Unpaired *t*-test was used to compare the difference between treatments and controls. 3,4-diOH PAA = 3,4-dihydroxyphenylacetic acid, EGC = epigallocatechin, EGCG = epigallocatechin 3-*O*-gallate. \* (*p* < 0.05). Error bars represent SEM.

(Fig. 4). Other PPs (3,4-dihydroxyphenylacetic acid, quercetin, EGC, EGCG and pyrogallol) showed no significant effect on biofilm maturation.



**Fig. 4** Effect of selected (poly)phenols on maturation of *S. mitis* biofilm. Standardized inoculum suspension ( $1 \times 10^7$  CFU  $\text{mL}^{-1}$ ) of *S. mitis* in artificial saliva and supplemented TSB was added to HA coated, 96-well plates and incubated for 4 h to allow adhesion and biofilm formation by *S. mitis*. After incubation, biofilm were washed twice with PBS before treating with PMIC of selected PPs for 20 h. Both positive and negative control were included. After 20 h incubation, media was removed and the well were gently washed with PBS. Biofilm biomass was then quantified by adding AlamarBlue® for 2 h and measuring absorbance at 570 and 600 nm using a microplate reader. The assay was performed on two independent occasions using six replicates. 3,4-diOH PAA = 3,4-dihydroxyphenylacetic acid, EGC = epigallocatechin, EGCG = epigallocatechin 3-*O*-gallate. Unpaired *t*-test was used to compare the difference between treatment and untreated controls. Error bars represent SEM, \*\**p* < 0.01, \*\*\**p* < 0.001.

**Table 5** Summary of the effect of selected PPs adsorption and effect on key phases of *S. mitis* biofilm formation and maturation<sup>a</sup>

PP	Adsorption	Biofilm formation	Biofilm maturation
Gallic acid			✓
3,4-DiOH PAA			
Quercetin			
Curcumin	✓		✓
Hesperitin			✓
Naringenin			
EGC	✓	✓	
EGCG	✓		
Pyrogallol	✓		
Pyrocatechol			✓

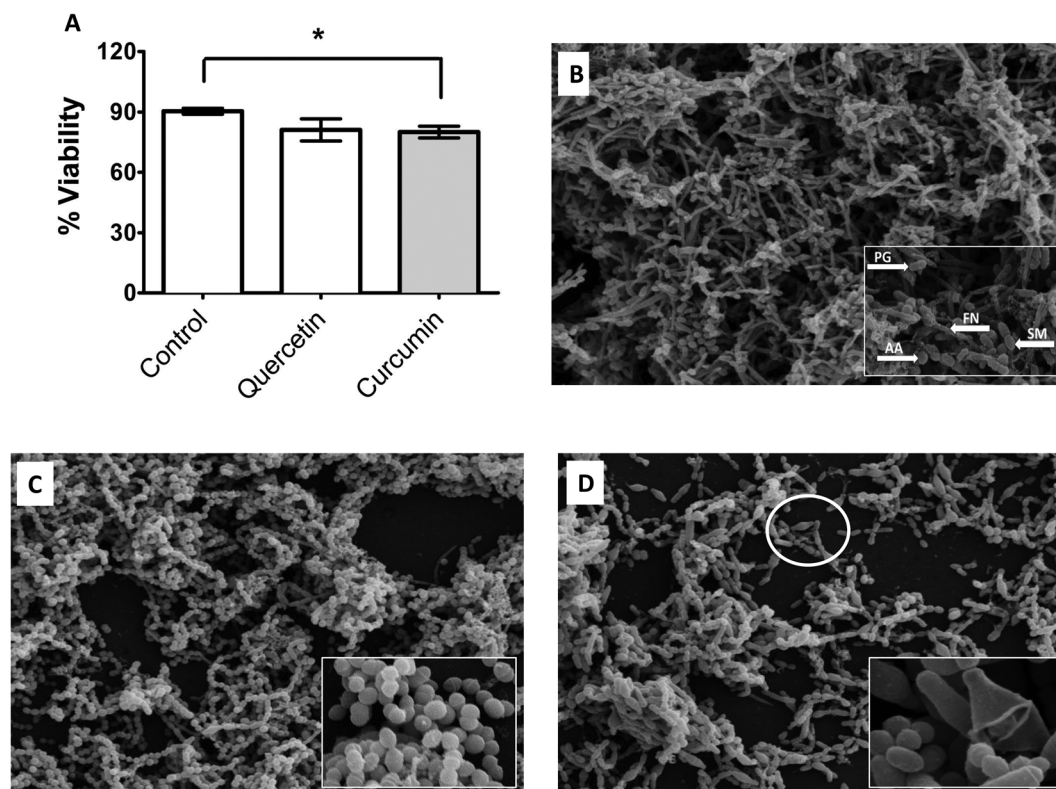
<sup>a</sup> 3,4-diOH PAA = 3,4-dihydroxyphenylacetic acid, EGC = epigallocatechin, EGCG = epigallocatechin 3-*O*-gallate.

A summary of the levels at which the ten selected PPs were effective in relation to adsorption to substrate and inhibition of biofilm formation or maturation is given in Table 5. It is interesting to note that none of the ten PPs were significantly involved in all three steps (adsorption, inhibition of formation or maturation).

#### Bactericidal effect of curcumin and quercetin on mature, multi-species, pathogenic biofilm and ultrastructure changes

The metabolic activity of complex 4-species biofilms was significantly decreased (*p* < 0.05) in curcumin-treated biofilms





**Fig. 5** (A–D) Effect of quercetin and curcumin on metabolic activity and ultra-structure changes in mature, multi-species biofilms. Multi-species (*S. mitis*, *A. actinomycetemcomitans*, *P. gingivalis* & *F. nucleatum*) biofilms were grown in 24 well plates containing HA discs or Thermanox™ coverslips in artificial saliva. Biofilms were allowed to mature for 7 days followed by treatment with PMIC concentration of curcumin and quercetin for 24 hours (A) After treatment, biofilm biomass was quantified adding AlamarBlue® and measuring absorbance at 570 and 600 nm using a microplate reader. Unpaired t-test was used to compare the difference between treatment and untreated controls. Error bars represent SEM, \* $p < 0.05$  (B–D) Biofilms grown on coverslips were processed, viewed under scanning electron microscope and images assembled using Adobe Photoshop. B = normal biofilm, C = curcumin-treated biofilm, D = quercetin-treated biofilm. PG = *P. gingivalis*, FN = *F. nucleatum*, AA = *A. actinomycetemcomitans*, SM = *S. mitis*.

compared with controls, but not quercetin-treated biofilms (Fig. 5A). SEM analysis of mature, multi-species biofilms treated with curcumin and quercetin are shown in Fig. 5B–D. Untreated biofilms covered the entire coverslip with all strains (*S. mitis*, *A. actinomycetemcomitans*, *P. gingivalis* & *F. nucleatum*) clearly visible and embedded in extracellular matrix (Fig. 5B), whereas curcumin-treated biofilms predominantly contain *S. mitis* (Fig. 5C) with no extracellular matrix visible. Biofilms treated with quercetin showed loss of biomass compared with untreated controls, and lack of *F. nucleatum* and extracellular matrix (Fig. 5D). Bacteria, especially *P. gingivalis*, appeared to swell and rupture (Fig. 5D, inset).

## Discussion

Global prevalence of PD, coupled with partial effectiveness of current treatments (due to development of antibiotic resistance), poses a significant challenge to scientists and oral health professionals across the world.<sup>40,41</sup> There is, therefore, an opportunity for identification and testing of natural, alternative, therapeutic agents for prevention and/or manage-

ment of the disease. Here, we have reported the antibacterial potential of PPs against key periodontal pathogens, which may be helpful in the prevention and possibly management of PD.

An important consideration for cell-based assays involving PPs is their solubility. Many PPs are water insoluble and solvents such as dimethyl sulfoxide (DMSO), methanol or ethanol are often used. However, the antibacterial activity of these solvents can often interfere with the interpretation of the data.<sup>42</sup> Keeping these limitations in mind, we first confirmed that DMSO in the concentration of 2.5% (v/v) was the most suitable solvent, having no discernible effect on the growth of the test strain (data not shown). Previously reported PMICs of PPs against *P. gingivalis* were noticeably different from our data (Table 1), *i.e.* gallic acid (MIC = 1 mg mL<sup>-1</sup>), quercetin (250 mg mL<sup>-1</sup>), naringin (250 mg mL<sup>-1</sup>), EGCG (500 µg mL<sup>-1</sup>) and EGC (1000 µg mL<sup>-1</sup>).<sup>21,23,26–28</sup> This is, most probably, due to variations in the methods used for susceptibility testing, some authors have used the agar dilution method, different test strains, and sources of PP (commercial or natural).

A species-dependent susceptibility of periodontal pathogens to PPs was observed, where the PMIC for Gram negative test strains (*A. actinomycetemcomitans*, *P. gingivalis* & *F. nuclea-*



tum) was lower than for the Gram positive test strain *S. mitis* (Table 2). Similar results have been obtained by Bakri and Douglas<sup>43</sup> where Gram negative oral bacteria (*A. actinomycetemcomitans*, *P. gingivalis* & *F. nucleatum*) were more susceptible to the action of allicin (present in garlic extract) than streptococci. This differential activity of PPs was further reflected in the time kill curves performed on Gram positive *S. mutans* and Gram negative *P. gingivalis*.<sup>43</sup> Among Gram-negative bacterial strains tested in our study, *F. nucleatum* was the least susceptible to the action of PPs and in some cases the PMIC value was approaching that for the Gram-positive strain (*S. mitis*). Interestingly, in addition to an outer membrane, *F. nucleatum* has a large periplasmic space surrounded by peptidoglycan layers, in-between the outer and inner cytoplasmic membranes.<sup>44</sup> Therefore, it is reasonable to speculate that decreased sensitivity of the bacterium could be partly due to its membrane structure, which could hamper access of PPs to the target enzymes in cytoplasmic and periplasmic space. Similarly, the diminished sensitivity of *S. mitis* may be explained by the presence of a thick peptidoglycan layer in the cell wall of Gram-positive bacteria.<sup>45</sup> The difference between PMIC and PMBC values reflect that the PPs tested in this study were bactericidal in their activity against the test strains. These results were in agreement with a recently published study.<sup>46</sup>

Our study also reported for the first time the relationship between the chemical structure of PPs and antibacterial activity against *A. actinomycetemcomitans*, the most susceptible of the test strains. We have shown that increased hydroxylation of the benzene ring in phenolic acids and benzene alcohols is associated with increases in antibacterial activity. The opposite association was reported previously for *E. coli*.<sup>29</sup> This difference could be due to the use of different antimicrobial susceptibility assays, in addition to test strains (*E. coli*) and growth conditions. We showed that PPs containing a 3,4,5-trihydroxyphenyl (pyrogallol) group in their chemical structure were more potent inhibitors (possessing lower PMICs) of the growth of *A. actinomycetemcomitans* than PPs containing 1,2-dihydroxyphenyl (pyrocatechol) groups. These findings are in agreement with Taguri *et al.*<sup>47,48</sup> who report an increased antibacterial potential of PPs containing a pyrogallol moiety against a number of food-borne pathogenic bacteria ( $n = 96$ ) including both Gram positive and Gram negative strains. We also observed a stronger antibacterial activity of aglycones than their corresponding PP glycosides. PPs present in foods are mainly found in the glycosylated form however, reports indicate that PP glycosides can be hydrolysed into aglycones by salivary enzymes and the oral microflora.<sup>49,50</sup> This further raises the possibility of increased antimicrobial activity of PPs in the oral cavity following dietary consumption that can also contribute to the prevention of oral infectious diseases including periodontitis.

The primary aetiological factor in PD is biofilm formation and its dynamic complexity. Theoretically, inhibition of the early steps in this process can result in the prevention of periodontal diseases, as has been the focus in developing vaccines against the pioneer species.<sup>51</sup> An important property of PPs is

their ability to adhere to oral hard and soft tissues, microbes and salivary proteins.<sup>52,53</sup> Our study has confirmed that PPs, especially curcumin, possess high adsorptive affinity for HA that can be employed in increasing the local (oral) availability of PPs. This can further help in preventing and inhibiting biofilm formation on tooth surface, either through physical inhibition<sup>54</sup> or high antimicrobial properties observed in this study and elsewhere.<sup>55</sup> Both curcumin and quercetin can also affect the metabolic activity and architecture of mature, multi-species, pathogenic biofilm. The activity of both curcumin and quercetin appeared to be selective based on imaging of the biofilms; depleting only specific pathogens in mature biofilms, leaving a biofilm composed mainly of *S. mitis*. This finding is interesting because *S. mitis* is a part of the normal oral flora, and biofilms containing only streptococci are inert and pose no threat *per se* to oral health. Also, swelling and rupture of *P. gingivalis* cells following treatment with quercetin may be a mechanism for the observed killing activity by quercetin.

Along with key strengths (the large screening, use of published guidelines, use of planktonic and biofilm assays, and single and multispecies models), this study presents some limitations. Adsorption of PPs onto HA-coated 96-well plates or commercial HA discs might be different than adsorption onto HA powder; due to differences in the available surface area and details (HA discs are more polished). Quantification of PP adsorption onto HA discs using an ellipsometer would be helpful in this regard, and further studies combining HA with simulated salivary pellicle as a more complex surface are warranted. In addition, the antimicrobial susceptibilities and relationship between PP structure and antimicrobial activity need to be further evaluated by using other species and clinical strains. Multispecies biofilm models have been extensively used in our group;<sup>38</sup> their construction however can differ, and ours is completely different (both in terms of the number of bacterial species and the sequence of addition of bacterial strains to produce a biofilm) from other recent work<sup>56</sup> reporting death of *S. mitis* in biofilm following addition of *P. gingivalis*.

Collectively our observations provide evidence that the PPs tested in this study may be important in preventing oral microbial diseases and maintaining oral health. Potential routes for exploitation of PP beneficial activities (antibacterial potential) include dietary intervention and pharmaceutical preparations. For the most active PPs, the PMICs were less than  $100 \mu\text{g mL}^{-1}$  which can be achieved through diet as they are present in commonly consumed foods. For example, tea (green, black, oolong), turmeric and onions are rich sources of curcumin, EGCG and quercetin.<sup>57</sup> It is estimated that one cup of tea (240 mL) contains 240 mg of EGCG.<sup>58</sup> Moreover, the estimated daily intake of turmeric in Nepal and India is approximately 1500–2500 mg per day corresponding to 50–100 mg of curcumin.<sup>59</sup> In the Dutch population, the estimated flavonoid intake, of which 70% is quercetin, is about 34 mg per day.<sup>60</sup> Oral availability of PP can be potentiated by their ability to adsorb onto the tooth surface.



Where adequate intake cannot be achieved by diet alone, there are opportunities to use PPs for pharmaceutical development of oral healthcare products. Indeed, PPs containing toothpaste and dental gel are available in the market (AO Pro-Toothpaste, AO ProVantage dental gel and Antioxidant Oral Care System marketed by PERIOSCIENCES®) containing natural (poly)phenols (phloretin & ferulic acid) with no clinical data so far supporting a PP-specific effect on oral health. A key consideration for oral healthcare is the potential for tooth staining, which was demonstrated for tea polyphenolic extracts.<sup>33,61</sup> Preparation of synthetic analogues with superior or optimal therapeutic properties without staining risk may be more suitable.<sup>62</sup> We have demonstrated the antibacterial potential of PPS *in vitro*, further cellular and *in vivo* studies are required for successful use of PPs in the prevention and treatment of periodontal diseases.

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